IMPLEMENTATION OF STUBBLE WASTE FOR BIOTRANSFORMATION TO INDUSTRIALLY IMPORTANT CHEMICALS

A Thesis Submitted
In Partial Fulfillment of the Requirements
for the degree of

DOCTOR OF PHILOSOPHY

by

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I Neha Kukreti hereby certify that the work which is being presented in the thesis entitled **"Implementation of stubble waste for biotransformation to industrially important chemicals"** in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy, submitted in the Department of Biotechnology, Delhi technological University is an authentic record of my own work carried out during the period from 01.08.2019 to 22.04.2024 under the supervision of Prof. Pravir Kumar, Department of Biotechnology, Delhi Technological University and co-supervision of Dr. Rashmi Kataria, School of Bioscience and Technology, Vellore Institute of Technology, Vellore, India.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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ACKNOWLEDGEMENTS

I express my sincere gratitude to people for all their contributions that helped me a lot to make this research a fabulous success.

First and foremost, praises and thanks to the God, the Almighty, for showering blessings throughout my research work to complete the research successfully. I am very grateful and thankful to the Vice- Chancellor of Delhi Technological University and HoD of Department of Biotechnology, Delhi Technological University for permitting me to pursue my Ph.D. research work and providing the laboratory facilities to carry out the work at Delhi Technological University.

I acknowledge and have high regards and respect for my supervisor, Professor Pravir Kumar, Dean International Affairs and former Head of Department, Department of Biotechnology, DTU for guidance, supervision and constant support throughout the research process.

I would also like to thank my co-supervisor, Dr. Rashmi Kataria, Department of Bioscience and Biotechnology, Vellore Institute of Technology for her enduring support for carrying the experiments and much appreciated advice throughout my research work.

I am thankful to my fellow Ph.D. research scholars – Mehar, Neetu, Rahul, Shefali, Shrutikirti in the laboratory and Ms. Sonika Kag for her support, assistance, and compatibility over the years. The acknowledgement would be incomplete without crediting the teaching and non- teaching staff in the Department of Biotechnology for their help in every possible way. Sincere gratitude to Department of Chemistry, DTU, AIIMS, JNU, MTCC and NCIM for helping in analysis facility to ensure the accuracy

and reliability of the research findings. Thanks to the funding agencies DBT and SERB, Govt. of India for providing the research grant to carry out research work.

Last but not the least, to my dearest parents for their deep consideration, support, encouragement, finances for me to achieve success and family members for their support throughout my Ph.D.

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- **Kukreti N**, Kumar P and Kataria R (2024), A sustainable synthesis of polyhydroxyalkanoate from stubble waste as a carbon source using Pseudomonas putida MTCC 2475. Front. Bioeng. Biotechnol. 12:1343579. doi: 10.3389/fbioe.2024.1343579 **(I.F-5.7)**

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IMPLEMENTATION OF STUBBLE WASTE FOR BIOTRANSFORMATION TO INDUSTRIALLY IMPORTANT CHEMICALS

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ABSTRACT

The lignocellulosic biomass - paddy straw, corn straw, wheat straw etc are known as stubble waste. Setting fire to stubble waste is the source of air pollution causing major risks to human health and the environment. The problem which occurs due to its burning has an impact on the local and regional level. It releases harmful gases in the atmosphere together with particulate matter and kills useful micro-organisms in the soil as well as causing soil erosion. The proper utilization of stubble waste is of utmost importance to reduce the strain on the environment. Thus, treating lignocellulosic waste as a valuable resource is an essential pillar in waste management. In the proposed project, compositional analysis will be done for homogenized corn stover. It was fermented using two methodologies – Solid state fermentation (SSF) and Submerged fermentation (SmF) to produce microbial enzymes and Polyhydroxyalkanoates (PHA).

Corn stover is the residue that is left in the field after the grain is harvested and thus, can be used as feedstock for valuable product formation. Corn stover undergoes pretreatment to break the recalcitrant and complex structure of biomass and expose the carbohydrate fractions. Pretreatment methods include acid / alkali hydrolysis, steam explosion, enzymatic hydrolysis and others. After the pretreatment method, cellulosic and hemi cellulosic fraction is hydrolyzed to attain monomeric sugars like glucose and

xylose. The sugar hydrolysate gained from the corn stover is then fermented using microorganisms to convert to PHA. The process of fermentation includes cultivating microorganisms under controlled parameters such as temperature, Ph, time and nitrogen / carbon supply. The extraction of PHA from the biomass by using methods such as solvent extraction, precipitation and cell disruption techniques. Furthermore, purification of the extracted PHA to remove the impurities.

Polyhydroxyalkonate are biodegradable polymers which can be produced by microorganisms from lignocellulosic biomass. Cheap and easily available raw material such as corn stover waste has potential to lessen the cost of PHA synthesis. In this research study, corn stover is utilized for sodium hydroxide alkali pre-treatment and optimization with central composite design (CCD) for high cellulose and low lignin followed by characterization using FT-IR, TGA and SEM. Design expert performed optimized condition of alkali pre-treated corn stover for high total reducing sugar (TRS) enhancement using CCD using Response surface methodology (RSM). The optimized condition by RSM produced total reducing sugar yield of 707.19 mg/g. Fermentation using corn stover hydrolysate by *Pseudomonas putida* MTCC 2475 gave mcl- PHA detected through GC-MS/MS and characterization of PHA film by DSC, FTIR and NMR. Thus, this research paper focus on use of agriculture (stubble) waste as alternative feedstock for PHA production.

Phanerochaete chrysosporium is a white rot fungus is capable to produce comprehensive extracellular enzymes. These microbial enzymes have important applications in disrupting complex structure of plant cell wall, decolourization of synthetic dyes, de-pulping and many more. Solid state fermentation is an economical and sustainable process. Hence it is used for high enzyme production yield by using lignocellulosic biomass as substrate. Lignocellulytic enzymes like are produced from

an agriculture waste. In this research paper, untreated as well as alkali pre-treated corn stover were used as substrate for enzyme production. Fungal strain was used for enzymes including cellulases and Manganese peroxidase production. The maximum endoglucanase production was observed 121.21 ± 0.90 U/ml on 9^{th} day and 79.75 ± 0.57 U/ml on 6^{th} day in untreated and treated biomass respectively. The maximum exoglucanase production was reached 2.46 ± 0.008 FPU/ml on 3^{rd} and 0.92 ±0.002 FPU/ml on 6^{th} day in untreated and treated biomass respectively. The maximum manganese peroxidase production was reached on 5076.81 U/L on 6^{th} and 1127.58 ±0.23 U/L on 3^{rd} day untreated and treated biomass respectively. Thus, corn stover is abundant renewable sustainable biomass for enzyme production.

The economic viability of using corn biomass for PHA and microbial enzymes production depends on many factors like the availability of cost stover, pretreatment efficiency, PHA / enzymes yield and market demand of the bioproducts. The sustainable practices and conversion technologies is crucial to bio transform corn stover to PHA and microbial enzymes with the help of commercially viable and environment friendly methods.

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LIST OF ABBREVIATIONS

Symbol or abbreviations	Description
LB	Lignocellulosic biomass
LB	Luria-Bertani media
PHA	Polyhydroxyalkonate
PHB	Polyhydroxybutyrate
SCL	Short chain length
MCL	Medium chain length
RSM	Response surface methodology
sp.	Species
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared spectroscopy
TGA	Thermal gravimetric analysis
GC-MS/MS	Gas chromatography Mass spectrometry-Mass spectrometry
NMR	Nuclear magnetic resonance
SEM	Scanning electron microscopy
CCD	Central composite design
DCW	Dry cell weight
DNS	Di-nitrosalicyclic acid
FPA	Filter paper assay
O.D.	Optical density
HCl	Hydrochloric acid
NaCl	Sodium chloride
NH_4SO_4	Ammonium sulphate
H_2SO_4	Sulfuric acid
$CHCL_3$	Chloroform
$CDCL_3$	Denatured chloroform
CH_3OH	Methanol
KBr	Potassium bromide
$CaCO_3$	Calcium carbonate
NaOH	Sodium hydroxide
SSF	Solid state fermentation
SmF	Submerged fermentation
mM	Milli meter
TRS	Total reducing sugars
MTCC	Microbial type culture collection
ºC	Degree Celsius
P. putida	*Pseudomonas putida*
α	Alpha
µ	Micron
U	Enzyme units
ml	Milli-litre
g	Gram
mg	Microgram

RPM	Rotation per minute
%	Percentage
NCIM	National Collection of Industrial Microorganisms
P.chrysosporium	*Phanerochaete chrysosporium*
nm	Nano meter
DW	Dry weight
CMC	Carboxymethyl cellulase
LiP	Lignin Peroxidase
MnP	Manganese peroxidase
Lac	Laccase
Cm	Centimeter
BG	Beta glucosidase
UV	Ultraviolet
Kg	Kilogram
L	Litre
β	Beta
PNPG	Beta nitrophenyl b-D-glucopyranoside
ABTS	2,3- ethylbenzothiazoline-6-sulphonic acid
$MnSO_4$	Manganese sulphate
Na_2SO_3	Sodium suphite

CHAPTER 1

INTRODUCTION

The economic development and population growth of the countries has increased the industrial activities worldwide which has shoot up the world's consumption of energy (Wijaya et al., 2014). This corresponds in the annual biomass production which is estimated to be 1×10^{11} tons (Baadhe et al., 2014). There are various options for renewable energy resources, but lignocellulosic biomass is economic, ample carbon source on earth, renewable, low cost, available in abundance and environment friendly due to their chemical composition(Saldarriaga-Hernández et al., 2020; Sud et al., 2008; Wijaya et al., 2014). Several types of biomass sources are available such as municipal waste, agricultural waste, forest waste, plastic waste, energy crops and animal waste which can be used as a sustainable and energy secured alternate carbon source (Gollakota et al., 2018). Lignocellulosic biomass can be classified as wood – energy crops such as switch grass, willow, poplar; agriculture waste such as sugarcane bagasse, wheat straw, corn stover, rice straw, corn stalk; municipal solid and industrial waste such as wastepaper, paper mill sludge recycled newspapers; forest waste such as mill scrap, wood waste, saw dust (Hossain et al., 2020).

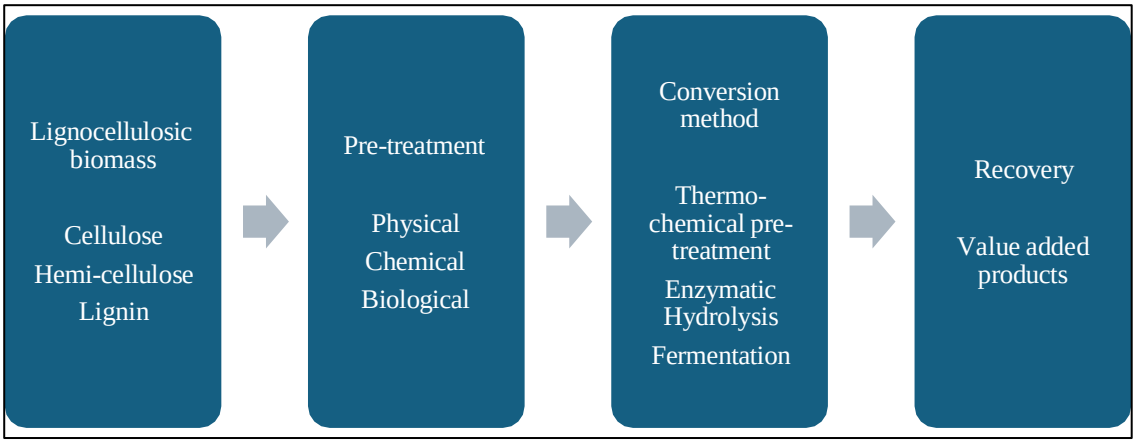


Figure 1. The main steps invoved in conversion of lignocellulosic biomass to value added products.

1.1 Lignocellulosic Biomass (LB) – Stubble Waste

Lignocellulosic biomass is highly recalcitrant and resistant and is difficult for enzymatic breakdown thus pre-treatment is important to enhance interaction of biomass to enzymes. The main objective of all the pre-treatment methods is to optimize the yield of the fermentable sugars from biomass. The sugar yield not only depends on the characteristics of the biomass but also on the interactivity with the conditions of the pre-treatment and formulations of enzymes. The different physio-chemical pre-treatment includes grind milling, auto/hot water hydrolysis, steam explosion, acid and alkali treatment, hydrothermal hydrolysis. Pre-treatment of biomass is an essential step to change the recalcitrant structure of biomass and to have high yield of products via bioconversion processes (Timung et al., 2015). The high recalcitrant structure of biomass is associated with covalent and hydrogen bonds formation between cellulose, hemi-cellulose and lignin. The solubilization of bio-polymeric structure is difficult due to the presence of lignin and thereby inhibits the hydrolysis of cellulose and hemicellulose (Baruah et al., 2018). Enzymatic hydrolysis is obstructed due to the complex structure of the lignocellulosic biomass (Moodley & Gueguim Kana, 2019).

1.2 Chemistry of LB

Lignocellulosic biomass is composed of cellulose, hemi-cellulose and lignin and is an excellent alternative to fossil feedstock which can be utilized for the synthesis of value-added products as shown in **Figure 1**. Cellulose $(C_6H_{10}O_5)n$ is a homopolysaccharide composed of linear chain of beta 1,4-linked d-glucose units (Baruah et al., 2018; Tathod C Dhepe, 2015). Hemicellulose $(C_5H_8O_4)n$ is a heteropolysaccharide including xylose, arabinose, mannose, galactose, glucose, fructose, glucuronic acid and galacturonic acid in various amounts depending on the source (Baruah et al., 2018; Machmudah et al., 2017). Lignin $[C_9H_{10}O_3(OCH_3)0.9{-}1.7]x$ is a complex polymer compose of aromatic monomers such as synapyl alcohol, coniferyl alcohol and p-coumaryl alcohol (Tathod & Dhepe, 2015; Yue & Economy, 2017).

1.3 Corn Stover – As an Agricultural Waste

Agricultural wastes are produced in vast amounts of two billion tons every year in the world (Millati et al., 2019). This waste has a diverse chemical composition, and after cultivation and manufacturing processes, a considerable amount of biomass is produced from major crops and with the help of pretreatment methods and technologies biomass can be converted to valuable products as shown in **Figure 2**. In the agricultural sector, crop waste is generated in the form of cornstalk, fruit peel, vegetable peel, sugarcane-bagasse, sunflower–stalk, and rice-stalk, rice husk, wheat straw and sugarcane etc (Millati et al., 2019; Sud et al., 2008). Among these, corn waste, also known as corn stover, consists of maize (corn) stalks, leaves, cobs and these lignocellulosic wastes constitute lignin, cellulose and hemicellulose. Cellulose, a homopolysaccharide made of a linear chain of ß-1,4- d-glucose units, is present in a significant amount in plant cell walls (Baruah et al., 2018; Tathod C Dhepe, 2015). Hemicellulose is a heteropolysaccharide of xylose, arabinose, mannose, galactose, glucose, fructose, glucuronic acid and galacturonic acid and present in variable amounts in plant cells (Baruah et al., 2018; Machmudah et al., 2017). Lignin is a complex polymer composed of aromatic monomers like synapyl alcohol, coniferyl alcohol and p-coumaryl alcohol (Tathod C Dhepe, 2015) as given in **Figure 3**. Corn cob is cut apart from corn kernels either manually or using machines during manufacturing processes (Millati et al., 2019). The composition of corn stalks is 35.0 cellulose (wt%), 14.4 hemicelluloses (wt%) and 21.5 lignin (wt%). The corn cobs are composed of 33.7 cellulose (wt%), 31.7 hemicellulose (wt%) and 6.1 lignin (wt%) (Ifeanyi et al., 2016). Corn is also rich in silica and carbon compounds. Corn stover is a sustainable resource for second-generation (Millati et al., 2019).

1.4 Processes Involved in the Conversion of Corn Waste

The biochemical steps for utilization of corn waste involve are pretreatment, saccharification of pretreated corn biomass and using enzymes like cellulases, xylanases and the last step is the fermentation of monosaccharide sugars. Different microbes are used for the production of desirable products (Khare et al., 2015). The

corn waste biomass is also utilized for solid-state fermentation for microbial production of enzymes. The overall scheme is shown in (Fig. 10.3).

1.4.1 Pretreatment

Pretreatment of lignocellulosic biomass such as corn biomass is an important step for removing lignin, decreasing the polymerization and crystallinity of cellulose, increasing surface area for enzyme activity, and releasing several reducing sugars (Behera et al., 2014). The resulted sugars are converted to value-added products and metabolites. There are different types of pretreatments, like physical, chemical or biological (Fig. 10.1). Physical pretreatment includes milling, grinding, mechanical extrusion, microwave, ultrasound, pyrolysis (Chen et al. 2019). Chemical pretreatment uses alkali, acid, oxidizing agents to degrade recalcitrant lignocellulose in corn waste with or without combination to temperature (Behera et al., 2014). There are structural changes in corn waste after the pretreatment step, which affects the enzymatic hydrolysis and microbial fermentation. The degradation of cellulose leads to the formation of glucose sugars, and hemicellulose degradation forms glucose, xylose, arabinose, mannose, galactose, and rhamnose sugars. Dilute acid pretreatment with HCl is the efficient industrially proven method for lignocellulosic biomass conversion and production of low-cost fermentable sugars (Zviely, 2013). Acidic pretreatments lead to disruption of van der Waals forces, hydrogen bonds and covalent bonds that hold the corn biomass components, which leads to the disruption and solubilization of hemicellulose fractions. Hemicellulose - xylan is hydrolysed during the acidic chemical pretreatment method. In the alkali chemical pretreatment, acetate group from the hemicellulose fraction is removed. Thus, an easy action of hydrolytic enzymes on carbohydrates polymer can take place; therefore, this process is effective for the delignification of corn waste (Bhutto et al., 2017). Biological pretreatment of lignocellulosic biomass is done with either microorganisms or enzymes and requires less energy. It is eco-friendly as there is no release of toxic compounds, effluents and fermentation inhibitors (Sindhu et al., 2016). Several enzymes involved in the pretreatment of corn waste are laccase, lignin peroxidase, manganese peroxidase and other microorganisms.

1.4.2 Hydrolysis

The production of value-added products from corn waste involves saccharification, leading to the bioconversion of complex sugars into simple sugars (Nagoor Gunny et al., 2019). The hydrolysis can be performed by enzymes such as cellulase, hemi-cellulases, ligninase or acids such as H_2SO_4, HNO_3 or alkali such as NaOH, KOH (Abdu et al., 2020). The concept of using essential enzymatic cocktails to have a higher yield during lignocellulosic biomass saccharification. Three major enzymes are involved in cellulosic biomass hydrolysis that is endo-glucanase, exo-glycanase and β-glucosidase. The repeating units of glucose are present in a linear chain linked by β-1,4- glycosidic bonds in cellulose structure. Different oligosaccharides are formed due to the action of endo -1,4-β glucanase and exo-1,4-β-glucanase on cellulose and cellobiose. β-glucosidase acts on cellobiose and forms glucose (Abdullah et al., 2021; Dhillon et al., 2011). Hemi-cellulases are a group of enzymes that are involved in the breakdown of galactans by D- galactanases, xylans by D-xylanases, mannans by D-manases and arabans by L-arabinose. The enzymes endo-xylanase and 1,4- β-D- xylan xylanohydolase hydrolyze the β-D- xylano pyranosyl linkages in xylan and forms xylo-oilgosaccharides. β-D- xylosidase hydrolyzes xylobiose to form D - xylose(Meena et al., 2017). The nature of the substrates and source of enzymes play a role in biomass hydrolysis (Maitan-Alfenas et al., 2015). The lignin part of lignocellulosic biomass is degraded by lignin peroxidase, manganese peroxidase and laccase enzymes (Khare et al., 2015).

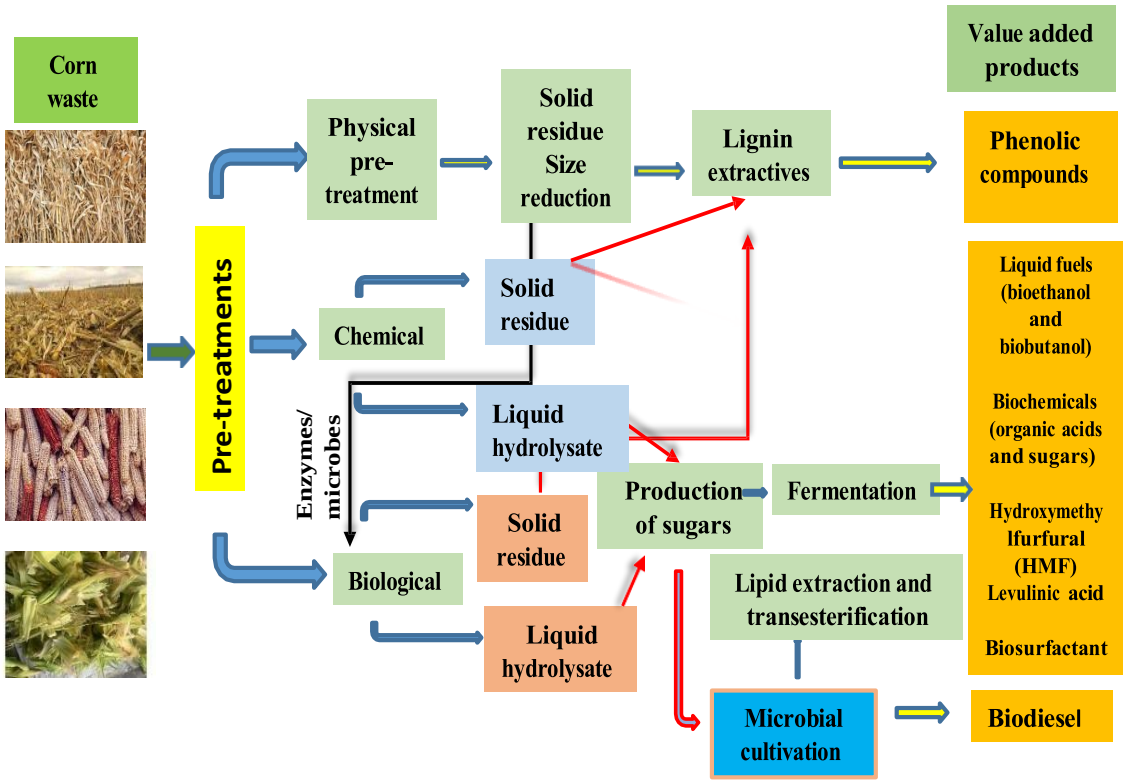


Figure 2. A roadmap of the trends and technologies involved in the corn waste valorization.

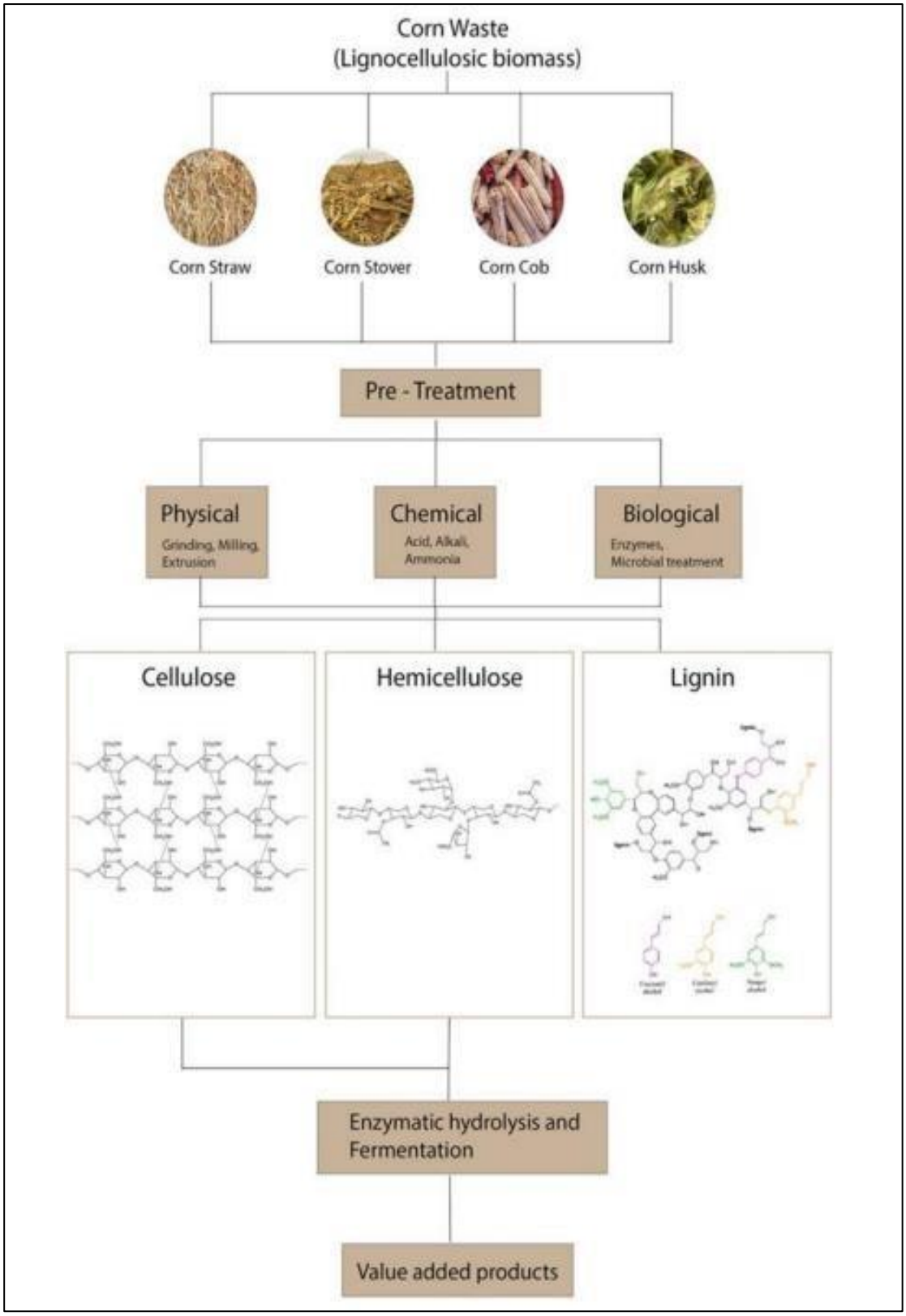


Figure 3. Pretreatment of corn waste (corn straw, stover and husk) to yield carbohydrate fraction and lignin for value-based chemicals.

1.4.3 Fermentation

Fermentation is a process having a series of chemical reactions that convert sugars into alcohols or acids with the help of yeast and bacteria. Corn waste, after physical and chemical pretreatment, forms liquid hydrolysate, which is composed of monomeric sugars, it is further fermented by microorganisms into valuable products (Khan et al., 2013). There are different types of fermentation methods such as solid-state fermentation, submerged fermentation, dark fermentation and photo-state fermentation. The solid-state fermentation (SSF) process uses solid support to produce various microbial products like antibiotics, single-cell protein, PUFA's, enzymes, organic acids, biopesticides, biofuels (Bhargav et al., 2008; Sukumaran et al., 2009). Submerged fermentation (SmF) uses free liquids such as molasses and broth where the product formed after fermentation is present in the broth (Suriya et al., 2016). The dark fermentation process occurs in the absence of light and uses anaerobic bacteria to degrade the organic content (Borole C Greig, 2019). In the Photo-fermentation process, sunlight is the energy source and green algae provide electrons for photosynthesis by breaking down the endogenous substrate (Sağır C Hallenbeck, 2019). Microbial fermentation has great potential in conversion of lignocellulosic biomass to bio-based products (Ma C Ruan, 2015; F. Q. Wang et al., 2013). Different fungi produce lignocellulolytic enzymes which degrade lignocellulosic matter in synergistic manner. Group of enzymes capable of degrading lignocellulosic material are called as lignocellulolytic enzymes. Cellulolytic enzymes are endoglucanase, exoglucanase and β-glucosidase. Hemi-cellulolytic enzymes are endoxylanases, arabinofuranosidase, β-xylosidases, feruloyl esterase. Ligninolytic enzymes are laccases, lignin peroxidase, manganese peroxidase, and versatile peroxidase (Jayasekara C Ratnayake, 2019). The popular way to utilize any biomass is by enzymatic conversion into fermentable sugars as given in **Figure 4**.

1.5 Value – Added Products from Corn Waste

1.5.1 Enzymes Production using LB

Corn stover biomass is abundantly available lignocellulosic waste across the world after harvesting the crop. The agriculture waste contains high total organic matter and is also decomposed slowly (H. Liu et al., 2018) and thus is worthwhile bioresource to be utilized for enzyme production by using solid state fermentation (SSF) approach (Kapoor et al., 2016). Corn stover has highly recalcitrant structure with three major components that is cellulose, hemicellulose and lignin as shown in **Figure 5** and due to rigid lignin component, access to cellulose and hemicellulose becomes difficult. This reduces the degree of hydrolysis. It is a potential carbon source for production of value-added products (Raja Sathendra et al., 2019). Pre-treatment is vital to disrupt crystalline macro as well as microfibrils and expose polymer cellulose and hemicellulose(Ahmed et al., 2019). Physical and chemical pre-treatment is requisite to disorganise lignocellulosic components of biomass. Biological pre-treatment is environment friendly method as no inhibitors are generated (Andlar et al., 2018). SSF has immense potential for the enzyme production and there are advantages of SSF over submerged fermentation (SmF) such as high productivity, high amount of product generation, lower effluent formation and need of less complicated equipment for fermentation (Patil, 2011). *Phanerochaete chrysosporium* is able to produce a group of enzymes like: cellulase, hemicellulose, ligninase, manganese peroxidase and amylase using SSF(Tirado-González et al., 2016). *P. chrysosporium*, is reported as most active ligninolytic micro-organism. It degrades lignin component and various aromatic compounds due to the presence of non-specific extracellular enzymes including MnP (Manganese peroxidase), LiP (Lignin peroxidase) and Lac (Laccase) (Min et al., 2022). The enzymes produced has role in breakdown decomposition of lignocellulosic complex structure of biomass as well as decolourization of dyes, bio pulping, bio bleaching used in industries. Biological pre-treatment method using micro-organism is environment friendly, efficient and requires low energy (Andlar et al., 2018). In present work, focus is on to use agriculture (stubble) biomass as substrate rather than media components as nutrient source for fungus and further to compare growth in untreated and treated corn stover biomass. This study describes

the utilization of economic abundant agricultural waste as raw material for enzyme production in untreated and alkali pre-treated biomass. It explains the efficacy of recalcitrant polysaccharide as substrate which lowers the cost of enzyme production. It also validates a novel sequential cultivation of *Phanerochaete chrysosporium* NCIM 1106 for cellulase and manganese peroxidase production.

Xylan is the second most abundant polysaccharide and is a major component of the plant cell wall. The binding properties are mediated by covalent and noncovalent interactions with lignin, cellulose and other polymers (Subramaniyan C Prema, 2002). The hydrolysis of xylan yields xylose. Plant feedstock including corn cob is composed of C6 sugars from cellulose and C5 sugars from hemicellulose. The production of corn cob from the processing of maize crop are nearly 180 kg of corn cob from each tonne of maize. The microorganisms for xylanase production are *Bacillus* sp., *Bacillus megaterium, Streptomyces viridochromoge, Aspergillus terri-cola, Aspergillus ochraceus, Humicola brevis, Penicillium sp., Aspergillus fumigatus* (Simair et al., 2018). Corn stover media is used for cellulase and xylanase production with white-rot fungi (Tirado-González et al., 2016). In a study, corn cob was used for xylanase production using wild type and UV mutated *Aspergillus niger.* The bio enzyme production from cheaper substrates such as corn cob is beneficial and reduces the negative environmental effects of these wastes. Xylanase can be produced from agricultural waste with new thermophilic *Bacillus cereus* strain TH-050 by solid substrate fermentation and non-sterilized solid substrate fermentation (Ire et al., 2021). The application of xylanase includes in paper and pulp industry, clarification of fruit juices and food industries. This enzyme is also used in agribusiness in animal feed to break down, such as arabinoxylans that reduce the viscosity of raw material (Simair et al., 2018).

Laccases are multicopper proteins found in higher plants, fungi, insects and bacteria. Cornstalk is used as the substrate for laccase production using solid-state fermentation. It's easily available and low cost play a major role in extracellular enzyme production from microorganisms. Pretreatment of the substrate is necessary before the fermentation process. The maximum laccase production from corn stalk waste observed as 6.88 U/m L using steam explosion pretreatment and

solid-state fermentation (Perdani et al., 2020). Corn steep liquor also demonstrated as a nutrient for improving laccase production by *Trametes versicolor* (F. Wang et al., 2014). Laccase has application in delignification of pulp, oxidation of organic pollutants, decolorization of and detoxification of industrial effluents. The application of laccase is in higher plants for lignification process and degradation of humic acids. The reactive radicals lead to the formation of monomers by the cleavage of covalent bonds. The ring cleavage of aromatic compounds is catalyzed by laccase and used to degrade xenobiotics like synthetic dyes and nitroaromatics (Kuddus et al., 2013).

The steps involved in the production of beta-glucosidase from corn cob are pretreatment, enzymatic hydrolysis and fermentation. There are mild conditions for enzymatic hydrolysis such as temperature 30–50°C and pH 5. In a study corn cob cut into small pieces, dried and pretreatment in autoclave conditions that were 121°C for 15 min to obtain carbohydrate fraction from the corncob biomass to remove lignin compounds. Fermentation was done using Aspergillus niger following solid-state fermentation. The nutrients and substrate were in the ratio 1:15. The crude enzyme fraction was extracted using 0.1 M phosphate buffer and pH maintained at 7. The samples for the analysis of beta-glucosidase during fermentation analyzed for enzyme activity. Fermentation using corn cob produced beta-glucosidase with enzyme activity 95.01 U/ml (Aliyah et al., 2017).

Beta-glucosidase is a component of the cellulase enzyme complex and is responsible for the complete hydrolysis of cellulose into glucose (Bai et al., 2013). It has industrial applications such as improved the conversion rate, easier separation of product from the reactant broth, low cost of commercial production (Hati et al., 2020; Jeng et al., 2011). This enzyme cleaves the beta-glycosidic linkage in disaccharides. It has other applications in medical, biotechnological, agricultural, industrial (Jeng et al., 2011).

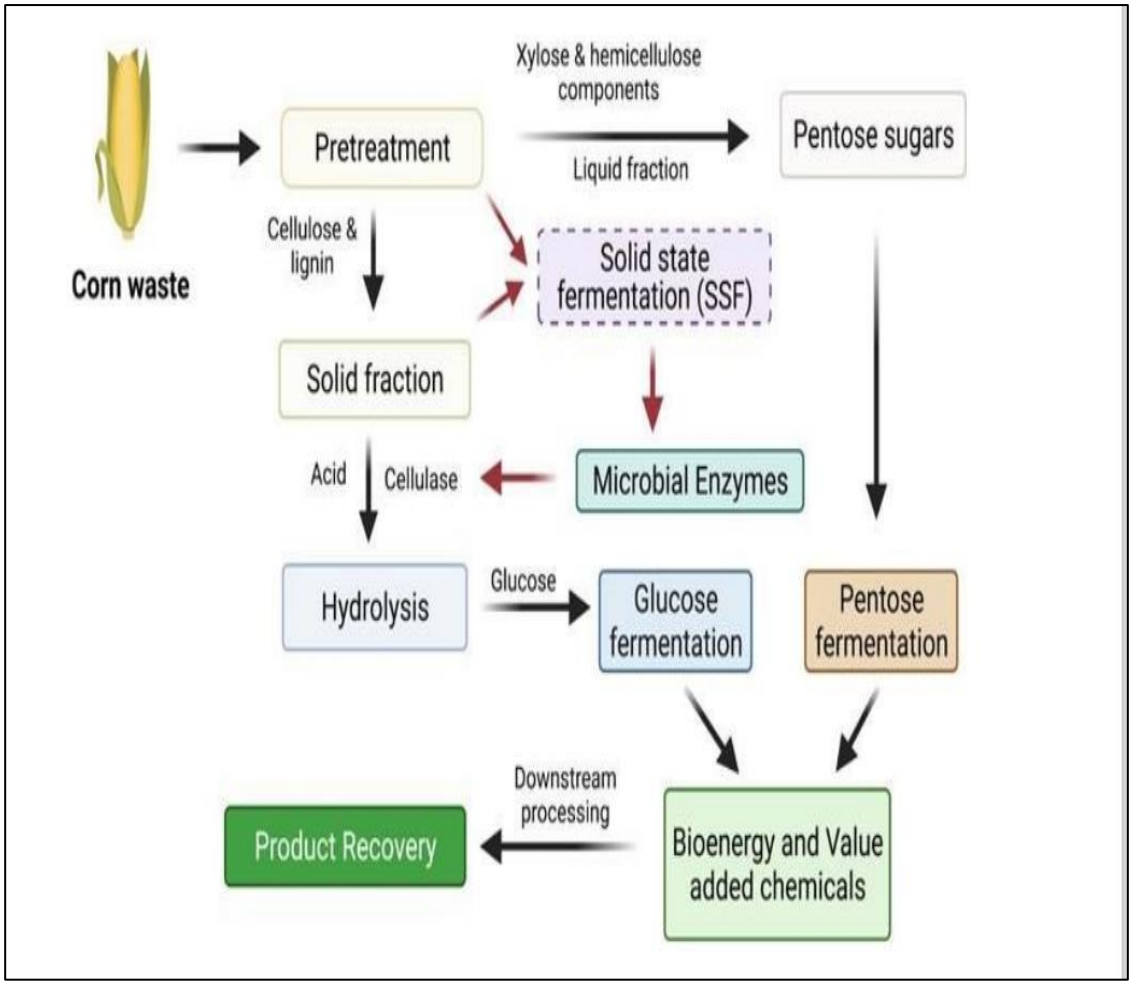


Figure 4. Overall steps involved in the corn waste carbohydrates to energy and metabolites production.

1.5.2 Polyhydroxyalkonate Production using LB

PHA, or polyhydroxyalkanoates, are a type of biodegradable and renewable biopolymer that hold great potential as a sustainable alternative to petroleum-based plastic. With plastic production reaching a staggering 448 million tons in 2015 and is estimated to double by 2050, it is imperative to find ecological solutions. Synthetic plastics not only pose a threat to the environment but also to all living organisms on Earth. The appeal of petroleum-based plastics lies in their lightweight nature and cheap manufacturing, making them abundantly usable (Phanisankar et al., 2020). To address the pressing issues surrounding the generation of petroleum-based plastics and the widespread use of single-use plastic, the production of biodegradable biopolymers becomes crucial (Ashby et al., 2022). Recently, there has been a growing interest in using lignocellulosic biomass (LB) as a carbon source

for the production of polyhydroxyalkanoates (PHA). LB is highly valuable due to its renewability, abundance, and easy availability. If left unutilized, LB is often discarded in fields or burnt, leading to pollution that poses risks to human health and the environment (Saldarriaga-Hernández et al., 2020). Therefore, corn stover, a type of LB, can be effectively utilized for PHA production. It offers advantages such as economic feasibility, easy availability, no transportation issues, carbon neutrality, and sustainability. India, being the third largest producer of corn globally, cultivates approximately 22.3 million metric tons of corn stover each year, making it a suitable and substantial resource for PHA production. Annual global production of corn stover is nearly 1 billion tons. Corn stover primarily consists of cellulose, hemicellulose, and lignin, with lignin being composed of polyphenolic compounds. The presence of lignin impedes enzymatic hydrolysis, necessitating pre-treatment for efficient sugar extraction from corn stover biomass (Baadhe et al., 2014). The conventional method of pre-treatment involves chemical treatment with alkali or acidic conditions to remove hemicellulose and lignin, which are the major components of the resistant structure of lignocellulosic biomass. Alkali pre-treatment specifically removes lignin while maintaining the carbohydrate (cellulose and hemicellulose) portion (Cheng et al., 2020). Approach leads to higher yields of reducing sugar during enzymatic saccharification and reduces the formation of inhibitors. To liberate reducing sugars from corn stover, a combination of pre-treatment methods is employed, resulting in the extraction of sugars such as glucose, xylose, arabinose, galactose, and mannose (Sawant et al., 2015).

Polymers play a critical role in the global industrial economy, and the production of biopolymers from biological sources offers an alternative to reduce reliance on fossil fuels and petroleum. Polyhydroxyalkanoates (PHA) are polyesters created using carbon and in nitrogen stress conditions. The production of PHA depends on the genetic makeup of the microbial strain and the substrate fed to the microorganisms depending on which the PHA produced has the properties such as rigid thermoplastic in short chain (scl-) PHA and flexible elastomers in medium chain (mcl-) PHA. Mcl-PHA has an advantage over scl-PHA. These biopolymers can degrade into carbon dioxide and water. Fermentation cost associated with PHA production is too high and therefore efforts are put to use inexpensive and

renewable corn stover(Ashby et al., 2022). *Pseudomonas putida* is the producer of mcl-PHA which is precursor of bioplastic (Kanavaki et al., 2021).

