Recovery of Cellulose and Bioethanol Production through Integrated Pretreatment of Corncob

Mihret Tekalgn Abiza

A Thesis Submitted to

The Department of Chemical Engineering

School of Mechanical, Chemical and Materials Engineering

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Chemical Engineering (Specialization in Process Engineering)

Office of Graduate Studies

Adama Science and Technology University

September, 2023

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master Thesis entitled **" Recovery of Cellulose and Bioethanol Production through Integrated Pretreatment of Corncob "** is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Mihret Tekalgn Abiza	_____	_____
Name of student	Signature	Date

RECOMMENDATION

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled **" Recovery of Cellulose and Bioethanol Production through Integrated Pretreatment of Corncob "** prepared under our guidance by Mihret Tekalgn submitted in partial fulfillment of the requirements for the degree of Masters of Science in Chemical Engineering. Therefore, we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

Dr. Zelalem Tumsa (Ph.D.)	_____	_____
Major Advisor	Signature	Date
Dr. Selvakumar Periyasamy (Ph.D.)	_____	_____
Co-advisor	Signature	Date

APPROVAL PAGE

We, the advisors of the thesis entitled entitled **" Recovery of Cellulose and Bioethanol Production through Integrated Pretreatment of Corncob "** and developed by **Mihret Tekalgn Abiza,** hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis

Dr. Zelalem Tumsa (Ph.D.)	_____	_____
Major Advisor	Signature	Date
Dr. Selvakumar Periyasamy (Ph.D.)	_____	_____
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Mihret Tekalgn have read and evaluated the thesis proposal entitled **" Recovery of Cellulose and Bioethanol Production through Integrated Pretreatment of Corncob "** and examined the candidate during the open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Chemical Engineering (Specialization in Process Engineering).

_____	_____	_____
Chairperson	Signature	Date
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Internal Examiner	Signature	Date
Dr. Wondalem Misganaw	_____	19-10-2023
External Examiner	Signature	Date

Finally, approval and acceptance of the thesis is contingent upon submission of its final Copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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Department Head	Signature	Date
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School Dean	Signature	Date
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Office of Postgraduate Studies, Dean	Signature	Date

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First and foremost, praises and thanks to **God**, the Almighty, for His showers of blessings throughout my research work to complete the research successfully. Next, I would like to express my deepest and heartfelt gratitude to my advisors, Dr. Zelalem Tumsa, and Dr. P. Selvakumar for their valuable guidance, assistance and continuous encouragement throughout this research.

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LIST OF ACRONYMS AND ABBREVIATION

AFEX	Ammonia Fiber Explosion
ANOVA	Analysis of variance
CC	Corncob
CCC	Corncob cellulose
CCD	Central Composite Design
CO_2	Carbon dioxide
$C_6H_{12}0_6$	Glucose
CR	Cellulose recovery
CrI	Crystallinity Index
CSAE	Central Statics Agency of Ethiopia
FTIR	Fourier Transform Infrared Spectroscopy
GHG	Green House Gases
GSR	Global Stats Repot
HMF	Hydroxmethyl furfural
H_2SO_4	Sulfuric Acid
LA	Levulinic acid
LCB	Lignocellulos Biomass
MSW	Municipal Solid Waste
MV	Microwave
MW	Mega Watt
NaOH	Sodium Hydroxide
NH_4OH	Ammonium Hydroxide
NREL	National Renewable Energy Laboratory
rpm	Revolutions per Minute
RCC	Raw corncob
RSM	Response Surface Methodology
UV-VIS	Ultraviolet–visible spectroscopy
XRD	X-Ray Diffraction

ABSTRACT

Corncob is one of the lignocellulosic wastes that contains a sufficient amount of cellulosic material, has potential as a feedstock for fermentable sugar, and can be utilized for bioethanol production. It required appropriate pretreatment methods to remove recalcitrant parts of the biomass (lignin and hemicellulose), maximizing cellulose recovery and fermentable sugars. In this research work, microwave-assisted binary alkaline ($NaOH+NH_4OH$) pretreatment was employed using corncob biomass to enhance cellulose recovery, fermentable sugar, and bioethanol production. Response surface methodology (RSM) with central composite design (CCD) was employed to investigate optimum conditions for microwave-assisted binary alkaline pretreatment of corncob. The effects of three parameters, such as alkaline concentration (1–3%, v/v), pretreatment time (5–15 min), and biomass loading ratio (0.05-0.15 g/mL), were evaluated to increase the cellulose recovery and hemicellulose removal. FTIR and XRD analyses were used to characterize the samples. The residues after the pretreatment were hydrolyzed using dilute sulfuric acid at concentrations of 1%, 2%, 3%, and 4% at temperatures (100 °C, 110 °C, 120 °C, and 130 °C) and times (15, 30, 45, and 60 min). Bioethanol fermentation was carried out at 30 °C with 200 rpm for 4 days with the selected hydrolysates using Saccharomyces cerevisiae. The maximum cellulose recovery and hemicellulose removal were 92.6% and 83.8%, respectively, at the optimum conditions of alkaline concentration (2%, v/v), biomass loading ratio (0.1 g/mL), pretreatment time (10 min), and 400W microwave power. An overall increase in crystallinity index from 37.29 to 56.41% was observed at the optimum condition. Moreover, FTIR spectra revealed the removal of hemicelluloses and lignin after pretreatment. The maximum glucose concentration of 58.16 mg/mL was released at 120 °C with an acid concentration of 3% (v/v) for 45 min of hydrolysis time, and the maximum bioethanol concentration was 23.64 mg/mL. The characterization of the bioethanol product was performed by FTIR, which revealed O-H, C-O, and C-H functional groups similar to those of standard ethanol. These results suggest that the microwave-assisted binary alkaline ($NaOH+NH_4OH$) pretreatment process is a novel pretreatment approach that increased cellulose recovery, improved fermentable sugar production, and could be used successfully for bioethanol production.

Keywords: *Bioethanol; Cellulose recovery; Corncob biomass; Hydrolysis; Microwave-assisted binary alkaline pretreatment*

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the Study

The energy demand is increasing rapidly with the growing world population. The economic growth of countries has been directly or indirectly related to their energy consumption. In today's world, environmental problems caused by the use of fossil fuels are a serious concern. The utilization of fossil fuels as a source of energy for economic development can cause high emissions of greenhouse gases (GHGs) in the Earth's atmosphere. However, concerns about the depletion of fossil fuels, increasing energy consumption, and industrialization have encouraged researchers to find alternative energy sources. (Hanif et al., 2019). One of the most promising biofuels is ethanol, produced from lignocellulosic biomass by microbial fermentation. As a result, some countries have managed to lessen their reliance on traditional energy systems through novel innovations and improved infrastructure, with socio-economic and environmental benefits. However, most countries continue to depend primarily, and in some cases, almost entirely, on fossil fuels. For example, in Ethiopia, transport energy demand is entirely dependent on petroleum imports (Benti et al., 2021).

Lignocellulosic waste is reasonable agro-industrial biomass that is available at an inexpensive price and is categorized as a renewable source. In general, lignocellulose biomass (LCB) can be classified into four main sources: agricultural and forest residues, energy crops, and cellulosic wastes. As a wide category of biomass, agro-industrial biomass incorporates both the food-based and the non-food-based portions of crops (Fabiana Meijon Fadul, 2019).

Bioethanol can be produced from unique biomass along with sugar or starchy materials in addition to lignocellulosic biomass, which might be rich in hexoses and pentose (Zabed et al., 2017). On the one hand, using sugary and starchy biomass as first-generation biofuel production leads to the high cost of bioethanol production due to the excessive price of raw materials (Robak & Balcerek, 2018). The use of food stocks like sugarcane, corn, and cereal grains would then again possibly cause a food crisis. Biofuels generated from lignocellulosic biomass (second-

technology biofuel) constitute one of the largest renewable sources of power that can be non-polluting and sustainable (S. Singh et al., 2015).

Corncob is one of the agricultural waste products of corn, which contains a large amount of sugar that can be further utilized to produce various compounds (Arumugam et al., 2021). The bioconversion of lignocellulosic biomass to biofuel from cheap, non-edible materials such as corncobs for renewable energy is imperative. Corncobs contain a sufficient amount of cellulosic material, which is the best source of fermentable sugars, and it is estimated that approximately 50 million tons of corncob residue are generated every year in Ethiopia (Du et al., 2020). So the production of bioethanol from corncob biomass seems very attractive and sustainable for several reasons. The renewable and ubiquitous nature of biomass and its non-competitiveness with food crops are the major ones. Another significant factor that adds value and importance to corncob bioethanol is the reduction in greenhouse gas emissions.

The three main steps in bioethanol production from LCBs are pretreatment, hydrolysis, fermentation, and distillation. Pretreatment is the most expensive and limiting stage in converting corncobs into bioethanol. Appropriate pretreatment methods can increase the concentration of fermentable sugars after enzymatic saccharification, which can increase the efficiency of the overall process. Corncob is an unavoidable part of the plant cell wall. It is a complex natural compound made up of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose, as two major polysaccharides in corncob, are highly associated with lignin and form a complex lignocellulosic network. This network is very robust and resistant to depolymerization (A. K. Kumar & Sharma, 2017). It should be mentioned that the effectiveness of pretreatment is a key issue to produce a higher yield of bioethanol. Therefore, manufacturing biofuel from LCB requires a complicated conversion process, which has made it commercially challenging (Moysés et al., 2016).

Based on the limitations of single pretreatment methods, many studies have carried out various pretreatment methods to increase cellulose content and to increase concentrations of fermentable sugars from the lignocellulosic biomass degradation or separation of cellulose, hemicellulose, and lignin components during the pretreatment process. In this study, the new approach (integrated microwave-assisted binary alkali pretreatment) process was optimized for a particular

biomass (corncob) pretreatment method to increase cellulose recovery, enhance the efficiency of acid hydrolysis, and produce high-yield bioethanol.

1.2. Statement of the Problem

The amount of corn produced in Ethiopia increases every year. Because of this, approximately 50 million tons of corncob residues are disposed of as waste. This waste has a high amount of cellulose wchich is more than other agricultural wastes such as fruit waste (Du et al., 2020). Therefore, pretreatment of corncobs is important for lignin removal and the breakdown of cellulosic matter. The economic production of gasoline and ethanol in Ethiopia relies specifically on molasses from sugar industries (Yimam & Ababa, 2022). Therefore, corncob could be a potential raw material for bioethanol production to resolve the problem related to energy safety.

Pretreatment is one of the most crucial steps in LCB conversion to bioethanol; however, the complicated structure and recalcitrant nature of LCB have presented pretreatment as the most important step during biomass conversion to bioethanol, and subsequently, intensive studies have been undertaken to improve the pretreatment efficiency. However, so far, most pretreatment methods have failed to achieve desirable cellulose recovery and hemicellulose removal in the biomass, which is necessary to develop the process economics and competitiveness of bioethanol. Most of the time, single pretreatment is less efficient in lignin removal, less effective in hemicellulose degradation, less cellulose recovery, and has a long reaction time (Nath et al., 2021). For that reason, in this study, combining physical and chemical pretreatment is a way to solve this problem. Furthermore, up to now, there is no harmonized pretreatment strategy to treat all kinds of lignocellulosic biomass. However, the use of an integrated microwave-assisted binary alkaline pretreatment strategy can extensively increase the performance of the system.

The researchers are paying more attention to hydrolysis parameter optimization. They suggest continuously optimizing the process parameters for biomass pretreatment processes (which are not always adapted properly) for bioethanol production, which significantly influences bioethanol yield. Besides, very few studies have been achieved to produce bioethanol from non-edible, lignocellulosic material, i.e., corncob, using a combined pretreatment method. Therefore, in this thesis, to overcome this issue, treating corncob biomass using the new approach of integrated microwave-assisted binary alkali pretreatment was used for optimizing the process

parameters (biomass loading ratio, alkaline concentration, and pretreatment time) for efficient cellulose recovery, enhancing hemicellulose solubilization, and improving bioethanol production.

1.3. Objectives

1.3.1. General objective

The general objective of this thesis is to investigate the microwave-assisted binary alkaline pretreatment of corncob biomass for efficient cellulose recovery to produce bioethanol.

1.3.2. Specific Objectives

- ✓ To analyze the proximate determinations and chemical composition of corncob biomass.
- ✓ To find the optimum pretreatment parameters such as binary alkaline concentration, pretreatment time, and biomass loading ratio on the pretreatment process using response surface methodology.
- ✓ To characterize raw, microwave-assisted binary alkaline treated corncob and corncob cellulose using FTIR, and XRD instruments.
- ✓ To investigate the effects of hydrolyzing parameters: hydrolysis time, acid concentration, and temperature on the glucose concentration.
- ✓ To evaluate the ethanol fermentation process from selected hydrolysates using a specific yeast organism and characterize the product using FTIR**.**

1.4. Significance of the study

Because of the strong natural recalcitrance of LCBs, it is highly unlikely that a single alkaline pretreatment would break all polysaccharide-lignin linkages. Thus, it is expected that not all polymeric sugars can be recovered. Therefore, the significance of this study was that combined microwave-assisted alkaline pretreatment was applied, and the operating conditions were optimized to obtain efficient cellulose recovery, enhanced fermentable sugar, and improved bioethanol production.

This study can also introduce a new approach for corncob biomass pretreatment techniques that enhance cellulose recovery to release improved fermentable sugar molecules like glucose. This improved the overall production of bioethanol.

This study significantly improved bioethanol from non-edible agricultural waste (corncob), which is abundantly available in Ethiopia. Bioethanol production from corn cobs is considered a 2nd generation biofuel process since it has no direct conflict with human food, as is the case with 1st generation biofuels produced from agricultural crops such as corn and sugarcane. It could sustain the economy by reducing the cost of imported petroleum and emitting neutral CO_2. Moreover, it enhances the economy by providing value-added market opportunities for the agricultural and transportation sectors.

Furthermore, this study also highly contributes to the substitution of fossil fuels with biofuel. Fossil fuels are being depleted due to extensive and continuing overutilization. If consumption goes at this rate, the fossil fuel reserve will be depleted completely within a short period of time. As a renewable and non-food-competitive feedstock, it is desirable for the production of alternative fuel oils such as bioethanol.

1.5. Scope of the Study

Due to the strong natural recalcitrance of corncob biomass, conventional pretreatment is less efficient in lignin removal, less effective in hemicellulose degradation, less cellulose recovery, and has a long reaction time. In this work, the proximate analysis, the chemical composition of corncob, and the selection of an appropriate binary alkaline ratio to treat the corncob biomass. Furthermore, the integrated microwave-assisted binary alkaline pretreatment was optimized from corncob biomass for efficient cellulose recovery, improving glucose concentration, and bioethanol production. The experiments were analyzed to study the effect of process parameters (pretreatment time, biomass loading ratio, and alkaline concentration) on cellulose recovery, hemicellulose removal, and also characterizing the raw, microwave-assisted binary alkaline-treated corncob and corncob cellulose by using instrumental techniques such as Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD). In addition, the pretreated corncob was hydrolyzed using dilute sulfuric acid hydrolysis to produce glucose. Following hydrolysis, the identification of the reducing sugar of the hydrolyzed corncob was conducted using the phenol-sulfuric acid method. Finally, the fermentation of glucose was converted to bioethanol, characterizing the produced bioethanol.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Introduction

Energy is a critical factor that controls the socio-economic development of a country. The world is having difficulties with energy problems due to the depletion of fossil fuels. Fossil fuels are in finite amounts and cause severe environmental pollution, such as an increase in greenhouse gases, in particular carbon dioxide (CO_2). These greenhouse gases are responsible for the global warming of the earth and the climatic deregulation observed in recent decades (Abo et al., 2019). One viable solution to this problem is to make use of alternate and renewable resources. Many countries have implemented policies for bioethanol production to replace petrol-based engines. As per the Global Status Report (GSR) on energy, the large majority, i.e., 78.4%, is met by non-renewable fossil fuels such as petroleum, coal, and natural gases, and merely 19% from renewable resources such as solar, hydropower, wind, and biomass (B. Kumar et al., 2020). The mentioned renewable resources can satisfy energy demand in the power sector. Still, the transportation sector mainly depends on liquid fuels, which cannot be produced from other renewable sources except biomass (David et al., 2021).

Biomass energy, or 'Bioenergy', is one of the most challenging renewable energies in modern fuels. The challenge is to ensure that biomass energy is produced on a large scale and to compensate for the increased price of food. In other words, bioenergy should do a better job than fossil fuels in terms of its production, efficiency, and availability. Modern bioenergy resources include liquid biofuels, biomass-fired electricity, or methane from animal wastes. It also includes cellulosic ethanol and Fischer-Tropsch (Anca-couce et al., 2021). There are three generations of biofuels that can be used: the first, second, and third. First-generation biofuels are currently produced in large commercial quantities in many countries from agricultural crops such as sugarcane, maize, soybeans, and jatropha through well-established technologies such as hydrolysis, fermentation, and trans-esterification. Second-generation biofuels can be made from lignocellulosic biomass, inclusive of agricultural crop residues, forestry and timber processing wastes, organic components of municipal solid waste (MSW), and power crops, such as

Mischantus giganteus, using either thermochemical or biochemical processes and 3rd-generation biofuels are produced from feedstocks that include micro- and macro-algae, vegetable oils, and biodiesel. The second generation of biofuel is more promising in terms of efficiency and low cost of raw materials as compared to the first and third generations of biofuel. (Duque et al., 2017).

2.2. Bio-ethanol and its characteristics

Bioethanol, also known as ethyl alcohol (C_2H_5OH), is a liquid obtained from fermented sugar after distillation. It can also be obtained from various resources, such as agriculture and forestry residue, dedicated starchy crops, woody and herbaceous crops, and the organic portion of municipal solid waste. In comparison to gasoline, bioethanol has a higher octane number, broader flammability limits, faster flames, and higher heat of vaporization. Ethanol is an industrial chemical with significant utilization. Ethanol is an industrial chemical with significant utilization. It can be utilized in the production of pharmaceutical products, dyes, perfumes, the transportation sector, and many other products. Under normal circumstances, ethanol is a volatile, flammable, transparent, and colorless chemical compound. Ethanol is one of the most interesting synthetic oxygen-containing organic chemicals because of its unique combination of properties as a solvent, a beverage, an antifreeze, and more especially due to its versatility as a chemical intermediate for other chemicals.

2.2.1. Ethiopia's status of bio-ethanol production

In order to increase agricultural development and agro-processing, job creation, export revenue, and import substitution of petroleum goods, ethanol production in Ethiopia is to be connected to factories. However, only a small portion of the potential is now being used, and an alternate 5% and 10% ethanol blend has been made available in the capital of the country. Furthermore, the only sugar factories in the country that are producing bioethanol are Finchaa and Metehara. About 20.5 million liters of ethanol were used in the transportation sector in 2014/15 (8 million liters came from the Fincha sugar factory and 12.5 million liters from the Metahara sugar factory) and were provided to the energy system of the country (Benti et al., 2021). Currently, in Ethiopia, 13 sugar factories are deliberately, under construction, or running, with a projected 3.6 million mt of sugar, 339 million liters of ethanol, and 250 MW of excess power for cutting-edge production, about 400,000 mt of sugar and 21 million liters of ethanol (Fuel, 2021). Biofuel is considered an opportunity for providing domestic energy security, rapid economic development,

and the creation of wealth. There are great expectations that biofuel could contribute to solving the main development challenges the country is facing today. In 2007, the government created a strategic plan that took jatropha as the main feedstock for the manufacture of biodiesel and sugarcane as the main feedstock for the production of bioethanol. The plan emphasized, among other things, the establishment of a biofuel program, the promotion of feedstock development, the stimulation of customer demand, the conception and promotion of biofuels, and the renewal of energy policy to incorporate bioenergy in detail. As a continuation of this endeavor, there have been repeated efforts to initiate the use of bioethanol for domestic use, particularly blending 5% of ethanol with gasoline in 2008, followed by 10% in 2011, and increasing the percentage in the years to come. Currently, in Ethiopia, 13 sugar factories are deliberately, under construction, or running with a projected 3.6 million metric tons of sugar, 339 million liters of ethanol, and 250 MW of excess power for cutting-edge production of about 400,000 metric tons of sugar and 21 million liters of ethanol (Fuel, 2021). Table 2.1 below shows the current production and projected bioethanol concentration (Adapted from (Selvakumar et al., 2022)).

Table 2.1 Shows current production and projected bioethanol concentration in (million /liter)

Sugar factory	Total ethanol production amount
Finchaa Sugar Factory	20 million liters (producing)
Metahara Sugar Factory	12 million liters (producing)
Wonji Shoa Sugar Factory	12.8 million liters (projected)
Omo-Kuraz Sugar Factories I, II, III and IV	140 million liters (projected)
Kessem Sugar Factory	30 million liters (projected)
Tendaho sugar Factory	31 million liters (projected)
Arjo Diddessa	12 million liters (projected)
Wolkaiyt Sugar Factory	41.6 million liters (projected)
Tana Beles Sugar, 2 plants	40 million liters (projected)
Total Production in 2019	21 million liters
Eventual capacity	339 million liters

2.3. Feedstock for bioethanol production

Generally, bio-ethanol feedstocks can be divided into three major groups: (i) sucrose-containing feedstock (sugarcane, sugar beet, and sweet sorghum); (ii) starch-containing feedstock (wheat, corn, and cassava) and (iii) cellulose-containing feedstock (agricultural wastes, paper, straw, grasses, wood), etc**.**

2.3.1. Sugar-containing feedstock

Microorganisms that are involved in fermentation use the fermentable sugars as food, and as a result, ethanol and other byproducts are produced. These microorganisms can usually use 6-carbon sugars, among which glucose is the most common. As a result, the biomass materials that are most readily converted into ethanol are those with high glucose content or glucose precursors. Sugarcane is one of the best sugar-containing feedstocks for bioethanol (Canilha et al., 2012). Sugar cane and beet are the most important sugar-producing plants in the world and are easily hydrolyzed by the enzyme invertase, which is synthesized by most *Saccharomyces* species. Typically, sugarcane contains 12–17% total sugars on a wet weight basis with 68–72% moisture. About 90% of the sugars are sucrose with glucose and fructose making up the balance. The yeast S. cerevisiae easily produces ethanol by fermenting all three of these sugars. During the fermentation process, the yeast produces the enzyme invertase and uses it to convert sucrose to glucose and fructose (Hoang & Nghiem, 2021).

2.3.2. Starch-containing feedstock

Starch-rich crops like corn, wheat, barley, potatoes, and cassava can be used to make bioethanol. Corn, commonly known as maize, is the second-largest feedstock for the manufacture of bioethanol and has been used extensively in the USA (J. Li et al., 2022). The long chain of glucose molecules that make up starch are joined together by glycosidic bonds (1,4-glycosidic). In comparison to sugarcane and sugar beet, the manufacturing of ethanol from cereal grains like corn, wheat, and barley requires more processing steps to convert the feedstock to sugar units. Grain milling is the first step in the procedure, followed by the hydrolysis of starch polymers to sugar units. The other phases of the process are comparable to those of other feedstocks typically utilized for the production of bioethanol. For instance, after grinding in a dry mill, reacting with

diluted acid, and then reacting with amylases such α-amylase and glucoamylase, the starch in maize is transformed into glucose. The fermentation and distillation processes are comparable to those used in the production of bioethanol from sugarcane. (Kassim et al., 2022).

2.3.3. Lignocellulosic biomass

Agro-industrial biomass comprised of lignocellulosic waste is inexpensive, renewable, abundant, and provides a unique natural resource for large-scale and cost-effective bio-energy collection (Anwar et al., 2014). On Earth, lignocellulosic biomass is the most abundant type of organic material. It comes from forests, agricultural and forest cultivation, harvest, forestry and industrial residues, and cellulose and recycled paper (Hadar, 2013). Agricultural wastes such as corn Stover, corn stalks, rice and wheat straws, as well as sugarcane bagasse, and forest residues, are the main lignocellulosic biomasses used as feedstock for bioethanol production. Moreover, municipal and industrial solid wastes are also a prospective pathway for biofuel production (Achinas & Euverink, 2016). For second-generation biofuel production, utilization of renewable biomass resources has received major focus in the world (J. Kumar & Reetu, 2015).

2.4. Structural Components and Composition of LCB

The main components of LCB are cellulose, hemicelluloses, lignin and inorganic materials. Lignocellulosics are the most abundant source of unutilized biomass and their availability does not impact land use. Biomass in general consists of 40-50% cellulose, 25-30% hemicelluloses, and 15-20% lignin and other extractable components (Scordia et al., 2014). The percent composition of these complex carbohydrate polymers such as cellulose, hemicellulose, lignin, and other extractives in lignocellulose biomass are explained in Table 2.1 (Mohan et al., 2015).

