PROCESS IMPROVEMENT FOR ETHANOL PRODUCTION USING LIGNOCELLULOSIC BIOMASS

A

Thesis

Submitted towards the Requirement for the Award of the Degree of

Doctor of Philosophy

in

BIOTECHNOLOGY ENGINEERING

Under the Faculty of Engineering and Technology

By

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ACKNOWLEDGEMENT

This Ph.D. journey has been a profound and transformative experience filled with intellectual challenges, moments of doubt, growth, and, ultimately, accomplishment. The completion of this thesis would not have been possible without the support, guidance, and encouragement of many individuals, each of whom played an important role in shaping this journey. With heartfelt gratitude, I wish to express my sincere thanks to all who have contributed to my academic and personal development during these formative years.

First for most I would like to thanks almighty god for perseverance, courage and strength bestowed on me to undertake this project.

Furthermore, I extend my sincere thanks to the **Shri Jagdeesh Prasad Sharma**, Chancellor, P.K. University, Shivpuri (M.P.) for providing me necessary infrastructure and facilities that were essential during my research work.

My profound sense of gratitude, faith, and awe also to **Prof. (Dr.) Yogesh Chandra Dubey**, Vice Chancellor, P.K University, Shivpuri, (M.P.) for his generous and valuable support throughout my research work.

I take this pleasant opportunity to express my deep sense gratitude and reverence to his holiness **Dr. Jitendra Kumar Mishra**, Director (Admin), P.K University, Shivpuri (M.P.) for his constant encouragement and providing me with the required facilities that enabled me to complete my research work.

For most, I extend my deepest appreciation to my esteemed Supervisor, **Dr. Manish Kumar**, Associate Professor, Faculty of Biotechnology Engineering, P.K University, Shivpuri (M.P.), without whom this work would not have come to fruition.

His wisdom, patience, and insight have guided me from the very beginning of my research work. Your unwavering support, constructive criticism, and encouragement have been instrumental in shaping not only this research work but also my academic identity.

I would like to extend my gratitude and deep appreciation to respected **Prof. (Dr.) Bhaskar Nalla,** Dean Research & I/c Ph.D cell, P.K University, Shivpuri (M.P.).

I also extend my deepest appreciation and thanks to **Prof. (Dr.) Jitendra Kumar Malik,** Dean of Faculties, P.K University, Shivpuri (M.P.) for his valuable support and encouragement throughout my research work.

I express my gratitude and thanks to **Dr. Aiman Fatima,** Dean Academics, P.K University, Shivpuri (M.P.).

I also extend my deepest appreciation and thanks to **Prof. (Dr.) Balveer Singh,** Faculty of Engineering and Technology, P.K University, Shivpuri (M.P.) for his valuable support and encouragement throughout my research work.

Dr. Deepesh Naamdev, Registrar, P.K University, Shivpuri (M.P.) who has inspired me with his encouraging guidance and support right from the beginning of this research work in spite of his busy schedule.

To the members of my Research Advisory Committee, and Research Cell, thank you for your valuable inputs at various stages of the project. Your questions, suggestions, and recommendations helped in my work.

I also express my gratitude to **Miss. Nisha Yadav,** Librarian, P.K University, Shivpuri (M.P.) along with **Mr. Anand Kumar**, Assistant Librarian, and Mr. Ashish Gupta supporting staff of Research cell and other staff of P.K University.

I am also thankful to Mr. Alok Kahre, and Mr. Purushottam I.T Cell of P.K University, Shivpuri (M.P.). I would also like to acknowledge external examiners whose detailed review and constructive criticism during the final stage of this journey helped me view my work from a fresh perspective and improve its scholarly merit.

I am profoundly grateful to **P. K University, Shivpuri, (M.P.)** and the Department of Biotechnology Engineering for providing an intellectually stimulating environment and the resources needed to pursue my research.

I wish to thank all of my fellow colleagues of Bundelkhand University for their cooperation and support.

I am profoundly grateful to my brother **Mr. Shailendra Shukla**, his wife **Mrs. Smita Shukla** and my wife **Mrs. Suhasini Shukla** for their unwavering support, love, and encouragement during this challenging yet fulfilling journey. Their belief in me has been my greatest strength.

My thanks and best wishes go to my friends Dr. Yashodhara Sharma, Dr. Zakir Ali, Dr. Sadik Khan, Mr. Om Prakash Chaubey, Dr. Manoj Malhotra, Mr. Abu Arshad Khan, and Mr. K.M. Mishra for their inspiration.

I wish to acknowledge and extend my special thanks to Dr. Gaurav Srivastav, Er. Sandeep Kumar, Er. Ajay Tiwari, Er. Kuldeep Mishra and Miss Shivangi Suman have helped me immensely in writing my thesis and have provided continuous moral support.

At last, I thank **all those who, some or other way, helped** in my research work but somehow missed a mention.

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LIST OF ABBREVIATIONS

Abbreviations	Definition
WS	Wheat straw
SMS	Spent Mushroom Substrate
R.S.	Rice Straw
IEA	International Energy Agency
mM	Millimolar
SDGs	Sustainable Development Goals
GHG	Greenhouse Gas
CO_2	Carbon Dioxide
ILUC	Indirect land-use change
SSF	Simultaneous Saccharification and Fermentation
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
3G	Third-generation
LCA	Life cycle assessments
PBRs	Photobioreactors
RED	European Union's Renewable Energy Directive
AFEX	Ammonia fiber expansion
nM	Nanometer

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Paper/Submission ID	4418513
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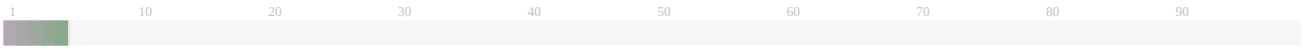

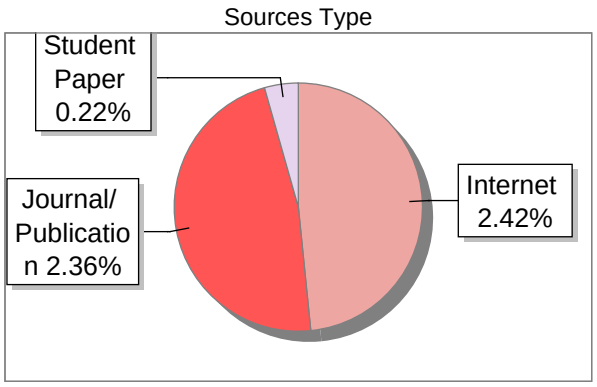

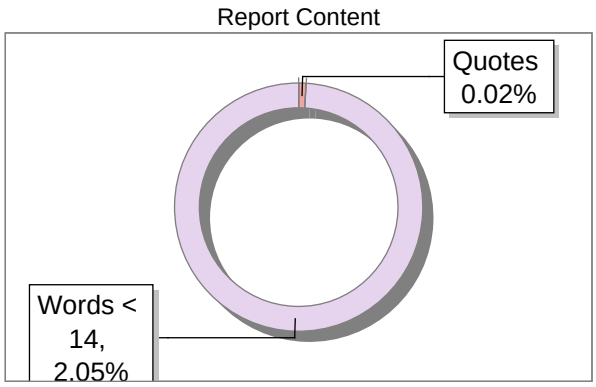

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ABSTRACT

Bioethanol production from lignocellulosic biomass has gained significant attention, particularly in the context of the Indian government's initiative to blend 20% bioethanol with gasoline, aimed at reducing reliance on fossil fuels, lowering carbon emissions, and effectively managing agricultural waste.

In this research, bioethanol production was explored using two prominent lignocellulosic substrates: Wheat Straw (WS) and Spent Mushroom Substrate (SMS). A multi-step process was employed, including pretreatment, enzymatic saccharification, and fermentation. Alkaline peroxide pretreatment was used to enhance the release of fermentable sugars, followed by hydrolysis using enzymes produced by Aspergillus niger (MTCC 2196) and Trichoderma viride (MTCC 800).

Comparative analysis demonstrated that SMS yielded superior bioethanol production, achieving 15.11%, compared to 10.63% from WS. These findings highlight the critical influence of pretreatment and enzymatic saccharification on optimizing bioethanol yields, while also acknowledging the economic challenges posed by enzyme costs. The study suggests that producing enzymes in-house can significantly reduce expenses, making the overall process more viable. This research underscores the potential of lignocellulosic biomass, particularly SMS, as a cost-effective and sustainable feedstock for renewable energy production, offering valuable insights for the advancement of bioethanol production technologies.

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CHAPTER 1

INTRODUCTION

1.1 Global Energy Demand

The global demand for energy is growing rapidly, driven by population growth, industrialization, and increasing energy needs in transportation, manufacturing, and other sectors. According to the International Energy Agency (IEA), the world's energy consumption is expected to rise significantly in the coming decades. One of the primary drivers behind this is the growth of developing economies, where rising living standards and urbanization are fueling the demand for electricity, fuel, and industrial energy. Global energy demand is projected to increase by 30% by 2040 if current consumption trends continue. (Chauhan et al, 2024)

The United Nations General Assembly adopted the Sustainable Development Goals (SDGs) in 2015 to inspire international cooperation to create a sustainable future for the planet. The goal of the SDGs by the end of 2030 is to eradicate extreme poverty, reduce inequality and injustice, and the main goal is to protect the planet by improving sustainable energy**.** *Energy Technology Perspectives 2008: Scenarios & Strategies to 2050: in Support of the G8 Plan of Action*. OECD/IEA.

A rapid global shift in energy systems is crucial to ensure that the increase in the average global surface temperature remains below 2°C.The Paris Agreement significantly impacts the energy sector, effects that are not yet fully incorporated into most current energy scenarios (IRENA and International Energy Agency (IEA)**.** At the heart of this change is a movement from fossil fuels to low-carbon energy alternatives, as energy-related carbon dioxide (CO_2) emissions represent roughly two-thirds of total greenhouse gas (GHG) emissions.

The reliance on fossil fuels remains high, but the environmental consequences of this dependence, including greenhouse gas (GHG) emissions and climate change, have catalyzed interest in renewable energy sources, such as bioethanol, solar, and wind.

One of the sectors facing the highest energy demand growth is transportation, which still depends largely on petroleum-based fuels like gasoline and diesel. The transportation sector currently consumes about 50% of the world's oil supply and is responsible for approximately 25% of global energy-related CO_2 emissions (Chang et al, 2017**).** As a result, enhancing energy security and reducing greenhouse gas (GHG) emissions and air

pollution from vehicles have become key priorities. This has driven governments to explore alternatives to petroleum-based fuels, which continue to dominate the transportation industry (Milovanoff et al, 2020). Efforts to decarbonize transportation have led to the adoption of renewable fuels such as bioethanol. Bioethanol, a type of biofuel derived primarily from biomass like corn, sugarcane, and lignocellulosic feedstocks, has emerged as a promising alternative to fossil fuels. Countries with high bioethanol production, such as the United States and Brazil, are among the leaders in utilizing biofuels to meet growing energy demands while striving for energy security and reducing reliance on imported oil (https://www.watertechnologies.com/blog/increasing-ethanol-production-efficiency-and-yiel d-unlock-sustainability-biofuels). Increased energy demand has necessitated the search for renewable and sustainable energy options, as fossil fuel combustion contributes significantly to global warming. Bioethanol's potential to be integrated into existing energy systems, particularly in the transportation sector, has positioned it as a key player in addressing global energy challenges. Bioethanol can be blended with gasoline in various proportions (e.g., E10, E85), offering the advantage of reducing GHG emissions while utilizing existing infrastructure such as fuel distribution networks. (Chauhan et al, 2024)

However, to meet the growing global energy demand sustainably, the production of bioethanol must be scaled up without causing environmental harm. As demand for bioethanol rises, innovations in feedstock selection and more efficient conversion processes are critical to ensuring bioethanol remains a viable solution for reducing the carbon footprint of the energy sector. (Merrit et al, 2023)

In addition to these considerations, advancements in biotechnological research have made it increasingly feasible to utilize agricultural residues and industrial waste, such as spent mushroom substrate (SMS), for sustainable bioethanol production. These second-generation feedstocks not only reduce competition with food resources but also help manage agricultural waste effectively. Moreover, recent innovations in enzymatic hydrolysis and microbial engineering have improved the sugar yield and conversion rates from lignocellulosic materials. Government initiatives, policy incentives, and cross-sector collaborations are also playing a pivotal role in promoting bioethanol as a mainstream fuel option. As countries continue to set net-zero carbon targets, the integration of biofuels into national energy strategies is likely to accelerate, making bioethanol a critical element in the global transition towards a low-carbon future.

1.2 Environmental Impact of Bioethanol Production

Bioethanol production offers significant environmental benefits, particularly in reducing greenhouse gas emissions. When bioethanol is burned as a fuel, the carbon dioxide (CO_2) released is roughly equivalent to the CO_2 absorbed by the plants used to produce it. This creates a closed carbon cycle that can significantly lower net emissions compared to fossil fuels, which release carbon stored in the earth for millions of years. Studies have shown that bioethanol can reduce GHG emissions by 50-70% compared to gasoline, depending on the feedstock and production method used. (Broda et al. 2022)

One of the most notable environmental impacts of bioethanol is its role in mitigating climate change. Transportation is a major contributor to GHG emissions, particularly in the form of CO_2. By replacing gasoline with bioethanol, it is possible to reduce emissions substantially. For instance, in states like California and Oregon, where clean fuel standards are in place, bioethanol has reduced GHG emissions from transportation by 42-45%**.** (https://www.watertechnologies.com/blog/incre asing-ethanol-production-efficiency-and-yiel d-unlock-sustainability-biofuels).

On a global scale, bioethanol production is projected to increase, helping to displace fossil fuels and lower the overall carbon footprint of the transportation sector (https://www .iea.org/reports/renewables-2023/transport-biofuels). Additionally, bioethanol production can utilize various feedstocks, including agricultural residues, municipal waste, and dedicated energy crops. The use of non-food-based (second-generation) feedstocks, such as lignocellulosic biomass, offers even greater environmental benefits by reducing competition with food production and minimizing the environmental impact of land-use changes. (Broda et al. 2022) second-generation bioethanol to be truly viable at a commercial scale, it must be integrated into a broader, sustainable, and low-carbon energy framework. This means more than just producing fuel-it involves optimizing the entire production chain to reduce costs, environmental impact, and resource intensity, while also aligning with global climate and economic goals.

One key area is techno-economic optimization. Advances such as enzyme recycling, improved hydrolysis strategies, and integrated fermentation methods like Simultaneous Saccharification and Fermentation (SSF) or Simultaneous Saccharification and Co-Fermentation (SSCF) significantly enhance yield while reducing input costs. These innovations not only improve

conversion efficiency but also help reduce enzyme usage, which remains a major cost factor in lignocellulosic ethanol production.

Equally important is policy and infrastructure support. Governments play a crucial role by introducing regulatory incentives such as blending mandates (e.g., E10, E20 fuel standards), tax credits, carbon pricing, and subsidies for biofuel technologies. Investment in distribution infrastructure, feedstock logistics, and public-private research partnerships is also essential to help bring advanced bioethanol into the mainstream energy mix.

Beyond energy, environmental co-benefits strengthen the case for bioethanol. Utilizing agricultural residues like wheat straw and rice husk not only diverts waste from landfills but also helps reduce air pollution from open-field burning. This can lead to improved air quality, especially in regions where residue burning contributes significantly to seasonal smog. Additionally, establishing biomass collection networks and decentralized processing facilities creates rural economic opportunities, offering new income streams for farmers and generating employment in bioenergy supply chains. By embedding second-generation bioethanol within a circular, low-carbon economy, it becomes more than a fuel-it becomes a tool for climate action, economic development, and sustainable agriculture.

These advanced feedstocks can be sourced from agricultural byproducts like wheat straw or corn stover, which would otherwise be discarded or burned, leading to further emissions reductions. However, bioethanol production is not without its environmental challenges. First- generation ethanol was primarily derived from plant-based sugars or starches. These biofuels are produced directly from food crops, with corn, wheat, and sugarcane serving as the main feedstocks. Sugar-based ethanol is primarily produced from plants like sugarcane, with Brazil being the leading producer using this method. In contrast, starch-based ethanol is mainly derived from corn and other grains, with the United States being the largest contributor. Other notable producers of starch-based ethanol include China, Canada, France, Germany, and Sweden. (Niphadkar et al, 2020).

First-generation bioethanol has raised concerns about food security, land-use changes, and water consumption. As of early 2016, first-generation bioethanol continued to account for the majority of the 25 billion gallons produced globally. The production was primarily attributed to the United States and Brazil, which accounted for about 85%, mainly utilizing corn and sugarcane, respectively. (36) Another important aspect to consider is that the primary feedstocks for first-generation biofuels are also food sources, potentially leading to competition between food and fuel production. Although only 2%

of the world's arable land is allocated to growing biomass feedstocks for first-generation biofuels (OECD/IEA 2008), this limited use may still contribute to rising prices of food and animal feed commodities. However, the direct or indirect influence of biofuels on food price increases remains uncertain.

(36) The diversion of food crops to bioethanol production has led to debates about the trade- off between using land for food versus fuel. There are also concerns about the indirect land- use change (ILUC), where the expansion of bioethanol feedstock cultivation can lead to deforestation or the displacement of other land uses, indirectly contributing to carbon emissions. (Abbas et al, 2017)

Third-generation bioethanol is derived from algae, offering a sustainable and non-food alternative to traditional feedstocks. Algae grow rapidly, require no arable land, and can utilize wastewater or saline water, minimizing resource competition. Both microalgae and macroalgae contain fermentable carbohydrates like starch, cellulose, and alginate, which can be hydrolyzed and fermented into ethanol. Ethanol yields of up to 64 g/L have been reported, showing promising conversion efficiency. However, challenges such as high cultivation costs, energy-intensive harvesting, and limited large-scale infrastructure remain. Advancements in strain engineering and integrated biorefineries are essential for making algae-based bioethanol economically viable and environmentally scalable.

1.3 Advantages of Algal Biomass for Bioethanol

i) Cultivation on Non-Arable Lands

Algae thrive in a variety of non-freshwater environments-such as seawater, brackish water, or nutrient-rich wastewater-eliminating competition with agricultural areas and reducing freshwater use. This flexibility supports sustainable biomass production without sacrificing arable acreage or food security. (Abbas et al, 2017).

ii) Rapid Growth & High Productivity

This flexibility supports sustainable biomass production without sacrificing arable acreage or food security. As a result, biomass yields per hectare can be notably higher, with multiple harvest cycles possible each year (Agwa et al., 2017).

iii) Rich and Diverse Carbohydrate Profiles

Algae, particularly macroalgae, contain polysaccharides like alginate, laminarin, mannitol, and sulfated glycans, in addition to starch and cellulose. These varied carbohydrate sources

provide multiple fermentable substrates, enhancing flexibility in hydrolysis and fermentation strategies. (Müller et al., 2023)

iv) Year-Round Cultivation and CO_2 Mitigation

Algae grow continuously throughout the year in suitable climates and can be cultivated in controlled photobioreactors. This continuous production, combined with their ability to capture CO_2 from industrial emissions, aligns with global decarbonization goals.

1.3.1 Cultivation and Pretreatment of Algal Biomass Cultivation Systems

Algal biomass can be cultivated using open pond systems or closed photobioreactors (PBRs)- each presenting unique advantages and challenges:

- Open ponds (e.g., raceway ponds) are simple and cost-effective, requiring minimal infrastructure like paddle-wheel mixing and shallow basins. They're widely favored for large-scale operations due to low capital and operational costs. However, they are prone to contamination, water evaporation, and limited control over environmental parameters like temperature, light, and CO_2.
- Photobioreactors (PBRs) are enclosed systems, such as tubular, flat-plate, or panel designs, that allow precise control over light exposure, gas exchange, temperature, and nutrient input. (Ekin, n.d.) This enables higher biomass densities, improved product quality, and reduced contamination. The trade-off is higher capital and operating costs, as well as challenges with scaling up due to light penetration issues and oxygen accumulation.
- Hybrid approaches combining both open and closed systems aim to leverage the fast growth in PBRs and low-cost harvesting in ponds, improving productivity and lipid yields.

1. 3.1.1 Pretreatment and Hydrolysis

Before fermentation, algal polysaccharides must be converted into fermentable sugars through pretreatment**,** typically using dilute acid and/or enzymatic methods:

- For example, the macroalga *Nizimuddinia zanardini* was treated with 7% H_2SO_4 at 100 °C**,** resulting in high sugar recovery, including glucan and mannitol release, with minimal inhibitor formation. (Ekin, n.d.)

- Follow-up enzymatic hydrolysis using cellulase and β-glucosidase achieved up to 80–82% of theoretical glucose yield, particularly following hot-water or acid pretreatment

- Dilute acid pretreatment effectively breaks down complex carbohydrates like laminarin and alginate, while minimizing the formation of inhibitory compounds such as HMF and furfural, critical for maintaining downstream fermentation efficiency.

1.3.1.2 Fermentation and Ethanol Yields

In third-generation bioethanol production, saccharification and fermentation often mirror those used with terrestrial feedstocks, typically employing yeast in processes like Simultaneous Saccharification and Co-Fermentation (SSCF). A landmark study using the red macroalga Kappaphycus alvarezii demonstrates SSCF's high potential: following dilute acid pretreatment, the biomass produced 64.3 g/L ethanol, equivalent to about 105 L per ton of dry seaweed. This yield translates to approximately 0.43 g of ethanol per gram of substrate, signaling efficient sugar recovery and conversion. (Tong et al., 2024) In that study, the acid pretreatment created two distinct streams: a galactose-rich liquid and a cellulose-rich solid. SSCF of both fractions together outperformed separate fermentation trials, achieving 64.3 g/L ethanol compared to 38 g/L from the liquid stream and 53 g/L from the solid stream individually. This synergy highlights SSCF's advantage in maximizing sugar utilization by combining saccharification, fermentation, and co-fermentation.

These results underscore the effectiveness of SSCF for macroalgal substrates rich in diverse sugars. They validate algae's potential as a robust ethanol feedstock and the value of integrated processing approaches that streamline steps and boost overall yield.

Despite challenges, such as the need for optimized pretreatment, sugar balance, and inhibitor management, achieving ethanol titers exceeding 60 g/L positions algae-based bioethanol as a promising complement to traditional first and second-generation biofuels. Future research should aim to improve pretreatment selectivity, enhance microbial tolerance to mixed sugars, and develop SSCF configurations that maximize both yield and economic feasibility.1.6 Integrating Bioethanol into a Sustainable Framework. (W. Wu et al., 2018)

1.3.1.3 Environmental Considerations of Third-Generation Bioethanol

Third-generation (3G) bioethanol, derived from macroalgae, provides environmental benefits compared to first- and second-generation biofuels. Life cycle assessments (LCA)

have shown that macroalgal ethanol exhibits significantly lower environmental burdens, thanks to minimal arable land use, reduced freshwater demand, and the absence of energy-intensive lignin processing steps typical of terrestrial biomass processing Many macroalgae species lack lignin entirely, eliminating a common bottleneck in enzymatic breakdown and chemical use Moreover, macroalgae growth captures atmospheric or industrial CO_2, contributing to greenhouse gas mitigation.

However, environmental trade-offs remain. Energy demands for dewatering, drying, and harvesting algae-especially in photobioreactor systems-can offset some benefits. Open-pond systems are resource-efficient but suffer from evaporation and contamination issues. Photobioreactors, while more controlled, are energy-intensive and rely on artificial lighting. Both cultivation modes introduce complexities reflected in LCA hotspots related to infrastructure and resource use. Environmental impacts can be softened by using wastewater or industrial CO_2 streams, but comprehensive cradle-to-grave assessments are essential to validate sustainability claims.

Overall, macroalgal bioethanol can offer robust environmental benefits compared to land-based biofuels. Yet, realizing these gains demands a holistic production framework that balances cultivation, processing, and resource inputs to ensure a net positive impact.

Despite clear environmental promise, the economic viability of third-generation bioethanol remains uncertain. Major cost drivers include cultivation infrastructure, harvesting, dewatering, and downstream processing. Macroalgal cultivation often relies on photobioreactors or offshore systems, which are expensive to build and maintain. Harvesting operations-whether through centrifugation, flocculation, or screening-can consume as much energy as the biofuel produced, posing a significant economic barrier.

Techno-economic assessments suggest that process cost dominates the total production cost, often making macroalgal ethanol substantially more expensive than first- or second-generation alternatives. For example, producing microalgal biodiesel can reach $3.90 per liter, though co-production strategies-such as combining biofuel with high-value products like pigments or nutraceuticals-can reduce costs to $0.54 per liter. This indicates that integrated biorefinery models could bridge the price gap.

Future advancements aim to reduce costs through several pathways:

- Optimizing high-density, lower-cost cultivation using wastewater or nutrient-rich land.
- Energy-saving downstream techniques for harvesting and pre-treatment.

- Genetic engineering of algae and fermentation microbes to improve carbohydrate yield and stress resilience.
- Co-product valorization, producing proteins, pigments, or feed alongside bioethanol.

Overall, while macroalgal ethanol shows improved environmental sustainability, economic feasibility demands integrated, multi-product biorefineries and optimized processes-without these, 3G bioethanol is unlikely to compete at scale.

Table 1: Comparison of First, Second, and Third Generation Substrates for Bioethanol Production

Parameter	First Generation	Second Generation	Third Generation
Feedstock Source	Food-based crops	Lignocellulosic biomass (non-food residues)	Algae and aquatic biomass
Examples	Corn, sugarcane, wheat, cassava	Wheat straw, rice husk, sawdust, SMS	Microalgae, macroalgae
Key Advantages	High sugar/starch content, easy to ferment, mature technology	Abundant, avoids food conflict, waste valorization	No land use, fast growth, CO_2 fixation, high yields
Key Limitations	Competes with food, needs arable land, and high-water use	Requires pretreatment, enzyme cost, and inhibitors	High processing cost, water-intensive, and infrastructure limits
Fermentation Complexity	Low-direct sugar/starch fermentation	High – requires pretreatment & hydrolysis	Moderate to high – needs hydrolysis & detoxification
Sustainability	Moderate – food vs fuel concerns	High agricultural waste utilization	Very high potential – non-land-based, carbon reduction

To mitigate these concerns, many countries are promoting second-generation bioethanol, which uses non-food biomass and offers a more sustainable option. Additionally, policies are being introduced to ensure that bioethanol production adheres to sustainability criteria. For example, the European Union's Renewable Energy Directive (RED) includes sustainability standards for biofuels, ensuring that bioethanol contributes positively to environmental and climate goals. (Abbas et al, 2017).

In conclusion, while bioethanol production offers substantial environmental benefits in terms of GHG reductions and the potential to mitigate climate change, its sustainability depends on responsible feedstock sourcing, efficient production methods, and adherence to environmental regulations.

1.4 Lignocellulosic Biomass

As nations progress and living standards rise, energy demand increases significantly. Simultaneously, the depletion of fossil fuels is creating an energy shortfall, highlighting the urgent need for alternative energy sources. A promising solution to bridge this energy gap is the utilization of sustainable and renewable resources, such as lignocellulosic biomass. (Agbor et al, 2011)

Lignocellulosic biomass (LCB), also referred to as lignocellulose, is the most plentiful renewable material found on Earth. It is generated through photosynthesis, a process in which plants absorb sunlight and utilize it to transform carbon dioxide (CO_2) from the atmosphere and water into organic compounds. This biomass is composed of a complex mixture of compounds, primarily polysaccharides, phenolic polymers, and proteins, which are key components of plant cell walls, particularly in woody plants. (Bajpai et al, 2016a)

The structure of LCB is intricate and highly organized. At its core, cellulose-a long-chain carbohydrate polymer-serves as the primary structural material. Surrounding the cellulose fibers is hemicellulose, another type of carbohydrate polymer, which binds with the cellulose. Further encasing these polysaccharides is lignin, a tough and rigid aromatic polymer that gives wood its strength and resistance to degradation. This tightly bound structure of cellulose, hemicellulose, and lignin makes lignocellulosic biomass both resilient and challenging to break down.

The makeup of these components can differ significantly between plant species. For instance, hardwoods tend to have higher cellulose content, while materials like wheat

straw and leaves contain larger amounts of hemicellulose. Within a single plant, the proportions of these constituents also shift based on factors such as the plant's age, growth stage, and environmental conditions. These polymers are interconnected in a complex matrix, with the degree of association and their relative composition varying depending on the biomass's type, species, and even the specific source from which it is derived. (Bajpai et al, 2016b).

In recent years, lignocellulosic biomass has gained significant interest not only as a key resource for second-generation bioethanol production but also for its potential in advancing the biorefinery concept, where a single feedstock can be utilized to produce various value- added products.Beyond biofuels, LCB can be converted into bioplastics, organic acids, animal feed, and biochar, making it a versatile resource in the circular bioeconomy. Its availability in large quantities as agricultural residues-such as wheat straw, corn stover, bagasse, and spent mushroom substrate-adds to its sustainability appeal by reducing competition with food crops and supporting waste valorization. Additionally, advances in pretreatment technologies and enzyme engineering have enhanced the feasibility of LCB utilization by improving sugar recovery and reducing process inhibitors. As research progresses, the integration of lignocellulosic feedstocks into decentralized energy systems, especially in rural and agrarian regions, may contribute to both energy security and rural economic development, making it a cornerstone of future renewable energy strategies.

Additionally, advances in pretreatment technologies, enzyme engineering, and microbial fermentation systems have improved the conversion efficiency of lignocellulosic feedstocks into fermentable sugars. Emerging research also explores the integration of genetically modified microorganisms and adaptive process optimization using AI and machine learning, aiming to minimize cost and maximize yield. As research progresses, the integration of lignocellulosic feedstocks into decentralized energy systems, particularly in rural and agrarian communities, may offer a dual advantage of energy independence and local economic upliftment. Therefore, LCB is poised to play a pivotal role in future renewable energy strategies and sustainable industrial development.

Table 2: The levels of cellulose, hemicellulose, and lignin in various types of lignocellulosic biomass.

Types of ligno-cellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	40-50	25-35	25-35
Corn cobs	45	35	15
Wheat straw	30	50	15
Switchgrass	45	31.4	12

Constituents of lignocellulosic biomass- The following are the constituents of the lignocellulosic Biomass

1.4.1.1 Cellulose

Cellulose, the world's most prevalent organic substance, is a complex carbohydrate found in plant structural frameworks, including wood, cotton, and grasses. Its linear chains of glucose molecules, joined by beta-1,4-glycosidic linkages, provide strength, stiffness, and insolubility in water. These qualities make it useful in industries such as paper and textile manufacture, food additives, and bioplastics. However, its most promising use is bioethanol production. (Bai et al,2022) To convert cellulose into fermentable sugars, a multistep process is required. To begin, pretreatment degrades the hard structure, allowing enzymes to enter. Cellulases, which are specialized enzymes, then hydrolyze cellulose to yield glucose monomers. Finally, glucose molecules are fermented by yeast or bacteria to produce ethanol. While this technology provides a renewable and sustainable alternative to fossil fuels, it confronts several obstacles, including high production costs due to the energy-intensive nature of pretreatment and hydrolysis, lower ethanol yields compared to starch-based sources, and competition from other cellulose uses. Despite these hurdles, researchers and developers are constantly looking for ways to increase the efficiency and cost-effectiveness of cellulose-based bioethanol production, paving the way for a more sustainable energy future.

Cellobiose, the smallest repeating unit of cellulose, can be broken down into glucose molecules. The cellulose-hydrolyzing enzymes (i.e., cellulases) are classified into three major groups: endoglucanases, exoglucanases, and β-β--β-glucosidases.

The endoglucanases catalyze arbitrary fractionalization of internal bonds of the cellulose chain, while cellobiohydrolases (exoglucanases) attack the chain ends, releasing

cellobiose. (Chang et al, 2011), (Ilić et al. 2023)

In addition to their individual roles, these enzymes often work synergistically to achieve effective cellulose breakdown. Endoglucanases initiate the process by opening up internal regions of the cellulose microfibrils, creating new chain ends for exoglucanases to act upon. Exoglucanases, in turn, cleave cellobiose units from the exposed ends, and β-glucosidases complete the conversion by hydrolyzing cellobiose into glucose monomers. The efficiency of this enzymatic cascade is highly dependent on the substrate's crystallinity, porosity, and degree of polymerization. Native cellulose, especially in biomass with high lignin content, is often recalcitrant to enzymatic action, which is why pretreatment is a crucial prerequisite.

Furthermore, advancements in enzyme engineering have led to the development of thermostable and pH-tolerant cellulases, which can operate under industrial conditions and reduce process costs. The application of genetically modified microbial strains capable of overproducing cellulases is also being explored to boost hydrolysis efficiency. The integration of these technologies into biorefineries not only increases glucose yield but also enhances the overall economic viability of lignocellulosic ethanol production, aligning with global goals for clean energy and circular bioeconomy.

1.4.1.2. Hemicellulose

Hemicellulose, constituting approximately 20-50% of lignocellulosic biomass, is the second most abundant polymer. Unlike cellulose, hemicellulose is not chemically uniform and is characterized by branched structures with short lateral chains composed of various sugars. These monosaccharides include pentoses (such as xylose, rhamnose, and arabinose), hexoses (such as glucose, mannose, and galactose), and uronic acids (including 4-O-methyl glucuronic, D-glucuronic, and D-galacturonic acids). The hemicellulose backbone can be a homopolymer or heteropolymer, with short branches connected through beta (1,4)-glycosidic and occasionally beta (1,3)-glycosidic bonds. (Sarip et al, 2016) Hemicellulose can also exhibit acetylation, as seen in heteroxylan. Compared to cellulose, hemicellulose has a lower molecular weight, with short lateral chains that are easily hydrolyzed. Its composition varies across biomass sources; for instance, hemicelluloses in agricultural residues like straw and grasses are primarily composed of xylan, while softwoods predominantly contain glucomannan. In many plants, xylans are heteropolysaccharides with a backbone of 1,4-linked beta-D-xylopyranose units, and may also contain arabinose, glucuronic acid or its 4-O-methyl ether, acetic acid, ferulic acid, and p-coumaric acid. Xylan can be extracted under acidic

or alkaline conditions, while glucomannan extraction typically requires stronger alkaline conditions. Hemicelluloses are among the most thermochemically sensitive components of lignocellulosic materials. They are thought to coat cellulose fibrils within plant cell walls, and it has been suggested that removing at least 50% of hemicellulose significantly enhances cellulose digestibility. (Porninta et al. 2024), (Bhatia et al. 2020) However, pretreatment conditions must be carefully controlled to avoid the formation of degradation products such as furfural and hydroxymethylfurfural, which are known to inhibit fermentation. Therefore, pretreatment parameters are often optimized to balance sugar recovery. Depending on the pretreatment method, hemicellulose can be recovered either as a solid fraction or as a mixture of solid and liquid fractions. (Chang et al, 2012). In addition to serving as a barrier to enzymatic cellulose hydrolysis, hemicellulose plays a critical functional role in maintaining plant structural integrity and regulating water retention. Its branched and amorphous nature allows it to act as a binding matrix between cellulose and lignin, facilitating flexibility and strength within plant cell walls. From a biofuel perspective, the hydrolysis of hemicellulose yields a diverse mixture of fermentable sugars, primarily xylose and arabinose, which are particularly valuable in second-generation bioethanol production. However, most industrial yeast strains like Saccharomyces cerevisiae are naturally inefficient at fermenting pentose sugars. To overcome this limitation, extensive metabolic engineering and adaptive evolution techniques have been applied to develop recombinant strains capable of utilizing xylose and arabinose more effectively. Moreover, recent research has highlighted the potential of hemicellulose-derived oligosaccharides in producing value- added biochemicals, such as xylitol and lactic acid, offering additional economic incentives for biorefinery development. As interest grows in fully valorizing lignocellulosic biomass, hemicellulose is increasingly viewed not just as a secondary component but as a key contributor to integrated bioeconomy strategies aimed at maximizing resource efficiency, reducing carbon emissions, and enhancing the overall sustainability of biofuel systems.

1.4.1.3 Lignin

Lignin, the third most abundant natural polymer, is a complex macromolecule made up of crosslinked phenolic monomers. Plant cell walls include lignin, which offers stiffness, impermeability, and resistance to microbial destruction and oxidative stress. It is an important structural component of the primary cell wall, helping maintain the plant's structural integrity and infection resistance. Lignin is made up of three primary phenylpropanoid alcohols: coniferyl alcohol (guaiacyl propanol), coumarin alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). Typically, herbaceous

plants like grasses have lower lignin concentrations, whereas softwoods have higher levels. . **(**Chauhan et al, 2024**),** (Broda et al. 2022), (Mnich et al, 2020) Lignin serves as the "glue" that binds the components of lignocellulosic biomass, making it insoluble in water and posing a considerable barrier to enzymatic and microbial degradation. Cellulose microfibrils are tightly bound to lignin, a complex polymer that acts as a barrier, hindering the breakdown of biomass for biofuel production. Removing lignin has been shown to significantly improve digestibility, as it directly impedes the enzymes responsible for breaking down cellulose. Lignin's negative impacts extend beyond simply being a physical barrier; it actively interferes with the enzymes by binding to them non-productively and releasing toxic byproducts that inhibit microbial activity. (Cheah et al, 2020) (Yuan et al,2021).

Different feedstocks have varied quantities of lignin, which must be decreased during pretreatment to improve biomass digestibility. During pretreatment, lignin is thought to melt and re-solidify when cooled, modifying its characteristics and allowing it to precipitate. Delignification, or the chemical extraction of lignin, has various advantages, including biomass swelling, disruption of the lignin structure, increased internal surface area, and better accessibility of cellulolytic enzymes to cellulose fibers. Although not all pretreatment procedures remove substantial amounts of lignin, they may alter the chemical structure of lignin, increasing digestibility even if the overall lignin content remains unchanged relative to untreated biomass. In recent years, considerable interest has emerged in valorizing lignin as a byproduct rather than discarding it. Lignin-derived compounds can be transformed into a variety of high-value chemicals, such as vanillin, phenolic resins, adhesives, and carbon-based materials. These valorization pathways offer economic incentives to biorefineries and reduce waste output. Furthermore, the structural complexity of lignin-once considered an obstacle-is now viewed as a resource for generating aromatic building blocks that are difficult to derive from petroleum alternatives. Additionally, advanced analytical techniques such as nuclear magnetic resonance (NMR) and Fourier-transform infrared spectroscopy (FTIR) are being used to monitor lignin composition and structural changes post-pretreatment, aiding in the design of more efficient biomass processing methods. The continued development of mild, selective delignification techniques (e.g., organosolv, oxidative delignification) is also enhancing lignin removal without excessive degradation of cellulose or hemicellulose. Understanding lignin’s chemical reactivity and role in plant defense is crucial for the future of biofuel research, as it enables tailored pretreatment strategies and supports the development of lignin-tolerant microbial strains for more robust fermentation processes.

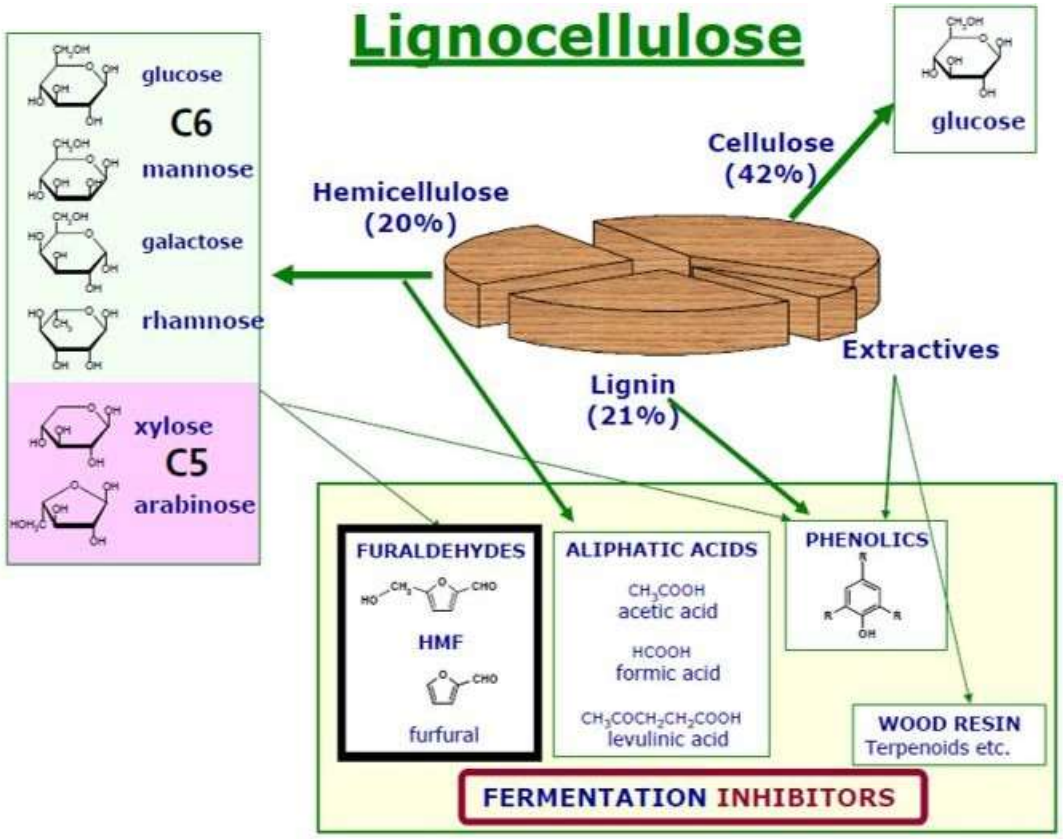

Fig. 1: Lignocellulosic Biomass composition

1.5 Bioethanol from Spent Mushroom Substrate and Wheat Straw

Wheat straw is a highly underutilized lignocellulosic resource that holds great potential for bioethanol production, along with spent mushroom substrate, whose significant use in bioethanol production is not widely documented. Both of these materials are often discarded as waste, contributing to environmental pollution, yet they are rich in cellulose and hemicellulose, key components required for bioethanol production.

1.5.1 Spent Mushroom Substrate (SMS)

SMS is the leftover material after mushroom cultivation. It typically contains a mixture of organic materials like straw, sawdust, and animal manure that have been partially decomposed by the fungi. Although rich in organic matter, spent mushroom substrate (SMS) is commonly regarded as agricultural waste. In many countries, SMS disposal poses a significant challenge for mushroom farms due to environmental regulations and high waste management costs. (Jordan et al, 2008)

Spent Mushroom Substrate (SMS), the residual material left after mushroom harvesting, represents a valuable yet largely untapped resource for bioethanol production. Typically composed of partially degraded straw, sawdust, poultry manure, and other organic

amendments, SMS is enriched with microbial enzymes and partially digested lignocellulosic matter. During the mushroom cultivation cycle, fungi such as *Pleurotus ostreatus* or *Agaricus bisporus* secrete ligninolytic and cellulolytic enzymes to access nutrients, leading to the partial breakdown of cellulose and lignin in the substrate. This biological degradation process preconditions the biomass, reducing its recalcitrance and enhancing its suitability for enzymatic hydrolysis and fermentation.