The present research work focuses on the utilization of corn stover, an agricultural waste product, as a carbon source for PHA production. The process involves two main steps - alkali pretreatment and enzymatic saccharification - to convert the corn stover into sugar hydrolysate. The alkali pretreatment is optimized to achieve maximum lignin removal and to enhance the depolymerization and disintegration of the tough corn stover structure. The optimized condition of alkali pre-treated corn stover is characterized using Fourier transform infrared (FT-IR) spectroscopy, thermogravimetric analysis (TGA), and scanning electron microscopy (SEM). The resulting residue is then subjected to enzymatic saccharification using commercial cellulase to produce high-reducing sugars. Submerged fermentation with *Pseudomonas putida* MTCC 2475 is conducted using the sugar hydrolysate obtained from corn stover to produce PHA. The monomers of PHA were detected using GC-MS/MS, and the extracted PHA film is characterized using Differential scanning calorimetry (DSC), Fourier transform infrared (FT-IR) spectroscopy, and Nuclear magnetic resonance (NMR). Lastly, a mass balance study is conducted on a bench scale to estimate the potential large-scale production of PHA. The novelty of the work lies in using for the first-time wild type of *Pseudomonas putida* MTCC 2475 strain for mcl-PHA production using simple carbon source from corn stover.

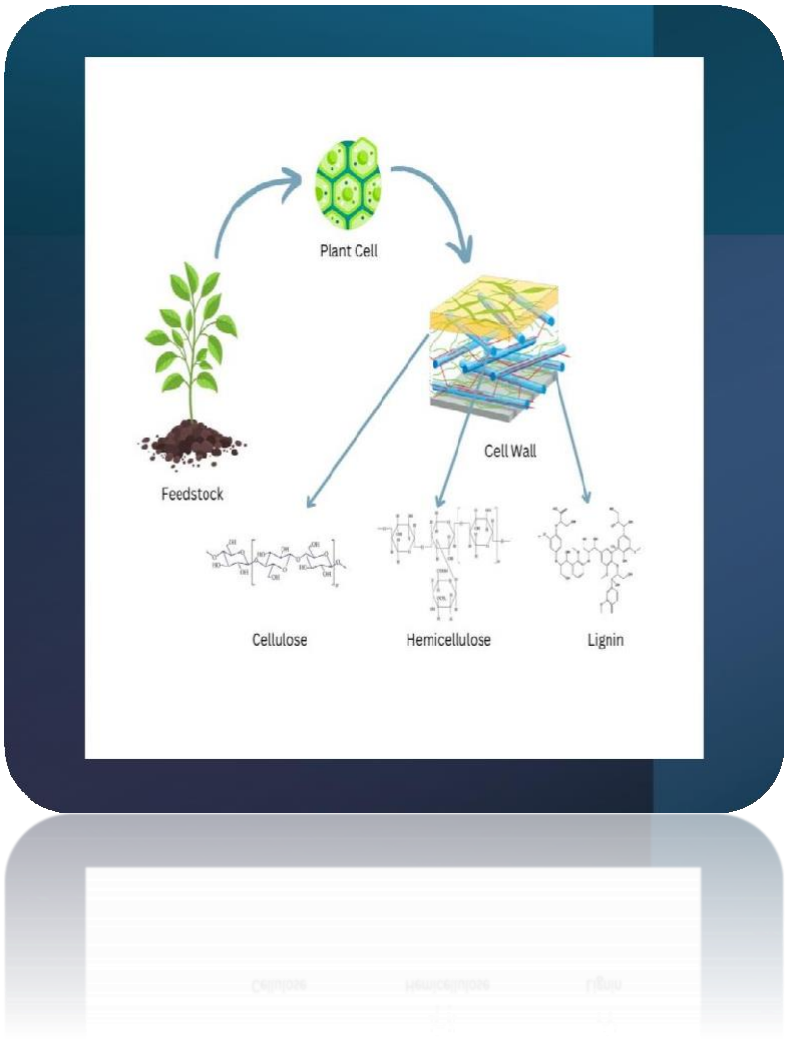


Figure 5. Composition of lignocellulosic biomass – cellulose, hemicellulose and lignin.

1.6 Benefits and Challenges in Converting Corn Waste to Value-Added Products

The advantages of using corn waste are high biomass productivity and availability of economic, effective, renewable, easily available and carbon–neutral biomass (Bhutto et al., 2017).There is no additional use of land to produce corn stover, corn cob and corn straw and no direct competition of corn stover with food as this is not useful as food for human beings. There is an additional benefit to the farmers as the straw or husk is used for high-value products. Valorizing corn waste is important rather than disposing of it as waste; it serves as an alternate substrate in place of depleting fossil fuels (Loong et al., 2021). The limitation of using corn stover biomass is due to the recalcitrant and resistant nature of lignocellulosic biomass cellulose, hemicellulose and lignin (Bhutto et al., 2017). This structure of corn stover biomass restricts the interaction between the enzymes or microbial strain with the cellulose and thus is not easily converted to various metabolites. The main important step before hydrolysis, fermentation and purification steps is the pretreatment method which disrupts the crystalline and polymeric structure of corn stover. Extensive research is required for the cost-effective utilization of corn stover and corn cob for the viable production of chemicals and bioenergy. The saccharification process is still a critical bottleneck and an ideal method should be generated for stoichiometric amounts of fermentable monomeric sugars out of the lignocellulosic complex. The other problem which needs to be solved is regarding the search for kinetically more efficient cellulase (Khare et al., 2015).

1.7 Scope of the Study

Agricultural production has increased over the last 50 years and corn waste is an abundant, renewable carbon resource that may be utilized which otherwise causes resource waste or environmental deterioration. The burning of crop waste is the major cause of worsening air quality in different countries worldwide. Biorefinery is a major direction for crop straw use reasonably and efficiently. Corn waste is unfermentable due to the primary cellulosic, hemi cellulosic and lignin components and most microbes cannot degrade it and thus, pretreatment plays a vital role in overcoming the recalcitrant and resistant nature of the biomass. Wide

conversion technologies are used for making value-added products from corn waste. Biorefineries enable sustainable processing of biowaste into a wide range of marketable products, bioenergy and biobased products. Nowadays, research focus on the high yield of products with less energy consumption. Different types of products just as biofuels, organic acids, bio enzymes, phenolic compounds, bio-based polymers, sugar alcohols, and other industrially important chemicals are obtained from corn waste. Optimizing, scaling, implementing and integrating parameters and techniques will lead to enhanced and efficient bioproducts for corn waste. In the present time, efforts are put to replace fossil fuels and fossil fuel-based products with renewable biomass as it is considered a better energy resource due to its low cost, availability and carbon neutrality. Effectual use of biomass feedstock, mainly lignocellulosic biomass is of great importance worldwide. Thus, lignocellulosic biomass can be utilized by its breakdown into simple sugars and further used by microbes for bioenergy, bio-products, bio-enzymes production etc. The by-products from the agricultural field contribute majorly to global production of waste. Therefore, is an abundant, easily available, economic biomass to be transformed to valuable products. Enzyme is high value biocatalyst with extensive applications and play key role in hydrolysis of substrate. Bioplastic has tremendous potential to decrease pollution and improve biodegradability by replacing synthetic fibres in bioplastic.

1.8 Objectives of the Study

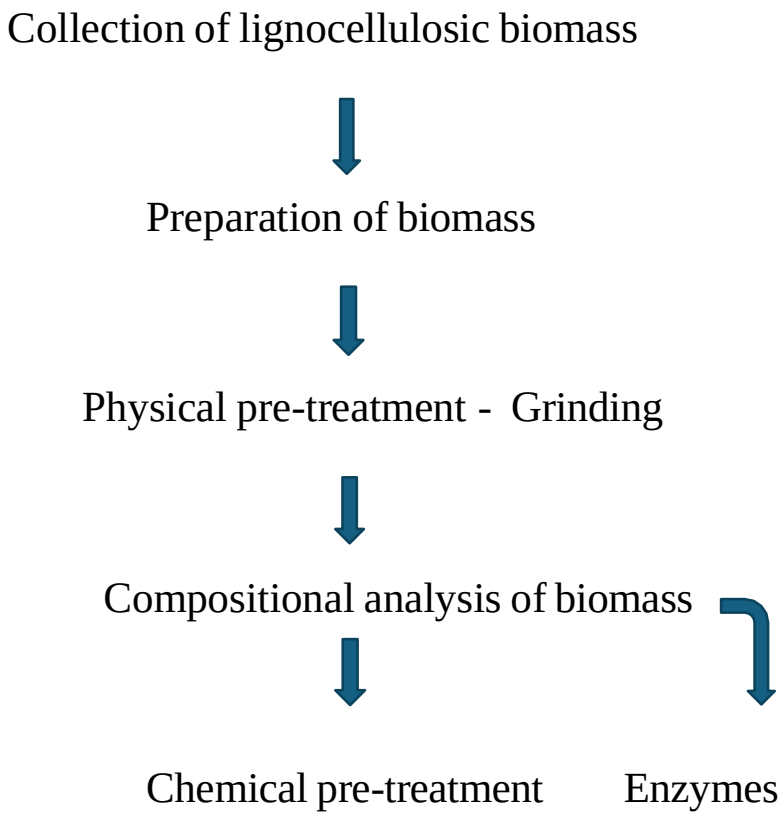
1. Compositional analysis and Pre-treatment of biomass

2. Enzymatic hydrolysis of pre-treated biomass

3. Value added – product

4. Production of crude enzymes from potential substrate

1.9 Overview of Thesis



1.10 Literature Review

This section enlists the various research work done by researchers biorefinery field-

Baadhe, R. R., Potumarthi, R., & Mekala, N. K. (2014). Influence of dilute acid and alkali pretreatment on reducing sugar production from corncobs by crude enzymatic method: A comparative study. Bioresource Technology, 162, 213–217. https://doi.org/10.1016/j.biortech.2014.03.117 Corncob as the raw material is gaining a lot of attention these days for the production of valuable products. Efforts are put to find the suitable method for pre-treatment. In this study, different concentrations of dilute H_2SO_4 and NaOH for the pre-treatment of corn cobs. The solid and liquid ratios were varied to find the effect on the concentration of sugar. After the chemical pre-treatment, pre-treated biomass was subjected to enzymatic hydrolysis. The amount of sugar released is increased with an increase in solid/liquid ratio between 0.03 to 0.2. NaOH pre-treated corn cobs released 350mg/ml and H_2SO_4 pre-treated corn cobs released 415.12mg/ml of reducing sugars at solid/liquid ratio 0.05. The maximum amount of sugar is released with 0.25M sulfuric acid. The highest sugar that is 398.5 mg/ml released during enzymatic hydrolysis of 0.25M acid pre-treated corn cob. NaOH pre-treatment released low amount of sugars as compared with H2SO4 pre-treatment and with increase in concentration of NaOH, the sugar liberation decreased. The solid to liquid ratio (1:20) removed more xylan part and improved the enzymatic hydrolysis to produce reducing sugars. Lower reducing sugars were produced in case of NaOH pre-treatment rather than H2SO4 pre-treatment. The concentration of fermentable sugars was higher in enzymatic hydrolysate from H2SO4 pre-treated corncobs.

Liu, J., Yang, J., Wang, R., Liu, L., Zhang, Y., Bao, H., Jang, J. M., Wang, E., & Yuan, H. (2020). Comparative characterization of extracellular enzymes secreted by *Phanerochaete chrysosporium* during solid-state and submerged fermentation. International Journal of Biological Macromolecules, 152, 288–294. https://doi.org/10.1016/j.ijbiomac.2020.02.256 The popular way to utilize any biomass is by enzymatic conversion into fermentable sugars. Various filamentous fungi are used for the production of lignocellulose degrading enzymes. Two

important ways for enzyme production are solid-state fermentation and submerged fermentation. This study has done comparative secretome analysis of *Phanerochaete chrysosporium* during both solid state and submerged fermentation while using corn stover as the carbon source. In solid state and submerged fermentation, 110 and 64 extracellular carbohydrate active enzymes were identified respectively. Among which 57 enzymes were common in both secretomes. *Phanerochaete chrysosporium* secreted more cellulases and hemicellulases in solid state fermentation while carbohydrate binding module was higher in submerged fermentation. The results from this study reveal that enzyme production by *Phanerochaete chrysosporium* is influenced by fermentation conditions.

Zhao, L., Cao, G. L., Wang, A. J., Ren, H. Y., Dong, D., Liu, Z. N., Guan, X. Y., Xu, C. J., & Ren, N. Q. (2012). Fungal pretreatment of cornstalk with *Phanerochaete chrysosporium* for enhancing enzymatic saccharification and hydrogen production. Bioresource Technology, 114, 365–369. https://doi.org/10.1016/j.biortech.2012.03.076 Fossil fuels have environmental impacts as well are limited energy resources. Lignocellulosic biomass is a good option for hydrogen production due to its low cost, environment friendly and large-scale availability. In China, corn stalk is an agriculture residue that has high potential for hydrogen production. Pre-treatment methods that are used are dilute acid, hydrothermolysis, alkali extraction and steam explosion. Zhao et al., 2012 study is about fungal pre-treatment to improve enzymatic saccharification and Hydrogen production from cornstalk. The fungal pre-treatment of cornstalk was done with *Phanerochaete chrysosporium* and the maximum enzymatic saccharification was 47.3% which was 20.3% higher than control that is without pre-treatment. The yield of hydrogen was 80.3 ml/g pre-treated stalk upon fermentation of enzymatic hydrolysate.

Wang, F. Q., Xie, H., Chen, W., Wang, E. T., Du, F. G., & Song, A. D. (2013). Biological pretreatment of corn stover with ligninolytic enzyme for highly efficient enzymatic hydrolysis. Bioresource Technology, 144, 572–578. https://doi.org/10.1016/j.biortech.2013.07.012 The utilization of lignocellulose to be biotransformed to bio-based chemicals and liquid biofuel is the area of

research. The development of techniques for degradation of crystalline structure of cellulose and to separate lignin and hemi-cellulose from cellulose. this research paper by Wang et al aims at increasing the yield of sugars from corn stover by biological pre-treatment. Crude ligninolytic enzymes from *Phanerochete chrysosporium* and *Coridus versicolor* were used to break the lignin structure in corn stover. The sugar yield was increased by 2-day bio-treatment and enzymatic hydrolysis.

Lemechko, P., Fellic, Magali., & Bruzaud, S. (2019). Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) using agro-industrial effluents with tunable proportion of 3-hydroxyvalerate monomer units. International Journal of Biological Macromolecules, 128, 429-434. Bioplastics is an important concern to the scientists and industries. PHA (polyhydroxyalkanoates) are class of polyester which are a bioresource and biodegradable. Microorganisms such as bacteria are used to biosynthesized PHA which has thermal and mechanical properties and still a challenge for large scale production. The use of waste substrates for the bacteria decreases the high production cost. Agro-industrial effluents was used a carbon source and used for the growth of *Halomonas sp*. SF 2003 to produce poly (3-hydroxybutyrate). The PHA productivity was 1.3 g/L in 40 hours and molecular weight was 342, 000 g/mol. Addition of valeric acid with the agro-industrial effluent substrate poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) and hydroxyvalerate (HV) monomers were present. The thermal and mechanical properties were estimated by HV amount and results obtained showed decrease in glass transition and melting temperatures and Young modulus showed high HV content increase. Thus, PHB was obtained from agro-industrial effluents using marine bacterial strain. Decrease in molecular weight is because of the process and nature of the substrate used for PHB production. The PHBHV produced from the marine bacterial strain had 35% of HV present.

CHAPTER 2
MATERIALS AND METHODS

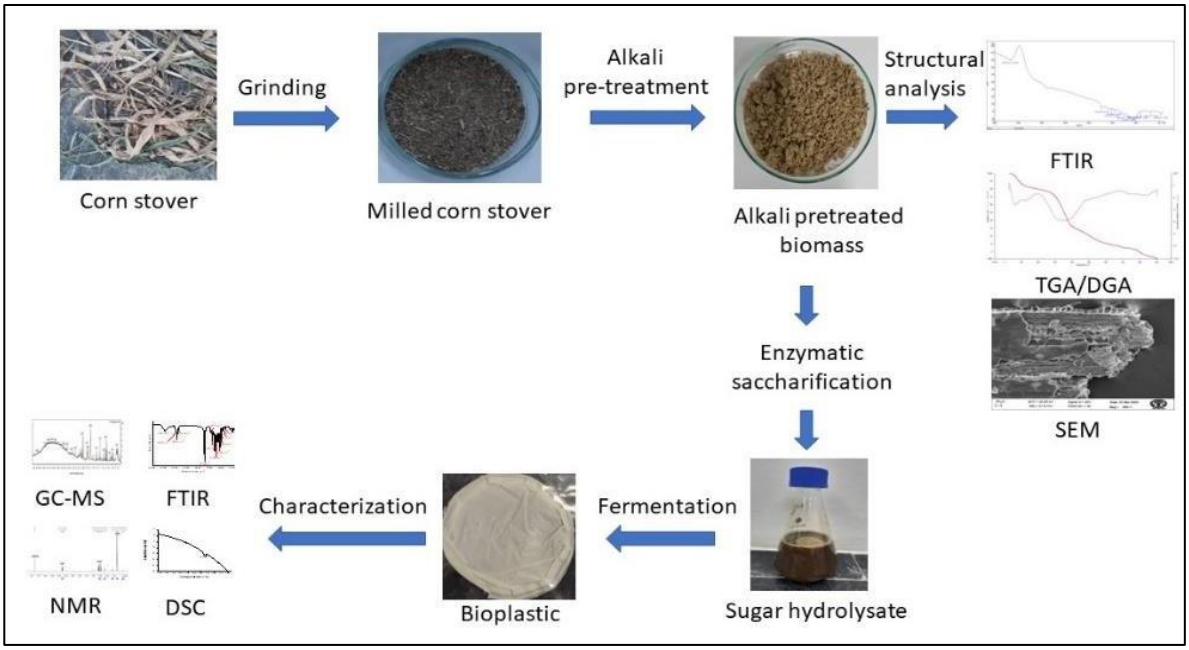


Figure 6. Overall steps involved in PHA production using corn stover.

2.1 PHA Production using Corn Stover

2.1.1 Collection and Bioprocessing of Corn Stover

The corn stover in the research study was collected from the fields of Uttar Pradesh, India. The sample was kept in microprocessor-controlled oven (Metrex Scientific Instruments, New Delhi, India) at 35°C for drying until constant weight, then cut in 2-4cm size, pulverised through grinder (Bajaj GX3701), sieved to 1mm and kept in airtight containers for further experimental analysis. The entire chemical used in the study were from ThermoFisher Scientific and SRL. The overall steps for the PHA production using corn stover is shown in **Figure 6**.

2.1.2 Compositional Analysis of Corn Stover

Structural carbohydrates and lignin were determined using National Renewable Energy Laboratory (NREL) (Sluiter et al., 2012). Crystalline cellulose content was estimated using Updegraff reagent method (Bauer C Ibá, 2014). Ash content was

determined using AOAC, 2000 with some modifications. 1g sample weighed in crucible and kept in muffle furnace at 550°C for 8h (AOAC 2000).

2.1.3 Optimization of Alkali Pre-treatment Conditions of Corn Stover by Central Composite Dign (CCD) using Minitab

In the experimental design, Response surface methodology (RSM) was used to optimize two parameters that were cellulose and lignin content. The design consisted of 13 sets of experiments involving five replicates at centre points. Two parameters were varied – (A.) Alkali concentration 0.25%, 1.625% and 3% NaOH (w/v), B. Time (15, 45, 75 minutes) based on reported research literature. The alkali pre-treatment optimization for high cellulose and low lignin was carried out by Minitab and temperature 121°C was taken fixed condition for all the 13-run order.

The alkali pre-treatment of corn stover was performed using Sodium hydroxide (NaOH) in autoclave with experimental conditions designed with different alkali concentrations and time by RSM. 10g sample was taken in reagent bottles of 500 ml (Borosil) with different alkali concentrations 0.25%, 1.625% and 3 % NaOH (w/v) (pH -10.8, 12.6 and 13.7) respectively were added and kept in autoclave (Baadhe et al., 2014; Modenbach, 2014). After pre-treatment, the solid and liquid biomass were separated using muslin cloth. The solid biomass was neutralized with milli-Q water and liquid recovered was neutralized with 1N HCl. The liquid hydrolysate was filtered using 0.2-micron syringe filter and solid biomass was dried in oven at 40°C until constant weight and stored for further experiments. Solid biomass recovered was utilized for cellulose and total lignin content. The solid biomass was tested for cellulose content by Updegraff method and total lignin by NREL protocol (Sluiter et al., 2012).

2.1.4 Characterization of Alkali Pre-treated Corn Stover Before and After Pre-treatment

Fourier transform infrared spectroscopy **(**FTIR**)** was conducted using FTIR spectrophotometer (Perkin Elmer Frontier) for functional groups in the frequency range 4000-400 cm^{-1}. The pellet was prepared using KBr (FTIR grade) and sample thoroughly crushed and mixed in mortar and further pressed using pellet press machine to form pellet (Zhang et al., n.d.). Thermal gravimetric analysis (TGA) for thermal stability of untreated and treated sample that were used for experiments was analysed using Thermogravimetric analyser (Perkin Elmer/ TGA 4000) using maximum temperature of 900°C and heating rate of 10°C min^{-1} under nitrogen atmosphere. The weight change of corn stover biomass was recorded in accordance with temperature and time. 2g sample of dried powder were placed in the crucible in the analyser (Zhang et al., n.d.). The morphology of untreated and treated samples was determined by Scanning electron microscope (ZEISS EVO 18 Research) (Zhang et al., n.d.).

2.1.5 Enzymatic Saccharification of Alkali Pre-treated Corn Stover

The alkali pre-treated biomass with high cellulose and low lignin content was subjected to enzymatic saccharification using commercial cellulase (Meicellase) from *Aspergillus niger,* 13000CMC U/g by Sisco Research laboratories (SRL) for cellulose conversion to reducing sugars. As reported 20 U/g of commercial cellulase was used for enzymatic saccharification (Y. Wu et al., 2021). Thus, experiments were designed for optimization of conditions with biomass loading % and enzyme U. Total reducing sugar was estimated by Di-nitro salicylic acid (DNS) method (Lorenz Miller, n.d.) and further corn stover hydrolysate was kept in 4°C for its use in PHA production.

2.1.6 Optimization of Alkali Pre-treated Biomass for Enhanced Enzymatic Saccharification with Commercial Cellulase for Maximum TRS Yield

To optimize the enzymatic saccharification conditions for high total reducing sugar from alkali pre-treated corn stover content was carried out by Design Expert. Central composite design with 2 factors Biomass loading (%) and Enzyme units (U) were taken to analyse enzymatic saccharification for total reducing sugar (TRS). Temperature 50°C, 0.05M citrate buffer (pH – 4.8), 200 rpm and 72h were the fixed parameters. The selected variables were

represented in alpha, highest, central and lowest (-alpha, -1, 0, +1, +alpha). All the experiments were performed in triplicates to check the deviation in the results.

$$\boldsymbol{Saccharification} = \frac{\boldsymbol{Total\ reducing\ sugars}\ (\boldsymbol{g/L}) \times \boldsymbol{0.9} \times \boldsymbol{dilution\ factor}}{\boldsymbol{biomass}\ (\boldsymbol{g})}$$

The factor 0.9 is taken as it converts polysaccharides to monosaccharides considering the water uptake in the hydrolysis process (Alrumman, 2016).

2.1.7 Microbial Strain, Media Preparation and Bacterial Growth Curve

Pseudomonas putida MTCC 2475 was procured from MTCC (The Microbial Type Culture Collection and Gene Bank), Chandigarh, India. Long term storage of the pure strain was done using 50% glycerol stock and stored at -20°C. Luria-Bertani (LB) agar with 10g/l tryptone, 5g/l yeast extract, 10g/l NaCl and 15g/l agar was used for maintenance of cells. The seed culture composition consisted of 10g/l tryptone, 5g/l yeast extract and 10g/l NaCl. The pH of the seed culture was balanced to 7 and autoclaved at 121°C for 15 minutes. The seed culture was inoculated with microbial strain (*Pseudomonas putida* MTCC 2475) and kept in orbital shaker at 30°C, 150rpm for 16h till the O.D. @600nm reached 0.8. Four different types of media conditions (LB broth, only hydrolysate, modified media with synthetic glucose and modified media with glucose replaced with hydrolysate) were inoculated with 1% bacterial suspension to compare the bacterial growth. The bacterial suspensions were incubated in 37°C and growth curve, dry cell weight and sugar consumption were measured at 2h interval. Sugar consumptions were analysed with Di-nitro salicylic method (Lorenz Miller, n.d.).

2.1.8 PHA Production Using Corn Stover Hydrolysate

The production media experiments were performed in 2L shaking flasks using 1L media with nitrogen stress - 1g/L ammonium sulphate, 5g/L sodium chloride and 10g/L hydrolysate. Corn stover hydrolysate after enzymatic saccharification was used as carbon source. Ammonium sulphate $(NH4)_2\,SO_4$ was used for nitrogen content. Sodium chloride (NaCl) was added to maintain the osmotic balance. Sodium chloride and ammonium sulphate were sterilized in autoclave at 121°C for 20 minutes and hydrolysate was sterilized through 0.2µ syringe filter and added in the flask prior to inoculation. In the flask, 1g/L ammonium sulphate was added to maintain C/N ratio 10:1. The flasks were

inoculated with 1% of overnight seed culture of *P. putida* and grown for 24h, 36h, 48h, 60h and 72h at 30°C and 150 rpm. The bacterial cells were harvested by centrifugation and pellets were washed with milli-Q water and dried till constant weight.

2.1.9 Extraction of PHA and GC-MS/MS for Monomer Composition

Methanolysis method was performed for PHA extraction with 20 mg dried cells with 2ml chloroform for extraction in 15 ml Borosil sealed culture tube with 2ml acidified methanol (85% v/v methanol and 15% v/v sulphuric acid) and incubation at 100°C for 140 minutes (Mahato, 2021) and polymer PHA fragmented in fatty acids methyl esters monomeric components. After cooling, added 2ml milli-Q water for organic phase separation (Mahato, 2021). The organic layer transferred in another vial, filtered through 0.2µ syringe filter, and kept in GC vials. The organic layer was examined by GC-MS/MS (Agilent GC7000D/TQ) to analyse the monomers of PHA. Injection volume 2µl was injected in gas chromatograph with inert helium taken as carrier gas, flow rate 2ml/minute and oven temperature were at 200°C with N-hexane used as blank. NIST library database was used for PHA monomers compound identification (Mahato, 2021).

2.1.10 Characterization of Extracted PHA Film

Thermal analysis of extracted PHA film by Differential Scanning Calorimetry (DSC) were performed using 5mg sample placed in crucible and loaded into the sampler port of PerkinElmer DSC 8000. The PHA film were heated from 30°C to 250°C at 10°C/min. Nitrogen gas was used for purging (Dartiailh et al., 2021). Structural analysis by FTIR spectrometer of PHA for functional group identification with 1mg extracted sample with KBr to form the pellet. Analyzation of PHA polymer by Perkin Elmer Frontier FTIR spectrophotometer in the range 4000-450 cm^{-1} (Mahato, 2021). ^{1}H-NMR was performed using Brucker 500Mhz spectrometer to analyse the chemical structure as well as the monomer composition of extracted PHA film. 4mg of extracted PHA film was dried and dissolved in 600µl $CDCl_3$ (Dartiailh et al., 2021).

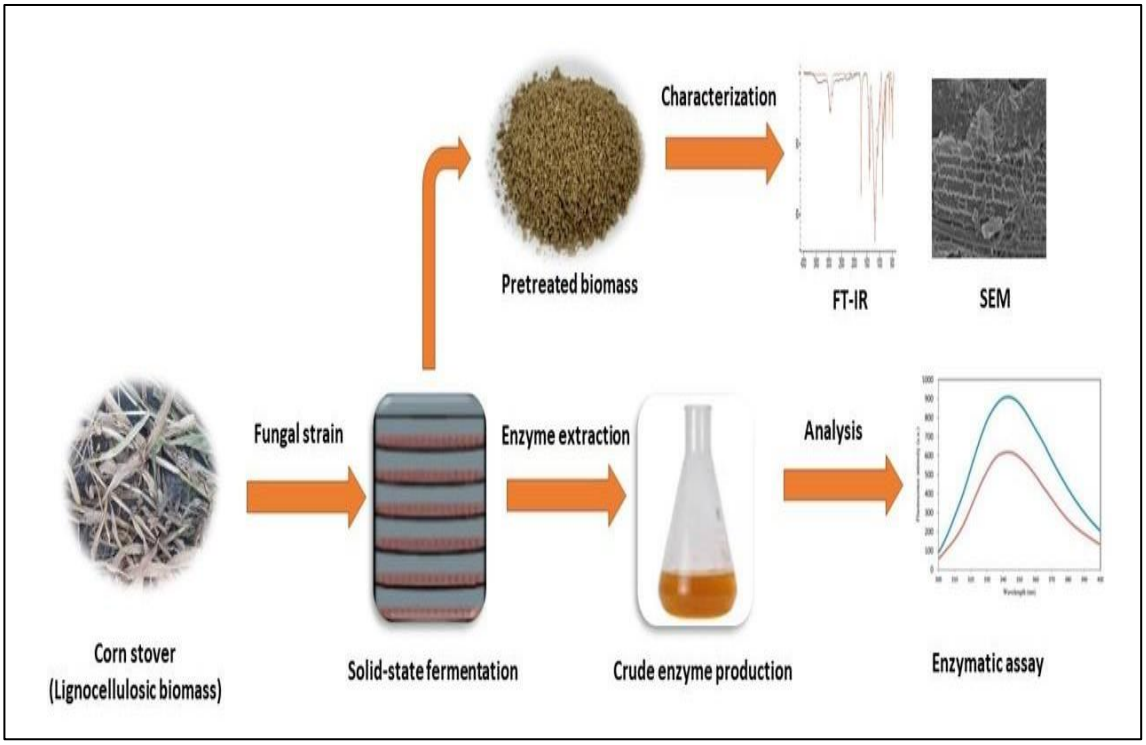


Figure 7. Overall stepd involved in enzymes production using corn stover.

2.2 Enzymes Production from Corn Stover

2.2.1 Substrate and Composition analysis

Corn stover biomass was used in the study was collected from the fields of Uttar Pradesh, India. Substrate was kept in microprocessor-controlled oven at 35-40°C for drying till constant weight, pulverised and kept in airtight containers for further experimental analysis. All the chemicals used in the study were procured from ThermoFisher Scientific and Sigma. NREL (National Renewable Energy Laboratory) protocol was followed for structural carbohydrate and lignin content in corn stover. Cellulose was estimated using Updegraff method and ash content following (AOAC 2000). The overall steps in enzymes production using corn stover is shown in **Figure 7**.

2.2.2 Sodium Hydroxide Pre-treatment of Corn Stover Biomass

Corn biomass was grinded, and alkali pretreatment was done with 3% NaOH (w/v) for

15 minutes in an autoclave at 121°C. In a beaker (500ml) 10gm corn sample and 100ml 3% NaOH (w/v) was taken and pretreated. After pretreatment liquid hydrolysate was separated and solid biomass neutralized with milli-Q water. The solid biomass was dried in oven at 35°C till constant weight and stored at room (Phuong Vi Truong & Kim, 2018).

2.2.3 Fungal Culture

Live culture of *Phanerochaete chrysosporiym* NCIM 1106 was obtained from NCL Pune, India and maintained on Potato dextrose agar media at 30°C as given in **Figure 8**. Sporulated slants were obtained after growth for 10 days and stored at 4°C (L. Liu et al., 2019).

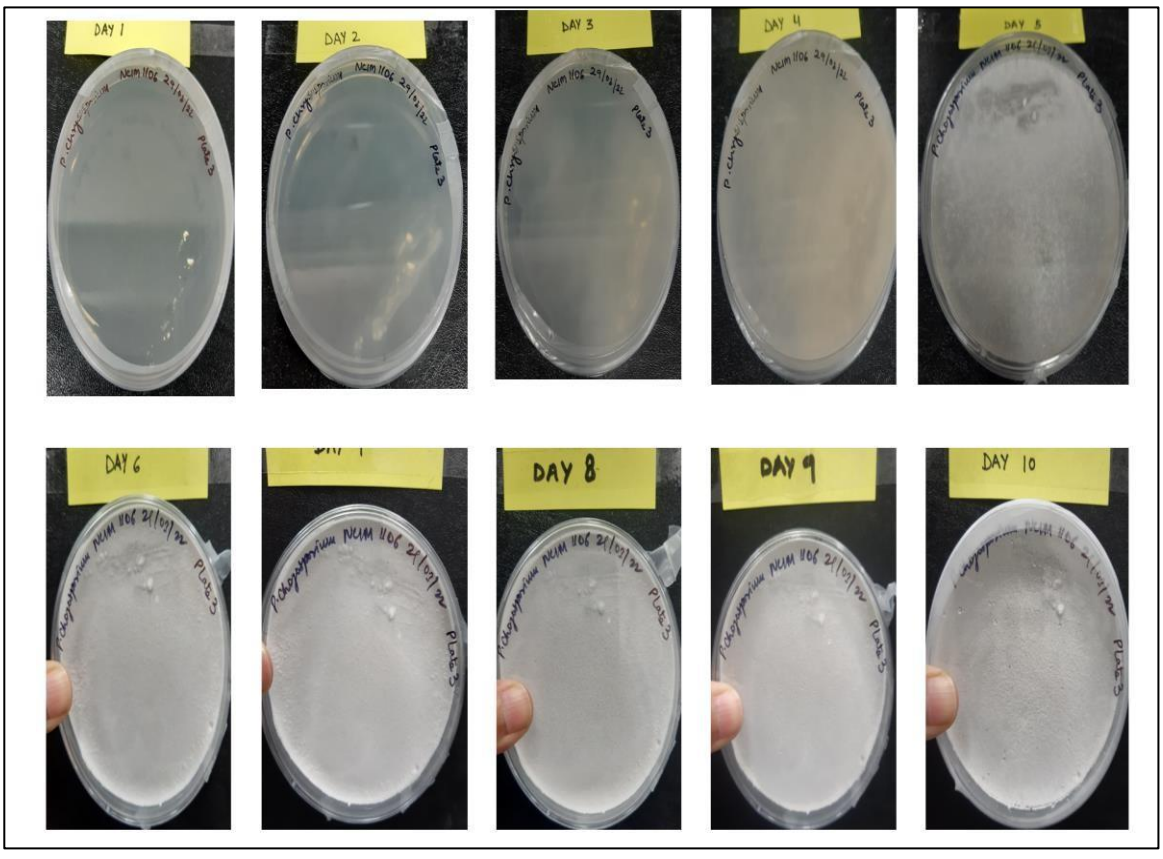


Figure 8. Growth of white rot fungus *Phanerochaete chrysosporium* on potato dextrose agar (PDA) plate for 10 days.

2.2.4 Preparation of Inoculum Suspension

White lawn of mycelia was formed on PDA agar plates incubated at 28°C for 10 days. Spore was harvested delicately to prevent detachment of the mycelium by adding 10

ml of sterile distilled water and then collected in sterilized bottles, it was used as an inoculum for enzyme production, the suspension was diluted by 1:100 (v/v), and the spore count was performed in a counting chamber (Tirado-González et al., 2016).

2.2.5 Solid - State Fermentation for Enzyme Production

Untreated and alkali pre-treated biomass used in Fermentation process, in 250 ml Erlenmeyer flasks with 5 gm milled corn stover substrate, were initially moistened to 60% w/v as shown in **Figure 9 and 10**. After closing with cotton plug flasks autoclaved at 121°C for 20 minutes. Sterilized flasks with solid biomass were inoculated with spores of *Phanerochaete chrysosporium* NCIM 1106 at a density of 1×10^4 spores/ml and further incubated at 30°C in incubator without shaking (F. Q. Wang et al., 2013). Moisture content was maintained regularly 60% using autoclaved tap water. Flasks were taken out at 3 days interval for further enzyme extraction and estimation. 6 flasks each for both untreated and treated biomass were kept being taken out on respective day of analysis.

Figure 9. Solid state fermentation flask with untreated corn stover for different days of analysis.

Figure 10. Solid state fermentation flask with alkali treated corn stover for different days of analysis.

2.2.6 Enzyme Extraction

For extraction of enzymes from the fermentation media, milli-Q water was added in the ratio of 1:10 (Substrate: milli Q water). 50ml of autoclaved milli-Q water was used in each flask. The flasks were retained in an incubator shaker at 150 rpm for 60 minutes. Then, the mixtures were filtered with Whatman filter paper and centrifuged 5000 rpm for 10 minutes. Further, liquid hydrolysate was filtered through 0.2um syringe filter and liquid was used in the enzyme assay and protein estimation. Solid biomass was used for solid biomass weight loss and lignin estimations (Sukumaran et al., 2009).

2.2.7 Enzymatic Assay

2.2.7.1 Estimation of CMCase Activity

The total assay volume was taken as 1000 µl. Reaction mixture with 250µl carboxymethylcellulase (2% w/v) solution, 10µl crude enzyme mixture and 240 µl sodium acetate buffer (50 mM, pH 5.0) was incubated at 50°C for 30 minutes. It was

cooled at room temperature to stop the reaction. 500 µl Di-nitrosalicyclic reagent was added to estimate total reducing sugar using glucose as standard. One unit of endocellulase activity (U) was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute (Chen et al., 2013).

2.2.7.2 Filter Paper Assay

Whatman filter paper No. 1 were cut 1.0 × 6.0 cm long strips which weighed 50 mg, were taken as substrates. National Renewable Analytical Laboratory (NREL) - Laboratory analytical procedure for measurement of cellulase activity were followed for analysis. Firstly, in a test tube filter paper strip 1 × 6 cm, 1ml Sodium citrate buffer (0.05M) and 50 µl crude enzyme were added. The Number of dilutions is to be made for each cellulase sample. Dilutions should be more and less than 2.0 mg of glucose concentration. The test tubes were labelled and incubated at 50ºC for 1 hr. After the incubation time period, test tubes were cooled, and reaction was stopped by adding 3.0 ml of freshly prepared Di-nitro salicyclic acid (DNS). Test tubes were kept in water bath at 100ºC for 10 minutes. Absorbance taken with UV-vis spectrophotometer at 540 nm. For determination of cellulase concentration, it is marked that release exact 2.0mg of glucose. A curve was plotted between absorbance at 540 nm and glucose released. Filter paper Unit estimated with formula = 0.37/ concentration of cellulase needed to release 2.0 mg glucose (Adney & Nrel, 2008).

2.2.7.3 Manganese Peroxidase Assay

The manganese peroxidase (MnP) activity was estimated using manganese sulfate ($MnSO_4$) as substrate (Kuan et al., 1993). The enzymatic assay was performed in a reaction mixture with 250 ml of sodium lactate buffer solution (0.2 mol/L and pH 4.5, 100 ml of $MnSO_4$ (0.4 mol/L), 100 ml of crude enzymatic extract, 20 mL of hydrogen peroxide (H_2O_2, 0.1 mol/L) with 530 ml of distilled water, incubated at 40°C for 10 minutes (Nugraha et al., 2020). Substrate oxidation process was monitored by absorbance at 270 nm. 1 unit of enzyme activity (U) taken as quantity of enzyme used to produce 1 u mol of substrate per minute.

2.2.8 Characterization of Corn Stover Biomass After Fungal Pre-treatment

Solid biomass recovered after fungal pre-treatment was characterized through Scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). SEM was used to characterize surface structure of the corn stover in untreated and alkali pre-treated after fungal pre-treatment by ZEISS EVO,18 Research. FTIR was performed through Perkin Elmer Frontier to study the changes in the functional groups within the frequency range 4000 to 400 cm^{-1}. KBr pellet was used as standard, and sample was refined through mortar pestle and mixed with KBr and pressed by pellet pressing machine to make pellets.

2.2.9 Reducing Sugar Analysis

Total reducing sugar is measured with Di-nitro salicylic (DNS) method (Lorenz Miller, n.d.). DNS reagent was freshly prepared by 1.0 g 3,5 – di-nitro salicylic acid, 0.05 gm sodium sulphite, 18.2 gm sodium potassium tartarate, 1.0 gm (NaOH) sodium hydroxide and 0.2 g phenol and adjust the volume with deionized water to 100ml. Thermo scientific (Genesys 50) UV- vis spectrophotometer was used to take absorbance at 540nm. Standard curve was prepared with glucose and all the experiments were performed in triplicates (Irfan et al., 2016).

2.2.10 Analysis of Statistical Data

All the results are represented as mean and with standard deviation (SD).

2.2.11 Effect of Incubation Time on Enzyme Activity

Enzyme activity of cellulase and manganese peroxidase were estimated on various incubation time varying 3^{rd} ,6^{th} ,9^{th} ,12^{th} and 15^{th} days.

2.2.12 Estimation of Residual Solid Biomass

Recovered solid biomass after SSF, filtered by Whatman filter paper no. 1, followed by washing with milli-Q water and drying in oven till obtained constant weight. Recovery was calculated by taking weight before and after the biological pre-treatment (Sala et al., 2020)

Chapter 3

RESULTS AND DISCUSSION

3.1 PHA Production using Corn Stover

3.1.1 Composition of Corn Stover

Corn stover was characterized for composition % (w/w) as follows: cellulose, 41.67%; total lignin, 24.59%; ash, 11.26%; and other components, 3.51%, as indicated in **Table 1 and Figure 11**. Generally, in other research studies untreated corn stover exhibited cellulose content of 36.5%, hemicellulose content of 22.1%, and lignin content of 18.8% which highlights its high carbohydrate content available for bioproduct generation as shown in **Figure 12 - 16**. Thus, corn stover is a key lignocellulosic biomass for sugar recovery and value-added product generation (Yang et al., 2016) (C. Li et al., 2020).

