OPTIMIZATION STUDY AND KINETIC MODELING IN THE SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) OF CORN COB TO PRODUCE BIOBUTHANOL

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FACULTY OF ENGINEERING

UNIVERSITY OF BENIN

EDO STATE, NIGERIA.

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CERTIFICATION

This is to certify that this research project was carried out by Anesi Precious Emike with matriculation number ENG200212 in the Department of Chemical Engineering, University of Benin, Benin City, Edo State Nigeria.

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DEDICATION

This work is dedicated first to the almighty God for his love, guidance, protection and abundance of provision for me, then to my mother Mrs. Anesi for her care, love and support that have seen me through my stay in the University of Benin and to my uncle Mr. Kadiri for his support.

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My utmost gratitude goes to God almighty, for he has kept me safe and sound all through my stay in this Great University of Benin and in the cause of writing this project. For all the glory belongs to him.

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I am grateful to God for my mother, Mrs. Anesi Rabi Usman, without you I would neither be in this world nor in this great institution, I would never have made it anywhere in life, your support, advices and encouragement kept me pushing through difficulties, My brother, Mr Anesi Oshione Divine for your push and support and my Uncle Mr. Kadiri for his financial support and assistance in this project.

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ABSTRACT

Biobutanol is a renewable biofuel characterized by high energy density, low volatility, and compatibility with existing petroleum infrastructure. Despite these advantages, its large-scale production remains limited by high feedstock costs, low microbial tolerance, and process inefficiencies.

This study investigates the conversion of corn cob, an abundant lignocellulosic residue, into biobutanol through Simultaneous Saccharification and Fermentation (SSF) using *Clostridium beijerinckii*. The corn cob was pretreated with dilute sulfuric acid to enhance enzymatic accessibility, followed by detoxification and enzymatic hydrolysis using a cocktail of cellulase, β-glucosidase, and pectinase.

The hydrolysate obtained served as the substrate for SSF, and the key operational parameters were optimized using Response Surface Methodology (RSM). Fourier Transform Infrared (FTIR) spectroscopy confirmed effective delignification and structural modification after pretreatment. Optimum conditions of pH 5.48, inoculum size 9.04% (v/v), and temperature 37.45 °C produced a maximum butanol concentration of 15.60 g/L. Kinetic modeling empirical (quadratic fits / RSM) kinetic analysis accurately described substrate utilization and solvent formation.

The results demonstrate that corn cob is a viable low-cost feedstock for sustainable biobutanol production, and that the integrated SSF approach offers an efficient and environmentally responsible pathway for renewable fuel generation and agricultural waste valorization in Nigeria.

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ACRONYMS

ABE – Acetone–Butanol–Ethanol

ANOVA – Analysis of Variance

CCD – Central Composite Design

DNS – Dinitrosalicylic Acid

DOE – Design of Experiments

FTIR – Fourier Transform Infrared Spectroscopy

ISPR – In Situ Product Recovery

LAP – Laboratory Analytical Procedure

NREL – National Renewable Energy Laboratory

RSM – Response Surface Methodology

SEM – Scanning Electron Microscopy

SSF – Simultaneous Saccharification and Fermentation

SSB – Solid State Bioconversion

TS – Total Solids

VS – Volatile Solids

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

The global imperative to find sustainable energy solutions due to increasing energy demand and escalating climate change concerns has driven intense research into biofuels, a renewable liquid fuels from biological sources. These fuels are classified into three generations, with second-generation biofuels derived from non-food lignocellulosic biomass like agricultural residues standing out as particularly promising. This generation avoids competition with food crops and offers access to vast, low-cost raw materials.

Among these advanced options, biobutanol (n-Butanol) is highly attractive as a fuel alternative to gasoline. Its superior properties include a higher energy content energy density ($\approx 29\ MJ \cdot L^{-1}$), lower volatility and water absorption, and greater compatibility with existing fuel infrastructure and engines compared to ethanol. Despite these benefits, efficient large-scale production remains hampered by technical and economic hurdles like low fermentation yields, product inhibition, and expensive recovery processes.

Lignocellulosic biomass, which forms the structural components of plants (composed mainly of cellulose, hemicellulose, and lignin), can be converted into fermentable sugars via pretreatment and enzymatic hydrolysis. These sugars are then used to produce biofuels like butanol.

Corn cob, the residue left after maize kernel removal, is an excellent lignocellulosic resource for second-generation biofuels, especially in maize-producing regions like Nigeria. It is abundant and particularly well-suited for conversion due to its high cellulose and hemicellulose content and relatively low lignin. Studies show that a two-step process which includes dilute-acid pretreatment followed by enzymatic hydrolysis can yield sugar concentrations suitable for acetone–butanol–ethanol (ABE) fermentation using *Clostridium* species.

In Nigeria, the significant annual maize yield generates a massive volume of corn cob waste, which is often mismanaged (e.g., burned or discarded), contributing to pollution. Utilizing corn cob for butanol production offers a dual benefit: mitigating agricultural waste's environmental impact while creating a sustainable, locally-sourced raw material for renewable energy. This conversion provides a viable path for Nigeria to achieve energy diversification, reduce its reliance on imported petroleum, and foster rural economic growth.

1.11 TECHNICAL CHALLENGES AND SSF APPROACH

Efficient conversion requires overcoming the natural recalcitrance of the biomass. Pretreatment, such as dilute-acid hydrolysis, is crucial for breaking down the complex lignocellulosic matrix and making the polysaccharides accessible to enzymes. The resulting sugars are then fermented by bacteria like *Clostridium beijerinckii*, known for its high butanol selectivity in the ABE pathway.

The Simultaneous Saccharification and Fermentation (SSF) technique integrates hydrolysis and fermentation into a single step, promoting immediate sugar consumption, minimizing enzyme inhibition, reducing overall processing time, and potentially boosting butanol yield. Remaining challenges include the formation of inhibitory compounds during pretreatment (e.g., furfural), butanol toxicity, and the high cost of enzymes. To combat toxicity, in situ product recovery methods (like gas stripping or pervaporation) are being developed to continuously remove butanol from the fermentation broth and enhance overall productivity.

Investigating the SSF of corn cob using *Clostridium beijerinckii* is a relevant and timely endeavor. It directly addresses Nigeria’s need for sustainable, locally-sourced energy, offers an environmentally sound method for waste management, and contributes to the global advancement of second-generation biofuels and national goals for energy security.

1.2 PROBLEM STATEMENT

Nigeria's national economy and energy security are deeply vulnerable due to its heavy and persistent dependence on fossil fuels. As the primary economic engine, petroleum accounts for a staggering majority of commercial energy consumption, exceeding 85%, and contributes over 90% of the nation's export earnings (NNPC, 2022). This overdependence directly exposes the country to crippling vulnerabilities, including drastic fluctuations in global oil prices, persistent foreign exchange instability, and chronic domestic supply insecurity. Furthermore, the environmental costs of this reliance are severe, with fossil fuel combustion being a major contributor to greenhouse gas emissions, widespread urban air pollution, and the accelerating impacts of climate change. Consequently, a shift toward renewable, and sustainable energy alternatives is no longer merely an option, but an urgent national priority for economic stability and ecological health.

Concurrently with this energy crisis, Nigeria faces a significant, yet largely unaddressed, agricultural waste management challenge. The country's extensive maize cultivation generates millions of tonnes of lignocellulosic residues annually, including vast quantities of corn cobs. These corn cob residues, a potentially valuable domestic resource, are currently grossly underutilized. Instead, they are typically discarded, subjected to open-air burning, or simply left to decompose. These practices not only consume valuable land but also directly contribute to environmental degradation by releasing potent greenhouse gases, namely carbon dioxide and methane, thereby exacerbating the waste management burden on rural communities. Although corn cob possesses a composition rich in fermentable polysaccharides, specifically cellulose and

hemicellulose—its capacity as a sustainable raw material for advanced biofuel production remains practically untapped within Nigeria's industrial sector.

Globally, biobutanol has been validated as a superior next-generation biofuel when compared to bioethanol, primarily due to its higher energy density, lower vapor pressure, and inherent compatibility as a "drop-in" replacement for existing gasoline infrastructure (Dürre, 2007). However, achieving commercial-scale biobutanol production from lignocellulosic sources is obstructed by three interconnected and severe technical bottlenecks.

1.2.1 Lignocellulosic Recalcitrance: The dense, protective, and interwoven structure of the biomass requires highly effective, yet cost-efficient, pretreatment methods to successfully deconstruct the matrix and fully release the fermentable sugars.

1.2.2 Fermentation Inhibitions: The Acetone–Butanol–Ethanol (ABE) fermentation process utilizing *Clostridium* species is severely limited by the toxicity of the butanol product itself, leading to low solvent tolerance in the microorganism and drastically reduced final yields.

1.2.3 Process Economics: The necessary downstream recovery and purification of butanol from the fermentation broth are often highly energy-intensive and economically burdensome, further undermining the commercial viability of the entire process.

In the Nigerian context, these global challenges are magnified by a critical deficit in local research capacity and technological infrastructure. There is currently almost no commercial-scale technology or optimized process dedicated to addressing these integrated conversion challenges. This creates a clear and significant gap between the vast and accessible supply of domestic corn cob feedstock and the technical feasibility of economically producing high-value biobutanol via high-efficiency processes such as Simultaneous Saccharification and Fermentation (SSF). Without targeted, applied research focused on optimizing every stage from pretreatment and microbial fermentation kinetics to cost-effective recovery strategies, Nigeria will continue its dual failure: wasting an abundant, valuable biomass resource while remaining perpetually vulnerable to the volatility of imported petroleum products.

The central problem this study addresses is the lack of an optimized, integrated, and locally validated process for the effective and economically viable conversion of corn cob — a ubiquitous Nigerian agricultural residue — into biobutanol. This gap specifically necessitates research to define the optimal parameters for Simultaneous Saccharification and Fermentation (SSF) using *Clostridium beijerinckii*,.

1.3 AIM AND OBJECTIVES OF STUDY

AIM

The aim of this study is to optimize biobutanol production and develop kinetic modelling by employing Simultaneous saccharification and fermentation (SSF) of pretreated corn cob.

OBJECTIVES

To achieve this aim, the study is guided by the following specific objectives:

1. Characterization/compositional analysis of feedstock.
2. Acid pretreatment of corn cob
3. Optimization of SSF to get optimal conditions to give maximum ABE
4. Develop and validate kinetic models describing the effects of PH and Inoculum Size on biobutanol oncentration.
5. Quantification of Biobutanol in optimal ABE solvent .

1.4 SIGNIFICANCE OF STUDY

This research possesses substantial significance, offering tangible benefits and implications across scientific, socio-economic, and environmental domains for researchers, industry stakeholders, and policymakers alike.

From a scientific perspective, this study is essential for generating foundational experimental data on the potential of converting corn cob into biobutanol using the integrated approach of Simultaneous Saccharification and Fermentation (SSF) with *Clostridium beijerinckii*. The precise quantitative findings—including measured sugar yields, detailed quantification of butanol in ABE solvent that characterize the efficiency of the microbial process will serve as a crucial technical benchmark. This data is invaluable for the broader scientific community, directly informing and accelerating future research focused on optimizing biomass pretreatment protocols, developing more effective enzyme formulations, and genetically improving microbial strains for enhanced lignocellulosic biofuel production.

On the economic and social front, the utilization of corn cob, a plentiful yet currently underexploited agricultural byproduct presents clear opportunities for local value creation. This conversion pathway can actively promote the establishment of small-scale, decentralized bioenergy ventures, thereby diversifying economic activity beyond traditional farming. By creating a commercial demand for corn cob, the study offers a critical avenue for generating additional income for maize farmers and simultaneously assists local governments by lowering operational waste management costs in rural communities. Ultimately, demonstrating a viable, locally sourced, advanced biofuel route substantially bolsters Nigeria's national efforts to diversify its energy mix and sustainably boost rural economies.

Environmentally, the study champions circular economy principles by transforming an environmental liability into a valuable energy asset. Converting corn cobs into biofuel, rather than letting them decompose or be openly burned, directly reduces the release of potent greenhouse gases and significantly curbs air pollution, thereby contributing effectively to sustainable energy goals and climate change mitigation. For industry leaders and policy architects, this research delivers essential practical insights and validation necessary to guide real-world deployment. The findings can be leveraged to inform and justify investment strategies for pilot-scale implementation and to support the technology uptake required for scaling up bioenergy production. In essence, the study successfully bridges fundamental laboratory innovation with critical national development priorities, facilitating the essential transition toward cleaner, renewable, and locally sustained energy systems.

1.5 SCOPE OF STUDY

This research is meticulously defined by its focus on the laboratory-scale conversion of corn cob biomass into biobutanol via the Simultaneous Saccharification and Fermentation (SSF) approach. The scope encompasses the entire sequence of the lignocellulosic bioconversion chain, explicitly including the critical stages of pretreatment, enzymatic hydrolysis and fermentation with a central goal of optimizing the dynamic interaction between these processes to maximize the final butanol yield.

The study initiates with the pretreatment stage, employing dilute acid hydrolysis to disrupt the complex, recalcitrant lignocellulosic matrix of the corn cob. This essential step enhances the accessibility of the embedded cellulose and hemicellulose for enzymatic action. Key pretreatment parameters, including the specific acid concentration, reaction temperature, and holding time, are strategically selected based on established literature and previous optimization studies (Eboka & Egharevba, 2023) to ensure both effective delignification and the minimal formation of inhibitory compounds.

Following pretreatment, the enzymatic hydrolysis phase is carried out using a specialized cocktail of cellulolytic and accessory enzymes, primarily consisting of cellulase, β-glucosidase, and pectinase. These enzymes function to catalyze the breakdown of pretreated cellulose into fermentable monosaccharides, chiefly glucose and xylose, which serve as the essential substrates for microbial metabolism. During this phase, critical factors such as enzyme dosage, reaction PH, and temperature are rigorously maintained under controlled laboratory conditions to ensure optimal saccharification efficiency.

The fermentation stage integrates the saccharification reaction with microbial conversion using *Clostridium beijerinckii*, a well-characterized solventogenic bacterium selected for its high selectivity toward butanol within the ABE (acetone–butanol–ethanol) pathway. The utilization of the (SSF) configuration is central to this study, enabling the continuous release and immediate utilization of sugars, which effectively mitigates end-product enzyme inhibition and contributes to enhanced solvent productivity. Key operational parameters, including the broth PH, temperature, size of the microbial inoculum, and agitation rate, are systematically monitored throughout the duration of the process.

The concluding phase of this study centers entirely on the quantification of the solvents produced during ABE fermentation, rather than focusing on large-scale recovery or purification. Following fermentation, the liquid broth is prepared by centrifugation to isolate the cells from the clarified supernatant. This liquid phase is then rigorously analyzed using High-Performance Liquid Chromatography HPLC with a refractive index detector. This precise analytical method is

employed to determine the exact concentrations of butanol, acetone, and ethanol within the medium. These quantified solvent levels are crucial: they provide the necessary data to evaluate overall process performance, including conversion efficiency, microbial productivity, and the effectiveness of the SSF system.

It is important to state that the scope of this research is strictly confined to bench-scale laboratory experimentation under controlled environmental parameters. This investigation specifically excludes any extension to pilot- or industrial-scale production, comprehensive techno-economic evaluations, or detailed life-cycle assessments. Nonetheless, the resulting findings are anticipated to generate reliable baseline experimental data on the technical feasibility of converting this abundant local feedstock into biobutanol via SSF, thereby providing valuable scientific knowledge crucial for the future development of sustainable biofuel technologies in Nigeria.

CHAPTER TWO

LITERATURE REVIEW

2.1 BIOFUELS

Biofuels function as vital renewable energy carriers derived from diverse biological materials, spanning agricultural crops, forestry and agricultural residues, various industrial by-products, and microorganisms. These energy sources are generally classified into three distinct generations, with categorization based primarily on the type of feedstock utilized and the technological maturity of the conversion process. The first generation originates from edible feedstocks, such as starch from maize, sugar from sugarcane juice, or oil from oil-bearing seeds. Classic examples include ethanol derived from corn and biodiesel from vegetable oils. While these fuels were instrumental in the initial development of the bioenergy sector, their fundamental reliance on food-based raw materials has ignited major concerns regarding food security, competition for limited arable land, and the overall sustainability of large-scale production.

2.11 SECOND AND THIRD GENERATION BIOFUELS

The second generation of biofuels provides a more sustainable path by utilizing lignocellulosic biomass, encompassing crop residues, various grasses, and forestry wastes. These materials are characterized as being non-edible, widely available, and comparatively inexpensive, thus serving as superior, more sustainable alternatives to food-based feedstocks. Crucially, the conversion of lignocellulosic biomass actively promotes waste valorization and sound environmental management by effectively transforming what would otherwise be agricultural waste into valuable energy resources. Moving further into advanced technology, the third generation of biofuels is sourced from highly advanced and specialized biological systems, such as microalgae and genetically engineered microorganisms. These sources offer the promise of greater productivity potential and significantly reduced land requirements compared to conventional energy crops. However, the widespread commercialization of third-generation biofuels remains constrained by elevated cultivation and processing costs, necessitating further significant technological advancements (Naik et al., 2010; Zabed et al., 2017). Globally, the combined forces of rising energy demand, deepening climate change concerns, and the threat of fossil fuel depletion have intensified the search for sustainable alternatives. Biofuels stand out as promising renewable and carbon-neutral options that simultaneously offer a route to reducing petroleum dependence, stimulating rural development, and improving waste management (Serrano-Ruiz & Dumesic, 2011).

