PRODUCTION OF BIOETHANOL FROM RICE STRAW USING BIPHASIC SYSTEM AT PILOT SCALE

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Botany

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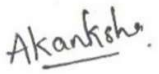
DECLARATION

I hereby declared that the presented work in the thesis entitled "**Production of bioethanol from rice straw using biphasic system at pilot scale**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of **Dr. Anand Mohan**, working as **Associate Professor**, in **School of Bioengineering and Biosciences Department** of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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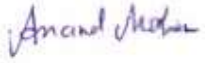
CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "**Production of bioethanol from rice straw from rice straw using biphasic system at pilot scale**" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy** in the Department of Botany, School of Bioengineering and Biosciences, is a research work carried out by **Akanksha Shukla**, **11916900**, is Bonafede record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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Abstract

In this day and age, the research is headed toward constructing a circular bioeconomy and the ultimate step towards a sustainable agricultural waste reduction process. It is considered that the bioeconomy is focussed on both food and non-food materials associated with conserving natural as well as restoring non-renewable resources. There is a need of transforming a linear economy into a circular bioeconomy as well as a sustainable integrated approach towards bioenergy development using agro-industrial, food, textile, and microalga as a source of circular bioeconomy. Bioenergy is considered a vital source of renewable energy formation as well as electricity production for industries, biofuel for the transportation sector, and decarbonization of various sectors to achieve net zero emission for the achievement of sustainable development goals, an exclusive method for effective waste reduction and management. The waste reduction process can be directed towards biorefinery pathways associated with biofuel production that give rise to a sustainable environment and circular bioeconomy. Furthermore, various perspectives in establishing a link between circular, green, and bio-based economies are developed with the major aim of meeting the demands of emerging population. There is an emergent need for development of biofuel production from the available agricultural waste to reduce greenhouse gas emissions.

Bioethanol production is somehow subjected to various challenges firstly due to the complex structure of lignocellulosic biomass (LCB) and secondly the adoption of a suitable technique for its conversion to affordable biofuel. LCB is considered a potential substrate for bioethanol production, the carbon-neutral source for creating green chemicals and sustainable energy. The techniques for bioethanol production involve pretreatment, saccharification, and fermentation process. In this present work, various physiochemical pretreatment techniques were utilized with different acid impregnation at different time intervals or in varied ratios. These involve steam explosion and liquid hot water (LHW) pretreatment with ratios of different chemicals as given under-

- Steam explosion impregnation with 0.05% H_2O_2
- Steam explosion impregnation with 0.1% H_2O_2
- Steam explosion impregnation with 0.25% H_2O_2
- Steam explosion impregnation with 0.5% H_2O_2
- Steam explosion impregnation with 0.75% H_2O_2
- Steam explosion impregnation with 1% H_2O_2
- Steam explosion impregnation with HPCA in the ratio 1:1
- Steam explosion impregnation with HPCA in the ratio 1:2
- Steam explosion impregnation with HPCA in the ratio 2:1
- LHW treatment impregnation with 1M HNO_3 at 80, 100, 120 and 140°C
- LHW treatment impregnation with 1M HCl at 80, 100, 120 and 140°C

- LHW treatment impregnation with 1M H_2SO_4 at 80, 100, 120 and 140°C
- LHW treatment impregnation with 1M Formic acid at 80, 100, 120 and 140°C
- LHW treatment impregnation with 1M Oxalic acid at 80, 100, 120 and 140°C
- LHW treatment impregnation with 1M Acetic acid at 80, 100, 120 and 140°C
- LHW treatment impregnation with 0.5M Oxalic acid
- LHW treatment impregnation with 0.75M Oxalic acid
- LHW treatment impregnation with 1.5M Oxalic acid

The pretreatment process and enzyme utilized for its conversion to reducing sugar played a vital role in enhancing the production of sugar and the improvement in these parameters would be considered further for the production of bioethanol. The main focus is on breakdown of recalcitrant structures comprised of cellulose and hemicellulose to fermentable sugar that is needed for efficient bioethanol production. Not only glucose but also xylose, are recognized as essential fermentable sugars derived from lignocellulose biomass for the production of bio-based products. In this work, a bi-phasic system was utilized with the extraction of both pentose and hexose sugar from the SPS method and enzymatic hydrolysis respectively. This SPS method helped in the reduction of time utilized for the pretreatment and saccharification process. Similarly, for the utilization of both hexose and pentose sugar, a co-fermentation process was used to enhance the production of bioethanol. During the co-fermentation process, there is a requirement for bioconversion of both glucose and xylose to develop a profitable process of bioethanol production. Still, there is an insufficiency of vigorous microorganisms or fermentation process to transform both pentose and hexose sugar which reliably compromises the complete fermentation yield. So, to deal with the problem, many techniques are adopted with an approach accessible for its bioconversion is the co-fermentation process using both hexose and pentose sugar fermenting yeast strains for effective conversion of reducing sugar to bioethanol. This research work is focused on various physiochemical pretreatment techniques that will easily be implemented for large-scale production of bioethanol. It is also focused on various co-fermentation yeast strains, and their nutrition media along with an evaluation of the amount of bioethanol produced from the pretreated biomass are demonstrated. In this research, the best pretreatment method was scaled while comparing the experimental findings to those of laboratory-scale pretreatment.

Major challenges associated with biofuel production are-

1. Integration of various processes to cut down the steps involved in it,
2. Selection of appropriate microorganisms with maximum tolerance to inhibitors
3. Extraction of fermentable sugars both hexose and pentose from LCBs by improving the hydrolysis process,
4. Similarly, finding suitable fermenting yeast that ferments all sugar present in the hydrolysate.

The major challenges mentioned above are incorporated while performing the present work through various integrated processes, as it is required to develop innovative strategies that will further be incorporated with industrial applications. As a result, the current research may be regarded as a proof-of-concept for overcoming the bottlenecks mentioned above. Bioethanol minimizes the discharge of hazardous pollutants and GHG emissions and increases energy security and employment and lessens the country's reliance on oil imports. The government is currently engaged in an ethanol blending program; nevertheless, there is a need to emphasize further developing technology, where cost economics plays an important part. If a policy is adopted and assesses insufficiency and restrictions involved in production, with a focus on specific criteria involved at the commercial level, the country will benefit more.

This thesis is divided into five chapters. Chapter one illustrates the introduction of the research that covers global biofuel demand, geographical expansion of biomass-based energy towards biofuel regulation with the recent updates in biofuel policy in India, fuel property of bioethanol, major source of bioethanol using rice straw as lignocellulosic biomass with the estimated rice straw production from 2010 to 2021, lignocellulosic biomass structure with various techniques used for conversion of biomass to bioethanol. Chapter Two shows a critical review of literatures whereas the research methodology used in the research is discussed in Chapter Three. Chapter four presents an extensive discussion of various obtained experimental data from various techniques used during the process. The summary and conclusions have been illustrated in chapter five.

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LCB: Lignocellulosic biomass

HP: Hydrogen peroxide

HPCA: Hydrogen peroxide and citric acid treatment

SPS: Simultaneous pretreatment and saccharification

RS: Rice straw

OA: Oxalic acid

LHW: Liquid hot water pretreatment

DNS: 3,5- Dinitrosalicyclic acid reagent

FTIR: Fourier transform infrared spectroscopy

TGA: Thermogravimetric analysis

XRD: Powder X-ray diffraction analysis

CrI: Cellulose crystallinity index

FESEM: Field emission scanning electron microscopy

HPLC: High-performance liquid chromatography

GC: Gas chromatography

EH: Enzymatic hydrolysis

AH: Acidic hydrolysis

TRS: Total reducing sugar

EIA: Energy Information Administration

GHG: Greenhouse gas emissions

SDG: Sustainable development goal

EBP: Ethanol blended petrol

CAGR: Compound annual growth rate

FAME: Faster adoption and manufacturing of Hybrid and electric vehicles scheme

XOS: Xylo-oligosaccharide

MSP: Minimum support price

ISMA: Indian Sugar Mills Association

CDRI: Coalition for Disaster Resistant Infrastructure

NCAP: New Car Assessment Program

AFEX: Ammonia fibre explosion

5-HMF: 5-hydroxymethyl furfural

YPD: Yeast peptone dextrose

IRRI: Indian rice research institute

ISA: International Solar Alliance

CHAPTER-1

INTRODUCTION

Preamble

Biorefineries are assisted by the conversion of biomass into a wide range of value-added byproducts including biofuels, significant chemical compounds, bio-pesticides, and bioenergy. In general, a biorefinery utilizes hybrid technologies to directly convert into marketable bioproducts with the production of least quantity of non-biodegradable waste. Bioenergy production has diversified agricultural production systems by reducing greenhouse gas (GHG) emissions as well as a fossil-fuel dependency that reduces the climatic effect on environment. Lignocellulosic biomass is gaining traction as a viable alternative to non-renewable petroleum-based energy. Biomass includes a wide range of feedstocks, comprising various lignocellulosic biomass, agrochemical biomass, and bio-waste, that are transformed by biochemical, chemical, physical, or thermochemical techniques. The utilization of feedstock for the production of affordable fuel and a sustainable approach towards bioethanol production is due to the fluctuating price of gasoline as well as the limited availability of oil and the need to mitigate the effect of GHG emissions. Thus, upgrading agricultural waste into valuable products will provide benefits to the farmer as well as reduce the import of crude oil (Periyasamy et al., 2022). The valorization of available agricultural residue to produce major motor fuel that has 80% less carbon emission than that of conventional fuel. Ethanol blending is mandatory as it is one of the ways to reduce carcinogenic substances in the form of unburned hydrocarbon, greenhouse gas and sulphur dioxide (major components of acid rain) (Amândio, Rocha, & Xavier, 2023).

Nowadays, with an upsurge in the global demand for energy and fuel in India, there is a requirement for sustainable forms of energy that will subsequently enhance the overall development of India. There is a need for sustainable development of available resources due to the emerging belief that the global climate is changing as a result of various anthropogenic activities, one being the excessive utilization of fossil fuels as primary sources of energy, such as natural gas, petroleum and coal, that emit a potent GHG. One technique to reduce crude oil usage and pollution is to produce bioethanol from agricultural residues. According to the Ministry of New and Renewable Energy, the total estimated biomass generation in India is 750 million tons per year, agricultural waste has an estimated biomass readily available of around 230 MMT per year, or a potential energy generation capacity of approximately 28 GW (Shukla & Arora, 2019). Due to its high-octane number, bioethanol is suitable to be used as a blended fuel in gasoline engines, whereas its less cetane number and intense heat of vaporization prohibit self-ignition in diesel engines. When employing gasoline-bioethanol-blended fuel it can improve ignition in the engine, surface ignition, glow plugs, and pilot injection that further facilitate self-ignition (Sanap, Diwan, & Mahajan, 2023).

Researchers and technology developers have put a lot of effort into improving biofuel manufacturing processes. Research on this topic aims at lowering environmental effects leading to a more energy-viable society because it is made from renewable sources. Ethanol has proven among the most popular options within this tendency framework. There is a need for an alternative development route that facilitates

advancement in 2G bioethanol production from LCB such as agricultural waste, energy grasses and woody crops. Thus, biomass is considered a reliable source of energy production. Based on the source of raw material available for bioethanol production, these are categorized as first-generation (1G), second-generation (2G) and third-generation (3G) bioethanol. 1G bioethanol was produced using starch and sugar-based feedstock such as sugarcane and maize seeds as the raw substrate for its production but due to limited food for everyone in most of the countries, its production was a major concern. 2G bioethanol is generated from lignocellulosic feedstock is an inedible agricultural residue left after harvesting of crops in the form of corn stover, sugarcane bagasse, rice husk, rice straw and wheat straw that is feasible to convert cellulose from residues to ethanol, which can be further blended with conventional fuels. There have been various discussions over the relative sustainability, economic viability, and yield potential of 1G and 2G bioethanol. Many studies have concluded that as 2G bioethanol production makes better use of 1G municipal solid waste, residues, and biomass, it can be seen as a technological breakthrough that tackles environmental problems (Jiradechakorn et al., 2023). While 3G bioethanol is produced from the algae and has the advantage of not competing with human sustenance maintaining fiscal viability is the major concern. Thus, 2G bioethanol production has been promoted to convert residues into energy. Lignocellulosic biomass is a possible replacement for fossil fuels due to its substantial abundance, ability to regenerate, and low level of pollution (Tomar et al., 2023).

To avoid pollution, these residues should be disposed of as soon as possible, or they can be used to make bioenergy and bio-products. The availability of biomass and the requirements for liveliness are often factors in these transformation methods. These transformation methods not only depend on the source of biomass but also the technology involved in the conversion of bioenergy (Rather et al., 2022). The implementation of biorefineries utilizing bio-based feedstocks is among the intervention strategies highlighted in the Bio-economy vision. World consumption of fuel, environmental quality, and energy security have sparked interest in liquid biofuels like biodiesel and bioethanol. Governments worldwide have implemented various policy measures, such as mandatory fuel blending programs, subsidies for foldable fuel vehicles, and farm subsidies for farmers (Kumbhar, 2023).

1.1. Global demand for biofuel

The worldwide demand for biofuel is set to increase day by day and it is expected to rise by 28% i.e., 48 billion liters from 2021-2026. On a global basis, the bioethanol produced in 2018 was 110 billion liters and is expected to increase to 140 billion liters with a growth rate of 7.6% annually due to the feasibility of the process with the major powerhouse country in bioethanol production is Brazil, US, China, European Union and Canada (Cavelius et al., 2023). According to the Energy Information Administration (EIA), by 2035 the number of automobiles will have enhanced to 1.7 million. Furthermore, the transportation sector is predicted to consume the most liquid fuel, accounting for almost 73% of overall consumption. Liquid fuel

demand is projected to increase from 19.1 billion barrels per day in 2019 to reach 21.9 million gallons per day by 2035. As a result, the transportation industry's increasing demand for bioethanol is offering various potential prospects (Bioethanol Market Size, GIOF, 2022). Bioethanol as a biofuel production market is experiencing strong growth as a result of increased environmental concerns among nations, which is driving a shift in demand for renewable energy sources over fossil fuels (Biofuels, Renewables 2021 Analysis - IEA).

1.2. Geographical expansion of biomass-based energy toward biofuel regulation

The global bioethanol market is expected to grow considerably during the projected period due to the growing demand for a sustainable and clean energy source. Bioethanol is a sustainable and clean energy resource yielded from sugar fermentation and various chemical processes performed on lignocellulosic biomass. Because of its high-octane value and lower greenhouse gas emissions, bioethanol is a feasible substitute for conventional gasoline sources. Moreover, the reduction in traditional energy resources and a rising emphasis on renewable energy sources are likely to drive the bioethanol market growth from 2019 to 2025 (Kumbhar, 2023). Biofuel has the lion's share for the bioenergy development from the available renewable waste source to meet the global energy demand of the transport sector by making it mandatory to blend it with petrol according to various countries' requirements. It is estimated that during the period from 2020-2025, the biofuel industry is expected to grow with a CAGR of less than 8%. The demand for sustainable and clean sources of energy will increase by up to 28% by 2040 (Biofuels Market Analysis, 2022-27). The collaboration of developing countries in the step towards globalization along with the integration of advanced technologies towards biofuel production and management of foreign capital towards economic growth and stability in the specific country. Different countries have planned their different area of biofuel blending strategies to mitigate the greenhouse gas mission as well as achieve SDG 2030 (Subramaniam & Masron, 2021). The major blending updates of various developed countries such as the U.S., Brazil and China have mandated 15-27% blending in 2020-2022 (Biofuels Market Size, IOR, 2022-27). 84% of the total biofuel is produced by the U.S. and Brazil and the feedstock used for it is sugarcane with the involvement of various advanced technologies (Nystrom, 2019). The regulatory mechanisms of different countries with their biofuel policy tool will support the fuel market. The national biofuel policy of India in 2018 mandated the blending of 20% bioethanol with petrol as well as 5% biodiesel blending with diesel by 2030 (S. Das, 2020a). Similarly, Brazil with the regulatory framework RenovaBio launched in December 2017 mainly emphasizes carbon footprint and development of advanced biofuel technology with the involvement of the biogas sector (Grangeia et al., 2022). While in Cambodia sugar and palm sector is used to enhance the bioethanol and biodiesel production industry respectively. From 2008 to 2018, approx. 90% of total palm and sugar are used as substrates for the production of biofuel with the establishment of a main market deal with biofuel (Palacio-Ciro et al., 2020). Also, the EU's multimodal techniques of transport sector with 10% blending with conventional fuel by 2020 and also integrated various industries (Harnesk,

2019). The target of biofuel blending was already decided in 2003 while the biomass action plan was adopted in 2005 for the implementation of bioenergy policy in the different member countries (Takaes Santos, 2020). In 1975, a national fuel alcohol program was adopted in Brazil for the production of ethanol from sugarcane which will provide economic benefits. In 2004, the national biodiesel production and utilization program was initiated in the matrix of all national energy production, and the completion of the B5 prototype by 2013 (Saravanan et al., 2020). Table 1.1 illustrates the biofuel mandate policy of various countries.

Table 1.1- Biofuel policy tool with its mandate blending criteria of different countries

Countries	**Policy tool (in year)**	**Bioethanol blending**	**Biodiesel blending**
India	Blending of bioethanol with petrol (2018)	20% in petrol	5% in diesel
Canada	Renewable fuel legislation	5% in gasoline	2% in diesel
Argentina	Biofuel law	12% in gasoline	10% in diesel
France	Finance bill (2019)	7.9% biofuel blending in 2019, 8.2% in 2020	
Malaysia	National biofuel policy 2006	5% palm oil blending in diesel	
Brazil	RenovaBio	27% in gasoline	11% in diesel
Philippines	The biofuel Acts 2006	10% in petrol	5% in diesel
United Kingdom	Renewable transport fuel obligation	2.5% by 2008 and 5% by 2011	
EU	Directive 2003	2% by 2005 and 5.75% by 2010	
	Directive 2009	10% biofuels in fossil fuel by 2020	
Colombia		10% in gasoline	10% in diesel
Germany	2009 to 2014	2.8% in gasoline	4.4% in diesel

1.3. Recent updates in the bioethanol production in India

Bioethanol, a gasoline substitute, is a renewable resource naturally generated from food crops including rice, sugarcane, wheat, corn and maize, but with the development of 2G and 3G biofuel technologies, the reliance of bioethanol production on food crops has reduced. The major aim of the manufacturers is to produce bioethanol from agricultural and forest leftovers, as well as energy crops including sugarcane bagasse, switchgrass and miscanthus. As biofuel is obtained from agricultural wastes is considered to be a sustainable alternative and less expensive than commercially available petrol. With this adoption, not only are environmental conditions being improved but also farmers benefit from it as the MSP

of these crop residues is much higher than that of commercial price. Bioethanol is produced with the diversification of agricultural waste towards empowerment of farmers, power and energy sectors as well as generation of rural employment.

In June 2017, ethanol production from agricultural waste and segregated municipal waste was considered a 'game changer' for the farmers as it diversified the farm sector. The government has started an initiative for the development of bioethanol plants with 15 industrial units. In the city of Nagpur, around 55 air-conditioned public buses are running with the usage of 100% bioethanol engines. The automotive industry grew at a rate of 22% per year (Williams & Blyth, 2023). According to the Ministry of Road Transport and Highways, the government is providing sugar mills with export subsidies ranging from 3,000 to 6,000 crore to liquidate excess sugar stocks. With the emergence of 100% bio-ethanol flexi-fuel vehicles, demand for ethanol will immediately increase by 4 to 5 times. Since 2018, the government has launched innumerable ethanol prices based on the biomass utilized for ethanol production. The surplus stock of sugar at around 45 to 60 lakh metric tonnes will enhance the ethanol production quality by 30% due to the better quality of raw material. With this in Dec 2021, India set an example of adopting e-vehicles in the world, a step towards the reduction of pollution and conversion of construction material to greener options. The FAME India scheme aims to convert vehicle engines to electric engines. Along with the concept of electric engines, vehicles with flexi engines that use 100% ethanol or the blending of 22% bioethanol with petrol and 15% biodiesel with diesel are also encouraged which can replace normal engines. With the introduction of flexi engines, the existing rate of bioethanol production will be increased to 4000 cr from the current 400 cr liters of bioethanol (Mohammadi & Saif, 2023).

According to the report given by Energy Information Administration, it is supposed that by 2035 number of vehicles will surpass 1.7 million and utilization of liquid fuel will be 73% by the transport sector which will be around 21.9 million gallons per day (Bioethanol MS, GIOF, 2022). A sustainable development goal is one of the mandatory actions towards reduction in global warming as well as mitigating climatic changes. Thus, it is required to enhance the utilization of alternative fuel i.e., bioethanol replacing petroleum products. The global ethanol market in 2015 is expected to be worth $5652 million and will be enhanced to $ 9544 million by 2022 which is expanding at 7.6% CAGR from 2016 to 2022.

Automobiles running on 100% ethanol blending and then with hydrogen as the major renewable fuel will be considered the future of mobility. The major steps taken by Government of India along with the technological tie-up with Brazil towards the aviation industry by blending 50% bioethanol with conventional jet fuel and will reduce 80% of greenhouse gas emissions. It was estimated that the E-20 fuel program would increase the blending by 20% by 2025 and also increase the economy generated from ethanol from 20000 cr to 20 lakhs cr. For 20% ethanol blending, around 10 billion liters of ethanol will be utilized by 2025. Various industries such as the praj industry, India glycol, Balram Chinni and Shree Renuka Sagar benefitted

from such an initiative. Thus, the Shree Renuka Sagar industry planned to enhance the production of bioethanol from 24 cr liters per annum to 33 cr liters by 2023 and the Balram Chinni industry expected to increase its production from 18 cr liters to 30 cr liters. This 100% ethanol blending will save the amount of 30000 cr being wasted in the import of fossil fuels (Plaza, Complex, Towers, & Secretary, 2022).

In the Indian Sugar Mills Association (ISMA) conference of Oct 2021, the Ministry of Road Transport and Highway stated that bioethanol production from agricultural waste will generate additional income for the farmer. For this Ethanol blended Petrol (EBP) and E20 fuel program. with this program, it is estimated that India will produce 10 billion liters of ethanol by 2025. Till 2019, India has touched only 5.6% blending with petrol. According to the Ministry of Road Transport and Highways of India, India will be the world's leading automobile production hub. These automobiles operate on all types of fuel and also utilize hydrogen as an alternative fuel. It is meant to increase the production and usage of biofuel in the country. Recently, 2G ethanol was constructed at approximately 900 Cr by the Indian Oil Corporation Ltd. in Panipat. This achievement is to transform the energy sector into a more accessible, affordable, sustainable and efficient one. It is a step towards India waste waste-to-wealth mission, utilizing 2 lakh tonnes of rice straw to produce 3 cr liters of ethanol annually. This project employs people who are involved in plant operation and indirect employment will be generated for farmers who are involved in rice straw cutting, handling and storage. It will reduce global greenhouse emissions by up to 3 lakh tonnes of CO_2 emission and replace 63000 cars annually (Mohammadi & Saif, 2023).

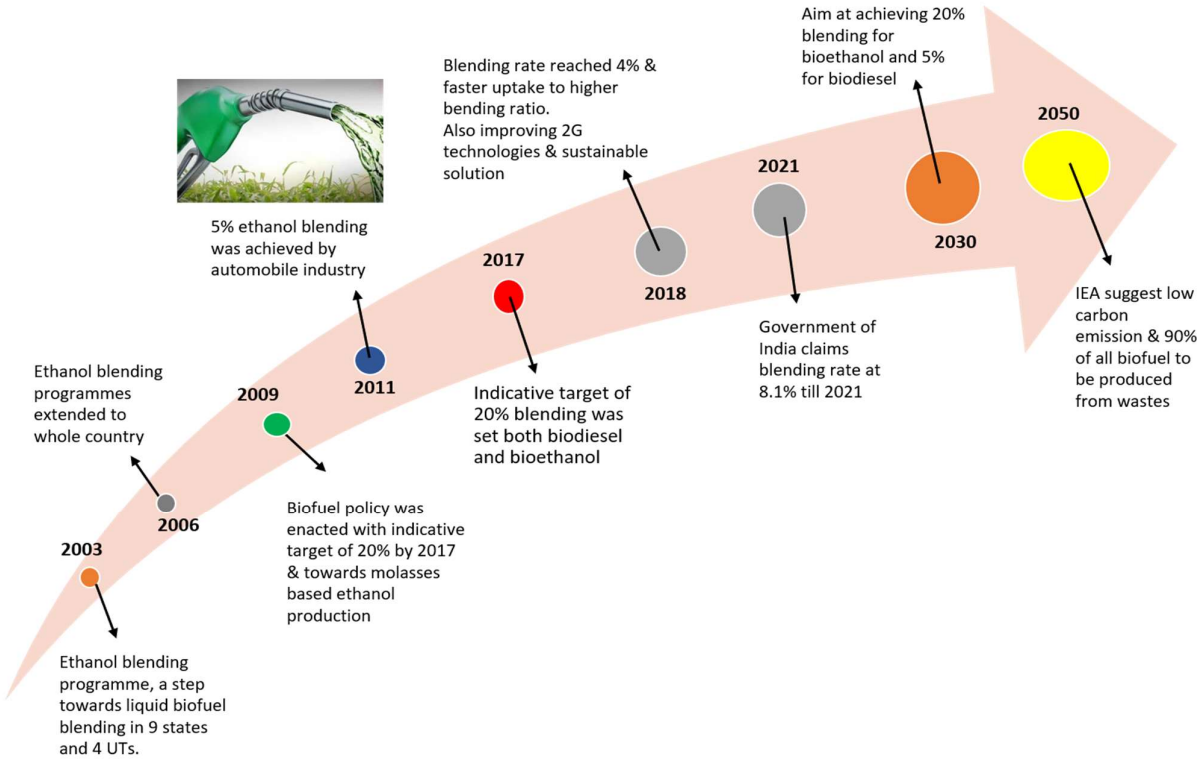


Figure 1.1- Illustration of timeline for ethanol blending policy in India

According to Oil Ministry, ethanol will account for one-fifth of all gasoline by 2025 and from April 2023, India will begin supplying gasoline containing 20% ethanol. This is to reduce India's reliance on oil imports and address environmental concerns. "10% ethanol blend transformed into a forex impact of more than Rice straw 41,500 crore, lowered greenhouse gas emissions by 27 lakh tonnes, and resulting in farmers receiving over Rice straw 40,600 crore expeditiously," said the Ministry of Petroleum and Natural Gas. Blending ethanol reduces not only vehicular pollution but also import dependency and farmer income. The prime minister of India claims that it helps to conserve valuable foreign exchange that would otherwise be used on crude oil imports. Figure 1.1 represents the timeline for national biofuel policy in India.

In 2022, the minister of road transport and highways of India has started a step towards Bharat NCAP (New Car Assessment Program) which is meant to star rating Indian vehicles based on their performance in the crash test. It has been troubled for decades by natural resource depletion, the ensuing pollution, and the global warming induced by the usage of fossil fuels. The only way to tackle this problem is to power automobiles and other machines with renewable and ecologically favorable resources such as biofuels. India is heading towards flexi engines or electric hybrid engines that utilize 100% ethanol for their operation, 40% on electricity and almost on petrol. Further moves towards bio-LNG and bio-CNG using rice straw. In winter, 5 tonnes of rice straw are usually burned by farmers generating hazardous pollutant that produces 1 tonne of bio-LNG. Along with this biofuel production, green hydrogen production using sewage waste has also gained pace. Recently, India's automobile industry, Toyota launched its first green hydrogen car on 18th March 2022. India leads global efforts to combat climate change through partnerships with nations like Denmark, the ISA, and the Coalition for Disaster Resistant Infrastructure (CDRI). These aid in preserving peace and stability within the immediate region including its surrounding areas. The quick progress of the railway projects for linking with Sittwe Port in Bangladesh, Nepal and Myanmar was highlighted. Inland water projects with energy grids are also being developed in Myanmar and Bhutan. In the Sugar and Ethanol India Conference 2022 held in Mumbai, the Ministry of Road Transport and Highways of India addressed the conference by saying that in the next few years, India will be a large fuel exporter rather than an importer in the fuel industry by enabling bio-diesel, bioethanol, bio-LNG, bio methanol, green hydrogen along with an expansion of utilization of electric vehicles in the transport sector. During the conference, the emphasis was on converting farmers from anna data to urja data with the diversification of agricultural feedstock towards power and energy generation. At present, India is producing 465 cr liters of bioethanol. An increase in the consumption of ethanol will reduce pollution, generate jobs for young youths as well and bring down the import of bioethanol. Owning this, recently during the 63rd annual meeting of the company, Indian Oil has planned to invest Rs. 2 trillion to achieve net zero carbon emission by 2046. This biofuel, green hydrogen, offsetting of carbon and renewable energy approach are considered emission reduction pathways with the help of ecosystem restoration as well as utilization and storage of carbon capture (Reuker, Lao, & Edwards, 2018). According to the Ministry of Petroleum and Natural Gas

report released in 2023, India's present capacity to produce ethanol, 1,364 crore liters, is adequate to achieve the fuel blending requirements. It was found that Karnataka, Maharastra and Uttar Pradesh were ethanol-surplus states. As per the strategy, oil marketing corporations have managed to blend 10% ethanol in 2021–2022 and 12% in 2022–2023 (Report MPNG).

1.4. Fuel properties of bioethanol

Bioethanol would indeed be a reduced carbon emitting substrate since CO_2 emissions through consumption would be offset by CO_2 absorption by biomass. Bioethanol also has a high-octane rating (113) with a boiling point of 78.5℃ and a freezing point of -114.1℃ and emits no harmful substances. It can be used both in the form of gasoline blended fuel or in the form of hydrous. Due to the higher oxygen concentration of ethanol in the former scenario, hydrocarbon combustion is more efficient, which results in lower GHG emissions. Moreover, it adheres to the most cutting-edge government regulations in terms of gasoline blended fuel. Further, It prevents the heating up of the engine as the combustion rate is very low, the engine shows a faster rate of cooling and cleaner ejaculation nozzle of the engine as the performance of the engine increases with the increase in octane number (Bioethanol Fuel Production and Properties Report, 2015). It acts as an octane number ameliorator for petrol. Bioethanol has a significantly reduced amount of energy than gasoline (about three-quarters of the latter's energy content on a volume basis). This indicates that for mobility purposes, for a specific tank volume, the vehicle's range is reduced proportionally. Because ethanol has a greater octane number than gasoline, it offers superior antiknock properties. This improved fuel quality may be taken advantage of if the engine's compression ratio is modified suitably. This improves the engine's fuel efficiency. The oxygen concentration of ethanol also contributes to improved efficiency, resulting in an environmentally friendly combustion process at a lower temperature range. (Bioethanol–EBIA, 2016). Fuel's physical properties depend on the blending of fuel with the air on sufficient atomization or dispersion of fuel and easy combustion potential of fuel. Ethanol has the maximum latent heat of vaporization that that of gasoline i.e., more energy is required to vaporize ethanol than conventional fuel. This is the reason 70-85% ethanol blending ensures an adequate amount of gasoline to vaporize by frequently fixing with air and allowing the engine to start immediately when cooled. For the adequate blending of ethanol with gasoline, the biochemical properties of ethanol in comparison with gasoline are illustrated in Table 1.2.

Table 1.2- Biochemical properties of gasoline and bioethanol

Physical property	Gasoline	Ethanol
Lower heating value (MJ kg^{-1})	42.7-44	26.9
Air/fuel ratio	14-15	9-10

Latent heat of vaporization (kJ kg $^{-1}$)	350-356	842
Reid vapour pressure (kPa)	48-103	22
Octane number	91-99	107-111
Oxygen content (% by mass)	0	34.8
Cetane rating	0	29
Solubility in water	Insoluble	Soluble

Ethanol can be utilized as a transportation fuel to substitute petroleum, a thermal combustion fuel for power production, a thermochemical reaction fuel for fuel cells, a fuel for cogeneration systems, and a feedstock for the chemical industry. Due to its high octane number, ethanol works best in spark-ignition motors. It is less suited for diesel engines because it has a low cetane number and weak combustion quality. Since pure ethanol has a low vapour pressure and a high latent heat of vaporization, using it in spark-ignition engines is typically not feasible because it makes the initial start challenging. The most economical solution is to blend ethanol with a tiny quantity of flammable fuel, like gasoline (Alternative Fuels Data Center, 2020). As a result, different bioethanol and petrol or diesel fuel mixtures have been utilized. By percentage, the most well-known mixes are:

- E5G to E26G (5-26% ethanol, 95-74% gasoline)
- E85G (85% ethanol, 15% gasoline)
- E15D (15% ethanol, 85% diesel)
- E95D (95% ethanol, 5% water, with ignition improver)

Bioethanol has been widely tested as E85G in light-duty flexible-fuel cars. ETBE is utilized in gasoline blends of 10-15% to increase octane rating and minimize pollutants. Gasoline blends containing up to 22% ethanol (E22G) may be utilized in spark ignition engines with no material or operational issues. Diesel blends containing up to 15% ethanol (E22D) do not cause difficulties in the technical engine and do not improve the ignition engine.

1.5. Rice as the major source of bioethanol production in India

Among various agricultural wastes available and utilized for bioethanol production, rice straw has a moderate level of lignin and enhanced energy value. Rice is the staple crop mostly grown in tropical countries such as India with typically hot and moist climatic conditions. The temperature required for rice cultivation is 16-27ºC and an average rainfall of 100-200 cm. The harvesting temperature for rice is 20-25℃ with very little rainfall during the harvesting season. The soil required for its cultivation is fertile clayey,

loamy and black lava soil. The major rice-producing states are West Bengal, Kerala, Bihar, Tamil Nadu, Odisha, Andhra Pradesh, Uttar Pradesh and Assam with West Bengal (14.24%) and Punjab (6.41%) being the largest producers of rice per hectare (Rice, 2019). India is an agriculturally enriched country with an enhanced number of remnants after the harvesting of crops. Approximately 512.8 MT of rice is produced globally every year and according to IRRI, the typical rice grain-to-straw production ratio is 0.7:1.4. Thus, it is estimated that 1025.6 MT of straw produced is burned by the local farmers which if utilized rationally could add up to the global bioethanol production. Globally, India is the second largest rice producer in the world after China. According to the Ministry of Agriculture and Farmers Welfare, the average production in 2020-21 was 124.37 million tonnes of rice while in 2021-22, it will be 121.10 million tonnes of average rice production in India (MAFWD, AFW Directorate 2021-22). So, total straw production in the year 2020-21 was 248.74 MT while in the year 2021-22, it was 242.20 MT. A year's worth of 2.1 BT of ethanol could be made from this rice straw (Sharma et al., 2015). The graphical representation of rice production from 2010 to 2021 is illustrated in Figure 1.2.

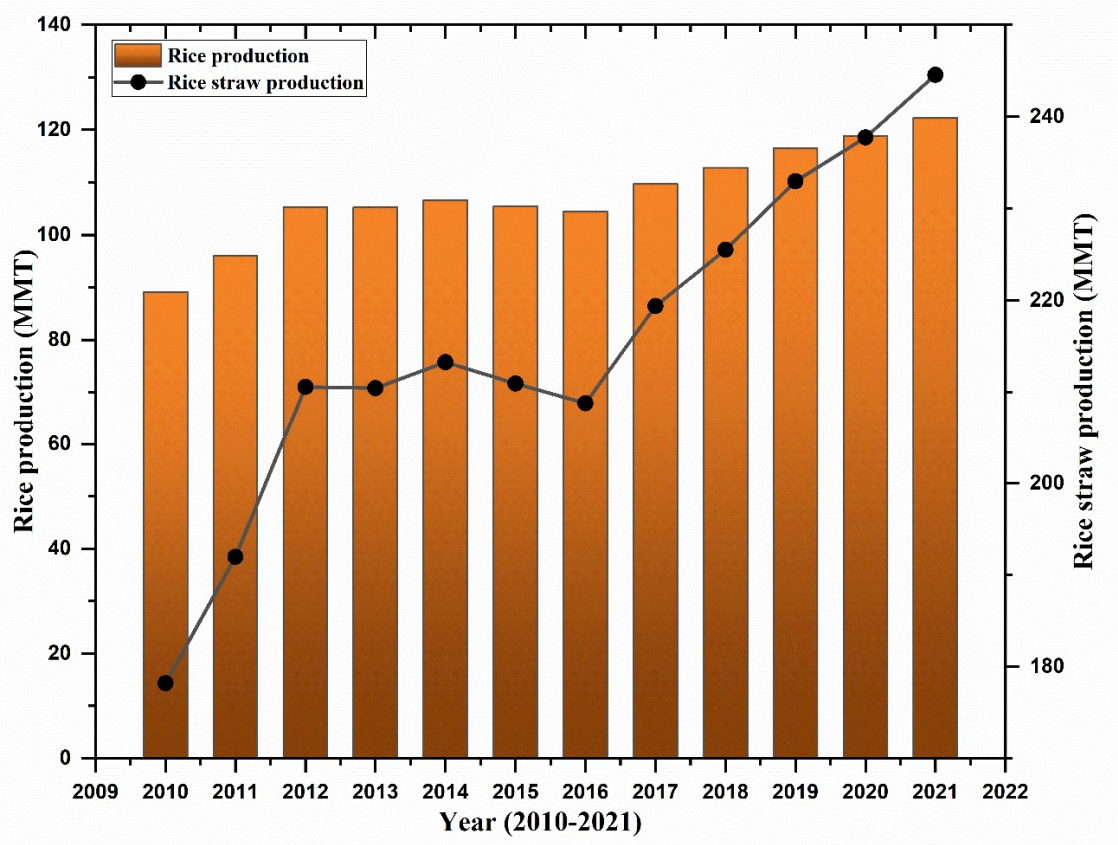


Figure 1.2- Illustration of rice and rice straw production in India from 2010-2021

1.6. Other major agricultural biomass used as a substrate for bioethanol production

As a lignocellulosic biomass source, bamboo has been suggested as a viable option in confronting energy problems and climate change. Bamboo's rapid expansion and valuable outputs make it a promising

substrate for bioenergy production. It is also one of the most well-known biomass resources due to its high productivity, self-regeneration and ability to withstand deficient soil, which allows it to flourish on degraded land along with, it has an enormous quantity of cellulose and hemicellulose, which may be transformed into monosaccharides (Rathour et al., 2022). Bamboo has good fuel qualities, such as a low alkali index, ash content, lower moisture content and a lower heating value that is higher than that of grasses, straw and other biomass but lower than that of many woody biomasses (Krishnamoorthi et al., 2023; Liang et al., 2023). The disadvantages of bamboo biomass include land occupation, which also shows detrimental effects on the environment, such as excessive water usage, so using bamboo as feedstock for energy needs has to be carefully considered to reduce any ill effects on the environment. The bamboo biomass needs extra pretreatment measures to increase its digestibility because of its inherent resistance and high lignocellulosic content. This can lower the viability of producing second-generation bioethanol economically (Bernard & Lucotte, 2022). The amount of lignin, cellulose, hemicellulose linkages and crystalline structure all have an impact on the conversion efficiency of lignocellulosic biomass, which in turn affects biomass digestibility. Bamboo biomass is not sufficient to provide all of the world's energy needs. To fully utilize their potential and offer a sustainable energy supply, it must be integrated with other sources. Comparing bamboo to trees, which take decades to reach full maturity, bamboo is known around the world for growing at the fastest rate among all the plant kingdom. They grow in a variety of locations, are tolerant of poor soil conditions, require little to no watering to thrive, and self-regenerate quickly after harvest. They also require little to no chemical pesticides and fertilizers. Since bamboo can grow at various geographical locations and have multiple applications, which leads to high biomass availability that is year-round and can fulfill the nation's future energy requirements. Bamboo is a non-food crop with a high lignocellulose content that makes it a prospective source of second-generation biofuels. These qualities make it an appealing feedstock for the production of bioethanol (Ha truong an, 2014; Liang et al., 2023). Since bamboo biomass can absorb carbon dioxide (CO_2) and is a potentially renewable substrate that can be used to produce biofuels and chemicals, the biorefinery technique has drawn a lot of attention. Thus, bamboo biomass is regarded as an exceptional substrate when compared to other probable lignocellulosic biomasses (LCBs) (Ding et al., 2023).

1.7. Lignocellulosic biomass (LCB) structure

LCB is considered a carbon-neutral source for creating green chemicals and sustainable energy that is a probable substrate for bioethanol generation. LCB is a complex structure with entangled fibers consisting of fermentable and non-fermentable parts that provide rigidity to the plant cell walls. The fermentable part is cellulose and hemicellulose which is embedded with the non-fermentable part, i.e., lignin. Cellulose is the most abandoned LCB (lignocellulosic biomass) with a compositional analysis of 33-47% that is utilized for further process of hydrolysis (Singh et al., 2016). Another abandoned compound in the lignocellulosic biomass is hemicellulose (19-27%) in composition. Non-fermentable part is the lignin (5-

24%) and silica (18.3%) component which forms a lignin-carbohydrates complex and hinders the further process of hydrolysis by binding with cellulose, reducing the exposed surface area for enzymatic action (Akhtar et al., 2017) as well as forms a hindrance against external encroachment and prevents degradation. Due to its rigid structure consisting of a carbohydrate polymer matrix, it is required to evaluate the efficient pretreatment methods that can be used to release the carbohydrate part from its lignin counterpart and to make both cellulose and hemicellulose accessible to enzymatic action. Both hemicellulose and lignin form a covering over the cellulosic portion of biomass and reduce the efficiency of enzymatic hydrolysis and fermentation which ultimately lowers the product yield. It is a prerequisite to have the region-wise analysis of biomass as LCB (lignocellulosic biomass) is a versatile resource not only used for biofuel production but also to account for the production of varied profit-based industrial products. With its high economic value, it is required to estimate the economic viability of the biofuel industry (Kumar et al., 2019; Y. Singh et al., 2024).

1.7.1. Cellulose

The largest carbohydrate constituent of LCB is a polymer of anhydrous-D-glucose with a lengthy structural chain constituent of β-glucose monomers having an affinity with β-(1,4)-glycosidic bond and gathered together into microfibril bundle (Sebayang et al., 2016). The linear cellulosic chain is associated together with inter- and intramolecular hydrogen bonds presenting different degrees of polymerization. This hydrogen bond forms a highly ordered crystalline region that makes it accessible for the activity of the hydrolytic enzyme (Shukla et al., 2023). Some regions in the cellulosic structure are less crystalline-amorphous regions that make it resistant to biodegradation and the enzyme can easily bind to cellulose in these regions to start the hydrolysis process. It has been visualized that feedstock with more cellulosic content is accessible for bioethanol production (Pinto et al., 2022).

1.7.2. Hemicellulose

A hemicellulose is a group of polysaccharides that consists of a short branched chain of sugars such as arabino-glucouronoxylan, arabino-4-O-methyl-glucuronic- xylan, glucourono-xylan, arabino-xylan, and galactic-arabino-glucorono-xylan. In other words, it is the polymer edifice of both pentose sugars (D-xylose and L-arabinose), hexose sugars (D-glucose and D-galactose) and acetylated sugars (Sebayang et al., 2016). It is a random structure containing five or six carbons of sugar. It is the second most abandoned polymer in the plants' secondary cell wall.

Both cellulose and hemicellulose microfibrils are linked together by hydrogen bonds and thus form a strong covalent connection between them that gives strength and toughness to the structural plant cell wall. Hence, making a barrier for enzyme accessibility that converts biomass to fuels. For developing strains for cellulosic ethanol production, necessary to have deep knowledge of the structure of hemicellulose and its by-products. The main hemicellulose in plant cell walls is in the form of xylan, which gets converted into its by-product xylose in the hydrolysis process utilized for strain development in biomass (Rocha-Meneses et al.,

2020). Thus, acetylation frequently takes place during the biosynthesis of galactose residue and another by-product such as acetic acid formed by hydrolysis of hemicellulose that hinders the growth of microbes and fermentation of ethanol (Akhtar et al., 2017). Thus, to inhibit the formation of by-products, it is required to maintain the temperature and retention time of hemicellulose degradation. Since hemicellulose has a branched-chain structure with a short lateral chain and low molecular weight, it can be easily hydrolyzed (Deng et al., 2023; Singh et al., 2015).

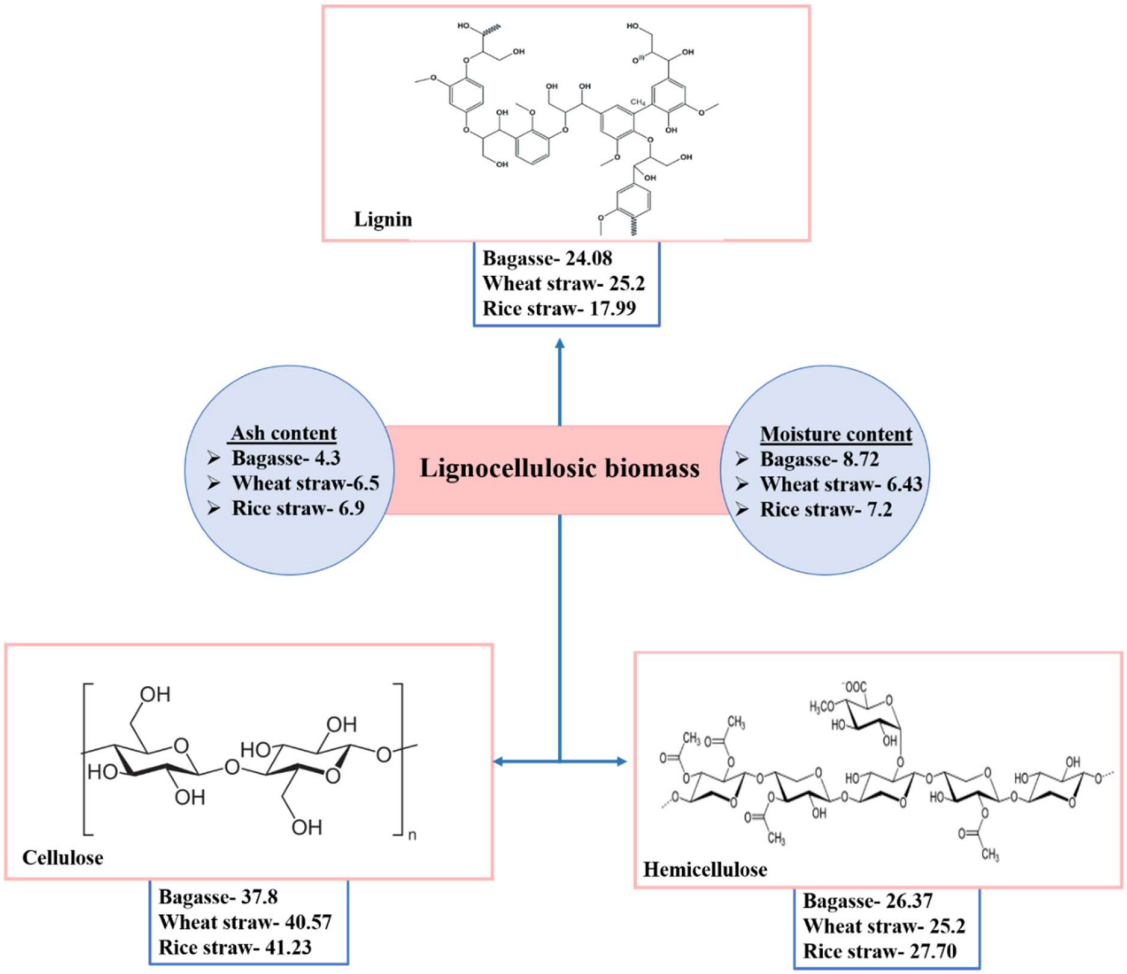


Figure 1.3- Representation of varying compositions of commonly available lignocellulosic biomass

1.7.3. Lignin

It is a heteropolymer and complex structure containing three types of monomers such as p-coumarin, synapyl alcohols and coniferyl that are formed by the oxidative coupling of three basic units including guaiacyl (G), p-hydroxyphenyl (H) and syringyl (S) (Moreira-Vilar et al., 2014; Vu et al., 2020). These monomers have a high ambivalence, which is responsible for ensuring strength and stiffness to the biomass. It is a natural polymer of LCB (lignocellulosic biomass) with a highly cross-linked structure synthesized from a phenyl propanoic unit (Rezania et al., 2020). It is a barren sugar-based edifice having a 3-dimensional structure that possesses both cellulose and hemicellulose embedded in it. Lignin generally acts as an

'adhesive' between cellulose and hemicellulose, that retards the production of bioethanol. Thus, several physical, biological, chemical and physiochemical pretreatments are enacted to loosen the strong interactions among these LCB (lignocellulosic biomass) and remove lignin to increase the accessibility of carbohydrates for further process of ethanol production (J. Cai et al., 2017). The lignin component of biomass must be removed and separated before biochemical conversion since microorganisms are unable to break it down during fermentation to maximize the yield of ethanol. Figure 1.3 depicts the widely varying composition of commonly available lignocellulosic sources.