Corn stover sample

Grinded corn stover sample

Figure 11. Corn stover sample taken from the fields and grinded corn stover sample after physical pre-treatment.

Table 1. Composition of corn stover.

Parameters	Corn stover (%)
Total structural carbohydrate	59.60 ± 0.13
Cellulose	41.67 ± 0.59
Total lignin	24.59 ± 0.34
Acid insoluble lignin	22.05 ± 0.34
Acid soluble lignin	02.54 ± 0.00
Ash	11.26 ± 0.12

Results are expressed as mean standard followed by standard deviation.

Corn sample before ash analysis

Corn sample after ash analysis

Figure 12. Corn sample before and after ash analysis.

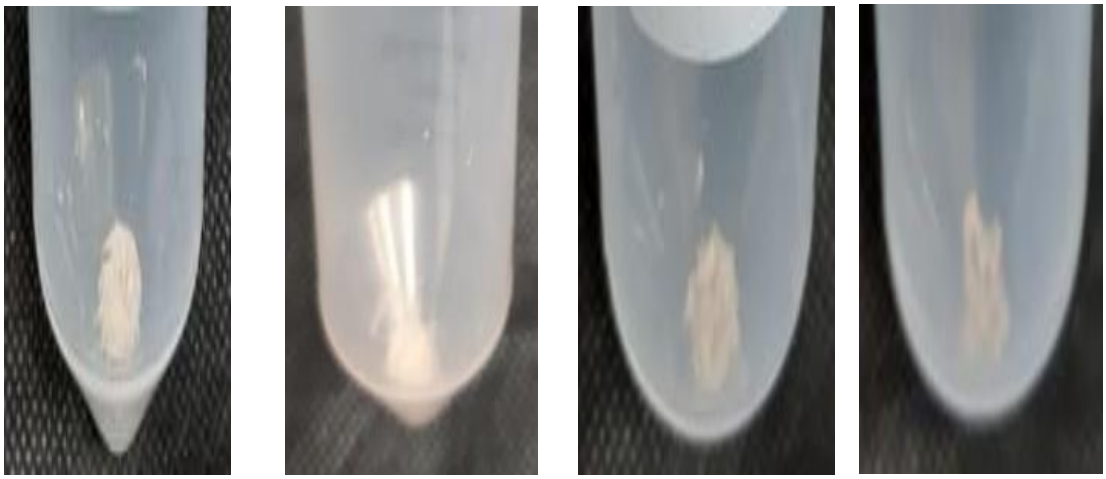


Figure 13. Corn sample untreated and treated conditions after Saeman hydrolysis for estimation of crystalline cellulose content.

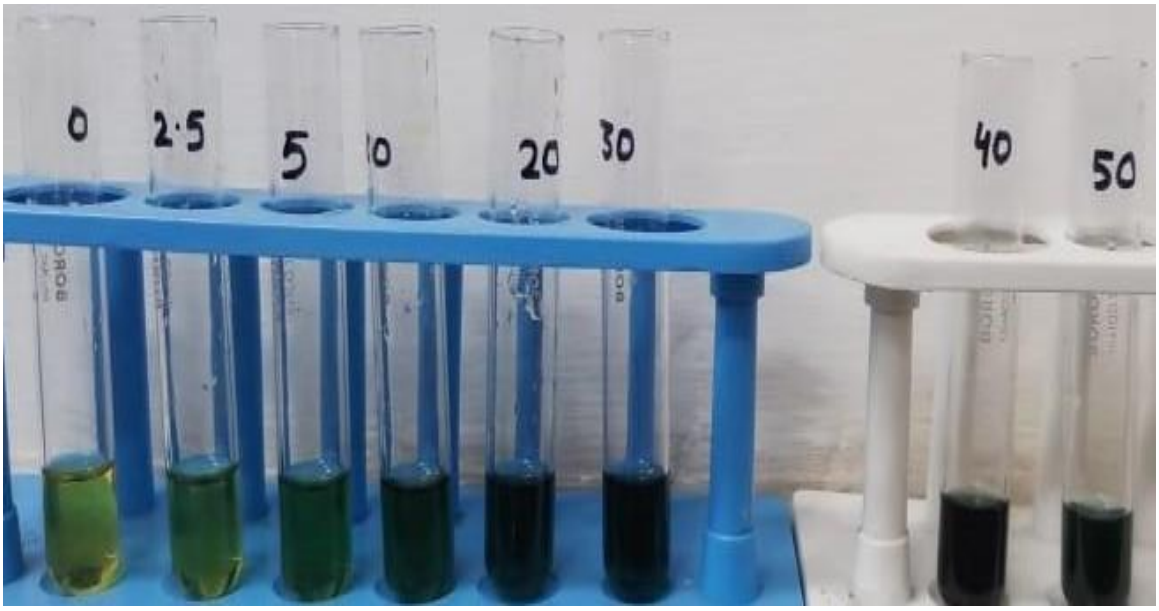


Figure 14. Standard test tubes after anthrone assay for cellulose estimation by Updegraff method.

Figure 15. Sample test tubes after anthrone assay for cellulose estimation by Updegraff method.

Figure 16. Protein and nitrogen estimation by Kjeldahl method involving digestion, distillation and titration method.

3.1.2 Optimization of Alkali Pre-treated Corn Stover by Response Surface Methodology

Response surface methodology is a mathematical and analytical tool to analyze the data using models. It has optimization approaches for setting variables and get responses with maximum and minimum value. Central composite design (CCD) is factoral response surface design with centre, axial points to analyze the curvature (Sharif et al., 2023).

Alkali pretreatment with Sodium hydroxide can increase the effectiveness of enzymatic saccharification of corn stover for PHA production. Therefore, CCD was used to optimize cellulose and lignin content in corn stover by optimizing alkali concentration (%) and time (minutes) in pretreatement process as shown in **Figure 17 - 32**. The optimization conditions and responses were cellulose % and lignin content % present after the alkali pretreatment as shown in **Table 2 - 5.** Comparing the cellulose and lignin content before and after the pretreatment it was found that the cellulose content in untreated corn stover was 41.69 % and after alkali-pretreated 3% NaOH (w/v) 15-minutes corn stover was 83.58%. Thus, an increase in cellulose content in alkali pretreated corn stover. Further, the lignin content in untreated corn stover was 24.59 % and after alkali pretreated 3% NaOH (w/v) 15 minutes corn stover was 7.17 %. There is a decrease in lignin content as the long-term NaOH chemical pre-treatment along with high temperatures causes exposure of free hydrogen bonds in cellulosic structure which causes an increase in affinity with water and accumulation of corn stover particles agglomerates. Sodium hydroxide pretreatment also solubilize, extract lignin from lignocellulosic biomass by affecting the acetyl and ester group, decrease the degree of polymerization and breaks the bonds between lignin and other carbohydrate polymers. Therefore, alkali pretreatment is an utmost step to increase cellulose digestibility and improving lignin solubilization (Klongklaew et al., 2023; Venturin et al., 2018).

Solid biomass recovered after different alkali pretreatment conditions are given in **Table 6.** The total reducing sugars recovered after alkali pretreatment is given in **Figure 7 – 8.** The solid recoveries after the pretreatment are decreasing with increase

in alkali concentration and time as the pretreatment conditions involves increasing NaOH solutions which increase the solubility of lignin and carbohydrate components and further leads to loss of solid biomass in the pretreatment process. Alkali solutions partially dissolve the solid cellulose component and decreases the solid biomass recovery. Alkali pretreatment also shows structural modifications in the solid biomass after pretreatment like damage of cell walls or degradation of microfibrils which causes difficulty to recover the solid biomass. In the SEM images of solid residue remained after the pre-treatment conditions 1.625% and 3% NaOH (w/v) shows rough and exposed structure whereas untreated corn stover shows smooth surface as in **Figure 46 - 50**.

The optimized condition obtained for high cellulose and low lignin content after alkali pretreatment was at 3% NaOH (w/v) with 15.9 minutes. The optimized condition was carried out with 100g corn stover with 3% NaOH (w/v) and 15.9 minutes and had solid recovery of 55.8g with 82.91% cellulose and 7. 53% lignin content. (Yang et al., 2022) reported the mechanochemical pretreatment combined with alkaline pretreatment of corn stover. The highest glucose yield was 91.9% at 3% NaOH and ball milling for 10 minutes. The optimal condition had 44.4% lignin removal and 86.6% cellulose retention. According to (Gao et al., 2020), pretreatment of silvergrass at 4% NaOH concentration, cellulose removal was 45.2%, hemicellulose removal was 74.4% and lignin removal was 92.7% and on increasing the concentration from 4% to 5% NaOH, hydrolysis efficiency decreased due to increase in cellulose crystallinity. There is no change in LB degradation by varying the solid to liquid ratio ($p > 0.05$) and also no change in cellulose degradation by increasing the residence time ($p > 0.05$). The lignin removal enhanced by increasing time to 90 minutes ($p > 0.05$). Furthermore, regression equation obtained by RSM are given as -

$$\begin{aligned} &\boldsymbol{Regression\ equation\ for\ Lignin}\ (\%) \\ &\quad = \mathbf{25.251} - \mathbf{9.231}\ \boldsymbol{NaOH} - \mathbf{0.0339}\ \boldsymbol{Time} \\ &\quad + \mathbf{1.213}\ \boldsymbol{NaOH} \times \boldsymbol{NaOH} + \mathbf{0.000281}\ \boldsymbol{Time} \times \boldsymbol{Time} \\ &\quad - \mathbf{0.01152}\ \boldsymbol{NaOH} \times \boldsymbol{Time} \end{aligned}$$

***Regression equation for cellulose* (%) = 33.72 + 43.04 NaOH + 0.166 Time – 8.005 NaOH × NaOH – 0.00004 Time × Time – 0.2063 NaOH × Time**

A P-value of more than 1 tells not significant model terms. In the case of the studied factors in our research study, the maximum F-value in cellulose and lignin implies the most significant variable and the lowest F-value implies the least significant factor. The regression coefficient (R-square value) of the lignin % model is 99.52 % and predicted R-square value is 99.17% and the adjusted R-square value is 98%. The regression coefficient (R-square value) of the cellulose % model is 96.59%. The predicted R-square value is 94.15% and the adjusted R-square value is 89.86%. **Figure 33 and 36.** describes the Pareto charts of the standardized effects with responses (lignin and cellulose %). The Pareto chart shows the reference line to detect which factors are statistically significant and bars that are crossing the reference line are significantly important. In the Pareto chart **Figure 33** reference line 2.36 crosses factors A, AA and B and in **Figure 36** reference line 2.365 crosses factors A, AA, AB and B representing these statistically significant factors. Therefore, the factors represent statistical significance at the 0.05 level with current model terms. **Figure 34** and **37** depicts the response contour plots of cellulose % concerning NaOH and time and lignin (%) concerning NaOH and time. In **Figure 34** the high cellulose content area is shown in dark green with nearly 1.7 to 3% NaOH (w/v) and time (10-30) minutes. In **Figure 37** less lignin content is shown in light green with no effect of time. **Figure 35** and **38** shows the response heat maps with maximum cellulose and less lignin content in 3% NaOH (w/v) for 15 minutes. **Table 9-12** represents the optimized values of cellulose % after alkali pretreatment. **Table 13-16** represents the optimized values of lignin % after alkali pretreatment. The response optimization is given in **Table 17 – 21.** In this research study, lignin content decreased to 7.17%, lignin removal was 70.84% and cellulose content increased to 83.58% in corn stover after the alkali pretreatment which indicates the major role of alkaline pretreatment in removing lignin from corn stover and exposing large portion of biomass for effective enzymatic saccharification.

Figure 17. Comparison of solid corn stover biomass after different alkali pre-treatment conditions by RSM using Central composite design. Untreated sample and alkali pre-treated with 0.25%, 1.625% and 3% NaOH (w/v). Increasing the alkali concentration leads to delignification and further exposed the cellulose for utilization enzymatic saccharification. Alkali pre-treatment affects the structural properties of the corn stover, which is also studied by FTIR, TGA and SEM.

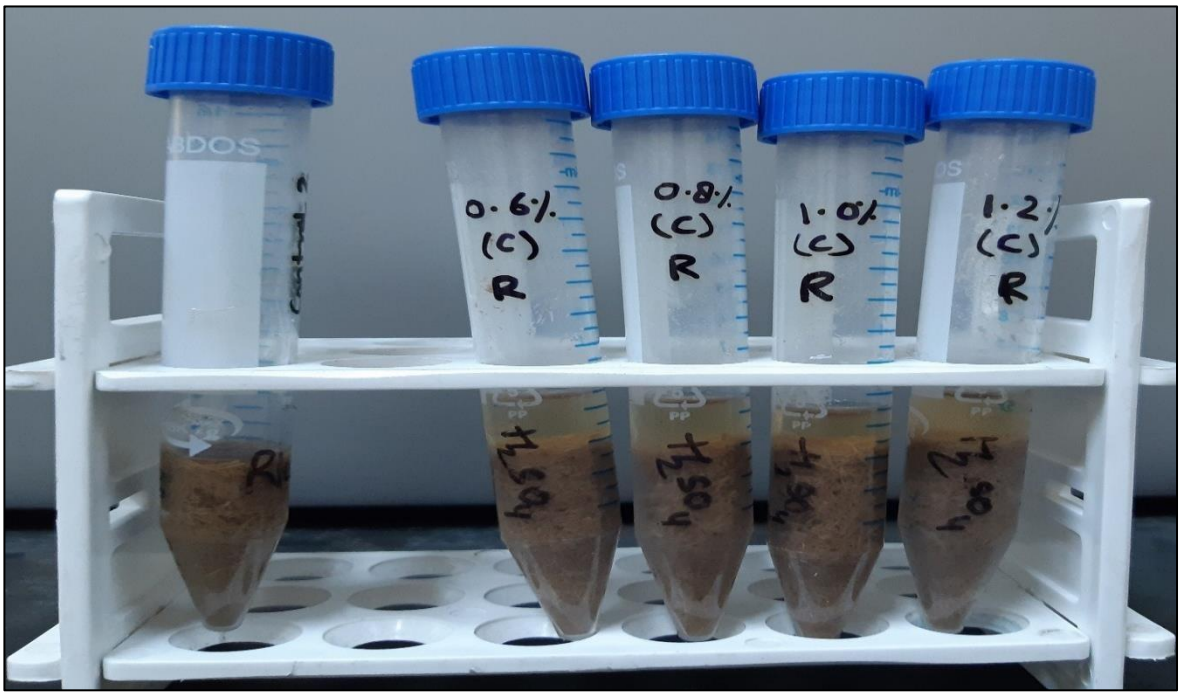


Figure 18. Test tubes after thermochemical pre-treatment.

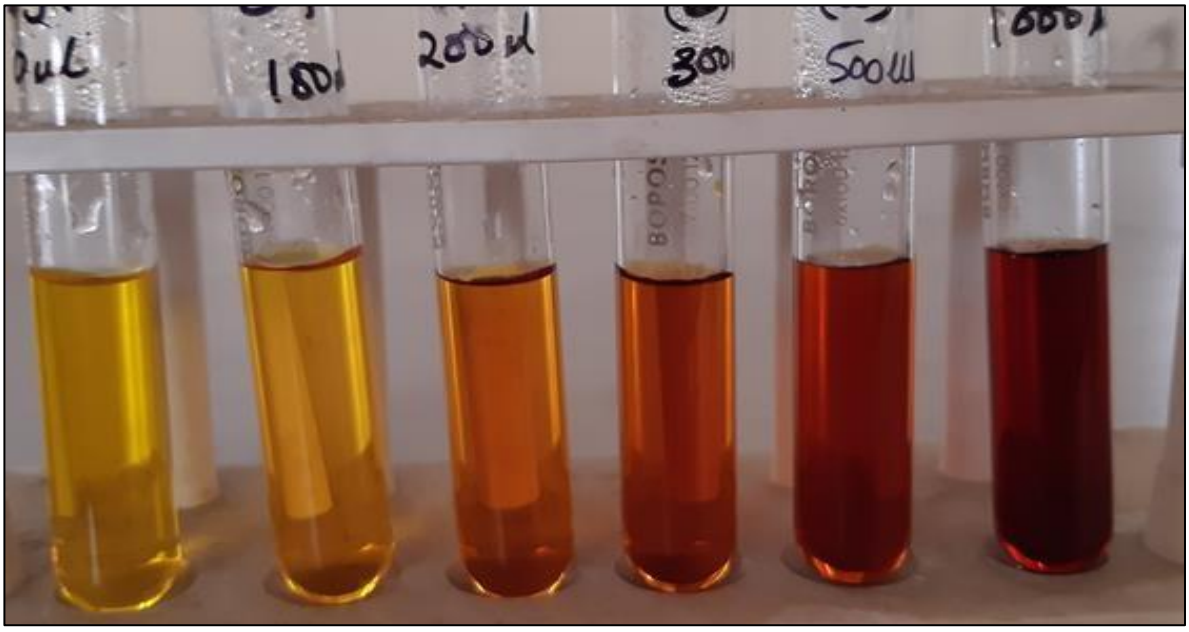


Figure 19. Standard glucose test tubes showing color after DNS test for total reducing sugar analysis.

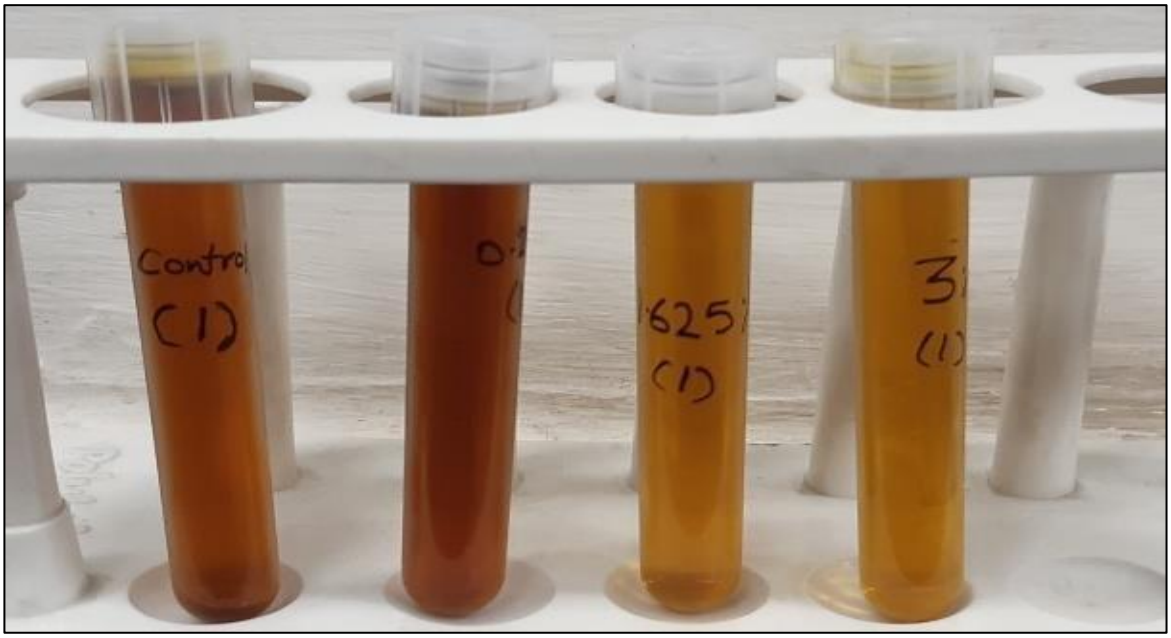


Figure 20. Sample test tubes of control, 0.25%, 1.625%, 3% NaOH (w/v) after 15 minutes of thermochemical pre-treatment.

Figure 21. Sample test tubes of control, 0.25%, 1.625%, 3% NaOH (w/v) after 45 minutes of thermochemical pre-treatment.

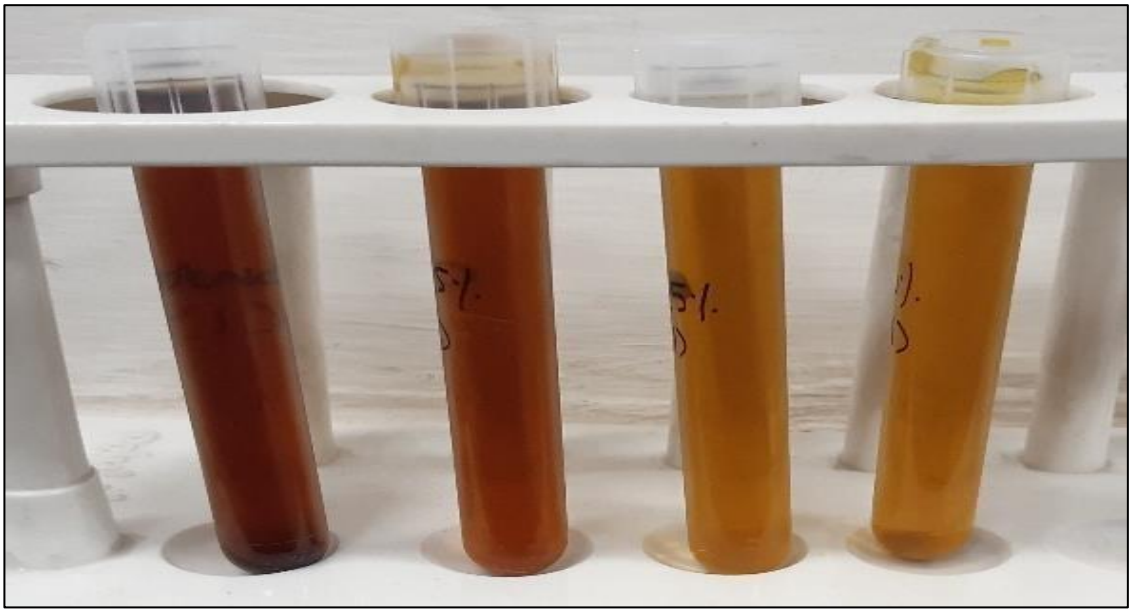


Figure 22. Sample test tubes of control, 0.25%, 1.625%, 3% NaOH (w/v) after 15 minutes of thermochemical pre-treatment.

Table 2. Optimization condition with alkali concentration and time.

Variable	Symbol	Coded levels		
		-1	**0**	**+1**
Alkali concentration (%)	**A**	0.25	1.625	3
Time (minutes)	**B**	15	45	75

Table 3. Design summary.

Parameters	Number
Factors	2
Replicates	1
Base runs	13
Total runs	13
Base blocks	1

Total blocks	1

Table 4. Point Types with parameters.

Parameters	Number
Cube points	4
Center points in cube	5
Axial points	4
Center points in axial	0

Table 5. Experiments designed by Minitab.

Std order	Run order	Pt type	Blocks	Alkali concentration (%)	Time (minutes)
2	1	1	1	3	15
6	2	-1	1	3	45
11	3	0	1	1.625	45
12	4	0	1	1.625	45
4	5	1	1	3	75
8	6	-1	1	1.625	75
1	7	1	1	0.25	15
7	8	-1	1	1.625	15
3	9	1	1	0.25	75
13	10	0	1	1.625	45
10	11	0	1	1.625	45
5	12	-1	1	0.25	45
9	13	0	1	1.625	45

Table 6. Central composite design and the response for cellulose (%) and lignin (%) with different alkali concentration (%) and time (minutes).

Run Order	Alkali concentration (%)	Time (minutes)	Cellulose (%)	Lignin (%)
1	3 (+1)	15 (-1)	83.62±0.15	7.17±0.49
2	3 (+1)	45 (0)	71.23±0.65	6.54±0.30
3	1.625 (0)	45 (0)	81.02±1.88	11.92±0.07
4	1.625 (0)	45 (0)	71.59±0.94	11.98±0.15
5	3 (+1)	75 (+1)	56.23±0.35	4.69±0.27
6	1.625 (0)	75 (+1)	68.80±0.36	11.32±0.51
7	0.25 (-1)	15 (-1)	47.03±0.63	22.4±0.26
8	1.625 (0)	15 (-1)	79.11±1.81	13.17±0.24
9	0.25 (-1)	75 (+1)	53.65±0.40	21.85±0.10
10	1.625 (0)	45 (0)	72.89±1.06	11.39±0.02
11	1.625 (0)	45 (0)	75.90±1.98	11.64±0.50
12	0.25 (-1)	45 (0)	46.51±0.33	22.03±0.15
13	1.625 (0)	45 (0)	74.67±0.02	10.68±0.60

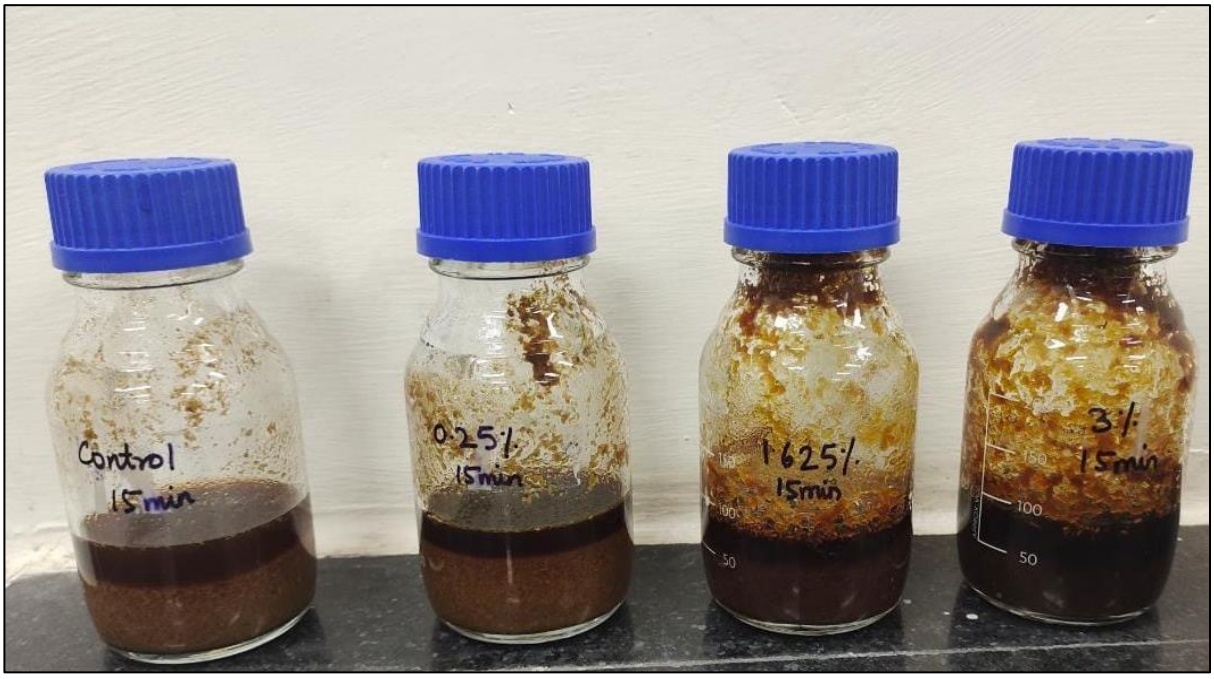


Figure 23. Thermochemical pretreatment of corn stover with NaOH at different RSM conditions using Minitab.

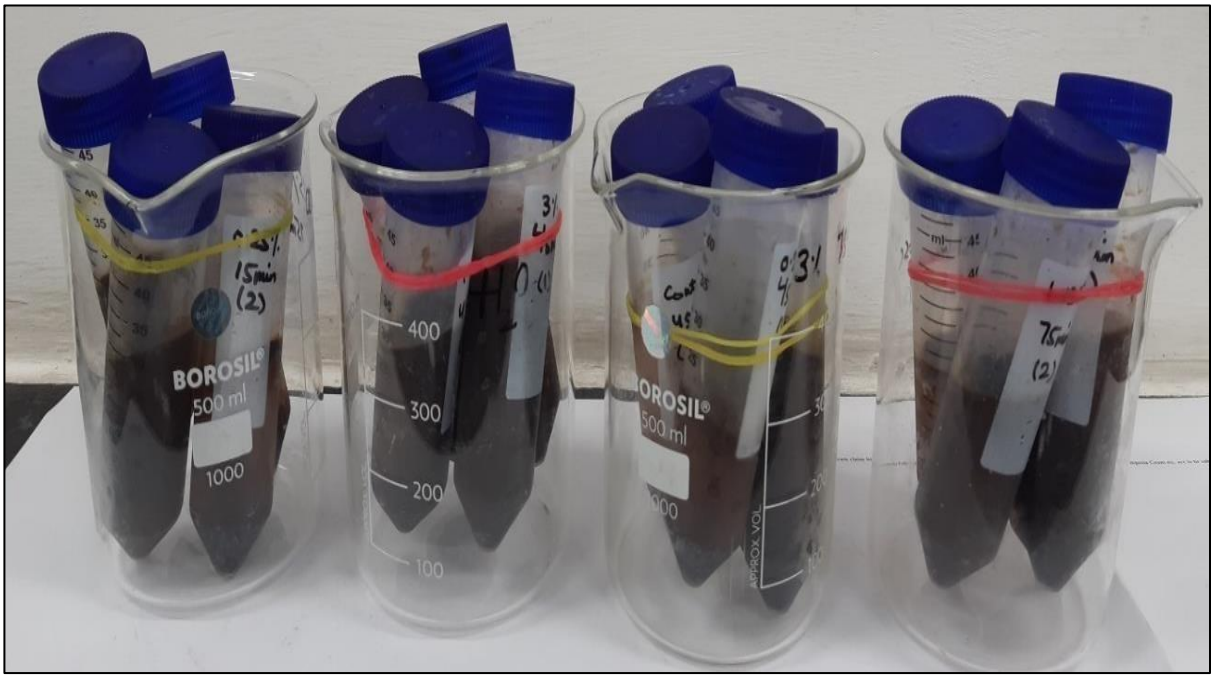


Figure 24. Liquid hydrolysate after Na OH pre-treatment.

Optimized condition 3% NaOH, 15 minutes 50g in 500ml

Optimized condition 3% NaOH, 15 minutes, solid biomass recovery

Optimized condition 3% NaOH, 15 minutes, liquid recovery

Figure 25. Large scale optimized condition of 3% NaOH (w/v) for 15 minutes to be used for enzymatic hydrolysis.

Table 7. Results of total reducing sugar after alkali pre-treatment.

Tubes	Glucose concentration(mg/ml)	Absorbance (540nm)	Absorbance (540nm)	Average absorbance (540nm)
1	0	0	0	0
2	0.1	0.0527	0.0547	0.0537
3	0.2	0.1653	0.1546	0.15995
4	0.3	0.2562	0.2323	0.24425
5	0.4	0.3189	0.3309	0.3249
6	0.5	0.4256	0.4357	0.43065
7	0.6	0.4711	0.4614	0.46625
8	0.7	0.616	0.6301	0.62305
9	0.8	0.6638	0.6797	0.67175
10	0.9	0.8328	0.8213	0.82705

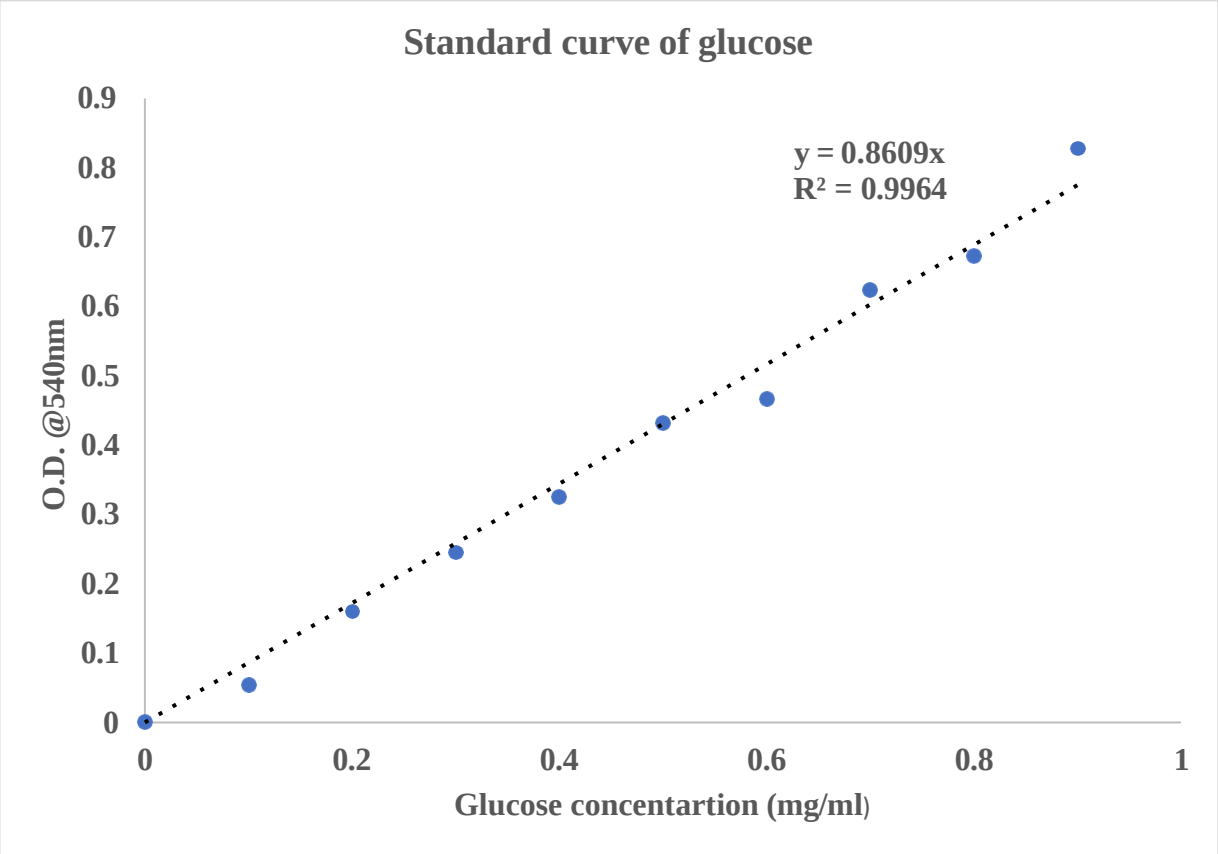


Figure 26. Glucose standard curve for DNS test.

Table 8. Total reducing sugars at different pretreatment conditions.

S.No.	Conditions	Average TRS (mg/ml)	Liquid recovery (ml)	Neutralization with 1N HCl, vol. added (ml)	Final liquid hydrolysate (ml)	Dilution factor	TRS mg/ml	TRS mg/g
1	0.250%, 15min	1.658	29	0.05	29.05	1.00	1.661	4.817
2	1.625%, 15min	0.435	29	3.9	32.9	1.13	0.493	1.432
3	3.000%, 15min	0.437	26	9	35	1.34	0.588	1.531
4	0.250%, 45min	1.557	28	0.04	28.04	1.00	1.560	4.368
5	1.625%, 45min	0.530	29.9	4.4	34.3	1.14	0.608	1.820
6	1.625%, 45min	0.539	30.5	4.5	35	1.14	0.619	1.889
7	1.625%, 45min	0.511	30.3	4.4	34.7	1.14	0.585	1.773
8	1.625%, 45min	0.502	30	4.4	34.4	1.14	0.576	1.728
9	1.625%, 45min	0.506	29.9	4.4	34.3	1.14	0.581	1.738
10	3.000%, 45min	0.480	27	10	37	1.37	0.658	1.779
11	0.250%, 75min	1.860	28	0.04	28.04	1.00	1.863	5.217
12	1.625%, 75min	0.624	26	3	29	1.11	0.696	1.810
13	3.000%, 75min	0.547	24	7.4	31.4	1.30	0.716	1.720

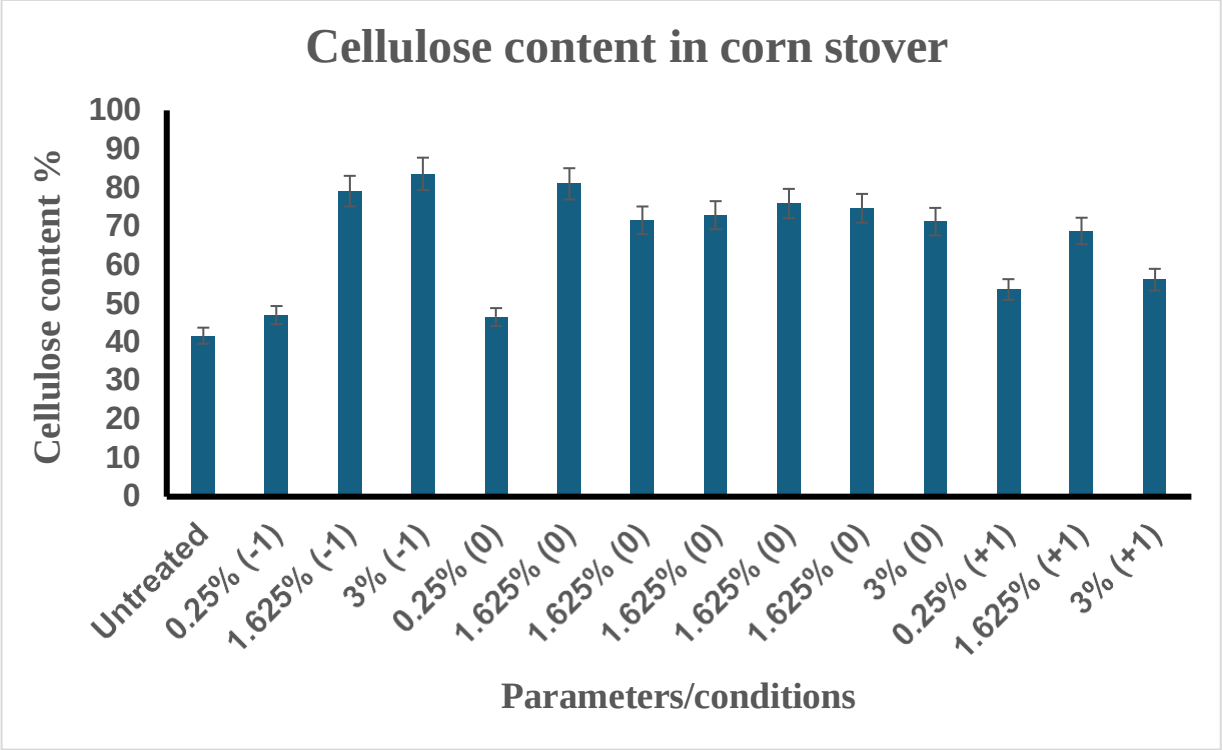


Figure 27. Cellulose content after alkali pre-treatment in corn stover at different RSM conditions.

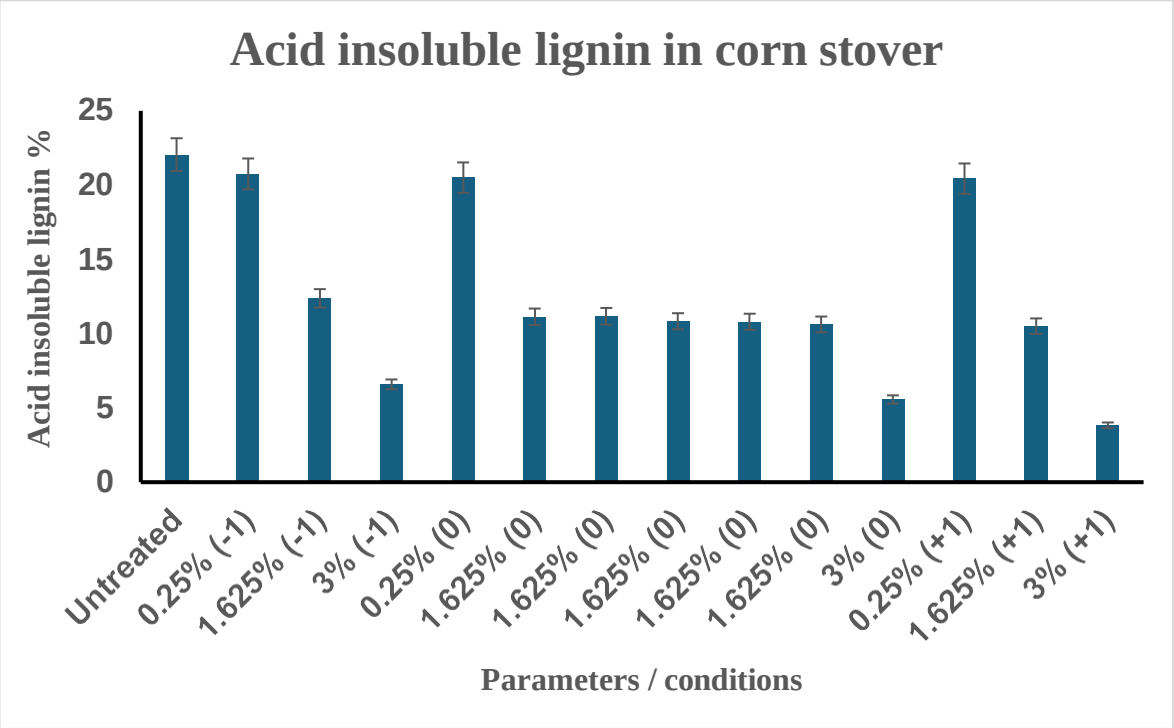


Figure 28. Acid insoluble lignin content after alkali pre-treatment in corn stover at different RSM conditions.