2.12 THE SUPERIORITY AND CHALLENGES OF BIOBUTANOL

Among the range of available biofuels, biobutanol has increasingly garnered attention due to its distinctly favorable fuel properties compared to bioethanol. Butanol exhibits a higher energy alongside a lower vapor pressure and reduced hygroscopicity—meaning it absorbs less atmospheric water. These beneficial features enhance butanol's suitability for storage and transport, and crucially, make it more compatible with existing petroleum infrastructure, including pipelines and conventional combustion engines (Dürre, 2007; Qureshi et al., 2014). Furthermore, butanol can be blended with both gasoline and diesel at higher volumetric proportions without provoking engine corrosion or requiring significant engine modifications, establishing it as an exceptionally attractive alternative transportation fuel.

Despite these clear technical advantages, the large-scale commercial production of butanol remains limited. Industrially, butanol is primarily manufactured via the Acetone–Butanol–Ethanol (ABE) fermentation process, utilizing solventogenic *Clostridium* species such as *C. beijerinckii*. Though historically important in the early 20th century, this process waned with the rise of cheaper petrochemical synthesis. In recent decades, however, renewed interest in sustainable fuels has spurred a research revival into (ABE) fermentation. Major challenges persist, including inherently low yields and productivity—typical fermentations rarely exceed 10–20 g/L of butanol (Zhang et al., 2012)—and the high cost burden associated with utilizing food-based substrates. A significant biological barrier is product inhibition, where butanol becomes toxic to the producing microbes at concentrations above approximately 13–15 g/L severely limiting fermentation efficiency. Finally, the energy-intensive nature of downstream recovery and purification of butanol from highly dilute fermentation broths adds substantial cost, further hindering commercial viability (Liu et al., 2015).

2.13 STARTEGIES FOR PROCESS OPTIMIZATION

Current research efforts are strategically directed at overcoming the persistent challenges discussed above through several key approaches. A primary strategy involves pivoting to the use of low-cost lignocellulosic feedstocks, such as corn cobs, sugarcane bagasse, and wheat straw, which offer a sustainable, economically viable alternative to expensive food-based substrates. Another major area of focus involves the metabolic and genetic engineering of *Clostridium* species to specifically enhance their solvent tolerance and intrinsic productivity. Furthermore, researchers are actively developing integrated biorefinery systems that seamlessly combine pretreatment, hydrolysis, fermentation, and solvent separation into single, streamlined processes, thereby substantially improving energy efficiency and reducing overall production costs (Ezeji et al., 2010). Advances in separation technologies, including advanced methods like gas stripping, pervaporation, and adsorption, are also being thoroughly investigated to minimize the significant energy consumption associated with butanol recovery. The future prospects for biobutanol are

highly encouraging. Ongoing pilot-scale and demonstration projects continue to report tangible improvements in process integration, enzyme efficacy, and microbial performance. With sustained advancements in biotechnology and process optimization, biobutanol is well-positioned to become a major contributor to the global renewable energy sector. Its large-scale development promises to not only reduce worldwide carbon emissions but also significantly enhance energy security, particularly in agricultural economies like Nigeria, where abundant and underutilized lignocellulosic feedstocks such as corn cob are readily available.

2.2 CORN COB (LIGNOCELLULOSIC BIOMASS)AS FEEDSTOCK

Lignocellulosic biomass constitutes the structural backbone of plants and represents the most abundant renewable feedstock for producing second-generation biofuels. It is mainly composed of three key components: cellulose (30–50%), hemicellulose (20–35%), and lignin (10–25%). Cellulose is a linear polymer made up of glucose units that can be hydrolyzed into fermentable sugars. Hemicellulose is a heterogeneous branched polymer containing various sugars such as xylose, arabinose, mannose, and galactose, while lignin is a complex aromatic polymer responsible for providing structural rigidity and protection against microbial degradation.

Due to its dense crystalline structure and the presence of a lignin barrier, lignocellulosic biomass is naturally resistant to enzymatic breakdown. As a result, pretreatment is a critical step that helps to disrupt the compact structure, enhance surface accessibility, and facilitate the release of fermentable sugars for further biochemical conversion (Agrawal et al., 2020). Typical lignocellulosic feedstocks include agricultural and forestry residues such as corn stover, rice husk, wheat straw, sugarcane bagasse, cassava bagasse, elephant grass, and sawdust (Zhang et al., 2012; Yildirim et al., 2014). These materials are abundant, inexpensive, and non-edible, which makes them both economically viable and environmentally sustainable. Their utilization reduces competition with food crops and promotes sustainable waste management, presenting a clear advantage over first-generation biofuel feedstocks.

Among these biomass sources, corn cob has emerged as a particularly promising material for biofuel production. The corn cob is the woody central core of the maize ear that remains after the kernels are removed. With global maize production exceeding one billion metric tons each year, vast amounts of corn cobs are generated as agricultural residues (FAO, 2021). In maize-producing countries such as Nigeria, corn cobs are often discarded, burned, or used as low-grade fuel, resulting in environmental pollution and loss of potential value (Obi et al., 2016).

In terms of composition, corn cobs are rich in cellulose (35–40%) and hemicellulose (25–30%) while containing relatively low levels of lignin (15–20%), making them ideal for enzymatic hydrolysis and fermentation into biofuels (Boonsombuti et al., 2023). Studies have shown that dilute acid pretreatment of corn cobs can yield hydrolysates with sugar concentrations of up to 49 g/L, which are highly suitable for Acetone-Butanol-Ethanol (ABE) fermentation using Clostridium beijerinckii (Zhang et al., 2012; Boonsombuti et al., 2023).

The use of corn cobs as a biofuel feedstock offers both environmental and economic benefits. It provides a cost-effective and renewable raw material for biobutanol production while also minimizing agricultural waste and reducing open-field burning, which contributes to lower greenhouse gas emissions. From a circular economy perspective, converting corn cobs into biofuels adds value to what would otherwise be an underutilized agricultural residue.

In the context of Nigeria, corn cob-based biofuel production holds considerable promise. Given the country's extensive maize cultivation and the consistent availability of corn cob residues, converting this biomass into biobutanol aligns with national goals to diversify energy sources, reduce dependence on imported fossil fuels, and promote rural industrialization through bio-based technologies. This approach not only supports environmental sustainability but also strengthens energy security and stimulates local economic development.

Figure 2.1: Dried corn cob as feedstock

2.3 PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

The transformation of lignocellulosic biomass into biofuels such as biobutanol is often restricted by the rigid and intricate composition of plant cell walls. The main limiting factor is lignin, a complex aromatic polymer that surrounds cellulose and hemicellulose, thereby protecting them from enzymatic degradation. Without pretreatment, enzymes cannot efficiently access or break down these polysaccharides into fermentable sugars, resulting in poor hydrolysis and fermentation performance. Consequently, pretreatment is a vital step in the bioconversion process, designed to improve the accessibility and digestibility of the structural components of biomass.

The principal aims of pretreatment include increasing the surface area and porosity of the biomass, altering or removing lignin, decreasing cellulose crystallinity, and reducing the formation of inhibitory compounds that can impede microbial activity during fermentation (Agbor et al., 2011; Sun & Cheng, 2002). Accomplishing these objectives enhances the effectiveness of subsequent enzymatic hydrolysis and fermentation stages, ultimately improving overall conversion efficiency.

Pretreatment is necessary because lignocellulosic materials are naturally resistant to enzymatic breakdown. The cellulose microfibrils within the biomass are tightly organized in a crystalline form and embedded in a matrix of hemicellulose and lignin, forming a complex and recalcitrant structure that limits enzyme penetration. This inherent resistance reduces the conversion of cellulose into glucose and, by extension, decreases the availability of fermentable sugars for downstream fermentation. Pretreatment helps to overcome this challenge by disrupting the dense structure of the biomass, exposing cellulose and hemicellulose, and enhancing the efficiency of enzymatic saccharification.

An efficient pretreatment process significantly increases sugar yield, which directly boosts biobutanol production during fermentation (Alvira et al., 2010). Therefore, optimizing pretreatment techniques and conditions is a critical step in advancing cost-effective and sustainable bioconversion technologies for lignocellulosic feedstocks.

2.3.1 PRETREATMENT METHODS

Pretreatment is a vital step in converting plant materials, known as lignocellulosic biomass, into biofuels. It helps weaken the tough structure of plant cell walls so that enzymes can easily reach and convert the complex sugars into fermentable forms. Pretreatment methods are generally classified into four main categories—physical, chemical, physicochemical, and biological—and are sometimes combined to achieve better efficiency and results.

Physical pretreatment involves the use of mechanical actions such as cutting, grinding, or milling to reduce the size of the biomass and increase its surface area. This improves the accessibility of enzymes to the material. However, these methods often require a large amount of energy and are not effective in removing lignin, the protective compound that makes plant materials rigid.

Chemical pretreatment, on the other hand, uses various chemicals to alter the structure of biomass. Dilute acid pretreatment, typically using sulfuric acid (H_2SO_4), breaks down hemicellulose into simple sugars, thereby improving the exposure of cellulose for enzymatic action. However, this process may also produce harmful by-products like furfural that can inhibit fermentation. Alkaline pretreatment, which commonly employs sodium hydroxide (NaOH), is effective at removing lignin and enhancing enzymatic digestibility. Oxidative pretreatment methods, which use agents such as ozone, can also break down lignin effectively, though their high cost makes them unsuitable for large-scale applications.

Physicochemical pretreatment methods combine physical factors such as heat and pressure with chemical agents to efficiently disrupt the plant structure. In the steam explosion process, biomass is subjected to high-pressure steam and then rapidly depressurized, which breaks apart hemicellulose and loosens the lignin structure. The liquid hot water (LHW) method uses only pressurized hot water to dissolve hemicellulose without the need for added chemicals. The ammonia fiber expansion (AFEX) process treats biomass with ammonia to increase its porosity and reduce cellulose crystallinity, making it easier for enzymes to act during subsequent hydrolysis.

Biological pretreatment represents a more environmentally friendly approach. It uses microorganisms such as fungi or enzymes like laccases to naturally degrade lignin over time. This method is low-cost and safe for the environment but much slower compared to chemical and physicochemical alternatives.

Each pretreatment method offers distinct advantages and limitations. The choice of method depends on several factors, including the type of biomass being processed, the desired biofuel product, and the overall cost and efficiency of the process. When properly optimized, pretreatment greatly enhances the conversion of lignocellulosic materials into valuable biofuels like biobutanol.

2.3.2 ACID PRETREATMENT OF CORN COB

Corn cob is widely recognized as a highly suitable feedstock for acid pretreatment, a preferred method for breaking down the rigid structure of lignocellulosic biomass to release fermentable sugars. This process involves treating the biomass with dilute sulfuric acid (H_2SO_4) at elevated temperatures. The acid primarily hydrolyzes hemicellulose, one of the main structural components of corn cob, leading to the release of simple sugars such as xylose and arabinose. These fermentable sugars serve as essential substrates for microbial fermentation in the production of biofuels like biobutanol.

During acid pretreatment, the acid penetrates the corn cob’s cell wall matrix and disrupts the chemical bonds connecting cellulose, hemicellulose, and lignin. This disruption weakens the

overall structure of the biomass, increasing the accessibility of cellulose for subsequent enzymatic hydrolysis. To achieve optimal sugar yield and minimize the formation of unwanted by-products, process parameters such as temperature, acid concentration, and reaction time must be carefully controlled.

The effectiveness of this method for corn cob has been confirmed through several studies. Boonsombuti et al. (2023) reported that dilute acid pretreatment using 2.8% H_2SO_4 at 122 °C achieved a maximum sugar concentration of 46 g/L, creating ideal conditions for fermentation by Clostridium species. Similarly, Zhang et al. (2012) observed that applying a detoxification step—also known as overliming, which involves treating the hydrolysate with calcium hydroxide ($Ca(OH)_2$)—greatly enhanced sugar utilization and improved Acetone-Butanol-Ethanol (ABE) fermentation efficiency by Clostridium beijerinckii.

Despite its high efficiency, acid pretreatment is often associated with the formation of inhibitory compounds that can negatively affect fermentation. Under the combined effects of heat and acidity, sugars and lignin fragments degrade to form toxic substances such as furfural, 5-hydroxymethylfurfural (HMF), acetic acid, and phenolic derivatives. These compounds are harmful to fermenting microorganisms, leading to reduced cell growth, slower sugar consumption, and lower solvent yields.

To address these challenges, several detoxification strategies are employed. The most widely used method is overliming, in which $Ca(OH)_2$ is added to neutralize acidity and precipitate toxic compounds. Another effective technique is activated carbon adsorption, which removes inhibitors through surface binding. Additionally, biological detoxification methods utilize microorganisms or enzymes to naturally degrade inhibitory compounds in an eco-friendly manner (Palmqvist & Hahn-Hägerdal, 2000).

Acid pretreatment remains one of the most efficient and globally recognized techniques for processing corn cob into fermentable sugars. Its ability to achieve high sugar recovery makes it a cornerstone of lignocellulosic biofuel production. However, achieving sustainable and efficient biobutanol production requires a delicate balance—optimizing pretreatment conditions to maximize sugar release while integrating effective detoxification steps to minimize inhibitor formation and ensure smooth microbial fermentation.

2.3.3 ADVANCEMENTS AND OPTIMIZATION STRATEGIES

In recent years, notable progress has been achieved in enhancing the efficiency of pretreatment processes for lignocellulosic biomass. Efforts have mainly focused on lowering production costs, reducing the formation of harmful by-products, and improving sugar release for fermentation. Scientists are now applying modern optimization techniques and eco-friendly technologies to refine existing pretreatment methods and make them more practical for industrial-scale biofuel production.

A key optimization tool in this field is the Response Surface Methodology (RSM). This statistical method helps determine the best combination of process variables such as temperature, acid concentration, and reaction time to achieve the highest sugar yield. By studying how these

variables interact, RSM allows researchers to identify the most efficient pretreatment conditions while saving time and resources. According to Boonsombuti et al. (2023), RSM has proven highly effective in predicting and optimizing pretreatment outcomes for corn cob and other biomass materials.

Another major improvement involves enzyme synergy, which focuses on using multiple enzymes together to achieve better breakdown of plant materials. While cellulases are mainly responsible for converting cellulose into glucose, their performance improves greatly when used alongside accessory enzymes like xylanases and β-glucosidases. These additional enzymes help decompose hemicellulose and remove inhibitory by-products such as cellobiose. The combined action of these enzymes enhances the rate of hydrolysis, increases sugar yield, and improves the overall efficiency and cost-effectiveness of the process (Agrawal et al., 2020).

In addition, there has been growing interest in environmentally friendly pretreatment techniques designed to make biofuel production more sustainable. Innovative methods like ionic liquid and deep eutectic solvent (DES) pretreatments are showing promise because they can break down lignocellulosic structures under mild conditions while producing fewer inhibitors. These solvents are reusable, non-toxic, and require less energy compared to traditional chemical treatments. Likewise, biological pretreatment methods that use fungi or enzymes to degrade lignin are being explored as green alternatives. Although these processes are safer and cheaper, they tend to be slower and less scalable for industrial applications.

Looking toward the future, the success of lignocellulosic biofuel production will depend on combining affordable pretreatment technologies with advances in microbial and process engineering. Integrating optimized pretreatment with engineered microbes capable of withstanding inhibitors and fermenting multiple types of sugars can make production faster, more stable, and economically feasible. Continued research and technological innovation in these areas are expected to promote the development of large-scale, sustainable biorefineries capable of converting agricultural residues like corn cob into renewable fuels such as biobutanol.

2.3.4 CHALLENGES AND CONSIDERATIONS IN PRETREATMENT

Although significant progress has been achieved in developing pretreatment technologies for lignocellulosic biomass, several obstacles still limit their industrial application. One of the main issues is the generation of inhibitory compounds, which is common in chemical pretreatments like acid hydrolysis. During these processes, the combined effects of heat and acidity can cause sugars and lignin components to degrade into harmful substances such as furfural, hydroxymethylfurfural (HMF), and phenolic compounds. These by-products negatively affect microbial activity during fermentation, leading to lower fuel yields.

Another major limitation involves the high energy and chemical demands of many pretreatment methods. Physical and chemical approaches often consume large amounts of energy or require expensive reagents, increasing operational costs. In addition, incomplete lignin removal remains a recurring challenge, as residual lignin can block enzyme access to cellulose and reduce the overall sugar recovery rate. Environmental concerns also arise due to the use and disposal of

strong acids, alkalis, and other hazardous chemicals, underscoring the need for eco-friendly and sustainable pretreatment alternatives that minimize waste and pollution.

Scaling up these processes from laboratory experiments to industrial systems poses further difficulties. Variations in heat transfer, mixing efficiency, and reaction kinetics at larger scales can reduce process performance and reliability. To address these issues, ongoing research focuses on process integration, reactor design, and optimization strategies that can lower costs while maintaining high efficiency.

In conclusion, pretreatment plays a crucial role in transforming lignocellulosic feedstocks like corn cob into fermentable sugars suitable for biofuel production. The selection of an appropriate pretreatment method depends on several factors, including the type of biomass, the target sugar yield, economic feasibility, and compatibility with subsequent fermentation steps. While chemical and physicochemical methods remain widely used for their effectiveness, biological and green pretreatments offer promising, environmentally friendly alternatives. Recent developments—such as Response Surface Methodology (RSM) for optimizing reaction conditions and enzyme synergy for improving hydrolysis efficiency—have further enhanced sugar release while minimizing the formation of inhibitors.

Ultimately, the advancement of industrial-scale biobutanol production depends on achieving a balance between high performance, low cost, and environmental sustainability. Continued innovation aimed at overcoming challenges related to inhibitors, energy use, scalability, and waste management will be vital for establishing pretreatment as a reliable and sustainable foundation for future biofuel production.