What distinguishes SMS from untreated agricultural waste is this inherent biological pretreatment, which lowers the requirement for harsh chemical or thermal processing. As a result, the energy input and chemical load associated with SMS-based bioethanol production can be significantly reduced compared to conventional lignocellulosic feedstocks. Moreover, SMS is available in large volumes in mushroom-producing regions, and its disposal is a growing environmental challenge. Utilizing it for bioethanol production offers a dual benefit: mitigating waste management issues and generating renewable energy.

In terms of sugar release and fermentation efficiency, studies have reported promising glucose yields from SMS following mild enzymatic treatment. This highlights SMS as a low-cost, sustainable, and regionally abundant feedstock with high potential for integration into second- generation biofuel systems.

However, SMS is an excellent candidate for bioethanol production because its partial decomposition by fungi can enhance its digestibility. The lignocellulosic structure is already partially broken down during mushroom growth, reducing the need for intensive pretreatment processes that are typically required for bioethanol production. This makes SMS a low-cost, readily available feedstock that can be converted into fermentable sugars more easily compared to other raw lignocellulosic materials. (Oguri et al, 2011) (Chen et al, 2022)

1.5.2 Wheat Straw

Wheat straw, another lignocellulosic agricultural residue, is also highly underutilized. Global wheat production reached 670 million tons in 2012 and continues to rise annually. Assuming a ratio of 1.3 for straw to grains, the residual weight is 887 million tons. Wheat straw can be used for a variety of purposes, including soil erosion prevention, tillage, burning, bedding, and forage for ruminants. It is estimated that around 400 million tons of wheat straw will go unused, presenting an opportunity for its utilization as biomass for ethanol production. It is

produced in vast quantities during wheat cultivation, with a large portion left in the fields after harvest **(**Mankar et al, 2021**).** In many regions, wheat straw is either burned in open fields or used as low-value animal bedding, contributing to air pollution and wasted potential.

Instead of burning or discarding it, wheat straw can be converted into bioethanol. Its high cellulose and hemicellulose content makes it a valuable resource for biofuel production, while its low economic value as a waste product makes it an attractive option for industries seeking sustainable feedstocks.

The use of wheat straw in bioethanol production not only adds value to this agricultural byproduct but also helps reduce environmental pollution caused by open-field burning.

Wheat straw's structural composition-typically consisting of 35–45% cellulose, 20–30% hemicellulose, and 15–20% lignin-makes it particularly suitable for enzymatic hydrolysis following appropriate pretreatment. The fibrous and porous structure allows relatively good accessibility for enzymes, especially after lignin disruption through alkaline or oxidative pretreatment methods. Studies have shown that wheat straw pretreated with alkaline hydrogen peroxide or dilute acid can yield significant amounts of fermentable sugars when subjected to enzymatic saccharification.

Furthermore, wheat straw’s widespread availability in major wheat-producing countries such as China, India, Russia, the United States, and Canada provides logistical advantages for establishing decentralized bioethanol production facilities. Its potential is further enhanced by its compatibility with co-fermentation processes when combined with other lignocellulosic feedstocks like rice straw or spent mushroom substrate (SMS), improving overall process economics and sugar recovery. Additionally, integrated biorefinery concepts are increasingly exploring wheat straw as a multipurpose feedstock, not just for ethanol but also for co-products like biogas, organic acids, and lignin-derived biochemicals. As such, wheat straw holds significant promise as a cornerstone material in the global shift toward renewable, biomass- based energy systems.

1.5.3 Environmental and Economic Benefits

Utilizing both SMS and wheat straw for bioethanol production offers several benefits. It aligns with the principles of the circular economy by turning waste materials into valuable energy resources. This reduces waste, minimizes environmental harm, and adds economic value to agricultural and industrial byproducts

https://www.iea.org/energy- system/renewables/bioenergy. Moreover, it provides an alternative to using food crops for bioethanol, thus avoiding the food-vs-fuel debate while contributing to cleaner energy production.

In conclusion, SMS and wheat straw represent underutilized, renewable resources with great potential for bioethanol production. Their use not only mitigates waste management issues but also contributes to the development of sustainable energy solutions.

1.6 Definition and Composition of Spent Mushroom Substrate (SMS)

Spent Mushroom Substrate (SMS) is the organic material that remains after the cultivation of mushrooms, serving as a byproduct of the mushroom farming process. This substrate typically consists of a variety of organic materials that provide nutrients for mushroom growth, such as straw, sawdust, poultry manure, cottonseed hulls, and other agricultural residues. Once the mushrooms are harvested, the remaining substrate is considered "spent," though it still contains valuable organic matter that can be repurposed. (Jordan et al, 2008)

Despite being termed “spent,” SMS retains a substantial portion of its original chemical and structural composition, including unutilized cellulose, hemicellulose, and partially degraded lignin. The fungal activity during mushroom growth alters the substrate’s physical and biochemical characteristics, often improving its porosity and increasing the digestibility of lignocellulosic components. As a result, SMS is not only suitable for agricultural applications such as compost or animal feed but also shows high potential as a feedstock in bioenergy production, particularly bioethanol.

The exact composition of SMS varies depending on the mushroom species cultivated and the substrate formulation used. For instance, SMS from oyster mushroom cultivation tends to have higher levels of residual carbohydrates and ligninolytic enzyme activity, while button mushroom SMS may contain more organic nitrogen due to the inclusion of manure. Physicochemical analysis of SMS reveals it is rich in total organic carbon, with a moderate C:N ratio, which supports microbial fermentation processes. Additionally, its fibrous texture and improved enzymatic accessibility post-harvest make it a strong candidate for pretreatment and enzymatic hydrolysis in lignocellulosic biorefineries. Utilizing SMS not only contributes to waste valorization and circular agriculture but also helps mitigate environmental problems associated with its bulk disposal, such as methane emissions from uncontrolled decomposition.

1.6.1 Composition of SMS

The primary components of SMS are cellulose, hemicellulose, and lignin, which are common in lignocellulosic biomass. These components are partially broken down by the fungi during the mushroom growing process, making SMS a rich and relatively accessible source of fermentable sugars for bioethanol production.

Cellulose: Cellulose is a polysaccharide made of glucose units linked together in a linear chain. It forms the structural component of plant cell walls and constitutes around 30-40% of SMS. This high cellulose content is crucial for bioethanol production, as it can be enzymatically hydrolyzed into glucose, which can then be fermented into ethanol by microorganisms like *Saccharomyces cerevisiae*.

Hemicellulose: Hemicellulose is another polysaccharide, but unlike cellulose, it is branched and made up of various sugars such as xylose, mannose, and galactose. It accounts for 20-30% of SMS and is more easily degraded than cellulose. The partial breakdown of hemicellulose during the mushroom cultivation process improves its accessibility during bioethanol production

Lignin: Lignin is a complex aromatic polymer that provides rigidity to plant cell walls and is resistant to enzymatic breakdown. While it is less useful for bioethanol production, lignin can be utilized for energy production or as a byproduct in other industrial processes. In SMS, lignin accounts for 10-15% of the substrate. The partial decomposition of lignin during the mushroom growth process makes the cellulose and hemicellulose more accessible for enzymatic conversion. (Vasilakis et al, 2023)

In addition to these primary constituents, SMS often contains residual fungal biomass, trace minerals, and microbial metabolites. These components, though present in smaller amounts, may play roles in downstream processing by influencing microbial growth and enzyme efficiency during hydrolysis and fermentation. For example, residual nitrogen from the mushroom substrate or fungal cell walls may support microbial metabolism, reducing the need for nutrient supplementation. Furthermore, due to partial biological pretreatment by fungi, SMS often exhibits lower recalcitrance than untreated lignocellulosic residues, which improves enzyme binding and activity. The porosity and surface area of SMS are also enhanced post-harvest, aiding in moisture retention and enzyme penetration. These changes not only improve sugar yields during saccharification but also reduce the severity and duration of external pretreatment requirements. Understanding the full compositional profile of SMS is essential for optimizing its use

as a bioethanol feedstock, allowing for tailored process parameters that increase efficiency and cost-effectiveness in biofuel production.

1.6.2 Potential as a Bioethanol Feedstock

SMS is particularly attractive as a feedstock for bioethanol production because it has already undergone partial decomposition by the fungi, reducing the need for extensive pretreatment processes that are usually required for lignocellulosic materials. The mushrooms degrade some of the complex structures, especially lignin, which acts as a barrier to the hydrolysis of cellulose and hemicellulose.

Moreover, using SMS for bioethanol production offers significant environmental and economic benefits. Mushroom farming generates large volumes of SMS, which are often discarded or used as low-value compost or animal feed. This leads to waste management challenges and environmental concerns, such as greenhouse gas emissions from decomposing organic material. Converting SMS into bioethanol not only reduces waste but also provides a renewable source of energy, making the process more sustainable and economically viable.

Additionally, SMS is readily available in mushroom-producing regions, and its use does not compete with food production, as it is an agricultural byproduct. This makes it a promising alternative to other first-generation bioethanol feedstocks, such as corn or sugarcane, which are associated with the food vs. fuel debate.

In summary, SMS is a valuable and underutilized resource with significant potential for bioethanol production due to its rich content of lignocellulosic materials. Its partial decomposition during mushroom cultivation makes it easier to process than other biomass feedstocks, reducing the cost and complexity of converting it into ethanol. By using SMS, bioethanol production can be made more sustainable while addressing waste management issues in the mushroom farming industry.

Additionally, the microbial and enzymatic profile of SMS further enhances its suitability as a feedstock. SMS often retains a consortium of beneficial microbial communities and fungal enzymes-such as laccases, manganese peroxidases, and lignin peroxidases-that continue to exhibit residual activity even after mushroom harvesting. These native enzymes can contribute to further degradation of lignocellulosic fibers during bioethanol processing, thus enhancing saccharification efficiency. Furthermore, due to its porous and fibrous structure, post- mushroom cultivation, SMS exhibits improved water retention and better enzyme accessibility during hydrolysis. Recent studies have also

reported that the carbon-to-nitrogen (C: N) ratio of SMS is favorable for microbial fermentation, supporting robust growth of fermentative organisms like Saccharomyces cerevisiae. Ongoing advancements in biotechnological processing, such as consolidated bioprocessing (CBP) and simultaneous saccharification and fermentation (SSF), could make SMS-based bioethanol production even more viable by integrating multiple steps into a single process. Ultimately, SMS not only represents an economical and efficient bioethanol feedstock but also plays a role in circular bioeconomy models, supporting both waste valorization and renewable energy generation.

1.7 Composition of Wheat Straw

Wheat straw, an abundant agricultural residue, is primarily produced during the cultivation of wheat, one of the most widely grown crops globally. Regions with intensive wheat farming, such as China, India, the United States, and Europe, generate massive quantities of wheat straw annually. After harvesting wheat, the straw, which constitutes about 50% of the wheat plant's total biomass, is left behind in the fields. For every ton of wheat produced, approximately 1.5 tons of straw are generated.

Given the global production of wheat, wheat straw is available in immense quantities and has the potential to be a key feedstock for bioethanol production.

Wheat straw is mainly composed of lignocellulosic materials, primarily including cellulose, hemicellulose, and lignin. These components are critical for bioethanol production because they can be broken down into fermentable sugars through pretreatment and enzymatic hydrolysis. Specifically:

- Cellulose (approximately 30-60% of wheat straw) is a polymer of glucose units that can be converted into fermentable sugars, making it the most valuable component for bioethanol production.
- Hemicellulose (about 20-40%) is a heteropolymer composed of various sugars, such as xylose and arabinose. It is more readily hydrolyzed than cellulose and plays a role in bioethanol production as well.
- Lignin, comprising approximately 15–25%, is a complex aromatic polymer responsible for giving structural strength to plants. Although lignin is not directly useful for ethanol production, it can be separated and used as a source of heat or chemicals in the bioethanol production process. (Hendriks et al, 2009)

Given its high cellulose and hemicellulose content, wheat straw is a promising raw material for bioethanol production. Its widespread availability makes it a low-cost,

renewable feedstock, particularly in regions where wheat farming is a significant part of the agricultural economy.

1.7.1 Current Utilization of Wheat Straw

In many wheat-producing regions, wheat straw is often considered a waste byproduct of agriculture. Its current utilization is limited and not optimized for sustainability or energy generation. The most common uses for wheat straw include:

- Animal Bedding: Wheat straw is often used as bedding for livestock. However, this represents a low-value use, and only a small percentage of the total straw produced is used in this way.
- Mulching and Soil Amendment: Some farmers leave wheat straw on the field as mulch to improve soil quality, retain moisture, and prevent erosion. While this is an environmentally friendly practice, it only accounts for a fraction of the total straw produced.
- Burning: A significant portion of wheat straw is burned in the fields, particularly in regions with limited agricultural space or where immediate field clearance is necessary for subsequent planting. This practice contributes to environmental pollution, as the combustion of wheat straw releases large quantities of carbon dioxide (CO_2), particulate matter, and other harmful pollutants into the atmosphere. (He et al, 2020)
- Field burning of wheat straw is a major source of air pollution, particularly in countries like India and China, where agricultural burning is common. It has been linked to severe air quality issues, causing smog, respiratory problems, and even contributing to climate change through the release of greenhouse gases. For instance, in northern India, wheat straw burning during the post-harvest season is a significant factor in the region's annual air quality crisis. In addition to CO_2, burning straw releases black carbon, a potent climate pollutant that accelerates global warming. (He et al, 2020), (Adam et al, 2020) (Tripathi et al, 2024).

Given these environmental challenges, finding alternative uses for wheat straw, such as bioethanol production, has significant potential to reduce pollution and create a renewable energy source.

1.7.2 Wheat Straw as a Feedstock for Bioethanol Production

Bioethanol production from wheat straw represents an effective solution for utilizing this abundant agricultural residue while addressing environmental issues. Wheat straw's

high cellulose and hemicellulose content make it an ideal candidate for bioethanol production through biochemical conversion processes.

The bioethanol production process typically involves the following steps:

1. Pretreatment: Wheat straw undergoes pretreatment to break down the lignocellulosic structure, particularly lignin, which acts as a barrier to the enzymatic breakdown of cellulose and hemicellulose. Pretreatment methods include acid or alkali treatments, steam explosion, or biological processes using microorganisms.
2. Enzymatic Hydrolysis: After pretreatment, enzymes are added to hydrolyze cellulose and hemicellulose into simple sugars such as glucose and xylose.
3. Fermentation: The resulting sugars are fermented by microorganisms, typically *Saccharomyces cerevisiae* (yeast), which converts them into ethanol.
4. Distillation and Purification: The ethanol produced during fermentation is separated from the mixture through distillation and purified to achieve the desired fuel-grade ethanol content. (J. Li et al., 2015)

Wheat straw's potential as a feedstock for bioethanol production is significant for several reasons:

- Abundance and Low Cost: Wheat straw is widely available and relatively inexpensive, especially compared to other bioethanol feedstocks like corn or sugarcane.
- Sustainability: Utilizing wheat straw for bioethanol production contributes to the development of second-generation biofuels, which do not compete with food crops. This alleviates the food vs. fuel debate that has been a major criticism of first-generation bioethanol feedstocks.
- Reduction of Environmental Pollution: By diverting wheat straw from being burned in the fields to bioethanol production, harmful emissions such as CO_2, methane, and particulate matter can be reduced. This not only helps mitigate air pollution but also reduces the overall carbon footprint of agriculture.
- Renewable Energy Source: Bioethanol produced from wheat straw is a renewable energy source that can reduce dependence on fossil fuels. It is a cleaner alternative to gasoline, emitting fewer greenhouse gases during combustion. (Talebnia et al, 2010).

1.7.3 Challenges and Opportunities

While the benefits of utilizing wheat straw for bioethanol production are clear, several challenges must be addressed. One major challenge is the cost of pretreatment. Lignocellulosic

biomass, such as wheat straw, necessitates rigorous pretreatment to disrupt the complex structure of cellulose and hemicellulose. Developing cost-effective and energy-efficient pretreatment methods is crucial for making bioethanol production from wheat straw economically viable

Another challenge lies in infrastructure and technology. Most bioethanol production facilities are designed to process first-generation feedstocks like corn or sugarcane. Retrofitting these facilities to handle wheat straw or building new plants designed for lignocellulosic feedstocks requires significant investment. However, advancements in biochemical conversion technologies, such as improved enzymes for hydrolysis and more efficient fermentation processes, are helping to reduce costs and improve the yield of bioethanol from wheat straw

Wheat straw, as an abundant and underutilized agricultural residue, holds immense potential for bioethanol production. Its high cellulose and hemicellulose content makes it an attractive feedstock for second-generation bioethanol, providing a sustainable alternative to fossil fuels while addressing the environmental challenges of wheat straw disposal. By diverting wheat straw from being burned or left to decompose, bioethanol production can help reduce greenhouse gas emissions, improve air quality, and promote renewable energy development.

While challenges remain, particularly regarding the cost of pretreatment and infrastructure needs, ongoing research and technological advancements are likely to make bioethanol production from wheat straw increasingly feasible and economically viable. As the global demand for renewable energy grows, wheat straw represents a valuable resource that can contribute to a more sustainable and cleaner energy future.

1.7.4 Current Utilization of Wheat Straw and Environmental I20mpact

Wheat straw is a major agricultural byproduct, particularly in regions with intensive wheat farming such as India, China, Europe, and the United States. After wheat is harvested, a significant amount of straw is left in the fields, where it is typically either allowed to decompose naturally or burned. These practices, though common, have

significant environmental consequences and represent a missed opportunity to utilize this abundant biomass more sustainably.

1.7.5 Field Burning and Environmental Pollution

In many countries, particularly in parts of Asia, burning wheat straw is a common practice to clear fields quickly for the next crop cycle. However, this method contributes significantly to air pollution. The open burning of wheat straw releases large amounts of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O), all of which are potent greenhouse gases that contribute to global warming. Additionally, the combustion process emits particulate matter (PM), which can cause severe air quality problems and respiratory issues in humans. (He et al, 2020).

For instance, in northern India, post-harvest burning of wheat straw is a major contributor to smog during the winter months, with harmful levels of particulate matter that affect millions of people. The practice has also been linked to soil degradation, as burning removes essential nutrients that could otherwise be returned to the soil.

1.8 Bioethanol Production: A Cleaner Alternative

Rather than burning wheat straw, utilizing it for bioethanol production presents a more environmentally friendly solution. Wheat straw is rich in cellulose and hemicellulose, which can be converted into fermentable sugars and subsequently into ethanol. By redirecting wheat straw from burning to bioethanol production, several environmental benefits can be achieved:

- Reduction of Greenhouse Gas Emissions: Instead of releasing CO_2 and other harmful gases into the atmosphere, converting wheat straw into bioethanol provides a renewable fuel that emits less CO_2 during combustion compared to fossil fuels.
- Air Quality Improvement: Avoiding the open burning of straw can significantly reduce air pollution and its associated health risks.
- Sustainable Energy Source: Bioethanol produced from wheat straw is a second-generation biofuel, meaning it does not compete with food crops, unlike corn or sugarcane-based ethanol

In summary, shifting the utilization of wheat straw from burning to bioethanol production offers a sustainable alternative that not only addresses waste management issues but also reduces environmental pollution while providing a renewable energy source.

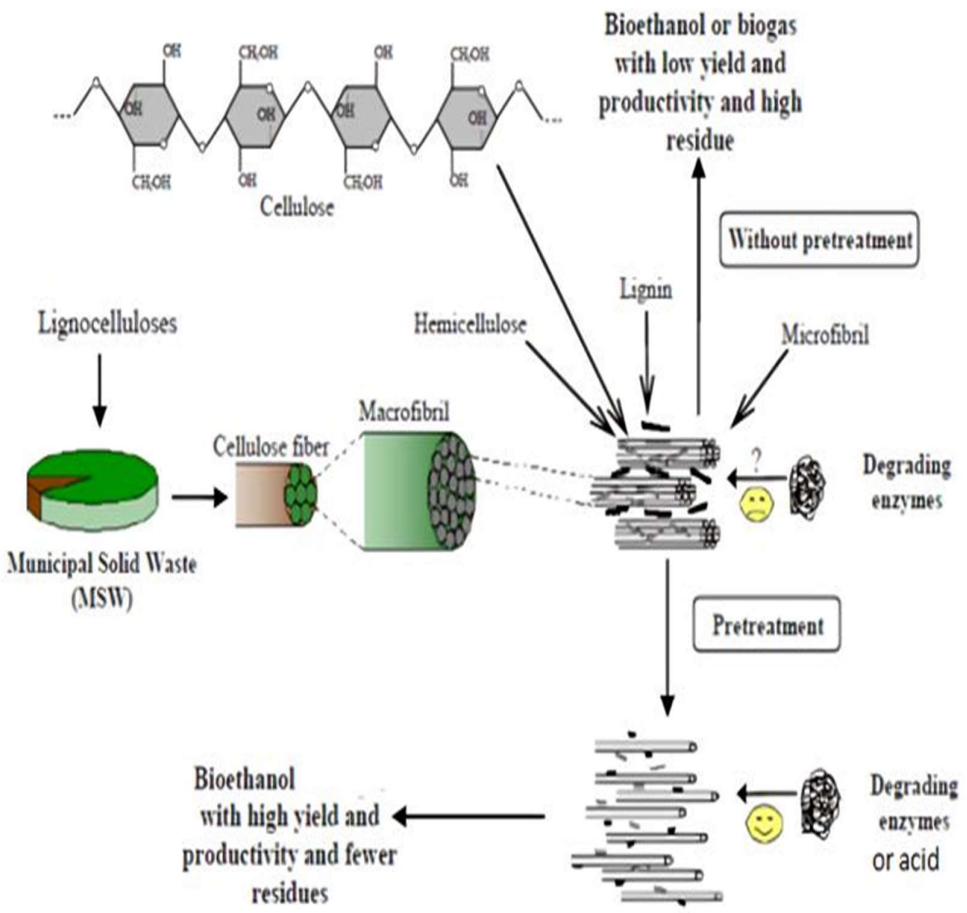

Fig 2: Schematic Representation of Lignocellulosic Biomass Structure and the Role of Pretreatment in Enhancing Bioethanol Production

OBJECTIVES:

1. Selection of suitable and potential raw material (Lignocellulosic biomass) and its compositional analysis.
2. Optimization of pretreatment for lignocellulosic biomass to maximize delignification and with maximal retention of overall sugars.
3. Optimization of hydrolysis with enhanced sugar recovery, minimizing degradation products.
4. Media formulation for the ethanol fermentation.
5. Optimization of the fermentation process for maximal yield and productivity.

CHAPTER 2

LITERATURE REVIEW

2.1 Bioethanol Production from Lignocellulosic Biomass

Bioethanol production from lignocellulosic biomass offers a promising solution for creating renewable energy while utilizing abundant agricultural residues, such as wheat straw, corn stover, and wood chips. Lignocellulosic biomass consists mainly of cellulose, hemicellulose, and lignin, with cellulose and hemicellulose being valuable sources of fermentable sugars. These sugars can be converted into ethanol through biochemical processes, making lignocellulosic bioethanol a second-generation biofuel.

Unlike first-generation biofuels derived from food crops like corn or sugarcane, bioethanol from lignocellulosic biomass does not compete with food production, addressing concerns related to the food vs. fuel debate.

The use of agricultural and forestry residues, which are often treated as waste, makes bioethanol production from lignocellulosic sources more sustainable and environmentally friendly. Additionally, bioethanol has the potential to reduce greenhouse gas emissions significantly when used as a fuel in the transportation sector, providing a cleaner alternative to gasoline.

The production process generally involves four key stages:

1. Pretreatment: The lignocellulosic biomass is treated to break down the complex structure of cellulose, hemicellulose, and lignin, improving the accessibility of enzymes that convert these polymers into simple sugars.
2. Enzymatic Hydrolysis: Specific enzymes, such as cellulases, are used to break down the cellulose and hemicellulose into fermentable sugars like glucose and xylose.
3. Fermentation: The sugars are fermented by microorganisms, typically *Saccharomyces cerevisiae* (yeast), which convert them into ethanol.
4. Distillation and Purification: The ethanol is separated from the fermentation broth through distillation and purified to fuel-grade ethanol.
5. Lignocellulosic bioethanol is seen as a crucial element in the shift toward renewable energy.

However, challenges remain in terms of optimizing the process for large-scale commercial

production, particularly regarding pretreatment and enzyme costs. Technological advancements are continuously improving the efficiency of these steps, making bioethanol from lignocellulosic biomass more competitive with conventional fuels.

Before we get into the details of how lignocellulosic biomass is pretreated, it's important to first understand the types of materials being used in this study. The way each biomass breaks down during bioethanol production-whether during enzymatic hydrolysis or fermentation- depends a lot on its structure. Things like how tightly packed the cellulose is, how much lignin it contains, or whether it has ash or silica all affect how easily it can be processed. That's why the next section takes a closer look at five specific types of biomasses: wheat straw, rice straw, rice husk, spent mushroom substrate, and sawdust. Comparing their properties helps us figure out which materials are most promising for bioethanol production and how best to process each one in the steps that follow.

2.2 Selection and Evaluation of Biomass Feedstocks

i) Wheat Straw

Wheat straw ranks among the most plentiful agricultural residues globally. Composed of approximately 30–40% cellulose, 20–30% hemicellulose, and 10–20% lignin, it represents an ideal second-generation feedstock-non-food, readily available, and rich in fermentable carbohydrates (Goodman, 2020). Its widespread availability, notably in countries such as India, China, and across Europe, has driven significant interest in valorizing wheat straw for bioethanol production.

Pretreatment methods-including dilute acid, alkali, and steam explosion-are routinely employed to dismantle its lignocellulosic structure, improving enzyme accessibility while minimizing the formation of inhibitory compounds such as furfural and hydroxymethylfurfural (HMF). Following pretreatment, enzymatic hydrolysis using cellulases and hemicellulases converts the polysaccharides into glucose and xylose. Reported glucose yields range from 70% to 95%, with subsequent fermentation by *Saccharomyces cerevisiae* or engineered yeasts yielding ethanol in the range of 65% to nearly 100% of the theoretical maximum.

Despite its promise, wheat straw presents challenges: efficient collection and cost-effective pretreatment are crucial to overcome its relatively recalcitrant nature. Moreover, variability in composition due to geography and agricultural practices affects process consistency and yield.

ii) Rice Straw

Rice straw, a major by-product of rice cultivation-especially across Asia-is chemically similar to wheat straw, containing ~32–47% cellulose, 19–27% hemicellulose, 5–24% lignin, and 6– 12% silica ash. (Zhong et al., 2009) (Verma et al., 2022) Despite its abundance, it is often discarded or burned in fields, exacerbating air pollution and wasting a valuable feedstock.

Its cellulose and hemicellulose contents (~35–45%) make it suitable for bioethanol production, yet the silica-rich outer layers can inhibit enzyme access and damage equipment (Verma et al., 2022). Pretreatments such as alkaline peroxide combined with ionic liquid, ammonia fiber expansion (AFEX), and organic acid effluent have been applied to disrupt lignin–cellulose bonding and mitigate silica effects. For instance, an alkaline-peroxide + IL pretreatment achieved ~63.8% lignin removal and 92.1% saccharification, yielding 91% of theoretical ethanol at high solids loading (Hong et al., 2019)**.** Similarly, AFEX pretreatment resulted in ~80.6% glucan and ~89.6% xylan conversions, with 95% ethanol yield using *S. cerevisiae* 424A (Zhong et al., 2009). Acid-steam + microwave/alkali pretreatment led to
~84% saccharification and 0.41 g/g ethanol yield. (Sidhu & Jaspreet, n.d.)

Even more innovative, popping pretreatment yielded 87.2% glucose recovery and 0.44 g ethanol/g glucose in 24 h fermentation (Gon Wi et al., 2013). Green pretreatment using alkaline wastewater from petha production removed ~90% silica and boosted sugar release fivefold (Kumari & Singh, 2022). A weak acid–mechanocatalytic process achieved nearly complete hydrolysis-98.3% within 12 hours-yielding over 500 mg/g sugar. (P. Yu et al., 2024)

These studies clearly demonstrate that, despite the challenge of silica contamination, rice straw can achieve high saccharification (80–98%) and ethanol yields (70–95% theoretical) when using tailored pretreatment strategies. Ongoing research works to refine these methods to limit inhibitors, improve enzyme access, and reduce costs-ultimately enabling large-scale, low- impact applications.

iii) Rice Husk

Rice husk, the protective outer layer of rice grains, stands apart in composition: while containing modest amounts of cellulose and hemicellulose (~25–35%), it features substantial lignin (~13–35%) and a notably high silica ash content (15–20% or more). This makes it chemically quite different from typical straw and forest residues.

The high silica and ash content introduces major challenges for biochemical processing. These components not only hinder enzymatic access but also damage equipment and reduce conversion efficiency. For example, rice husk's dense lignin structure and silica layers result in poor saccharification and higher inhibitor production-even when using identical hydrothermal pretreatment methods applied to rice straw. (J. Wu et al., 2018)

As a result, research focuses on silica and lignin removal before fermentation. Alkaline-hydrogen peroxide and aqueous ammonia pretreatments have achieved up to ~82% lignin reduction and near-complete silica elimination at moderate conditions (80 °C over 48 h), significantly improving enzymatic digestibility (Novia et al., 2022). Additionally, acid-catalyzed ionic liquid (IL) pretreatment at 130 °C for 30 min disrupted both crystalline cellulose and silica layers, enhancing cellulose conversion by over 500% compared to untreated biomass. (Y. J. Wang et al., 2021) Approaches combining ILs and alkali also facilitate recovery of both fermentable sugars and functional silica nanoparticles-a promising route for integrated biorefineries.

Valorization strategies extend beyond fermentation substrates. Rice husk has been converted into nanocellulose, high-purity amorphous silica, and activated carbons, broadening its application in materials science (Ludueña et al., 2011). However, for the specific goal of bioethanol production, aggressive pretreatment is essential to detoxify the feedstock and make it fermentable. In many cases, the energy and chemical costs required shift the focus toward thermochemical routes or material derivation rather than fermentation.

In summary, while rice husk presents technical challenges due to silica-driven recalcitrance and ash issues, targeted pretreatment strategies-such as alkaline peroxide, ILs, and ammonia- can selectively remove inhibitors and unlock its potential for fermentation. Nevertheless, its high silica content often leads researchers to prefer value-added material production (e.g., silica, activated carbon) unless integrated biorefinery designs rationalize the extra chemical and energy inputs.

iv) Spent Mushroom Substrate (SMS)

SMS is the leftover lignocellulosic material after mushroom cultivation, commonly based on wheat straw, sawdust, and animal manure. Its defining feature is that it has undergone biological pretreatment by fungi (notably white-rot species like *Pleurotus spp.*), resulting in partial lignin degradation and enhanced substrate digestibility. (Chen et al. 2022), (Berglund et al., 2024).

Studies report high saccharification efficiency: up to 80–90% glucan digestibility with commercial enzyme cocktails (Chen et al., 2022), even without harsh chemical pretreatment. Ryden et al. (2017) showed ethanol yields reaching ~47 g/L (≈187 g ethanol/kg DM) when fermenting sorghum-based SMS, using hydrothermal pretreatment and robust yeast strains. (Ryden et al., 2017) Similarly, oyster mushroom SMS hydrolyzed under dilute alkali conditions produced fermentable sugars with cellulose content of ~36%, hemicellulose of ~22%, and residual lignin ~12%, leading to viable ethanol production. (Grover et al., 2015).

V) Sawdust

Sawdust abundant by-product of the timber and woodworking industries-varies in composition depending on the wood species, but generally contains 35–50 % cellulose, 20–30 % hemicellulose, and 20–30 % lignin. However, the dense lignin–carbohydrate complex and compact structure of wheat straw hinder enzymatic accessibility, requiring more intensive pretreatment compared to less recalcitrant feedstocks such as grasses or spent mushroom substrate (SMS).

2.3 Pretreatment

Aggressive pretreatment strategies such as steam explosion, wet explosion, alkaline peroxide, organosolv, and ionic liquid (IL) pretreatments effectively disrupt lignin–carbohydrate complexes, improving enzyme accessibility and sugar yield. (Ben Atitallah et al., 2022) For instance, wet explosion pretreatment of hybrid poplar sawdust achieved up to ~75% cellulose and ~83% hemicellulose digestibility under optimized conditions (177 °C, 7.5% O_2, 30 min) (Biswas et al., 2020). Alkaline peroxide pretreatment of shea-tree sawdust resulted in improved enzymatic sugar conversion and higher delignification when combined with *Trichoderma* enzyme cocktails and *Saccharomyces cerevisiae* fermentation (Ayeni et al., 2016). Similarly, steam explosion combined with organosolv pretreatment demonstrated effective results in eucalyptus sawdust, enhancing subsequent hydrolysis and sugar release. Furthermore, IL pretreatment (e.g., [Emim][OAc]) boosted glucose yields by 60% for oak and 50% for spruce sawdust, although residual ILs can be inhibitory at higher concentrations.

Despite these methods, enzyme accessibility still lags behind that of grasses or SMS-derived substrates. Even after pretreatment, sawdust often demands higher enzyme loads to achieve comparable sugar yields. (Sridevi et al., 2015) One study using crude *Aspergillus niger* cellulase on alkali-pretreated sawdust observed a threefold increase in sugar release compared to untreated material, yet overall yields remained modest-about 14 % sugar release versus 5.4 % for raw sawdust. (Sridevi et al., 2015).

To alleviate this recalcitrance, some studies explore co-processing sawdust with other residues. For example, co-cultivation of mushrooms on sawdust–rice husk mixtures enhanced fungal delignification, suggesting biological pretreatment routes that could lower chemical usage (Ben Atitallah et al., 2022). Additionally, simultaneous saccharification and fermentation (SSF) protocols using *Trichoderma* exo-enzymes and *S. cerevisiae* have shown promise-though ethanol titers remained lower than those from straw-based systems unless high pretreatment severity was used.

A recent study using levulinic acid pretreatment demonstrated greener methods gaining traction: levulinic acid–pretreated sawdust achieved efficient SSF-based ethanol production, highlighting new potential for eco-friendly pretreatment chemistries. (Nawaz et al., 2022)

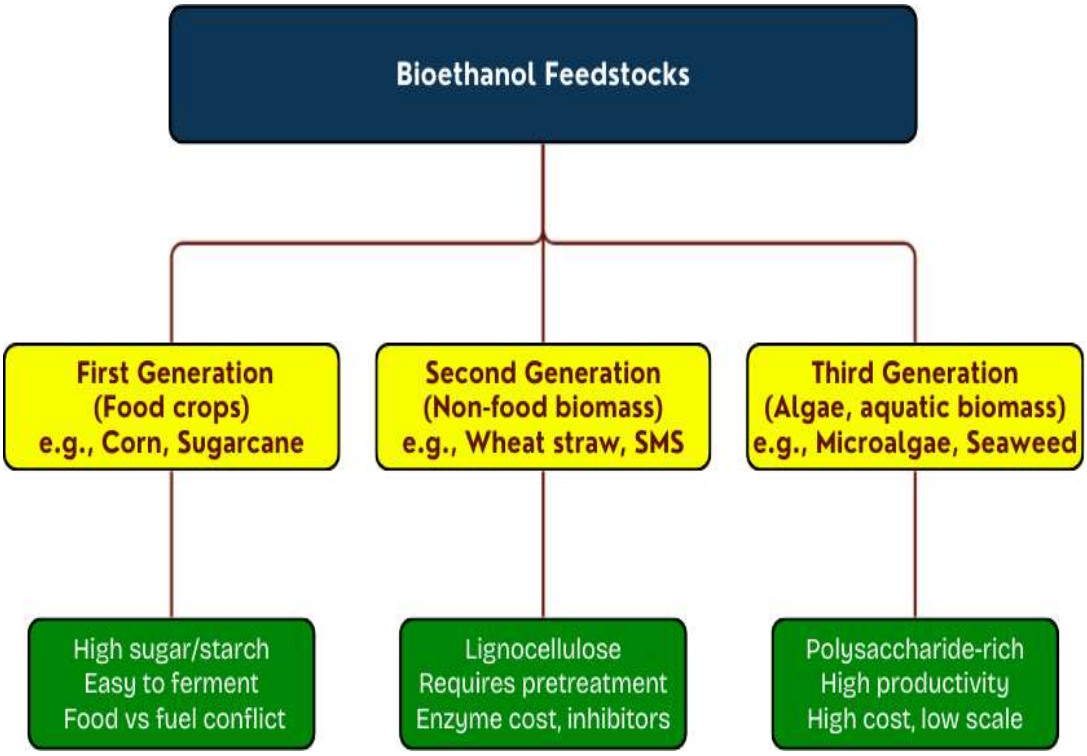

Fig 3: Bioethanol Feedstocks: First, Second, and Third Generation Sources

Pretreatment plays a vital role in bioethanol production from lignocellulosic biomass by breaking down the natural barriers of plant cell walls. Lignocellulosic biomass consists of a tightly bound matrix of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are polysaccharides that can be hydrolyzed into fermentable sugars, but lignin acts as a barrier, preventing enzymes from accessing these sugars. Therefore, pretreatment is

essential to disrupt this structure and increase the efficiency of subsequent hydrolysis. Several pretreatment methods are used in bioethanol production, including:

- Physical Pretreatment: Techniques such as milling and grinding reduce the particle size of the biomass, increasing its surface area and making it more accessible to enzymes.
- Chemical Pretreatment: Methods such as acid hydrolysis, alkaline pretreatment, and steam explosion help break down lignin and hemicellulose. Acid pretreatment hydrolyzes hemicellulose, while alkaline methods, such as sodium hydroxide treatments, dissolve lignin
- Biological Pretreatment: Involves the use of fungi or bacteria to degrade lignin and hemicellulose naturally, though it is slower compared to other methods.
- The efficiency of pretreatment significantly affects the overall bioethanol yield and production cost. An effective pretreatment method can increase sugar release, reduce enzyme requirements, and improve fermentation efficiency. Therefore, optimizing pretreatment is a focus of ongoing research to make bioethanol from lignocellulosic biomass commercially viable.

The effectiveness of the pretreatment process has a major impact on both the final bioethanol yield and the overall production cost. Therefore, optimizing pretreatment is a focus of ongoing research to make bioethanol from lignocellulosic biomass commercially viable.

In recent years, hybrid or integrated pretreatment approaches have garnered attention. These combine physical, chemical, and biological techniques to capitalize on the advantages of each method while minimizing their limitations. For instance, combining alkaline treatment with mild enzymatic or fungal pretreatment can effectively remove lignin while preserving carbohydrate content. Additionally, advancements in green chemistry have led to the exploration of ionic liquids and deep eutectic solvents, which are environmentally friendly agents that selectively dissolve lignin and hemicellulose while leaving cellulose relatively intact.

Another evolving trend is the recycling and reuse of pretreatment chemicals, which not only reduces environmental burden but also lowers operational costs. The effectiveness of pretreatment is often evaluated by parameters such as delignification percentage, enzymatic digestibility, and overall sugar yield. Moreover, feedstock-specific optimization is increasingly emphasized, as different types of lignocellulosic biomass (e.g., agricultural residues, energy crops, or spent mushroom substrate) respond differently to the same pretreatment protocol. Understanding these nuanced responses is essential for

designing scalable, efficient, and economically feasible biorefineries that can contribute meaningfully to sustainable fuel production.

2.2.1 Physical pretreatment

Physical pretreatment is one of the most fundamental and widely used steps in preparing lignocellulosic biomass for further chemical or enzymatic processing. Its primary goal is to reduce the particle size of the biomass, thereby increasing the surface area and improving the accessibility of cellulose and hemicellulose to hydrolytic enzymes. Common physical techniques include mechanical milling, grinding, shredding, and extrusion, which help to break down the rigid structure of plant cell walls. Advanced methods such as ultrasonication, microwave irradiation, and steam explosion have also been explored for their ability to disrupt the crystalline structure of cellulose and partially alter lignin, improving enzymatic digestibility. In many cases, physical pretreatment is used in conjunction with chemical or biological treatments to enhance overall efficiency. While physical methods alone may not significantly break down lignin, they provide a necessary preparatory step that facilitates the effectiveness of subsequent pretreatment processes and improves bioethanol yield**.**

2.2.1.1 Milling Pretreatment

Milling is a way to reduce This can be done by biomass particle size. various methods such as grinding, tearing, cutting, etc. Biomass produces particles from 0.2 to 2 mm in size to 10 to 30 mm in size. The advantages of this method are: (Broda et al. 2022), (Chauhan et al, 2024) reducing cellulose crystallization, (Gielen et al, 2019) increasing the surface for enzymatic hydrolysis, (*Scenarios & Strategies to 2050: in Support of the G8 Plan of Action*. OECD/IEA) reducing the rate of cellulose polymerization, and (Chang et al, 2017) increasing mass transfer due to the reduction of particles. Mechanical pretreatment is usually performed before the next processing step, and the desired particle size depends on these subsequent steps.

The main drawback of the ball technique is great energy consumption, which represents approximately 33% of the total energy required for the general process. Another important barrier is a non-reprime lignin during the process. Lignin present in biomass leads to a reduction in the accessibility of enzymes that hydrolyze cellulose and hemicellulose (Mankar et al, 2021)

In recent years, hybrid pretreatment approaches that combine milling with other methods have gained attention as a means to overcome the limitations of mechanical size reduction alone. For instance, milling coupled with alkaline or dilute acid treatment has shown improved delignification and sugar recovery due to the synergistic effect of mechanical disruption and chemical solubilization. This combined approach helps in loosening the lignin-carbohydrate complex and reducing the recalcitrance of the biomass. Moreover, innovations such as cryo- milling, where biomass is ground at extremely low temperatures using liquid nitrogen, have shown to significantly reduce particle crystallinity without excessive thermal degradation. Similarly, technologies like vibro-energy mills and planetary ball mills are being evaluated for their ability to reduce energy consumption while maintaining effective particle size reduction. Additionally, advancements in pre-processing analytics, such as real-time particle size monitoring and energy balance tracking, are helping researchers optimize milling parameters to improve efficiency and sustainability. Furthermore, the integration of mechanical pretreatment with downstream processes, such as simultaneous saccharification and fermentation (SSF), is also being investigated to reduce processing steps and operational costs. These evolving strategies not only help in improving the overall digestibility of biomass but also align with the goal of making lignocellulosic bioethanol production commercially viable and environmentally sustainable on a larger scale.

2.2.1.2 Microwave Pretreatment

Microwave pretreatment is an emerging technology used to improve the efficiency of bioethanol production from lignocellulosic biomass. It involves exposing the biomass to microwave radiation, which generates heat rapidly and uniformly within the material. The rapid heating causes the disruption of the lignocellulosic structure, particularly weakening the bonds between cellulose, hemicellulose, and lignin, which enhances the accessibility of cellulose for enzymatic hydrolysis

Microwave pretreatment is typically performed at high temperatures, ranging between 150°C and 230°C, and often in the presence of a chemical catalyst such as acid or alkali to further accelerate the breakdown of biomass. The microwave radiation penetrates the material, causing water molecules within the biomass to vibrate, which results in internal heating. This heating process helps break down the lignocellulosic matrix, disrupts the crystalline structure of cellulose, and degrades hemicellulose into simple sugars.

The advantages of microwave pretreatment include its speed and efficiency. Microwave treatment requires less time compared to conventional thermal treatments, and the

uniform heating reduces the risk of overheating or incomplete biomass breakdown. It also requires lower energy input when optimized, reducing operational costs. Studies have shown that microwave pretreatment can significantly increase the yield of fermentable sugars, making it a promising approach for bioethanol production.