The first step towards utilization of LBs is the disruption of the natural boundaries to extract the cellulose and hemicellulose, which become the substrate for further process of saccharification. At present, this approach is to break the barrier of LCB degradation through pretreatment that can eliminate lignin and hemicellulose along with rupturing of the linkage with cellulose to destroy its crystalline structure and contract its degree of polymerization (Zhang et al., 2020). It was shown that using 2% NaOH (sodium hydroxide) at 121°C for 1 hr removed the lignin content with slight effect on cellulose and hemicellulose as compared with increasing concentration of H_2SO_4 in which cellulose and hemicellulose content increased while reversed with lignin content. Thus, using acid pretreatment, hemicellulose can easily be hydrolyzed (Jin, Song, & Liu, 2020) and further it is required to evaluate the correct compositional analysis of lignocellulosic biomass for maximum conversion yield and to determine the economic process of bioethanol conversion. There are some methods for compositional analysis of LCBs, these are sulfuric acid hydrolysis method, kinetic analysis methods and near-infrared spectroscopy methods (J. Cai et al., 2017; W. Cai et al., 2023).

1.8. Pretreatment process

Pretreatment is the prior and one of the essential and crucial steps towards bioethanol production by disintegration of recalcitrant structure of LCB, simultaneously increasing the porosity and reducing its resistance to deconstruction. These processes can be expensive and energy-intensive regardless of the type and complexity of pretreatment (Shukla et al., 2023). An ideal pretreatment step dwindles the connective link between lignocellulosic recalcitrant structure and makes feedstock available for further process, i.e., enzymatic accessibility and saccharification process with less inhibitor formation and increase in the recovery rate of cellulose and hemicellulose (Kumar et al., 2020). The primary goal of the pretreatment stage is to eliminate lignin and modify the crystallinity of cellulose, which might result in a large surface area, making biomass highly porous. Pretreatment is an energy-intensive phase in the conversion of biomass to bioethanol. In general, four approaches are used, including the physical, chemical, physiochemical, and biological procedures (Figure 1.4). Thus, there is a requirement to make the process cost-effective by deploying advanced techniques of pretreatment. According to various reports, effective pretreatment reduces the size of the biomass, minimizes sugar loss, and maximizes lignin removal along with a reduction in the formation of inhibitors, thereby making the process economical (Solarte-Toro et al., 2019). Pretreatment is

required to disintegrate the lignin structure and to make the cellulosic complex more accessible for hydrolysis by enhancing enzyme accessibility. It is used to reinforce the accessibility and conversion of cellulose to glucose, thus making it more accessible to the enzymatic action by hydrolysis of hemicellulosic content and by solubilization of lignin content in the biomass (Rocha-Meneses et al., 2020). The pretreatment methods show the following effect on the lignocellulosic biomass by comparing its pretreatment efficiency both before and after the pretreatment process. The pretreatment is considered to disrupt the compositional analysis of the biomass and enhances the adaptation towards available biomass with the main emphasis on particle size, and degradation of lignin, hemicellulose, and cellulose for subsequent processing (Guo et. al., 2023). This will enhance the formation of reducing sugar and compatibility towards fermentation, further morphological analysis using XRD, TGA, FESEM, and FTIR spectra show the variation in the structural composition of biomass both before and after the pretreatment process. The efficient pretreatment has minimum sugar degradation with a slight formation of toxic compounds.

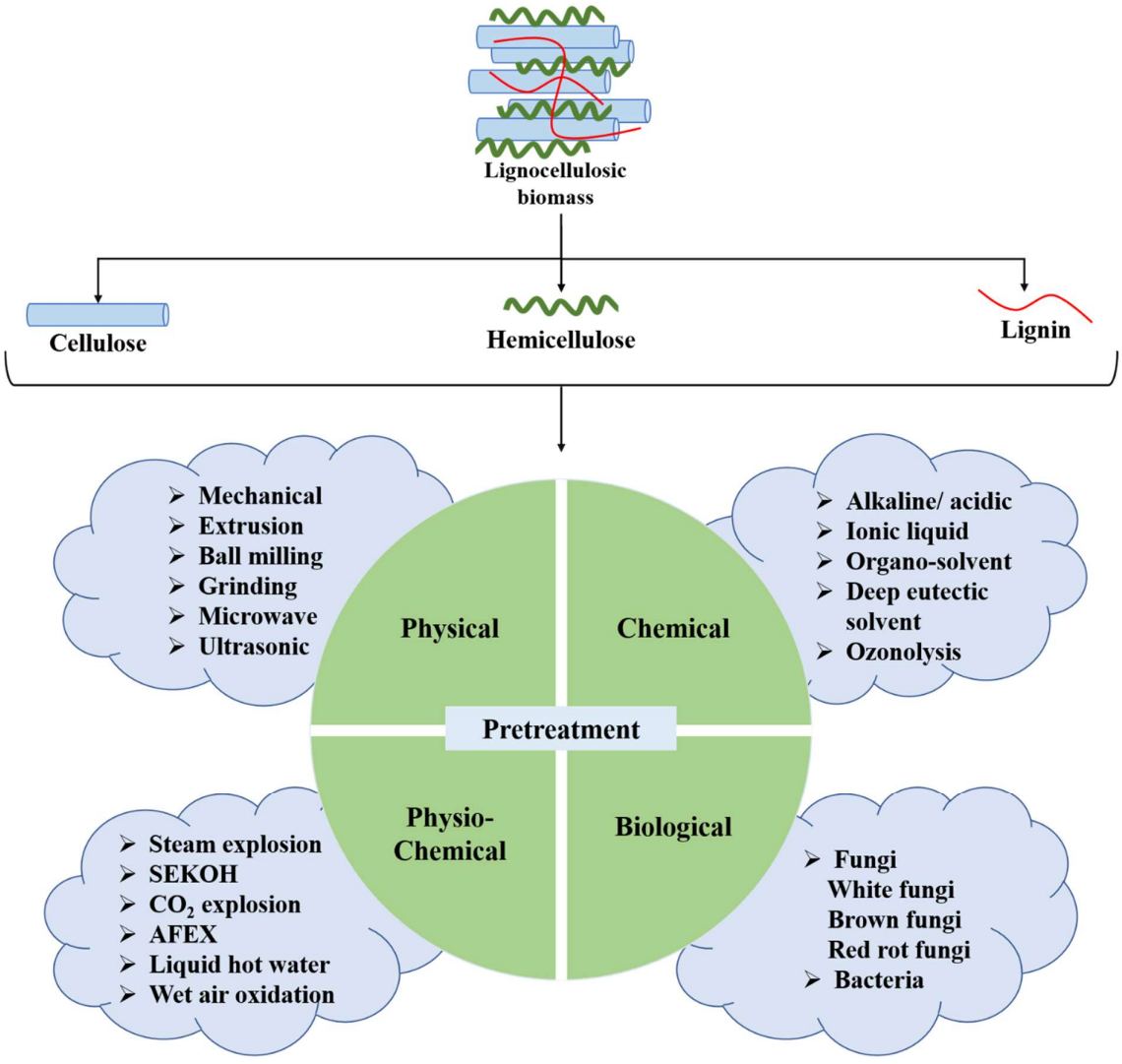


Figure 1.4- Illustration of various available pretreatment techniques

1.9. Saccharification process

The hydrolysis process can be applied to LCBs obtained after various treatment methods, which are required to hydrolyze the treated lignocellulosic to yield reducing sugars. This includes mainly two processes i.e., acid hydrolysis and enzymatic hydrolysis (Auxenfans et al., 2017). Right now, the most frequently used procedure of obtaining cellulosic-based ethanol is the most prominent enzymatic-based process as it is one of the most environmentally friendly processes and ultimately leads to more hydrolysis yield than acidic hydrolysis. Enzymatic hydrolysis is the incorporation of the following enzymes β-1,4-exoglucanase, β-1,4-endoglucanase, β-glucosidase, and β-1,4-endoxylanase for enzyme-based substrate system. Enzymatic hydrolysis is a comparatively expensive process due to the excessive utilization of enzymes for conducting the process. Whereas acidic hydrolysis is performed using various acids such as HCl or H_2SO_4, it may dilute one or concentrate one which can hydrolyze the hemicellulosic part at a lower temperature. It is somehow an accessible process but it is prohibited due to the formation of non-selective by-products such as furfurals, acetic acid and phenolic compounds (Keshav et al., 2016). A dilute acidic hydrolysis process using 0.197 M H_2SO_4 along with 2-methyltetrahydrofuran/H_2O will enhance hemicellulose-derived monosaccharides production to 20.04 g/l of dry biomass with less production of degrading products and only 85.3% removal of furan generated during hydrolysis process (Dávila, Diaz, & Labidi, 2021). Therefore, acidic hydrolysis is confined to the neutralization process and formation of byproducts in the form of inhibitors. Whereas, in the case of enzymatic hydrolysis with enzyme loading lower than the threshold limit will limit the conversion of cellulose and increase the duration of subsequent process (Suresh et al., 2020). So, the saccharification process depends on the specificity of various enzymes used for the process but it is hindered due to the requirement of high fermentation time. It is also noted that in-house production of enzymes will reduce production costs along with the separation, storage and transportation of enzymes (Jin et al., 2020).

1.10. Co-fermentation process

The efficient ethanol production from hydroxylates using acidic or enzymatic saccharification that releases reducing sugar and conversion of all obtained reducing sugar to ethanol is preferred. So, there is a need to find a co-fermentation process that can utilize both hexose and pentose counterparts to their respective by-products (Malik et al., 2021). Various yeast are available for the fermentation process that produces various cellulolytic enzymes required for bioethanol production from the reducing sugar obtained after the hydrolysis process. Now, a day there is a trend of utilizing both pentose and hexose metabolizing strains as well as genetic engineering microorganisms in a single pot. This is basically to increase the yield by fermenting both sugars present in it. Some fermenters can ferment only glucose to ethanol, these traditionally used fermenters are *S. cerevisiae and Z. mobilis* while some of the pentose-utilizing fermenters are *Pichia stipitis, Candida shehatae, and Pachysolen tannophilus.* While some yeast ferment xylose to

ethanol these are *Pichia stipitism, Pachysolen tannophilus, Candida Shehatae,* and *Candida tropicalis* (Pereira et al., 2011a). The efficient ethanol production from hydrolysates using acidic or enzymatic saccharification that releases reducing sugar and conversion of all obtained reducing sugar to ethanol is preferred (Queiroz et al., 2023). So, there is a need to find a co-fermentation process that can utilize both hexose and pentose counterparts to their respective byproducts. Co-fermentation process using immobilized yeast for fermentation has adopted various advantages aspects which include

1. Lowering of microbial contamination due to more cell density and its activity towards the fermentation process
2. Unexpected ethanol production
3. Absorbing of substrate at higher rate
4. High resistance toward substrate concentration
5. Reducing end-product inhibition
6. Protecting cells from inhibitor formation (Malik et al., 2021)

1.11. Bioethanol as a sustainable form of energy

2G bioethanol production from agricultural waste, municipal and industrial waste, and non-food crops benefits the environment more effectively by reducing greenhouse gas emissions while competing for the available agricultural land for food supply. The LCBs utilized for biofuel generation are considered waste being burned by farmers daily. The extraction of ethanol from LCB has the potential to become the leading global sustainable aviation biofuel, necessitating the development of sophisticated biofuel technology (Bansal et al., 2012; Muktham et al., 2016; Soni, Sharma, & Soni, 2023). India has started an aggressive path of renewable energy formation to reduce carbon dioxide emissions in the battle against global warming and to reduce the dependency on the import of crude oils which is the largest expenditure of our economy. National biofuel policy has mandated to blending of 10% green biofuels into petrol (S. Das, 2020a). But India today barely manages to achieve 3% of blending with petrol. So, to meet the requirement, there is a need for 2G biofuels to achieve 10% blending and beyond in the near future. 2G biofuels are extracted from the wastes that don't influence the human food chain (S. Das, 2020b; Tantipaibulvut et al., 2015). Waste materials include trash and sugarcane bagasse in Uttar Pradesh, Punjab, Tamil Nadu, and Maharashtra; rice straw in Punjab and Haryana; cotton and castor stalks in Gujarat and Maharashtra; bamboo in Assam, West Bengal, and Odisha; and cotton and castor stalks in Gujarat and Maharashtra are put together and have potential to meet all the Petro-derived demand to renewable green fuel. However, this path is far from simple, these might be years of effort and amount spent by government and industry worldwide. Currently, there is a total of eight bioethanol plants that are running at a commercial scale utilizing a variety of feedstock available in India. As of now, various bioresources are available that have the potential for bioethanol production, these include industrial waste, agricultural biomass (cotton stalk, rice husk, rice

straw, sorghum stalk, corn cob, wheat straw, sugarcane bagasse, and jatropha pruning), energy crops, and woody biomass (Soni et al., 2023). Figure 1.5 illustrates the mechanism of pretreatment techniques towards bioethanol production and its effect on feedstock with a main emphasis on size reduction, and cellulose disruption along hemicellulose and lignin depolarization.

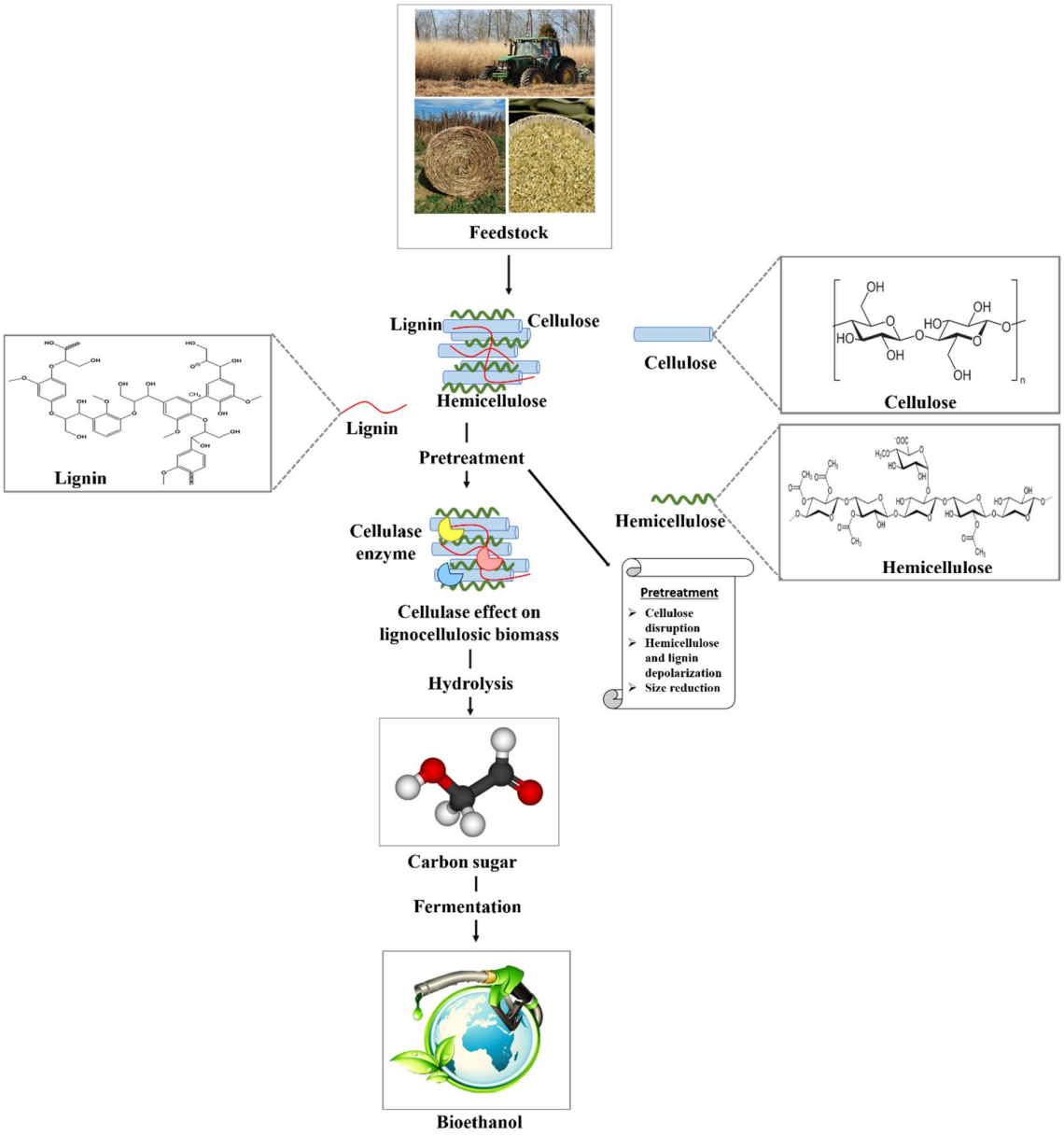


Figure 1.5- Illustrate mechanism of pretreatment techniques toward bioethanol production

1.12. Issues related to bioethanol production

Rice straw produced after harvesting rice in October and November is occasionally burned in the field or used as feedstock for cattle rearing or discarded. This burning will cause inevitable environmental consequences. Thus, rice straw consists of more content of lignocellulosic material that is utilized for the production of low-cost bioethanol.

In the bioenergy and bioproducts industry, time is a critical component. Longer processing time is the biggest roadblock in making bioethanol more cost-effective at the pilot scale. Thus, the integration of various process units was mainly expected to curb the overall processing time. The biomass pretreatment process plays a vital role in bioethanol production (Rathankumar et al., 2020).

The high cost of cellulase enzymes is another bottleneck in the production of bioethanol from lignocellulosic biomass. In this, the main focus is on using low enzyme loading that will increase the glucose obtained from the pretreatment opted for breakdown of lignocellulosic biomass structure (Kaur et al., 2020).

The co-fermentation of xylose and glucose remains an issue for the cellulosic biofuel industry. Optimization may be more challenging in co-culture processes since there are two distinct microorganisms involved, each with a different optimal condition. It was advised that different yeast has different oxygen and sugar absorption in the co-culture system, this would be maximized by increasing the effectiveness of the co-fermentation process (Das et al., 2013).

One option is to develop a simple and environment-friendly approach for reducing the hemicellulose content of the biomass residue after pretreatment. Because plant cell walls are made up of three essential components that are all interconnected, the precise mechanisms through which hydrolysis improves after pre-treatment is unknown.

1.13. Overcome issues related to bioethanol production

Separate pretreatment and saccharification processes are time-consuming and enzyme-intensive processes. Thus, to reduce capital cost towards bioethanol production, there is a requirement to maintain single pot reaction conditions for multiple processes to be performed at a time as well as two phasic hydrolysis processes are to be performed to extract both pentose and hexose sugar from the acidic and enzymatic hydrolysis of obtained pretreated rice straw. Another bottleneck is related to the utilization of total sugar in the fermentation, for this co-fermentation process is incorporated with both hexose and pentose utilizing yeast for the fermentation of desired sugar. The current study is based on an eco-friendly, less tedious, single-pot methodology to reduce the time required for processing.

CHAPTER-2

LITERATURE REVIEW

2.1. Literature review

An extensive review of literature has been supported to understand the method of bioethanol production and mass yield of produced bioethanol from various agricultural waste available with different compositions of cellulose, hemicellulose, and lignin that have been reviewed in detail.

2.1.1. Review on physiochemical pretreatment

2.1.1.1. Steam explosion pretreatment along with acid impregnation

Steam explosion pretreatment is similar to the autohydrolysis technique, which is one of the basic and widely accepted physio-chemical pretreatment methods, due to its environment-friendly nature. It is widely considered a highly cost-effective option over other pretreatment methods. In the steam explosion process, the lignocellulosic biomass (LCB) is promptly heated with saturated steam at high pressure for a short period, probably for a minute, followed by the sudden release of pressure causing expansion of steam within lignocellulosic material, which results in the detachment of individual fibers by interrupting the cell wall structure and solubilizing hemicellulose and lignin. Y. Huang et al., (2015) and Zhao et al., (2023) performed a steam explosion impregnated with sequential 1% H_2SO_4 on cotton stalks biomass, which would increase the digestibility with the highest sugar-ethanol conversion rates, while in comparison with the highest concentration of NaOH (16% w/w) resulted in the highest hexose yield with the lowest conversion rates and highest saccharification yield. The steam explosion pretreatment along with lower concentration of H_2SO_4 had increased the accessibility of enzymatic hydrolysis by 5-6 times from untreated biomass as well as enhanced sugar-ethanol conversion rate. The higher concentration of NaOH showed the highest accessibility towards enzyme but reduced sugar-ethanol conversion rate. Thus, it was found that acid treatment leads to fractional liberation of lignin, oligosaccharides and monosaccharides while alkali causes the disintegration of entire polymers. A similar process of steam explosion with 1% H_2SO_4 was carried out by Semwal et al., (2019), evaluating maximum glucan conversion of up to 89.6% using 5 FPU/g of cellulase enzyme using rice straw as the biomass. It was evaluated that by altering the process parameters at 180℃ temperature for 10 min at 1.5 MPa pressure, greater particle size may be handled with less energy consumption and lower production costs. Further, El Harchi et al. (2018) performed thermal acid hydrolysis using H_2SO_4 and HCl as acid catalysts for the production of bioethanol from green macroalgae *Ulva rigida*. An optimized condition for thermal hydrolysis was 4% (v/v) H_2SO_4 with 10% (w/v) biomass loading for 1 hr incubation time, had resulted in 34.25 mg/ml of reducing sugar concentration and fermentation was performed using *Pachysolen tannophilus* yeast, capable of fermenting both pentose and hexose reducing sugar. The acidic hydrolysis residence time had a significant effect on reducing sugar decomposition. This 60 min of hydrolysis time resulted in 342 mg/l of reducing sugar with 64% of hydrolysis efficiency. Further, the steam explosion was performed in the presence of acid catalysts such as H_2SO_4 and H_3PO_4 by Fockink et al. (2018). The optimized condition for pretreatment was 195℃ for 7.5 min on sugarcane bagasse using 9.5

mg of H_3PO_4. It was evaluated that the emergence of sustainable biorefineries depends on the overall carbohydrate recovery and sugar yield during the pretreatment process. In this total sugar released was 69.4% after the process of pretreatment and hydrolysis. It was stated that steam explosion generated the desirable substrates for enzymatic hydrolysis with 15% of total sugar recovered, although pretreatment required longer residence periods and higher temperatures.

Another study on the production of oligosaccharides and reducing sugar from the species named *Miscanthus* using the steam explosion technique was performed by Bhatia et al., (2020). The optimum temperature maintained during the process was 200℃ at 15 bar pressure for 10 min. The xylo-oligosaccharides produced up to 52% (w/w) of the biomass's original xylan. Chipped biomass particles (10 to 30 mm) produced greater XOS yields (55% w/w of original xylan) than milled and smaller biomass particles (0.18 to 0.85 mm) under identical SE pretreatment conditions (200°C; 15 bars; 10 min) (49 percent w/w). Similarly, Zhang et al., (2022) studied the impact of SE performed at 1.5 MPa for 5 min on defatted soybean meal. Before experimenting, biomass was soaked in water (1:15 w/w) for 2 hrs. After pretreatment, morphological analysis was performed using SEM analysis showing cracks, on the biomass surface with an irregular void by increasing pressure due to saturation of water vapour. It was estimated that increasing pressure up to 1.5 MPa degraded hemicellulose and cellulose to 71.1% and 50.7% respectively. Sharma et al., (2015) and Zhao et al., (2023) facilitated steam explosion technique using water, 0.5% H_2SO_4 and 0.5% H_3PO_4 as the reaction media using rice straw as a feedstock for bioethanol production. Cellulase enzyme with 578 FPU/g was used for the saccharification process. The greatest sugar yield was 86% after enzymatic saccharification of solid fraction remained after pretreatment at 200℃ for 10 min retention time. The concentration of glucose obtained after hydrolysis was 51.5 g/l after 72 hrs of reaction. It was evaluated that higher oligomers significantly reduce the amount of sugar produced by enzymes, as was seen in the case of water. It was observed that total saccharification yield was determined by mass balance experiments for water and SA-assisted SE to be 81.8 and 77.1%, respectively. Further research using thermochemical pretreatment operating at 192℃ on pine, poplar and wheat straw with acidic hydrolysis using H_2SO_4 was performed by Cornejo et al., (2019). It was estimated that harsh pretreatment condition was suitable for glucan conversion during enzymatic hydrolysis while mild condition was suitable for xylan conversion to furfural during the process. It was evaluated that 50 kg of biomass was pretreated to form 12.6 kg of glucose and 2.5 kg of furfural from subsequent xylan conversion. Another study performed by Kaur et al., (2022) using 1% HNO_3 at 121℃ for 30 min, resulted in 71.32% of xylan conversion which was considered an efficient process in the production of xylitol from pretreatment process of rice straw. It was evaluated that HNO_3 pretreatment was claimed as the preferred approach due to many advantages such as a shorter duration of processing time with better sugar yields and the generation of substantially fewer inhibitory chemicals than H_2SO_4. Furthermore, HNO_3 is less corrosive and has a higher efficiency for removing hemicellulosic compounds than HCl and H_2SO_4. Similar work was performed by Kim et al., (2014) and Wang et al., (2023)

using HNO_3 as an acid catalyst for pretreatment of rice straw. It was evaluated that 0.65% HNO_3 at 158.8℃ temperature for 5.86 min, resulted in 86.5% of xylose yield from liquid fraction with 83% of enzymatic digestibility. Further, nitrate extracted from HNO_3 used as a catalyst facilitates increment of ethanol yield to 14.50 g/l using *Pichia stipitis* as a fermenting yeast. Thus, it was concluded that HNO_3-based treatment served as a nitrogen source for further processing steps and ultimately reduced the production cost of bioethanol. More intriguingly, steam explosion requires less energy than that of mechanical pretreatments, which means it could do away with the need for recycling and lower environmental costs associated with chemical pretreatments (Hoang et al., 2023). Depending on the distribution and chemistry of the lignin component in the steam-treated rice straw fibers, steam explosion can provide materials with less moisture absorption, better strength characteristics, stronger thermal stability and lesser cellulose density (Shangdiar et al., 2023).

2.1.1.2. Steam explosion pretreatment along with alkaline impregnation

Various researchers have performed alkaline impregnation along with steam explosion. C. Zhao et al., (2018) performed NaOH pretreatment on rice straw and corn stover followed by enzymatic hydrolysis using cellulase enzyme obtained by *Trichoderma reesei* and *Aspergillus niger*. With enzymatic hydrolysis, the yield of up to 81.5% and 70.5% from corn stover and rice straw respectively containing glucose and xylose. The fermentation process using *S.cerevisiae* produced 27.6 g/l and 21.7 g/l of ethanol. It was evaluated that 33.1 gm of ethanol from 113.20 gm of corn stover and 26 gm of ethanol from 117.58 gm of rice straw. Co-fermentation using *S.cerevisiae* with xylose fermenting capability surges fermentation after 9 hrs while *P.stipitis* has the capability for maximum ethanol yield of 2.51 times and is considered to be more effective towards the production of bioethanol. Similarly, Liu et al., (2020) studied steam explosion assisted 1.2% CaO, w/v and alkaline treatment with 0.8% NaOH on corn stalks, at 50 Hz frequency and 2200 W for 1 hr was utilized for the pretreatment process. Enzymatic hydrolysis was performed using 22.5 FPU/gm biomass of cellulase dosage for 48 hrs at 50℃ resulting in a reduced sugar yield of 335.09 mg/l biomass. Thus, a cheap pretreatment process meets the requirement of industrialization. Further, steam explosion with prior soaking in choline chloride in the ratio 1:2.2 (w/w) with the reaction temperature at 184ºC for 15 min at the pressure of around 1.0 MPa resulting in 84.7% delignification of corn stover biomass, obtained by Nasir et al., (2020)**.** The SE-ChCl pretreatment resulted in the highest lignin and xylan removal while maximum recovery of glucan up to 74.59%. The enzymatic hydrolysis was performed at 20 FPU/g of Novozyme incubated at 50ºC at 170 rpm for 72 hrs. The SEM analysis showed the expulsion in the lignin and hemicellulose structure that resulted in the loosening of the recalcitrant biomass structure and led to the exposure of the interior structure for further enzymatic hydrolysis. The XRD analysis showed the cellulose crystallinity from 27.8% to 38.8%. The cellulose conversion at a higher concentration of ChCl to 84.2% at subsequent low enzyme loading. This ChCl resulted in efficient lignin removal with reduced enzyme loading with enzyme efficiency up to 6.4 times higher than single-step processing. A similar study was performed by

Chu et al., (2018) using two-stage pretreatment using steam explosion and alkaline sulphonation on Eucalyptus woody biomass. This showed the alkaline sulphonation along with steam explosion resulted into 69.37% of lignin removal from the biomass. This rise in delignification was due to first-stage of alkaline pretreatment, which had the potential to noticeably increase substrate porosity, making the substrate more porous and hence more amenable to the second-stage steam treatment. Enzymatic hydrolysis was performed using 10 U of β-glucosidase and 20 FPU of cellulase in 50 mM acetate buffer with pH 4.8 at 50℃ of incubation time. In this sequential fermentation process that was carried out by *S. cerevisiae* and *Pichia stipitis*. The glucose fermentation was performed using *S. cerevisiae* at pH 5.5 at 30℃ for 24 hrs while xylose fermentation was performed using *Pichia stipitis* at 30℃ for 36 hrs. During the process 96.74% of cellulose recovery was obtained with delignification of 57.09% and the obtained final concentration of sugar was about 77.04 g/l from enzymatic hydrolysate. This resulted in 74.24 g/L ethanol production after depletion of the accessible glucose during 24 h glucose fermentation, with 0.46 g/g of sugar-ethanol conversion rate.

2.1.1.3. Steam explosion pretreatment along with H_2O_2 impregnation

Steam explosion impregnation with H_2O_2 is used for delignification due to the presence of highly reactive radicals namely superoxide anion radical ($O2^-$) and hydroxyl radical (OH-). These forms of active radical delignification of lignocellulosic biomass structure through the process, namely, oxidation and degradation (Muthuvelu et al., 2019). Alexandropoulou et al., (2023) obtained bioethanol from willow saw dust and date palm fibers using a combination of alkaline hydrogen peroxide and sodium hydroxide pretreatment. Pretreatment was performed at 180°C with 4% (g/g) of H_2O_2 loading for 10, 20, 30, and 60 min of treatment time. It was found that 60 min of pretreatment time had led to 88% of glucomannan removal from water-insoluble solid. It was suggestive that total cellulosic content of 44.2% is present in hydrolysate. The water-insoluble solids yield decreased from 91.9% to 69.9% with the increase in the concentration of H_2O_2. It was concluded that H_2O_2 dosage and pretreatment time had little influence on acetic acid concentrations, indicating that acetic acid was primarily produced through acetyl group breakage. Similarly, C. Huang et al., (2020) performed pretreatment using modified alkaline hydrogen peroxide (MAHP) at a mild condition of 100℃ with 3 wt% H_2O_2 along with 1 wt% ethanol concentration showed 79.25% lignin removal. The extensive study on XRD analysis showed that cellulose crystallinity (CrI) increased from 57.04% to 66.24% when there was an increase in pretreatment temperature and chemical loading that occurred by lignin and hemicellulose removal from LCB. Enzymatic hydrolysis for 72 hrs increased glucan and xylan to 96.76% and 97.38% with H_2O_2 pretreated sample at 100℃ temperature. Further, research done by Verardi et al., (2018) to study the effect of steam explosion assisted with hydrogen peroxide at 0.2% and 1% concentration on sugarcane bagasse. The inclusion of hydrogen peroxide with steam explosion methods has increased the glucose yield by up to 12% and the xylose yield by up to 34%. H_2O_2 acts as a strong oxidizing agent that promotes the loosening of LCB along with lignin solubilization.

The impregnation of H_2O_2 resulted in a decrease in the formation of arabinose and mannose by-products with an increase in the hydrolysis time along with a decrease in the yield of cellobiose to about 30%. The maximum glucose yield was obtained at steam explosion technique using 210℃ for 15 min with impregnation using 1% H_2O_2. Similarly, Bazargan et al., (2020) studied alkaline peroxide pretreatment on rapeseed straw using 1% (v/v) at 50℃ for 1 hr showing 71.78% of delignification was obtained along with 88.47% of silica removal with 8% NaOH treatment followed by 0.5 M $MgSO_4$ treatment that resulted in an enhancement of efficiency to 0.92% ethanol production. It was estimated that at lower temperatures, maximum reducing sugar was obtained due to decomposition of H_2O_2 to water at increasing temperature while the increase in pretreatment time had no significant effect on the saccharification process. The combined fermentation utilizing *P.stipitis* and *S.cerevisiae* for 48 hrs resulted in 17.39 g/100 g biomass. The alkaline peroxide pretreatment approach, which effectively reduced the production of furfural and HMF has been utilized in several studies. Thus, alkaline peroxide pretreatment was considered an effective method for reduction in the formation of inhibitors for bioethanol production. A further efficient process using alkaline H_2O_2 was performed by Yuan et al., (2018) using 40 mg of H_2O_2 per 1 gm of wheat straw at 50℃ for 7 hrs to develop digestible biomass for efficient hydrolysis with a sugar conversion rate of about 92.4% was achieved. Co-fermentation process using *S.cerevisiae* was performed and yielded 31.1 g/l of ethanol and 86.4% of silica was removed from wheat straw. 0.2 M NaOH treatment was used to remove 91% of silica after 5 hrs of treatment at 30℃ resulted in recovery of 86.4% of silica and 54.1% of lignin from the H_2O_2 pretreated wheat straw. From the above study, it was evaluated that the impregnation of H_2O_2 resulted in an increase of fermentable sugar with no residues in the biomass and also led to the reduction in the formation of inhibitors. Above all, the cost of H_2O_2 was comparatively lower than that of other chemicals used for the pretreatment process.

2.1.2. Liquid hot water (LHW) pretreatment process

Lyu et al., (2018) proposed a two-phase extraction of pentose and hexose sugar from the cassava biomass, firstly the biomass was treated with varied temperatures ranging from 180℃ for 60 min and secondly the biomass was treated with 200°C for 30 min. Further enzymatic hydrolysis was performed for the extraction of hexose sugar. It was found that the yield of C5 and C6 sugar was 66.48% and 45.62%. The amount of C5 sugars produced was increased by 14.66% and the amount of C6 sugars produced was increased by 39.4% compared to one-step pretreatment. Two-phasic sugar extraction aimed to enhance the hexose and pentose sugar yield to maximize the yield of bioethanol. Similarly, Imman et al. (2021a) produced bioethanol using pineapple leaves due to high cellulosic content i.e., 62.37% in it. LHW pretreatment was performed in a high-pressure reactor at 130℃ for 40 min along with 0.6 M H_2SO_4, releasing 91.54% of glucose yield. XRD analysis showed a rise in *CrI* from 54.34% of the untreated sample to 65.37% of the pretreated sample, this concluded that amorphous structures including lignin and hemicellulose were eliminated from the crystalline cellulose as the main part in pretreated biomass. The

above research resulted in less solid biomass loading which yielded 94.68% of ethanol. It was estimated that acid-impregnated LHW pretreatment resulted in the enhancement of reducing sugar recovery during enzymatic hydrolysis. Another research on hydrothermal pretreatment was performed by Syaftika & Matsumura, (2018) using rice straw as substrate for bioethanol production. The optimum condition for pretreatment was 150℃ and 250℃ for 30 min. It was evaluated that the highest yield of glucose was obtained at a temperature up to 180℃ followed by enzymatic hydrolysis and further yield decrease was observed with the increase of temperature to 200℃, 230℃ and 250℃. Thus, optimum pretreatment temperature must be maintained while performing physiochemical pretreatment methods. Further work performed by Shang et al., (2019) stated that increasing the temperature from 150 to 225℃ as well as retention time from 5 to 60 min, resulted in hemicellulose degradation from 27.69% to 99.07% but it was also cleared that pretreatment at 225℃ had a negative effect for any pretreatment time utilized for biomass breakdown. Thus, the effective temperature for pretreatment process during liquid hot water pretreatment was at 175℃ for 30 min.

Another work performed by Imman et al., (2015), utilized alkaline-assisted liquid hot water treatment using rice straw as a feedstock for production of bioethanol. The optimum condition maintained at 140℃ for 10 min using 0.25% NaOH as a catalyst, this was resulted in 49.3% of pentose yield and 71.8% of glucose yield after enzymatic hydrolysis. Thus, the addition of other chemicals during liquid hot water treatment led to an enhancement of the yield of xylose in obtained hydrolysate after pretreatment and increased the solid digestibility during enzymatic action. Additionally, combining the process of hydrothermal as well as alkaline treatment performed by Mariano et al., (2021), demonstrating 40 min liquid hot water treatment followed by 1% NaOH treatment on coconut pulp waste, resulted in a total reducing yield of 257.14 g/l after enzymatic hydrolysis that was carried out using 2% cellulase enzyme loading. The fermentation process was performed using *S.cerevisiae* placed in an incubator at 30℃ for 72 hrs, resulting in 27.19 g/l of ethanol yield with 51.83% of fermentation efficiency and 0.57 g/l/h of ethanol productivity. Thus, elevating the alkaline concentration also enhances sugar production, suggesting that NaOH is required in the pretreatment process. Meanwhile, enzymatic hydrolysis supports low alkali concentrations due to lignin polymerization caused by enhancing pretreatment severity. Similarly, Liu et al., (2020a) performed liquid hot water treatment assisted with $NaOH/O_2$ at an optimum condition of 180℃ for 60 min. Enzymatic hydrolysis was performed using 3 FPU/g of cellulase loading along with PEG-6000 to stimulate the fermentation process. It was evaluated that from 100 gm of biomass, 40.2 g/l of bioethanol concentration was obtained with 83.7% of bioethanol production yield. Thus, the utilization of PEG-6000 overall reduces the cellulase loading during the hydrolysis process and ultimately, reduces the bioethanol production cost. Further study performed by Imman et al., (2014) stated the effect of acid and alkali on the pretreated rice straw, using 0.25% H_3PO_4, HCl, H_2SO_4, oxalic acid and NaOH. The optimum conditions for pretreatment were maintained at 140-180℃ for 5-20 min of residence time. It was evaluated that under optimal LHW conditions at 160 °C, oxalic

acid and NaOH was used as notable promoters, resulting in 84.2% glucose yield and 91.6% glucose recovery through enzymatic hydrolysis with the least furans formation. Thus, acids treatment is significant in hydrothermal pretreatment because they break down H-bonds present in hemicellulose, resulting in cellulosic partial hydrolysis and a portion of soluble lignin.

Table 2.1- Recent studies on LHW pretreatment on various LCB

Pretreatment method used	Biomass used	Required conditions	Enzymatic hydrolysis	Effect of pretreatment on biomass and sugar recovery	Ref.
LHW	Corncob	160°C, 10 min	5% biomass with 10 FPU/g Celluclast in 50 mM Sodium acetate buffer, incubated at 50°C, pH 5, 72 hrs	CrI increases up to 73.6% Glucose recovery up to 81.8% and pentose recovery up to 71.2%	(Imman & Laosiripojana, 2017)
LHW treatment impregnated with 0.25% H_2SO_4, HCl, H_3PO_4, oxalic acid and NaOH	Rice straw	160°C, 20 min	5% biomass loading, 10 FPU/g cellulase, 330 IU/g β-glucosidase and 120 IU/g Hemicellulase in 50 mM sodium acetate buffer, pH 5 at 30 rpm for 72 hrs	Increase in CrI up to 68.6-70.3 %. Oxalic acid shows better results with the smallest loss of glucose during glucan hydrolysis	(Imman et al., 2014)
LHW pretreatment assisted with 0.3 M H_2SO_4, 0.9 M HCl, 0.6 M HNO_3	Pineapple leaves	143.2°C for 38.4 min	5% biomass with 25 FPU/g of Cellic Ctec2, Novozymes in 50 mM sodium citrate buffer, pH 4.8 with 1% sodium azide, 50°C at 30 rpm for 72 hrs	Higher ethanol yield of 94.68%	(Imman et al., 2021b)
LHW pretreatment assisted with	Reed	180°C for 60 min	Cellulase loading- 3 FPU/g with 0.01	Glucose concentration 3.24	(Lu et al., 2020b)

$NaOH/O_2$			g/g PEG 6000	g/l, Ethanol concentration 26.45 g/l	
LHW pretreatment	Cassava straw	1st stage- 180°C for 60 min 2nd stage- 20°C for 20 min	Enzyme CTec2 from Novozymes dosage at 31 mg/g of glucan, pH 4.8, incubated at 50°C for 72 hrs at 120 rpm	Yield of C5 sugar 83.15% and C6 85.02%	(Lyu et al., 2018)
LHW pretreatment assisted with Na_2CO_3/O_2	Reed	170°C for 60 min	21.5% biomass loading with 20 FPU/g cellulase, sodium citrate buffer, pH 4.8, incubated at 50°C at 150 rpm for 72 hrs	Glucose conc. 16.3 g/l, Bioethanol conc. 66.5 g/l and yield of 0.133 g/g	(Lu, Song, et al., 2020)

2.1.3. Utilization of various chemicals for pretreatment process

Ebrahimi et al. (2017) analyzed acidified aqueous glycerol and glycerol carbonate pretreatment on rice husk at 90℃ and 130℃ for 60 min and performed enzymatic saccharification using 10 FPU/gm glucan of cellulase that had resulted into digestibility of glucan to 78.2% and 69.7% in 72 hr rice straw. Simultaneous saccharification and fermentation were performed utilizing *S. cerevisiae* yielded in 11.58 g/l of ethanol concentration after 3 days of incubation, from 2.34 g/l of ethanol concentration produced from untreated biomass. It was evaluated that enhancing pretreatment time over 60 min had led to more loss in the glucan content as in this method with 69.70% of glucan digestibility obtained at 60 min of reaction time. Another researcher Zhao et al. (2018) performed supercritical CO_2 at low temperatures around 50-80℃ and pressure up to 17.5-25 MPa for 12- 60 hrs pretreatment time on various agricultural biomass. $ScCO_2$ was considered an effective pretreatment method to increase the enzymatic accessibility of cellulosic biomass towards further processing. Further, it was estimated that with further increase in pressure leads to a decrease in enzymatic hydrolysed sugar yield while the increase in the temperature for pretreatment of rice straw increases sugar yield up to 100°C and then decrease with further increase in the pretreatment temperature. Thus, in this study lower temperature pretreatment was considered effective towards enzymatic hydrolysis and also prevented degradation of hemicellulose. An alternative method using deep eutectic solvent for

pretreatment utilized by Liu et al., 2021 demonstrated bioethanol production from sugarcane bagasse in the form of triethyl benzyl ammonium chloride/ lactic acid that increases cellulose digestibility by 88.23% with 4 hrs of pretreatment time in the ratio 1:15 at 120℃ reaction temperature. Enzymatic hydrolysis was performed using cellulase and β-glucosidase at a higher temperature of 140℃ led to higher lignin and xylan removal 94.13%, 89.48% due to breakage of the bond between cellulose, hemicellulose and lignin while the temperature at 120℃ had resulted into maximum cellulose recovery of 95%. The CrI of untreated sample was 50.96% and enhanced to 69.47% on pretreatment at 140℃ of treatment time. In this study, 82 CBU/gm glucosidase and 25 FPU/g cellulase were used to evaluate total sugar yield after enzymatic hydrolysis. It had been noted that fermentable sugar from DES pretreated sample enhanced the accessibility of enzyme up to 237%. The fermentation process increased the ethanol concentration of DES pretreated sample to 16.84 g/l, it was evaluated that sugar concentration remains constant on increasing the fermentation time. Additionally, Madu & Agboola, (2018) demonstrated bioethanol production using rice husk pretreated with sodium hydroxide, hydrochloric acid and ferric chloride for 15 min at 121℃ temperature. Separate saccharification and fermentation were performed using cellulase from *Trichoderma reesei* and *S.cerevisiae* for 48 hrs in a shaking incubator that resulted in a maximum sugar yield of up to 3.875 mg/ml of glucose and the highest ethanol yield of 3.802%. It was found that increasing the fermentation period up to 72 hr, resulted in the formation of acetic acid from $FeCl_3$ and NaOH pretreated biomass. Further, Jiradechakorn et al., 2023 performed alkaline pretreatment as a measure to remove lignin via the saponification process. It was found that alkaline pretreatment has the potential to increase pore size and swell structure, which would improve enzyme accessibility as well as lead to breakage of cellulosic glycosidic linkage and is hydrolyzed further to reduce the polymerization of cellulose. The result shows that the highest reducing sugars were obtained by soaking biomass in NaOH for 30 minutes, yielding 0.4513 kg reducing sugar/kg biomass. This was followed by ball-milling for 18 hours which produces the highest energy efficiency. Thus, using alkaline pretreatment as a sustainable and environmentally friendly technology integrates a hydrochemo-mechanical pretreatment process into the biorefinery framework for cellulosic bioethanol production.

Table 2.2- Recent research done on steam explosion pretreatment techniques on various LCB

Pretreatment techniques	**Biomass**	**Required condition**	**Degradation in cellulose & hemicellulose**	**Major findings**	**Ref.**
Steam explosion	*Miscanthus*	200℃, 15 bar, 10 min	Xylo-oligosaccharide yield 55%, w/w	Larger biomass (10-30 mm) particles led to higher XOS yield (55%, w/w)	(Bhatia et al., 2020)

Steam explosion & catalysed with H_3PO_4 & H_2SO_4	Sugarcane bagasse	195°C for 7.5 min	69.4% of free sugar released after pretreatment	Maximum mass recovery in presence of acid catalysts and higher xylan content up to 6.6 wt.%	(Fockink et al., 2018)
Steam explosion + 0.5% H_2SO_4	Rice Straw	200°C/ 100 bar/ 10 min	Increase sugar concentration to 12.4 g/l	Max. sugar yield 86%	(Sharma et al., 2015)
Steam explosion	Defatted soybean meal	1.5 Mpa/ 193.1°C for 5 min	Cellulose- 50.7% Hemicellulose- 71.1%	At high pressure, biomass saturated with water vapour, the fiber network gets relaxed and lignin content migrates	(Zhang et al., 2022)
Steam explosion + 0.8% w/v NaOH + 1.2% w/v CaO	Corn stalk	2.4 gm NaOH, 3.60 gm CaO in 5:1 ratio (v/w), 130 kPa pressure for 1 hr	54.7% reducing sugar yield	*CrI* has increased little due to an increase in the order of amorphous cellulose and degree of freedom.	(C. Liu et al., 2020)
Stem explosion + sulphonation (Na_2SO_3 (12%, w/w) + $NaHCO_3$ 4%, w/w)	Woody Eucalyptus	210°C for 5 min	Glucan Hydrolysis increased to 57.89% from 12.23%	Solubilization of hemicellulose Hydrolysis yield was higher at 210°C as compared to 200°C steam	(Chu et al., 2018)
Steam explosion + 1%, w/w, dil. H_2SO_4	Rice straw (10 mm particle size)	200°C for 10 min	Glucan conversion to 89.6%	Large particle size consumes less energy & low production cost	(Semwal et al., 2019)

2.1.3.1. Pretreatment process using hydrogen peroxide impregnated with acids

Further combined process of H_2O_2 with other acids was incorporated and used for the pretreatment of biomass. The most commonly used was HPAC i.e. Hydrogen peroxide with acetic acid and phosphoric acid.