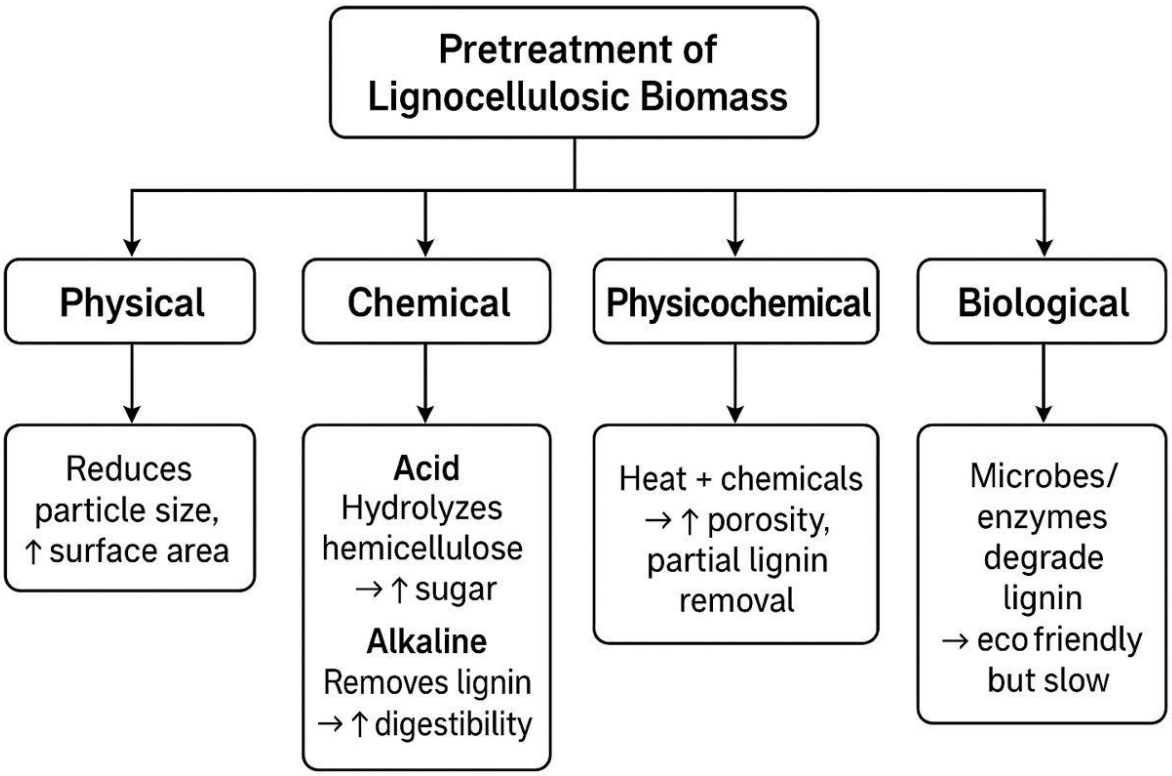

Figure 2.2: pretreatment of lignocellulosic biomass

2.4 ENZYMATIC HYDROLYSIS

After the pretreatment stage, which helps to loosen the rigid structure of lignocellulosic biomass, the next essential step in converting it into biofuel is enzymatic hydrolysis. This process uses enzymes to break down the complex carbohydrates—mainly cellulose and the remaining hemicellulose—into simple fermentable sugars such as glucose and xylose. These sugars serve as the primary carbon source for microorganisms during fermentation, making enzymatic hydrolysis a critical stage in the overall biobutanol production process.

Also known as saccharification, enzymatic hydrolysis relies on a group of enzymes called cellulases that work together to decompose the cellulose chains into smaller sugar molecules. The cellulase system typically includes endoglucanases, which cut internal bonds within cellulose fibers to create new chain ends; exoglucanases (cellobiohydrolases), which remove cellobiose units from these ends; and β-glucosidases, which further convert cellobiose into glucose that can be readily fermented by microbes such as Clostridium beijerinckii. This coordinated enzyme activity ensures the complete and efficient degradation of cellulose under mild, controlled conditions (Singhania et al., 2017; Agrawal et al., 2018).

Compared to chemical hydrolysis, enzymatic hydrolysis offers several advantages. It operates under moderate temperatures (typically 40–50°C) and near-neutral pH levels, avoiding the harsh

conditions that often lead to sugar degradation in acid or alkali hydrolysis. The mild environment minimizes the formation of inhibitory compounds such as furfural and hydroxymethylfurfural (HMF), ensuring better sugar recovery and improved fermentation outcomes. Additionally, because enzymes act selectively on cellulose and hemicellulose, the process is more precise and environmentally friendly, producing fewer waste products and requiring less energy input.

2.4.1 IMPORTANCE OF ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis is essential because it determines how efficiently cellulose can be converted into glucose, which directly affects the yield of biobutanol. Although pretreatment processes (for example, dilute acid pretreatment of corn cob) expose cellulose fibers and partially remove hemicellulose and lignin, the tightly packed crystalline structure of cellulose still prevents direct microbial access. Enzymatic hydrolysis resolves this challenge by gradually cleaving the cellulose molecules, ensuring a steady and controlled release of glucose throughout the process. This continuous sugar availability supports effective fermentation and higher solvent yields during simultaneous saccharification and fermentation (SSF).

Incomplete hydrolysis, on the other hand, results in less glucose production and limited substrate availability for fermentation, which reduces biobutanol yield. Therefore, optimizing factors such as enzyme loading, hydrolysis time, temperature, and pH is crucial to achieve maximum sugar recovery.

Recent research and technological advancements have further improved the efficiency and cost-effectiveness of enzymatic hydrolysis. The development of enzyme mixtures that combine cellulases with accessory enzymes such as xylanases and ligninases has enhanced the breakdown of complex biomass structures. Moreover, new approaches like enzyme immobilization and the use of genetically engineered microorganisms capable of secreting multiple enzymes simultaneously are being explored to improve reaction efficiency and reduce production costs.

In summary, enzymatic hydrolysis represents a vital step in transforming lignocellulosic materials such as corn cob into fermentable sugars for biobutanol production. Its mild operational conditions, high specificity, and ability to preserve sugar integrity make it a superior alternative to chemical methods. With continuous improvements in enzyme technology and process optimization, enzymatic hydrolysis is becoming increasingly feasible for large-scale, sustainable biofuel production.

2.4.2 ENZYME COMPLEXES AND ACCESSORY ENZYMES

The enzymatic breakdown of cellulose into fermentable sugars, referred to as saccharification, is driven by a group of enzymes collectively known as **cellulases**. These enzymes act synergistically to hydrolyze both the crystalline and amorphous regions of cellulose into simple sugars that microorganisms can utilize. The cellulase complex is made up of three major enzymes, each performing a distinct but complementary function. **Endoglucanases (EC 3.2.1.4)** initiate the reaction by randomly cleaving internal β-1,4-glycosidic bonds within the cellulose

polymer, thereby producing new chain ends. **Cellobiohydrolases (EC 3.2.1.91)** then act progressively on these free ends to release **cellobiose**, while **β-glucosidases (EC 3.2.1.21)** convert cellobiose and other short oligosaccharides into **glucose**, which serves as the primary substrate for acetone–butanol–ethanol (ABE) fermentation by *Clostridium beijerinckii* (Singhania et al., 2017).

In lignocellulosic materials such as corn cob, cellulose fibers are tightly bound within a matrix of hemicellulose, lignin, and pectin, which hinders enzyme access and limits hydrolysis efficiency. Consequently, complete conversion requires the presence of accessory enzymes such as **xylanases**, **pectinases**, and **laccases**, which help break down the surrounding non-cellulosic components. Xylanases hydrolyze the hemicellulosic fraction, pectinases degrade pectin linkages, and laccases act on lignin to loosen the plant cell wall structure. Through this cooperative activity, cellulases and accessory enzymes work in synergy to enhance cellulose accessibility and significantly increase overall sugar yield (Agrawal et al., 2018).

In the present study, saccharification was carried out using a composite enzyme cocktail under Simultaneous Saccharification and Fermentation (SSF) conditions. The cocktail comprised cellulase, β-glucosidase, and pectinase, which were selected for their complementary functions and compatibility with the fermentation environment. The addition of β-glucosidase minimized product inhibition by rapidly converting cellobiose to glucose, while pectinase aided in the breakdown of pectic substances that can obstruct enzymatic access to cellulose. This customized enzyme mixture provided a balanced catalytic system that promoted continuous sugar release during fermentation, thereby supporting microbial activity and improving butanol formation by *Clostridium beijerinckii*.

Employing enzyme cocktails offers several advantages over single-enzyme systems in lignocellulosic bioconversion. These mixtures simulate the cooperative action observed in natural microbial consortia, enabling multiple degradation pathways to occur simultaneously. Such synergy enhances hydrolysis efficiency, reduces enzyme loading requirements, shortens reaction time, and ensures a more complete depolymerization of the biomass. In this work, the enzyme cocktail was particularly effective in sustaining a steady release of fermentable sugars during the SSF process, which in turn enhanced solvent yield and overall process efficiency. The saccharification of cellulose is catalyzed by cellulase complexes, which comprise three main enzymes with complementary roles. Endoglucanases randomly cleave internal β-1,4-glycosidic bonds in the cellulose chain, generating new chain ends and increasing the number of accessible sites for further enzymatic action. Cellobiohydrolases act processively from these chain ends to release cellobiose units, while β-glucosidases hydrolyze cellobiose into glucose, the fermentable sugar required for ABE fermentation (Singhania et al., 2017).

In addition to cellulases, accessory enzymes such as xylanases, laccases, and pectinases play a critical role in removing residual hemicellulose and lignin, which can otherwise hinder cellulase access to cellulose fibers. This synergistic action between cellulases and accessory enzymes ensures more complete hydrolysis of pretreated biomass and enhances sugar yields (Agrawal et al., 2018). Studies have shown that combining these enzymes significantly improves hydrolysis efficiency, especially for lignocellulosic residues with high hemicellulose content, such as corn cobs.

2.4.4 CHALLENGES AND CONSIDERATIONS ASSOCIATED WITH ENZYMATIC HYDROLYSIS

Despite the effectiveness of enzymatic hydrolysis, several challenges must be addressed to achieve efficient and cost-effective sugar release. Enzymes can be expensive, and their activity may decline under industrial operating conditions, including variations in pH and temperature. Product inhibition, particularly by glucose and cellobiose, can significantly reduce hydrolysis efficiency if not managed properly. Furthermore, the choice of pretreatment method directly impacts enzyme accessibility; incomplete removal of lignin or residual hemicellulose can limit enzyme action (Agrawal et al., 2018). To overcome these limitations, integrated strategies that combine optimized pretreatment, enzyme supplementation, and fermentation conditions are essential for maximizing sugar release and ensuring high biobutanol yields.

Enzymatic hydrolysis is a critical link between pretreatment and fermentation in the production of biobutanol from corn cob. Through the action of cellulases and accessory enzymes, cellulose is converted into glucose under controlled conditions, with kinetics governed by substrate accessibility and enzyme activity. Effective hydrolysis ensures a sufficient supply of fermentable sugars for Clostridium beijerinckii, enabling efficient ABE fermentation and maximizing butanol production. Optimizing enzyme usage, process conditions, and integration with fermentation is key to industrial feasibility.

2.5 MICROORGANISM FOR ABE FERMENTATION: CLOSTRIDIUM BEIJERINCKII

Solventogenic species of the genus *Clostridium* are strictly anaerobic, spore-forming bacteria renowned for their ability to synthesize industrially valuable solvents through the Acetone–Butanol–Ethanol (ABE) fermentation pathway. Among these microorganisms, *Clostridium beijerinckii* has emerged as one of the most promising biocatalysts for biobutanol production. Its popularity stems from its strong physiological robustness**,** broad substrate utilization range**, and** enhanced tolerance to toxic compounds and inhibitory substances commonly present in lignocellulosic hydrolysates (Jones & Woods, 1986; Dürre, 2007).

Compared with the more extensively characterized *Clostridium acetobutylicum*, *C. beijerinckii* demonstrates greater metabolic versatility, enabling it to efficiently ferment both hexose sugars (such as glucose and mannose) and pentose sugars (including xylose and arabinose) (Boon et al., 2011; Dürre, 2007). This wide substrate adaptability is particularly advantageous when processing lignocellulosic materials such as corn cob, which yield mixed sugar streams after pretreatment and enzymatic hydrolysis. The organism's capacity to co-utilize these sugars minimizes residual substrate accumulation, shortens the fermentation lag phase, and enhances overall solvent yield. Additionally, *C. beijerinckii* exhibits notable resilience to environmental fluctuations—particularly changes in pH and solvent concentration—making it highly

compatible with Simultaneous Saccharification and Fermentation (SSF) systems, where sugars are gradually released and immediately metabolized by the cells.

2.5.1 BIPHASIC METABOLISM

The Acetone–Butanol–Ethanol (ABE) fermentation process in *Clostridium beijerinckii* proceeds through two main metabolic stages: acidogenesis and solventogenesis. In the first stage, acidogenesis, the microorganism directs its metabolic activity toward the production of short-chain organic acids—primarily acetic and butyric acids—which leads to a gradual decline in the culture's pH. This initial phase is essential for energy generation and the synthesis of intermediate metabolites that serve as precursors for solvent production (Kang et al., 2023).

When the culture environment becomes sufficiently acidic, typically around pH 4.5, *C. beijerinckii* undergoes a physiological shift to the solventogenic phase. During this stage, the acids produced earlier are reassimilated and transformed into neutral solvents such as acetone, butanol, and ethanol (Jones & Woods, 1986; Dürre, 2007).

The smooth progression from acidogenesis to solventogenesis is a determining factor for overall solvent yield and productivity. Any delay or inhibition in this metabolic transition—caused by unfavorable conditions or the accumulation of toxic by-products—can markedly reduce butanol formation. Factors including temperature, pH, nutrient levels, and the presence of inhibitory compounds originating from lignocellulosic hydrolysates strongly influence this acid-to-solvent conversion (Huang et al., 2013). A thorough understanding of this biphasic metabolic behavior is therefore crucial for optimizing fermentation performance, particularly under Simultaneous Saccharification and Fermentation (SSF) conditions, where sugars are steadily liberated from biomass and immediately utilized by the fermenting microorganism.

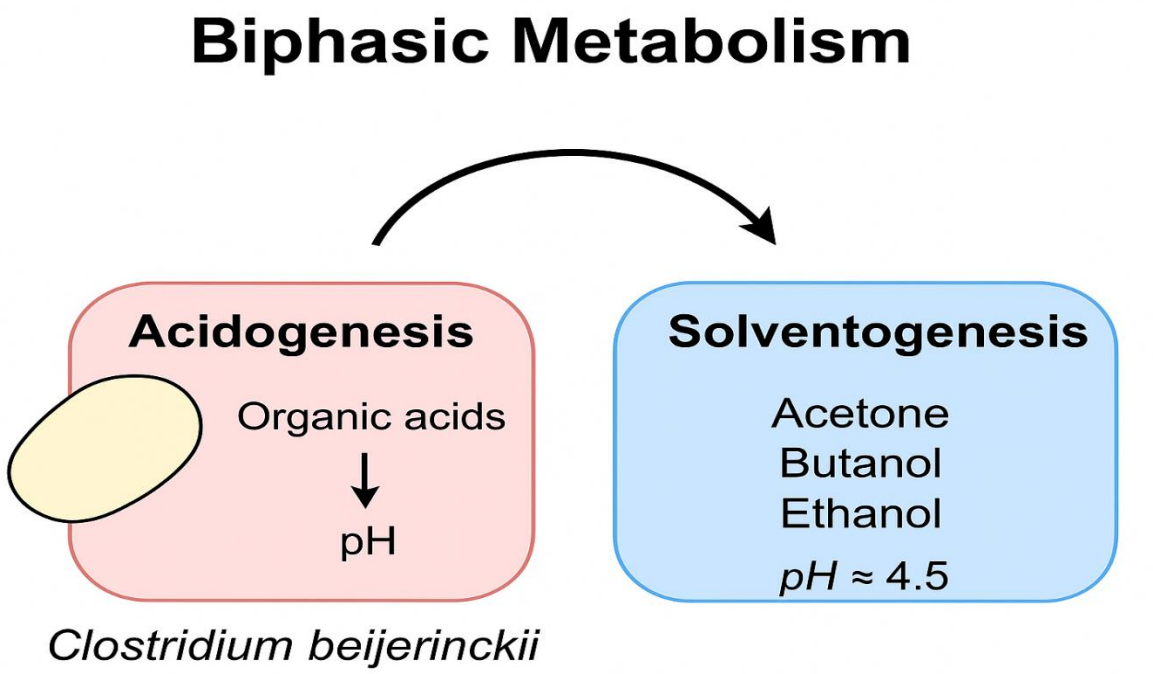

Figure 2.3: Biphasic Metabolism

2.5.2 PERFORMANCE OF MICROORGANISM WITH COR NCOB HYDROLYSATE

Corn cobs represent a valuable lignocellulosic feedstock rich in fermentable sugars once subjected to pretreatment and enzymatic hydrolysis, making them highly suitable for biobutanol production by *Clostridium beijerinckii*. Several studies have demonstrated the effectiveness of this organism, noting its inherent tolerance to mild fermentation inhibitors. For instance, Boonsombuti et al. (2023) reported a butanol concentration of 6.26 g L^{-1} using a direct Simultaneous Saccharification and Fermentation (SSF) process with corn cob hydrolysate, even without prior detoxification. This outcome highlights the strain's robustness against inhibitory substances such as acetic acid and phenolic compounds. In contrast, research by Zhang et al. (2012) revealed that conditioning or detoxifying the feedstock can further enhance fermentation efficiency, achieving butanol titers up to 16 g L^{-1} when hydrolysates were treated with $Ca(OH)_2$.

A key metabolic advantage of *C. beijerinckii* lies in its ability to utilize both hexose and pentose sugars (Jones & Woods, 1986; Dürre, 2007). This flexibility enables the microorganism to fully metabolize the mixed sugar profile released from the cellulose and hemicellulose fractions of corn cob biomass—an obstacle for many other fermentative species. By efficiently converting this diverse range of sugars into solvents, *C. beijerinckii* achieves higher total butanol yields, reinforcing its promise as a robust biocatalyst for large-scale lignocellulosic biobutanol production.