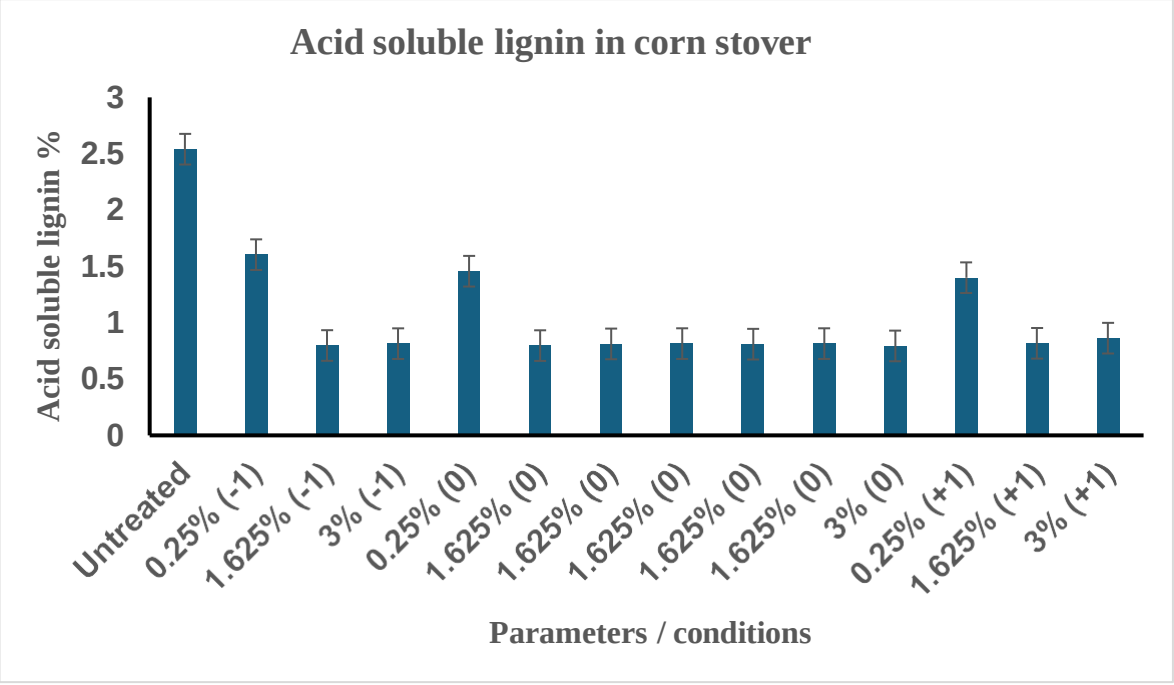


Figure 29. Acid soluble lignin content after alkali pre-treatment in corn stover at different RSM conditions.

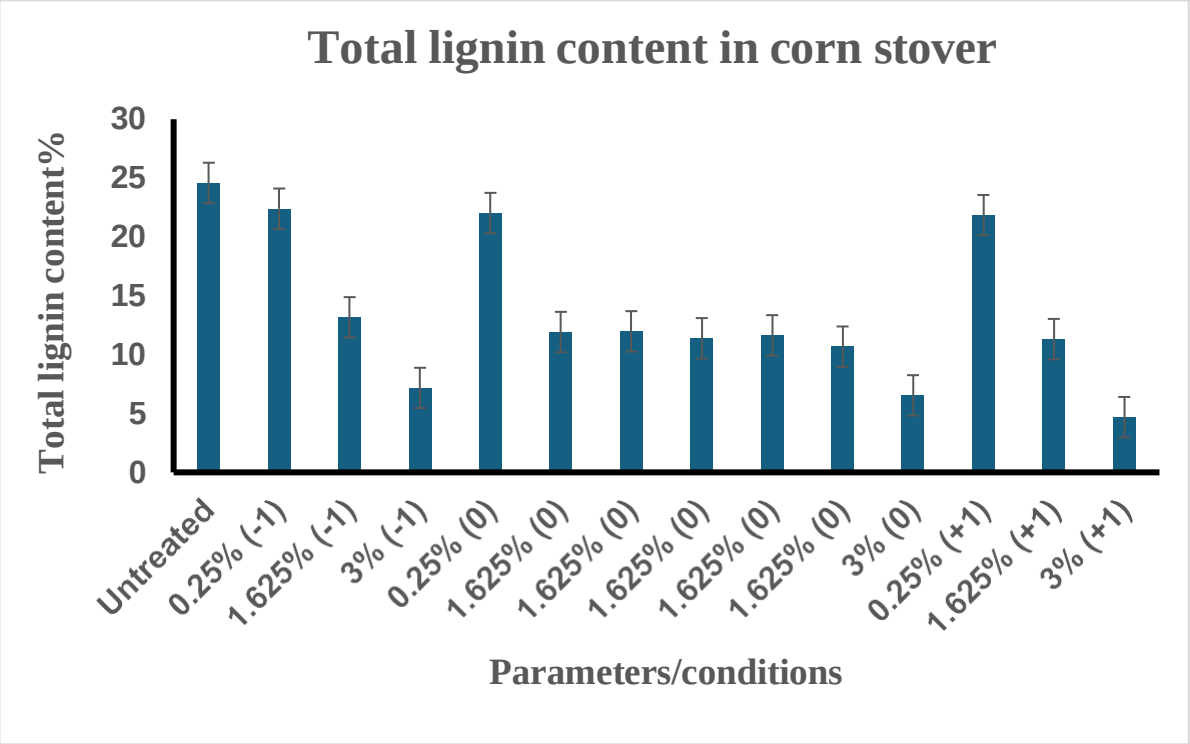


Figure 30. Total lignin content after alkali pre-treatment in corn stover at different RSM conditions.

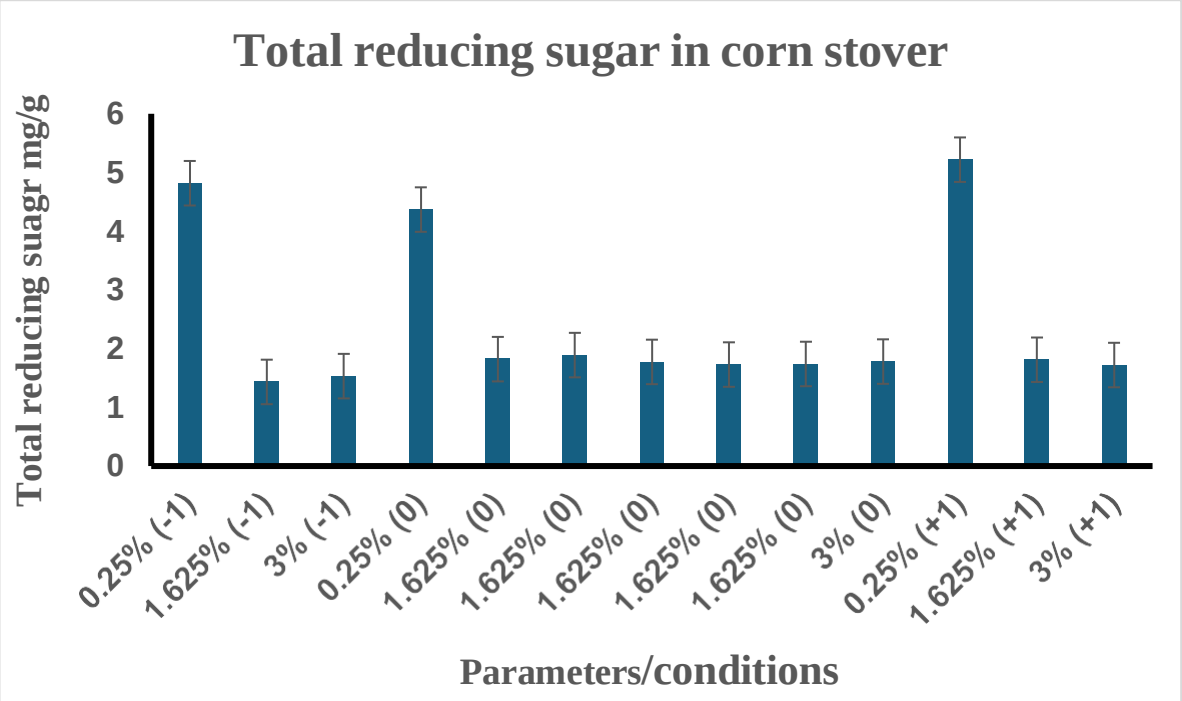


Figure 31. Total reducing sugars after alkali pre-treatment in corn stover at different RSM conditions.

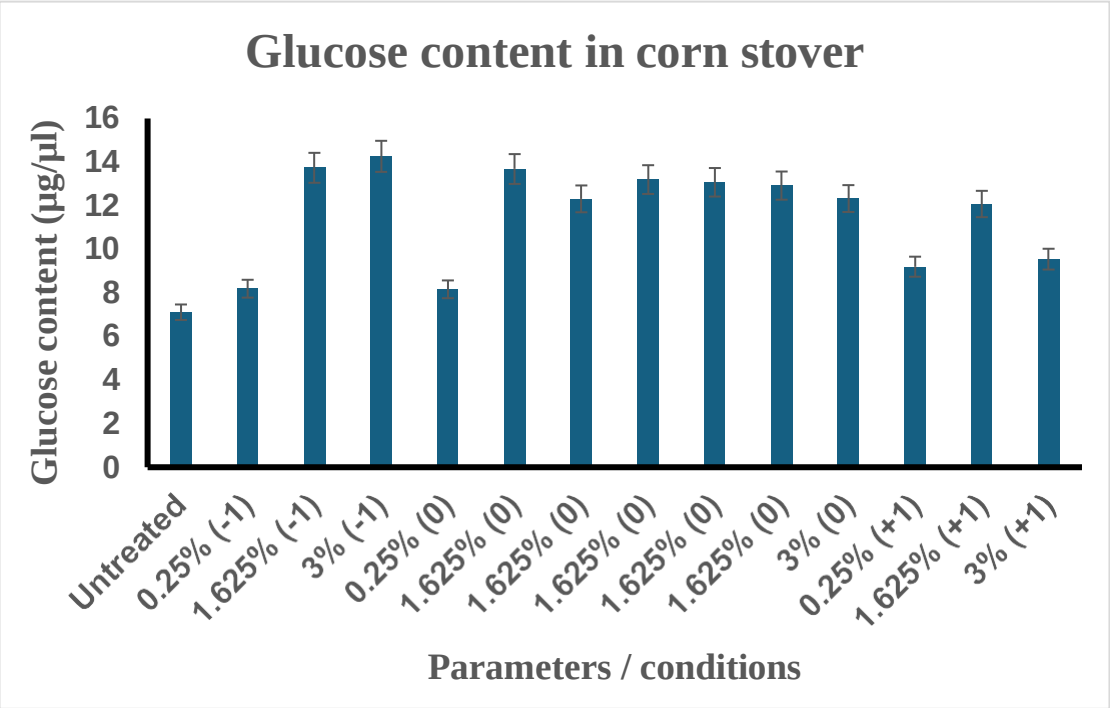


Figure 32. Glucose concentration after alkali pre-treatment in corn stover at different RSM condition.

Table 9. Response surface regression: Lignin (%) versus NaOH and Time.

Term	Coef	SE Coef	T-value	P-value	VIF
Constant	11.656	0.220	52.94	0.000	
NaOH	-7.985	0.216	-36.89	0.000	1.00
Time	-0.818	0.216	-3.78	0.007	1.00
NaOH*NaOH	2.293	0.319	7.19	0.000	1.17
Time*Time	0.253	0.319	0.79	0.453	1.17
NaOH*Time	-0.475	0.265	-1.79	0.116	1.00

Table 10. Model summary.

S	R-sq	R-sq(adj)	R-sq(pred)
0.530273	99.52%	99.17%	98.00%

Table 11. Analysis of variance of lignin content.

Source	SS	Df	Mean square	F-value	p-value	
Model	406.11	5	81.222	288.85	0.000	Significant
Linear	386.579	2	193.29	687.4	0.000	
NaOH	382.561	1	382.561	1360.51	0.000	
Time	4.018	1	4.018	14.29	0.007	
Square	18.628	2	9.314	33.12	0.000	
NaOH*NaOH	14.525	1	14.525	51.66	0.000	
Time*Time	0.177	1	0.177	0.63	0.453	
2-way intercation	0.902	1	0.902	3.21	0.116	
NaOH*Time	0.902	1	0.902	3.21	0.116	
Error	1.968	7	0.281		0.000	
Lack of fit	0.86	3	0.287	1.03	0.467	
Pure Error	1.108	4	0.277		0.000	
Model statistics						
R2	99.52					
Adj-R2	99.17					
Pred-R2	98					

Regression equation in uncoded units

Lignin (%) = 25.251 – 9.231 NaOH – 0.0339 Time + 1.213 NaOH*NaOH + 0.000281 Time*Time – 0.01152 NaOH*Time

Table 12. Fits and diagnostics of unusual observations.

Observations	Lignin (%)	Fit	Residue	Std Residue
7	10.680	11.656	-0.976	-2.02

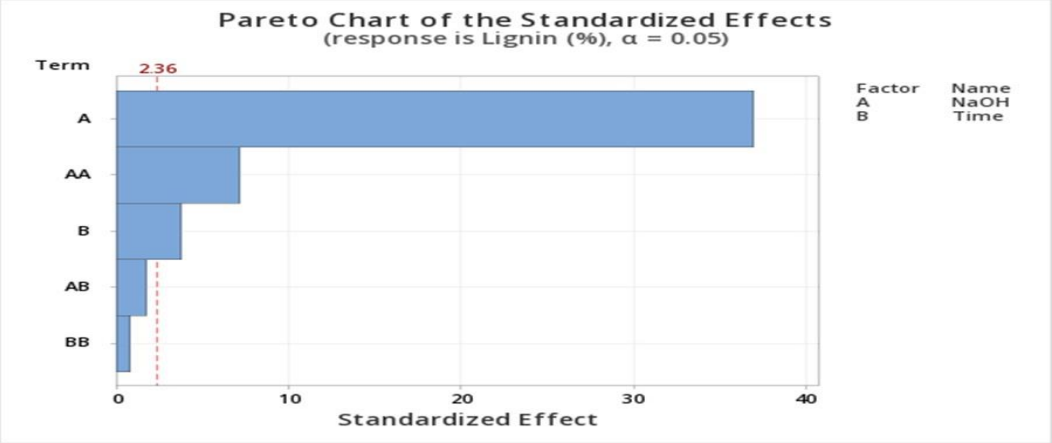


Figure 33. Pareto charts of the standardized effect with two factors NaOH and time showing response of cellulose (%).

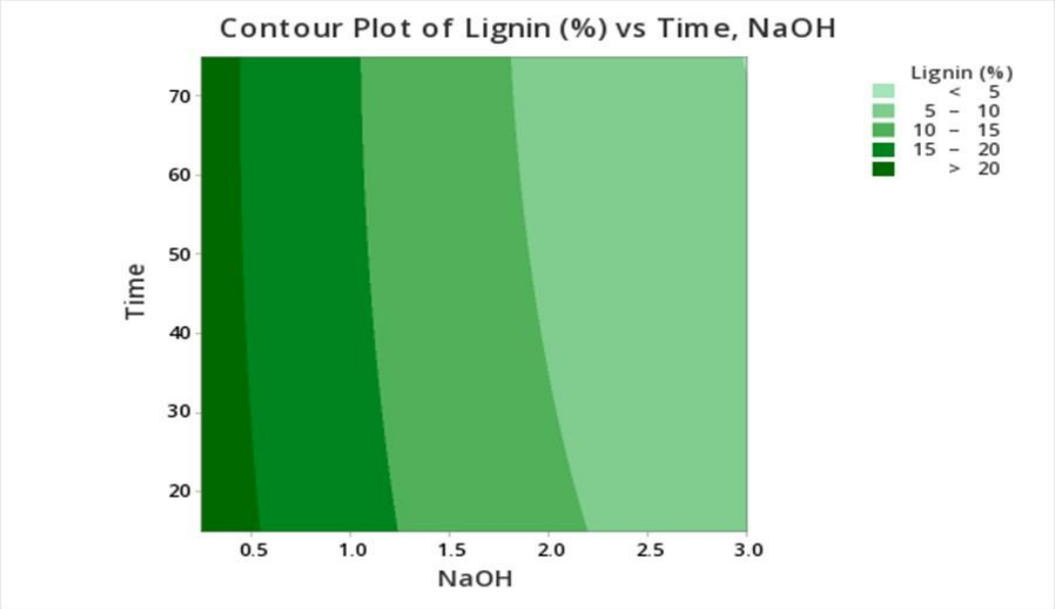


Figure 34. Response contour plots for alkaline pretreatment of corn stover describing the interaction of time (minutes) and 3% NaOH (w/v) i.e. effect on cellulose (%) vs Time and NaOH.

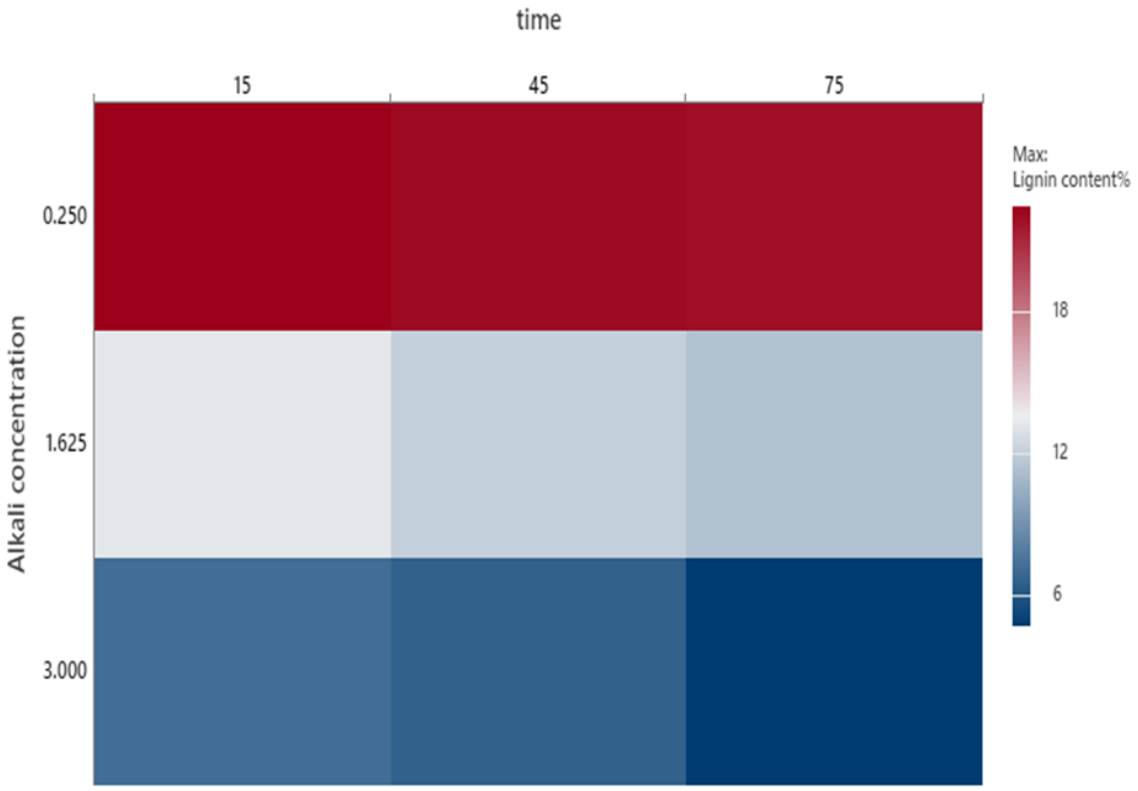


Figure 35. Response heat maps for alkaline pretreatment of corn stover showing interaction between 3% NaOH (w/v) and time (minutes) with maximum cellulose content (%) respectively.

Table 13. Response surface regression: Cellulose (%) versus NaOH and Time.

Term	Coef	SE Coef	T-value	P-value	VIF
Constant	74.83	1.28	58.64	0.000	
NaOH	10.64	1.25	8.48	0.000	1.00
Time	-5.18	1.25	-4.13	0.004	1.00
NaOH*NaOH	-15.13	1.85	-8.18	0.000	1.17
Time*Time	-0.04	1.85	-.002	0.984	1.17
NaOH*Time	-8.51	1.54	-5.54	0.001	1.00

Table 14. Model summary.

S	R-sq	R-sq(adj)	R-sq(pred)
3.07306	96.59%	94.15%	89.86%

Table 15. Results of analysis of variance of cellulose content.

Source	SS	Df	Mean square	F-value	p-value	
Model	1871.73	5	374.346	39.64	0.00	Significant
Linear	840.67	2	420.336	44.51	0.00	
NaOH	679.47	1	679.47	71.95	0.00	
Time	161.20	1	161.202	17.07	0.004	
Square	741.38	2	370.688	39.25	0.00	
NaOH*NaOH	632.55	1	632.549	66.98	0.00	
Time*Time	0.00	1	0.004	0.00	0.984	
2-way intercation	289.68	1	289.68	30.67	0.001	
NaOH*Time	289.68	1	289.68	30.67	0.001	
Error	66.11	7	9.444			
Lack of fit	13.12	3	4.374	0.33	0.805	
Pure Error	52.98	4	13.246			
Model statistics						
R2	96.59					
Adj-R2	94.15					
Pred-R2	89.86					

Regression equation in uncoded units

Cellulose (%) = 33.72 + 43.04 NaOH + 0.166 Time – 8.005 NaOH*NaOH -0.00004 Time*Time – 0.2063 NaOH*Time

Table 16. Fits and diagnostics of unusual observations.

Observations	Lignin (%)	Fit	Residue	Std Residue
2	80.97	74.83	6.14	2.20

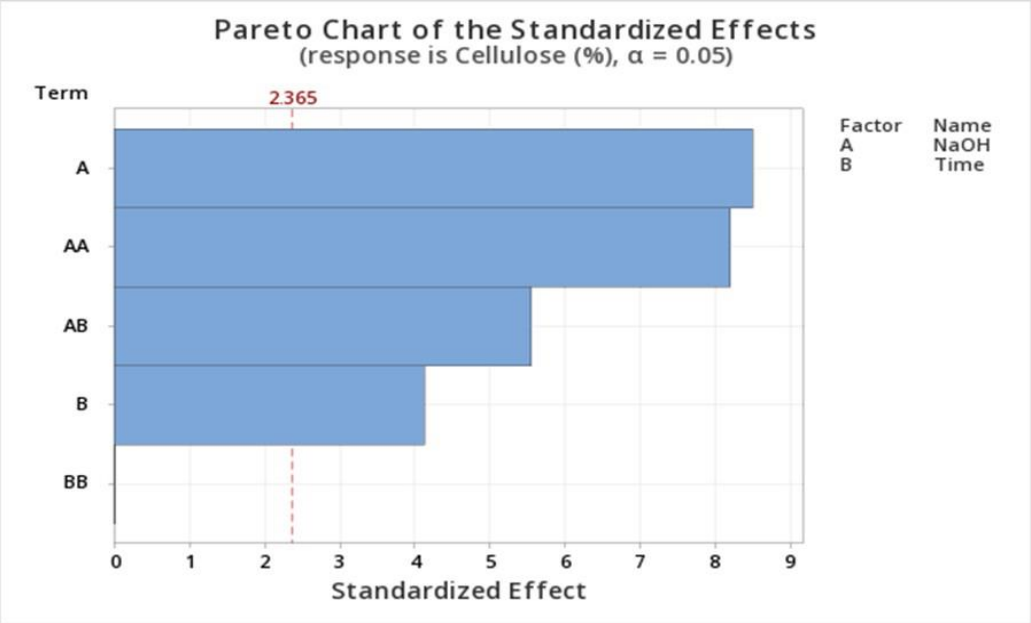


Figure 36. Pareto charts of the standardized effect with two factors NaOH and time showing response of lignin (%).

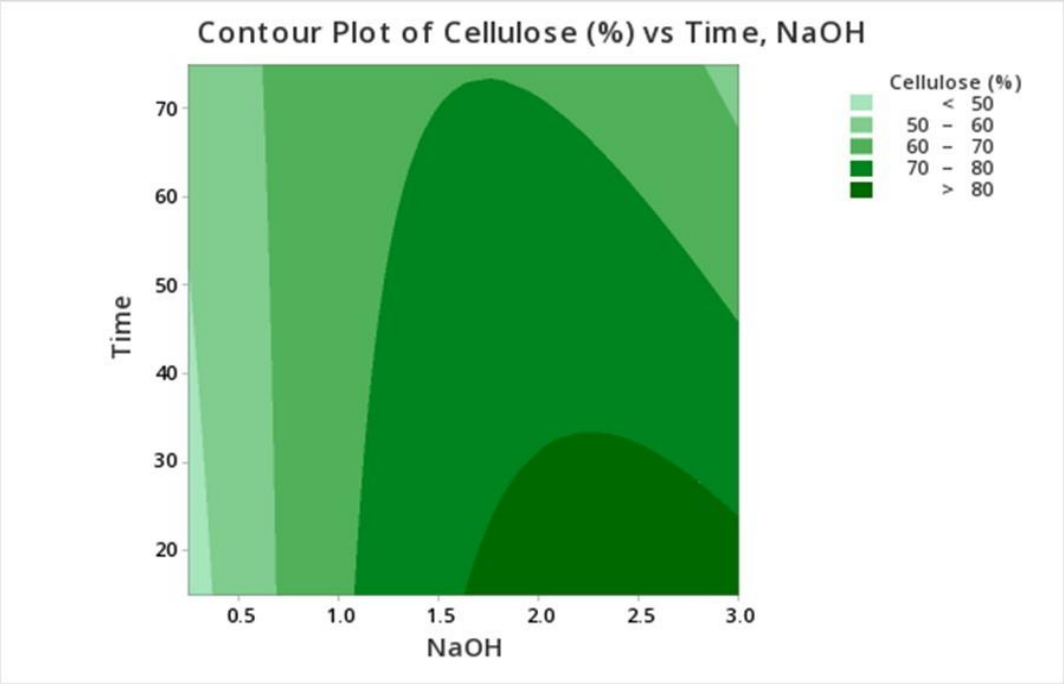


Figure 37. Response contour plots for alkaline pretreatment of corn stover describing the interaction of time (minutes) and 3% NaOH (w/v) i.e. effect on lignin (%) vs Time and Na OH.

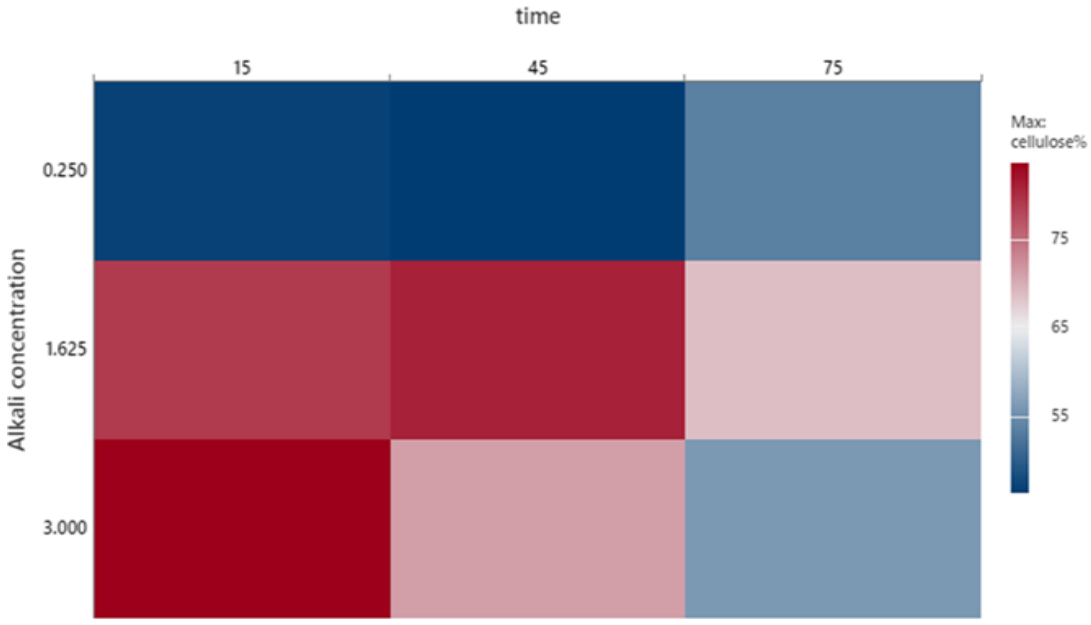


Figure 38. Response heat maps for alkaline pretreatment of corn stover showing interaction between 3% NaOH (w/v) and time (minutes) with minimum lignin content (%) respectively.

Table 17. Response optimization: Cellulose and Lignin (%).

Response	Goal	Lower	Target	Upper	Weight	Importance
Cellulose (%)	Maximum	46.48	83.58	-	1	1
Lignin (%)	Minimum	-	4.69	22.43	1	1

Table 18. Solution with cellulose (%) and lignin (%).

Solution	NaOH	Time	Cellulose (%) Fit	Lignin (%) Fit	Composite Desirability
1	3	15.9122	83.5797	7.45660	0.918717

Table 19. Multiple response prediction with variables - NaOH and time.

Variable	Setting
NaOH	3
Time	15.9122

Table 20. Response prediction of cellulose (%) and lignin (%).

Response	Fit	SE Fit	95% CI	95% PI
Cellulose (%)	83.58	2.67	(77.28, 89.88)	(73.96, 93.20)
Lignin (%)	7.457	0.460	(6.369, 8.544)	(5.797, 9.116)

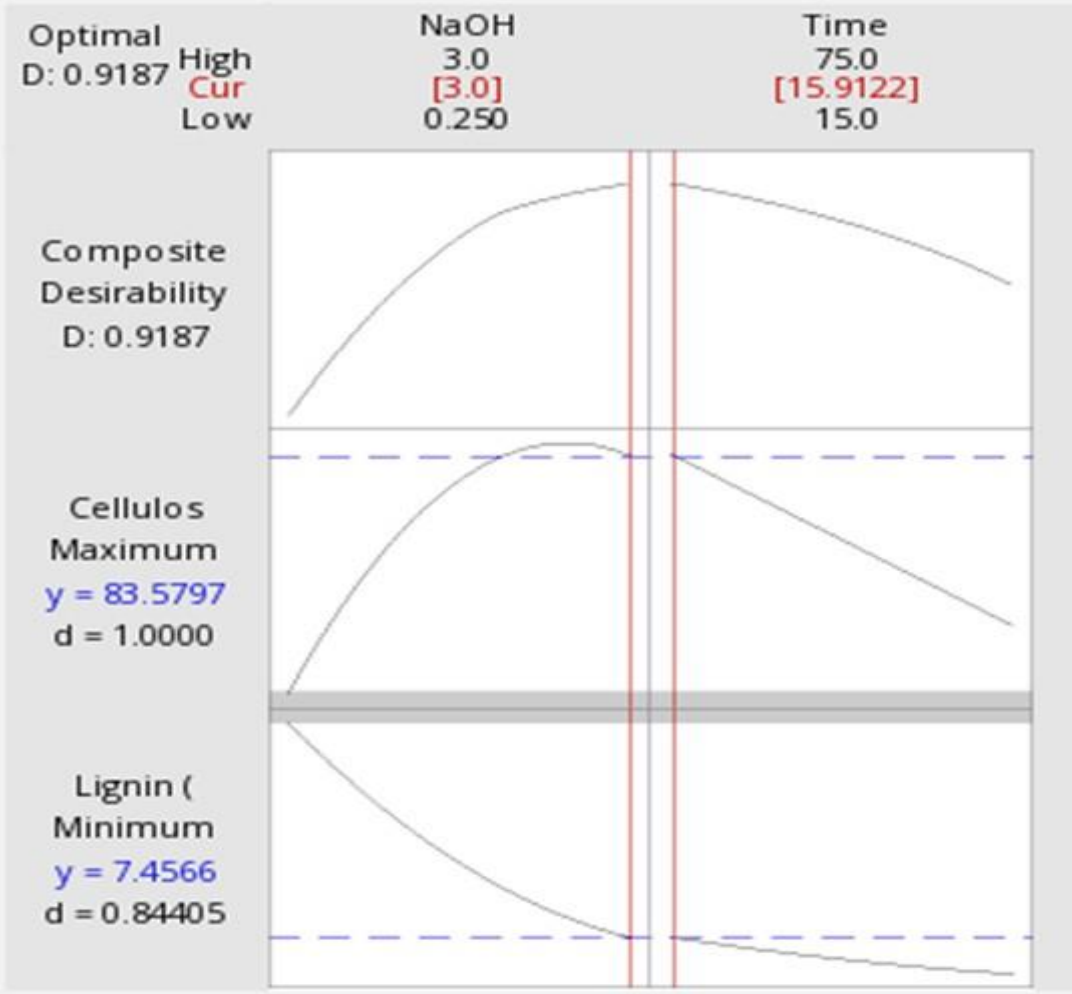


Figure 39. Optimization graph with maximum cellulose and minimum lignin.

Table 21. Experimental and predicted values of cellulose (%) and lignin (%)

NaOH (%)	Time(minutes)	Cellulose (%)		Lignin (%)	
		Experimental	Predicted	Experimental	Predicted
3	15.9	82.91	83.57	7.53	7.45

3.1.3 Characterization of Alkali Pre-treated Corn Stover Before and After Pre-treatment

Characterization of untreated and alkali pre-treated corn stover was done to understand the physiochemical changes before and after alkali pretreatment. Therefore, changes in cellulose crystallinity, thermal characteristics and surface elemental composition were measured.

3.1.3.1 Thermal Stability Analysis (TGA)

Figure 40 - 43 shows the TGA and DTG curves which shows the thermal characteristics of untreated, and alkali optimized treated corn stover. In untreated corn stover, the sample started with 99.441% initial weight at 29.62°C and the weight reduced to 50.984% at 352.21°C and at 796.54°C the weight was 0.024%. In the optimized sample at 3% NaOH (w/v) 15 minutes, the initial sample weight was 99.121% at 29.58°C and the weight was 50.927% reduced at 366.76°C and further to 0.000% at 682.97°C. The relationship is drawn between the change in weight and temperature to check the thermal behaviour. As in Shubhedar et al., 2018, the first stage below100°C in both untreated and treated corn stover is due to moisture loss. The second stage between 200-400°C is representing nearly 55-60% weight loss due to degradation of carbohydrates (hemicellulose and cellulose) and it denotes the fastest region of weight loss. The decomposition beyond 400°C represents the mass loss due to lignin residues. The lignin residue being less in the treated sample became more susceptible to fast thermal degradation (Subhedar et al., 2018).

3.1.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

Figure 44 - 45 shows the FTIR spectra recorded between 4000 to 400 cm^{-1}. The two FTIR spectra of untreated corn stover sample and 3% NaOH (w/v) 15.9 minutes optmized treated corn stover sample was done to study the structural changes in corn stover, changes in crystallanity, chemical functional groups, structure of lignin, bonding of carbohydrate and lignin complex. An increase in width and symmetry between 3200 and 3400cm^{-1} indicates dissociation of the cellulosic structure, whereas changes in the peak intensity at 2915cm^{-1} indicates $–CH_2$ stretching and reupture of cellulose (Sharma et al., 2016). Decrease in intensity at approximately 1652cm^{-1}

demonstrates lignin removal and this peak associates to stretching vibration of aromatic rings and phenyl ester side chain C=O bonds of lignin. The peak at 1056cm-1 indicates to removal of amorphous cellulose. The peak around 1732cm-^{1} is due to C=O stretching vibration, 1515cm-^{1} is due to C=C aromatic symmetrical stretching, 1464cm-^{1} is due to HCH and OCH in plane bending vibration, 1248 cm-^{1} is due to G ring stretching, 1161cm-^{1} is due to stretching of uncounjugated C-Obonds, 1039 cm-^{1} is due to aromatic C-H deformation (Woźniak et al., 2021) and therefore, band intensities are reduced after alkali pretreatment. FTIR analysis of treated corn stover sample show the disappereance of many peaks due to removal of lignin and hemicellulose compoment from the biomass after NaOH pretreatment (Fan et al., 2012).

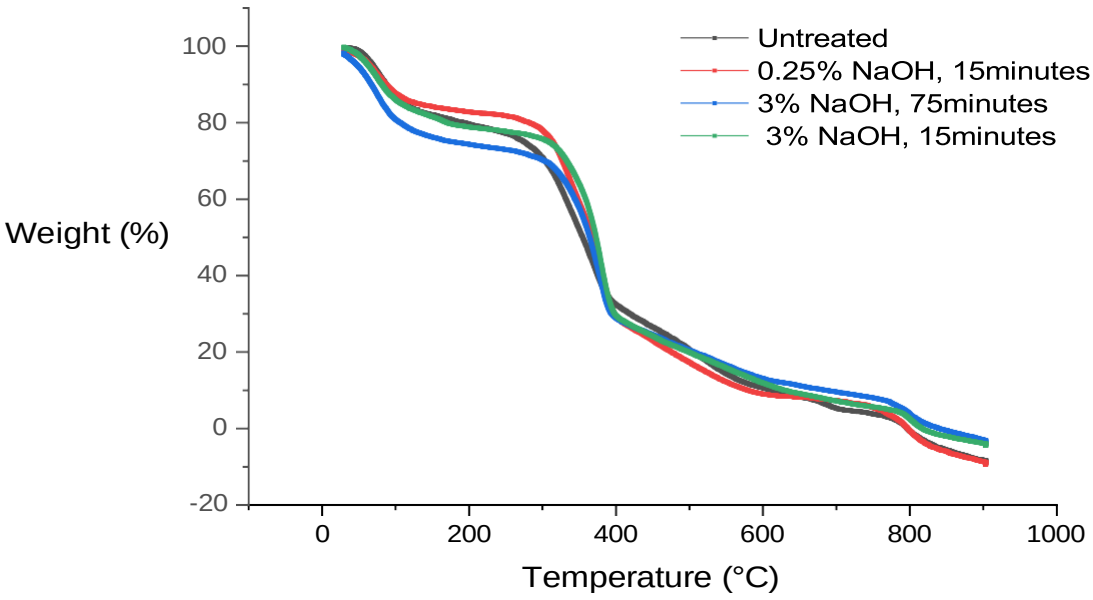


Figure 40. TGA graph of different conditions of corn stover after alkali pre-treatment.

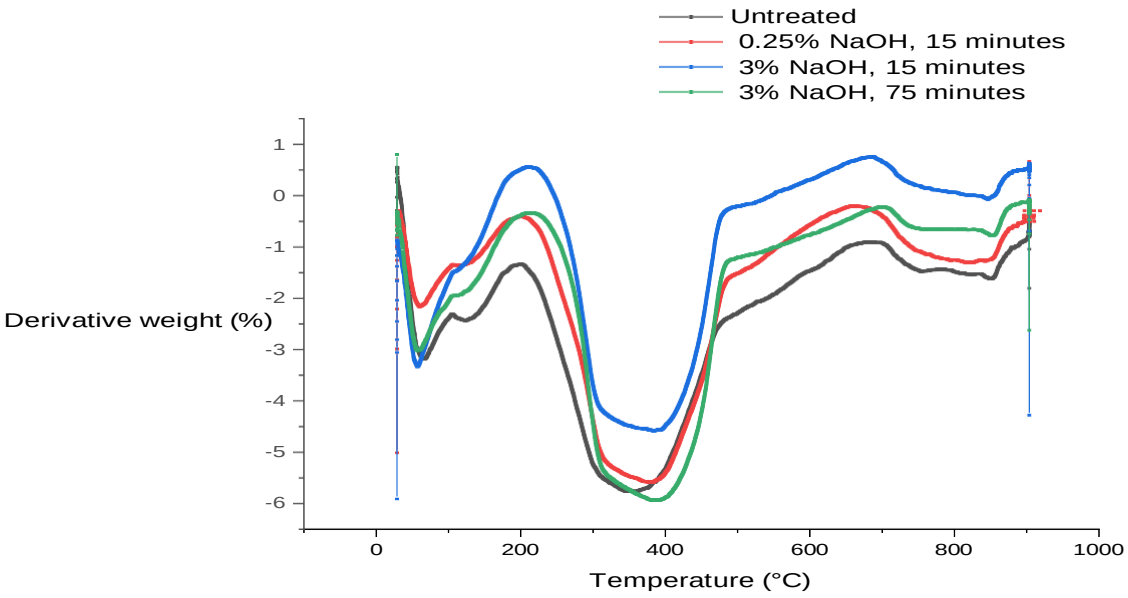


Figure 41. DGA graph of different conditions of corn stover after alkali pre-treatments.

