OPTIMIZATION OF ENZYMATIC HRDROLYSIS OF CASSAVA BAGASSE USING CELLULASE AND PECTINASE

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CERTIFICATION

This is to certify that this research project was carried out by **TONWE OROMEWUN ASANTORITSE** (ENG1804698), a student of the Department of Chemical Engineering, University of Benin, Benin City, Edo State Nigeria.

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DEDICATION

I dedicate this project to the Almighty God who saw me through every step of this project and to the Chemical Engineering Department of the University of Benin for training me to be able to effectively carry out this research

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ABSTRACT

The process of enzymatic hydrolysis of cassava bagasse is a potentially effective method of producing biofuel, as cellulase and pectinase are essential for the conversion of biomass. The goal of this work was to compare the hydrolysis effectiveness of cellulase in isolation and in combination with pectinase over a range of time periods (6 and 24 hours). Separately, under controlled conditions, cassava bagasse was used to incubate both cellulase alone and the cellulasepectinase combo. The yield of fermentable sugars was determined by analyzing the resultant hydrolysates, which gave information about how well each combination of enzymes worked. In the first six hours, the yields of cellulase by itself were higher than those of the cellulasepectinase combination. This is explained by the greater starting activity of cellulase in hydrolyzing cellulose, the main ingredient in cassava bagasse. But compared to cellulase alone, the cellulasepectinase combination showed better hydrolysis efficiency over the longer 24-hour period, producing more fermentable sugars. Pectinase was added to help break down pectin barriers, which improved cellulase access to cellulose fibers and eventually raised hydrolysis efficiency. These findings emphasize the significance of reaction time and enzyme synergy in biomass conversion processes. The combination of pectinase and cellulase maximizes the yield of fermentable sugars from cassava bagasse over extended periods of time, despite the fact that cellulase alone may produce larger initial yields. By providing insightful information on how to best utilize enzymatic hydrolysis processes for producing biofuel, this work advances the creation of efficient and sustainable bioenergy technology.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND TO THE STUDY

The hunt for alternative energy sources has intensified due to several nations' pursuit of energy independence and the general understanding of the need to cut greenhouse gas emissions. Biofuels are anticipated to lower greenhouse gas emissions and other pollutants, lessen reliance on imported petroleum and the political and economic risk that goes along with it and boost demand and pricing for agricultural products, all of which will help to stimulate the economy.

Because its energy density, vaporization heat, air-fuel ratio, and octane number are comparable to those of gasoline, n-butanol, a typical four-carbon alcohol that is currently primarily used as an industrial solvent (Kumar et al., 2013), is regarded as an advanced transportation fuel (Xue et al., 2017; Zhao et al., 2013). Consequently, there is a great deal of interest in using fermentation to produce n-butanol from biomass (Cheng et al., 2019). But one major obstacle to producing biobutanol is the comparatively high cost of available fermentation substrates and enzyme selection for the optimal yield. (Lee et al., 2016).

It is desirable to produce biobutanol from alternative low-cost feedstocks with wide availability, such as agricultural residues, woody biomass, and industrial wastes (Gottumukkala et al., 2017; Huzir et al., 2018; Jang et al., 2012; Wang et al., 2014). Increased production of first-generation biofuels, such as ethanol and butanol, which are mostly derived from corn and sugarcane sugars, would constrain the supply of these food crops, and greatly increase food prices (Jang et al., 2012; Kumar et al., 2013; Mariano et al., 2013). It is typically disposed of in landfills as solid trash, which has a significant negative impact on the environment. Cassava bagasse hydrolysate (CBH) is an inexpensive raw material for industrial fermentation that mostly consists of glucose with a small proportion of xylose. CBH can be used to manufacture a wide range of high-value bio-based chemicals, such as organic acids, biopolymers, and biofuels (Carta et al., 1999; John et al., 2006; Jyothi et al., 2005; Lu et al., 2012; Prado et al., 2005; Thongchul et al., 2009; Woiciechowski et al., 2004; Zhang et al., 2016). Because the synthesis of butanol from cassava bagasse does not conflict with the availability of food, it is a promising feedstock for the cassava and biofuel industries.

For lignocellulosic biomass to be efficiently converted into biobutanol, enzyme synergy is essential. Biobutanol is produced primarily by the enzymatic saccharification and fermentation process, for which Clostridia is the most crucial industrial production strain. (Guo et al., 2022). The synergistic enzymatic saccharification and fermentation of lignocellulosic biomass can lead to efficient hydrolysis and fermentation of the biomass, resulting in higher yields of biobutanol.

The optimization of enzyme loading and pretreatment methods is essential for efficient hydrolysis of the biomass. For example, the optimization of sodium hydroxide pretreatment and enzyme loading for efficient hydrolysis of rice straw has been shown to improve succinate production. Similarly, biobutanol production from 2-year-old willow biomass by acid hydrolysis and acetone-butanol-ethanol fermentation has been reported. In addition to the optimization of enzyme loading and pretreatment methods, the use of microbial consortium for biobutanol production with bioaugmentation has been shown to enhance the bioconversion of hemicellulosic biomass for biobutanol production. The use of microbial consortium can lead to more efficient utilization of the biomass, resulting in higher yields of biobutanol. The regulation and engineering of metabolic pathways for biobutanol production has also been studied. For example, the endogenous n-butanol production pathway in Saccharomyces cerevisiae has been shown to be dependent on the catabolism of threonine. The optimization of the keto-acid pathway for n-butanol synthesis has been achieved through the metabolic engineering of the threonine biosynthetic pathway.

In summary, the synergistic enzymatic saccharification and fermentation of lignocellulosic biomass, the optimization of enzyme loading and pretreatment methods, the use of microbial consortium for biobutanol production, and the regulation and engineering of metabolic pathways for biobutanol production are all important factors for efficient biobutanol production. These factors can lead to more efficient utilization of biomass, higher yields of biobutanol, and more sustainable and cost-effective biobutanol production processes.

The study's multiple contributions to tackling urgent global concerns and seizing opportunities make it important for manufacturing biobutanol from cassava bagasse.

There are various advantages of biobutanol, including its potential to be used as an alternative to gasoline (Ibrahim et al., 2018). Because of its increased energy content and decreased tendency to pollute, biobutanol is a possible alternative to gasoline. It has a larger caloric value than bioethanol and hence performs better as a fuel. Biobutanol, a biofuel derived from renewable sources, presents a range of advantages that position it as a promising alternative to traditional fossil fuels.

The first and most important advantage of biofuels and in this case bio butanol is lower emissions, when used as a fuel it produces fewer greenhouse gases and pollutants compared to the conventional gasoline, which in turn contributes to cleaner air and a healthier environment. Biobutanol production can boost energy security by creating jobs, utilizing different raw materials, and decreasing reliance on foreign oil supply.

- **Infrastructure and compatibility**: in contrast to ethanol, biobutanol is less corrosive and can be utilized in already-existing pipelines, tanks, filling stations, and pumps without the need for changes.
- **Possibility for Greenhouse Gas Reduction**: Biobutanol helps to mitigate climate change by absorbing carbon dioxide during feedstock growth and lowering total greenhouse gas emissions.
- **Versatility in Blending**: Without requiring major changes to cars or engines, biobutanol can be blended or utilized straight with gasoline at different concentrations.
- **Transport Options**: Due to its immiscibility with water, biobutanol can potentially be transported through pipelines, offering cost-effective transportation methods. (Hauwa et al., n.d.)

1.2 PROBLEM STATEMENT

Research on the synergy of enzymes with cassava bagasse aims to tackle the problem of producing bio-butanol from lignocellulosic biomass efficiently. A widely available agricultural residual, cassava bagasse contains cellulose, hemicellulose, and lignin that can be fermented to produce fermentable sugars and then bio-butanol. Nevertheless, the intricate composition of lignocellulosic biomass renders it impervious to enzymatic hydrolysis, hence restricting sugar yields and the efficiency of bio-butanol production.

Studies on enzyme synergy look into how several enzymes, including hemicellulases, cellulases, and auxiliary enzymes like pectinases, work together to enhance the hydrolysis of cassava bagasse.

The goal of these investigations is to increase the yields of bio-butanol by breaking down cellulose and hemicellulose into fermentable sugars by optimizing enzyme combinations and reaction conditions.

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1.3 AIM AND OBJECTIVES

The aim of this project is to carry out enzyme synergy studies which is key to effectively breaking down the complex lignocellulosic structure of biomass into fermentable sugars efficiently,

OBJECTIVES

- Carrying out a preliminary assessment on a feedstock for biobutanol production based on availability, environmental impact, chemical composition, and economic feasibility.
- To ascertain the chosen feedstock's carbohydrate content.
- To compare the feedstock's characteristics before and after pretreatment in order to assess the impacts of the treatment.
- To ascertain the feedstock's yield for reducing sugar.

1.4 SCOPE

First and foremost, the study's cautious selection of cassava bagasse as the main feedstock is one of its many important facets. As a crucial component of the next bioconversion stages, cellulose accessibility can be improved by enhancing the pretreatment procedure.

It also includes strategies for minimizing the formation of undesirable byproducts and effectively managing them.

The study only looks at one feedstock; it doesn't represent second-generation feedstocks in general. The feedstock transportation and distribution necessary for the last stages of the biobutanol production process are not included in the research, which is limited to gate-to-gate analysis, which comprises feedstock pretreatment, ABE fermentation, and simultaneous recovery. Because the study is being carried out on a laboratory basis, the outcomes might not be consistent with what is needed for large-scale production. Lastly, the study was carried out to the best of existing technology and knowledge, and if biobutanol production improves, the results and data may no longer be accurate.

1.5 RELEVAVANCE OF THE STUDY

Because enzyme synergy studies optimize the enzymatic hydrolysis process, they are essential to the manufacture of biobutanol from a variety of feedstocks. The efficient conversion of biomass's complex lignocellulosic structure into fermentable sugars—a crucial stage in the synthesis of biobutanol—requires these investigations(Li et al., 2020a). Through pretreatment and investigation of mixed bacterial microflora, I hope to increase the overall efficiency of biobutanol production from various feedstocks by increasing the glucose yield from enzymatic hydrolysis.

The background highlights several crucial elements that highlight the significance of this study:

Substrate Recalcitrance: Because of its intricate structure and lignin content, cassava bagasse shows resistance to enzymatic hydrolysis. Using a mixture of enzymes that can effectively break down cellulose, hemicellulose, and other biomass components, enzyme synergy studies seek to overcome this recalcitrance.

Poor Sugar Yields: The amount of fermentable sugars available for the fermentation of bio-butanol is limited by the inefficient hydrolysis of cellulose and hemicellulose, which results in poor sugar yields from cassava bagasse. Studies on enzyme synergy aim to maximize the conversion of biomass into soluble sugars in order to raise sugar yields.

Cost and Efficiency: Enzyme combinations that are inefficient may result in excessive enzyme usage without a corresponding increase in sugar yields. This is especially true for enzyme cocktails used for biomass hydrolysis, which can be expensive.

CHAPTER TWO

2.1 ENERGY TRANSITIONS

Three primary elements are associated with energy needs: population increase, economic development, and technological advancement. Energy consumption has been rising steadily since the Industrial Revolution, keeping up with the trend of rising energy consumption per capita. It is currently generally agreed upon that developed nations should make a moral compromise in order to protect the energy supplies of the nearly 2 billion people who do not currently have access to commercial energy, and that every effort should be made to pursue the time-bound commitments on pollutant emissions reduction that were outlined and signed by the majority of nations at the Kyoto Summit in 1997.

It is convenient to identify two major transitions that contributed to the structural changes that occurred in the energy systems: the first was the development of the steam engine, which was linked to coal; the second was the diversification of primary energy sources and the related development of technologies for final use. The first transition was associated with the first and second technological transformations, whereas the second transition was somewhat associated with the third and fourth technological transformations, involving the internal combustion engine and electricity as a means of energy transportation.(de Oliveira Matias & Devezas, 2007)

Notable are a few significant factors.