2.4.1. Cellulose

Cellulose $(C_6H_{10}O_5)_n$ is produce from a linear chain of D-glucose linkages by β-(1,4)-glycosidic bonds. The cellulose fiber is created by this linear chain altogether. The cellulose structure appears to be flat sheet-type with glucose monomer units. Moreover, many cellulose strands are packed into crystalline fibrils. Cellulose structure consists of many intra-molecular and intermolecular hydrogen bonds with the glucose unit bound tightly. The strong forces of interaction between cellulose sheets are Vander Waal forces. The cellulose polymeric units simultaneously include two different types of linkages, including a glycosidic linkage (also

known as an ether bond) and a hydrogen bond linkage between two hydroxyl groups (Ginni et al., 2021). The glycosidic bond binds the glucose units, while the hydrogen bond connects the straight chains of the polymer. Cellulose has a high degree of polymerization, due to which it has low flexibility, and is primarily insoluble in water, and most solvents (Devi et al., 2021a). The prominent advantages of cellulose over other polymers are its hydrophilic nature, non-toxicity, biodegradability, biocompatibility, eco-friendly, easy derivatization, etc. Due to these unique features, it has been widely used for industrial applications like bioethanol production, petroleum mining, plastics, medicine, and synthesis resins (Tapia-Orozco et al., 2016). At low temperatures, cellulose is also insoluble in dilute acid solutions. The solubility of the polymer is powerfully connected to the degree of hydrolysis achieved. As a result, parameters that affect the hydrolysis rate of cellulose also affect the solubility that takes place, however, with the molecule being in a different form than the native one. At higher temperatures, it becomes soluble as the energy supplied is enough to break the hydrogen bonds that hold the crystalline structure of the molecule. The structure of single-molecule cellulose is given in Fig. 2.1.

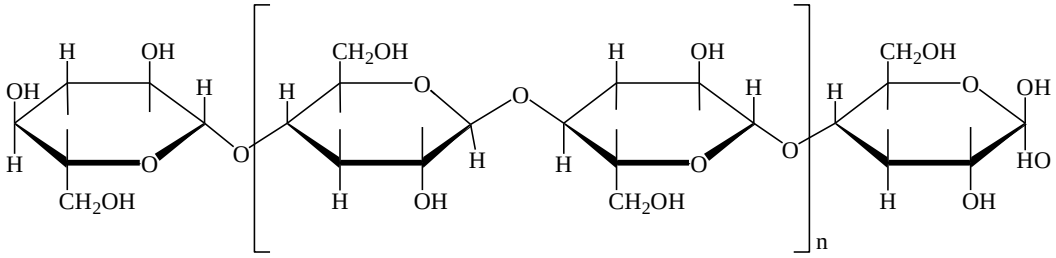

Figure 2.1 Structure of cellulose (Adapted from (Vasić et al., 2021))

2.4.2. Hemicellulose

Hemicelluloses $(C_5H_8O_4)n$, located in secondary cell walls, are heterogeneous branched biopolymers containing pentoses (β-Dxylose, α-L-arabinose), hexoses (β-D-mannose, β-Dglucose, α-D galactose), and/or urgonic acids (α-D-glucuronic, α-D-4-O-methylgalacturonic, and a-D-galacturonic acids) (Mohan et al., 2015). Due to their amorphous, branching structure (with a short lateral chain) and reduced molecular weight, they are relatively simple to hydrolyze. In order to increase the digestibility of cellulose, large amounts of hemicelluloses must be removed as they cover cellulose fibrils, limiting their availability for enzymatic hydrolysis (D. Sun et al., 2019). As compared to cellulose, hemicellulose is easily hydrolyzed

due to the lesser degree of polymerization. The hemicellulose structure is illustrated in Figure 2.2. These amorphous hemicellulose structures make them partially soluble in water.

2.4.3. Lignin

Lignin $(C_{31}H_{34}O_{11})_n$ is the most complex natural polymer. It is a three-dimensional amorphous polymer with phenylpropane units as the main building blocks. More specifically, p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol are the most frequently observed. Lignin in wood is an insoluble, three-dimensional network. It plays a vital role in the endurance and development of cells, as it affects the transport of water, nutrients, and metabolites in the plant cell. It serves as a binding agent between the cells, forming a composite material with outstanding resistance to compression, bending, and impact. Low molecular alcohols, dioxane, acetone, pyridine, and dimethyl sulfoxide are among the solvents that have been found to significantly dissolve lignin. Furthermore, it has been observed that at elevated temperatures, thermal softening of lignin takes place, which allows depolymerization reactions of an acidic or alkaline nature to accelerate (Gumel et al., 2018).

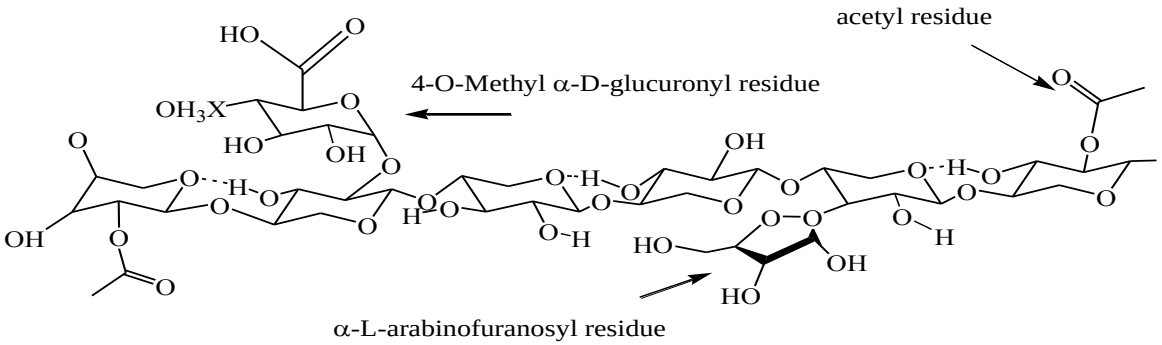

Figure 2.2 structure of hemicellulose (Adapted from (Y. D. Singh & Satapathy, 2018))

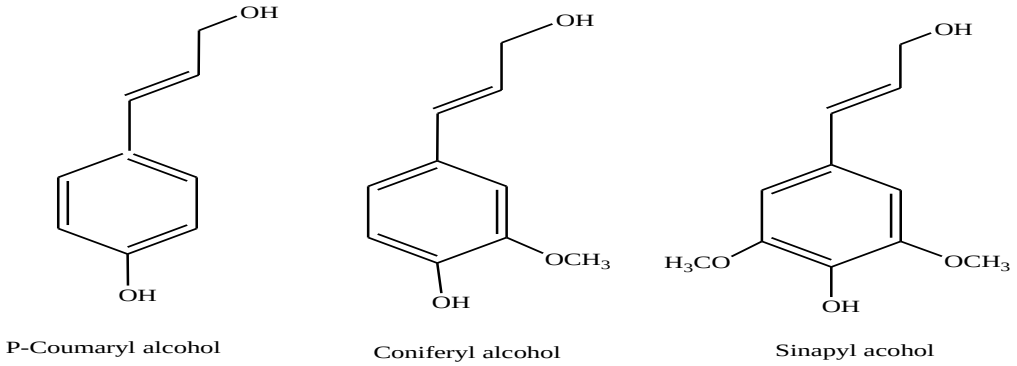

Figure 2.3 Structure of lignin (Adapted from (Devi et al., 2021b))

2.4.4. Extractives

Extractives are non-structural components of lignocellulosic biomass, and they are easily extracted from the biomass by water or neutral organic solvents such as dichloromethane, ethanol, toluene, acetone, and hexane. Extractives are sources of diverse biopolymers such as terpenes, terpenoids, steroids, fats, lipids, proteins, gums, waxes, and phenolic compounds such as lignans, tannins, and stilbenes, and their amounts vary between different lignocellulosic biomass (Okolie et al., 2021). (Okolie et al., 2021). Biomass extractives usually make up a minimum proportion (0–15 wt%), and they are mainly composed of some organic and inorganic compounds.

Table 2.2 Composition of various lignocellulosic biomasses

Material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Sugar Cane Bagasse	32 – 48		23 – 32	(Jaffar et al., 2019)
Barley straw	44	27	7	(Iroba et al., 2013)
Corn Stover	38–40	28	7–21	(Gao et al., 2012)
Rice straw	33	26	7	(C. Bai et al., 2016)
Sweet sorghum	45	27	21	(Gomes et al., 2019)
Barley hull	34.0	36.0	13.8-19	(Verardi et al., 2012)
Hardwood stems	40 – 55	24 – 40	18 – 25	
Softwood stems	45 – 50	5 – 35	25 – 35	
Nut shells	25 – 30	25 – 30	30 – 40	
Corn cobs	40 – 45	31 – 39	14 – 18	(Kucharska et al., 2020)

Grasses	25 – 40	35 – 50	10 – 30	
Paper	85 – 99	0	0 – 15	
Wheat straw	30	50	15	
Sorted refuse	60	20	20	
Leaves	15 – 20	80 – 85	0	
Cotton seed hair	80 – 95	5 – 20	0	
News paper	40 – 55	25 – 40	18 – 30	(Verardi et al., 2012)
Waste paper from chemical pulps	60 – 70	10 – 20	5 – 10	
Primary wastewater solids	8 – 15	Na	Na	
Solid cattle manure	1.6 – 4.7	1.4 – 3.3	2.7 – 5.7	
Coastal Bermuda grass	25	35.7	6.4	
Switch grass	45	31.5	12	
Swine waste	6.0	28	Na	

2.5. Corncobs as a feedstock for bioethanol production

Corncob (CC) is an agricultural residue that is generated from maize and remains after removing the grains. It is mainly used as a feedstock for household fuel. It contains hemicelluloses, cellulose, and lignin and consists of monomer molecules (Ranum et al., 2014). Corncobs were found to yield higher glucose concentrations than other corn residues, like stalks or leaves, and were removed from the fields during conventional harvest (Luque et al., 2016). Corncobs form about 30% of maize agro-wastes of which applications in the bio-fuel industry are focused on many studies aimed at achieving an effective and efficient waste management scheme. It contains 32.3–45.6% cellulose, 39.8% hemicelluloses, mostly composed of pentosan, and 6.7–13.9% lignin and can be converted to fermentable sugar for ethanol production. The chemical

properties and physical characteristics of corn cobs make them suitable as feedstocks for energy generation.

2.5.1. Corn (maize) production in Ethiopia

In Ethiopia, maize is one of the main foods and feed crops and is the lowest-cost caloric source among all major cereals, which is significant given that cereals dominate household diets. Its production in Ethiopia has shown a considerable increase over the last 15 years, with a drastic increment in recent years. Production increased from as low as 3.1 million tons in 2000 to over 7.2 million tons in 2014 (Negatu et al., 2016). Currently, in 2020, maize production in Ethiopia was 8,600 thousand tones.

Ethiopia is among the major maize producers in Sub-Saharan African countries, where smallholder farmers dominate the majority of production. Though maize is widely grown in Ethiopia, only three regional states contribute to 94% of the total annual production. These three regions are Southern Nations, Nationalities, and People's Region (SNNP), Oromia, and Amhara. According to five-year (2003/2004 - 2007/2008) Central Statics Agency of Ethiopia (CSAE) data, the share of Oromia region was on average, 60% of the total maize production in the country. This was followed by Amhara region with 21.67% and SNNP region with 12.55**%** (Gebreselassie et al., 2015).

2.6. Process overview of bioethanol production

Ethanol can be produced in two different ways. Either chemically, by the hydration of ethylene, which is produced from crude oil or natural gas, or through the fermentation of sugar that contains sugar, starchy feeds, or lignocellulosic feeds. About 5%–10% of the ethanol produced in the world is a petroleum product. Catalytic hydration of ethylene using sulfuric acid as a catalyst results in the production of petroleum ethanol. Additionally, it can be obtained from other sources, such as calcium carbide, coal, oil, and gas, via ethylene or acetylene. The two primary ways of producing fuel ethanol from cellulosic feedstock are the biochemical conversion process and the thermochemical conversion process (Nitsos et al., 2013), (Mekonnen, 2010).

2.7. Biochemical conversion process

Biomass biochemical conversion technologies refer to the conversion of biomass into corresponding products through certain physical, chemical, and biological pretreatments. Biochemical conversion processes have the objective of hemicellulose and cellulose polymer fractionation into sugar molecules (Nitsos et al., 2013). For the production of bioethanol from cellulosic feedstock, four major steps are required: pretreatment, hydrolysis, fermentation, and distillation of the product mixture to separate ethanol, as presented in Figure 2.4 (Braide et al., 2016).

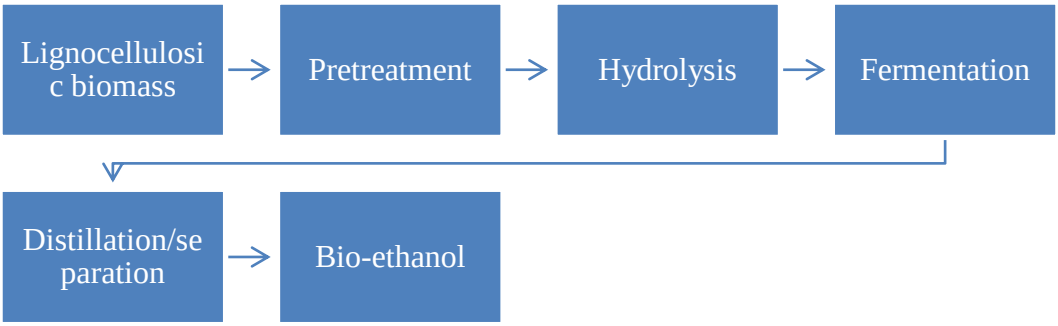

Figure 2.4 general steps during bioethanol production from lignocellulosic biomass

2.7.1. Pretreatment

Pretreatment is One of the most common and important steps in the biochemical conversion of biomass into ethanol is pretreatment technology. However, LCBs are resistant to chemical and biological breakdown, known as biomass recalcitrance The recalcitrance biomass must be overcome for effective utilization of lignocellulosic feedstocks, is caused by several factors, including the crystalline structure of cellulose, the degree of delignification, and the structural heterogeneity and complexity of cell-wall components (Baruah et al., 2018). The purpose of the pretreatment technology is to loosen the strong bond held between the cellulose fibers. The pretreatment induces the breaking of the bonds that bind with lignin, cellulose, and hemicelluloses. A pretreatment process is necessary to achieve higher enzymatic degradation in the production of ethanol from LCB.

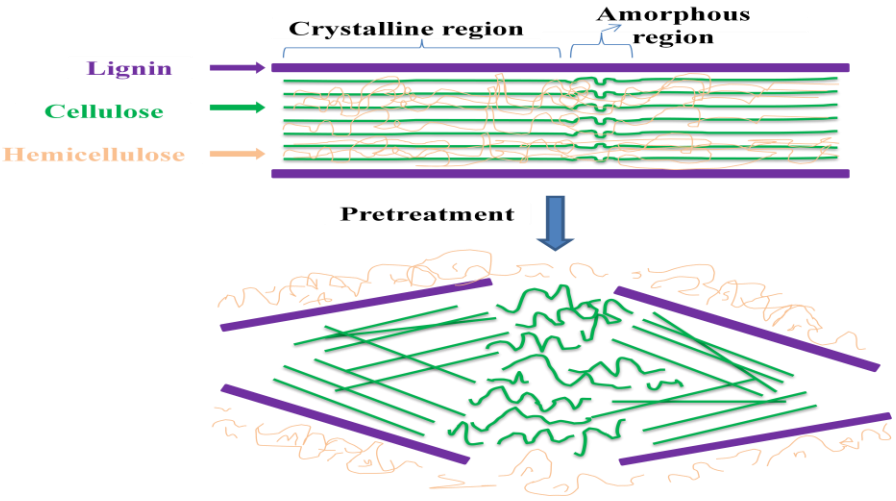

Figure 2.5 Schematic of pretreatment to disrupt the physical structure of biomass

Pretreatment is important because it allows enzymes to attack, removing the formation of inhibitory compounds; hemicellulose and cellulose recovery; size reduction; and reducing the cost of materials for the construction of fermentation reactors. It also increases the size of the pore (Y. D. Singh & Satapathy, 2018). Several pretreatment methods are identified in the laboratory or on an industrial scale for the conversion of lignocellulosic biomass into bioethanol. However, different researchers are still working on searching for effective pretreatment methods that improve the cellulose yield as well as increase the total sugar recovery for subsequent improved bioethanol production. These pretreatment methods can broadly be divided into four different categories, such as physical pretreatment, chemical pretreatment, physico-chemical pretreatment, and biological pretreatment as presented in Figure 2.6 (A. K. Kumar & Sharma, 2017).

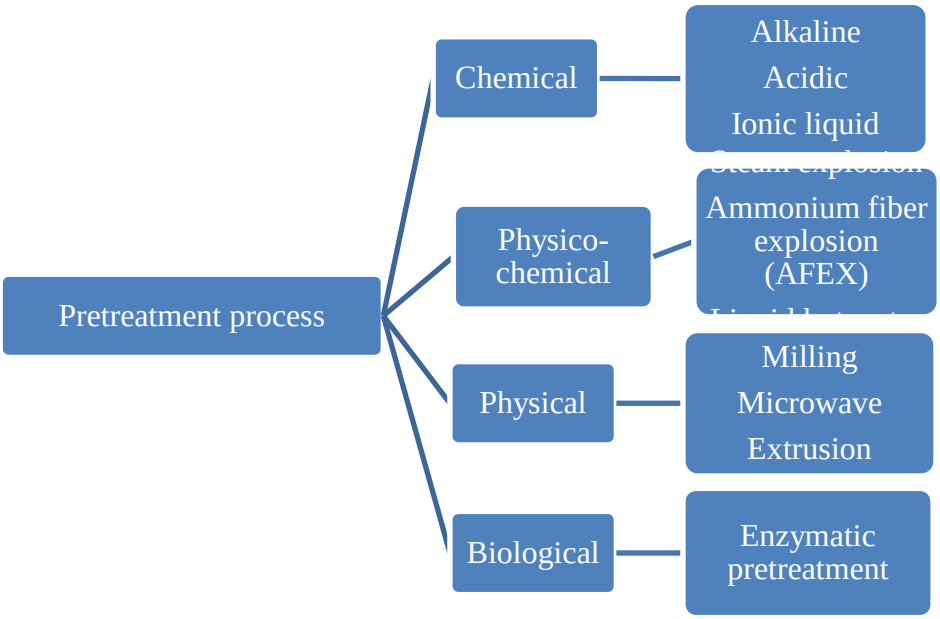

Figure 2.6 Lignocellulosic biomass pretreatment techniques

2.7.1.1. Physical pretreatment

Physical pretreatments are primarily carried out to reduce the particle size, which results in an increase in the surface area of lignocellulosic materials to enzymes by reducing their particle size or disrupting their structural regularity and decreasing the degree of polymerization and crystallinity. Physical pretreatment is the process of applying mechanical force, such as mechanical chipping, grinding, or milling, and irradiation (gamma rays, electron beams, or microwaves), to improve the digestibility of lignocellulosic materials (S. Sun et al., 2016).

A. Milling

Milling is important to reduce the crystallinity and particle size of lignocellulosic biomass. There are several types of milling pretreatment methods used in bioethanol production. Ball milling, two-roll milling, hammer milling, colloid milling, and disk milling are commonly used pretreatment methods. This method reduces the size of the biomass by applying physical forces. The size of the particle obtained from milling pretreatment depends on the methods enforced. However, 10–30 mm and 0.2-2 mm of particle size have been achieved in chipping (Y. D. Singh & Satapathy, 2018). For milling pretreatment, factors like capital costs, operating costs, scale-up possibilities, and depreciation of equipment are very important. High energy consumption is the main drawback of milling. To overcome this, wet disk milling has been introduced as a more acceptable mechanical pretreatment in terms of energy consumption. However, the yields of

glucose and xylose after enzymatic hydrolysis of some pretreated biomass by wet disk milling are lower than using ball milling (Baruah et al., 2018).

B. Microwave-assisted pretreatment

Microwave heating has been found to be a better alternative to conventional heating methods. Because of its rapid heating and simple operation, it has been extensively used in various fields. Microwave-assisted pretreatment utilizes both thermal and non-thermal effects generated by an extensive intermolecular collision because of the realignment of polar molecules, such as water, with microwave oscillations. Recent studies on microwave-assisted pretreatment of different substrates include wheat straw, corn stover, rice straw, soy hull, and rice hull. Although the operating factors, such as microwave power, exposure time, and solid loading, were varied, the general perception is that microwave heating can disrupt the matrix structure of lignocellulose and enhance the release of reducing sugar (Ethaib et al., 2017). Microwave irradiation alters the ultrastructure of cellulose, partially removes lignin and hemicelluloses, and disrupts the silicified waxy surface (H. Ma et al., 2009). The advantages of this method include a short process time, high uniformity and selectivity, and less energy input than conventional heating (Cheng et al., 2011).

Microwave irradiation alters the ultrastructure of cellulose, partially removes lignin and hemicelluloses, and disrupts the silicified waxy surface (H. Ma et al., 2009). Short process times, good uniformity and selectivity, and less energy input are the advantages of this method compared to conventional heating. Microwave-assisted pretreatment is more energy efficient as well as faster than conventional pretreatment due to its unique heating mechanism, which causes a direct connection between the polar part of the biomass and the electromagnetic field. The results of this work present promising potential for using microwaves to assist biomass thermo-chemical pretreatment (Z. Zhu et al., 2015).

2.7.1.2. Chemical Pretreatment

This method is based on the utilization of chemicals for pretreatment. During these pre-treatments, different chemical reagents such as acids, bases, oxidizing agents, ionic liquids, organic solvents, and ozone changes are used. These can make the biomass more suitable for enzymatic saccharification (hydrolysis). The chemical agents break the hemicellulose linkages

under mild temperature conditions, increasing biomass porosity. Some chemicals are used for the pretreatment of LCB and are explained below.

A. Alkaline pretreatment

Alkali pretreatment is a chemical pretreatment technique that has received a lot of attention because it relies on the solubilization of lignin in the alkali solution. Sodium, potassium, calcium, and ammonium hydroxides are among the numerous alkaline reagents frequently employed for alkali pretreatment. Sodium hydroxide was discovered to be the most efficient of these (Kim et al., 2016). When an alkali is used, the ester and glycosidic side chains degrade, changing the structure of the lignin and causing swelling and partial crystallization of the cellulose. Due to the degradation of the heterogeneous matrix, a suitable condition was created for enzymatic hydrolysis and the fermentation process. Most of the time, alkali pretreatment occurs at low temperatures, but it can be performed with an approximately long duration and a high concentration of the base. Furthermore, these methods work by improving the accessible surface area, reducing cellulose crystallinity, disordering lignin, removing the acetyl group, and cleaving uronic acid from the polysaccharides. Sodium hydroxide is the most commonly used alkali for a wide range of biomass preprocessing during the generation of bioethanol.

Ammonia-based treatments have received the greatest attention among alkaline pretreatments for a variety of reasons. It is easily recoverable, non-corrosive, and non-toxic. It is inexpensive to the extent that the majority (80%) of ammonia is produced and is currently used for the production of fertilizer. The main effects of ammonia pretreatment methods include the removal of lignin, an increase in surface area or pore size, and the modification of cellulose and hemicellulose structures. When ammonia is present in aqueous form, it causes the biomass to swell, significantly altering its morphology. In comparison to other alkaline pretreatment techniques, it also affects the degree of crystallinity of the integral biomass, if not the core of cellulose fibers. Ammonia pretreatment opens up the structure of the biomass, making it amenable to enzymatic hydrolysis (Kim et al., 2016). In the study (Carvalho et al., 2016) alkaline pretreatment of sugarcane bagasse using NaOH 15% at 175 ^{o}C; 1.5 h, a glucose yield of 5.29 g/l, and 8.8 g/L bioethanol yield were achieved in the study. and also in another study, increased cellulose concentration up to 56.65% w/w due to improvement of cellulose crystallinity with low hemicellulose solubilization of 10.7–33.1% (w/w) is obtained by using (2% w/w) sodium

hydroxide (Shahabazuddin et al., 2018). The vital advantage of alkaline pretreatment is its ability to remove lignin and acetyl groups and different uronic acid substitutions that inhibit the saccharification of cellulose so that its accessibility as well as its partial decrystallization can be efficiently increased. But as compared to acid or hydrothermal processes, the solubilization of hemicelluloses and cellulose in this method is less (X. Li et al., 2010). More specifically, delignification results from disruption of the ester bond cross-linking lignin and xylan. Comparatively, there is no requirement for complex reactors for alkaline pretreatment, and it is also operated at a lower temperature (Balat, 2011), which makes it easy to employ. However, it has drawbacks such as a long residence time (from hours to days) and the requirement of neutralization of the pretreated slurry (Wan et al., 2011).