2.6 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF)

The **Simultaneous Saccharification and Fermentation (SSF)** process offers several important advantages over the traditional **Separate Hydrolysis and Fermentation (SHF)** approach, making it an appealing technique for biofuel and biochemical production (Olofsson et al., 2008; Jørgensen et al., 2007). In SSF, the enzymatic breakdown of cellulose and the microbial fermentation of the resulting sugars occur in a single integrated step, leading to greater process efficiency and improved product yields.

One of the most notable benefits of SSF is its ability to **eliminate product inhibition**. In the SHF process, intermediates such as glucose and cellobiose tend to accumulate during enzymatic hydrolysis. High concentrations of these sugars inhibit the cellulase enzymes responsible for cellulose degradation, thereby slowing the reaction. In contrast, SSF avoids this problem because the fermenting microorganisms rapidly consume the sugars as soon as they are released, preventing their buildup and allowing the enzymes to maintain a consistently high level of activity (Alvira et al., 2010; Kristensen et al., 2009).

By preventing inhibition, SSF also helps to **reduce the amount of enzyme required** for effective saccharification. Since the enzymes remain active for longer periods, a smaller total dosage can achieve the same level of cellulose conversion. This reduction in enzyme usage

directly lowers operating costs, which is especially important in large-scale biofuel production where enzymes represent a major expense (Olofsson et al., 2008).

Additionally, integrating both hydrolysis and fermentation within a **single reactor system** simplifies plant design and operation. The process eliminates the need for separate hydrolysis and fermentation vessels, associated piping, and intermediate handling steps. This simplification shortens the overall processing time and significantly reduces capital and maintenance costs (Ballesteros et al., 2004; Jørgensen et al., 2007).

Finally, the close coordination between enzymatic hydrolysis and microbial fermentation in SSF enhances overall conversion efficiency and product yield. The simultaneous removal of inhibitory sugars allows both reactions to proceed smoothly, resulting in more complete utilization of the cellulose substrate. This synergy often leads to higher solvent or ethanol output compared to conventional multi-step processes, underscoring SSF's potential as a cost-effective and efficient configuration for lignocellulosic biofuel production (Olofsson et al., 2008; Alvira et al., 2010).

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2.6.2 LIMITATIONS OF SSF

Although the Simultaneous Saccharification and Fermentation (SSF) technique offers several advantages over conventional processes, it still faces key limitations that constrain its efficiency and industrial application. One of the primary challenges is the incompatibility of optimal conditions required by the enzymatic and microbial systems involved. The cellulase enzymes responsible for cellulose hydrolysis exhibit peak catalytic activity at relatively high temperatures, typically between 45–50 °C, and under mildly acidic conditions (pH 4.8–5.0). Conversely, *Clostridium beijerinckii*, the solventogenic bacterium widely used in Acetone–Butanol–Ethanol (ABE) fermentation, grows best at a lower temperature range of 35–37 °C and under nearly neutral pH conditions (6.0–6.8) (Boon et al., 2011). This mismatch necessitates the use of compromise operating conditions in SSF systems, which are suboptimal for one or both components. Consequently, the process often suffers from decreased enzymatic activity, slower hydrolysis rates, and reduced microbial performance, ultimately leading to lower solvent yields.

Another major obstacle is the presence of inhibitory compounds that are generated during the pretreatment of lignocellulosic feedstocks. Substances such as furfural, hydroxymethylfurfural (HMF), and various phenolic compounds, released from the breakdown of hemicellulose and lignin, can significantly impede microbial metabolism. These inhibitors suppress solvent formation, delay the onset of solventogenesis, and reduce butanol productivity (Palmqvist & Hahn-Hägerdal, 2000). Even at relatively low concentrations, they can interfere with enzymatic reactions, disrupt nutrient uptake, and diminish cell viability, thereby complicating both saccharification and fermentation stages.

In addition to biological constraints, the scale-up of SSF operations introduces several engineering difficulties. Maintaining efficient mixing and mass transfer in high-solids systems, ensuring strict anaerobic conditions for *Clostridium* cultures, and minimizing shear stress that can damage microbial cells and enzymes are among the major design challenges. The high viscosity of lignocellulosic slurries further complicates reactor operation by hindering uniform enzyme distribution and causing localized substrate inhibition. Addressing these biochemical and engineering bottlenecks remains central to advancing SSF toward reliable and economically viable large-scale biobutanol production.

2.6.3 STRATEGIES FOR OPTIMIZATION OF SSF

Enhancing the efficiency of the Simultaneous Saccharification and Fermentation (SSF) process requires addressing its fundamental limitations—chiefly the mismatch between the optimal operating conditions for cellulase enzymes and those for fermenting microorganisms. To overcome these challenges and improve the overall performance of SSF, researchers have focused on biological, process, and engineering-based optimization strategies that make the system more robust, productive, and economically viable.

One of the most promising approaches lies in enzyme engineering, which aims to improve the characteristics of cellulases so they can function efficiently under the moderate conditions required for microbial fermentation. Conventional cellulases perform best at higher temperatures and slightly acidic pH values, conditions that are often detrimental to the growth of solvent-producing microorganisms such as *Clostridium beijerinckii*. Through protein engineering and directed evolution, scientists have developed cellulase variants that are both thermostable and pH-tolerant, meaning they retain high activity at lower temperatures and near-neutral pH levels typical of SSF operations (Bornscheuer et al., 2014). By tailoring the enzyme properties to fit the shared environment of the SSF reactor, this approach effectively reduces the trade-off between enzymatic efficiency and microbial viability, leading to smoother hydrolysis and fermentation synergy.

Another important area of advancement is process engineering optimization, which involves refining reactor conditions and feeding strategies to enhance performance. For instance**,** fed-batch SSF allows biomass to be added gradually rather than all at once, preventing excessive viscosity that can impede mixing and mass transfer. This controlled feeding maintains the concentrations of sugars and inhibitors within ranges that favor both enzyme activity and microbial metabolism. In addition**,** high-solids SSF has emerged as an efficient strategy for improving productivity, as it uses higher substrate concentrations to achieve greater sugar conversion and higher solvent or ethanol yields. This approach not only increases final product titers—thereby lowering the energy demand for downstream recovery—but also minimizes water usage, making the process more sustainable (Zhang et al., 2010).

Alongside enzyme and process improvements**,** microbial strain development plays a crucial role in optimizing SSF. Since microorganisms are responsible for converting sugars into the final biofuel products, enhancing their tolerance and productivity is essential, particularly in butanol-producing systems where solvent toxicity is a major limitation**.** Strain improvement can be achieved through two complementary strategies**:** metabolic engineering, which involves the targeted modification of genes and metabolic pathways to increase yield and resistance, and adaptive laboratory evolution, where microbes are exposed to gradually increasing concentrations of inhibitory compounds until more robust strains naturally emerge (Lütke-Eversloh & Bahl, 2011). These improved strains show greater resilience to environmental stressors, solvents, and pretreatment-derived inhibitors, thereby maintaining stable fermentation performance even under challenging SSF conditions.

Finally, integration with in situ product recovery (ISPR) techniques has become a pivotal strategy to counteract product inhibition during fermentation. In SSF systems producing solvents like butanol, the accumulation of the product in the broth can be toxic to the microorganisms, drastically reducing their activity and the final yield. ISPR technologies, such as gas stripping**,** pervaporation**,** and adsorption, allow continuous removal of the solvent as it forms, thereby maintaining low product concentrations in the medium (Qureshi & Blaschek, 2001). This not only alleviates toxicity but also enables microbes to continue fermenting for longer periods, resulting in higher overall conversion rates and productivity. The combination of SSF and ISPR represents a powerful integrated approach that enhances both the biological and engineering efficiency of the process.

Collectively, these strategies—spanning enzyme optimization, process design, microbial improvement, and product recovery integration—are transforming SSF from a laboratory-scale concept into a feasible and scalable platform for sustainable biofuel production. Ongoing research continues to refine these methods to achieve higher yields, lower costs, and greater environmental benefits in lignocellulosic biobutanol and bioethanol production systems.

2.6.4 IN SITU PRODUCT RECOVERY (ISPR)

An effective approach to improving Acetone–Butanol–Ethanol (ABE) fermentation performance is the implementation of In Situ Product Recovery (ISPR**)**, where product removal techniques are directly coupled with the fermentation process. This integrated strategy allows for the **continuous extraction of inhibitory solvents** during fermentation, reducing toxicity within the broth and extending the period of active microbial metabolism (Qureshi & Blaschek, 2001; Xue et al., 2017).

Among the different ISPR techniques, gas stripping has proven particularly successful in enhancing butanol productivity. In this method, an inert gas is passed through the fermentation medium to remove volatile solvents such as butanol, acetone, and ethanol as they form. Studies have shown that incorporating gas stripping into fermentation can increase butanol yields by over 50% compared to conventional batch processes (Qureshi & Blaschek, 2001). Similarly, coupling Simultaneous Saccharification and Fermentation (SSF) with pervaporation, a membrane-based solvent separation process, has been reported to raise overall solvent productivity by minimizing feedback inhibition and maintaining favorable fermentation conditions (Boonsombuti et al., 2023; Ezeji et al., 2007).

In addition to improving microbial activity and solvent yield, ISPR integration provides notable economic advantages. The continuous removal of solvents produces a more concentrated product stream, which reduces the energy and cost requirements of downstream purification steps (Gapes, 2000; Oudshoorn et al., 2009). Furthermore, integrating recovery systems into fermentation enhances process stability by preventing the accumulation of inhibitory metabolites that could suppress solventogenesis.

However, several engineering and biological challenges must be addressed to optimize ISPR for industrial use. Reactor design must facilitate effective mass transfer while preserving the strict anaerobic conditions necessary for *Clostridium beijerinckii* growth. Likewise, the compatibility of extraction solvents or membrane materials with the microbial culture must be ensured to prevent cellular toxicity or enzyme inactivation (Xue et al., 2017). Despite these constraints, ISPR remains one of the most promising methods for achieving high butanol yields, improved process efficiency, and reduced operational costs in integrated biobutanol production systems.

2.7 QUANTIFICATION OF BUTANOL IN ACETONE-BUTANOL-ETHANOL (ABE) SOLVENT

Quantification of butanol in Acetone–Butanol–Ethanol (ABE) solvent is a key step in evaluating process efficiency and microbial performance. In contrast to ethanol fermentation, where product concentrations often exceed 50 g/L, butanol levels in ABE systems rarely surpass 20 g/L due to

its toxicity to solvent-producing microorganisms (Xue et al., 2017). Measuring these low concentrations accurately is therefore vital for determining fermentation efficiency, microbial productivity, and the overall performance of configurations such as Simultaneous Saccharification and Fermentation (SSF). Quantification also provides insights into substrate conversion efficiency and the metabolic shift between acidogenesis and solventogenesis, making it an important indicator for assessing process optimization and improvement potential (Ezeji et al., 2004).

In this study, butanol, along with acetone and ethanol, was quantified using High-Performance Liquid Chromatography (HPLC). This technique offers a precise and dependable method for identifying and quantifying volatile solvents, even at the relatively low concentrations produced during batch and SSF fermentations (Qureshi & Blaschek, 2001). The procedure includes centrifuging the fermentation broth to remove cells and suspended solids, followed by filtration of the supernatant before injection into the HPLC system. Detection is performed using a Refractive Index Detector (RID), which responds to differences in refractive properties caused by solvent components (Xue et al., 2017).

The accuracy and reproducibility of HPLC make it particularly suitable for analyzing lignocellulosic fermentations, where impurities or residual inhibitors can interfere with simpler detection methods. The technique also enables simultaneous measurement of multiple components, providing a complete solvent profile that includes acetone, butanol, and ethanol. This allows for precise yield determination and a better understanding of the fermentation balance between substrate utilization and solvent formation.

Although this research does not include product recovery or purification, solvent quantification is central to evaluating the performance and feasibility of the SSF process. The concentrations obtained through HPLC reflect both the efficiency of microbial metabolism and the effectiveness of enzymatic hydrolysis under the given operating conditions. These data are crucial for comparing fermentation approaches, identifying inhibitory effects, and optimizing key parameters such as temperature, pH, and enzyme concentration. Consequently, accurate butanol quantification serves as an essential performance metric for assessing the potential of corn cob-based biobutanol production and provides a foundation for future investigations into integrated recovery systems and process scale-up (Agrawal et al., 2020; Qureshi & Blaschek, 2001).

2.8 FUTURE DIRECTIONS IN THE PRODUCTION OF BIOBUTANOL

Biobutanol production from lignocellulosic resources such as corn cob remains one of the most promising approaches to advancing sustainable biofuel development. Nevertheless, the transition from laboratory-scale experiments to economically feasible industrial operations continues to pose substantial challenges. Future research must therefore be directed toward overcoming the technical, biological, and economic limitations that currently hinder large-scale deployment.

A key area for improvement involves the co-optimization of enzymes and microorganisms used in the Simultaneous Saccharification and Fermentation (SSF) process. The mismatch between the optimum operating conditions of cellulolytic enzymes and solvent-producing *Clostridium*

species continues to constrain overall process performance. Developing thermotolerant strains of *Clostridium beijerinckii* that can grow efficiently at temperatures favorable for enzymatic hydrolysis (approximately 45–50 °C) could eliminate the need for compromise conditions. Likewise, advances in protein engineering aimed at producing cellulases with enhanced stability and activity near neutral pH would allow more effective saccharification and fermentation in a single bioreactor (Bornscheuer et al., 2014). Combining such biological innovations would likely boost solvent productivity and shorten total fermentation time.

Further progress can also be achieved through enzyme cocktail optimization. Adjusting the ratio of cellulases, hemicellulases, and accessory enzymes such as laccases and pectinases can significantly improve the hydrolysis of complex lignocellulosic structures like corn cob. Developing synergistic multi-enzyme systems alongside adaptive microbial cultures offers a promising route to maximizing sugar release while reducing enzyme loading and cost.

From an engineering perspective, scaling up SSF requires rethinking reactor design and control systems. Novel configurations that accommodate high-solids operations, ensure efficient mixing, and support continuous processing could substantially increase volumetric productivity. Integrating SSF with in situ product recovery (ISPR) remains another promising avenue; methods such as gas stripping and pervaporation have demonstrated the ability to mitigate butanol toxicity while reducing downstream separation energy requirements (Qureshi & Blaschek, 2001; Xue et al., 2017). Hybrid systems combining these strategies could yield significant improvements in overall process efficiency.

Moreover, the use of techno-economic analysis (TEA) and life-cycle assessment (LCA) will be vital for guiding commercial implementation. These tools can identify cost-intensive stages—such as pretreatment, hydrolysis, or recovery—and help prioritize areas for optimization while quantifying the environmental benefits of biobutanol relative to fossil fuels.

Expanding the feedstock base through the utilization of agricultural residues will also enhance sustainability. Corn cob, a readily available and often underexploited by-product of maize farming, represents a valuable resource, but future studies should also explore mixed-feedstock systems, seasonal biomass variability, and region-specific supply chains to ensure continuous and adaptable production—particularly in agricultural economies like Nigeria.

Ultimately, continued advancement in lignocellulosic biobutanol production will depend on integrating biological innovation, process optimization, and comprehensive sustainability assessment. Through collaborative research and technological refinement, biobutanol derived from renewable materials such as corn cob can progress from experimental development to a commercially viable and environmentally beneficial energy alternative.

Future research is focusing on hybrid recovery technologies that combine the strengths of multiple methods. For instance, coupling pervaporation with gas stripping can increase recovery rates while lowering overall energy input. Similarly, integrating adsorption with distillation has been proposed to pre-concentrate solvents before final separation.

Another area of advancement lies in membrane materials. Novel nanocomposite membranes with higher selectivity and resistance to fouling are being tested for pervaporation and membrane distillation. Likewise, the development of eco-friendly extractants, such as ionic liquids and deep eutectic solvents, offers a pathway toward safer and greener liquid–liquid extraction.

Beyond technology development, techno-economic analysis (TEA) and life cycle assessment (LCA) are critical for determining which recovery strategies are most viable at scale. These tools can identify the best trade-offs between energy efficiency, cost, and environmental impact. Ultimately, the future of biobutanol recovery will depend on integrating process intensification with sustainability goals to make lignocellulosic butanol competitive with petrochemical and ethanol-based alternatives.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1. MATERIALS AND REAGENTS

The reagents used are listed below along with their purposes/use;

Table 3.1: Materials and Reagents

Reagents	**Source**	**Purpose/Use**
Sulfuric acid (H_2 SO_4)	Loba Chemie, Mumbai, supplied by Finlab Nigeria Ltd, Lagos.	Used for acid pretreatment of cassava bagasse.
Sodium Hydroxide (NAOH)	BDH Chemicals, England, supplied by Pascal Scientific,	Use in pretreating the cassava bagasse for hydrolysis

	Lagos.	
Distilled water	Laboratory distilled (In-house).	Used for making solutions, standards, and to rinse the biomass after pretreatment.
Cellulase, xylanase, pectinase, laccase, beta-glucosidase	Sigma-Aldrich enzymes supplied by Quest Laboratory Supplies, Lagos.	A suite of enzymes used for the enzymatic hydrolysis of the pretreated biomass.
Cellulase	Sigma-Alddrich (via Quest Laboratory Supplies by Finland Nigeria Ltd.	Breaks down the crystalline structure of cellulose into **cellobiose and glucose**.
β-Glucosidase	Loba Chemie _ supplied by Finlab Nigeria Ltd.	• Hydrolyzes **cellobiose (a product of cellulase action)** into **glucose**. • Prevents accumulation of cellobiose, which otherwise **inhibits cellulase activity**.
Xylanase	Novozymes (distributed by Pascal Scientific Ltd, Lagos.	Breaks down the hemicellulosic matrix that surrounds cellulose fibers, making cellulose more **accessible to cellulase**.
Pectinase	Lova Chemie – Supplied by Finlab Nigeria Ltd.	• Breaks down **pectin**, a heteropolysaccharide found

		in cassava cell walls. • Degradation of pectin **loosens the cell wall structure**, releasing cellulose and hemicellulose for further hydrolysis.
Laccase	Sigma-Aldrich enzyme (via authorized distributor, Lagos).	• Removes lignin that blocks access to cellulose and hemicellulose. • Helps improve the **efficiency of cellulase and xylanase** action.
Sodium citrate & Citric acid	BDH Chemicals Ltd, England – via Finlab Nigeria Ltd.	Used to prepare the sodium citrate buffer, which maintains the pH of the hydrolysate at 4.8 during enzymatic hydrolysis.
Sodium azide	Sigma-Aldrich reagent (via Pascal Scientific, Lagos.	Added to the buffer to prevent microbial contamination during the hydrolysis process.
Bovine Serum Albumin (BSA)	Thermo Fisher Scientific-supplied by Quest Laboratory	Used to prepare the standard curve for the Bradford assay,

	Supplies Lagos.	which determines protein concentration.
Bradford reagent	Bioo-Rad Laboratories reagent (via camlab Nigeria Ltd).	Used in the Bradford assay to determine the protein content of the enzymes.
3,5-dinitrosalicylic acid (DNS)	Labo Chemicals – supplied by Finlab Nigeria Ltd.	A key component of the DNS reagent used to determine the amount of reducing sugars.
Sodium potassium tartrate tetrahydrate	BDH Chemicals, England – via Finlab Nigeria Ltd.	Used in the preparation of the DNS reagent.
Sodium hydroxide (NaOH)	BDH Chemicals – via Pascal Scientific Ltd, Lagos.	Used in the preparation of the DNS reagent.
D-glucose	Loba Chemie – supplied by Finlab Nigeria Ltd.	Used to create the standard curve for the reducing sugar test.