- The lengthy and slow replacement of primary energy sources, such as the traditional renewable fuel of wood being replaced by fossil fuels;

- The long-term dominance of coal, which provided more than two thirds of the primary energy consumed;

- The arrival of natural gas practically simultaneously, which was initially used as a byproduct of oil extraction and then as a principal energy source;

- The oil crisis that coincided with the 1970s oil consumption peak;

- Lastly, there has been some volatility in the principal energy sources' consumption dynamics over the past 20 years.

It is simple to see that, in the 250 years from the start of the Industrial Revolution, at least five significant technological advancements have occurred in the field of socioeconomic growth. The development, synergy, and entrenchment of a cluster of innovations at each following wave, or "technosphere," causes these transitions to occur sporadically with a frequency of roughly fifty years and to be associated with long waves in economic activity [4]. The purpose of the ensuing section is to illustrate the connection between the global socioeconomic development and the replacement of primary energy sources. (de Oliveira Matias & Devezas, 2007)

The discovery of significant fossil fuel reserves, British urbanization, commerce, and technological advancements all contributed to the energy shift away from wood and toward fossil fuels. The shift started in earnest in the early 1700s, and by the end of the 19th century, nearly two centuries later, fossil fuels had surpassed the whole supply of biomass fuels to take the lead as the world's main source of energy.

But the rates of change varied per industry, ranging from 80 to 400 years (Fouquet, 2010). The lengthy and painful transformation that happened as the process of industrialization played out in the UK was sparked by high labor costs, dwindling regional wood sources, and challenges exporting wood because of its low energy density (Smil, 1994).

While wood costs skyrocketed, the rapid rise of towns like London, Manchester, Birmingham, and Liverpool unleashed a torrent of inexpensive northeastern British coal (Allen, 2009).

The UK was the first region to switch from wood to coal as its main fuel source during the first Industrial Revolution (Fouquet, 2010). The primary energy sources prior to the Industrial Revolution were human labor and the extraction of wind and water's kinetic energy. Both windmills and watermills had spread quickly throughout the United Kingdom by the eleventh century, with watermills being the most common technique. In southern and eastern England, there were 5624 watermills, or one for every 350 persons, according to the Domesday Book tally of 1086 (Smil, 1994).

Particularly paid labor played a major role in commerce throughout this transformation. While wages in the rest of Europe declined to low levels, earnings in Northwestern Europe remained strong.

When land area is fixed, it is usually only possible to employ a greater labor force if wages decrease. Nonetheless, larger towns and higher agricultural production in the growing English economy of the 1700s led to scale economies, greater efficiency, and higher wages than in other regions (Allen, 2009). Put otherwise, there was a movement towards the right in the labor demand curve. This pushed British companies to replace labor with cheaper energy sources, typically coal, and technology.

2.2 BIOBUTANOL AS AN ALTERNATE FUEL SOURCE

The reality that the world's oil reserves are running out has led to a general agreement among society that contemporary fuel and energy procurement methods are imperative. Social, economic, and political decisions are influenced by climate change and energy security, which are at the forefront of national interests. As a result, more attention needs to be paid to the study and creation of novel energy alternatives that may be able to avert issues with energy security and climate change Li et al. (2020b)

As a result, scientists are focusing on biofuels and other fuel sources as potential substitutes for oil. Numerous advantages of biofuels can hasten the global adoption of biofuels. As a result, there is a greater chance of developing sustainable and renewable fuels, less reliance on imported fossil fuels, and a larger worldwide market for agricultural products. It also reduces emissions of NOx, CO, CO2, HC, and PM. By generating jobs, the biofuels industry will also help local workers' financial situation. Currently the most popular alternative fuel are first-generation biofuels, such as bioethanol and biodiesel made from food sources. There is a competing usage for these fuels, which cannot be sustained over time, because the raw material for them can also be used to produce food and feed. As a result, it may have an impact on food supply reduction and price increases. Li et al. (2020b)

In response to this struggle between food and fuels, interest is starting to focus on waste biomass, biomass that is not used for food, and waste that is a byproduct of other processes that can also be utilized to generate energy without competing with food sources. The same-quality biogas, biodiesel, and bioalcohols can be produced by processing them. This is also the main distinction between alcohol and bioethanol, or more generally, bioalcohol and bioethanol. The chemical is typically referred to as "ethanol" or "alcohol" when it comes from sources like oil, but it can also be referred to as "bioethanol" or "bioalcohol" when it comes from biological matter or plant sources. Other than that, their physical and chemical compositions are identical. In addition to benefits like lowering pollution emissions, promoting the circular economy, lowering reliance on outside sources, and decreasing landfilling, fuels made from waste or byproducts have no influence on the availability of food. We refer to these fuels as second-generation biofuels.

Because of its exceptional fuel qualities (high energy density, excellent mixability, low volatility, and low corrosiveness), butanol has garnered a lot of attention as a valuable biofuel and significant platform chemical.2,3 However, for large-scale practical applications, butanol fermentation is costly due to high substrate cost, solvent toxicity, low butanol titre, and low productivity.4 Acetone-butanol-ethanol (ABE) fermentation has historically been used to produce butanol from sugars or starch as substrates. However, the cost of the substrates (approximately 60% of the total cost of ABE fermentation) greatly reduced the competitiveness of ABE fermentation, to the point where biobutanol made by Clostridium is unable to compete with petrochemical butanol. (Guo et al., 2022)

The choice of viable substrates is crucial to the manufacture of butanol. As of right now, butanol is produced using four generations of feedstocks, the advantages and disadvantages of which are outlined in Table 1 Among these, rice straw, corncobs, wheat straw, barley straw, sorghum bagasse, and various wood kinds including pine and elm have all been studied as inexpensive feedstock for the synthesis of biobutanol. Since lignocellulosic biomass is the most prevalent, economical, and sustainable type of biomass, its valorization into biofuel has the potential to yield significant advantages such as (i) fixing atmospheric CO_2, (ii) lowering air pollution from burning, and (iii) improving energy security for nations that import oil. (Guo et al., 2022)

Table 1: Different feedstocks with their benefits and limitations (Guo et al., 2022)

FEEDSTOCK	BENEFITS	LIMITATIONS
First Generation Feedstock Starch and Sucrose Feedstock	High butanol yield. Sufficient fermentable substrates through simple pre-treatment processes.	Occupies potential cropland. Competes with food supply. Significantly increases the overall cost of fermentation.
Second Generation Feedstock Lignocellulose Biomass	Cost-effective, huge carbon resources. No competition with food supply.	It is difficult to achieve sufficient fermentable sugars from complex and recalcitrant biomass. Requires complex pre-treatment and detoxification process.
Third Generation Feedstock Algal Biomass	No competition with cultivation land and food supply. Fewer or no lignin and fermentation inhibitors.	Significantly increases the cost of downstream processes due to low production and productivity of butanol. Difficult to obtain sufficient fermentable sugars.
Fourth Generation Feedstock Syngas	Increased CO_2 capture ability. Directly utilizes *clostridia* with a high production rate.	Still in its infancy as a technology. Several unknown key parameters that limit butanol production. Poor mas transfer from gas to liquid.

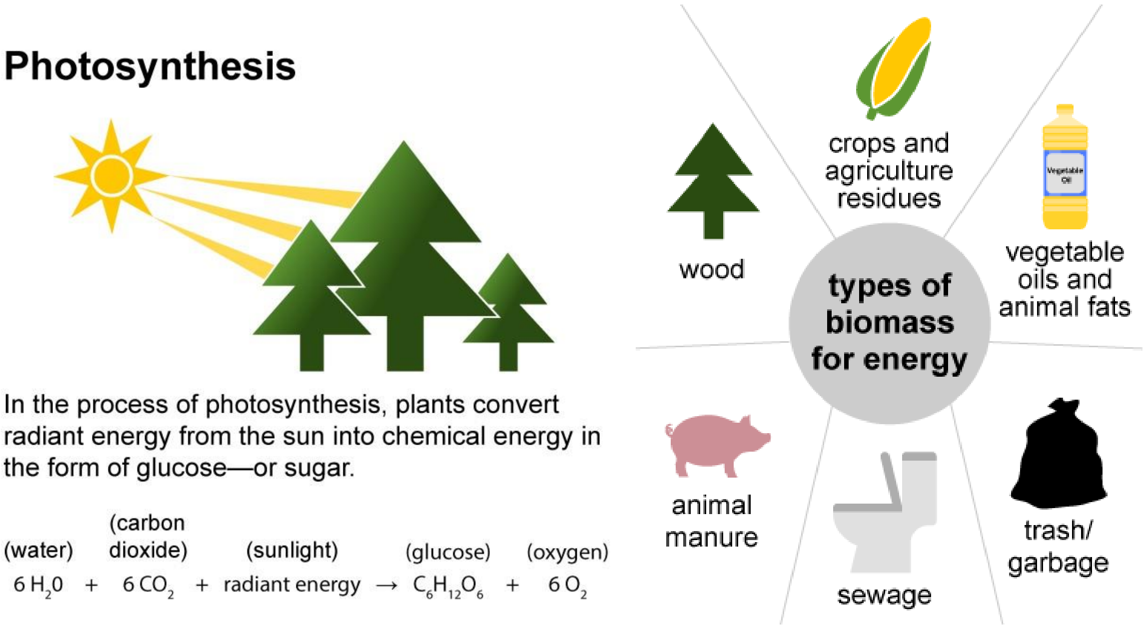
2.3 BIOMASS OVERVIEW

Renewable organic material derived from plants and animals is called biomass. The chemical energy that plants make during photosynthesis is stored in biomass. Various techniques can be used to convert biomass into liquid and gaseous fuels, or it can be burned directly for heating.

Up until the middle of the 1800s, biomass was the main source of the nation's annual energy consumption. By 2022, the United States' primary energy consumption was about five percent from biomass. Biomass is a fuel for transportation as well as a means of heating and producing energy. In many nations, biomass is a vital fuel, particularly in underdeveloped nations where it is used for heating and cooking.

2.3.1 SOURCES OF BIOMASS

- **Wood and wood processing waste**—firewood, wood pellets, and wood chips, lumber and furniture mill sawdust and waste, and black liquor from pulp and paper mills
- **Agricultural crops and waste materials**—corn, soybeans, sugar cane, switchgrass, woody plants, algae, and crop and food processing residues, mostly to produce biofuels
- **Biogenic materials in** municipal solid waste—paper products; cotton and wool products; and food, yard, and wood wastes
- Animal manure and human sewage for producing biogas (renewable natural gas)



In the process of photosynthesis, plants convert radiant energy from the sun into chemical energy in the form of glucose—or sugar.

$$6\,H_2O + 6\,CO_2 + \text{radiant energy} \rightarrow C_6H_{12}O_6 + 6\,O_2$$

Figure 1: Biomass energy formation and types of biomass

2.3.2 BIOMASS ENERGY CONVERSION

Energy is produced from biomass via a number of techniques, such as:

- Direct burning or combustion to generate heat

- Fuels can be produced by thermochemical conversion as liquids, gases, and solids.
- Transformation chemically to create liquid fuels biological reaction that yields gaseous and liquid fuels

The most popular technique for turning biomass into useable energy is direct burning. Any type of biomass can be burned directly to heat buildings and water, supply process heat for industry, and power steam turbines

Gasification and pyrolysis are two methods used in the thermochemical conversion of biomass. Both procedures involve heating biomass feedstock materials to high temperatures in closed, pressure tanks known as gasifiers in order to initiate thermal decomposition. The two primary differences between the processes are the temperatures and the concentration of oxygen during conversion.

- Pyrolysis entails heating organic materials to between 800° F and 900° F (400° C and 500° C) in the nearly complete absence of free oxygen. Biomass pyrolysis produces fuels such as charcoal, bio-oil, renewable diesel, methane, and hydrogen.