However, challenges include the high cost of microwave equipment and potential degradation of sugars at extremely high temperatures, which can affect the fermentation process. Continued research is focusing on optimizing parameters like temperature, time, and catalyst concentration to improve efficiency and scalability.

2.2.1.3 Ultrasound Pretreatment Method

Ultrasound pretreatment involves using high-frequency sound waves to break down lignocellulosic biomass for bioethanol production. In this method, ultrasonic waves, typically in the range of 20 kHz to 100 kHz, are applied to the biomass in a liquid medium, causing the formation and collapse of microbubbles in a process called cavitation. This cavitation creates intense localized pressure and temperature changes, which disrupt the biomass structure.

The primary effect of ultrasound pretreatment is the disruption of the lignocellulosic matrix, particularly breaking down the lignin that encases cellulose and hemicellulose. This mechanical action increases the surface area of the biomass and enhances the penetration of enzymes during the subsequent hydrolysis process. It can also reduce the crystallinity of cellulose, making it easier to convert into fermentable sugars. Ultrasound pretreatment is often combined with other chemical or biological methods to enhance its effectiveness. For example, when used in conjunction with alkaline pretreatment, ultrasound can further improve the delignification process, leading to a higher release of fermentable sugars.

The main advantages of ultrasound pretreatment are its low energy consumption, short treatment time, and its ability to improve the efficiency of enzymatic hydrolysis. Ultrasound is also considered an environmentally friendly method since it typically does not require harsh chemicals. However, its effectiveness can vary depending on the biomass type, and the high capital cost of ultrasonic equipment can be a limitation for large-scale operations.

Ongoing research is focused on optimizing ultrasound parameters such as frequency, intensity, and duration to maximize its potential for bioethanol production.

In light of its numerous advantages, ultrasound pretreatment is gaining traction as a supplementary method to conventional biomass pretreatment techniques. Its ability to induce both physical and chemical alterations in lignocellulosic material through non-thermal, solvent-minimal means positions it as a greener alternative for improving biomass digestibility. Nevertheless, to translate this technology from the lab scale to industrial applications, several practical considerations must be addressed. These include developing cost-effective, energy-efficient ultrasound equipment, determining optimal operational parameters for different feedstocks, and evaluating long-term mechanical durability and maintenance needs of ultrasonic reactors. Moreover, the integration of ultrasound pretreatment with other unit operations-such as enzymatic hydrolysis, fermentation, and downstream processing-should be explored to determine the feasibility of continuous biorefinery setups. There is also increasing interest in applying ultrasound in tandem with emerging green technologies, including ionic liquids and deep eutectic solvents, to further boost delignification and sugar yields while minimizing environmental impact. From a sustainability perspective, coupling ultrasound pretreatment with on-site renewable energy sources could lower its carbon footprint and align with broader goals of eco-friendly biofuel production. Overall, while ultrasound technology alone may not replace traditional pretreatment methods, its complementary role in integrated bioconversion systems holds substantial promise for advancing the bioethanol industry toward cleaner, more efficient, and scalable production models.

2.2.2 Physicochemical Pretreatment

Physicochemical pretreatment involves following techniques:

2.2.2.1 Steam Explosion

Steam explosion is a widely studied physicochemical method for the pretreatment of lignocellulosic biomass, often referred to as autohydrolysis due to the changes occurring during the process. In this method, biomass is typically chopped, ground, or conditioned before being exposed to high-pressure steam, ranging from 0.7 to 4.8 MPa, and heated to 160– 240°C. (Bajpai et al, 2016a) The pressure is sustained for a short duration-ranging from a few seconds to several minutes-promoting the breakdown of hemicellulose and transformation of lignin. Following this, the pressure is rapidly released, leading to the disruption of the biomass structure.

The high temperature in the steam explosion facilitates the degradation of hemicellulose and alters lignin, making cellulose more accessible to enzymes for subsequent hydrolysis. Studies have shown that steam explosion can improve hydrolysis efficiency significantly; for example, poplar chips pretreated with steam explosion achieved a 90% enzymatic

hydrolysis rate in 24 hours compared to only 15% hydrolysis in untreated chips.

Several factors influence the effectiveness of this pretreatment method, including moisture content, temperature, residence time, and the size of the biomass particles. Optimal conditions for hemicellulose breakdown are achieved either through high temperatures with short residence times (e.g., 270°C for 1 minute) or lower temperatures with longer times (e.g., 190°C for 10 minutes). The latter method is preferred to minimize the formation of sugar degradation products, which can inhibit fermentation.

Acetic acid and other organic acids produced from hemicellulose during pretreatment contribute to further hydrolysis of the biomass. The addition of catalysts such as sulfuric acid, carbon dioxide, or sulfur dioxide can improve hemicellulose sugar recovery, reduce inhibitors, and enhance enzymatic hydrolysis. Steam explosion is particularly effective for agricultural residues and hardwoods, but less so for softwoods unless combined with an acid catalyst. (Bajpai et al, 2016b)

Despite its advantages, the implementation of steam explosion at an industrial scale poses several operational and economic challenges. One of the primary limitations is the energy requirement to generate and maintain high-pressure steam, which can raise processing costs. Additionally, the sudden pressure release can cause mechanical stress on equipment, necessitating robust reactor design and frequent maintenance. The production of inhibitory compounds such as furfural and hydroxymethylfurfural (HMF), especially under harsh conditions, can also adversely affect subsequent microbial fermentation. To mitigate these effects, process optimization and detoxification steps may be required, which can further complicate the workflow. Nonetheless, ongoing research focuses on integrating steam explosion with other pretreatment or enzymatic techniques to maximize sugar recovery and minimize the formation of inhibitors. Hybrid approaches, such as combining steam explosion with dilute acid treatment or enzymatic conditioning, have shown promise in improving the selectivity and efficiency of biomass fractionation. Furthermore, the suitability of steam explosion for a variety of feedstocks, particularly low-cost agricultural residues, adds to its appeal as a scalable pretreatment technology. Future improvements in process control, energy integration, and reactor design may enhance its commercial viability. As a result, steam explosion continues to be a compelling option in the pursuit of economically feasible and environmentally sustainable bioethanol production from lignocellulosic biomass.

2.2.2.2 Liquid Hot Water (LHW) pretreatment

Liquid Hot Water (LHW) pretreatment is a biomass processing method similar to steam explosion but uses hot water at high temperatures, typically between 160°C and 230°C, instead of steam. This approach is also known by other names such as solvolysis, hydrothermolysis, aqueous fractionation, or aquasolv. LHW pretreatment is effective in breaking down the lignocellulosic structure of biomass by removing lignin and hydrolyzing hemicellulose, which increases the accessibility of cellulose for enzymatic hydrolysis.

Compared to steam explosion, LHW uses lower temperatures (optimal for corn stover is 180– 190°C), resulting in fewer fermentation inhibitors such as furfural and 5-hydroxymethyl furfural, which are formed at higher temperatures. By maintaining the pH between 4 and 7, the degradation of sugars can be minimized, thereby reducing the formation of these inhibitors.

LHW pretreatment is typically conducted in different reactor configurations, including cocurrent, countercurrent, and flow-through reactors, depending on the movement of water and biomass within the system. Flow-through reactors, where water is passed over a stationary bed of biomass, have been shown to be more effective for the removal of hemicellulose and lignin.

One of the key advantages of LHW is that it avoids the use of chemicals other than water, eliminating the need for neutralization or washing after pretreatment, thus reducing costs. However, LHW is more energy-intensive than steam explosion due to the large volumes of water involved. Although this technique dissolves more components, the concentration of the resulting products is less than that obtained by steam explosion. LHW pretreatment has shown promise in laboratory-scale applications and has been successfully scaled up for treating large quantities of biomass, such as corn fiber slurry, indicating its potential for industrial bioethanol production.

Despite its promising results in enhancing enzymatic digestibility and reducing fermentation inhibitors, Liquid Hot Water pretreatment still presents certain limitations that must be addressed for effective commercial application. One major consideration is the high water-to- biomass ratio required, which increases the energy demand for heating and water handling, thereby affecting the overall process economics. Moreover, although LHW pretreatment reduces the production of furans and phenolic inhibitors, other soluble degradation compounds, including weak acids and oligosaccharides, may still interfere with downstream fermentation processes. These components may require additional

conditioning or detoxification steps, which can increase operational complexity. To overcome such barriers, researchers have explored process intensification strategies such as integrating LHW with pressure filtration or membrane separation systems to concentrate solubilized sugars and reduce downstream volume. Additionally, coupling LHW with enzymatic pretreatment or microbial consortia has shown improvements in hydrolysis efficiency and product yield. The adaptability of LHW to different reactor configurations also offers flexibility in optimizing residence time and temperature gradients for diverse feedstocks. With continued innovation in reactor design, heat recovery systems, and process integration, LHW pretreatment holds considerable potential as a sustainable and scalable option for bioethanol production. Its chemical-free nature and minimal environmental impact make it particularly attractive for future biorefinery platforms aiming for green, low-emission biofuel technologies.

2.3.3. Chemical Pretreatment

Various acids, alkali and other chemicals are used to pretreat different lignocellulosic biomasses

2.3.3.1 Acid Pretreatment

Acid-based pretreatment is a common method for removing hemicellulose from lignocellulosic biomass (LCB), which enhances enzyme accessibility to cellulose (Jordan et al, 2008). Frequently employed acids include sulfuric, acetic, and phosphoric acids (Vasilakis et al, 2023). This pretreatment can be done in two main ways: dilute acid pretreatment, using low acid concentrations (0.1%) at high temperatures above 200°C, or concentrated acid pretreatment, which involves using stronger acid concentrations (30-70%) at lower temperatures below 50°C (Hendriks et al, 2009).

Each method has distinct benefits and drawbacks. Dilute acid pretreatment requires less acid but is energy-intensive due to the need for high temperatures. Conversely, concentrated acid pretreatment is more energy-efficient as it operates at lower temperatures, but the strong acid can lead to the generation of fermentation inhibitors like furfural and 5- hydroxymethylfurfural. These inhibitors can severely impact microbial activity in the fermentation process, damaging DNA, reducing RNA synthesis, and thus limiting enzyme efficiency (Lorenci Woiciechowski et al., 2020). Additionally, high acid concentrations increase the risk of corrosion in reaction vessels.

Several studies have explored different acid pretreatment conditions. For instance, Prasad et al. (2018) used 2% dilute sulfuric acid at 180°C for 10 minutes to pretreat wheat straw, recovering 43.1% of total soluble sugars and achieving an ethanol yield of 5.2% (v/v). In

another study, Santos et al. (2018) treated elephant grass with 20% sulfuric acid at 121°C for 30 minutes, yielding 76% glucose for the whole plant and up to 89% for its leaf fraction, though the stem portion, with its higher lignin content, had lower glucose yields. Similarly, Kuglarz et al. (2018) pretreated rapeseed straw with 1% sulfuric acid at 180°C for 10 minutes, leading to 84.6% sugar yield after enzymatic hydrolysis with CTec2 and HTec2 enzymes.

Although acid pretreatment is highly effective in solubilizing hemicellulose and enhancing enzymatic access to cellulose, its practical implementation must balance efficiency with economic and environmental considerations. The generation of inhibitory compounds, especially under severe conditions, remains one of the major bottlenecks, as these substances can hinder microbial fermentation and lower ethanol yields. To mitigate these issues, detoxification steps such as overliming, activated carbon treatment, or biological conditioning are often employed post-pretreatment, but they introduce additional cost and complexity to the process. Moreover, the requirement for acid recovery and neutralization generates significant amounts of chemical waste, which can pose environmental risks if not managed properly. The type of biomass and its compositional heterogeneity also influence the efficacy of acid pretreatment. For example, substrates with high lignin content tend to produce more inhibitors, requiring tailored strategies for optimal performance. Despite these challenges, acid pretreatment remains one of the most studied and commercially explored options for biomass conversion. Innovations such as flow-through acid pretreatment systems, continuous reactors, and integration with membrane separation technologies are currently under investigation to improve sugar recovery and minimize the environmental footprint. Therefore, while acid pretreatment is not without limitations, continued advancements in process optimization and inhibitor mitigation may secure its place as a vital step in lignocellulosic bioethanol production.

2.3.3.2. Alkaline Pretreatment

Alkaline pretreatment is an important chemical approach for biomass deconstruction, utilizing bases such as sodium hydroxide, potassium hydroxide, calcium hydroxide (lime), or ammonium hydroxide, and is sometimes enhanced by the addition of agents like hydrogen peroxide. This process causes the biomass to swell, expanding its surface area while reducing polymerization and cellulose crystallinity. The alkali disrupts lignin structure and breaks the bonds between lignin and other carbohydrates in the biomass, enhancing accessibility to the remaining polysaccharides. As lignin is removed, polysaccharide reactivity increases, and hemicellulose's acetyl and uronic acid groups, which block enzyme access, are eliminated.

The effectiveness of alkaline pretreatment varies by biomass type, generally performing better on low-lignin content materials like hardwoods, herbaceous crops, and agricultural residues than on softwoods with higher lignin content. For example, Millet et al. (1976) found that treating hardwood with sodium hydroxide improved digestibility from 14% to 55%, with a lignin reduction from 24-55% to 20%. In contrast, softwoods with over 26% lignin content showed limited improvement.

Alkaline pretreatment is flexible in terms of the severity of conditions, depending on the biomass and desired results. For instance, lime pretreatment was used by Kim and Holtzapple (2005) to remove up to 87.5% of lignin from corn stover at 55°C over 4 weeks with aeration. Playne (1984) also showed that lime pretreatment at ambient temperature improved sugarcane bagasse digestibility from 20% to 72%. Sodium hydroxide was particularly effective, achieving an 85% increase in glucose yield when used on rice straw.

Lime pretreatment, often using calcium hydroxide, has been reported to improve biomass digestibility. Calcium hydroxide is cost-effective and can be regenerated by converting it into calcium carbonate with carbon dioxide and reprocessing it in a lime kiln. Pretreatment with lime can be performed at a range of temperatures, from ambient to high heat, depending on the biomass and desired results. Chang et al. (1997) reported that lime pretreatment can solubilize significant amounts of hemicellulose and lignin, and oxidative conditions, such as the introduction of oxygen at high pressure, can further enhance the effectiveness of lime treatment.

Although alkaline pretreatment offers several advantages, including lower temperatures and pressures than other methods and less sugar degradation, it has some drawbacks. These include the conversion of alkali into irrecoverable salts, challenges in managing the salts produced, and reduced effectiveness with high-lignin content biomass. Additionally, the process may require long pretreatment times, ranging from hours to weeks, depending on the specific conditions.

In summary, alkaline pretreatment, especially with agents like sodium hydroxide and lime, plays a crucial role in improving the digestibility of lignocellulosic biomass. By removing lignin and acetyl groups, it enhances enzyme access and facilitates the breakdown of cellulose and hemicellulose into fermentable sugars. However, its limitations, such as salt management and effectiveness with high-lignin biomass, must be considered for large-scale applications.

Recent advances in alkaline pretreatment research have aimed to optimize the process for broader applicability and economic feasibility, particularly in the context of bioethanol production from diverse biomass sources. Hybrid techniques combining alkaline agents with oxidative compounds like hydrogen peroxide (alkaline hydrogen peroxide, AHP) have shown enhanced lignin degradation while minimizing carbohydrate loss. Additionally, alkali-assisted steam pretreatment and microwave-assisted alkali treatments are emerging approaches that aim to reduce reaction times and chemical consumption. The integration of such advanced techniques holds promise in overcoming limitations like long reaction durations and inefficient delignification in high-lignin feedstocks. Furthermore, efforts are being made to recover and recycle alkali reagents, thereby addressing environmental concerns related to salt accumulation and water contamination. For instance, the reuse of lime through carbonation and calcination cycles offers a sustainable solution, especially when applied in closed-loop systems. Researchers are also exploring the synergy between alkaline pretreatment and downstream enzymatic hydrolysis, focusing on enzyme optimization to complement the structural changes induced by alkali exposure. As global interest in lignocellulosic biofuels intensifies, alkaline pretreatment continues to evolve into a more adaptable and cost-effective platform. Its ability to significantly enhance biomass digestibility with relatively mild operating conditions supports its potential in integrated biorefinery models, contributing meaningfully to sustainable bioethanol production.

2.3.3.3. Organosolv pretreatment

The organosolv pretreatment method utilizes organic solvents such as methanol, ethanol, tetrahydrofurfuryl alcohol, ethylene glycol, and acetone to treat biomass. Occasionally, organic acids like acetylsalicylic, oxalic, and salicylic acids, or bases such as sodium hydroxide and lime, are used as catalysts. This process breaks the bonds between lignin and hemicellulose, increasing cellulose surface area and making it more accessible for enzymatic hydrolysis (Balat et al, 2011). Tang et al. 2017) (Keller et al (2003) tested a combination of 60% aqueous ethanol and n-propylamine (10 mmol/g dry biomass) as a base catalyst for corn stover pretreatment at 140°C for 40 minutes. They achieved an 83.2% sugar yield and 81.7% lignin removal. The n-propylamine acted as a dual-function catalyst, promoting hydroxide ion generation, which cleaved ester bonds between lignin and hemicellulose through saponification. It also disrupted hydrogen bonding within cellulose by introducing competition from its –NH2 group and the hydroxyl groups of cellulose.

Mirmohamadsadeghi et al. (2014) used 75% aqueous ethanol with 1% sulfuric acid to pretreat different biomass types for methane production at 150–180°C for 30–60

minutes. The pretreatment led to significant lignin removal: 27% in Elmwood, 21% in pinewood, and 37.7% in rice straw, with maximum methane yields of 93.7, 71.4, and 152.7 L/kg carbohydrates, respectively.

A study in 2016 investigated the effects of various solvents, including ethanol, 1-pentanol, 1-butanol, 1-propanol, and 2-propanol, on the pretreatment of sorghum bagasse at 180°C for 45 minutes with 1% sulfuric acid. Solvents with higher partition coefficients, such as 1-butanol (0.88) and 1-pentanol (1.51), led to the formation of three distinct fractions: solid, liquid, and black liquor. In contrast, solvents with lower partition coefficients, like ethanol, 1-propanol, and 2-propanol, produced only two fractions: solid and liquid. The ethanol yields were notably higher for 1-butanol (43.1 g/L) and 1-pentanol (47.2 g/L) (Teramura et al., 2016). Despite its effectiveness, organosolv pretreatment has drawbacks, including high costs, flammability, volatility, and challenges in solvent recovery, making it energy-intensive and expensive.

In recent years, advancements in organosolv pretreatment have focused on overcoming the economic and operational limitations of the process. One major area of improvement is solvent recovery systems, where closed-loop distillation and membrane-based recovery methods are being explored to reduce energy consumption and improve process sustainability. Additionally, co-solvent systems, such as ethanol–water or acetone–water mixtures, have shown promising results in enhancing delignification efficiency while lowering solvent usage. Researchers are also examining the role of ionic liquids and deep eutectic solvents as alternative organosolv agents due to their low volatility, high thermal stability, and recyclability. These greener solvents offer the potential to dissolve lignin more selectively and operate under milder conditions, making them suitable for large-scale applications. Furthermore, integrating organosolv pretreatment into biorefinery frameworks allows for simultaneous valorization of all biomass components. For instance, the lignin fraction extracted during organosolv can be utilized in producing high-value products such as bioplastics, adhesives, and carbon fibers. This holistic approach enhances economic feasibility while supporting zero-waste principles. Overall, while the initial capital and operational costs of organosolv pretreatment remain a challenge, ongoing innovations in solvent design, recovery technologies, and process integration are making it an increasingly viable option for efficient and sustainable lignocellulosic biomass conversion.

2.3.4. Biological Pretreatment

The biological pretreatment method for lignocellulosic biomass utilizes microorganisms, primarily fungi, to break down lignin and hemicellulose, enhancing the accessibility of cellulose for subsequent hydrolysis. This method is considered eco-friendly and operates under mild conditions, without the need for harsh chemicals or extreme temperatures, making it an energy-efficient alternative to chemical and physical pretreatments.

White-rot fungi, such as *Phanerochaete chrysosporium* and *Trametes versicolor*, are the most commonly used microorganisms for lignin degradation. They produce lignin-degrading enzymes like lignin peroxidase, manganese peroxidase, and laccase, which selectively degrade lignin

while minimizing cellulose loss. Brown-rot and soft-rot fungi, though less selective, also play a role by degrading both lignin and carbohydrates to some extent.

During biological pretreatment, fungi colonize the biomass and secrete these enzymes, breaking the lignin network and disrupting lignin-carbohydrate bonds. This allows for better penetration and activity of cellulolytic enzymes in subsequent steps, improving the overall efficiency of biomass conversion into biofuels or other bioproducts.

Biological pretreatment, especially using white-rot fungi, has gained increasing attention due to several inherent advantages: (i) it is a safe and environmentally friendly approach; (ii) it requires low energy and is cost-effective; (iii) it offers selective lignin degradation; (iv) in some cases, the pretreated biomass can directly undergo enzymatic conversion or fermentation; and (v) it improves the cellulose digestibility of various agricultural wastes and forages (Mirmohamadsadeghi et al, 2014)

The biological approach has unique characteristics that have sparked growing interest among researchers. Numerous studies have demonstrated the effectiveness of biological pretreatment, which has advanced significantly. Additionally, organosolvents like ethanol, methanol, butanol, ethylene glycol, and n-butylamine are sometimes combined with biological treatment to enhance lignin degradation, hemicellulose removal, and disruption of cellulose's crystalline structure. (Monrroy et al, 2010)

However, biological pretreatment has some limitations, including long treatment times (weeks to months) and relatively low efficiency compared to other methods. To enhance its effectiveness, researchers are investigating ways to optimize conditions such as moisture content, pH, temperature, and the selection of fungal strains. Additionally,

combining biological pretreatment with other methods, like chemical or mechanical processes, can lead to faster lignin removal and better overall performance.

In summary, biological pretreatment offers a sustainable and environmentally friendly option for lignocellulosic biomass processing, with ongoing research focused on overcoming its slow processing speed and enhancing efficiency.

To further enhance the utility of biological pretreatment, emerging research is focused on integrating advanced biotechnological tools and genetic engineering strategies. For instance, modifying fungal strains through genetic manipulation can lead to higher expression levels of ligninolytic enzymes, thereby accelerating lignin degradation and reducing the time required for treatment. Synthetic biology approaches are also being explored to engineer microbial consortia that combine the benefits of different organisms, such as white-rot fungi and cellulolytic bacteria, in a synergistic system. Moreover, omics technologies-like transcriptomics, proteomics, and metabolomics-are being employed to better understand the metabolic pathways and regulatory mechanisms involved in lignin degradation, allowing for more precise control and optimization of the process. Another promising direction is the immobilization of fungi or enzymes on solid supports, which can increase enzyme stability, reduce contamination risks, and enable continuous processing. Additionally, researchers are investigating the use of agricultural and industrial wastes as substrates for fungal cultivation, further enhancing the sustainability and cost-effectiveness of the process. As these innovations mature, they could significantly improve the commercial viability of biological pretreatment, making it a competitive alternative to conventional methods. With its minimal environmental footprint and potential for integration with other pretreatment technologies, biological pretreatment remains a key focus area in the development of green biorefinery systems.

2.3.4.1 Types of Biological Pretreatment

Biological pretreatment methods can be broadly categorized based on the type of microorganisms used and their specific modes of action. The most common types include fungal, bacterial, enzymatic, and microbial consortia-based pretreatments.

i) Fungal Pretreatment

Fungal pretreatment is considered one of the most effective and environmentally benign biological methods for delignifying lignocellulosic biomass. It predominantly utilizes white- rot fungi such as *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Pleurotus ostreatus*, known for their remarkable ability to degrade lignin while leaving cellulose

relatively intact. These fungi produce an array of oxidative enzymes, including lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, which work synergistically to cleave the complex and recalcitrant lignin polymer into smaller, less inhibitory molecules. White-rot fungi are unique in their selective lignin degradation capability, making them especially useful for improving the enzymatic digestibility of cellulose in biomass.

Other fungal groups, such as brown-rot fungi and soft-rot fungi, also contribute to biomass degradation. However, these fungi tend to break down both lignin and polysaccharides, including cellulose, which may not be ideal for bioethanol production where cellulose preservation is critical. Brown-rot fungi primarily depolymerize cellulose through non- enzymatic mechanisms like Fenton reactions, while soft-rot fungi modify lignin structure to a lesser extent and are more commonly found in wetter environments. Despite their differences, fungal pretreatment, especially with white-rot fungi, remains a promising low-energy, chemical-free strategy for enhancing the efficiency of lignocellulosic biomass conversion.

ii) Bacterial Pretreatment:

Bacterial pretreatment of lignocellulosic biomass is an emerging strategy that leverages the metabolic capabilities of certain bacterial species to degrade lignin and hemicellulose. Among the most commonly studied genera are *Bacillus*, *Pseudomonas*, and *Streptomyces*, each known for producing a range of ligninolytic and hemicellulolytic enzymes. These enzymes include cellulases, xylanases, and laccases, which can disrupt the lignin-carbohydrate complex and enhance the release of fermentable sugars from biomass.

One key advantage of bacteria is their ability to thrive in a variety of environmental conditions, such as extreme temperatures, pH ranges, and oxygen levels, making them suitable for diverse operational setups. In addition, bacteria generally have faster growth rates than fungi and are more amenable to genetic engineering, allowing for the optimization of enzyme expression and metabolic pathways to target specific biomass components.

However, bacterial delignification tends to be less efficient than fungal pretreatment, often requiring extended time periods to achieve substantial lignin removal. To address this, researchers are exploring the use of mixed bacterial cultures or co-cultures with fungi to enhance the overall pretreatment efficiency. With advances in microbial biotechnology and process engineering, bacterial pretreatment holds promise as a sustainable and scalable method for improving lignocellulosic biomass conversion to bioethanol.

iii) Enzymatic Pretreatment:

Enzymatic pretreatment involves the application of specific enzymes to modify or degrade lignocellulosic biomass, primarily targeting lignin and hemicellulose. These enzymes are typically purified from naturally occurring microbial sources or produced through recombinant DNA technology in controlled environments. Common enzymes used in this process include laccases, peroxidases (such as lignin peroxidase and manganese peroxidase), and hemicellulases like xylanase and mannanase. These enzymes selectively break down the non-cellulosic components of biomass, thereby improving the accessibility of cellulose to hydrolytic enzymes used in subsequent saccharification steps.

One of the primary benefits of enzymatic pretreatment is the high level of specificity and control it offers. Unlike whole-cell biological methods, enzymatic pretreatment does not introduce living organisms into the system, significantly reducing the risk of microbial contamination and competition for sugars during fermentation. It also enables process conditions-such as pH, temperature, and enzyme dosage-to be finely tuned for optimal performance.

However, enzymatic pretreatment is often considered economically challenging due to the high cost of enzyme production and purification. Despite this limitation, advancements in enzyme engineering and cost-effective production systems are steadily improving feasibility. When integrated with other pretreatment techniques, enzymatic pretreatment can greatly enhance biomass conversion efficiency in a sustainable and controlled manner.

iv) Microbial Consortia

Microbial consortia refer to the deliberate use of mixed cultures of fungi, bacteria, or both, to pretreat lignocellulosic biomass. This approach leverages the synergistic interactions between different microorganisms, allowing them to complement each other's enzymatic capabilities for more effective degradation of lignin, hemicellulose, and cellulose. For instance, fungi such as *Trametes versicolor* can break down lignin effectively, while bacteria like *Bacillus subtilis* or *Pseudomonas fluorescens* contribute to hemicellulose deconstruction and facilitate further cellulose accessibility.

The combination of organisms with diverse enzymatic portfolios not only accelerates biomass decomposition but also improves yield and reduces the time required for pretreatment. Such consortia can adapt to varying environmental conditions and offer

Table 3: Comparison of Pretreatment Methods for Lignocellulosic Biomass

Physical	Milling, Grinding, Extrusion	Reduces particle size, increases surface area	Simple, enhances enzyme accessibility, no chemical use	High energy consumption , limited delignification
	Steam Explosion	Rapid decompression of steam-treated biomass	Partially removes hemicellulose, increases porosity	Generates inhibitors (furfural, HMF), incomplete lignin removal
Chemical	Dilute Acid	Hydrolyzes hemicellulose into monomers	Efficient sugar release, rapid action	Inhibitor formation, equipment corrosion, and acid recovery are needed
	Alkaline (e.g. NaOH, ammonia)	Solubilizes lignin and disrupts lignin–carbohydrate bonds	Effective lignin removal enhances enzymatic hydrolysis	Long reaction times, high water usage, and chemical recovery required
	Organosolvo	Uses organic solvents to dissolve lignin	High delignification , lignin recovery is possible	Solvent cost, safety, and recycling issues
	Ionic Liquid	Disrupts crystalline cellulose and dissolves lignin	High efficiency, selective fractionation	Expensive, toxic solvents, recycling challenges
Biological	Fungal (white-rot, brown-rot fungi)	Enzymatic lignin and hemicellulose degradation	Low energy, environmentally friendly	Slow process, sensitive to conditions, requires long residence time
	Bacterial	Enzyme- producing microbes degrade hemicellulose/lign in	Mild conditions, minimal inhibitor formation	Lower delignification efficiency, scalability issues

better resilience than single strains when processing heterogeneous biomass types. Additionally, microbial consortia can self-regulate their population dynamics, optimizing enzyme production based on substrate availability.

Recent research has shown that fine-tuning the composition of microbial communities and controlling operational parameters such as pH, moisture, and aeration can significantly enhance delignification efficiency. This method is especially promising for large-scale and cost-effective bioethanol production due to its potential to minimize chemical inputs and energy requirements. Overall, microbial consortia represent a biologically robust and ecologically sustainable strategy for improving lignocellulosic biomass pretreatment.

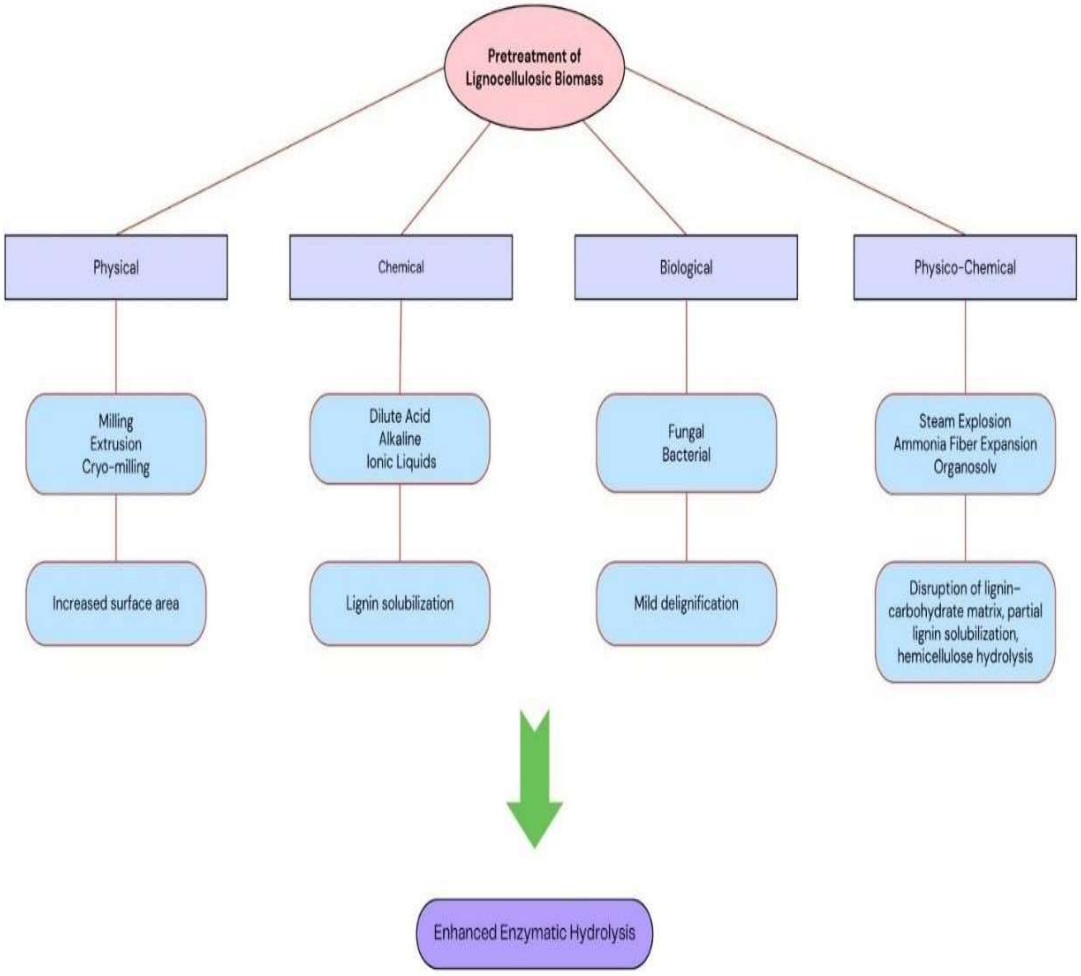

Fig 4: Classification of Pretreatment Methods for Lignocellulosic Biomass

2.4 Enzymatic Hydrolysis

Enzymatic hydrolysis is a critical stage in converting lignocellulosic biomass into fermentable sugars for bioethanol production. Following pretreatment, which opens up the biomass structure and removes lignin barriers, specific enzymes are introduced to break

down cellulose and hemicellulose into simple sugars like glucose and xylose. This step is especially important for the substrates used in this study-wheat straw, rice straw, rice husk, spent mushroom substrate (SMS), and sawdust-because their composition and pretreatment efficiency directly influence hydrolysis outcomes.

Cellulose hydrolysis typically involves a synergistic action of three enzyme groups: endoglucanases, which randomly cleave internal bonds within cellulose chains; exoglucanases, which release cellobiose units from chain ends; and β-glucosidases, which convert cellobiose into glucose monomers. (Yuan et al., 2021a) For hemicellulose, accessory enzymes like xylanases and mannanases are necessary, particularly for substrates like wheat and rice straw.

Hydrolysis efficiency depends on factors such as cellulose crystallinity, surface area, enzyme loading, and residual lignin. High lignin content in sawdust and rice husk can bind enzymes non-productively, reducing sugar yields (Mosier et al., 2005a) . In contrast, SMS, having undergone fungal degradation, typically shows improved digestibility and requires lower enzyme doses.(Lin et al., 2021)

To optimize hydrolysis, reaction conditions like pH (4.8–5.5), temperature (45–55°C), and solid loading must be carefully adjusted. Additionally, surfactants or enzyme recycling techniques may help lower costs and improve conversion rates. Ultimately, efficient enzymatic hydrolysis lays the foundation for high ethanol yields in downstream fermentation.

2.4.3. Types of Hydrolysis

1. Chemical Hydrolysis: Chemical hydrolysis involves the use of acids or bases to break down the polysaccharides in lignocellulosic biomass into simple sugars. Two major types of chemical hydrolysis are:

a. Acid Hydrolysis: This process can be carried out using either concentrated acids or dilute acids.

i) Concentrated Acid Hydrolysis: This method uses strong acids like sulfuric acid (H_2SO_4) at high concentrations to directly hydrolyze cellulose and hemicellulose into sugars. It is highly effective but requires large amounts of acid, which are corrosive and need careful handling and recovery, making it expensive and environmentally challenging. The high sugar yield and minimal formation of inhibitors are advantages, but neutralization and recovery costs are significant drawbacks.

ii) Dilute Acid Hydrolysis: In this approach, dilute acids (usually sulfuric or hydrochloric acid) are applied at higher temperatures and pressures to break down the biomass. While it is more cost-effective and safer than concentrated acid hydrolysis, it can lead to lower sugar yields and the formation of inhibitory byproducts, such as furfural and hydroxymethylfurfural (HMF), which can inhibit downstream fermentation.

b. Alkaline Hydrolysis: Alkaline hydrolysis uses bases like sodium hydroxide (NaOH) or lime ($Ca(OH)_2$) to break down the ester bonds between lignin and hemicellulose, making the

cellulose more accessible. Alkaline hydrolysis is less aggressive than acid hydrolysis and is more effective at removing lignin, but it can also lead to the loss of some carbohydrates.

2. Enzymatic Hydrolysis: Enzymatic hydrolysis is a biological process where cellulase enzymes break down cellulose into simple sugars, primarily glucose. Hemicellulase enzymes are also used to degrade hemicellulose into xylose, mannose, arabinose, and other sugars.

❖ Cellulases: These enzymes work in a coordinated way, with endoglucanases breaking internal cellulose bonds, exoglucanases cleaving off sugar units from the ends of cellulose chains, and β-glucosidases converting cellobiose into glucose.

❖ Hemicelluloses: These enzymes break down hemicellulose, which contains a mixture of different sugars, into its sugar components.

Enzymatic hydrolysis is highly selective and operates under mild conditions, making it more environmentally friendly than chemical hydrolysis. However, the cost of enzymes is a limiting factor, and the process is relatively slow. Pretreatment of the biomass is essential to improve enzyme accessibility, as the natural lignin and hemicellulose barriers must be disrupted for efficient enzyme activity.

2.4.4. Paradigm Shift: Pretreatment as a Prerequisite

Before enzymatic hydrolysis can effectively convert lignocellulosic biomass into fermentable sugars, the rigid structure of the biomass must be disrupted. This is achieved through pretreatment methods like dilute acid, alkaline, steam explosion, ammonia fiber expansion (AFEX), hydrothermal, or ionic liquid techniques. These methods work to remove lignin, increase porosity, lower cellulose crystallinity, and reveal the cellulose and hemicellulose fibers, making them accessible to enzymes.(Baruah et al., 2018). Without any

pretreatment, hydrolysis yields are extremely low-often below 20% of the theoretical maximum-because the enzymes cannot reach or interact efficiently with the polysaccharide chains. However, once effectively pretreated, sugar recovery dramatically improves, often achieving saccharification rates of 70–90% across a wide variety of feedstocks**.**

For example, steam explosion-one of the most widely used industrial methods-effectively fractures the plant cell wall using high temperatures and pressure, increasing enzyme accessibility and sugar yield. Similarly, alkaline and acid pretreatments selectively dissolve

lignin and hemicellulose, respectively, while hydrothermal (hot water) and ionic liquid strategies disrupt the overall network, exposing cellulose (Yu et al., 2018)

These pretreatments not only enhance sugar yields but also reduce costs in downstream processing by minimizing enzyme load and cutting detoxification steps. They are therefore the essential first step before enzymatic hydrolysis can proceed efficiently in the production of bioethanol from substrates like wheat straw, rice straw, rice husk, spent mushroom substrate, and sawdust.

2.4.3 Key Factors Influencing Hydrolysis

Efficient enzymatic hydrolysis of lignocellulosic biomass depends not only on pretreatment but also on a set of critical substrate and process characteristics. This section delves into five key factors-particle size, crystallinity, accessible surface area & porosity, synergistic enzyme action, and inhibition via non-productive binding-to highlight their impact on sugar recovery.

a) Particle Size & Surface Area

Reducing lignocellulosic biomass to finer particles plays a pivotal role in improving enzymatic hydrolysis efficiency. By increasing external surface area and shortening diffusion paths, smaller particles enable better enzyme access to internal cellulose fibres. For instance, sub- millimetre milling (≤ 0.5 mm) has demonstrated an approximate 10% increase in enzymatic conversion efficiency compared to larger, millimetre-sized particles (Achinas et al. 2016), (Sun et al. 2024). A detailed study by Yang et al. reported that corn stover particles sized at 0.25–0.5 mm achieved roughly 5–10% greater pretreatment effectiveness and a similar rise in enzymatic hydrolysis compared to 1–4 mm particles. However, the overall sugar recovery did not significantly improve due to lower mass retention after pretreatment.

Scanning electron microscopy (SEM) of corn stover has provided visual evidence that the internal pore surfaces-rather than external dimensions-largely determine enzyme access. Although external surface area scales with smaller particle size, the internal porosity remains the main contributor; reducing particle size from 1.42 to 0.21 mm increased external surface area, but total surface area (including internal pores) saw minimal change, around 2% (H. Li et al., 2015) . This explains why further size reduction, beyond a threshold, yields diminishing returns on hydrolysis unless accompanied by strategies that enhance internal porosity.

In practical terms, moderate particle sizes (~0.5–1.0 mm) often strike the optimal balance between enhanced hydrolysis and energy savings from milling. Excessively fine grinding consumes more power without proportionate gains in sugar yield. This insight is critical when designing cost-effective biomass-to-ethanol processes.

b) Crystallinity

The degree of crystallinity in cellulose significantly affects its breakdown by enzymes. Highly crystalline cellulose, with tightly packed and ordered chains, resists enzymatic attack, whereas amorphous regions-where chains are more disordered-are more readily hydrolyzed. Pretreatments such as alkaline or organosolv processing help disrupt this crystalline structure, increasing enzyme access.(Xu et al., 2019)

In one study on loblolly pine, organosolv pretreatment reduced crystallinity and yielded higher glucose recovery during hydrolysis, demonstrating the importance of altering crystalline structure (Xu et al., 2019)**.** Similarly, a multivariate analysis of corn stover revealed that lower crystallinity index (CrI) values consistently correspond with increased sugar release, underscoring this parameter as critical in substrate digestibility assessments.

However, caution is necessary: excessive pretreatment can lead to cellulose re-crystallization or condensation, potentially creating new resistant structures and reducing overall hydrolysis efficiency (Pardo et al., 2019)**.** For example, sugarcane residue treated only with organosolv showed unexpectedly higher crystallinity-likely due to removal of amorphous components-yet still achieved good hydrolysis from better lignin removal (Pardo et al., 2019)

Hence, an optimal pretreatment must strike a balance, sufficiently reducing crystallinity to enhance enzyme access without triggering re-crystallization. This balance varies depending on each substrate's initial composition and pretreatment method.

c) Accessible Surface Area & Porosity

Accessible surface area and pore volume are crucial for effective enzymatic hydrolysis, as they determine how deeply enzymes can penetrate the biomass matrix. Studies using BET (Brunauer- Emmett-Teller) analysis have consistently shown a strong correlation between increased porosity and improved sugar yields during enzymatic hydrolysis. Enzymes require sufficient access to cellulose and hemicellulose chains, and porosity greatly facilitates this interaction. (He et al., 2014)

Mechanical refining methods-such as disc refining, fibrillation, and ball milling-enhance porosity by separating fibers and disrupting the lignocellulosic structure, thereby improving

enzyme penetration. (Y. Li et al., 2018) For example, refining of wheat straw increased BET surface area by nearly 3.5-fold and significantly enhanced hydrolysis efficiency when paired with alkaline pretreatment. Another study on poplar demonstrated that refining coupled with steam explosion yielded a 40% increase in glucose production.

However, accessible surface area alone does not guarantee high sugar recovery. If lignin and hemicellulose are not adequately removed or altered during pretreatment, they can obstruct pores and prevent enzymes from accessing the substrate. Thus, effective biomass conversion strategies must integrate mechanical and chemical approaches to maximize both internal porosity and biochemical accessibility.

d) Synergistic Enzyme Action

Enzymatic hydrolysis of lignocellulosic biomass requires a well-orchestrated mixture of enzymes-commonly referred to as an enzyme cocktail-to effectively break down cellulose and hemicellulose.

