Saccharification of pre-treated rice straw by recombinant cellulases

DISSERTATION

Submitted by

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In partial fulfilment for the award of the degree of

Master of Science in Biotechnology

Under the guidance of

Prof. Dinesh Goyal

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July 17, 2023

CERTIFICATE

This is certified that the thesis entitled **"Saccharification of pre-treated rice straw by recombinant cellulases"** submitted by **Ms. Shivangi Singh (302101025)**, in partial fulfilment of requirement for the award of degree of Master of Science in Biotechnology at Thapar Institute of Engineering & Technology (TIET), Deemed to be University, Patiala is a record of student's bonafide work carried out under my supervision and guidance. The matter embodied in the thesis has not been submitted in part or full to any other university or institute for award of any other degree.

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DECLARATION

I hereby declare that I have personally worked on the research project entitled **"Saccharification of pre-treated rice straw by recombinant cellulases"** in partial fulfilment of requirement for the award of degree of Master of Science in Biotechnology at Thapar Institute of Engineering & Technology (TIET), Deemed to be University, Patiala is an authentic record of my own work during the period from January 2023 to July 2023, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, TIET. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad. Any data or information in this report, which has been collected or borrowed from other agency, has been duly acknowledged.

Place: Patiala

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Dedicated to my family & siblings

For constant support

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Place: Patiala

Date:

Shivangi Singh

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List of abbreviations

AIL	Acid insoluble lignin
ASL	Acid soluble lignin
BG	β-glucosidase
BSA	Bovine serum albumin
CBH	Cellobiohydrolase
CH_4	Methane
CHP	Combined heat and power
CI	Crystallinity index
CMC	carboxymethylcellulose
CO_2	Carbon dioxide
CtCBH5A	Cellobiohydrolase
CtCel8A	Endo-β-1,4-glucanase
$CuSO_4.5H_2O$	Copper (II) sulphate
D	Dextro
DNSA	3,5-Dinitrosalicyclic acid
EG	Endoglucanase
Et al.,	And all others
$FeCl_3$	Ferric chloride
FPU	Filter per unit
GH	glycoside hydrolase
GHGs	Greenhouse gases
H_3BO_3	Boric acid
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HtBgl	β-1,4-glucosidase
IL-US	Ionic liquid-Ultra Sound
IPTG	isopropyl β-D-1-thiogalactopyranoside

LB	Luria broth
LBM	Lignocellulosic biomass
$NaClO_2$	Sodium chlorite
Na_2CO_3	Sodium carbonate
$NaHCO_3$	sodium bicarbonate
N_2O	Nitrous oxide
Na_2SO_3	Sodium sulphite
NaOH	Sodium hydroxide
pNP	p-nitrophenyl
RCF	Relative centrifugal field
RS	Rice straw
RPM	Revolution per minute
RSM	Response surface method
TGA	Thermal gravimetric analysis
TRS	Total reducing sugar
UV	Ultraviolet

List of symbols

α	alpha
β	beta
γ	gamma
ºC	degree Celecius
cm	centimetre
FPU/g	filter per unit/gram
g	gram
g/L	gram/litre
hrs	hours
kg	kilogram
kGy	kilogray
kHz	kilohertz
L	litre
%	percentage
nm	nanometre
µg	microgram
µg/mL	microgram/millilitre
µL	microlitre
µm	micrometre
mg	milligram
mg/g	milligram/gram
mg/mL	milligram/millilitre
min	minute
mL	millilitre
mm	millimetre
psi	pound-force per square inch
pH	potential of hydrogen

sec	second
U/g	unit/gram
v/v	volume/volume
W	Watt
wt	weight
w/v	weight/volume

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ABSTRACT

This study investigated the saccharification of delignified rice straw using recombinant cellulases to release reducing sugars. Compositional analysis of the native rice straw revealed presence of 35.5% cellulose, 20% hemicellulose, and 22.2% lignin. Acid and alkali pre-treatment were applied to non-grinded, grinded and trash rice straw. Non-grinded biomass had the highest cellulose content of 68%. Delignification and pre-treatment efficiencies were 94.6% and 97.3% respectively for non-grinded biomass. The enzymatic saccharification of pre-treated non-grinded rice straw using recombinant cellulases yielded 79 mg/g total reducing sugar using enzyme ratio of CtCel8A:CtCBH5A:HtBgI (250:20:30 U/g) after 12hrs at 50°C. This study demonstrates the potential of recombinant cellulases for efficient saccharification after delignification of pretreated rice straw as a valuable method for the production of fermentable sugar for sustainable production of bioethanol.