- Hydrotreating is used to process bio-oil (produced by *fast pyrolysis*) with hydrogen under elevated temperatures and pressures in the presence of a catalyst to produce renewable diesel, renewable gasoline, and renewable jet fuel.

- Gasification entails heating organic materials to between 1,400° F and 1,700 F (800° C and 900° C) with injections of controlled amounts of free oxygen or steam into the vessel to produce a carbon monoxide- and hydrogen-rich gas called synthesis gas or *syngas*. Syngas can be used as a fuel for diesel engines, for heating, and for generating electricity in gas turbines. It can also be treated to separate the hydrogen from the gas, and the hydrogen can be burned or used in fuel cells. The syngas can be further processed to produce liquid fuels using the Fischer–Tropsch process.

To create fatty acid methyl esters (FAME), which are then utilized to manufacture biodiesel, vegetable oils, animal fats, and greases are converted chemically through a procedure called transesterification.

Anaerobic digestion yields biogas and fermentation yields ethanol as two examples of biological conversion of biomass. Vehicle fuel is made of ethanol. Biogas, also known as renewable natural gas or biomethane, is created in anaerobic digesters at sewage treatment facilities as well as dairy and cattle farms. Additionally, solid waste dumps are where it forms and may contain it. The uses of properly processed renewable natural gas are identical to those of natural gas used as a fossil fuel.

2.4 COMPOSITION OF BIOMASS

Transportation fuels made from lignocellulosic biomass—woody materials like hardwoods, softwoods, pulp, or forestry residues; and herbaceous materials like grasses, straws, or dedicated energy crops—can help to support rural economies, lessen reliance on finite petroleum reserves, and cut down on greenhouse gas emissions. These biological, non-food materials are made up of cellulose and hemicellulose, two structural carbohydrates, as well as lignin and other components. Fuels can be produced from the structural carbohydrates in lignocellulosic biomass by fermentation, catalysis, or other methods if they can be effectively solubilized. Recalcitrance is the word for biomass's resistance to solubilization and degradation, and it represents a significant obstacle to the production of affordable fuels. Products with great volume but low cost, like fuel for transportation, need high feedstock-to-fuel conversion yields as essential to process economics. A high monomer sugar yield is also an important parameter for metabolic conversion pathways.

In order to ascertain the reasons behind the uncertainty in the minimum ethanol selling price (MESP) and the impact of biomass variation on MESP, summative compositional analysis techniques are employed. High-quality analytical data for the feedstock and process intermediate samples collected during the biofuel conversion process are necessary to determine these conversion yields. The overall amount of carbohydrates present in feedstocks and process intermediate samples can be found using these sulfuric acid hydrolysis techniques, but the source of the polymers or the carbohydrate links within the materials cannot be found. Therefore, when hydrolyzed collectively, glucose released from starch, cellulose, or hemicellulose is not sourcespecific but rather counts toward the total pool of glucan that is available for conversion. These biomass techniques are employed to evaluate component balance around biorefinery unit activities, compare the compositions of various lignocellulosic feedstocks, and calculate conversion yields in biomass-to-biofuel processes. These data can be used to develop or improve biofuel production processes by identifying better feedstocks and conversion pathways.(Templeton et al., 2016)

To account for all the various components (such as extractives, structural carbohydrates, lignin, protein, and ash) contained in lignocellulosic biomass, a variety of analytical techniques, both gravimetric and instrumental, are required. With these biomass approaches, the primary difficulty is in the discrete isolation and quantification of each component without any duplication of counts. Based on sulfuric acid wood lignin isolation techniques, a suite of techniques is employed at the National Renewable Energy Laboratory (NREL) and has been documented elsewhere. NREL and others have modified this suite of techniques to analyze the whole composition of herbaceous lignocellulosic biomass.

In order to measure additional components that are frequently present in herbaceous material, new tests have to be developed when converting the analytical procedures from woody to herbaceous feedstocks. (Templeton et al., 2016)

Table 2: Composition of lignocellulose on a dry basis (Sharma et al., 2019)

Lignocellulosic Materials	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Corncob	45	35	15
Wheat straw	30	50	15
Switch grass	45	31.4	12
Sugarcane bagasse	32	25	20

Other analytical methods exist to analyze biomass for different purposes such as:

X-Ray Diffraction Analysis

The amount of cellulose that is in a crystalline state as opposed to an amorphous state is measured by the degree of crystallinity, which can be determined by XRD examination.

Several pretreatment techniques, including hot water (HW), extreme-low acid (ELA), and alkaline hydrogen peroxide (AHP) pretreatment, might influence the crystallinity of cellulose. For instance, XRD research has demonstrated that while the AHP pretreatment has minimal impact, the HW and ELA pretreatments can enhance the crystallinity of cassava residue. (Jia et al., n.d.). XRD works by irradiating a material with incident X-rays and then measuring the intensities and scattering angles of the X-rays that leave the material.

Fourier Transform Infrared Spectroscopy (FTIR)

Compounds having a dipole moment absorb infrared (IR) light in a specific way based on the functional groups that make them unique, such as amide, hydroxyl, and nitrogen. Plotting the absorbance against wavelengths ranging from 700 to 400 000 nm and wave numbers from 14 000 to 25 cm^{-1} is known as an IR spectrogram. Depending on its geometry, the molecule vibrates more as the components absorb energy, stretching and bending. The absorption spectrum's pattern, which may be used for both quantitative and qualitative analysis, is similar to a molecule's fingerprint. While the intensity is dependent on the following, the IR absorption frequency is determined by the vibrational modes. (Guerrero-Pérez & Patience, 2020)

Scanning Electron Microscopy (SEM)

Across several disciplines, scanning electron microscopy (SEM), often known as SEM analysis or SEM technology, has been employed globally. It can be considered a useful technique for analyzing materials on the nanoscale to micrometer (μm) scale, both organic and inorganic. With a high magnification of up to 300,000x and even 1000000 (in some contemporary models), SEM can produce incredibly exact images of a wide variety of materials. Together with SEM, energy

dispersive X-ray spectroscopy (EDS) yields qualitative and semi-quantitative data. When combined, these methods may offer basic details on the material makeup of scanned
specimens that are not possible to obtain from standard laboratory testing. (Mohammed & Abdullah, n.d.)

2.5 BIOMASS PRETREATMENT

Thirty to fifty percent starch, ten to twenty percent cellulose, ten to twenty percent hemicellulose, and a trace quantity of lignin are found in cassava residue (Canilha et al. 2011; Wu et al. 2011; Zhao et al. 2012). According to earlier lab studies, cellulo-starch waste from the cassava starch industry has the potential to be an inexpensive substrate for the synthesis of bioethanol (Lin et al. 2016; Jia et al. 2017). Owing to its high starch concentration and low cellulose and hemicellulose contents, cassava residue could be readily converted using reasonably priced methods to produce high-value products. .. Pretreatment is typically necessary to separate the refractory structure of cassava residue, which is a lignocellulosic substrate with a complex and hard structure. This makes the cellulose and non-cellulosic fractions more accessible to the hydrolytic enzymes that can produce fermentable sugars.

According to Gallo et al. (2013) and Ge et al. (2016), pretreatments can be mechanical, thermal, chemical, or biological in nature. For instance, lignocellulosic materials treated with alkaline hydrogen peroxide (AHP) have higher glucose yields because the lignin is removed, increasing cellulose accessibility and hydrolysis degree.

Furthermore, AHP causes cellulose to swell, increasing the interior surface area that is accessible for an enzymatic treatment (Rabelo et al. 2011).

Another useful technique for dissolving hemicellulose and keeping most of the cellulose component is a diluted acid pretreatment (Llyod and Wyman 2005). Partial hemicelluloses may be hydrolyzed during the acid pretreatment, which can increase the cellulose's accessibility to enzymes. Sulfuric acid is usually used in diluted form at concentrations of 0.4 to 2.5% (w/w) and at temperatures between 100 and 200 °C. The hydrolysis of cellulosic materials has been enhanced by the use of high temperature and very low acid (ELA) (less than 0.1%) procedures, leading to even higher glucose yields.

For instance, Kim et al. (2001) reported that pure cellulose hydrolysis can yield yields of about 61%, while Lee et al. (2013a) reported that glucan yields were 4.2 and 2.4 times higher than the untreated substrate after Spiraea japonica was pretreated with ELA and then hydrolyzed enzymatically. Furthermore, Lee et al. (2013b) discovered that pretreating Lonicera japonica with ELA might significantly increase its glucan fraction and enzymatic digestibility. The moderate acidity of ELA conditions, which resemble those of a neutral aqueous system, simplifies downstream operations like neutralization and waste treatment while also significantly lowering equipment costs (Thomsen et al. 2010).

In hot water (HW) pretreatments, water serves as the reaction medium; no other chemicals are needed. According to reports, they have a low recycling and environmental cost and can release organic acids by cleaving the hemiacetal links (Laser et al. 2002; Zhang et al. 2011). However, high temperatures (140 to 220 °C) are typically used for the HW pretreatment. In general, the stabilities of cellulose, hemicellulose, and lignin to acid vary, with hemicellulose decomposing more easily than cellulose or lignin. Furthermore, biomass recalcitrance can be decreased by removing significant amounts of lignin and the majority of hemicellulose prior to cellulose degradation in the presence of diluted acid or HW pretreatment conditions (Xiao et al. 2013).

Table 3:Methods for biomass lignocellulosic pretreatment (Kumar et al., 2009)

		Operating conditions	**Advantages**	**Disadvantages**
Physical	Chipping Grinding Milling	Room temperature Energy input < 30Kw per ton biomass	Reduces cellulose critallinity	Power consumption higher than inherent biomass energy
Physio- chemical	Steam pretreatment	160-260°C (0. 69-4.83MPa) for 5-15 min	Causes hemicellulose auto hydrolysis and lignin transformation; cost-effective for hardwoods and agricultural residues	Destruction of a portion of the xylan fraction; incomplete distruption of the lignin-carboydrate matrix; generation of inhibitory compounds; less effective for softwoods
	AFEX (Ammonia fiber explosion method)	90°C for 30 min.1- 2kg ammonia /kg dry biomass	Increases accessible surface area, removes lignin and hemicellulose;	Do not modify lignin neither hydrolyzes hemicellulose;
	ARP (Ammonia recycle percolation method)	150-170°C for 14 min Fluid velocity 1cm/min	Increases accessible surface area, removes lignin and hemicellulose;	Do not modify lignin neither hydrolyzes hemicellulose;
	CO_2 explosion	4kg CO_2/kg fiber at 5.62 Mpa 160 bar for 90 min at 50 °C under supercritical carbon dioxide	Do not produce inhibitors for downstream processes. Increases accessible surface area, does not cause formation of inhibitory compounds	It is not suitable for biomass with high lignin content (such as woods and nut shells) Does not modify lignin neither hydrolyze hemicelluloses
	Ozonolysis	Room temperature	Reduce lignin content; does not produce toxic residues	Expensive for the ozone required;
	Wet oxidation	148-200°C for 30 min	Efficient removal of lignin; low formation of inhibitors; low energy demand	High cost of oxygen and alkaline catalyst
Chemical	Acid hydrolysis: dilute-acid pretreatment	Type I: T>160°, continuous-flow process for low solid loading 5-10%,)- Type II: T<160°C, batch process for high solid loadings (10-40%)	Hydrolyzes hemicellulose to xylose and other sugar; alters lignin structure	Equipment corrosion; formation of toxic substances

		Operating conditions	**Advantages**	**Disadvantages**
	Alkaline hydrolysis	Low temperature; Long time high. Concentration of the base; For soybean straw: ammonia liquor (10%) for 24 h at room temperature	Removes hemicelluloses and lignin; increases accessible surface area	Residual salts in biomass
	Organosolv	150-200 °C with or without addition of catalysts (oxalic, salicylic, acetylsalicylic acid)	Hydrolyzes lignin and hemicelluloses	High costs due to the solvents recovery
Biological		Several fungi (brown-, white- and soft-rot fungi	Degrades lignin and hemicelluloses; low energy requirements	Slow hydrolysis rates
Electrical	Pulsed electrical field in the range of 5-20 kV/cm,	~2000 pulses of 8 kV/cm	Ambient conditions; disrupts plant cells; simple equipment	Process needs more research

2.6 HYDROLYSIS OF LIGNOCELLULOSE BIOMASS

Generally speaking, lignocellulosic biomass consists of inedible plant material, such as wastes from agro-forests and specific crops of grass and wood. The primary components of lignocellulosics are cellulose, hemicellulose, and lignin.