Liao et al., (2021) performed alkaline peroxide-acetic acid along with NaOH pretreatment that tend to remove 94.1% of lignin with the removal of acetyl group from the recalcitrant structure of the biomass. It was found that NaOH treatment reduces the treatment time by up to 50% and HPAC loading by up to 35% without effecting the yield of hydrolysis. The CrI was increased to 46.5%-66.7% from 48.8% while after alkaline pretreatment it changed to 66.9% because of the exclusion of amorphous components like lignin and hemicellulose from the pretreated biomass. Similarly, Song et al., (2020) performed HPAC pretreatment on waste bamboo for bioethanol production with xylose and ethanol yield of 76.7% and 83.1% respectively and both were separated from the pervaporation technique. During HPAC pretreatment, the hydrolysis efficiency was increased by 95% of the conversion rate. The 10% of biomass was soaked in 1:1 HPAC solution with 30% H_2O_2 boiled at 85℃ for 2 hrs of pretreatment time. The enzymatic saccharification was performed by utilizing sodium citrate buffer using 50 FPU/g of cellulase loading and was carried out in a shaking incubator at 200 rpm at 45℃ for 48 hrs. The further analysis of sugar was performed by GC and xylose was estimated to be 28.9 g/l in the pretreated biomass. Further, HPAC pretreatment was performed by Ying et al., (2021) on poplar biomass with 85.8% of glucan content present in it. It was evaluated that 250.8 g/l of glucose concentration was achieved with 40% of biomass loading. It was estimated that HPAC was an effective method for bioethanol production from the lignocellulosic waste. This further processing provides various value-added products such as xylitol and xylulose obtained from xylose that improve the cost-competitiveness of the production of bioethanol. Another research performed by Mota et al., (2019) extensively studied biomass delignification using HPAC with an efficiency of 45-75%, enhancing the saccharification efficiency from 2.6 to 7.1 folds along with adsorption capacities of enzyme of pretreated maize straw, eucalyptus bark, and sugarcane bagasse. The HPAC pretreatment was performed in an incubator at 80℃ for 2 hrs reaction time. The reduction in lignin content following HPAC pretreatment led to an increased polysaccharides cell wall surface area. This, in turn, enhanced the adsorption of enzymes on to pretreated feedstock, resulting in higher hydrolysis yield. Thus, it was considered as valuable method towards 2G ethanol production. While impregnation of H_2O_2 with phosphoric acid was studied by Yao et al., (2019) on wheat straw used as the biomass. The optimum condition for pretreatment was maintained at 40.2℃ for 2.9 hrs with 67.8% of lignin removal. The enzymatic saccharification was carried out for 240 hrs at 140 rpm with cellulase loading at 20 FPU/g and 1 g/l of Tween 80 used as an additive during the hydrolysis process. Another study was performed by Phan & Tan, (2014) using pressurized CO_2 with 20.6 MPa with 1% H_2O_2 at 273 K temperature. The enzymatic hydrolysis was performed with cellulase loading of 15 FPU/g cellulose. It was found that the above pretreatment condition resulted in 97.8% of glucose recovery. Under the mentioned condition, the glucose recovery rate was 97.8%, this outcome suggests that superoxide anion radicals (O^{2-}) and hydroxyl radicals (HO^-), generated through the alkaline breakdown of H_2O_2 were able to degrade and oxidize lignin, which led to increase in glucose recovery. Furthermore, the step involving $scCO_2$ treatment expands the surface area available for H_2O_2 treatment. This facilitates the

breakdown of hemicellulose and the removal of lignin, as well as the potential for enzymatic hydrolysis of pretreated biomass.

Table 2.3- Recent research on H_2O_2 and HPAC pretreatment performed on various LCB

Pretreatment methods	Biomass	Required pretreatment condition	Changes after pretreatment	Ref.
Alkaline hydrogen peroxide treatment	Bamboo	3%, v/v H_2O_2, 100°C, 2.2% w/v NaOH	76.5% of glucan, 56% of xylan recovered & 79.25% of lignin removal	(Huang et al., 2020)
HPAC	Maize straw, Sugarcane Bagasse, Eucalyptus Bark	10 ml HPAC (1:1, v/v), incubated at 80°C for 2 hr	Cellulose crystallinity increases from 34% to 53%	(Mota et al., 2019)
Alkaline hydrogen peroxide	Wheat straw	0.2 mol/l NaOH at 30°C for 5 hr 20 mg H_2O_2/g biomass 50°C for 7 hrs	42.7% of lignin removal	(Yuan et al., 2018)
Hydrogen peroxide-acetic acid (75% HPAC)	Poplar	30%, w/w H_2O_2 & 99%, w/w acetic acid in 1:1 ratio 100 mM H_2SO_4 as catalyst (1:10, w/v) 80°C, 2 hr	delignification from 28.2% to 3.1% Glucan content increased from 40% to 67.2%, with 11.8% glucan removal	(Liao et al., 2021)
HPAC	Bamboo	30% H_2O_2: CH_3-COOH (1:1, v/v)	Reducing sugar yield increases by more than 1.3-fold while cellulosic content increases by 1.7-fold	(Song et al., 2020)
H_2O_2 + H_3PO_4 (PHP)	Wheat straw	74.92 gm H_3PO_4 (85%, w/w) & 5.08 gm H_2O_2 (30%, w/w)	54.1% of lignin removal, with 92.4% of sugar conversion	(Yao et al., 2019)
Alkaline hydrogen	Sugarcane	Pressured CO_2 –	97.8 %, w/w of glucose	(Phan &

peroxide+ supercritical CO_2 + ultrasound	bagasse	20.6 Mpa, 453 K temperature for 1 hr, 0.6% H_2O_2 treatment for 9 hr and 4 hr ultrasound retention time	recovery	Tan, 2014)
Combined process of acetic acid and H_2O_2 (AC-HPAC)	Poplar	5%, v/v Acetic acid with solid to liquid ratio of 1:10 100 ml HPAC (80%, v/v) & 100 mM H_2SO_4 at 60℃ for 2 hr.	Glucan content increased from 42.1% to 54.5% with 85.8% glucan content	(Ying et al., 2021)

2.1.3.2. Oxalic acid pretreatment on various LCB

Lee et al., (2011) performed oxalic acid pretreatment on corn cob using 30 g/l of oxalic acid. Further, simultaneous saccharification and fermentation process was performed using β-glucosidase and cellulase, and *Pichia stipitis* for the fermentation process performed in an orbital shaker at 30℃ at 150 rpm. The total fermentable sugar of 35.29 g/l was generated from 80.3 kg of corncob and yielded ethanol up to 10.3 grams per liter. These findings indicated that pretreatment utilizing oxalic acid could be explored further for pilot-scale research, and then for large-scale operations. Another research following with recycling was oxalic acid after the pretreatment process was earlier performed by Lee et al., (2013). It was reported that recovery of oxalic acid after the pretreatment process through electrodialysis led to the removal of inhibitors during the fermentation process and further improved the fermentability of the process. This will further enhance the hydrolysis efficiency as well as bioethanol yield up to two times higher than the originally obtained hydrolysate. Further, Kundu & Lee, (2015) performed oxalic acid treatment of yellow poplar that was earlier deacetylated using 0.8% NaOH at 60℃ for 80 min. At the same time, Simultaneous fermentation and saccharification of biomass was performed using β-glucosidase and cellulase and *Pichia stipitis* was used for fermenting sugar to ethanol. The optimum condition for pretreatment was 0.1 M oxalic acid at 150℃ for 45 min of treatment which resulted in 14.50 g/l of xylose and a further increase in the pretreatment time resulted in a decrease in xylose concentration. Thus, it was evaluated that pretreatment time and concentration of oxalic acid are important independent factors influencing biomass recovery following pretreatment. The recovery of biomass and xylan content was directly associated with increased production of ethanol. Another study lists the synergistic impact of oxalic acid in the pretreated biomass and on its further conversion process by Zhuang et al., (2022). It was found that peracetic acid and oxalic acid treatment at 140℃ for 60

min resulted in delignification of 85.4%, xylan removal of 93.9% and a further resulted in 758 mg/g of obtained biomass. Enzymatic hydrolysis was performed using 20 FPU/g of cellulase loading in 50 mM sodium citrate buffer. It was found that CrI of oxalic acid pretreated biomass was 58.1% cellulose accessibility towards enzymatic action and the rise in glucan content up to 58.6-76.2% rise in glucan content with total 355 mg/g of glucose released during enzymatic action. Thus, this work revealed that pretreatment using acidic oxidative reaction led to fractioned, oxidized, and deconstructed lignin due to the breakdown of C-O and C-C bonds in lignin. Further, Kundu & Lee, (2016) performed oxalic acid pretreatment with 100 mM concentration at 170℃ for 50 min and further detoxification was carried out using resin adsorption (XAD-4). The *CrI* of pretreated biomass was 56.93% showing a reduction in the amorphous region after pretreatment. It was evaluated that after 96 hrs of fermentation, the net yield of ethanol decreased after detoxification. Thus, it was found that detoxified hydrolysates have higher ethanol fermentability than the original hydrolysate. Another study performed by Chotirotsukon et al, (2019) using 300 mM oxalic acid at 170℃ pursued by extraction of acetone at 30℃ led to 71.7% of cellulose recovery from sugarcane trash biomass. It was found that increasing the temperature of reaction mixture led to a decrease in the overall solid recovery of pretreated biomass and also reduced the excessive cellulosic degradation. The enzymatic hydrolysis was performed using 5 mg/g of Novozymes in 50 mM sodium acetate buffer. The optimised pretreatment was performed by Ramaraj & Unpaprom, (2019) using oxalic acid**.** The study suggests that at the time of pretreatment, the rate of degradation was effected by xylan breakdown. This caused the quantity of glucan that remained in the pretreated biomass to be greater than that of the native poplar biomass. The pretreated biomass resulted in a higher ethanol yield of 34.54 g/l with 95.21% of fermentation efficiency. This finding may be valuable in determining the viability of oxalic acid pretreatment toward the production of bioethanol. Further, oxalic acid-assisted ball milling pretreatment was performed by Deng et al., (2016) using the hydrothermal treatment method. It was found that oxalic acid treatment for 60 min at 130℃ resulted in a maximum of 86.10% of xylose yield, further, it was estimated that particular optimized temperature and time resulted in reduced production of inhibitors and acetic acid during further processing steps. Another researcher Scordia et al, (2013) performed pretreatment using 2% oxalic acid concentration at 190℃ for 40 min pretreatment time. Further, the SPS process was performed using Accelerase enzyme with *Pichia stipitis* that resulted in 20.2 g/l of ethanol concentration with 0.28 g/l/h of ethanol productivity. In this above-mentioned study using Miscanthus & giganteus that is a triploid, sterile and interspecific hybrid, used as LCB appears appealing because of higher biomass production and maximum carbohydrate content, used as non-food crop that can be cultivated with little agricultural supply.

Further hydrothermal processing of eucalyptus biomass was performed by Da Silva et al., (2019) using 50 mmol/l of oxalic acid at 140℃ for 50 min that resulted in 65% of glucan yield. The viability of hydrothermal pretreatment of eucalyptus biomass under oxalic acid was demonstrated in this study. The combined usage of oxalic acid and ethanol at elevated temperatures yielded a greater glucan quantity in the

partial hydrolysis of the treated substrate. Despite its denser structure, eucalyptus biomass had the largest increments at 180℃. Temperature had the greatest independent influence on the chemical changes in biomass, followed by oxalic acid treatment. Another study based on the effect of pretreatment was demonstrated by Lim et al., (2013) using oxalic, maleic and H_2SO_4 as acid catalysts. The optimum condition maintained during the process was 170℃ for 60 min which resulted in 69.9% of glucan content and complete degradation of xylan content from treatment with all acid used during the process. It was evaluated that dicarboxylic acid has a higher susceptibility towards cellulose and hemicellulose hydrolysis. This finding may be valuable in determining the commercial viability of acid treatment for bioethanol generation from agricultural waste. Another study was based on the effect of H_2SO_4, H_3PO_4 and oxalic acid performed by Ibrahim et al, (2020) to study the effect of pretreatment on beech wood biomass. It was evaluated that oxalic acid treatment resulted in an enhanced amount of carboxyl groups and showed enzymatic digestibility of up to 86.3 g/l with increased glucan content of up to 75.6 g/l.

2.1.4. Simultaneous pretreatment and saccharification (SPS) methods developed

The main focus of the present study was on SPS method with the incorporation of pretreatment and saccharification in the single pot that will further reduce the processing time of bioethanol production. Various researchers have performed SPS method, Sindhu et al., (2016) studied the combined pretreatment and hydrolysis process on rice straw biomass using an ultrasonic cell disrupter along with cellulase and surfactant (Tween 80). The maximum reducing sugar yield of 0.374 g/g with 6 min of ultrasonic pretreatment along with 9 hrs of incubation time was obtained with biomass loading of 3% w/w along with 0.25% w/w concentration of surfactant with 20 FPU of cellulase loading. The micrographs showed highly deformed structure as well as an increase in the exposure of microfibrils by enhancing surface area and porosity of the biomass. Fermentation was performed utilizing a co-culture system of *Pichia stipitis* and *S. cerevisiae* with a fermentation efficiency of 61.25%. This experiment showed that the combining process was an economically viable option for the commercialization of produced bioethanol due to the elimination of the detoxification process as the reaction was devoid of inhibitors. Further, the SPS method was performed by Masran et al., (2020) on an oil palm fruit branch using both cellulase and laccase cocktail in a single pot at 45 U/g:25 FPU/g. In this, method laccase was synthesized by white rot basidiomycetes fungi that degrade the lignin barrier in lignocellulosic biomass with 8.3% lignin removal. The simultaneous method would reduce the steps to carry out the reaction. The reducing sugar yield was 10.9 g/l with 32.62% hydrolysis yield and subsequently minimizing the need for additional vessels. It was found that with the involvement of SPS process, the hydrolysis time was reduced from 144 hrs to 72 hrs. This study also indicated that the simultaneous method additionally produces enhanced saccharification yield compared to individual saccharification and pretreatment. As a result, it was proposed that this study be applied and investigated further because of its good characteristics in terms of processing effectiveness and conversion time. Similarly, the Laccase enzyme was used as a detoxifying agent with the eliminated half of phenolic

content by Dhiman et al., (2015) during SPS method. The conversion efficiency of sugar shows 72.4% from the liquid hydrolysate to bioethanol. The SPS method was an eco-friendly process performed in a single pot and considered as less labourism and less time-consuming process. It was found that the surfactant loading at lower doses stimulated the enzymatic hydrolysis, making the solution less viscous and preventing the tying of cellulase to its hydrophobic lignin part. Additionally, an intermixture of both laccases obtained from *Pleurotus djamor* and cellulase-xylanase obtained from *Trichoderma reesei was* performed by Kumar et al., (2017). It was found that after SPS method, 503.16 mg/l of reducing sugar was obtained after 8 hrs of incubation time with a saccharification efficiency of 72.44%. The fermentation process was performed using *C. beijerinckii* at 37℃ for 72 hrs with 6.45 g/l of biobutanol yield and a conversion efficiency of 45.98% from sugar to biobutanol. Additionally, Rathankumar et al., (2020) studied SPS process using both Laccase for delignification of lignocellulosic biomass and cellulase for the saccharification process. FESEM images show a highly damaged outer surface of pretreated biomass due to the delignification of biomass. With 15% biomass loading yielded 392.96 g/kg of fermentable sugar. It was concluded that with rise in loading of biomass up to 25%, followed in the reduction of fermentable sugars yield. Acid treatment in SPS method was performed by Rehman et al, (2013) incorporating sonification process along with 10% H_2SO_4 concentration resulting in a total reducing sugar yield of 31.78 g/100 g of rice straw. The required sonification time was 50 min at 80℃ with acidic hydrolysis using 10% H_2SO_4 in a single pot. It was estimated that combining two processes proved to be economical with the elimination of one complete process and improves the reducing sugar yield. Another work performed by Dessie et al., (2022) using 2% oxalic acid treatment at 121℃ for 30 min on pomelo fruit and industrial hemp residues resulted in 39.49 g/l of sugar production from industrial hemp biomass. It was evaluated that higher sugar yield was obtained at 16 hrs and declined thereafter due to inhibition of enzymes byproducts. Further, 0.30 mol/L lactic acid-choline chloride for 120℃ for 3 hrs with 15% biomass loading was performed by Huang et al., (2020). The net yield of reducing sugar was 49.9% higher than that of a separate process. It was concluded that pretreatment with less biomass loading and higher reaction temperature resulted into more lignin removal that would subsequently lead to release of higher sugar and more digestibility of polysaccharides. Another study based on ionic liquid along with cellulase loading of 5 U/mg and β-glucosidase loading of 66.6 U/g on sawdust biomass during SPS method was carried by Auxenfans et al., (2017). The pretreated biomass was subjected to NMR spectral analysis that showed 38% of cellulose crystallinity. The enzymatic hydrolysis resulted in 37.6% of sugar yield.

Further, Karimi et al., (2017) studied SPS method using combined biological delignification of lignocellulosic biomass and enzymatic saccharification in a single pot. The maximum delignification efficiency of 74% with 8.52 g/l of sugar concentration using *Trichoderma viride* fungus for biological pretreatment. The enzymatic saccharification of delignified rice straw showed 81% saccharification efficiency. Similarly, Ma & Ruan, (2015) performed research work using simultaneous bio-delignification

and saccharification by co-cultivation of *Coprinus comatus* and *Trichoderma reesei* producing laccase, xylanase and CMCase enzyme. Maximum delignification up to 66.5% showed at the enzymatic activity at 50℃ reaction temperature. It was considered an innovative method for the development of ligninolytic enzymes with 82% maximum polysaccharide yield after delignification and saccharification in a single reactor. A similar study was performed by Potumarth et al., (2013) on rice husks using white rot fungus *Phanerochaete chrysosporium* with maximum reducing sugar yield on the 18th day of fungal treatment. This reduced the operational cost that was incorporated with continuous washing as well as the removal of inhibitors. It was estimated that the yield of reducing sugar yield was 44.75% greater than that of other combined processes but low as compared to that of conventional chemical pretreatment. This process reduces the operational cost due to combining delignification and saccharification in a single pot. Additionally, Sophanodorn et al., (2022) used tobacco stalk as a substrate for bioethanol production that was hydrothermally pretreated at 100℃ for 30 mins, after that, it was subjected to alkaline treatment and hydrolysis using 2% (v/v) CaO at 60℃ for 24 hrs and 2% (v/v) cellulase enzyme. The total reducing sugar estimation was 27.97 g/l. Further, fermentation was carried out using 2% (v/v) *S.cerevisiae* for 72 hrs at 30℃. The high amount of ethanol output was observed in the fermentation process after 48 hrs and remained consistent after 72 hrs. It had been evaluated that a combination of pretreatment procedures was employed to boost bioethanol production by degrading biomass and improving accessibility to abundant sugars. Another research performed by Rajak & Banerjee, (2020) employed wasteland weed, *Saccharum spontaneum* for 2G bioethanol production using a single processing step. Fermentation was carried out using *S. cerevisiae* incubated at 37℃ for 24 hrs. Finally, ethanol was estimated using the potassium dichromate method with 59.96 g/l of ethanol concentration and 60% of conversion efficiency. Thus, this research shows the integration of various steps together to be feasible toward reduction in processing time.

2.1.5. Saccharification process

The saccharification technique can be used on LCBs generated by pretreatment processes that hydrolyze the treated lignocellulose to maximize the yield of reducing sugar. This includes mainly two processes i.e., acid hydrolysis and enzymatic hydrolysis (Auxenfans et al., 2017). Right now, the most frequently used procedure of obtaining cellulosic-based ethanol is the most prominent enzymatic-based process as it is considered one of the most environmentally sustainable processes and ultimately leads to more hydrolysis yield than acidic hydrolysis. Enzymatic hydrolysis is the incorporation of the following enzymes β-glucosidase, β-1,4-endoglucanase, β-1,4-exoglucanase and β-1,4-endoxylanase for enzyme-based substrate system. Enzymatic hydrolysis is a comparatively expensive process due to the excessive utilization of enzymes for conducting the process. Whereas acidic hydrolysis is performed using various acids such as HCl or H_2SO_4, it may dilute one or concentrate one which can hydrolyze the hemicellulosic part at a lower temperature. It is somehow an accessible process but it is prohibited due to the development of non-selective by-products such as furfurals, acetic acid and phenolic compounds (Keshav et al., 2016). A dilute acid

hydrolysis process using 0.197 M H_2SO_4 along with 2-methyltetrahydrofuran/H_2O will enhance hemicellulose-derived monosaccharides production to 20.04 g/l of dry biomass with less production of degrading products and only 85.3% removal of furan generated during hydrolysis process (Dávila et al., 2021). Therefore, acidic hydrolysis is confined to the neutralization process and formation of by-products in the form of inhibitors. Whereas, in the case of enzymatic hydrolysis with enzyme loading lower than the threshold limit will limit the conversion of cellulose and increase the duration of the subsequent process (Suresh et al., 2020). So, enzymatic hydrolysis depends on the specificity of various enzymes used for the process but it is hindered due to the requirement of high fermentation time. It is also noted that in-house production of enzymes will reduce production costs along with the separation, storage and transportation of enzymes (Jin et al., 2020). The flowchart showing bioethanol production using two techniques SHF and SSF is represented below in fig. 03. Further, to make the process cost-effective and less time-consuming, it is required to follow the strategies that will be applied at the earlier stage of fermentation. These include:

2.1.5.1. Separate hydrolysis and fermentation process

In this process, both saccharification and fermentation are carried out in an individual vessel under optimal conditions. During this process, higher saccharification efficiency from the higher sugar content feedstock. In this process, firstly starch available in the feedstocks is subjected to a gelatinization process that succeeded in the liquefaction process of biomass along with enzymes and heat utilization for the effective process to stimulate the rate of hydrolysis process (Szambelan et al., 2018). This process requires 196 hrs giving an ethanol concentration of 99 g/l as compared to 105 hr with an ethanol concentration of 108 g/l in SSF process with a PEG concentration of 2.5%. this difference in the concentration of bioethanol is due to the inhibition of glucose from high glucose concentration as it exceeds the glucose inhibition level at 15% (w/v) (Jawad, Madhab, & Murthy, 2019). It is estimated that the feedstock with more starch content exhibits more saccharification efficiency that ultimately confirms more ethanol yield. The feedstock used in this process has a high density of reducing sugar that is obtained after the galvanization process which is only applicable with SHF (Szambelan et al., 2017).

2.1.5.2. Simultaneous saccharification and fermentation process

Biofuel production is mainly dependent on the degradation of cellulose to subsequent reducing sugar i.e., hydrolysis along with the conversion of reducing sugar to final bioethanol using various agents i.e., fermentation. Thus, it is estimated that the production of bioethanol is widely dependent on the availability of reducing sugar (Phitsuwan et al., 2016). The sequential process for conventional bioethanol production is pretreatment followed by liquefaction and saccharification and lastly fermentation. For glucose production, both amylase and glucoamylase are required at a high temperature which requires more energy for the process. Thus, SSF is considered an alternative source to minimize enzyme requirements, reduce energy demand, and subsequently reduce production costs (Izmirlioglu & Demirci, 2017). Similarly, performing SSF at dry solid loading i.e., dry SSF (DSSF) can yield ethanol at a higher concentration of more than 40g/l

which reduces the energy demand for the process. Thus, DSSF is preferred over SSF due to less capital and production cost, less water and fewer equipment requirements for the process (Molaverdi, Karimi, & Mirmohamadsadeghi, 2019). The next process is the SSSF (solid-state simultaneous saccharification and fermentation) estimated to produce maximum ethanol concentration at low enzyme and solid loading. As the enzyme loading reduces from 20 to 2.5 FPU/g i.e., approx. 87.5% reduction, when subjected to 72 hrs of SSSF resulted in more ethanol yield of up to 40.4 g/l while enzyme loading of 40 FPU/g yielded 37.5 g/l of ethanol (Molaverdi et al., 2019). Similarly, another process is the semi-simultaneous saccharification and fermentation (SSSF) process in pre-saccharification is performed before SSF. This SSSF process of ethanol production results in higher potency and outcome as compared to both SHF and SSF. This ought to be only when the time for pre-saccharification is suitable (Sivarathnakumar et al., 2019). It is estimated that ethanol yield and productivity (g/l/h) using the SSSF strategy is quite higher than that of SSF using *P. stipitis, S. cerevisiae* and *Z. mobilis*. This higher fermentation efficiency is due to the application of a short pre-saccharification period that can maximize the solubility and substrate along with the conversion of cellulose to glucose and subsequently to ethanol and will result in less formation of inhibitors during the hydrolysis process (Gonçalves et al., 2016).

The saccharification of pretreated biomass lets off mainly hexose and pentose sugar that covers up arabinose, mannose, xylose, glucose and galactose. It is noted that some reducing sugars are present in an abundant amount such as glucose and xylose. Thus, it is required to ferment it efficiently so that there will be more production of bioethanol from the available feedstock (Y. Wang et al., 2017).

2.1.6. Various fermenting yeast and required nutrients

This part consists of a review of various fermenting yeast required for the conversion of sugar to ethanol. Ethanol fermentations are carried out using yeast with high optical density under anaerobic conditions. It is estimated that the fermentation efficiency mainly depends on the attenuation property of a particular yeast strain used for the process stated by Walker & Walker, (2018). B. Singh and Kumar, (2020) performed a sodium carbonate (1% w/v) pretreatment process on biomass, used as a substrate for bioenergy production. The SEM and FTIR analysis showed the morphological changes in the pretreated rice straw. Enzymatic hydrolysis was performed using cellulase (20 U/gm) in 0.1 M sodium acetate, pH 5 and incubation at 60℃ for 150 rpm for 6 hrs. Fermentation was performed using *Saccharomyces cerevisiae* in a shaking incubator at 150 rpm at 35℃ for 96 hrs of fermentation time. The reducing sugar analysis was performed using HPLC with 531.20 mg/g of reducing sugar yield and 18.07 g/l of bioethanol yield after 72 hrs of fermentation time. In this work, pretreatment process was performed using sodium hydroxide on rice straw was considered an ideal pretreatment method with increased liberation of reducing sugar. Another study was performed by Hickert et al., (2013) using co-culture fermentation using *S. cerevisiae* and *C. shehatae*. Using a substrate rice hull hydrolysate (RHH) production of ethanol, the feasibility of both *Candida shehatae* and *S. cerevisiae* was investigated under both oxygen-limited and anaerobic conditions in

RHH, synthetic media. The estimated xylitol yield of approx. 0.20 g/l & 0.13 g/l in both synthetic and RHH media was obtained. As arabinose was also partially converted to xylitol, it increased subsequent yield. The estimated ethanol yield of 0.44 to 0.48 g/l in RHH media while 0.51 g/l under synthetic media was observed with little manipulation in oxygen enriched and oxygen limited process. Apart from it, it was noted that fungus as a dimorphic species have capacity to produce maximum ethanol yield. The fungus *Mucor indicus* has large amount of proteins and lipids, causing high temperature resistant capacity and lower contamination risk, along with higher performance towards fermentation process leading to utilization of xylose along with glucose (Molaverdi et al., (2019).

Hickert et al., (2013) performed co-fermentation and saccharification in a single pot using double microbial strain, tending to metabolize pentose and hexose to ethanol and xylitol. The co-fermentation showed a better result without further detoxification of hydrolysate. The combined process of hydrolysis and fermentation increased the production of ethanol faster as the obtained reducing sugar was simultaneously fermented to glucose in a single pot. So, co-fermentation provides an economical process where both hexose and pentose utilizing yeast can be used for fermenting available hydrolysate. This bioconversion can be implemented effectively only by maintaining the oxygenation rate and inhibiting furanic formation in the media. The required microaerophilic condition was maintained at 37°C (pH 6.5), to ferment available C5 sugar counterparts to bioethanol. Further studies by Todhanakasem et al. (2019) were carried out by using two strains of *Z. mobilis* in a biofilm reactor for the fermentation of hydrolysate obtained after enzymatic hydrolysis. It was shown to have advantages over other processes, with higher ethanol yield, high fermentation productivity, with broad pH range of 3.5-7.5 to ferment sugar and lastly having maximum tolerance towards inhibitors. It was estimated that *Z. mobilis* is considered an ethanologenic microbe that tended to grow in glucose, fructose and sucrose along with high ethanol production of up to 97%.

Another thermo-tolerant yeast *Kluyveromyces marxianus* utilized in the study performed by Sivarathnakumar et al., (2019) due to its ability to ferment both pentose and hexose sugar and yield a maximum ethanol concentration of 21.45 g/l from 35.5 g/l of hydrolysate. The growth media was maintained with pH 4.9, temperature around 41°C along with substrate and inoculum concentrations of 2% and 3% (v/v) respectively. It was estimated that during 72 hrs of fermentation time, the maximum yield was obtained after 12 hrs of processing, yeast started to multiply rapidly and reached maximum intensity at 72 hrs. Another yeast strain of *Wickerhamomyces anomalus* X19 used by Ben Atitallah et al., (2019), known as non-*saccharomyces* or wild yeast cultured on an agar plate using YPD media. This isolated yeast led to maximum ethanol production of 44.9 g/l from 100 gm of total initially produced sugar with 72.38% fermentation efficiency. Another thermotolerant yeast *Pichia kudriavzevii* was used by Sunkar & Bhukya, (2021) which resulted in 11.98 g/l of ethanol with an efficiency of 82.56% at the optimum temperature of 42°C. It was demonstrated that this yeast strain would ferment both detoxified and undetoxified hydrolysate of xylose. Workers performed a biphasic hydrolysis process using corncobs and chemical pretreatment was

performed using NaOH, KOH, $NaClO_2$, Na_2SO_3, and $Na_2S_2O_4$ at varied concentrations of 0.5, 1.0, 2.0, 3.0% (w/v). The biomass was incubated at 55℃ for 4 hrs, and slurry was filtered out for the subsequent hydrolysis process. In this ultrasonic-assisted acidic hydrolysis was performed using HNO_3, HCl, H_2SO_4, and H_3PO_4, Further, incubation was carried out at 100℃ for 120 min with the ultrasonic frequency of 40 kHz. For detoxification of the sample, activated charcoal was used at different concentrations that further enhanced the bioethanol yield.

Table 2.4- Illustration of different fermenting yeast utilized in previous study

Fermenting yeast	Nutrient media required	Amount of sugar utilised	Ethanol fermentation efficiency	Ref.
Saccharomyces cerevisiae	5% w/v glucan, 1% w/v yeast extract, 2% w/v peptone,	67.70 g/l	47.78% (10.61 g/l)	(B. Singh & Kumar, 2020)
Mucor indicus	YPD medium- 20 g/l agar 40 g/l glucose, 10 g/l peptone at 32℃ for 5days	89.2 g/l	89.5% 99.4 g/l	(Molaverdi et al., 2019)
Zymomonas mobilis	yeast extract, 10 g/l; KH_2PO_4, 2 g/l; $(NH_4)_2SO_4$, 1 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; glucose, 20 g/l. Z. mobilis was cultured at 30℃ for 12 h	Glucose 14.34 g/l Xylose 3.56 g/l	84.56% (10.96 g/l)	(Todhanakasem et al., 2019)
Kluyveromyces marxianus	YMP agar media-3 g/l yeast extract, 3g/l malt extract, peptone 5 g/l, agar 20 g/l, pH 5.5, temperature 30℃.	35.5 g/l	72%, 21.45 g/l	(Sivarathnakumar et al., 2019)
Pichia kudriavzevii	peptone, 10.0; yeast extract, 5.0: $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2.2H_2O$, 0.1;	27.33 g/l	85.95%, 11.98 g/l	(Sunkar & Bhukya, 2021)

	$ZnSO_4 \cdot 7H_2O$, 0.01			
Pichia stipitis	Yeast extract 10 g/L, peptone 20 g/L, and xylose 20 g/L under aerobic condition at 30℃	Glucose 31.82 g/l Xylose 13.75 g/l	50.2% (17.37 g/l)	(Toquero & Bolado, 2014)
Saccharomyces tanninophilus	3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone, 10.0 g/L glucose and 20 g/L agar at 30℃ for 24 h	443 gm	83.5% (189 gm) 9.45 g/l	(Jin et al., 2020)
Candida shehatae	Xylose 60g/l, yeast extract 10, $MgCl_2$ 1g/l, KH_2PO_4 1, $(NH_4)_2SO_4$ 1, pH 5.5	81.11%	26.19 g/l	(Mishra & Ghosh, 2019a)

2.1.7. Co- fermentation process

After the process of saccharification, the hydrolysate releases both pentose and hexose sugar which includes glucose, mannose, galactose, xylose, and arabinose. Among these fermentable sugars, glucose and xylose are the most abandoned while others are present in trace amounts, effective way of fermentation process is the utilization of both sugars by using co-fermentation process. For the optimum conversion of hydrolysate obtained from saccharification process, it is required to convert all fermentable sugar to ethanol. Suriyachai et al., (2013) performed pretreatment method using 5% NaOH on rice straw at 90℃ for 20 min. Enzymatic hydrolysis was carried out using Accellerase 1500 enzyme in a 50 mM sodium citrate buffer incubated at 50℃ for 72 hrs. Further co-fermentation process was performed using hexose and pentose utilizing yeast at 30℃ after 48 hrs yielding 14.11 g/l of bioethanol concentration with a conversion efficiency of 97% from the total reducing sugar available was 19.09 g/l. It was evaluated that optimizing hydrolysis and pre-hydrolysis duration and temperature was proposed to reduce the consistency of high biomass fermentation to optimize ethanol output. A similar study was proposed by Raina et al., (2020) using sawdust of Sal residue from the furniture industry. Enzymatic hydrolysis was performed using cellulase and pectinase enzyme with a total reducing sugar concentration of 19.09 g/l after 48 hrs of the hydrolysis process. The co-fermentation process was performed using *S. cerevisiae* (MTCC-36) inoculated in YPD media and *P. stipitis* NCIM-3498 strain inoculated in MGYP media, producing ethanol yield of 11.64 g/l from 19.09 g/l of sugar with fermentation efficiency of 88% from the acid hydrolysate of alkali pretreated biomass. According to the findings of this study, HCl pretreated biomass displayed improved release of reducing sugars, proving an accurate bioethanol production process from SS. A further study performed by

Li Y et al., (2011) employed a co-fermentation process using *P. stipitis* and *S. cerevisiae*. This inactivation led to the entire transformation of both glucose and xylose obtained at approx. 80 hrs as its inactivation reduces the wastage of xylose when it is fermented by *P. stipitis* after fermentation of glucose. Thus, a simple pretreatment procedure not only significantly improved the efficiency of saccharification of glucan and xylan in rice straw, but it also retained all of the monosaccharides and oligosaccharides produced throughout the pretreatment process in the vessel. Similarly, Naseeruddin et al., (2017) executed a delignification process using 2% $Na_2S_2O_4$ at 30℃ for 18 hrs on *Prosopis juliflora* biomass. 1% H_2SO_4 was used for the acidic hydrolysis process at 110℃ for 30 min and enzymatic hydrolysis was performed using cellulase loading of 28.66 IU and supplemented by 1% Tween 80 incubated at 50℃ for 36 hrs at 150 rpm. Both acidic and enzymatic hydrolysate were subjected to a co-fermentation process utilizing *P. stipitis* NCIM3498 and *S. cerevisiae* VS3, which produced 10.85 g/l of bioethanol with 87.34% fermentation efficiency, with ethanol yield of 0.445g/g of biomass. It was estimated that the gap of standard 18 hrs had to be maintained so that hexose and pentose sugar were utilized by respective yeast one at a time. This process would increase the ethanol yield up to 10.85 g/l, which afterward started to decline as the sugar metabolism decreased and the yeast would further shift from exponential stage to the stationary stage after 36 hrs of fermentation.

Another study was performed by Ndaba et al, 2014 using a mixed culture of *Zymomonas mobilis* and *Saccharomyces cerevisiae* at 5:10 g/l. It was estimated that *S. cerevisiae* was susceptible to a glucose concentration greater than 5 g/l which reduced the catabolism and usage of xylose. Co-fermentation of both yeasts occurs in the same broth to ferment both xylose and glucose. This would yield higher ethanol of 9.3 g/l, where glucose was continuously fermented to ethanol while xylose content reduced after 48 hrs. The maximum yield in comparison to the previous work was due to inoculum ratio for fermentation along with the effect of perpetrator strain of microorganisms used for the process.

Further, Mishra & Ghosh, (2019a) performed co-fermentation process utilising *Z. mobilis* with *Scheffersomyces shehatae* biomass for bioethanol production. Initially, *S. shehatae* was cultured at 20 g/l xylose for 20 hrs yielded 7.84 g/l of ethanol, after which *Z. mobilis* was included in co-culture system. In the specified work, both xylose and glucose rich fraction were used as xylose bioconversion was inhibited or delayed in fermentation process yielding higher glucose-rich fraction. So, it was recommended that 87.33% of XRF and 92.08% of GRF, yielded 25 g/l of ethanol under synthetic conditions. Thus, by co-culturing system the problem of adopting glucose as carbon source was eliminated. Another research by Singh, Majumder, & Ghosh, (2014), used co-culture system of *Pichia stipitis* in xylose-rich media with *Zymomonas mobilis*. This sequential system, using synthetic fermentation media both at flask and bioreactor level, was adopted due to catabolite repression of xylose utilized by *P. stipitis* in existence of high glucose concentration utilized by *Z. mobilis*. Also, concentration of glucose or even higher suppress the activity of xylose thus resulted into delay of fermentation as well as less ethanol by-product. Thus, in this sequential

system *P. stipitis* utilising xylose as a carbon source managed earlier than that of Z. *mobilis* that utilizes glucose as a carbon source. The overall sugar utilized in the above process was 97.2% whose consumption resulted in 57.8 g/l of ethanol yield. Similarly, Fu et al. (2009) performed co-culturing of *Z. mobilis* and *P. stipitis* with glucose and xylose at 20 and 30 g/l respectively, fermented completely to ethanol with 1.277 g/l/h of ethanol productivity in 19 hr of reaction time. In this sugarcane bagasse was pretreated with 2% H_2SO_4 and further hydrolysis was performed by cellulase and Novozyme loading at 2% at 60℃ for 24 hrs at 200 rpm in a shaking incubator. The obtained hydrolysate was subjected to co-fermentation process using *Z.mobilis* and *P.stipitis* for glucose and xylose fermentation respectively. From the above process, 50 g/l of sugar was obtained and was fermented to ethanol yielding 0.47 g/l with productivity of ethanol up to 0.83 g/l/h. Thus, the efficacy of the immobilization might be enhanced with an efficient co-culture system.

Additionally, Rojas-Chamorro et al., (2020) performed H_3PO_4 and H_2SO_4 pretreatment and co-fermentation process using co-culture of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* strains along with hydrolysis performed using *E. coli* resulted in 39 g/l of ethanol yield which 76% more than that of theoretical value obtained after 56 hrs of fermentation. This process is supplemented with sodium metabisulphite to decrease the hydrolysate toxicity during chemical pre-treated biomass and improve the metabolism of xylose by *E. coli*. Similarly, co-culturing of *Pichia stipitis 3498* strain along with *S. cerevisiae* NCIM 3090 in YPD media incubated at 30℃ for 36 hrs at 5.5 pH was carried out. To enhance the capability of xylose fermentation, detoxification of hydrolysate is required using activated charcoal along with over-liming that will increase the sugar loss with elevated pH (Deshavath et al., 2021). Another dynamic-based co-fermentation process that involves a continuous, fed batch process along with recycling of effluents from the reactor which will enhance the yield of ethanol to 0.18 kg of dry biomass has been carried out. In this study, one mathematical model was involved that will integrate the process of hydrolysis and co-fermentation and minimize the involvement of equipment with better yield (Rodriguez et al., 2011). Various co-culturing of yeast for desired ethanol production with its sugar utilization and efficiency of particular yeast strains towards ethanol production are represented below in table 2.5.

Table 2.5- Tabular representation of various co-fermentation processes used

Co-fermentation	**Amount of sugar utilized**	**Max. conversion efficiency**	**Ethanol yield (ethanol concentration)**	**Ref.**
Saccharomyces cerevisiae & *Spathaspora arborariae*	Glucose-20 g/l Xylose- 13 g/l	-	14.5 g/l	(Hickert, Souza-Cruz, et al., 2013)
Saccharomyces cerevisiae & *Scheffersomyces stipitis*	60.4 g/l	85%	14.8 g/l	(Suriyachai et al., 2013)

*Saccharomyces cerevisiae (*MTCC-36) *& Pichia stipitis* (NCIM- 3498)	19.09 g/l	97%	9.43 g/l	(Li et al., 2011)
Saccharomyces cerevisiae & Zymomonas mobilis	24.9 g/l	37.64%	40.1 g/l	(Ndaba et al., 2014)
Pichia stipitis & Zymomonas mobilis	32.84 g/l	91.2%	56.9 g/l	(L. K. Singh et al., 2014)
Zymomonas mobilis & Pichia stipitis	37.5 g/l	-	0.45 g/l (44.3 g/l)	(Fu et al., 2009)
Saccharomyces cerevisiae & Scheffersomyces stipitis CBS 6054	50.8 g/l	68%	17.5 g/l (227 L)	(Rojas-Chamorro et al., 2020)
Saccharomyces cerevisiae & Pichia stipitis	71.83 g/l	78%	31.01 g/l	(Santosh et al., 2017)
Zymomonas mobilis & Candida shehatae	59.74 g/l	82.45%	67.28 g/l	(Mishra & Ghosh, 2019b)
Zymomonas mobilis & Scheffersomyces shehatae	Xylose- 21.8 g/l Glucose-40.32 g/l	78.6%	25 g/l	(Mishra & Ghosh, 2020)
Saccharomyces cerevisiae & Pachysolen tannophilus	22.65 g/l	33.62%	(0.46%) 9.21 g/l	(Malik et al., 2021)

The production of bioethanol from the study done by Da Cunha-Pereira et al., (2011a) using *Saccharomyces cerevisiae* and *Spathaspora arborariae.* The obtained rice hull hydrolysate was efficiently converted to both xylose and arabinose efficiency of 39% and 31% respectively, this led to the yield of xylitol and ethanol to 0.39 g/g xylitol with 3 g/l concentration and 0.48 g/g at 11 g/l concentration and. In this xylitol production reaches up to 8.2 g/l using rice hull hydrolysate, this is due to yeast *S. arborariae* metabolism under any toxic compound present that shows about 35% conversion of furfural and acetic acid along with maximum xylitol yield. An alternative strategy for co-fermentation was performed using cellulolytic strain of *Clostridium thermocellum* as well as non- cellulolytic strain of *Thermoanaerobacter* X514 & 39E. This will intensify the ethanol production up to 62% in comparison with using individually.

The cellulosic ethanol production using economically viable methods by fermenting both pentose and hexose utilizing yeast from the available feedstock using different strains of both *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* was performed by Santosh et al., (2017) with maximum ethanol production of 31 g/l. The concentration of ethanol produced directly depends on sugar present experiment

vessel with maximum fermentation efficiency of 78%. This efficiency is due to the attenuation properties of different yeast strains used for the process. Another study was performed by Malik et al. (2021) using a co-fermentation process with the yeast strains of *Saccharomyces cerevisiae* YPH499 and *Pachysolen tannophilus* ATCC32691. The consortium of both the yeast is maintained at 1:1 ratio at substrate level with the ethanol concentration obtained at 9.81 g/l from 266.6 mg/g of sugar obtained after NaOH pretreated biomass with conversion efficiency of 35%. Another Mishra and Ghosh, 2019a performed a combined approach of fractional hydrolysis along with co-fermentation of *Zymomonas mobilis* & *Candida shehatae* for glucose and xylose sugar fermentation to bioethanol. The microbial strain of *Z. mobilis* shows effective ethanol tolerance of up to 10% while utilizing only 93.6% of glucose utilization with ethanol production of 26.14 g/l from 60 g/l of sugar while in the case of *C. shehatae* NCIM-3502 strain shows tolerance of up to 6% with minimum ethanol production. During co-fermentation process, glucose was preferred as a carbon source to xylose. It was estimated that xylose conversion using *C. shehatae* would be prohibited at an enhanced concentration of glucose. Within 48 hrs of the fermentation process, hydrolysate media produced 67.28 g/l of ethanol from 93.28% of the xylose-rich substrate and 95.44% of the glucose-rich substrate. Thus, the fractional hydrolysis technique would eliminate the use of glucose as a carbon source by microorganisms and generate xylose and glucose rich fraction sugar which would eliminate excessive use of glucose during fermentation. This resulted in the efficient use of both glucose and xylose during co-fermentation process. It is estimated that adding ampicillin at concentration of about 50 mg/L prevents bacterial contamination. With the increase in concentration of wash liquid from 0% to 100% will significantly reduce the ethanol yield with maximum sugar accumulation and cell inactivation during the fermentation process. Qin et al., (2017) isolated wash liquorice straw from the dilute acid and ethylenediamine pretreated corn stover and utilized it for evaluation of the effect of soluble materials on simultaneous saccharification and co-fermentation process. In this genetically modified *S. cerevisiae* SyBE005 cultured at YPX agar plate. It was found that genetically modified yeast had capability of consuming both xylose and glucose sugar, further it was found that dual effect of soluble materials stimulates the fermentation process without adding the nutrient and inhibits the enzymatic hydrolysis process.

2.2. Motivation and background (Research gap)

For the bioconversion of lignocellulosic biomass to ethanol, it is required to develop an economical and efficient pretreatment method to disrupt the lignocellulosic biomass. As to the latest trend in the formulation of bioethanol blending with petrol along with the development of methods that will be efficiently adopted by the respective industry at the commercial scale, the huge demand for bioethanol consumption as well as a reduction in the utilization of fossil-based conventional fuels and to mitigate the global pollution that has led to greenhouse gas emissions.

1. According to a literature survey conducted, the Simultaneous Pretreatment and saccharification (SPS) method included in one of the biphasic systems is untouched and needs more research.
2. Till now the work on SPS method has been performed using a fungal consortium. As in the SPS method, pretreatment had already been performed using chemical methods, which results in the extensive use of chemicals and hence leads to environmental degradation because of poor discharging techniques, whereas on the other hand, unexplored combined pretreatment processes have an upper hand in this regard, as it can help to improve the environmental conditions along with ensuring the safety of lab-workers.
3. Another loophole is in the fermentation process which is carried out using co-culture. Using the advantages of co-culture which is still not performed alongside the SPS method and can prove to be beneficial in yield and time consumption.
4. The effective pretreatment procedure in the hydrolysis step is typically regarded as one of the process's rate-limiting steps. As a result, a variety of pretreatments involving chemical, thermal and biological methods are used to disturb specific structural characteristics of biomass to improve the accessibility of enzymes to hydrolyze polysaccharides into sugar monomers.
5. Through the use of improved feedstocks, pretreatment methods that produce fewer byproducts, more effective enzymes, and adaptable fermenting yeast, innovative ways can aid in cost reduction. It is possible to employ a variety of biomass substrates to produce energy. The main sources of bioethanol in India are sugarcane bagasse and sweet sorghum, however, due to strong industrial demand, it is not practical to combine it with gasoline. Therefore, it is significant to enhance the bioethanol production from lignocellulosic agricultural leftovers by using more native crops, forestry, and perennial herbaceous feedstocks.

2.3. Scope of the study (Hypothesis)

The main hypothesis of the present work is to develop an efficient process that has more economic value and can be utilized at a commercial scale with a broader viewpoint. The agricultural waste can be used as a substrate for bioethanol production as it has more cellulosic content. Rice straw, a non-food crop, has been selected as lignocellulosic biomass for bioethanol production. It has been hypothesized that rice straw contains maximum cellulosic content and is used as a substrate for further processing steps in bioethanol production. In contrast, rice straw that has been utilized in the present study has maximum hemicellulosic content that has led to the development of bi-phasic system with the utilization of pentose and hexose sugar evolved from cellulose and hemicellulosic part. The first and most intense stage is the pretreatment process, which makes the cellulose in the biomass more readily available to enzymes by infringing the lignin enclosing, allowing for the fast processing of carbohydrates into fermentable sugars with substantial yield. Various physiochemical pretreatment processes will be utilized under SPS method and obtained hydrolysate with xylose sugar extraction will be further subjected to detoxification method for co-fermentation process.

The pretreated solid fraction will be further subjected to enzymatic hydrolysis for the extraction of hexose sugar. The obtained hydrolysate from both processing steps will lead to a co-fermentation process with the ability to utilize both hexose and pentose sugar in order to obtain maximum bioethanol production yield. The present study will apply existing potential in the estimation of rice straw as a substrate for 2G production of bioethanol. Thus, acid impregnation with steam explosion pretreatment methods would be regarded as an evaluation tool for this study since these have been widely employed in the processing of rice straw to 2G production of bioethanol at a pilot scale. Prior to the production of a commercial rice straw-based bioethanol facility, data on additional agricultural residues must also be collected. These include trade-offs addressing farmers' readiness or unwillingness to plant rice, generation of straw, food production vs energy difficulties, economic viability analyses, and societal projections will be analyzed. As a result, site-specific techno-economic studies will be advised further.