Keywords: Rice straw; cellulose; saccharification; reducing sugar.

Introduction

From ancient times, mankind has been dependent on plants for food, flavors, medicinal and other uses. And food crops are the crops whose final product used as food for human consumption and that is also the main source of energy for world's population. Now a days, there are total five major crops grown all over the world on which humans are dependent, including *Zea mays* (maize), *Oryza sativa* (rice), *Triticum* (wheat), *Manihot esculenta* (Cassava), *Solanum tuberosum* (potato).

1.1 Cereal grains

The cereal grains are the one of the important and major source of human food source and animal feed throughout the world. And the annual grain yields exceeding 2300 million tone all over the world. These crops are well adapted beyond the continents in a different environment. Starch is the major component of cereal grains **(Farooq *et al.*, 2021)**.

1.2 Rice cereal grain

Oryza sativa commonly known as "rice" is edible starchy cereal grain from family Poaceae, grown in warmer part of the world like all of the east and south Asia. Approx one-half of the world population, wholly dependent on this staple food **(Slayton *et al.*, 2008)**. Approximately 600 million tons are harvested annually worldwide **(Chen *et al.*, 2014)**. The milling of paddy rice has nearly a 70% yield and its major products. Although there are some unconsumed portions of the rice produced, for example rice husk, rice bran and rice germ. Most of the rice by-products, including rice husk and rice bran, are used as animal feeds **(Wilson *et al.*, 2002)**. Rice straw is residual by-product of rice production after harvesting. The major components of dietary fibre in rice by-products are cellulose, hemicellulose and lignin. The main component in crops straw is cellulose, which is a compact structure of hemicelluloses and in close association with lignin **(Sun & Sun, 2002)**. They can be classified into two different types, depending upon their solubility in water. The structural or matrix fibres, such as lignin, cellulose and some hemicelluloses, are insoluble and the minor components are the natural gel-forming fibres including pectin, gums, mucilage and the remainder of the hemicelluloses are soluble **(Mohd *et al.*, 2016)**. In many countries, straw is an abundant cellulosic by-product from crop production like wheat, rice, corn and soybean. Managing rice straw remains a challenge in Asia and countries too where the rice production is huge and hence, more straw is grown each year to meet rising demand.

1.3 Rice straw and it's management

The production of rice grain is expected to increase significantly in future with major demand from the Asian and African countries. Approx, 660 million tons of rice produced by paddy rice and also 800 million dry tons of agriculture residues, mainly the straw (**Domínguez-Escribá *et al.*, 2010)**. Augmentation of rice-cropping systems has been associated with the use of high-yielding and short-duration varieties with shorter turn-around time between crops in multi-cropping systems. Moreover, rapid introduction of combine harvesters constitutes a game changer because of the larger amounts of straw that are left spread out on the field.