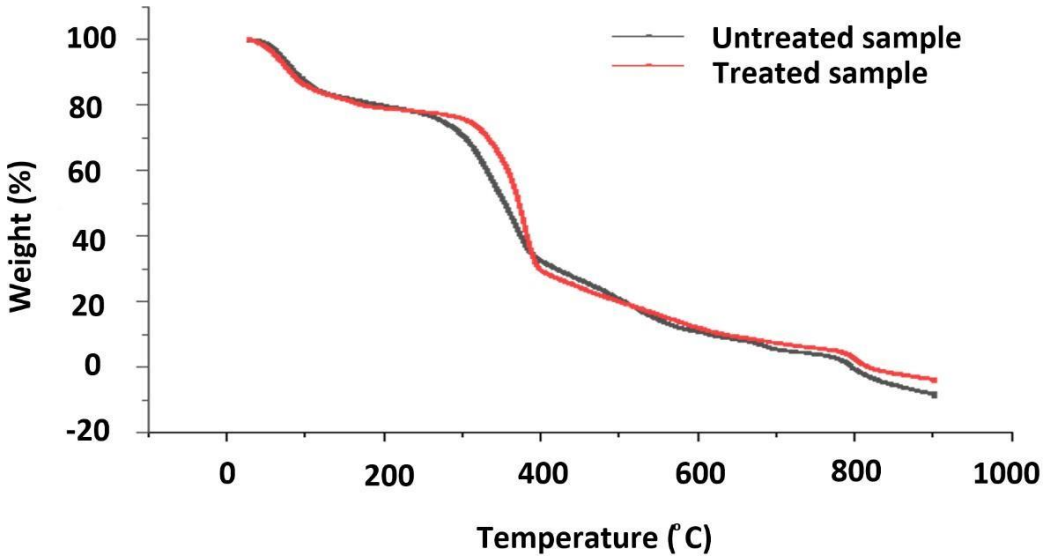


Figure 42. TGA graph of untreated and treated corn stover sample after alkali pretreatment.

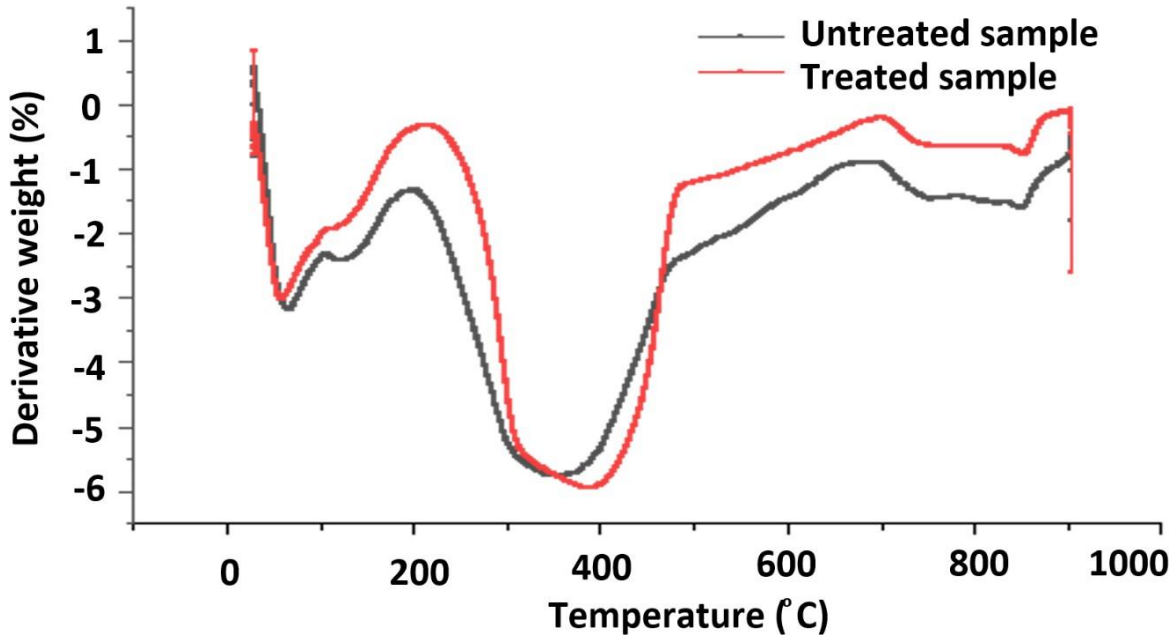


Figure 43. DGA graph of untreated and treated corn stover sample after alkali pretreatment.

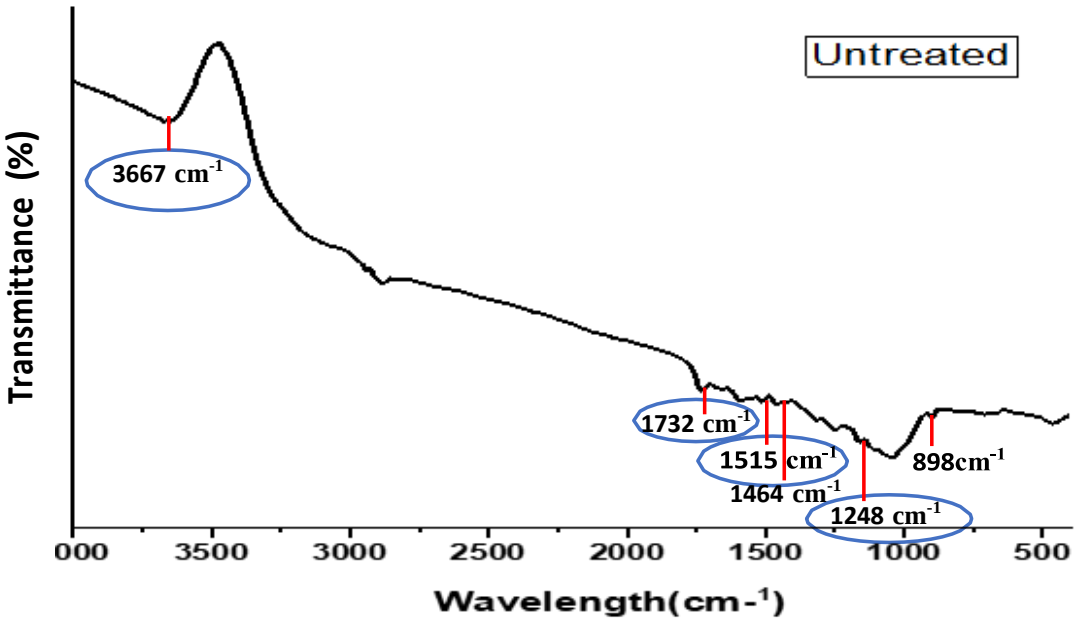


Figure 44. FTIR graph of untreated corn stover sample after alkali pretreatment.

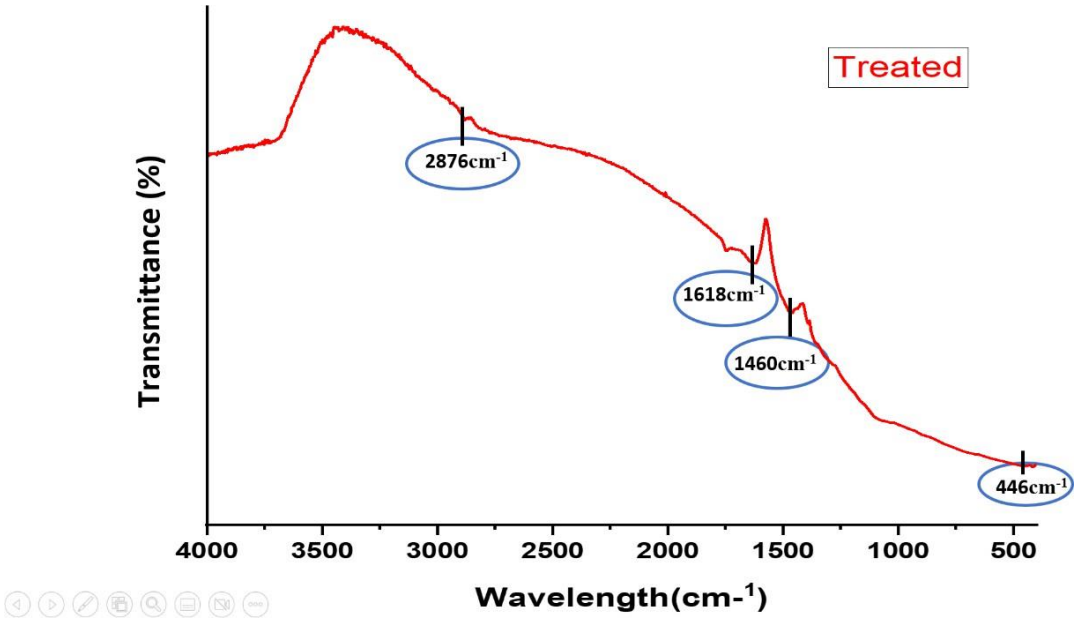
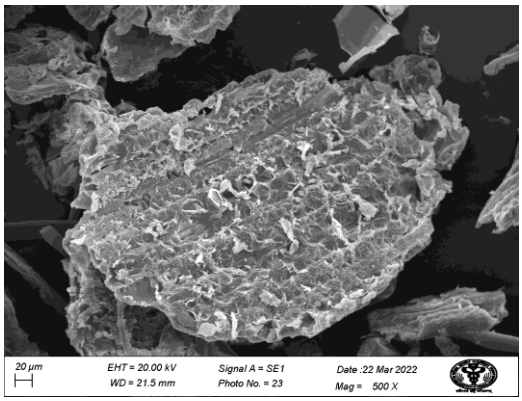


Figure 45. FTIR graph of pretreated corn stover sample after alkali pretreatment.

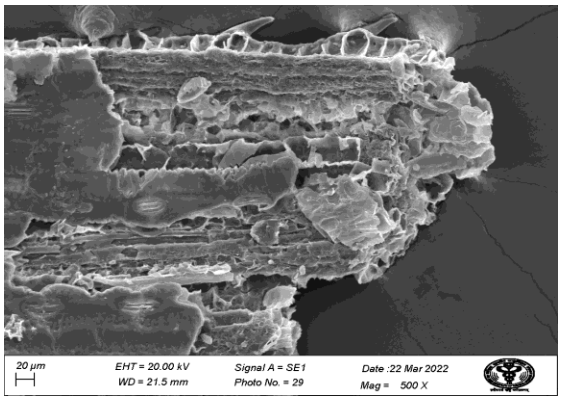
3.1.3.3 Scanning Electron Microscopy (SEM)

SEM analysis of untreated and optimized treated corn stover samples. An untreated corn stover sample shows the compact structure of lignocellulosic biomass, which is covered with lignin, and forms a smooth structure. After NaOH, pre-treatment at a high concentration that is 3% (w/v) lignin had broken down and condensed in the form of spiral sheets, which is not present in the untreated samples. The chemical hydrolysis has created a large surface area on the biomass for better enzyme saccharification and the structure has loosened up. SEM results explain that NaOH pre-treatment was effective in removing the lignin from corn stover biomass (Mpho et al., 2020) (Yang et al., 2022). The biomass characterization results demonstrate that alkali solution has disrupted the lignin component and exposed cellulose and hemicellulose in corn stover making the enzymes accessible during enzymatic saccharification. After the pretreatment, the enzymes can efficiently catalyze the saccharification process and will result in higher sugar yield. Therefore, alkali pretreatment is an important step in

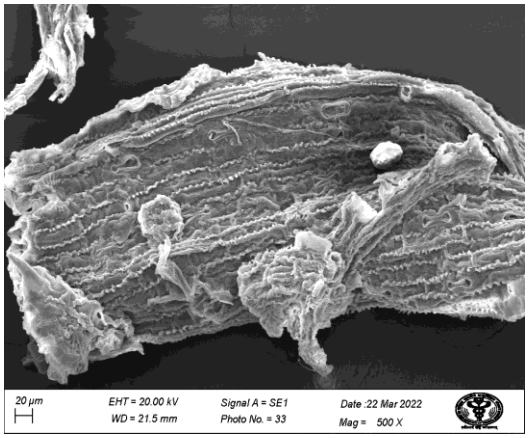
conversion of corn stover to PHA production (Sabeeh et al., 2020).



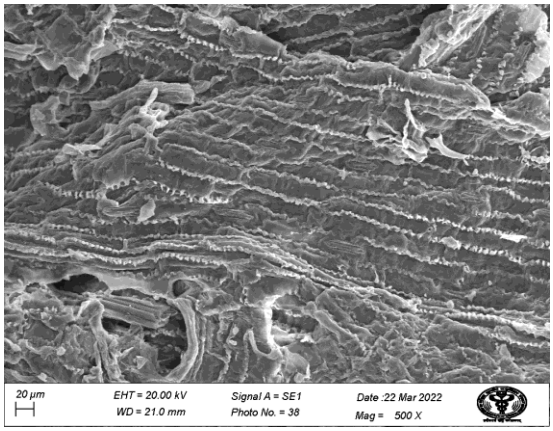
46 a. Untreated corn stover (500x)



46 b. 0.25% NaOH, 15 minutes (500x)

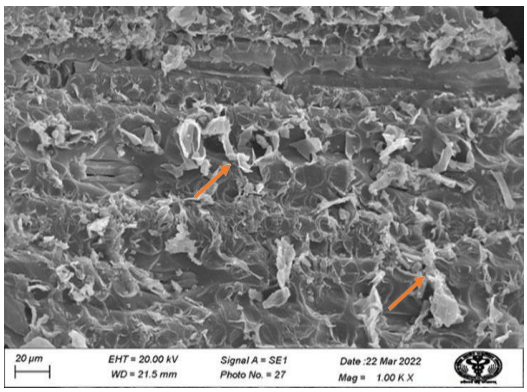


46 c. 3% NaOH, 15 minutes (500x)

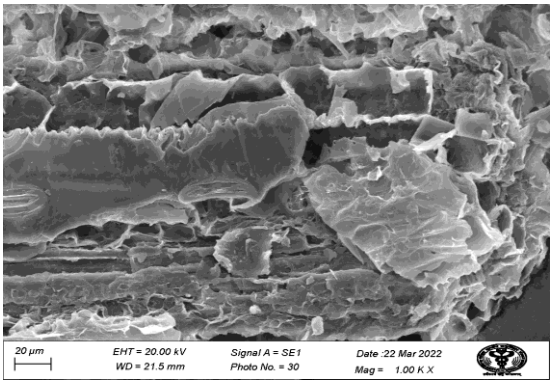


46 d. 3% NaOH, 15 minutes (500x)

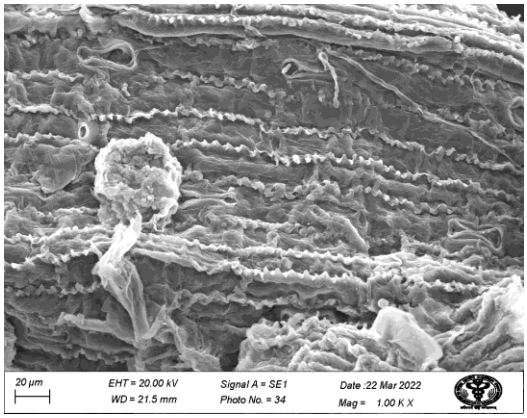
Figure 46. SEM images of untreated and treated corn stover at 500x.



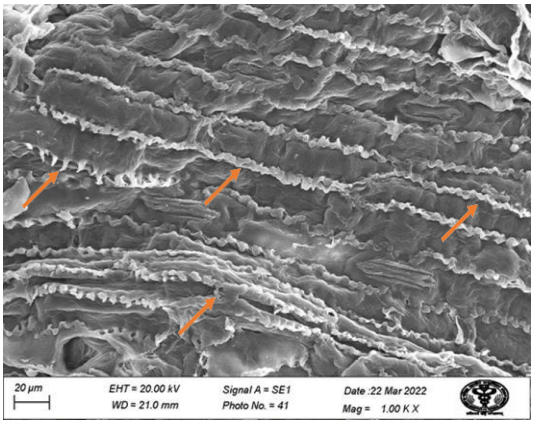
47 a. Untreated corn stover (1000x)



47 b. 0.25% NaOH, 15 minutes (1000x)

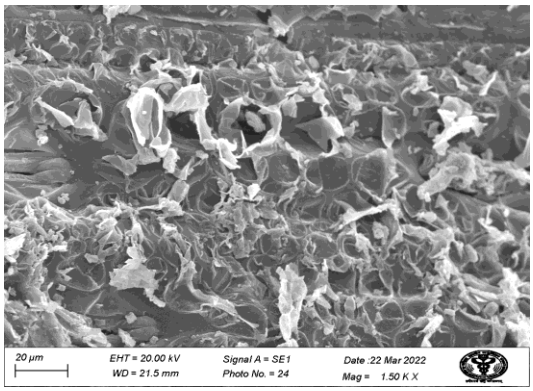


47 c. 3% NaOH, 15 minutes (1000x)

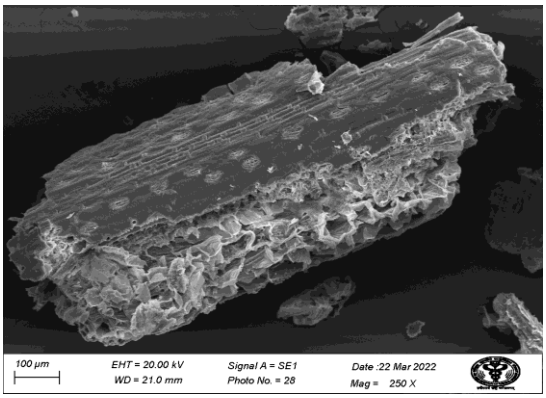


47 d. 3% NaOH, 15 minutes (1000x)

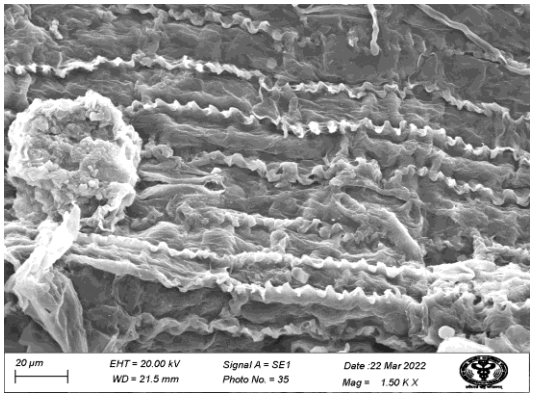
Figure 47. SEM images of untreated and treated corn stover at 1000x.



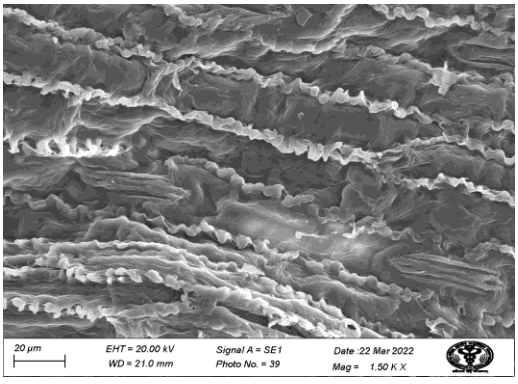
48 a. Untreated corn stover (1500x)



48 b. 0.25% NaOH, 15 minutes (250x)

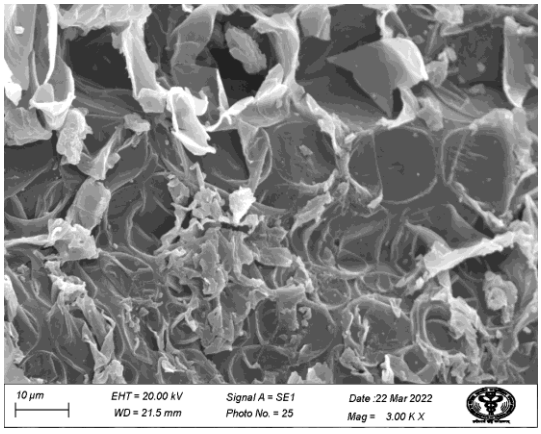


48 c. 3% NaOH, 15 minutes (1500x)

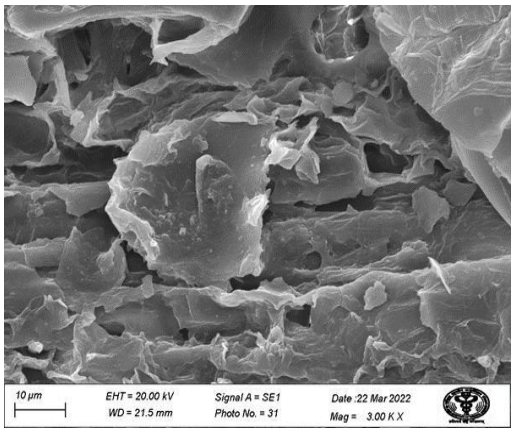


48 d. 3% NaOH, 15 minutes (1500x)

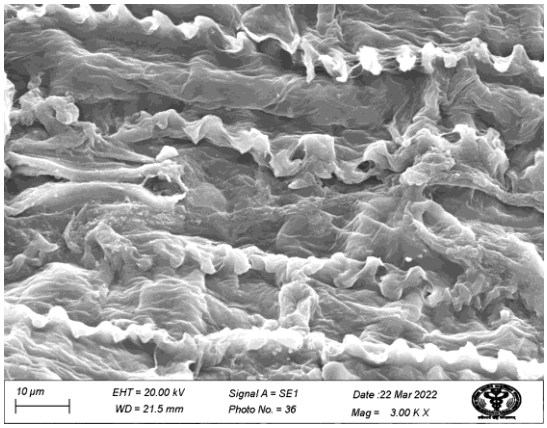
Figure 48. SEM images of untreated and treated corn stover at 1500x.



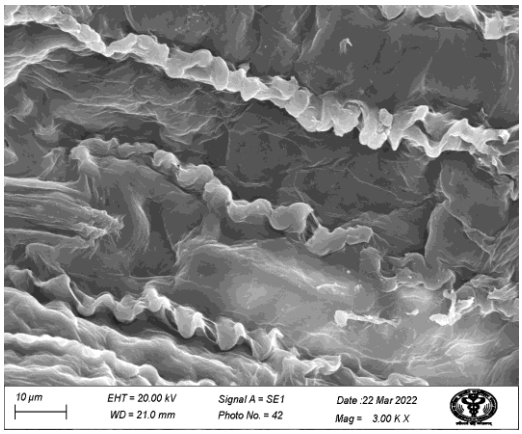
49 a. Untreated corn stover (3000x)



49 b. 0.25% NaOH, 15 minutes (3000x)

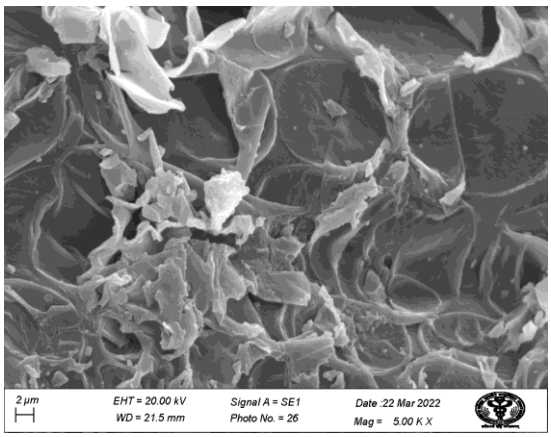


49 c. 3% NaOH, 15 minutes (3000x)

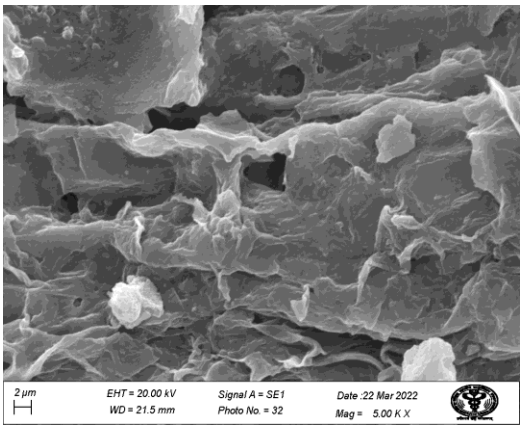


49 d. 3% NaOH, 15 minutes (3000x)

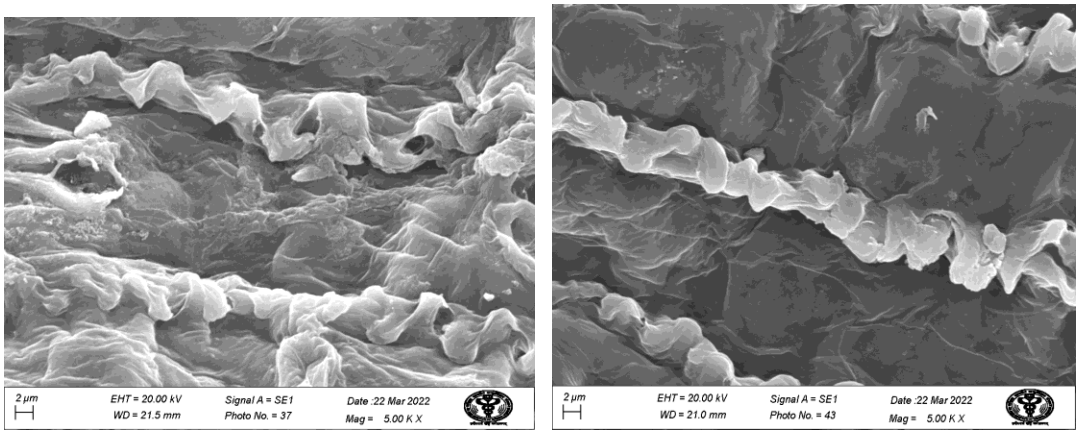
Figure 49. SEM images of untreated and treated corn stover at 3000x.



50 a. Untreated corn stover (5000x)



50 b.0.25% NaOH, 15 minutes (5000x)



50 c. 3% NaOH, 15 minutes (5000x) **50 d.** 3% NaOH, 15 minutes (5000x)

Figure 50. SEM images of untreated and treated corn stover at 5000x.

3.1.4 Enzymatic Saccharification of Alkali Pre-treated Corn Stover for Enhanced Total Reducing Sugar Recovery

Alkali pretreatment was performed as it helps in partially breaking down lignin and carbohydrate components in corn stover and make it more accessible for the enzymes. Enzymatic saccharification uses enzymes for the complete conversion of cellulose into glucose and analysis by DNS method as shown in **Figure 51**. This step was also optimized for high total reducing sugars in which alkali pre-treated corn stover 55.8g was digested with commercial cellulase (Meicellase) from *Aspergillus niger.* 13 sets of experimental designs were obtained by RSM with two parameters of biomass loading (%) and enzyme units (U) and response was total reducing sugar (%) as shown in **Table 22 -27**. The maximum TRS concentration reached up to 719.22 mg/g. The minimum TRS concentration was 94.76 mg/g at 5% biomass loading and 20U enzyme. The response optimization values are given in **Table 28 -31**. Further, experimental and predicted values of TRS mg/g is given in **Table 32 –36**. As the biomass loading was high and enzyme concentration was less, the TRS was the lowest. The effect of enzyme concentration and biomass loading were examined to analyze whether higher enzyme concentration leads to high TRS recovery. (Modenbach et al., 2017) reported lower cellulose conversion at 60 FPU/g than in 5.2 FPU/g at 5% biomass loading. Increasing

the solid loading also decreased the cellulose conversion and higher enzyme to substrate ratio limits the diffusion process.

Delignified LB gives higher glucose concentration than non-delignified LB as lignin component hampers the enzymatic hydrolysis. Increasing the enzyme concentration did not enhance glucose concentration as the higher enzyme results in adsorption on the surface of the substrate restricting the diffusion through the LB structure. Alkaline delignification enhances enzymatic saccharification. According to (Klongklaew et al., 2023), the pretreated CS has increased sugar when the enzyme concentration increased from 10-40 FPU/g. The maximum sugar released was at 40 FPU/g after pretreatment with 1% sulfuric acid. (Q. Li et al., 2019) reported 100g/l glucose by treating corn stover with 72wt. (%) sulfuric acid but concentrated acid pretreatment yields high sugar and generates high inhibitory compounds. Thus, alkali pretreatment is better for delignification of LB. Also, pre-treatment of biomass is important before enzymatic saccharification due to lignocellulosic biomass's recalcitrant and tough nature. Pre-treatment provides easy accessibility to enzymes for their action on the biomass. After enzymatic saccharification, cellulose converts to C-6 hexose sugars (e.g., Glucose) and hemicellulose to C-5 pentose sugars (xylose and arabinose). As reported corn stover hydrolysate is composed of mainly glucose and xylose (J. Wu et al., 2021). The corn stover hydrolysate from the optimized condition was 707.19 mg/g and stored at -20°C for its use in PHA production.

$$\boldsymbol{Regression\ equation\ of\ coded\ levels\ TRS}\ (\frac{\boldsymbol{mg}}{\boldsymbol{g}})$$
$$= \mathbf{714.2} - \mathbf{216.3}\ \boldsymbol{Biomass\ loading}\ \% + \mathbf{11.78}\ \boldsymbol{Enzyme\ U}$$
$$+ \mathbf{10.65}\ \boldsymbol{Biomass\ loading}\ \% \times \boldsymbol{Biomass\ loading}\ \%$$
$$- \mathbf{0.1325}\ \boldsymbol{Enzyme\ U} \times \boldsymbol{Enzyme\ U}$$
$$+ \mathbf{0.044}\ \boldsymbol{Biomass\ loading}\ \% \times \boldsymbol{Enzyme\ U}$$

Moreover, according to the two factors studied that are the biomass loading %, enzyme U, biomass loading × biomass loading, and enzyme U × enzyme U are significant as their p-values are less than 0.100. Biomass loading % plays the most significant role as its F–value is maximum. The regression coefficient (R-square value) of the total reducing sugar (mg/g) model is 99.57%, the predicted R-square value is 97.64% and

the adjusted R-square value is 99.26%. Therefore, we can infer that pretreated corn stover has enhanced enzymatic hydrolysis and efficient removal of lignin. In **Figure 52**, the contour plot represents a dark green area as the region of maximum TRS production depicting the interaction between biomass loading and enzyme U (Sahare et al., 2012; Yang et al., 2022). In **Figure 53**, the response surface plot for enhanced total reducing sugar (mg/g) with 3% NaOH (w/v) pretreated corn stover describing the interaction of enzyme (U) and biomass loading (%). In the pareto chart, **Figure 54**, the reference line 2.36 crosses the factors A, B, AA and BB representing the statistically significant factors. **Figure 55 and 56** represents pareto chart of Total reducing sugars (mg/g) from lowest to highest sugar concentration and **Figure 57 and 58** represents the scatter plots of TRS (mg/g) vs Enzyme (U) and TRS (mg/g) vs Biomass loading (%) respectively and **Figure 59** represents optimization graph with maximum total reducing sugar (mg/g).

Table 22. Optimization condition with biomass loading (%) and enzyme (U).

Variable	**Symbol**	**Coded levels**				
		-alpha	**-1**	**0**	**+1**	**+alpha**
Biomass loading (%)	**A**	1.37	2	3	5	5.62
Enzyme (U)	**B**	11.715	20	40	60	68.28

Table 23. Design summary.

Parameters	**Number**
Factors	2
Replicates	1
Base runs	13
Total runs	13
Base blocks	1
Total blocks	1

Alpha = 1.41421

Two-level factorial: Full factorial

Table 24. Point types with different parameters.

Parameters	Number
Cube points	4
Center points in cube	5
Axial points	4
Center points in axial	0

Table 25. Experiments designed by Minitab.

Std order	Run order	Pt type	Blocks	Biomass loading (%)	Enzyme (U)
10	1	0	1	3.50	40.00
8	2	-1	1	3.50	68.28
9	3	0	1	3.50	40.00
13	4	0	1	3.50	40.00
4	5	1	1	5.00	60.00
3	6	1	1	2.00	60.00
12	7	0	1	3.50	40.00
2	8	1	1	5.00	20.00
11	9	0	1	3.50	40.00
6	10	-1	1	5.62	40.00
5	11	-1	1	1.37	40.00
7	12	-1	1	3.50	11.71
1	13	1	1	2.00	20.00

Table 26. Results of Total reducing sugar (TRS) after enzymatic saccharification of alkali pre-treated corn stover.

Tubes	Glucose concentration(mg/ml)	Absorbance (540nm)
1	0	0.001
2	0.25	0.109
3	0.5	0.239
4	1	0.578
5	1.25	0.765
6	1.5	0.98

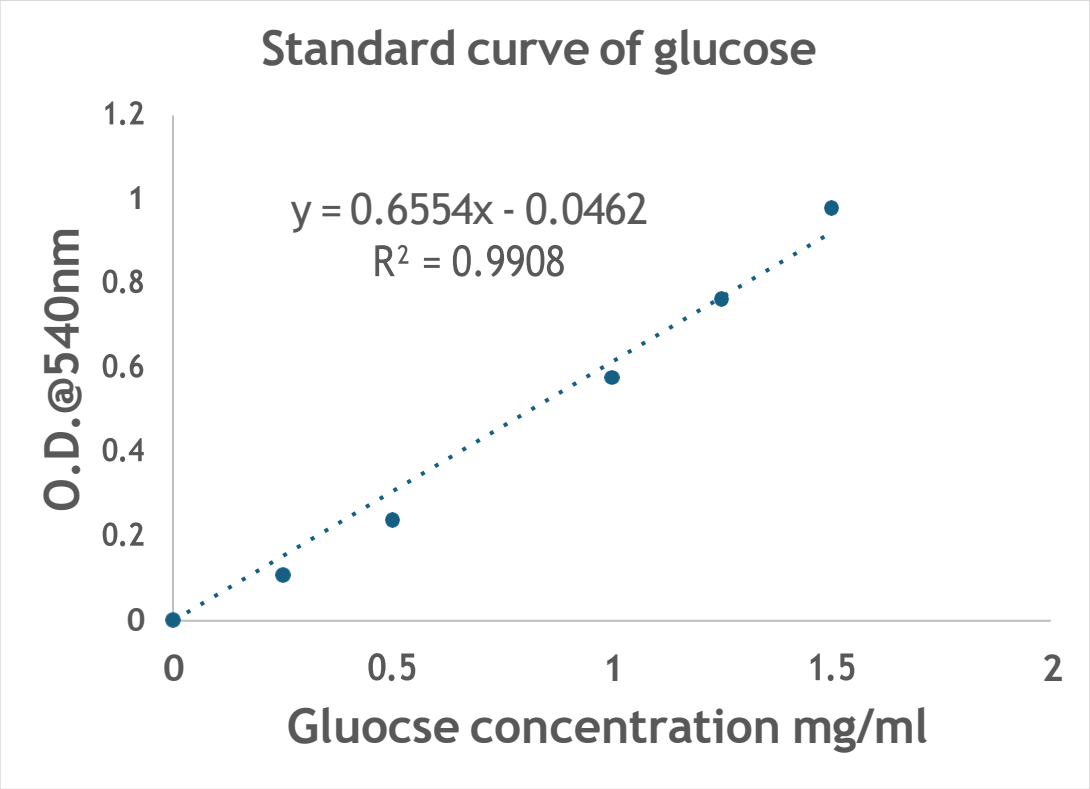


Figure 51. Glucose standard curve for DNS test.

Table 27. Central composite design and responses of total reducing sugar (mg/g) with different biomass loading (%) and enzyme units (U).

S.No.	Biomass loading (%)	Enzyme units (U)	TRS (mg/g)
1	3.5 (0)	40 (0)	339.49
2	1.37 (-alpha)	40 (0)	719.22
3	3.5 (0)	40 (0)	360.74
4	2 (-1)	60 (+1)	540.08
5	3.5 (0)	40 (0)	343.76
6	3.5 (0)	68.28 (+alpha)	294.90
7	5 (+1)	20 (-1)	94.76
8	3.5 (0)	11.715 (-alpha)	209.70
9	5.62 (+alpha)	40 (0)	94.87
10	3.5 (0)	40 (0)	365.46
11	3.5 (0)	40 (0)	355.96
12	5 (+1)	60 (+1)	143.66
13	2 (-1)	20 (-1)	496.46

Table 28. Response surface regression: TRS (mg/g) versus Biomass loading and Enzyme units.

Term	Coef	SE Coef	T-value	P-value	VIF
Constant	353.75	6.90	51.29	0.000	
Biomass loading (%)	-297.57	7.72	-38.55	0.000	1.00
Enzyme (U)	37.65	7.71	4.88	0.002	1.00
Biomass loading (%) * Biomass loading (%)	48.1	11.7	4.11	0.005	1.02
Enzyme (U)* Enzyme (U)	-106.0	11.7	-9.06	0.000	1.02
Biomass loading (%) *Enzyme(U)	2.6	15.4	0.17	0.869	1.00

Table 29. Model summary.

S	R-sq	R-sq(adj)	R-sq(pred)
15.4227	99.57%	99.26%	97.64%

Table 30. Analysis of variance for linear model of total reducing sugar (mg/g) after enzymatic saccharification.

Source	SS	Df	Mean square	F-Value	p-value	
Model	5	385511	77102	324.15	0.000	Significant
Linear	2	359244	179622	755.16	0.000	
Biomass loading%	1	353572	353572	1486.48	0.000	
Enzyme U	1	5672	5672	23.85	0.002	
Square	2	26343	13171	55.37	0.000	
Biomass loading%*Biomass loading	1	4019	4019	16.90	0.005	
Enzyme U*Enzyme U	1	19543	19543	82.16	0.000	
2-way intercation	1	7	7	0.03	0.869	
Biomass loading*Enzyme U	1	7	7	0.03	0.869	
Error	7	1665	238			
Lack of fit	3	1173	391	3.18	0.146	
Pure Error	4	492	123			
Model statistics						
R2	99.57					
Adj-R2	99.26					
Pred-R2	97.64					

Regression equation in uncoded units

TRS (mg/g) = 714.2 – 216.3 Biomass loading % + 11.78 Enzyme U + 10.65 Biomass loading % * Biomass loading % -0.1325 Enzyme U * Enzyme U + 0.044 Biomass loading % * Enzyme U

Table 31. Fits and diagnostics of unusual observations.

Observations	Lignin (%)	Fit	Residue	Std Residue
2	719.22	699.43	19.79	2.10 R
4	540.09	559.20	-19.11	-2.02 R

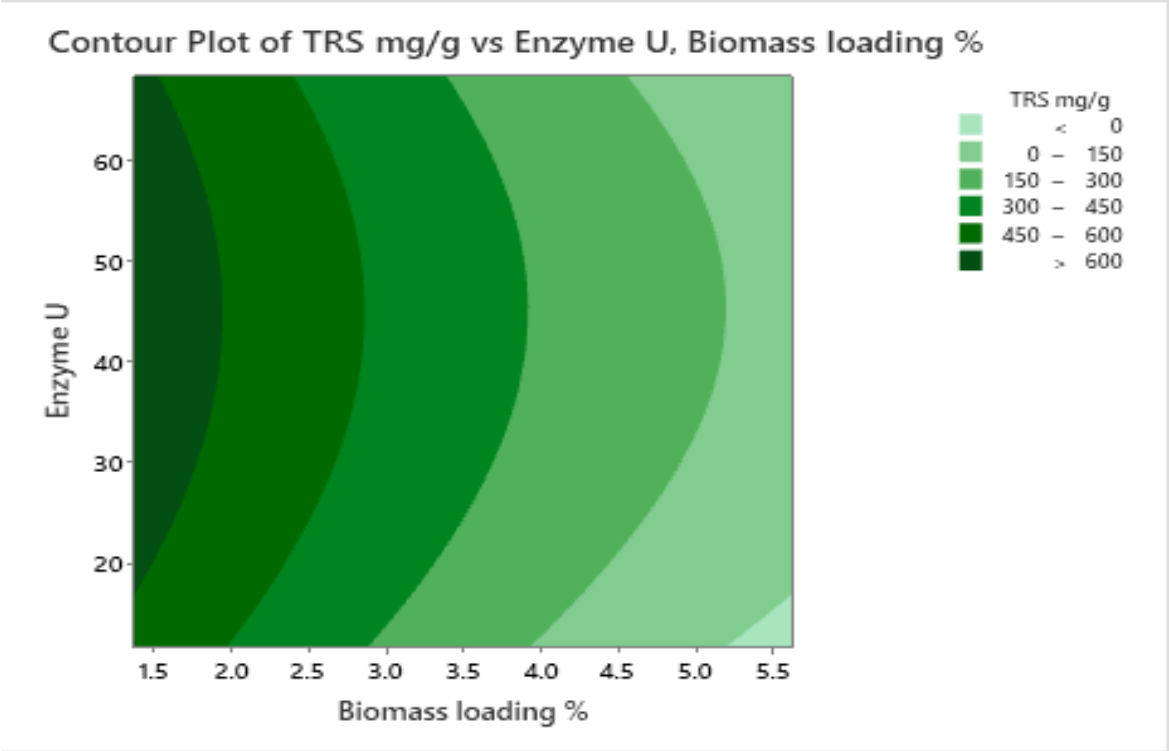


Figure 52. Response contour plot for enhanced total reducing sugar (mg/g) with 3% NaOH (w/v) pretreated corn stover describing the interaction of enzyme (U) and biomass loading (%).