A homopolysaccharide, cellulose is made up of □-D-pyranose units connected by ┘-1, 4-glycosidic linkages. The smallest repeated unit is called cellobiose, which is made up of two glucose monomers. Microfibrils made of long-chain cellulose polymers are assembled through hydrogen and van der Waals bonding. The microfibils are covered by lignin and hemicellulose (Fig. 2). A combination of polysaccharides, such as pentoses, hexoses, and uronic acids, makes up hemicellulose. The most complicated natural polymer is lignin, which mostly consists of phenylpropane units as its building component. (Verardi et al., n.d.)

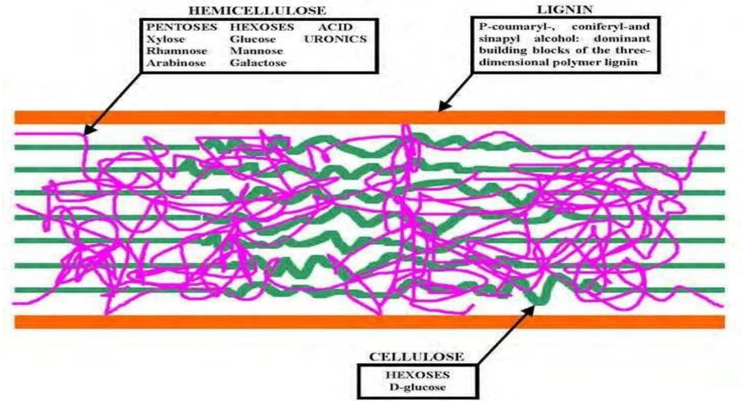


Figure 2: Lignocellulosic materials: composition of major compounds (Kumar, 2009)

Table 3: Composition of some common sources of biomass (Sun and Cheng, 2002)

Lignocellulosic materials	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Coastal bermudagrass	25	35.7	6.4
Corn Cobs	45	35	15
Cotton seed hairs	80-95	5-20	0
Grasses	25-40	35-50	10-30
Hardwoods steam	40-55	24-40	18-25

Leaves	15-20	80-85	0
Newspaper	40-55	25-40	18-30
Nut shells	25-30	25-30	30-40
Paper	85-99	0	0-15
Primary wastewater solids	8-15	NA	24-29
Softwoods stems	45-50	25-35	25-35
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Sorted refuse	60	20	20
Swine waste	6.0	28	NA
Switchgrass	45	31.4	12.0
Waste papers from chemical pulps	60-70	10-20	5-10
Wheat straw	30	50	15

The biorefinery concept suggests that lignocellulosic biomass can serve as a possible source for a number of bio-based products. At the moment, the production of the chemical industry is mostly unrepresented by the products derived from bioresources. However, the fast rising barrel costs and growing worry over the near-term depletion of fossil fuels have spurred interest in bio-based goods (Hatti-Kaul et al., 2007). The production of energy and chemicals from various biomass feedstocks by combining various technologies is the aim of the biorefinery approach (FitzPatrick et al., 2010). A general biorefinery scheme for producing fuel, composite materials, or specialized polymers is shown in Figure 3 (FitzPatrick et al., 2010).

Several additional products can be obtained after the hydrolysis of the carbohydrates in the lignocellulosic materials, in addition to ethanol. For example, the xylan/xylose found in hemicelluloses can be thermally changed into cheto compounds (hydroxy-1-propanone, hydroxy1-butanone), short chain organic acids (formic, acetic, and propionic acids), and furans (2furfuraldeyde, hydroxymethil furfural) (Güllü, 2010; Bozell & Petersen, 2010).

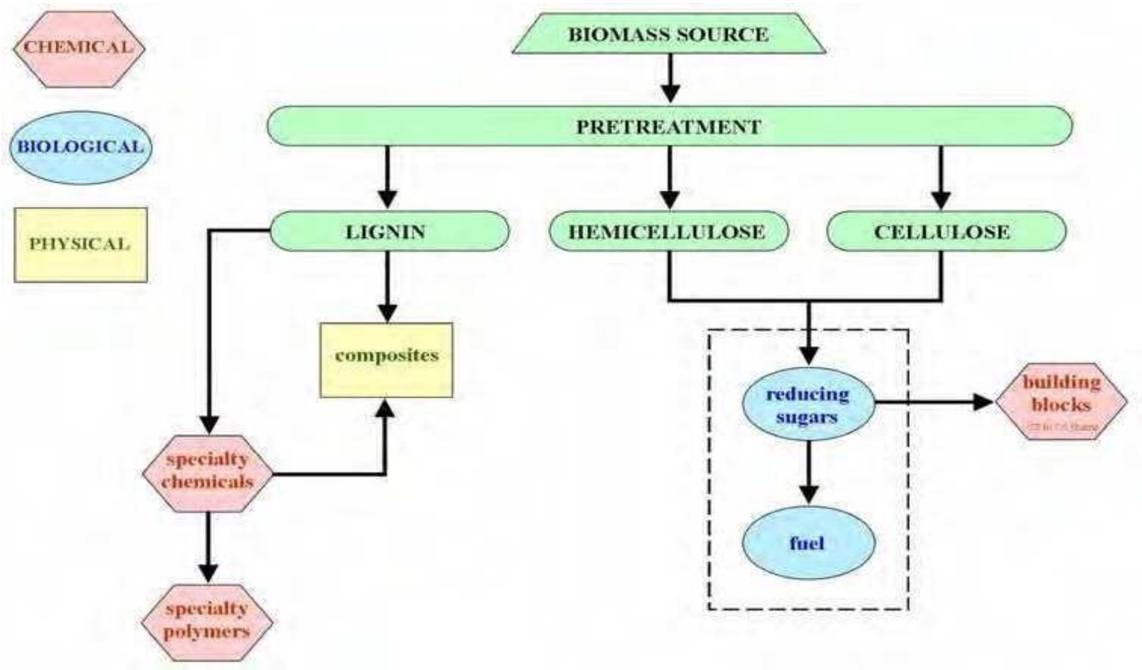


Figure 3: Scheme of a lignocellulosic biorefinery. The shape of each step describes the type of process used, chemical, biological, and physical (legend) (FitzPatrick et al., 2010)

The most widely used renewable fuel that is acknowledged as a possible substitute for transportation fuels generated from petroleum is ethanol. It can be made from lignocellulosic materials in a number of ways, but they all involve the same processes: fermentation, product recovery, and the hydrolysis of cellulose and hemicellulose to monomeric sugars (fig 4). The primary distinctions are found in the hydrolysis step, which can be carried out enzymatically, with concentrated acid, or with diluted acid (Galbe & Zacchi, 2002).

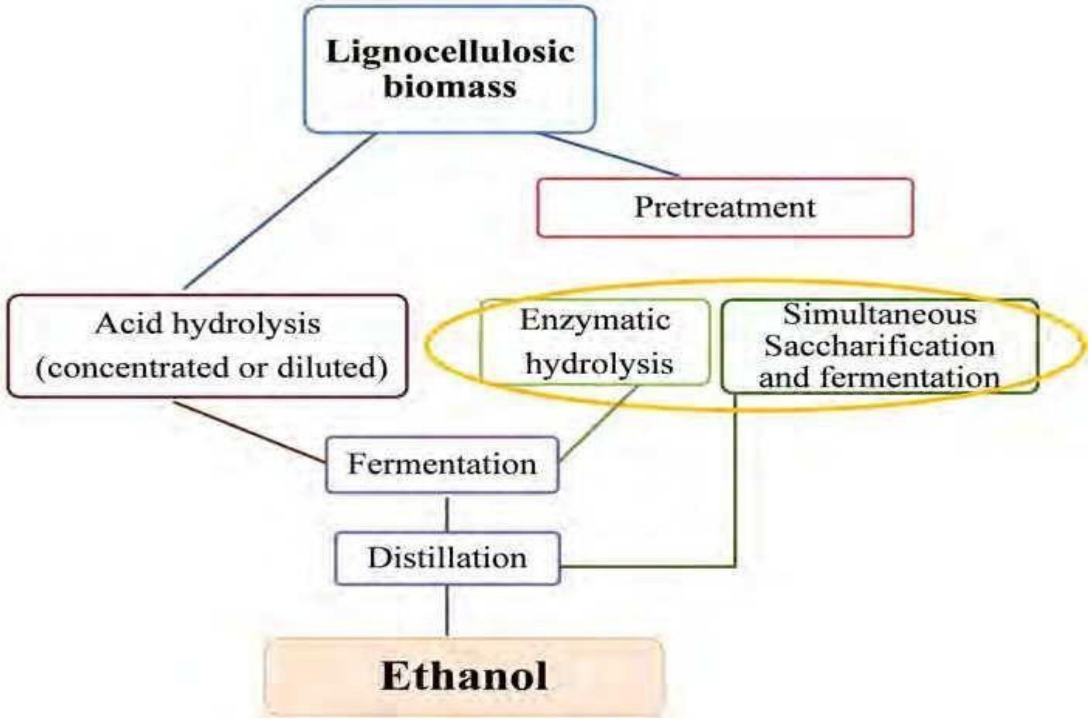


Figure 4: Process for production ethanol from lignocellulosic biomass. The circle in the scheme indicates two alternative process routes: simultaneous hydrolysis and fermentation (SSF); separate hydrolysis and fermentation (SHF).

2.6.1 ACID HYDROLYSIS

The primary benefit of acid hydrolysis is its ability to break down cellulose and hemicellulose polymers into individual sugar molecules by penetrating lignin without the need for any prior preparation of biomass. Numerous acids, either concentrated or diluted, including phosphoric, nitric, hydrocloric, hydrofluoric, sulfuric, and formic acid, can be utilized (Galbe & Zacchi, 2002). The most widely employed catalysts for the hydrolysis of lignocellulosic biomass are hydrochloric and sulfuric acids (Lenihan et al., 2010).

In the concentrated acid hydrolysis procedure, an acid concentration of between 10 and 30 percent is employed. Low temperatures throughout the procedure result in high cellulose hydrolysis yields, or 90% of the theoretical glucose yield (Iranmahboob et al., 2002).

Nevertheless, this procedure calls for a lot of acids, which can cause equipment corrosion. The primary benefit of the diluted hydrolysis method is the small amount of acid (2–5%) needed.

However, in order to achieve acceptable rates of cellulose conversion, this process is done at high temperatures. High temperatures accelerate the breakdown of hemicellulose sugars, leading to the creation of hazardous substances including 5-hydroxymethyl-furfural (HMF) and furfural. These substances reduce the rate at which ethanol is produced by inhibiting yeast cells and the fermentation process that follows (Larsson et al., 1999; kootstra et al., 2009). Furthermore, these substances result in a decrease in fermentable sugars (Kootstra et al., 2009). Furthermore, heat causes equipment to corrode more quickly (Jones & Semrau, 1984).