And the manual collection of the straw in the field is expensive because of the high labor cost and incorporation in the soil poses challenges in intensive systems with two to three cropping rounds per year, due to the insufficient time for decomposition, leaving the straw with poor fertilization properties for the soil and hindering crop establishment. Therefore, open-field burning of straw has increased dramatically over the last decade, despite being banned in most rice growing countries because of pollution and the associated health issues. Therefore, it is important to look for sustainable solutions and technologies that can reduce the environmental footprint and add value by increasing the revenues of rice production systems **(Gummert *et al.*, 2020).** Two effective methods for hydrolysis of cellulosic material are acid hydrolysis and enzymatic hydrolysis, in between them enzymatic hydrolysis is more convenient method the reason is it has low environmental impact and the process can be done under mild conditions and also, acid hydrolysis reacts much faster than enzymatic saccharification but they generally produce inhibitors like acids, furan derivatives, phenolic compounds that needs detoxification before fermentation and limits enzyme saccharification **(Silva-Fernandes *et al.*, 2017).** Enzymatic saccharification, effective & green process to enhanced production of reducing sugars from polymeric sugars in lignocellulosic biomass **(Manisha and Yadav, 2017; Nizami *et al.*, 2017).** Cellulose, hemicellulose and lignin constitute about 85–90% of lignocellulosic biomass also organic extractives and inorganic minerals constitute the rest. Cellulose is the major structural polymer of a plant cell wall and generally exists as long thread like fibers known as microfibrils. It is a linear polysaccharide consisting of monomeric units of anhydro-d-glucose units with a β-(1 → 4) glycosidic linkages **(Mohan *et al.*, 2006, Pérez *et al.*, 2002).** The chemical composition and nature of the biomass polymers differ significantly with their respective biomass types. On a dry weight of lignocellulosic biomass, it contained 40–50% of cellulose, 25–35% of hemicellulose and 16–33% of lignin **(Mohan *et al.*, 2006).**

REVIEW OF LITRATURE

2.1 Composition of lignocellulosic biomass

Lignocellulosic biomass, mainly composed of cellulose, hemicelluloses and lignin, a complex assembly of these polymers naturally intransigent to enzymatic conversion **(Zhao *et al.*, 2012; Kumar and Sharma, 2017)**. The composition of the biomass is highly dependent on the plant varieties, sources, growth conditions, and many other factors **(Bhatia *et al.*, 2020).** The lignocellulosic biomass, mainly composed of 25–30% hemicellulose, 40–50% cellulose, 15–20% lignin, and traces of pectin, nitrogen compounds, and inorganic ingredients (figure 1). These polymers can be cross-linked to build a complex and recalcitrant polymer network that can naturally resist biological attack and provide support to plant **(Holwerda *et al.*, 2019; Lorenci Woiciechowski *et al.*, 2020).**

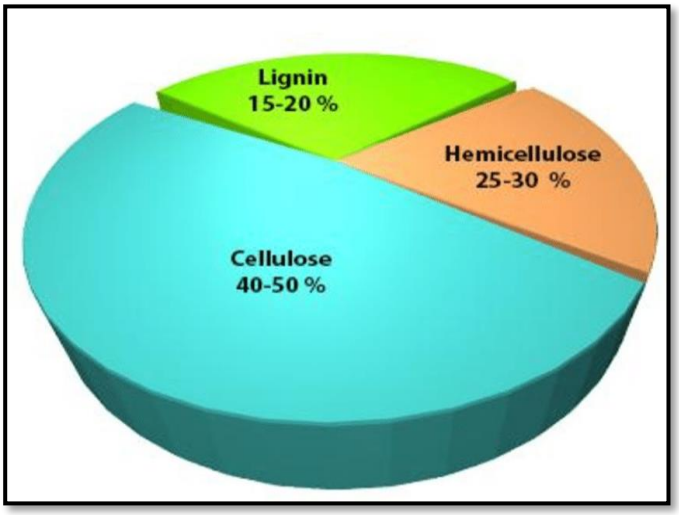

Figure 1: General composition of lignocellulosic biomass

2.1.1 Cellulose

Cellulose, the most abundant lignocellulosic biomass polymer, representing 40–60% in dry weight **(Sharma *et al.*, 2019)**, and it consists of ß-D-glucopyranose units linked via ß-(1,4) glycosidic bonds, with cellobiose as the fundamental repeating unit. Cellulose is more commonly considered as linear chain of several hundred to 10 thousand recurring D-glucose units, linked by ß,1-4 glycosidic linkages **(Delmer *et al.*, 1995; Zhang *et al.*, 2004).** Cellulose fibrils are embedded in a lignocellulosic matrix that makes it resistant from the enzymatic hydrolysis. It is assumed that long cellulose chains contain more hydrogen bonds and are difficult to hydrolyze, whereas shorter chains of cellulose contain a weaker hydrogen-bonding

system and therefore are believed to facilitate enzyme accessibility **(Hallac and Ragauskas, 2011; Meng *et al.*, 2017).**