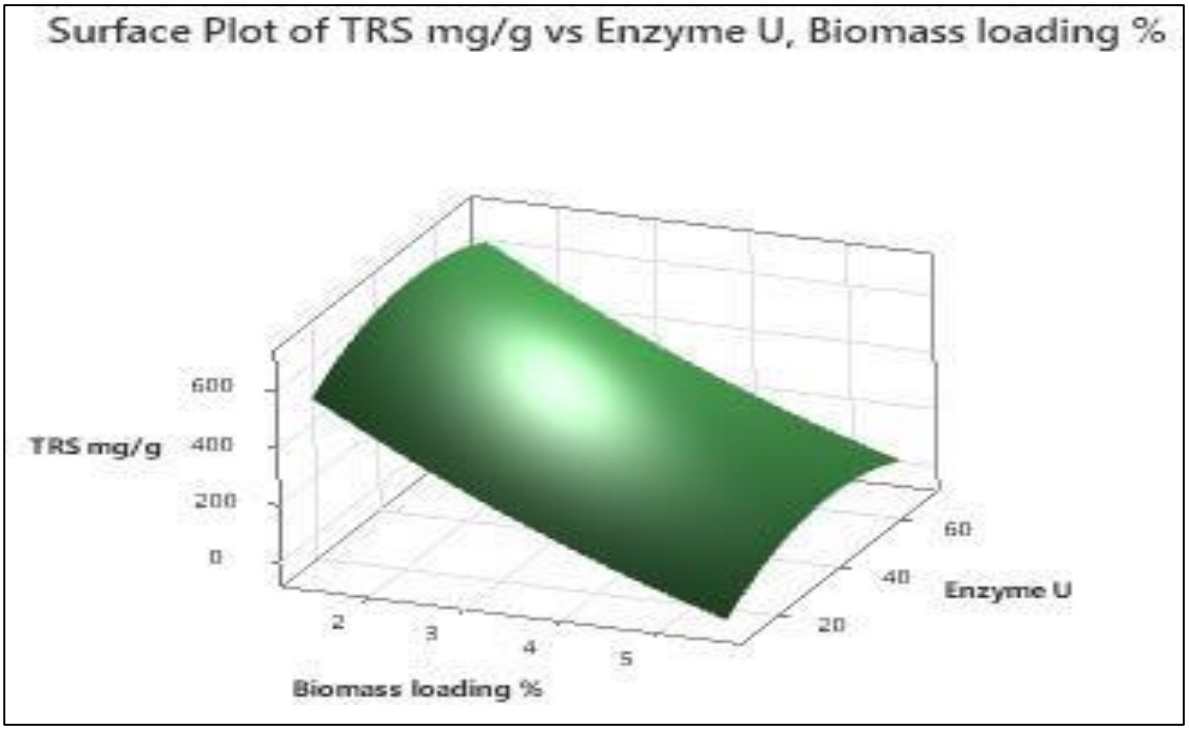


Figure 53. Response surface plot for enhanced total reducing sugar (mg/g) with 3% NaOH (w/v) pretreated corn stover describing the interaction of enzyme (U) and biomass loading (%).

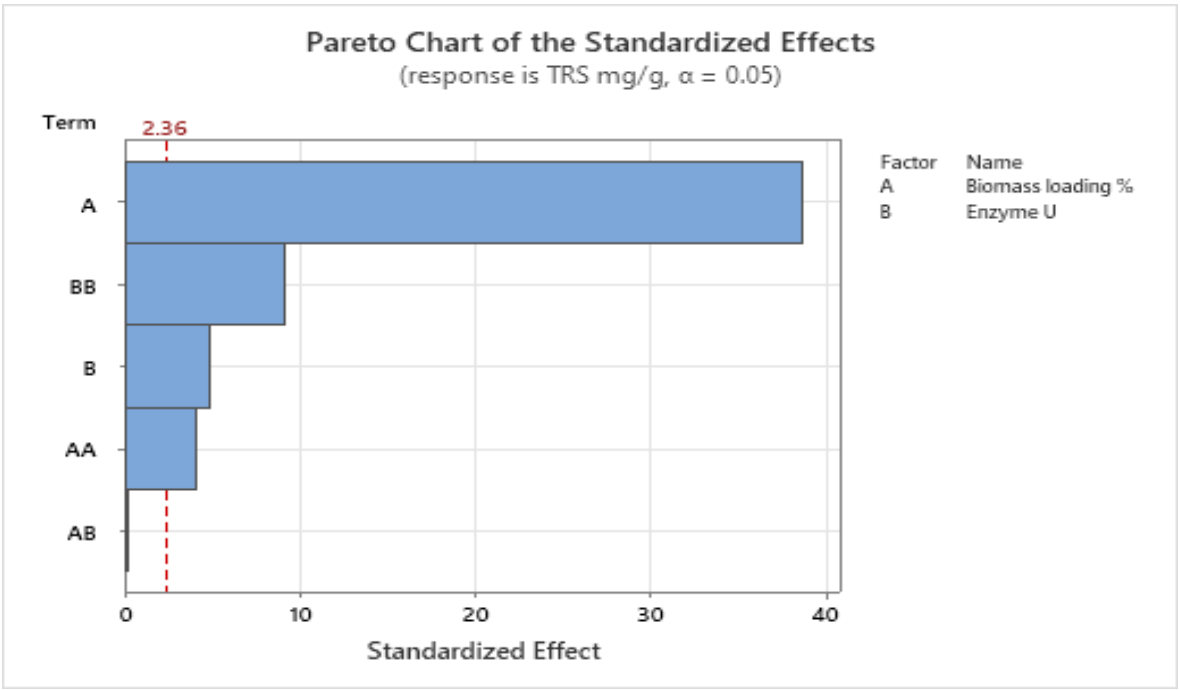


Figure 54. Pareto charts of the standardized effect with two factors biomass loading (%) and enzyme (U) showimg response TRS (mg/g).

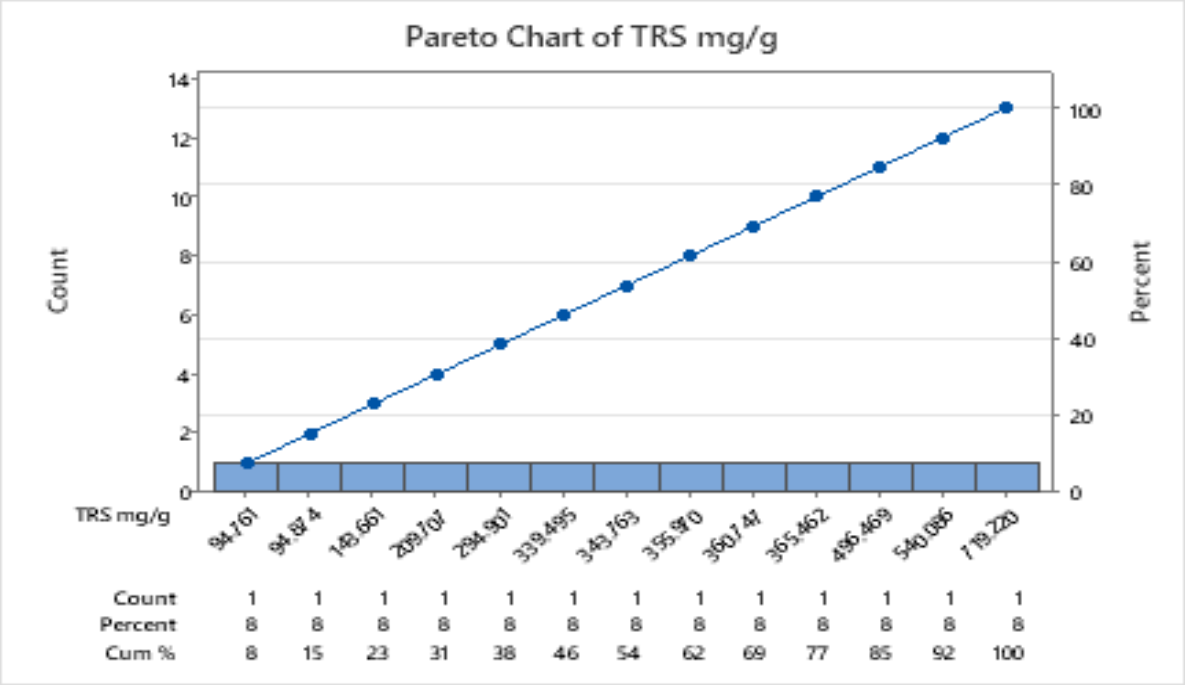


Figure 55. Pareto chart of Total reducing sugars (mg/g) from lowest to highest sugar concentration.

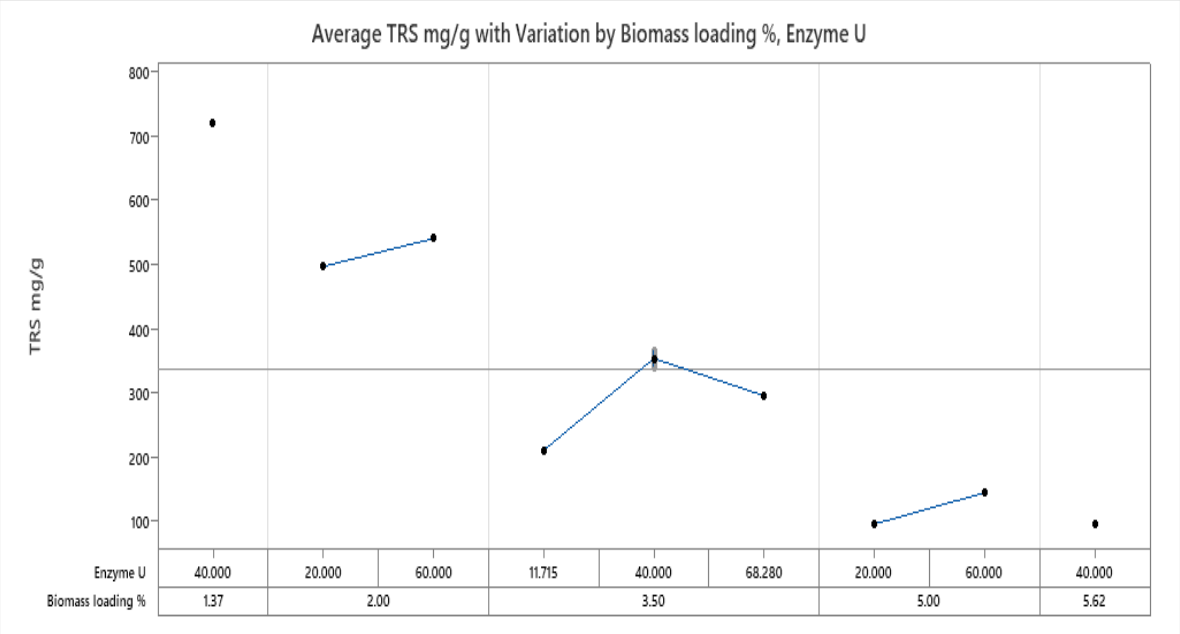


Figure 56. Graph showing average TRS (mg/g) with variation by biomass loading (%) and Enzyme (U).

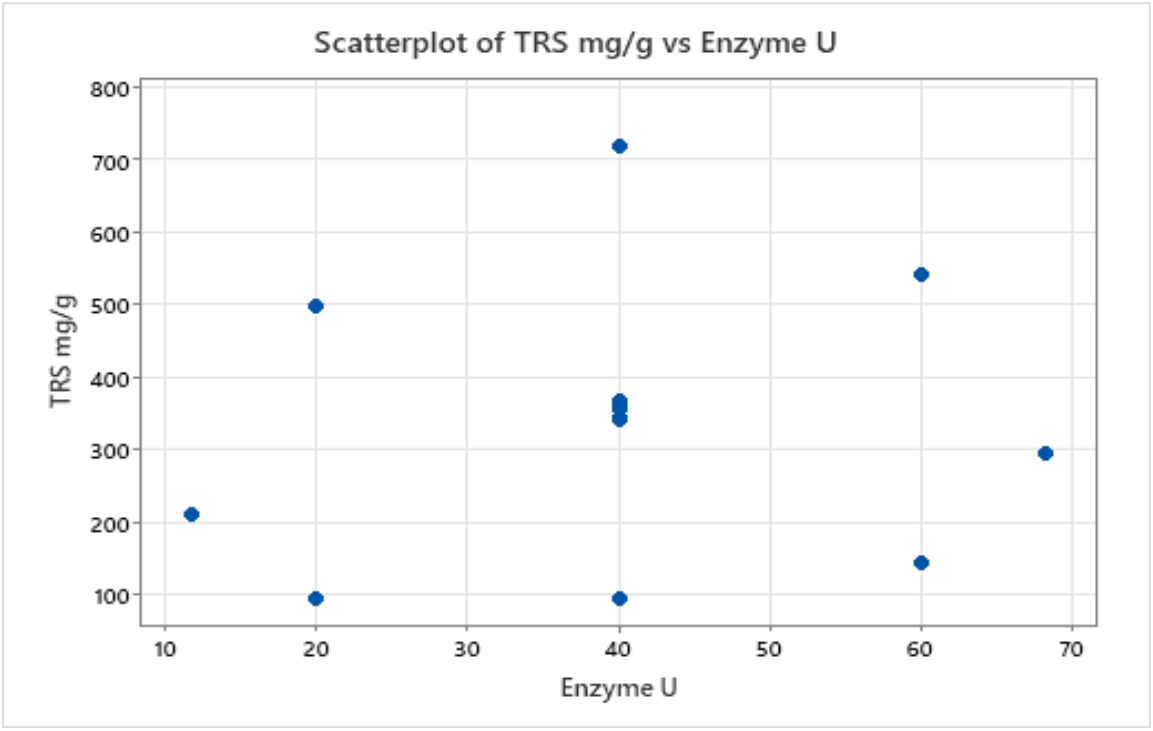


Figure 57. Scatter plot graph of TRS (mg/g) vs Enzyme (U).

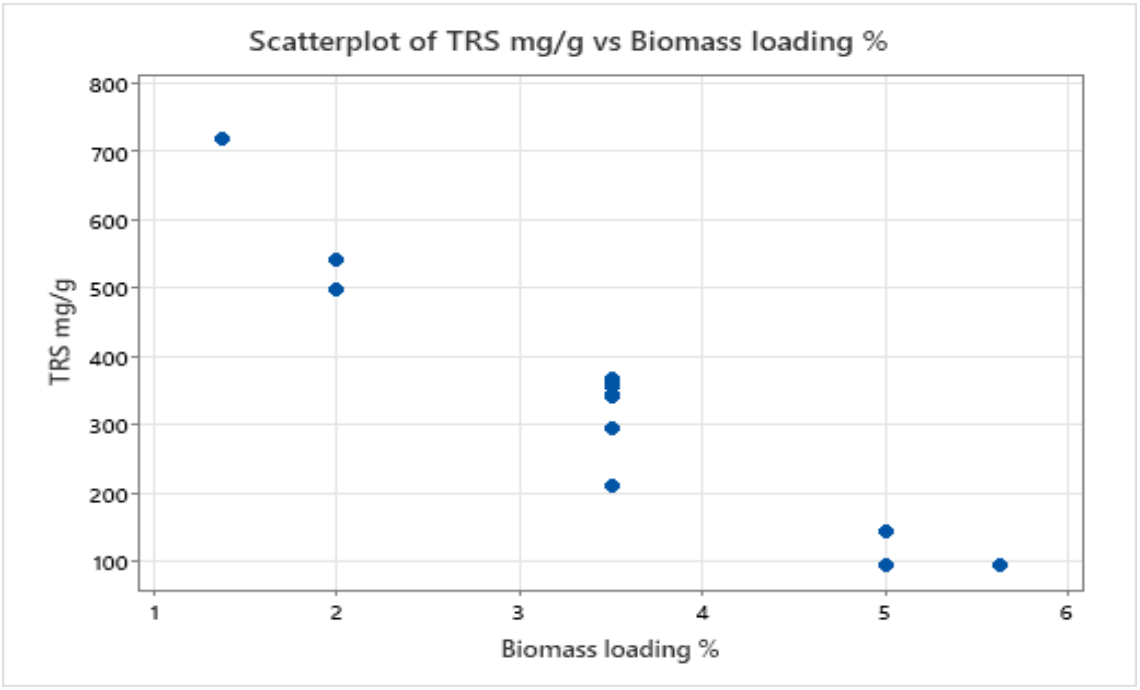


Figure 58. Scatter plot graph of TRS (mg/g) vs Biomass loading (%).

Table 32. Response optimization: Total reducing sugar (TRS) mg/g.

Response	Goal	Lower	Target	Upper	Weight	Importance
TRS (mg/g)	Maximum	94.7614	719.220	-	1	1

Table 33. Solution of biomass loading (%) and enzyme (U).

Solution	Biomass loading (%)	Enzyme (U)	TRS (mg/g) Fit	Composite Desirability
1	1.37	44.8541	83.5797	0.972930

Table 34. Multiple response prediction.

Variable	Setting
Biomass loading (%)	1.37
Enzyme (U)	44.8541

Table 35. Response prediction of TRS (mg/g).

Response	Fit	SE Fit	95% CI	95% PI
TRS (mg/g)	702.3	12.5	(672.7,731.9)	655.4,749.3)

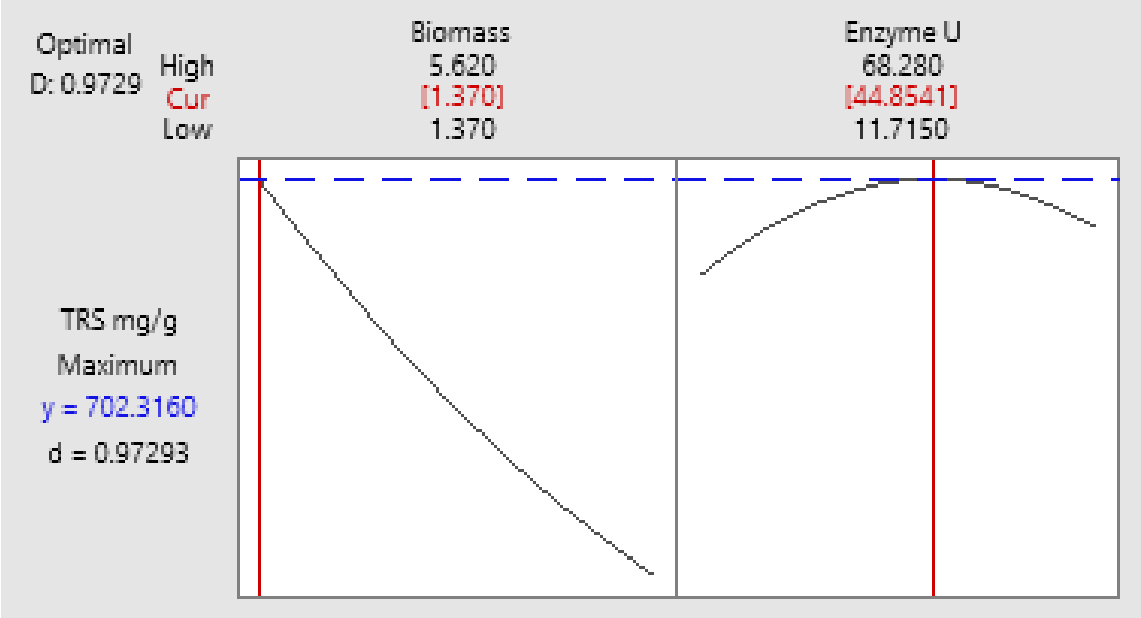


Figure 59. Optimization graph with maximum total reducing sugar (mg/g).

Table 36. Experimental and predicted values of TRS (mg/g).

Biomass loading (%)	Enzyme(U)	TRS (mg/g)	
		Experimental	**Predicted**
1.37	44.85	707.195	702.316

3.1.5 PHA Production by *P. putida* Using Corn Stover Sugar Hydrolysate

In this study, commercial cellulase was applied for enzymatic saccharification after alkali pre-treatment of corn stover. The optimized condition obtained by RSM for the total reducing sugar was at 1.37% biomass loading and 44.85 U which yielded 707.19 mg/g sugar. Firstly, the growth of bacteria was tested in four conditions as seen in **Table 4** for growth curve, residual sugar (g/l) and DCW (g/l). The result indicates the maximum DCW (g/l) in D media conditions which is modified media with hydrolysate (Sodium chloride, Ammonium sulphate and hydrolysate). **Figure 60 – 63** represents Growth of *P. putida* in LB broth, only hydrolysate, modified media with glucose and modified media with hydrolysate and showing residual sugar (g/L), O.D. @ 620nm

and DCW (g/L) respectively. The dry cell weight is highest in modified medium with hydrolysate as corn stover hydrolysate is lignocellulosic which is composed of D-glucose, D-xylose, L-arabinose and other sugars (Baptista et al., 2018). As Pseudomonas is known for its metabolic versatility and adaptability it utilizes different carbon sources and is efficient in capturing and metabolizing the hydrolysate components. While in case of modified media with glucose, it is just utilizing one type of sugar and has less dry cell weight. Also, it is known that limiting the nitrogen availability and providing abundant carbon source induces PHA production which clearly indicates bacteria uses excess carbon for PHA synthesis. All the above analysis demonstrates that nitrogen 1g/l and carbon (sugar) 10g/l as given in our study is favorable for PHA production.

Now, modified media with hydrolysate was used as production media with ammonium sulphate supplemented to maintain nitrogen as a limiting factor with other components. In the production media (g/l), 1% sugar was used for PHA production from the sugar obtained after enzymatic saccharification. Only 1% sugar was used in the experiment as low sugar concentration decreases the growth of bacterial cells which results in slow cell division. This favors the bacterial cells to use the resources for PHA synthesis rather than biomass accumulation. Carbon limitation stimulates a metabolic shift and enhances pathways to generate PHA as intracellular carbon and energy storage substance. Limiting sugar concentration optimizes the PHA production process and avoids inhibitory role of high sugar concentrations. High sugar concentration can be used for economic reasons of PHA production, but bench scale research mainly focusses on low sugar concentrations to have an idea of metabolic pathways and optimized condition for PHA production (de Souza et al., 2020).

The maximum DCW was 2.74 g/l, PHA accumulation 24.4% and residual sugar of 0.89 g/l was observed at 48h. After 48 h cultivation period, corn stover hydrolysate which was used as a carbon source remained at 0.89%. Then bacterial cells were harvested and further subjected to methanolysis for extraction of PHA. The PHA accumulation decreased after 48 h as shown in **Table 37 and Figure 64.** There are many research reports on the major role of carbon and nitrogen in PHA production (Valentino et al., 2015)(F. Wang C Lee, 1997). The limitation of nitrogen and the

presence of carbon sources increase PHA productivity (Mahato, 2021). The overall steps from fermentation to PHA film production is given in **Figure 65 – 69.**

(Ashby et al., 2022) reported PHA production using corn stover hydrolysate and Levulinic acid (Lev A). Corn stover was acid pretreated to recover sugars from cellulosic and hemi cellulosic fraction. *Burkholderia sacchari* utilized the non-detoxified hydrolysate to achieve maximum PHA titer 1.2 g/l. Bacterium *Azohydromonas lata* induced bacterial growth and PHA production only in detoxified hydrolysate. According to (Rebocho et al., 2019) apple pulp waste from the fruit processing industry was used as feedstock for mcl-PHA production. *Pseudomonas citronellolis* has a polymer content of 30% wt. and PHA productivity 0.025 g/l/h. The polymer is composed of 3-hydroxydecanoate (68% mol), 3-hydroxyactanoate (22% mol), 3- hydroxydodecanoate (5% mol), 3-hydroxytetradecanoate (4% mol) and 3-hydroxyhexanoate (1% mol) and molecular weight 3.7×10^5 Da. The thermal degradation of 296ºC. Thus, apple pulp is suitable feedstock for mcl-PHA production. In a study by (Lemechko et al., 2019), agro-industrial effluents were used for poly (3-hydroxybutyrate-co-hydroxyvalerate) by *Halomonas* sp. SF2003 and had PHA yield of 31% and PHA productivity of 1.89 g/l.

Moreover, we can infer that LB (corn stover hydrolysate) is more favorable for the growth of microbial strain *P. putida* MTCC 2475 and the synthesis of biopolymer. The major challenge in production of PHA is the high production cost which can be highly reduced using easily available and renewable carbon sources. The results also demonstrate that enzymatic saccharification is favorable for enhanced sugar recovery. **Table 40** also denotes the different PHA content in other research studies and earlier reports using corn stover as hydrolysate. Therefore, corn stover is an economical and sustainable substrate for PHA content.

$$\boldsymbol{Residual\ biomass}\ (\boldsymbol{g/L}) = Dry\ biomass\ cell\ weight\ (g/L) - Extracted\ qiantity\ of\ PHA\ (g/l)$$

$$\boldsymbol{PHA\ accumulation}\ (\%) = Extracted\ quantity\ of\ PHA\ (g/L)/\ Dry\ biomass\ cell\ weight\ (g/L) * 100$$

Table 37. Cell growth of *P. putida* in different media conditions.

S.No.	Media condition	Time (h)	O.D. (600nm)	Residual sugar (g/l)	DCW (g/l)
1	A	34	1.81 ± 0.006	1.19 ± 0.154	1.01 ± 0.000
2	B	34	0.86 ± 0.006	3.27 ± 0.007	0.45 ± 0.003
3	C	34	0.77 ± 0.003	5.19 ± 0.001	0.40 ± 0.07
4	D	34	2.47 ± 0.023	1.70 ± 0.015	1.41 ± 0.013

.

- ***A.*** *LB broth* ***B.*** *only hydrolysate* ***C.*** *Modified media with synthetic glucose* ***D.*** *Modified media with hydrolysate.*
- **A.** Composition of LB broth (Tryptone, Sodium chloride, yeast extract), **B.** Only hydrolysate, **C.** Modified media with synthetic glucose (Sodium chloride, Ammonium sulphate and glucose) and **D.** Modified media with hydrolysate (Sodium chloride, Ammonium sulphate and hydrolysate)

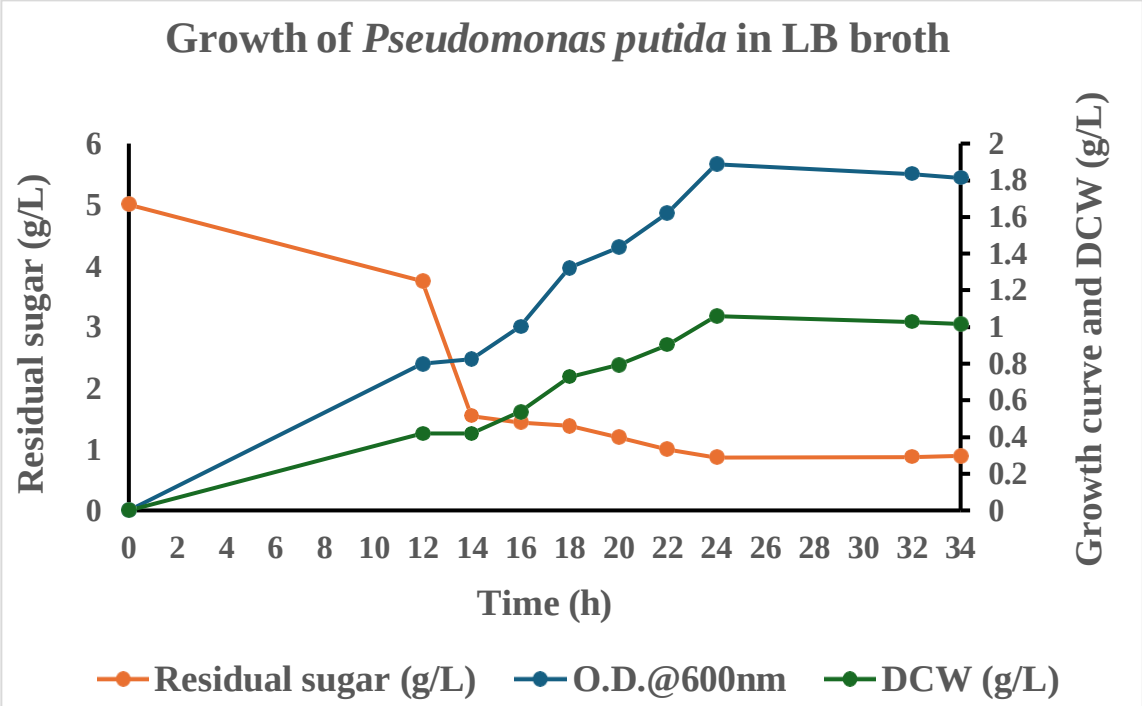


Figure 60. Growth of *P. putida* in LB broth and showing residual sugar (g/L), O.D. @ 620nm and DCW (g/L).

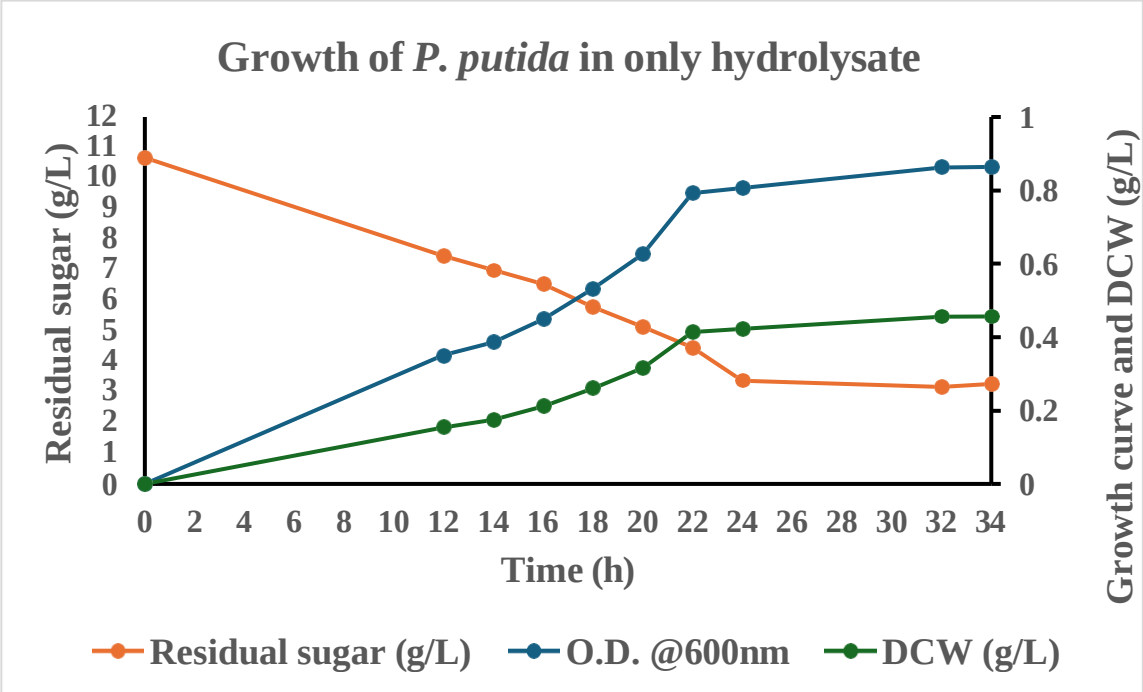


Figure 61. Growth of *P. putida* in only hydrolysate and showing residual sugar (g/L), O.D. @ 620nm and DCW (g/L).

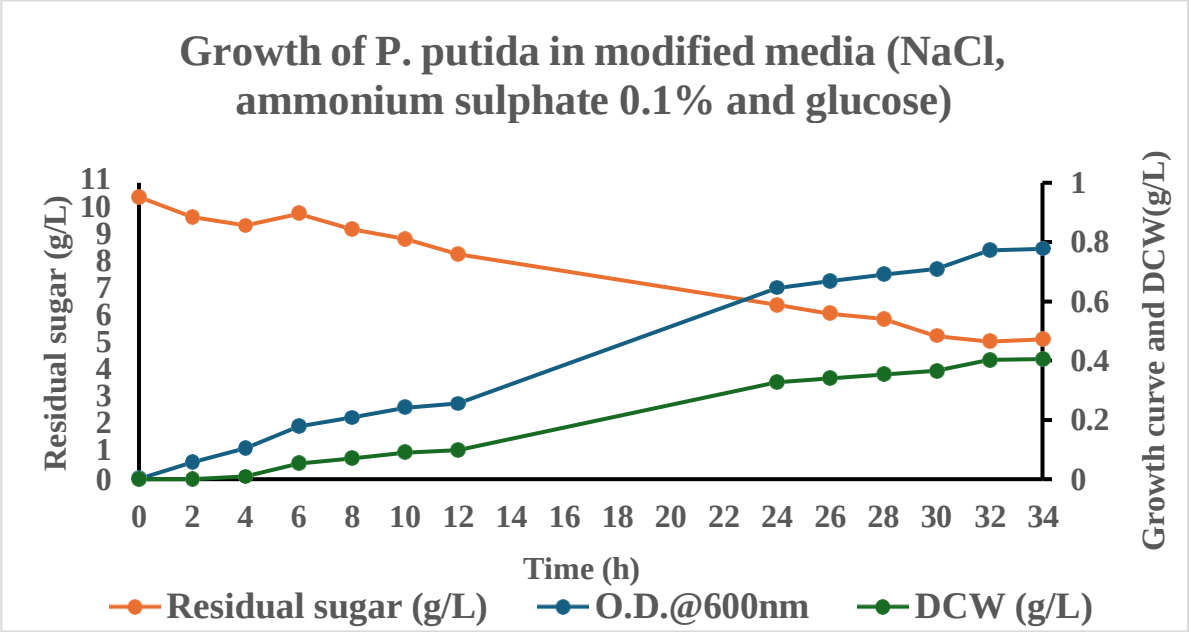


Figure 62. Growth of *P. putida* in modified media (composition: Sodium chloride, Ammonium sulphate 0.1% and glucose) and showing residual sugar (g/L), O.D. @ 620nm and DCW (g/L).

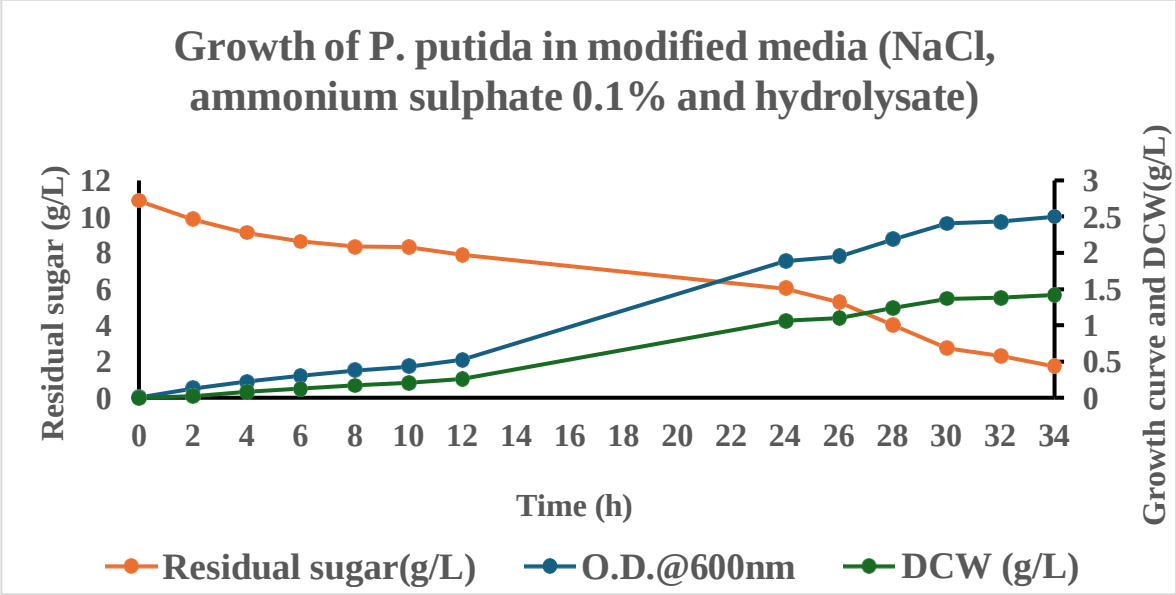


Figure 63. Growth of *P. putida* in modified media (composition: Sodium chloride, Ammonium sulphate 0.1% and hydrolysate) and showing residual sugar (g/L), O.D. @ 620nm and DCW (g/L).

Table 38. Biosynthesis of PHA by *P. putida* using 10g/L hydrolysate as carbon source.

Modified media with hydrolysate	Time (h)	Residual sugar (g/L)	DCW (g/L)	PHA accumulation (g/g)
A	24	2.78 ± 0.00	1.05 ± 0.00	0.067 ± 0.00
B	36	1.76 ± 0.00	1.98 ± 0.06	0.145 ± 0.02
C	48	0.89 ± 0.03	2.74 ± 0.07	0.244 ± 0.03
D	60	0.58 ± 0.08	1.84 ± 0.05	0.170 ± 0.00
E	72	0.29 ± 0.02	1.65 ± 0.06	0.144 ± 0.00

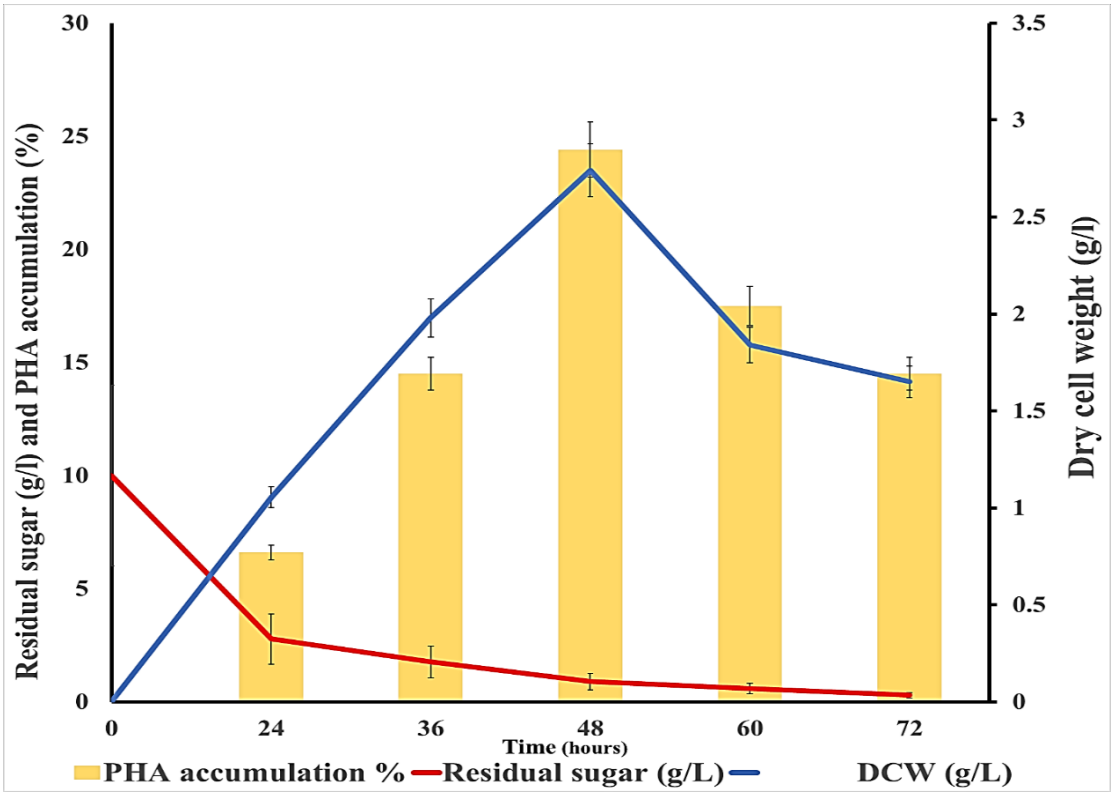
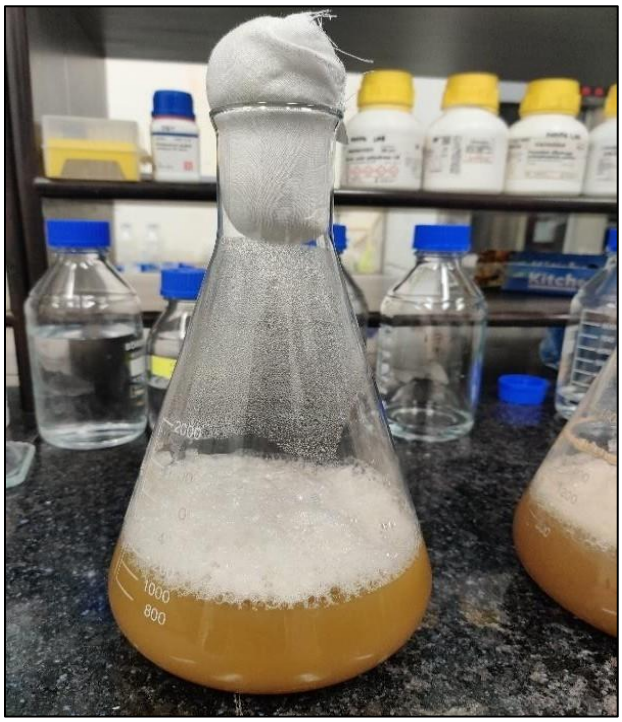


Figure 64. Graph showing dry cell weight (g/l), residual sugar (g/l) and PHA accumulation (%) in modified media with hydrolysate at different time intervals.

46 a.

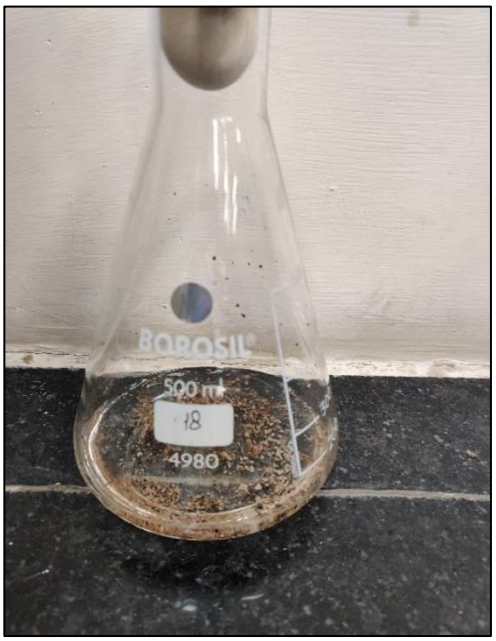


46 b.