B. Acidic Pretreatment

Acid pretreatment is a well-known method for producing bioethanol from LCBs. Pretreating the LCB with acid causes more hemicellulose to be broken down. The acid solubilizes mainly hemicellulose, improves biomass porosity, and renders cellulose more accessible to enzyme attack. The temperature range for dilute acid pretreatment is usually 120–200 °C, depending on the pretreatment severity. Acid pretreatment can be performed either under dilute acid at a high temperature or under concentrated acid at a lower temperature (Wu et al., 2020). Acid pretreatment is a highly effective chemical technique used to disrupt the lignocellulosic matrix with the aid of the cleavage of glucosidic bonds. In this process, different organic acids, such as acetic, formic, and oxalic acids, as well as inorganic acids such as sulfuric acid, hydrochloric acid, maleic acid, nitric acid, nitrous acid, and phosphoric acid, are used for biomass degradation (X. Bai et al., 2016). However, sulfuric acid and hydrochloric acid are the two acids that are most frequently utilized. The advantages of the acid pretreatment method over other methods could be attributed to the disruption of the matrix structure of the lignocellulosic biomass and amorphous cellulose conversion. However, this method has some disadvantages, such as the expensive cost of acid recovery and the formation of inhibitors such as furfural and 5-hydroxymethyl furfural. Therefore, it is worth discerning that the economic and environmental features of acid pretreatment have improved over time. Pretreatment generally aims to maximize product yields while minimizing costs during subsequent enzymatic hydrolysis and fermentation processes. Recently, it has been demonstrated that dilute acid hydrolysis can achieve high reaction rates in a

short time and significantly improve cellulose hydrolysis (Chaturvedi & Verma, 2013). According to (Chiesa & Gnansounou, 2014) 85.5% glucose yield was achieved by using dilute acid pretreatment of empty fruit bunches from oil palm tree at 1.51% H_2SO_4, 161.5°C and 9.44min. And also in another study 89.5% hemicellulose solubilization and 86.11% by using diluted sulfuric acid pretreatment of sugarcane bagasse at 0.5% (v/v), 140°C and 15min and e pentose-fermenting yeast Spathaspora passalidarum NRRL Y-27907 respectively (Dionísio et al., 2021).

2.7.1.3. Physico-chemical pretreatment

Physico-chemical pretreatment methods combine both chemical and physical processes to enable ethanol production. Steam explosion, catalyzed (SO2 or CO2) steam explosion, ammonia fiber explosion (AFEX), liquid hot water, and microwave-chemical pretreatment are some of the most significant procedures in this area.

A. Steam explosion pretreatment

Steam explosion is a thermo-physical-chemical process that provides a mechanical deconstruction of LCBs by combining vapor cracking and explosive decompression steps. This pretreatment method allows the opening of the biomass fibers and makes the biomass polymers easily accessible by enzymes in hydrolysis, fermentation, or densification processes. Steam explosion is one of the most attractive pretreatment processes due to its minimum chemical and energy requirements as well as its effective biomass disruption characteristics. Compared with the processes using mechanical size reduction prior to chemical pretreatment with large liquid-to-wood solid ratios, steam explosion is relatively energy efficient (J. Y. Zhu & Pan, 2010). However, incomplete lignin removal and the production of some toxic chemicals during the process are the disadvantages of this method (Tabka et al., 2006)

B. Ammonia Fiber Explosion (AFEX) Pretreatment

Ammonia fiber explosion is a physice-chemical pretreatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. The AFEX method and steam explosion are highly comparable. In the AFEX pretreatment process, biomass is heated to the desired temperature before coming into contact with anhydrous liquid ammonia under pressure. The main advantages

of the AFEX treatment include its dry-to-dry nature, which eliminates the need for a washing step, the low production of inhibitory compounds, the lack of a detoxification step, the retention of a high cellulose and hemicellulose content, and the requirement for a moderate temperature and short residence time. The disadvantages of this method are the high cost of ammonia, the requirement of a highly controlled environment, and the need for sophisticated equipment requiring higher capital and utility cost (B. Kumar et al., 2020).

2.7.2. Hydrolysis

The manufacture of bioethanol requires a hydrolysis step that aims to depolymerize cellulose and/or hemicelluloses into simple fermentable sugars. Because of its crystalline structure, cellulose hydrolysis is more difficult than that of hemicellulose. Therefore, the hydrolysis step can be performed in different ways: either by an acid, which is called chemical hydrolysis or by specific enzymes, which is then called enzymatic hydrolysis (also called biochemical hydrolysis or saccharification) (Modenbach, 2013)

$$\text{Cellulose} \xrightarrow{\text{Hydrolysis}} \text{Glucose} \qquad (2.1)$$

$$\text{Hemicellulose} \xrightarrow{\text{Hydrolyses}} \text{Pentose and Hexoses} \qquad (2.2)$$

2.7.2.1. Chemical hydrolysis

These methods are performed based on revealing lignocellulosic materials to a chemical for a certain period of time at a specific temperature and resulting in monomeric sugar from cellulose and hemicellulose. Acid hydrolysis is perhaps now the most technologically matured chemical method of sugar release from biomass. Sulfuric acid and hydrochloric acid are the two most commonly used acids for hydrolysis. Acid hydrolysis can be of two types: dilute acid hydrolysis and concentrated acid hydrolysis. Depending on the concentration of the acid and the other parameters can be used: dilute acid maybe used at high temperature and pressure while concentrated acids maybe used at very low temperature and pressure (Karim et al., 2014). For example, in the case when sulfuric acid can be used as concentrated (25-80%) or dilute (3-8%), measured as the weight of acid in the weight of acidified aqueous solution that is present with the feedstock (Mushimiyimana & Tallapragada, 2016).

Table 2.3 current studies of biomass pretreatment for cellulose recovery with hemicellulose as well as lignin removal during bioethanol production

Raw material	Pretreatment type	Pretreatment condition	Recovered cellulose%	Hemicellulse removal (%)	Delignification rate (%)	Bioethanol yield (g/L)	Reference
Sugarcane bagasse	NaOH 5% (w/w)	175 ^{o}C; 1.5 h	11	45	90	8.8	(Carvalho et al., 2016)
Sugarcane bagasse	NaOH 2w%	121 ^{o}C; 30 min	46.6	-	86.6	-	(Wunna, 2021)
Wheat straw	Microwave-assisted NaOH (1.5% NaOH)	At 160 ^{o}C;15 min	74.15%	38.34%	69.49%	68.2%	(Tsegaye et al., 2019)
Sweet sorghum bagasse	microwave-assisted ammonia 28% v/v NH3	at 160 ^{o}C.;1 h	90%	35%	48%	(2.1 g ethanol/1g dry biomass	(Chen et al., 2012)

Corncob	H2SO4-NH3·H2O	(2 wt% H2SO4+15 wt% NH3·H2O 1:6), 121°C , 1h	78.53	83.46	85.4	17.9 g/l	(Xie et al., 2014)
Corncob	2%(w/w)H_2O_2 with 5M NaOH	50 °C, 6h	81.3	38.7	75.4	-	(Su et al., 2015)

2.7.2.2. Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose and hemicelluloses can be performed under mild conditions, such as temperature from 40 to 50 ^{o}C and pH around 4.5 and 5. The pretreatment of lignocellulosic biomass plays a crucial part in the effectiveness of hydrolysis. This pretreatment procedure also involves enzyme loading, removal of lignin, increasing hemicellulose solubility, and hydrolysis time (Sources & Vasi, 2021).

The enzymatic hydrolysis of complex polymer structure is the biochemical transformation of cellulose and hemicelluloses into simple sugars with the aid of enzymes secreted by microorganisms (bacteria and fungi). Cellulases break down cellulose during enzymatic hydrolysis to create fermentable reducing sugars. During enzymatic hydrolysis, some of the highly specific enzymes promote 100% transformation of cellulose into glucose without the formation of undesirable products (inhibitors), which is what makes this process very attractive compared to chemical hydrolysis (Abo et al., 2019).

Table 2.4 Acid hydrolysis of lignocellulosic biomass at different conditions to release sugar

Raw material	Acid type	Hydrolysis condition	Reducing sugars	Reference
Wheat straw	H_2SO_4	2%, 180^{o}C, 10 min	43 mg/mL	(Radhakrishnan, 2018)
pineapple leaves	H_2SO_4	12%, 100^{o}C,20min	(1.62±0.20g/L	(Ariffin et al., 2020)
Corncob	H_2SO_4	2%, 110^{o}C;5h	2.8g/L	(Fournier & Corre, 2017)

2.7.3. Fermentation

The term fermentation denotes the enzyme-catalyzed energy-yielding pathway in cells involving the anaerobic breakdown of molecules such as glucose. After acidic or enzymatic hydrolysis of lignocellulosic biomass, the hydrolysate is obtained, and then it is subjected to fermentation by microorganisms such as yeast to produce bioethanol. The fermentation of ethanol is the biological process that transforms fermentable sugars like glucose and xylose into cellular energy

by using microorganisms that produce waste byproducts such as ethanol and carbon dioxide anaerobically through the metabolic pathways of carbohydrates. Microorganisms should be needed to effectively ferment these sugars because this lignocellulose hydrolysate contains not just glucose but also a number of monosaccharides, including xylose, mannose, galactose, arabinose, and oligosaccharides.

Saccharomyces cerevisiae is the most widely used and studied species for the production of bioethanol because it is vigorous and suitable for the fermentation of glucose from the hydrolysis of lignocellulosic biomass. However, it is not able to ferment the pentose sugar that is released from the hydrolysis of the hemicelluloses, so the fermentation of pentose requires another microorganism (Environment, 2011). In general, the conversion of lignocellulosic material to sugar and then ethanol is governed by (equation 2.3) below:

$$(C_6H_{10}O_5)\,n + nH_2O \longrightarrow nC_6H_{12}O_6 + \text{yeast} \longrightarrow 2nC_2H_5OH + 2nCO_2 \qquad (2.3)$$

The first process is hydrolysis, and the second step is fermentation, according to the equation above. The overall reaction of this fermentation of hexose sugar (glucose) by yeast has been expressed by Gay-Lussac, which forms the basis of calculating fermentation efficiency as:

$$3C_5H_{10}O_5 \longrightarrow 5C_2H_5OH + 5CO_2 \qquad (2.4)$$

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 \qquad (2.5)$$

Saccharomyces cerevisiae is the most preferred organism for ethanol synthesis from hexoses. Both hexose (glucose) and pentose (xylose) carbohydrates can be fermented by P. stipitis and Candidashehatae to produce ethanol(Joshi et al., 2011). Bacteria, yeast, and filamentous fungi are all groups of microorganisms that can ferment xylose. Currently, xylose fermenting bacteria include both native and genetically engineered organisms, and many have characteristics useful for simultaneous saccharification and fermentation (Kefale et al., 2012).

Due to its high bioethanol synthesis from hexoses and high tolerance to bioethanol and other inhibitory chemicals in the acid hydrolysates of lignocellulosic biomass, one of the most efficient bioethanol-producing yeasts, S. cerevisiae, has a number of advantages. However, because this yeast's wild-type strains are unable to utilize pentoses like xylose and arabinose, the amount of bioethanol that can be produced from a lignocellulose hydrolysate is insufficient. For xylose-

using S.cerevisiae, high bioethanol yields from xylose also require metabolic engineering strategies to enhance the xylose (Kefale et al., 2012).

2.7.4. Distillation

Distillation is one of the purification processes. The process of distillation is used to separate two liquids according to their different boiling points. However, several distillations are necessary in order to achieve great purity. This is because, during distillation, two components will co-distill because all materials have intermolecular interactions with one another. In other words, the ratio of two materials, in this case ethanol and water, can be adjusted while maintaining the existence of two materials in layers, namely the liquid and vapor layers.

Various research papers have been published on the areas of the production of bioethanol from lignocellulose biomass, such as those by Araujo et al., (2019), (Sajad et al., 2019) and Nath et al., (2021). We observe that one pretreatment method is not suitable for all types of lignocellulose biomass. Most of them are did not optimize the process parameters during the pretreatment these can reduce the yield of cellulose recovery as well as the respective hemicellulose removal. Since none of these studies were able to provide the maximum cellulose recovery as well hemicellulose recovery from the pretreatment steps. For example, the main disadvantage of alkaline pretreatment is less cellulose recovery and the long operation time (from hours to days). According to Pryor et al. (2012) at a condition of 16.75 w/v solid loading, 60 °C temperature, and 8 hr reaction time, using aqueous ammonia solution, pretreatment of switchgrass resulted in 65.6% cellulose recovery, 67.7% hemicellulose removal, and 46.95 lignin removal. This method shows less recovery of cellulose and a long residence time. And also in other studies, the optimized sequential pretreatment of sugarcane bagasse was achieved at 10% (w/v) of raw sugarcane bagasse loading at 1% (w/v) NaOH (50 °C, 2 h), followed by treatment with organosolv (85%, v/v phosphoric acid, 50 °C, 1 h) with chilled acetone. This sequentially pretreated sugarcane bagasse showed delignification of 83.2% (w/w) and cellulose recovery of 66.1% (w/w) (Nath et al., 2021). As a result, in order to reduce the aforementioned gap in the literature a new approach, which is integrated microwave-assisted binary alkaline pretreatment of corncob with appropriate binary alkaline ratios was used in this study. This method allowed for the efficient recovery of cellulose as well as the efficient release of fermentable sugars, which increased the overall production of bioethanol.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Materials

The major equipment and chemicals used in this study are presented below

Table 3.1 Major equipment used

Equipment and materials	Purpose
• Plastic bag • Mortar and Ball mill • Sieve analyzer • Oven • Weighing balance	Raw material collection and sample preparation
• Microwave oven • Weighing balance • Desiccator • Centrifuge & Centrifuge tubes • Refrigerator and water bath • Muffle furnace and autoclave • Vacuum Filter and Digital pH meter • Flasks and Measuring cylinders • Petri dish • Cotton v Aluminum foil • Glove • Filter paper v Spatula	Proximate analysis, pretreatment and characterization sample for cellulose recovery as well as hemicellulose and lignin removal.

• Autoclave • Hot-air Oven • Measuring cylinder • Flasks • Micropipettes	Hydrolysis of cellulose to glucose
• Autoclave • Shaking incubator • Water bath • Flasks • Cotton and Aluminum foil • Glove • Filter paper and Spatula	Fermentation and determination of bioethanol
• Fourier transform infrared spectroscopy (FTIR) • X-ray diffraction (XRD) • Scanning electron microscopy (SEM)	Characterization of raw corncob, pretreated corncob, corncob cellulose and produced bioethanol
• UV-spectrophotometer (UVD-3200)	Determination of glucose and bioethanol concentration during fermentation

Table 3.2 Chemicals used

Chemical used	Purpose
• Toluene (99%) • Ethanol (98%)	Extraction
• Sodium hydroxide • Ammonium hydroxide (25%) • Sulfuric acid (H_2SO_4) (98%) • Acetic acid (99.8%) • Distilled water • Nitric acid	Pretreatment for cellulose recovery as well as hemicellulose and lignin removal, hydrolysis, determination of sugar concentration, pH adjustment and determination of bioethanol
• Phenol solution • Glucose ($C_6H_{12}O_6$)	Determination of sugar concentration
• Dextrose sugar, • Yeast extract, • Urea, $MgSO_4.7H_2O$, • Yeast extract agar, • Yeast (*Saccharomyces cerevisiae*), • Potassium dichromate.	Media preparation, fermentation, and determination of bioethanol

3.2. Methods

3.2.1. Sample collection and preparation

The raw corncob was collected from Melkasa Agricultural Research Center (MARC) and it was packed in plastic bags, and transported to the laboratory. Firstly, it was washed with deionized water to remove unwanted particles, like dust and aerosol parts. The corncob was cut with a knife into pieces of about 3-5 cm in length and oven-dried at 50 ℃ for 24 hours. The size-reduced corncob was ground further using a milling machine, and the size was passed through a 60-mesh screen (Jiang & Hsieh, 2015). Finally, the obtained powder, which has less than 250µm was transferred and packed in a polyethylene bag.

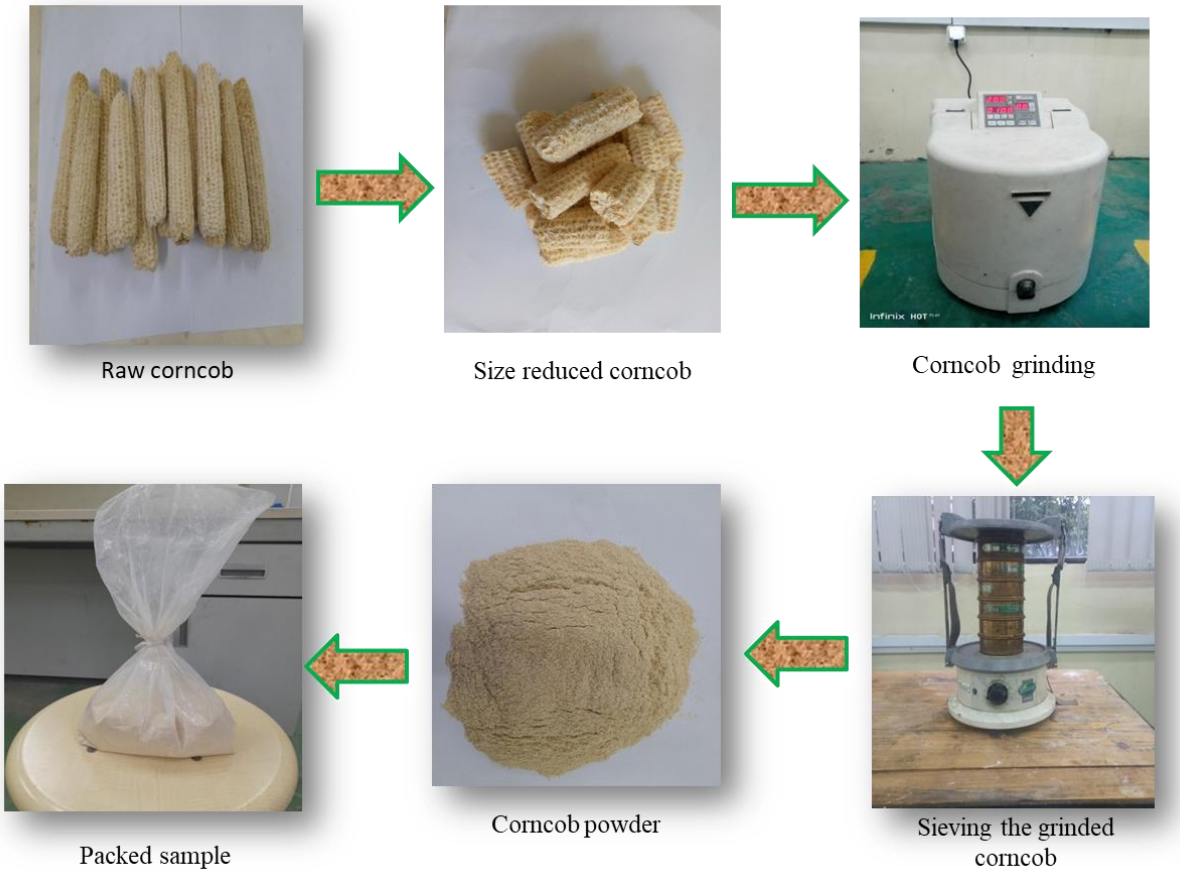

Figure 3.1 Sample collection and preparation of corncob powder

3.2.2. Proximate analysis of corncob

The proximate analysis such as moisture content, fixed carbon content, ash content, and volatile matter content was characterized based on the procedure of the National Renewable Energy Laboratory (NREL), United States of America (Ayeni et al., 2015).

Moisture Content

To determine the moisture content, two grams of the corncob were weighed in a clean, preheated crucible of known weight by using a digital balance. The sample and crucible were kept in an oven at 105 °C for three hours. The crucible was covered, transferred to desiccators, and weighed after reaching room temperature. The crucible was heated in the oven for another hour at the same temperature and reweighed. It was repeated until a constant weight was obtained. The percent moisture content was determined using the following formula:

$$\text{Moisture content } (\%) = \frac{W_1 - W_2}{W_1} * 100\% \qquad (3.1)$$

Where, W_1 = Initial weight; W_2 = Weight after oven-dried

Ash content

The empty crucible was weighed, and then two grams of oven-dried sample was placed on it. The sample in the crucible was placed in a muffle furnace and was heated for 2 hours at 550 ℃. The crucible was taken from the furnace and was placed in a desiccator to cool the sample and weighed. The percent ash content was determined using the following formula:

$$\text{Ash content } (\%) = \frac{W_2}{W_1} * 100\% \qquad (3.2)$$

Where, W_1 = Original sample weight; W_2 = Weight of ash was burned on the furnace

Volatile matter

The empty crucible was weighed, and then oven-dried samples were put in it. The sample and crucible were placed in a muffle furnace for 30 min at 600 ℃. The crucible was removed from the furnace and placed in a desiccator for cooling, then reweighed. The same procedure was repeated until a constant weight was obtained. The volatile content of the sample was calculated as follows:

$$\text{Volatile content } (\%) = \frac{W_1 - W_2}{W_1} * 100\% \qquad (3.3)$$

Where, W_1 = Original weight of oven-dried sample; W_2 = Weight after heating in the furnace

Fixed Carbon Content

This is the residue left after the moisture, volatile, and ash content was given up from the sample. It was deduced by subtracting from 100, the percentage of moisture, volatile matter, and ash content. The fixed carbon content was estimated using the formula,

$$\text{Fixed Carbon} = 100 - (\%\ \text{moisture} + \%\ \text{volatile matter} + \%\ \text{ash}) \quad (3.4)$$

3.2.3. Chemical composition of corncob

The chemical composition of the corncob such as Extractive, Cellulose, Hemicellulose and Lignin Content was analyzed using the standard gravimetric method under the conditions (Ayeni et al., 2015).

3.2.3.1. Extractive determination

5 grams of the oven-dried raw sample were placed into the thimble. 300 mL of ethanol and toluene mixture was measured in a 500 mL conical flask as solvent (ratio, 2:1), and the Soxhlet extractor was set up with the boiling flask positioned on the heating mantle set at 100 °C for 4 hours. After extraction, the sample was air dried at room temperature for a few minutes, and it was placed in an oven at 105 °C until the moisture content was completely removed. The percentage of extractive content was evaluated as the difference in weight between the raw extractive-laden biomass and extractive-free biomass.

$$\text{Extractive content}\ \% = \frac{W_1 - W_2}{W_1} * 100 \quad (3.5)$$

Where, W_1 = weight of dry biomass before extraction in grams, W_2 = weight of dry biomass after extraction in grams

3.2.3.2. Cellulose determination

First 1.5 grams of extractive-free corncob biomass were added to a 250 ml volumetric flask. After that, acetic-nitric acid reagent (30 ml of a mixture containing 150 ml of 80% acetic acid and 15 ml of concentrated nitric acid) was added into a 250 ml volumetric flask and perfectly mixed. Then it was tightly capped and heated in a water bath at 100 °C for 30 min (Bauer & Ibáñez, 2014) After that, 100 ml distilled water was added to cool and dilute the sample. The diluted sample was centrifuged at 5000 rpm for 20 min and the supernatant was discarded.

Finally, the residual was washed with distilled water until pH was seven and the obtained residual was oven-dried at 150 °C for 3hr. Cellulose quantity was determined according to the method of (Vallejo et al., 2021). The cellulose content was calculated as

$$\text{Cellulose content } \% = \frac{W_2}{W_1} * 100 \qquad (3.6)$$

Where, W_1 = Weight of dry biomass before treatment; W_2 = Weight of dry biomass after treatment

3.2.3.3. Hemicellulose Content

To determine the hemicellulose content, 1 g of extractive-free biomass was transferred into a 250 mL conical flask, and then 150 mL of a 0.5 mole/L NaOH solution was added. The mixture was boiled at 100 °C for 3.5 hours with distilled water to increase the heating effect. After boiling, the mixture was cooled, filtered using vacuum filtration, and washed until a neutral pH was achieved. The residue was dried at 105 °C in a convection oven and cooled in a desiccator. The difference between the sample weight before and after this process is the hemicellulose content, which was calculated as follows:

$$\text{Hemicellulose content}, \% = \frac{W_1 - W_2}{W_1} * 100 \qquad (3.7)$$

Where, W_1 = Weight of dry biomass before treatment; W_2 = Weight of dry biomass after treatment

3.2.3.4. Lignin determination

0.3 g of dried extractive-free biomass was weighed in glass test tubes. 3 mL of 72% H_2SO_4 was added, and the sample was kept at room temperature for 2 hours by carefully shaking at 30 min intervals to allow for complete hydrolysis. After the initial hydrolysis, 84 ml of distilled water was added to the sample to dilute it to 4% and put in an autoclave for 1 hour at 121°C. The slurry was cooled at room temperature. The hydrolysates were filtered through a vacuum filter using a filtering crucible. The acid-insoluble lignin was then determined by drying the residues at 105 °C and accounting for ash by incinerating the hydrolyzed samples at 575 °C in a muffle furnace. The lignin quantity was determined using Equation (3.8) (Song et al., 2019).

$$\text{Lignin content } \% = \frac{W_2}{W_1} * 100 \qquad (3.8)$$

Where, W_1 = Weight of the initial sample mass; W_2 = Weight of obtained lignin mass after pretreatment

3.3. Pretreatment of sample

3.3.1. Selection of significant parameters

For efficient cellulose recovery as well as maximum hemicellulose and lignin removal, the integrated microwave-assisted binary alkaline (NaOH and NH_4OH) method was selected for the pretreatment of the corncob biomass. A microwave reactor (household microwave oven) was used to treat the corncob biomass. For the selection of an appropriate binary alkaline ratio, different experiments with various binary alkaline ratios (1:1), (1:2), (1:3), (2:1), and (3:1) were done, and the other parameter was constant, such as (1% v/v) alkaline concentration, (0.1 g/mL) biomass loading, 240W power, and 4 min operating time for effective cellulose recovery with maximum hemicellulose as well as lignin removal. In addition to this, for the selection of appropriate power, four powers were used (80W, 240W, 400W, and 640W), and the other parameter was constant, such as (1% v/v) alkaline concentration (2:1 ratio), (0.1 g/mL) solid loading ratio, and 4 min operating time for effective cellulose recovery. However, parameters were selected based on (Sewsynker-Sukai & Gueguim Kana, 2018; Tsegaye et al., 2019) and the preliminary experiments. And also, compare the single alkaline (NaOH) and (NH_4OH) with combined alkaline (NaOH and NH_4OH) using a microwave reactor based on their cellulose recovery and hemicellulose removal.