The core cellulase system includes:

- Endoglucanases: These enzymes randomly cleave internal β1,4 glycosidic bonds in cellulose, generating new chain ends and disrupting crystalline regions. Their action is essential for increasing enzyme accessibility to cellulose fibres.(Ramírez Brenes et al., 2023)
- Exoglycanases (cellobiohydrolases): These processively remove cellobiose units from the reducing or nonreducing ends of cellulose, further breaking down the polymer chain.

- β-Glucosidases: These enzymes hydrolyse cellobiose into glucose monomers, alleviating product inhibition from cellobiose build-up and completing the cellulolytic cycle.

This sequential strategy-moving from polymer to monomer-is highly synergistic, meaning the combined action of the enzyme suite yields far more sugar than each enzyme alone. To enhance this system, hemicellulases (e.g., xylanases, mannanases) and debranching enzymes like deacetylases and glucuronidases are added. They remove side chains from hemicelluloses, reducing steric hindrance and increasing cellulase efficiency[4].

A breakthrough has been the inclusion of lytic polysaccharide monooxygenases (LPMOs), which utilize controlled H_2O_2 to oxidatively cleave crystalline cellulose, dramatically improving hydrolysis rates. For instance, controlled H_2O_2 supply increased saccharification yields by nearly two orders of magnitude in Avicel and industrial substrates such as spruce and birch under optimized conditions[1]. LPMO effectiveness can be further enhanced when combined with cellulases, provided the oxidative environment is tightly regulated to avoid enzyme inactivation. Effective hydrolysis thus relies on carefully balanced enzyme cocktails, often quantified by the Degree of Synergism (DS) metric, which compares combined activities against individual enzyme contributions[4]. Tailoring enzyme blends-including cellulases, hemicellulases, and LPMOs-to specific biomass types (e.g., wheat straw, rice husk) ensures maximum glucose yield. (Ramírez Brenes et al., 2023).

e) Inhibitors & Non-Productive Binding

Residual lignin, pseudo-lignin, and lignin-derived phenolics often bind enzymes non-productively, nullifying hydrolysis potential. Lignin's hydrophobic and electrostatic interactions with cellulases inhibit enzyme activity, lowering conversion yields (Yuan et al., 2021). Pretreatment strategies aim to not only remove lignin but also modify its structure to reduce enzyme adsorption.

Additives like Tween-80, BSA, PEG, or soluble lignin derivatives (e.g., lignosulfonates) can block lignin-binding sites, improving efficiency. Surfactant use, however, must be optimized: excessive supplementation may lead to enzyme denaturation or competition for binding sites. Additionally, LPMOs provide oxidative cleavage by targeting crystalline cellulose facets, offering a solution to bypass lignin interference. (Mafa et al., 2021).

Table 4: Key Structural and Biochemical Factors Influencing Enzymatic Hydrolysis Efficiency

Factor	Impact on Hydrolysis
Particle Size	Small particles boost surface area but energy-efficient size thresholds exist
Crystallinity	Lower crystalline cellulose enhances enzyme penetration and speed
Surface Area & Porosity	Essential for deep enzyme access within biomass structure
Enzyme Synergy	Balanced enzyme cocktails and LPMO inclusion maximize breakdown efficiency
Lignin Inhibition & Binding	Managing enzyme-lignin interactions through additives and pretreatment improves yields

2.4.4. Process Configuration& Optimization

Effective enzymatic hydrolysis depends not only on the choice of enzymes and pretreatment but also on how the process is configured. Key factors include whether hydrolysis and fermentation are conducted separately (SHF) or simultaneously (SSF), the method of enzyme recycling, the strategy for substrate feeding, and the use of additives like surfactants. These elements influence reaction conditions, reduce product inhibition, and improve enzyme efficiency. Proper configuration can lower operational costs, enhance sugar yields, and improve overall bioethanol productivity, especially when working with complex feedstocks like wheat straw, rice husk, or sawdust.

2.4.5 Modes of Hydrolysis& Fermentation

The configuration of enzymatic hydrolysis and fermentation, whether performed as separate or combined steps, significantly impacts the efficiency, yield, and cost of bioethanol production from lignocellulosic biomass. In the Separate Hydrolysis and Fermentation (SHF) approach, each step is carried out under its optimal conditions, allowing precise control over temperature and pH. However, this increases the risk of contamination, prolongs processing time, and requires more equipment. In contrast, Simultaneous Saccharification and Fermentation (SSF) combines both steps in a single reactor. This simplifies the process and reduces product inhibition, as sugars are fermented as soon as

they are released. While SSF lowers capital and operational costs, it requires a temperature compromise-enzymes perform best around 50 °C, while yeast thrives at 30–35 °C. Each method has advantages depending on the feedstock and process goals, but ongoing innovations, such as fed-batch strategies and engineered microbes, are increasingly favoring SSF for industrial-scale bioethanol production.

2.4.5.1. Separate Hydrolysis and Fermentation (SHF)

The Separate Hydrolysis and Fermentation (SHF) configuration is a traditional and widely used approach in lignocellulosic bioethanol production. In this method, enzymatic hydrolysis and microbial fermentation are performed in two distinct steps and reactors. Hydrolysis is typically carried out at around 50 °C and pH 4.8–5, conditions that favor the activity of cellulolytic enzymes such as endoglucanases and β-glucosidases. Following hydrolysis, the resulting sugar- rich hydrolysate is transferred to a second vessel, where fermentation occurs at lower temperatures, typically 30–35 °C, which is optimal for the growth and ethanol production of yeasts like *Saccharomyces cerevisiae*. (Olofsson et al., 2008). One of the main advantages of SHF is the ability to independently optimize each step. By separating the processes, it becomes possible to maximize enzyme efficiency without compromising yeast viability. This flexibility is particularly useful when using genetically modified microbial strains or enzymes that function best under tightly defined conditions. Furthermore, SHF enables detailed monitoring and control of sugar conversion and ethanol yield at each phase.

However, SHF has several limitations. The most significant is product inhibition. During hydrolysis, sugars such as glucose and cellobiose accumulate in the reactor, which can feed back-inhibit cellulase activity, slowing the breakdown of cellulose. Since fermentation does not begin until hydrolysis is complete, these inhibitors remain in the system for extended periods.(Mosier et al., 2005) SHF also requires longer processing time, more complex sterilization protocols, and increased risk of contamination due to multiple material transfers. Additionally, it demands a higher capital investment, as two sets of reactors, heating systems, and control units are needed.

Despite these challenges, SHF remains relevant in pilot and commercial setups where specific operational controls, microbial engineering, or enzyme recovery strategies are necessary.

Table 5: Advantages and Disadvantages of Separate Hydrolysis and Fermentation (SHF)

Advantages	Disadvantages
Allows independent optimization of hydrolysis and fermentation conditions	Requires longer total processing time
Enables the use of specialized enzymes and microbial strains	Increased contamination risk due to multiple handling stages
Better process control and monitoring of each stage	Product inhibition occurs during hydrolysis due to sugar accumulation
Flexibility in adjusting temperature, pH, and residence time for each step	Higher capital cost due to the need for separate reactors and associated equipment
Useful for experimental setups and strain development	Inefficient enzyme usage if inhibitors are not promptly removed

2.4.5.2. Simultaneous Saccharification and Fermentation (SSF)

Simultaneous Saccharification and Fermentation (SSF) is a widely adopted process configuration in lignocellulosic bioethanol production. In SSF, enzymatic hydrolysis and microbial fermentation occur concurrently in a single bioreactor. As enzymes break down cellulose and hemicellulose into simple sugars, fermenting microorganisms such as *Saccharomyces cerevisiae* simultaneously convert these sugars into ethanol. This real-time conversion alleviates one of the major limitations of SHF-product inhibition. Since sugars like glucose and cellobiose are consumed as they are released, they do not accumulate to levels that inhibit enzyme activity, resulting in higher hydrolysis efficiency (Olofsson et al., 2008).

Another key advantage of SSF is its operational simplicity. Conducting both steps in the same vessel reduces the number of equipment units, lowers energy input, and simplifies sterilization procedures. This streamlined approach leads to reduced capital and operational costs, making SSF particularly attractive for industrial-scale applications (Öhgren et al., 2006).

Table 6: Advantages and Disadvantages of Simultaneous Saccharification and Fermentation (SSF)

Advantages	Disadvantages
Reduces product inhibition by converting sugars as they are released	Requires compromise in operating temperature between enzymes and microbes
Simplifies process flow with fewer reactors and steps	Limited ability to independently optimize the hydrolysis and fermentation stages
Lower capital and operational costs due to integrated design	Difficult to recycle enzymes and microbial cells efficiently
Suitable for high-solids operations with fed-batch feeding	Enzyme performance may be suboptimal at fermentation-compatible temperatures
Reduced contamination risk with fewer transfers and open stages	May require engineered yeast strains for temperature and inhibitor tolerance

Despite these benefits, SSF is not without its challenges. The most significant is the temperature conflict: cellulases function optimally around 50°C, while most ethanologenic yeasts perform best between 30–35°C. SSF typically operates at a compromise temperature of 37–39°C, which is suboptimal for both the enzymes and the microorganisms, potentially limiting conversion rates.(Z. H. Liu et al., 2014) Additionally, recycling enzymes and yeast from the slurry is complex due to the physical and chemical variability of the combined medium.

Nevertheless, SSF has demonstrated excellent results, especially in high-solids fermentations. For example, steam-pretreated corn stover at 12% glucan loading, treated with Tween-20 surfactant and fermented at 39°C, achieved 79% glucan conversion and ethanol concentrations of nearly 60 g/L-an 18% improvement over SHF under comparable conditions[3]. These results highlight SSF's potential as a more efficient, cost-effective configuration for bioethanol production, particularly when paired with surfactants and process optimizations.

2.4.5.3 Strategic Considerations and Advances

While both SHF and SSF configurations offer distinct advantages, they are not without challenges. Common issues include sugar inhibition during hydrolysis, high enzyme costs, and complex reactor strategies. To address these, researchers have explored hybrid processes and optimized operational strategies to combine the strengths of each approach.
i) Hybrid Hydrolysis and Fermentation (HHF) is one such innovation. HHF begins with enzymatic hydrolysis under optimal temperature and pH conditions, then transitions mid-

process to a combined SSF mode. Early hydrolysis reduces recalcitrant solids, preparing the feedstock for fermentation, while the SSF phase makes efficient use of microbial uptake of sugars and helps reduce product inhibition. Although less common in industrial applications, HHF has shown promising results in pilot trials using corncob and softwood, delivering improved enzyme efficiency and ethanol yields comparable to SSF, but with greater flexibility in operation conditions. (Öhgren et al., 2006).

ii) Fed-Batch SSF represents another important advance. Here, substrates and enzymes are fed incrementally to the reactor rather than introduced all at once. This approach helps manage the viscosity of high solids slurries-crucial for industrial-scale ethanol production-and controls the build-up of inhibitory compounds like phenolics and furans. Studies have reported ethanol

yields of approximately 80% of theoretical values when SSF is performed at 17% solid loading using a fed-batch protocol, demonstrating the scalability of this approach.

iii) Additives and Surfactants, particularly Tween-20, have also become key tools for enhancing performance. Surfactants work by reducing non-productive binding of cellulase enzymes to lignin, preserving enzyme activity and improving overall sugar yields. For example, SSF trials using steam-pretreated corn stover with Tween-20 demonstrated a notable 18% improvement in ethanol output over surfactant-free controls.

Finally, strain and temperature engineering continue to hold significant promise. Engineered yeast strains capable of fermenting at higher temperatures (up to 39°C) and tolerating inhibitors like furfural are allowing SSF processes to be operated closer to the enzyme's optimal temperature. This reduces the typical compromise temperature issue and significantly boosts conversion efficiency, even without additional pretreatment modifications. (Z. H. Liu et al., 2014)

Both SHF and SSF approaches have distinct advantages and challenges. SHF offers strict process control and optimized conditions at a higher capital cost and contamination risk. SSF simplifies operations and reduces capital exposure, but requires a temperature and condition trade-off. Recent innovations-such as high-solid SSF, surfactant inclusion, fed-batch feeding, and advanced yeast/enzyme engineering-continue to close the performance gap, making SSF increasingly competitive for high-yield, low-cost bioethanol production from substrates like wheat straw, rice husk, sawdust, and spent mushroom substrate.

2.4.6 Enzyme Recycling

Enzymes account for a substantial 30–40% of total costs in enzymatic hydrolysis, so improving enzyme reuse is vital for economic viability. Two main recycling strategies are currently explored:

1. Soluble Enzyme Recovery

This method targets enzymes remaining in the liquid fraction after hydrolysis. Unfortunately, many cellulases and hemicellulases adsorb onto residual solids or become inactivated during the process, making recovery from the liquid challenging and generally inefficient. (Bootsma et al., 2008).

2. Solid-Phase Enzyme Recycling

A more promising strategy involves retaining the enzyme-coated, unhydrolyzed biomass solids and reusing them in subsequent hydrolysis cycles. Studies demonstrate that this method can save approximately 40–50% of enzyme dosage over five consecutive batches without significant loss in sugar yield[2]. By keeping enzymes physically bound to the substrate, solid- phase methods reduce the need for repeated enzyme additions, significantly lowering overall costs (Du et al., 2011).

To further enhance this approach, the addition of mild surfactants such as Tween-20 has proven effective in reducing enzyme binding to lignin. Surfactants help maintain free enzyme activity in the liquid, ensuring higher catalytic availability in each cycle[2]. Implementing surfactants during enzyme recycling can recover approximately 10–15% more enzymatic activity compared to non-supplemented controls.

3. Feeding Strategy & Product Removal

A well-established solution is fed-batch substrate addition. Instead of introducing all the biomass at the start, small batches are added gradually. This method lowers initial viscosity, improves enzyme distribution, and reduces the risk of clogging. Studies using pretreated sugarcane bagasse show that starting at 12% solids and incrementally feeding fresh material up to 33% solids results in high sugar yields (~230 g/L total sugars, ~60% glucan conversion) by optimizing mixing and diffusion. (Y. Liu et al., 2015)

Another approach is in-situ product removal, such as gas stripping or liquid–liquid extraction, which continuously removes sugars or ethanol from the reactor. By lowering local product concentrations, these strategies help maintain enzyme activity and relieve fermentation organisms from inhibitory stress.

Together, fed-batch feeding and continuous product removal help address key challenges at industrial scale: reducing slurry thickening, improving mass transfer, and maintaining enzyme efficiency-all of which are critical for delivering economically viable, high-yield bioethanol processes.

2.4.7 Additives to Enhance Performance

Adding specific compounds like surfactants, proteins, or lignin derivatives can dramatically reduce enzyme loss and boost sugar yield during enzymatic hydrolysis and fermentation.

Surfactants such as Tween-20, Tween-80, polyethylene glycol (PEG), and bovine serum albumin (BSA) play a key role by blocking the hydrophobic binding sites on lignin. This prevents enzymes from adhering non-productively, which increases the amount of active enzyme available for hydrolyzing cellulose. Studies have shown that adding Tween-20 during SSF of steam-exploded wheat straw can enhance sugar release and ethanol yield by up to 15% compared to control runs without additives. Similar benefits have been observed with PEG and BSA, with yields improving by 10–20% depending on substrate lignin content.

Proteins or soluble lignin derivatives-like lignosulfonates or soy protein-work through a similar mechanism. By adsorbing to lignin surfaces, they effectively shield cellulases from non-productive binding. In one study involving corn stover, supplementing with soy protein increased enzymatic efficiency by 12%, enabling a 20% reduction in enzyme loading while maintaining high sugar yields. Lignosulfonates have also shown stabilizing effects and improved enzyme performance in several trials. In summary, these additives optimize enzyme effectiveness and cost-efficiency, especially when treating lignin-rich feedstocks.

2.4.8 Integrated Best Practices

Optimizing bioethanol production requires a holistic design that combines various process strategies, particularly in SSF performed at ~39 °C using lignocellulosic substrates such as wheat straw or corn stover.

A robust configuration includes:

1. Surfactant addition (e.g., Tween-20) to minimize enzyme-lignin binding.
2. Fed-batch feeding to control slurry viscosity and inhibitor concentration.
3. Solid-phase enzyme recycling to reclaim enzymes from unhydrolyzed solids.

This integrated approach has yielded significant performance gains. For example, fed-batch SSF of wheat straw using surfactant and enzyme recycling demonstrated an 18–40% boost in ethanol yield compared to conventional SHF at 12% solids loading. These gains are attributed to reduced enzyme loss, sustained enzyme activity through multiple cycles, and improved

mass transfer. In industrial trials, such process integration not only elevated ethanol concentration but also shortened processing time and cut enzyme costs.

2.5 Fermentation Process

The fermentation of lignocellulosic biomass includes turning the simple sugars produced by the hydrolysis of cellulose and hemicellulose into biofuels, primarily ethanol, or other important compounds. Fermentation is often carried out by microorganisms such as yeast or bacteria, which use anaerobic respiration to convert carbohydrates into alcohol or other compounds. This step is critical in the generation of bioethanol and biochemicals from lignocellulosic biomass.

Fermentation process steps include pretreatment and hydrolysis. Before fermentation begins, the lignocellulosic biomass must be prepared and hydrolyzed. Pretreatment alters the structure of the biomass, providing access to cellulose and hemicellulose. Hydrolysis subsequently converts these complex carbohydrates into simple fermentable sugars like glucose and xylose, which are necessary for fermentation.

Fermentation is the final and essential step in the bioethanol production process, where fermentable sugars-mainly glucose and xylose-are biologically converted into ethanol by microorganisms. This stage follows enzymatic hydrolysis and is critical for determining the final yield and overall efficiency of the process. The type of fermentation setup chosen- Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), or Simultaneous Saccharification and Co-Fermentation (SSCF)-influences process performance.

In SHF, hydrolysis and fermentation are performed in separate reactors under conditions optimized for each step. SSF combines both steps in one reactor, allowing sugars to be fermented as soon as they are released, which reduces sugar inhibition and simplifies processing. SSCF is an advanced method where both hexose (e.g., glucose) and pentose (e.g., xylose) sugars are fermented simultaneously using specially adapted or engineered microorganisms.

Choosing the right fermentation strategy depends on factors such as feedstock composition, microorganism selection, and process economics. Effective fermentation integration is crucial for maximizing ethanol output, especially at high-solid loadings.

2.5.1 Fermentation key Steps

Fermentation process steps include pretreatment and hydrolysis. Before fermentation begins, the lignocellulosic biomass must be prepared and hydrolyzed. Pretreatment alters the structure of the biomass, providing access to cellulose and hemicellulose. Hydrolysis subsequently converts these complex carbohydrates into simple fermentable sugars like glucose and xylose, which are necessary for fermentation.

1. Microbial Fermentation: Microorganisms ferment the sugars released during hydrolysis to yield ethanol or other desirable compounds. There are two major types of microorganisms used:
Saccharomyces cerevisiae is the most often used yeast for converting glucose to ethanol. It effectively converts glucose to ethanol in anaerobic circumstances. Bacteria: *Zymomonas mobilis* and *Escherichia coli* may ferment hexoses (C6 sugars like glucose) and pentoses (C5 sugars like xylose).

2. Co-Fermentation (Simultaneous Fermentation): Co-fermentation (or co-culture) is used to enhance the utilization of all available sugars by combining two or more microbial strains. One strain may specialize in fermenting glucose, whereas another ferments xylose. This method boosts overall ethanol yield by using all sugar fractions generated from hemicellulose and cellulose.

3. Simultaneous Saccharification and Fermentation (SSF): SSF combines enzymatic hydrolysis and fermentation in the same reactor, saving time and money. Cellulase enzymes degrade cellulose into glucose, while yeast converts glucose to ethanol in parallel. SSF minimizes product inhibition while simplifying the process by consolidating two processes into one.

4. Separate Hydrolysis and Fermentation (SHF): This classic method has two stages: first, hydrolysis is performed, and then the sugars are fermented in a separate step. While this approach provides more control over each stage, it may result in decreased efficiency due to inhibitor accumulation and longer processing times.

2.5.2. Selection of Fermenting Microorganisms

Choosing the right microorganisms is essential for efficient ethanol production from lignocellulosic biomass, where both hexose and pentose sugars must be utilized effectively.

Saccharomyces cerevisiae, the most widely used yeast in conventional ethanol production, is known for its high ethanol yield and strong inhibitor tolerance. However, it cannot naturally ferment pentose sugars like xylose, a significant portion of lignocellulosic hydrolysates. To overcome this limitation, metabolic engineering has introduced heterologous pathways such as xylose reductase (XR) and xylitol dehydrogenase (XDH) from *Scheffersomyces stipitis*, or bacterial xylose isomerase (XI) into S. cerevisiae. These genetic modifications allow S. cerevisiae to co-ferment glucose and xylose, producing ethanol yields up to ~0.47 g/g of consumed sugar in engineered industrial strains-a significant step toward near-theoretical maximum conversion efficiency. (Romaní et al., 2015)

Scheffersomyces stipitis is a naturally xylose-fermenting yeast with superior performance on pentose sugars but less inhibitor tolerance and ethanol productivity compared to S. cerevisiae. In contrast, the bacterium *Zymomonas mobilis* excels in efficient glucose fermentation using the Entner-Doudoroff pathway, producing high ethanol titres with lower biomass production and higher yield (up to 98%). However, wild-type *Z. mobilis* cannot ferment pentoses, making its application in lignocellulosic processes limited unless engineered for xylose utilization.

Recent research explores microbial consortia, such as combining *S. stipitis* and engineered *Z. mobilis*, to balance glucose and xylose fermentation. Using this dual approach has improved xylose consumption by ~5% and boosted ethanol titers by ~6%, compared to monocultures. (Sun et al., 2021).Overall, while S. cerevisiae remains the workhorse, pentose-fermenting yeasts and bacteria, and engineered or mixed systems play growing roles in efficiently converting mixed-sugar lignocellulosic hydrolysates.

2.5.2.1 Hexose and Pentose Sugar Utilization

Efficient bioethanol production from lignocellulosic biomass demands leveraging both hexose (C_6) and pentose (C_5) sugars. Cellulose hydrolysis yields glucose, whereas hemicellulose releases pentoses like xylose and arabinose. Maximizing ethanol yield thus depends on effectively converting both streams, reducing waste, and enhancing economic viability. (C. G. Liu et al., 2019).

Importance of C_6 and C_5 Sugar Conversion

One of the central challenges in lignocellulosic bioethanol production is the complete utilization of all fermentable sugars released from the biomass. Lignocellulose consists of two major carbohydrate polymers: cellulose, which yields glucose (a hexose or C_6 sugar), and hemicellulose, which primarily yields xylose and arabinose (pentoses or C_5 sugars).

While Saccharomyces cerevisiae efficiently ferments glucose to ethanol with yields close to 0.51 g/g, it cannot naturally metabolize pentose sugars. These pentoses can make up 20–30% of the total sugar content of lignocellulosic hydrolysates. If they are not utilized, a substantial fraction of the biomass is wasted, lowering the overall ethanol yield and the economic feasibility of the process.

To address this, metabolic engineering has focused on enabling microorganisms-especially S. cerevisiae-to co-utilize glucose and xylose. When both sugar types are fermented effectively, total ethanol production can increase by up to 30%, depending on the feedstock. This improvement significantly reduces the per-liter cost of ethanol and enhances the viability of second-generation biofuels. Beyond the yield benefit, pentose conversion also improves process sustainability, reducing waste streams and maximizing the energy recovered per ton of biomass processed. Thus, tapping into the full sugar potential of lignocellulosic biomass is not just advantageous but essential for commercial-scale bioethanol production.

2.5.2.2. Pathway Engineering: XR/XDH and XI Systems

To enable Saccharomyces cerevisiae to ferment xylose-an essential pentose derived from hemicellulose-two major metabolic engineering strategies have been developed: the XR/XDH pathway and the XI pathway.

XR–XDH Pathway

The xylose reductase (XR)–xylitol dehydrogenase (XDH) pathway introduces two enzymes- usually from *Scheffersomyces stipitis*-into S. cerevisiae: XR, which reduces xylose to xylitol using NADPH, and XDH, which oxidizes xylitol to xylulose using NAD^+. (Qui et al. 2023) While this makes xylose metabolism possible, the differing cofactor requirements create a redox imbalance. Under anaerobic conditions, NAD^+ regeneration is limited, leading to NADH build-up and accumulation of xylitol as a by-product, which can suppress ethanol formation (Kwak & Jin, 2017) To address this, researchers have engineered enzymes to shift

cofactor specificity (e.g., XR from NADPH to NADH), overexpressed XDH, or introduced redox-balancing systems, resulting in reduced xylitol and improved ethanol yields (Kwak & Jin, 2017)

2.5.2.3 XI Pathway

In contrast, the xylose isomerase (XI) pathway involves a single enzyme that directly converts xylose into xylulose, bypassing cofactor use and redox issues (Lee et al., 2012)

While the theoretical ethanol yield can reach up to 0.51 g/g, XI-based systems in S. cerevisiae often struggle with low enzyme activity and slow sugar uptake at first. Through directed evolution, thermostable XIs from organisms like *Piromyces* have been improved, showing an 8-fold increase in xylose consumption and ethanol production in engineered strains. Additionally, co-expression with transporters and downstream enzymes (e.g., xylulokinase) further boosts flux through the pentose phosphate pathway. (Lee et al., 2012)

2.5.2.4 Comparative Performance

While XR/XDH strains consume xylose more rapidly initially, their efficiency is hampered by redox imbalance. XI strains deliver higher ethanol yield with less xylitol, but often require evolutionary adaptation to reach industrial performance (Lee et al., 2012) Some novel designs even combine both pathways, leveraging the speed of XR/XDH and the yield advantages of XI, to enhance performance on non-detoxified biomass (Cunha et al., 2019)

By integrating pathway engineering, strain adaptation, and redox balancing, researchers aim to develop industrial yeast strains capable of co-fermenting glucose and xylose efficiently, ultimately unlocking the full bioethanol potential of lignocellulosic feedstocks.

Challenges in Co-Fermentation: Redox Imbalance and Glucose Repression

Despite the success of engineering yeasts to metabolize both hexoses and pentoses, co-fermentation poses several biochemical and regulatory challenges that affect efficiency. The first major barrier is glucose repression, where the presence of glucose suppresses the uptake and metabolism of xylose. This delay in xylose fermentation often leads to extended process times, incomplete sugar utilization, and lower overall ethanol productivity. To overcome this, researchers have engineered yeast strains with modified regulatory networks or introduced xylose-specific transporters that function effectively even when glucose is present.

Table 7: Comparison and Significance of C_6 and C_5 Sugar Conversion in Bioethanol Production

Parameters	C_6 Sugars (e.g. Glucose)	C_5 Sugars (e.g. Xylose,Arabinose)	Significance in Bioethanol Process
Source of biomass	Primarily from cellulose	Primarily from hemicellulose	Reflects the dual polysaccharide structure of lignocellulosic biomass
Natural fermentability by *S. cerevisiae*	Yes	No	Limits ethanol yield if only C_6 sugars are fermented
Typical proportion in biomass	~60–70% of total carbohydrates	~20–30% of total carbohydrates	Pentoses represent a significant untapped sugar fraction
Ethanol yield (g/g sugar)	~0.51	Up to ~0.49 (with engineered strains)	High yields are achievable if both sugars are fermented efficiently
Engineering required	None for conventional strains	Requires pathway engineering (XR/XDH or XI)	Increases process complexity but is essential for full sugar utilization
Challenges	Minimal	Redox imbalance, glucose repression, slower uptake	Co-fermentation strategies needed for simultaneous sugar utilization
Impact on overall ethanol yield	High, but limited if C_5 sugars are ignored	Essential to maximize ethanol from total sugar content	Co-utilization improves yield, efficiency, and economic viability

The second and more critical issue lies in the redox imbalance observed in yeasts expressing the XR/XDH pathway for xylose metabolism. Xylose reductase (XR) uses NADPH to convert

xylose into xylitol, while xylitol dehydrogenase (XDH) uses NAD^+ to convert xylitol into xylulose. This cofactor mismatch creates an excess of NADH and leads to the accumulation of xylitol, a non-fermentable byproduct, ultimately reducing ethanol yield. Strategies to address this include engineering XR to prefer NADH instead of NADPH or co-expressing NADH oxidases to rebalance intracellular cofactors.

Other approaches involve the use of xylose isomerase (XI), which directly converts xylose into xylulose without involving cofactors, thereby eliminating redox imbalance. However, XI- based systems often suffer from low enzyme activity in S. cerevisiae. Continued progress in strain engineering aims to combine cofactor balance, derepression

of xylose metabolism, and robust enzyme expression for high-yield, co-fermenting yeast suitable for industrial application.

Strategies such as modulating XR/XDH enzyme expression ratios, introducing cofactor regeneration (e.g., NADH oxidases), and engineering promoters for enhanced XI expression have shown progress in reducing xylitol yield and improving ethanol production from xylose[4]. Recent strains expressing optimized XI and pentose pathway enzymes achieved 0.49 g ethanol per gram xylose, highlighting the path toward near-theoretical yields.

2.5.3. Challenges in Lignocellulosic Fermentation

1 Furfural, hydroxymethylfurfural (HMF), acetic acid, and phenolic compounds are among the many inhibitory chemicals that are frequently generated during the pretreatment and hydrolysis processes. Lower ethanol yields can arise from these inhibitors' ability to impede microbial growth and decrease fermentation efficiency. Sometimes successful fermentation requires the hydrolysate to be detoxified first.

2 Utilization of C5 and C6 Sugars: Lignocellulosic biomass contains both pentose (C5) and hexose (C6) sugars, predominantly from hemicellulose and cellulose, respectively. While yeasts such as S. cerevisiae are capable of successfully fermenting the C6 sugar glucose, they are typically not able to metabolize the C5 sugar xylose. To increase ethanol output, research into creating or engineering microbes that can co-ferment both kinds of sugars is essential.

3 Low Pentose Yield: Compared to hexose sugars, the pentose portion of lignocellulosic biomass ferments less effectively. The most prevalent C5 sugar, xylose, is more challenging for naturally occurring fermentation microbes to digest. S. cerevisiae and other strains are being modified through genetic engineering to increase their capacity to ferment C5 sugars, which will increase the amount of ethanol produced overall.

4 Fermentation Efficiency and Process Integration: To optimize fermentation efficiency in industrial settings, variables including pH, temperature, nutrient delivery, and inhibitor removal must be carefully controlled. Integration of hydrolysis and fermentation, as in SSF, can improve process efficiency, but requires optimization of the microbial strains and enzymes used.

2.5.3 Advances in Lignocellulosic Fermentation

Genetically Modified microbes: Thanks to developments in metabolic engineering, microbes that can co-ferment C5 and C6 sugars have been created. To increase the yield of ethanol, yeasts such as S. cerevisiae have undergone genetic modification to enable them to ferment both glucose and xylose. In a similar vein, bacteria like *Z. mobilis* and *E. coli* have been modified to produce more ethanol and better utilize sugar.

Consolidated Bioprocessing (CBP): CBP uses designed microorganisms that can both break down lignocellulose and ferment the sugars that are left over. This allows CBP to combine the synthesis of enzymes, hydrolysis, and fermentation into a single process. This decreases costs and eliminates the requirement for additional enzyme addition, making the process more feasible commercially.

2.5.4 Fermentation Configurations: SHF vs SSF vs SSCF

In bioethanol production, choosing the appropriate fermentation configuration is essential for maximizing efficiency and yield. The three primary setups-Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), and Simultaneous Saccharification and Co-Fermentation (SSCF)-differ in how enzymatic hydrolysis and sugar fermentation are integrated. SHF allows optimal conditions for each step but requires more equipment and time. SSF simplifies processing by combining both steps in one reactor, though it requires temperature compromises. SSCF further enhances efficiency by co-fermenting hexose and pentose sugars simultaneously, improving sugar utilization and ethanol output, especially from lignocellulosic biomass.

2.5.4.3 Separate Hydrolysis and Fermentation (SHF)

Separate Hydrolysis and Fermentation (SHF) represents the traditional, sequential approach to converting complex carbohydrates, like those found in lignocellulosic biomass, into fermentable sugars and then into desired products. As the name suggests, this process is divided into two distinct stages, each typically carried out in separate reactors under optimized conditions.

Stage 1: Hydrolysis (Saccharification) In the initial stage, the raw material, often pretreated lignocellulosic biomass (e.g., agricultural residues, forestry waste), undergoes enzymatic hydrolysis. This involves using enzymes, primarily cellulases and hemicellulases, to break down complex polysaccharides (cellulose and hemicellulose) into their constituent monomeric sugars, such as glucose, xylose, and arabinose. This stage is usually performed at higher temperatures (e.g., 45-55°C) and a specific pH (e.g., pH 4.5-5.5) to maximize

enzyme activity and efficiency (Kaur et al., 2018).

Stage 2: Fermentation Once a sufficient concentration of fermentable sugars is achieved in the hydrolysate, the liquid is transferred to a separate fermentation vessel. Here, microorganisms (e.g., *Saccharomyces cerevisiae* for ethanol production) are introduced to convert these sugars into the target product. This stage typically operates at lower temperatures (e.g., 30-37°C) and a different pH (e.g., pH 4-5) which are optimal for microbial growth and product formation (Kaur et al., 2018).

Advantages of SHF:

- Optimized Conditions**:** The primary advantage of SHF lies in its ability to allow each step to operate under its ideal conditions. This means hydrolysis can proceed at temperatures and pH levels that maximize enzyme activity and sugar yield, while fermentation can occur at conditions optimal for the chosen microorganism, leading to higher fermentation efficiency and product titers (Koppram et al., 2013)
- Flexibility in Microorganism Selection: Since the hydrolysis and fermentation steps are physically separated, there is greater flexibility in choosing microorganisms that are highly efficient in sugar conversion but might not tolerate the higher temperatures or other conditions required for enzymatic hydrolysis.
- Reduced Enzyme Inhibition: The accumulation of sugars during hydrolysis can inhibit cellulase activity, a phenomenon known as product inhibition. In SHF, the sugars are produced and then moved for fermentation, potentially mitigating this inhibition during the hydrolysis phase.

Disadvantages of SHF:

- Higher Capital and Operational Costs: Operating two separate reactors, each with its own set of optimal conditions and control systems, increases the capital investment and operational costs. This includes the cost of heating/cooling, pH adjustment, and potential sterilization for both stages.
- Longer Process Time: The sequential nature of SHF inherently leads to a longer overall process time compared to integrated approaches.
- Risk of Contamination: The transfer of the sugar-rich hydrolysate between reactors can increase the risk of microbial contamination, requiring stringent sterilization procedures.
- Inhibitor Accumulation: Pretreatment of lignocellulosic biomass can generate inhibitory compounds (e.g., furans, phenols) that can negatively impact both enzymes during hydrolysis and microorganisms during fermentation. While SHF allows for some detoxification between stages, high concentrations can still be problematic.

• Industrial Applications: SHF has been a long-standing method, particularly in industries where raw material hydrolysis is a distinct and crucial step, such as in the production of glucose syrups from starch before fermentation for beverages or some biochemicals. However, for large-scale cellulosic ethanol production, its economic drawbacks often make it less favorable than more integrated approaches.

2.5.4.4 Simultaneous Saccharification and Fermentation (SSF)

Simultaneous Saccharification and Fermentation (SSF) is a more integrated approach that combines the enzymatic hydrolysis of complex carbohydrates and the fermentation of the resulting sugars into a single vessel. This means that enzymes and microorganisms are introduced concurrently into the reactor, and both processes occur simultaneously.

Mechanism: In SSF, as the enzymes break down the cellulose and hemicellulose into sugars, the fermenting microorganisms immediately consume these sugars. This continuous consumption of sugars by the microbes helps to alleviate product inhibition of the hydrolytic enzymes, thereby promoting a faster and more complete saccharification process.

Advantages of SSF:

- Reduced Capital and Operational Costs: By combining two stages into one reactor, SSF significantly reduces capital investment and operational costs associated with separate vessels, pumping, and heating/cooling systems. This integration also leads to a simpler overall process
- Reduced Product Inhibition: The immediate consumption of sugars by the microorganisms prevents the build-up of glucose, which is a potent inhibitor of cellulase enzymes. This can lead to higher overall hydrolysis rates and better enzyme efficiency.
- Lower Risk of Contamination: The presence of ethanol (or other fermentation products) and the lower sugar concentrations due to immediate consumption can create an environment less favorable for contaminants, thus reducing the risk of unwanted microbial growth.
- Shorter Process Time: The simultaneous nature of SSF generally results in a shorter overall processing time compared to SHF.

Disadvantages of SSF:

- Suboptimal Conditions: A major challenge in SSF is finding a compromise in operating conditions (temperature and pH) that are suitable for both the hydrolytic enzymes and the fermenting microorganisms. The optimal temperature for most

cellulases is typically higher (e.g., 45-55°C) than that for common industrial yeasts like *Saccharomyces cerevisiae* (e.g., 30-37°C). This often means one or both processes operate at suboptimal efficiency.

- Limited Microorganism Selection: The need for microorganisms that can tolerate the conditions required for enzymatic hydrolysis (e.g., higher temperatures, presence of inhibitors) can limit the choice of suitable strains. This often necessitates the use of thermotolerant or engineered microorganisms.
- Challenging Process Control: Controlling and optimizing two concurrent biological processes within a single reactor can be more complex than managing them separately.

- Industrial Applications: SSF is widely explored and implemented for the production of biofuels, especially bioethanol from lignocellulosic biomass, due to its economic advantages. It is a more established technology than some newer configurations and has seen commercial deployment.

2.5.5.3. Simultaneous Saccharification and Co-fermentation (SSCF)

Simultaneous Saccharification and Co-fermentation (SSCF) is an advanced modification of the SSF concept, specifically designed to address the challenge of utilizing all available sugars in lignocellulosic biomass. While SSF primarily focuses on the fermentation of hexose sugars (like glucose), lignocellulosic biomass also contains significant amounts of pentose sugars (like xylose and arabinose) derived from hemicellulose. Most traditional fermenting organisms, such as *Saccharomyces cerevisiae*, cannot efficiently ferment these pentose sugars.

Mechanism: SSCF involves the simultaneous enzymatic hydrolysis of cellulose and hemicellulose into both hexose and pentose sugars, followed by the concurrent fermentation of *both* types of sugars by specialized or engineered microorganisms. This means the chosen microbial strain or consortium must possess the metabolic pathways to efficiently convert both glucose and xylose/arabinose into the desired product. Pretreatment strategies for SSCF are often designed to retain hemicellulose in the solid phase for co-processing.

Advantages of SSCF:

- Improved Biomass Utilization: The most significant advantage of SSCF is its ability to utilize a broader spectrum of sugars present in the biomass, including both hexoses and pentoses. This leads to higher overall product yields per unit of biomass, making the process more resource-efficient and economically attractive.

- Enhanced Productivity and Yield: By converting more of the available carbon sources, SSCF can achieve higher titers and productivity of the target product compared to SSF, which might leave pentose sugars unutilized.
- Reduced Product Inhibition (Similar to SSF): Like SSF, the continuous consumption of sugars prevents their accumulation and the associated product inhibition of hydrolytic enzymes.

Disadvantages of SSCF:

- Requirement for Specialized Microorganisms: The major bottleneck for SSCF is the need for robust microorganisms capable of efficiently co-fermenting both hexose and pentose sugars. Naturally occurring organisms with this capability are often less efficient or robust than traditional yeasts, and genetically modified organisms (GMOs) are frequently required. The challenge lies in avoiding preferential hexose consumption (catabolite repression) and ensuring efficient pentose fermentation.
- More Complex Process Control: Managing the metabolic activity of microorganisms that are simultaneously fermenting multiple sugar types, while also balancing enzyme activity, adds a layer of complexity to process control and optimization.
- Potential for Inhibitor Sensitivity: The specialized microorganisms used in SSCF might be more sensitive to inhibitory compounds generated during biomass pretreatment, which can negatively impact their fermentation performance.
- Sterilization Needs: SSCF processes generally require sterile conditions to prevent contamination by other microorganisms that could compete for sugars or produce unwanted byproducts.

Industrial Applications: SSCF is a highly promising configuration for the production of advanced biofuels (e.g., cellulosic ethanol) and other bio-based chemicals from lignocellulosic feedstocks. While still facing challenges in large-scale implementation, ongoing research and development are focused on engineering more efficient and robust co-fermenting microorganisms and optimizing process conditions.

The choice among SHF, SSF, and SSCF fermentation configurations depends on a multitude of factors, including the type of feedstock, the desired product, economic considerations, and the availability of suitable enzymes and microorganisms. SHF, with its sequential nature, offers independent optimization but at higher costs. SSF integrates hydrolysis and fermentation, reducing costs and mitigating enzyme inhibition, but demands a compromise in optimal conditions. SSCF builds upon SSF by enabling the

utilization of all major sugars (both hexoses and pentoses) in biomass, leading to higher yields but requiring specialized or engineered microorganisms and more complex process control.

Recent advances in fermentation technology, including predictive microbiology models, high- throughput screening for microbial strains, advanced bioreactor designs, and the application of artificial intelligence and machine learning, are continuously pushing the boundaries of all these configurations. As the demand for sustainable and cost-effective bioproducts grows, further innovation in fermentation configurations and microbial engineering will undoubtedly play a critical role in realizing the full potential of bio-based industries. The trend is clearly towards more integrated and efficient processes that maximize substrate utilization and minimize operational complexities, with SSCF representing a significant step in this direction for comprehensive biomass valorization.

Table 8: Comparative Summary of SHF, SSF, and SSCF Fermentation Configurations in Bioethanol Production.

Configuration	Temperature	Integration	Yield	Strengths	Trade-Offs
SHF	50 °C / 30 °C	Separate	High	Optimal control	High capital & inhibition risks
SSF	37-39 °C	Integrated	70–90%	Low cost, reduced inhibition	Temperature compromise
SSCF	37-39 °C	Integrated & co-fermentation	~75%	Uses both sugars	Requires engineered strains

2.5.5 Inhibitor Tolerance and Detoxification

During the pretreatment of lignocellulosic biomass, harsh chemical or thermal conditions break down structural components like cellulose, hemicellulose, and lignin. While this process releases fermentable sugars, it also generates a range of toxic byproducts, including furfural, hydroxymethylfurfural (HMF), acetic acid, and various phenolic compounds such as vanillin. These inhibitors originate from the degradation of pentoses and hexoses (furfural and HMF), the deacetylation of hemicellulose (acetic acid), and the breakdown of lignin (phenolics). Once released into the hydrolysate, these compounds negatively affect yeast metabolism by disrupting membrane integrity, damaging enzymes, and impairing DNA and protein synthesis. As a result, ethanol yield and fermentation rates are significantly reduced. Additionally, enzymes used during hydrolysis can be deactivated by these inhibitors, lowering sugar conversion efficiency. Therefore, mitigating the impact of

these compounds is essential for ensuring robust microbial performance and maintaining the economic viability of lignocellulosic bioethanol production. (Yang et al., 2018).