2.6.2 ALKALINE HYDROLYSIS

A pretreatment of lignocellulosic materials with certain bases can be applied, and the amount of lignin in the materials determines how well an alkaline pretreatment works. Compared to other pretreatment technologies, alkali pretreatment techniques use lower temperatures and pressures It is possible to do alkali pretreatment in ambient setting; however, the process takes hours or days as opposed to minutes or seconds. Alkaline techniques degrade sugar less than acid processes, and many of the caustic salts can be recovered or regenerate. Alkaline pretreatment treatments such as sodium, potassium, calcium, and ammonium hydroxides are appropriate. The most research has been done on sodium hydroxide out of these four.(Kumar et al., 2009)

Slake lime, or calcium hydroxide, has, nevertheless, been demonstrated to be a successful pretreatment agent and is the cheapest hydroxide per kilogramme. By neutralizing it with cheap carbon dioxide, calcium can be recovered from an aqueous reaction system as insoluble calcium carbonate. The calcium hydroxide can then be recycled using well-established lime kiln technology.

The process of lime pretreatment involves slurrying the lime with water, spraying it onto the biomass material, and storing the material in a pile for a period of hours to weeks. The particle size of the biomass is typically 10 mm or less. Elevated temperatures reduce contact time. The enzymatic hydrolysis of lime-treated biomass is affected by structural features resulting from the treatment. These are the extents of acetylation, lignification, and crystallization. Lime pretreatment removes amorphous substances (e.g., lignin and hemicellulose), which increases the crystallinity index. (Chang et al.) reported correlations between enzymatic digestibility and three structural factors: lignin content, crystallinity, and acetyl content. They concluded that extensive delignification is sufficient to obtain high digestibility regardless of acetyl content and crystallinity, delignification and deacetylation remove parallel barriers to enzymatic hydrolysis; and crystallinity significantly affects initial hydrolysis rates but has less of an effect on ultimate sugar yields. These results indicate that an effective lignocellulose treatment process should remove all of the acetyl groups and reduce the lignin content to about 10% in the treated biomass. Therefore, alkaline pretreatment can play a significant role in exposing the cellulose to enzyme hydrolysis. Lignin removal increases enzyme effectiveness by eliminating nonproductive adsorption sites and by increasing access to cellulose and hemicellulose. (Kim et al)

2.6.2 ENZYMATIC HYDROLYSIS

The cellulase-producing microorganisms use different strategies for the degradation of cellulose: anaerobic cellulolytic microorganisms produce complexed cellulase systems, known as cellulosomes; aerobic bacteria and fungi secrete soluble extracellular enzymes known as non complexed cellulase systems (Sun et al., 2002). Two recently identified bacteria, the anaerobic Fibrobacter succinogenes and the aerobic Cytophaga hutchinsonii, were shown to degrade cellulose. A third theory was put out to explain this behavior (Ilmén et al., 1997).

A straightforward cellulase mechanism. The Trichoderma reesei model is one of the noncomplexed cellulase systems that has been thoroughly studied. The saprobic teleomorph Hypocrea jecorina, or T. reesei, is well-known for its ability to synthesize extracellular enzymes with high efficiency (Bayer et al., 1998). At least seven endoglucanases, multiple glucosidases, and two cellobiohydrolases make up its non-complexed cellulase system. ß-glucosidase in T. reesei cellulases, however, is present in less amounts than those required for the effective hydrolysis of cellulose into glucose. Cellobiose is thus the main byproduct of hydrolysis. This glucose dimer exhibits high inhibition against both endo- and exoglucanases, causing the hydrolysis process to be markedly slowed down by the buildup of cellobiose (Gilkes et al., 1991).

Observations indicate that the mechanism of cellulose enzymatic hydrolysis by T. reesei comprises three concurrent processes (Ting et al., 2009):

1. Alterations occur in both the chemical and physical properties of the cellulose solid phase. Chemical changes involve adjustments in polymerization degree, while physical modifications relate to alterations in the accessible surface area. This stage is primarily facilitated by endoglucanase enzymes.

2. Primary hydrolysis, a gradual process, entails the liberation of soluble intermediates from the cellulose surface. Cellobiohydrolase activity is instrumental in this phase.

3. Subsequent hydrolysis, termed secondary hydrolysis, involves the further breakdown of soluble fractions into lower molecular weight intermediates, eventually yielding glucose. This step, notably swifter than primary hydrolysis, relies on the action of β-glucosidases.

Moreover, a complexed cellulase system known as cellulosomes is primarily synthesized by anaerobic bacteria. While predominantly found in Firmicutes, these systems have also been identified in select anaerobic fungi species such as Neocallimastix, Piromyces, and Orpinomyces. Cellulosomes, protrusions developed on the cell wall of cellulolytic bacteria cultivated on cellulosic substrates, comprise stable enzyme complexes securely attached to the bacterial cell wall(Verardi et al., n.d.). These complexes exhibit flexibility, enabling strong binding to cellulose. A cellulosome consists of two subunit types: non-catalytic scaffoldins and enzymatic subunits. Scaffoldins serve as functional units containing multiple cohesins that selectively interact with domains of the enzymatic subunits, namely CBD (cellulose binding domains) and CBM (carbohydrate binding modules). These cohesins have complementary counterparts known as dockerins, specific to each bacterial species (Gilligan & Reese, 1954; Lynd et al., 2002; Arai et al., 2006;).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 LIST OF MATERIALS AND EQUIPMENT

Table 4:List of materials and their uses

S/N	**MATERIAL**	**USE**
1	Cassava bagasse	Lignocellulosic biomass used.
2	Sulphuric acid (H_2SO_4)	Used for acid pretreatment.
3	Distilled water	Used for washing biomass after pretreatment and neutralizing the acid.
4	Cellulase and pectinase	Enzymes used for enzyme synergy studies and hydrolysis of the biomass.
5	Sodium citrate buffer	Buffer solution used to maintain pH.
6	Bovine Serum Albumin (BSA) and Bradford reagent	Used for determining amount of protein present in the enzymes.
7	3,5-dinitrosalicylic acid (DNS)	Used for determination of reducing (glucose) sugar.
8	Filter paper	Used for filtering pretreated biomass.
9	Litmus paper	Used to check for pH of the pretreated slurry.
10	Paper tape	For labelling samples.

11	Aluminum foil	Used for protection of samples against external variables like insects.

Table 5:List of equipment and their uses

S/N	EQUIPMENT	USE
1	Autoclave chamber	Used for carrying out pretreatment of biomass.
2	Beakers, conical flasks	Used for holding pretreated and hydrolysate samples.
3	Pipette	Used for drawing fluids like enzymes, acid.
4	Measuring cylinder	For measuring fluid level.
5	Stirrer	Used for stirring solutions.
6	Weighing balance	Used for accurately measuring amounts of solids.
7	pH scale	Used for checking pH of solutions.
8	Spectrophotometer	Used for carrying out absorbance test.
9	Water bath shaker	Provides a warm environment for carrying out enzymatic hydrolysis.

3.2 METHODOLOGY

The aim of this project is to carry out enzyme synergy studies which is key to breaking down the complex lignocellulosic structure of biomass into fermentable sugars efficiently, The use of enzymatic hydrolysis to convert lignocellulosic biomass into fermentable sugars is important for efficient biobutanol production. The optimization of enzyme loading and pre-treatment methods, such as sodium hydroxide pre-treatment and acid hydrolysis, can lead to efficient hydrolysis of the biomass and higher yields of biobutanol.

3.3 PRELIMINARY STUDY

Before choosing cassava bagasse as a feedstock for the synthesis of biobutanol, a preliminary investigation was carried out. To evaluate the applicability of cassava bagasse, this study entails obtaining crucial data on availability, composition, economic viability, and environmental impact.

The key components of this preliminary study are as follows:

3.3.1 CASSAVA BAGASSE AVAILABILITY

This involved reaching out to cassava processing facilities, farms, and other locations to estimate the amount of cassava bagasse generated to evaluate the local availability of cassava bagasse.

3.3.2 CHEMICAL COMPOSITION

Detailed research was carried out on the analysis of the chemical composition of cassava bagasse to determine the percentage of cellulose and lignin as these components are crucial for subsequent conversion into fermentable sugars. Also, the composition of cassava bagasse was compared with other potential feedstock to assess its sustainability for biobutanol production.

3.3.3 ECONOMIC FEASIBILITY STUDY

An estimate of the cost was made, considering both transportation and processing costs for cassava bagasse. An extensive analysis was carried out with the objective of assessing the economic feasibility of using cassava bagasse by contrasting these expenses with those related to alternative feedstocks.

3.3.4 ENVIRONMENTAL IMPACT

A preliminary impact assessment was undertaken to evaluate the sustainability of using cassava bagasse considering factors such as energy consumption, greenhouse gas emissions and waste generation.

A thorough grasp of the practicality and feasibility of employing cassava bagasse as a feedstock for biobutanol synthesis is provided by the preliminary investigation. It provides a basis for well-informed decision-making and aids in locating potential opportunities and problems related to the selected feedstock. The project to produce biobutanol was designed and implemented in accordance with the study's conclusions.

3.5 PROTEIN ANALYSIS

Bradford reagent, BSA, and distilled water were used for the protein analysis. First, 0.1g of BSA and 10ml of distilled water were combined. Next, 200µl of Bradford reagent was added. The process was then repeated using BSA in the following weights: 0.2g, 0.3g, 0.4g, and 0.5g. To check for absorbance, each mixture was put in a different test tube and brought to the spectrophotometer after standing for ten minutes. Next, a graph was created using the absorbance values of the standards, with the y-axis denoting absorbance and the x-axis signifying concentration. The Bradford assay standard curve equation was subsequently computed.

3.5 PREPARATION OF CASSAVA BAGASSE HYDROLYSATE (PRETREATMENT)

The dried cassava bagasse from a starch factory was milled into a fine powder about (about 50–100 µm in diameter) and was pretreated by autoclaving biomass immersed in 2.06% concentrated H_2SO_4 at 117.347^{o}c for 30.98 mins, which produced a yield of 798.524g/l.

Filter paper was used to filter the pretreatment slurry, and the liquid fraction that was left over was carefully gathered into plastic bottles in preparation for a more thorough investigation. This step's goal was to separate the liquid from the solid residue so that the solubilized chemicals could be examined more closely.

After the filtering operation, distilled water was used to thoroughly wash the solid residue. Through this washing process, the solid residue's soluble components were to be eliminated, further purifying the leftover material. The purest water available, distilled water was selected to minimize any potential interference with the ensuing analyses.

This methodological approach was created to ensure the isolation and preservation of distinct components obtained during the pretreatment process, laying the groundwork for subsequent investigations and analyses aimed at understanding the composition and characteristics of the treated cassava bagasse. Following the washing process, the wet solid residue was subjected to an additional filtration step to eliminate excess moisture. This meticulous filtration procedure contributed to obtaining a more concentrated and consistent solid sample, free from undue moisture content that could potentially affect subsequent stages of the experimental process. The collected liquid fraction and the purified solid residue were then carefully stored for further analyses.

3.6 HYDROLYIS

500 ml conical flasks were used for enzymatic hydrolysis, and they were kept in a water bath shaker with a temperature of 50ºC. At pH 4.8, the enzymatic hydrolysis was carried out in a 0.05M sodium acetate buffer. 800ml of distilled water was used to dissolve 7.676 grams of sodium citrate and 4.4 grams of citric acid, with the volume then adjusted to one litre. The buffer's pH was meticulously brought down to 4.8 to foster the best possible enzyme activity.

A 1% sodium azide supplement was added to the buffer solution to guard against microbial contamination during the hydrolysis process. This microbial inhibitor was essential in preserving the enzymatic hydrolysis system's sterility.

10% (w/v) on a dry weight basis was the solid loading for enzymatic hydrolysis. To ensure a constant and regulated experimental setting for enzymatic activity, this % was computed using the substrate's starting dry weight. Enzymatic hydrolysis reactions were contained in conical flasks that were placed inside a water bath shaker. The procedure was followed precisely to break down cellulose and hemicellulose into sugars that could be fermented. To best liberate sugar from the pretreated cassava bagasse and get it ready for further fermentation procedures, this phase of enzymatic hydrolysis was carried out.