3.1.2. APPARATUS USED FOR THIS EXPERIMENT

Table 3.2: Apparatus used for this experiment

S/N	APPARATUS	MANUFACTURER/MODEL	USES/FUNCTIONS
1	Autoclave chamber	Ocean Med+ England, Model: YX-18LD	Used for carrying out the acid pretreatment process.
2	Kern Electronic Balance	Kern & Sohn Germany, Model: ALS-160	Used for accurately measuring the amounts of solid materials.
3	pH scale	Local/Generic(commonly China), Model: PH-009(1)A	Used for checking the pH of solutions, including the pretreated

			slurry
4	Visible Spectrophotometer	Model: 721S	Used to measure the absorbance of solutions for both protein analysis and the reducing sugar test
5	Water bath shaker	Labtech/ DK series (China/Korea), Model: DK420 U-Clear	Creates the optimal environmental temperature for enzymatic hydrolysis and provides continuous agitation
6	Beakers, Conical flasks	Generic Laboratory Glassware Manufacturer.	Used for holding pretreated and hydrolysate samples.
7	Pipette	Generic Laboratory Pipette Manufacturer (Eppendorf).	Used for drawing fluids, such as enzymes and acid.
8	Measuring cylinder	Generic Laboratory Glassware Manufacturer (Pyrex).	Used for measuring fluid levels.
9	Beakers	Pyrex (USA)	Used for measuring and mixing liquid.
10	Litmus paper	BOENMED	Used to check for the pH

			of the pretreated slurry to ensure neutralization.
11	Local attrition mill	Locally fabricated.	Used to grind the sun-dried cassava bagasse into a fine powder.
12	Chromatograph	Chinese HPLC Manufacturers, Model-3528D HPLC Chromatograph	Used for stripping Biobutanol from the mixture of Acetone, Biobutanol and Ethanol
13	Oven	OHASUS pioneer	For drying pretreated samples
14	Stirrer	FALC Instruments	Used to stir liquid and solute to get an even solution
15	Filter Paper	Whatman Ltd, Model: Whatman No.4	Used for separating solute and filtrate from solution
16	Heating Mantle	FALC Instruments	Used for gradual heating of samples

3.1.3 FEEDSTOCK: CORN COB

Corn cobs were chosen as the main lignocellulosic feedstock because they are readily available as an agricultural by-product in Nigeria, especially in areas with high maize production. The corn cob used for this project was locally sourced from a Garri processing mill in Warri, Delta State.

Upon collection, the corn cobs were thoroughly washed to eliminate dirt and impurities, air-dried until they reached a constant weight, and then mechanically milled into fine particles using a hammer mill. The resulting biomass was sieved to achieve a uniform particle size of ≤2 mm, which enhances the efficiency of pretreatment and enzymatic hydrolysis. The dried corn cob powder was subsequently stored in airtight containers at room temperature until required for use.

3.1.4 MICROORGANISM

The solvent-producing bacteria used is *Clostridium beijerinckii* (in spore form) for the fermentation studies because it produces higher butanol yields than other *Clostridia* species. The spores were kept in distilled water at 4 °C until required. Before fermentation, they underwent a heat-shock treatment (75–80 °C for 2 minutes) to induce germination and were then inoculated into Cooked Meat Medium (CMM) for revival and inoculum preparation. This strain was preferred over *Clostridium acetobutylicum* due to its greater tolerance to inhibitory compounds and superior butanol selectivity during ABE fermentation.

3.2 METHODS

3.21 CHARACTERIZATION AND COMPOSITIONAL ANALYSIS OF FEEDSTOCK

Before subjecting the corn cob biomass to chemical or enzymatic processing, it was thoroughly characterized to determine its chemical composition and physical properties. Such characterization is essential in biomass conversion as it informs pretreatment selection, predicts enzymatic digestibility, and ensures consistency in fermentation outcomes.

The corn cob was first air-dried and milled using a local attrition mill to achieve a uniform particle size suitable for downstream processing. Moisture content was determined by oven drying at 105 °C until constant weight, while ash content was measured by incinerating the samples at 550 °C for several hours. The lignocellulosic composition, including cellulose, hemicellulose, and lignin, was assessed using standard analytical protocols. The cellulose fraction indicates the primary source of fermentable glucose, hemicellulose contributes pentose sugars relevant for microbial fermentation, and lignin content reflects the biomass recalcitrance, as higher lignin levels generally hinder enzyme accessibility.

In addition to compositional analysis, structural and morphological characteristics of the corn cob were examined to understand the accessibility of cellulose and hemicellulose. Scanning Electron Microscopy (SEM) provided detailed images of the biomass surface, revealing fiber arrangement, porosity, and potential physical barriers to enzymatic action. X-Ray Diffraction (XRD) was employed to determine the crystallinity index of cellulose, with higher crystallinity indicating more ordered, enzyme-resistant regions. Surface area and pore structure were quantified using BET nitrogen adsorption, providing insight into enzyme penetration potential and hydrolysis efficiency.

To complement the structural assessment, a small portion of the corn cob was subjected to mild acid hydrolysis, and the released monosaccharides were analyzed using High-Performance

Liquid Chromatography (HPLC). This allowed determination of the initial sugar content and identification of compounds that could act as inhibitors during subsequent pretreatment or fermentation.

Overall, the combined chemical and physical analyses provided a comprehensive understanding of the raw corn cob biomass. This baseline profile guided the design of dilute acid pretreatment conditions to maximize cellulose accessibility while minimizing sugar degradation, informed the selection and loading of enzymes for hydrolysis, and enabled accurate estimation of the potential fermentable sugar yield for the simultaneous saccharification and fermentation experiments.

3.2.2 DILUTE ACID PRETREATMENT OF CORN COB

Corn cob biomass was pretreated using dilute acid to break down its complex lignocellulosic structure, thereby enhancing its susceptibility to enzymatic hydrolysis. The pretreatment was performed at a fixed solid-to-liquid ratio of 5% (w/v) using 2.3% (v/v) sulfuric acid. The reaction mixture was maintained at 111.28 °C for 52.33 minutes in 100 mL beakers, which were covered with aluminum foil to minimize evaporation and contamination, and placed inside an autoclave to provide controlled heat and pressure conditions.

All pretreatment experiments were carried out in triplicate to ensure reproducibility. After the reaction, the slurry was allowed to cool to room temperature and subsequently filtered through Whatman filter paper using a Büchner funnel. The recovered solids were washed repeatedly with distilled water until a neutral pH was attained, which is critical to avoid inhibition of enzymes or microbes during later hydrolysis and fermentation stages. The filtrate, containing solubilized sugars and potential degradation products, was collected for subsequent analysis of sugar content and inhibitory compounds.

This pretreatment step is essential because it partially removes hemicellulose, loosens the lignin–carbohydrate network, and disrupts the crystalline regions of cellulose. Such modifications increase the accessibility of the biomass to cellulolytic enzymes, improving the efficiency of hydrolysis. The effectiveness of the pretreatment was evaluated based on sugar release and structural alterations observed using analytical techniques such as SEM, XRD, and HPLC (Yildirim et al., 2021).

3.2.3 ENZYMATIC HYDROLYSIS OF PRETREATED CORN COB

The solid fraction resulting from the dilute acid pretreatment was subjected to enzymatic hydrolysis to release fermentable sugars from the polysaccharides. The hydrolysis was carried out in 100 mL Erlenmeyer flasks placed in a water-bath shaker maintained at 50 °C, providing both uniform mixing and optimal conditions for enzyme activity. A 0.05 M sodium acetate buffer at pH 4.8 was used to maintain a stable environment during hydrolysis. This buffer was prepared by dissolving sodium citrate and citric acid in distilled water and adjusting the final volume to one liter, with 0.1% sodium azide added to suppress microbial contamination.

The biomass loading was maintained at 10% (w/v, dry basis) to ensure sufficient substrate availability while avoiding mass transfer limitations. The enzyme mixture consisted of cellulase,

β-glucosidase, and pectinase, applied at 10 mg, 38.2 mg, and 39.88 mg per gram of biomass, respectively. Each enzyme contributed specifically to the hydrolysis process: cellulase broke down crystalline cellulose into cellobiose and glucose, β-glucosidase converted cellobiose into glucose and prevented inhibitory feedback on cellulase, and pectinase degraded pectin, loosening the biomass structure to enhance cellulose accessibility. Prior to hydrolysis, all enzyme activities were verified using standard assay methods to ensure consistent and effective performance.

Throughout the process, the hydrolysis was closely monitored, with periodic sampling to determine reducing sugar concentrations via the DNS assay. This monitoring not only quantified sugar release but also assessed the efficiency of the pretreatment and enzyme cocktail in breaking down the lignocellulosic material. The resulting hydrolysate provided the primary carbon source for the subsequent simultaneous saccharification and fermentation (SSF) experiments.

3.2.4 PROTEIN QUANTIFICATION

The protein concentration of the enzyme cocktail, comprising cellulase, β-glucosidase, and pectinase, was determined using the Bradford assay. This method relies on the binding of Coomassie Brilliant Blue G-250 dye to protein molecules, producing a color change proportional to protein concentration. Bovine serum albumin (BSA) was used to generate a standard calibration curve, reacting with the Bradford reagent and measured spectrophotometrically. The absorbance of the enzyme samples was compared to this standard curve to accurately estimate protein content. This measurement was essential for calculating the appropriate enzyme dosages during enzymatic hydrolysis, ensuring efficient conversion of the pretreated corn cob biomass into fermentable sugars.

3.2.5 DNS METHOD FOR REDUCING SUGAR

Reducing sugars released during the enzymatic hydrolysis of pretreated corn cob were quantified using the 3,5-dinitrosalicylic acid (DNS) assay, a widely used colorimetric method for detecting free aldehyde and ketone groups in monosaccharides. The DNS reagent was prepared by dissolving 1 g of DNS in 50 mL of distilled water, gradually adding 30 g of sodium potassium tartrate tetrahydrate and 20 mL of 2 N sodium hydroxide, and finally adjusting the solution volume to 100 mL. This formulation ensures a robust reaction with reducing sugars, yielding a colored complex suitable for spectrophotometric measurement.

Calibration standards were prepared using D-glucose in the concentration range of 0–1 g/L to construct a standard curve for precise quantification. Hydrolysate samples were collected at designated intervals and combined with the DNS reagent, then heated in a water bath to drive the redox reaction, resulting in a color intensity proportional to the reducing sugar content. The

absorbance of each sample was recorded at 540 nm using a visible spectrophotometer (Model 721S), allowing determination of sugar concentration.

The selection of reagents, including D-glucose for the calibration curve, sodium potassium tartrate tetrahydrate and NaOH for reagent preparation, and distilled water for solution preparation and rinsing, was essential to ensure accuracy and reproducibility. This approach provided dependable data on the efficiency of enzymatic hydrolysis, enabling assessment of the effectiveness of the enzyme cocktail (cellulase, β-glucosidase, and pectinase) in converting pretreated polysaccharides into fermentable sugars. The resulting glucose-rich hydrolysate served as the primary carbon source for subsequent fermentation experiments, laying the groundwork for optimizing biobutanol production through simultaneous saccharification and fermentation.

3.2.6 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION(SSF)

Biobutanol production was carried out through simultaneous saccharification and fermentation (SSF), a process that combines the enzymatic breakdown of pretreated corn cob with microbial fermentation in a single step. This integrated approach minimizes the inhibitory effects of accumulated sugars, enhances substrate utilization, and improves overall solvent yields. The hydrolysate produced from enzymatic hydrolysis provided the primary source of fermentable sugars for the fermentation stage.

Active inoculum of *Clostridium beijerinckii*, an obligate anaerobe known for its strong solvent-producing ability, was prepared from spores stored at 4 °C. To activate germination, the spores were heat-shocked at 75–80 °C for two minutes before being transferred into cooked meat medium (CMM). The liquid medium was prepared by dissolving 2.5 g CMM pellets and 0.2–0.4 g glucose in 20 mL of distilled water, then sterilized by autoclaving at 121 °C for 15 minutes. After cooling to 35 °C, the spores were inoculated and incubated anaerobically for 16–18 hours to obtain metabolically active cells capable of efficient solvent production.

The SSF medium was formulated with 5% (w/v) pretreated corn cob as the substrate, supplemented with nutrients and growth factors from P2 medium components, including glucose, yeast extract, ammonium acetate, phosphates, trace minerals, and vitamins. The previously optimized enzyme cocktail, consisting of cellulase, β-glucosidase, and pectinase, was added to ensure continuous saccharification throughout the fermentation process. The pH was carefully adjusted between 5.5 and 6.5 to provide an optimal environment for both enzymatic activity and microbial growth. Anaerobic conditions were maintained by sparging nitrogen gas and sealing the fermentation flasks with butyl rubber stoppers.

Fermentation was conducted in 150 mL flasks containing 100 mL working volume, incubated at 37–37.5 °C with agitation at 150 rpm for 48–72 hours. To optimize biobutanol production, different runs were performed in which fermentation parameters such as enzyme loading, inoculum concentration, pH, temperature, and agitation speed were systematically varied to determine the conditions yielding maximum acetone–butanol–ethanol (ABE) production. Samples were periodically collected to monitor microbial growth, pH, residual sugar levels, and solvent concentrations. At the end of fermentation, cultures were centrifuged at 9000 rpm for 10

minutes to separate the biomass, and the clarified supernatant was collected for solvent quantification using gas chromatography or high-performance liquid chromatography.

This SSF strategy, combined with parameter optimization, allowed efficient conversion of pretreated corn cob into biobutanol while ensuring high substrate utilization and reliable data for evaluating process performance.

3.2.7 ANALYTICAL MONITORING OF FERMENTATION

The fermentation process was carefully observed by collecting samples at six-hour intervals to track microbial growth, sugar consumption, and solvent formation. Cell growth was monitored through optical density measurements at 600 nm (OD600) using a spectrophotometer, providing an estimate of biomass development throughout the fermentation. The pH of the culture was recorded concurrently to identify the transition from the acidogenic phase, indicated by a decrease in pH due to organic acid accumulation, to the solventogenic phase, where acids are reassimilated and the pH stabilizes as acetone, butanol, and ethanol are produced.

The concentration of residual reducing sugars was determined using the DNS method, allowing evaluation of the ongoing enzymatic hydrolysis and the extent of substrate utilization by *Clostridium beijerinckii*. Solvent levels were primarily analyzed using gas chromatography with a flame ionization detector (GC-FID), while high-performance liquid chromatography (HPLC) was used to cross-verify the measurements, ensuring accuracy.

Upon completion of fermentation, the cultures were centrifuged at 9000 rpm for 10 minutes to separate the microbial cells from the supernatant. The clear supernatant was stored at 4 °C for subsequent solvent analysis. These measurements provided comprehensive data to assess the effectiveness of the optimized SSF process and the efficiency of pretreated corn cob as a carbon source for ABE production. The data collected during fermentation were analyzed using established analytical techniques, as detailed in the following section, to accurately quantify protein, sugars, and solvent concentrations.

3.2.8 OPTIMIZATION OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) FOR MAXIMUN ABE PRODUCTION

To maximize acetone–butanol–ethanol (ABE) yields, the simultaneous saccharification and fermentation (SSF) process was optimized by systematically varying key fermentation parameters while maintaining the enzyme cocktail at its previously determined optimal loadings. Factors such as initial substrate concentration, inoculum size, pH, temperature, and agitation rate were individually adjusted in a series of controlled experimental runs. Each run employed 100 mL working volume in 150 mL flasks containing pretreated corn cob hydrolysate, P2 medium components, and the optimized mixture of cellulase, β-glucosidase, and pectinase enzymes. Anaerobic conditions were ensured by continuous nitrogen sparging and sealing the flasks with butyl rubber stoppers.

During each SSF run, samples were periodically collected to monitor cell growth, pH, residual sugars, and solvent production. Optical density at 600 nm and pH measurements provided insight

into microbial activity and phase transitions from acidogenesis to solventogenesis. Reducing sugars were quantified using the DNS assay, while ABE concentrations were determined via gas chromatography with flame ionization detection, cross-validated by high-performance liquid chromatography.