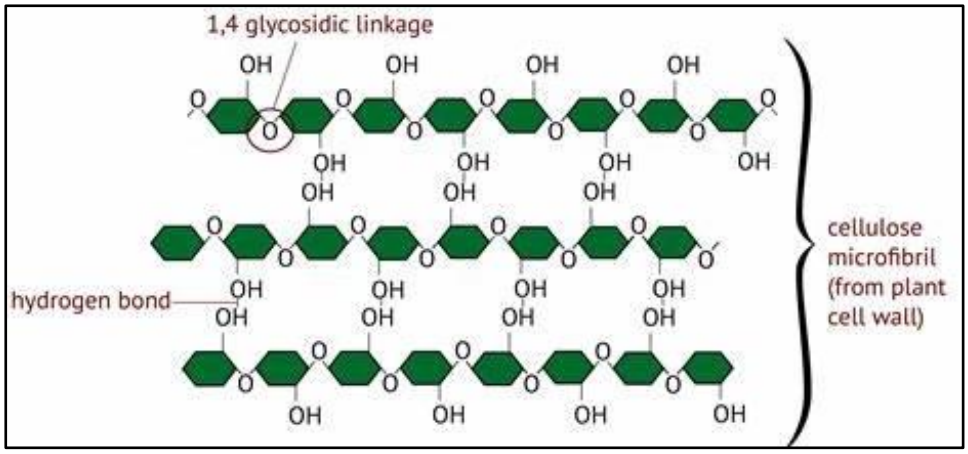

Figure 2: Structure of cellulose

2.1.2 Hemicellulose

The second most abundant natural polymeric carbohydrate on the earth is hemicellulose **(Hendriks *et al.*, 2009; Agbor *et al.*, 2011).** Hemicelluloses are heterogeneous groups of biopolymers, in plant cell walls that have ß-1,4 linked, copolymer of C5 and C6 sugar and it represents 20–35% of the biomass weight **(Chandel *et al.*, 2018).**

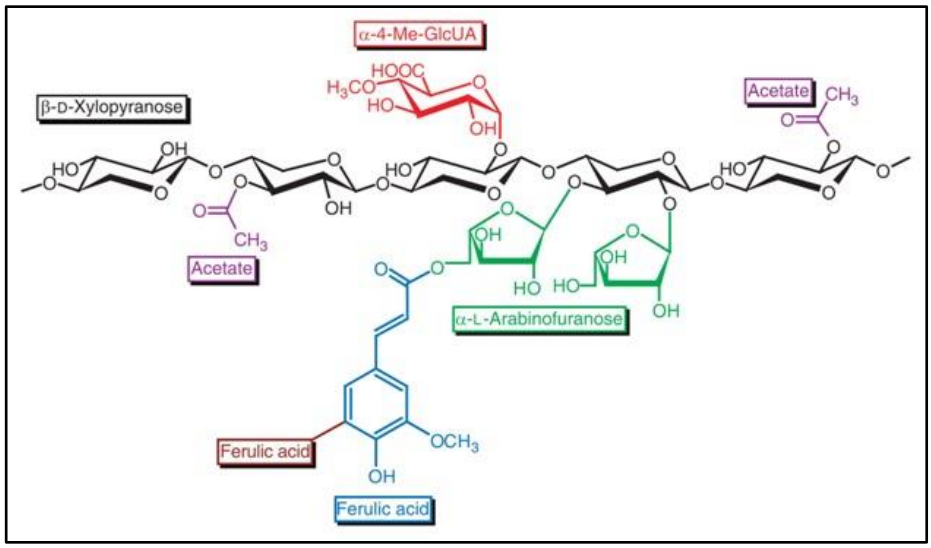

Figure 3: Structure of hemicellulose

It is a complex of polymeric carbohydrate like, xylan which is heteropolymer of D-xylose and D-glucose, glucomannan the heteropolymer of D-glucose and D-mannose and D-mannose and arabinogalactan the heteropolymer of D-galactose and arabinose **(Shallom *et al.*, 2003).** Hemicellulose is amorphous, with slightly physical strength. It is readily hydrolysed with the

help of diluted acids or bases, and also by hemicellulase enzymes **(Isikgor and Becer, 2015).** Hemicelluloses act as a physical barrier limiting the accessibility of enzymes. It has been reported that removal of hemicelluloses by dilute acid pre-treatment could increase cellulose conversion by improving the accessibility of enzymes to cellulose which effect cellulose hydrolysis **(Auxenfans *et al.*, 2017; Herbaut *et al.*, 2018; Santos *et al.*, 2018).**