Figure 65. Flasks containing production media (a) before and (b) after fermentation for PHA production.

Figure 66. Microbial dry cell biomass recovery from modified media with hydrolysate for extraction of PHA after 24 hrs.

Figure 67. Microbial dry cell biomass recovery from modified media with hydrolysate for extraction of PHA after 48 hrs.



68 a. **68 b.**

Figure 68 a. Flask containing dry cell biomass in chloroform **b**. Chloroform-methanol extraction of PHA from microbial dry cell biomass.

Figure 69. PHA film obtained from *P. putida* from modified media with hydrolysate by solvent evaporation method.

Table 39. GC-MS/MS for monomer detection.

S.No.	Compound	Formula	RT	CAS	Peak area%
1	Octanoic acid, 3-hydroxy-, methyl ester	$C_9 H_{18} O_3$	3.183	7367-87-5	6.40
2	Methyl 3-hydroxytetradecanoate	$C_{15} H_{30} O_3$	8.581	55682-83-2	4.14
3	Methyl 3-hydroxydodecanoate	$C_{13} H_{26} O_3$	10.343	72864-23-4	2.79

3.1.6 PHA monomeric composition and distribution

Figure 70 represents the steps involved in downstream processing for biopolymer production. The monomeric composition of PHA can be detected by GC-MS and NMR. In case of chromatography methods, intact polymer cannot be detected therefore, depolymerization of polymer along with chemical derivatization is necessary (Tan et al., 2014). Polyhydroxyalkonate extracted from *Pseudomonas putida* strain contained (3HO) 3- hydroxyoctanoic acid methyl ester (6.40%), (3HTD) 3-hydroxytetradecanoate methyl ester (4.14%) and (3HDD) 3-hydroxydodecanoate methyl ester (2.79%) as given in **Table 39**. These compounds proves that monomers were present of biodegradable polyester family and confirmed through GC-MS/MS results **Figure 71** The prominent peaks of (3HO) 3 hydroxyoctanoic acid methyl ester, (3HDD) 3hydroxydodecanoate methyl ester, (3HTD) and 3-hydroxytetradecanoate methyl ester appeared at 3.183, 8.581and10.343 minutes as given in **Figure 71** and their molecular formula $C_9H_{18}O_3$, $C_{15}H_{30}O_3$ and $_{13}H_{26}O_3$ respectively. *Pseudomonas* sp. mainly synthesize mcl PHAs (Knabaki et al., 2021) which is hence proved by the 3 monomers present in GC-MS/MS results. By gravimetric analysis, PHA accumulation was shown to be 24.40%. NMR technique explains the intact PHA chemical structure and provides topology as well as functional group in the molecule. 1H-NMR is sensitive, high proton abundance in nature and performed in short analytical time. NMR depicts saturated and unsaturated PHA analysis. According to reports in other research papers, peaks at 5.3 corresponds to the protons beside carboxyl group and chemical changes in peak 5.3 ppm specify the presence of

unsaturated group in the monomers (such as methine group -CH-). The spectrum shows a doublet at 2.5ppm comparable to methylene group (-CH_2). The peak at 1.6 ppm indicates the first CH_2 of the side chain and the peak at 1.3 ppm is for the side chain CH_2 group such as methyl group (Muangwong et al., 2016) (Salgaonkar et al., 2013) **Figure 72 – 75.** According to Tan et al., 2014 GC-MS/MS results along with NMR spectrum is pre-eminent analytical tool for the investigation of PHA (Tan et al., 2014).

3.1.7 Characterization of Extracted PHA Film

3.1.7.1 Thermal Property of PHA Film

The thermal property of extracted PHA film was analysed by DSC (Differential scanning calorimetry) as shown in **Figure 76.** According to Vostrejs et al., 2020, medium chain length polymers (mcl) polymers as detected by GC-MS/MS in this research paper are amorphous PHAs. It does not possess the ability to crystallize due to the chemical structure of PHAs. The benefits of amorphous PHAs are in its flexibility and other mechanical stricture as same as elastomers (Vostrejs et al., 2020). The extracted PHA film from *P. putida* has melting temperature (Tm) peak of 171.8ºC. Tm values for PHA is in the range from non-observable to 177°C (Tan et al., 2014). The variation in the Tm depends on the composition of monomers in biopolymer of PHA. The decomposition of the extracted PHA started at 200°C and lost its mass at 240°C (Sedlacek et al., 2020). Thermodegradation temperature (T_d) for PHA is between 227 to 256°C as reported by Tan et al., 2014. T_d of extracted PHA film is 240°C in our study.

3.1.7.2 Structural Property of PHA Film

Structural property of extracted PHA from *P. putida* was analysed by FTIR (Fourier transform infrared spectroscopy) and the spectra was scanned from 4000 to 400cm-^{1} as shown in **Figure 77**. PHA biopolymers span from non-crystalline to highly crystalline. In FTIR spectrum, PHA depicts characteristic infrared absorption bands at wavenumbers which are in relation to crystallinity (Tan et al., 2014). The presence of broad characteristic peak of hydroxyl group is at 3437cm-^{1} (López-Cuellar et al., 2011). The presence of IR spectra at 2961, 2926 cm-^{1} is due to stretching of C-H of methyl and ethylene group. The peak at 2855cm-1 indicated the $–CH_2$-CH_3. The prominent absorption bands at 1724cm-^{1} and 1230cm-^{1} have shown the relevance of –C=O stretching and –C-O-C-(Tanikkul et al., 2020). Furthermore, the absorption band at 1724cm-^{1} have specified the PHA marker band with the carbonyl C=O stretching vibrations of ester group. The peaks at 1110cm-^{1} and 797cm-^{1} are due to vibrations in –C-O- and –C-C- respectively (Sathiyanarayanan et al., 2017). Also, according to Tanikkul et al., 2020, the peaks near 2961, 2926, 1724 and 1058cm-^{1} confirms the structure of mcl-PHA (Tanikkul et al., 2020). The presence of $–CH_3$ group at peak 1380cm-1 is confirmed from literature (Shamala et al., 2009). Mass balance study of utilizing corn stover as substrate for PHA production is shown in **Figure 78**.

Furthermore, a mass balance study of utilizing corn stover as a substrate for PHA production is done to obtain and calculate the PHA derived from 100g of corn stover as shown in **Figure 78.** 100g corn stover was alkali pretreated and solid biomass 55.8 g was used for enzymatic saccharification using Meicellase from *Aspergillus niger* at 50 ºC, 200 rpm and 72 hrs and further liquid hydrolysate was taken for fermentation using *P. putida* at 30 ºC, 170 rpm and 48 hrs. Dry cell biomass recovered after fermentation was 10.2 g and 2.4 g PHA was extracted. Furthermore, the major challenge in commercial PHA production is the high production which is reduced in this study due to use of inexpensive carbon source. The overall process of PHA production from corn stover is feasible as the raw material is highly and globally produced agricultural waste. Finding the alternative cheaper carbon source downsizes the production cost of PHA production. Moreover, the wild type *Pseudomonas puitda*

has stable genomes and less genetic instability and optimizes production efficiency and can utilize different carbon sources such as readily available substrate.

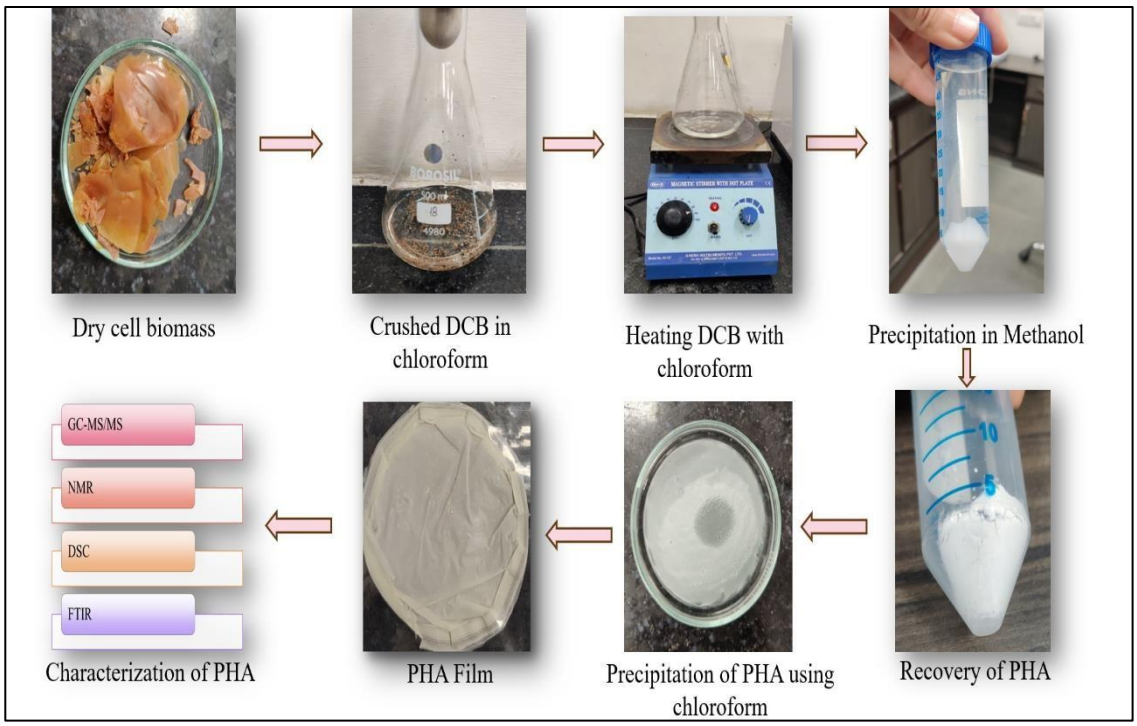


Figure 70. Downstream processing of biopolymer production.

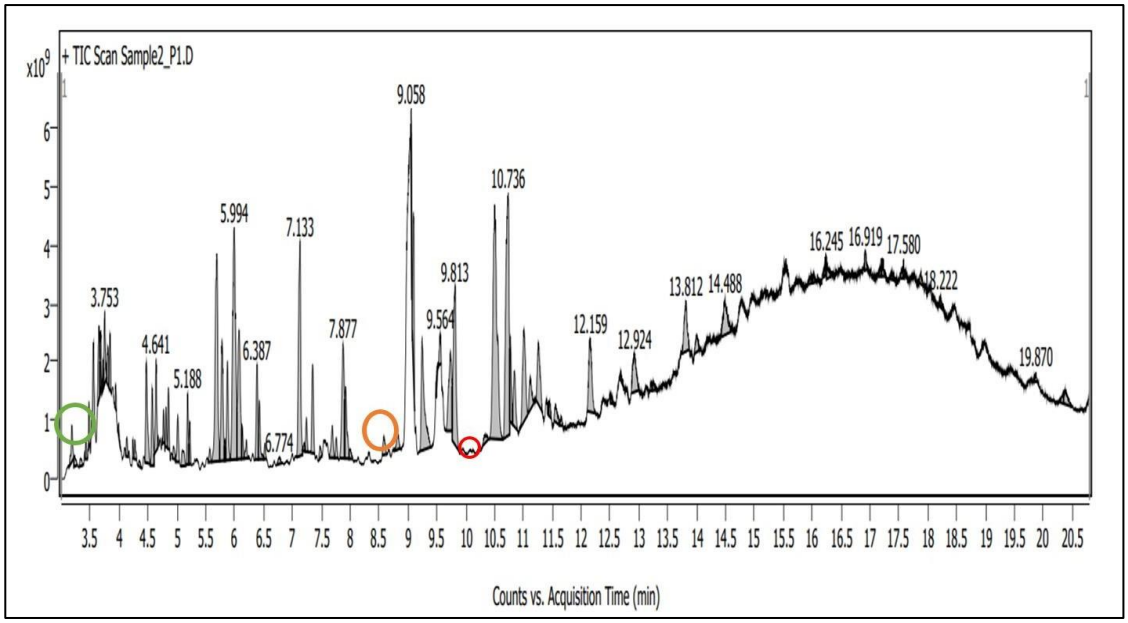


Figure 71. Result of GC-MS/MS chromatograms with three monomers of PHA (3-hydroxy octanoic acid methyl ester, 3-hydroxy tetra decanoate methyl ester and 3-hydroxy dodecanoate acid methyl ester).

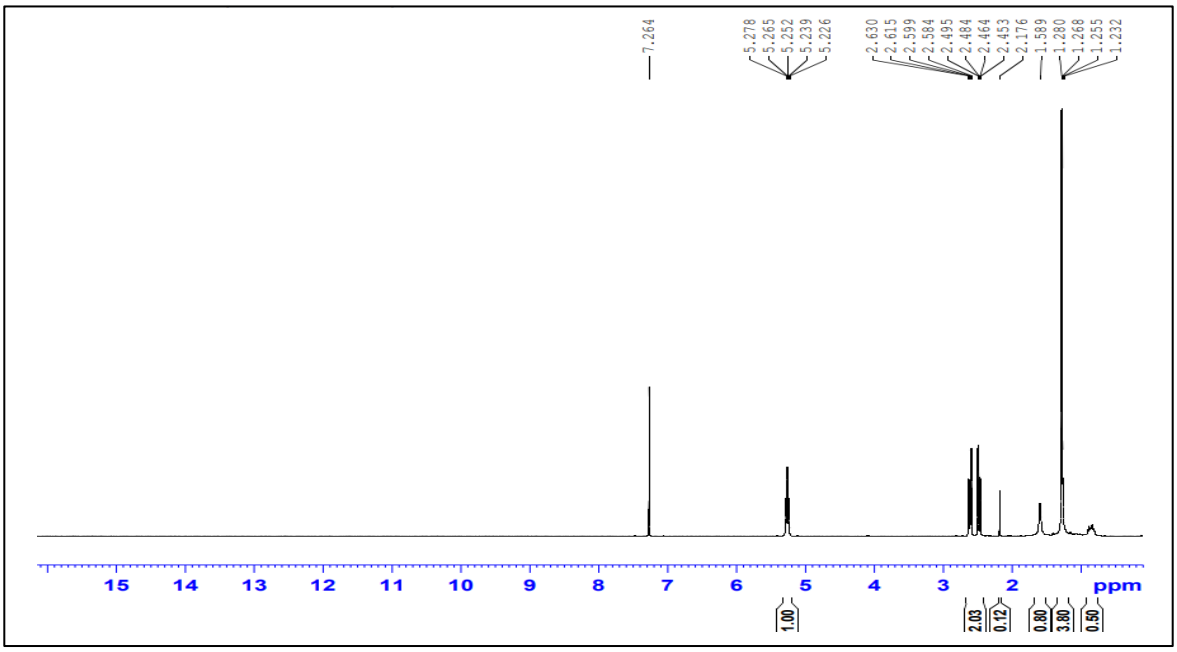


Figure 72. 1H NMR spectrum of PHA film from *P. putida* from 2 to 15ppm.

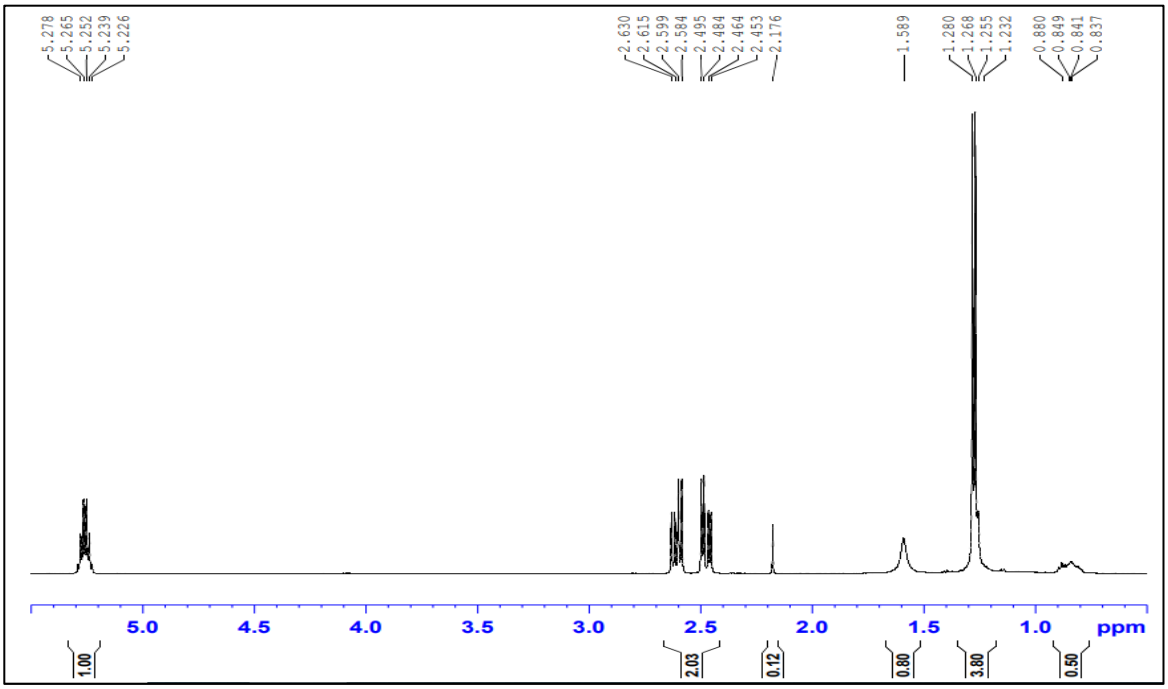


Figure 73. 1H NMR spectrum of PHA film from *P. putida* from 1 to 5 ppm.

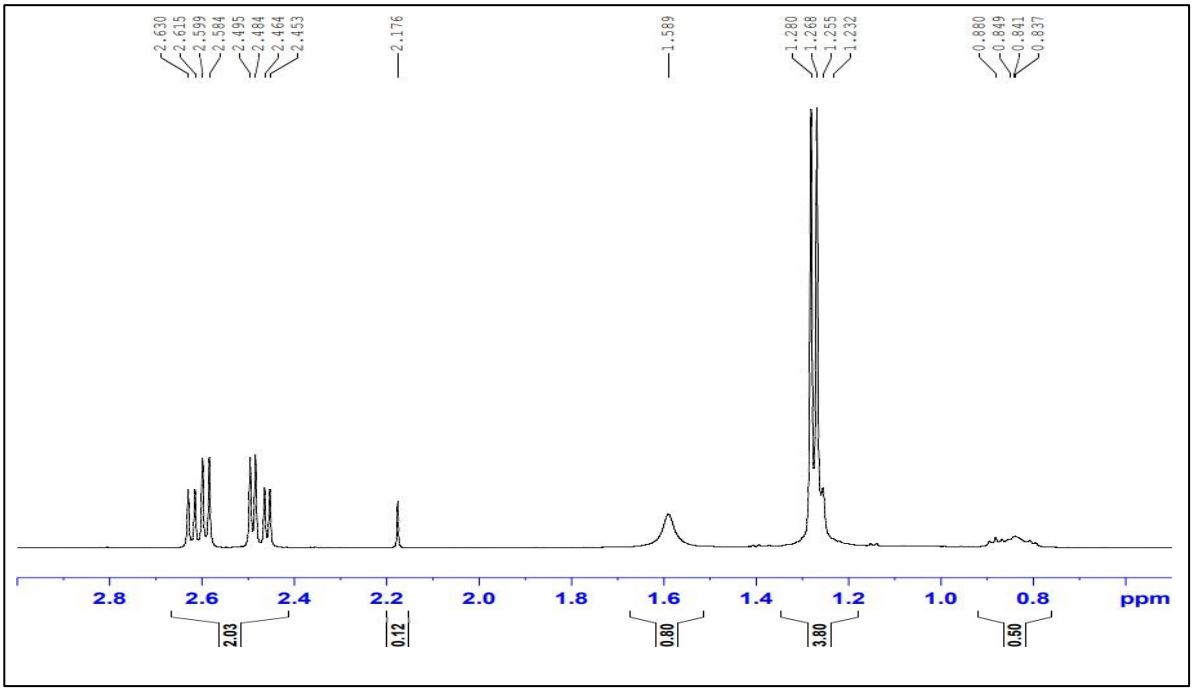


Figure 74. 1H NMR spectrum of PHA film from *P. putida* from 0.8 to 2.8 ppm.

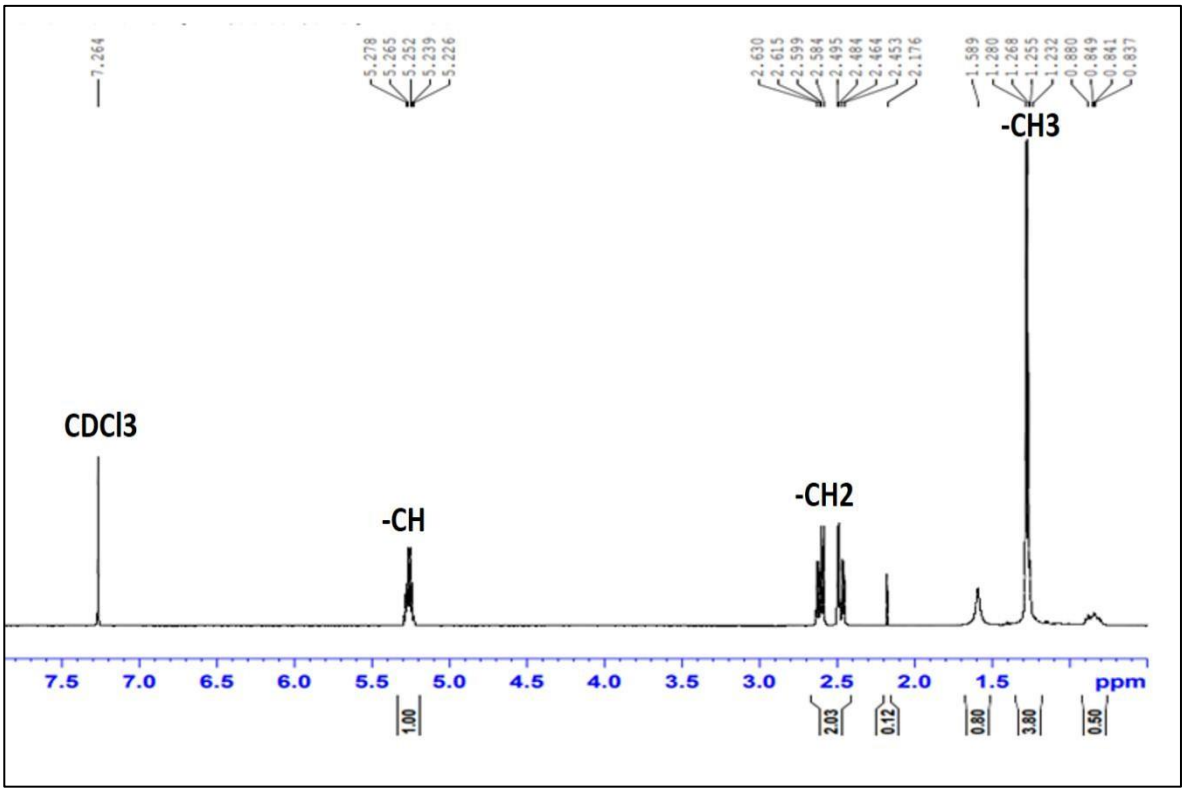


Figure 75. 1H NMR spectrum of PHA film from *P. putida* from 1.5 to 7.5 ppm.

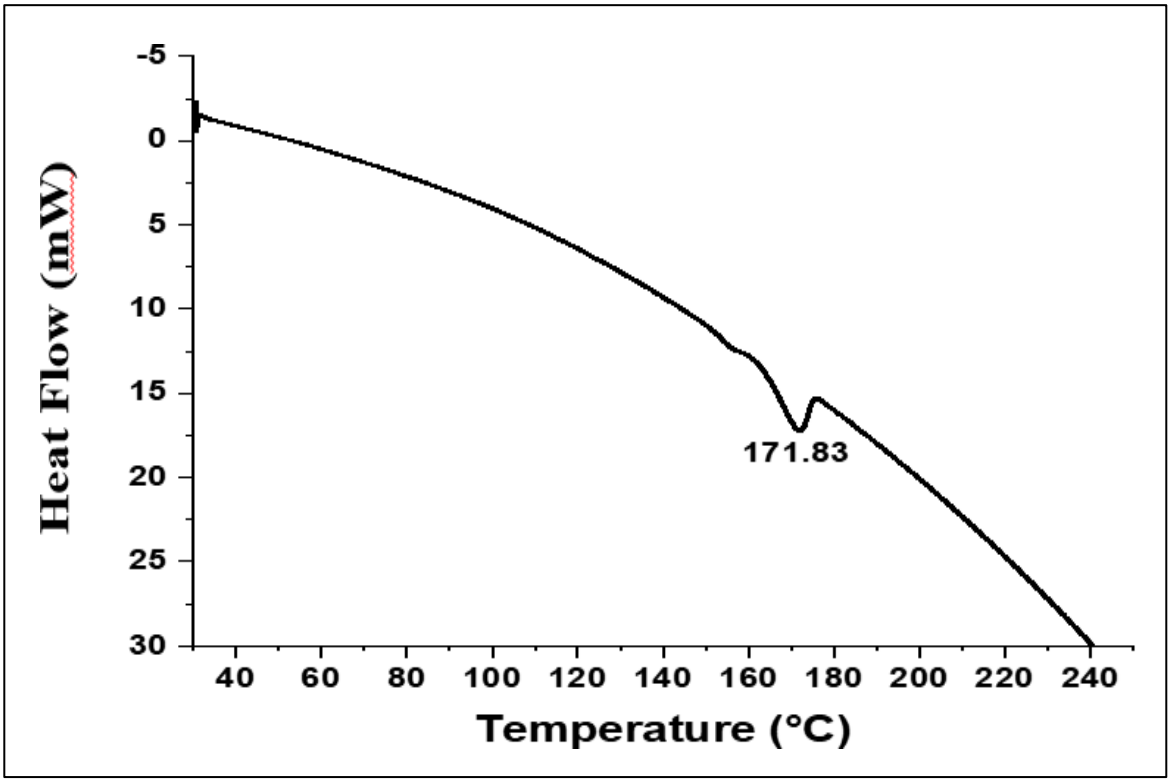


Figure 76. DSC thermograms of extracted PHA film.

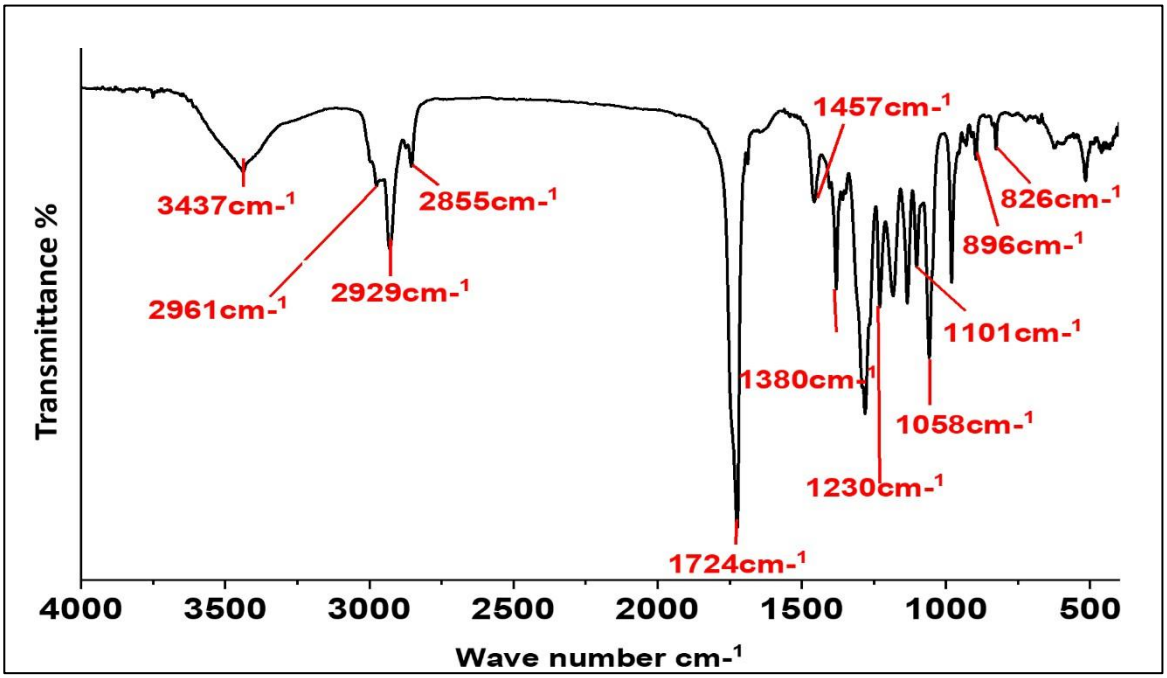


Figure 77. FTIR spectrum of extracted PHA film.

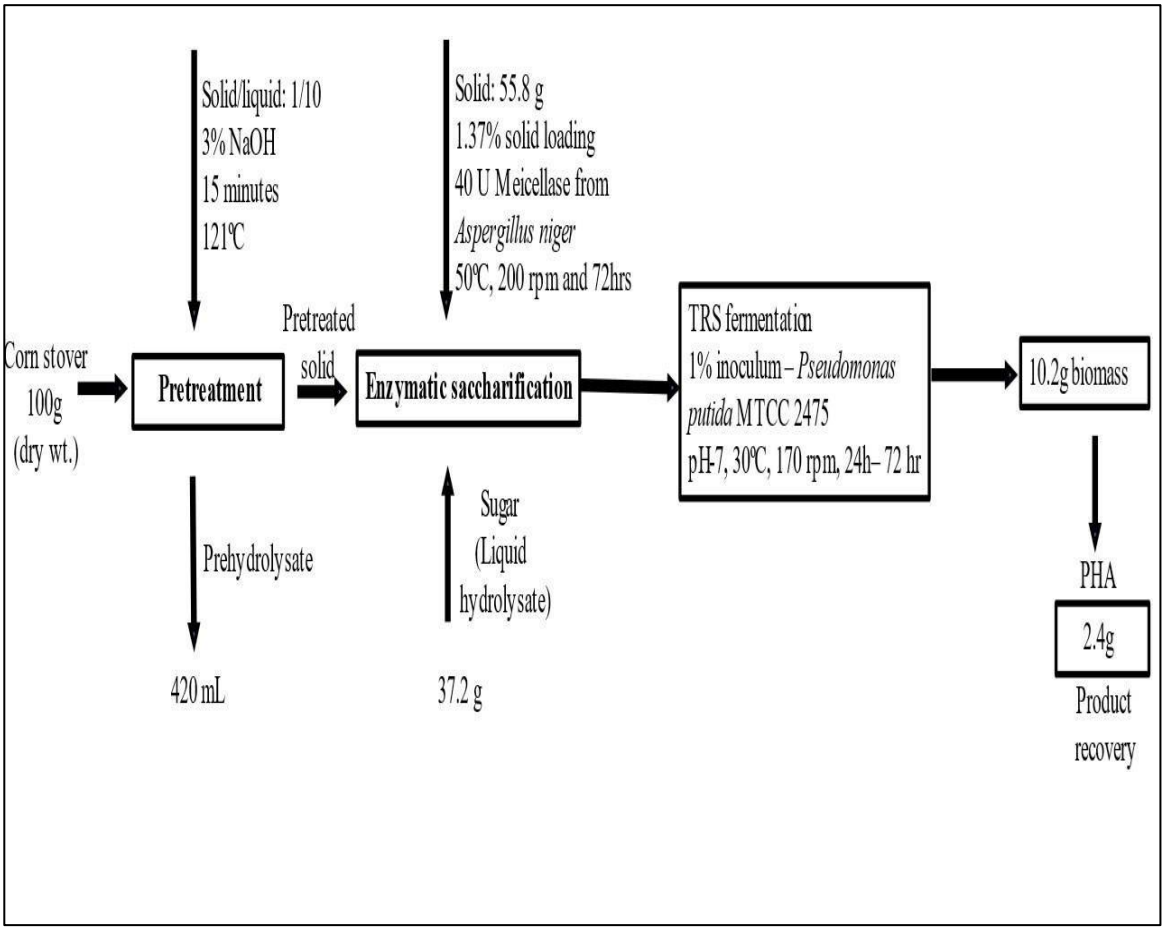


Figure 78. Mass balance study of utilizing corn stover waste as substrate for PHA production.

Table 40. Summarized table of the different PHA content in other research studies and earlier reports using corn stover as hydrolysate.

S.No.	Substrate	Pre-treatment/ Saccharification	Microorganism for PHA production	Biopolymer	Polymer content	References
1	Corn stover hydrolysate	Alkaline pretreatment, densification and enzyme digestibility	Metabolically engineered *Escherichia coli* WJ03-02	Poly(3-hydroxybutyrate-co-lactate)	19.00 wt.%	(J. Wu et al., 2021)
2	Corn stover hydrolysate	1%(v/v) sulfuric acid pretreatment and absence of LevA	*Azohydromonas lata* DSM 1122	Short chain polyhydroxyalkanoate	No polymer production	(Ashby et al., 2022)
3	Corn stover hydrolysate	1%(v/v) sulfuric acid pre-treatment and absence of Lev A	*Burkholderia sacchari* DSM 17165	Short chain polyhydroxyalkanoate	17.90%	(Ashby et al., 2022)
4	Corn stover hydrolysate	Alkaline (3%sodium hydroxide) and enzymatic saccharification with cellulase from *Aspergillus niger*	*Pseudomonas putida* MTCC 2475	Medium-chain Polyhydroxyalkonate	24.40%	This study

3.2 Enzymes Production using Corn Stover

3.2.1 Composition of Corn Stover

Composition of untreated corn stover dry weight % is as follows cellulose 43.18%, structural carbohydrate 62.92%, lignin 23.70%, ash 11.76% and other components 1.61% is shown in **Table 41.**

Table 41. Composition analysis of corn stover before and after fungal treatment.

Component	Untreated biomass before fungal pre-treatment (%)	Untreated biomass after fungal pre-treatment (%)
Cellulose	43.18±0.11	38.98±0.07
Structural carbohydrate	62.92±0.66	56.43±0.05
Acid soluble lignin	2.41±0.00	1.53±0.02
Acid insoluble lignin	21.30±0.39	16.43±0.06
Ash	11.76±0.74	10.76±0.09
Other components	1.61±0.00	-

3.2.2 Sodium Hydroxide Pre-treated Biomass

Corn stover was cut and grinded for further use in experiments as shown in **Figure 79**. Corn stover was alkali pre-treated to remove lignin content and to expose cellulosic structure of the biomass (Zheng et al., 2010). In this study, cellulose content was increased, and lignin content was decreased with exposure to sodium hydroxide pre-treatment. Results demonstrates that alkali pre-treatment significantly effectively removed lignin from corn stover sample. Carbohydrate content is exposed for efficient hydrolysis/saccharification as pre-treatment has removed significant amount of lignin(Nwankwo et al., 2021). Thus, the study conducted of growth and enzyme production by *P. chrysosporium* in untreated and treated biomass demonstrates less

enzyme production in alkali treated biomass than untreated. The growth of white rot fungus took nearly 10 days and forms white lawn and then, spores are recovered for experimental analysis. Schematic of steps performed in enzyme production from corn stover utilizing *Phanerochaete chrysosporium* NCIM1106 by solid state fermentation is given in **Figure 80.**

Figure 79 (a). Corn stover taken from the field **(b).** Grinded corn stover sample to be used for further analysis.

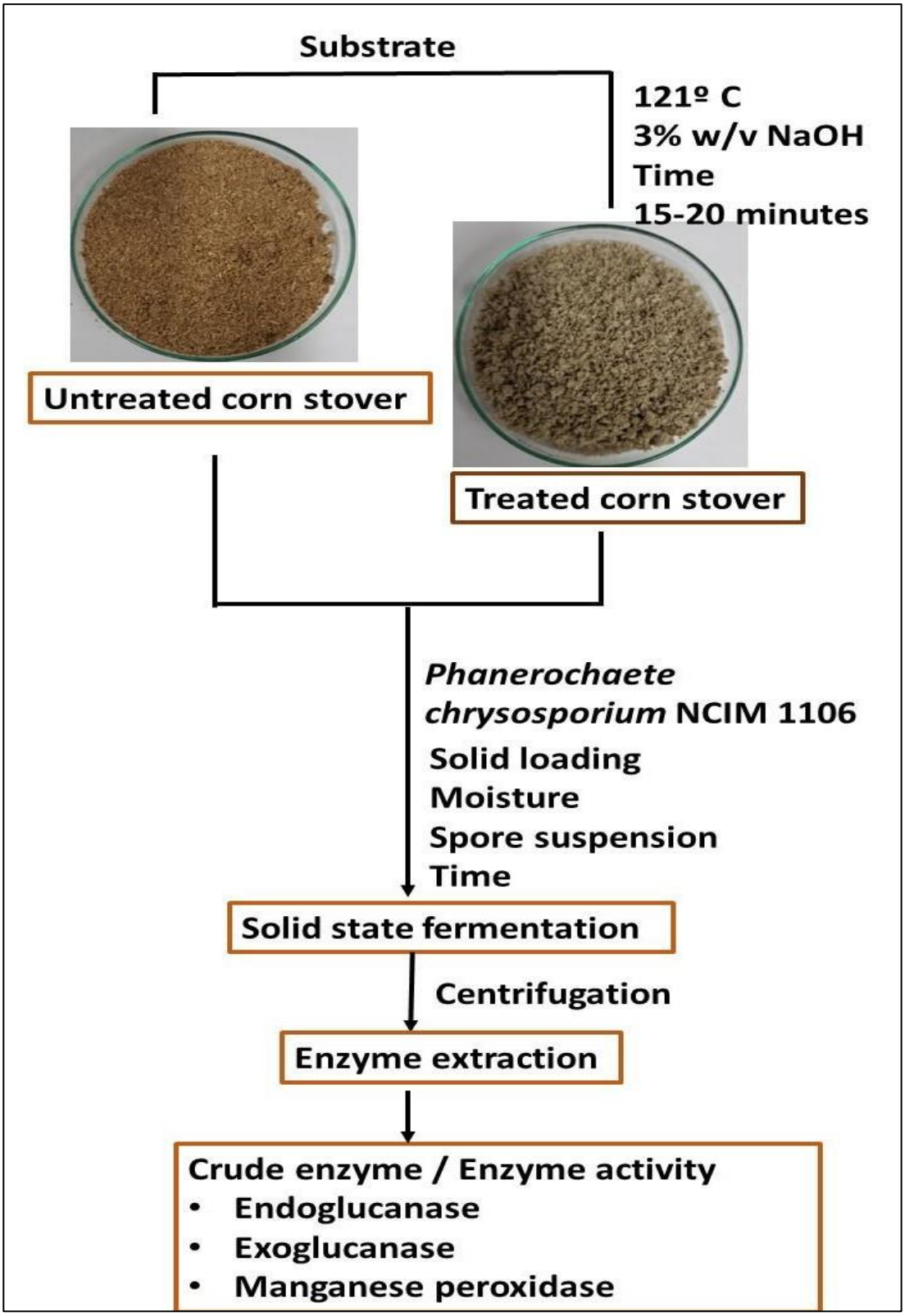


Figure 80. Schematic of steps involved in solid state fermentation for conversion of corn stover biomass to enzymes using untreated and treated substrate.

3.2.3 Cellulase Production in Untreated and Treated Corn Stover

The cellulolytic activity of exoglucanase and endoglucanase from fungal strain depends on the source and type of biowaste from the environment (Islam et al., 2018) and in this study, enzyme production of endoglucanase was estimated by carboxymethyl cellulose assay (CMCase) and exoglucanase by filter paper assay (FPA). Thus, cellulolytic enzymes help in breaking down carbohydrate component into glucose and xylose. Maximum endoglucanase activity 121.21 ± 0.90 U/ml was achieved on day 9^{th} in untreated biomass. Maximum exoglucanase activity 2.46 ± 0.008 FPU/ml was achieved on day 3^{rd} in untreated biomass as shown in **(Figure 81.a and 81.b) / (Table 42).** Generally, in comparison of untreated and treated biomass, *P. chrysosporium* showed more growth in untreated biomass. In a study NaOH concentration higher than 2% showed lower enzyme production. This is due to higher lignin removal, loose composition of lignin and hemicellulose content (Bandikari et al., 2014). The internal structure of substrate is highly disrupted and have high porosity in substrate. Maximum exoglucanase and endoglucanase activity 79.75 ± 0.57 U/ml and 0.92 ± 0.002 U/ml observed in treated biomass. Thus, the results obtained from this study shows that cellulolytic extracellular enzymes are produced by *P. chrysosporium* by utilizing agricultural waste. As reported, cellulase produced by *Trichoderma reseei* was 131 U/gds using lignocellulosic biomass(Dhillon et al., 2011). However, 150 U/gds cellulase production from *Cladosporium cladosporioides* using sugarcane bagasse as substrate by SSF was observed (Srivastava et al., 2020). According to (Abdullah et al., 2021) endoglucanase was 0.1 U/ml synthesised by *Talaromyces thermophilus* using *Saccharum spontaneum* as substrate by solid state fermentation (Abdullah et al., 2021). Thus, the results attained in this research study is in line with the other reported studies.