2.4. Work plan

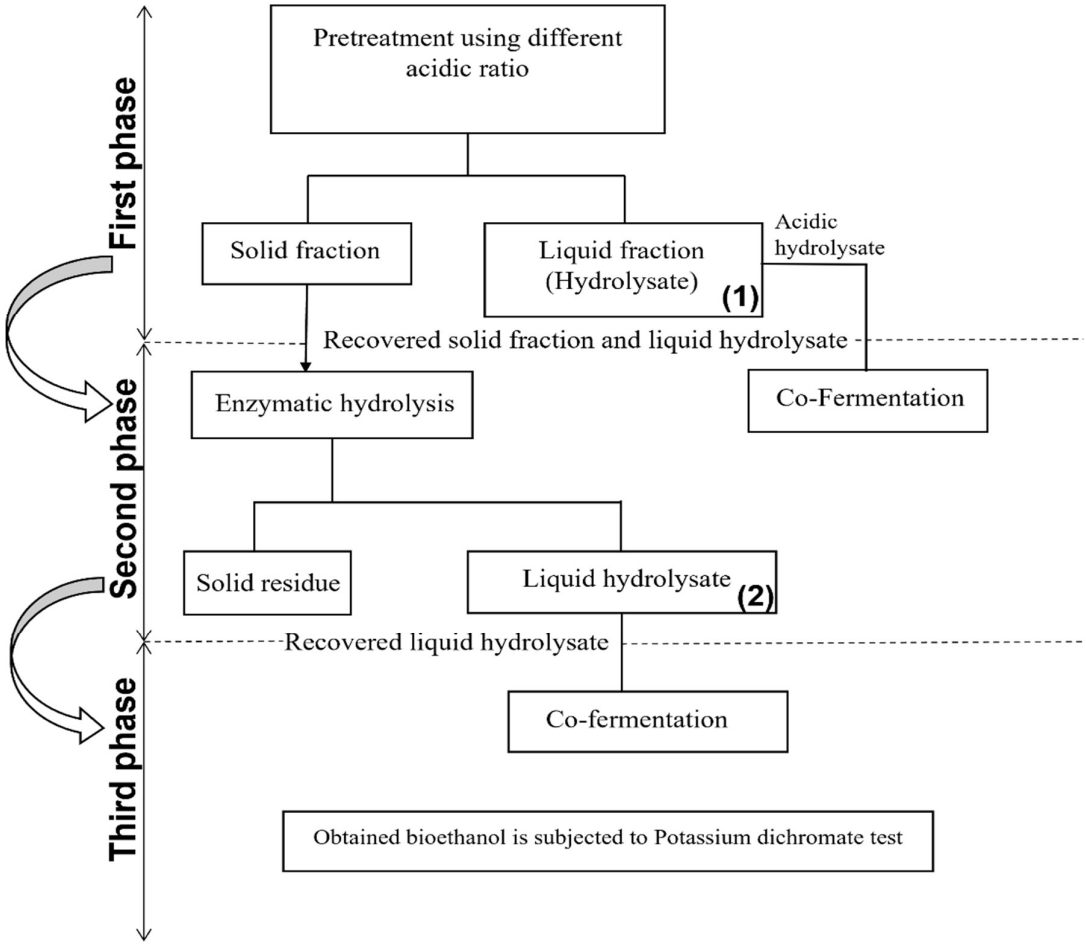


Figure 2.1- Diagrammatical illustration of work plan for bioethanol production

2.5. Objective of the study

1. Determination of viability for physiochemical pretreatment methods for simultaneous pretreatment and saccharification (SPS) method for hydrolysis of rice straw
2. Determining the accessibility of acid hydrolysis and enzymatic hydrolysis for efficient hydrolysis process during SPS method.
3. Verification of the biphasic system using rice straw.
4. Determination and characterization of pentose and hexose utilizing yeast for performing fermentation process to maximize yield of bioethanol obtained at pilot scale.

CHAPTER-3

METHODS AND MATERIALS

3.1. Introduction

This research required involvement of biphasic system i.e., simultaneous pretreatment and saccharification methods for bioethanol production using rice straw. The feasible method than that of separate pretreatment and saccharification as the retention time for the reaction gets reduced and yields higher reducing sugar. Current research has been emphasized on two phasic hydrolysis processes. This includes the extraction of pentose sugars from the first phase using the physiochemical method impregnated with acidic treatment as well as hexose sugars from the second phase using enzymatic hydrolysis. Further, the co-fermentation process was incorporated with both hexose and pentose utilizing yeast that can ferment both hexose and pentose sugar to bioethanol.

Objective 1- Determination of viability for physiochemical pretreatment methods for simultaneous pretreatment and saccharification (SPS) method for hydrolysis of rice straw

Under this objective, various physiochemical pretreatment was performed in a reactor impregnated with various concentrations of acid. In this research, steam explosion and liquid hot water treatment impregnated with different concentrations of acids was performed as a physiochemical pretreatment method.

3.2. Collection of rice straw

The rice straw used during the present study was collected from the local farms of Kanpur, Uttar Pradesh. As rice (*Oryza sativa)* is the main staple food grown in most of northern India. The obtained rice straw was washed continuously 3-4 times with tap water to remove dust and impurities present in it. After that, rice straw was dried in the air in the presence of natural sunlight at 35℃ temperature for 3-4 days. After drying up in the air it is further dried in the oven at 60℃ for a duration of 12 hrs. The dried biomass was mechanically grinded to 3-5 µm particle size and stored in the Duran bottle at room temperature of 25±2℃ till further use. The silica content was removed by treating rice straw with 0.2 M NaOH as the protocol suggested by Yuan et al., (2018). The compositional analysis of native rice straw was carried out using an acid hydrolysis process as per protocol developed by the National Renewable Energy Laboratory (NREL) which has been discussed in a further section of composition analysis of Rice Straw. The obtained grinded rice straw was subjected to physiochemical pretreatment process using steam explosion and liquid hot water. The illustration of different stages of obtained rice straw from the local farm to the grinded one that was used for further processing is depicted in Figure 3.1.

Figure 3.1. (a) Rice straw obtained from a local farm (b) Dried rice straw in Hot air oven (c) Grinded Rice Straw stored in a Duran bottle

3.3. Compositional analysis of Rice straw

3.3.1. Determination of Moisture content in the native rice straw

The moisture content in the rice straw is determined by drying the rice straw in hot air oven to obtain the constant dried weight of the rice straw. Petri plates dried in hot air oven with the initial weight noted down were used. 1 gm of rice straw in the same petri plate was dried at 135℃ for 2 hr in hot air oven. After that, rice straw was kept in a desiccator to maintain the normal temperature. The final weight of the dried sample is obtained using the following formula

$$Moisture\% = \frac{W_r - (W_f - W_i)\, X\ 100}{W_r}$$

Where W_r is the weight of the initial rice straw, W_f is the weight of dried petri plate along with initial weight of the rice straw, W_i is the initial weight of the petri plate.

3.3.2. Determination of Extractive present in the native Rice straw

Soxhlet extraction was set up to obtain extract-free biomass. Compositional analysis of desired biomass for the determination of hemicellulose, cellulose, and lignin content was carried out. 11.06 gm of rice straw was taken in a cellulose thimble using acetone as a solvent for extraction. The sample was maintained at the required temperature (40-50℃) for 8 hrs and the extract-free rice straw was dried at 60℃ in a hot air oven. The weight of the extract present in the rice straw was measured before and after the Soxhlet extraction to find out the weight of extract available in rice straw as suggested by Ayeni et al. 2015.

3.3.3. Determination of ash content present in the native rice straw

The ash content present in the native rice straw was obtained using a muffle furnace. 2 gm of native rice straw was taken in the crucible and kept in the muffle furnace at 575℃ for 3 hr. After that, it was taken out and kept in a desiccator to maintain normal temperature. The final weight of the crucible was measured out, to get the percentage of ash content present in the native rice straw.

3.3.4. Determination of hemicellulose present in the native Rice straw

For hemicellulose determination, 1 gm of the extract-free sample was taken in 200 ml of Erlenmeyer flask along with 100 ml of 450 mol/m^3 (1.8 gm) NaOH solution. The sample was boiled for 3.5 hrs at 80℃ and repeatedly washed with distilled water to obtain neutral pH followed by drying at 105℃ until the dried biomass was obtained to determine the constant weight of hemicellulose (%w/w) present in rice straw.

3.3.5. Determination of lignin present in the native Rice straw

Lignin determination was carried out by taking 1 gm of extract-free sample which was dissolved in 50 ml of 96% H_2SO_4 at ambient temperature for 2 hrs for acidic treatment. The 84 ml of distilled water was added to the slurry and was autoclaved for 1 hr. The hydrolysate was further separated using vacuum filtration and washed with distilled water to eliminate sulphate ions from the biomass. The amount of acid-insoluble lignin (AIL) was determined by obtaining the constant weight of rice straw. The acid-soluble lignin (ASL) was evaluated by measuring its absorbance at 205 nm wavelength of obtained hydrolysate. Afterward, it was dried at ambient temperature to obtain the constant weight of lignin present in the rice straw (Yao et al., 2019).

3.3.6. Determination of cellulose present in the native rice straw

The extract-free rice straw was further subjected to cellulose determination as suggested by Rajeswari et al., 2019. 2 gm of rice straw was dissolved in 5 ml of 70% HNO_3 and 50 ml of 80% Acetic acid, incubated in hot water bath at 70℃ for 20 min. The solid residue was filtered and alternative washing was done using 95% ethanol along with distilled water until neutral pH was obtained. After that slurry was dried at 60℃ overnight to obtain a constant dried weight of rice straw biomass. The cellulosic content was obtained using

$$Cellulose\ content\ (\%) = \frac{Final\ dried\ weight\ of\ RS}{Initial\ weight\ of\ RS} \times 100$$

3.4. Method of synthesis

3.4.1. Pretreatment

Several pretreatment methods have been suggested by previous workers, which include physical (mechanical and extrusion), chemical (acid, alkali, organic solvent, and ionic liquid), biological, and other combined pretreatment processes such as steam explosion, AFEX, LHW and wet air oxidation. The current

research work utilized steam explosion and liquid hot water impregnation with different acids which are described below

The prior step of pretreatment was performed using physiochemical pretreatment on mechanically grinded rice straw. The pretreatment utilized processes viz., steam explosion impregnated with H_2O_2 and liquid hot water impregnated with H_2SO_4, HNO_3, HCl, oxalic acid, formic acid and acetic acid. Further, a steam explosion impregnated with different concentrations of oxalic acid was performed as well.

3.4.1.1. Steam Explosion

a). Stem explosion impregnated with H_2O_2

The grounded agricultural waste i.e., rice straw was subjected to physiochemical pretreated using a steam explosion technique impregnated with various concentrations of H_2O_2 (0.05%, 0.1%, 0.25%, 0.50%, 0.75%, 1%) in an autoclave at 121℃ for 1 hr residence time at 15 psi pressure. The solid fraction and liquid hydrolysate were filtered using vacuum filtration and the solid fraction was dried overnight in hot air oven at 60℃. The liquid fraction was stored at 4℃ for further sugar analysis and the solid fraction was stored for further morphological and structural changes in the pretreated biomass using FESEM, FTIR, TGA and XRD. The experimental design with optimal conditions is illustrated in table 3.1 below.

b). Steam explosion impregnated with H_2O_2 and Citric acid (HPCA)

After evaluating the presence of sugar in H_2O_2 pretreated rice straw, it was further combined with citric acid in the ratio 1:1, 2:1 and 1:2. A similar process of section (a) for separation was performed to evaluate sugar analysis in obtained hydrolysate and solid fraction was subjected to morphological analysis using TGA, XRD, FESEM, and FTIR. The solid fraction was stored at 4℃ for further processing step.

Table 3.1- Illustration of experimental design of steam explosion assisted with H_2O_2 and HPCA

Pretreatment methods	Acid/alkali used	Solid: liquid ratio	Concentration Of acid/ alkali used/ ratio	Reaction time (min)	Temp.(℃) / pressure
Steam explosion	H_2O_2	1:10	0.05%	1 hr	121℃/15 psi
			0.1%		
			0.25%		
			0.50%		
			0.75%		
			1%		
Steam explosion	H_2O_2 and citric acid	1:10	1:1	1 hr	121℃/15 psi
			1:2		
			2:1		

3.4.1.2. Liquid hot water

Liquid water pretreatment was performed with impregnation of rice straw in 1 M H_2SO_4, HCl, HNO_3, formic acid, acetic acid and oxalic acid, carried out in 100 ml Teflon coated hydrothermal with 50 ml as a working volume of reactor used. The pretreatment process was performed at rice straw-to-water ratio of 1:10 and was further kept in hot air oven for 1 hour at varying temperatures with the range of 80-140℃ (Table 3.2). The reactor was doused in a water bath at the end of the reaction until the temperature reached 25℃. The liquid and solid fractions were separated using vacuum filtration and were stored in the refrigerator at 4℃. The liquid and solid fractions were utilized further for reducing sugar estimation and morphological changes in the biomass structure, respectively. The solid fraction was subjected to further analysis of FESEM, FTIR, TGA and XRD.

Table 3.2- Illustration of optimum condition maintained during liq. Hot water pretreatment

Pretreatment methods	Acid/alkali used	Biomass: liquid ratio	Concentration of acid/ alkali used	Reaction time (min)	Temp.(℃) / pressure
Liq. Hot water	H_2SO_4	1:10	1 M	1 hr	80℃, 100℃, 120℃, 140℃
	HCl	1:10	1 M	1 hr	80℃, 100℃, 120℃, 140℃
	HNO_3	1:10	1 M	1 hr	80℃, 100℃, 120℃, 140℃
	Formic acid	1:10	1 M	1 hr	80℃, 100℃, 120℃, 140℃
	Oxalic acid	1:10	1 M	1 hr	80℃, 100℃, 120℃, 140℃
	Acetic acid	1:10	1 M	1 hr	80℃, 100℃, 120℃, 140℃

3.4.1.3. Liquid hot water impregnated with oxalic acid

Liquid hot water pretreatment was performed using different concentrations of oxalic acid (0.5 M, 0.75 M, 1 M, 1.5 M) using a hydrothermal reactor at 120℃ for 1 hr residence time. The solid and liquid fraction was separated and stored at 4℃ for further processing steps.

3.5. Determination of cellulose, hemicellulose and lignin after pretreatment

The compositional analysis of rice straw after different pretreatment methods was determined as suggested by Singh & Kumar, 2020. The hemicellulosic content was determined by taking 1 gm of pretreated rice straw and further treating it with 200 ml of 0.5 M NaOH at 80℃ for 3 hrs. The obtained slurry was filtered and washed continuously with distilled H_2O until a neutral pH was obtained. The solid residue was dried overnight in hot air oven at 60℃ to obtain constant weight of biomass. The initial and final weight of rice straw gives the amount of hemicellulose present in the pretreated rice straw.

Further, approximately 0.5 gm of hemicellulose-free rice straw was taken and dissolved in 15 ml of 98% H_2SO_4 and heated at 30℃ in a heating mantle for 2 hrs to dissolve the sulphate anion into the biomass.

After that distilled water of up to 352 ml was added to dilute it to 4% H_2SO_4, the obtained slurry was autoclaved for 1 hr and the solid fraction was filtered out. The filtered aliquots were taken to measure the OD at 205 nm wavelength in a UV-spectrophotometer until 0.2-0.8 absorbance was obtained using 4% H_2SO_4 as blank. After the required absorbance was obtained, the aliquot was filtered out and the rice straw was dried to obtain the constant weight of biomass that was considered as the obtained acid-soluble lignin present in the various pretreated rice straw. Further, subtracting the obtained dried weight of hemicellulose and lignin gives the cellulosic content in the pretreated rice straw.

Objective 2- Determining the accessibility of acid hydrolysis and enzymatic hydrolysis for an efficient hydrolysis process during SPS method

Under this objective, the extraction of pentose sugars from the first phase using liquid hydrolysate after pretreatment as well as the extraction of hexose sugars from the second phase of enzymatic hydrolysis. The liquid hydrolysate obtained after the pretreatment process was subjected to the Molisch test to determine the presence of sugar in it. Further, DNS reagent test for determining the total obtained reducing sugar, hexose estimation was performed using the Anthrone reagent method while pentose estimation was performed using the orcinol method. The absorbance was measured using a UV-spectrophotometer at 540 nm and 620 nm respectively. The standard graph for various sugars was obtained using a linear regression.

3.4.2. Saccharification process

3.4.2.1. Simultaneous pretreatment and saccharification method

During simultaneous pretreatment and saccharification method, the delignified biomass was subsequently used for saccharification adopting a bi-phasic method for complete sugar extraction. In the first phase, physiochemical treatment aided with acidic treatment was used to extract pentose sugars, accompanied by cellulase hydrolysis in the second phase to extract hexose sugars. The detoxification of obtained acidic hydrolysate was performed using CaO under continuous stirring at room temperature till pH 10 which was further incubated for 2 hr at 30°C with periodic stirring to precipitate inhibitors present in hydrolysate. The obtained hydrolysate was subjected to filtration to obtain clear filtrate. A similar protocol with little modification was obtained from Naseeruddin et al., (2017). Further, the detoxification of hydrolysate was subjected to obtain the desired pH 6 using 1N H_2SO_4 and again filtered to eliminate the traces of salt formed during the process and was further used for the fermentation process. The quantification of pentose sugar was done using the orcinol method against the obtained standard curve of xylose.

3.4.2.2. Enzymatic Hydrolysis

Enzymatic hydrolysis was performed using commercially available cellulase enzyme from Aspergillus niger that was supplied by TCI with a specific activity of 17000 Units/g; one unit liberates 10 µg of glucose from carboxymethyl cellulose per min. at pH 4.5 at 40°C. The different pretreated rice straw was

subjected to enzymatic hydrolysis using 20 U of cellulase enzyme i.e., 1.117 mg was dissolved in 50 ml of 0.05 M sodium citrated buffer (pH 4.5) and 0.02% of sodium azide to prevent microbial growth. The reaction was incubated at 40°C performed in a rotatory incubator at 150 rpm. The 2 ml hydrolysate was sampled at different time intervals i.e., 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs of hydrolysis time. The obtained hydrolysate was boiled in a boiling water bath for 8-10 min to denature the enzymatic activity. The sample was cooled at room temperature and centrifuged at 4000 rpm for 20 min. The obtained supernatant was subjected to reducing sugar analysis using the Molisch test to determine the presence of sugar and the DNS reagent test to quantify the total sugar obtained after hydrolysis, elucidating R^2= 0.9746 and Y= 0.0019X-0.0071 obtained from the glucose standard curve.

a). Molisch reagent test

2 ml of hydrolysate was taken in a test tube, added with 2-3 drops of 1% α-Naphthol and mixed well by shaking the test tube in Vortex shaker. 2 ml of concentration H_2SO_4 was added in the solution slowly from the walls of test tube. After 50 secs, a violet-purple line appeared at the junction of the two solutions that showed the presence of sugar in the sample.

b). DNS Reagent test

The DNS reagent test was performed to quantify the amount of reducing sugar present in the hydrolysate. 1 ml of aliquot was taken in the test tube and 2 ml of DNS reagent was added to it. The test tube was kept at 80°C for 5 min in hot water bath and colour changed was observed. Further, the absorbance was measured at 540 nm wavelength in UV-spectrophotometer. After taking the absorbance of different samples, the quantity of reducing sugar was determined using R^2 and Y values obtained from the glucose standard curve.

<u>Objective 3</u>- Verification of the biphasic system using rice straw

The current objective involved quantification of produced sugar from the two-phasic system i.e., pentose sugar from acidic treatment during the SPS method and hexose sugar from the enzymatic treatment of obtained pretreated rice straw. The obtained hexose and pentose were quantified using the Anthrone and orcinol methods respectively. The detailed process step is explained below

1). Orcinol reagent test

This test has been used for quantification of pentose sugar (Xylose) obtained from the first phase of hydrolysis process. 1 ml of obtained hydrolysate was reacted with 5 ml of freshly prepared orcinol reagent and mixed properly in a vortex. The test tubes were kept in hot water bath at 100°C for 10 min. After cooling at room temperature, the absorbance was measured at 671 nm wavelength and the quantification of xylose was performed against the obtained standard curve.

2). Anthrone reagent method

This method has been used for the quantification of hexose sugar (glucose and galactose) obtained after the second phase of hydrolysis. 1 ml of hydrolysate was taken in a test tube with 5 ml of freshly prepared Anthrone reagent and was mixed properly in vortex. The test tubes were kept in hot water bath at 100°C for 10 min. After cooling at room temperature, the absorbance was measured at 620 nm wavelength and the quantification of glucose and galactose was performed against the obtained standard curve.

Objective 4- Determination and characterization of pentose and hexose utilizing yeast for performing fermentation process to maximize the yield of bioethanol obtained at pilot scale

Under this objective, various hexose and pentose-utilizing yeast were identified by reviewing various published papers on co-fermentation process that were further implemented in this research work. Some yeast can ferment only glucose to ethanol, these traditionally used yeasts are *S. cerevisiae and Z. mobilis* while some of the pentose-utilizing yeast are *Pichia stipitis, Candida shehatae, and Pachysolen tannophilus* and *Candida tropicalis* (Da Cunha-Pereira et al., 2011a).

3.4.3. Yeast culture

After quantifying the reducing sugar present in the hydrolysate, the fermentation process was performed using hexose and pentose utilizing yeast namely *Saccharomyces cerevisiae* (MTCC173) and *Zymomonas mobilis* (MTCC91). The co-fermentation process was performed using a combination of the following yeast *S. cerevisiae* and *Z. mobilis*.

3.4.3.1. *Saccharomyces cerevisiae* nutrient culture media

Saccharomyces cerevisiae growth was performed on YPD broth containing 10 g/l yeast extract, 5 g/l peptone and 10 g/l dextrose for yeast growth. Firstly, 5 gm of YPD broth was dissolved in 100 ml of distilled water, it was further autoclaved for 15 min at 15 psi pressure. *S. Cerevisiae* MTCC173 culture was inoculated in the broth and was kept in an incubator at 30°C for 24 hrs. After the growth of yeast was observed, it was stored in a 50% glycerol solution at 4°C in a refrigerator and further revived again to obtain the pure yeast culture. The yeast culture was used further for fermenting the sugar hydrolysate obtained after saccharification process. The synthetic nutrient media was prepared in 50 ml Erlenmeyer flask containing 3 g/L yeast extract, 4.8 g/L peptone, 0.25 g/L $CaCl_2$, 1.5 g/L KH_2PO_4, and 0.4 g/L $MgCl_2$ in distilled water with 10 ml hydrolysate.

3.4.3.2. *Zymomonas mobilis* nutrient culture media

Similarly, *Z. mobilis* yeast culture was performed in YPS broth containing 10 g/l yeast culture, peptone 10 g/l, sucrose 20 g/l was dissolved in 100 ml of distilled water and was autoclaved for 15 min at 15 psi pressure to sterilize the growth media. The yeast was inoculated in the YPS broth and was kept in an

incubator at 30°C for 24 hours in an orbital shaker at 130 rpm. The synthetic nutrient media was prepared using 60 g/l glucose, 10 g/l of yeast extract and 1 g/l each of $MgCl_2$, KH_2PO_4, $(NH_4)_2SO_4$ with pH adjusted to 5.5. It was further stored at 4°C in a 50% glycerol stock solution and was revived again to obtain pure yeast culture.

3.4.4. Co-fermentation process

The fermentation process was performed in a 50 ml glass bottle containing 25 ml of liquid hydrolysate from the pretreated sample obtained after centrifugation of the slurry at 6000 rpm for 20 min. The bottle was autoclaved at 121°C for 15 min and was cooled at room temperature, then inoculated with *Saccharomyces cerevisiae* MTCC173 and *Zymomonas mobilis* MTCC92 yeast strain. For bioethanol production, 50 ml of hydrolysate was inoculated with 10% (v/v) yeast inoculum and incubated in a rotatory shaker at 150 rpm for 72 hrs at 30°C (Lee et al., 2022). The liquid fraction was collected every 24 hrs and was subjected to estimation of bioethanol production using potassium dichromate reagent and the absorbance was measured at 590 nm wavelength, elucidating R^2=0.9773 and Y=3.633X+0.0726 obtained from the ethanol standard curve (A. Kumar et al., 2020; Suresh et al., 2020).

3.5. Method of preparation of various reagents used for performing the test

3.5.1. 0.05 M Sodium citrate buffer

For cellulase activity, 0.05 M sodium citrate buffer was used with pH 4.5. 3.378 gm of Sodium citrate dihydrate was added to 400 ml of distilled water. Then, 2.596 gm of citric acid was added to the above solution with pH 3.6, it was adjusted to pH 4.5 using 2N NaOH. The distilled water was added to make the final volume 500 ml and was stored in a refrigerator for further usage.

3.5.2. DNS reagent

For preparing 100 ml DNS reagent for estimation of reducing sugar in the hydrolysate, 30 gm of Potassium sodium tartrate tetrahydrate was dissolved in 20 ml of distilled water. 1 gm of 3,5-dinitrosalicyclic acid was dissolved in 50 ml of distilled water, the solution was mixed continuously using a magnetic stirrer in hot water at 95°C temperature. With a continuous stirring of solution, both the above-mentioned solution was mixed gradually in a beaker. After that 2N NaOH solution was prepared by dissolving 1.6 gm NaOH in 20 ml distiller water. The 2N NaOH was poured gradually into the solution by continuously mixing in a magnetic stirrer. After the solution was properly mixed, the final solution was filtered using Whatman filter paper and finally prepared DNS reagent was stored in a dark glass bottle at ambient temperature.

3.5.3. Anthrone reagent

The Anthrone reagent was prepared by dissolving 2 gm of Anthrone in 1000 ml of H_2SO_4 while the glucose stock solution was prepared by dissolving 20 mg of glucose in 100 ml of distilled water. The

Anthrone reagent test was performed by pipetting out the varied concentrations of glucose from the stock solution in a test tube and 5 ml of Anthrone reagent was added by mixing it properly in a vertex. The galactose standard curve was obtained by measuring the absorbance at 620 nm wavelengths and the quantification of hexose sugar was done by elucidating the linear regression curve.

3.5.4. Orcinol reagent

The orcinol reagent was prepared by dissolving 1.5 gm of reagent grade orcinol and 0.10 gm of ferric chloride ($FeCl_3$) in 500 ml of HCl, 12.2M to form yellow coloured solution. The orcinol reagent was prepared with little modification as suggested by Pham et al., (2011).

3.5.5. Molisch reagent

Molisch reagent test was performed to determine the presence of sugar in the hydrolysate. 3.75 gm of α-Naphthol was dissolved in 25 ml ethanol to produce 1% α-Naphthol and was used as a Molisch reagent to determine the presence of sugar in the liquid hydrolysate.

3.5.6. Potassium dichromate reagent

100 ml of potassium dichromate reagent was prepared by dissolving 6.8 gm of potassium dichromate in 100 ml of water, after that 65 ml of H_2SO_4 was dissolved slowly and cooled down. Further, the orange-red colour of the reagent was obtained and was used for the analysis of ethanol present in the sample. Tri-n-butyl phosphate (TBP) was further used to extract the ethanol from the sample of concentrations 1%, 2%, 3%, 4% and 5% of absolute ethanol (99.9%). 1 ml of standard solution and 1 ml of TBP were taken in a test tube and vortexed vigorously until the mixture was separated into upper and lower phases that were transparent and turbid respectively. From that transparent phase, 500 µl solution was taken in another test tube with 500 µl of potassium dichromate reagent in it. It was further vortexed vigorously for 20 min, the obtained blue-green colour was diluted 5 times and optical density was measured at 590 nm wavelengths. The quantitative analysis of unknown sample was determined from the obtained standard curve.

3.6. Instruments

Table 3.3 – Instrument used in the present research work

Sr. No.	Instrument	Model number
1.	Autoclave	NSW-227
2.	Hot air oven	YSI 431
3.	pH meter	Model no. 361
4.	Shaking incubator	REMI RIS 24BL
5.	Spectrophotometer	Model: LI-2800 Ex
6.	UV laminar air flow	STI-164
7.	Deep freezer (-80ºC)	ULT-490

8.	Water bath	NSW-125
9.	Weighing balance	PG8220
10.	Refrigerator	KS201EBR
11.	Mixer grinder	MG-1080
12.	Vacuum pump	SAV15-230
13.	Muffle furnace	STXMF145
14.	Hot plate Magnetic stirrer	VTMS 200
16.	Centrifuge	REMI R-8C
17.	Incubator	YSI 438D
18.	Hydrothermal	TI010

3.7. Characterization Analysis

3.7.1. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed using the Perkin Elmer spectrum with highly sophisticated infrared spectroscopy (CIF lab, LPU). The 0.3 mg dried rice straw was weighed and agitated for 2 min with 50 mg of KBr as a grid of FTIR analyzer. The structural variation occurs between 4000-400 cm^{-1} wavelength, with a maximum resolution of 4 cm^{-1}. On average 20 scans were performed with different peaks resembling the stretching of the different functional groups of both native and pretreated rice straw. Table 3.4 below illustrates the bond stretching of constituents present in the LCB at different wavelengths.

Table 3.4- Illustrating bond stretching during FTIR analysis at different wavelengths

Wavelength	Bond stretching	Constituent of LCB
3421 cm^{-1}	O-H bond	Lignin
2937 cm^{-1}	Asymmetric C-H bond	Lignin
1735 cm^{-1}	C=O bond	Hemicellulose (carboxyl, carbonyl, acetyl group)
1682 cm^{-1}	Unconjugated C=O bond	Lignin
1593 cm^{-1}	Aromatic vibration of C=O bond	Lignin
1466 cm^{-1}	Deformation of C-H bond	Carbohydrate and lignin
1422 cm^{-1}	Deformation of plane C-H bond	Lignin
1380 cm^{-1}	Bending of C-H bond	Cellulose and hemicellulose

1370 cm^{-1}	Stretching of aliphatic C-H bond present in CH_3	Cellulose
1327 cm^{-1}	Stretching of syringyl derivative of C-O bond	Cellulose, hemicellulose and lignin
1263 cm^{-1}	C-O bond	Lignin
1200 cm^{-1}	O-H bond	Cellulose and hemicellulose
1160 cm^{-1}	C-O-C bond	Cellulose and hemicellulose
1050 cm^{-1}	C-O bond	Cellulose and hemicellulose
1035 cm^{-1}	C-C-O, C-O, C=C bond	Cellulose, hemicellulose and lignin
896 cm^{-1}	Deformation of C-H bond	Cellulose

3.7.2. Field emission scanning electron microscopy (FESEM)

Field emission scanning electron microscopy was used to analyze the sample based on elemental and topographical analysis at the magnification of 50X to 15000X resolution. FESEM analysis was performed using JEOL model JSM-7610F Plus available at CIF lab, LPU. It works on the principle of field emission cathode that was first developed in 1936 by Erwin Muller and resulted in higher-resolution images. FESEM uses a field emission gun as an electron source to emit an electron beam that can visualize very fine topographic characterization of biomass. A thin layer of gold coating can be applied to produce high-resolution pictures.

3.7.3. Thermogravimetric analysis (TGA)

Thermal degradation of native and pretreated biomass was performed using Perkin Elmer TGA 4000 (CIF Lab, LPU). It was utilized to analyze degradation in the weight, both before and after pretreatment by heating the biomass from 50°C to 600°C at 10°C/min. Throughout the onset of the temperature rise, weight loss was significantly recorded with a constant heating rate.

3.7.4. Double beam UV-vis spectrophotometer

The microprocessor UV-vis double beam spectrophotometer model LI-2800 Ex was used to determine the absorbance obtained from the sample. The cuvette with 2-3 µl of sample was placed in the holder and the dispersion and scattering of UV-light. Based on absorption of ultraviolet light by the solvent absorption spectra versus specific wavelength was obtained. The absorbance obtained was further used for the quantification of an unknown sample against the standard curve of the known solvent.

3.7.5. X-ray diffraction (XRD)

The **c**ellulose crystallinity index of both native and pretreated samples was analyzed using the Bruker D8 Advance XRD system (CIF Lab, LPU). X-pert pro diffractometer with scanning range 2θ within 5-40° at

0.03°/s using Cu-Ka radiation X-ray. The crystallinity index was calculated from PXRD analysis using a formula earlier developed by Sindhu et al., (2016),

$$CrI\ (\%) = [(I_{002} - I_{am})/I_{002}] \times 100 \quad \text{--1}$$

Where *CrI* shows the crystallinity index, I_{002} shows maximum intensity in the crystalline region of cellulose at the 002 planes while I_{am} shows the minimum intensity in the amorphous region of cellulose between its lattice planes.

3.8. Calculation used during the research

3.8.1. Conversion rate

$$Biomass\ conversion\ rate = \frac{Total\ reducing\ sugar\ produced\ (g)}{Biomass\ (g)} \times 100$$

$$Sugar - ethanol\ conversion\ rate = \frac{Ethanol\ produced\ (g)}{total\ reducing\ sugar\ produced\ (g)} \times 100$$

3.8.3. Compositional changes in untreated and pretreated biomass

$$Hemicellulose\ removal\ \% = \frac{H_i - (H_f \times {\%soild\ recovery\ of\ RS}/{100}}{H_i} \times 100$$

$$Lignin\ removal\ \% = \frac{L_i - (L_f \times {\%solid\ recovery\ of\ RS}/{100}}{L_i} \times 100$$

3.8.2. Glucan and xylan content

$$Glucan\ content\ present\ in\ biomass = \frac{conc.of\ glucose\ \left(\frac{mg}{ml}\right) \times 86.73 \times 0.9}{final\ weight\ of\ obatined\ RS} \times 100$$

$$Xylan\ content\ present\ in\ biomass = \frac{conc.of\ xylose\ \left(\frac{mg}{ml}\right) \times 86.73 \times 0.88}{final\ weight\ of\ obtained\ RS} \times 100$$

Here, 86.73 is the volume of acid hydrolysis liquid

0.88 is the conversion factor for pentose sugar

0.9 is the conversion factor of hexose sugar

3.8.3. Ethanol yield

$$Ethanol\ Production\ yield\ (EPY)\ (\%)_{AB} = \frac{Ethanol\ concentration\ (\frac{g}{l})_{AB}}{TRS_{AB}} \times 100$$

$$Ethanol\ Production\ yield\ (\%)_{CD} = \frac{Ethanol\ concentration\ (\frac{g}{l})_{CD}}{TRS_{CD}}$$

$$Overall\ ethanol\ yield\ (\%)\ (E_O) = \frac{EPY_{AB}(g) + EPY_{CD}(g)}{Raw\ Biomass\ (g)} \times 100$$

$$Maximum\ ethanol\ yield\ (\%)\ (E_{max}) = \frac{(C_A + H_A) \times 0.568}{Raw\ biomass\ (g)} \times 100$$

$$Fermentation\ efficiency\ (\%) = \frac{E_0}{E_{max}} \times 100$$

CHAPTER-4

RESULTS AND DISCUSSION

Introduction

This chapter deals with the structural and morphological changes that occurred during the physiochemical pretreatment of rice straw using various chemical impregnations as well as the incorporation of pretreatment and saccharification in a single pot. The results of total sugar formed after two-phase hydrolysis process are covered in this chapter along with the yield of bioethanol produced from the co-fermentation process using both hexose and pentose utilizing yeast. The detail of various physiochemical pretreatment techniques is already described in Chapter 3 (section 3.4.1), the saccharification process in section 3.4.2 and co-fermentation was covered in section 3.4.3. All the obtained results are analyzed and described along with findings obtained during the present research are described in this chapter.

4.1. Collection and compositional analysis of Rice straw

The rice straw utilized for the process was collected from the local farm in February 2022 from Kanpur (26.42°N, 80.38°E), Uttar Pradesh. Kanpur has a tropical temperature with hot summer (42°C) and foggy and cold winters with temperatures dropping down to 5°C. The Compositional analysis of biomass plays a vital role in the selection of effective pretreatment techniques. It was found that physiochemical pretreatment with impregnation of different acids has resulted in the breakdown of hemicellulose and lignin crosslinked structure and increased the accessibility of cellulose towards enzymatic hydrolysis (Shukla et al., 2023). Based on rice straw dry weight, the chemical composition of untreated biomass was assessed for its internal constituents, including cellulose, hemicellulose, lignin, extractives and ash content. Following processing, 60-75% of solid recovery were obtained after pretreatment with different acid used. Upon finding compositional analysis of native rice straw, cellulose account for 32.4±0.017% (w/w), hemicellulose 57±0.011% (w/w), lignin 12.5±0.021% (w/w), extractive 10.12±0.08% (w/w) and ash 7.4% (w/w) content. The high cellulosic and hemicellulosic content in untreated rice straw makes it, a promising source for bioethanol production using the two-phase technique of biomass conversion to ethanol. The majority of lignocellulosic biomass is composed of the polysaccharide cellulose and hemicellulose, as well as the aromatic polymer lignin. All of these components were meticulously woven together to offer unwavering support to the plant cell wall. This complicated ambiguity of LCB components impeded enzyme degradation into a low-molecular building block. The composition of the rice straw used in this study was initially compared to previous studies (Table 4.1), and it was discovered to contain primarily hemicellulose (57% w/w), cellulose (32.4% w/w), lignin (12.5% w/w), extractive (10.12% w/w) and ash (7.4% w/w). Semwal et al., (2019) reported a higher cellulosic content (37.8%) for the rice straw biomass along with hemicellulose (21.6% w/w), lignin (13.6% w/w), and ash (13.2% w/w) content. Ayeni et al., (2015) reported higher cellulosic contents for the rice straw they used. Indeed, such varied compositional analyses of lignocellulosic biomass due to soil type, nitrogen fertilization, and harvest time, all had a strong influence on biochemical composition (Bhatia et al., 2020). The presence of both lignin and hemicellulose had the potential to reduce

overall efficacy in conventional bioethanol synthesis from cellulose alone, as these additional components could impair both the sample pretreatment and the enzymatic hydrolysis phases (Syaftika & Matsumura, 2018). In this study, physiochemical pretreatment followed by enzymatic saccharification was applied to the rice straw biomass and described in further section.

Table 4.1- Comparison of compositional analysis of Rice straw used in previous studies

Component	Present study	Syaftika & Matsumura, (2018)	Semwal et al., (2019)
Cellulose (Glucan)	32.4	28	37.8
Hemicellulose (Xylan)	57	55	21.6
Lignin	12.5	11	13.6
Ash	7.4	6	13.2
Extractive	10.12	NA	16.1

4.2. Physiochemical pretreatment

4.2.1. Steam Explosion impregnated with different concentrations of H_2O_2

After steam explosion pretreatment attributed with different concentrations of H_2O_2, the solid and liquid samples were filtered and the solid fraction was subjected to morphological analysis using FTIR, TGA, XRD and FESEM analysis. The obtained hydrolysate was subjected to a DNS reagent test using UV-spectrophotometer and absorbance was measured at 540 nm wavelength. The result was being discussed below with respect to functional group stretching at different absorption peaks, thermal decomposition and weight loss at different time intervals, cellulose crystallinity was determined using intensity at I_{am} (18.5) and I_{002} (22.5), and structural morphology of the different pretreated sample.

(a). FTIR analysis

The changes in chemical characteristics brought on by pre-treating the biomass were investigated by studying the absorption band of FTIR spectra (Figure 4.1). The band from 4000-1000 cm^{-1} showed the region with vibration of functional group and 1000-400 cm^{-1} is considered as the fingerprint region. The key features in the FTIR spectra were attributed to functional groups found in cellulose, hemicelluloses, and lignin. According to FTIR spectra, the greatest significant transmittance was found for both native and pre-treated biomass at around 3306, 2916, 2355, 1625, 1056 and 480 cm^{-1}wavelength. Similar peaks were attributed by Akhtar et al., (2017). The broad band between 3200-3400 cm^{-1} was attributed to OH bond stretching in cellulose that indicates the stretching vibrations of H-bonds made with hydroxyl groups, most likely from cellulose's β-1,4 glycosidic connections or its alcoholic and phenolic groups. This band is more prominent in 0.05% H_2O_2 pretreated biomass and it got flattened on increasing the concentration of H_2O_2.

The absorption band at 2916 cm^{-1} represent the stretching of C-H and CH_2 bond present in cellulose and hemicelluloses that represent rapture of methyl group present in cellulose and hemicellulose while band at 1625 cm^{-1} represent the stretching of C=C bond vibration in lignin and carboxyl groups demonstrating hemicellulose-lignin bond. The stretching region at 1037 cm^{-1}, 1375 cm^{-1} and 788 cm^{-1} related to O–H, C–O–C, and C=C stretching vibrations at β-glycosidic linkages in the structure of cellulose and hemicellulose. Similarly, stretching of the band at 1056 cm^{-1} represents C-O bond vibration in cellulose and hemicellulose while stretching at 480 cm^{-1} represents C-H deformation in cellulose. The bond detachments and reorganizations are seen by these peak changes. The removal of lignin and hemicellulose from the linkages is thought to be the cause of their weakening and cleavage. The prominent peak at around 1500-1100 cm^{-1} and 900-600 cm^{-1} in pretreated rice straw showed the existence of crystalline cellulose (type 1) and amorphous cellulose (type 2), this represents the enhancement in the amorphous cellulose in the pretreated rice straw. The increased intensity of peak at 1500-1100 cm^{-1} in pretreated rice straw demonstrates stretching of the C-O bond and the distortion in cellulose and lignin structure that maximizes the pore-like structure in the pretreated biomass.

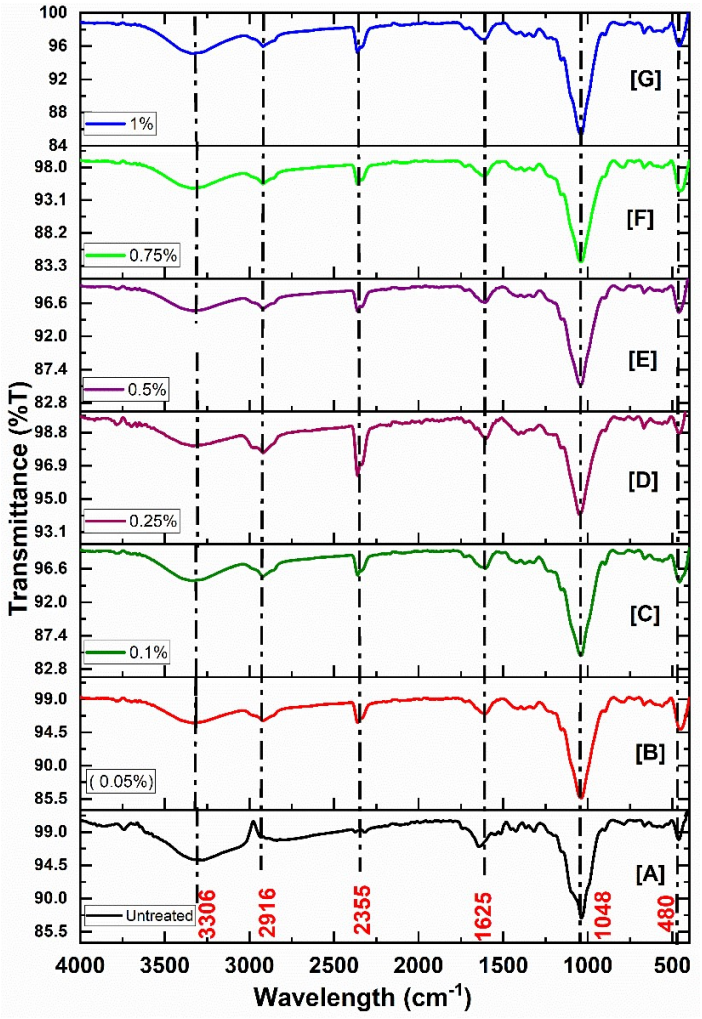


Figure 4.1. FTIR analysis of untreated and pretreated rice straw using different concentration of H_2O_2

(b). Thermogravimetric analysis

TGA analysis was critical to investigate the thermal degradation of the treated sample, which was linked to the chemical structure of LCB. TGA analysis was carried out to effectively convey the thermal information of various pretreated biomass as shown in Figure 4.2. The TGA analysis result showed three major weight loss regions (A-zone, B-zone, C-zone) of native and pretreated rice straw that mainly correspond to moisture removal (dehydration), thermal decomposition (volatile material removal), and solid disintegration respectively, as similar findings revealed by Monir and his team mates (Monir, Aziz, & Yousuf, 2022). The slight weight loss at 60°C of all pretreated rice straw was due to evaporation of moisture content. The maximum degradation occurred at a temperature between 260ºC and 365°C. It was noted from the graph that cellulose and hemicellulose started degrading at around 267°C and 360°C for all concentrations of H_2O_2 with an average weight loss of 0.08 (wt.%/°C) at 296°C and 0.06 (wt.%/°C) at 320°C temperature. The maximum weight loss percentage of raw rice straw was obtained at 365°C while the pretreated sample showed maximum weight loss at 376ºC and 378°C temperatures and a similar finding was earlier observed by C. Huang et al., (2020). This was mostly due to the pretreatment elimination of a specific percentage of hemicellulose and lignin, which had a stochastic amorphous structure and was rendered obsolete with the rising temperature. After raising the temperature from 376°C leaving behind the minimum traces of ashes (14%) while highest lignin breakdown occurred after 400°C with the highest weight loss of 0.96% which was attributed to the breakdown of rice straw fraction to gaseous compounds. It was also discovered that the lignin component of biomass was the most difficult counterpart to degrade, and its breakdown occurred very gradually throughout the entire temperature profile (up to 600°C). It was found that B and C-zone show the maximum weight loss, as B-zone showed up to 52-70% in comparison to untreated rice straw that showed up to 44.73% of weight loss while C-zone that is attributed to lignin degradation resulted in 47-66.6% of weight reduction from pretreatment with different concentration of H_2O_2. It is considered that lignin has a multifaceted molecular structure made up of phenolic hydroxyl, resulting in higher molecular weights due to the presence of intermolecular C-C bonds that manifest a greater degree of stability and begin to degrade at temperature ranges from 100 to 600°C.

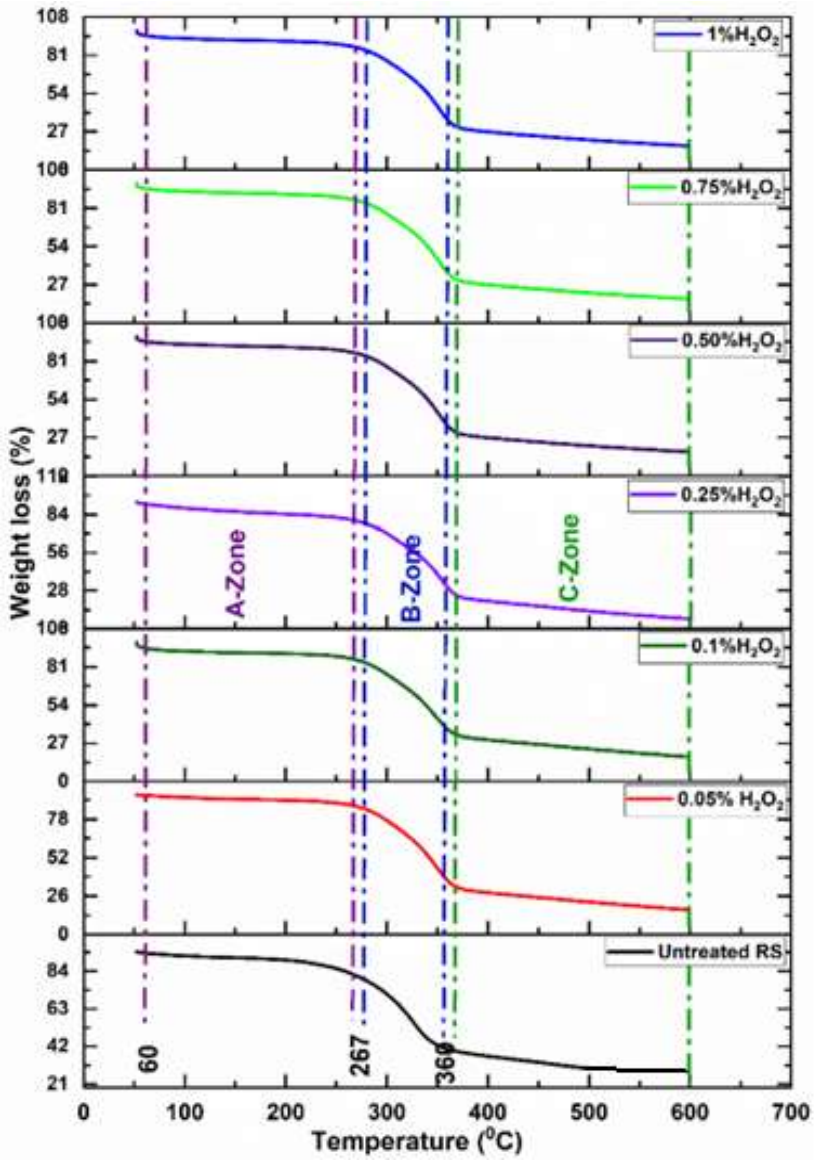


Figure 4.2- TGA analysis of untreated and pretreated rice straw using different concentrations of H_2O_2

(c). FESEM analysis

FESEM micrograph was illustrated in Figure 4.3 (A) at 400X magnification and (B) at 2000X magnification. It was shown that pretreated rice straw shows irregular, bulging and swelling in the outer surface of rice straw. Similar observations were discussed by Zhao et al., (2017) when H_2O_2 presoaking was performed prior to the AFEX pretreatment process. It was stated that after pretreatment external accessible surface area was enhanced and porous structure was modified during the process. The segments were exposed after being detached from the initial linked structure, enhancing the porosity and exterior surface area. This would promote enzymes interacting with the inner connection, hastening the degradation process. It was evaluated that 0.05% H_2O_2 pretreated rice straw, exhibited physical changes as a result of pre-treatment that was evidenced by roughening, inconsistency owing to silica layer breakdown, and microfibril distortion due to dissolution of hemicellulosic structure in the obtained hydrolysate (S. Kaur et al., 2022).