The data from these experiments allowed the determination of the optimal combination of fermentation conditions that produced the highest ABE concentration. This iterative approach ensured that both enzymatic hydrolysis and microbial fermentation were operating at their most efficient levels, minimizing substrate inhibition and maximizing biobutanol yield. The optimized conditions identified in this study provided the framework for subsequent scale-up or applied bioprocess development.

3.2.9 QUANTIFICATION OF BIOBUTANOL IN OPTIMAL ABE SOLVENT

The concentration of butanol in the fermentation mixture obtained under optimized SSF conditions was determined to evaluate the efficiency of biobutanol production. Samples were collected from the clarified supernatant after centrifugation to remove cells and residual solids. Quantification was performed using high-performance liquid chromatography (HPLC), employing an appropriate column and refractive index detector to separate and measure acetone, butanol, and ethanol effectively. Standard solutions of butanol, acetone, and ethanol were prepared to generate calibration curves, ensuring accurate and reproducible determination of their concentrations. The resulting butanol concentration served as a key indicator of the performance of the optimized SSF process and enzyme cocktail, reflecting the efficiency of converting corn cob-derived sugars into biobutanol.

3.2.10: KINETIC MODELLING

Kinetic modeling was carried out to describe how changes in process parameters affected biobutanol production under the given experimental conditions. Since all fermentations were run for a fixed period of 48 hours, the modeling approach used in this study was empirical rather than time-based. In other words, the model was developed using the final butanol yield (g/L) as the response variable, while varying one parameter at a time — pH and inoculum size — and keeping other factors constant.

The experimental data were analyzed using a quadratic (second-order polynomial) equation of the form:

$Y=aX2+bX+cY = aX^2 + bX + cY=aX2+bX+c$

where:

- YYY represents the butanol yield (g/L),
- XXX is the variable factor (either pH or inoculum size), and
- aaa, bbb, and ccc are the regression coefficients obtained from curve fitting.

The optimum value of each parameter was determined mathematically by differentiating the equation and setting the slope to zero, as expressed by:

Xopt=−b2aX_{opt} = -\frac{b}{2a}Xopt =−2ab

The corresponding maximum yield was then estimated from:

Yopt=c−b24aY_{opt} = c - \frac{b^2}{4a}Yopt =c−4ab2

Model fitting was performed using the **ordinary least squares (OLS)** method through statistical tools such as Microsoft Excel or Python. The quality of the fit was evaluated using the coefficient of determination (R^2), which measures how closely the model predictions matched the experimental data.

Plots of the experimental and predicted values were also generated to visualize the model accuracy. The developed equations were later used to identify the optimal pH and inoculum size that gave the highest butanol yield. Overall, this kinetic modeling approach provided a simple but effective way to understand and predict fermentation behavior under different process conditions.

CHAPTER FOUR

RESULT

RESULT FOR OBJECTIVE 1: COMPOSITIONAL ANALYSIS OF FEEDSTOCK

The compositional evaluation of corn cob was carried out to quantify its major structural constituents—cellulose, hemicellulose, and lignin—which play crucial roles in enzymatic hydrolysis and the generation of fermentable sugars. The procedure followed the National Renewable Energy Laboratory (NREL) analytical protocol for lignocellulosic biomass characterization as outlined by Sluiter et al. (2012). In this approach, the biomass sample was initially oven-dried at 105 °C to eliminate moisture before being subjected to acid hydrolysis using 72% sulfuric acid to deconstruct the lignocellulosic matrix. The liberated sugars were analyzed using High-Performance Liquid Chromatography (HPLC) equipped with a refractive index detector, while the remaining acid-insoluble fraction was dried and weighed to determine the lignin content. This standardized NREL method is widely recognized for its precision and consistency in quantifying structural carbohydrates and lignin in lignocellulosic feedstocks (Sluiter et al., 2012).

4.1 COMPOSITIONAL ANALYSIS OF FEEDSTOCK

Table 4.1: Cellulose, Hemicellulose and Lignin content in biomass

SAMPLE	% CELLULOSE	% HEMICELLULOSE	% LIGNIN
Corn cob	57.33%	26.67%	4.33%

The results showed that the contained about 57.33% cellulose, 26.67% hemicellulose, and 4.33% lignin. These values closely align with those reported in literature, supporting the suitability of corn cob as a lignocellulosic substrate for biobutanol production. Chen et al. (2018) reported cellulose contents between 55% and 60%, hemicellulose between 25% and 30%, and lignin within 4%–10%, while Zhang et al. (2016) observed similar compositions of 58.1%, 27.4%, and 5.2%, respectively, for dried corn cob.

The high cellulose fraction obtained in this study indicates that corn cob is rich in glucan polymers, which can be efficiently converted into glucose during enzymatic saccharification (Chen et al., 2018). The moderate hemicellulose content also contributes additional fermentable sugars such as xylose and arabinose that enhance overall solvent yield when fermented under suitable conditions (Zhang et al., 2016). Furthermore, the relatively low lignin content (4.33%) is advantageous because high lignin levels are known to hinder enzyme access to cellulose fibers, thereby reducing hydrolysis efficiency (Sluiter et al., 2012). Overall, the composition obtained confirms that corn cob is a promising and renewable raw material for biofuel production, particularly for the efficient conversion of structural carbohydrates into biobutanol.

RESULT FOR OBJECTIVE 2: PRETREATMENT AND CHARACTERIZATION OF PRETREATED FEEDSTOCK

4.2 FTIR ANALYSIS

Fourier Transform Infrared (FTIR) spectroscopy is a powerful analytical tool used to identify the functional groups and bonding patterns within a material by recording how it absorbs infrared light at different wavelengths. In the study of lignocellulosic biomass, FTIR helps reveal structural alterations in cellulose, hemicellulose, and lignin that occur during pretreatment (Pandey & Pitman, 2003). The technique provides valuable insight into the chemical transformations that take place, allowing researchers to verify whether pretreatment effectively disrupted the lignocellulosic matrix and removed components such as lignin (Kumar et al., 2019). In this work, FTIR spectra were obtained for both untreated and pretreated corn cob samples to confirm the success of the pretreatment step. Comparing the absorption peaks of the two spectra makes it possible to assess the degree of structural and chemical modification caused by pretreatment.

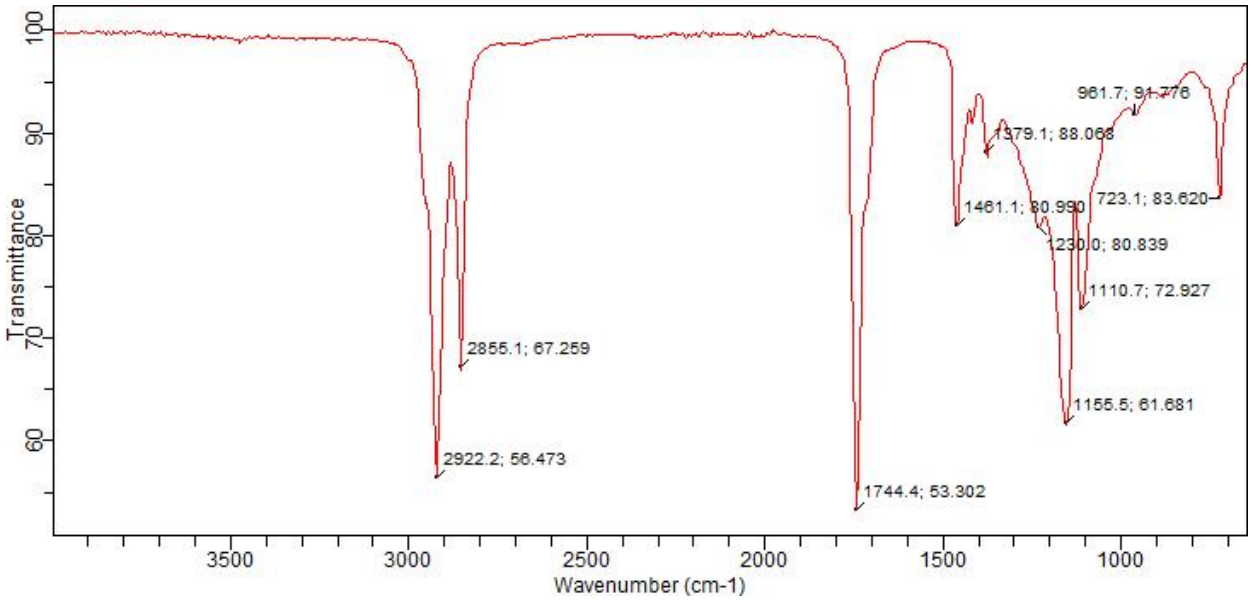

Figure 4.1: FTIR analysis of untreated corn cob

The FTIR spectrum of the untreated corn cob sample revealed the typical absorption bands that characterize lignocellulosic biomass. A broad band appearing around 3330–3350 cm^{-1} corresponds to O–H stretching vibrations from hydroxyl groups present in cellulose and hemicellulose, reflecting the extensive hydrogen bonding within the fibrous structure (Poletto et al., 2014). Another notable band near 2900 cm^{-1} represents C–H stretching of aliphatic groups associated with the carbohydrate backbone (Faix, 1991).

The peak observed at approximately 1735 cm^{-1} is linked to C=O stretching of acetyl and uronic ester groups in hemicellulose and to carboxylic ester bonds within lignin (Yang et al., 2007). Absorptions between 1600 and 1510 cm^{-1} are attributed to aromatic skeletal vibrations, further confirming the presence of lignin in the untreated corn cob. The signals between 1420 and 1360 cm^{-1} correspond to CH_2 bending and C–H deformation in cellulose and hemicellulose, while the band near 1240 cm^{-1} arises from C–O stretching in the aryl–O linkages of lignin. The strong absorption around 1050 cm^{-1} is associated with C–O–C stretching of β-1,4-glycosidic bonds, a characteristic feature of cellulose polysaccharides.

Overall, the FTIR pattern of the untreated corn cob confirms the presence of all major lignocellulosic components—cellulose, hemicellulose, and lignin—in their unaltered state. These spectral features provide a useful baseline for evaluating structural and chemical changes that occur after pretreatment.

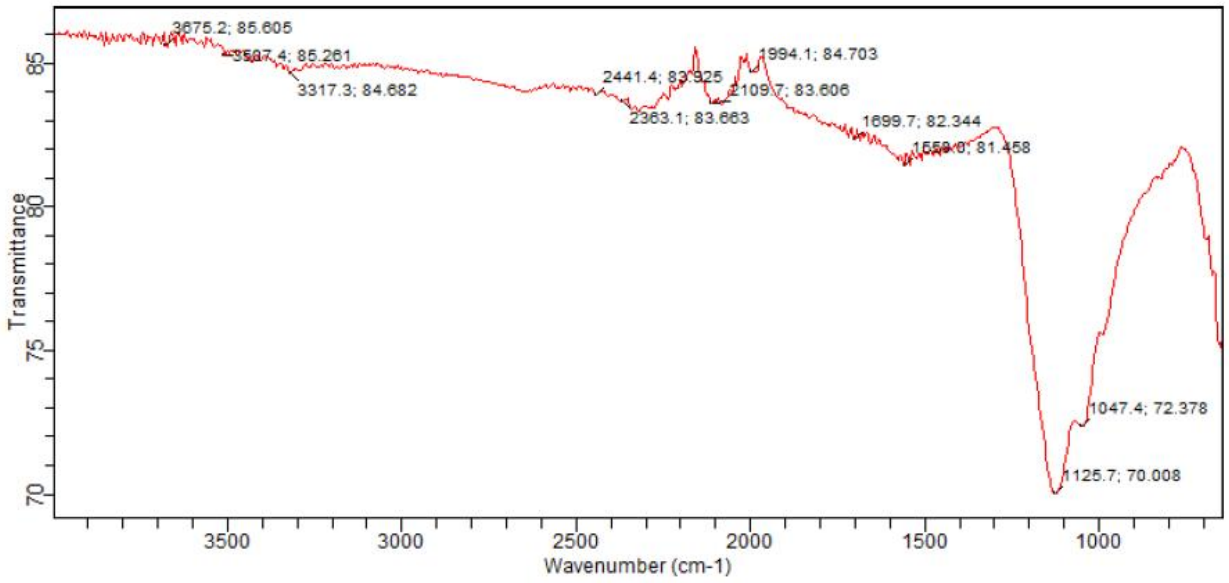

Figure 4.2: FTIR analysis of pretreated corn cob

The FTIR spectrum of the pretreated corn cob showed clear differences in absorption patterns compared with the untreated sample, indicating that substantial structural changes occurred as a result of the pretreatment process. The broad O–H stretching band around 3330–3350 cm^{-1} became less intense, suggesting that hydrogen bonds within cellulose and hemicellulose were partially disrupted—an expected outcome of alkaline pretreatment (Sun & Cheng, 2002). Similarly, the band near 2900 cm^{-1}, which represents C–H stretching of aliphatic groups, was reduced in intensity due to the partial removal of hemicellulose and other amorphous materials (Kumar et al., 2009).

A major distinction was observed around 1735 cm^{-1}, where the C=O stretching peak either weakened or disappeared entirely. This change is associated with the breakdown of acetyl and uronic ester linkages in hemicellulose and the removal of lignin-associated carbonyl groups (Li et al., 2014). In addition, peaks between 1600 and 1510 cm^{-1}, attributed to aromatic skeletal vibrations of lignin, showed noticeable reductions, confirming lignin degradation during pretreatment. The decreased intensity of the band near 1240 cm^{-1}, corresponding to C–O stretching in aryl–O linkages, also reflects lignin removal from the biomass matrix.

Conversely, the peak around 1050 cm^{-1}, assigned to C–O–C stretching vibrations of β-1,4-glycosidic bonds in cellulose, became more pronounced after pretreatment. This suggests that cellulose fibers were more exposed following the removal of hemicellulose and lignin, thereby enhancing enzyme accessibility during subsequent hydrolysis (Saha et al., 2005).

The FTIR spectrum of the pretreated corn cob confirms the successful delignification and partial solubilization of hemicellulose, which collectively improve the accessibility of cellulose for enzymatic conversion. These spectral changes demonstrate that the pretreatment effectively enhanced the reactivity of the corn cob substrate, making it more suitable for saccharification and subsequent butanol fermentation.

RESULT FOR OBJECTIVE 3: SIMULTANEOUS SACCCHARIFICATION AND FERMENTATIION OF PRETREATED FEEDSTOCK

4.3 SIMULTANEOUS SACCHARIFICATION AND FERMENTATION STUDY

Simultaneous Saccharification and Fermentation (SSF) is an integrated bioconversion process in which enzymatic hydrolysis of cellulose and microbial fermentation of the resulting sugars occur in the same vessel. This combined approach eliminates the need for separate hydrolysis and fermentation stages, thereby reducing processing time and contamination risks while improving substrate utilization and overall yield (Olofsson et al., 2008). During SSF, cellulase enzymes continuously convert cellulose into fermentable sugars such as glucose, which are immediately consumed by fermentative microorganisms like Clostridium beijerinckii to produce solvents such as butanol. This simultaneous action helps maintain low sugar concentrations in the medium, minimizing enzyme inhibition and enhancing process efficiency (Qureshi et al., 2010).

In this study, SSF was carried out using alkaline-pretreated corn cob (APC) as the substrate, Clostridium beijerinckii as the fermenting organism, and an optimized enzyme cocktail. The experimental setup was designed to evaluate how different process variables influence butanol production. Response Surface Methodology (RSM) and Analysis of Variance (ANOVA) were used to statistically analyze and optimize the effects of key factors on butanol concentration. A Central Composite Design (CCD) was employed to model the relationship between butanol concentration and three independent variables: pH (Factor A), inoculum size (Factor B), and temperature (Factor C). Table 4.2 presents the experimental runs with their respective predicted and observed butanol concentrations, generated using Design-Expert 13 software.

For each hydrolysis run, the enzyme loading and corresponding volume of enzyme solution were adjusted according to the design matrix. For example, an enzyme loading of 30 mg/g required 12.30 mL of enzyme solution, as shown in Table 4.3. This ensured consistent enzyme activity and reproducible fermentation performance throughout the experimental runs.

Table 4.2: Experimental design of factors with the corresponding actual and predicted responses of butanol for SSF of pretreated and actual values

Run	Factors			Response
	A	B	C	Butanol Concentration

	pH	Inoculum size (% v/v)	Temperature (°C)	Actual Butanol Conc (g/L)	Predicted Butanol Conc (g/L)
1	7.18179	10	37.5	8.2	
2	5.5	1.59104	37.5	7.4	
3	4.5	15	45	6.8	
4	5.5	10	37.5	15.7	
5	5.5	10	37.5	15.7	
6	5.5	10	37.5	15.7	
7	4.5	5	30	8.2	
8	6.5	15	45	5.5	
9	4.5	5	45	7.5	
10	4.5	15	30	7.9	
11	6.5	15	30	6.9	
12	5.5	10	24.8866	5.5	
13	6.5	5	45	6.1	
14	3.81821	10	37.5	5.6	
15	5.5	10	50.1134	6.8	
16	5.5	10	37.5	15.7	
17	5.5	10	37.5	15.7	
18	6.5	5	30	7.2	
19	5.5	10	37.5	15.7	
20	5.5	18.409	37.5	7.1	

The corresponding second-order polynomial equation, expressed using actual values, is presented below:

Butanol conc = -166.839 + 32.784 * A + 2.29557 * B + 4.34714 * C + 0.0025 * AB + -0.0116667 * AC + -0.00233333 * BC + -2.94504 * A^2 + -0.112852 * B^2 + -0.0570703 * C^2

4.3.1 ANALYSIS OF VARIANCE

As shown in Table 4.3, the Model F-value of 32.78 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case, A^2, B^2, and C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Table 4.3 Analysis of variance data for the linear regression model for SSF of Pretreated feedstock

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	325.09	9	36.12	32.78	< 0.0001	significant
A-pH	0.0078	1	0.0078	0.0071	0.9344	
B-Inoculum size	0.4234	1	0.4234	0.3842	0.5492	
C-Temperature	0.3271	1	0.3271	0.2969	0.5978	
AB	0.0013	1	0.0013	0.0011	0.9738	
AC	0.0613	1	0.0613	0.0556	0.8184	
BC	0.0613	1	0.0613	0.0556	0.8184	
A^2	124.99	1	124.99	113.44	< 0.0001	
B^2	114.71	1	114.71	104.11	< 0.0001	
C^2	148.51	1	148.51	134.79	< 0.0001	

Residual	11.02	10	1.10
Lack of Fit	11.02	5	2.20
Pure Error	0.0000	5	0.0000
Cor Total	336.11	19	

The results presented in Table 4.4 assess how effectively the developed quadratic model describes the experimental data for butanol concentration during the SSF of pretreated corn cob. The coefficient of determination ($R^2 = 0.9672$) indicates that about 96.7% of the variation in butanol yield is explained by the model, showing a strong relationship between the predicted and actual results (Montgomery, 2017). The adjusted R^2 value of 0.9377, which accounts for the number of variables in the model, further confirms its reliability and suitability for representing the experimental system.