2.5.6.1. Common Inhibitor Types

Pretreatment of lignocellulosic biomass, particularly using acid hydrolysis or steam explosion, frequently leads to the formation of microbial inhibitors that pose significant challenges during fermentation. Furfural and hydroxymethylfurfural (HMF) are two of the most common inhibitors. Furfural forms from the dehydration of pentose sugars, while HMF originates from hexose sugars under high-temperature, acidic conditions. Both compounds are highly toxic to fermenting microorganisms. They inhibit glycolytic enzymes, damage nucleic acids, and disrupt metabolic pathways, leading to slower fermentation, prolonged lag phases, and reduced ethanol yields. (Senatham et al., 2016)

i) Acetic acid is released during the deacetylation of hemicellulose, particularly xylan. Under low pH conditions typical of fermentation, acetic acid exists in its undissociated form, which diffuses into yeast cells and dissociates internally, lowering cytoplasmic pH. This stresses microbial metabolism, drains ATP as cells try to restore pH homeostasis, and slows ethanol production.

ii) Phenolic compounds, such as vanillin, syringaldehyde, and p-coumaric acid, are byproducts of lignin degradation. These molecules interact with microbial membranes and proteins, causing oxidative stress and damaging key cellular structures. Their hydrophobic nature makes them particularly disruptive to membrane integrity and function, compounding stress in already vulnerable cells. Together, these inhibitors reduce both the rate and extent of sugar conversion to ethanol, making their mitigation a critical step in the lignocellulosic ethanol process.

iii) Detoxification Methods: Detoxification is a critical step in preparing lignocellulosic hydrolysates for fermentation, as untreated biomass slurries often contain inhibitors like furfural, HMF, acetic acid, and phenolic compounds. Several physical and chemical methods have been developed to reduce these inhibitors, but each comes with trade-offs in terms of efficiency, cost, and sugar preservation.

iv) Over liming is one of the most widely used methods. It involves raising the pH of the hydrolysate using lime ($Ca(OH)_2$) to around 10–11, then allowing the solution to settle before readjusting the pH back to optimal fermentation levels. This high pH environment precipitates and neutralizes inhibitors such as furans and phenolics, significantly improving fermentation performance. However, overliming can lead to sugar degradation or loss, especially if exposure to high pH is prolonged. It also introduces additional steps and

increases processing time and costs.(Olofsson et al., 2008).

v) Activated carbon offers a more selective approach. Its high surface area and porosity allow it to adsorb a wide range of inhibitory compounds while leaving most fermentable sugars intact. This makes it effective in removing furans and phenolics. Nonetheless, it introduces additional material costs, and removing spent carbon from the slurry can complicate downstream processing.

vi) Evaporation, particularly under vacuum conditions, is another viable method. It targets volatile inhibitors such as furfural and acetic acid, which can be vaporized and condensed out of the hydrolysate. This method typically preserves sugars well, but its energy requirements are high, and it is less effective against non-volatile inhibitors like phenolics.

While these methods can significantly improve fermentation outcomes, they often add operational complexity and cost. Thus, balancing inhibitor removal with sugar preservation and economic feasibility is crucial in designing scalable bioethanol processes.

2.5.7 Inhibitor-Tolerant Yeast Strains

One of the most effective and sustainable solutions to counteract fermentation inhibitors in lignocellulosic bioethanol production is the use of inhibitor-tolerant yeast strains. These strains are either naturally resistant or have been enhanced to withstand toxic compounds such as furfural, hydroxymethylfurfural (HMF), acetic acid, and phenolic derivatives. Several strategies are used to develop such tolerance. Adaptive Laboratory Evolution (ALE) exposes yeast to gradually increasing inhibitor concentrations, selecting for robust variants. Genetic engineering enables targeted overexpression of detoxifying genes. Physiological pre-conditioning primes cells to resist toxins through controlled pre-exposure, while omics-guided engineering helps identify and modify genes critical to inhibitor response. These approaches improve ethanol yield and process stability, reducing the need for costly detoxification steps.

1. Adaptive Laboratory Evolution (ALE)

Adaptive Laboratory Evolution (ALE) is a powerful method for enhancing microbial tolerance to fermentation inhibitors. It involves repeatedly culturing yeast or bacteria in gradually increasing concentrations of toxic compounds such as furfural, HMF, and acetic acid, allowing only the most resilient mutants to survive and proliferate. Over time, this selection pressure drives the accumulation of beneficial genetic mutations that confer increased tolerance and stability under stress. (Nitiyon et al., 2016) (Yao et al., 2023)

For example, *Kluyveromyces marxianus* evolved through ALE to tolerate both inhibitor-rich hydrolysates and elevated temperatures of 42 °C. The evolved strain exhibited a 3.3-fold faster growth rate and nearly 80% higher ethanol productivity than its non-adapted parent, highlighting ALE's effectiveness.

Similarly, Zymomonas mobilis subjected to ALE in media containing furfural and acetic acid evolved into strains with significantly improved fermentation capacity, even in the presence of these inhibitors. ALE-derived strains often retain their enhanced phenotypes over time, making them suitable for repeated or long-term industrial use.

Because ALE mimics natural evolutionary processes without requiring genetic engineering, it is relatively easy to implement and scale up, especially for industrial fermentation setups that deal with minimally detoxified lignocellulosic biomass.

2. Genetic and Regulatory Engineering

Genetic and regulatory engineering offers a precise and effective approach to improving yeast tolerance to common lignocellulosic fermentation inhibitors such as furfural, HMF, and acetic acid. This strategy involves the targeted overexpression or modification of specific genes that play a role in detoxification, stress response, or redox balance.

One notable example is the overexpression of ARI1, a gene encoding an aldehyde reductase. When overexpressed in *S. cerevisiae*, ARI1 accelerates the conversion of furfural into less toxic alcohol derivatives, significantly reducing its inhibitory effects. This enhances yeast growth and ethanol productivity even in furfural-rich media. (Opaliński et al., 2018).

Other genes, such as PRO1 and INO1, are involved in the biosynthesis of proline and myo- inositol, respectively-molecules that function as antioxidants and osmoprotectants. Their overexpression helps mitigate oxidative stress and maintain cellular homeostasis in the presence of toxins like HMF and phenolics (X. Wang et al., 2015)

Additionally, the global regulatory gene IrrE, originally identified in *Deinococcus radiodurans*, has shown promise when expressed in *S. cerevisiae*. This transcription factor can upregulate multiple stress-response pathways, resulting in broad-spectrum resistance to furfural and other inhibitors. (Patel et al. 2021), (Ren et al., 2024).

This multifaceted approach-combining specific detoxification genes with broader stress regulators-has proven effective in enhancing yeast resilience. Genetically engineered strains with these traits offer more stable and efficient fermentation performance, especially in minimally detoxified lignocellulosic hydrolysates.

3. Physiological Pre-adaptation

Physiological pre-adaptation is a simple yet effective method to enhance yeast tolerance to fermentation inhibitors. This technique involves pre-conditioning yeast by either growing them to the early stationary phase or exposing them to low concentrations of inhibitors such as furfural, HMF, or acetic acid before actual fermentation. Such mild stress exposure stimulates cellular defense mechanisms, including the induction of detoxification enzymes, heat shock proteins, and repair pathways, which help the cells cope more efficiently when later exposed to higher toxin levels.

Research has shown that pre-adapted *Saccharomyces cerevisiae* cells enter the fermentation phase with significantly reduced lag time and enhanced survival, resulting in improved sugar consumption rates and higher ethanol productivity in inhibitor-rich hydrolysates. (X. Wang et al., 2015) These cells are metabolically more active and maintain better membrane integrity under stress.

Since this approach does not require genetic modification or additional chemicals, it is cost- effective and easy to implement at both lab and industrial scales. It provides a practical bridge between strain engineering and detoxification, enhancing overall fermentation robustness.

4. Omics-Guided Engineering

Omics-guided engineering is a cutting-edge approach that utilizes systems biology tools such as transcriptomics, proteomics, and metabolomics to identify molecular targets involved in stress responses. These high-throughput methods allow researchers to study how yeast and other fermenting organisms react at a cellular level when exposed to common lignocellulosic inhibitors like furfural, HMF, acetic acid, and formate.

For example, transcriptomic studies have shown that genes like ADH6, which encode aldehyde dehydrogenase, are consistently upregulated in the presence of furfural and HMF. This enzyme helps convert these toxic aldehydes into less harmful alcohols, reducing their negative impact on yeast metabolism. Similarly, TKL1, a key enzyme in the pentose phosphate pathway, supports the generation of NADPH, a crucial molecule for maintaining redox balance and driving antioxidant defenses. FDH1, which encodes formate dehydrogenase, plays a vital role in metabolizing formate into CO_2, thereby neutralizing its inhibitory effects.

The integration of these findings enables rational strain engineering, where multiple genes associated with tolerance can be simultaneously overexpressed or regulated. This multi-target approach is far more effective than modifying a single gene, as it enhances the

overall robustness and adaptability of the microorganism.

Ultimately, omics-guided engineering facilitates the design of industrial yeast strains capable of thriving in minimally detoxified or crude hydrolysates. These engineered strains not only tolerate high concentrations of inhibitors but also maintain high ethanol yields, making the process more efficient, reliable, and cost-effective for large-scale bioethanol production.

Table 9: Summary of Strategies for Enhancing Yeast Tolerance to Fermentation Inhibitors

Strategy	Mechanism	Advantages
ALE	Adaptive mutations via long- term culture	Broad-spectrum tolerance, stable traits, and low cost
Genetic engineering	Overexpression of detoxifying enzymes and regulators	Targeted resilience against specific inhibitors
Pre-conditioning	Culturing cells to induce detoxification pathways	Reduced lag phase, practical to implement pre-fermentation
Omics-guided design	Integration of multiple pathways informed by global profiling	Holistic, data-driven strain improvement

In short, developing yeast strains that can tolerate fermentation inhibitors-through ALE, gene engineering, pre-conditioning, or omics-guided techniques-is essential to processing minimally detoxified biomass. Such strains make the fermentation phase more robust, economically viable, and suitable for industrial-scale bioethanol production.

2.5.8 Process Parameter Optimization

Optimizing key process parameters is critical for the success of Simultaneous Saccharification and Fermentation (SSF) and Simultaneous Saccharification and Co-Fermentation (SSCF) systems. Parameters such as temperature, pH, oxygen availability, fermentation time, and ethanol concentration thresholds directly impact the efficiency of enzymatic hydrolysis and microbial fermentation. For instance, maintaining a temperature range of 37–39 °C helps balance enzyme performance with yeast viability, while pH between 4.5–5.0 ensures enzyme stability and yeast growth. Controlled micro-aeration can enhance cofactor regeneration, and a well-chosen fermentation duration

(typically 36–48 hours) ensures maximum sugar-to- ethanol conversion. Achieving ≥40 g/L ethanol is also crucial for economically viable downstream recovery.

i) Temperature: Temperature plays a critical role in the success of SSF and SSCF processes, as it directly affects both enzyme activity and microbial fermentation performance. In traditional enzymatic hydrolysis, cellulases work most efficiently at around 50 °C, while common fermenting microbes like *Saccharomyces cerevisiae* prefer lower temperatures, typically between 30–35 °C. In SSF and SSCF, where both hydrolysis and fermentation occur simultaneously, a compromise temperature is required-usually in the range of 37–39 °C.

ii)This temperature window has been widely studied and found to offer the best balance between enzyme function and microbial viability. For instance, operating at 38 °C has been shown to result in higher ethanol titers in fed-batch SSF systems. This moderate elevation from typical yeast temperatures is enough to sustain reasonable enzyme activity without severely compromising yeast health or ethanol production efficiency (Z. H. Liu et al., 2014)

iii) If the temperature drops too low, enzyme activity slows, limiting sugar release. Conversely, exceeding 39–40 °C risks thermal stress on yeast, reducing viability and fermentation rate. Therefore, the 37–39 °C range represents an operational sweet spot that maximizes sugar conversion and ethanol output in a single, integrated process, making it ideal for industrial- scale SSF and SSCF configurations.

i) pH Control and Oxygen: In SSF and SSCF processes, maintaining an optimal pH range of 4.5 to 5.0 is essential for efficient bioethanol production. This range is favorable for both cellulolytic enzymes, which require slightly acidic conditions to function effectively, and yeast strains like *Saccharomyces cerevisiae*, which show stable growth and fermentation performance within this window. Deviations from this range can be harmful-too low pH can denature enzymes, while too high pH can stress microbial cells, leading to reduced ethanol yields.

ii)Oxygen availability also influences fermentation dynamics. While ethanol fermentation is typically anaerobic, introducing micro-aeration-small, controlled amounts of oxygen-can enhance yeast performance by supporting the regeneration of essential cofactors like NAD^+, particularly important when fermenting pentoses in SSCF. However, excessive oxygen can shift yeast metabolism from ethanol production to biomass growth, lowering product yield. Thus, careful pH and oxygen control are vital for maximizing both sugar conversion and ethanol output.

iii) Fermentation Time: The duration of fermentation is a crucial parameter in SSF and

SSCF processes, directly affecting ethanol yield and overall process efficiency. For systems operating at 10–20% solid loading, the typical fermentation time to achieve maximum ethanol concentration ranges from 36 to 48 hours. Within this time frame, both enzymatic saccharification and microbial conversion of sugars to ethanol occur concurrently.

To enhance efficiency, many setups incorporate a pre-hydrolysis phase of approximately 24 hours before introducing the fermenting microorganism. During this stage, enzymes act on the lignocellulosic biomass without microbial interference, breaking down complex carbohydrates into fermentable sugars. This step helps reduce viscosity, improve mass transfer, and increase the availability of glucose and xylose for fermentation. Including pre-hydrolysis has been shown to significantly improve both ethanol titers and sugar conversion rates in the subsequent fermentation phase. (Z. H. Liu et al., 2014) When properly timed, it shortens the overall fermentation cycle and leads to a more efficient and productive bioethanol process.

2.5.9 Inoculum Density and Ethanol Recovery Thresholds

In SSF and SSCF processes, selecting an appropriate yeast inoculum density is critical for efficient fermentation. A commonly used benchmark is an optical density (OD_{600}) of around 4.0, which translates to a sufficient cell concentration to initiate rapid sugar conversion. This level ensures that yeast quickly dominates the system, reducing the risk of contamination and minimizing the lag phase. Importantly, it also avoids excessive biomass formation, which could lead to non-productive sugar consumption. If too many yeast cells are present, a greater portion of the available sugars may be directed toward cell growth instead of ethanol production, lowering overall yield.

From a downstream processing perspective, achieving a sufficient ethanol concentration is vital for cost-effective recovery. Industrial distillation becomes economically viable only when ethanol concentrations reach at least 4% w/w (about 40 g/L). Below this threshold, the energy required to separate ethanol from water exceeds the value of the ethanol recovered. Optimized SSF systems, particularly those incorporating pre-hydrolysis and surfactants, have been shown to routinely achieve ethanol concentrations in the 40–60 g/L range within 36–48 hours of fermentation. (Olofsson et al., 2008)

Achieving both the right inoculum density and ethanol titer is therefore essential for ensuring both technical efficiency and economic feasibility in lignocellulosic bioethanol production. These parameters are especially important at high solid loadings where fermentation inhibition and viscosity pose additional challenges.

2.5.10 Ethanol Recovery Thresholds

In industrial bioethanol production, reaching a minimum ethanol concentration of ≥4% w/w (approximately 40 g/L) is crucial for economical distillation. Below this threshold, the energy required to separate ethanol from water becomes too high, making the process financially unviable. Therefore, fermentation strategies must be designed to consistently achieve or exceed this level.

Optimized Simultaneous Saccharification and Fermentation (SSF) systems, especially those employing pre-hydrolysis, high-solid loadings, and surfactant additives, have demonstrated the ability to produce ethanol concentrations between 40 and 60 g/L within 36–48 hours of fermentation. (Olofsson et al., 2008). These yields not only support efficient distillation but also indicate high sugar conversion efficiency and robust microbial performance. Achieving this threshold is a key metric of process success and directly impacts the scalability and profitability of lignocellulosic ethanol production.

Table 10: Optimized Process Parameters for SSF and SSCF in Lignocellulosic Ethanol Production

Parameter	Optimized Range/Condition	Purpose/Impact
Temperature	37–39 °C	Balances enzyme activity (optimal ~50 °C) with yeast fermentation needs (~30 °C)
pH	4.5–5.0	Supports enzyme stability and microbial performance
Oxygen Supply	Micro-aerobic (limited aeration)	Enhances redox balance for cofactor regeneration without triggering biomass growth
Pre-hydrolysis	~24 hours at 50 °C before SSF	Improves saccharification and sugar availability before fermentation
Fermentation Time	36–48 hours	Allows complete sugar conversion and ethanol accumulation
Inoculum Density	OD_{600} ~4.0	Ensures rapid yeast dominance and efficient fermentation
Ethanol Yield Goal	≥40 g/L (4% w/w)	Minimum concentration for cost- effective distillation and industrial viability

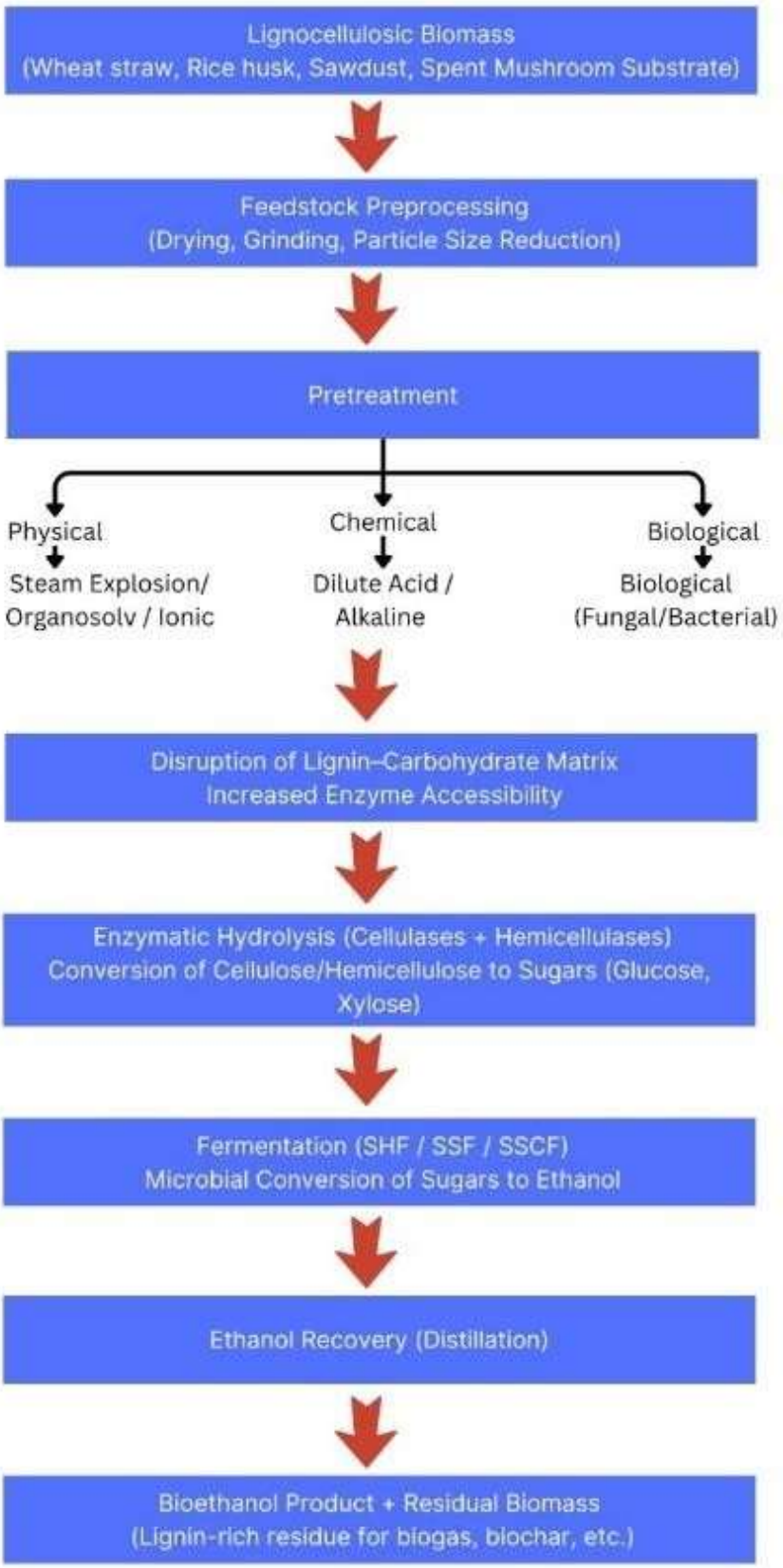

Fig 5: Bioconversion of Lignocellulosic Biomass to Bioethanol

Each experiment described in the following sections was performed three times to ensure accuracy, reproducibility, and statistical reliability. Standard laboratory procedures were adhered to for sample preparation, analysis, and data gathering. Suitable controls were upheld throughout the research to confirm the findings. Unless stated otherwise, all reagents and chemicals utilized were of analytical quality, and all experiments were conducted in sterile environments to avoid contamination.

3.1 Substrate Selection

In this study, lignocellulosic biomass used for bioethanol production includes wheat straw, spent mushroom substrate (SMS), sawdust, rice husk, and rice straw, all of which were processed using distinct methods.

3.1.1 Wheat Straw Processing

For the present study, wheat straw was selected as one of the primary lignocellulosic biomass sources due to its abundance in agricultural regions and its well-documented composition of cellulose, hemicellulose, and lignin. The straw was collected from nearby farms after the harvesting season in local agricultural fields, ensuring that the material was fresh and representative of typical post-harvest residues. The proximity of the fields to the research facility helped in minimizing the degradation or contamination of the straw during transportation and storage.

Upon collection, the wheat straw was manually sorted and thoroughly cleaned. This step was necessary to remove unwanted extraneous matter such as soil particles, dried leaves, leftover grains, dust, and other field debris that might have clung to the straw during harvesting. The cleaning process involved shaking, brushing, and occasionally wiping off visible contaminants to obtain a relatively clean and consistent batch of straw. This preparatory step is crucial, as the presence of impurities can interfere with experimental accuracy and potentially introduce variables not intended for analysis.

Once the cleaning was completed, the straw was subjected to mechanical grinding using a laboratory-scale mixer grinder. The grinding process was carefully controlled to reduce the straw to a consistent particle size of approximately 0.82 millimeters. This specific size was chosen deliberately based on previous studies and preliminary trials, as it facilitates better interaction with chemicals or microbial agents during further pretreatment or fermentation steps. A uniform particle size ensures reproducibility and consistency across all experimental runs, which is essential for maintaining the reliability

of results.

Additionally, the fibrous texture and internal structure of the ground wheat straw were observed and recorded in detail. Particular attention was given to the length, surface texture, and fragmentation patterns of the fibers, which are known to play a significant role in determining how effectively the straw interacts with other substances in a composite or bio- conversion matrix. These structural characteristics were documented to gain a deeper understanding of the wheat straw's behavior when used in various biotechnological or bioenergy processes.

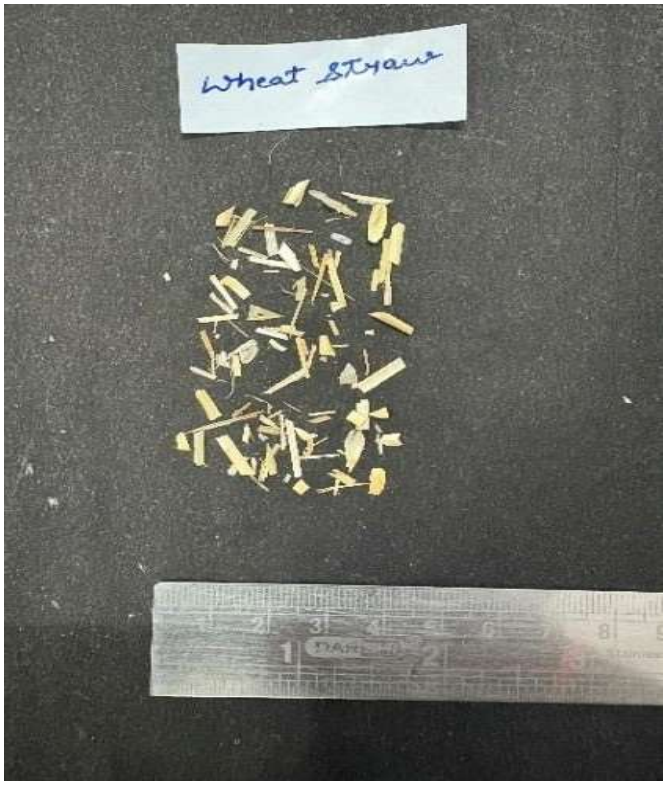

Fig 6: Particle size of Wheat Straw

The processed wheat straw was then stored in clean, moisture-free containers under ambient laboratory conditions to preserve its quality until further use. No chemical treatment or modification was carried out at this stage, as the focus was on evaluating the material in its most natural and untreated state, following standard physical preparation protocols.

3.1.2 Spent Mushroom Substrate (SMS) Processing

Spent Mushroom Substrate (SMS) was obtained as a byproduct from in-house mushroom cultivation trials conducted under controlled laboratory conditions. Oyster mushrooms (*Pleurotus ostreatus*) were grown on wheat straw, which served as the primary substrate.

The cultivation process was carried out in sterile trays under monitored environmental conditions to ensure uniform fungal growth and substrate degradation.

Fig. 7: Particle size of SMS

Following the completion of the fruiting phase, the remaining biomass-comprising partially decomposed wheat straw interwoven with fungal mycelium-was collected as SMS. To prepare it for experimental use, the SMS was first air-dried at room temperature to reduce residual moisture. It was then manually broken down into smaller fragments to achieve a relatively uniform texture. No chemical pretreatment or additional processing was performed at this stage, and the material was stored in sealed containers until further use.

3.1.3 Sawdust

The sawdust used in this study was collected from a local sawmill situated in the Jhokanbagh area of Jhansi, Uttar Pradesh. Since sawdust is a by-product generated during the cutting and processing of wood, it was already in a finely powdered form when collected. This made it unnecessary to grind or mechanically reduce its size further. However, to ensure the quality and cleanliness of the material before its application in the experiments, a few basic preparation steps were carried out.

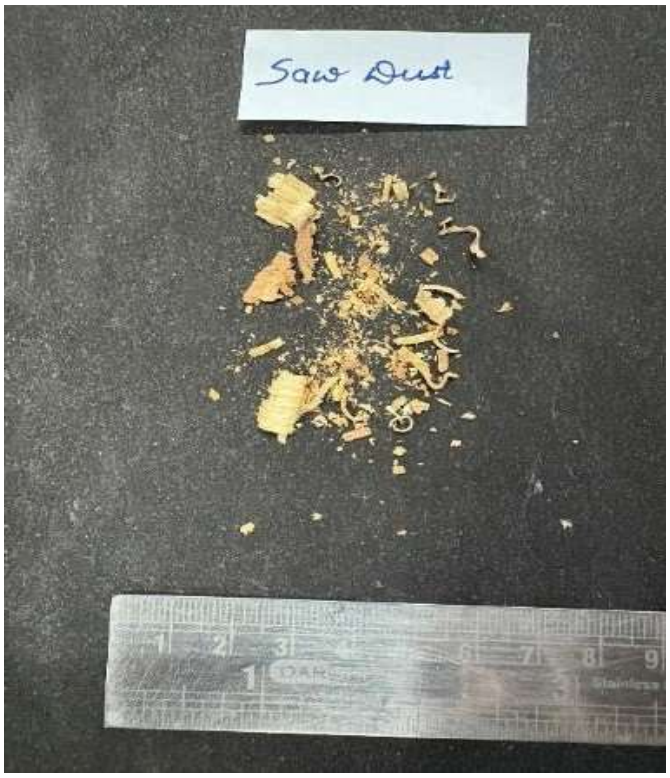

Fig. 8: Particle size of Saw Dust

Firstly, the sawdust was passed through a fine mesh sieve to remove any noticeable physical contaminants such as small pieces of bark, wood chips, or foreign debris that may have been unintentionally mixed during the sawing process. This sieving ensured a more uniform texture and composition. After sieving, the material was spread out on clean trays and left to air-dry under ambient conditions. This air-drying process helped in reducing the natural moisture content of the sawdust, which is essential for accurate and consistent experimental results. No chemical treatment, mechanical alteration, or further processing was conducted on the sawdust. It was used in its natural, untreated form as a lignocellulosic substrate for the study.

3.1.4 Rice Straw

The rice straw used for experimental purposes was gathered from agricultural fields in the vicinity, immediately after the harvesting of rice crops. These fields are located near the same region, which helped in maintaining consistency in sample sourcing. Rice straw, which consists of the dried stalks left behind once the grain has been harvested, is one of the most abundantly available agricultural residues in India and holds significant potential for bioresource conversion.

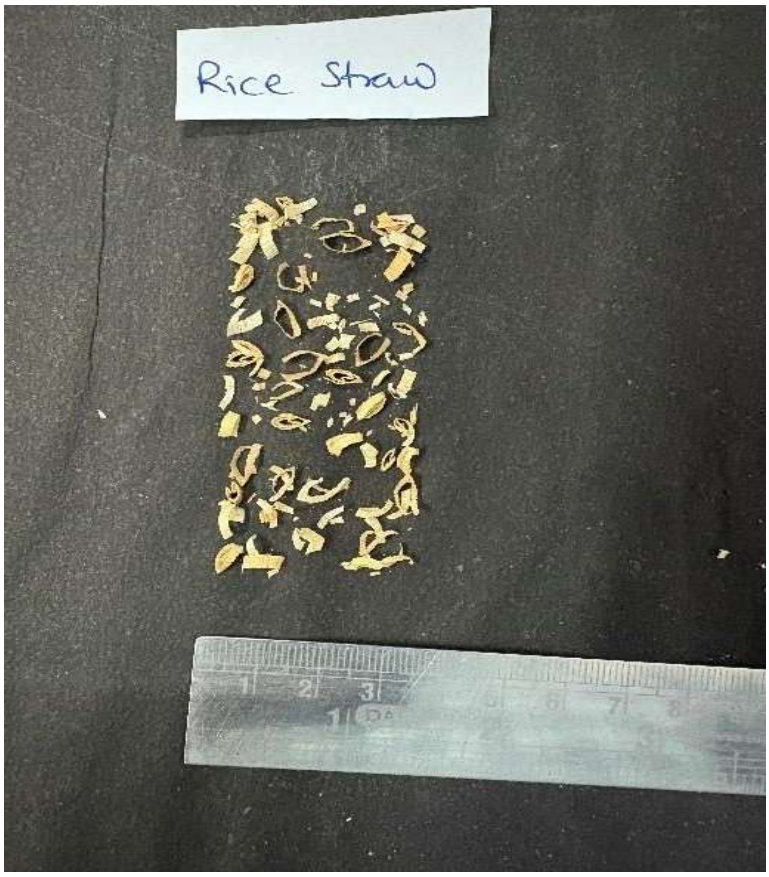

Fig.9: Particle size of Rice straw

Before using it in the laboratory, the collected rice straw was manually cleaned to remove any dirt, soil particles, and other unwanted materials such as dried leaves, weed fragments, or broken grain husks that might have been mixed in during field collection. This cleaning was done carefully by hand to maintain the structural integrity of the straw while ensuring it was free from contaminants that could affect the accuracy of results.

Once cleaned, the straw was dried under natural conditions and then processed using a laboratory-grade grinder. This grinding step reduced the straw to a uniform particle size of about 0.82 millimeters. The chosen particle size allowed for better mixing and handling during experimental procedures and ensured that all samples were treated under uniform physical conditions. This level of fineness is also ideal for improving the efficiency of chemical and biological treatments applied in later stages of processing.

3.1.5 Rice Husk

Rice husk, which is the hard outer layer that encases individual rice grains, was selected as another lignocellulosic material for this study due to its high availability and potential as a bioresource. The rice husk samples were sourced from a rice processing mill located in Bada Bazar, a region in Jhansi, India known for its agricultural and milling activity. Procuring the material from this region helped maintain a steady and reliable source of

husk in sufficient quantities.

Upon collection, the rice husk was visually inspected and then sieved to remove coarse particles, pebbles, broken grains, and other contaminants that could interfere with the experimental process. Sieving also helped in obtaining a more homogenous sample suitable for repeatable lab trials. After sieving, the rice husk was spread out in open trays and air-dried under shaded but well-ventilated conditions. This drying process helped to reduce its inherent moisture content, making it more stable and suitable for storage and experimental handling.

No chemical treatment or grinding was applied to the rice husk in its preparation. It was used in its natural state following the above cleaning and drying steps, making it representative of the material's real-world application potential.

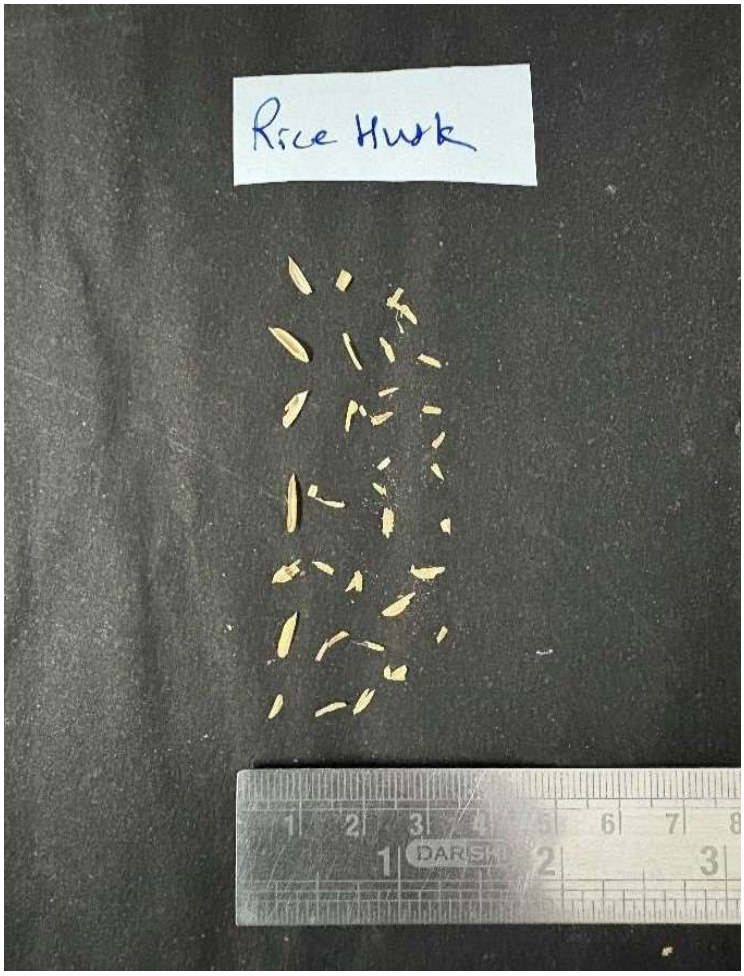

Fig.10: Particle size of Rice Husk

3.2 Oyster Mushroom Cultivation Using Wheat Straw as a Substrate

The cultivation of oyster mushrooms (*Pleurotus ostreatus*) was carried out using wheat straw as the primary lignocellulosic substrate due to its wide availability, cost-effectiveness, and proven suitability for mushroom growth. The process involved a series of carefully controlled steps aimed at optimizing mycelial colonization, minimizing microbial contamination, and achieving consistent mushroom yields under laboratory conditions.

Initially, wheat straw was chopped into smaller segments approximately 2–4 cm in length. This size reduction was done manually and served to increase the surface area accessible to the fungal mycelium, thereby facilitating more efficient colonization. The chopped straw was then subjected to chemical pretreatment using Bavistin, a commercially available systemic fungicide (active ingredient: carbendazim). This treatment was performed by soaking the straw in a Bavistin solution for a specific duration to inhibit the growth of competing molds and other unwanted microorganisms, which often pose a challenge during the early colonization phase.

After chemical treatment, the straw was thoroughly rinsed with distilled water and subsequently sterilized through autoclaving. The autoclaving process was conducted at 121 °C under 15 psi pressure for approximately 25 minutes. This step ensured the complete elimination of microbial contaminants that could hinder or outcompete the growth of the target mushroom species. Additionally, autoclaving helped to soften the fibrous structure of the straw, making it more amenable to enzymatic degradation by fungal enzymes during colonization**.** (Mahari et al. 2020)

Once sterilized, the straw was allowed to cool down to ambient temperature under sterile conditions to avoid thermal shock to the mushroom spawn and to prevent post-sterilization contamination. After cooling, excess moisture was drained from the straw to maintain an optimal moisture content-neither too dry to inhibit fungal growth nor too wet to encourage bacterial proliferation.

In a sterile environment (typically within a laminar airflow cabinet), the prepared straw was inoculated with oyster mushroom spawn at a rate sufficient to ensure even colonization. The spawn was thoroughly mixed into the substrate and the inoculated mixture was then packed into perforated polypropylene bags or containers. The perforations facilitated air exchange, which is critical for aerobic fungal growth during the spawn run.

The packed bags were placed in a dark incubation chamber maintained at a temperature range of 20–24 °C with relative humidity above 80%. This incubation period lasted for approximately 15 to 21 days, during which the fungal mycelium spread rapidly and colonized the entire substrate. The progress of colonization was visually monitored through the transparent bags, and fully colonized blocks appeared white and compact due to dense mycelial growth.

Following successful colonization, the bags were transferred to a fruiting chamber, where environmental parameters were adjusted to trigger the formation of fruiting bodies. Conditions in the fruiting chamber were modified to 15–20 °C with increased light exposure (natural or artificial indirect light) and high relative humidity of 85–95%. Regular misting or humidification was carried out to maintain consistent moisture levels.

Under these favorable conditions, pinheads (initial mushroom formations) began to appear within a few days, followed by the development of mature oyster mushrooms. The mushrooms were harvested at the appropriate stage of development, typically when the caps were fully expanded but before spore release. Multiple flushes (harvest cycles) were obtained from the same substrate over the following weeks, making the process both productive and resource- efficient.

This method ensured reliable and reproducible oyster mushroom production under controlled laboratory conditions, allowing for the consistent generation of spent mushroom substrate (SMS) for further experimental use in this study**.** (Girmay et al. 2016).

3.2.1 Utilization of Spent Mushroom Substrate for Bioethanol Production

Following the final harvest of mushrooms, the leftover organic residue, known as spent mushroom substrate (SMS), was gathered and utilized for bioethanol production**.** Since SMS consists of partially decomposed lignocellulosic material, it presents an attractive feedstock for enzymatic hydrolysis and fermentation. The timing of SMS collection was crucial to preserving its chemical composition and ensuring its effectiveness as a bioethanol substrate.

Fig 11: Oyster Mushroom

3.2.2.1 Justification for Substrate Selection

Both wheat straw and SMS were chosen based on several factors, including abundant availability, low cost, and environmental benefits. These feedstocks offer a cost-effective and sustainable approach to bioethanol production while promoting efficient waste valorization. A comprehensive analysis of both materials was conducted to evaluate their suitability for bioethanol production, focusing on physical properties, biochemical composition, and overall potential to enhance biofuel yield.

3.3. Substrate Screening and Selection Criteria

In order to identify the most suitable lignocellulosic feedstocks for bioethanol production, five different substrates-namely wheat straw, spent mushroom substrate (SMS), sawdust, rice husk, and rice straw-were initially subjected to a uniform chemical pretreatment process. The primary goal of this pretreatment was to disrupt the rigid lignocellulosic structure of the materials, allowing for greater accessibility of cellulose and hemicellulose components, which are essential for downstream enzymatic hydrolysis and fermentation.

Each substrate was pre-weighed to maintain consistency across samples. Precisely 10 grams (on a dry weight basis) of each material were taken and soaked in 100 mL of distilled water to form a semi-liquid slurry. This initial soaking helped in softening the fibers and facilitating even penetration of the pretreatment chemicals throughout the biomass matrix.

To initiate the oxidative delignification process, hydrogen peroxide (H_2O_2) was added to the slurry at a concentration of 2.5% (v/v). Hydrogen peroxide acts as an effective oxidative agent, capable of breaking down lignin-the complex aromatic polymer that encases cellulose and hemicellulose-thereby increasing the exposure of fermentable sugars in the substrate. The use of hydrogen peroxide is also considered a relatively eco-friendly and mild pretreatment strategy compared to strong acids or harsh alkalis.

Next, the pH of each slurry was carefully adjusted to 11.5 using a sodium hydroxide (NaOH) solution. Alkaline conditions enhance the activity of hydrogen peroxide by facilitating the cleavage of ester linkages within lignin and by swelling the plant cell wall structure. The combination of oxidative and alkaline conditions promotes the selective removal of lignin while minimizing degradation of the carbohydrate fraction.

Following pH adjustment, the slurries were transferred into 250 mL Erlenmeyer flasks, each sealed with cotton plugs or caps to maintain a sterile environment and prevent evaporation. The flasks were placed in a rotary shaker incubator set to 25 ± 1°C and agitated at a constant speed of 250 revolutions per minute (rpm) for a total duration of 24 hours. Agitation ensures proper mixing and uniform exposure of the biomass to the pretreatment solution, while the chosen temperature and duration were optimized based on literature and preliminary trials to ensure efficient lignin disruption without compromising sugar integrity.

Upon completion of the 24-hour incubation period, the contents of each flask were filtered to separate the solid fraction (pretreated biomass) from the liquid. The solid residue was

then visually inspected for changes in physical characteristics such as color, texture, and structural breakdown. Effective delignification typically results in noticeable bleaching of the biomass and a softened texture, both of which were used as qualitative indicators of pretreatment success.

To further evaluate the effectiveness of the pretreatment, a preliminary quantification of reducing sugars released during the process was carried out using the 3,5-dinitrosalicylic acid (DNS) method. This colorimetric assay measures the amount of free reducing sugars, an indirect indicator of how accessible the carbohydrate portion of the biomass has become following pretreatment.

Based on both visual assessment and reducing sugar analysis, a comparative evaluation of all five substrates was performed. Three substrates-those showing minimal structural disintegration or lower sugar release-were excluded from further investigation. These substrates were deemed less efficient in terms of their potential for enzymatic hydrolysis and fermentation.

The remaining two substrates, which exhibited significantly better performance in terms of delignification and sugar availability, were shortlisted for subsequent detailed studies. These included saccharification and fermentation steps to evaluate their actual ethanol yield potential. The selection of only the top-performing substrates ensured that further experimentation would be both cost-effective and scientifically meaningful.