3.3.2. Optimization of Microwave-assisted binary alkaline (NaOH + NH4OH) pretreatment

An important statistical and mathematical method for optimization is Response Surface Methodology (RSM). The microwave-assisted binary alkaline (NaOH + NH_4OH) method was used to pretreat the corncob biomass in order to maximize cellulose recovery and removal of hemicellulose and lignin while minimizing sugar loss. The pretreatment was affected by three operating variables: alkaline concentration, solid loading ratio, and microwave pretreatment time. To achieve efficient cellulose recovery as well as maximum hemicellulose removal, the pretreatment method was adjusted between alkaline concentrations from 1% to 3% (v/v), time (5–15 min), biomass loading (0.05-0.15 g/ml), and power (400 W). These parameter ranges were

chosen following extensive literature (Sewsynker-Sukai & Gueguim Kana, 2018; Tsegaye et al., 2019) and from the preliminary experiment. Design Expert software version 13 was used to design the experiments and analyze the results. The response variable was cellulose recovery. To maximize the cellulose recovery of corncob, a central composite design (CCD) was applied. Three process variables (microwave pretreatment time, solid loading ratio, and alkaline concentration) were studied at five levels, as shown in Table 3.3. The effects and impacts of each variable and level, as well as interactions between variables and levels, on the responses to cellulose recovery, were analyzed by analysis of variance (ANOVA). Then the cellulose recovery and hemicellulose reduction can be calculated as follows:

$$\text{Cellulose recovery (\%)} = \frac{C_1}{C_o} * 100 \quad (3.9)$$

Where C_o = Cellulose before pretreatment; C_1 = Cellulose after pretreatment

$$\text{Hemicellulose reduction (\%)} = \frac{H_o(g) - H_1(g)}{H_o(g)} * 100\% \quad (3.10)$$

Where H_o = Hemicellulose before pretreatment; H_1= hemicellulose after pretreatment

Table 3.3 Values for each variable of the central composite design (CCD) during microwave binary alkaline pretreatment of corncob

Parameters	Levels				
	-2	-1	0	1	2
Alkaline concentration (%, v/v)	0.32	1	2	3	3.7
Biomass loading ratio (g/mL)	0.02	0.05	0.1	0.15	0.18
Time (min)	1.6	5	10	15	18.4

3.3.3. Microwave-assisted binary alkaline (NaOH + NH4OH) pretreatment of corncob biomass

Corncob biomass was pretreated in a microwave reactor (household microwave oven). The pretreatment was carried out in a 500 ml glass three-necked round-bottom flask placed in the microwave oven and equipped with a reflux condenser set. During the pretreatment, the condenser kept the solution it was holding without losing steam. The substrate continued to vaporize and condense after 5 minutes of microwave heating, and the condensate was recycled into the pretreating flask. The experiments were conducted with biomass loadings ranging from 0.05 to 0.15 g/ml and alkaline concentrations ranging from 1% to 3% (v/v). The microwave power for all the experiments was set at 400W for heating times of between 5 and 15 min. These parameter ranges were chosen following extensive literature (Sewsynker-Sukai & Gueguim Kana, 2018; Tsegaye et al., 2019) and from the preliminary experiment. After each pretreatment, the slurry was filtered and the solid material was washed with deionized water to remove NaOH and NH_4OH remained after pretreatment. After that, it was dried at 105^oC for 24 h until its moisture content was less than 10% w/w, and the dried sample was milled to a size of 250 μm in the milling machine. The dried solid sample was retreated to analyze its cellulose content before hydrolysis. The diagrammatic representation of different stages of biomass pretreatment is illustrated in the figure below.

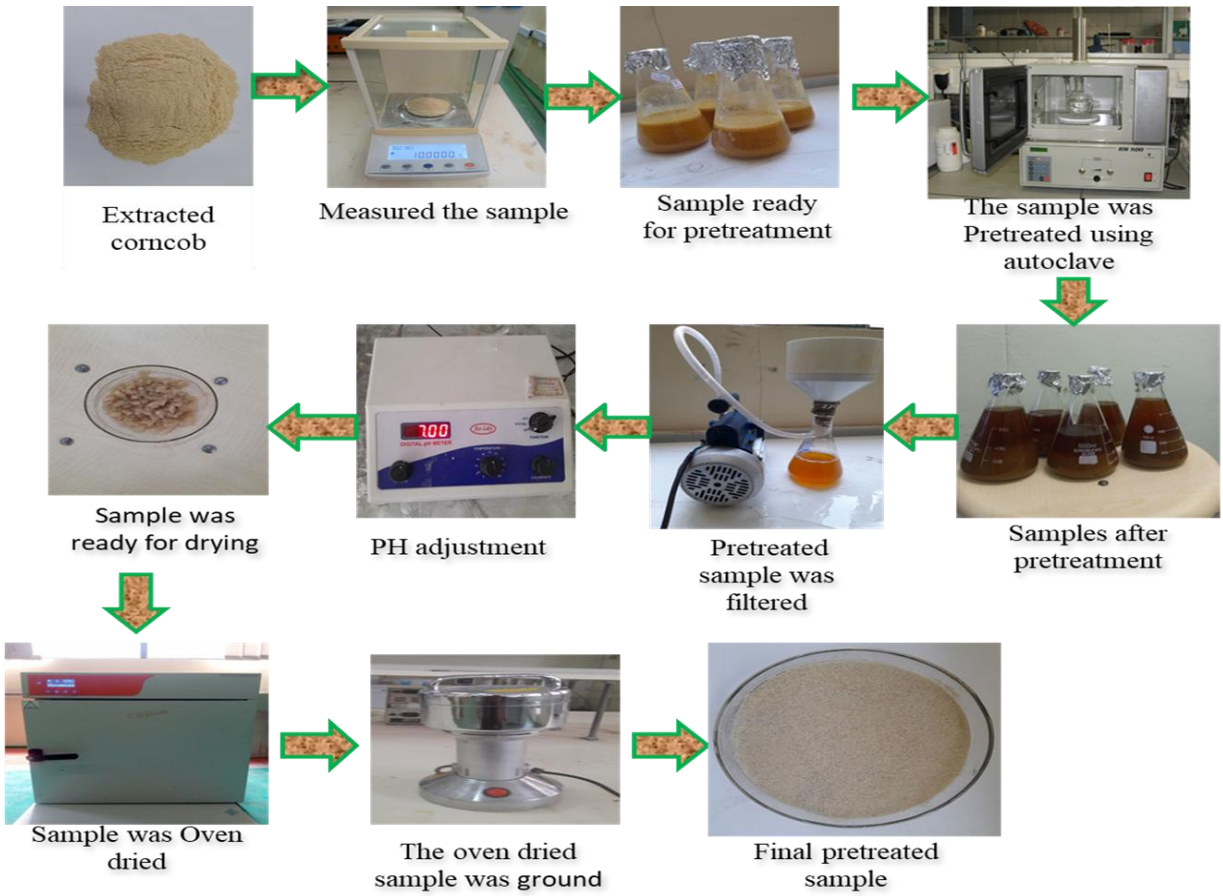

Figure 3.2 Diagrammatic representation of different stages of biomass pretreatment

3.4. Characterization of raw and pretreated biomass

3.4.1. X-ray diffraction (XRD) analysis

X-ray diffractogram analysis (XRD model Shimadzu XRD-7000) was used to determine the crystallinity of raw, microwave-assisted binary alkali-treated and corncob cellulose. The X-ray diffractograms of all materials were recorded at room temperature using Cu-K radiation with a wavelength of 1.54, a current of 30 mA, and an accelerating voltage of 40 kV. Diffraction intensities were scanned in the range of 2θ =10 to 40° with a scanning rate of 3^{o} min^{-1} in continuous scan mode and using a sampling pitch of 0.0200 (deg) and preset time of 0.40 (sec) (Binod, Satyanagalakshmi, Sindhu, Janu, et al., 2012). The crystallinity index (CrI) measurement showed a decrease in amorphous cellulose and an increase in crystalline cellulose. The ratio of the crystalline components of samples to their amorphous portions was indicated by the term

"crystallinity index" (CrI). The crystallinity of cellulose was determined according to the empirical method proposed by(Binod, Satyanagalakshmi, Sindhu, & Janu, 2012).

$$\mathrm{CrI}\,\frac{\mathrm{Fc}}{\mathrm{Fa}+\mathrm{Fc}} * 100 \qquad (3.11)$$

Where CrI is the crystalline index, Fc and Fa are the areas of crystalline and non-crystalline (amorphous) regions respectively.

3.4.2. Fourier transform infrared spectroscopy (FTIR) analysis

Fourier Transform Infrared spectroscopic with model name FTIR-6600 type A and serial number A013861790 analysis was conducted to detect changes in functional groups that may have been caused by the pretreatment. In this study, FTIR spectroscopy discusses the functional group of the change of raw corncob, microwave-assisted binary alkali-treated corncob and corncob cellulose was investigated. FTIR analysis was performed to investigate the removal of lignin by preserving cellulose during microwave-assisted alkali pretreatments. The ground and dried samples were pelletized with KBr and the spectra were obtained in the wavelength range 4000 – 500 cm^{-1} at a resolution of 4 cm^{-1}(Araújo et al., 2019).

3.5. Hydrolysis

Acid hydrolysis (dilute sulfuric acid) is most frequently employed in the hydrolysis step because it separates cell wall components with great efficiency. The long chains of simple sugar that make up the cellulose molecules were reduced to simple sugar before it was fermented to produce alcohol. After being sun-dried, the pretreated sample was weighed for hydrolysis. The ratio of pretreated sample to distilled water was 1:10 w/v. For maximum hydrolysis of cellulose to glucose (reducing sugar), the hydrolysis step was conducted in an autoclave at different acid concentrations (1, 2, 3, and 4%), different times (15, 30, 45, and 60 min), and different temperatures (100, 110, 120, and 130 °C) (Selvakumar et al., 2022). To investigate the effects of each hydrolysis parameter on the maximum conversion of cellulose to glucose (reducing sugar), hydrolysis was carried out by using one factor at a time. Next, vacuum filter the hydrolysate to separate the solid components from the liquid (and remove the non-fermentable lignin component). After separating the solid portion, wash the solid portion with distilled water and remove it. The filtrate portion was ready for subsequent procedures (Gebrekidan, 2019).

3.5.1. Determination of glucose concentration

The phenol sulfuric acid method was used to calculate the total amount of carbohydrates in each hydrolysate. The highest absorption occurs at 490 nm and results in a green-colored product when combined with phenol (Solms et al., 2020).

3.5.1.1. Preparation of reagents

To make a 5% phenol solution, 5 grams of phenol were weighed and mixed with 100 mL of distilled water, whereas 96% sulfuric acid was made by combining 97.96 mL of 98% sulfuric acid with 2.04 mL of distilled water.

3.5.1.2. Standard Preparation

The standard stock solution of glucose was prepared by combining 10 g of glucose with 100 mL of distilled water. The final concentration of the stock solution was then adjusted to 100mg/ml, and it was diluted to produce 6 standard solutions. Working standards were prepared by pipetting aliquots of the standard stock solution containing 0, 20, 40, 60, 80, and 100 mg/mL into distinct 100 ml volumetric flasks, and then diluting them with distilled water to a constant volume. Table 3.4 shows how the standard solutions were prepared.

Table 3.4 standard solutions preparation

Dilutions	Volume of solution (mL)	Volume of distilled water	Volume of stock that was taken (mL)	Concentration (mg/mL)
1	10	10	0	0
2	10	8	2	20
3	10	6	4	40
4	10	4	6	60
5	10	2	8	80
6	10	0	10	100

For the standard glucose preparation, a total of six tubes were prepared; one tube for the blank and five tubes for the glucose standard. 4 mL aliquot of each dilution was pipetted into a separate

test tube, and 1 mL of 5% phenol was then added and mixed with all the other tubes. Then 5 mL of sulfuric acid with a 96% concentration was added. Simultaneously, the tubes were shaked to effect fast and complete mixing. For color display, they were placed in a water bath set at 30 °C for 10 minutes. The identical procedure was used to make blank solutions, with the exception that distilled water was used in place of the 4ml standard solution The absorbance was determined using UV spectrophotometry at 490 nm. The prepared standard graph of sugar concentration and its absorbance was used to calculate the total quantity of reducing sugar present in our samples.

The total amount of reducing sugar found in our samples was determined as,

$$y = mx + b \tag{3.12}$$

Where; Y = is absorbance; m = is the slope; X = concentration and b is y-intercept

$$\text{Con. of unknown sample (X)} = \frac{\text{absornace of unknown sample} - (\text{y intercept})}{\text{slop}} * 100 \tag{3.13}$$

$$\text{Glucose yield \%} = \frac{\text{sugar produced}}{\text{raw material used}} * 100 \tag{3.14}$$

But, the raw material used can be calculated as,

$$\text{Raw material used} = \frac{\text{gram of sample used during hydrolysis}}{\text{milliliter of solution}} \tag{3.15}$$

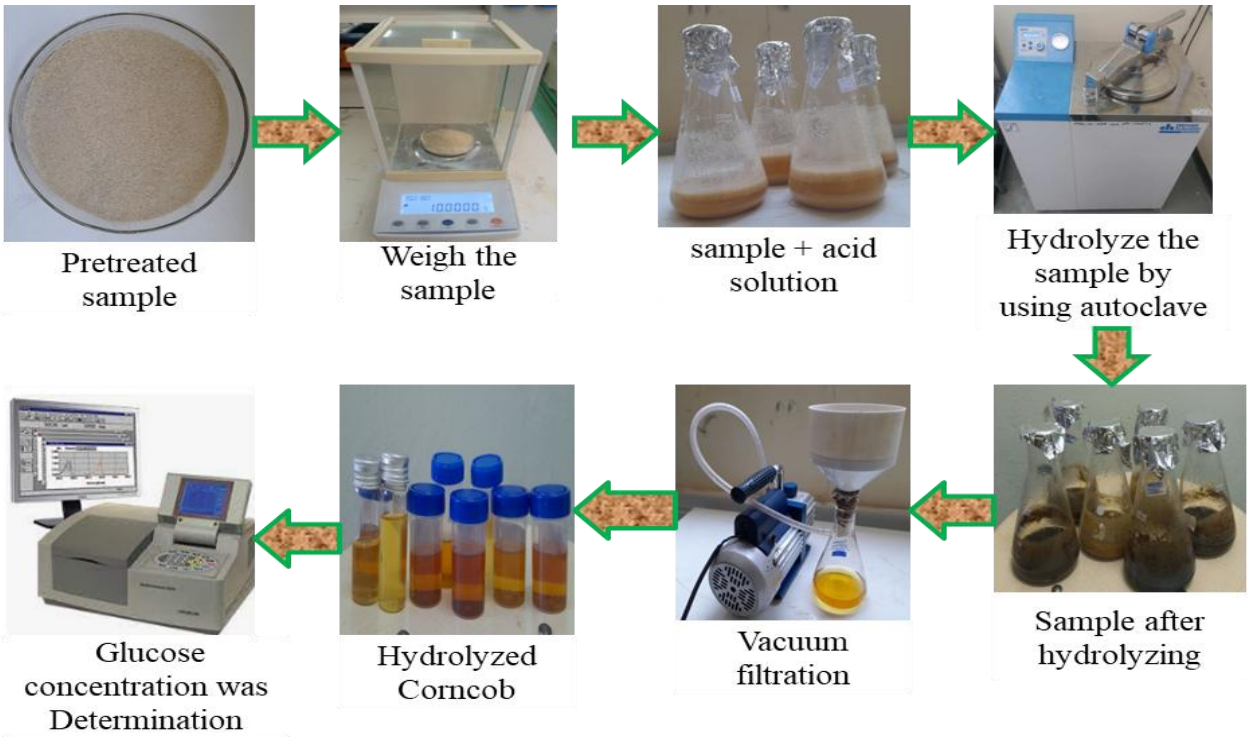

Figure 3.3 General experimental process for producing glucose from corncob cellulose

3.6. Fermentation

3.6.1. Preparation of the inoculum

For fermentation, the yeast strain S. cerevisiae was purchased from the Adama Regional Biotechnology Laboratory in Adama, Ethiopia. A 250 ml conical flask containing 100 mL of distilled water was used to generate the inoculum medium by adding glucose (20 g/L), peptone (5g/L), and yeast extract (3 g/L), the pH of the medium was adjusted to 7.0. It was then autoclaved for 15 minutes at 121 °C and 15 psi using an autoclave (Zhao et al., 2018). After sterilization, the yeast strain was transferred at room temperature from the stock culture to the inoculum medium. For fermentation purposes, the inoculum medium was incubated at 30 °C for 24 hours at 200 rpm in an orbital shaking incubator.

3.6.2. Fermentation media preparation

A 500 mL flask was used to prepare the fermentation media in 400 mL of distilled water. The media content included yeast extract agar (1 g) as a source of vitamins and minerals, corncob

hydrolysate (20 g) as a source of carbon, urea (4 g) as a source of nitrogen, and fast growth and MgSO4.7H2O (4 g) as a source of magnesium (Selvakumar et al., 2022). The inoculum was added to this media, and the pH was adjusted to 5.5. It was then sterilized for 15 minutes at 121 °C (Khalil et al., 2015)

3.6.3. Sample preparation

The materials used for fermentation were sterilized in an autoclave at 121 ℃ for 15 minutes before the fermentation step was started. Then the prepared media to sample were mixed in the 500 ml flasks with the proportion of 1:10 (prepared media to sample ratio) (Cai et al., 2012). Then, placed on a shaking incubator at a temperature of 30°C and 200 rpm for 96 hours and samples taken at times 12 h intervals, were centrifuged at 2000 rpm and analyzed for ethanol concentration (Mitiku & Hatsa, 2020).

3.7. Distillation

Distillation is the method used to separate two liquids based on their different boiling points. At a temperature of 78°C for two hours, the fermented product was distilled using a rotary evaporator (RE-2000B). The distillate obtained from the rotary evaporator was used to determine bioethanol concentration. After that, the ethanol concentration was evaluated by preparing an ethanol standard curve using a potassium dichromate reagent (Khalil et al., 2015).

3.7.1. Determination of standard bioethanol concentration

In this work, the alcohol content of our sample was determined by UV-visible spectrophotometer quantitative analysis of alcohols using potassium dichromate reagent. In a 1L volumetric flask, 400 mL of distilled water was used to dissolve about 34 grams of potassium dichromate. 325 mL of concentrated H_2SO_4 was added drop-by-drop into the volumetric flask while it was in an ice bath to produce the minimum amount of heat. Add 10 mL of the dichromate reagent after pipetting 1 mL of the sample into the 100 mL volumetric flask. The flask was then kept at 60°C for 20 minutes while being incubated in a water bath. Volume was increased using distilled water to 50 ml (El-Mekkawi et al., 2019). The linearity curve was plotted by taking concentrations of 5, 10, 15, 25, and 30 mg/mL of standard ethanol. The blank solution was prepared with distilled water. The absorbance of each concentration was measured at 587nm using a UV-visible spectrophotometer.

3.7.2. Sample preparation

1 ml of each produced bioethanol sample collected from distillation was poured into test tubes. Then 10 ml of chromium reagent was added. Then the mixture was allowed to water bath at 60℃ for 20min. then the tube was removed from the bath and 50 ml of distilled water was added to stop the reaction. Then the absorbance was measured at 587 nm using the UV-VIS spectrophotometer. Then the amount of concentration of ethanol in the test sample was determined by UV from the linearity Curve plotted at 587 nm. The produced bioethanol was characterized by FTIR and ethanol yield was calculated by Eq. (3.17).

$$\text{Ethanol yield } \% = \frac{\text{Ethanol produced, mg/mL}}{\text{Initial glucose concentration} \times 0.511, \text{mg/mL}} \qquad (3.16)$$

Where 0.511 is the theoretical conversion yield of glucose to ethanol

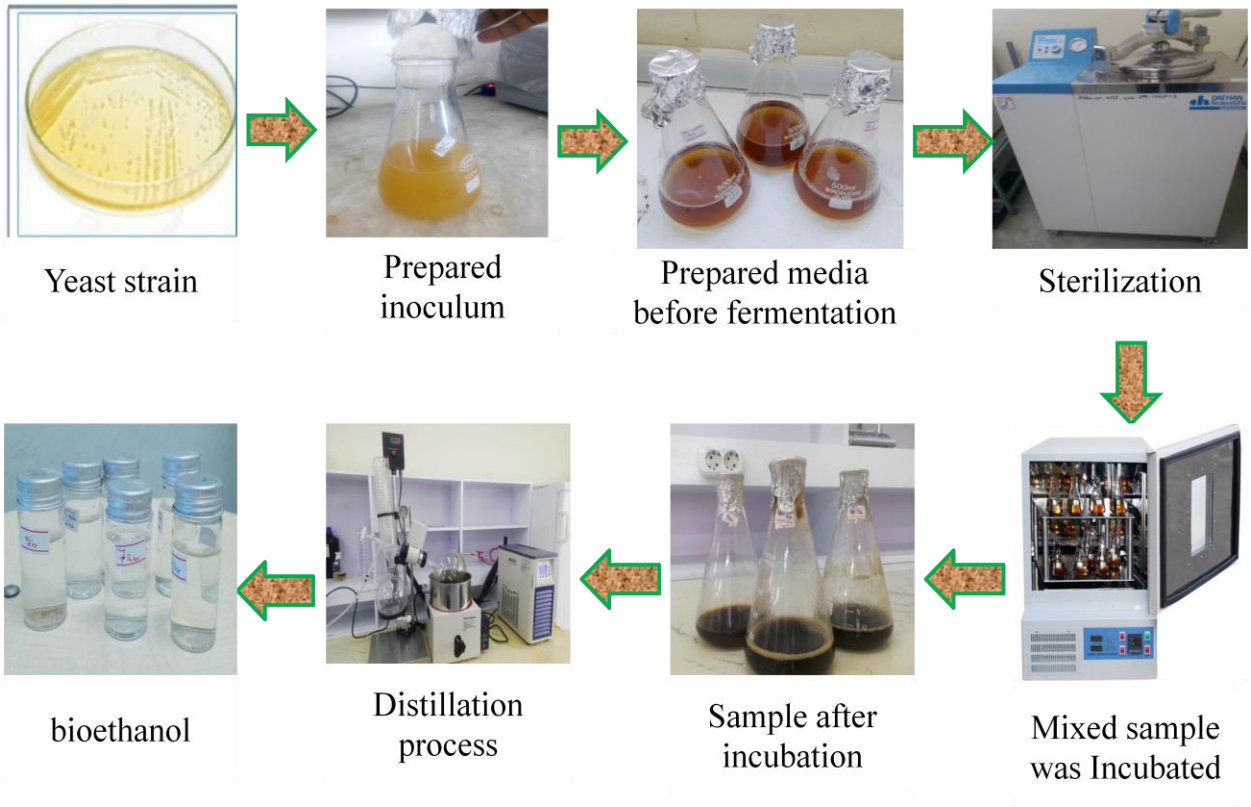

Figure 3.4 Overall experimental procedure of fermentation process during bioethanol production

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1. Characterization of corncob

4.1.1. Proximate analysis of corncob

The moisture content, ash content, volatile matter, and fixed carbon content of corncob obtained in this study are displayed in Table 4.1.

Table 4.1 Proximate analysis of corncob sample

Proximate Analysis	Weight percentage (%wt. dry basis)
Moisture content	6.82±0.1%
Ash content	2.02±0.25%
Volatile matter	82.2±0.2%
Fixed carbon	8.96±0.3%

Data (mean ± SD) at ($P \leq 0.05$)

The moisture content is a measure of the amount of water in the corncob (Dutta & Kumar, 2022). In the pretreatment and hydrolysis methods, moisture content analysis is used to calculate the proportionality of the solid-to-liquid ratio and increasing moisture content affecting the product quality. The sample with the higher moisture content requires more heat to vaporize the moisture (Oyelaran & Tudunwada, 2015). The moisture content of corncob in this study was (6.82±0.1%) slightly lower than the values reported in earlier work adapted by (Awosusi et al., 2017) which was (7.53 ± 0.4) and (Selvakumar et al., 2022) obtained (8.2 ± 0.26%). The amount of inorganic, noncombustible material that a sample contains was determined by its ash content. Ash content refers to the remnants left over after a sample has been entirely burned. These remnants can include minerals and inorganic salts found in fresh tubers, fertilizer use, as well as contamination from the air and soil during processing (Perea-Moreno et al., 2018). The ash content of the corncob in this study was (2.02±0.25%) which was slightly less than previously reported results (Okpalla, 2020) where it was (4.0%) and by (Awosusi & Ayeni,2017) where it was (2.09 ± 1.8).