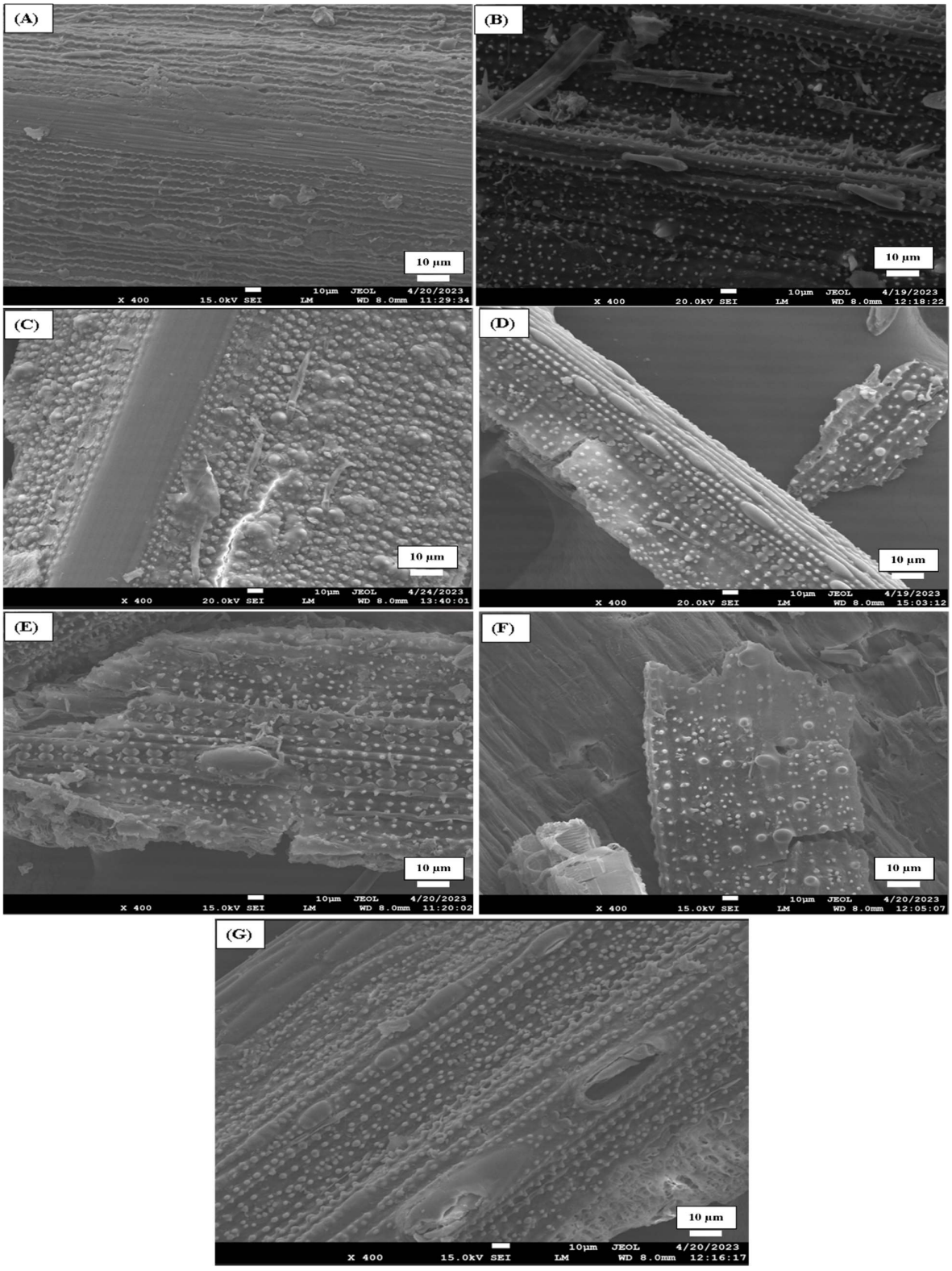


Figure 4.3 (A)- FESEM analysis of untreated and pretreated rice straw at 400X magnification (A) Untreated rice straw (B) 0.05% H_2O_2 (C) 0.1% H_2O_2 (D) 0.25% H_2O_2 (E) 0.5% H_2O_2 (F) 0.75% H_2O_2 (G) 1% H_2O_2 pretreated rice straw

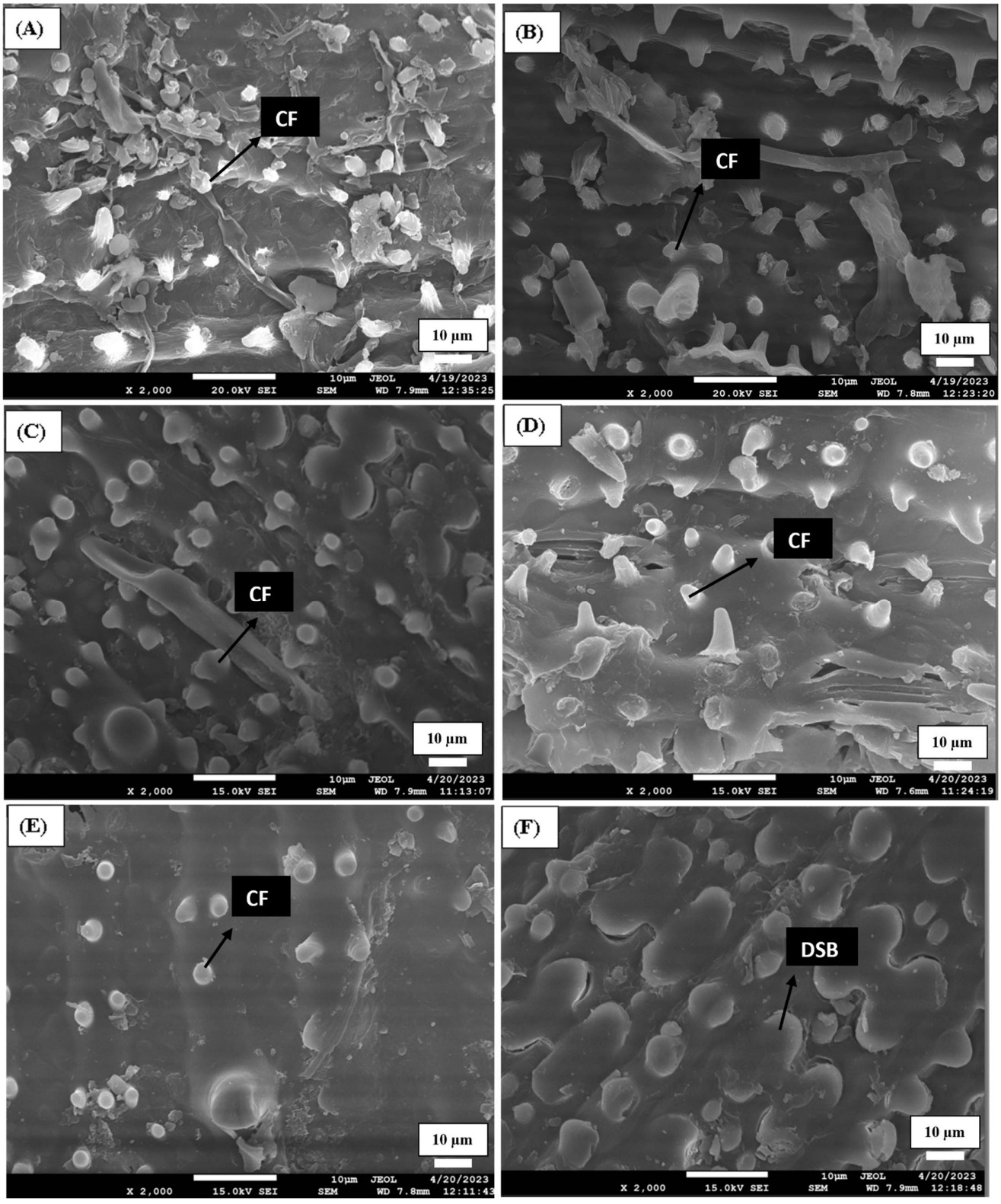


Figure 4.3 (B)- FESEM analysis of pretreated rice straw at 2000X magnification (A) 0.05% H_2O_2 (B) 0.1% H_2O_2 (C) 0.25% H_2O (D) 0.5% H_2O_2 (E) 0.75% H_2O_2 (F) 1% H_2O_2 (CF- Cellulose fibrils, DSB- Dumb-bell shaped silica layer)

(d). XRD Analysis

The cellulose crystalline index (CrI) was a key factor that influences lignocellulosic biomass enzymatic digestibility. The crystalline nature of cellulose varies depending on the biomass, and PXRD was used to analyze the variations in the crystallinity index of cellulose both for native and pretreated rice straw. There are crystalline and amorphous forms present in the cellulosic part of lignocellulosic biomass. To prevent cellulose degradation, the crystalline structure features a large intramolecular hydrogen bonding that was earlier confirmed by Liu et al., (2020). The research carried out by Zhang et al., (2022) studied the sharp high-intensity peaks that indicate the crystalline nature of all the samples while a broad array of peaks in all biomass samples indicate that they were amorphous in nature. The strong diffraction peak at various 2θ values corresponds to the (110), (200), and (004) crystal phase of the biomass as illustrated in Figure 4.4, an analogous finding was earlier obtained by Malgas et al., (2020). The CrI was calculated using the intensity range of both amorphous and crystalline cellulose at the strong diffraction peak range of (200) and (110) respectively. It was estimated that the pretreated sample showed slightly higher CrI than the native rice straw. It was reported that the CrI value of native and 0.05% H_2O_2 samples was 49.4% and 57% respectively and thereby started decreasing with an increase in the concentration of H_2O_2. The *CrI* calculation based on intensity values at 18.5 (I_{am}) and 22.5 (I_{002}) was presented in Table 4.2. This reduction in CrI of the pretreated sample suggests that it was extremely amorphous, indicating that the lower concentration of H_2O_2 has broken down intra- and interchain H-bonding in the crystalline structure of cellulose. The XRD pattern's large diffraction peak signifies that the crystalline form of processed biomass has undergone significant modifications. The rise in CrI was attributed mostly to the removal of lignin and hemicellulose from the amorphous area. Thus, similar findings from Paramasivan et al., (2021) revealed that more amorphous cellulose was generated in the presence of greater surface accessibility, implying that more cellulolytic activity was potentially possible.

Table 4.2- Cellulose crystallinity Index derived from XRD analysis of H_2O_2 pretreated rice straw

Different pretreated sample	**Intensity at I_{am}**	**Intensity at I_{002}**	***CrI***
Untreated biomass	9.4	18.6	49.4
0.05% H_2O_2	10.3	24	57
0.1% H_2O_2	10.4	23.1	54.9
0.25% H_2O_2	10.26	22.7	54.8
0.5% H_2O_2	9.6	20.7	53.6
0.75% H_2O_2	10.7	22.8	53.07
1% H_2O_2	11.4	23.8	52.1

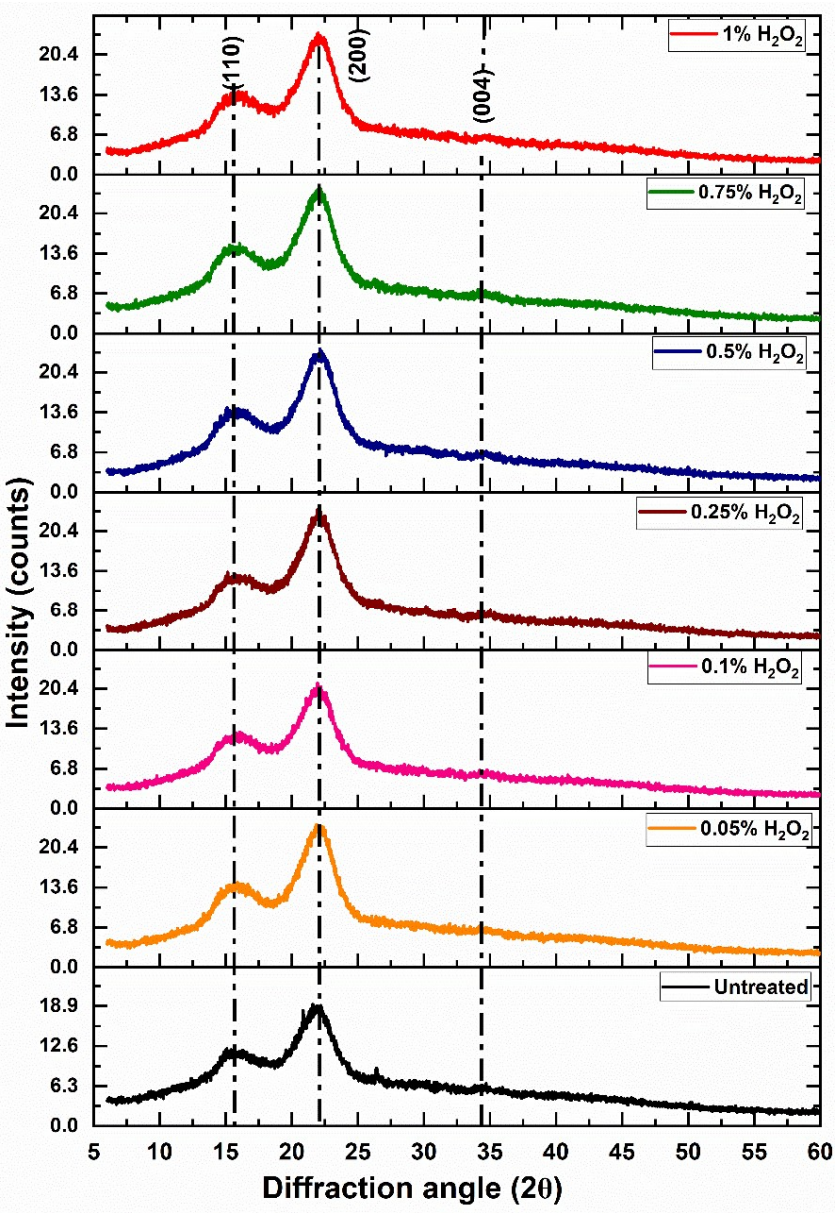


Figure 4.4- XRD analysis of untreated and pretreated rice straw using different concentrations of H_2O_2

4.2.2. Steam explosion with H_2O_2 along with citric acid (HPCA) in different ratio

The native rice straw was subjected to steam explosion pretreatment impregnated with HPCA in the ratio 1:1, 1:2 and 2:1. The pretreated biomass was dried in a hot air oven overnight at 60°C and was stored in a plastic bag at 4°C for further analysis including FESEM, FTIR, XRD and TGA. This was done to analyze the morphological and structural changes that occur after the pretreatment of rice straw. The result was discussed below under a specific section.

(a). FTIR analysis

FTIR absorption peak on native and pretreated rice straw was illustrated in Figure 4.5 (A). The major absorption of peak at different wavelengths 3334, 2924, 2347, 1617, and 1037 cm^{-1} resembles stretching of different functional group namely O-H bond, C-H bond for CH_2 and CH_3 that show deformation of methoxyl

and methylene group, this indicates slight changes in the aromatic structure of lignin during the process of delignification, the reduction in the peak height at 1617 cm^{-1} illustrate the delignification of lignocellulosic biomass, C-O stretching of cellulose, while stretching at 1037 cm^{-1} shows the vibration of C-O bond present in the cellulose. Similarly, various peaks at different wavelengths corresponding to various bond stretching among lignocellulosic biomasses were observed. A shift in the interactions among sugar molecules and intermolecular disintegration in the structure of hemicellulose was indicated by a reduction in the band at 804 cm^{-1}, which shows the existence of predominant β-glycosidic linkages between both the sugar units in hemicellulose and cellulose. In this, the intensity of various peaks is increased as compared to raw and H_2O_2 pretreated rice straw which represent the enhancement in porous structure and glucan content in pretreated rice straw.

(b). TGA analysis

It was critical to investigate the thermal properties of the pretreated sample, which were linked to the chemical structure of LCB. TGA analysis was brought out to effectively convey the thermal information of various samples as shown in Figure 4.5 (B). The initial A-zone shows 7.6-16% of total weight loss of different pretreated rice straws. From the graph, it was evaluated that the initial degradation was observed at around 60°C that represents evaporation of moisture content while maximum weight loss was observed at around 250-375°C temperature up to 68% in the B-zone. This region showed the degradation in the cellulose and hemicellulose structure. The maximum weight loss percentage of raw rice straw was obtained at 365°C while the pretreated sample shows maximum weight loss at 272°C temperatures and a similar finding was earlier observed by C. Huang et al., (2020). The C-zone represent percentage weight loss up to 62.7-74% due to lignin degradation, as its degradation started from the 100°C and reached maximum up to 500°C with 85% of total weight loss in the pretreated rice straw. The maximum weight loss was attributed with biomass pretreated with HPCA in the ratio 1:2. This shows that citric acid concentration with H_2O_2 had major effect in the degradation of biomass in the respective zones.

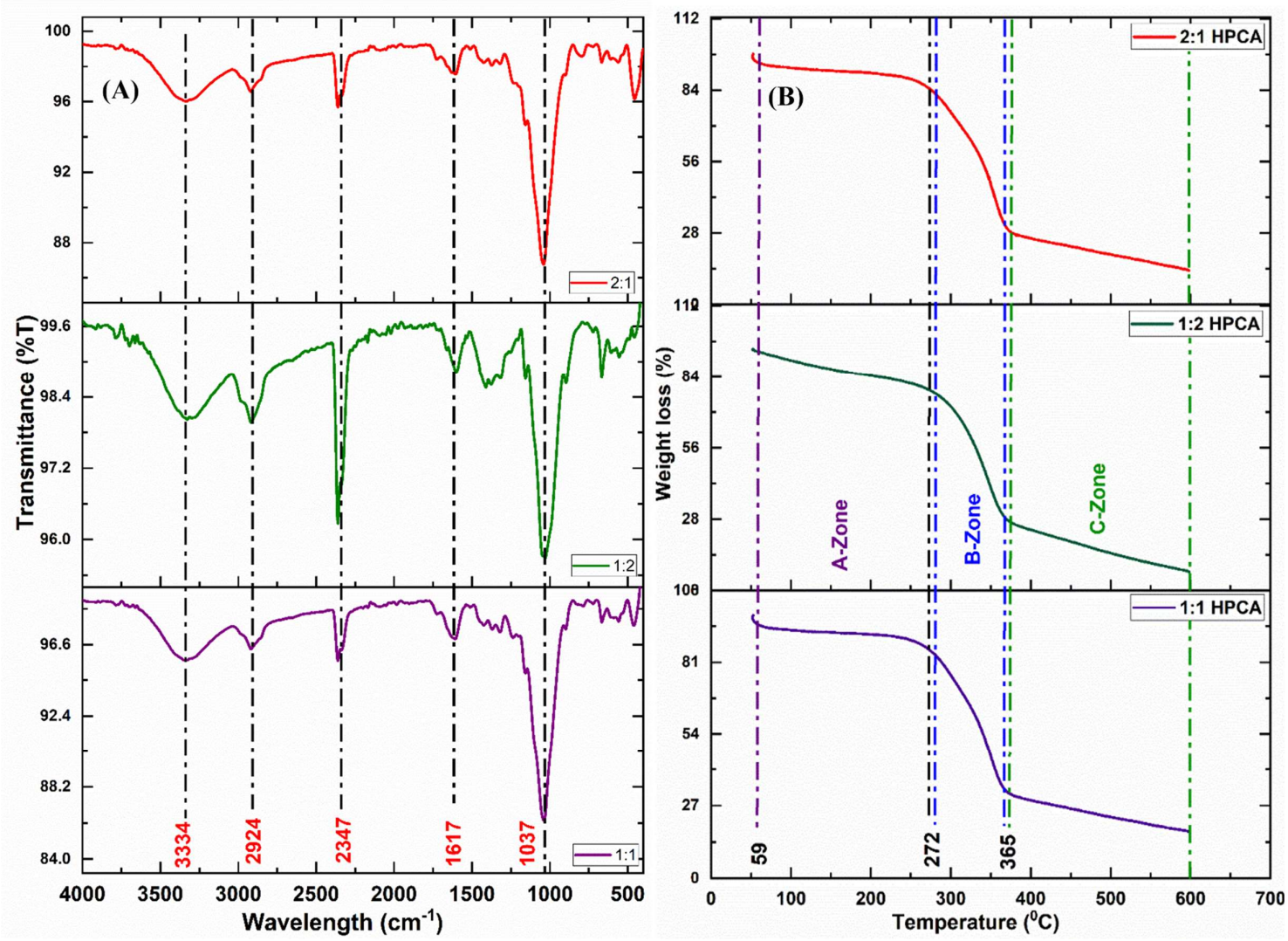


Figure 4.5- (A) FTIR analysis **(B)** TGA analysis of untreated and pretreated rice straw using HPCA in different ratio

(c). FESEM analysis

The FESEM micrographs of HPCA pretreated rice straw at 1000X magnification are illustrated in Figure 4.6. The bulging and irregular structure was visible in the HPCA pretreated rice straw in the ratio 1:1 and 1:2. Fibers were extensively degraded and smooth thin-layer surfaces emerged in the case of acid-impregnated steam explosion process due to deep penetrations caused by the combined influence of acids and temperature. This is almost probably going to result in the breakdown of hemicellulose and solubilization of lignin. The pretreatment process results in the reduction of particles of rice straw that eventually reduces the polymerization of substrate, ultimately enhancing the internal surface area that would increase the proper accessibility of cellulase enzyme during the hydrolysis process (Pant et al., 2021).

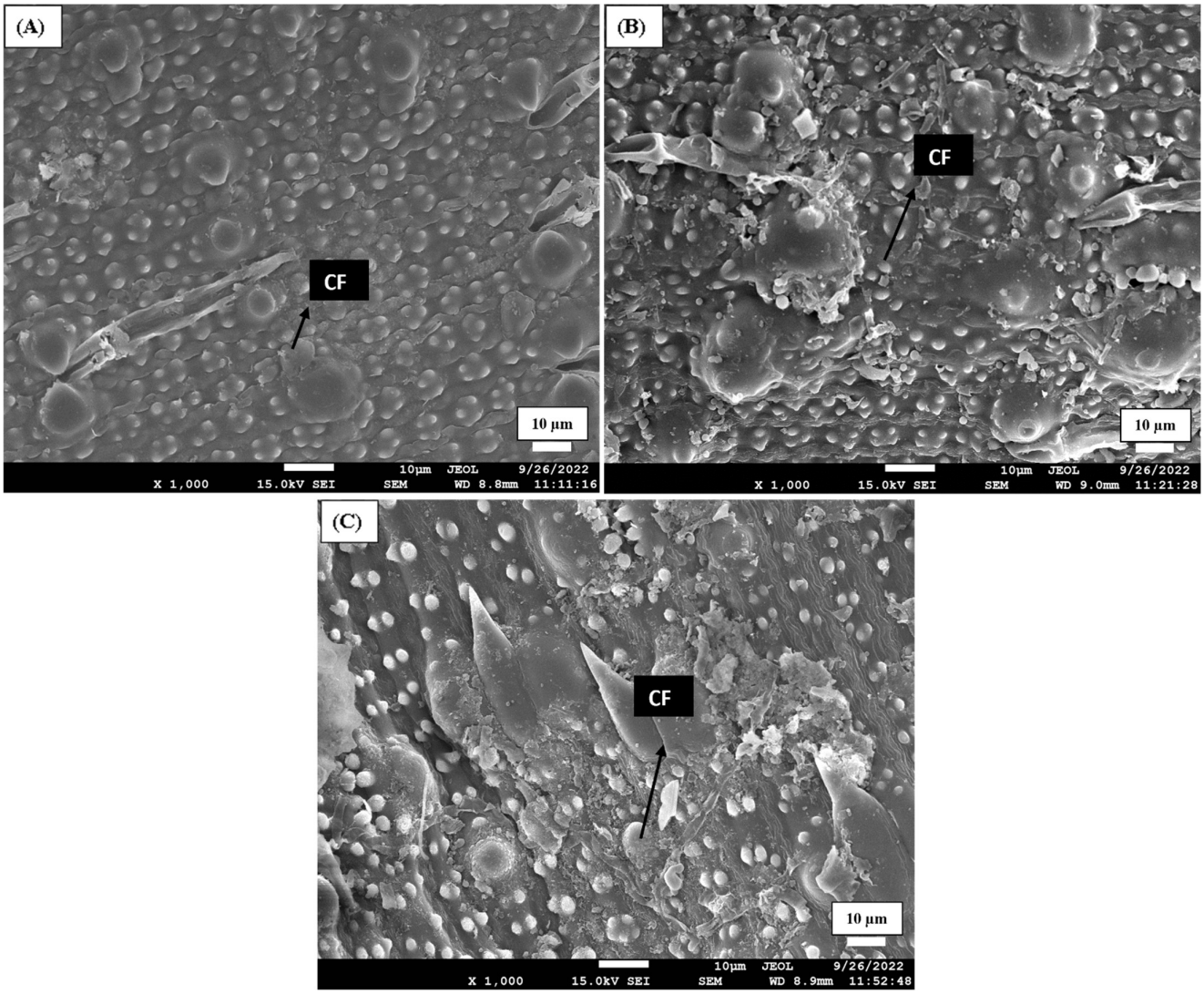


Figure 4.6- FESEM analysis of HPCA pretreated rice straw in different ratios at 1000X (A) 1:1 (B) 1:2 (C) 2:1 (CF- Cellulose fibrils)

(d). XRD analysis

From the obtained graph on XRD (Figure 4.7) the *CrI* calculation based on intensity values at 18.5 (I_{am}) and 22.5 (I_{002}) are presented in Table 4.3, it was evaluated that cellulose crystallinity of various HPCA pretreated biomass in the ratio 1:1, 1:2 and 2:1 showed 61.5, 53.4 and 52.8. The crystalline nature of cellulose varies depending on the concentration of acid used during the treatment method. It was found that HPCA pretreated in the ratio 1:1 showed maximum CrI of 61.5%, which shows 19.6% rise in cellulose crystallinity while 13.3% rise in crystallinity on treating with 0.05% H_2O_2 due to delignification of biomass. The maximum digestibility of biomass towards enzymatic activity, represent cellulosic and amorphous region of lignocellulosic biomass. Similar findings from Paramasivan et al., (2021) revealed that more amorphous cellulose was generated in the presence of greater surface accessibility, implying that more cellulolytic activity was potentially possible. The earlier study performed by Xu et al., (2023) shows a

similar rise in cellulose crystallinity up to 61.8% when sugarcane bagasse was treated under high temperature and pressure, this shows the rise in cellulose content in the biomass. Furthermore, it was evaluated that biomass crystallinity is determined by two contrasting components such as the swelling and dissolving of the crystalline cellulose part and the elimination of the amorphous lignin and xylan. The higher CrI values show the elimination of amorphous components whereas the expansion of cellulose fiber shows a decrease in CrI (Z. Zhao et al., 2018). Thus, it was found that the rise in CrI was attributed mostly to the separation of lignin and hemicellulose from the amorphous area.

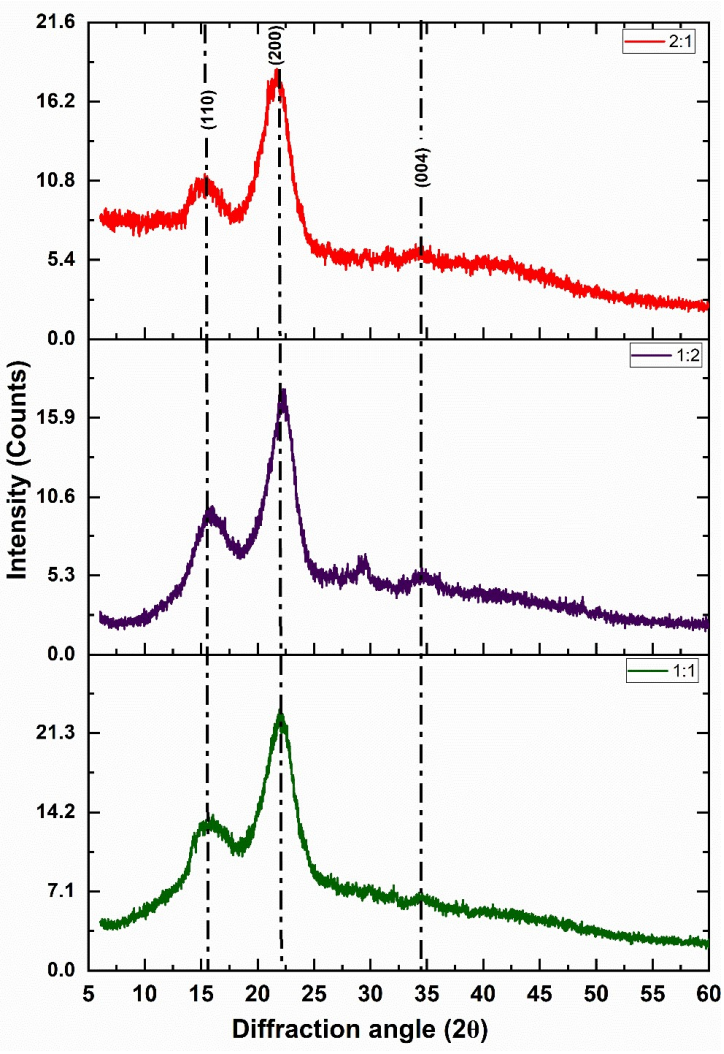


Figure 4.7- XRD analysis of HPCA pretreated rice straw

Table 4.3- Cellulose crystallinity index (*CrI*) of various HPCA pretreated rice straws in different ratio

Different pretreated sample	Intensity at I_{110}	Intensity at I_{200}	*CrI*
1:1	6.8	17.7	61.5
1:2	8.06	17.3	53.4
2:1	10.7	22.7	52.8

4.2.3. Compositional changes after H_2O_2 and HPCA pretreated rice straw

The steam explosion pretreatment coupled with H_2O_2 can break the bond amid the recalcitrant structure of lignin due to an enhance in the degradation of lignin content along with oxidation of lignin. Thus, greater the lignin degradation higher the accessibility of hydrolytic enzymes to access the hemicellulose and cellulose to loosen the recalcitrant structure. In previous literature, the steam explosion was considered to be abrupt an initial explosion and fragmentation of the biomass cell wall structure into finer constituents by improving shear strength, compression, bulk density and mean particle size (Sharma et al., 2015). Higher hemicellulose degradation of up to 71% would occur at 1.5 MPa pressure during steam explosion methods due to partial hydrolysis of hemicellulose along with polymerization of lignin in the biomass, as was reported by Zhang et al., (2022). Thus, the steam explosion was an effective pretreatment as it enhanced the water solubility of rice straw and also enhanced the utilization of polysaccharides for the further process of hydrolysis. There were mainly two stages in the process, the biomass was firstly subjected to extremely saturated steam for a few minutes before being abruptly released, leading to a significant alteration in the composition and structure of the lignocellulosic material. The hemicelluloses are partially hydrolyzed to yield monomeric and oligomeric sugars due to the utilization of excessive pressure steam, owing to the emission of acetic as well as other organic compounds in the reaction environment (Fockink et al., 2018). The high-pressure steam explosion of 2.5 MPa for 1 min led to a higher degree of fragmentation of lignocellulosic biomass and also eliminated the intracellular structure of biomass (Q. Ma et al., 2021). Thus, it was earlier noted that steam explosion impregnated in H_2O_2 resulted in enhancing the glucose concentration of up to 12% and xylose content up to 34% while a 30% decrease in cellobiose yield during the pretreatment process, the presence of H_2O_2 reduces the accumulation of lignocellulosic by-products (Verardi et al., 2018). The maximum mass removal of 66.8% of 0.05% H_2O_2 pretreated rice straw shows the maximum removal of xylan depending on the acidic nature of the solution in the acidic hydrolysis process. The maximum removal was assumed due to electrophilicity of H_2O_2 in acidic circumstances as it acts as a reaction agent or catalyst while in alkaline circumstances, it acts as a nucleophile. Further, work performed by Gustavo & Miranda, (2023) stated that HPCA can supply a significant amount of radical in the form of HO^- and HOO^- for the pretreatment of the biomass. These species can attack the phenolic and non-phenolic units as well as the carbonyl structure of lignin, causing the lignin structure to be oxidized and fragmented. HPCA can react to produce more oxygen radicals with the same amount of acid, which can break more lignin structures into little pieces and result in a high degree of delignification. It was also found that citric acid contains three carbonyl groups that can react further to produce more oxygen radicals for the delignification of biomass. Thus, H_2O_2 has been effective for biomass pretreatment due to its efficiency in removing hemicelluloses and lignin. However, most non-phenolic lignin is unreactive to alkaline H_2O_2 regardless of high temperature.

Table 4.4- Illustration of solid recovery and removal of H_2O_2 pretreated rice straw

Sr.No.	Different pretreated sample	Initial weight (W_1) (gm)	Final weight (W_2) (gm)	Solid recovery (%)	Removal (%)
1.	0.05% H_2O_2	2.0007	1.3295	33.5	66.8
2.	0.1% H_2O_2	2.0015	1.2462	37.69	62.34
3.	0.25% H_2O_2	2.0004	1.2682	36.59	63.41
4.	0.5% H_2O_2	2.0012	1.3192	34.09	65.91
5.	0.75% H_2O_2	2.0010	1.2194	39.06	60.94
6.	1% H_2O_2	2.0020	1.3056	34.7	65.3
7.	1:1	2.0003	1.1201	43.9	56.05
8.	1:2	2.0017	1.1591	37.09	57.91
9.	2:1	2.0015	0.8591	57.07	42.93

4.2.4. Impact of steam explosion pretreatment on H_2O_2 and HPCA impregnated rice straw

Impregnation of rice straw in diluted acids prior to steam explosion has been observed to be efficient for obtaining high sugar yield, which can increase the effectiveness of pretreatment. The steam explosion pretreatment coupled with H_2O_2 can break the bond amid the recalcitrant structure of lignin due to an enhanced in the degradation of lignin content along with oxidation of lignin. Thus, the greater the lignin degradation higher the accessibility of hydrolytic enzymes to access the cellulose and hemicellulose to loosen the recalcitrant structure. In previous literature, the steam explosion was considered to be abrupt an initial explosion and fragmentation of the biomass cell wall structure into finer constituents by improving shear strength, compression, bulk density and mean particle size (Sharma et al., 2015). Higher hemicellulose degradation of up to 71% would occur at 1.5 MPa pressure during steam explosion methods due to partial hydrolysis of hemicellulose along with polymerization of lignin in the biomass, as was reported by Zhang et al., (2022). Thus, SE was an effective pretreatment method, as it enhances the water solubility of rice straw and also enhances the utilization of polysaccharides for the further process of hydrolysis. There were mainly two stages in the process, the biomass was firstly subjected to extremely saturated steam for a few minutes before being abruptly released, leading to a significant alteration in the composition and structure of the lignocellulosic material. The hemicelluloses are partially hydrolyzed to yield monomeric and oligomeric sugars because of the utilization of excessive pressure steam, owing to the emission of acetic as well as other organic compounds in the reaction environment (Fockink et al., 2018). The high-pressure steam explosion of 2.5 MPa for 1 min led to a higher degree of fragmentation of lignocellulosic biomass and also eliminated the intracellular structure of biomass. The current research on the impact of steam explosion on various LCBs is illustrated in Table 4.4. Thus, it was earlier noted that steam explosion impregnated in H_2O_2 resulted in an

increase in 12% glucose and 34% xylose content while a 30% decrease in cellobiose yield during the pretreatment process, the presence of H_2O_2 reduces the accumulation of lignocellulosic by-products (Verardi et al., 2018).

4.2.5. Liquid Hot Water pretreatment impregnated with different acid

Liquid hot water pretreatment was performed on rice straw dissolved in different concentrations of 1M H_2SO_4, HCl, HNO_3, formic acid, acetic acid and oxalic acid. The solid fraction was subjected to FTIR, XRD, TGA, and FESEM analysis.

(a). FTIR analysis

Utilizing FTIR, lignocellulosic biomasses were assessed for changes in chemical composition brought on by pretreatment. FTIR spectra of different pretreatment methods used in the present study are illustrated in Figure 4.8. The absorption peak shows the stretching of different bonds present between cellulose, hemicellulose and lignin. The absorption peak at around 1040 cm^{-1} showed the stretching of C-OH bond equivalent to the alcohol group prominent in cellulose. Similarly, the stretching of C=O at around 1645 cm^{-1} peak showed the alteration in the structure of lignin. The peak at 1645 cm^{-1} got weaker after pre-treating rice straw with different acids that showed pretreatment strategy, demonstrate positive results towards delignification and xylan removal. The absorption peak from 1500-1100 cm^{-1} resembles the stretching of C-O-C ring vibrational group present in the hemicellulosic complex structure prominent in rice straw. Various small peak around 1300 cm^{-1} resembles the deterioration of lignin structure due to alteration in the phenolic group with the stretching of CH_2 and CH bond (Pant et al., 2021). It was proven that no cellulose derivatives were formed when comparing the spectra of pretreated sample in the peak region of 1300-800 cm^{-1} resembling cellulosic content present in the biomass. The change in absorption peak corresponding to different functional groups showed destruction in the lignin structures, revealing more cellulose permeability to enzymatic hydrolysis (Paramasivan et al., 2021). The peak characterized for lignin stretching corresponds to 1554 cm^{-1} to 1280 cm^{-1}, 1645 cm^{-1} attributed to C=C stretching of lignin structure and 1438 cm^{-1} peak stretching of aromatic structure C-C bond.

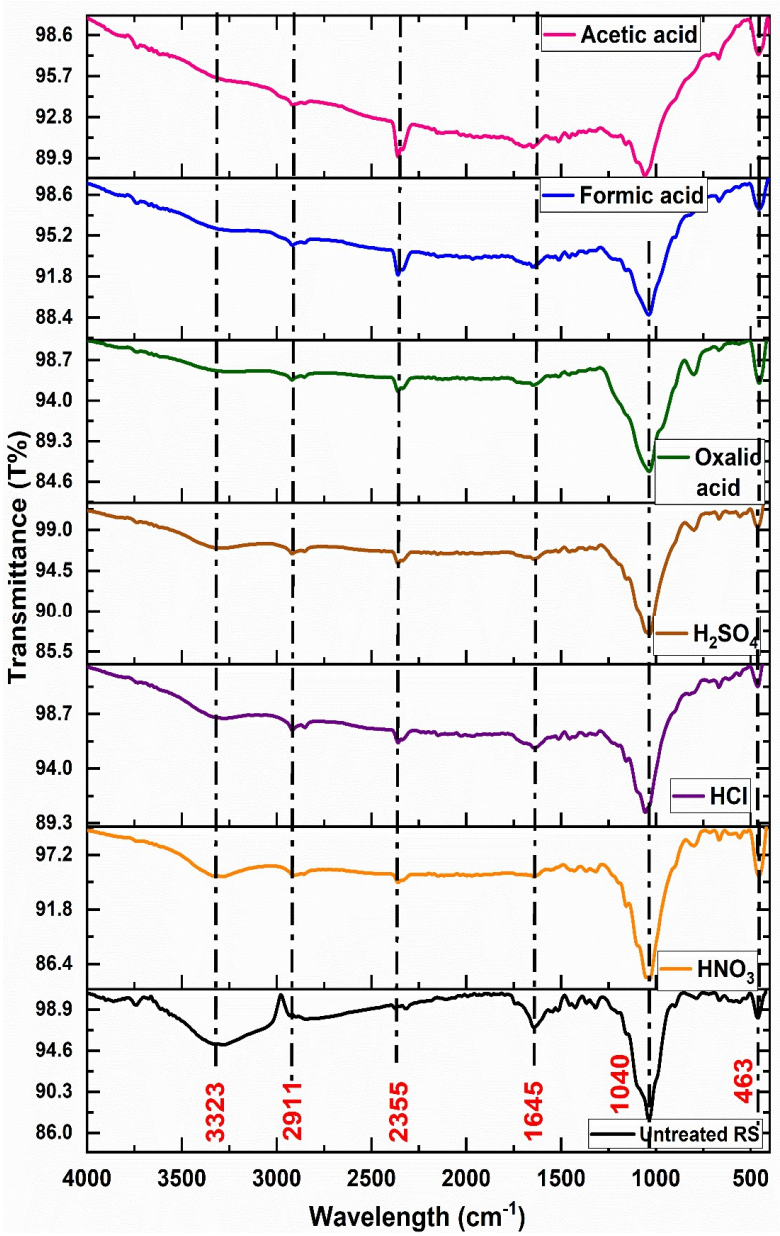


Figure 4.8- FTIR analysis of different acid pretreated Rice straw

(b). TGA analysis

Thermal degradation of both native and pretreated rice straw to determine the degree of cellulose, hemicellulose and lignin degradation was analyzed using TGA with a heating rate of 10°C/min was plotted in Figure 4.9 (A). The maximum weight loss was obtained in B and C-zone with a temperature range of 180-275°C that representing loss of xylan sugar and cellulose. It was earlier reported that hemicellulose, cellulose and lignin had typical peak temperatures of 180-300°C, 300-350°C and 370-550°C respectively (Ebrahimi et al., 2017). However, the wide range of temperatures at which lignin degrades from 100 to 700°C is due to its complex molecular structure, which includes phenolic hydroxyl, larger molecular weights brought on by

intermolecular C-C bonds, and better stability. One of the important factors that influence the thermal stability and behaviour of cellulose nanofibers, in addition to the amount of carboxyl groups present, is the degree of crystallinity. According to several research, cellulose nanofibers having maximum CrI showed greater thermal stability (Ji et al., 2019). In HNO_3 pretreated biomass, a weight loss of 41.7% was observed in A-zone corresponding to hemicellulose degradation while C-zone showed a weight loss of 41.94% with degradation of both cellulose and lignin components. In HCl-pretreated biomass, the A-zone showed less degradation at about 5.42% while the B-zone showed 50.7% weight loss while the C-zone indicated 88.1% weight loss corresponding to lignin degradation. In other pretreated biomass with H_2SO_4, A-zone showed 6.26% of weight loss. Among various acid-pretreated biomass, HNO_3 exhibits maximum weight loss in the A-zone and shows maximum hemicellulose degradation while the C-zone shows maximum degradation of 89.2% from oxalic acid pretreated rice straw.

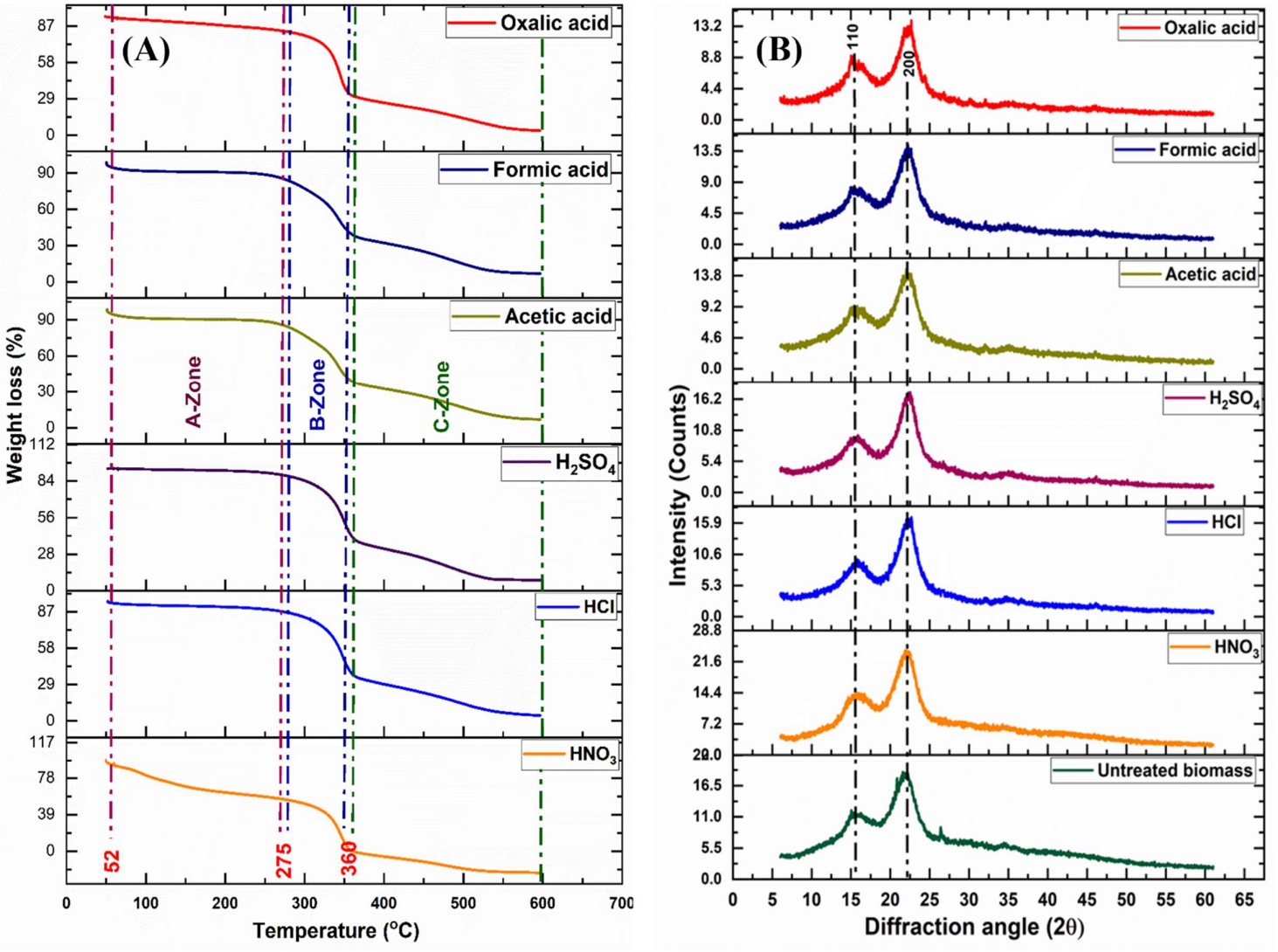


Figure 4.9- (A) TGA analysis of different acid-pretreated rice straws (B) XRD analysis of untreated and different acid-pretreated rice straws

(c). XRD analysis

XRD analysis is well known for cellulose crystallinity determination, as one of the significant elements influencing the rate of hydrolysis of cellulose, which is measured using intensity peaks at 18.5 and 22.5 both before and after pretreatment. It was found that the cellulose peak became sharper, indicating an increase in the glucan contents of the biomass. The X-ray diffractograms and cellulose crystallinity index of native and pretreated rice straw are illustrated in Figure 4.9 (B) and Table 4.5. The diffraction intensity of crystalline region 002 decreased more quickly than that of crystalline region 101, implying that the crystalline region 002 was readily impacted by LHW-acid treatment. The peaks in the crystallization regions (101 and 002) were reduced. However, the substrate compositions have a significant impact on *CrI*. The *CrI* values determine the proportion of crystalline cellulose in lignocellulosic biomass. As a result, the significant rise in *CrI* is obtained by pretreatment with HNO_3 and Oxalic acid i.e., 62.6% and 60.4% respectively. This shows a 21.08% and 18.21% rise in *CrI* from untreated rice straw, which is because of the elimination of amorphous region i.e., hemicellulose and lignin from untreated rice straw. 1M acidic pretreatment with acid for 1 hr might have to change the crystallinity of cellulose. Wu, Zhao, & Liu stated that the crystallinity of cellulose was somewhat reduced when the treatment was extended to 1.5 hrs. This was most likely due to the possibility that the formylation of cellulose might alter the hydrogen bond network, changing crystallinity in the process (Wu et al., 2016).