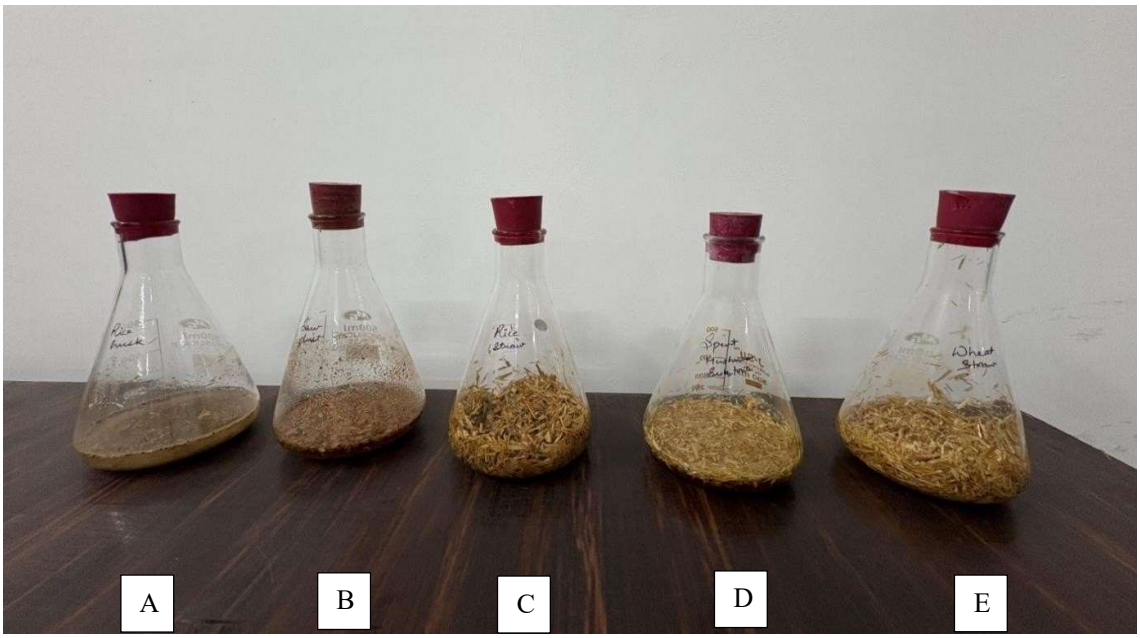

Fig.12: Different Substrates used for the selection criteria

(A) Rice husk (B) Sawdust (C) Rice straw (D) SMS (E) Wheat Straw

3.4 Analyzing and Improving Pretreatment Techniques for Effective Biomass Utilization

In an effort to enhance the accessibility of fermentable sugars from lignocellulosic biomass, two widely recognized pretreatment strategies were employed and systematically compared: dilute acid pretreatment and alkaline peroxide pretreatment. These methods were chosen due to their established effectiveness in disrupting the complex lignin–cellulose–hemicellulose matrix present in plant-based residues. The selected substrates for this comparative study were wheat straw and spent mushroom substrate (SMS), both of which are abundant agricultural wastes and hold significant promise as raw materials for second-generation bioethanol production.

3.4.1 Dilute Acid Pretreatment

The dilute acid pretreatment method was designed to hydrolyze hemicellulose and partially solubilize the lignin components, thereby exposing the cellulose fraction for further enzymatic action. For this process, 10 grams of air-dried and finely ground substrate (wheat straw or SMS) were accurately weighed and mixed with 100 mL of 5% (w/v) sulfuric acid (H_2SO_4). This acid concentration was selected based on previously established protocols (Gonzales et al., 2017) that demonstrated its effectiveness in breaking down hemicellulosic bonds without causing excessive degradation of sugars.

The acid-substrate mixture was then subjected to thermal hydrolysis by autoclaving in a sealed, heat-resistant vessel. The autoclave conditions were set at 121 °C and 15 psi pressure for 30 minutes, a temperature-time combination known to sufficiently disrupt lignocellulosic structures while preserving monomeric sugars. After the heating cycle, the slurry was carefully removed and allowed to cool to room temperature under sterile conditions.

Importantly, no filtration or washing of the treated sample was performed after autoclaving. This approach was intended to retain both the solid and solubilized fractions of the biomass, including any reducing sugars that may have leached into the liquid phase during acid hydrolysis. This ensured a more accurate representation of total sugar yield for downstream analysis.

Prior to estimating the sugar content, the pH of the liquid fraction was adjusted to 5.0 using 1 N sodium hydroxide (NaOH). Neutralization of the sample was a critical step, as the 3,5- dinitrosalicylic acid (DNS) assay employed for quantifying reducing sugars is highly pH- sensitive, exhibiting optimal color development and accuracy within a

slightly acidic to neutral pH range. The adjusted liquid was then used directly, without further dilution or purification, for quantifying reducing sugars released during the acid pretreatment process.

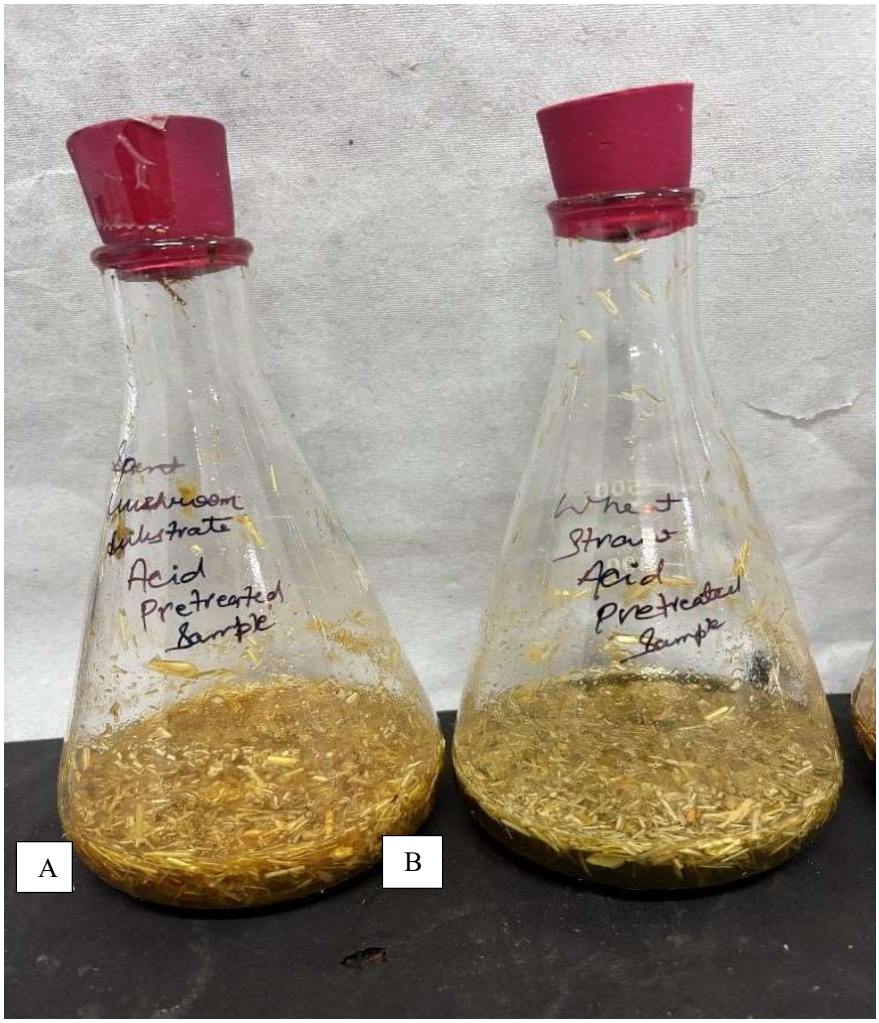

Fig 13: Acid pretreated sample (A) SMS (B)Wheat Straw

3.4.2 Alkaline Peroxide Pretreatment

In parallel, the alkaline peroxide pretreatment method was applied to the same substrates, providing an oxidative alternative aimed more at lignin solubilization than hemicellulose hydrolysis. This method is often regarded as a milder and more environmentally friendly approach compared to acid-based techniques.

To begin, a slurry was prepared by adding 10 grams (dry weight) of substrate to 100 mL of distilled water, maintaining a biomass concentration of approximately 10% (w/v). To this mixture, 2.5% (v/v) hydrogen peroxide (H_2O_2) was added, serving as the oxidative agent responsible for breaking down lignin bonds. Hydrogen peroxide was selected due to its ability to produce reactive oxygen species under alkaline conditions, which are known

to attack and fragment lignin polymers.

Fig 14: Alkaline Pretreated Sample (A) SMS (B)Wheat Straw

The pH of the slurry was then carefully increased to 11.5 using a sodium hydroxide (NaOH) solution. Alkaline conditions activate the hydrogen peroxide, creating a synergistic environment that promotes efficient delignification while preserving the polysaccharide content of the biomass. The mixture was then transferred to 250 mL Erlenmeyer flasks and incubated in a rotary shaker set at 250 rpm and 25 °C. The duration of incubation varied between 3 to 24 hours, depending on the level of structural breakdown desired. This range allowed for an observation of how extended exposure to oxidative conditions influenced substrate digestibility.

After the incubation period, the pH of the pretreated slurry was lowered to 5.0 using concentrated hydrochloric acid (HCl). This adjustment was crucial to bring the treated slurry into the appropriate pH range for enzymatic hydrolysis and reducing sugar assays. Unlike the acid method, which involves thermal treatment, the alkaline peroxide method

operates effectively at ambient temperatures, making it potentially more energy-efficient for large- scale applications. (Saha & Cotta, 2006).

3.4.3 Rationale for Dual Approach

By applying both pretreatment methods under controlled but distinct chemical environments, this comparative study aimed to evaluate the efficacy of each technique in improving sugar accessibility and breaking down the structural integrity of complex lignocellulosic biomass. Furthermore, keeping the substrates unfiltered and unwashed post-treatment in both methods allowed for a more comprehensive analysis of the total sugar yield, including both soluble and insoluble sugar fractions. This dual-method framework provided insight into how the structural and compositional differences between wheat straw and SMS influence their response to different pretreatment strategies, which is essential for selecting the most efficient, cost-effective, and scalable approach for bioethanol production.

3.5 Optimized Pretreatment Strategy: Alkaline Peroxide Treatment

The Pretreatment is done by the Alkaline Peroxide method. This is a multistep process that involves several carefully controlled steps to enhance the breakdown of the material, making it more suitable for enzymatic conversion. This process begins by creating a slurry of wheat straw and water, with the straw concentration adjusted to 10% (w/v). To this slurry, hydrogen peroxide (H_2O_2) is added in varying concentrations, typically between 0% and 4.3% (v/v), depending on the requirements of the experiment. Hydrogen peroxide plays a critical role in breaking down lignin, a major component of plant cell walls that obstructs enzyme access to cellulose.

The slurry's pH was gradually adjusted to 11.5 by adding a sodium hydroxide (NaOH) solution. The alkaline conditions created by NaOH allow for better penetration of hydrogen peroxide into the biomass. This slurry is then placed in an incubator and agitated at a constant speed of 250 rpm. The incubation temperature was 25°C and the time duration depends to the extent of desired lignin removal from 3 to 24 hours.

Following the pretreatment, the pH of the mixture is lowered to 5.0 using concentrated hydrochloric acid (HCl). This adjustment is crucial because the enzymes that break down cellulose into fermentable sugars-used in the subsequent saccharification process-work optimally at a pH of around 5.0. After adjusting the pH, the enzymatic hydrolysis phase begins, converting the treated straw into fermentable sugars.

To lower production costs, the enzymes used for this process are produced internally, reducing reliance on expensive commercial enzymes while maintaining high efficiency. Different wheat straw materials, such as ground straw and spent mushroom substrate, exhibit distinct characteristics before and after treatment. A 24-hour pretreatment time has been shown to provide the highest sugar yield, with only minor gains observed beyond that period.

3.6 Enzymatic Saccharification and In-House Enzyme Production

Saccharification is a crucial step in bioethanol production, where complex polysaccharides in pretreated biomass are broken down into fermentable sugars using enzymatic hydrolysis. The performance of the process is largely determined by the specific enzymes selected and their respective concentrations. However, the high cost of commercially available enzymes poses a significant challenge for large-scale biofuel production. To address this issue, in-house enzyme production was explored as a cost-effective alternative. This section details the saccharification process, the enzymatic hydrolysis conditions, and the production of enzymes in the laboratory to optimize sugar release while minimizing costs.

3.7 pH Adjustment and Initiation of Enzymatic Hydrolysis

Following the pretreatment, the pH of the mixture is lowered to 5.0 using concentrated hydrochloric acid (HCl). This adjustment is crucial because the enzymes that break down cellulose into fermentable sugars-used in the subsequent saccharification process-work optimally at a pH of around 5.0. After adjusting the pH, the enzymatic hydrolysis phase begins, converting the treated straw into fermentable sugars.

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3.8 Microorganisms for Enzyme Production

To achieve a cost-effective and efficient enzymatic hydrolysis of pretreated wheat straw, the enzymes were produced in-house using two fungal strains, *Aspergillus niger* (MTCC 2196) [obtained from the Microbiology laboratory of Department of Biotechnology, P.K. University.] and *Trichoderma viride* (MTCC 800*)* [obtained from the Microbiology

laboratory of Department of Biotechnology, P.K. University]. These fungi are well-known for their ability to generate a variety of cellulolytic and hemicellulolytic enzymes necessary for breaking down complex polysaccharides into fermentable sugars.

Trichoderma viride can be utilized in fermentation processes due to its ability to produce cellulases and other hydrolytic enzymes that break down plant biomass. It produces cellulases, xylanases, and other enzymes that are capable of breaking down cellulose and hemicellulose into fermentable sugars, making it a viable option for biomass applications. In this regard, *Aspergillus niger* has been extensively studied for its capacity to produce a broader range of enzymes, including higher levels of β-glucosidase, which is crucial for converting cellulose into glucose. Additionally, enzymes produced by *Aspergillus* species tend to be more thermostable, enhancing their industrial relevance.

Fig 15: ***Trichoderma Viride***

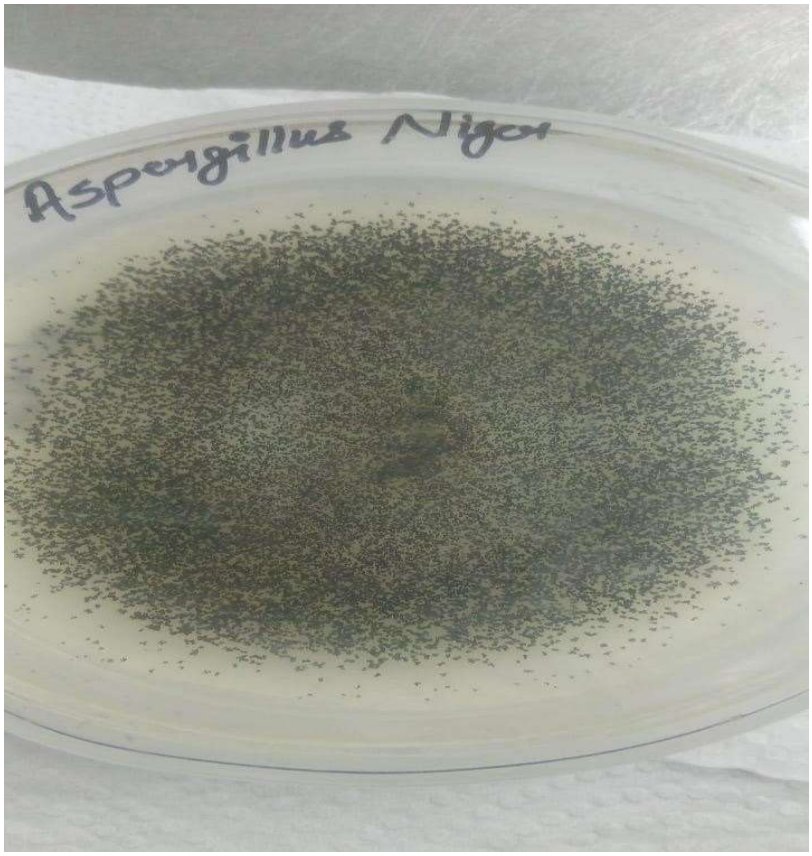


Fig 16: ***Aspergillus Niger***

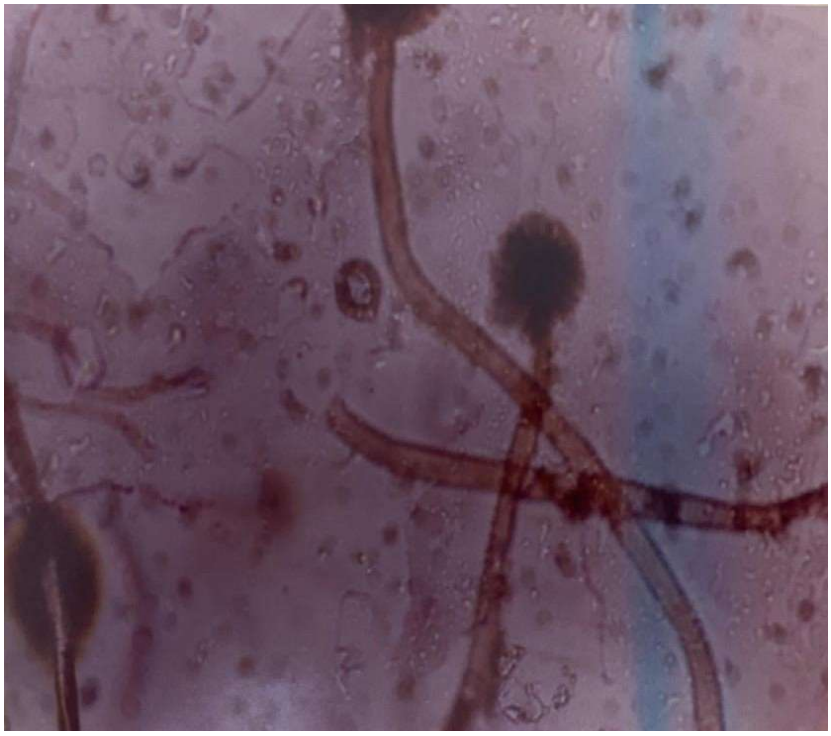


Fig 17: Photograph of ***Aspergillus niger*** **with sporangiophores and spores under light- microscope**

3.8.1 Cultivation of *Aspergillus niger*

The fungal strain *Aspergillus niger* (MTCC 2196) used in this study was sourced from the culture collection maintained by Microbiology laboratory of Department of Biotechnology, P.K.University. This strain was selected due to its well-established role in biomass degradation and its relevance in various biotechnological applications, particularly in enzyme production.

Cultivation was initiated by aseptically inoculating sterilized Potato Dextrose Agar (PDA) plates with fungal spores. All inoculation procedures were carried out inside a laminar airflow cabinet to maintain sterile conditions and prevent any form of external contamination. PDA was used as the growth medium because it provides a nutrient-rich environment that supports the robust growth of fungal colonies.

Following inoculation, the plates were incubated at a stable temperature of 28 °C for a period of 5 to 7 days. This incubation period allowed for the gradual development of fungal mycelium and the formation of characteristic colonies. As growth progressed, typical black, powdery spore formations were observed, which are visually distinct features of Aspergillus niger colonies.

Once mature colonies had developed, a single, healthy, and well-isolated colony was selected and subcultured onto fresh PDA plates. This subculturing step was performed to ensure the purity of the strain by eliminating the possibility of mixed or contaminated growth. The fresh plates were incubated under the same conditions (28 °C for 5–7 days), and the resulting colony was monitored to confirm its morphological consistency and purity.

For long-term storage and repeated use in experiments, the purified strain was preserved on PDA slants. These slants were stored at 4 °C in a refrigerator to slow down metabolic activity, thereby maintaining the viability and integrity of the fungal culture over an extended period. This method of preservation allowed for easy retrieval and reactivation of the culture whenever required during the course of the study.

Through this careful and sterile cultivation process, an active and uncontaminated stock culture of *Aspergillus niger* was successfully maintained, ensuring consistent and reliable results in all subsequent experimental applications.

3.8.2 Cultivation of *Trichoderma viride*

The fungal strain *Trichoderma viride* (MTCC 800) used in this study was procured from the Microbiology Laboratory of the Department of Biotechnology, P.K. University. This strain is widely known for its cellulolytic and ligninolytic enzyme production, and is frequently used in biodegradation and bioconversion studies due to its robust growth and competitive colonization ability.

To initiate cultivation, the fungal spores were aseptically inoculated onto freshly prepared Potato Dextrose Agar (PDA) plates. All inoculation procedures were performed under sterile conditions within a laminar airflow cabinet to avoid microbial contamination and ensure the integrity of the culture. PDA was selected as the medium because it provides a rich nutritional base that supports the rapid growth and sporulation of *Trichoderma viride*.

The inoculated plates were incubated at a constant temperature of 28 °C for a period of 5 to 7 days. This incubation period allowed for the development of well-defined fungal colonies. Characteristic features of *Trichoderma viride* were observed during this phase, including the formation of dense, fluffy mycelial growth with a distinct green pigmentation that gradually intensified as sporulation occurred. Additionally, the fungus exhibited rapid radial expansion across the surface of the agar, a trait commonly associated with this species.

To confirm purity and prevent cross-contamination, a healthy and well-isolated colony was selected and transferred to a fresh PDA plate using sterile techniques. This subculturing process was repeated at least once to ensure a pure culture, free from any unwanted microbial growth. The subcultured plates were again incubated under identical conditions (28 °C for 5– 7 days) to confirm consistent morphology and growth behavior.

For long-term storage and ease of access during the course of the study, the purified strain was preserved on PDA slants. These slants were sealed properly and stored at 4 °C in a refrigerated environment. Cold storage helped to maintain the viability of the strain while slowing down metabolic activity, thereby preventing overgrowth or nutrient exhaustion during storage.

This cultivation protocol ensured the continuous availability of an active, pure, and contamination-free culture of *Trichoderma viride*, which was subsequently used for enzyme production, biomass degradation studies, and other experimental applications throughout the research.

3.9 Inoculum Preparation for Enzyme Production

The enzyme production process is taken from the protocol given by Winarsih and Siskawardani (2020). First, inoculum preparation involved collecting conidia from *T. viride* and *Aspergillus niger* was cultured on Potato Dextrose Agar (PDA) plates. Using an inoculating loop, the conidia were scraped from the mold surface and suspended in 10 mL of physiological saline solution (0.85% NaCl). From this, approximately 2 mL of the spore suspension was transferred to 50 mL of sterile culture medium designed for mold growth. This medium contained yeast extract (4 g/L), malt extract (10 g/L), and glucose (4 g/L), providing essential nutrients for fungal development.

The cultures were incubated in a water bath shaker set at 30°C with a constant shaking speed of 120 rpm for three days. After this incubation period, the spore density was determined using a hemocytometer. Once the spore concentration reached 10^7 spores per mL, the inoculum was deemed ready for enzyme production, ensuring an optimal concentration for enzyme synthesis.

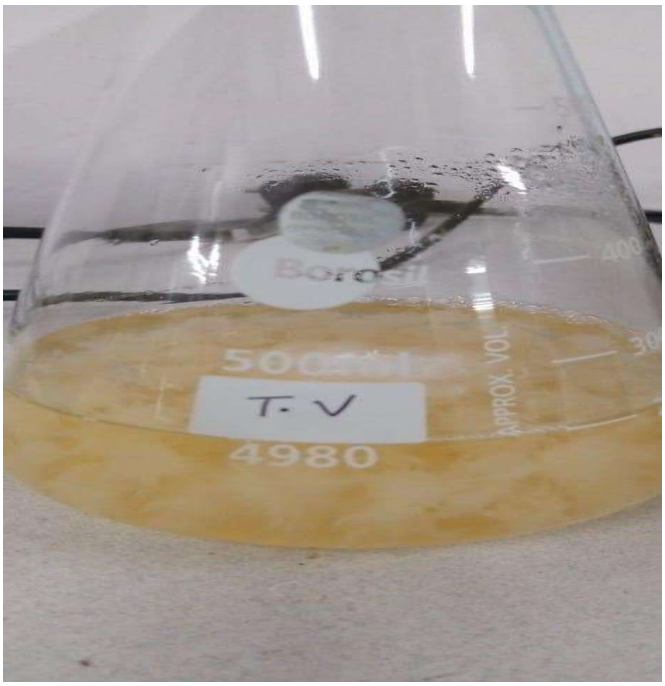


Fig 18: Spores of *Trichoderma Viride*

3.10 Enzyme Production

Solid-state fermentation (SSF) for enzyme production was conducted using wheat straw as a substrate. The process involved placing the wheat straw in 500 mL Erlenmeyer flasks, and the moisture content of the straw was adjusted to 60%. This was done by adding a 0.9% (w/v) ammonium sulphate solution that had been prepared using 0.1 mol/L hydrochloric acid (HCl)..

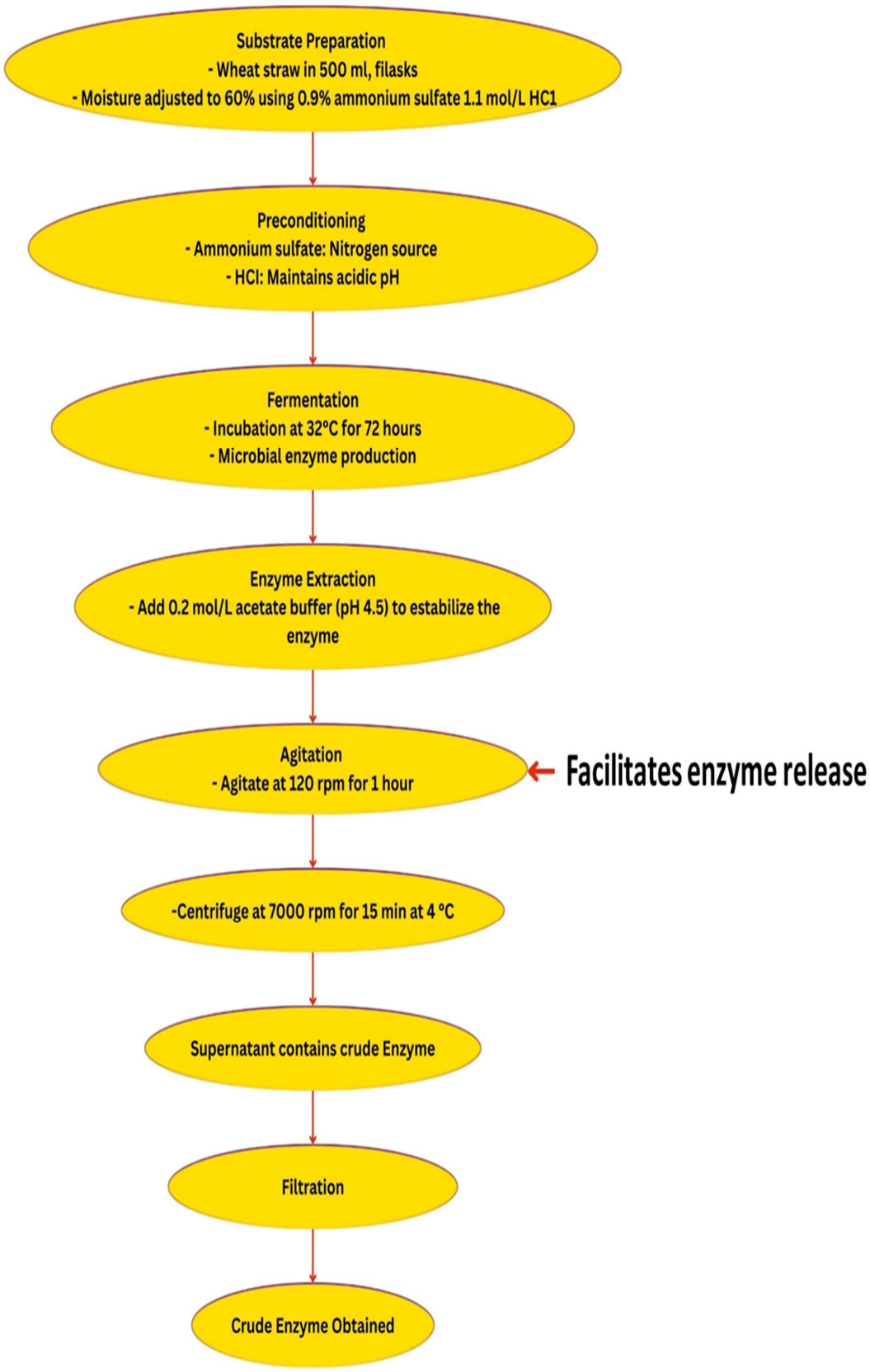


Fig 19: Enzyme Production Overview

Ammonium sulphate serves as a nitrogen source, enhancing microbial growth and enzyme production, while HCl ensures the proper acidic environment for fermentation. The fermentation process was carried out at a temperature of 32°C and continued for 72 hours. This period allows microorganisms, such as fungi or bacteria, to break down the wheat straw and produce the desired enzymes. After the 72-hour fermentation, enzyme extraction was performed by adding a 0.2 mol/L acetate buffer solution at a pH of 4.5 to the solid substrate. This buffer is ideal for stabilizing the enzymes during extraction.

To facilitate enzyme release from the substrate, the mixture was agitated at 120 revolutions per minute (rpm) for one hour, ensuring adequate mixing and promoting efficient extraction.

After agitation, the solution containing the enzymes was filtered to remove any solid particles, yielding a clear enzyme extract. The extracted enzymes were then stored at -18°C to preserve their activity for further analysis. This method is efficient for producing enzymes from agricultural residues like wheat straw in a controlled laboratory environment.

3.11 Enzyme Saccharification

The enzymatic saccharification of alkaline peroxide-pretreated wheat straw was carried out under controlled conditions to efficiently convert the complex carbohydrates in the biomass into fermentable sugars. The process began by gently shaking the pretreated wheat straw mixture at 100 revolutions per minute (rpm) while maintaining a temperature of 45°C. These conditions are optimal for enzymatic activity, ensuring effective breakdown of the substrate.

To ensure the enzymes functioned in an ideal environment, the pH of the mixture was adjusted to 5.0 using hydrochloric acid (HCl). Maintaining the correct pH is critical, as it influences enzyme stability and activity. Once the pH was stabilized, enzymes were added to the mixture at a concentration of 4 mL per 100 g of wheat straw. This dosage provides an adequate amount of enzyme to interact with the pretreated biomass, ensuring efficient saccharification.

The enzymatic saccharification process was allowed to proceed for 72 to 120 hours, depending on the specific experimental goals. During this period, enzymes, such as cellulases and hemicellulases, broke down the complex carbohydrates-mainly cellulose and hemicellulose-into simple fermentable sugars, like glucose and xylose. These sugars

are vital intermediates in the bioconversion process, particularly for biofuel production. At various time points during the saccharification, samples were collected for analysis to monitor the progress and efficiency of the conversion. This approach represents a key step in biomass-to- biofuel processes, as it enables the transformation of lignocellulosic material into valuable sugars (Saha & Cotta, 2006).

3.12. Incorporation of Spent Mushroom Substrate as a Biologically Modified Feedstock

In this study, Spent Mushroom Substrate (SMS) was utilized alongside wheat straw as a lignocellulosic feedstock for enzymatic hydrolysis. The SMS used had previously served as a cultivation bed for *Pleurotus ostreatus* (oyster mushroom), a white-rot fungus renowned for its ability to degrade lignin through the secretion of oxidative enzymes. During mycelial colonization and fruiting body development, oyster mushrooms release key ligninolytic enzymes such as laccases, manganese peroxidases (MnP), and lignin peroxidases (LiP). These enzymes facilitate the breakdown of complex aromatic structures within lignin by catalyzing radical-mediated oxidative reactions, thereby enhancing the accessibility of cellulose and hemicellulose components for further enzymatic action. (W. Wu et al., 2018)

The enzymatic hydrolysis of SMS was carried out under standardized conditions to evaluate the impact of prior biological lignin degradation on sugar recovery. Both SMS and wheat straw were subjected to hydrolysis using in-house produced crude enzymes derived from *Aspergillus niger* and *Trichoderma viride*. These fungal strains were chosen for their potent cellulolytic and hemicellulolytic enzyme systems, which include endoglucanases, exoglucanases (cellobiohydrolases), β-glucosidases, and xylanases. These enzymes synergistically degrade cellulose and hemicellulose into monomeric sugars such as glucose and xylose (Saha & Cotta, 2006).

The hydrolysis setup included incubation of the pretreated biomass at 45°C and 100 rpm, with the pH adjusted to 5.0 using HCl to create optimal conditions for enzymatic activity. Enzymes were applied at a rate of 4 mL per 100 g of biomass, and the reaction was allowed to proceed for 72 to 120 hours. Periodic sampling was conducted to monitor reducing sugar concentrations using the DNS method.

SMS exhibited a markedly improved saccharification profile compared to wheat straw. This enhancement is attributed to the partial lignin depolymerization during mushroom cultivation, which rendered the substrate more amenable to enzymatic attack. By reducing lignin-related steric hindrance and increasing porosity, the biological

preconditioning effectively boosted enzyme-substrate interaction.

Thus, the integration of SMS into the enzymatic hydrolysis process not only supports the valorization of agricultural waste but also leverages natural fungal activity to improve downstream bioconversion efficiency.

3.13 Fermentation

Fermentation is a key biological process in bioethanol production, wherein specific microorganisms, such as *Saccharomyces cerevisiae* (yeast) and *Zymomonas mobilis* (bacteria), convert simple sugars into ethanol and carbon dioxide under anaerobic conditions. This transformation is central to the valorization of lignocellulosic biomass-such as wheat straw and spent mushroom substrate-by converting the reducing sugars released during saccharification into a valuable renewable fuel.

The process occurs in the absence of oxygen, allowing microorganisms to utilize the available glucose, xylose, and other fermentable sugars for ethanol production through glycolysis followed by alcoholic fermentation. Among the commonly used organisms. Saccharomyces cerevisiae is widely preferred in fermentation processes because of its strong ethanol resistance, fast sugar-to-ethanol conversion capability, and established effectiveness in industrial applications. *Z. mobilis*, on the other hand, offers advantages in terms of higher sugar uptake rates and ethanol yields, although it is less commonly used at a large scale due to limited substrate range and genetic tools.

Fermentation serves not only as a biological route to produce bioethanol but also plays a crucial role in sustainable energy generation. It enables the conversion of agricultural residues into energy-rich fuel, reducing environmental pollution and minimizing greenhouse gas emissions when compared to fossil fuels. Moreover, the use of waste biomass like wheat straw and spent mushroom substrate adds value to underutilized agro-industrial byproducts, promoting circular bioeconomy principles.

Optimizing fermentation parameters-such as pH, temperature, inoculum concentration, and fermentation time-can significantly influence ethanol yield and productivity. Additionally, enhancing the performance of microbial strains through adaptation or genetic modification can improve sugar utilization and ethanol tolerance, thereby improving the overall efficiency and economic feasibility of the bioethanol production process.

3.14 Yeast Culturing and Preparation for Fermentation

In the process of bioethanol production, microorganisms such as yeast and certain bacteria play a fundamental role due to their ability to efficiently convert sugars into ethanol through fermentation. Among these, *Saccharomyces cerevisiae*, commonly known as baker's yeast, is one of the most widely used organisms. Its popularity stems from its high ethanol tolerance, fast fermentation rate, and well-documented performance in industrial-scale bioethanol production systems. In this study, *S. cerevisiae* was selected as the primary fermenting organism because of its consistent and reliable ability to metabolize simple sugars like glucose into ethanol under anaerobic conditions.

To begin the fermentation process, it was essential to culture and prepare a healthy and active population of yeast cells. The initial step involved growing the yeast on Potato Dextrose Agar (PDA) plates. PDA is a nutrient-rich medium that promotes rapid growth and supports the development of robust yeast colonies. The solid medium also provides an easy way to isolate pure cultures and visually assess the health and uniformity of the growing yeast.

Once sufficient colony growth was observed on the PDA plates, the yeast cells were transferred into a liquid medium known as yeast extract peptone (YEP) broth to prepare them for fermentation. This medium was specifically prepared by dissolving 1 gram of yeast extract, 2 grams of peptone, and 2 grams of glucose in 100 milliliters of distilled water. Each component of this medium plays a specific role in supporting yeast metabolism: yeast extract provides essential growth factors such as vitamins and amino acids, peptone acts as a nitrogen source necessary for cell development, and glucose serves as a readily fermentable carbon source that fuels cellular activity and proliferation.

The inoculated broth was incubated under appropriate conditions to allow the yeast to multiply. During this phase, the population of yeast cells was carefully monitored, and a cell count of approximately 13 × 10^7 cells per milliliter was recorded. This high cell density was considered optimal for initiating fermentation, as a large number of actively growing cells increases the rate and completeness of sugar conversion into ethanol. A higher yeast biomass not only shortens the fermentation time but also reduces the chances of contamination and incomplete sugar utilization.

By combining both solid (PDA) and liquid (YEP) culturing techniques, this approach ensured that the yeast cells were healthy, active, and present in sufficient numbers before the fermentation process began. Maintaining a strong and viable yeast culture is critical

in fermentation-based bioethanol production, as it directly influences the overall ethanol yield and process efficiency.

This preparation strategy provided a consistent foundation for the subsequent fermentation experiments, supporting the goal of optimizing ethanol production from the fermentable sugars obtained from lignocellulosic biomass. It also aligns with the broader aim of improving the cost-effectiveness and sustainability of second-generation biofuel production systems by maximizing microbial performance under controlled laboratory conditions.

3.15 Batch Fermentation Setup and Conditions

To evaluate ethanol production from lignocellulosic biomass-derived sugars, batch fermentation experiments were carried out using a controlled laboratory setup. The experiments were performed in 500 mL Erlenmeyer flasks**,** each specifically prepared to support yeast fermentation under semi-anaerobic conditions. These flasks were chosen due to their suitability for small-scale fermentation trials and their ability to provide sufficient headspace for gas exchange, which is important when mimicking the conditions found in industrial fermentation systems.

Each flask was filled with 150 mL of fermentation medium, carefully leaving a 350 mL headspace. This unfilled volume was necessary to allow for the buildup and escape of gases such as carbon dioxide that are naturally released during the fermentation process. It also helped in replicating semi-anaerobic conditions**,** where oxygen was not fully eliminated but limited. Such a setup closely mimics the oxygen-restricted environment required by *Saccharomyces cerevisiae* for effective ethanol production, as complete anaerobic conditions are difficult to maintain without sophisticated equipment.

The fermentation medium consisted of Wheat Straw hydrolysate and Spent Mushroom Substrate, which were obtained through the enzymatic saccharification of alkaline peroxide-pretreated Wheat Straw and Spent Mushroom Substrate. These hydrolysates served as the main carbon and nutrient sources**,** containing a mixture of fermentable sugars - primarily glucose-along with trace amounts of xylose and other monosaccharides. These sugars are the key substrates for *S. cerevisiae*, which metabolizes them to produce ethanol, carbon dioxide, and other secondary metabolites. The choice of Wheat Straw hydrolysate and Spent Mushroom Substrate hydrolysate also supported the study's goal of utilizing low-cost, renewable feedstock for sustainable biofuel production.

A critical parameter in any fermentation process is the pH of the medium, which significantly influences microbial activity, enzyme stability, and product yield. In this experiment, the pH of the fermentation broth was continuously monitored and adjusted throughout the process to maintain it within the optimal range for yeast activity, typically around pH 4.5 to 5.5. Deviations from this range can hinder yeast metabolism and reduce ethanol output. To control pH effectively, a 4 M solution of potassium hydroxide (KOH) was used to neutralize acids generated during fermentation and maintain a stable environment for yeast function. This addition was made carefully to avoid sharp fluctuations, ensuring a consistent and conducive environment for ethanol synthesis.

The yeast strain used in this experiment was *Saccharomyces cerevisiae*, known for its high ethanol tolerance, robustness, and long-standing industrial relevance. Prior to fermentation, the yeast was cultured and prepared using both solid and liquid media (as described in Section 3.4.1), ensuring high viability and active biomass before inoculation into the flasks.

By optimizing parameters such as substrate composition**,** pH control**,** and aeration conditions, the experimental setup provided a reliable model for studying yeast-based ethanol fermentation. This approach enabled efficient sugar conversion and aimed to closely replicate real-world fermentation systems on a laboratory scale. Furthermore, using Wheat Straw hydrolysate and Spent Mushroom Substrate aligns with the broader objectives of reducing production costs, valorizing agricultural waste, and developing a more environmentally sustainable method for bioethanol generation.

3.16 Ethanol Quantification Using the Dichromate Method

The quantification of ethanol in the distilled fermentation broth was carried out using a classical spectrophotometric method based on the oxidation of ethanol by potassium dichromate in a strongly acidic medium. This method is well-documented in analytical chemistry and was adapted from the protocol described by Pilone et al. (1985). The principle of the method involves the colorimetric detection of a chemical change, specifically the reduction of orange-colored dichromate ions ($Cr_2O_7^{2-}$) to green-colored chromium ions (Cr^{3+}), which occurs when ethanol is oxidized to acetic acid. The intensity of the color change is directly proportional to the ethanol concentration in the sample. The complete experimental procedure was carried out in six sequential steps, as detailed below:

Step 1: Preparation of Dichromate Reagent

The dichromate reagent used in this experiment was freshly prepared to ensure its stability and reactivity. Potassium dichromate ($K_2Cr_2O_7$), a bright orange crystalline solid, was carefully weighed using an analytical balance to maintain precision. It was then dissolved in a concentrated sulfuric acid (H_2SO_4) solution. The acidic medium is critical because it facilitates the redox reaction by protonating the dichromate ions, enhancing their oxidizing power. The reaction that underpins this method is:

$$C_2H_5OH + 2Cr_2O_7^{2-} + 16H^+ \rightarrow 2CH_3COOH + 4Cr^{3+} + 11H_2O$$

This step was performed under a fume hood due to the corrosive and toxic nature of sulfuric acid and potassium dichromate. Proper personal protective equipment (PPE), including gloves, goggles, and a lab coat, was used to minimize exposure risks. The reagent was prepared just before use and stored in a dark, amber-colored glass container to protect it from light-induced decomposition.

Step 2: Mixing of Ethanol Sample with Dichromate Reagent

An aliquot of the distilled ethanol sample was carefully measured using a micropipette or graduated pipette, ensuring volumetric precision. An equal volume of the freshly prepared dichromate reagent was added to the ethanol sample in a clean, dry reaction vessel. The vessel was immediately sealed with a stopper or Parafilm to prevent the evaporation of ethanol, which is highly volatile and could otherwise lead to underestimation of the actual concentration. Gentle shaking was performed to ensure homogeneity of the reactants.

Step 3: Incubation of Reaction Mixture

The sealed reaction vessel containing the mixture of ethanol and dichromate reagent was then incubated in a temperature-controlled water bath or incubator maintained at 37 ± 1°C for a period of 10 minutes. The specific temperature and time were chosen based on the optimization suggested in previous literature to ensure complete oxidation of ethanol without degradation of other potential sample constituents. During this incubation period, ethanol (C_2H_5OH) is oxidized to acetic acid (CH_3COOH), while the dichromate ions are simultaneously reduced to chromium ions (Cr^{3+}).

This redox process results in a notable color change from bright orange to varying shades of green, depending on the amount of ethanol present. The reaction is stoichiometric, and thus, the amount of chromium (III) formed is directly proportional to the amount of ethanol oxidized. This visible color transition forms the basis for spectrophotometric quantification.

Step 4: Dilution and Thorough Mixing

Following incubation, an equal volume of pre-warmed distilled water (also at 37°C) was added to the reaction mixture. This dilution step is essential to reduce the concentration of the acidic medium and to stabilize the absorbance reading by minimizing any residual heat effect. The solution was mixed thoroughly using a vortex mixer or by inversion to ensure that the contents were homogeneous before spectrophotometric analysis. Proper mixing helps eliminate any concentration gradient or color variation that could affect absorbance readings.

Step 5: Spectrophotometric Measurement

The final step of the assay involved the measurement of absorbance at **600 nm** using a calibrated UV-visible spectrophotometer. This wavelength was selected as it corresponds to the maximum absorbance for the green-colored Cr^{3+} ion, ensuring optimal sensitivity and specificity of the assay.

The spectrophotometer was first blanked using a reagent blank (prepared in the same manner but without ethanol) to nullify any background absorbance due to the reagent itself. After zeroing the instrument, the sample cuvette was inserted, and the absorbance was recorded.