The differences between these values may be due to the source of the corncob, the type of corncob, and the handling conditions. In this study, the low ash content of corncob constituents was observed and it can decrease sludge formation in ethanol production, and the high ash content of biomass makes it less desirable as a fuel. The volatile matter and carbon content in this study were 82.2±0.2% and 8.96±0.3%, respectively, which was expected because of the organic nature of the material. The volatile matter and carbon concentration in another study (Selvakumar et al., 2022) were (79.85±0.15%), (10.15±0.4%) respectively. These values slightly agree with the findings of our investigation. Biomass has a higher volatile matter content, and its combustion and gasification rates are correspondingly higher (Martinez et al., 2019).

4.1.2. Chemical composition analysis of corncob

This study confirmed that the corncob used to produce bioethanol displayed the following chemical composition: cellulose 42.53±0.47%wt%, hemicellulose 34.8±0.2% wt%, lignin 18±0.2%wt%, and extractives 4.08±0.18%wt%. According to Kucharska et al., (2020) composition of corncob, i.e. 40–45% cellulose, 31–39% hemicellulose, and 14–18% lignin content. Also in other studies performed that (45%) cellulose, (35%) hemicelluloses, and (15%) lignin, were previously examined (A. K. Kumar & Sharma, 2017). The results obtained from this study are comparable range with other literature. Therefore, the determination of cellulose and hemicellulose can be applied to quantify the theoretical production of ethanol, and the presence of high cellulose content on corncob leads to the release of high fermentable sugar. Lower the lignin content the easier hydrolysis condition, and decrease the formation of toxic chemicals such as, aromatic, polyaromatic, phenolic and aldehydic (Wei et al., 2021). Table 4.2 displays the results of chemical components analysis of the raw corncob.

Table 4.2 Chemical components analysis of corncob

Chemical composition analysis	Weight percentage (%wt. dry basis)
Cellulose	42.53±0.47%
Hemicellulose	34.8±0.2%
Lignin	18±0.2%
Extractive	4.08±0.18%

Data (mean ± SD) at ($P \leq 0.05$)

4.2. Pretreatment of corncob

4.2.1. Selection of significant parameters

In this study, the appropriate power and binary alkaline ratio among the various power and binary alkaline ratios were selected for efficient recovery of cellulose as well as for effective removal of hemicellulose during biomass pretreatment. The binary alkaline ratio (2:1) shows better cellulose recovery and hemicellulose removal as shown below in Table 4.3. In addition to this compared the single alkaline sodium hydroxide (NaOH) and ammonium hydroxide (NH_4OH) with the binary alkaline ($NaOH+NH_4OH$) pretreatment method by using a microwave reactor. The maximum cellulose recovery (72.68%) was observed in microwave-assisted binary alkaline ($NaOH+NH_4OH$) pretreatment. In addition, the experimental result as shown in Table 4.4 below 400W power gives better cellulose recovery (73.76%) as compared to other power. To increase the cellulose recovery, hemicellulose removal, and lignin removal, operating parameters were optimized, and intensive cellulose recovery was used as the response.

Table 4.3 Chemical components analysis of pretreated corncob by different binary alkaline ratios

Microwave-assisted binary alkaline	Conditions	Cellulose weight after treatment (g/g)	Hemicellulose weight after treatment (g/g)	Cellulose Recovery (%)	Hemicellulose Removal (%)
MV-NaOH	1%, 240W,4min and 0.1g/ml	0.2840	0.1845	66.77	46.98
MV-NH_4OH	1%, 240W, 4min and 0.1g/ml	0.2967	0.1817	69.76	47.79
	1% (1:1), 240W, 4min and 0.1g/ml	0.303	0.1634	71.24	53.05

MV $NaOH+NH_4OH$	1% (1:2), 240W, 4min and 0.1g/ml	0.3079	0.1584	72.4	54.48
	1% (1:3), 240W, 4min and 0.1g/ml	0.3019	0.1754	71	49.58
	1% (2:1), 240W, 4min and 0.1g/ml	0.3091	0.1223	72.68	64.85
	1% (3:1), 240W, 4min and 0.1g/ml	0.296	0.1547	69.6	55.5

Table 4.4 Chemical components analysis of pretreated corncob by different power

Microwave-assisted binary alkaline	Ratio	cellulose weight after treatment (g/g)	Hemicellulose weight after treatment (g/g)	Cellulose Recovery (%)	Hemicellulose Removal (%)
MV-assisted NaOH+NH4OH	1% (2:1), 80W,4min	0.2953	0.1555	69.43	55.31
	1% (2:1), 240W, 4min	0.3037	0.1356	71.42	61.03
	1% (2:1), 400W, 4min	0.3137	0.1311	73.76	62.32
	1% (2:1), 640W, 4min	0.3064	0.1377	72.05	60.43

The initial weight of cellulose, hemicellulose, and lignin was 42.53%, 34.8%, and 18%, respectively. These values were obtained after analysis of the corncob sample by standard method. To investigate the effect of microwave-assisted binary alkaline pretreatment process parameters on the cellulose recovery and hemicellulose removal were calculated from equations 3.9 and 3.10 respectively.

4.2.2. Optimization of pretreatment parameters of microwave-assisted binary alkaline ($NaOH + NH_4OH$) (2:1) ratio for efficient cellulose recovery

In order to identify the optimal values of each factor that jointly optimizes the set of desired responses (cellulose recovery and hemicellulose removal) a multi-objective numerical optimization was performed using RSM. The objectives were to enhance cellulose recovery, hemicellulose solubilization, and lignin removal in the corncob. To obtain higher cellulose recovery, it is necessary to standardize the pretreatment method for each type of biomass. In this study, microwave-assisted binary alkaline ($NaOH + NH_4OH$) (2:1)) were selected for pretreatment because ammonium hydroxide (NH_4OH) has been found to give the highest cellulose recovery in the pretreatment of corncob (Chen et al., 2012). At the same time, the sodium hydroxide (NaOH) enables the degradation of hemicellulose simultaneously with great delignification at a low alkaline concentration (Martelli-Tosi et al., 2017). Therefore, this binary alkaline ratio was used for optimization to achieve high cellulose recovery and hemicellulose removal after pretreatment, the pretreatment process was set between alkaline concentration (1-3 % v/v), biomass loading ratio from (0.05 - 0.15 g/mL). The microwave power for all the experiments was set at 400 W for heating times of between 5 and 15 min. However, the ranges for the three parameters were selected based on the preliminary experiments and according to (Sewsynker-Sukai & Gueguim Kana, 2018; Tsegaye et al., 2019). The results of microwave-assisted binary alkaline pretreatments of corncob at each point based on the experimental central composite design template are given in Table 4.7. After microwave-assisted binary alkaline pretreatment, corncob composition was significantly improved. Based on the results of the experiment the cellulose recovery in the solid fraction ranged from 67.4-92.6 %, while hemicellulose solubilization ranged between 50-83.8 %. The pretreatment condition at 10min pretreatment time, 2 % alkaline concentration, and 0.1g/mL biomass loading preserved high cellulose recovery and removed high hemicellulose. At this condition, 92.6 % cellulose recovery was obtained while 83.8 % hemicellulose was removed. This point is set as the optimum

condition for microwave-assisted binary alkaline pretreatment of corncob. Alternatively, the lowest yield of cellulose recovery was 67.4 % and occurred under conditions of 18.4miin time, 2 % alkaline concentration and 0.1 g/mL biomass loading. In this case, an increase in time decreased the cellulose recovery of pretreated corncob; it is probably due to the cellulose content decomposition and derivatization to other compounds. We concluded that microwave-assisted binary alkaline pretreatment was more promising for the retention of high cellulose content in corncob biomass. Furthermore, high removal of hemicellulose was also observed. This overall increase in cellulose content was a result of the opening up of the complex lignocellulosic structure and the significant removal of hemicellulose and lignin portions. Lignin removal will improve the efficiency of hydrolysis of lignocellulosic biomass. This study demonstrated that effective depolymerization of corncob was achieved after microwave-assisted binary alkali pretreatment at an optimal condition this enhances saccharification efficiency of the biomass during hydrolysis and ethanol yield by increasing cellulose yield. From Table (4.7) below the experimental result shows that the maximum cellulose recovery, and the maximum hemicellulose, removal were 92.6 %, and 83.8 % respectively. This result somewhat coincided with other literature as shown below in Table 4.14.

Table 4.5 Chemical components analysis of Untreated and pretreated

Corncob by microwave-assisted binary alkaline method

Corncob component	Untreated sample Wight (%)	Pretreated sample (%)
Cellulose (%)	42.53 ± 0.47 %	92.6 %
Hemicellulose (%)	34.8 ± 0.2 %	83.8 %

4.3. Statistical Analysis of the Experimental Results

Essential process parameters (binary alkaline concentration, solid loading ratio and time) that affected the cellulose recovery yield during pretreatment were examined via RSM. The inputs for design experts are binary alkaline concentration (%v/v), biomass loading ratio (g/mL) and microwave pretreatment time (min) as independent variables and cellulose recovery yield dependent variable. The above-mentioned parameters were fed into design expert software and

generated the build information of the experimental analysis of the responses cellulose recovery process as shown in Table below.

Table 4.6 Information given for building experimental analysis of cellulose recovery yield.

Study type	Response surface
Initial point	Central composite design
Center point	6
Design Model	quadratic polynomial
Run	20
Blocks	No

Table 4.7 Cellulose recovery yield and hemicellulose removal at different conditions

Std	Run	Factor 1A: Time (min)	Factor 2 B: Alkaline concentration (%)	Factor 3 C: Biomass loading (g/mL)	Response 1 CR (%)	Response 2 HR (%)
11	1	10	0.32	0.1	81.7	75
7	2	5	3	0.15	86.4	56
4	3	15	3	0.05	70.2	63
8	4	15	3	0.15	89.87	59.2
14	5	10	2	0.18	80.5	50
5	6	5	1	0.15	70.04	54.3
16	7	10	2	0.1	89.98	79.9
18	8	10	2	0.1	91.9	82.6
9	9	1.6	2	0.1	73.4	58
2	10	15	1	0.05	80.6	76.2
10	11	18.4	2	0.1	67.4	77.5
12	12	10	3.7	0.1	85.2	60
3	13	5	3	0.05	70.3	54

20	14	10	2	0.1	92.6	83.8
19	15	10	2	0.1	91.82	83
17	16	10	2	0.1	92.4	78.6
6	17	15	1	0.15	68.9	70.4
15	18	10	2	0.1	92.2	82.8
13	19	10	2	0.02	77.8	64
1	20	5	1	0.05	85.7	62

Analysis of Variance (ANOVA)

Analysis of variance (ANOVA) generated by Design Expert software version 13 is used to study the model adequacy, model significance, model fitness and correlations between the predicted and observed values. The summary of the analysis of variance (ANOVA) is given in Table 4.8.

Table 4.8 ANOVA for response surface quadratic model for cellulose recovery yield

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F	
Model	1572.89	9	174.77	118.04	< 0.0001	significant
A-Time	12.30	1	12.30	8.31	0.0163	
B-AC	22.21	1	22.21	15.00	0.0031	
C-BL	12.28	1	12.28	8.29	0.0164	
AB	11.54	1	11.54	7.80	0.0190	
AC	7.09	1	7.09	4.79	0.0535	
BC	498.17	1	498.17	336.47	< 0.0001	
A^2	783.40	1	783.40	529.12	< 0.0001	
B^2	109.71	1	109.71	74.10	< 0.0001	
C^2	263.91	1	263.91	178.25	< 0.0001	
Residual	14.81	10	1.48			
Lack of Fit	10.32	5	2.06	2.30	0.1905	not significant
Pure Error	4.48	5	0.8962			
Cor Total	1587.69	19				

The significance of the model was tested by p-value (also known as the 'Prob > F' value). The p-value is less than 0.05, which implies that 5% occur due to noise whereas values greater than 0.1 indicate the model terms are not significant. Additionally, the 'Prob > F' values were used to determine the significance of each linear, quadratic, and interaction term's effects on the responses; the model's prediction equations were improved by removing the factors that were not significant. In this case, A, B, C, AB, BC, A^2, B^2 and C^2, were significant model terms whereas AC was not significant. If the value is more than 0.1000, the model terms are no longer considered relevant. Model reduction may enhance your model if there are numerous unimportant model terms (except those needed to maintain hierarchy). As indicated in Table 4.8, the ''Model F-value" of 118.04 indicated the model was significant and there was only a 0.01% chance that a ''Model F-value" this large could occur because of noise. The "Lack of Fit F-value" of 2.30 indicated that the lack of fit was not significant when compared to the pure error, and there was a 19.05% probability that noise may be the cause of such a high "Lack of Fit F-value."

Adjusted R-squared, a measure of the amount of variation in new data explained by the model, and Adequate precision, which is a signal-to-disturbance ratio due to random error, presented in Table 4.9 below, are used to decide whether the model can be used or not.

Table 4.9 Model adequacy measures

Std. Dev.	1.22	R^2	0.9907
Mean	81.95	Adjusted R^2	0.9823
C.V. %	1.48	Predicted R^2	0.9466
		Adeq Precision	27.7063

The goodness of fit of the model was examined by the determination coefficient (R^2 = 0. 0.9907), which implied that the sample variation of more than 99.07 % was attributed to the variables. The predicted determination coefficient (Predicted R^2 = 0.9466) was in reasonable agreement with the adjusted determination coefficient (Adjusted R^2 = 0.9823); i.e., the difference is less than 0.2, which was also sufficient to validate the model's significance. A lower value of coefficient of variation (CV = 1.48%) showed the experiments conducted were precise and reliable. "Adeq Precision" measured the signal-to-noise ratio. A ratio greater than 4 is desirable.

The ratio of 27.706 indicated an adequate signal. Therefore, model could be used to navigate the design space.

Regression model equation

Table 4.10 Regression coefficients in terms of coded factors

Factor	Coefficient Estimate	Degree of freedom	Standard Error	95% CI Low	95% CI High	VIF
Intercept	91.79	1	0.4963	90.68	92.89	
A-Time	-0.9490	1	0.3293	-1.68	-0.2154	1.0000
B-AC	1.28	1	0.3293	0.5416	2.01	1.0000
C-BL	0.9483	1	0.3293	0.2147	1.68	1.0000
AB	1.20	1	0.4302	0.2427	2.16	1.0000
AC	0.9413	1	0.4302	-0.0173	1.90	1.0000
BC	7.89	1	0.4302	6.93	8.85	1.0000
A^2	-7.37	1	0.3205	-8.09	-6.66	1.02
B^2	-2.76	1	0.3205	-3.47	-2.04	1.02
C^2	-4.28	1	0.3205	-4.99	-3.57	1.02

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. In an orthogonal design, the intercept is the overall average response of all runs. The coefficients are modifications based on the factor settings that are made around that average. VIFs are 1 when the factors are orthogonal; VIFs more than 1 imply multi-collinearity; the higher the VIF, the more severe the factor correlation. VIFs less than 10 are generally acceptable.

The regression coefficient of the quadratic equation describing the response cellulose recovery to the independent variables (factors) in terms of the coded and actual factors from corncob during microwave-assisted binary alkaline pretreatment process was described by Equation (4.4) and (4.5): Regression coefficients in terms of coded factors were shown in table 4.10.

The final equation in terms of coded factor

$$CR = 91.79 - 0.9490A + 1.28B + 0.9483C + 1.20AB + 0.9413AC + 7.89BC - 7.37A^2 - 2.76B^2 - 4.28C^2 \quad (4.4)$$

Where, A: is microwave pretreatment time (min), B: is binary alkaline concentration (wt %) of the reaction and C: is the biomass loading ratio (g/mL).

The final equation in terms of actual factor

Cellulose Recovery = 71.72651 + 4.85156Time - 5.87339alkaline concentration + 8.01512 Biomass loading + 0.240250Time * Alkaline concentration + 3.76500Time * Biomass loading + 157.82500 Alkaline concentration * Biomass loading - 0.294918Time^2 - 2.75908 Alkaline concentration ^{2} - 1711.74525 Biomass loading ^{2} (4.5)

The equation in terms of actual factors can be used to make predictions about the response for different levels of each factor.

4.3.1. Actual versus predicted plot

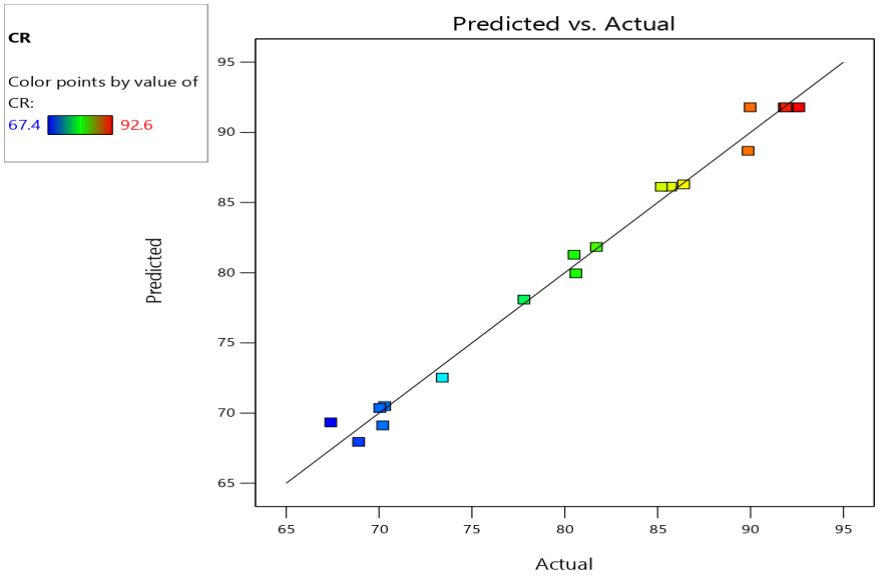

Figure 4.1 Actual versus predicted values for cellulose recovery

In Figure (4.1 above predicted versus the actual value of cellulose recovery was plotted. The plot shows how precisely the model) is modeled. The purpose is to detect a value or group of values, that the model does not easily predict. The points show how the predicted value and actual values of each run approach the straight line. The straight line shows how the predicted and actual values are closer to each other. When the point is above the straight-line predicted value is greater than the actual value, the reverse is also true If the scatter of the plot lies almost on the diagonal line, this shows that the model is designed very well, i.e., the experimental data is closely related to the data predicted from the model. As shown in Figure (4.1) all the data points are arranged near a straight line

which represents that there is no variation effect between the experimental response and predicted response.

4.4. Effects of pretreatment conditions on the composition of corncob

The effects of individual factors and the interaction effects of the various combinations of factors were taken into consideration in the model. The response surface plots of cellulose recovery were generated by feeding the experimental result into design expert software version 13 to study the effect of process parameters on cellulose recovery. The process parameter optimization aimed to maintain high cellulose recovery and high hemicellulose as well as high lignin removal. So the effect of pretreatment time, the effect of alkaline concentration, and the effect of biomass loading ratio on the response cellulose recovery were discussed below.

4.4.1. The effect of pretreatment time on the composition of corncob during pretreatment

Cellulose recovery of the corncob was increased to 67.4–92.6 % after microwave-assisted binary alkaline pretreatments. Fig 4.2 shows that at longer and shorter pretreatment time lower cellulose was recovered. At a pretreatment time of 5 min about 85.36% cellulose was preserved while about 83.46% cellulose was preserved at 15min pretreatment time. Too high pretreatment time solubilizes the released cellulose and facilitates the formation of glucose or furfural and furfural derivative compounds. Too short a pretreatment time is inefficient to depolymerize the biomass components (Tsegaye et al., 2020). The maximum cellulose recovery (92.6 %) was observed at the center points (10min). High cellulose recovery is important for increasing the yield of fermentable sugars and ethanol. The recovery of cellulose was mainly related to the solubilization of lignin.

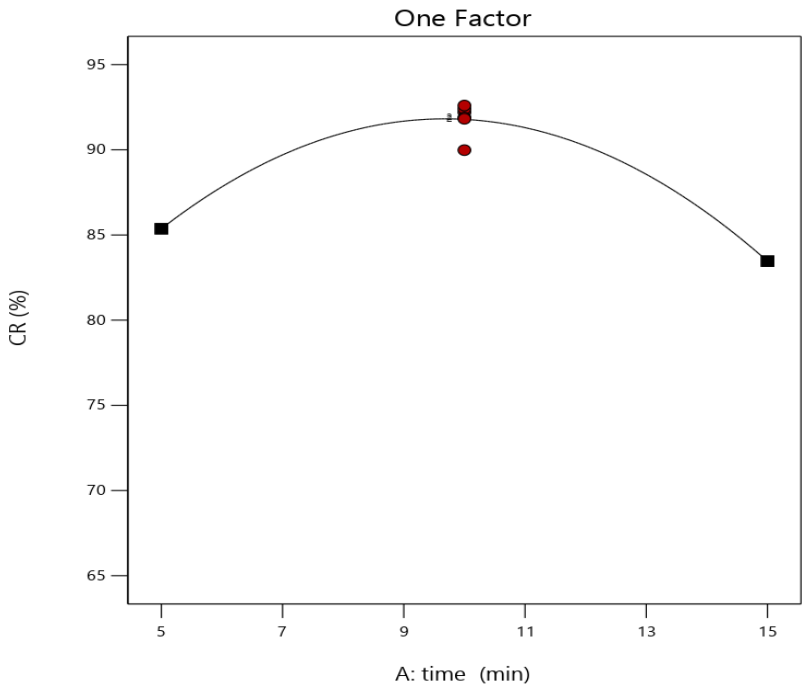

Figure 4.2 Effect of time on the yield of cellulose recovery

4.4.2. The effects of binary alkaline concentration on the composition of corncob powder

The concentration of binary alkaline was related to the solubilization, which is closely related to hemicellulose removal and delignification (Martelli-Tosi et al., 2017) Thus the pretreatment conditions optimization process aimed to maintain the high cellulose recovery and high hemicellulose removal. The influence of alkaline concentration on the microwave-assisted binary alkaline pretreated residue is shown in Figure (4.3). It shows that at higher and lower alkaline concentrations lower cellulose was obtained. Alkaline concentration at 1%, about 87.75 % cellulose was preserved while about 90.3 % cellulose was preserved at 3 % alkaline concentration. This demonstrates that the alkaline concentration increased and the cellulose recovery was increased up to the center points but slightly decreased cellulose recovery after the center point because the alkaline concentration further increased after the center point. Too high alkaline concentration solubilizes the released cellulose and facilitates the formation of glucose or furfural and furfural derivative compounds. Too low alkaline concentrations are inefficient to depolymerize the biomass components (Tsegaye et al., 2020). The maximum cellulose recovery (92.6 %) was observed at the center points (2%). This indicates that the concentration of binary alkaline had a significant influence on cellulose recovery yield. Furthermore, the hemicellulose

removal and delignification were considerably augmented with the increased binary alkaline dosage until the center point.

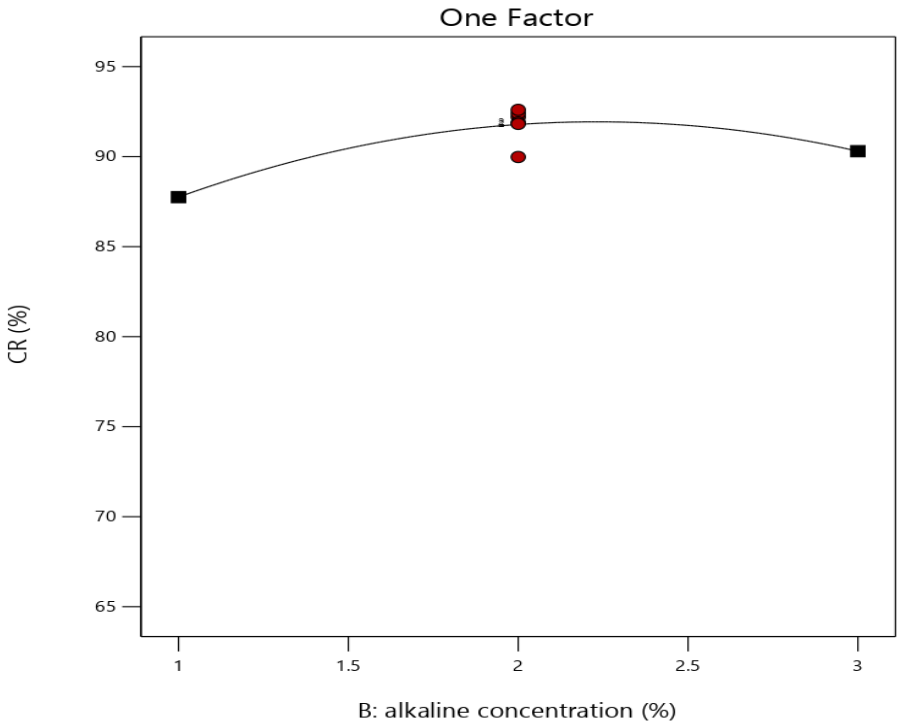

Figure 4.3 Effect of alkaline concentration on yield of cellulose recovery

4.4.3. Effects of biomass loading ratio on the composition of corncob during pretreatment

Biomass loading was associated with heat and mass transfer efficiencies, which had an effect on delignification (L. Ma et al., 2018). Therefore, Solid loading ratio (g/mL) was an important influence factor for evaluating this microwave-assisted binary alkaline pretreatment process. In this study, considering the p-value of cellulose recovery percentage all three variables are significant. Fig. 4.4 shows the main effect plots of cellulose recovery versus biomass loading. It was observed that the maximum amount of cellulose was recovered (92.6 %) at (0.1 g/ml,). The plots show that increasing biomass loading increased the cellulose recovery of pretreated corncob up to the center points, but slightly decreased cellulose recovery after center point due to further increasing biomass loading. For example, i.e. at 0.05 g/ml and 0.15 g/ml biomass loading about 86.55 % and 88.45 % of cellulose were recovered respectively. Extreme, too-high, or too-low biomass loading has adverse effects on cellulose recovery. It was possibly because of the accessibility of alkaline pretreatment, which led to more linkages among the three contents, be broken. A high biomass loading ratio decreases the alkaline accessibility to break the bond

between the three components (Loow et al., 2016). According to the study, pretreatment around the center points produced more cellulose than the lower and higher extreme conditions.