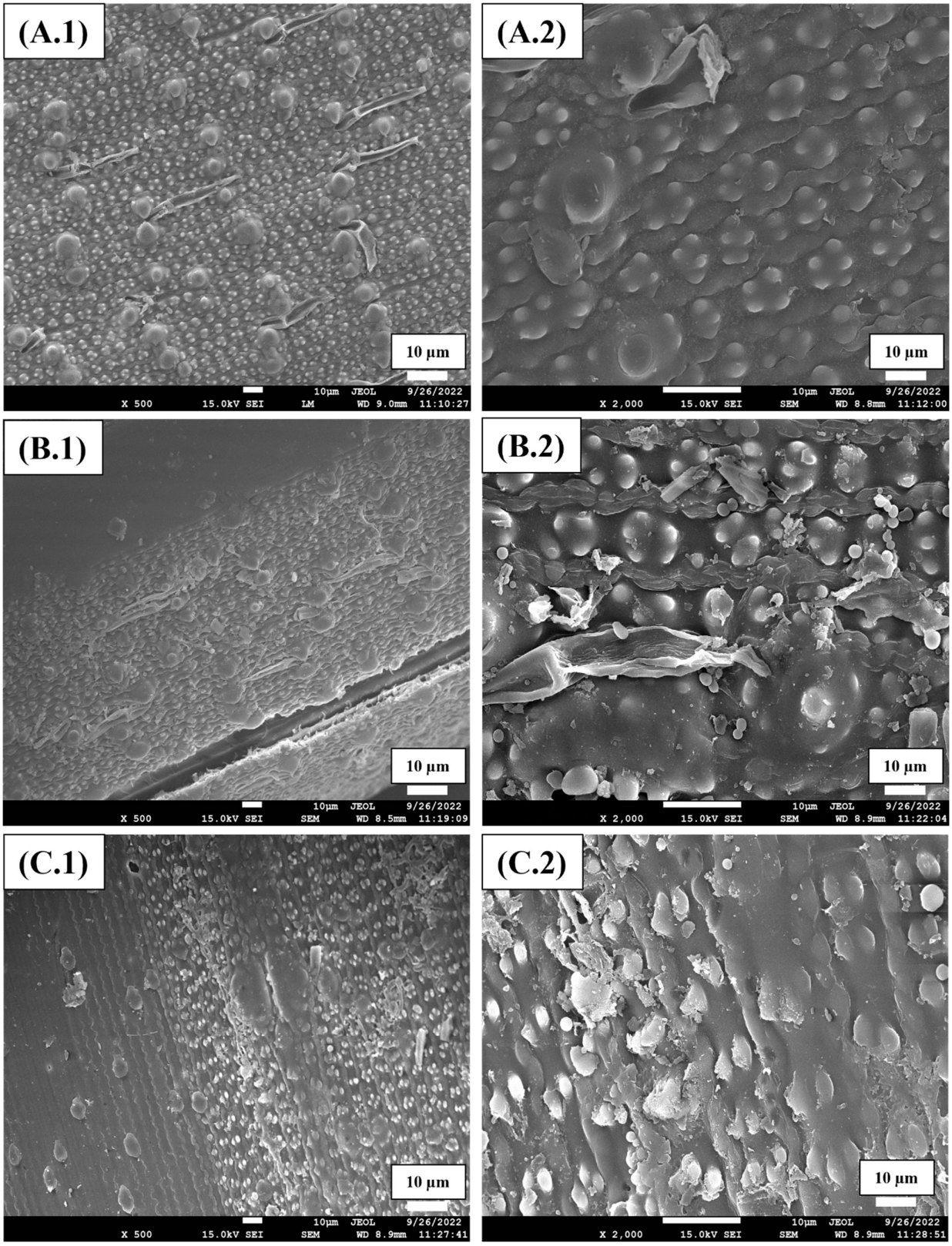
Table 4.5- *CrI* of untreated and different acid-pretreated rice straw

Different Pretreated sample	Intensity at I_{am}	Intensity at I_{002}	*CrI*
Native rice straw	9.4	18.6	49.4
HCl	10.3	23.4	55.9
HNO_3	6.01	16.1	62.6
H_2SO_4	6.3	16.5	61.8
Acetic acid	6.9	13.6	49.2
Oxalic acid	5.3	13.4	60.4
Formic acid	5.2	12.4	58.06

(d). FESEM Analysis

The physical structure of pretreated rice straw was analyzed using FESEM. SEM analysis provided a thorough understanding of the morphological alterations caused by pretreatment. The micrograph of pretreated rice straw with H_2SO_4, HCl, HNO_3, formic acid, oxalic acid and acetic acid, was obtained using SEM analysis and illustrated in Figure 4.10. FESEM analysis of native rice straw revealed a smooth and compact surface, without any fissures or voids on the microstructure. It was found that raw rice straw was not affected by the milling process and was consistent with less surface area accessible for adsorption by

cellulase enzyme. A similar type of morphology was earlier reported by Imman et al., 2015. The pre-treated residue, on the other hand, had highly separated fibrils and deep cavitation, which assists in the effective digestion of biomass. Furthermore, the micrograph of pretreated rice straw residue shows that most hemicelluloses have been removed, leaving just the cellulose portion of the biomass. It shows noticeable surface disturbance that makes the interior structures visible as well as segmented, this structure revealed the loosening of filamentous network among fibres with uneven, rough, nanostructured and fractured surfaces (Imman et al., 2021b). The SEM micrograph depicts the porous biomass of pretreated rice straw with an increase in the surface area that was directly revealed for the enzyme to interact with, hence increasing the efficiency of saccharification. The FESEM micrograph of native rice straw showed a smooth and compact structure, without any fissures or voids on the microstructure. It was found that raw rice straw was not affected by the milling process and was consistent with less surface area accessible for adsorption by cellulase enzyme. A similar type of morphology was earlier reported by Imman et al., (2015). However, physiochemical pretreatment, which influenced the removal of hemicellulose and lignin, altered the microstructures of rice straw. This caused the surface lignin to peel off, making the interior cellulose microfibers more accessible, which is connected with a larger extent of enzymatic digestibility. These modifications in the microstructure of rice straw matched those found in diverse agricultural residues that had undergone various pretreatment techniques (Imman & Laosiripojana, 2017). A similar, micrograph was attributed by (Imman et al., 2015) representing the peeling of lignin surface, improving access to cellulose microfibers in the biomass. While the lignin-hemicellulose complex was removed, the majority of micro-fibrous cellulose structures were highly preserved. The creation of papillae structures and extensive surface peeling effects were developed during the pretreatment process.


(A.1)
10 µm
X 500 15.0kV SEI LM 10µm JEOL WD 9.0mm 9/26/2022 11:10:27
(A.2)
10 µm
X 2,000 15.0kV SEI SEM 10µm JEOL WD 8.8mm 9/26/2022 11:12:00
(B.1)
10 µm
X 500 15.0kV SEI SEM 10µm JEOL WD 8.5mm 9/26/2022 11:19:09
(B.2)
10 µm
X 2,000 15.0kV SEI SEM 10µm JEOL WD 8.9mm 9/26/2022 11:22:04
(C.1)
10 µm
X 500 15.0kV SEI SEM 10µm JEOL WD 8.9mm 9/26/2022 11:27:41
(C.2)
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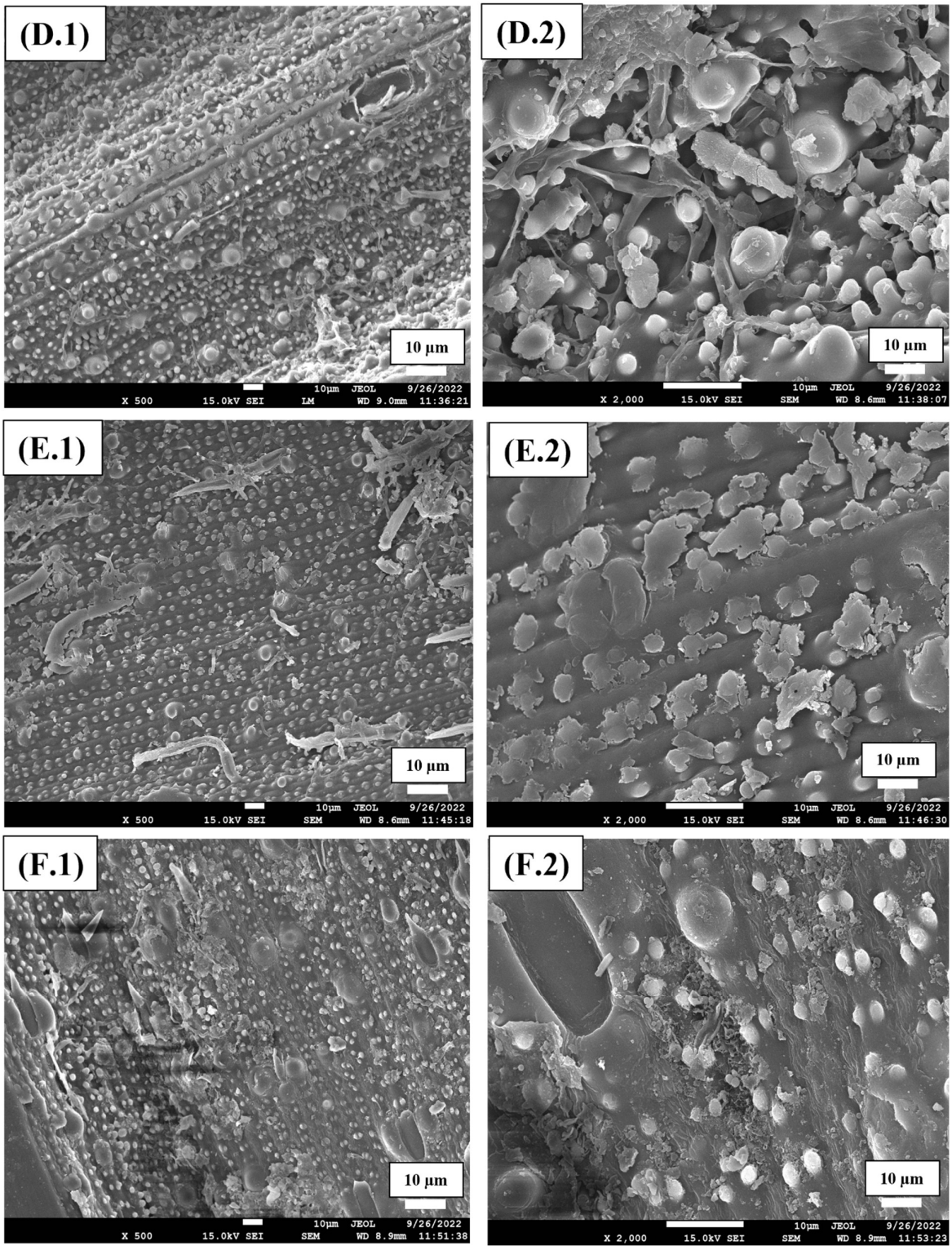

Figure 4.10- FESEM micrograph (A) HNO_3 pretreated rice straw (B) HCl pretreated rice straw (C) H_2SO_4 pretreated rice straw (D) Acetic acid pretreated rice straw (E) Formic acid pretreated rice straw (F) Oxalic acid pretreated rice straw

4.2.6. Compositional changes after pretreatment with different acids on rice straw

The goal of the pretreatment in this research is to open the lignin complex and disturb its crystalline structure to enable cellulose and hemicellulose easily accessible for more effective enzymatic hydrolysis. More precisely, the best pretreatment is intended to destroy the most lignin while preserving the most cellulose and hemicellulose content in the biomass. Solid recovery and removal of non-fermentable parts were analyzed in different pretreated samples and illustrated in Table 4.6. During the study, maximum removal was attributed to HNO_3 and oxalic acid among various strong and weak acids utilized for the pretreatment process. Raw rice straw after being treated with 1 M HNO_3 at 120°C for 1 hr, resulted in 55.15% removal with a solid recovery of 34.25%. HNO_3 is a strong acid catalyst that helps rice straw release its xylose and facilitate effective enzymatic hydrolysis and further neutralization of HNO_3 leads to the formation of nitrate that is used as a nitrogen source during the fermentation process (Abdul Manaf et al., 2022). The solid recovery of different pretreated samples ranges from 44.8% to 83.7%, indicating solubilization of biomass by enhancing the level of solubilization for the hemicellulosic fraction with maximum pretreatment severity (Kim et al., 2015). This process demonstrated that the presence of sulfuric components in the form of H_2S, thiols, and methanol resulting from H_2SO_4 pretreatment was prevented by the use of HNO_3 (He et al., 2023). According to the Mayer bond level analysis and electron localization function, H_2SO_4 mostly contributes to the reaction through the lone pair electrons in the oxygen atom and the hydrogen protons in the hydroxyl group. H_2SO_4 is a potent catalyst because of its high proton transfer capacity. Furthermore, because the S-O bond present in H_2SO_4 is longer than the OH bond present in H_2O, it was found that H_2SO_4 breaks spatial barriers, which significantly lowers the synthesis of anhydrous-disaccharides with α-linkage (He et al., 2023). Acids used in the pretreatment process protonate the hydroxyl group found in the C5 sugars, which hydrolyses the hemicellulose structure. As a result, the nitric acid pretreatment process has a wide range of potential applications when used with distinct kinds of cellulosic biomass (Skiba et al., 2022). It had been earlier reported by Zhao and co-workers (2014) that fast protonation of the glycosidic oxygen with the production of a conjugated acid initiates the acid-catalyzed breaking of the glycosidic link between components of lignocellulosic biomass. It had been earlier stated that the conversion of hemicellulose to xylose might range from 24.8% to 89.9% depending on the applied pretreatment process. Because the process of acid pretreatment is mainly to dissolve hemicellulose and promote enzymatic hydrolysis, the quantity of xylose in the liquid fraction can be utilized as a measure of pretreatment efficiency (B. Liu et al., 2022).

Table 4.6- Solid recovery (%) and removal (%) of LCB of different acid-treated rice straw

Sr.No.	Different pretreated sample	Initial weight (W_1) (gm)	Final weight (W_2) (gm)	Solid recovery (%)	Removal (%)
1.	HCl	2	1.564	78.2	21.8

2.	HNO_3	2	1.315	34.25	65.75
3.	H_2SO_4	2	1.392	61.7	38.3
4.	Acetic acid	2	1.210	60.5	39.5
5.	Oxalic acid	2	1.087	49.35	50.65
6.	Formic acid	2	1.674	83.7	16.3

3.3. Effect of simultaneous pretreatment and saccharification on LHW at 120°C

Simultaneous pretreatment and saccharification were performed using high temperature and pressure along with acidic hydrolysis using different weak and strong acids using 1M H_2SO_4, HNO_3, HCl, Acetic acid, formic acid and Oxalic acid at different time intervals for 1 hr. It was evaluated that enhancing pretreatment time over 60 minutes led to more loss in glucan content (Ebrahimi et al., 2017). It was found that 120°C of pretreatment time led to maximum sugar yield which further decreased with the increase in the temperature. The total reducing sugar 202.53 mg/l, 312.74 mg/l, 252.27 mg/l,152.15 mg/l, 270.52 mg/l, and 175.36 mg/l were obtained from HCl, HNO_3, H_2SO_4, acetic acid, oxalic acid and formic acid pretreated rice straw at 120°C temperature. This is because galactose, glucose, and 5-HMF levels drop at a temperature higher than 130°C as well as galactose's breakdown into various by-product substances. Levulinic acid and 5-HMF are the two primary by-products of sugar breakdown, while 5-HMF is a by-product of hexoses like glucose and fructose (Meinita et al., 2015). Levulinic acid and 5-HMF that are produced during acidic hydrolysis at elevated temperatures may have a negative effect on yeast growth as well as ethanol production. It was found that maximum sugar yield was obtained from 120°C pretreated rice straw. However, to attain a maximum glucan conversion rate in the LHW pretreatment, it is not practical to use a strategy that entails raising the pretreatment temperature. The majority of the xylan amount can be removed from LHW, however, a significant amount of lignin remained present in the liquid fraction (Lu, Liu, Song, et al., 2020b). It is widely known that liquid hot water behaves like acid when heated to high degrees, resulting in the generation of oxidation products and by-products in the form of Organic acids (such as formic, acetic, and lactic acids) that function as catalysts in the breakdown of hemicellulose (Lyu et al., 2018). It was found that during acidic hydrolysis of polysaccharides, not only hydrolysis of polysaccharides to monosaccharides but also the subsequent degradation process of monosaccharides into Levulinic acid and 5HMF has taken place. The fact that HCl is a weaker acid than H_2SO_4 must be the main cause of the decreased monosacchari de breakdown in biomass. In most cases, carbonyl group protonation is the first step in the breakdown of monosaccharides in acidic conditions. HCl can be used in acid catalyzed processes, while in others stronger conjugate base is needed to regenerate the catalyst and extract the proton. Each monosaccharide degraded si gnificantly more slowly in HCl than in H_2SO_4 because HCL is weaker than H_2SO_4 (Imman et al., 2021b). During formic acid treatment, the formyl group might increase the diameter of the cellulosic chain, which minimizes the chance of entering cellulase through the catalytic domain. Thus, the ability of cellulase to

recognize specific cellulose substrates is interfered, when hydroxyl group in cellulose is replaced with a formyl group. It could prevent cellulose from forming productive hydrogen bonds (binding) with cellulases' catalytic domain (Dong et al., 2017). Figure 4.11 illustrate the solid fraction obtained after different acid treatment at varied temperature ranges from 80-140°C.

The delignification of the pretreated rice straw represents the lignin removal from the biomass. It was evaluated that a maximum delignification of 74.81% was obtained from HNO_3 pretreated rice straw, 1M H_2SO_4 pretreated rice straw resulted in 60.90% of lignin removal. The earlier reported work by Chin et al., 2019 investigated maximum delignification of 53.2% when biomass was pretreated with 2% H_2SO_4, this shows that H_2SO_4 was effective in reducing the ash content up to 70%. In this work, HNO_3 pretreated rice straw has maximum delignification representing a strong catalyst for the degradation of lignin content from the biomass.

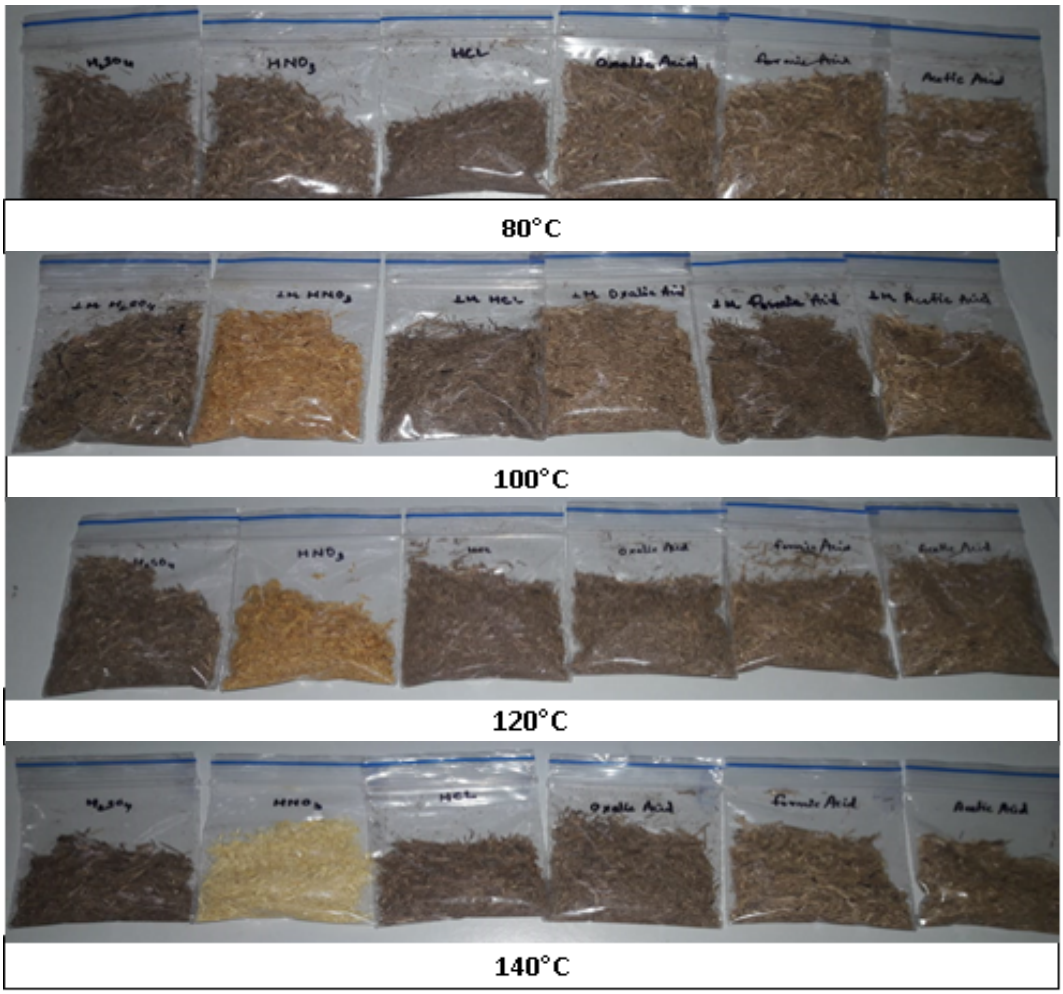

Figure 4.11- Solid fraction of different acid-pretreated rice straw at varied temperatures

4.2.8. Liquid hot water treatment with different concentrations of Oxalic acid

After evaluating the result, the aforementioned pretreatment process that was performed on rice straw, liquid hot water treatment with impregnation of different concentrations of oxalic acid (0.5 M, 0.75 M, 1 M, 1.5 M) was performed. It was evaluated that oxalic acid is considered a dicarboxylic acid that showed strong chelation activity due to which dissolution of excessive metal ions from the outer layer reactor surface was done. The results after pretreatment were evaluated by FTIR, XRD, TGA and FESEM analysis.

(a). FTIR analysis

The FTIR analysis of oxalic acid pretreated rice straw is illustrated in Figure 4.12 (A) representing the highest variation in the bond stretching in pretreated rice straw that leads to modification in the composition of chemicals as well as structural alteration. The reference for FTIR analysis was suggested by Dessie et al., 2022; Kundu & Lee, 2015; Tantayotai et al., 2022. The transmittance peak for untreated and pretreated rice straw was at 2498, 1646, 1043 and 447 cm^{-1} wavelength. The peak at 2498 cm^{-1} wavelength was assigned to C-H stretching in the cellulosic structure of rice straw. This peak is more prominent in pretreated rice straws and is not available in untreated rice straws. The peak corresponding to 1646 cm^{-1} was attributed to C=O stretching of the acetyl group in hemicellulose and lignin. As compared to untreated rice straw, the peak became weaker on pre-treating the rice straw, indicating that certain pretreatment procedures were effective in removing lignin and xylan. The peak is more prominent in 0.5 M Oxalic acid pretreated rice straw due to maximum delignification in the pretreated rice straw. The peak at 1043 cm^{-1} represents the stretching of C-O group among cellulose and hemicellulose structures in LCB. The figure shows the transmittance peak at this wavelength was lowered by increasing the concentration of oxalic acid and change in the peak intensity which represents the alteration in cellulose and hemicellulosic structure after pretreatment. Peaks of physiochemically treated rice straw in the fingerprint area (1800-500 cm^{-1}) differed significantly from other samples in form, band intensity, and % transmittance. The peaks at 900 and 1098 cm^{-1} show amorphous and crystalline cellulose C-O bond vibration. The ability of oxalic acid to degrade cellulose as well as hemicellulose can illustrate its intriguing multifaceted applications in the domains of lignocellulosic biomass pretreatment. The significant C-H bond stretching in methyl/methylene groups of cellulose at 2498 cm^{-1} wavelength was observed in 0.5 M oxalic acid pretreatment, earlier reported by Lim et al., (2013) from oxalic acid pretreated biomass. Condensation reactions and breaking of lignin aliphatic side chains are attributed to position reductions or shifts. The findings provide strong evidence that lignin breakdown capacity differs depending on acid catalysts. The ability of oxalic acid to degrade cellulose as well as hemicellulose can illustrate its exciting multifunctional applications in lignocellulosic biomass pretreatment (Dessie et al., 2022).

(b). TGA analysis

Thermal gravimetric analysis of oxalic acid pretreatment is illustrated below in Figure 4.12 (B). TGA graph is represented in three zones namely A-zone, B-zone and C-zone which represent moisture degradation, cellulose and hemicellulose degradation and further lignin degradation. The untreated rice straw represents 7.8% weight loss in A-zone, the maximum weight loss occurs from 350-400°C temperature with 52.185% degradation in B-zone with 0.5 M oxalic acid pretreated rice straw. This region showed the degradation of lignin in the pretreated rice straw with 44.73% of weight loss in untreated rice straw. This showed a rise in weight loss of up to 29.6% due to pretreatment of biomass. A similar result of weight loss pattern was earlier demonstrated by Da Silva et al., (2019), an investigation during regulated and progressive heating occurring due to heterogenous content from organic molecules with varying structural and chemical characterization, that the LCB exhibits. As a result, thermogravimetric analysis can provide valid assumptions about these compositions. The residual mass loss in thermogravimetric analysis (TGA) over 400℃ is mostly the result of residual lignin, being the most stable organic substance at that temperature. In addition to lignin, inorganic elements may contribute to residual mass at 400℃, which might explain the increased residual mass due to the pretreatment with oxalic acid leading to a rise in lignin content from pretreated rice straw. Earlier reported work by Del Castillo-Llamosas et al., (2021) shows an intense weight loss of 54.42% when the avocado peel waste was hydrothermally treated with the degradation of cellulose, hemicellulose and lignin.

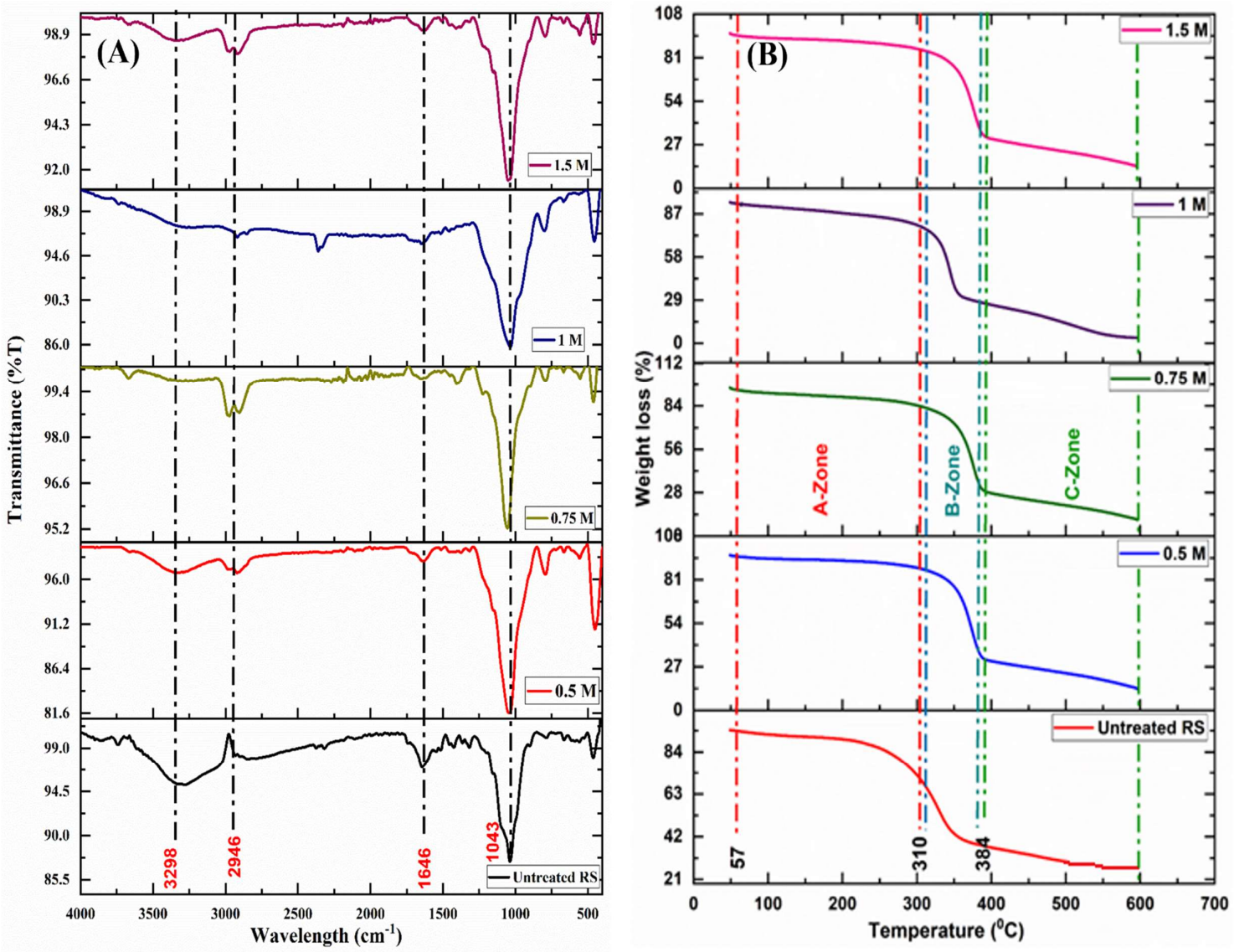

Figure 4.12 – (A) FTIR analysis of untreated and pretreated rice straw with different concentrations of oxalic acid **(B)** TGA analysis of untreated and pretreated rice straw with different concentrations of oxalic acid

(c). XRD analysis

The XRD analysis of raw and pretreated rice straw using different concentrations of oxalic acid is illustrated in Figure 4.13. The diffraction peak at 2θ is 15.1 for 110 plane and 22.4 for 200 plane. The peak intensity at 15.1 and 22.4 was used to calculate *CrI* value and was illustrated in table 4.7. A similar peak intensity was reported earlier by Deng et al., (2016) for oxalic acid-pretreated corncob biomass. The data showed that CrI calculated using the intensity of amorphous and crystalline region at 15.1 and 22.4 demonstrate that *CrI* increases from 46.4 of untreated rice straw to 60.9 of 0.5 M oxalic acid pretreated rice straw. There is an increase in the *CrI* value of pretreated rice straw from 55.1-60.9% as compared to untreated rice straw which is only 46.4%. This increase in CrI value illustrated the reduction in the amorphous regions of pretreated rice straw due to the degradation of hemicellulosic content from the

biomass. Similar results were obtained by Kundu & Lee (2016) in which untreated eucalyptus biomass showed a 49.79% *CrI* value while pre-treating biomass with 100 mM oxalic acid enhanced the *CrI* value to 56.93%. After pre-treating rice straw with different concentrations of oxalic acid, there was an increase in the *CrI* value up to 18.8% and on increasing the concentration of oxalic acid, the rate of increase in *CrI* value occurs to 10.3%. This increase in *CrI* value indicates hemicellulose was released from the cell wall of rice straw biomass, degraded to the soluble part in the obtained hydrolysate, and the part of cellulose in the amorphous region of biomass degenerated to glucose. It was found that the maximum hemicellulosic portion was removed during the pretreatment process which increased the crystallinity of rice straw. Moreover, the glycosidic bond that played an important role during the binding of recalcitrant structure of lignocellulosic biomass was destructed during pretreatment increasing *CrI* that represents the removal of amorphous components in biomass such as hemicellulose and lignin.

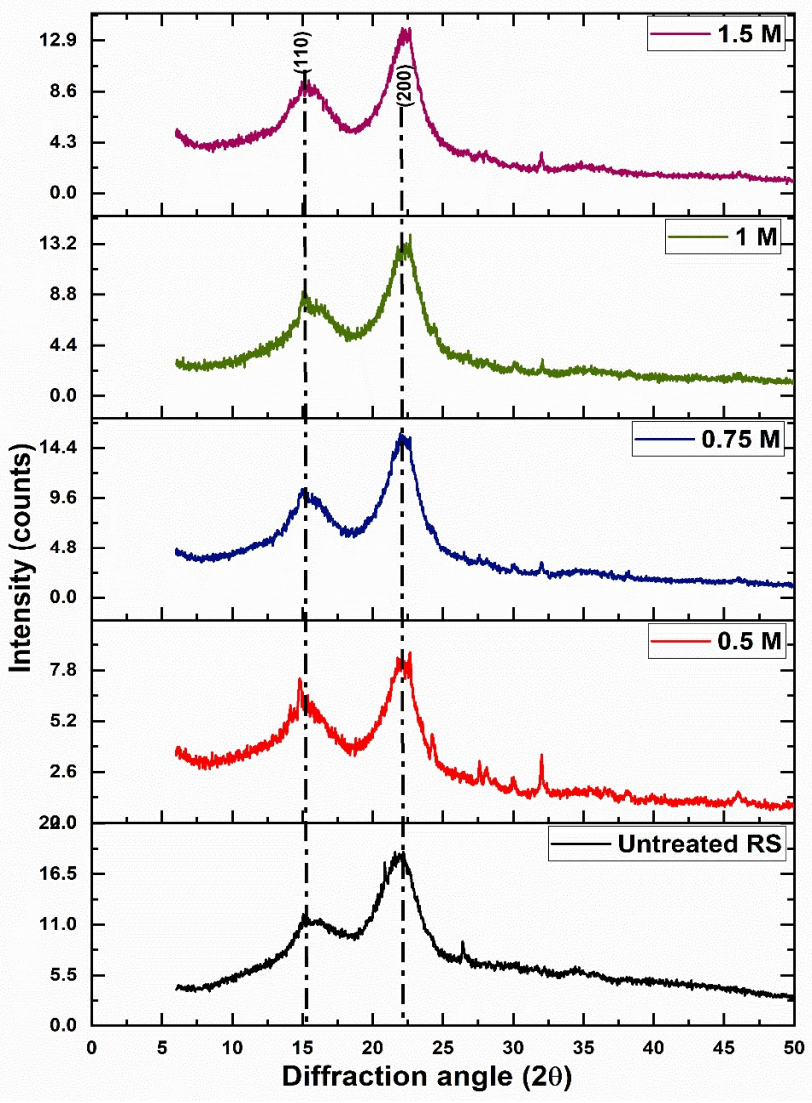

Figure 4.13- XRD analysis of Untreated and pretreated rice straw with different concentrations of Oxalic acid

Table 4.7 – CrI of untreated and pretreated rice straw with different concentrations of oxalic acid

Different Pretreated sample	**Intensity at I_{am}**	**Intensity at I_{002}**	***CrI***
Native RS	9.4	18.6	49.4
0.5 M	5.2	13.3	60.9
0.75 M	6.2	15.3	59.4
1 M	5.3	13.4	60.4
1.5 M	3.6	8.03	55.1

(d). FESEM analysis

The FESEM micrographs of rice straw pretreated with different concentration of oxalic acid is illustrated in Figure 4.14. It was demonstrated that raw rice straw showed regular, compact and ordered structure while oxalic acid pretreated rice straw showed irregularities in the outer surface of biomass. The oxalic acid pretreated rice straw has a rough surface with cracks and tiny fragments. The surface of the samples had a lot of micropores after steam explosion treatment. As the reaction time increased, the surface of the treated rice straw was gradually damaged. The removal of hemicelluloses from the cell wall of rice straw by steam explosion treatment was most likely responsible for the alteration in morphological features. A similar result of oxalic acid-assisted ball milling pretreatment on corn cob was obtained by Deng et al., (2016).

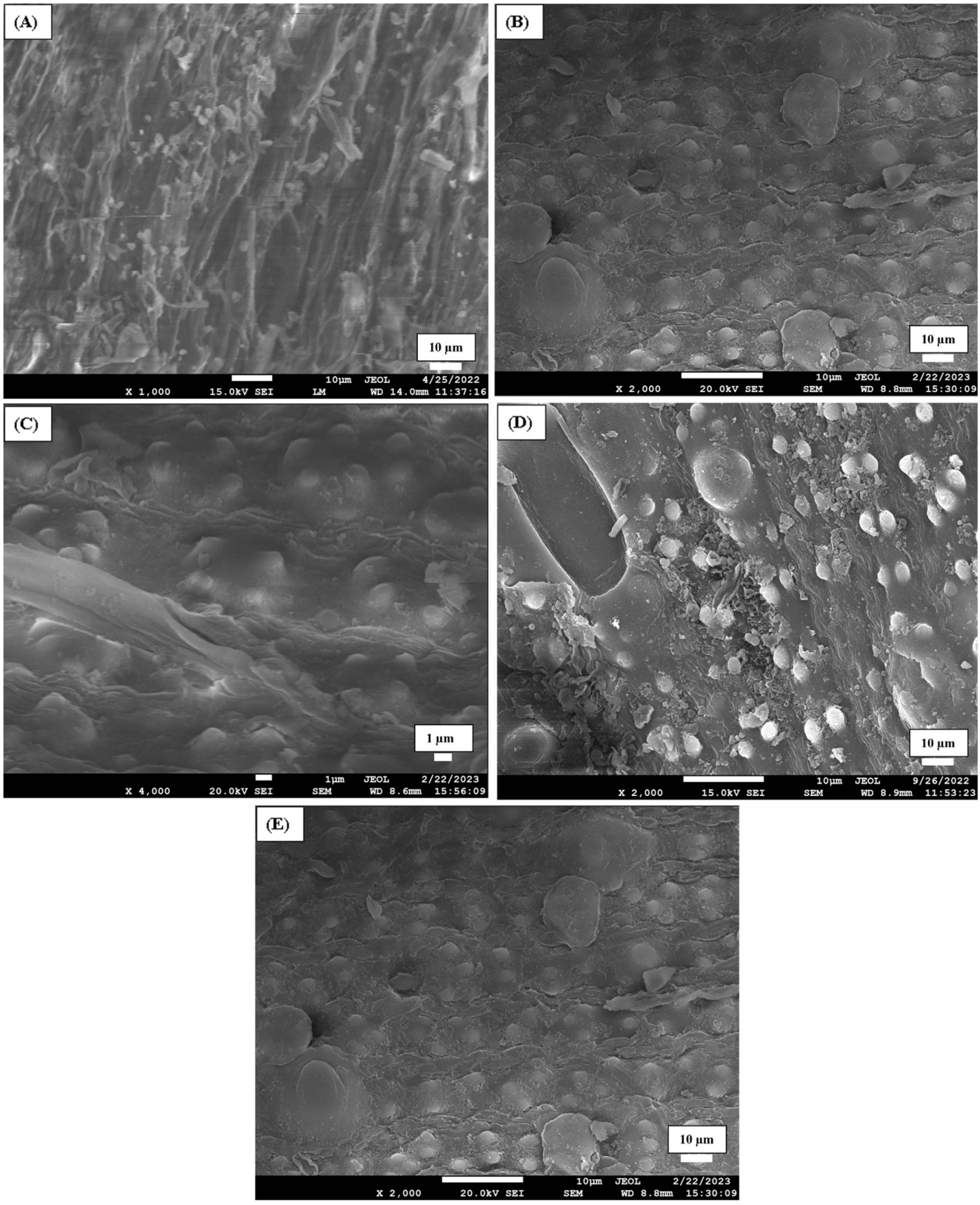

Figure 4.14- FESEM micrographs of pretreated rice straw using different concentrations of oxalic acid (A) Untreated rice straw (B) 0.5 M (C) 0.75 M (D) 1 M (E) 1.5 M

4.2.9. Composition of different oxalic acid pretreated rice straw

Modification occurs in the compositional analysis of LCB with changes after pretreatment during solid recovery and removal, caused in a portion along with specific saccharification and breakdown of certain components in the biomass. This solid removal was mostly due to the removal of xylan present in the rice straw (Ebrahimi et al., 2017). The amount of material that remains after pretreatment depending on the initial weight followed by oven drying is biomass recovery (%). It depends upon the degradation rate of cellulose, hemicellulose and lignin. From Table 4.8 below, a maximum recovery of 52.73% was observed in the maximum concentration of oxalic acid due to the severe effect of oxalic acid treatment on biomass. The degradation of biomass is consistent with the rise in the concentration of oxalic acid. The breakdown of hemicelluloses during pretreatment led to a rise in the amount of glucan in the pretreated rice straw. The high concentration of oxalic acid caused glucan and lignin to partially degrade due to the breakdown of hemicellulosic structure, which indicates the rise in percentages of inhibitors in the hydrolysate. Further, the maximum removal of 52.96% was observed in 0.05 M oxalic acid pretreated rice straw, this is due to the removal of xylan from the pretreated rice straw that occurs due to a process related to acid hydrolysis and acidity of the obtained hydrolysate.

Table 4.8- Solid recovery and biomass removal in oxalic acid pretreated rice straw

Sr. No.	Different pretreated sample	Initial weight (W_1) (gm)	Final weight (W_2) (gm)	Solid recovery (%)	Removal (%)
1.	0.5 M Oxalic acid	2.0002	1.0592	47.04	52.96
2.	0.75 M Oxalic acid	2.0005	0.9825	50.8	49.2
3.	1 M Oxalic acid	2.0002	0.9513	52.45	47.55
4.	1.5 M Oxalic acid	2.0007	0.9487	52.73	47.26

4.2.10. Impact of different concentrations of oxalic acid on pretreated rice straw

From the above analysis of the morphological and structural changes on the untreated and pretreated rice straw with different concentrations of oxalic acid, oxalic acid was an alternative organic acid, that is less corrosive than H_2SO_4 and improves the ability to regulate the biodegradability of material. Additionally, it is made from sustainable resources and bio-based components. Oxalic acid is considered a dicarboxylic acid. Oxalic acid showed strong chelation activity due to which the dissolution of excessive metal ions from the outer layer reactor surface occurs. Weak dicarboxylic acids have also gained popularity as a promoter due to decreased toxicity towards particular yeast strains during fermentation process and in compliance with a further breakdown of the solubilized contents through fermentation or catalytic activities (Imman et al., 2014). According to Ibrahim et al., (2020), both hemicellulose and lignin are interlinked by ether bonds that

are disrupted when acid is utilized as a catalyst during the pretreatment process. The increased hemicellulose removal during prehydrolysis leads to higher lignin recovery during LHW treatment. It is possible due to the repolymerization of lignin with the formation of carbonium ion intermediates, which promotes the synthesis of new C-C bonds in the form of β-β, C_1-β and C_5-β. As a result, the extraction of lignin is disrupted during SPS process. Thus, due to the corrosiveness of another strong acid, oxalic acid can be considered as best acid catalyst, leading towards enhanced glucose recovery from hydrolysis of pretreated solid fraction contrary to least solubilization as occurs in the case of alkali treatment. Oxalic acid shows appealing chemical and practical properties in the form of regulated progressive acidity, biodegradability, easy handling and storage with minimal corrosive behavior (A. Deng et al., 2016).

4.3. Standard curve

4.3.1. Standard curve of total reducing sugar

The standard curve (Figure 4.15.1) of reducing sugar is obtained using different concentrations of glucose by DNS reagent test and the absorbance was measured at 540 nm wavelengths. The linear regression was obtained by plotting the reducing sugar concentration against obtained absorbance elucidating Y=0.0019X-0.0017 with R^2=0.9746. Further, the quantification of produced total reducing sugar after acidic and enzymatic hydrolysis of different pretreated rice straws was performed.

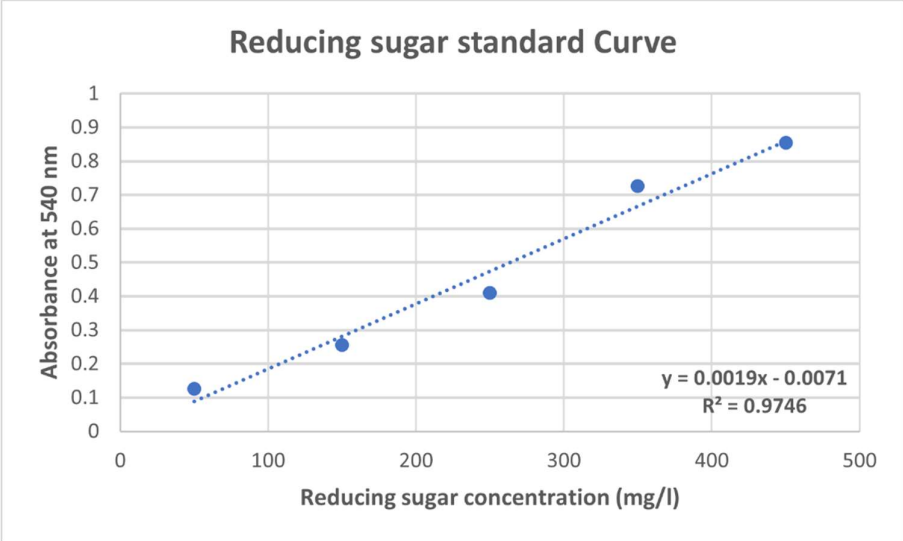

Figure 4.15.1- Standard curve of total reducing sugar (TRS)

4.3.2. Standard curve of hexose sugar

The standard curve (Figure 4.15.2) of hexose sugar (glucose) produced after enzymatic hydrolysis is quantified using the Anthrone reagent test and absorbance was measured at 620 nm wavelength. The standard curve was drawn with a concentration of glucose (µg/ml) on X-axis against absorbance on Y-axis.

The linear regression was obtained elucidating Y=0.0009X-0.0224 with R^2=0.9963. Further, the quantification of produced glucose was done after enzymatic hydrolysis from the obtained standard curve.

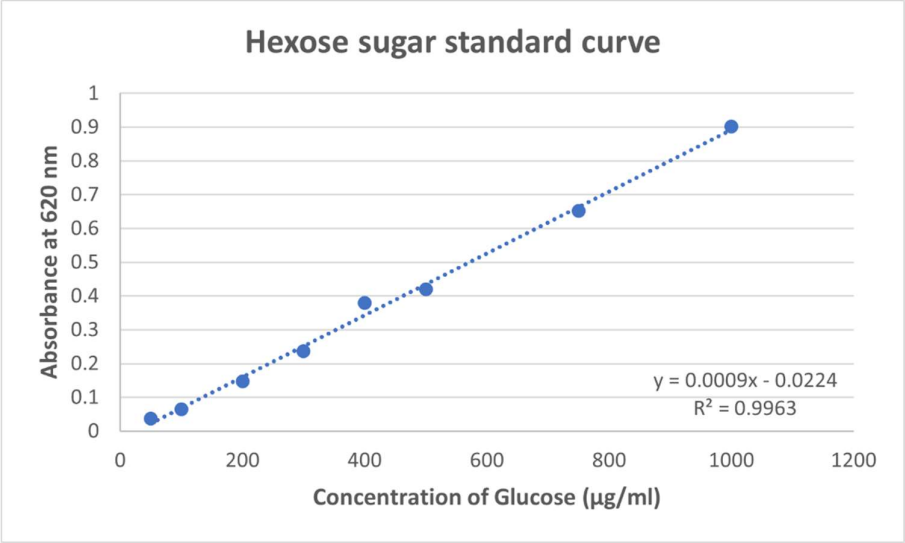

Figure 4.15.2- Standard curve of hexose sugar using Anthrone reagent test

4.3.3. Standard curve of pentose sugar

The standard curve (Figure 4.15.3) of xylose is obtained using the orcinol reagent test and was measured at 671 nm wavelengths, taking different concentrations of xylose on X-axis against absorbance on Y-axis. The linear regression equation elucidating Y=0.006X+0.1669, R^2=0.9505 was obtained to quantify the xylose concentration of the unknown sample.

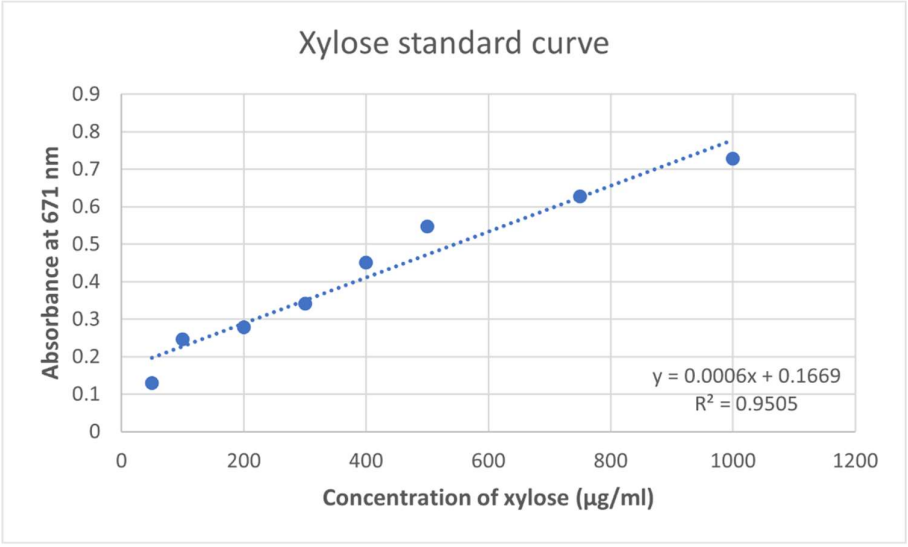

Figure 4.15.3- Standard curve of pentose sugar using orcinol test

4.3.4. Standard curve of Ethanol

The standard curve (Figure 4.15.4) of ethanol was drawn using different concentrations of ethanol against absorbance at 590 nm wavelength. It was obtained using the potassium dichromate test and linear regression was obtained elucidating Y=3.633X+0.0726 with R^2=0.9773. The following trendline equation was used to quantify the obtained bioethanol after the co-fermentation process.