3.7 SYNERGY STUDIES

The pre-treated biomass was first hydrolysed with cellulase with different quantities for 6 and 24 hours, then the sugar yield gotten from this process was measured using the reducing sugar test and the results were recorded.

I then proceeded to combine cellulase with an accessory which in this case was pectinase and using a 1:1 ratio and different quantities (5 to 125mg protein per gram of biomass), and using the same procedure when working with cellulase alone, it was used to hydrolyze the pretreated biomass for the same variety of time (6 and 24 hrs). The sugar yielded from this was also measured and recorded using the same methods.

A two-dimensional plot of the sugar yield was plotted against enzyme concentration for each case scenario to determine the synergy between cellulase and pectinase.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 PROTEIN ANALYSIS

The protein analysis results showed how much enzyme, measured in milliliters (ml), will be used for each sample during the experiment. The different volume samples of cellulase enzyme would be employed for each biomass sample used in the cellulase hydrolysis process;

Table 6:Volume of cellulase samples needed for hydrolysis

mg/g of biomass	volume of cellulase enzyme (ml)
0	0.00
5	1.34
10	2.69
20	5.38
25	6.72
30	8.06
40	10.75
50	13.44
75	20.16
125	33.60

The same method was employed to get the volume of pectinase

Table 7: Volume of pectinase samples needed for hydrolysis

mg/g of biomass	**volume of pectinase enzyme (ml)**
0	0.00
5	1.14
10	2.27

20	4.55
25	5.68
30	6.82
40	9.09
50	11.36
75	17.05
125	28.41

To obtain the volume of cellulase and pectinase components needed for the synergy analysis, the individual volumes of cellulase and pectinase were added and then the average of this sum was taken to obtain the values of enzyme needed because of the 1:1 ratio

Table 8:Volume of cellulase and pectinase samples needed for synergy experiment

mg/g of biomass	**volume of enzyme (cellulase) in ml**	**volume of enzyme (pectinase) in ml**	**Total volume of enzyme cocktail needed for synergy analysis**
0	-	-	-
5	1.24	1.24	2.48
10	2.48	2.48	4.96
20	4.97	4.97	9.93
25	6.2	6.2	12.4
30	7.44	7.44	14.88
40	9.92	9.92	19.84
50	12.4	12.4	24.80
75	18.61	18.61	37.21
125	31.01	31.01	62.01

4.1 CHARACTERIZATION OF CASSAVA BAGASSE

The chemical and physical composition of cassava bagasse was analyzed using various methods and the results obtained before pretreatment and after pretreatment are represented by graphs and images in Figure 2-7. The methods used for the analysis are:

4.1.1 X-ray diffraction (XRD)

The method of X-ray diffraction (XRD) is used to find out if organic substances contain cellulose. The crystalline structure of cellulose usually produces distinct diffraction peaks in the XRD pattern. The common 2θ values for cellulose peaks are 14.8°, 16.3°, and 22.6°. An XRD pattern can be used to determine whether cellulose is present in a sample by comparing it to a known cellulose diffraction pattern.

4.1.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR, or Fourier Transform Infrared Spectroscopy, is used to find out whether cellulose is present in biomass samples. One well-known FTIR property of cellulose is its infrared absorption. Strong bands approximately 3300 cm^{-1} (from O-H stretching) and 2900-2800 cm^{-1} (from C-H stretching) are present.

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4.1.3 Scanning Electron Microscopy (SEM)

Key information regarding the morphological structure of materials can be obtained through the use of scanning electron microscopy, or SEM. SEM can examine the fibrous or rod-like form of cellulose under high magnification. SEM cannot be used as the major signal for determining the presence of cellulose in an organic compound, even if it can assist in identifying its presence based on its usual form. FTIR and X-ray diffraction (XRD) are two further analytical techniques that are commonly used to confirm the existence of cellulose and distinguish it from other materials that have similar structural characteristics.