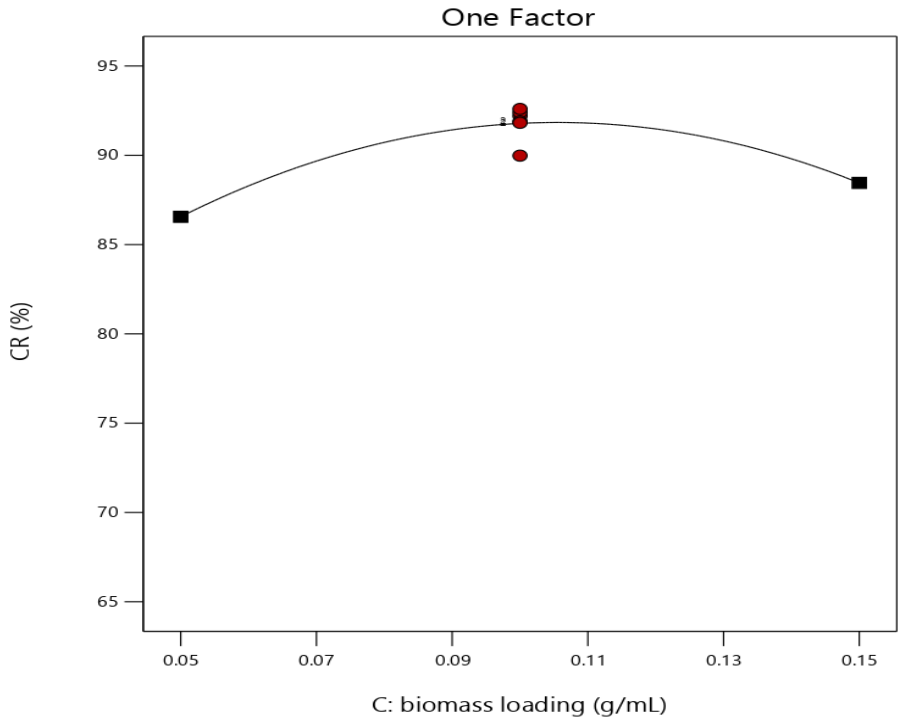

Figure 4.4 Effect of biomass loading ratio on yield of cellulose recovery

4.5. Interaction effect of pretreatment conditions on the yield of cellulose recovery

To observe the interactive effect of pretreatment time, alkaline concentration and biomass loading on cellulose recovery from corncob biomass, 3D response curve was drawn. The 3D response plots were used to study the interactive effects of pretreatment parameters on response (Tsegaye et al., 2019). The shapes of 3D response plots determine the extent and nature of interactions between the process variables (pretreatment time, alkaline concentration and biomass loading). If the nature of contour plates is circular, it indicates the interactions are negligible or very weak. Strong or noticeable interactions are identified by the elliptical nature of the contour plots. Main effect plots were generated by considering the effects of two factors at a time while keeping the third factor at the center (Tsegaye et al., 2019).

4.5.1. Effects of binary alkaline concentration and pretreatment time on the cellulose recovery yield

Figure 4.5 represents the amount of cellulose recovery after microwave-assisted alkaline pretreatment when the biomass loading was at the center, 0.1g/mL while varying pretreatment time and binary alkaline concentration. It shows that with higher and lower pretreatment time and alkaline concentration, lower cellulose was obtained. At a pretreatment time of 5.21 min, about 83.18% cellulose was preserved at 1.02% alkaline concentration. The amount increased to 85.94% at 2.03 % and then decreased to 84.49 % at 2.96 % alkaline concentration. About 78.99 %, 84.62 % and 83.8 % of cellulose recovery were obtained at 1.01%, 2.01% and 2.9 % alkaline concentration respectively at 14.8 min. The maximum amount of cellulose recovery (92.6 %) was obtained at 2 % and 10 min. This demonstrates that alkaline concentration and cellulose recovery have a direct relationship up to the center points and are inversely proportional after the center point. At higher binary alkaline concentration and higher pretreatment time possible for the formation of other molecules from the recovered cellulose, or the recovered cellulose was further hydrolyzed to glucose. Lower binary alkaline concentration and lower pretreatment time are inefficient to depolymerize the biomass components (Selvakumar et al., 2022).

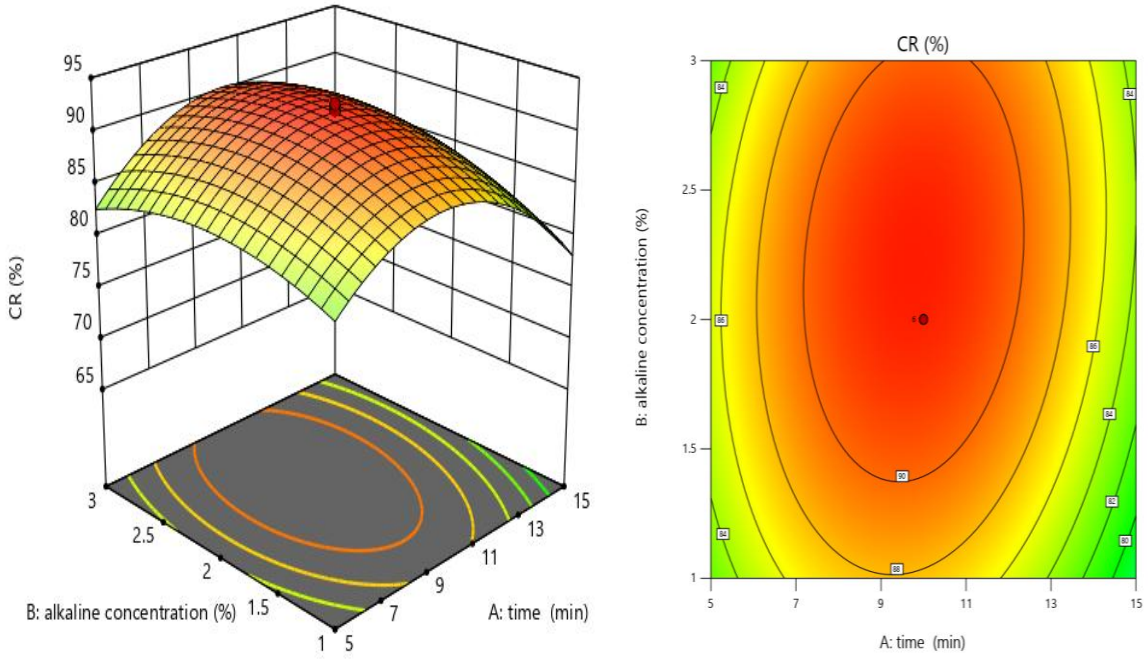

Figure 4.5 Effects of binary alkaline concentration and pretreatment time on the cellulose recovery yield

4.5.2. Effects of pretreatment time and biomass loading ratio on the cellulose recovery yield

The effects of pretreatment time and biomass loading ratio on the cellulose recovery yield when the alkaline concentration was the actual factor as shown in Figure (4.6) below. The maximum yield of cellulose recovery (92.6 %) was observed at the center points (biomass loading ratio (0.1 g/mL) and at pretreatment time (10 min)). The relationship of their interaction effect on the yield cellulose recovery was also shown from the regression coefficient of their interaction in equation (4.4). From the equation, the interaction of biomass loading ratio and time has positively affected cellulose recovery yield. Fig. 4.6 shows that at higher and lower pretreatment conditions lower cellulose was obtained.

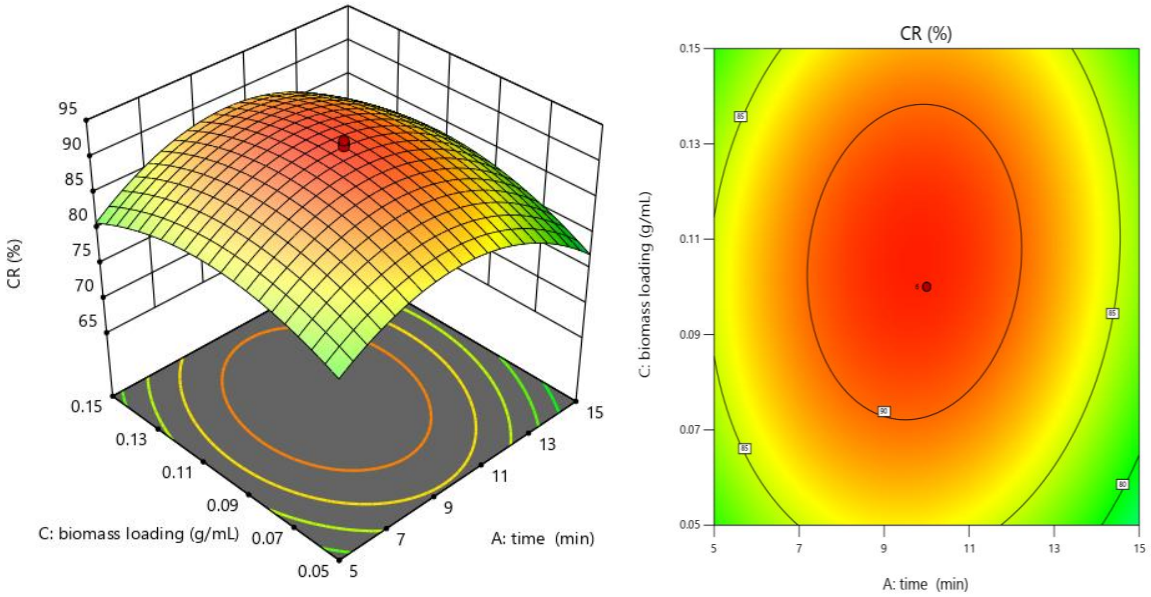

Figure 4.6 Effects of pretreatment time and biomass loading ratio on the cellulose recovery yield

4.5.3. Effects of alkaline concentration and biomass loading ratio on the cellulose recovery yield

The effect of binary alkaline concentration and solid loading ratio on the yield of cellulose at a constant 10 minutes was shown in Figure (4.7). The Figure depicted that a maximum cellulose yield was obtained at the center points with 2 % alkaline concentration and 0.1g/mL biomass loading. The relationship of their interaction effect on the yield was also shown from the regression coefficient of their interaction in equation **(**4.4**).** From the equation, the interaction of binary alkaline concentration and solid loading ratio positively affected cellulose recovery yield.

At alkaline concentration 1% about 90.41% cellulose was recovered at 0.05g/mL while about 76.52% cellulose was preserved at 0.15g/mL. About 77.1% and 91.9% cellulose were obtained at 0.05g/mL and 0.15g/mL respectively after 3%. This indicates that high cellulose was recovered at the condition of the interaction between high alkaline concentration and high biomass loading or low alkaline concentration and low biomass loading ratio but the interaction between high alkaline concentration and low biomass loading or vice versa low cellulose was obtained. In all pretreatment conditions, maximum cellulose recovery was observed at the center points (10 min, 0.1g/ml and 2% alkaline concentration). High cellulose recovery is important for increasing the yield of fermentable sugars and ethanol (Scopel & Rezende, 2021).

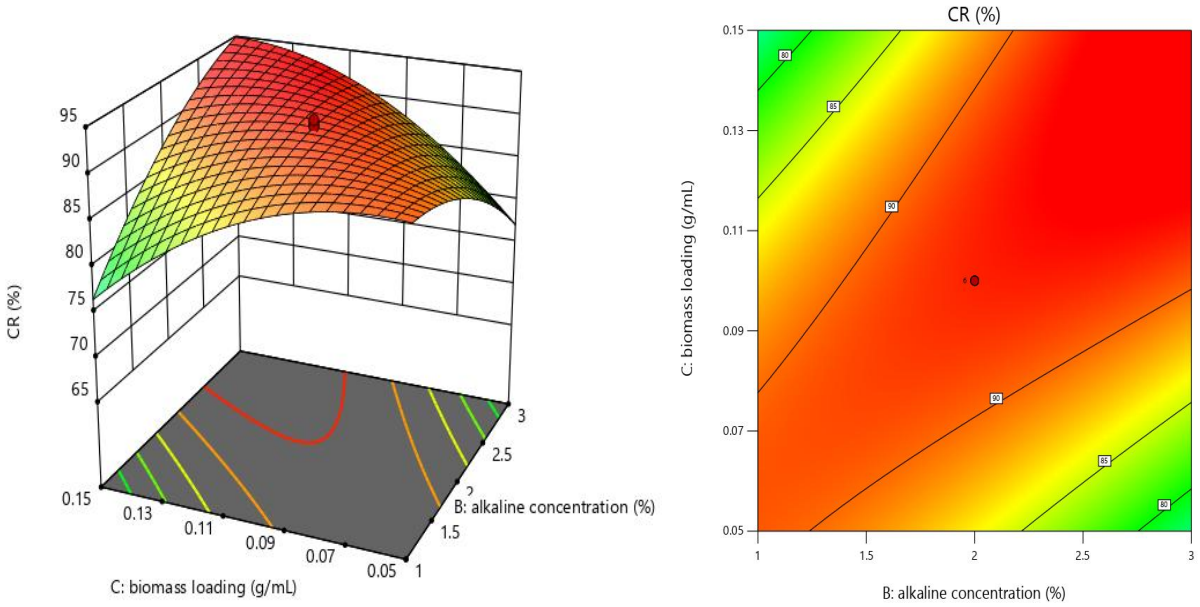

Figure 4.7 Effects of alkaline concentration and biomass loading ratio on the cellulose recovery yield

4.6. Optimization of the pretreatment conditions

The main objective of optimization is the production of corncob samples which are easily prone to hydrolysis and bioethanol production. Corncob having high cellulose, low hemicellulose, and lignin content and low crystallinity index are quickly well hydrolyzed (Louis & Venkatachalam, 2020). As the structural characteristics of lignocelluloses are somehow qualitative parameters, the cellulose recovery and hemicellulose solubilization of the samples are considered as responses for the process optimization. Optimization aims to gain a sample having maximum cellulose recoveries and hemicellulose as well as lignin removal. The optimum pretreatment conditions resulting from response surface method are summarized in Table 4.7. Optimization of

pretreatment process factors (binary alkaline concentration, solid loading ratio, and time) and response (cellulose recovery) was carried out by a multiple response method called desirability (D) function to optimize the different combinations of process parameters.

Table 4.11 Constraints for the factors and responses in numerical optimization

Name	Goal	Lower Limit	Upper Limit
Time	is in range	5	15
Alkaline concentration	is in range	1	3
Biomass loading	is in range	0.05	0.15
Cellulose Recovery	maximize	67.4	92.6

According to the experimental work in Table (4.7), the maximum yield of cellulose recovery 92.6%, was obtained at the interaction parameter of binary alkaline concentration (2%), biomass loading ratio (0.1 g/mL) and time (10 min). Whereas from the response optimization technique used in this study, the yield of cellulose recovery was optimized to 94.33%, at a desirable parameter interaction of binary alkaline concentration (2.889 v/v %), biomass loading ratio (0.149/mL) and reaction time (11.031min), as shown in the Appendix-2. From the 100 possible solutions of optimization, the table below is the optimized solution at 1 desirability.

Table 4.12 Optimized solution of cellulose recovery yield from corncob substrate

Number	Alkaline Concentration (%)	Biomass Loading (g/mL)	Time (min)	Cellulose Recovery (%)	Desirability	
1	2.889	0.149	11.031	94.33	1.000	Selected

Based on the analysis best local maximum cellulose recovery yield of 94.33% was found at alkaline concentration (2.889 v/v %), solid loading ratio (0.149g/mL), and time (11.031min).

4.6.1. Validation of the model

To confirm the validity of the optimized model, the actual validation experiment was carried out at optimum conditions that a design expert obtained. These optimum points were binary alkaline concentration (3%), biomass-loading ratio (0.15g/mL), and time (11 min). To validate the optimum conditions predicted by the response surface methodology (RSM) model results,

triplicate experiments were conducted at the above specified optimum process conditions predicted by the model. After optimization, triplicate experiments were performed using these optimized process conditions. At this condition, the mean percentage of yield obtained was 93.503±0.21%, as shown in Table 4.13, and which was then related to the data obtained from optimization analysis using the desirability function.

Table 4.13 Result of optimization and model validation

Number	Alkaline Concentration (%)	Biomass Loading (g/mL)	Time (min)	Cellulose Recovery (%)
Predicated	2.889	0.149	11.031	94.33
Experimental	3	0.15	11	93.503 ± 0.21 %

The mean percentage of cellulose recovery yield obtained by triplicate experiments was 93.503±0.21%, which is not significantly different from the predicted value of 94.33% cellulose recovery obtained at the Optimal conditions of binary alkaline concentration (2.889%), biomass loading ratio (0.149/mL) and reaction time (11.031min). As a result, the model was valid and capable of predicting the highest cellulose recovery yield; i.e., numerical optimization can be considered an optimal value because the predicted value was close enough to the experimental value. In summary, this study shows that binary alkaline concentration, biomass loading ratio and time could be used for the optimization of cellulose recovery yield from corncob biomass. The yield could be optimized by tuning binary alkaline concentration, biomass loading ratio and time parameters.

Table 4.14 Comparisons of cellulose recovery and hemicellulose removal of Corncob residues pretreated by different methods.

Pretreatment method	Pretreatment condition	Cellulose recovery (%)	Hemicellulose removal (%)	Reference
H_2SO_4-$NH_3 \cdot H_2O$	2 wt% H_2SO_4+15 wt% $NH_3 \cdot H_2O$ 1:6,	78.53	83.46	(Xie et al., 2014)

	121°C , 1h			
Alkaline hydrogen peroxide	2% (w/w) H_2O_2,1:20 50°C, 6h	81.3	38.7	(Su et al., 2014)
Combined dilute-alkaline and green pretreatments	(NaOH-LHW-CC) ,2 wt.% 90°C,1.5h	84.73	90	(Araújo et al., 2019)
Microwave-assisted binary alkaline pretreatment	2%v/v(NaOH+NH_4 OH), 400W, 10min, 0.1g/mL	92.6	83..8	This study

4.7. Effect of microwave-assisted binary alkaline pretreatments on physico-chemical features of corncob

4.7.1. X-ray diffraction (XRD) analysis

The amount of crystalline cellulose in a lignocellulosic material is determined by the crystallinity index. The CrI represents the ratio of crystalline to amorphous components in biomass. Crystallinity is considered an essential component during hydrolysis of cellulose. Various pretreatments can alter cellulose crystal structures by interrupting inter- and intra-chain hydrogen bonding in cellulose fibrils (Nasirpour & Mousavi, 2018). The crystallinity of the microwave-assisted binary alkaline treated samples was extensively investigated by XRD. The XRD profiles of untreated and microwave-assisted binary alkaline treated corncobs in optimal conditions are shown in Figure. 4.8. The crystalline index of the microwave-assisted binary alkaline pretreatment samples was found to be higher than that of the raw corncob. The diffraction patterns and data showed that the CrI of corncob increased from 37.29% to 56.41% as the cellulose content increased. These results show that microwave-assisted binary alkaline pretreatment can increase the crystallinity of raw materials, due to an increase in cellulose content. The partial solubilization of amorphous polysaccharide components, as well as a significant amount of lignin solubilization, led to the increased crystalline index. Several other researchers noticed a similar increase in crystallinity index after pretreatment in different lignocellulosic materials and the results were compared to the current study in Table 4.15.

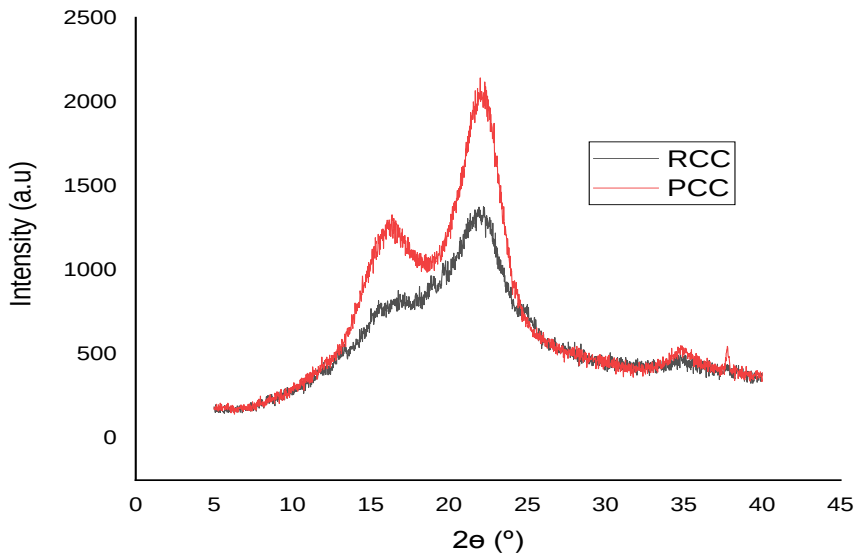

Figure 4.8 X-ray diffraction patterns of raw corncob and pretreated corncob

The removal of amorphous components such as hemicellulose and lignin results in an increase in crystallinity index after pretreatment. The increased values indicate the effect of microwave-assisted binary alkaline treatment and are in agreement with the biochemical characterization results, which show a slight reduction in hemicellulose and lignin concentration after pretreatment. The main diffraction peaks at 2θ values of 16.34°, 22.22°, and 34.56°. Figure 4.9 shows the X-ray diffraction of raw corncob, pretreated corncob and corncob cellulose in optimum conditions.

The crystallinity index (CrI) is calculated by using equation (3.12). The calculated values were tabulated in Appendix 3. The CrI of RCC, PCC and CCC are 37.29 %, 56.41 % and 66.25 %. After microwave-assisted binary alkaline treatments, increase the crystallinity index of corncob due to the removal of amorphous components such as hemicellulose and lignin. However, it was lower than that of the commercial micro-cellulose. According to Selvakumar et al., (2022), the crystallinity index of commercial micro-cellulose was 71.2 %.

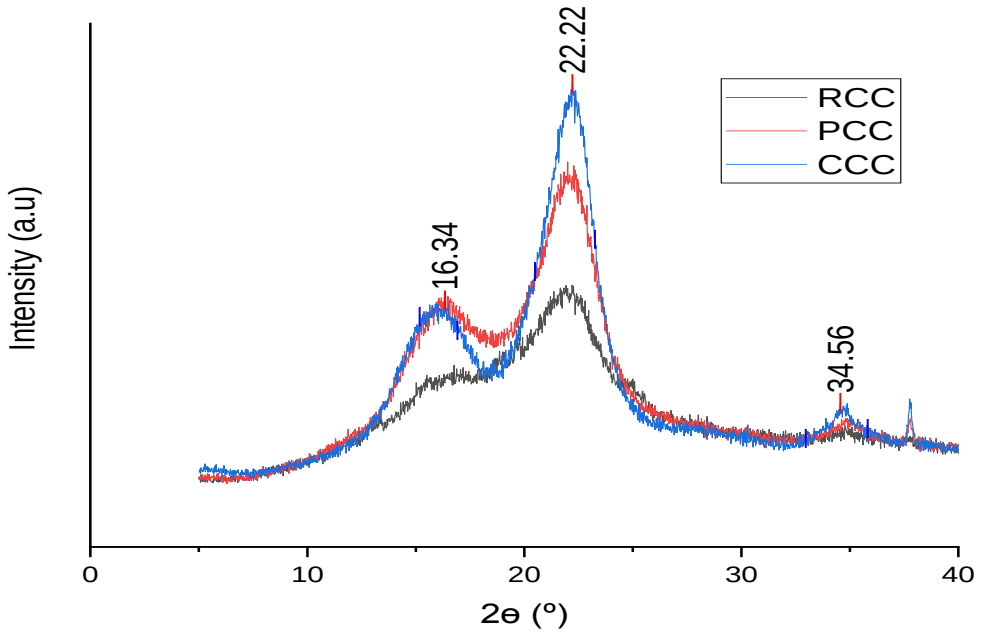

Figure 4.9 X-ray diffraction of raw corncob and pretreated corncob and corncob cellulose

Hemicellulose and lignin are amorphous components of lignocellulosic biomass, whereas cellulose is both crystalline and amorphous. Although hemicellulose and lignin are amorphous, cellulose is composed of linear chains that form a polycrystalline structure. Generally, high crystalline lignocellulosic materials are more stable and have lower reactivity than amorphous ones (Ponce et al., 2021). When compared to unpretreated biomass, the pretreatments encouraged the production of amorphous and crystalline regions. However, the crystalline region increased more than the amorphous region, resulting in a higher CrI compared with the unpretreated biomass. Therefore, the increase of the amorphous region in all pretreated samples promoted an increase in the hydrolysis process.