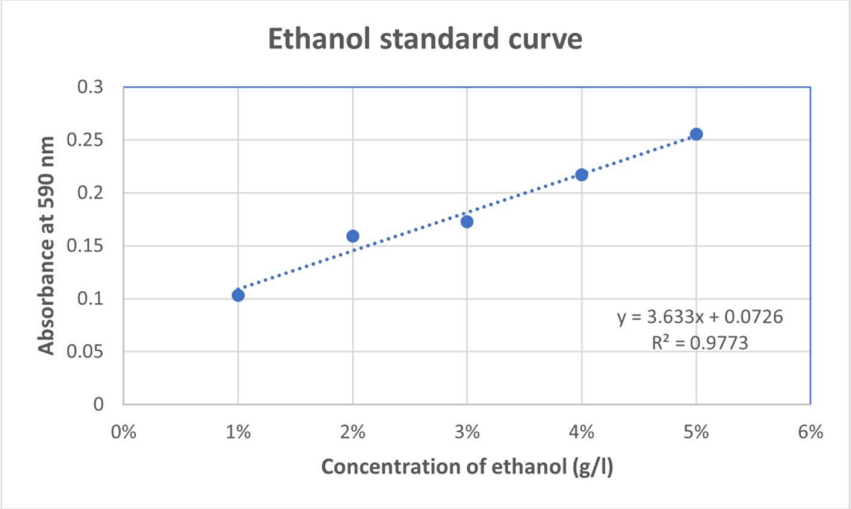

Figure 4.15.4- Standard curve of ethanol using the Potassium dichromate method

4.4. Saccharification process on pretreated rice straw

The enzymatic hydrolysis was performed on the pretreated rice straw following the protocol mentioned in the section (3.4.2.2) and the results obtained during the process are discussed below.

4.4.1. Acidic hydrolysis during SPS method of H_2O_2 pretreated rice straw

The acidic hydrolysis during SPS method resulted in xylose recovery from the liquid hydrolysate. From the present study, it was evaluated that maximum xylose removal was obtained from 0.05% pretreated rice straw in comparison with untreated rice straw. It was observed that the xylose concentration in 0.05% H_2O_2 pretreated rice straw was 380.02 µg/ml, there was a loss in the xylose concentration of up to 78.18% with 21.82% of xylan content while HPCA pretreated rice straw had 347.35 µg/ml of xylose concentration with 46.11% loss in the xylose concentration from the untreated rice straw. Similarly, in the case of reducing sugar obtained from acidic hydrolysate, it was observed that with a decrease in the concentration of H_2O_2, the obtained hydrolysate got turbid which confirms the presence of reducing sugar in the 0.05% (v/v) H_2O_2 pretreated sample. The highly reactive radical in the form of hydroxide and superoxide anion would delignify the lignocellulosic biomass by oxidation and degradation of the rice straw. It was observed that at 0.05% H_2O_2, 359.60 ± 0.012 mg/l of reducing sugar was quantitatively analyzed, and on increasing the

concentration of H_2O_2, the presence of reducing sugar gets reduced to 125.825 ± 0.024 mg/l from the obtained hydrolysate. Similarly, HPCA pretreatment in the ratio 1:1 yielded 361.12 ± 0.015 mg/l of reducing sugar. This shows the increment in sugar yield of up to 19.4% with the utilization of citric acid along with H_2O_2 impregnation in a 1:1 ratio. While 1:2 HPCA pretreated rice straw resulted in 367.19 ± 0.014 mg/l of reducing sugar. Furthermore, the increased proportion of monomeric sugar in the pretreated analytes indicates hemicellulose would easily dissolve at 121°C temperature. It was worth mentioning that an increased concentration of xylose recovery in the hydrolysate typically resulted in the maximum formation of inhibitors. While a maximum glucose concentration in the hydrolysate resulted in a lesser formation of Hydroxymethyl furfural (HMF). This might be because xylan was more unstable and unpredictable for saccharification than glucan. Earlier studies had reported similar results for the pattern of degradation of product formed during the process. A previous study conducted by Rabelo et al., (2011) found that the hydrolysate derived from sugarcane bagasse treated with H_2O_2 contained a lower level of xylose compared to the hydrolysate from bagasse pretreated with lime. The pretreatment with lime minimizes hemicellulose removal and the presence of xylose in the hydrolysate was attributed to the hydrolysis of the remaining hemicellulose in the bagasse post-treatment. This implies that enzymes were expected to exhibit high substrate specificities, and the cellulolytic complex was hydrolyzed hemicellulose. The quantification of reducing sugar and xylose is illustrated in Figures 4.16.1 and 4.16.2 respectively.

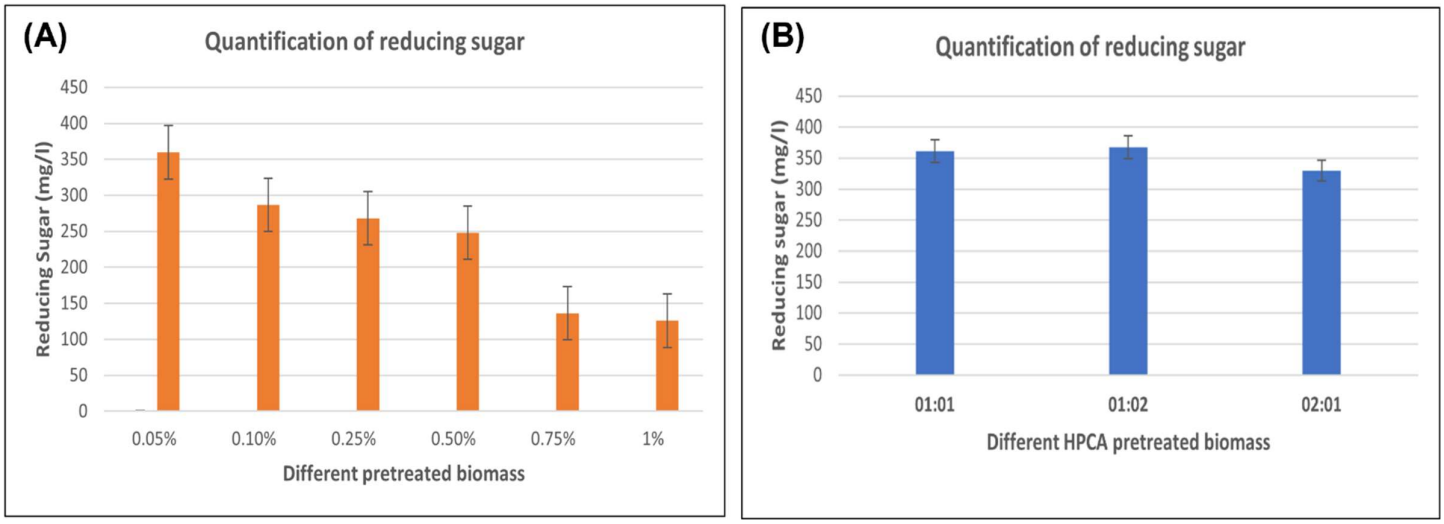

Figure 4.16.1- Quantification of total reducing sugar produced after SPS method (A) Different H_2O_2 pretreated biomass (B) Different HPCA pretreated biomass. The data is presented as an average of triplicates absorbance obtained for quantification of reducing sugar elucidating R^2=0.9746, Y=0.0019X-0.0071 with error bars showing S.D. ($p<0.05$)

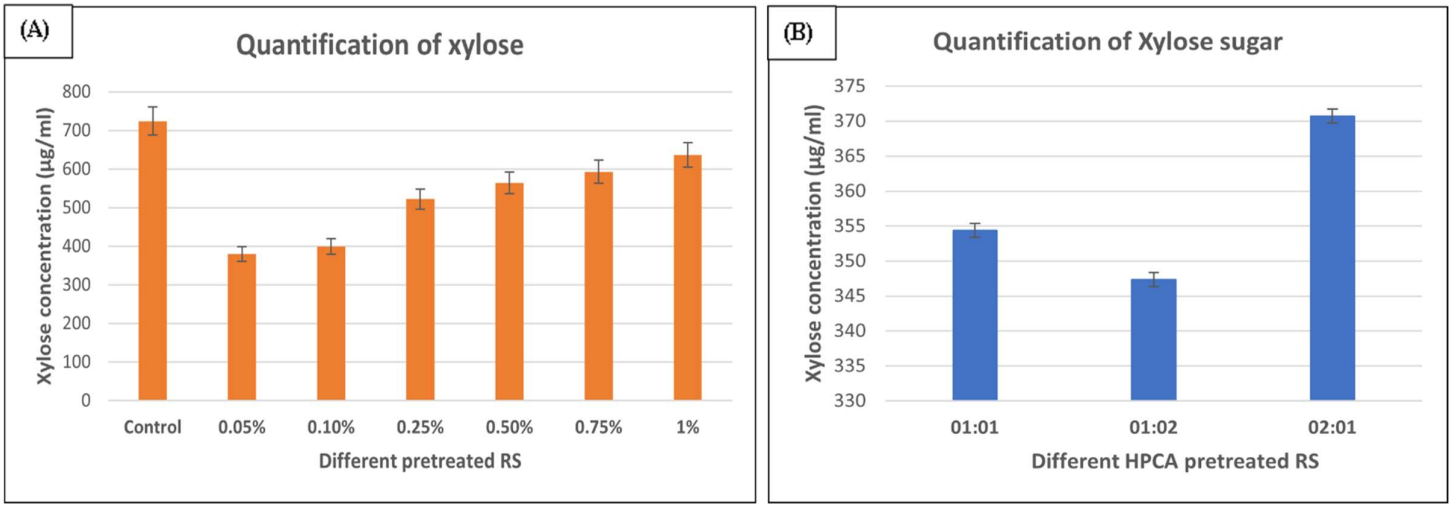

Figure 4.16.2- Quantification of xylose content (µg/ml) (A) Different H_2O_2 pretreated rice straw (B) Different HPCA pretreated biomass. The data is presented as an average of triplicates absorbance obtained for quantification of xylose sugar elucidating R^2=0.9505, Y=0.0006X+0.1669 with error bars showing S.D ($p<0.05$)

Table 4.9- Constituent of rice straw both before and after the pretreatment process using (A) Different concentrations of H_2O_2 and (B) Different HPCA pretreated RS in different ratios. The data is presented as an average of triplicates with error bars (±) showing S.D. ($p<0.05$)

Rice straw constituent	Unit	Before Pre-treatment	After pretreatment					
			0.05%	0.1%	0.25%	0.5%	0.75%	1%
Native RS[1]	g	2	1.3295±0.0029	1.2462±0.0003	1.2682±0.0192	1.3192±0.0291	1.2194±0.0018	1.3056±0.0062
Cellulose[1]	%	32.4±0.017	39.3±0.004	38.7±0.009	35.5±0.011	33.9±0.007	31.12±0.012	32.9±0.008
Hemicellulose[1]	%	57±0.011	40.96±0.013	46.21±0.021	42.97±0.014	48.25±0.003	51.68±0.024	49.41±0.018
Hemicellulose removal	%	NA	75.92	69.4	72.4	71.14	64.5	69.92
Lignin[1]	%	12.5±0.021	6.94±0.006	8.12±0.003	7.99±0.013	8.95±0.011	9.64±0.007	9.47±0.005
Lignin removal	%	NA	81.4	75.51	76.61	75.59	69.87	73.71
TRS[2]	mg/l	230±0.0035	359.60±0.0012	286.74±0.0041	268.01±0.0039	248.28±0.0017	136.452±0.0019	125.82±0.0024
Glucose[3]	µg/ml	696.78±0.0013	1438.41±0.016	1324.062±0.013	1205.368±0.005	1019.134±0.009	932.012±0.019	917.854±0.021
Glucan content[4]	%	43.99	84.45	82.93	74.17	60.29	59.66	54.87
Xylose[3]	µg/ml	724.30±0.004	380.02±0.002	399.47±0.011	522.37±0.009	564.37±0.006	593.15±0.014	636.71±0.007
Xylan content[4]	%	40.49	21.82	24.46	30.22	32.65	37.125	38.22

[1] On the total dry weight
[2] Present in the water extract as a soluble sugar
[3] These values consider simple sugars from enzymatic hydrolysis as well as those extracted from hydrolysate.
[4] Calculated value of glucose and xylose concentration from the final weight of pretreated rice straw

Rice straw constituent	Unit	Before Pretreatment	After pretreatment		
			1:1	1:2	2:1
Native RS[1]	Gm	2	1.1201±0.0016	1.1591±0.0019	0.8591±0.0021
Cellulose[1]	%	32.4±0.017	39.56±0.004	40.03±0.005	39.21±0.003
Hemicellulose[1]	%	57±0.011	38.19±0.025	36.28±0.017	42.64±0.013
Hemicellulose removal	%	NA	70.5	76.3	57.3
Lignin[1]	%	12.5±0.021	6.04±0.005	5.98±0.009	7.08±0.014
Lignin removal	%	NA	78.7	82.25	67.31
TRS[2]	mg/l	230±0.0035	356.059±0.0026	376.805±0.0031	329.240±0.0019
Glucose[3]	µg/ml	696.78±0.0013	1325.151±0.012	1454.746±0.009	1047.449±0.006
Glucan content[4]	%	43.99	90.29	91.32	68.97
Xylose[3]	µg/ml	724.0.004	623.49±0.015	632.05±0.005	611.82±0.011
Xylan content[4]	%	40.49	24.145	21.81	24.4

4.4.2. Enzymatic hydrolysis of H_2O_2 pretreated rice straw

The obtained pretreated solid fraction was subjected to enzymatic hydrolysis using commercially available cellulase enzyme (20 U/g of biomass). The sample was collected at different time intervals and was analyzed using the Anthrone reagent test. The total reducing sugar obtained after 72 hrs of enzymatic hydrolysis from 0.05% H_2O_2 pretreated rice straw shows 359.60±0.0012 mg/l of reducing sugar and decreases thereafter. The presence of reducing sugar has been decreased by increasing the concentration of H_2O_2. The glucose present in the H_2O_2 pretreated rice straw varies significantly, it was found that 1296.857±0.072 µg/ml of galactose present in the 0.05% H_2O_2 of pretreated rice straw with a maximum glucan content of 7.61% and decreases thereafter increasing the concentration of H_2O_2. Similarly, HPCA pretreated rice straw with a 1:2 ratio resulted in 9.68% glucan content and the concentration of glucose was 1438.41±0.018 µg/ml. The high glucose release from pretreated rice straw with lower xylan content as cellulose was surrounded by xylan hinders the cellulase accessibility towards cellulosic content in rice straw. The same amount of enzyme loading in different pretreated biomass that represents the delignification of pretreated rice straw has a positive impact on saccharification process. The removal of the physical barrier preventing cellulose from being accessed by cellulase and the ineffective binding to cellulase might account for this beneficial impact of delignification. It can be observed that the yield of glucose typically improves with time for cellulase loading because more enzyme loading can depolymerize cellulose to glucose with prolonged hydrolysis time (Tan & Lee, 2015). Thus, the process can be effective by utilizing less cellulase loading during enzymatic hydrolysis with more exposure time. The HPCA pretreated rice straw resulted in maximum glucose release after 72 hrs of enzymatic hydrolysis as compared to H_2O_2 pretreated rice straw. Karagöz et al., (2012) reported glucose and xylose yield of up to 12.79 g/l and 5.73 g/l from 2.5% H_2O_2 pretreated per 100 gm rapeseed straw while in this study 1.296 g/l of glucose was recovered from 0.5% H_2O_2 pretreated rice straw and the concentration of glucose reduces with the increase in the concentration of H_2O_2. The glucose concentration was further increased by the HPCA pretreatment process. The total reducing sugar obtained after enzymatic hydrolysis is illustrated in figure 4.17.1 and glucose recovery is illustrated in figure 4.17.2.

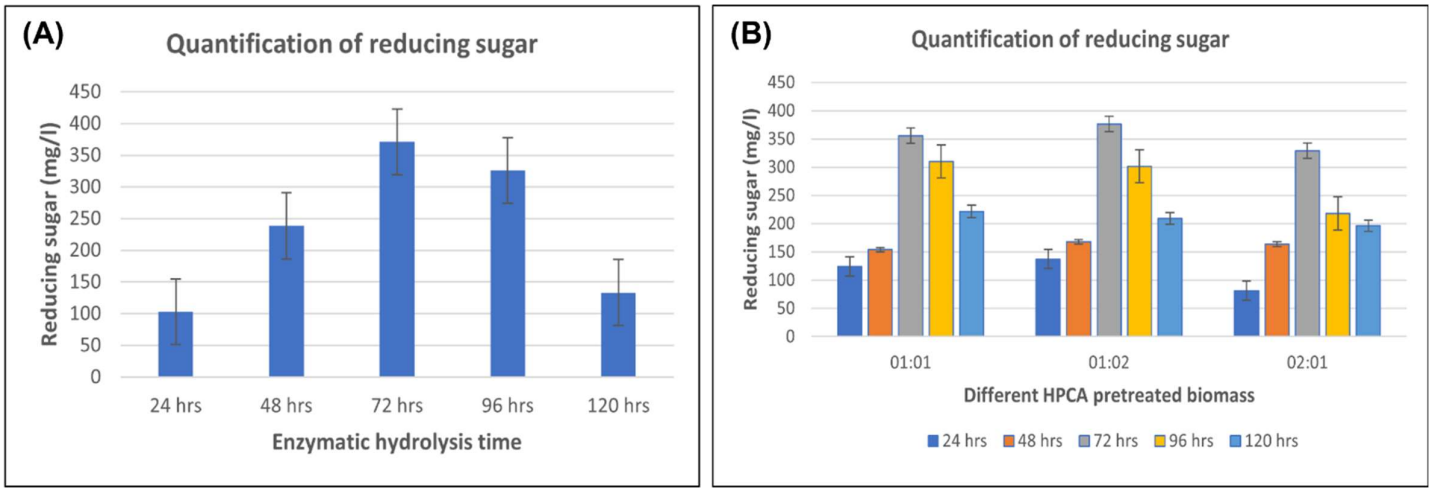

Figure 4.17.1- Quantification of total reducing sugar after enzymatic hydrolysis (A) 0.05% H_2O_2 pretreated rice straw (B) HPCA pretreated rice straw in different ratios. The data is presented as an average of triplicates absorbance obtained for quantification of reducing sugar elucidating R^2=0.9746, Y=0.0019X-0.0071 with error bars showing S.D. ($p<0.05$)

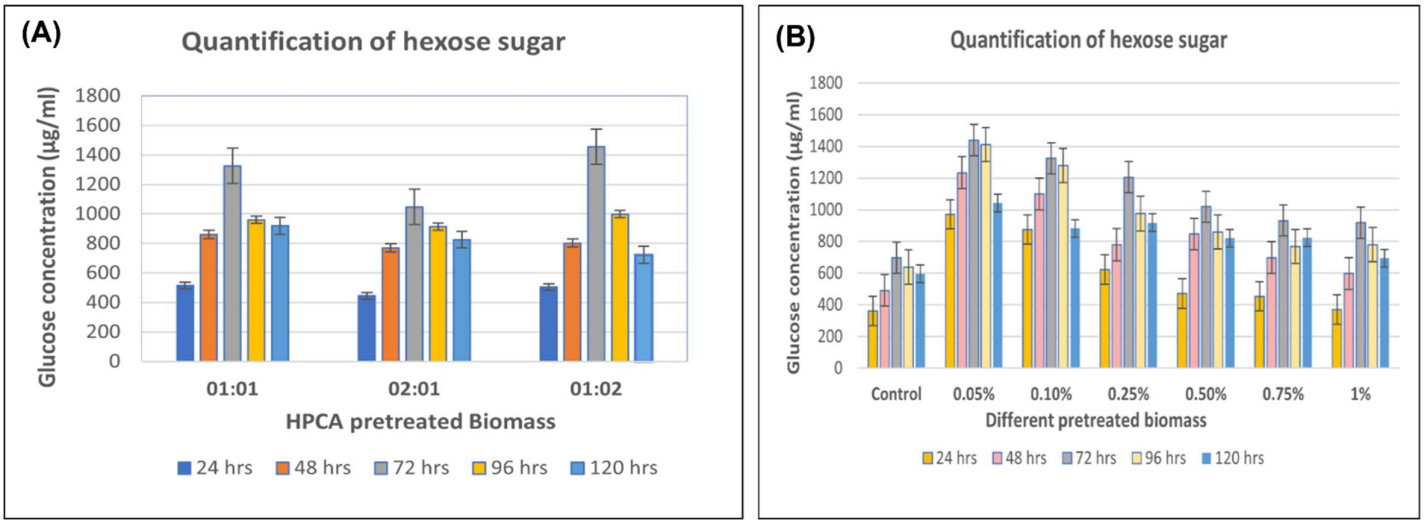

Figure 4.17.2- Quantification of Glucose released **(A)** HPCA pretreated biomass **(B)** H_2O_2 pretreated biomass. The data is presented as an average of triplicates absorbance obtained for quantification of hexose sugar elucidating R^2=0.9963, Y=0.0009X-0.0224 with error bars showing S.D. ($p<0.05$)

4.4.3. Acidic hydrolysis of Liquid hot water pretreated rice straw

The obtained hydrolysate of different pretreated rice straws was subjected to DNS reagent test. It was evaluated that at 120°C maximum reducing sugar of 150-320 mg/l of hydrolysate was obtained from different acid pretreatment. The maximum reducing sugar of 312 mg/l of hydrolysate from HNO_3 pretreated rice straw.

This is due to the presence of nitrate that has easily broken the strong interaction between the recalcitrant structure of lignocellulosic biomass. Among weak acids, oxalic acid pretreated rice straw yielded 268 mg/l of reducing sugar. It was evaluated that acidic hydrolysis might be completed within the stipulated reaction time to reduce the reducing sugar decompositions. Simultaneous pretreatment and saccharification were performed using high temperature and pressure along with acidic hydrolysis using different weak and strong acids using 1M H_2SO_4, HNO_3, HCl, Acetic acid, formic acid and Oxalic acid for 1 hr. It was evaluated that enhancing pretreatment time over 60 minutes led to more loss in the glucan content (Ebrahimi et al., 2017). The earlier reported work by Hu et al., (2018) with mild CaO treatment had resulted in the enhanced saccharification yield with an efficient sugar-ethanol conversion rate. The obtained xylose concentration from different pretreated biomass at varied temperature ranges showed a maximum xylose of 386.25 µg/ml present in HNO_3 pretreated biomass. This shows hemicellulose breakdown occurs further to pentose sugar releasing less xylan content. In comparison when acetic acid was used during SPS method Polysaccharide dissolutions were higher than in an aqueous solution due to the delignification effect using acetic acid. Acetic acid concentration had a substantial impact on the extent of acetylation of cellulose and carbohydrates (X. Zhao et al., 2014). Figure 4.18 (A) illustrates the effect of different substrates on the yield of reducing sugar with the temperature rise. It was found that 120°C of pretreatment temperature has led to maximum sugar yield which further decreases with the increase in the temperature. The total reducing sugar 202.53 mg/l, 312.74 mg/l, 252.27 mg/l, 157.14 mg/l, 270.52 mg/l and 175.36 mg/l were obtained from HCl, HNO_3, H_2SO_4, acetic acid, oxalic acid and formic acid pretreated rice straw at 120°C temperature. This is because galactose, glucose, and 5-HMF levels drop at a temperature higher than 130°C as well as galactose's breakdown into various by-product substances. Levulinic acid and 5-HMF are the two primary by-products of sugar breakdown, while 5-HMF is a by-product of hexoses like glucose and fructose (Meinita et al., 2015). Levulinic acid and 5-HMF that are produced during acidic hydrolysis at elevated temperatures may have a negative effect on yeast growth as well as ethanol production. However, to attain a maximum glucan conversion rate in the LHW pretreatment, it is not practical to use a strategy that entails raising the pretreatment temperature. The majority of the xylan amount can be removed from LHW, however, a significant amount of lignin remained present in the liquid fraction (Lu, Liu, Song, et al., 2020b). The xylose concentration is illustrated in figure 4.19 (B). It is widely known that liquid hot water behaves like acid when heated to high degrees, resulting in the generation of oxidation products and by-products in the form of organic acids (such as formic, acetic, and lactic acids) that function as catalysts in the breakdown of hemicellulose (Lyu et al., 2018). It was observed that during acidic hydrolysis of polysaccharides, not only hydrolysis of polysaccharides to monosaccharides but also the subsequent degradatio n process of monosaccharides into Levulinic acid and 5HMF occurs. The fact that HCl is a weaker acid than H_2SO_4 must be the main cause of the decreased monosaccharide breakdown in biomass. In most cases, carbonyl group protonation is the first step in the breakdown of monosaccharides in acidic conditions. HCl can be used in certain acid catalyzed processes, while in other cases stronger conjugate base is needed to regenerate

the catalyst and extract the proton. Each monosaccharide degraded significantly more slowly in HCl than in H_2SO_4 because HCl is weaker than H_2SO_4 (Imman et al., 2021b). While during formic acid treatment, formyl group might increase the diameter of cellulosic chain that minimises the chance of entering cellulase through the catalytic domain. Thus, the ability of cellulase to recognise specific cellulose substrates is interfered, when hydroxyl group in cellulose is replaced with a formyl group. It could prevent cellulose from forming productive hydrogen bonds (binding) with cellulases' catalytic domain (Dong et al., 2017). It was earlier evaluated that 0.65% HNO_3 pretreatment at 158.8°C for short duration of 5.86 min resulted in 86.5% of xylose yield during the processing step (I. Kim et al., 2014). In this present study, 1 M HNO_3 at 120°C for 1 hr has resulted in 22.11% of xylose content with 77.89% of xylan removal from the obtained liquid fraction.

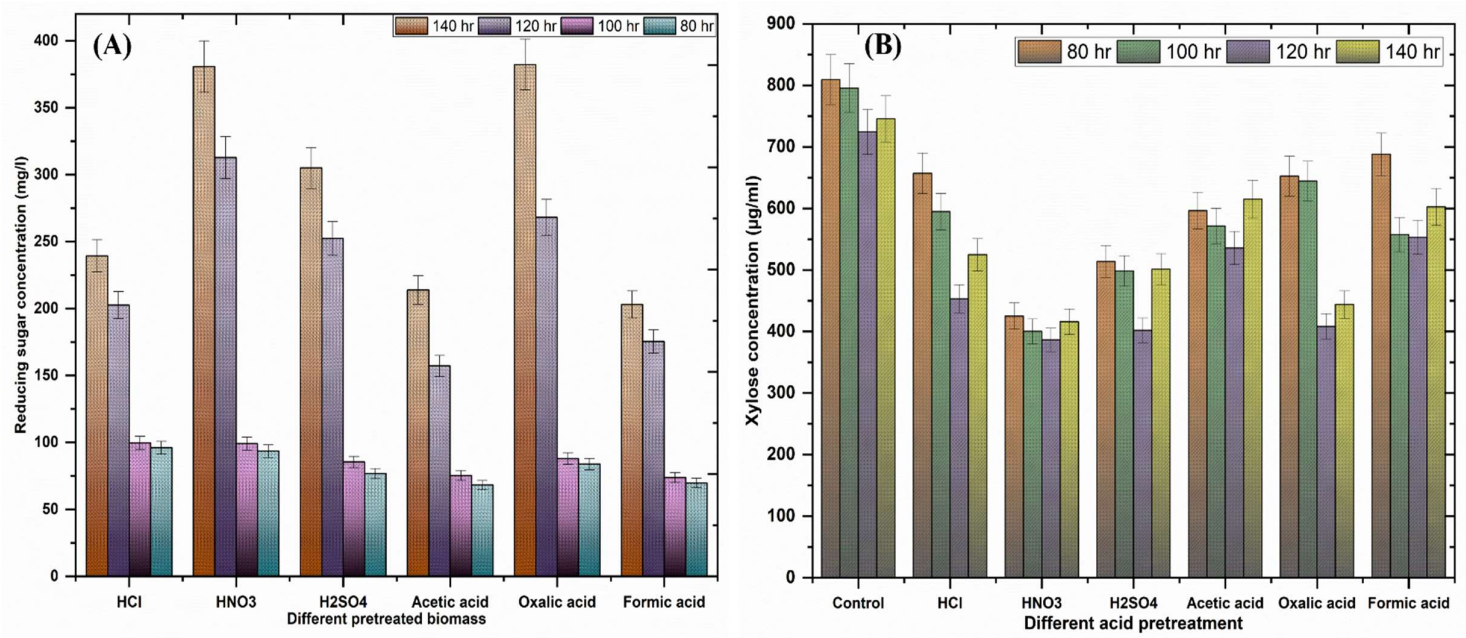

Figure 4.18- (A) Quantification of total reducing sugar after acidic hydrolysis during SPS method. The data is presented as an average of triplicates absorbance obtained for quantification of reducing sugar elucidating R^2=0.9746, Y=0.0019X-0.0071 with error bars showing S.D. ($p<0.05$) **(B)** Quantification of xylose obtained from different acid pretreated rice straw. The data is presented as an average of triplicates absorbance obtained for quantification of xylose sugar elucidating R^2=0.9505, Y=0.0006X+0.1669 with error bars showing S.D ($p<0.05$)

4.4.4. Enzymatic hydrolysis of Liquid hot water pretreated rice straw

Saccharification is the next essential step after the pretreatment of rice straw is indispensable for biomass conversion to bioethanol. This was used to examine the impact of pretreatment on cellulosic conversion efficiency by quantifying the total produced reducing sugar in the form of xylose, galactose, mannose, arabinose and cellobiose. Biomass content, pretreatment technique, and the kind of

enzyme employed for cellulosic hydrolysis are important variables that influence the enzymatic hydrolysis of cellulose (Paramasivan et al., 2021). The delignified biomass was afterward employed for saccharification using a bi-phasic approach for entire sugar extraction during the simultaneous pretreatment and saccharification procedure. Pentose sugars were extracted in the first phase using liquid hot water and acidic treatment. Hexose sugars were then extracted in the second phase using enzymatic hydrolysis. The total reducing sugar of 267.18 mg/l, 353.02 mg/l, 301.98 mg/l, 162.76 mg/l, 306.47 mg/l and 219.43 mg/l from HCl, HNO_3, H_2SO_4, acetic acid, oxalic acid and formic acid respectively. The obtained biomass conversion ratio was estimated to be 53.35%, 57.65%, 55.05%, 48.1%, 55.3%, and 40.95% respectively (Figure 4.28). This shows the maximum biomass conversion was obtained from HNO_3 pretreated rice straw. The yield of total reducing sugar after enzymatic hydrolysis is illustrated in Figure 4.19. The maximum reducing sugar yield was observed for HNO_3, oxalic acid, H_2SO_4, HCl, formic acid and acetic acid. The lower reducing sugar from HCl and acetic acid pretreated rice straw among strong and weak acid-catalyzed reactions was due to less release of cellulose during the pretreatment process. It has been evaluated that there is an enhancement of produced total reducing sugar up to 15-25% with the involvement of enzymatic hydrolysis for hydrolyzing hexose sugar as well. Similarly, as evidenced by the least amount of glucose lost during glucan hydrolysis, oxalic acid had the best degree of binding affinity between acid promoters. Lignin degradation was considerably accelerated in the alkali-catalyzed LHW process compared to the acid-catalyzed treatment reported by Imman et al., 2014. From the total produced sugar, it was found that 1366.535 µg/l of glucose recovered from the HNO_3 pretreated rice straw. It was found that 11.88% of glucan content was present in rice straw. The maximum glucan content present in enzymatic hydrolysis of acid-treated biomass was due to cellulose degradation during enzymatic hydrolysis. It was reported that there is variation in glucose content from 32-49% of glucose recovery from the untreated rice straw taken as a control. The earlier reported work by Hu et al., (2018) illustrated 31-38% of hexose recovery from the sugarcane bagasse biomass. In the present study, the decrease in the produced sugar concentration after 72 hrs was due to the inhibition of used enzymes by the products formed during enzymatic hydrolysis. Further, the slow hydrolysis rate of both acetic and formic acid pretreated biomass was due to acetylation and formylation of cellulose that was unavoidable during acetic acid and formic acid delignification, resulting in the replacement of a portion of the hydroxyl group with the acetyl and formyl groups. This change interfered with the cellulase enzyme identification of cellulose substrates, reducing the hydrolysis rate. It may prevent the establishment of active binding (H-bond) between cellulose and cellulases' catalytic domain (Wu et al., 2016). Further, cellulase loading has also effected the yield of bioethanol from the obtained hydrolysate that is due to the high viscosity of cellulose, increased cellulase loading may impair the enzyme's absorption efficiency, contributing to a reduced glucose output (Tan & Lee, 2015).

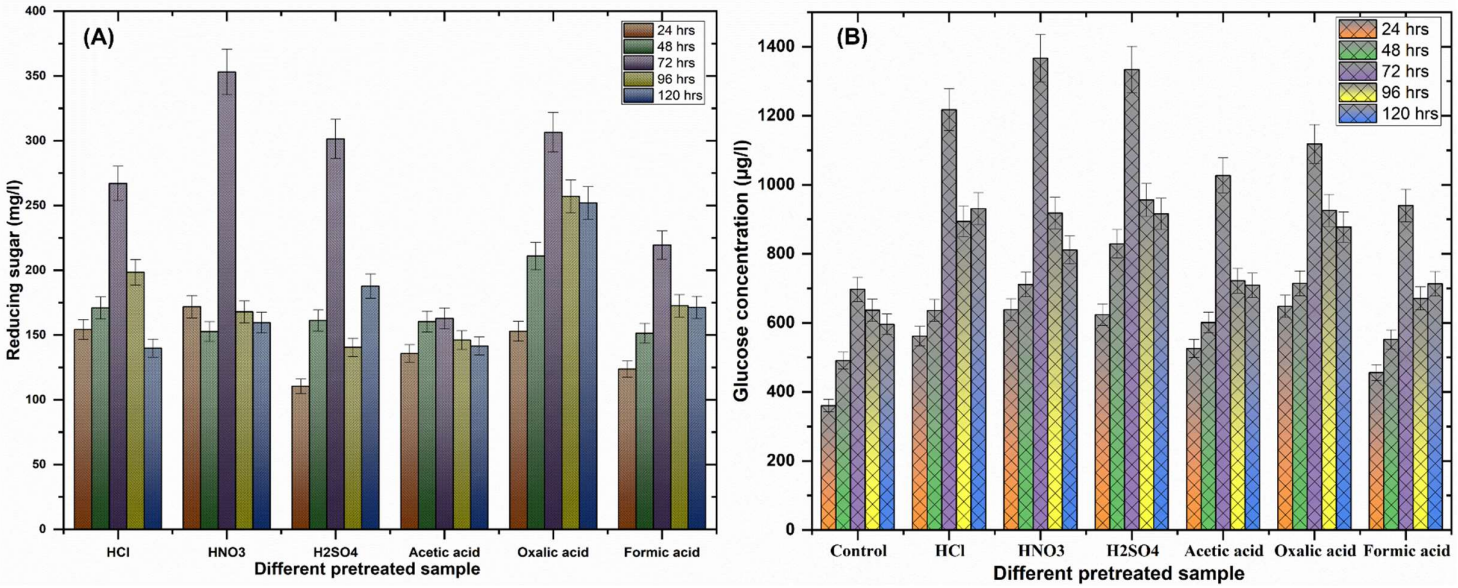

Figure 4.19- Quantification of sugar obtained pretreatment with different acids at different time intervals **(A)** Quantification of total reducing sugar in different acid pretreated rice straw. The data is presented as an average of triplicates absorbance obtained for quantification of reducing sugar elucidating R^2=0.9746, Y=0.0019X-0.0071 with error bars showing S.D. ($p<0.05$) (B) Quantification of glucose concentration. The data is presented as an average of triplicates absorbance obtained for quantification of hexose sugar elucidating R^2=0.9963, Y=0.0009X-0.0224 with error bars showing S.D. ($p<0.05$)

Table 4.10- Constituent of rice straw both before and after pretreatment using different acids. The data is presented as an average of triplicates with error bars (±) showing S.D. ($p<0.05$)

Rice straw constituent	Unit	Before Pre-treatment	After pretreatment					
			H_2SO_4	HCl	HNO_3	Acetic acid	Formic acid	Oxalic acid
Native RS[1]	G	2	1.234±0.038	1.564±0.059	0.897±0.008	1.210±0.062	1.674±0.041	0.987±0.071
Cellulose[1]	%	32.4±0.017	36.5±0.006	35.1±0.019	37.9±0.014	33.9±0.014	34.12±0.005	36.8±0.018
Hemicellulose[1]	%	57±0.011	35.67±0.006	34.02±0.009	34.19±0.003	39.98±0.013	40.28±0.008	38.04±0.007
Hemicellulose removal	%	NA	61.38	53.32	73.09	57.56	40.85	67.06
Lignin[1]	%	12.5±0.021	7.92±0.004	8.23±0.006	7.02±0.001	10.49±0.005	10.32±0.017	8.62±0.021
Lignin removal	%	NA	60.90	47.9	74.81	49.2	30.89	65.69
TRS (EH)[2]	mg/l	230±0.0035	301.41±0.0029	267±0.0017	352.02±0.0009	162.76±0.0015	219.43±0.0152	306.47±0.0021
Glucose[3]	µg/ml	696.78 ±0.0013	1333.86±0.0063	1217.338±0.0027	1366.53±0.0094	1026.76±0.0048	939.63±0.0021	1118.23±0.0037
Glucan content[4]	%	51.05	74.79	60.51	81.06	66.23	43.81	80.29
Xylose[3]	µg/ml	724.29±0.004	401.80±0.006	453.12±0.007	386.25±0.011	535.59±0.014	553.02±0.009	408.34±0.014
Xylan content[4]	%	40.49	22.03	24.11	22.4	33.78	25.21	28.671

4.4.5. Acidic hydrolysis of oxalic acid pretreated rice straw

The xylan concentration obtained after acidic hydrolysis during SPS method was 391.22 µg/ml of obtained hydrolysate from 0.5 M oxalic acid pretreated rice straw. It was found that xylan concentration was significantly increased with increasing concentration of oxalic acid which shows about 68.95% of xylan removal from the untreated rice straw. Oxalic acid is effective in hemicellulose depolymerization under moderate conditions, successfully avoiding cellulose breakdown. As a result, oxalic acid may be useful in removing hemicelluloses from lignocellulose biomass. It was earlier stated by Kundu & Lee, (2015) that xylan concentration has a negative effect on the hydrolysis of glucan to glucose using cellulase enzyme. Thus, effective removal of xylan was observed with 0.75 M oxalic acid treatment while only % of xylan removal was obtained from 1 M of oxalic acid-treated rice straw. The previous work reported by Kundu & Lee, (2015) showed the xylan concentration of 3.16% from 0.03 M oxalic acid-treated deacetylated biomass. Ibrahim et al., (2020) reported xylan removal of 6.5% from 100 mM oxalic acid pretreated beech wood at 175°C for 60 min. Thus, in this present study effective xylan removal was obtained under mild pretreatment conditions and a total xylose content of 28.19% from 0.5 M oxalic acid pretreated rice straw was achieved. Further, the total reducing sugar yield was estimated, and it was observed that 330.75 mg/l of reducing sugar concentration was obtained from acidic hydrolysis during SPS method. The total reducing sugar yield was enhanced by 42.79% after the pretreatment of rice straw, this occurred due to the xylan removal from pretreated rice straw making cellulose more accessible towards enzymatic hydrolysis. Due to the maximum xylan content in the obtained hydrolysate, the biphasic system of sugar breakdown was adopted in the present study. The quantification of reducing sugar and xylose from different oxalic acid pretreated rice straws from different concentrations of oxalic acid is illustrated in Figure 4.20.

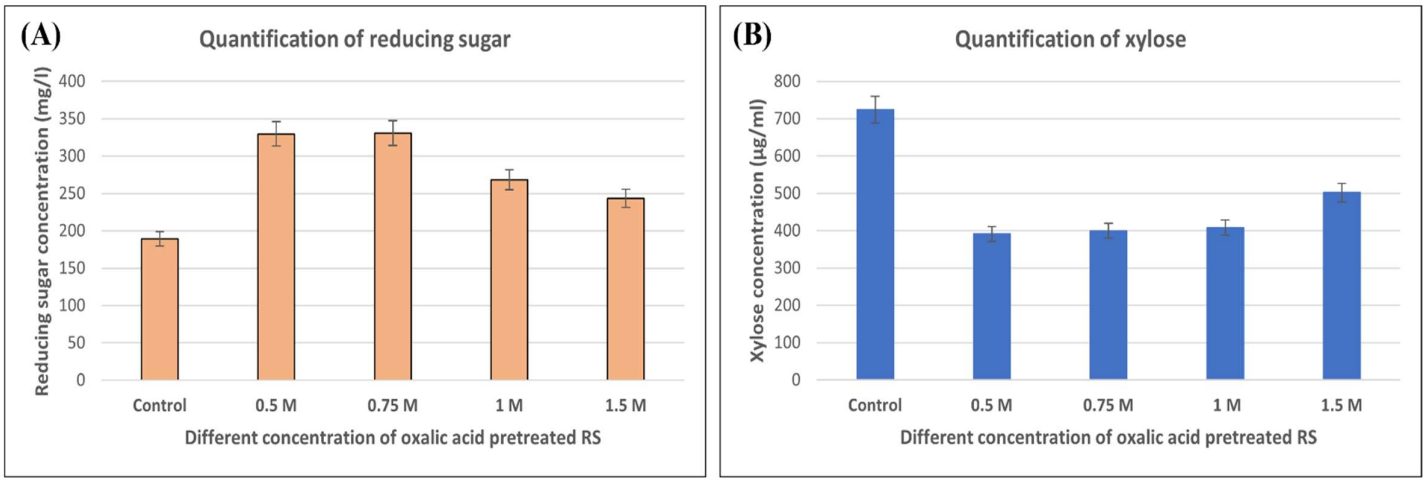

Figure 4.20- (A) Quantification of total reducing sugar after acidic hydrolysis during SPS method using different concentrations of oxalic acid. The data is presented as an average of triplicates absorbance obtained for quantification of reducing sugar elucidating R^2=0.9746, Y=0.0019X-0.0071 with error bars showing S.D.

($p<0.05$) **(B)** Quantification of xylose obtained after oxalic acid pretreatment during SPS method. The data is presented as an average of triplicates absorbance obtained for quantification of xylose sugar elucidating $R^2=0.9505$, $Y=0.0006X+0.1669$ with error bars showing S.D ($p<0.05$)

4.4.6. Enzymatic hydrolysis of oxalic acid pretreated rice straw

The hydrolysate obtained after enzymatic hydrolysis of pretreated rice straw contained 335.76 g/l of total reducing sugar out of which 1252.18 µg/ml was of glucose and 391.23 µg/ml of xylose from 0.05 M oxalic acid pretreated biomass with glucan and xylan content of 90.23% and 39.29% respectively. It was found that the amount of glucose increased significantly up to 36.88% with an increase in hydrolysis time. It was evaluated after 0.75 M oxalic acid pretreated rice straw resulted in 54.39% of glucan recovery. Earlier reported work by Kundu et al., (2016) showed 60.91% of glucan recovery from the deacetylated yellow poplar biomass of 0.16 M oxalic acid pretreatment at an optimum condition of 150℃ for 42 min. Oxalic acid is considered as strongest dicarboxylic acid with lower corrosivity (Ibrahim et al., 2020) and showed an intense hydrolysis efficiency than that of other strong acid. Because of the selective breakdown of hemicelluloses during pretreatment, the concentrations of glycan in pretreated rice straw were enhanced compared to raw material. It was evaluated that oxalic acid was proposed as a useful catalyst for the hydrolysis of β-(1-4) linkage while inhibiting subsequent dehydration processes (Chotirotsukon et al., 2019). Thus, the amount of fermentable sugar produced was diversified depending on the type of acid catalyst used during the process. Saccharification and fermentation were impacted by the structural alteration of pretreated biomass. The quantities of phenolic OH group in lignin, in particular, played a key influence in saccharification and ethanol fermentation. The obtained reducing sugar and hexose sugar concentration from the enzymatic hydrolysis is illustrated in figure 4.21. The present work focuses on the low enzyme loading with enhanced time exposure of the hydrolysis process. In other words, one of the most serious consequences of excessive enzyme loading is the ineffective adsorption of cellulase on the lignin surfaces, which reduces the enzyme's effective concentration (Lu et al., 2020).

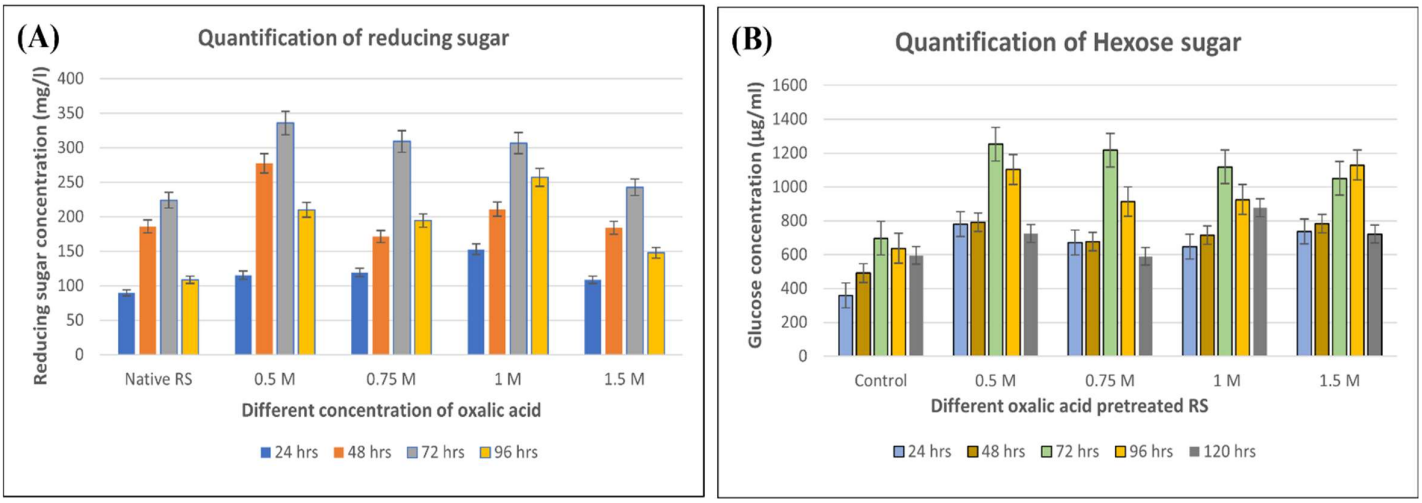

Figure 4.21- Quantification of sugar obtained after oxalic acid pretreatment **(A)** Quantification of total reducing sugar after enzymatic hydrolysis. The data is presented as an average of triplicates absorbance obtained for quantification of reducing sugar elucidating R^2=0.9746, Y=0.0019X-0.0071 with error bars showing S.D. ($p<0.05$) **(B)** Quantification of glucose concentration after enzymatic hydrolysis. The data is presented as an average of triplicates absorbance obtained for quantification of hexose sugar elucidating R^2=0.9963, Y=0.0009X-0.0224 with error bars showing S.D. ($p<0.05$)

4.5. Fermentation process of obtained liquid hydrolysate

4.5.1. Fermentation of hydrolysate obtained after acidic hydrolysis through SPS method of H_2O_2 pretreated rice straw

After the hydrolysis process, fermentation is required to transform the obtained fermentable carbohydrates into biofuels. The obtained liquid hydrolysate from acidic hydrolysis after SPS method of 0.05% H_2O_2 pretreated rice straw was subjected to a co-fermentation process using *S.cerevisiae* and *Z. mobilis* resulting in a maximum ethanol concentration of 0.075 g/l from pretreated rice straw after 72 hrs of co-fermentation process. The concentration of ethanol was reduced after enhancing the concentration of H_2O_2. During the fermentation process, both yeast *S. cerevisiae* and *Z.mobilis* used sugar as a reservoir of carbon and energy to produce bioethanol, the production of bioethanol increased whereas the reducing sugar content decreased. The ethanol production yield of 40.89% was obtained from 0.05% H_2O_2 pretreated rice straw, the ethanol concentration was further increased when rice straw was pretreated with HPCA in the ratio 1:2 resulting in 0.084 g/l of ethanol concentration with an ethanol production yield of 44.85% (Figure 4.22). The total calculated bioethanol production yield was increased to approximately 9% when HPCA in the ratio 1:2 was utilized with the fermentation efficiency of 70.75% and 87.56% of sugar to ethanol conversion rate.