Step 6: Calculation of Ethanol Concentration

The ethanol concentration in the unknown sample was calculated using a comparative method by referencing a standard ethanol solution of known concentration. The formula employed for this purpose is:

$$\% \text{ Ethanol } = \left(\frac{\text{Aunknown}}{\text{Astandard}}\right) 18.51$$

Where:

Aunknown = Absorbance value of the ethanol sample being analyzed

Astandard = Absorbance of a standard ethanol solution prepared with the same protocol

18.51 = A constant derived from the experimental conditions and calibration curve as reported by Pilone et al. (1985), which incorporates molar absorptivity and other proportional factors.

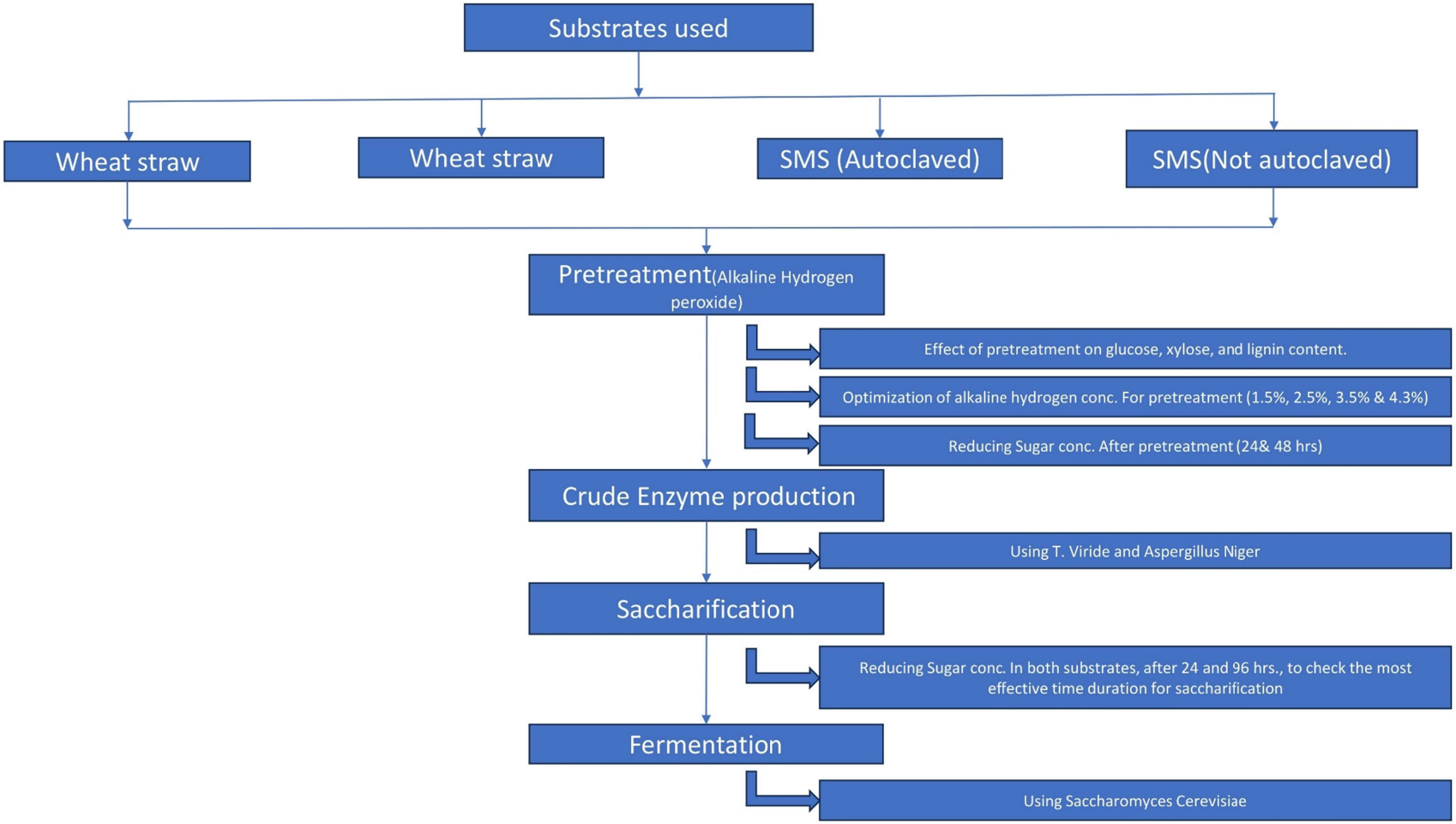


Fig 20: Overview of bioethanol production using lignocellulosic biomass

The primary aim of this study was to assess the rate and feasibility of bioethanol production from two abundant agricultural waste products: ground wheat straw and spent mushroom substrate (SMS). These substrates are both derived from wheat straw, with SMS representing a post-harvest waste product from mushroom cultivation. The comparison was conducted to determine which substrate yields a higher amount of bioethanol and demonstrates greater efficiency in the fermentation process.

4.1. Preliminary Screening of Substrates Using Alkaline Peroxide Treatment

To identify the most promising lignocellulosic materials for downstream bioethanol production, an initial comparative screening was conducted using five different agro-industrial residues: wheat straw, spent mushroom substrate (SMS), sawdust, rice straw, and rice husk. These substrates were selected based on their abundance, cost-effectiveness, and potential for bioconversion, and each was subjected to the same standardized alkaline peroxide pretreatment protocol to evaluate their structural digestibility and fermentable sugar potential.

For the pretreatment, exactly 10 grams of each dried and ground substrate were mixed with 100 mL of distilled water to form a slurry. To initiate oxidative delignification, 2.5% (v/v) hydrogen peroxide (H_2O_2) was added to each mixture. The pH of the solution was carefully adjusted to 11.5 using sodium hydroxide (NaOH), creating a strongly alkaline environment that promotes lignin disruption. The flasks containing the treated slurries were then incubated at a controlled temperature of 25 ± 1°C for 24 hours, with constant agitation at 250 rpm to ensure uniform exposure of the biomass particles to the reactive solution.

Following the 24-hour pretreatment, the substrates were filtered to separate the solid biomass, and visual observations were made regarding changes in color, texture, and structure. Effective delignification was generally indicated by a lighter coloration, softening of the biomass, and a more fragmented texture, all of which suggest improved accessibility of cellulose for enzymatic action.

To quantify the effectiveness of the pretreatment, the amount of reducing sugars released during the process was determined using the well-established DNS (3,5-dinitrosalicylic acid) assay. This colorimetric method provides a direct measurement of

fermentable sugar content in the liquid fraction and serves as a key indicator of how efficiently the biomass has been broken down.

The analytical results revealed a clear performance gradient among the five substrates. Wheat straw showed the highest reducing sugar concentration, with a yield of 6.63 ± 0.04 mM, followed closely by spent mushroom substrate (SMS), which released 5.85 ± 0.03 mM. Both substrates exhibited significant structural breakdown and color change, indicating successful pretreatment and high potential for subsequent enzymatic hydrolysis and fermentation.

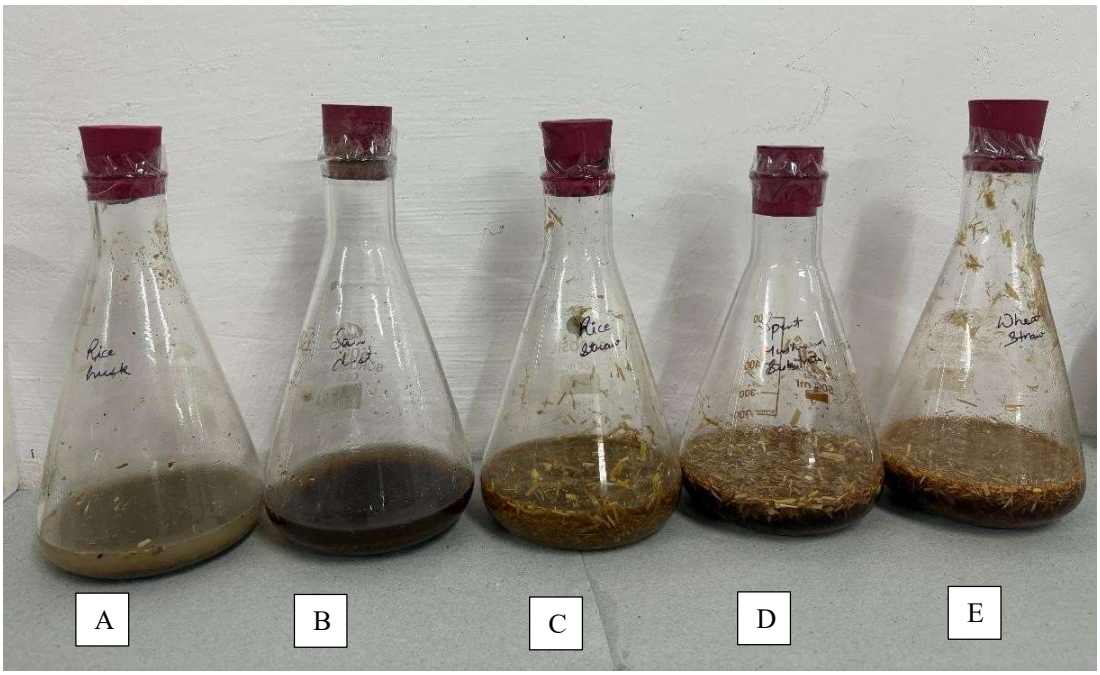


Fig. 21: Pretreated substrate to check reducing Sugar

(A) Rice husk (B) Sawdust (C) Rice straw (D) SMS (E) Wheat Straw

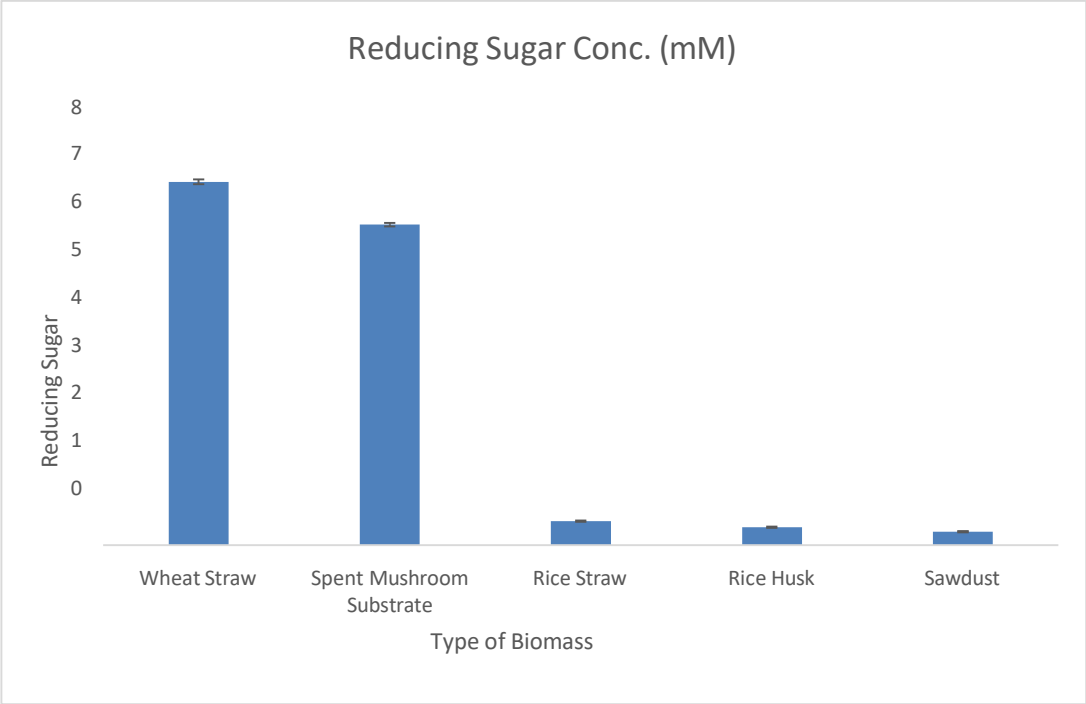
In contrast, the other three substrates-rice straw, rice husk, and sawdust-performed poorly under the same conditions. Rice straw released only 0.44 ± 0.01 mM of reducing sugars, while rice husk and sawdust yielded 0.33 ± 0.01 mM and 0.24 ± 0.012 mM, respectively. These values indicate limited delignification and a much lower degree of cellulose accessibility, likely due to the recalcitrant nature or denser composition of these materials.

Based on these findings, wheat straw and SMS were selected as the most suitable candidates for further optimization, saccharification, and fermentation experiments. This targeted selection allowed the study to focus on substrates that demonstrated the highest sugar yields and processing efficiency, ultimately improving the relevance and productivity of the bioethanol production trials.

This data-driven screening approach not only reduced experimental complexity but also highlighted the importance of choosing an appropriate feedstock for efficient biomass conversion. The significantly higher sugar recovery from wheat straw and SMS under identical treatment conditions reinforced their viability as cost-effective and renewable resources for second-generation bioethanol production.

Table 11: Substrate selection using Reducing Sugar

Substrate	Reducing Sugar in mM
Wheat Straw	6.63 ± **0.04**
Spent Mushroom Substrate	5.85 ± **0.03**
Rice Straw	0.44 ± **0.01**
Rice Husk	0.33 ± **0.01**
Sawdust	0.24 ± **0.012**



Graph 1: Reducing Sugar Concentration (mM) in Different Lignocellulosic Substrates

4.2 Comparative Analysis of Sugar Release from Pretreated Lignocellulosic Biomass

The comparative analysis of pretreatment methods revealed that **alkaline peroxide pretreatment was significantly more effective** than dilute acid pretreatment in enhancing the release of fermentable sugars from both **wheat straw (WS)** and **spent mushroom substrate (SMS)**. The differences in sugar yield were substantial and consistent across both substrates, underlining the superior efficiency of alkaline peroxide as a delignification and biomass-disrupting agent.

For wheat straw, the reducing sugar concentration obtained after alkaline peroxide treatment was **6.565 ± 0.12 mM**, which was markedly higher than the **4.02 ± 0.125 mM** obtained following dilute acid treatment. This difference represents an approximate **40% increase** in sugar yield, indicating that the alkaline oxidative environment was more effective at breaking down the complex lignocellulosic structure of wheat straw and enhancing the exposure of cellulose and hemicellulose to hydrolysis.

A similar trend was observed with spent mushroom substrate. When subjected to alkaline peroxide pretreatment, SMS produced a reducing sugar concentration of **6.015 ± 0.049 mM**, whereas the yield from acid pretreatment was only **3.52 ± 0.075 mM**. This corresponds to an improvement of nearly **41%**, further supporting the claim that alkaline peroxide conditions facilitate greater sugar recovery from this type of biomass.

These findings align well with previously published research in the field. Studies such as those by **Saha and Cotta (2006)** and **Zhang and Wu (2023)** have consistently reported higher sugar yields from lignocellulosic materials when treated with alkaline peroxide as opposed to dilute acids. For instance, Saha and Cotta (2005) demonstrated that a **2.15% hydrogen peroxide treatment** of wheat straw produced sugar concentrations ranging from **6 to 7 mM**, while **dilute sulfuric acid pretreatment** under similar processing conditions typically yielded only **3 to 4 mM**-a pattern very much mirrored in the present study.

One of the key advantages of alkaline peroxide pretreatment is its ability to **effectively remove lignin** while operating under relatively **mild conditions**-lower temperatures and neutral to mildly alkaline pH-thereby reducing the risk of sugar degradation and toxic by-product formation. This not only preserves the fermentable sugar content but also enhances the overall efficiency and sustainability of the bioethanol production process.

In conclusion, the **higher reducing sugar yields** observed with alkaline peroxide pretreatment in both wheat straw and SMS clearly justify its selection as the preferred

method for this study.Its effectiveness in **delignification**, combined with minimal loss of carbohydrates, makes it an ideal choice for preparing biomass feedstocks intended for bioethanol production. These findings contribute to the growing body of evidence supporting **alkaline peroxide** as a promising and practical approach for second-generation biofuel applications.

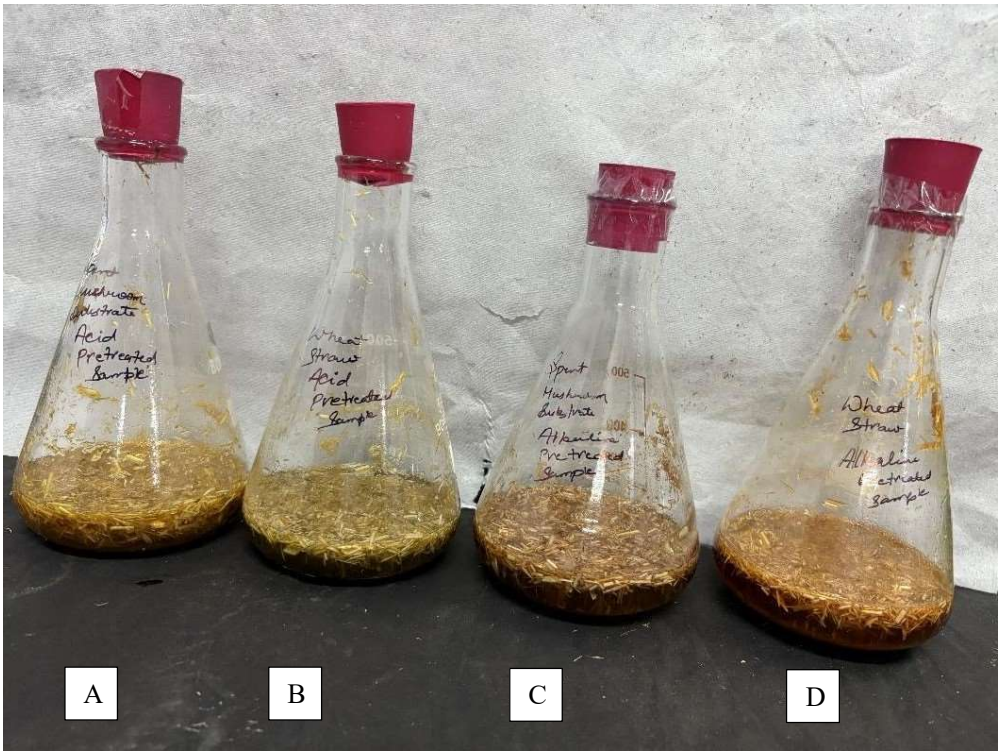
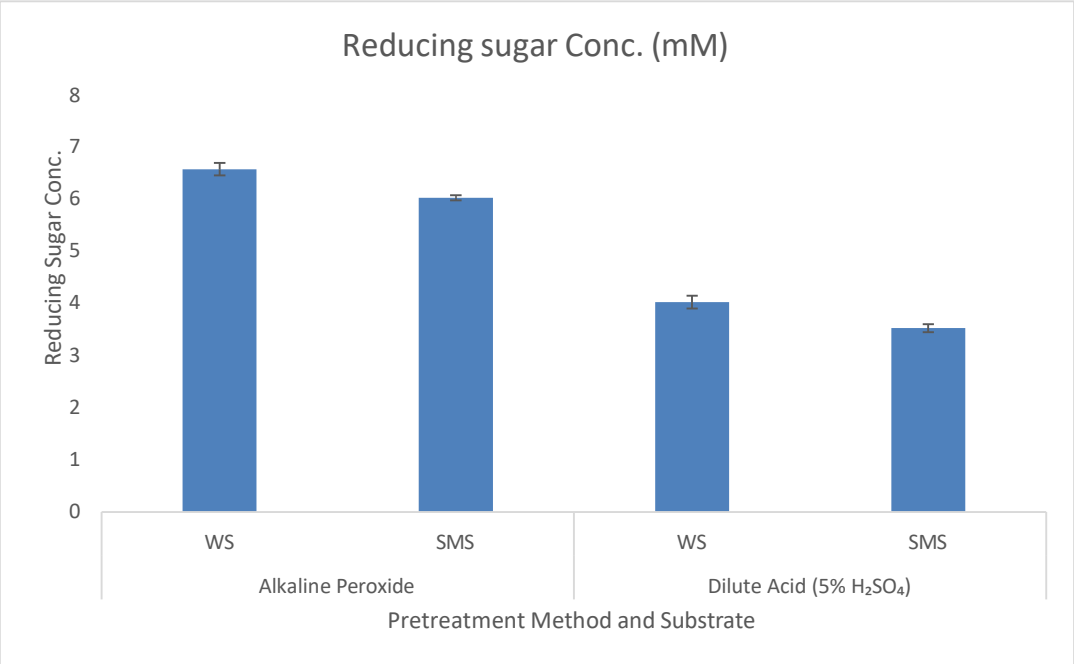


Fig. 22: Pretreatment Optimization for Method Selection

(A) SMS Acid pretreatment (B)WS Acid pretreatment (C) SMS Alkaline pretreatment (D)WS Alkaline Pretreatment

Table 12: Reducing Sugars Obtained from Wheat Straw (WS) and Spent Mushroom Substrate (SMS)

Pretreatment Method	Substrate	Reducing sugar concentration (mM)
Alkaline Peroxide	WS	6.565± 0.12
	SMS	6.015± 0.049
Dilute Acid (5% H_2SO_4)	WS	4.02± 0.125
	SMS	**3.52±0.075**



Graph 2: Effect of Pretreatment Methods on Reducing Sugar Concentration(mM) in Wheat Straw (WS) and Spent Mushroom Substrate (SMS)

4.3 Pretreatment

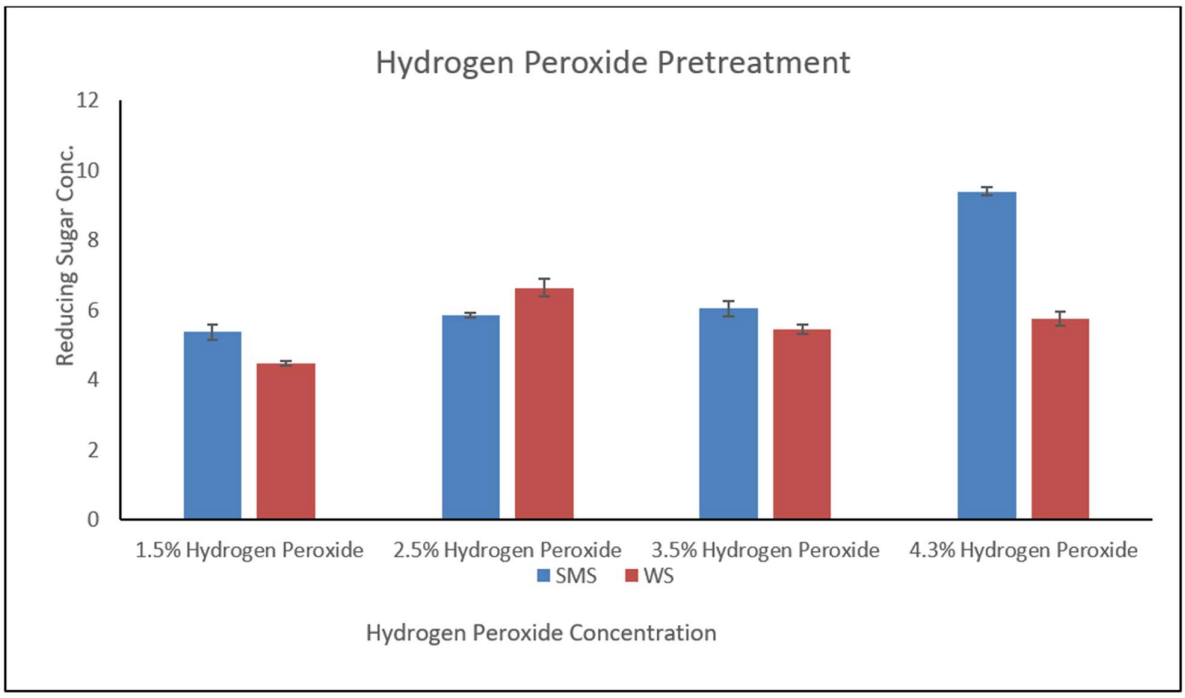
To evaluate the effectiveness of the alkaline peroxide pretreatment method, the production of reducing sugars was measured before and after the pretreatment. This method is critical as it helps to break down the complex lignocellulosic structure of the substrates, making the sugars more accessible for enzymatic hydrolysis and subsequent fermentation. The pretreatment process was explored over various durations to determine the optimal time frame for maximizing sugar yield. Additionally, the study investigated the influence of different concentrations of hydrogen peroxide (H_2O_2) on the efficiency of the pretreatment.

The findings revealed that the concentration of H_2O_2 played a significant role in enhancing sugar yield from both substrates. For ground wheat straw, the optimal concentration of hydrogen peroxide was found to be 2.15% (v/v), at which point a substantial increase in sugar release was observed. On the other hand, spent mushroom substrate required a higher concentration of 4.3% (v/v) H_2O_2 to achieve maximum sugar yield. These results indicate that while both substrates can be effectively pretreated with alkaline peroxide, they respond differently to varying concentrations of H_2O_2due to their distinct compositions and structural characteristics.

Consequently, for the remainder of the experiments, a hydrogen peroxide concentration of 2.15% (v/v) was adopted for wheat straw, and 4.3% (v/v) was selected for SMS. These optimized pretreatment conditions were crucial for ensuring high levels of sugar release, which is a key determinant in the efficiency of bioethanol production. The differential response to H_2O_2 concentration highlights the importance of tailoring pretreatment protocols to the specific characteristics of each substrate to maximize bioethanol yield.

Table 13: Reducing Sugar Concentration from different percentages of Hydrogen peroxide Pretreatment

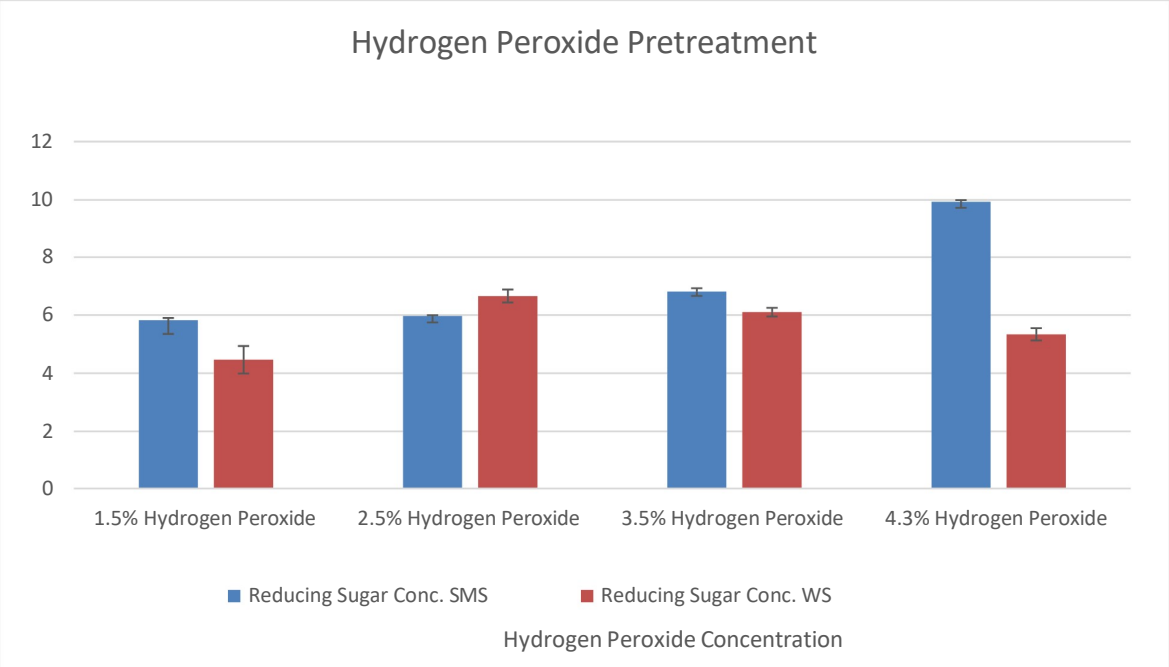
Hydrogen Peroxide Concentration	SMS (Reducing Sugar Conc. in mM)	WS (Reducing Sugar Conc. In mM)
1.5% Hydrogen Peroxide	5.377mM ± 0.20	4.480mM ± 0.07
2.5% Hydrogen Peroxide	5.854mM ±0.07	6.637mM ±0.24
3.5% Hydrogen Peroxide	6.044mM ±0.22	5.454mM ±0.12
4.3% Hydrogen Peroxide	9.404mM ± 0.121	5.763mM ±0.19



Graph 3: Effect of Varying Hydrogen Peroxide Concentrations on Biomass Pretreatment

Table 14: Reducing Sugar Concentration from different percentages of Hydrogen peroxide Pretreatment (Repeat)

HydrogenPeroxide Concentration	SMS(Reducing Sugar Conc. in mM)	WS (Reducing Sugar Conc. in mM)
1.5%	5.8283 ± 0.074	4.46445 ± 0.47
2.5%	5.97425 ±0.025	6.6617 ±0.22
3.5%	6.811725 ±0.11	6.103825 ±014
4.3%	9.9415 ±0.053	5.34027 ±0.21



Graph 4: Effect of Varying Hydrogen Peroxide Concentrations on Biomass Pretreatment (Repeat)

A recommended pretreatment duration for lignocellulosic biomass typically ranges between 3 and 24 hours, though evidence suggests that 24 hours yields the most favorable outcomes. While a slight increase in sugar concentration has been observed at the 48-hour mark, no significant additional gains are seen beyond that point. This indicates that extending the pretreatment period beyond 24 hours may not offer further benefits. The differences in substrate composition-such as ground wheat straw (WS) and spent mushroom substrate (SMS)-are significant, both before and after pretreatment. These variations are particularly noticeable in the levels of cellulose, hemicellulose, lignin, and other components.

4.4 Effect of Alkaline Peroxide Pretreatment on Biomass Composition

The lignocellulosic biomass, i.e., wheat straw and spent mushroom substrate, contains glucose, xylose, and lignin, and the pretreatment process significantly impacts these components. Prior to pretreatment, the levels of these components were considered 100%. Following pretreatment, glucose content increased to 127% in wheat straw and 137% in SMS, reflecting improved sugar availability due to the breakdown of complex carbohydrates. Similarly, xylose content rose to 115% in wheat straw and 128% in SMS, indicating enhanced hemicellulose hydrolysis. In contrast, lignin content decreased markedly, dropping to 57% in wheat straw and 58% in SMS, demonstrating the pretreatment's effectiveness in lignin removal. These changes emphasize the role of pretreatment in increasing fermentable sugar release while reducing lignin, making the biomass more suitable for biofuel production

4.5 Quantification of Sugar and Lignin:

4.5.1 Reducing Sugar Estimation Using the DNS Method

The DNS (3,5-Dinitrosalicylic Acid) assay was employed to measure reducing sugars in both pretreated and non-pretreated biomass hydrolysates. Initially, the hydrolysate samples were diluted 20 times with distilled deionized water (DDW) to guarantee that the sugar concentration was within the detectable limits of the assay. A calibration curve was created utilizing glucose standards made from a 20 mM stock solution, with final concentrations varying from 0 to 5 mM attained via serial dilutions. Every diluted sample and standard solution was placed into labeled test tubes, after which 150 µl of DNS reagent was added and mixed well. The tubes were subsequently immersed in a boiling water bath for 5 minutes, enabling the reducing sugars to interact with the DNS reagent and create a colored complex. Once cooled to room temperature, the absorbance of every sample was assessed at **540 nm** using a spectrophotometer, with a blank sample (0 mM glucose standard with DNS reagent) utilized for calibration. The absorbance readings of the glucose standards were graphed to create a calibration curve ($y = mx + b$), which was subsequently utilized to ascertain the concentration of reducing sugars in the hydrolysate samples. The ultimate sugar concentration measurements were modified according to the dilution factor, guaranteeing precise quantification. Data analysis included contrasting the reducing sugar levels in both pretreated and non-pretreated samples, and for experiments carried out at varying time intervals (e.g., 24 and 48 hours), monitoring changes in sugar concentration to assess the impact of pretreatment duration on sugar release.

4.6 Enzymatic Hydrolysis of SMS vs. Wheat Straw

The enzymatic hydrolysis of biologically pretreated Spent Mushroom Substrate (SMS) resulted in significantly higher reducing sugar yields compared to conventional wheat straw. This observation aligns with the hypothesis that the partial delignification mediated by oyster mushroom (*Pleurotus ostreatus*) during its growth phase enhances the digestibility of SMS. Quantitative analysis using the DNS method showed that SMS hydrolysates yielded up to 28– 35% more reducing sugars than untreated wheat straw when subjected to identical hydrolytic conditions.

The superior performance of SMS can be attributed to the residual ligninolytic enzymatic action exerted during the mushroom cultivation phase. Enzymes such as laccases and peroxidases degrade complex aromatic rings in lignin, reducing its shielding effect around polysaccharide chains. This partial degradation likely introduced structural alterations such as increased porosity, lower crystallinity, and disruption of hemicellulose-lignin associations, facilitating greater enzymatic access during saccharification.

Furthermore, the synergistic enzymatic system from *Aspergillus niger* and *Trichoderma viride* played a vital role in converting cellulose and hemicellulose into monomeric sugars. Particularly, *A. niger*'s β-glucosidase activity ensured the effective conversion of cellobiose into glucose, reducing product inhibition and driving the reaction forward. The extended saccharification time (up to 120 hours) allowed for near-complete conversion of accessible polysaccharides in SMS.

These findings are consistent with earlier reports demonstrating the efficacy of white-rot fungal preconditioning in improving hydrolysis efficiency (Bak et al., 2009; Jonathan & Fasidi, 2001). The dual approach-biological and enzymatic-significantly enhances sugar yield without necessitating severe chemical pretreatments, thereby lowering overall process energy and reagent requirements.

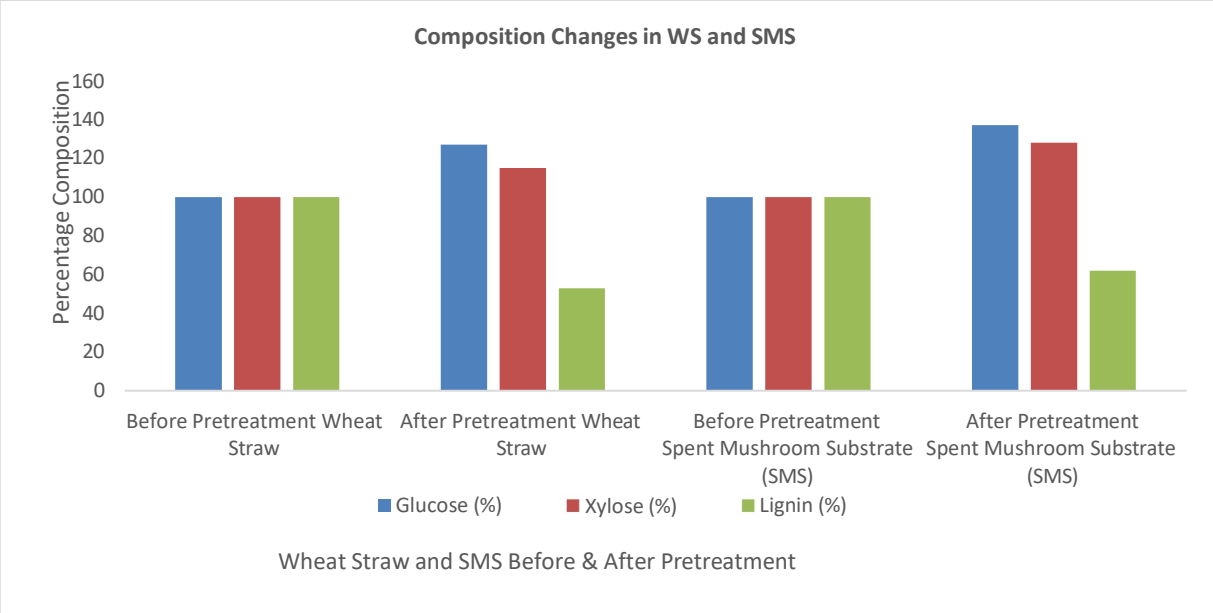
In conclusion, the incorporation of SMS not only supports sustainable biomass utilization but also offers an inherently pretreated substrate that improves saccharification efficiency. These advantages position SMS as a superior alternative or complementary feedstock to wheat straw in second-generation bioethanol production.

4.7 Lignin Quantification Using the Acetyl Bromide Spectrophotometric Method

Lignin quantification was performed using the Acetyl Bromide Spectrophotometric Method, a highly sensitive UV-Vis technique specifically suited for peroxide-pretreated lignocellulosic biomass. First, 5–10 mg of dried, pretreated biomass (wheat straw and spent mushroom substrate) was carefully weighed and transferred into a glass reaction vial. To ensure complete lignin solubilization, 1 mL of acetyl bromide (25% in glacial acetic acid) was added to the sample, and the reaction mixture was incubated at 50°C for 30 minutes. After incubation, the reaction mixture was allowed to cool to room temperature, and the volume was adjusted using acetic acid to achieve a standardized dilution. The absorbance of the resulting solution was measured at **280 nm** using a UV-Vis spectrophotometer, with appropriate blank and control samples to ensure accuracy (Barnes et al, 2024). The lignin concentration in the pretreated samples was determined by comparing the absorbance values with a standard calibration curve prepared using known lignin concentrations. This method allowed for precise quantification of lignin removal, which is essential for evaluating the efficiency of alkaline peroxide pretreatment in improving enzymatic hydrolysis and bioethanol production.

Table 15: Impact of Alkaline Peroxide Pretreatment on Glucose, Xylose, and Lignin Levels in Wheat Straw and Spent Mushroom Substrate

Substrate	Glucose (%)	Xylose (%)	Lignin (%)
Before Pretreatment Wheat Straw	100	100	100
After Pretreatment Wheat Straw	**127 ±0.01**	**115 ±0.011**	**53 ±0.25**
Before Pretreatment Spent Mushroom Substrate (SMS)	100	100	100
After Pretreatment Spent Mushroom Substrate (SMS)	**137 ±0.21**	**128 ±0.23**	**62 ±0.26**



Graph 5: Changes in WS and SMS Composition Before and After Pretreatment

4.8 Reducing Sugar Concentration in Both Substrates

The observed differences in sugar concentrations between SMS and WS highlight the importance of substrate variability, which can impact the overall efficiency of bioethanol production. This underscores the necessity of optimizing pretreatment based on the specific characteristics of each substrate to enhance bioethanol yield and process efficiency.

To accurately analyze the concentration of reducing sugars in pretreated and non-pretreated biomass samples, the 3,5-dinitrosalicylic acid (DNS) method was employed. The DNS assay is widely recognized for quantifying reducing sugars, with glucose used as the standard for calibration. Below is a detailed, step-by-step procedure of the assay to ensure proper execution, which can be used for inclusion in a thesis or scientific paper.

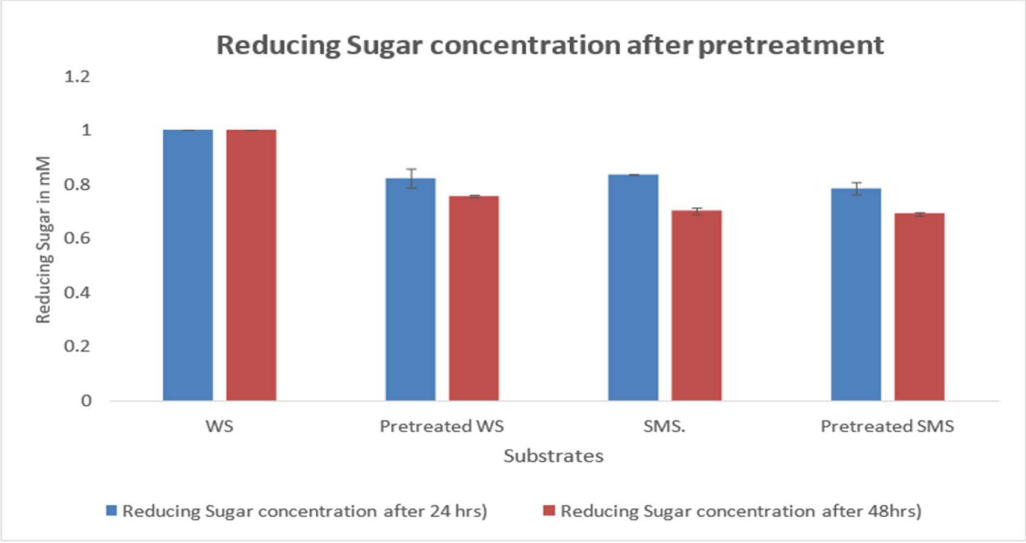
To evaluate the effect of pretreatment on SMS and WS, glucose concentrations were measured after both 24 and 48 hours of pretreatment. At the 24-hour mark, the pretreated SMS exhibited a glucose concentration of 5.135 mM, while the untreated SMS had a slightly higher concentration of 5.637 mM. Similarly, the pretreated WS showed a glucose concentration of 5.304 mM, whereas the untreated WS had a higher concentration of 6.752 mM. These results suggest a decrease in glucose concentration in both SMS and WS samples following pretreatment, compared to their untreated counterparts.

After 48 hours of pretreatment, the pretreated SMS showed a slight increase in glucose concentration, reaching 6.865 mM, although this was still lower than the untreated SMS, which had a concentration of 6.966 mM. On the other hand, the pretreated WS exhibited a glucose concentration of 7.493 mM, which was again lower than the untreated WS, which showed a concentration of 9.927 mM.

Overall, these results indicate that the glucose concentration in both pretreated SMS and WS decreased after 48 hours compared to the untreated samples. Additionally, only a slight increase in sugar levels was observed between 24 and 48 hours of pretreatment. This suggests that 24 hours of pretreatment is the optimal duration for maximizing sugar release, as extending the time beyond that point does not significantly increase sugar yield. The study highlights the importance of tailoring pretreatment duration based on substrate characteristics to achieve the best results in terms of sugar accessibility and overall bioethanol production efficiency.