2.1.3 Lignin

Lignin is the second most abundant polymer in lignocellulosic biomass after cellulose, represent 15–40% of dry weight of the lignocellulosic biomass **(Ragauskas *et al.*, 2014).** It is a very complex amorphous heterogeneous polymer of phenyl propane units linked with ether bonds **(*Agbor et al.*, 2011).** Lignin provides hydrophobicity and structural rigidity to the plant. It binds hemicelluloses to cellulose in the cell wall. And, it is already known that lignin limits the conversion of cellulose, influenced by several factors such as total lignin content, which is responsible to create barrier so that enzyme didn't comes in contact of cellulose **(Santos *et al.*, 2012).** Basically, lignin can physically limit polysaccharide accessibility because lignin plays a role as physical barrier which provide the plant resistance from disease but also it blocks the access of enzymes to cellulose. And, it can irreversibly adsorb cellulases and other enzymes during enzymatic hydrolysis due to its hydrophobic structural features and polyaromatic structures **(Kumar and Wyman, 2009; Zeng *et al.*, 2014)**. The removal of lignin which is known as pre-treatment of lignocellulosic biomass, disrupts the lignin-carbohydrates matrix and increases the porosity and reduces non-productive adsorption sites for enzymes which results in efficient hydrolysis **(Pihlajaniemi *et al.*, 2016; Kruyeniski *et al.*, 2019).**

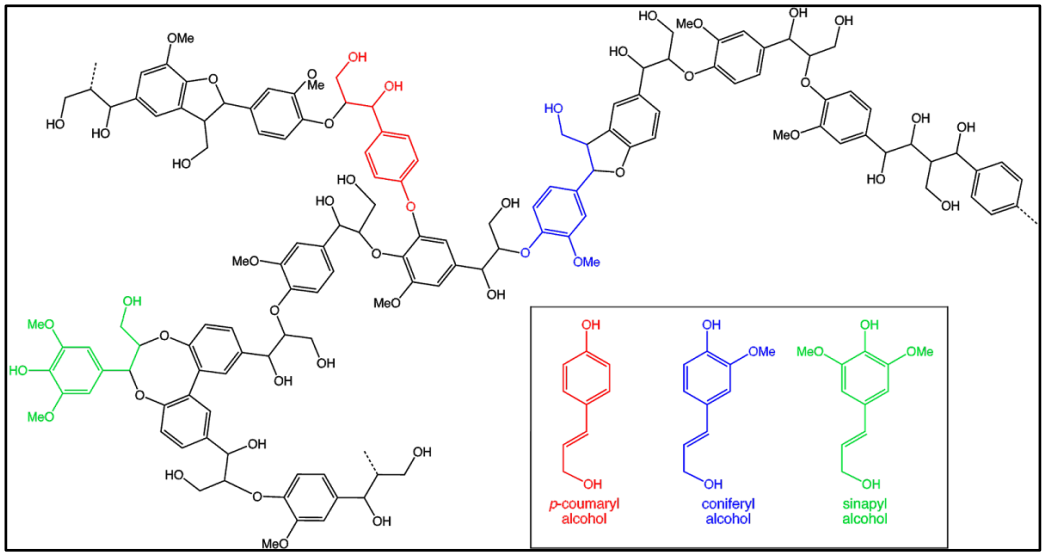

Figure 4: Structure of lignin

The composition of cellulose, hemicellulose and lignin varies in different lignocellulosic biomass (Table 1).