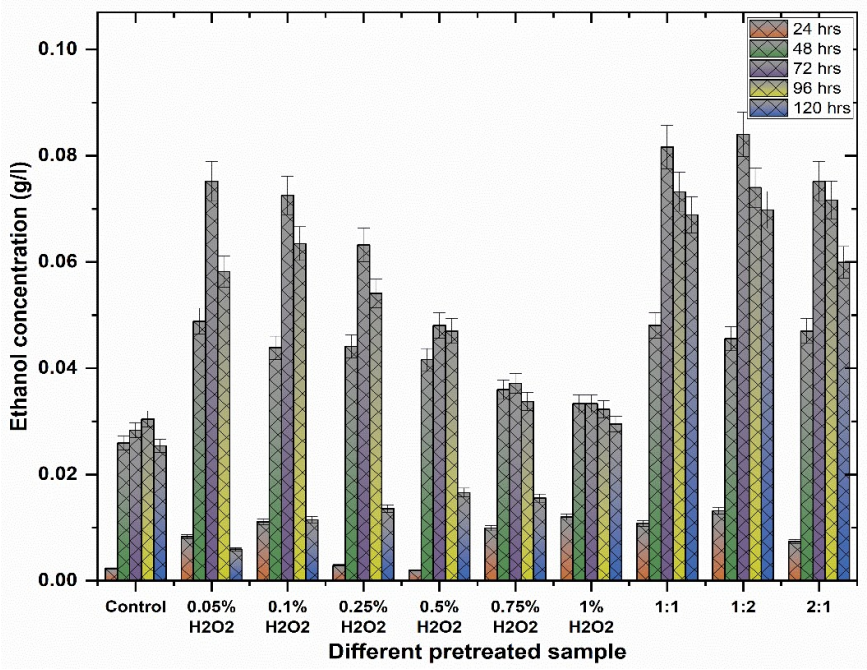

Figure 4.22- Quantification of produced ethanol from SPS method of different H_2O_2 and HPCA pretreated rice straw in the ratio 1:1, 1:2 and 2:1. The data is presented as an average of triplicates absorbance obtained for quantification of ethanol concentration elucidating R^2=0.9773, Y=0.3633X+0.0726 with error bars showing S.D. ($p<0.05$)

4.5.2. Fermentation of hydrolysate obtained after enzymatic hydrolysis of H_2O_2 pretreated rice straw

The obtained liquid hydrolysate after enzymatic hydrolysis was subjected to a co-fermentation process using *S. cerevisiae* and *Z. mobilis* and the ethanol concentration was analyzed after 24, 48, 72, 96 and 120 hrs using potassium dichromate test. It was evaluated that after 72 hrs of reaction time, maximum ethanol concentration up to 0.086 ± 0.013 g/l was obtained from 0.05% H_2O_2 pretreated rice straw which was further enhanced when rice straw was pretreated with HPCA in the 1:1 and 1:2 ratio having the same concentration of H_2O_2 and when the concentration of H_2O_2 was increased, the ethanol concentration was also reduced. The ethanol concentration from HPCA pretreated rice straw resulted in 0.091±0.003, 0.095±0.017 and 0.085±0.021 g/l from 1:1, 1:2 and 2:1 respectively (Figure 4.23). It was evaluated that 0.05% H_2O_2 pretreated rice straw resulted in an 84.8% rise in ethanol yield from untreated rice straw and a 13.9% rise in ethanol yield from various other pretreated methods utilized for the production of bioethanol.

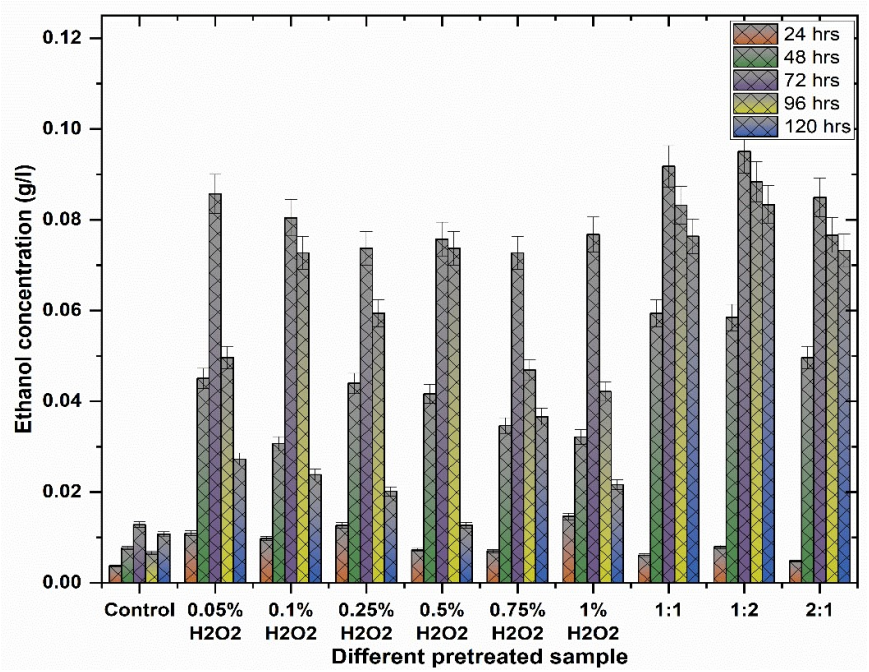

Figure 4.23- Quantification of produced ethanol from hydrolysate obtained after enzymatic hydrolysis of different H_2O_2 and HPCA pretreated rice straw in the ratio 1:1, 1:2 and 2:1. The data is presented as an average of triplicates absorbance obtained for quantification of ethanol concentration elucidating R^2=0.9773, Y=0.3633X+0.0726 with error bars showing S.D. ($p<0.05$)

4.5.3. Fermentation of hydrolysate obtained after acidic hydrolysis through SPS method of different acid-pretreated rice straw

This co-fermentation process was performed using *S.cerevisiae* and *Z.mobilis* of obtained reducing sugar from the simultaneous pretreatment and saccharification process. It was found that the greatest ethanol concentration was acquired from HNO_3 pretreated rice straw as after the neutralization process, nitrogen was used as a source for the subsequent fermentation process. The obtained ethanol concentration from SPS method of HNO_3 pretreated rice straw was 0.028 g/l from 2 gm of rice straw utilized for bioethanol production with an ethanol production yield of 17.8%. The produced reducing sugar from the acidic hydrolysis during the SPS method was 312.74 mg/l and was further fermented to ethanol. The acidic hydrolysis of produced pentose sugar to ethanol was analyzed and illustrated in Figure 4.24 (A).

4.5.4. Fermentation of hydrolysate obtained after enzymatic hydrolysis of different acid-pretreated rice straw

The co-fermentation process was carried out on liquid hydrolysate obtained from enzymatic saccharification of different acid-treated rice straw, the obtained total sugar ranges from 353 mg/l of biomass was fermented using *S.cerevisiae* and *Z. mobilis* and the concentration of ethanol at the end of 72 hrs was 0.0711 g/l obtained from HNO_3 pretreated rice straw with fermentation efficiency of 42.01%. The bioethanol yield from different pretreated biomass showed in this descending order HNO_3 > Oxalic acid > H_2SO_4 > HCl

> formic acid > acetic acid (Figure 4.24 (B). The native rice straw was used as the control. The result showed that HNO_3 pretreated rice straw was a suitable method for bioethanol production with a sugar-to-ethanol conversion rate of 73.2 g/g of sugar produced from the hydrolysis process. The yield of ethanol, fermentation time, and temperature during bioethanol production all depend heavily on the quantity of reducing sugars obtained after the hydrolysis process. Therefore, it is necessary to research the development of *Z. mobilis* and *S. cerevisiae* under various autophoretic settings as well as the growth rate kinetics of that particular yeast. Co-fermentation is a biological process using these yeast strain that has an innate affinity for sugar as a carbon source towards the production of bioethanol by inoculating it into obtained hydrolysate. It is considered that these yeast strains are widely available and easily cultured and these have strong inhibitor tolerance, and efficient ethanol generation in comparison to other yeast strains and bacteria, are also effective and extensively distributed globally for the bioethanol industries. It can be concluded from current work that ethanol production from HNO_3 pretreated rice straw was more than that of HCl and H_2SO_4 pretreated biomass. From the earlier reported work by Imman et al., (2021a), it was evaluated that the hydrolysate from HCl pretreated sample resulted in the formation of chloroethane and ethyl chloride was obtained as the final product of fermentation. The chemical reaction involved during its formation includes $C_2H_5OH + HCl \rightarrow C_2H_5Cl + H_2O$. Similar to the usual fermentation medium, nitric acid-pretreated samples may sustain fermentation effectively without the addition of nitrogen (Skiba et al., 2022). Nitric acid is neutralized to generate nitrate, which is the only source of nitrogen following neutralization that resulted in a higher ethanol yield with fermentation efficiency of 42.1% and 40.81% from HNO_3 and oxalic acid pretreated rice straw. The obtained ethanol concentration was 0.0711 g/l from 2 gm of native rice straw pretreated with HNO_3. A similar higher yield of bioethanol was obtained from HNO_3 pretreated corn stover by Abdul Manaf et al., (2022). Earlier reported work using LHW assisted with Na_2CO_3/O_2 resulted in an ethanol yield of 0.133 g/g of reed biomass under the optimum condition of 170°C for 60 min (Lu et al., 2020). Intense pretreatment procedures typically resulted in inhibitor production during the fermentation process in the form of furfural and 5-methyl furfural, generated by sugars and phenolic chemicals from the lignin complex structure. Thus, to avoid the production of inhibitors during the process, harsh pretreatment conditions are eliminated while performing this research.`

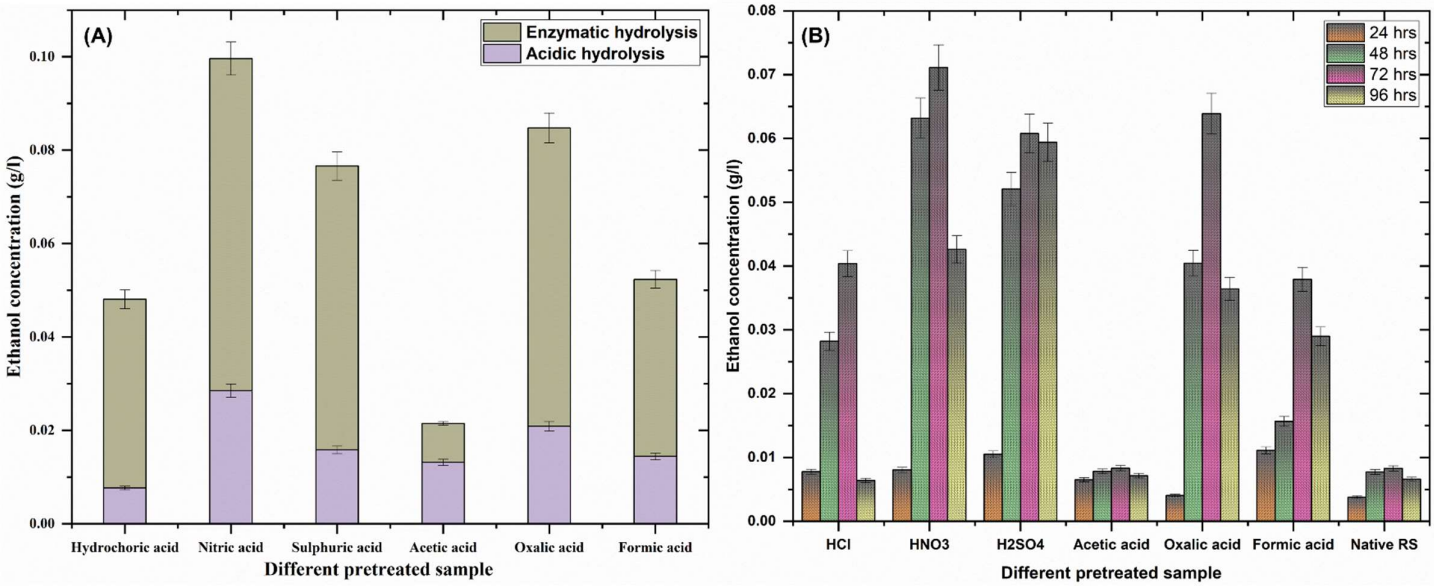

Figure 4.24- Quantification of ethanol concentration from hydrolysate obtained after **(A)** Acidic hydrolysis and enzymatic hydrolysis at 72 hrs **(B)** Enzymatic hydrolysate after different time intervals at 24hrs, 48 hrs, 72hrs and 96 hrs. The data is presented as an average of triplicates absorbance obtained for quantification of ethanol concentration elucidating R^2=0.9773, Y=0.3633X+0.0726 with error bars showing S.D. ($p<0.05$)

4.5.5. Fermentation of hydrolysate obtained after acidic hydrolysis through SPS method of oxalic acid pretreated rice straw

The obtained hydrolysate was subjected to a co-fermentation process after detoxification of the obtained hydrolysate. It was found that 0.059 g/l of ethanol concentration was observed from hydrolysate obtained from 0.75 M oxalic acid treated sample with 330.89 mg/l of reducing sugar concentration. Earlier reported work by Kundu & Lee, (2015), observed ethanol yield of 0.29 g/l to 0.49 g/g from hydrolysate obtained by 0.1 M oxalic acid pretreated deacetylated yellow poplar was carried out at 150℃ for 30 min. Similarly, in the present work, it was assumed that 0.35 g/g of ethanol yield from 0.75 M oxalic acid pretreated rice straw. The obtained ethanol yield ranges from 0.15 g/g to 0.35 g/g, it was decreased by enhancing oxalic acid concentration. It was observed that the detoxification of obtained hydrolysate had a synergistic effect on the quantification of obtained bioethanol (Kundu & Lee, 2016). The obtained results on ethanol concentration are illustrated below in Figure 4.25 and calculated results on ethanol yield and fermentation efficiency are illustrated in Figure 4.27 (B).

4.5.6. Fermentation of hydrolysate obtained after enzymatic hydrolysis of oxalic acid pretreated rice straw

The obtained hydrolysate after enzymatic hydrolysis was subjected to a co-fermentation process utilizing *Z. mobilis* and *S.cerevisiae*. The obtained ethanol concentration is illustrated in Figure 4.25. It was

observed that 0.073 g/l of ethanol concentration was obtained from 0.5 M oxalic acid pretreated rice straw. The observed ethanol yield of different concentrations of oxalic acid pretreated rice straw ranges from 0.43 g/g to 0.38 g/g of biomass. It was evaluated that the yield of bioethanol was enhanced after the enzymatic hydrolysis process of the obtained solid fraction after the pretreatment process. After enzymatic hydrolysis, the fermentation yield was enhanced up to 57.18% from 38.98%. The enhancement of fermentation efficiency was due to the more glucan content available as a source of glucose for the yeast utilized for the co-fermentation process. The earlier reported study by Kundu & Lee, (2016), evaluating original hydrolysate resulted in 0.22 g/g of ethanol yield that was further enhanced up to 0.40 g/g after XAD-electrodialysis of oxalic acid pretreated hydrolysate. It was observed that detoxification of obtained hydrolysate had a synergistic effect on the quantification of obtained bioethanol.

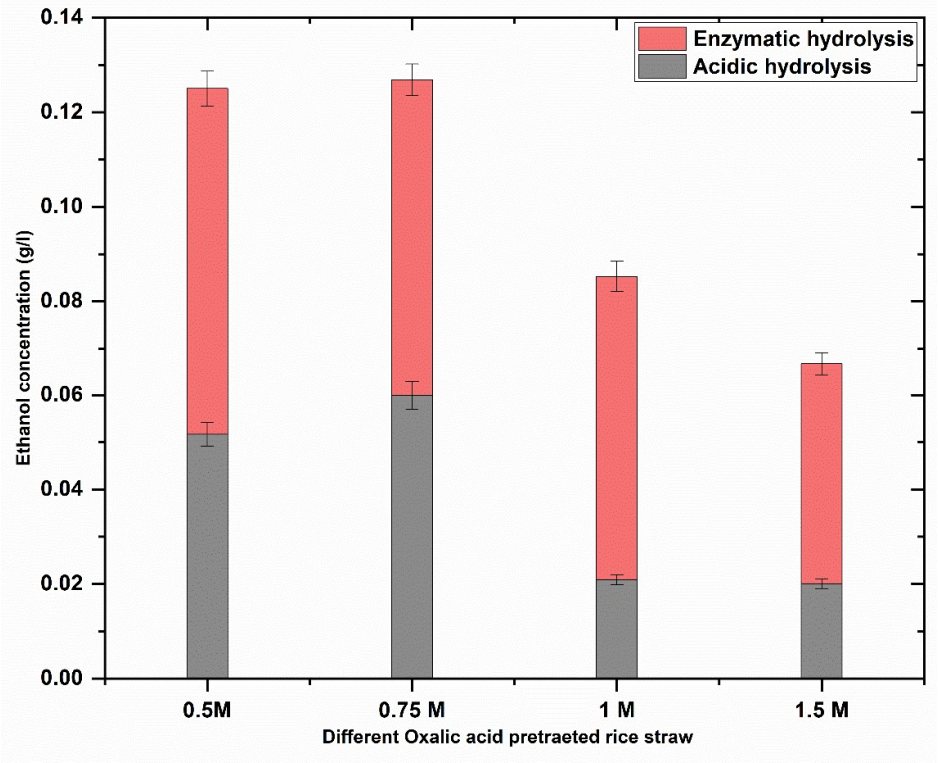

Figure 4.25- Quantification of ethanol concentration from oxalic acid pretreated rice straw after both acidic and enzymatic hydrolysis. The data is presented as an average of triplicates absorbance obtained for quantification of ethanol concentration elucidating R^2=0.9773, Y=0.3633X+0.0726 with error bars showing S.D. ($p<0.05$)

4.6. Conversion rate of different pretreated rice straw

The conversion of biomass to reducing sugar and further production of reducing sugar to ethanol using various acid-impregnated pretreatment processes is illustrated in Figure 4.26. From the obtained findings, it was observed that a maximum conversion rate of 68% from biomass to sugar was obtained from

HPCA pretreated biomass in the ratio 1:2. In contrast, 0.05% H_2O_2 pretreated biomass shows only 57.97% conversion. Further, with different acid treatments, it was evaluated that HNO_3 pretreated rice straw showed maximum biomass conversion to sugar as well as sugar conversion to bioethanol was up to 57.65% and 73.2% respectively. Among weak acids, oxalic acid showed the maximum conversion rate i.e., 55.3% and 68.53% of biomass to sugar and sugar to bioethanol respectively. Thus, H_2O_2 pretreated rice straw along with citric acid in the ratio 1:2 ratio resulted in the maximum conversion of biomass to sugar and further sugar to bioethanol. Oxalic acid pretreated rice straw resulted in a maximum 56.78% conversion from biomass to sugar and 71.8% conversion from sugar to ethanol was obtained, which was further decreased by increasing the concentration of oxalic acid.

Only the obtained solid fraction after pretreatment and washed biomass were employed to further the processing step, and significant quantities of sugars in the liquid portion of pretreated biomass, particularly xylose, were lost since the obtained hydrolysate with xylose enriched fraction was not added to the final liquid fraction used for the fermentation process. This was used separately to obtain maximum ethanol yield from the applied co-fermentation process and further enhance the conversion rate of sugar to ethanol (Kim et al., 2019).

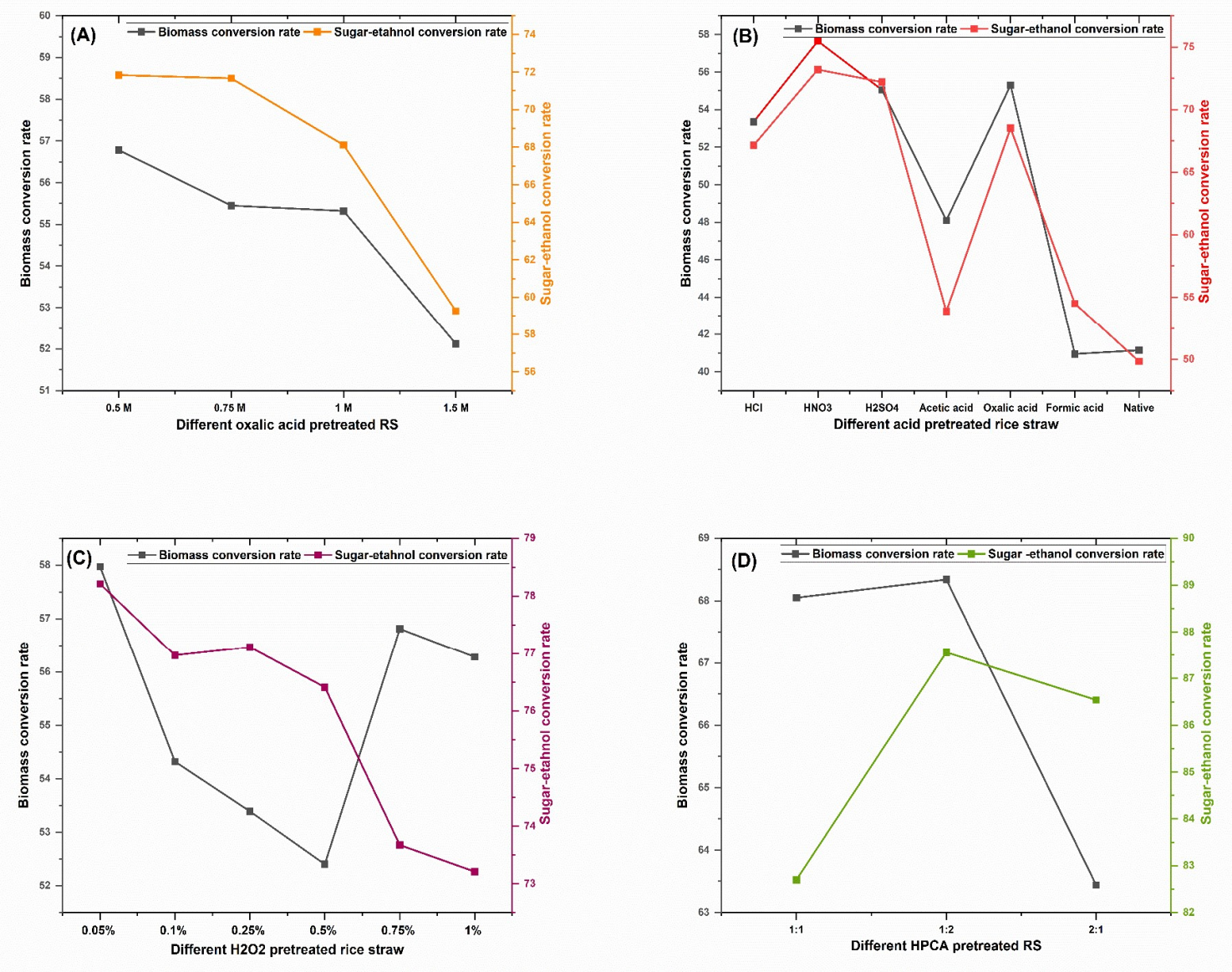

Figure 4.26- Biomass and sugar conversion rate **(A)** Oxalic acid pretreated rice straw **(B)** Different acid pretreated rice straw **(C)** H_2O_2 pretreated rice straw **(D)** HPCA pretreated rice straw

4.7. Bioethanol production from rice straw

The total bioethanol production yield and fermentation efficiency from the different acids, H_2O_2, HPCA and oxalic acid pretreated rice straw is illustrated in figure 4.27. The ethanol production yield was calculated based on ethanol concentration and total reducing sugar produced during the hydrolysis process, further based on it, maximum ethanol production was calculated from both hexose and pentose sugar obtained from SPS method and enzymatic hydrolysis process. Based on the theoretical yield of ethanol produced which was 65.71%, the fermentation efficiency was calculated. From the present study, it was evaluated that maximum ethanol production yield was obtained from H_2O_2 pretreated rice straw and a further increase was observed when rice straw was treated with HPCA in different ratios with a fermentation

efficiency of 74.73%. Citric acid is a weak poly-carboxylic acid, which is advantageous for preventing cellulose from being heavily degraded during acid hydrolysis and impacting the characteristics of modified non-cellulosic materials (Ji et al., 2019). However, the low acid ity of citric acid would make it difficult to hydrolyze the amorphous part of the cellulose, resulting in a low bioethanol production yield. Thus, utilizing HPCA is considered an effective method for enhanced bioethanol production. It was evaluated that under low xylan concentration in the pretreated rice straw, ethanol generation was increased under harsh pretreatment conditions. Further, HNO_3 pretreatment has been claimed as the preferred approach because of its shortened processing duration with better sugar production and the generation of substantially fewer inhibitory chemicals than sulfuric acid. Furthermore, HNO_3 is less corrosive and has a higher efficiency for removing hemicellulosic compounds than H_2SO_4 and HCl (S. Kaur et al., 2022). Earlier results demonstrated that nitrate released during HNO_3 treatment would serve as a nitrogen source to facilitate the fermentation process (Kim et al., 2014). Similarly, in this present study, HNO_3 pretreated rice straw resulted in a maximum bioethanol production yield with 42.5% fermentation efficiency.

Similar results were reported by Kundu & Lee, (2015) from deacetylated biomass along with 0.1 M oxalic acid pretreatment on yellow poplar at 150°C for 30 min, which resulted in ethanol production yield ranging up to 0.49 g/g from 0.29 g/g. In the present work, a pretreatment process with different concentrations of oxalic acid results in an overall maximum yield of 0.42 g/g biomass with 0.35 g/g of ethanol yield from acidic hydrolysate and 0.42 g/g of ethanol yield from enzymatic hydrolysate of 0.75 M oxalic acid pretreated rice straw.

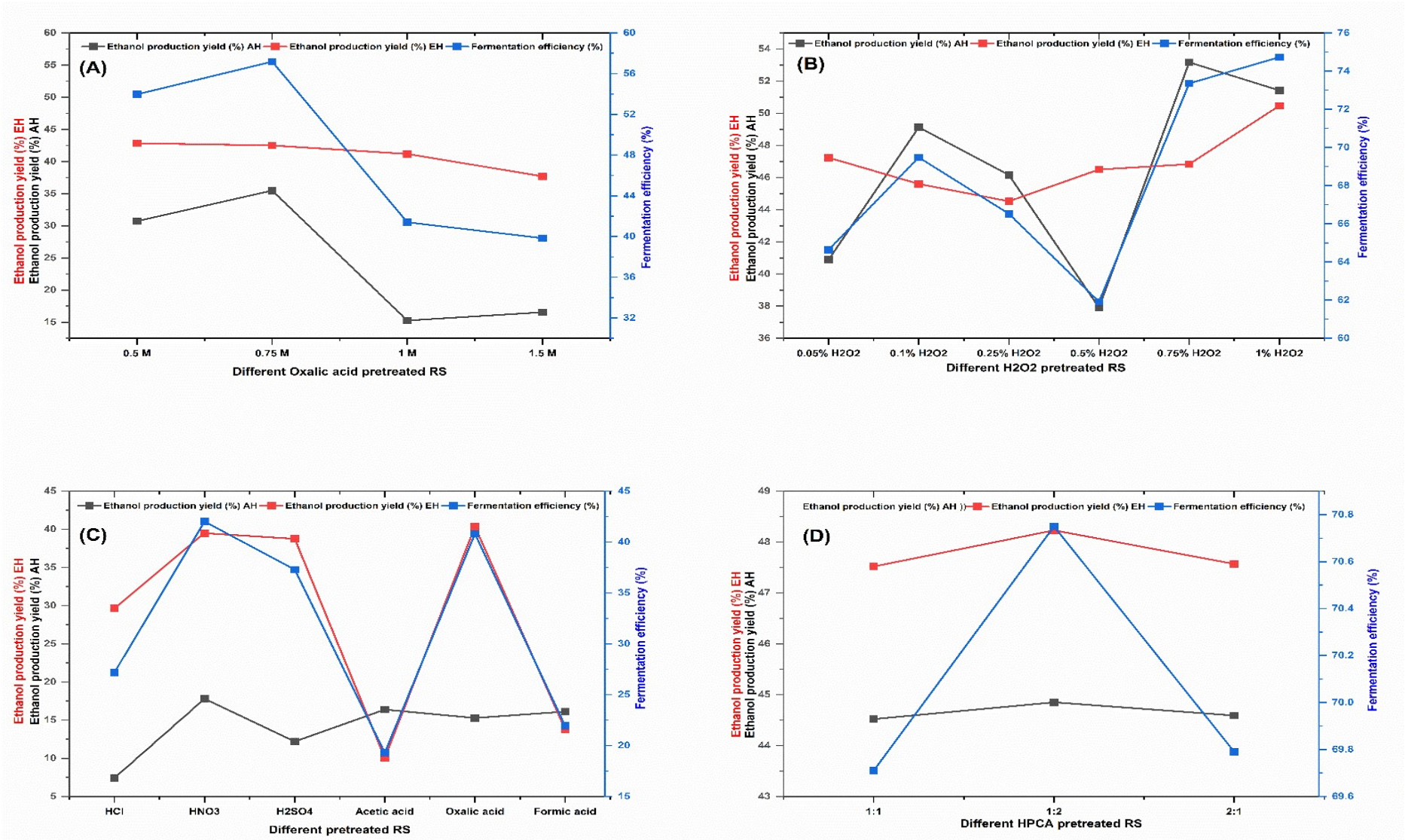

Figure 4.27- Ethanol production yield and fermentation efficiency (A) Different oxalic acid pretreated rice straw (B) Different H_2O_2 pretreated rice straw (C) Different acid pretreated rice straw (D) Different HPCA pretreated rice straw

4.8. Mass balance and cost analysis of bioethanol

The conceptual process design is used in the techno-economic analysis to determine the capital expenditure and operational expenditure of a manufacturing process of bioethanol production as well as mass balance analysis to implement present work to pilot scale. The quality of the criteria and assumptions determine how accurate the techno-economic study will be performed. This is based on a shaky hypothesis with an accuracy range of 60% which is considered as more precise criterion and assumption of this analysis. During conceptual process design, the experimental data or literature was evaluated with parameters for the required process performed as well as the operation cost of the major unit. Following this mass balance study, the bioethanol production cost from one kg of rice straw was dependent on the variable cost of different methods used for production. The overall mass balance analysis depends on the cost of chemicals used during the pretreatment process, enzymatic hydrolysis, fermentation and at last evaluation of total recovered sugar and ethanol. Accordingly, one kg of raw rice straw used for bioethanol production contains approximately 570 gm of hemicellulose, 125 gm of lignin and 324 gm of cellulose according to the biomass compositional analysis performed. It was found that native rice straw consists of 69.678 g/l of glucose and 72.43 g/l of xylose with an overall sugar release of 189.48 g/l, conforming that various pretreatment conditions can preserve the amount of sugar released. After various pretreatment processes were performed, approximately 369-570 gm of solid recovery was obtained. The overall sugar recovery after different pretreatment processes was quite higher than that of untreated rice straw. Similarly, among acidic treated biomass, 1 M HNO_3 pretreated rice straw has 31.2 g/l of total reducing sugar through the performed SPS method which was further enhanced to 35.3 g/l after enzymatic hydrolysis of pretreated rice straw. It was investigated that an 11.61% rise in glucose recovery occurs from the obtained hydrolysate.

The HNO_3-based pretreatment is expected to lessen the cost pressure on the cellulosic-based bioethanol production industry by streamlining after-pretreatment operations and providing a nitrogen supply for the subsequent fermentation process. Moreover, steam-exploded rice straw treated with H_2O_2 without adjustment of pH reduces the requirement for extra post-treatment operations. Similarly, the oxalic acid pretreatment was utilized by various researchers with the adaptivity of recycling of oxalic acid after the treatment process and was first utilized by Lee et al., (2013). This may boost the commercial feasibility of its usage. Further studies were done based on H_2SO_4 and HCl pretreatment evaluating that higher acid concentration i.e., >0.2M decreases the formation of 5-HMF. These findings might be attributed to the synthesis of Levulinic acid from 5-HMF, which is derived from monosaccharides (Meinita et al., 2015). Thus, to reduce the formation of inhibitors after SPS method using acid impregnation, 1 M acidic treatment was adopted throughout the study performed and which reduces the cost of acid utilized during the processing step. This exclusive hydrolysis process yields a high-quality hydrolysate that is fermentable to yield ethanol. The residual sugar in the acid-enzyme hydrolysate wasn't identified, implying that the earlier hydrolysis completely converted rice straw to fermentable sugar. In this study, it was demonstrated that

maximum sugar titers and yield could be obtained while reducing operational costs by utilizing both solid fractions as well as liquid hydrolysate obtained after SPS method using the whole slurry of pretreated rice straw.

From the present study of bioethanol production at pilot scale from 1 kg pretreated rice straw, it was assumed that 70 g/l of ethanol concentration was generated from 1 kg of rice straw from HNO_3 pretreated rice straw with 286 g/g of ethanol yield from pretreated rice straw while 85 g/l of ethanol was generated from H_2O_2 and 95 g/l of ethanol from HPCA pretreated rice straw in the ratio 1:2 with overall ethanol production yield of 480 g/g of pretreated rice straw. It was further assumed that the life cycle assessment (LCA) study of converting rice straw to 2G ethanol via the physiochemical pathway performed in laboratory has the potential to produce the greatest environmental sustainability in terms of GHG emissions reduction and resource depletion potentials when compared to other conversion routes and will be further implemented at large scale with the implementation of developed methods in the present study of biphasic system. As a result, converting rice straw to produce 2G ethanol appears to be quite appealing. Many rice-producing countries are interested in rice straws to generate bioethanol and reduce GHG emissions. The amount of chemicals used for the production of bioethanol at a large scale using 1 kg of rice straw and the cost involved during the production process is illustrated in Table 4.11.

Table 4.11- Usage and cost of chemicals during bioethanol production

Chemicals	Obtained from	Actual cost (Rs.)	Usage (gm or ml)	Unit price (Rs./gm)
Hydrogen peroxide	LOBA Chemie Pvt. Ltd.	472 (500 ml)	3.32 ml	3.1340
Citric acid anhydrous 99.5%	LOBA Chemie Pvt. Ltd.	354 (500 gm)	1.6409 gm	1.1617
H_2SO_4, 98%	LOBA Chemie Pvt. Ltd.	413 (500 ml)	55.29 ml	45.66
HNO_3, 69%	LOBA Chemie Pvt. Ltd.	366 (500 ml)	63.84 ml	46.7308
HCl, 98%	LOBA Chemie Pvt. Ltd.	380 (500 ml)	86.81 ml	65.97
Oxalic acid, 99.5%	LOBA Chemie Pvt. Ltd.	360 (500 gm)	63.03 gm	45.38
Acetic acid, 99.5%	LOBA Chemie Pvt. Ltd.	1534 (2500 ml)	57.42 ml	35.232
Formic acid, 85%	LOBA Chemie Pvt. Ltd.	230 (500 ml)	44.38 ml	20.4148
Cellulase enzyme	TCI chemicals Ltd.	2700 (1 gm)	1.17 mg	3.078
Sodium azide, 99%	LOBA Chemie Pvt. Ltd.	708 (100 gm)	0.833 gm	5.897
$MgCl_2.7H_2O$, 99.5%	LOBA Chemie Pvt. Ltd.	950 (500 gm)	1.5 gm	2.85
K_2HPO_4, 99%	LOBA Chemie Pvt. Ltd.	2900 (500 gm)	1.5 gm	8.7
$(NH_4)2SO4$, 99%	LOBA Chemie Pvt. Ltd.	3750 (250 ml)	1.5 gm	9.75
$CaCl_2.2H_2O$, 99.5%	LOBA Chemie Pvt. Ltd.	1500 (500 gm)	1.5 gm	4.5
Yeast extract	LOBA Chemie Pvt. Ltd.	1100 (500 gm)	10 gm	22

CHAPTER-5

SUMMARY

The current work attempts to address some of the challenges faced in the selection of high cellulosic agricultural waste to enhance the yield of bioethanol production with the utilization of both hexose and pentose sugar produced from the process of acidic and enzymatic hydrolysis respectively. Foremost is the compositional analysis of biomass utilized for bioethanol production which has played an important role in performing further processing steps. The obtained rice straw was subjected to compositional analysis resulted in 32.4% (w/w) cellulose, 57.08% (w/w) hemicellulose, 12.5% (w/w) lignin, 10.12% (w/w) extractive and 7.4% (w/w) ash content. The silica present in rice straw had a negative effect on enzymatic hydrolysis by requiring higher enzyme loading and ultimately increasing the production cost of bioethanol. Although, it has a significant role in rice production and acts as a shield for plants that ultimately hinders the pretreatment and enzymatic hydrolysis. Thus, extraction of silica prior to the pretreatment process enables higher recovery of lignin with low ash content. Among various pretreatment processes performed, it was illustrated that H_2O_2 pretreated biomass produces biodegradable material with higher recovery of glucose from the pretreated liquid. The use of H_2O_2 as an alternate impregnating agent for steam pretreatment seems desirable due to its low corrosivity and toxicity, as well as its ability to promote high cellulose conversion with less concentration of catalyst. Another vital factor is the potential of causing an alteration and oxidation in the structure of lignin, hence it becomes more effective as a metal chelating agent. In this present work, H_2O_2 pretreatment was preferred with citric acid (HPCA) making it a novel work and reducing the further production cost. This work was performed as an integrated method of physiochemical pretreatment technique and has proven to be an efficient method for breaking down lignocellulosic biomass structure. The optimum SE pretreatment condition was 103 kPa pressure maintained for 45 min with different concentrations of H_2O_2 along with HPCA. It was concluded that less concentration (0.05%) of H_2O_2 shows a maximum reducing sugar formation of 220.05 g/l of hydrolysate. Thus, steam explosion pretreatment of H_2O_2-impregnated rice straw shows better results with its ability to form radicals at higher concentrations of H_2O_2. The 0.05% (v/v) H_2O_2 loading along with citric acid in the ratio 1:1 was preferred due to its less toxicity and corrosivity than other pretreatment chemicals. The HPCA impregnation was performed using citric acid as one of the easily available weak acids to develop a cost-effective physiochemical pretreatment for bioethanol production. Thus, it was concluded that due to the strong oxidizing ability of H_2O_2, it was efficient for hemicellulose breakdown and delignification of lignocellulosic biomass, causing detachment and solubilization of lignin along with loosening of lignocellulosic recalcitrant structure.

Further, Liquid hot water treatment utilizing various acids with a main emphasis on HNO_3 and oxalic acid is considered an efficient pretreatment method. The two-phasic hydrolysis along with the co-fermentation process is considered the viable method of bioethanol production in terms of enhanced yield, productivity and concentration of obtained reducing sugar and bioethanol. The enhancement in the digestibility of rice straw was linked with the increase in the accessible surface as well as the removal of the non-cellulosic content of the substrate. This work is assumed to be an efficient method that makes biomass

highly reactive to enzymatic hydrolysis and easily feasible to produce glucose hydrolysate, which *S. cerevisiae* and *Z. mobilis* may effectively ferment into ethanol. The final biomass to sugar conversion rate was obtained to be 57.65% while the sugar ethanol conversion rate was estimated to be 73.2% from HNO_3 pretreated rice straw. The rice straw pretreated with HNO_3 and oxalic acid among strong and weak acids respectively were found to be efficient methods for the conversion of biomass to bioethanol. Consequently, the lower concentration of oxalic acid had maximum recovery of sugar with a maximum ethanol yield of up to 42.84% and fermentation efficiency of 57.18% from 0.75 M oxalic acid. It was evaluated that a maximum glucan yield of 90.23% with 28.19% of xylan content illustrates 43.63% of xylan removal after pretreatment of rice straw using 0.5 M oxalic acid. The present laboratory experiments should eventually be scaled up to examine the commercial processing of feedstocks into the intended products that involve biofuels and value-added products. Combining pretreatment with the saccharification process still needs more research, that will further enhance the implementation of bioethanol production at a commercial scale. Thus, the findings in this present work are intriguing, but more research into pretreatments to enhance simple sugar production while reducing inhibitor levels is needed further.

The longer time for the generation of value-aided bio-products has reduced the cost-effectiveness of desired product formation. It is required to integrate various time-consuming processes to reduce the time that plays an essential role in the bio-based product industry. So, to implement the process at a pilot scale there is the requirement for the development of various biphasic systems, that will benefit the industry on a larger scale and increase the yield of bioethanol by utilizing both hexose and pentose sugar. It was evaluated that during pretreatment, xylose was degraded to furfural due to maximum acid concentration, prolonged reaction temperature as well as time required for the pretreatment process. Thus, there is a requirement for maintaining the optimum reaction time and temperature to reduce inhibitor formation during the pretreatment process. In the present study, mild pretreatment conditions were utilized to avoid degradation of xylose to furfural and to use both cellulose and hemicellulose for further saccharification process using the selected conversion agent. Further, high-temperature treatment assisted with strong acids such as HNO_3, hydrolyses the hemicellulosic content and enhances further when treatment time was increased, due to hemicellulose removal, glucan and lignin content enhanced further in the solid fraction. In terms of compositional analysis of utilized chemicals and enzymatic hydrolysis of pretreated biomass, the performance of oxalic acid pretreatment employing recovered oxalic acid was quite steady. In this study, various SPS method was performed on rice straw, with shorter pretreatment time for various physiochemical method performed and mild CaO treatment on acidic hydrolysate resulting in enhancing the saccharification of biomass utilized for bioethanol production. Thus, it is required to optimize the pretreatment conditions to maximize the obtained sugar content and reduce the generation of degradation products in the form of inhibitors.

Among various acids utilized for the pretreatment process, it was earlier stated that HNO_3-based treatment offers a commercially viable option for pre-treating biomass and is completely consistent with the current circular economy paradigm. The type of acid catalyst required for SPS method was determined based on the amount of reducing sugar produced during the processing steps. This finding may be valuable in determining the commercial viability of acid pretreatment for ethanol production. In terms of environmental effects and operational costs, the lowest enzyme loading was 20 units which is capable of offering the optimum hydrolysis performance by enhancing the exposure of hydrolysis time. Further, optimized conditions for the pretreatment process played a significant role in bioethanol production. As, maintaining high temperature and pressure during the processing step is quite difficult, thus increasing the exposure time for the biomass to degrade is optimal way further with maximum lignin removal. In the present work, it was found that 120℃ is the optimum pretreatment temperature and further increasing the pretreatment temperature has led to the partial degradation of cellulose despite longer hydrolysis time.

CHAPTER-6
CONCLUSION

Second-generation (lignocellulosic) bioethanol production appears to be the most promising renewable feedstock for meeting Sustainable Development Goals. Several feedstock pretreatments have revealed process challenges in terms of yield and inhibitor formation during the process. However, the pretreatment of lignocellulosic biomass is a crucial step towards bioethanol production from available biomass due to the recalcitrant structure of LCBs. It is required for the delignification of biomass, i.e., the removal of lignin to make the availability of cellulose and hemicellulose for further processes of saccharification. Till now, the known pretreatment methods, i.e., physical, chemical, biological, and physiochemical approaches are enacted. Further advancement in these processes is required to develop the combined pretreatment for economically feasible processes. The main focus is to develop an efficient pretreatment method to remove the non-fermentable part of lignocellulosic biomass to get fermentable sugar. Subsequently, the combined process of pretreatment and saccharification has reduced the incubation time for process with more efficient desired outcomes. Thus, this will shorten the pretreatment time as well as, it will help in developing various new combined pretreatment processes at the required temperature, pH and retention time. Although the primary component of hemicellulose, xylose is the second-most prevalent and sustainable biomaterial, fewer of them have been implemented for producing bioethanol. This is probably because most yeast strains cannot transform xylose into ethanol. The economic viability of producing ethanol from lignocellulosic material would increase with the effective conversion of the pentose and hexose monomers. Recent research has centered on creating microorganisms that produce ethanol from xylose by adding xylose utilization mechanisms in yeast and other ethanol-producing organisms. Thus, a high concentration of sugar boosts the succeeding fermentation process's production of ethanol, which lowers the cost of product's distillation. It was evaluated that acidic treatment on the LCB resulted in lignin removal and hemicellulose degradation and formed amenable conditions for enzymatic hydrolysis and fermentation. The present study revolves around utilization of mild pretreatment conditions and mild acidic effect to produce environmentally sustainable process as well as an economically feasible process that will be further implemented at a commercial scale to enhance the overall production of bioethanol, utilizing two-phasic system in the way towards usage of both hexose and pentose formed from cellulose along with degradation of hemicellulose as well as delignification or removal of lignin component from the recalcitrant structure of utilized biomass.

To boost the economic viability of making bioethanol from lignocellulosic biomass, the generation of extra value-added chemicals from residual lignocellulose components such as hemicellulose or waste lignocellulosic biomass may be explored. As a result, in this research, the best pretreatment method was scaled while comparing the experimental findings to those of laboratory-scale pretreatment to pilot scale evaluating mass balance analysis along with the cost involved in the production of bioethanol. In the present study, a major outcome achieved was that a high aggregate ethanol yield was obtained by the two phasic systems of enzymatic hydrolysis and fermentation respectively from different pretreated rice straws,

indicating that the process was successfully scaled up. The present laboratory experiments should eventually be scaled up to examine the commercial processing of feedstocks into the intended products that involve biofuels and value-added products. Combining pretreatment with saccharification process still needs more research that will further enhance the implementation of bioethanol production at a commercial scale. Thus, the findings in this present work are intriguing, but more research into pretreatments to enhance simple sugar production while reducing inhibitor levels is needed further.

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List of publications

1. **A. Shukla**, D. Kumar, M. Girdhar, A. Kumar, A. Goyal, T. Malik, **A. Mohan**. Strategies of pretreatment of feedstocks for optimized bioethanol production : distinct and integrated approaches. Biotechnology for Biofuels Bioproduct, 2023:1–33. **Impact factor- 7.67**

DOI: 10.1186/s13068-023-02295-2

View online: https://doi.org/10.1186/s13068-023-02295-2

2. **A. Shukla**, D. Kumar, M. Girdhar, A. Sharma, **A. Mohan**. Steam Explosion Pretreatment with Different Concentrations of Hydrogen Peroxide along with Citric Acid : A Former Step towards Bioethanol Production. International Journal of Energy Research, 2023;13. **Impact factor- 4.67**

DOI: 10.1155/2023/2492528

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3). **A. Shukla**, R. Arora. Cost-effective production of environment-sustainable bioethanol from paddy straw by various pretreatment methods at a large scale. Journal of emerging technologies and innovative research, 2019;06. **UGC approved Journal**

View online: http://www.jetir.org/papers/JETIRDW06095.pdf

Oral presentation (conference proceedings)

1. Oral presentation at 3rd International Conference on Functional Materials Manufacturing and Performance (**ICFMMP-22)** with the topic "Review on sustainable approach towards waste management and bioenergy production: reducing fossil carbon emission". **The paper was accepted in API proceedings.** Held on 29th-30th July 2022.

2. Oral presentation at an international conference on Bioengineering and bioscience **(ICBB-22)** with the topic "Physiochemical pretreatment using steam explosion: An efficient process towards bioethanol production". Held on 18th- 19th Nov 2022.

3. Oral presentation in the international conclave on Materials, Energy and Climate **(ICMEC-22)** with the topic "Bioenergy resources in the way toward net-zero carbon energy systems". Held on 12th-14th Dec 2022.

International conference attended

1). Participated in **an** international conference on sustainability-life on Earth 2021 **(ICS-LOE 2021)** held on 17th- 18th Dec 2021 organized by the Department of Botany and Zoology, School of Bioengineering and Biosciences.

2). Participated in **"Advanced Composite & Functional Materials Congress-2023,** held from 27th- 30th April 2023

Workshop

1). Participated in a 3-day Indo-US workshop on the **Next Generation of STEM scientists (NGSS-2022)** held by Marwadi University in collaboration with NIT Uttarakhand, University of Virginia-USA, Lovely Professional University, Illinois State University USA on 4th-6th August 2022.

2). Participated in an International workshop on "References & Citations" organized by DIGISHAKSHAM on 27th March 2022.