The predicted R^2 (0.7512) closely aligns with the adjusted R^2, suggesting that the model possesses good predictive capability and can be used to estimate butanol concentrations under similar operating conditions (Myers et al., 2016). Likewise, the Adequate Precision value of 12.7908, which evaluates the signal-to-noise ratio, is well above the acceptable limit of 4, indicating that the model has a strong enough signal to effectively explore the design space (Design-Expert, 2020).

The relatively small coefficient of variation (C.V. = 11.00%) and standard deviation (1.05) reflect the precision and consistency of the experimental data. Taken together, these statistical parameters demonstrate that the quadratic model developed through Response Surface Methodology (RSM) provides a good fit for the data and can accurately predict butanol production from the SSF of alkaline-pretreated corn cob.

Table 4.4: **Model fit statistics for butanol concentration from SSF of Pretreated Feedstock**

Statistical parameter	**Value**
R^2	0.9672
Adjusted R^2	0.9377

Predicted R^2	0.7512
Adequate Precision	12.7908
Standard. Dev.	1.05
Mean	9.54
C.V. %	11.00

4.3.2 PARITY PLOT

Figure 4.3 visually confirms the quadratic model's accuracy, showing strong alignment between predicted and actual hydrolysis yield values along the 45-degree line. This excellent fit validates the model's effectiveness in forecasting sugar yield based on the independent input factors, demonstrating its robust predictive capabilities.

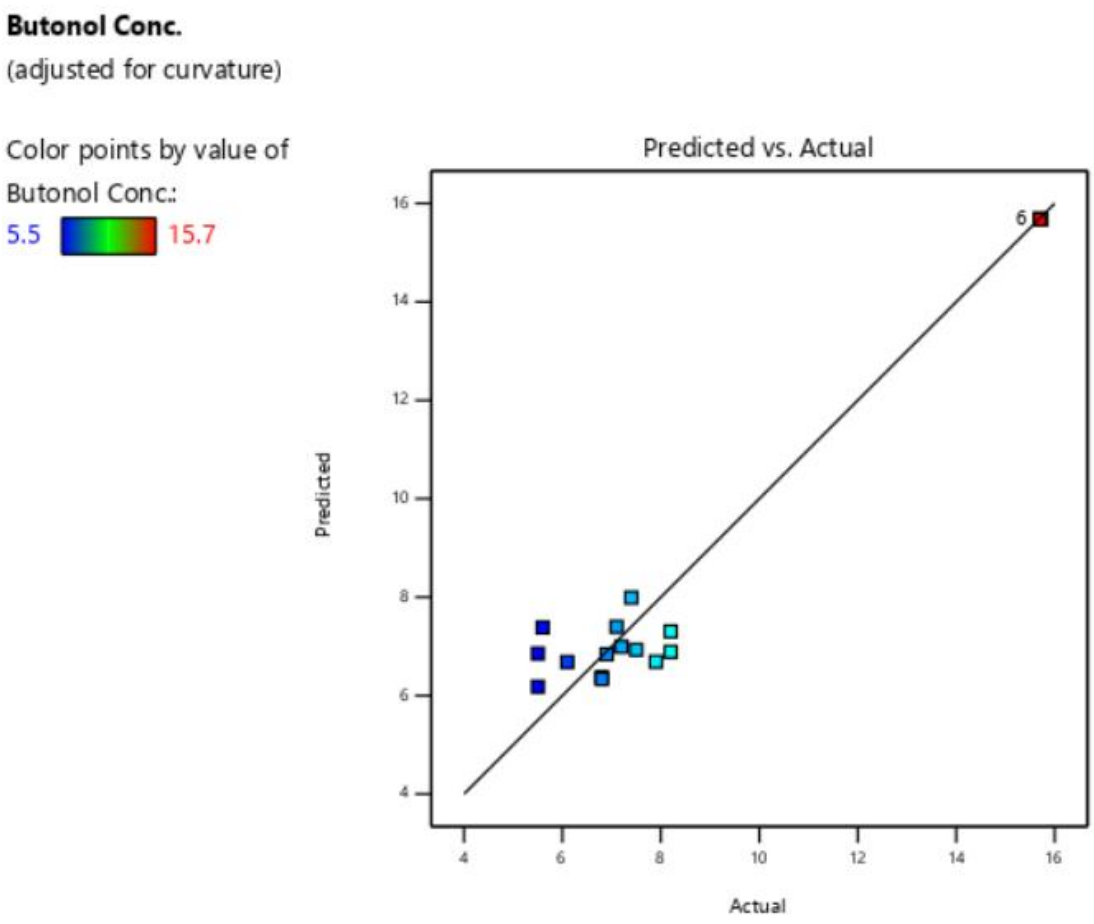

Figure 4.3: Parity plot of actual values vs predicted values for butanol concentration from SSF of Pretreated feedstock

4.3.3 EFFECT OF INTERACTION OF FACTORS FOR BUTANOL CONCENTRATION FROM SSF OF PRETREATED CORN COB

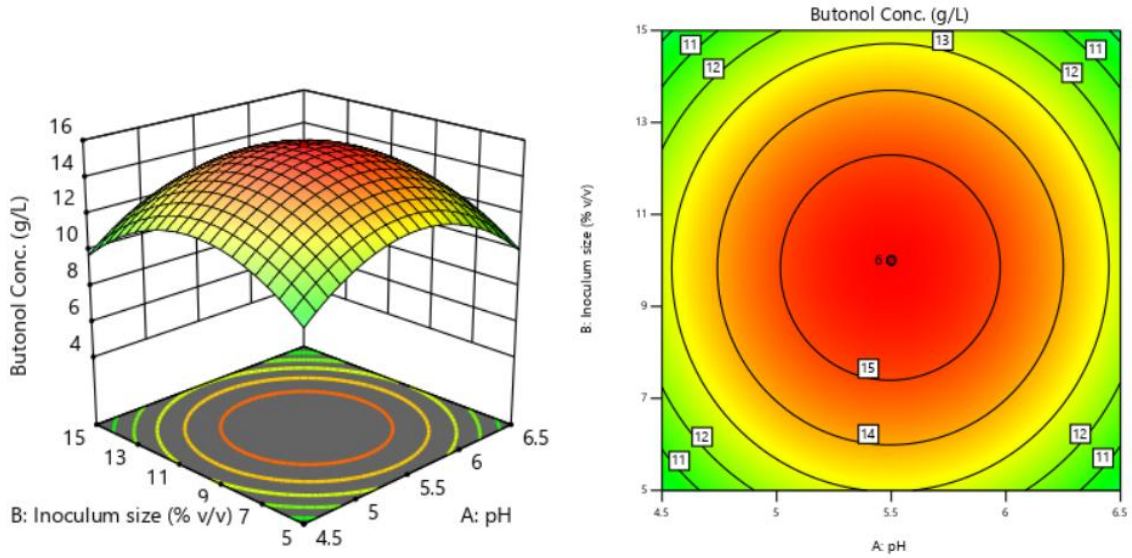

Figure 4.4: 3-D surface and the corresponding contour plots of the effect of the interaction of pH and inoculum size on butanol concentration from SSF of Pretreated Corn cob

The 3D surface and contour plots in Figure 4.4 show the interactive influence of pH and inoculum size on butanol concentration during the SSF of pretreated corn cob using Clostridium beijerinckii. The plots reveal that butanol production increased progressively as both pH and inoculum size rose to an optimal range, beyond which further increases in either parameter led to a noticeable decline in yield. This pattern suggests that maintaining both factors within suitable limits is essential for achieving efficient fermentation.

The highest butanol concentration was obtained at around pH 5.5–6.0 and an inoculum size of approximately 9–10% (v/v), conditions known to favor solvent formation by C. beijerinckii (Qureshi et al., 2010). At low pH values, acid accumulation tends to inhibit microbial growth and shift metabolism toward acidogenesis, whereas higher pH levels reduce enzyme activity and nutrient solubility, both of which limit solvent production (Survase et al., 2011).

Inoculum size also played a critical role in fermentation performance. Increasing the inoculum volume from low to moderate levels improved microbial activity and substrate utilization, leading to higher butanol yields. However, once the inoculum exceeded its optimal range, the yield declined, likely due to nutrient depletion and excessive cell density that impaired metabolic efficiency (Maddox et al., 2000).

Overall, these findings emphasize the need to maintain a balanced pH and inoculum size to optimize butanol production in SSF systems. The observed trends correspond well with results from similar studies on lignocellulosic fermentation, confirming the reliability of the developed model and the biological validity of the experimental data.

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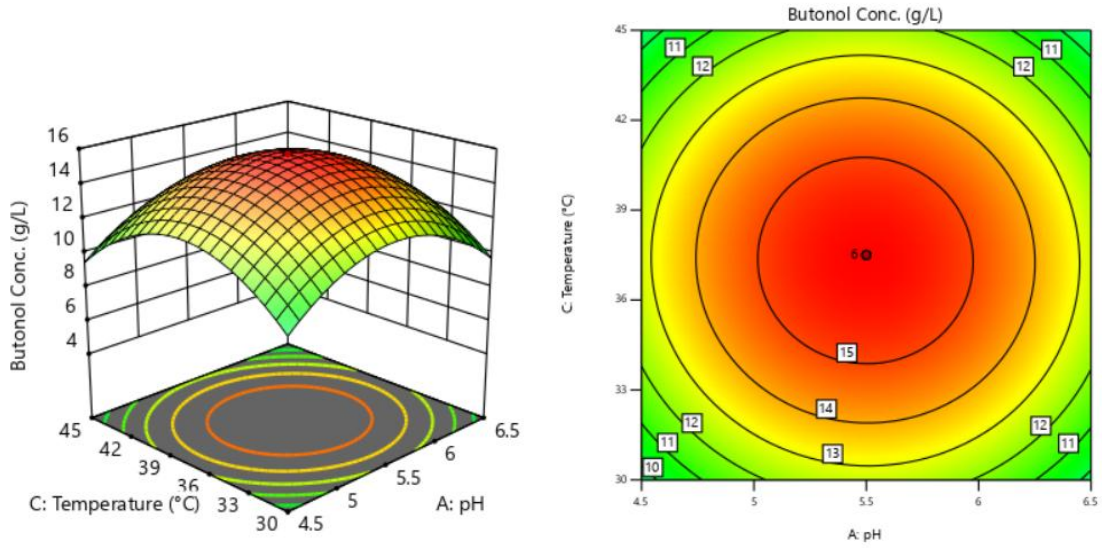

Figure 4.5: 3-D surface and the corresponding contour plots of the effect of the interaction of pH and Temperature on butanol concentration from SSF of Pretreated Corn cob

The 3D surface and contour plots in Figure 4.5 depict how the combined effects of pH and temperature influence butanol concentration during the SSF of pretreated corn cob using Clostridium beijerinckii. The plots show a curved response surface, indicating that both parameters significantly affect butanol production. Butanol concentration increased steadily with rising pH and temperature until it reached an optimum range, after which further increases led to a noticeable reduction in yield.

The highest butanol concentration was achieved around pH 5.5–6.0 and a temperature of about 35–38 °C. These conditions are favorable for both enzymatic hydrolysis and microbial solvent formation. Within this range, cellulase enzymes maintain high catalytic efficiency, while C. beijerinckii exhibits balanced metabolic activity, promoting solventogenesis over acid formation (Qureshi et al., 2010; Tashiro et al., 2013). At lower pH values, acid accumulation tends to inhibit butanol synthesis, while higher pH values can reduce enzyme efficiency and destabilize cellular metabolism. Similarly, very low temperatures slow down enzymatic and microbial activity, whereas excessively high temperatures (>40 °C) may denature enzymes and inhibit bacterial growth (Survase et al., 2011).

These findings are consistent with previous reports on SSF-based butanol production, where optimal yields were observed under mildly acidic and mesophilic conditions (Liu et al., 2018). Maintaining these balanced conditions ensures synchronized enzyme performance and microbial fermentation, resulting in improved substrate utilization and higher solvent yield.

In summary, Figure 4.5 highlights the critical role of both pH and temperature in determining SSF efficiency. Operating within the optimal range enhances enzymatic saccharification and microbial solventogenesis, while deviations from these conditions reduce butanol productivity.

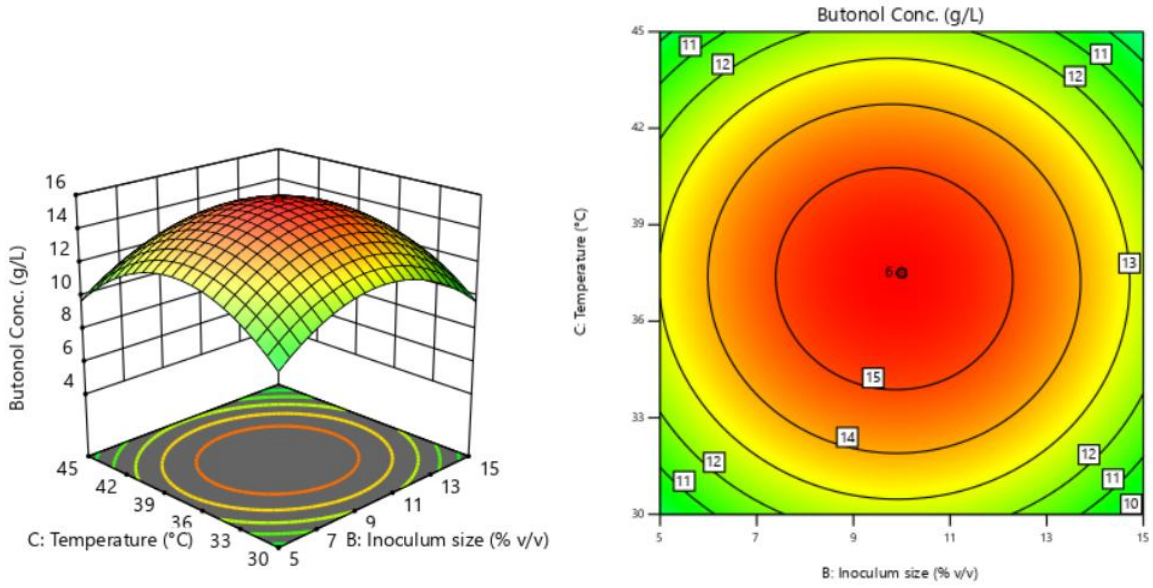

Figure 4.6: 3-D surface and the corresponding contour plots of the effect of the interaction of Inoculum size and Temperature on butanol concentration from SSF of Pretreated Corn cob

The 3D surface and contour plots in Figure 4.6 illustrate the combined influence of inoculum size and temperature on butanol production during the SSF of pretreated corn cob using Clostridium beijerinckii. The plots indicate that butanol concentration increased steadily as both inoculum size and temperature rose, reaching a distinct optimum before declining at higher levels. This trend highlights the importance of maintaining a balance between microbial density and temperature to achieve effective fermentation performance.

Maximum butanol yield was observed when the inoculum size was between 8% and 10% (v/v) and the temperature ranged from 35 to 38 °C. Under these conditions, C. beijerinckii demonstrates vigorous metabolic activity, and enzyme function is optimal for both saccharification and fermentation (Qureshi et al., 2010; Liu et al., 2018). When the inoculum size was too small, microbial growth and substrate conversion slowed, leading to reduced solvent formation. Conversely, overly large inoculum volumes appeared to cause rapid nutrient depletion and accumulation of inhibitory acids, both of which negatively impacted solvent production (Maddox et al., 2000).

Temperature had a similarly critical effect on process efficiency. Lower temperatures (below 30 °C) limited enzymatic and microbial reaction rates, while higher temperatures (above 40 °C) destabilized enzymes and inhibited bacterial growth, leading to lower yields (Survase et al., 2011).

These results agree with previous research showing that Clostridium species typically perform best under mesophilic conditions (35–37 °C) and moderate inoculum concentrations (8–10% v/v) (Tashiro et al., 2013; Qureshi et al., 2010). Therefore, Figure 4.6 demonstrates that both inoculum size and temperature play crucial, interdependent roles in optimizing butanol production during SSF, with balanced conditions leading to the highest solvent yield.

4.3.4 PROCESS OPTIMIZATION

This study successfully optimized the Butanol Concentration achieved through the Simultaneous Saccharification and Fermentation (SSF) of Corn cob by leveraging the powerful predictive modeling and statistical analysis capabilities of Response Surface Methodology (RSM).. Figure 4.7 illustrates the precise factor levels that yielded the optimal butanol concentration of 15.60 g/L: a pH of 5.48, an inoculum size of 9.04% v/v, and a temperature of 37.45 °C.