Table 1: Composition of lignocellulosic biomass

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Rice straw	38.02	18.30	21.60	Tsegaye *et al.*, 2019
Wheat straw	28	36	18	Akhtar and Goyal, 2014
Poplar wood	44	32	21	Meng *et al.*, 2012
Sugarcane bagasse	42	25	20	Kim and Day, 2011
Banana waste	13	15	14	Sanchez *et al.*, 2009
Corn strover	38	26	19	Zhu *et al.*, 2005
Hardwood	40	24	18	Malharbe and Cloete, 2002
Softwood	45	25	25	Malharbe and Cloete, 2002

2.2 Selection of feedstock

Khaire *et al.*, (2021) reported, the ceaseless exploitation of non-renewable energy resources and its continuous exhaustion is become a biggest concern in these times. The production of renewable fuels from agricultural waste is sustainable and also economically achievable. **Fahad *et al.*, (2019)** reported that, rice is being the second most consumed food, has an annual demand of approx 700 million Tons reported in 2018. And according to **FAO (2018),** rice is being grown globally with a gross cultivated area of nearly 160 million hectares and the production of around 760 million tonnes annually. **Gummert *et al.*, (2020)** reported rice straw is a residual by-product of rice production after harvesting. The total biomass of this residue depends on aspects such as varieties, soils and nutrient management and weather. At harvest,

rice straw is piled or spread all over the field depending on the harvesting methods used by farmers. The amount of rice straw taken off the field depends mainly on the cutting height. **Moraes *et al.*, (2014)**, in the industrial field each Ton of the processed rice paddy produces around 200 kg of rice husk, 140 kg of broken rice and 100 kg of rice bran. Annual agro wastes produce in India is shown in figure 5.

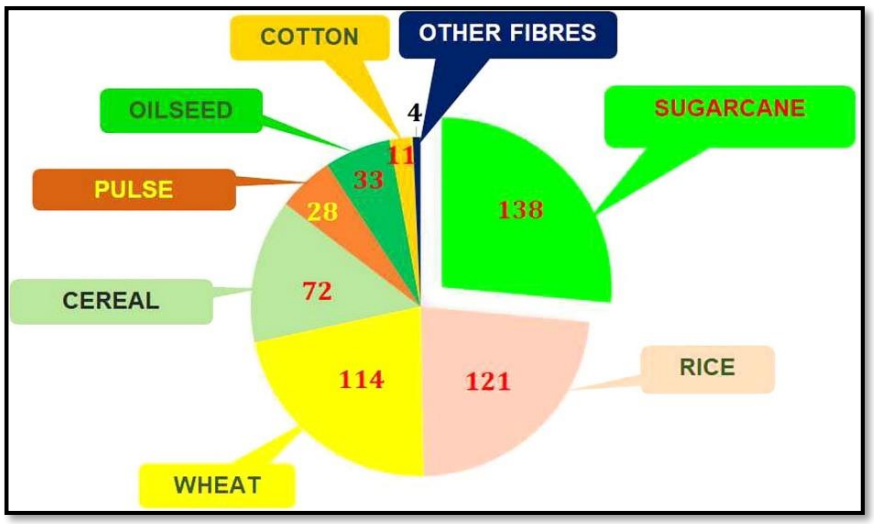

Figure 5: Annual agriculture waste production in India (in Million Tons)

(Report on State of Indian Agriculture 2015–16)

The rice straw left in the fields after harvesting can be collected, burned or mixed with the soil. According to a 2018 report by **Narayan *et al.*,** rice accounts for more than 11% of the world's agricultural land area and accounts for 10.1% of all agricultural greenhouse gas emissions. When considering the global anthropometric emissions, rice production accounts for 1.3% to 1.8% of the gross emissions. These emissions are generated by various aspects of the rice production process, including water management techniques, fertilization schemes, cultivation practices and post-harvest management of residue residues by precision field burning. **Arai *et al.*, (2015)** found that despite the known negative impacts on the environment, soil health and quality, most farmers prefer straw because of its lower cost, easier cultivation and fewer weeds in subsequent crops. Burning rice straw emits greenhouse gases (GHGs) such as CO_2, CH_4 and N_2O in addition to other rare gases. These gases are not only a potential source of global warming, but have been proven to adversely affect human health. **Singh *et al.*, (2021)** reported that open field burning is the most common alternative for agricultural residue straw management by farmers worldwide. Over the past decade, many countries have banned open burning, and governments have encouraged farmers to use fresh straw, store straw for use in electricity generation, and adopt alternatives such as composting or biochar.