3.2.4 Manganese Peroxidase Production in Untreated and Treated Corn Stover

The fungus produced MnP under solid state fermentation and MnP activity was highest 5076.81 ± 0.23 U/L on 6^{th} day and 1127.58 ± 0.23U/L on 3^{rd} day in untreated and treated biomass respectively as given in **Figure 81.c and Table 42.** Enzyme activity

was increased on day 6th and started declining after day 6th in untreated biomass as the activity of manganese peroxidase is dependent on hydrogen peroxide. Manganese peroxidase production is inactivated by high concentration of hydrogen peroxide. In the studies it is also cited that veratryl alcohol sometimes function as stabilizer of manganese peroxidase (Bermek, Li and Eriksson, 2002 In case of treated biomass, enzyme activity decreased after day 3rd as shown in **Table 42**. *P. chrysosporium* is the model organism to study MnP production but cellulase production is also reported (Thakkar et al., 2006). Oxidoreductases have immense fate to catalyse oxidation of organic molecules in presence of hydrogen peroxidase. MnP has less oxidant load on the environment and reduce the peroxidase environmental tolerance range. MnP are considered to be first series of proteins expressed in fungal strain catabolism of lignin and because of this application of lignin hydrolysis, MnP are of great interest for research (Nugraha et al., 2020). Huy et al., 2021 reported Manganese peroxidase 1.91 U/ml by *Fusarium sp*. using rice straw as biomass (Huy et al., 2021). In a study by (Urek C Pazarlioglu, 2007) MnP 356 U/L produced by *Phanerochaete chrysosporium* BKMF-1767 (ATCC 24725) using media components and different growth conditions(Urek C Pazarlioglu, 2007). In present study, media components have been replaced by lignocellulosic corn stover biomass and produced high MnP.

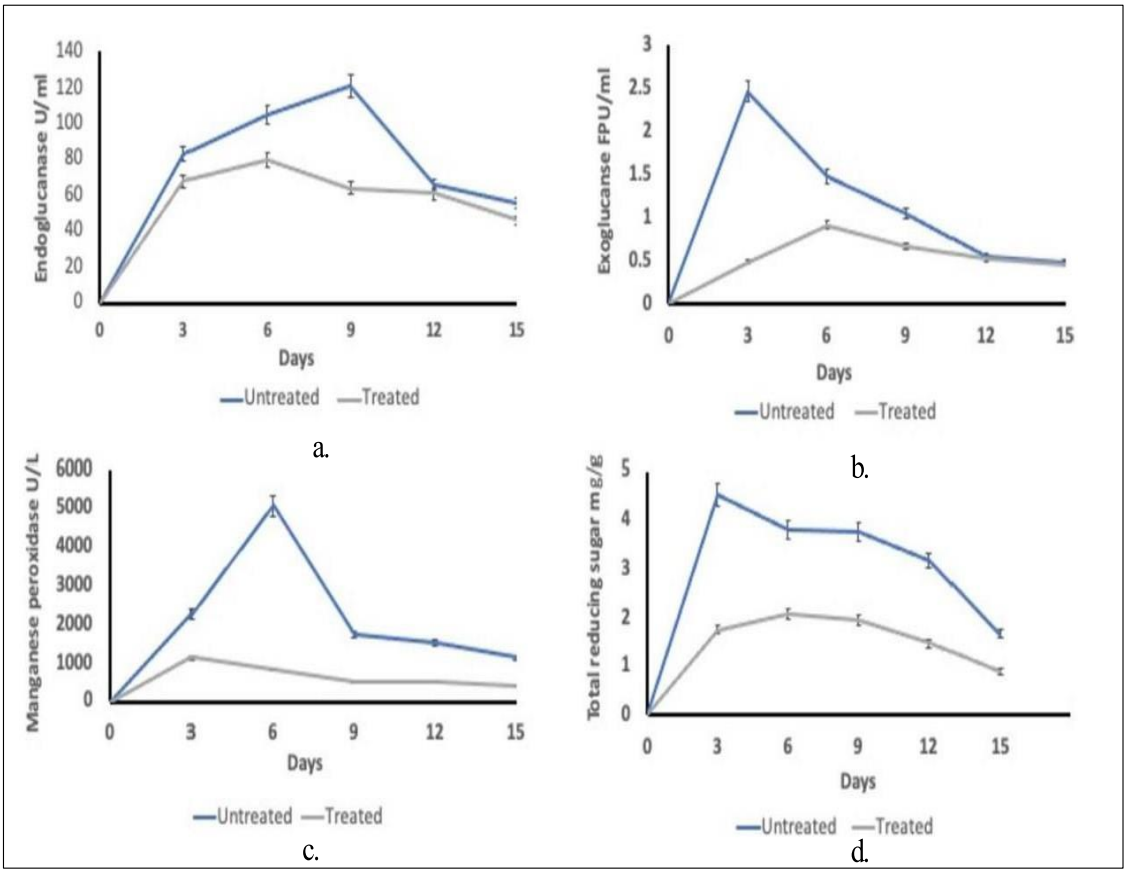


Figure 81 a. Endoglucanase**, b.** Exoglucanase and **c.** Manganese peroxidase produced by solid state fermentation on respective days as well as total reducing sugar (mg/ml) in untreated and 3% NaOH (w/v) treated sample after fungal pretreatment with *P. chrysosporium* NCIM 1106. **d**. TRS produced during the fermentation of corn stover in the solid substrate. All the data points were attained from experiments performed and presented as the mean standard deviation.

Table 42. Enzyme activity of the extracellular enzymes produced by solid-state fermentation in untreated and treated corn stover. All the data points were attained by experimental analysis in triplicate and shown as the mean standard deviation.

Substrate corn stover	No. of days	Moisture %	Spores (1×10^4 spores/ml)	Endoglucanase (U/gds)	Exoglucanase (FPU/ml)	Manganese peroxidase (U/L)
Untreated	3	60	1ml	82.95 ± 0.78	2.46 ± 0.008	2273.09 ± 0.41
Treated	3	60	1ml	68.37 ± 0.23	0.49 ± 0.006	1127.58 ± 0.23
Untreated	6	60	1ml	105.20 ± 0.13	1.48 ± 0.007	5076.81 ± 0.23
Treated	6	60	1ml	79.75 ± 0.57	0.92 ± 0.002	838.91 ± 0.86

Untreated	9	60	1ml	121.21 ± 0.90	1.05 ± 0.003	1747.20 ± 0.41
Treated	9	60	1ml	63.95 ± 0.63	0.67 ± 0.009	529.61 ± 0.63
Untreated	12	60	1ml	65.89 ± 0.74	0.56 ± 0.005	1519.12 ± 0.63
Treated	12	60	1ml	61.37 ± 0.95	0.52 ± 0.004	504.92 ± 0.61
Untreated	15	60	1ml	56.22 ± 0.22	0.49 ± 0.003	1127.75 ± 0.86
Treated	15	60	1ml	45.68 ± 0.81	0.46 ± 0.001	405.26 ± 0.22

3.2.5 Total Reducing Sugars (TRS) and Biomass Recovered in Untreated and Treated Corn Stover

The total reducing sugar (TRS) from the lignocellulosic substrate – untreated and treated corn stover was analysed by Di-nitro salicylic method (DNS) using glucose as standard. TRS production after biological pre-treatment was calculated on 5 days shown in **Figure 81.d.** TRS amount was highest 4.525 mg/g in untreated corn stover on 3rd day and was least on 15th day. The bioconversion of corn stover to fermentable sugars require various enzymes cellulolytic and ligninolytic enzymes. Enzymatic degradation has advantage over chemical hydrolysis as no inhibitors are generated. **Figure 82** shows untreated and treated flask on day 3rd and 15th and the mycelial growth in the form of white lawn in seen in untreated biomass on day 15th. Thus, it depicts that *P. chrysosporium* is model organism to study lignin degradation. Biomass recovered after the fungal treatment is shown in **Figure 83.** The utilization of biological process using microbes are good due to economic aspect and environment friendly (Min et al., 2022). Biomass recovered after fungal pre-treatment is reduced in untreated biomass as the fungus disrupts lignin and carbohydrate components to release reducing sugar, however in treated corn stover, biomass remained unutilized (Goukanapalle et al., 2020). Solid biomass of untreated and alkali pre-treated corn stover recovered after fungal pre-treatment was further analysed for composition after washing and drying till constant**. Table 41** shows the change in composition of untreated corn stover after fungal treatment as more enzyme is produced in untreated biomass. Comparative table of microbial enzyme production in this research study and other literature available is shown in **Table 43.**

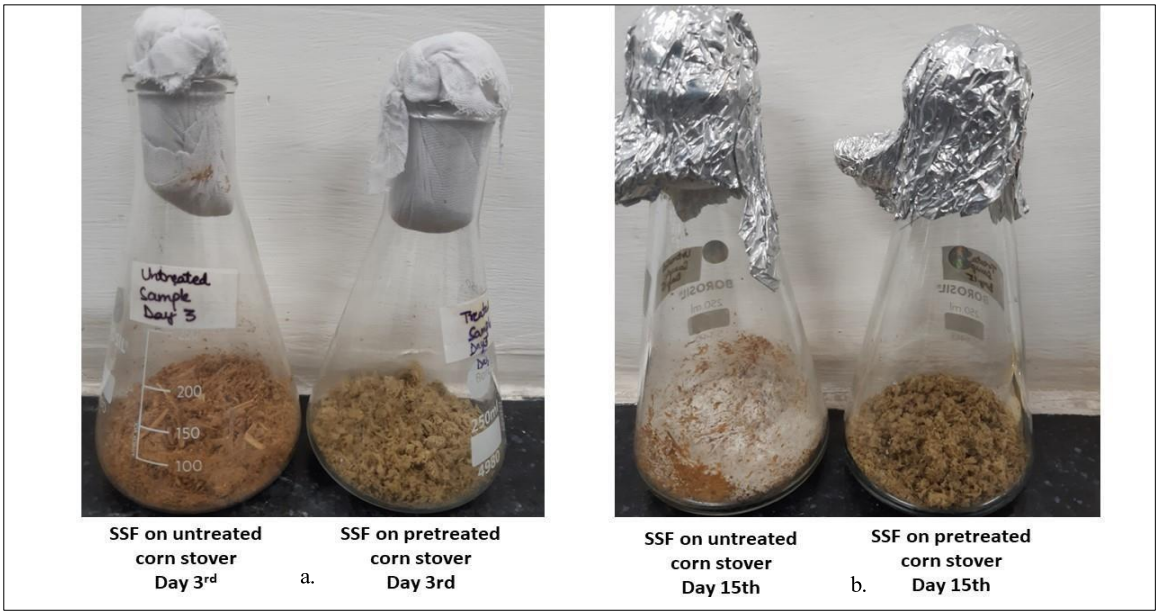


Figure 82 a and b. Untreated and 3% NaOH (w/v) treated corn stover sample with *Phanerochaete chrysosporium* NCIM 1106 on Day 3rd and Day15th respectively. P. chrysosporium mycelia are seen as white lawn on the untreated sample on Day15th but in case of the treated sample it is same on 3rd and 15th day respectively.

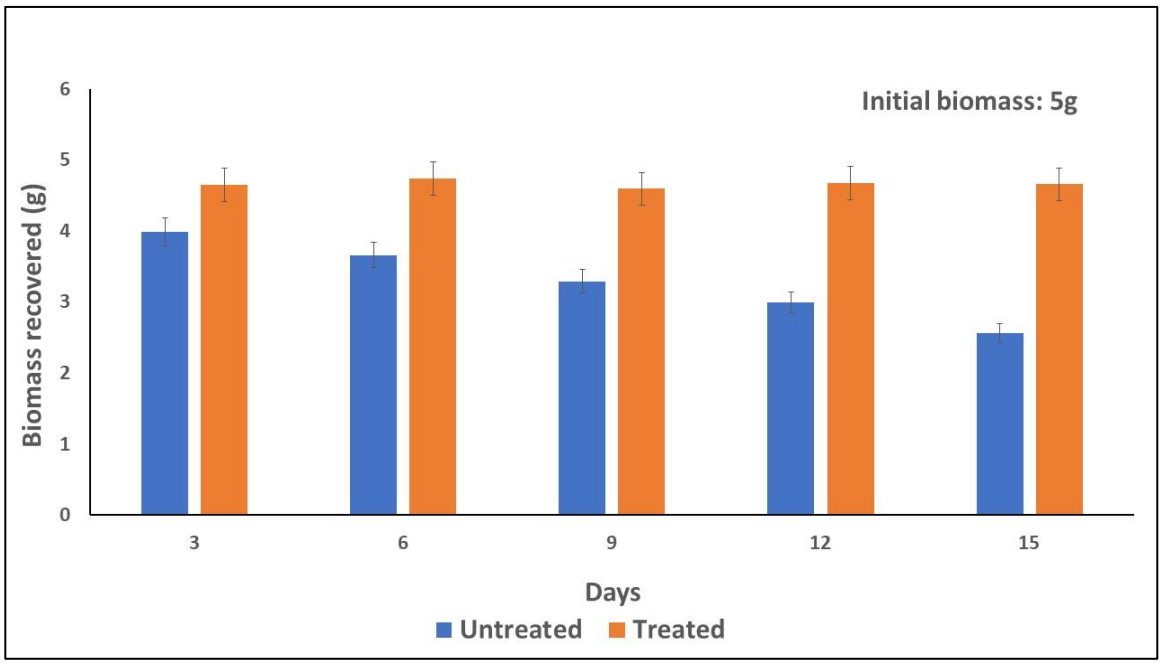
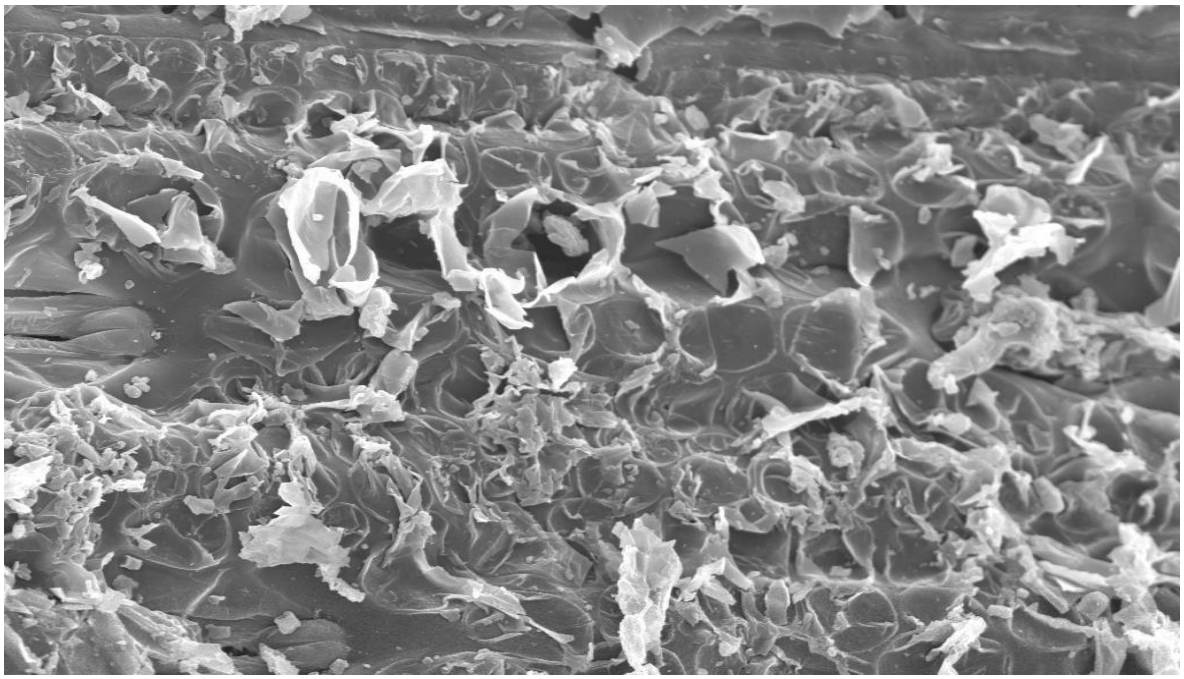


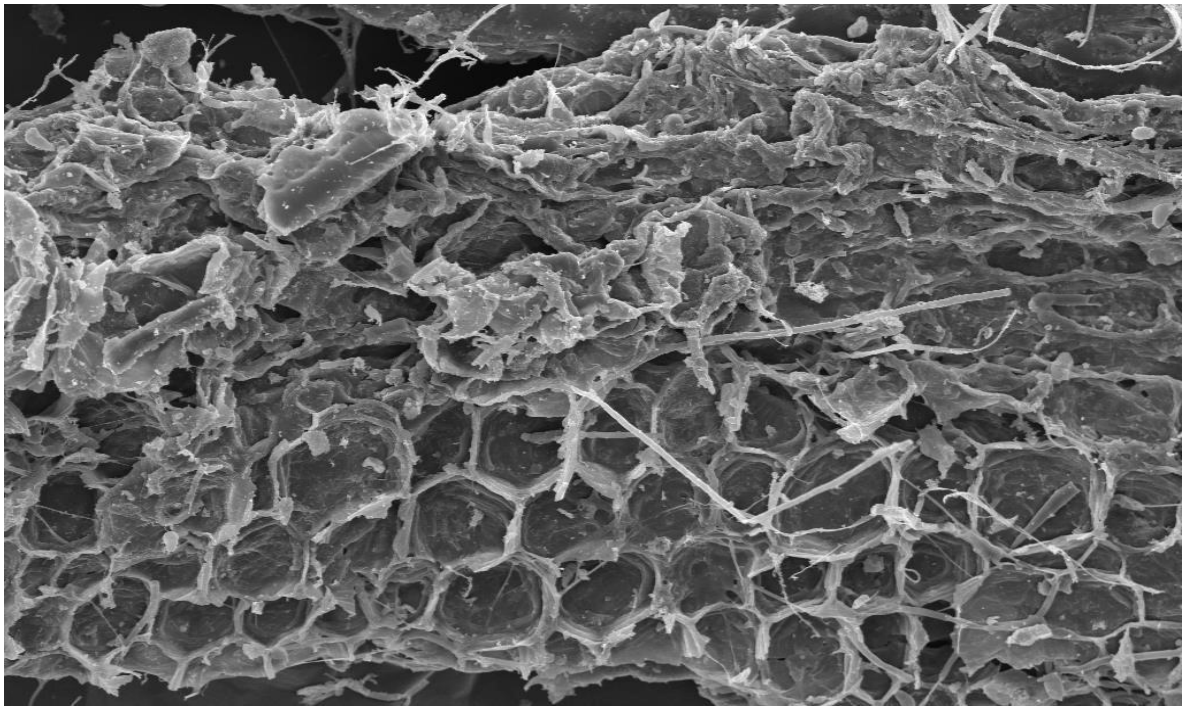
Figure 83. Biomass recovered after fungal pretreatment with *P. chrysosporium* NCIM 1106 in untreated and 3% NaOH (w/v) treated sample from day 3rd to 15th.

3.2.6 Characterization of Biomass After Fungal Pre-treatment by Scanning Electron Microscopy

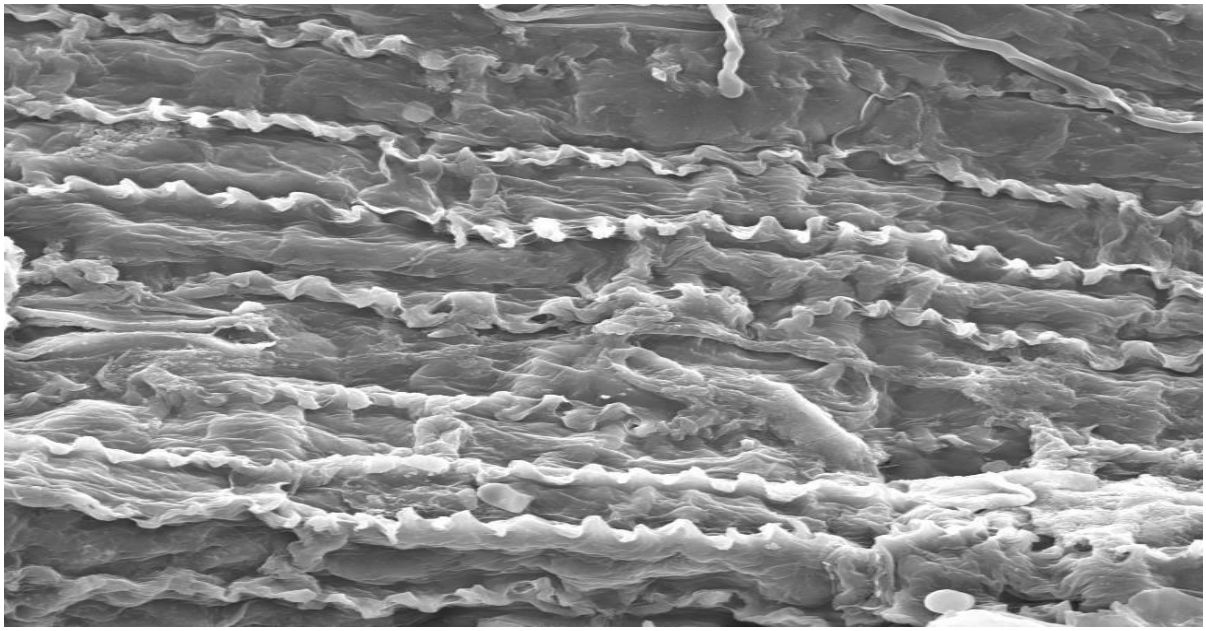
Figure 84 a and c shows the SEM images at 1000x of untreated and alkali treated corn stover biomass before fungal treatment. Untreated corn stover depicts the recalcitrant tough compact structure of lignocellulosic sample with intact lignin component and smooth structure of the biomass. After 3% (w/v) sodium hydroxide pre-treatment lignin component of the biomass disrupted and looks like condensed spiral ringlike structure and clearly different from untreated corn stover. After the alkali pre-treatment, the solid biomass recovered is light brown and effective for lignin removal from biomass (Li et al., 2015). **Figure 84 b and d** shows the SEM images at 1000x of the solid biomass recovered after the fungal treatment on untreated and alkali treated biomass. SEM images demonstrate the untreated corn stover after fungal treatment shows severe degradation and disruption in the intact structure of the lignocellulosic biomass. The penetration by the fungal hyphae contributes to the pore formation. Removal of cellulose and hemicellulose component increases the pore size of the biomass. The growth and disruption in the structure of the alkali treated biomass is less due to prior pre-treatment which causes larger particle size which is analysed by microscopic observation. There is significant difference in the internal structure of untreated and alkali treated corn stover.(Wendt et al., 2022).



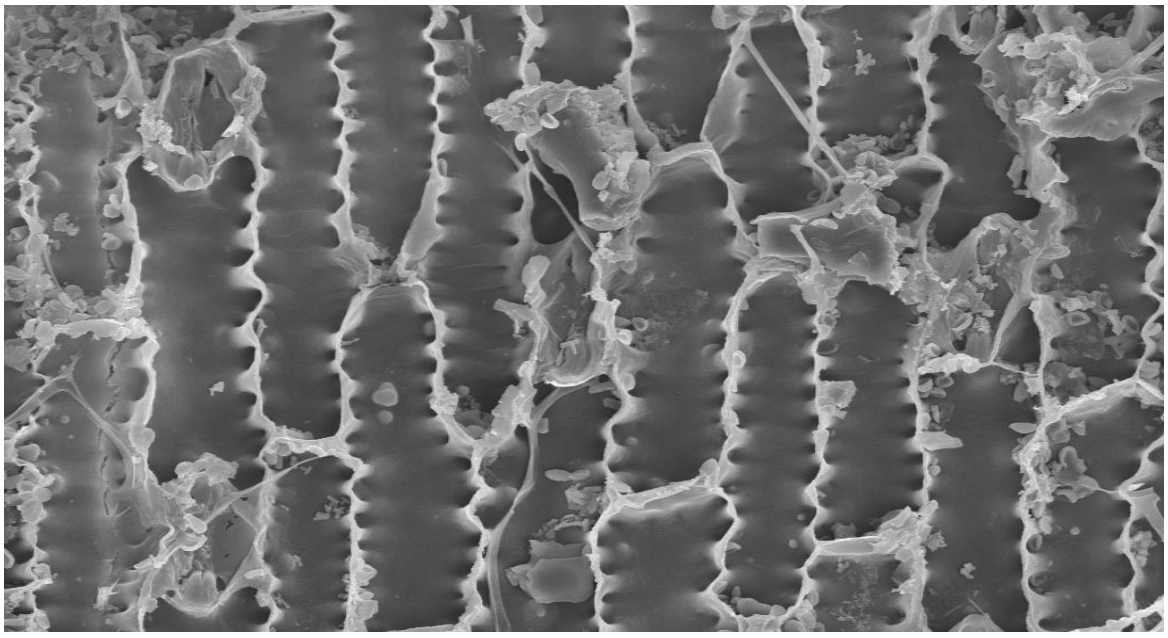
a. Untreated corn stover before fungal treatment



b. Untreated corn stover biomass after fungal pretreatment



c. Treated corn stover biomass before fungal pretreatment



d. Treated corn stover biomass after fungal pretreatment

Figure 84. a. SEM image of untreated corn stover before fungal treatment (1000x) **b.** Untreated corn stover after fungal treatment (1000x) **c.** Treated corn stover biomass before fungal pretreatment (1000x) and **d.** Treated corn stover biomass after fungal pretreatment (1000x).

3.2.7 Fourier Transform Infrared Spectroscopy Analysis after Fungal Pre-treatment

The FTIR spectrums of the different pre-treatment conditions, demonstrate the structure variation of corn stover biomass and composition of cellulose and lignin. To analyse the effect of pre-treatment on the corn stover, it is important to study the monomer units, functional group present and bonding of cellulose and lignin. The results are given as FTIR spectrums in **Figure 85**. The results are compared with untreated corn stover without any pre-treatment and biomass recovered after fungal treatment on untreated and alkali treated corn stover. The bond at 3386 cm^{-1} shows stretch of H bond with OH and 2901 cm^{-1} shows the C-H stretch(Sharma et al., 2016). The lignin component is shown as chemical functional groups at wavenumber between 1600 to 1400 cm^{-1} and depicting aromatic structure. The spectrums show the presence of lignin as aromatic ring like structure in three samples. The absorbance at 1647 cm^{-1} is lower in untreated biomass indicating that lignin had structural modifications during alkali pre-treatment and had high infrared absorption. The lignin and hemicellulose removal are shown at 1236 cm^{-1} which is -COO movement of acetyl groups and -C-O stretch of aryl group in hemicellulose and lignin respectively. The 1429 cm^{-1} shows the CH_2 bending. The wavenumber at 1050 cm^{-1} indicates the bend of hydroxyl group in lignin (Woźniak et al., 2021). Thus, the results dindicate that alkali pre-treatment has undergone condensation reaction with alcoholic hydroxy group of lignin structure to form aldehyde generated components.

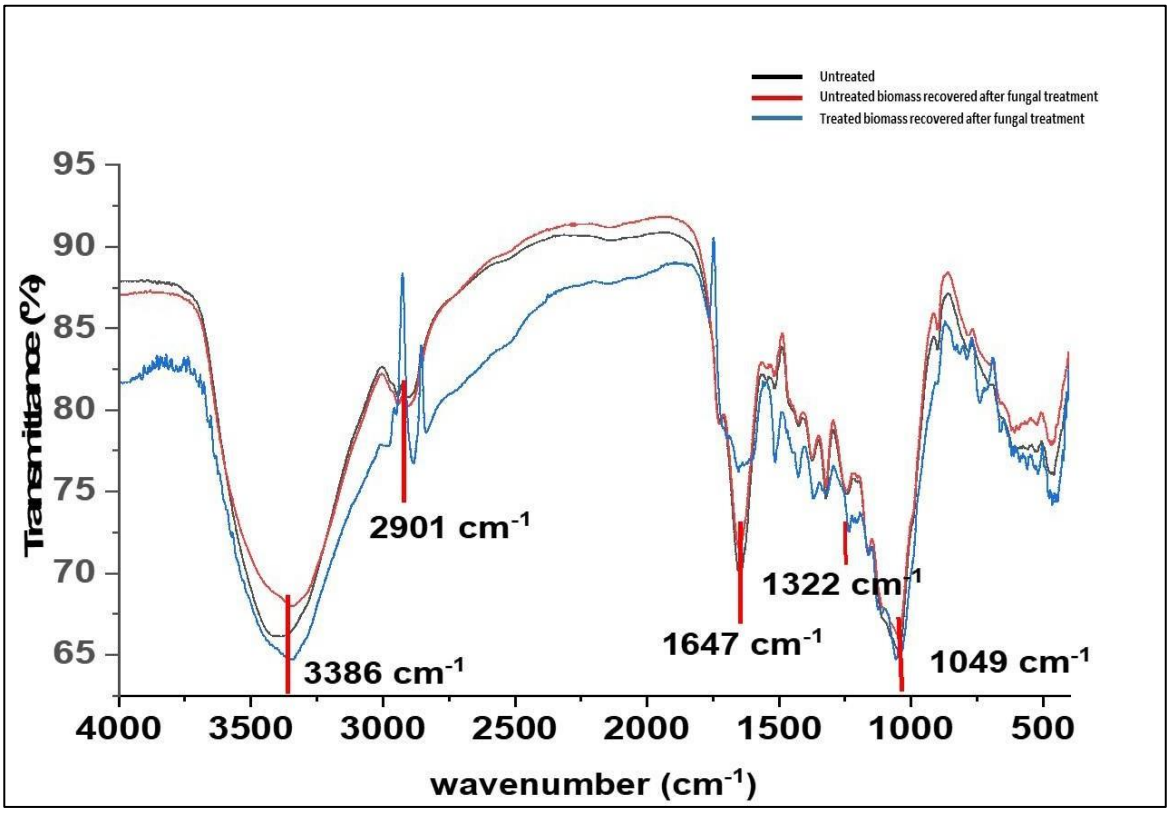


Figure 85. FTIR spectrum of untreated corn stover compared with biomass recovered of untreated and treated after fungal pre-treatment.

3.2.8 Biotechnological Based Application of Microbial Enzymes and Future Prospects

Cellulases and manganese peroxidase have several usages as biocatalyst for delignification of lignocellulosic biomass, biodegradation of dangerous pollutants, harmful dyes, phenolic groups, de-pulping, decolourization and biofuel production(Jayasekara C Ratnayake, 2019; Yafetto, 2022). Recombinant microorganisms can be constructed to enhance the stability, efficiency and yield of microbial enzymes. A recent study by (Chang et al., 2021) has taken functional recombinant MnP1 from *Phanerochaete chrysosporium*. Thus, these microbial enzymes have potential to be applied for biodegradation of hazardous environmental contaminants and use for bioremediation (Urek C Pazarlioglu, 2007)

Table 43. Comparative table of cellulase and manganese peroxidase production using different substrate and microorganism on solid state fermentation in other research studies.

S. No.	Microorganism	Substrate	Enzyme	Yield	Reference
1.	*Phanerochaete chrysosporium*	Empty fruit bunches of palm	Cellulase	0.36 U/ml	(Maceno et al., 2016)
2.	*Phanerochaete chrysosporium*	Rice straw	Cellulase	1.43 U/ml	(Potumarthi et al., 2013)
3.	*Phanerochaete chrysosporium*	Corn stover	Cellulase	1.57 U/ml (FPase)	This study
4.	*Fusarium sp.*	Rice straw	Manganese peroxidase	1.91 U/ml	(Nguyen, 2020)
5.	*Phanerochaete chrysosporium*	Corn stover	Manganese peroxidase	4.917 U/ml	This study
6.	*Trichoderma reseei*	Agricultural waste	Cellulase	131 U/gds (CMCase)	(Dhillon et al., 2011)
7.	*Cladosporium cladosporioides NS2*	Sugarcane bagasse	Cellulase	150 U/gds (CMCase)	(Srivastava et al., 2020)
8.	*Phanerochaete chrysosporium*	Corn stover	Cellulase	112.1 U/gds (CMCase)	This study

CHAPTER 4

CONCLUSION

Biodegradable PHA is alternate of conventional plastic and its production is limited because of its high cost. Corn stover stubble waste is utilized for total reducing sugar enhancement by optimizing conditions of alkali pre-treatment and enzymatic saccharification. Utilizing sugar *Pseudomonas putida* MTCC 2475 can synthesize PHA with heterogenous monomers. Thus, corn stover waste is renewable and easily available feedstock for bioconversion of lignocellulosic biomass into reducing sugar and minimizes the use of expensive substrates. In addition, a potential substrate for PHA production and up-scaling as well as optimization of conditions is further required for PHAs production at industrial platform.

The study highlights the potential of *P. chrysosporium* NCIM 1106 as a producer of cellulase and manganese peroxidase through solid-state fermentation (SSF). When alkali pre-treated corn stover is used as the substrate, it results in an increase in cellulose content and a decrease in lignin content, making it a suitable material for cellulase and manganese peroxidase production. *Phanerochaete chrysosporium* is well known for its lignin-degrading capabilities and superior ligninolytic properties. Interestingly, the study found that the maximum enzyme production occurs when using untreated corn stover biomass rather than alkali pretreated biomass. This research underscores the remarkable ability of white rot fungi to break down the complex structures found in plant cell walls. Moreover, the use of both untreated and alkali treated corn stover for cellulase and manganese peroxidase production has the potential to reduce agricultural waste and offer cost-effective solutions for enzyme-producing industries. In conclusion, corn stover emerges as an economically viable and environmentally sustainable substrate, offering a practical means of utilizing agricultural waste for enzyme production.

4.1 Future Scope of Research Work

- Lignocellulose is a kind of promising resource for biorefinery owing to their abundance, renewability and non-competition with human demands.

- Scaleup of the bioproducts from laboratory to industrial level and genetic manipulation of the microbes for large-scale production.
- To have economically competitive PHAs, production and the downstream need to be tackled simultaneously.
- As biotechnology advances genetic modifications, novel techniques, and enzyme engineering has a remarkable potential to boost enzyme yields.
- Circular economy is a waste reduction hypothesis emphasizing the reusing and recycling of available resources.

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LIST OF PRESENTATIONS IN CONFERENCES

- Oral Presentation: A Thermochemical Pre-treatment study of stubble waste for value added product. National conference on Bioengineering organized by NIT Rourkela, Odisha, India (December 10-11,2020)
- Abstract titled: A Comparative Study of Dilute Acid and Alkali Pre-treatement from Stubble waste to Renewable Chemicals Production published in the Conference Proceedings of the First International Conference on innovations in Biotechnology and Life Sciences (ICIBLS,2020) organized by Department of Biotechnology, Delhi Technological University, India (December 18-20,2020)
- Poster Presentation: Bio-based plastic from lignocellulosic biomass: Environment and Sustainable development presented in the BIOSPECTRUM International Conference on Biotechnology & Biological Sciences by University of Engineering and Management, Kolkata, India on 18th November to 20th November 2021

SEMINARS / WORKSHOPS

- On 21st May 2020 - Attended online scientific writing program on the topics structuring manuscript to impress journal editors and selecting a journal and preparing a great submission package organized by Editage.
- On 29th May 2020 - Attended Milli-Q webinar for research.
- On 12th June 2020 - Attended Online lecture on Prospects of Nanomaterials modified conducting Paper-based biosensors for cancer detection.
- From 10-14th June 2020 - participated in the TEQIP-III Sponsored online short-term course on "Trends and Prospects in Biorefinery" organized by Department of Biotechnology, Dr. B.R. Ambedkar National Institute of Technology Jalandhar, Punjab, India.
- On 16th June 2020 - attended webinar on Deployment of Bioenergy Combined with carbon capture and storage utilization by IEA Energy.
- From 28th June 2020 to 4th July 2020 - participated in the e-Faculty Development Program cum Workshop on "Waste to Bioenergy" organized by Department of Life Science, School of Basic Science and Research, Sharda University, Uttar Pradesh and Department of Agricultural Engineering, Maharashtra Institute of Technology, Aurangabad.
- From 13 to 17th July 2020 - attending e faculty development program cum training workshop on "Environment, Water and Disaster Risk Reduction' jointly organized by Sharda University, NCR, India and National Institute of Disaster Management, Ministry of Home Affairs, New Delhi.
- On 14th July 2020 - Attended National webinar on Recent trends in environment, health and technology by Mangalayatan University, Aligarh.

- On 9th August 2020 - Attended webinar on science of wood: its structure and significance.
- From 2nd - 6th September 2020 - Workshop on Computational tools for analysis of biological systems organized by Department of Biotechnology, Dr. B.R. Ambedkar National Institute of Technology Jalandhar, Punjab, India.
- On 16th September 2020 - Trends and drivers in alternate thermal conversion of waste confirmation by IEA Energy.
- From 19th - 21st September 2020 – attended webinar on Waste to energy organized by Centre for Environment, Institute of Science and Technology and Jawaharlal Nehru Technological University Hyderabad (JNTUH).
- On 9th December 2020 - attended webinar on NIR in feed and forage, grain milling oil and laboratories related applications along with best practices for NIR networking and management by FOSS India Pvt. Ltd.
- From 14th-18th December 2020 - attended international workshop on Bioinformatics by Department of Biotechnology, Delhi Technological University.
- Workshop on the Future of Bioenergy and Bio renewables by Pennsylvania State University, United States.
- From 18th-20th December 2020 - participated in International Conference on Innovation in Biotechnology and Life Sciences (ICIBLS).
- From 22nd to 23rd January 2020 - participated in the two-day International Webinar on "Plant Science Research Post Covid-19" organized by Department of Botany, Rajiv Gandhi University, India in collaboration with Tennessee State University, Nashville USA.

- On 11th February 2020 – attended international e-symposium on ‘women in science’ organized by the Department of Biotechnology, DTU.
- Two – weeks Comprehensive online Patent Information Course organized by Turnip Innovations.
- Attended The GASS National Research Online workshop on 6-7 May 2021organized by Truba Group of Institutes on How to write and publish.
- Attended webinar organized by National Productivity Council of India on Awareness on Energy Conservation on 2nd June 2021.
- Attended one day E-workshop on ‘Innovation and Entrepreneurship’ organized by Department of Biotechnology, Delhi Technological University on 18 September 2021.
- Poster presentation at - BIOSPECTRUM International Conference on Biotechnology & Biological Sciences by University of Engineering and Management, Kolkata, India on 18th November to 20th November 2021.
- Attended webinar on Motivational session by successful entrepreneurs and start up founders organized by Department of Biotechnology, Delhi Technological University on 25th November 2021.
- Attended webinar on the era of Indian Startups by Department of Biotechnology, Delhi Technological University on 15th October 2022.
- Attended seminar on Translational Bioinformatics organized by BIOSOC-DTU, Delhi Technological University on 20th October 2022.
- Attended sustainability webinar on Circular engineering approaches for environmental management by Prof. Brajesh K Dubey in IIT Gandhinagar on 09th March 2023.

- Attended lecture on Botany: From evolution and phylogeny to biodiversity and sustainability by Prof. J.S. (Pat) Heslop-Harrison, Cell Biology and Molecular Cytogenetics, Department of Genetics and Genome Biology, University of Leicester, UK in Department of Botany, University of Delhi on 25th Sept 2023.
- Attended lecture on Codes for making of a rice flowering stem: Roles for evolutionary conserved transcription factors by Prof. Usha Vijayraghavan, Ph.D. (Caltech), FNA, Indian Institute of Science, Bengaluru in Department of Botany, University of Delhi on 9th November 2023.
- Attended symposium on Biotechnology for Sustainable Development organized by Department of Biotechnology, Delhi Technological University on 23rd January 2024.
- Attended talk on Cancer Awareness and Palliative Care Sensitization organized by NSS – DTU & Department of Biotechnology, Delhi Technological University in association with DNip care on 24th January 2024.
- Attended webinar on Turnip, a National webinar on Patent Filing in India by Dr. Prachi Pandey on 19th July 2024.

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PLAGIARISM VERIFICATION

ANNEXURE-IV

DELHI TECHNOLOGICAL UNIVERSITY
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PLAGIARISM VERIFICATION

Title of the Thesis: Implementation of Stubble waste for biotransformation to industrially important chemicals

Total Pages: 149 Name of the Scholar: Neha Kukreti

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BRIEF PROFILE

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Department: Biotechnology

Ms. Neha Kukreti is a Ph.D. research scholar in the Department of Biotechnology, Delhi Technological University under the supervision of Prof. Pravir Kumar, Dean International Affairs and Former HoD, Department of Biotechnology, Delhi Technological University, Delhi and co-supervision of Dr. Rashmi Kataria, Associate Professor, School of Bioscience and Technology, Vellore Institute of Technology, Vellore, India. Her area of research is in the field of Biorefinery with sustainable utilization of biomass resource for the formation of value-added products such as bioplastics and crude enzymes. The entire process from biomass selection and collection, pretreatment, enzymatic conversion followed by fermentation and product recovery ad characterization. The title of the thesis – Implementation of stubble waste for biotransformation to industrially important chemicals. She has passed her Bachelor of Science (Honours) in Botany from Department of Botany, Gargi college, University of Delhi and Master of Science in Botany from Department of Botany, University of Delhi.