Table 4.15 A summary of the crystallinity indexes published in previous studies.

Lignocellulosic material	Crystallinity index (%)		References
	unpretreated	pretreated	
Cassava stem	39.56	47.15	(Kamalini et al., 2018)
Corncob	22.09	45.1065	(Mohanasundaram et al., 2023)

sugarcane bagasse	53.44	65.55	(Binod, Satyanagalakshmi, Sindhu, & Janu, 2012)
Corncob	36.78	51.01	(Kleingesinds et al., 2018)
Corncob	37.29	56.41	This study

4.7.2. Fourier transform infrared (FTIR) spectroscopy analysis

FTIR spectroscopy provides information on the functional groups present in biomass as well as changes in functional groups caused by pretreatment activities. The analysis of Fourier transform infrared spectroscopy in this study revealed the alterations in the fundamental components of corncob biomass (lignin, cellulose, and hemicellulose). This was noticeable by the appearance or disappearance of adsorption bands representing different functional groups. Figure 4.10 shows the FTIR spectra of raw corncob, pretreated corncob and corncob cellulose. The raw corncob contains functional groups with common absorption bands, such as the OH- stretching at 3343 cm^{-1} (Binod, Satyanagalakshmi, Sindhu, & Janu, 2012) and one at 2915 cm^{-1} to the C-H stretching of methyl, methylene, or methine group in the corncob

In general, the comparison of the three spectra suggests an intense change in the structure of the corncob pretreated sample in the optimum condition. A number of FTIR bands were employed to monitor the chemical changes in the structure of cellulose, hemicellulose, and lignin for comprehensive analysis. From the FTIR data in Figure 4.10 below the band that appeared at 3343 cm^{-1} represents OH stretching was shifted to higher frequencies in the spectrum of pretreated corncob showing an increase in the free hydroxyl groups and decreased intermolecular hydrogen bonding (Nasirpour & Mousavi, 2018). The extensive absorption at 2915cm^{-1} was attributed to C-H stretching in both samples and small shifting was observed in pretreated corncob and corncob cellulose (Ponce et al., 2021). As shown in Figure (4.10), the peak at 1730 cm^{-1} observed on raw corncob (RCC) is assigned to the indication of C = O stretching acetyl group of hemicellulose (Araújo et al., 2019). Whereas the shifting of this peak in pretreated corncob (PCC) indicates that hemicellulose was partially removed by microwave-assisted binary

alkaline treatment, this peak was completely absent in corncob cellulose (CCC due to effective hemicellulose removal by microwave-assisted binary alkaline treatment. The intensity band reduction observed at 1250 cm^{-1} for pretreated corncob (PCC) and on the corncob cellulose (CCC) confirms that the partial removal of C-O stretching of hemicellulose in the acetyl groups and C-O stretching in the aryl-ether group of lignin (Araújo et al., 2019). Another peak observed at 1641 cm^{-1} and 1637 cm^{-1} of pretreated corncob (PCC) represents the C = C stretching vibration of alkenes.

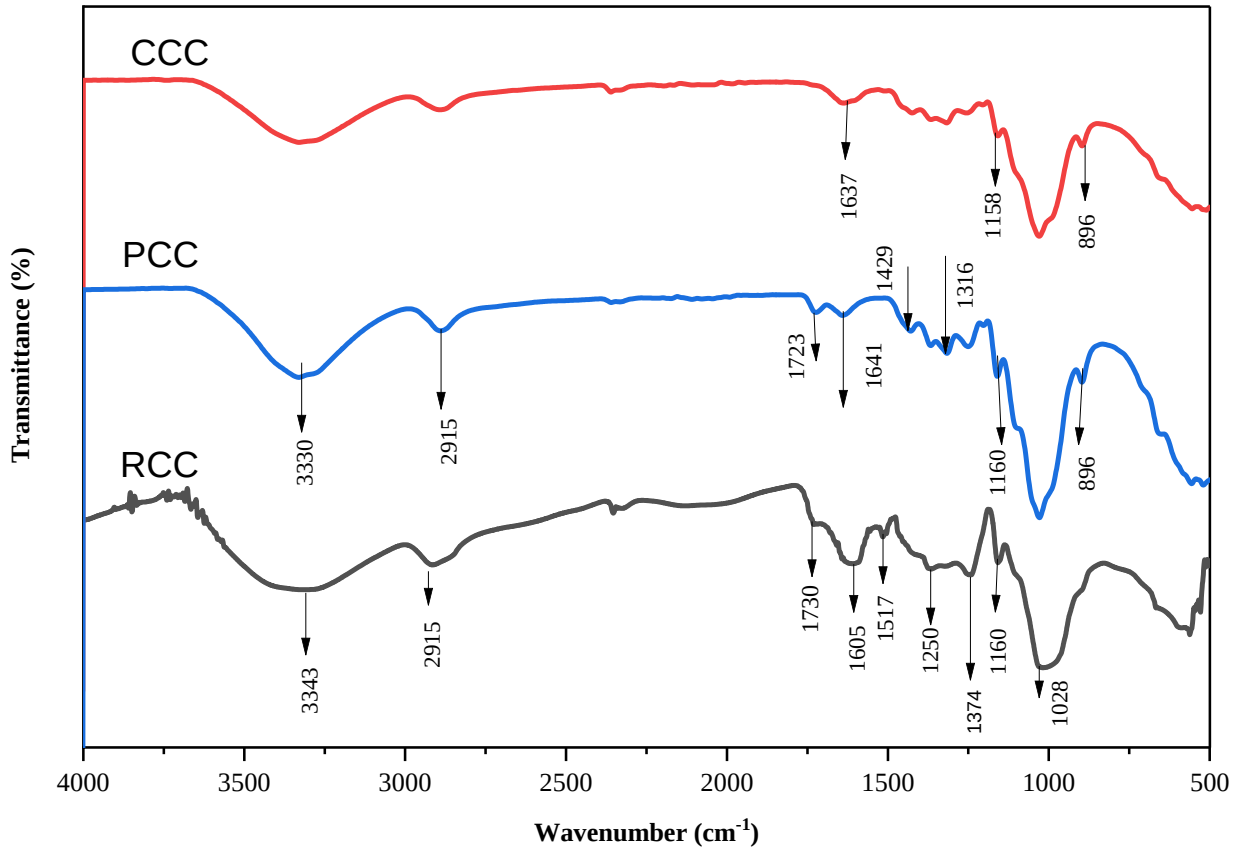

Figure 4.10 FTIR analysis of raw corncob, pretreated corncob and corncob cellulose

From Figure, it was observed that the peak at 1605cm^{-1} and 1517cm^{-1}, which were observed primarily in the raw corncob spectrum, were assigned to the indication of C-O and C = O stretching of aromatic skeletal vibrations in the lignin, respectively, which were attributed to the presence of hemicellulose and lignin. After microwave-assisted binary alkaline-treated corncob, the intensity of these bands decreased significantly (Reddy et al., 2017). Also, the total absence of this peak in

corncob cellulose (CCC) implies successful lignin removal, indicating that they are able to partially fractionate and remove non-cellulosic corncob components. The absorption bands in the spectra at 1429 cm^{-1} and 1316 cm^{-1} were assigned to symmetric CH_2 bending and wagging (Binod, Satyanagalakshmi, Sindhu, & Janu, 2012). The absorption band at 1028 cm^{-1} was associated with the vibration of C-O stretch in cellulose, and this peak became more pronounced in the treated sample, indicating that hemicellulose was removed and the distinctive cellulose peak was improved after microwave binary alkaline pretreatment (Souza et al., 2020). The absorption bands at 1158 cm^{-1} assigned to the indication C-O-C stretching at the β -(1-4) glycosidic linkages were observed in corncob cellulose (Binod, Satyanagalakshmi, Sindhu, & Janu, 2012). Finally, the presence of the peak at 896 cm^{-1} was attributed to the cellulose corresponding to the β-glycosidic link, which exists between the sugar units in hemicellulose and cellulose (Kamalini et al., 2018).

4.8. Effect of dilute acid hydrolysis of corncob cellulose on the glucose yield

Acid hydrolysis was utilized to convert carbohydrates in biomass (mostly starch and cellulose) to simple sugars for fermentative ethanol synthesis utilizing fermenting microorganisms (e.g., yeast) (Ho et al., 2013). Acid-catalyzed hydrolysis is often carried out at a mild temperature (100-250°C) (Kang et al., 2018). However, when cellulose is hydrolyzed at too high temperatures and acidic conditions, degradation products (5-HMF, levulinic acid (LA), and furfural) are produced, which may inhibit and disrupt microbial growth and metabolism throughout the fermentation process (Juarez et al., 2018). In this investigation, the pretreated corncob obtained at optimal pretreatment conditions, as mentioned above in Optimization, was used as the substrate for glucose synthesis. The cellulose in the pretreated corncob was hydrolyzed into glucose or reducing sugars using an acid solution.

The total carbohydrate content of the hydrolysates produced through microwave-assisted binary alkaline pretreatment and subsequent hydrolysis at different acid concentrations, time, and temperature was determined using phenol sulfuric acid method. To determine the total amount of glucose concentration obtained from microwave-assisted binary alkaline pretreated corncob cellulose, a standard glucose solution was prepared and the results of glucose concentration and its absorbance were tabulated in Appendix 5. The calibration curve of the glucose standard was sketched in excel, and the linear equation was generated. The linear equation and calibration

curve that were used to determine the unknown glucose concentration are shown in Appendix-6. The concentrations of unknown sugar samples were calculated using a calibration curve of glucose standard curve ($y = 0.0049x + 0.3503$; $R^2 = 0.9993$)

4.8.1. The effect of acid concentration on glucose concentration

Microwave-assisted binary alkaline pretreated corncob cellulose was further treated for determination of glucose concentration by using different sulfuric acid concentrations (1, 2, 3 and 4%) were heated in an autoclave at 120 °C for 45 minutes and the result was determined from the glucose standard curve and the result shown in Figure 4.13. The experimental result of the calculation is presented in Appendix-7. The effect of sulfuric acid concentration was evaluated from the sugar concentrations in (10 g/100mL) solid pretreated biomass slurry was contains concentrated corncob cellulose.

Table 4.16 Effect of acid concentration on glucose concentration

NO	Hydrolyzed at temperature of 120 °C for 45 min hydrolysis time	Concentration acid (%)	Absorbance at 490 nm	Glucose concentration (mg/mL)
1		1	0.5238	35.40
2		2	0.5343	37.55
3		3	0.6098	52.96
4		4	0.54079	38.90

Figure 4.11 below shows the amount of glucose concentration that was produced in the hydrolysis process. As shown from the plot increasing acid concentration from 1% to 3% the glucose concentration was increased from 35.4 to 52.96 mg/mL. However, further increases of the acid concentration (4%) decreased glucose concentration to 38.996 mg/mL. The decrease in sugar concentration with increasing acid concentration results from the degradation of monomeric sugars (xylose, glucose) to furfural and HMF, or from dehydrating or oxidizing glucose with sulfuric acid, or from the conversion of glucose to levulinic and formic acid, which results in a decrease in glucose yield (Zelelew et al., 2018). These may inhibit metabolism and

microbial growth and thus negatively affect the fermentation process (Sewsynker-Sukai & Gueguim Kana, 2017). The local optimum acid concentration was found to be 3 % and the yield of its acid concentration was 52.96 mg/mL. Similar work is also reported by (Dussan et al., 2014). As a result of (Figure 4.11), it was estimated that 3% acid concentration is the local optimal concentration for cellulose hydrolysis to glucose, and it was chosen for further investigation or to investigate the effect of pretreatment time.

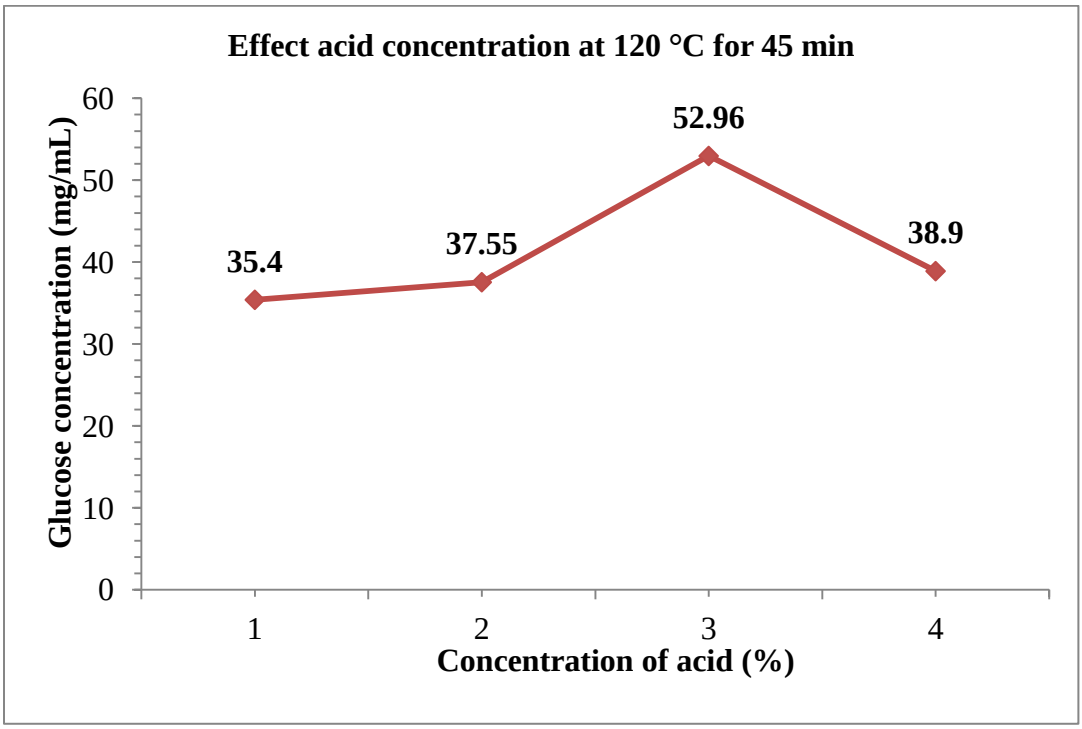

Figure 4.11 Effect of acid concentration on the glucose yield

4.8.2. The effect of hydrolysis time on glucose concentration

The microwave-assisted binary alkaline pretreated corncob cellulose, which contains hemicellulose and lignin residual, was further treated with a local optimum of acid concentration (3%) at various hydrolysis times (15, 30, 45 and 60 min) is shown in Table 4.17 below.

Table 4.17 Effect of hydrolysis time on glucose concentration

NO	Hydrolyzed at temperature of 120 °C acid concentration (3%)	Hydrolysis time (min)	Absorbance at 490 nm	Glucose concentration (mg/mL)
1		15	0.5385	38.40
2		30	0.5424	39.20
3		45	0.6353	58.16
4		60	0.5835	47.59

As shown in the Figure 4.12 the maximum sugar concentration (58.16) was obtained at the conditions of time 45 min, when acid concentration and hydrolysis temperature were constant at 3% and 120°C. The plot of hydrolysis time versus glucose concentration in Figure (4.12) shows that increasing the hydrolysis time increases the concentrations of glucose. When the hydrolysis time was increased from 15 to 45 min, the glucose concentration increased from 38.4 to 58.16 mg/mL. However, further increasing the hydrolysis time from 45 to 60 minutes reduces the glucose concentration to (47.56 mg/mL). This was due to the possibility of other molecules being formed instead of glucose or the conversion of glucose to other fermentation inhibitors such as furfural, 5-hydroxymethylfurfural (HMF), and levulinic acid (LA). These substances are harmful to yeast and can limit its growth. Therefore, the local optimum time was found to be 45min and the yield at this time was 58.16 mg/mL.

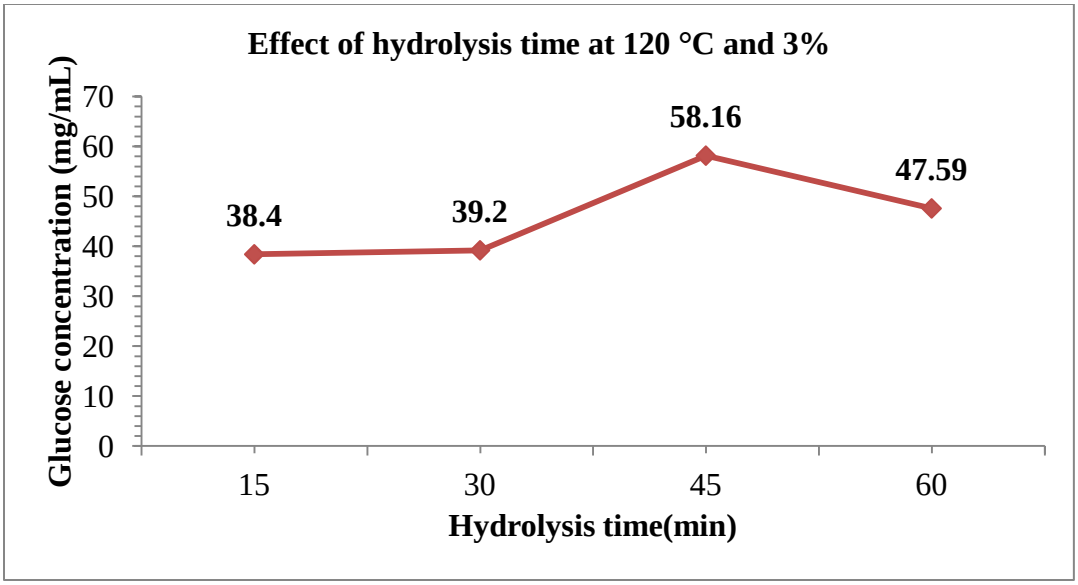

Figure 4.12 Effect of hydrolysis time on glucose concentration

4.8.3. The effect of hydrolysis temperature on the glucose concentration

The microwave-assisted binary alkaline pretreated corncob cellulose, which contains hemicellulose and lignin residual, was further treated with a local optimum of acid concentration (3%) and local optimum hydrolysis time (45min) at various heating temperatures (100,110,120 and 130 °C) is shown on the Table 4.18 below.

Table 4.18 Effect of hydrolysis temperature on glucose concentration

NO	Hydrolyzed at acid concentration (3%) local optimum hydrolysis time 45min	Heating temperature (°C)	Absorbance at 490 nm	Glucose concentration (mg/mL)
1		100	0.52276	35.20
2		110	0.53770	38.24
3		120	0.6189	54.81
4		130	0.61671	54.40

Figure 4.13 below shows the amount of glucose concentration that was produced in the hydrolysis process. As shown from the plot increasing heating temperature from 100 °C to 120 °C the glucose concentration was increased from 35.20 to 54.81 mg/mL. However, further

increases in the heating temperature (130 °C) slightly decreased glucose concentration (54.4 mg/mL). The decrease in sugar concentration with increasing heating temperature results from the degradation of obtained glucose molecules and produces byproducts 5-hydroxymethylfurfural, furfural and formic acid due to high temperature. Therefore, the optimum temperature was found to be 120℃ and the yield at this temperature was 54.81 mg/mL. Ta et al., (2016) also mentioned that an increase in temperature not only released more sugars during acid hydrolysis but also other toxic inhibitors such as furfural and 5- hydroxymethyl furfural (HMF) due to sugar degradation. Similar work is also reported by (Tizazu & Moholkar, 2018).

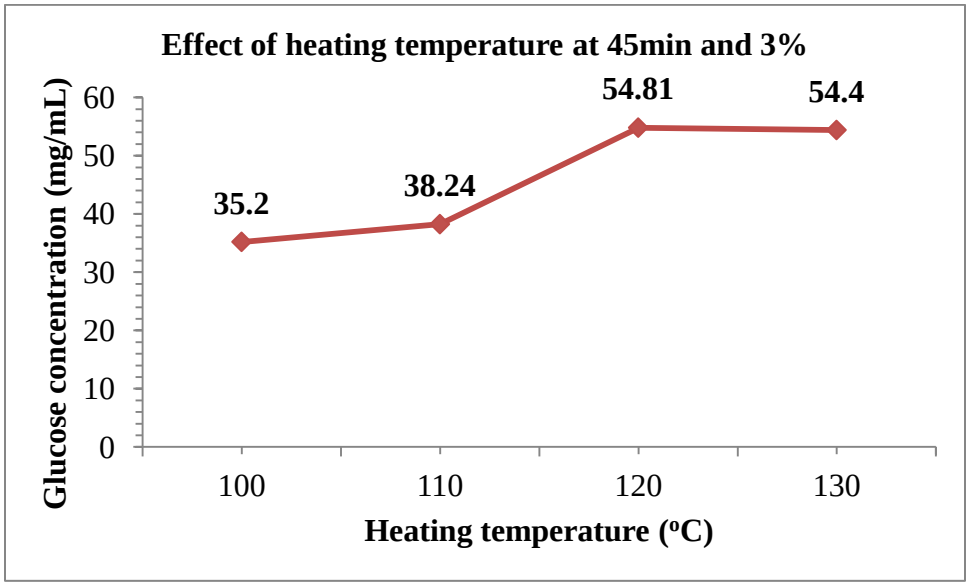

Figure 4.13 Effect of hydrolysis temperature

As shown in the above figures, the maximum sugar concentration (58.16mg/mL) was selected for fermentation purposes. It was shown that the maximum sugar concentration was obtained at the conditions of 3% acid concentration, 120 °C hydrolyzed temperature, and 45min hydrolyzed time. These results were in agreement with those of (Selvakumar et al., 2022), (Prasad et al., 2018), and other literature as shown below in Table 4.19.

Table 4.19 Summary of yield of reducing sugars reported in previous studies.

Raw material	Hydrolysis type	Hydrolysis Reaction condition	Reducing sugars	Reference
Wheat straw	Enzymatic	Cellulase enzyme (15 FPU/g	25.0	(Morikawa et

	hydrolysis	dry pretreated biomass solid), 50 ºC and 120 h	mg/mL	al., 2014)
Triticum spelta straw	Enzymatic hydrolysis	Cellulase enzyme (10 FPU/ g of dry pretreated biomass) 180 ºC and 48hr,	29.58 mg/mL	(de Barros et al., 2019)
Delonix regia Pods	enzymatic hydrolysis	Cellulase enzyme (74 filter per unit [FPU]), 50 °C and 72 h	55.57 mg/mL	(Iqbal et al., 2023)
Green seaweed Ulva rigida	Acid hydrolysis (H_2SO_4)	4% (v/v) , 120°C and 1h	34 ±0.25 mg/mL	(El Harchi et al., 2018)
Wheat straw	Acid hydrolysis (H_2SO_4)	2%, 180 ºC; 10 min	43 mg/mL	(Prasad et al., 2018)
Corncob	Acid hydrolysis (H_2SO_4)	3%v/v, 120 ºC for 45 min	55.4 mg/mL	(Selvakumar et al., 2022)
Corncob	Acid hydrolysis (H_2SO_4)	3%,120,45min	58.16mg/L	This study

As a result of the comparison, a reasonable value of glucose concentration was achieved from microwave binary alkaline pretreated corncob cellulose that was depolymerized by acid hydrolysis. According to this result, it is reasonable to conclude that the sugar concentration has been improved. This is because of the effective pretreatment performed on the corncob substrate to liberate cellulose, which releases fermentable sugar.

4.9. Fermentation of glucose to bioethanol

4.9.1. Determination of bioethanol concentration

To analyze the effectiveness of the microwave-assisted binary alkaline pretreatment on the conversion of glucose to cellulosic ethanol under predetermined conditions, the fermentation and bioethanol yield were determined. Fermentation was carried out at temperature (30°C), 200 (rpm) and the sample was taken at different fermentation periods. The results of bioethanol

concentration and its absorbance were tabulated in Appendix-8. And also standard calibration curve and standard bioethanol stock solution were prepared in order to determine the total concentration of bioethanol found in the fermented sample, as illustrated in Appendix-9. The fermentation was carried out for successive four days by *Saccharomyces cerevisiae*, which utilizes the recovered sugar as the nutrients and converts the glucose to bioethanol under anaerobic conditions. The result obtained during the production of bioethanol with the use of yeast *S. cerevisiae* was tabulated in Appendix-10. The result showed that the value of bioethanol production shows an increase in steady bioethanol concentration from 12 to 60 hours obtained from 3.41mg/mL to 21.93mg/mL. It shows after 60 hr a further slight increment in concentration up to 96 hours. There is a maximum bioethanol concentration (23.64mg/mL) was observed at 96 hour fermentation period. At all concentrations of the substrates, the bioethanol concentration increased steadily.