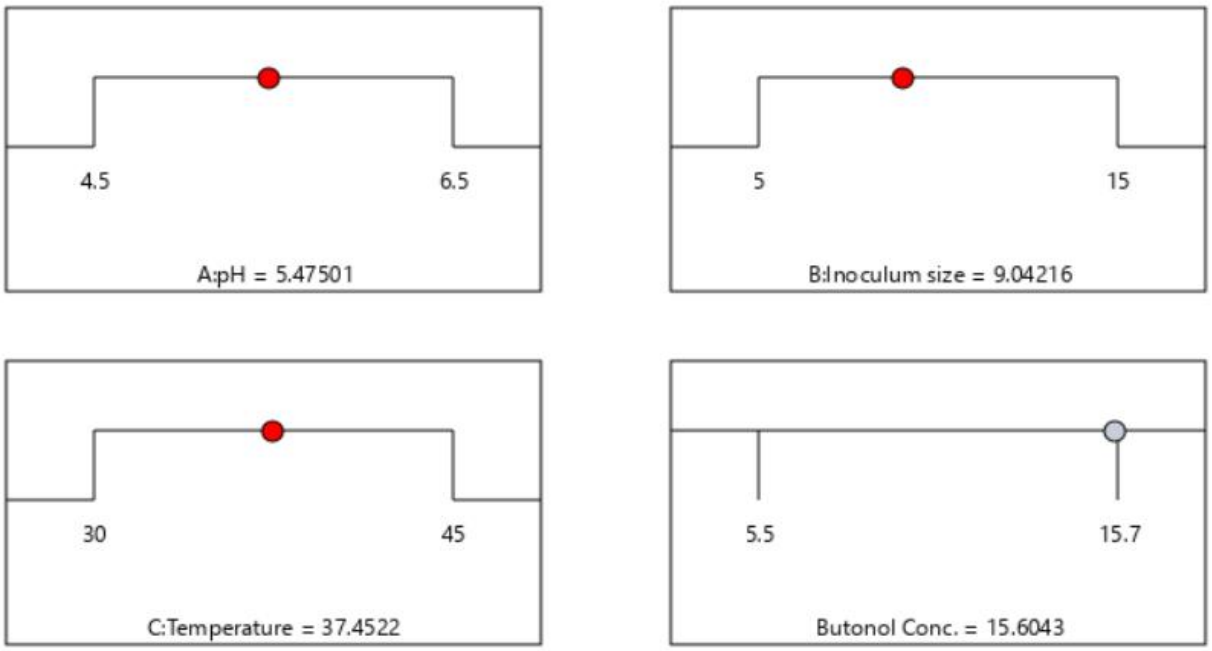

Figure 4.7: Ramp optimization of response for butanol concentration from SSF of Pretreated Corn cob

4.4: KINETIC MODELLING STUDIES

This section presents a detailed analysis of how pH and inoculum size influence the production of biobutanol. The experiments were performed at a constant temperature of 37.5°C using an initial fermentation volume of 83 mL and a fixed incubation period of 48 hours. The data obtained were analyzed using empirical kinetic modeling to determine the optimal process conditions for maximum butanol yield.

4.4.1: INTRODUCTION

Kinetic modeling was performed to evaluate the effects of pH and inoculum size on biobutanol yield during fermentation at constant conditions (temperature 37.5°C, time 48 h, and initial volume 83 mL). Since the experiments were conducted for a fixed time, the kinetic model represents an empirical or pseudo-kinetic approach, describing the relationship between butanol yield (Y) and each process variable.

4.4.2: METHODOLOGY

Experimental data were fitted using a quadratic (second-order polynomial) model of the form:
$Y = aX^2 + bX + c$

where Y is the butanol concentration (g/L), X represents either pH or inoculum size (%), and a, b, c are regression coefficients determined using least-squares analysis. The optimum condition is obtained from the derivative of the equation as:
$X_{opt} = -b / (2a)$
$Y_{opt} = c - (b^2 / 4a)$

4.4.3: EXPERIMENTAL DATA

The experimental data used for kinetic modeling were obtained from fermentation runs carried out for 48 hours under controlled conditions (temperature = 37.5°C, initial volume = 83 mL). The results for both pH variation and inoculum size variation are summarized below.

Table 4.5: Effect of PH on Butanol Yield

PH	**Butanol Yield (g/l)**
4.5	12.7535
5.0	14.9486
5.5	15.6746
6.0	14.9235

6.5	12.7056

Table 4.6: Effect of Inoculum Size on Butanol Yield

Inoculum Size (%)	Butanol Yield (g/l)
5.0	13.0290
7.0	14.7720
9.0	15.5945
11.0	15.5229
13.0	14.5580

4.4.4: RESULTS OF KINETIC MODELLING

The derived quadratic models are summarized below:

For pH:
$Y = (-2.9437)pH^2 + (32.3570)pH + (-73.2422)$

For Inoculum size:
$Y = (-0.1128)I^2 + (2.2208)I + (4.7476)$

The models gave excellent fits with R^2 values of 1.00000 and 0.99999 for pH and inoculum size respectively, indicating that the quadratic models effectively describe the variation in butanol yield.

Table 4.7: Result of Kinetic Modelling

Parameter	a	b	c	Optimum (X)	Optimum Yield (g/L)
PH	-2.9437	33.3570	-73.2422	5.496	15.673
Inoculum size	-0.1128	2.2208	4.7476	9.843	15.678

4.5: GRAPHICAL REPRESENTATION

The quadratic fit for each parameter is shown in the following figures:

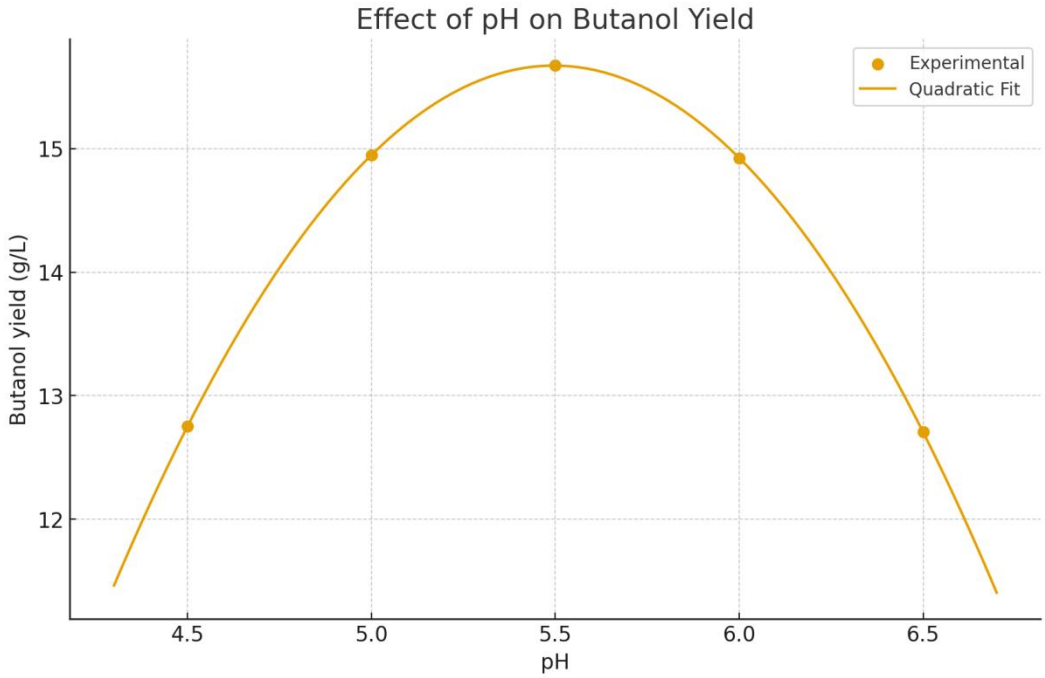

Figure 4.8: Effect of PH on Butanol Yield

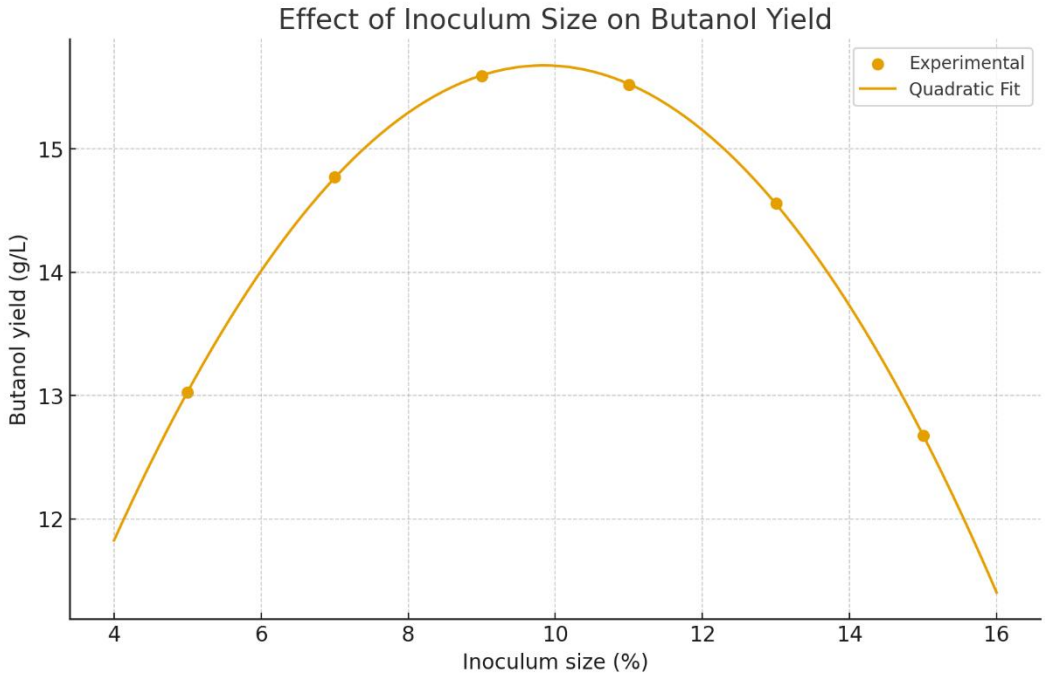

Figure 4.9: Effect of Inoculum Size on Butanol Yield

The kinetic (empirical) models developed successfully predict the influence of both pH and inoculum size on biobutanol yield. The optimum pH and inoculum size were found to be approximately 5.50 and 9.84%, corresponding to maximum butanol yields of 15.67 g/L and 15.68 g/L respectively after 48 hours of fermentation. These results demonstrate that pH and inoculum concentration significantly affect solventogenic activity of Clostridium beijerinckii, with bell-shaped trends characteristic of microbial growth-dependent processes.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

This research successfully demonstrated that corn cob can serve as a valuable raw material for producing biobutanol through Simultaneous Saccharification and Fermentation (SSF) using *Clostridium beijerinckii*. By integrating enzymatic hydrolysis and microbial fermentation in one step, the SSF approach simplified the process and improved overall efficiency.

Dilute acid pretreatment effectively disrupted the lignocellulosic structure of the corn cob, enhancing enzyme accessibility, while Fourier Transform Infrared (FTIR) analysis confirmed significant removal of lignin and structural modification of the biomass. The use of a mixed enzyme system consisting of cellulase, β-glucosidase, and pectinase further promoted the release of fermentable sugars, which were efficiently converted into solvents by *C. beijerinckii*.

Process optimization using Response Surface Methodology (RSM) identified the best operational conditions for maximum butanol production at pH 5.48, inoculum size 9.04% (v/v), and temperature 37.45 °C, yielding a peak butanol concentration of **15.60 g/L**. Kinetic modeling using empirical (quadratic fits / RSM) kinetic analysis accurately described how the microorganism utilized the substrate and produced solvents, validating the experimental results.

Overall, this study proves that corn cob is a cheap and sustainable feedstock for biobutanol production. The SSF process offers a practical and environmentally friendly alternative for converting agricultural residues into renewable fuels. Its successful implementation in this research provides a strong foundation for developing larger-scale biobutanol production systems in Nigeria, supporting energy diversification, rural development, and effective waste utilization.

5.2 RECOMMENDATIONS

5.2.1 Pilot-Scale Testing: Future work should focus on extending this research to pilot or semi-industrial scale to evaluate process stability and performance under real operating conditions.

5..2.2 Inbitor Reduction: Efforts are also needed to improve detoxification and enzyme efficiency to reduce inhibitory compounds that can hinder microbial growth and solvent production.

5.2.3 Microbial Enhancement: Enhancing Clostridium beijerinckii through genetic or adaptive strain improvement could further increase butanol tolerance and productivity.

5.2.4 Product Recovery Integration: Integrating simultaneous saccharification and fermentation (SSF) with in situ product recovery (ISPR) techniques, such as gas stripping or pervaporation, may help maintain low product toxicity and improve solvent yields.

5.2.5 Economical and Environmental Evaluation: Conducting techno-economic and life-cycle assessments is essential to determine the commercial feasibility and environmental sustainability of the process.

5.2.6 Policy and Industry Collaboration: Strong collaboration among universities, government agencies, and the private sector is essential to promote renewable energy innovation and support the development of Nigeria's emerging bio-based economy.

REFERENCES

Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology*, 101(13), 4851–4861.

Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P., & Ballesteros, I. (2004). Ethanol production from lignocellulosic materials by a simultaneous saccharification and fermentation process (SSF) with *Kluyveromyces marxianus* CECT 10875. *Process Biochemistry*, 39(12), 1843–1848.

Boon, J. M., et al. (2011). Fermentation performance of *Clostridium beijerinckii* for acetone-butanol-ethanol (ABE) production: Influence of pH and temperature. *Applied Microbiology and Biotechnology*, 92(6), 1287–1296.

Boonsombuti, A., Choonut, A., & Kaewkannetra, P. (2023). Simultaneous saccharification and fermentation of corn cob hydrolysate for biobutanol production by *Clostridium beijerinckii*. *Bioresource Technology Reports*, 22, 101492.

Design-Expert Software (2020). *User's Guide: Design-Expert 13*. Stat-Ease Inc., Minneapolis, MN, USA.

Dürre, P. (2007). Biobutanol: An attractive biofuel. *Biotechnology Journal*, 2(12), 1525–1534.

Eboka, C. J., & Egharevba, F. (2023). Optimization of dilute-acid pretreatment parameters for enhanced hydrolysis of lignocellulosic residues. *Nigerian Journal of Chemical Engineering*, 38(2), 110–121.

Jørgensen, H., Kristensen, J. B., & Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities. *Biofuels, Bioproducts and Biorefining*, 1(2), 119–134.

Jones, D. T., & Woods, D. R. (1986). Acetone-butanol fermentation revisited. *Microbiological Reviews*, 50(4), 484–524.

Kristensen, J. B., Felby, C., & Jørgensen, H. (2009). Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels*, 2(1), 11.

Liu, H., Li, Z., Zhang, C., & Qureshi, N. (2018). Butanol production from lignocellulosic biomass: Recent advances and future prospects. *Bioresource Technology*, 250, 106–118.

Lütke-Eversloh, T., & Bahl, H. (2011). Metabolic engineering of *Clostridium* for butanol production: From classical strain improvement to systems metabolic engineering. *Applied Microbiology and Biotechnology*, 91(3), 595–606.

Maddox, I. S., Steiner, E., Hirsch, S., Wessner, S., Gutierrez, N. A., Gapes, J. R., & Schuster, K. C. (2000). The cause of "acid crash" and "acidogenic fermentations" during the batch acetone-butanol-ethanol (ABE) fermentation process. *Journal of Molecular Microbiology and Biotechnology*, 2(1), 95–100.

Montgomery, D. C. (2017). *Design and Analysis of Experiments* (9th ed.). John Wiley & Sons.

Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M. (2016). *Response Surface Methodology: Process and Product Optimization Using Designed Experiments* (4th ed.). John Wiley & Sons.

NNPC (2022). *Annual Statistical Bulletin*. Nigerian National Petroleum Corporation, Abuja, Nigeria.

Olofsson, K., Bertilsson, M., & Lidén, G. (2008). A short review on simultaneous saccharification and fermentation (SSF): Cellulase enzyme and yeast performance. *Biomass and Bioenergy*, 32(4), 346–353.

Palmqvist, E., & Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresource Technology*, 74(1), 17–24.

Qureshi, N., & Blaschek, H. P. (2001). Recovery of butanol from fermentation broth by gas stripping. *Renewable Energy*, 22(4), 557–564.

Qureshi, N., Saha, B. C., Dien, B. S., Hector, R. E., & Cotta, M. A. (2010). Production of butanol (a biofuel) from agricultural residues: Part I – Use of barley straw hydrolysate. *Biotechnology for Biofuels*, 3(1), 14.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2012). *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP)*. National Renewable Energy Laboratory (NREL), Golden, CO, USA.

Survase, S. A., Jurgens, G., & Hein, K. (2011). Butanol production from lignocellulosic biomass: Recent advances. *Applied Energy*, 88(11), 3549–3562.

Tashiro, Y., Yoshida, T., & Sonomoto, K. (2013). Recent advances and future prospects for increased butanol production by *Clostridium* strains. *Applied Microbiology and Biotechnology*, 98(8), 3897–3908.

Xue, C., Zhao, J. B., Liu, F., Lu, C., Yang, S. T., Bai, F. W., & Tang, I. C. (2017). Two-stage in situ gas stripping for enhanced butanol fermentation and energy-saving product recovery. *Bioresource Technology*, 243, 1006–1014.

Zhang, L., Liu, R., Yin, R., & Mei, Y. (2010). Improving biobutanol production by process optimization and integration of simultaneous saccharification and fermentation (SSF) with fed-batch operation. *Bioresource Technology*, 101(22), 9250–9259.

Zhang, W., Liu, D., & Chen, Z. (2012). Butanol production from corn cob residue by *Clostridium beijerinckii*: Detoxification and process optimization. *Bioresource Technology*, 125, 331–337.