2.3 Pre-treatment of lignocellulosic biomass

The reason for pre-treatment is that, lignocellulosic biomass has polymeric sugars in cellulose and hemicellulose which bound tightly with lignin complex that are recalcitrant enzyme hydrolysis **(Zhang *et al.*, 2017).** The enzymatic saccharification is a green process for converting of polymeric sugars to mono sugars **(Tan *et al.*, 2016).** But, the gap between these two steps is filled with extensive pre-treatment process **(Mupondwa *et al.*, 2017).** Pre-treatment method is one of the important steps to improve biodegradability by altering its composition and structure of lignocellulosic biomass. It can remove lignin and hemicellulose to a certain extent, which improve the efficiency of cellulose hydrolysis, and also to develop effective pre-treatment methods that can deconstruct the lignocellulose structure. These methods can be classified into mainly physical, chemical, biological and physicochemical methods, and they are often used in combinations to improve the efficacy **(Akhtar *et al.*, 2016).**

2.3.1 Physical pre-treatment method

The physical pre-treatment involves the utilization of mechanical equipment for the reduction the size of biomass particles and also, increase the biomass porosity and surface area through physical forces **(Kim *et al.*, 2016).** Compared with chemical or biological methods, the lignin structure is altered less during physical pre-treatment.

Milling

According to **Akhtar *et al.*, (2016)** that reducing the crystallinity, particle size and degree of polymerization can make lignocellulose more sensitive to cellulase. This involves crushing, grinding, grinding, or a combination thereof to a mesh size of 0.2mm to 2mm. (**Andersen *et al.*, 2022),** mechanical pre-treatment by hammer mill was chosen as the simplest option from the point of view of process operation and design. To achieve good yield, the straw particle size is reduced from approximately 20 cm in length to an average particle size of 1 mm. Strong mechanical pre-treatment of straws by hammer mill results in insufficient fiber degradation to achieve full biodegradability up to 75%. However, straw fermentation with this process is the most cost-effective due to low capital constraints. In addition, the heat provided by the Combined heat and power (CHP) units used here can fully cover the given heat demand.

Electron beam irradiation

Li *et al.*, (2020), showed that the enzymatic hydrolysis performance of rice bran fibers was improved by electron beam pre-treatment. In addition, compositional analysis, structural

changes, thermal stability and its lignocellulosic fraction were investigated in this study. Cellulose content increased significantly ($P < 0.05$) with increasing radiation dose, from 42.13% to 52.31%. On the contrary, this pre-treatment method decreased the hemicellulose content. The infrared spectrum showed the disorder in the crystalline regions of cellulose and the disorder in the bond between lignin and hemicellulose. In addition, thermal gravimetric analysis (TGA) showed a decrease in the thermal stability of cellulose, lignin and hemicellulose under irradiation compared to untreated samples. It is worth noting that the glucose efficiency reached 34.99% in the radiation dose of 250 kGy.

Mechanical extrusion

Zhang *at al.*, (2020) reported, rice hulls are a plentiful and renewable biomass resource that contains carbohydrates and can serve as an alternative energy source. To effectively utilize this biomass energy, researchers employed extrusion and ultrasound technologies for pre-treating the rice hulls. The extrusion conditions were optimized using the response surface method (RSM), resulting in the following optimal parameters: material diameter of 60 mesh, material moisture content of 29%, extrusion temperature of 143°C, and screw speed of 350 rpm. These conditions led to a rice hull expansion degree of 2.16. Subsequently, the extruded rice hulls underwent ultrasound pre-treatment for 1.5 hours at 40 kHz/500W. After this pre-treatment, cellulase, β-glucosidase, and hemicellulose enzymes were utilized to hydrolyze the rice hulls, resulting in a conversion of 77.50% of the total cellulose and hemicellulose. The yield of reducing sugars, glucose, and xylose obtained was 381.59, 291.59, and 88.87 mg/g of rice hull, respectively.

2.3.2 Chemical pre-treatment method

Acid

Taherdank *et al.*, (2015), treating wheat plants with H_2SO_4 1% (v/v) at 121°C for 120 min reduced Crystallinity index (CI) by 23%, 84% of xylan decomposition and 15% of lignin were removed.