Table 16: Concentration of Reducing Sugar after 24 and 48 hours of Pretreatment

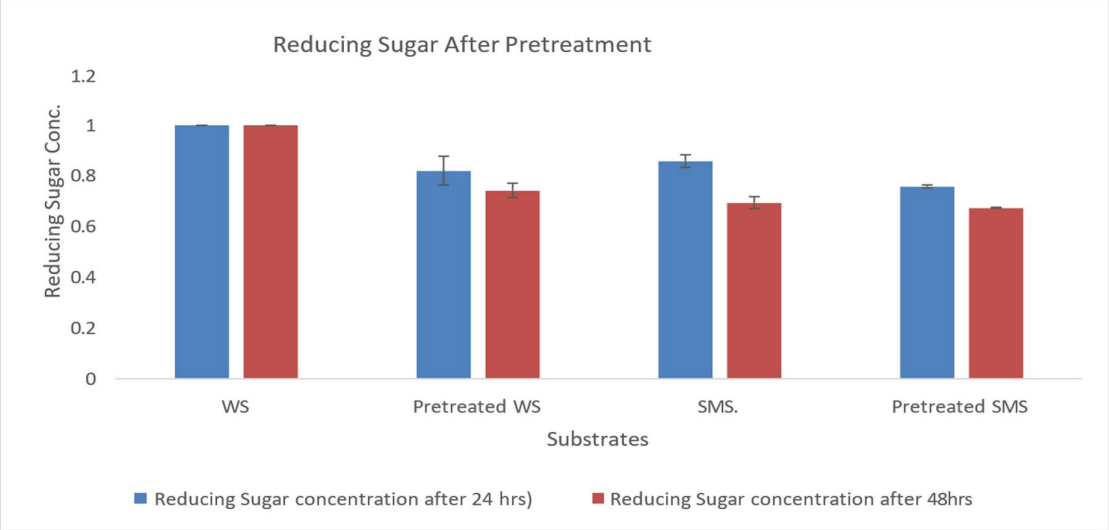
Samples	Reducing Sugar concentration(mM) after (24hrs)	Reducing Sugar concentration(mM) after (48hrs)
WS	1 ± 0	1 ± 0
Pretreated WS	0.820587001 ± 0.352	0.754859518 ± 0.004
SMS	0.834352368 ±0.004	0.701862976 ± 0.011
Pretreated SMS	0.782983252 ± 0.022	0.691686229 ± 0.006



Graph 6: Concentration of Reducing Sugar after 24 and 48 hours of Pretreatment

Table 17: Concentration of Reducing Sugar after 24 and 48 hours of Pretreatment (Repeat)

Samples	ing Sugar concentration after (24hrs)	ing Sugar concentration after (48hrs)
WS	1 ± 0	1 ± 0
Pretreated WS	0.820171724 ± 0.057	0.742023061 ± 0.028
SMS	0.858252258 ± 0.024	0.693721491 ± 0.023
Pretreated SMS	0.757709612 ± 0.005	0.673337076 ± 0.001



Graph 7: Concentration of Reducing Sugar after 24 and 48 hours of Pretreatment (Repeat)

Pretreatment is essential for breaking down lignin, as it improves the accessibility of biomass for enzymatic hydrolysis. The choice of pretreatment significantly influences the process. Similarly, efficient saccharification during hydrolysis is key in determining the feasibility and cost-effectiveness of producing bioethanol from lignocellulosic biomass. Together, these steps are crucial for optimizing biomass conversion and ensuring a practical approach to bioethanol production.

4.9 Saccharification

In this study, the saccharification process was investigated to understand how different time durations and pretreatments influence the release of reducing sugars from biomass, specifically Wheat Straw (WS) and Spent Mushroom Substrate (SMS). Saccharification,

which typically takes 72 to 120 hours, involves breaking down the biomass into fermentable sugars essential for bioethanol production. The DNS (3,5-dinitrosalicylic acid) reagent assay was used to quantify the reducing sugars, with the objective of determining the optimal saccharification time by observing when sugar levels stabilize.

After 24 hours of saccharification, the Wheat Straw without saccharification (WS) released 5.0339 mM in one dataset and 5.135 mM in the other, showing minimal variation. Similarly, WS+ Saccharification yielded 9.8519 mM in one case and 9.802 mM in the other. Pretreated Wheat Straw without saccharification (Pre WS) released 7.9448 mM in one dataset and 7.695 mM in the other. Meanwhile, Pre WS + Saccharification produced 9.2497 mM in one and 9.316 mM in the other.

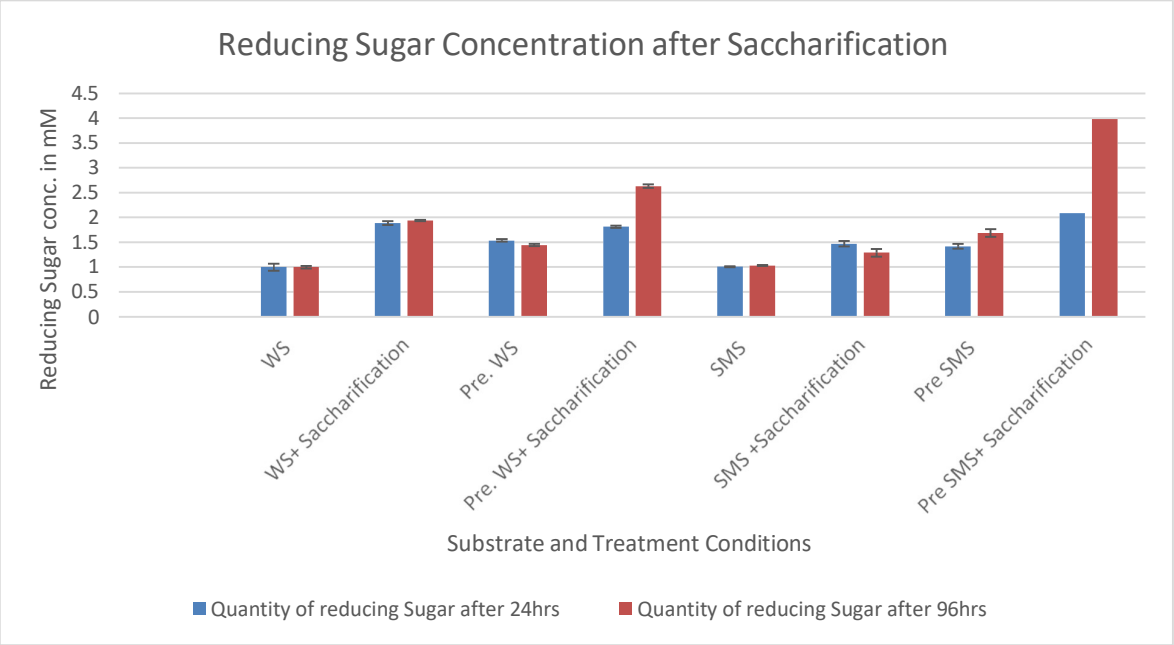
For Spent Mushroom Substrate (SMS), the untreated, non-saccharified SMS resulted in 5.1844 mM in one dataset and 5.3675 mM in the other, while SMS + Saccharification yielded 7.4429 mM and 7.8255 mM, respectively. Pretreated SMS without saccharification (Pre SMS) released 7.4429 mM in one case and 7.695 mM in the other. The highest reducing sugar release was observed in Pre SMS + Saccharification, with values of 10.7553 mM in one dataset and 10.7445 mM in the other.

After 96 hours, the untreated Wheat Straw (WS) yielded 6.0376 mM in one dataset and 6.3285 mM in the other, while WS + Saccharification produced 11.8595 mM in one and 11.985 mM in the other. Pre WS without saccharification led to 8.6474 mM in one case and 10.3895 mM in the other. For Pre WS + Saccharification, values were 16.0251 mM and 16.338 mM, confirming a significant increase in reducing sugar production.

For Spent Mushroom Substrate, the non-saccharified SMS resulted in 6.4391 mM in one dataset and 6.4055 mM in the other, while SMS + Saccharification produced 7.8444 mM and 8.1215 mM, respectively. Pre SMS without saccharification yielded 10.6550 mM in one dataset and 10.3895 mM in the other. Finally, Pre SMS + Saccharification showed the highest release of reducing sugar, with values of 24.5069 mM in one dataset and 24.293 mM in the other.

Table 18: Data of Saccharification After 24 and 96 hrs

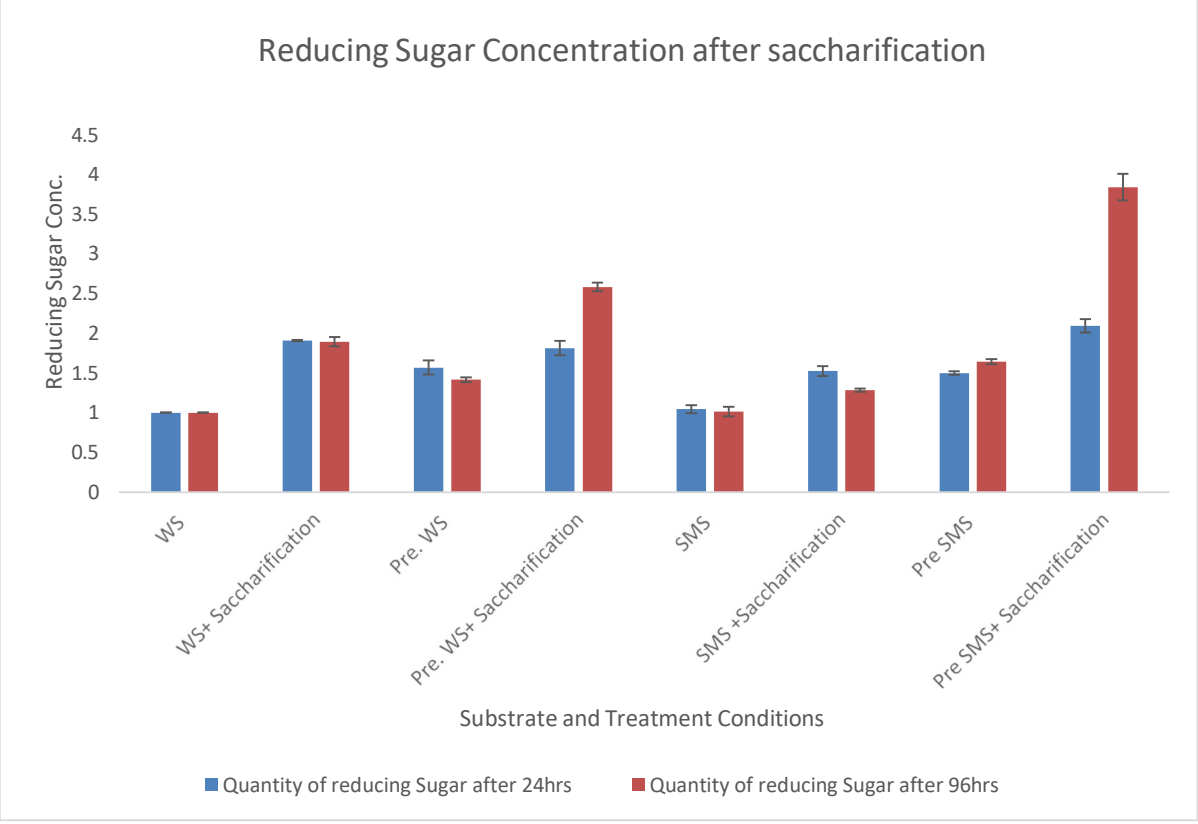
Sample	Quantity of reducing Sugar after 24hrs.	Quantity of reducing Sugar after 96 hrs.
WS	1 ± 0	1 ± 0
WS+ Saccharification	1.887309969 ± 0.06	1.939298567 ± 0.024
Pre. WS	1.537695981 ± 0.04	1.443706132 ± 0.011
Pre. WS+ Saccharification	1.812144642 ± 0.02	2.631650852 ± 0.022
SMS	1.008289774 ± 0.02	1.033267862 ± 0.033
SMS +Saccharification	1.470984829 ±0.007	1.29051886 ± 0.008
Pre+SMS	1.423326334 ±0.055	1.688482905 ± 0.076
Pre SMS+ Saccharification	2.088245206 ±0.048	3.983243935 ± 0.075



Graph 8: **Quantity of reducing sugar after Saccharification**

Table 19: Data of Saccharification After 24 and 96 hrs (Repeat)

Sample	Quantity of reducing Sugar after 24 hrs.	Quantity of reducing Sugar after 96 hrs.
WS	1 ± 0	1 ± 0
WS+ Saccharification	1.908860759 ± 0.007	1.8938137 ± 0.05
Pre. WS	1.568062317 ± 0.88	1.415027258 ±0.02
Pre. WS + Saccharification	1.814216164 ± 0.089	2.58165442 ±0.05
SMS	1.045277507 ± 0.05	1.01216718 ±0.06
SMS + Saccharification	1.523953262 ± 0.06	1.283321482 ±0.02
Pre SMS	1.498539435 ± 0.02	1.641700245 ±0.03
Pre SMS + Saccharification	2.092405063 ± 0.083	3.838666351 ±0.16



Graph 9: **Quantity of reducing sugar after Saccharification (Repeat)**

The results clearly demonstrate the significant impact of pretreatment and extended saccharification on the release of reducing sugars. After 96 hours, pretreated and saccharified samples produced the highest concentrations of reducing sugars, indicating that pretreatment plays a key role in improving saccharification efficiency. Among the substrates tested, Spent Mushroom Substrate (SMS) showed a greater potential for bioethanol production than Wheat Straw (WS) based on higher sugar release levels.

Additionally, the findings suggest that 96 hours is the optimal duration for saccharification, as sugar yields stabilize after this period, making further extension unnecessary. These observations emphasize the importance of both pretreatment and saccharification duration in optimizing bioethanol production from lignocellulosic biomass.

4.10 Fermentation

Table 5 provides a detailed overview of the bioethanol production outcomes when alkaline peroxide-pretreated and enzyme-saccharified wheat straw (WS) and spent mushroom substrate (SMS) were fermented using *Saccharomyces cerevisiae*. Enzymes for saccharification were derived from *Aspergillus niger* and *Trichoderma viride*. Both WS and SMS underwent identical processes of pretreatment, saccharification, and fermentation, ensuring consistency in experimental conditions.

Ethanol concentration in the distilled samples was measured using the spectrophotometric dichromate reagent method, which involved a simple distillation process in a hot water bath. Following distillation, the ethanol sample was mixed in equal proportions with dichromate reagent, sealed, and incubated at 37± 1°C for 10 minutes. After incubation, the mixture was diluted with water at the same temperature, stirred thoroughly, and the ethanol concentration was determined using a spectrophotometer set to 600 nm. For spectrophotometers lacking a concentration mode, ethanol content (% v/v at 15.56°C) was calculated using the formula: % EtOH = (A unknown/A std) × 18.51, following the method described by Pilone et al. (1985).

Several experimental setups were conducted to evaluate bioethanol yields under varying conditions. In the first setup, WS underwent pretreatment and fermentation, yielding 3.48% ethanol. Its negative control, which lacked pretreatment, produced only 0.41%. A second setup, which omitted the pretreatment step but included saccharification before fermentation, yielded 4.09% ethanol, with the corresponding negative control generating 0.68%. Another configuration, where WS was pretreated, saccharified, and fermented, resulted in a higher bioethanol yield of 10.63%, with its negative control producing

2.67%. In one experiment, WS was mixed with SMS, pretreated, and fermented, producing 5.8% ethanol. The control for this setup, which omitted the fermentation step, yielded only 0.93%.

Following the trials with WS, SMS was tested independently to assess its effect on ethanol production under different conditions. In the first experiment involving SMS, the substrate was autoclaved, pretreated, and fermented. The bioethanol concentration for this sample was 5.58%, while its negative control (autoclaved and pretreated but not fermented) yielded 2.95%. In the second setup, SMS was autoclaved and saccharified before fermentation, leading to 3.85% ethanol, with its negative control yielding 1.58%. A third configuration, where SMS was autoclaved, pretreated, saccharified, and fermented, produced the highest ethanol yield of 15.11%, while the negative control generated 3.6%.

In the final experiment, SMS was pretreated, saccharified, and fermented without autoclaving. This sample yielded 3.82% ethanol, while the control (pretreated but not fermented) produced 1.12%.

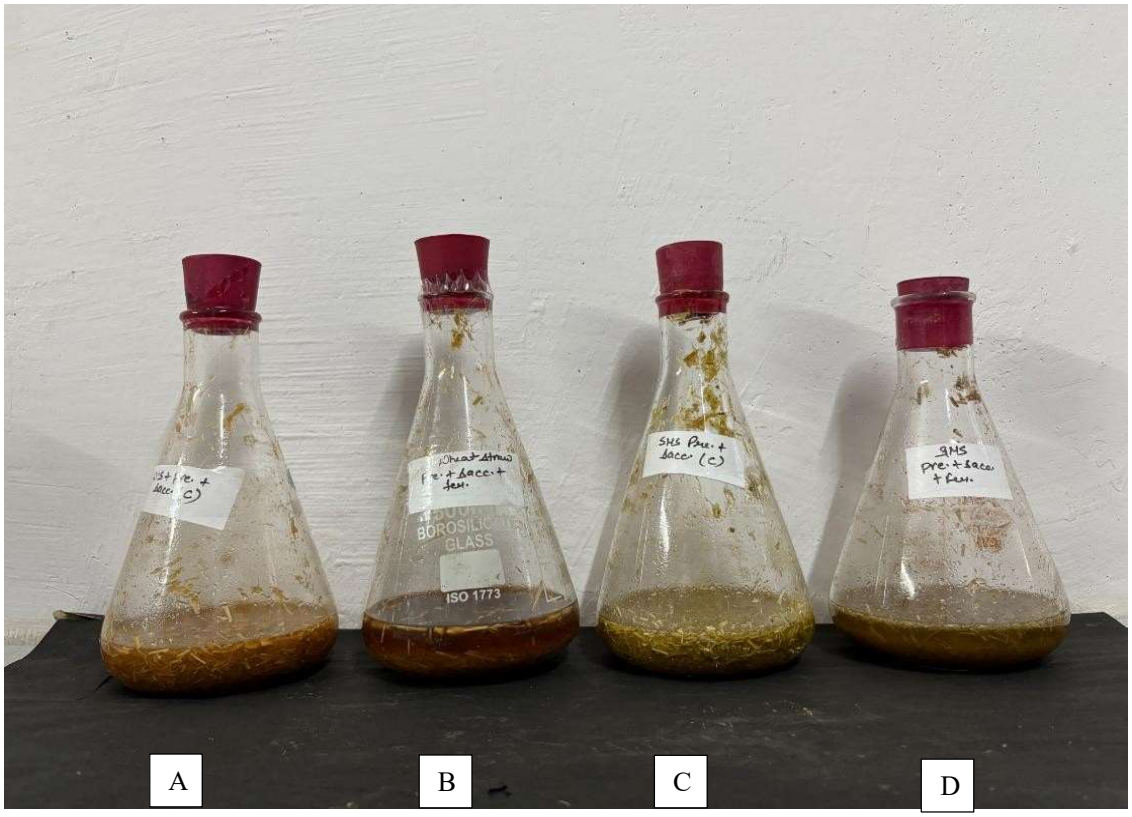


Fig. 23: WS & SMS Sample after Fermentation

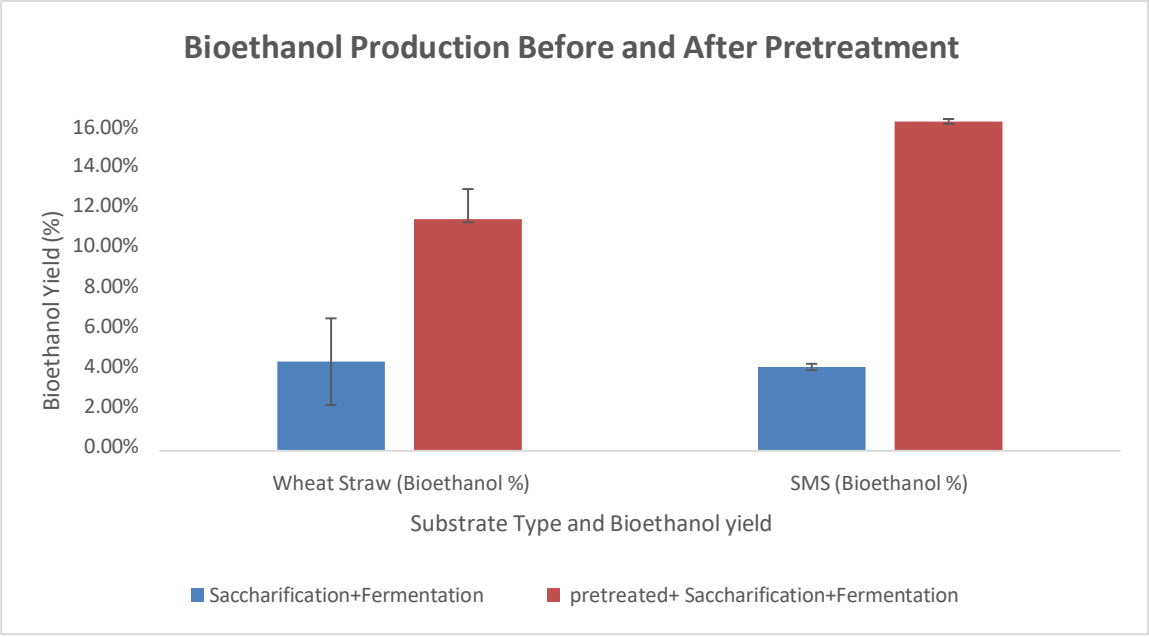
(A) WS + pretreatment + saccharification (control), (B) WS + pretreatment+ saccharification + fermentation, (C) SMS + pretreatment+ saccharification (control), (D) SMS+ pretreatment+ saccharification + fermentation

The experiment here clearly states that the pretreatment process shows improved bioethanol production in both WS and SMS. Before the process of pretreatment, WS subjected to saccharification and fermentation produced only 4.09% bioethanol, while after pretreatment, the outcome reached 10.63%. Similar results were observed in SMS, where the bioethanol production before pretreatment was 3.85% and following pretreatment, it became 15.11%.

This increase in bioethanol yield highlights the role of the alkaline peroxide pretreatment method in breaking down lignocellulosic biomass, enhancing the availability of fermentable sugars for enzymatic hydrolysis and fermentation.

Table 20: Impact of Pretreatment on Bioethanol Yield

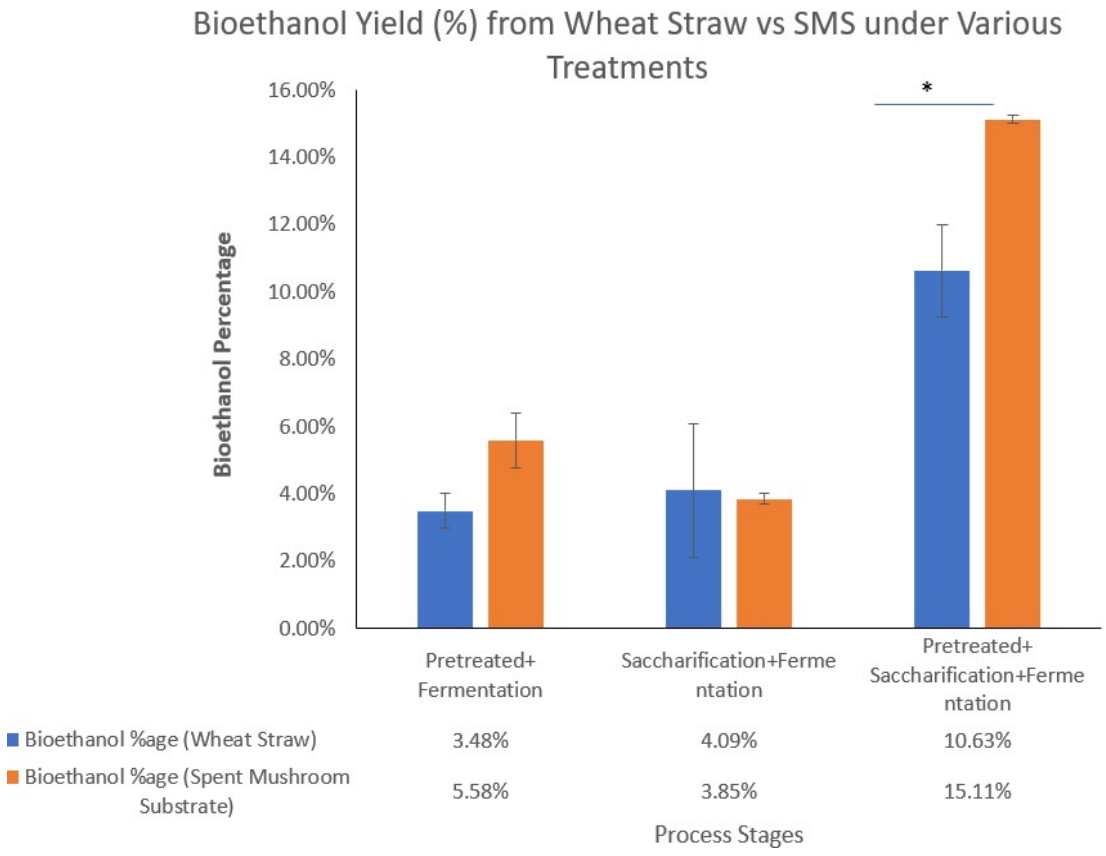
Treatment	Saccharification + Fermentation	Pretreatment+Saccharification + Fermentation
Wheat Straw (Bioethanol %)	4.09% ± 0.019	10.63% ± 0.013
SMS (Bioethanol %)	3.85% ± 0.0015	15.11% ± 0.001



Graph 10: Bioethanol Production Before and After Pretreatment

Table 21: Total Bioethanol Production via Different Experiments

Sample Description	Pretreatment	Scarification	Fermentation	Bioethanol Yield (%
Wheat straw (WS)	Yes	No	yes	**3.48 ± 0.005**
	No	Yes	Yes	**4.09 ±0.019**
	Yes	Yes	Yes	**10.63 ±0.137**
WS mixed with SMS	Yes	Yes	Yes	**5.8 ±0.002**
Spent Mushroom Substrate (Autoclaved)	yes	No	Yes	**5.58 ±0.008**
	No	Yes	Yes	**3.85 ±0.001**
	Yes	Yes	Yes	**15.11 ±0.001**



Graph 11: Graphical Representation of Bioethanol Production

These results highlight the critical role that substrate type and processing conditions play

in maximizing bioethanol yields. The combination of pretreatment, saccharification, and fermentation proved to be essential for enhancing the fermentability of both WS and SMS. The higher ethanol yield from SMS compared to WS suggests that SMS, when properly treated, is a more efficient feedstock for bioethanol production. Nonetheless, WS remains a valuable resource, especially considering its abundance and the substantial yield it can produce with comprehensive processing.

4.10.1 Optimization of Fermentation Conditions Using Response Surface Methodology (RSM)

To enhance ethanol production from spent mushroom substrate (SMS), a targeted optimization study was conducted using a simplified form of Response Surface Methodology (RSM). RSM is a well-established statistical approach used in experimental design to identify the optimal conditions for a process by evaluating the effects of multiple variables and their interactions. In this case, three key parameters that directly influence fermentation performance were selected for optimization: pH, temperature, and incubation time.

These three factors were chosen based on their known impact on the metabolic activity of *Saccharomyces cerevisiae*; the yeast strain used in the fermentation process. Small fluctuations in pH, temperature, or fermentation duration can significantly affect the efficiency of sugar utilization and the rate of ethanol production. Therefore, understanding how each of these factors influences the outcome was essential to improving bioethanol yield.

Initial observations and past laboratory trials had identified one specific combination-pH 5.0, a temperature of 30 °C, and a fermentation time of 48 hours-as the most favorable condition for ethanol production from SMS. Under this setting, the fermentation yielded 15.11% ethanol, the highest output recorded at the time. This condition was chosen as the baseline or central point for the optimization study.

To build on these findings, six additional fermentation experiments were designed where one or more of the three variables were altered within practical ranges:

- pH was varied from 4.0 to 6.0
- The temperature was adjusted between 25 °C and 35 °C
- Incubation time was modified from 24 to 72 hours

In each run, only one or two parameters were changed at a time, while the others were held constant. This approach simplified the experimental design while still allowing for meaningful insights into how each variable-and combinations of variables-affect ethanol production. This method is sometimes referred to as a "one-factor-at-a-time within RSM framework", which balances experimental efficiency with analytical rigor.

To ensure that the results reflected the true effects of these parameters, all other fermentation conditions were kept constant across the experiments. This included using the same optimized fermentation medium (based on prior media formulation studies), consistent inoculum volume, the same yeast strain (*S. cerevisiae*), and identical agitation and aeration conditions.

After each fermentation run, ethanol yield was measured and compared. This data was then analyzed to determine which specific set of conditions produced the highest ethanol output, and how sensitive the process was to changes in pH, temperature, and time. The aim was to find a sweet spot-a combination of conditions that consistently led to high ethanol yield with minimal energy and time investment.

The use of this structured yet simplified RSM approach proved to be very effective. It allowed for a better understanding of how fermentation performance responds to small environmental changes, and provided a practical guide for scaling up or further refining the process. Ultimately, this method helped in fine-tuning the fermentation environment for SMS, maximizing ethanol yield without the need for an excessively large number of experiments or resources.

4.10.2 Results of Fermentation Optimization Using SMS

To identify the best conditions for ethanol production from spent mushroom substrate (SMS), a series of fermentation experiments were performed where the pH, temperature, and incubation time were carefully varied. A total of five experimental runs were conducted, each designed to explore how small changes in these parameters affect ethanol yield. The values were chosen to surround a central condition-pH 5.0, temperature 30 °C, and fermentation time of 48 hours-which had been identified through literature review and preliminary trials as a potentially ideal setup for yeast fermentation.

This central combination produced the highest ethanol yield, reaching 15.11%, which marked it as the most effective among all the tested conditions. To evaluate its reliability, two lower and two higher values were tested for each of the three parameters, essentially forming a small “Response surface” around the central point.

The aim was to see whether these slightly altered conditions could produce equal or better yields, or if they negatively impacted fermentation.

The results clearly showed that deviating from the central values-either by lowering or increasing the pH, temperature, or duration-consistently led to a decrease in ethanol output. For example, reducing the pH to 4.0 or increasing it to 6.0 caused a noticeable decline in fermentation efficiency, likely due to unfavorable conditions for yeast activity.

Similarly, temperatures lower than 30 °C may have slowed down metabolic activity, while higher temperatures could have stressed the yeast cells. Extending the fermentation time beyond 48 hours also showed diminishing returns, with longer durations not translating to increased ethanol yield-possibly due to sugar depletion or the onset of inhibitory by-products.

The regression model used in this simplified form of Response Surface Methodology (RSM) was successful in predicting the trend observed experimentally. The model showed good alignment with the actual ethanol yields measured in the lab, suggesting that this focused optimization strategy was sufficient to accurately capture the interaction between the tested variables.

Overall, the data strongly confirmed that the combination of pH 5.0, 30 °C, and 48 hours represents the most favorable conditions for ethanol production from SMS under batch fermentation using *Saccharomyces cerevisiae*. These conditions provided a well-balanced environment that supported robust yeast metabolism, efficient sugar utilization, and minimal formation of inhibitory by-products.

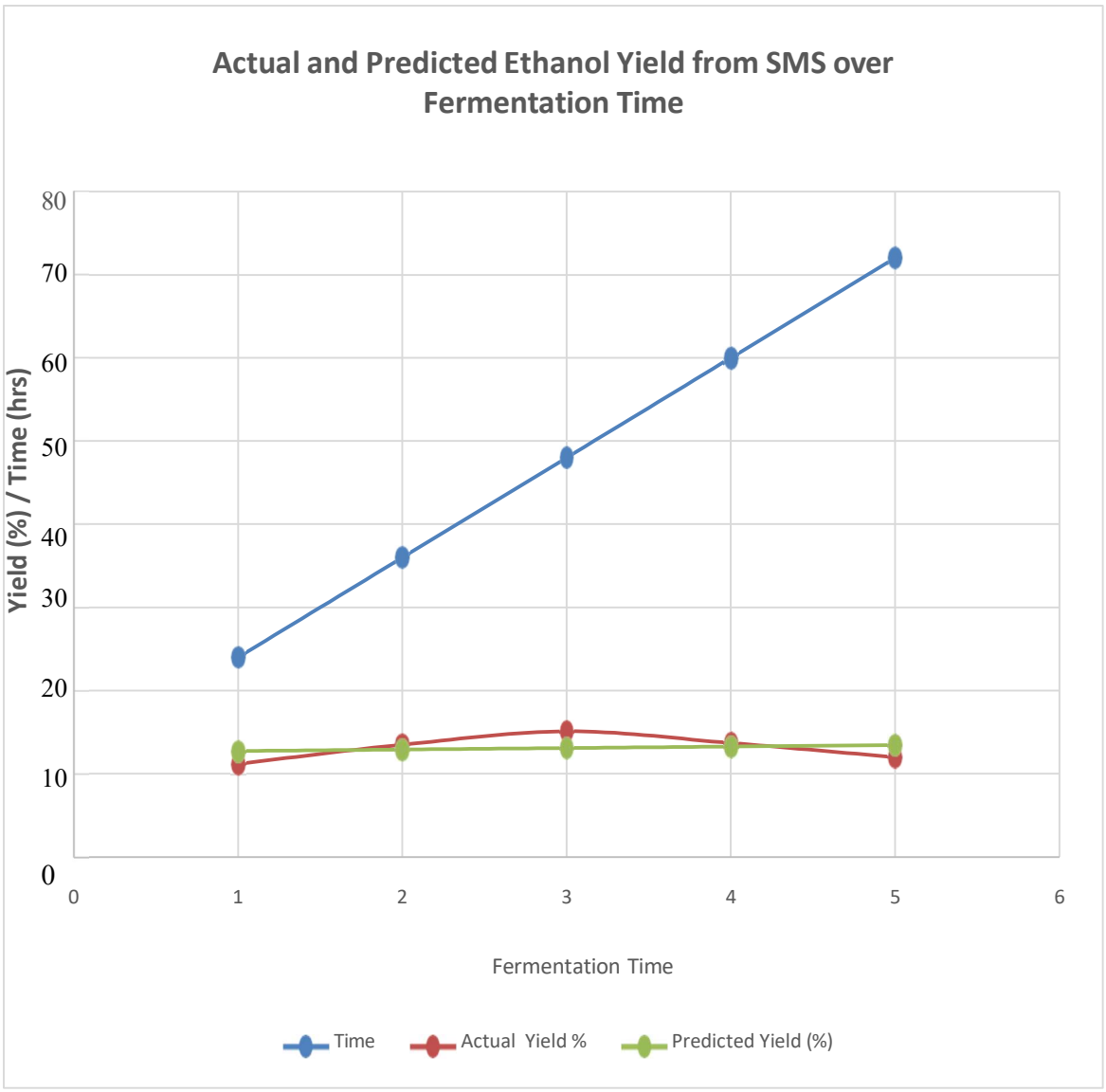
By avoiding excessive supplementation, extreme pH shifts, or unnecessarily long fermentation times, this optimized setting also supports economic feasibility and process efficiency, both of which are crucial for potential scale-up. This targeted optimization not only validated the selected parameters but also demonstrated the value of applying simplified RSM to fermentation process development, even with limited experimental runs.

The findings from this study underscore the importance of optimizing pretreatment and saccharification steps for both substrates to unlock their full bioethanol production potential. Additionally, the significant differences observed between untreated and treated samples indicate that refining these methods could lead to further improvements in bioethanol yields. Moving forward, continued exploration of these processing techniques will be vital in enhancing the viability of WS and SMS as renewable energy

sources, contributing not only to sustainable biofuel production but also to the reduction of agricultural waste and environmental impact.

Table 22: Effect of pH, Temperature, and Fermentation Time on Ethanol Yield in SSF Process

pH	Temp (°C)	Time (h)	Actual Ethanol Yield (%)	Predicted Yield (%)
4.0	25.0	24	11.20	12.74
4.5	27.5	36	13.50	12.92
5.0	30.0	48	15.11	13.10
5.5	32.5	60	13.70	13.28
6.0	35.0	70	12.0	13.46



Graph 12: Actual and Predicted Ethanol Yield from SMS over Fermentation Time

In the following sections, the discussion and conclusion will further explore the implications of these results, including a comparison of the environmental and economic benefits of WS and SMS in bioethanol production, as well as suggestions for future research directions.

CHAPTER 5

CONCLUSION AND FUTURE WORK

5.1Conclusion

The use of wheat straw (WS) and spent mushroom substrate (SMS) for bioethanol production presents significant advantages for both renewable energy generation and environmental management. Bioethanol, as a cleaner and sustainable fuel alternative, plays a vital role in reducing the dependency on fossil fuels and lowering greenhouse gas emissions. Utilizing agricultural residues like WS and SMS further enhances the sustainability of bioethanol production, transforming waste products into valuable energy sources.

Wheat straw, an abundant lignocellulosic residue, presently contributes to environmental pollution when burned post-harvest. https://en.wikipedia.org/wiki/Agricultural_waste Converting it into bioethanol can repurpose waste while reducing greenhouse gas emissions and particulate pollution. Meanwhile, SMS, a byproduct from mushroom cultivation (composted after approximately 5 kg of SMS per 1 kg of mushrooms),(Baptista et al., 2023) has often been underutilized or discarded with environmental consequences. (Leong et al. 2022)

Our study shows that SMS surpasses WS in ethanol yield: 15.11% compared to 10.63%, under identical processing conditions (Ursachi & Gutt, 2020). This matches literature indicating that SMS, thanks to the fungal degradation of lignin during cultivation, offers higher saccharification efficiency and better sugar release. Conversely, WS yields of 10–11% align with other studies reporting 74–99% (Leong et al. 2022**)** theoretical yield with effective pretreatment and saccharification (Talebnia et al. 2010). Both WS and SMS have shown promising results in bioethanol yield, with SMS particularly demonstrating high ethanol concentrations when processed through autoclaving, pretreatment, and saccharification. This makes these substrates ideal for bioethanol production, as they are readily available and offer competitive yields compared to other biomass feedstocks. Moreover, integrating WS and SMS into bioethanol production supports pollution management by reducing open burning of agricultural waste, minimizing landfill waste, and lowering the carbon footprint of energy generation**.** (Chen et al. 2022)

In addition to bioethanol, these substrates contribute to a circular economy by closing the loop on agricultural and industrial waste. Thus, WS and SMS not only serve as efficient feedstocks for bioethanol but also play a critical role in promoting environmental

sustainability and mitigating pollution through responsible biomass management.

In summary, all five objectives outlined for this study were successfully achieved. Suitable lignocellulosic biomasses were identified through the screening of five substrates, with wheat straw (WS) and spent mushroom substrate (SMS) selected based on compositional analysis, of which SMS demonstrated superior performance. Pretreatment protocols were optimized to achieve maximum delignification with minimal sugar loss, followed by hydrolysis optimization to enhance sugar recovery while limiting degradation products.

A suitable fermentation medium was formulated, and the fermentation process parameters were further optimized using Response Surface Methodology (RSM), resulting in maximal bioethanol yields of 15.11% from SMS and 10.63% from WS. These outcomes confirm that the research objectives were met comprehensively, providing a robust framework for efficient bioethanol production from lignocellulosic biomass.
Future studies should focus on optimizing the pretreatment and enzymatic hydrolysis steps to further enhance bioethanol yields from both WS and SMS, potentially making these agricultural residues more competitive in biofuel production.

5.2 Future of Bioethanol

The future of bioethanol production using substrates like wheat straw (WS) and spent mushroom substrate (SMS) holds significant promise, driven by the growing demand for renewable energy and the need for sustainable waste management. Both WS and SMS are abundant, low-cost agricultural and industrial residues that offer a dual benefit: reducing environmental waste and serving as valuable feedstocks for bioethanol production. As technology advances, the efficiency of converting these lignocellulosic materials into bioethanol is expected to improve, making the process more economically viable and environmentally friendly.

For WS and SMS, continued research into optimizing pretreatment and saccharification methods will be essential for maximizing ethanol yields. Enzyme technology, in particular, is expected to evolve, with more efficient and cost-effective enzymes becoming available to break down complex cellulose and hemicellulose into fermentable sugars. Additionally, advancements in microbial fermentation-such as the development of genetically modified strains of Saccharomyces cerevisiae or other bioethanol-producing microorganisms-could further enhance fermentation efficiency, increasing ethanol production from these substrates. (Topaloğlu et al. 2023). From a broader perspective, the overall future of bioethanol looks promising as governments and

industries continue to focus on reducing carbon emissions and transitioning to renewable energy sources. Second-generation bioethanol, produced from lignocellulosic materials like WS and SMS, is particularly appealing because it does not compete with food production, unlike first- generation bioethanol, which is derived from crops like corn and sugarcane. (Robak et al. 2018) With ongoing improvements in processing technologies, second-generation bioethanol could become a more dominant player in the biofuel market, contributing significantly to global energy needs while also supporting the circular economy. (Broda et al. 2022)

Additionally, bioethanol can play a key role in decarbonizing the transportation sector, especially as blending mandates increase around the world. Countries are increasingly adopting policies that mandate higher blends of ethanol in gasoline, driving up demand. Coupled with ongoing research into more efficient and sustainable production methods, the future of bioethanol looks promising, positioning it as a vital component of the global shift toward clean and renewable energy sources.

Converting spent mushroom substrate (SMS) and wheat straw (WS) into bioethanol not only valorizes abundant agricultural waste but also supports circular bioeconomy principles and sustainable energy production.

Studies demonstrate that hydrogen-peroxide pretreatment effectively breaks down lignin in SMS and WS, significantly boosting reducing sugar yields- SMS, in particular, shows superior delignification post-mushroom cultivation, making it a high-potential feedstock for ethanol conversion.

Review literature confirms that SMS averages 40–60% organic matter and contains ligninolytic enzymes, making it a versatile substrate for various bio-based applications, including bioethanol and biogas (Mahari et al. 2020), (Panaitescu et al. 2024).

Research on WS indicates that enzymatic pretreatment via white-rot fungi can degrade up to 80% of cellulose, increasing ethanol yield to over 10 g/L under optimized conditions (Ingrao et al. 2021), (Rusănescu et al. 2024).

Building on these strengths, combining oxidative pretreatment with fungal or enzymatic augmentation-especially using SMS-may improve lignocellulose accessibility and reduce processing severity. As global energy policies increasingly favor second-generation biofuels, feedstocks like WS and SMS offer the dual benefits of non-food competition and greenhouse gas mitigation(https://en.wikipedia.org/wiki/Cellulosic_ethanol). Moving forward, integrating SMS-based bioethanol production with lignin

valorization, enzyme recovery, and biogas co-generation can enhance economic returns and environmental sustainability-a promising step toward scalable, low-carbon biofuel systems.

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Example of Calculation for Obtaining Ethanol Yield (%)

Standard Ethanol (10–50% range)	
% Ethanol (Standard)	**Absorbance (Abs)**
10%	0.45
15%	0.675
18.51% (SRM)	**0.83295**
19%	0.855
20%	0.9
25%	1.125
30%	1.35
40%	1.8
50%	2.25
Composite value (Standard)	0.86265
SRM	**Standard Reference Material**

Bioethanol Yield Analysis under Different Substrate Processing Conditions

Sample Description	Pretreatment	Scarification	Fermentation	Absorbance (Unknown)	Calculation (EtOH%)	Bioethanol Yield (%)
Wheat straw (WS)	Yes	No	yes	0.162617	3.489254944	**3.48 ± 0.005**
	No	Yes	Yes	0.190615	4.090052339	**4.09 ±0.019**
	Yes	Yes	Yes	0.495411	10.63010214	**10.63 ±0.137**
WS mixed with SMS	Yes	Yes	Yes	0.2705	5.804155799	**5.8 ±0.002**
Spent Mushroom Substrate (Autoclaved)	yes	No	Yes	0.2601	5.581001565	**5.58 ±0.008**
	No	Yes	Yes	0.1795	3.851556251	**3.85 ±0.001**
	Yes	Yes	Yes	0.7046	15.11869936	**15.11 ±0.001**

$$\% \text{ Ethanol} = \left(\frac{\text{Aunknown}}{\text{Astandard}}\right) 18.51$$

$$\% \text{ Ethanol} = \left(\frac{0.162617}{0.862650}\right) 18.51$$

$$\% \text{ Ethanol} = 0.188508665\ X\ 18.51$$

$$\% \text{ Ethanol} = 3.489254944$$