BEFORE PRETREATMENT

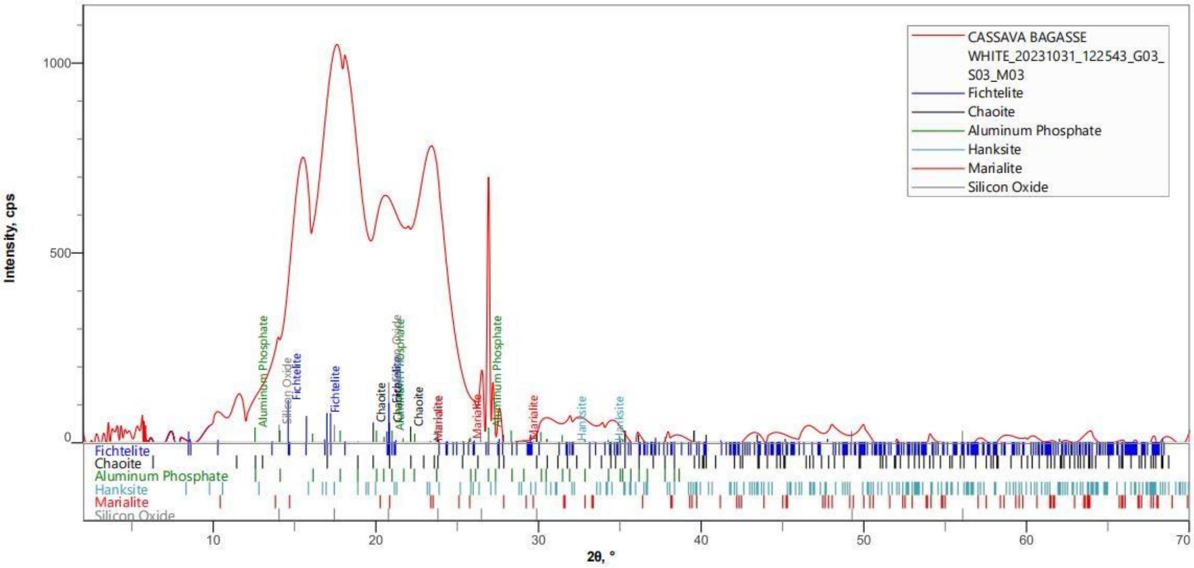


Figure 5: XRD analysis of untreated cassava bagasse

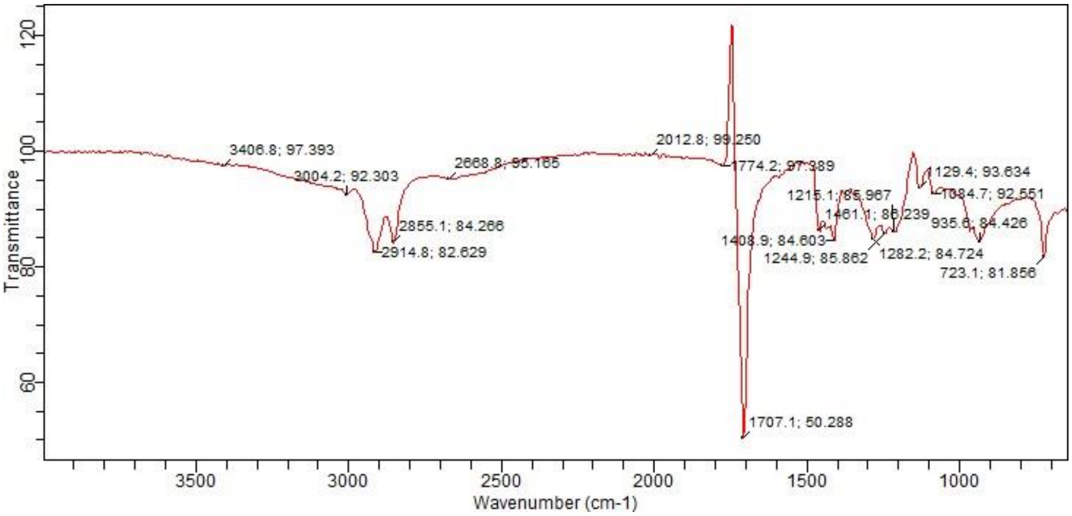


Figure 6: FTIR analysis of untreated biomass

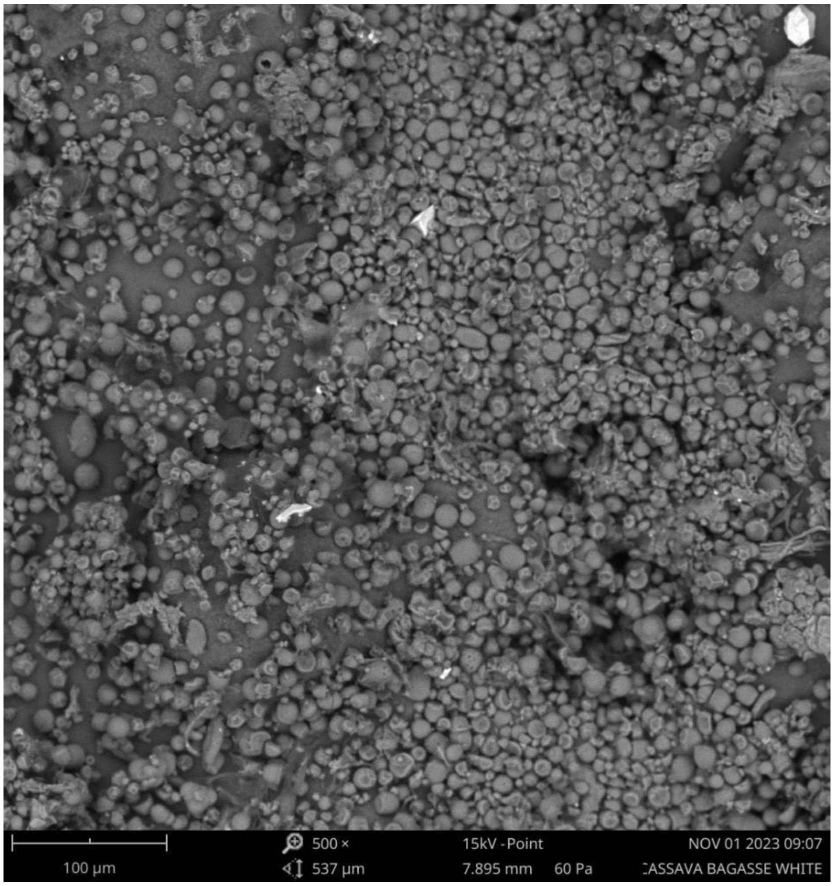


Figure 7: SEM analysis of untreated cassava bagasse

AFTER PRETREATMENT

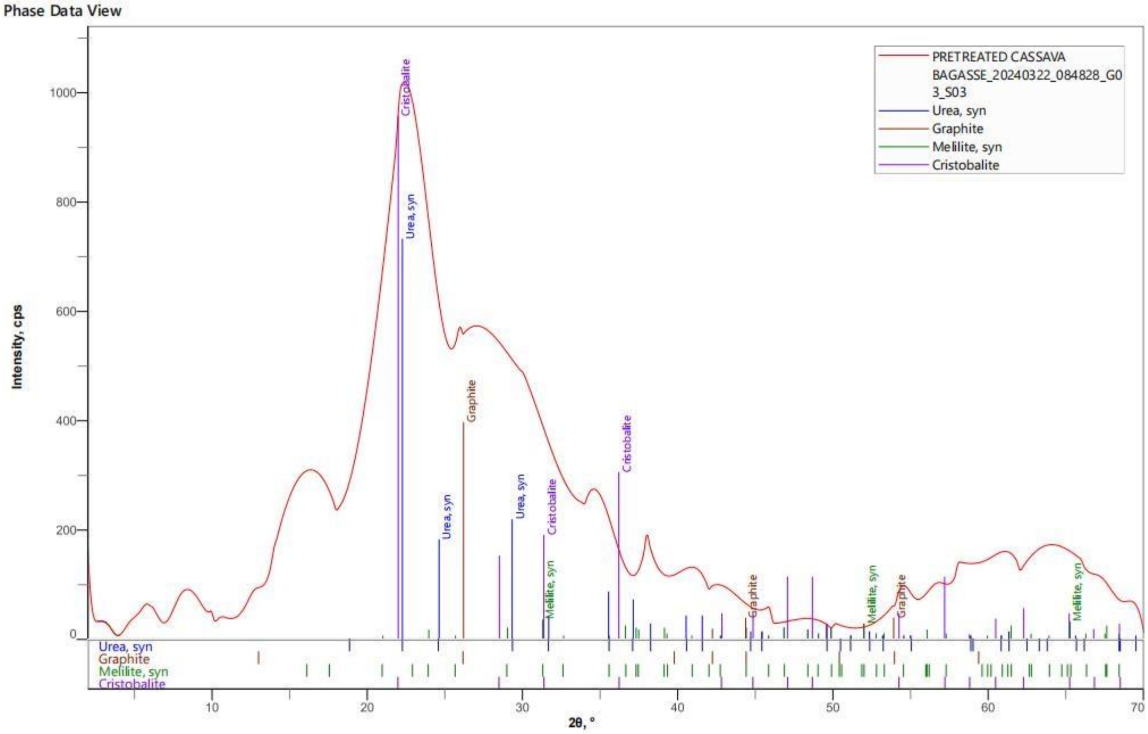


Figure 8: XRD analysis of pretreated cassava bagasse

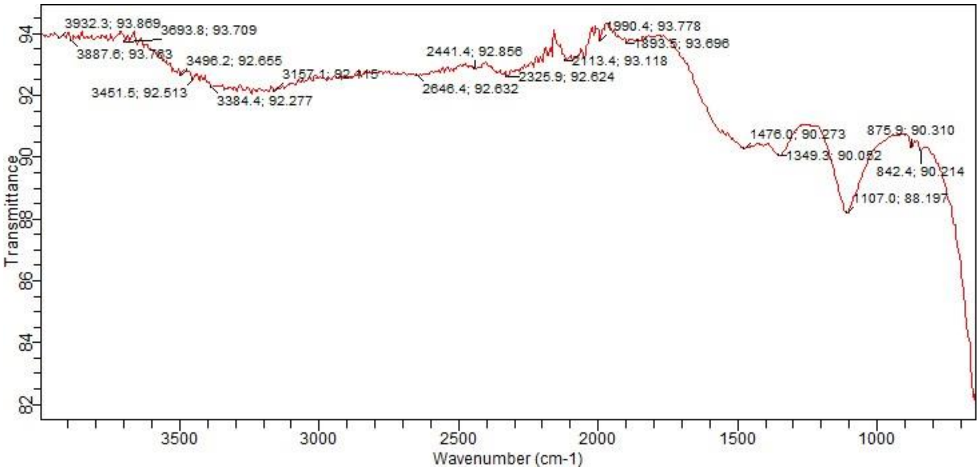


Figure 9: FTIR analysis of pretreated cassava bagasse

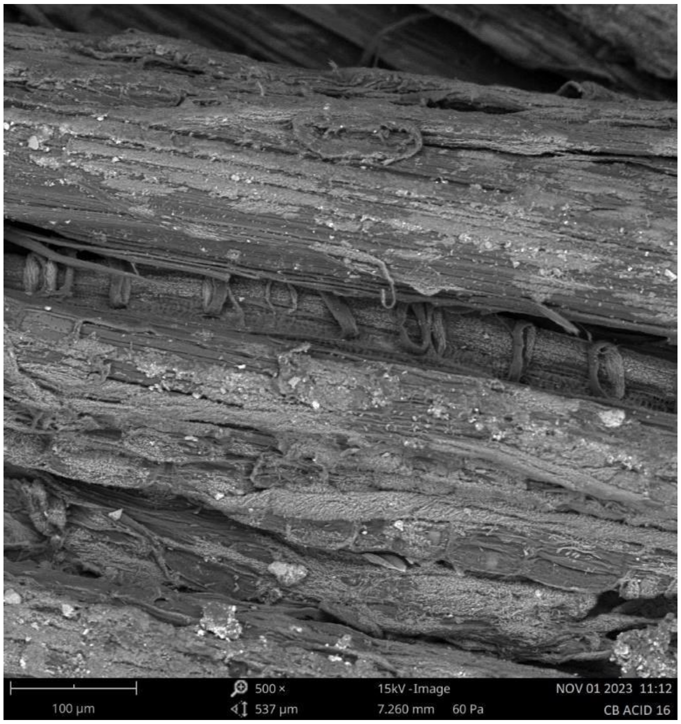


Figure 10: SEM analysis of pretreated cassava bagasse

Before pretreatment, the cellulose, hemicellulose, and lignin components of cassava bagasse usually show a complex and compact structure. The fibers contain ash and extractives, and they are covered in waxy materials. Enzymatic hydrolysis may find it challenging to access the interior structure and the surface is rough.

After pretreatment the rupturing of the lignocellulosic matrix enhances its porosity and surface area. This improves cellulose and hemicellulose accessibility for enzymes, resulting in more effective hydrolysis and increased yields of sugar. Pretreatment also results in a lower lignin content, which enhances enzyme accessibility and lessens the production of inhibitory chemicals during fermentation.

4.2 ENZYMATIC HYDROLYSIS

Cellulase alone was used to hydrolyze 5g of pretreated cassava bagasse for the selected time interval of 6hrs and then using a combination of cellulase and pectinase for the same time interval and the yield of reducing sugars for 6hrs time interval obtained are in Table 1 below

Table 9: *Concentration of reducing sugars and percentage hydrolysis for cellulase enzymatic hydrolysis for 6hrs time period*

mg/g of biomass	**Concentration of reducing sugars (g/L)**	**Percentage hydrolysis 6h (%)**
0	-	
5	0.472	5.21
10	0.918	10.14
20	0.560	6.18
25	0.855	9.44
30	1.774	19.59
40	1.276	14.09
50	1.868	20.62
75	2.114	23.35
125	2.379	26.27

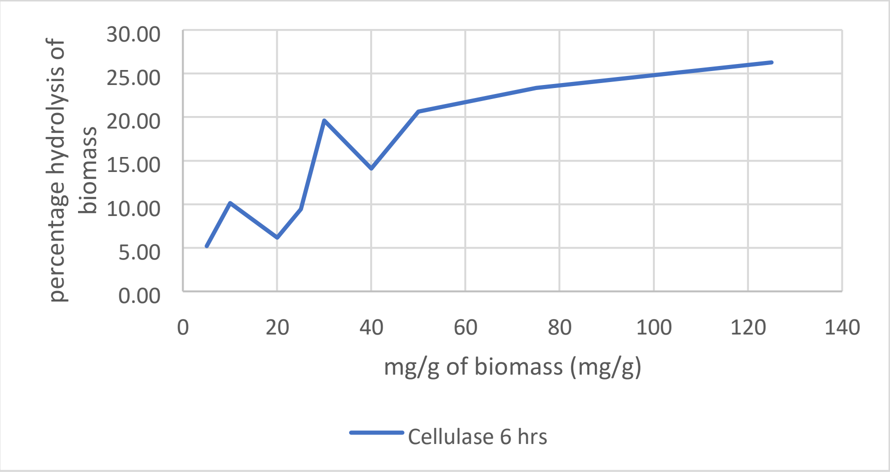


Figure 11:Graph of % hydrolysis against m/mg biomass concentration for cellulase enzymatic hydrolysis after 6 hours

Cellulase and pectinase was then used to hydrolyze also for the 6hrs time interval to compare with when cellulase was used alone

Table 10: *Concentration of reducing sugars and percentage hydrolysis for cellulase and pectinase enzymatic hydrolysis for 6hrs time period*

mg/g of biomass	**Concentration of reducing sugars (g/L)**	**Percentage hydrolysis 6h (%)**
0	-	
5	0.462	5.15
10	0.762	8.41
20	1.553	17.15
25	0.996	11.00
30	1.148	12.68
40	0.587	6.48
50	1.729	19.10
75	0.923	10.19
125	1.978	21.84

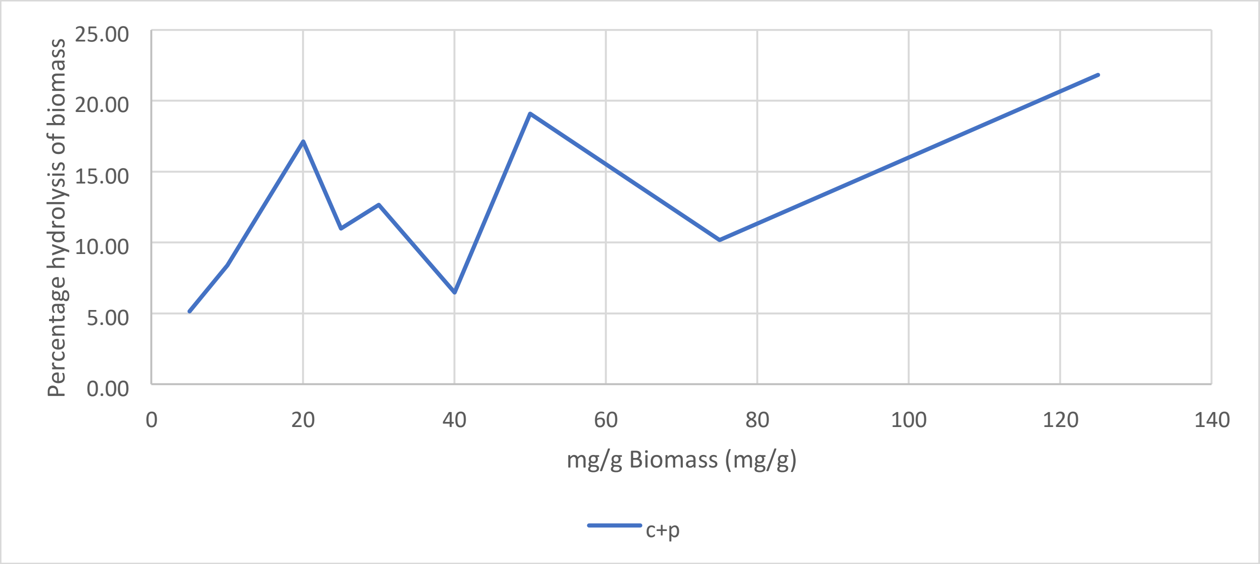


Figure 12:Graph of % hydrolysis against m/mg biomass concentration for cellulase and pectinase enzymatic hydrolysis after 6 hours

Table 11: *Concentration of reducing sugars and percentage hydrolysis for cellulase enzymatic hydrolysis for 24hrs time period*

mg/g of biomass	**Concentration of reducing sugars (g/L)**	**Percentage hydrolysis 24h (%)**
0	-	-
5	0.1202	1.33
10	0.4017	4.44
20	0.1868	2.06
25	0.2684	2.96
30	0.9481	10.47
40	0.7898	8.72
50	1.1264	12.44
75	1.0381	11.46
125	1.5378	16.98

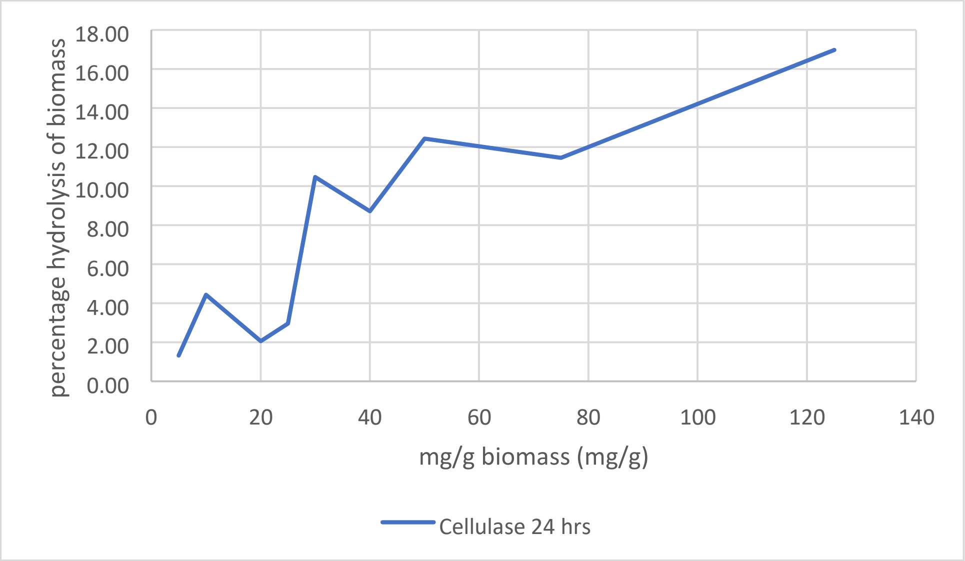


Figure 13: Graph of % hydrolysis against m/mg biomass concentration for cellulase enzymatic hydrolysis after 24 hours