The efficiency of ethanol fermentation depends on the raw material, especially on sugar content (Maceiras et al., 2021). The initial sugar concentration was 58.16mg/mL as shown in the above in the hydrolysis step. The conversion yield of ethanol during each fermentation period was calculated by using equation (3.16) and the final conversion yield of bioethanol 79.54% was observed in this study. The result in terms of bioethanol concentration (mg/mL) and ethanol conversion yield (%) reached 23.64 mg/mL and 79.54%, respectively. The results from this study indicated that the microwave-assisted binary alkaline pretreatment method was effective in corncob pretreatment and improved bioethanol yield.

The optimum concentration of ethanol (23.64mg/mL) using microwave-assisted binary alkali pretreated corncob after the fermentation process is in good agreement with the recent reports. For example, 25.2 mg/mL ethanol with a 31.03% theoretical yield was obtained after using microwave-alkali-acid pre-treated rice straw fermented by *S. cerevisiae* at 30 °C for 48 hr (Akhtar et al., 2017). Also in another study ethanol concentration 9.45 mg/mL was obtained at 12h from dilute NaOH pretreated rice straw fermented by Saccharomyces tanninophilus (Jin et al., 2020). Another study by Mikulski & Kłosowski, (2020) also observed that ethanol concentration was 20 mg/mL and a fermentation yield above 85% for wheat biomass. The ethanol concentration and conversion yield in this investigation were improved when compared to the mentioned literature. Studies have shown that the efficiency of microwave pretreatment

improved the fermentation yield because there would be more cellulose accessible for hydrolysis.

4.9.2. Produced bioethanol Characterization using FTIR

Alcohols have characteristic IR absorptions associated with the O-H, C-O and the C-H stretching vibrations. Alcohols have characteristic IR absorptions associated with the O-H, C-O and the C-H stretching vibrations. When run like a liquid film, the region 3500-3200 cm^{-1} with a very intense and broad band indicated the O-H stretch of alcohols, while the region 1260-1050 cm-**1** confirms the C–O stretch. The bands at around 2880 and 2930 cm-1 were identified as the symmetric stretching modes of the -CH_2 and -CH_3 groups, respectively (Sani et al., 2022). Due to the confirmation of these regions, as shown below, it was guaranteed that the product obtained from microwave-assisted binary alkaline pretreatment corncob was exactly ethanol.

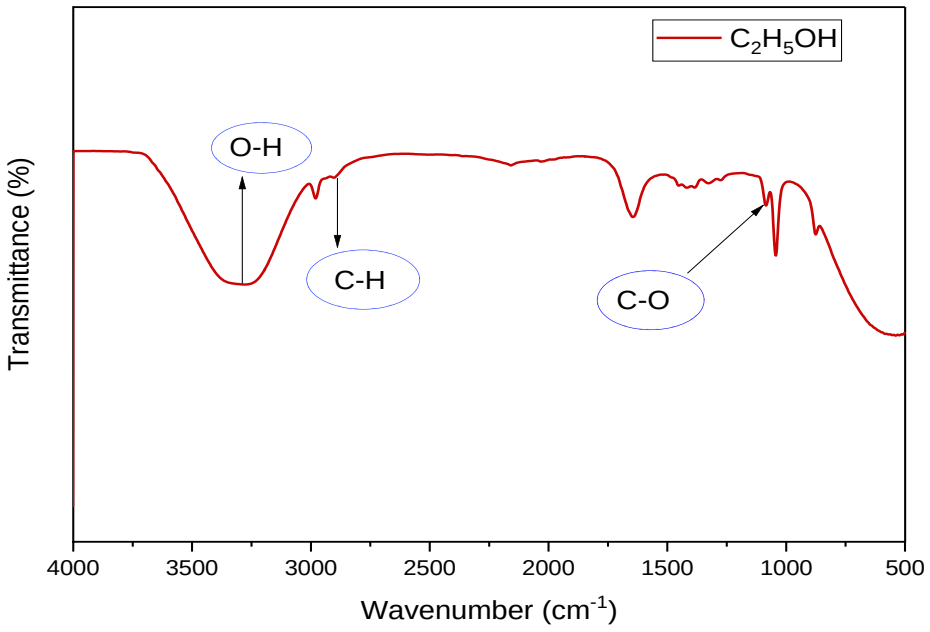

Figure 4.14 FTIR result of the bioethanol produced from microwave-assisted binary alkaline-treated corncob

CHAPTER FIVE

5. CONCLUSIONS AND RECOMMENDATION

5.1. Conclusions

Bioethanol is essential to reduce consumption of crude oil, to manage the use of resources and to reduce environmental pollution when they are produced from lignocellulosic biomass. Lignocellulosic biomass, as an accessible and cheap feedstock, is gaining popularity as a source of cellulose, hemicellulose, and fermentable sugars for the production of bioethanol. Corncobs have a high cellulose and hemicellulose content and high-molecular-weight constituents of lignocellulosic residues, which can broken down into simple sugars for microbial growth. It was easy and cost-effective for industrial bioethanol production. However, the complicated structure and recalcitrant nature of corncob was the main drawback in efficiently separating the fermentable cellulosic component. Pretreatment of corncob biomass is a precondition to overcome recalcitrance structure and effectively separate the major components (cellulose, hemicellulose, and lignin). It is one of the most crucial steps in corncob biomass conversion to bioethanol. This research focuses on developing an effective and new approach pretreatment method that efficiently separates the recalcitrant and the cellulosic components of corncob for improved bioethanol production. In this study, the application of response surface methodology on the effect of microwave-assisted binary alkaline pretreatment on corncob powder was investigated. Alkaline concentration is found to be the most influential factor in cellulose recovery and hemicellulose solubilization while all the three factors (alkaline concentration, heating time and biomass loading) showed a strong influence on cellulose recovery after pretreatment. The pretreatment condition at 400 W, 2% alkaline concentration, 10 min and 0.1g/mL biomass loading achieved high cellulose recovery and high hemicellulose removal. At this optimal pretreatment condition, 92.6% of cellulose recovery and 83.8% hemicellulose removal were obtained in the solid residue. The X-ray diffraction profile of raw, pretreated corncob and corncob cellulose showed that the crystallinity index of raw corncob was less compared to other pretreated corncob and corncob cellulose. This was due to the high recovery of cellulose in the solid residue after microwave-assisted binary alkaline treatment. The FTIR spectra confirmed that structural changes were observed due to effective removal of

hemicellulose and lignin during microwave-assisted binary alkaline treatment. The type of pretreatment and pretreatment parameters affected the glucose concentration in hydrolysates. Glucose concentration of hydrolysates was determined using phenol sulfuric acid method and gave a good glucose yield. The optimum operating condition was found to be at a temperature of 120℃, acid concentration 3%, and a hydrolysis time of 45 minutes. At these optimum operating conditions, 58.16mg/mL maximum yield of glucose concentration was obtained. Besides, surface area increment and removal of amorphous structures after microwave binary alkaline pretreatment in the corncob cellulose contributed to the improvement of the hydrolysis process for improved bioethanol production.

The investigation for bioethanol production from acid hydrolysis obtained from the pretreated corncob and fermentation by *S. cerevisiae* gave ethanol concentration of 23.64 mg/mL at operating conditions of pH 5.5, temperature 30 °C, agitation 200 rpm, and time 96 hr, which resulted in the ethanol yield of 79.54%. The characterization of produced bioethanol was performed by FTIR. From the result, the product indicated the presence of O-H, C-H, and C-O stretches similar to the standard ethanol's FTIR spectrum. This confirmed the presence of ethanol in the product.

Consequently, based on all these results, it can be concluded that microwave-assisted binary alkaline pretreatment methods were the most successful novel approach pretreatment methods for efficient cellulose recovery from corncob biomass and improved bioethanol production.

5.2. Recommendation

Based on the outcomes of this research work and overall understanding of the production process of bioethanol, the following recommendations are suggested.

- In this study, the combination of microwave and binary alkaline pretreatment methods was performed for efficient cellulose recovery. It is recommended that future studies should find other combination methods that may give the process a better efficiency.
- In the present investigation of microwave-assisted binary alkaline pretreatment of corncob, the effect of parameters such as alkaline concentration, time, and biomass loading on the recovery of cellulose and hemicellulose removal shows an excellent characteristic. However, the effect of parameters such as microwave power should be considered.
- The pretreatment process variables were optimized; It is also recommended that future studies should include optimization of hydrolysis parameters and optimization of fermentation process variables to obtain maximum yield of bioethanol from corncob.
- In this study, dilute acid hydrolysis was performed to convert pretreated samples to fermentable sugars. However, additional research on other methods of glucose extraction, such as enzymatic extraction methods, is needed to look into potential variations in the quality and yield of glucose.
- Further investigation should be done to analyze the potential of bioethanol production from corncob using a combination of yeast since xylose cannot be converted to sugar by *Saccharomyces cerevisae.*
- Further study is very important to explain how absolute bioethanol can be produced from corncob hydrolysate by using a rotary evaporator because it is challenging to produce pure ethanol since other chemicals can evaporate below the boiling point of ethanol (78 °C).

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APPENDIXES

Appendix-1. Properties of Bioethanol

Molecular formula	C_2H_5OH
Molar mass	46.07g/mol
Appearance	Colorless clear liquid
Density	0.789g/ml
Melting point	-114.3 °C, 159 K, -174 °F
Boiling point	78.4 °C, 352 K, 173 °F
Solubility in water	Fully miscible
Acidity	15.9
Viscosity	1.200 mPa·s (CP) at 20.0 °C
Dipole moment	5.64 fC·fm (1.69 D) (gas)

Appendix-2. Possible solutions of optimization

RUN	Time	Alkaline concentration(%)	Biomass loading (g/Ml)	CR (%)	desirability	
1	**11.031**	**2.889**	**0.149**	**94.333**	**1.000**	**Selected**
2	9.407	2.385	0.130	92.616	1.000	
3	9.584	2.712	0.149	93.500	1.000	
4	9.354	2.433	0.134	92.660	1.000	
5	8.950	2.939	0.148	94.079	1.000	
6	10.278	2.497	0.113	92.677	1.000	
7	9.845	2.675	0.140	93.644	1.000	
8	10.325	2.664	0.117	92.976	1.000	
9	12.658	2.979	0.149	93.314	1.000	
10	9.640	2.923	0.137	94.261	1.000	
11	9.901	2.648	0.123	93.349	1.000	
12	9.940	2.828	0.127	93.750	1.000	
13	11.589	2.852	0.130	93.414	1.000	
14	10.846	2.623	0.119	92.932	1.000	
15	10.037	2.528	0.139	93.085	1.000	

16	8.701	2.887	0.130	93.309	1.000	
17	11.492	2.896	0.141	94.011	1.000	
18	8.942	2.789	0.132	93.465	1.000	
19	10.774	2.736	0.128	93.572	1.000	
20	10.708	2.698	0.131	93.602	1.000	
21	8.371	2.804	0.147	93.019	1.000	
22	10.194	2.454	0.120	92.911	1.000	
23	9.021	2.457	0.128	92.690	1.000	
24	9.424	2.789	0.148	93.820	1.000	
25	10.307	2.594	0.121	93.156	1.000	
26	9.601	2.359	0.124	92.673	1.000	
27	9.861	2.365	0.127	92.698	1.000	
28	10.787	2.987	0.124	93.494	1.000	
29	8.848	2.638	0.121	92.793	1.000	
30	9.939	2.778	0.125	93.600	1.000	
31	10.739	2.472	0.126	92.899	1.000	
32	10.480	2.770	0.148	93.954	1.000	
33	10.517	2.507	0.131	93.092	1.000	
34	9.033	2.837	0.117	92.635	1.000	
35	10.962	2.937	0.119	92.925	1.000	
36	9.393	2.733	0.132	93.612	1.000	
37	10.355	2.829	0.129	93.836	1.000	
38	9.979	2.759	0.117	93.058	1.000	
39	10.719	2.955	0.132	94.099	1.000	
40	11.006	2.500	0.123	92.794	1.000	
41	10.778	2.506	0.128	93.002	1.000	
42	9.210	2.549	0.114	92.632	1.000	
43	9.987	2.431	0.118	92.841	1.000	
44	8.677	2.721	0.128	93.003	1.000	
45	12.464	2.871	0.137	92.849	1.000	
46	9.721	2.476	0.113	92.664	1.000	
47	9.670	2.658	0.121	93.210	1.000	
48	10.501	2.996	0.123	93.464	1.000	

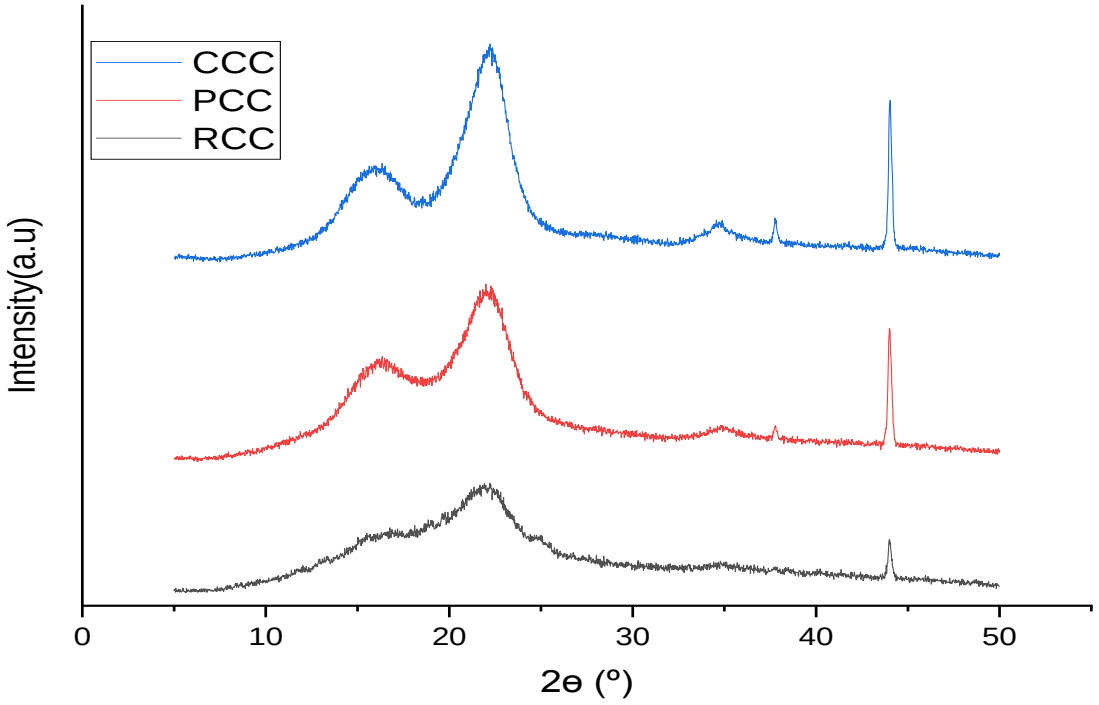
49	9.922	2.888	0.129	93.933	1.000	
50	10.947	2.556	0.126	93.034	1.000	
51	11.412	2.645	0.131	93.091	1.000	
52	9.362	2.522	0.120	92.929	1.000	
53	9.960	2.732	0.115	92.911	1.000	
54	8.422	2.625	0.127	92.603	1.000	
55	8.688	2.751	0.120	92.629	1.000	
56	9.393	2.463	0.128	92.896	1.000	
57	10.902	2.684	0.138	93.583	1.000	
58	10.491	2.823	0.145	94.216	1.000	
59	8.293	2.987	0.147	93.548	1.000	
60	10.354	2.721	0.133	93.799	1.000	
61	9.645	2.836	0.116	92.805	1.000	
62	9.303	2.425	0.115	92.628	1.000	
63	10.499	2.552	0.129	93.236	1.000	
64	10.146	2.352	0.122	92.695	1.000	
65	10.089	2.745	0.131	93.799	1.000	
66	9.612	2.676	0.134	93.595	1.000	
67	11.173	2.584	0.119	92.713	1.000	
68	10.924	2.636	0.134	93.392	1.000	
69	10.004	2.499	0.139	92.934	1.000	
70	9.644	2.498	0.120	92.963	1.000	
71	10.314	2.682	0.120	93.218	1.000	
72	9.515	2.365	0.117	92.642	1.000	
73	10.963	2.449	0.125	92.714	1.000	
74	9.993	2.521	0.113	92.736	1.000	
75	11.865	2.948	0.142	93.909	1.000	
76	9.880	2.724	0.122	93.361	1.000	
77	9.703	2.436	0.121	92.884	1.000	
78	10.173	2.483	0.132	93.058	1.000	
79	9.719	2.506	0.125	93.099	1.000	
80	9.741	2.445	0.112	92.612	1.000	
81	9.097	2.483	0.118	92.697	1.000	

82	10.694	2.437	0.116	92.636	1.000	
83	11.053	2.648	0.122	93.032	1.000	
84	9.540	2.556	0.117	92.895	1.000	
85	9.455	2.555	0.112	92.625	1.000	
86	10.980	2.568	0.119	92.793	1.000	
87	10.956	2.788	0.131	93.737	1.000	
88	10.061	2.387	0.134	92.627	1.000	
89	10.040	2.800	0.117	92.990	1.000	
90	10.612	2.952	0.117	92.806	1.000	
91	11.360	2.781	0.147	93.694	1.000	
92	9.240	2.956	0.120	92.968	1.000	
93	9.090	2.601	0.132	93.134	1.000	
94	11.014	2.924	0.131	93.915	1.000	
95	10.019	2.591	0.123	93.268	1.000	
96	9.955	2.396	0.118	92.773	1.000	
97	9.657	2.573	0.124	93.199	1.000	
98	9.977	2.685	0.129	93.603	1.000	
99	8.727	2.518	0.131	92.635	1.000	
100	9.871	2.878	0.129	93.866	1.000	

Appendix-3. Crystallinity index calculation for raw, microwave-assisted alkaline treated corncob and corncob cellulose

samples	Area of crystalline	Total area = (amorphous + crystalline)	CRI % = (Ac/At)*100
Raw Corncob	1920.098	5148.428	37.29
Microwave-assisted binary alkaline treated corncob	3435.606	6089.615	56.41
Corncob cellulose	5410.049	8165.74	66.25

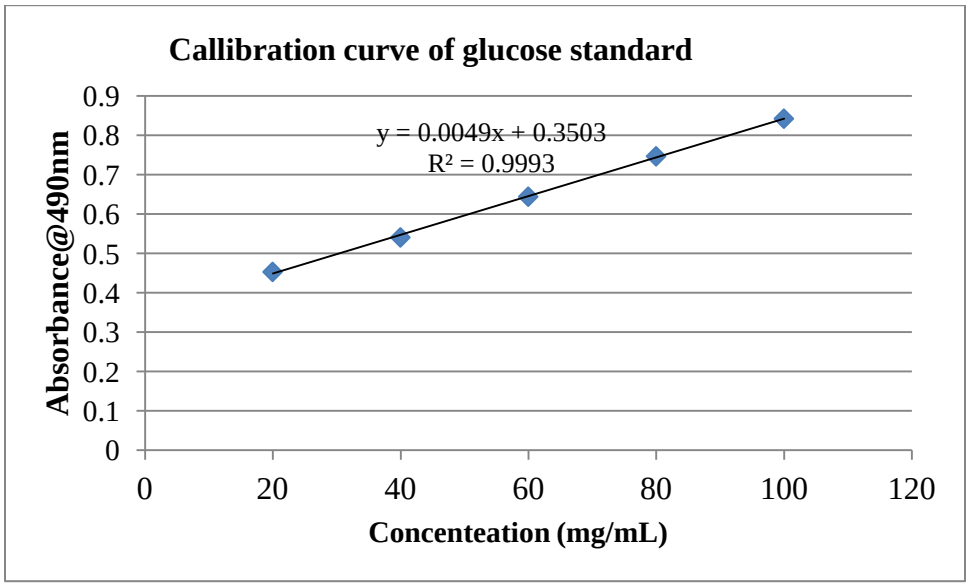
Appendix-4. XRD for raw corncob, microwave assisted alkaline treated corncob and corncob cellulose



Appendix-5. Glucose concentration and its absorbance

Glucose concentration (mg/mL)	Absorbance
20	0.4534
40	0.541
60	0.6446
80	0.7472
100	0.8428

Appendix-6. Calibration curve of glucose standard for determination of glucose content



Appendix-7. Calculation of glucose concentration and yield during acid hydrolysis

Concentrations and yield of glucose during hydrolysis

We have slope (m = 0.0049) and y intercept (b = 0.3503) then we can calculate the concentrations of unknown samples as follows:

$$\text{Con. of unknown (x)} = \frac{\text{absornace of unknown sample} - (\text{y intercept})}{\text{slope}} * 100$$

For effect of acid concentration

$$\text{Con} = \frac{0.5238 - 0.3503}{0.0049} = 35.4\text{mg/mL}$$

$$\text{Con} = \frac{0.5343 - 0.3503}{0.0049} = 37.55\text{mg/mL}$$

$$\text{Con} = \frac{0.6098 - 0.3503}{0.0049} = 52.96\text{mg/mL}$$

$$\text{Con} = \frac{0.54079 - 0.3503}{0.0049} = 38.90\text{mg/mL}$$

For effect hydrolysis time

$$\text{Con} = \frac{0.5385 - 0.3503}{0.0049} = 38.40\text{mg/mL}$$

$$\text{Con} = \frac{0.5424 - 0.3503}{0.0049} = 39.20\text{mg/mL}$$

$$\text{Con} = \frac{0.6353 - 0.3503}{0.0049} = 58.16\text{mg/mL}$$

$$\text{Con} = \frac{0.5835 - 0.3503}{0.0049} = 47.59\text{mg/mL}$$

For effect hydrolysis temperature

$$\text{Con} = \frac{0.52276 - 0.3503}{0.0049} = 35.20\text{mg/mL}$$

$$\text{Con} = \frac{0.53770 - 0.3503}{0.0049} = 38.24\text{mg/mL}$$

$$\text{Con} = \frac{0.6189 - 0.3503}{0.0049} = 54.81\text{mg/mL}$$

$$\text{Con} = \frac{0.61671 - 0.3503}{0.0049} = 54.4\text{mg/mL}$$

$$\text{Glucose yield } \% = \frac{\text{sugar produced}}{\text{Raw material used}} * 100$$

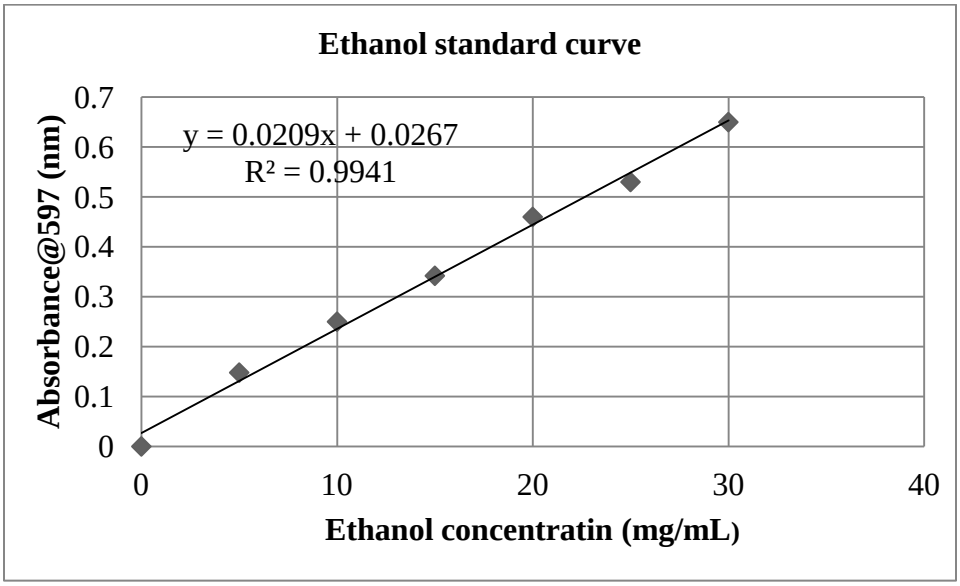
$$\text{Raw material used} = \frac{\text{gram of sample used during hydrolysis}}{\text{milliter of solution}} = \frac{5\text{g}}{50\text{ml}} = 100\ \text{mg/mL}$$

$$Glucose\ yeild\ (\%) = \frac{58.16mg/mL}{100mg/mL} = 58.16\%$$

Appendix-8. Standard ethanol concentration and its absorbance

Standard Ethanol concentration (mg/ml)	0	5	10	15	20	25
absorbance @587nm	0.0	0.148	0.250	0.342	0.460	0.53

Appendix-9. Ethanol standard curve



Appendix-10. Ethanol concentration and ethanol yield with their respective absorbance at constant temperature (30°C) and different fermentation periods

Time (hr)	12	24	36	48	60	72	84	96
Sample Ethanol absorbance @587nm	0.0981	0.1966	0.2866	0.3794	0.4581	0.4974	0.5047	0.5208
Sample ethanol Concentration (mg/mL)	3.41	8.12	12.43	16.87	21.93	22.52	22.87	23.64
Ethanol yield (%)	11.47	27.32	41.82	56.76	73.79	75.77	76.95	79.54