Table 12: *Concentration of reducing sugars and percentage hydrolysis for cellulase and pectinase enzymatic hydrolysis for 24hrs time period*

mg/g of biomass	**Concentration of reducing sugars (g/L)**	**Percentage hydrolysis 24h (%)**
0	-	-
5	1.769	23.25
10	1.764	23.18
20	2.456	32.27
25	2.101	27.60
30	1.646	21.63
40	1.260	16.55
50	2.174	28.57
75	1.818	23.88
125	2.211	29.05

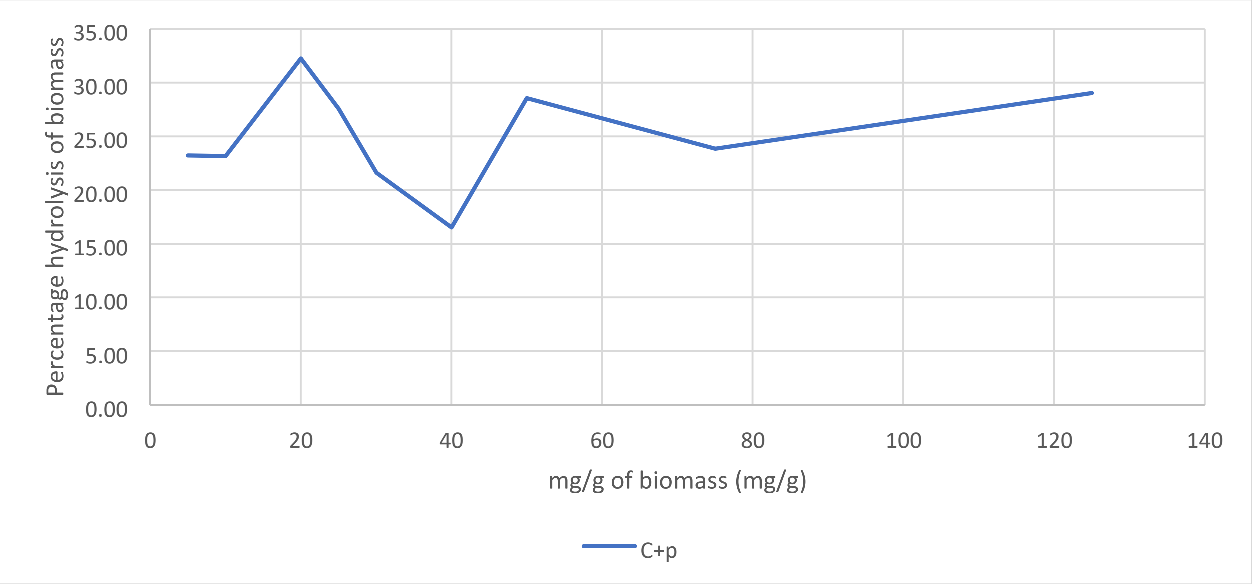
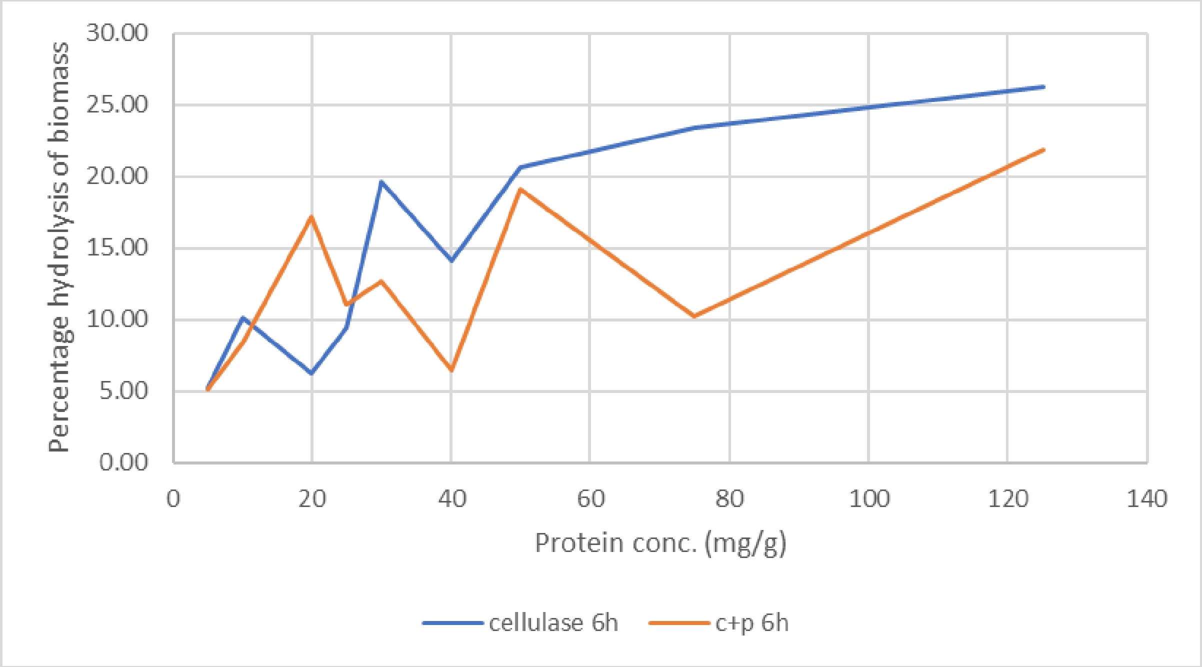
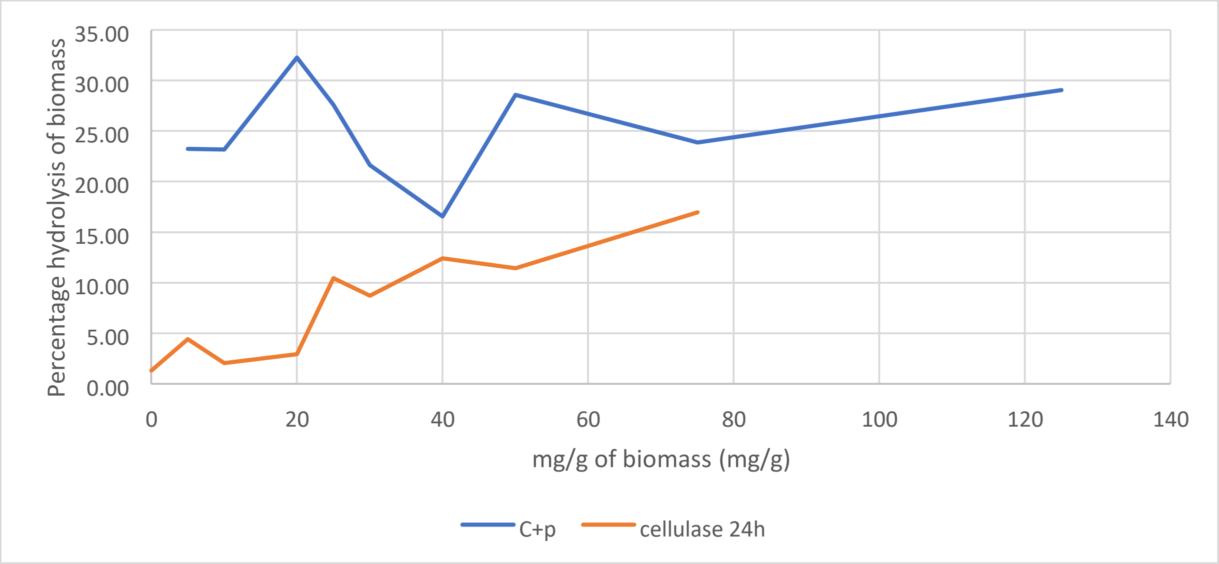


Figure 14: Graph of % hydrolysis against m/mg biomass concentration for cellulase and pectinase enzymatic hydrolysis after 24 hours





4.3 DISCUSSION

From the data in the tables showing the percentage hydrolysis firstly with cellulase alone for the 6hr and 24hr periods and secondly for a mixture of cellulase and pectinase for the same time period, it can be pictorially seen in the graphs that the mixture of pectinase and cellulase for the 6hr period has a slightly lesser percentage hydrolysis because the hydrolysis process may not have had enough time to fully take advantage of the synergistic effect of the two enzymes. As the hydrolysis time increases, the enzymes have more time to work together and break down a broader range of components, leading to a higher percentage hydrolysis for the mixture of cellulase and pectinase compared to cellulase alone for the 24hr period.

In my research and analysis, I also discovered that Enzyme activity and substrate selectivity are best achieved under distinct circumstances for cellulase and pectinase. Cellulase enzymes may hydrolyze cellulose more quickly and efficiently than pectinase in the short term (such as six hours). As a result, cellulase by itself could show somewhat greater hydrolysis percentages. On the other hand, the combined action of pectinase and cellulase over an extended period of time (like 24 hours) can result in increased hydrolysis efficiency and substrate breakdown.

Also the dynamics and kinetics of enzymes can change throughout time. Similar hydrolysis percentages for both enzyme conditions may arise from early enzymatic reactions that do not reach equilibrium or completely utilize the substrate in the near period in this case 6hts .But over an extended period of time (i.e 24 hours), the enzymes continue to catalyze events, which results in larger hydrolysis percentages and more widespread substrate destruction, especially when cellulase and pectinase work in tandem.

CHAPTER FIVE

CONCLUSION

The project aimed to determine the synergistic effect of pectinase and cellulase through enzymatic hydrolysis on cassava bagasse as a feedstock. The results of these tests were analyzed through calculated and plotted graphs for the different time intervals allowed for the enzymatic hydrolysis

Overall, the tests showed a good synergy between pectinase and cellulase particularly during the 24hr time interval. In comparison to the combination of pectinase and cellulase, cellulase alone demonstrated a greater yield during the first six hours of operation. There are multiple reasons for this. First off, the main component of cassava bagasse, cellulose, is hydrolyzed more quickly and efficiently by cellulase enzymes. Second, decreased yields in the presence of pectinase could have been caused by pectin, a polymer found in plant cell walls, which may have prevented cellulase from initially accessing cellulose fibers.

On the other hand, the combination of pectinase and cellulase showed a greater yield than cellulase alone during the longer 24-hour period. The synergistic effects of the enzymes cellulase and pectinase are responsible for this. Pectin is efficiently broken down by pectinase enzymes, which increases cellulase's accessibility to cellulose fibers and promotes a gradual improvement in hydrolysis efficiency. In addition, longer reaction times enable more thorough substrate degradation and enzyme utilization, which raises total yields when pectinase is present. These results highlight how crucial it is to take reaction time and enzyme synergy into account when converting biomass. The use of pectinase can greatly increase hydrolysis efficiency over longer times, even if cellulase alone may initially show larger yields. This will ultimately maximize the production of reducing sugars from cassava bagasse. This study contributes to the optimization of enzymatic hydrolysis processes for biofuel production, paving the way for more efficient and sustainable bioenergy technologies.

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