To recover sugars from corn stover, a two-stage pre-treatment process involving dilute hydrochloric acid followed by aqueous ammonia wet oxidation was employed **(An *et al.*, 2019)**. In the first stage of the process, conducted at 120°C for 40 min with 1 wt% HCl, a remarkable 82.8% xylan recovery was achieved. The second stage, performed under relatively mild reaction conditions, 130°C, 12.6 wt% ammonium hydroxide, 40 min, resulted in the

removal of 86.1% lignin. Subsequently, enzymatic hydrolysis using a low enzyme dosage (3 FPU·g−1) yielded a cellulosic component conversion of 71.5%. The effectiveness of the process can be attributed to its efficient hydrolysis of hemicellulose in the first stage and remarkable removal of lignin in the second stage.

A new approach for enhancing the biorefinery process of waste wheat straw using a natural surfactant called humic acid in combination with mild sulphuric acid pre-treatment was denoted by **Tang *et al.*, (2021)**. The humic acid surfactant proved effective in removing up to 40.6% of lignin and up to 96.2% of hemicellulose during the mild acid pre-treatment. These modifications led to a significant increase in enzymatic hydrolysis efficiency, reaching as high as 92.9%.

Alkali

Dong *et al.*, (2020) a new pre-treatment using NaOH/urea solution under cold winter outdoor conditions developed to promote enzymatic saccharification and arsenic production from rice straw. The results showed that the conversion rate of RS reducing sugar reached 90.02% after this pre-treatment at outdoor freezing temperature. Chemical composition analysis showed that lignin removal was up to 62.74% with cellulose and hemicellulose losses of 0.56% and 18.87% after pre-treatment with 3%-6% NaOH/urea for 3 months at 100% solids loading.

Organosolv pre-treatment

The method was developed specifically for softwood as raw material and mainly used ethanol as solvent. The Organosolv process has several desirable requirements for efficient pre-treatment, such as component selection, highly digestible cellulose, low inhibitor efficiency, and non-corrosive. This method generally dissolves lignin and hemicellulose in the liquid phase, yielding a solid cellulose fraction of high purity **(Sun *et al.*, 2022).** Despite, there are still research gaps that need to be covered in order to realize the full potential of the organosolv concept. It is not well known that the cost of pre-treatment and its environmental footprint are still important factors and cost-effective alternative solvents should be explored **(Rabelo *et al.*, 2023).**

Lou *et al.*, (2022) studied, the effects of organosolvoglycerol pre-treatment on major chemical constituents of corn. The results showed that the remaining corn stock after 80 wt% glycerol pre-treatment 30min at 220°C, produced 75.97% glucose and 78.21% xylose after enzymatic hydrolysis, which was 3.39 times compared to untreated corn and a 6-fold increase.

Xu *et al.,* (2023) reported pre-treatment of softwood with new solvents. In this study, an organosol pre-treatment using three green solvents including γ-valerolactone, tetrahydrofuran and 2-methyltetrahydrofuran was performed on Mason pine and compared with conventional pre-treatment. It was compared with ethanol pre-treatment. The results showed that including γ-valerolactone pre-treatment performed best in lignin removal from masonpin and achieved a relatively high lignin removal rate of 87.2% at 150°C and 75 mM H_2SO_4. Similarly, the highest glucose yield of 76.8% was observed for enzymatic hydrolysis of including γ-valerolactone-pre-treated Mason pine at relatively low cellulase loading, 20 FPU/g glucan.

2.3.3 Biological pre-treatment method

Akhtar *et al.,* (2015) reported white, brown and soft rot fungi are mainly used for biological pre-treatment to release cellulose from hemicellulose and lignin matrices. Pre-treatment breaks down lignin and hemicellulose, but leaves the cellulose intact. Biological pre-treatment requires low energy and mild operating conditions. However, the rate of biological hydrolysis is usually low and requires long retention times.

Vasco-Correa *et al.,* (2019) reported, the fungal pre-treatment with white rot fungus *Celiporiopsis subvermispora* was carried out in non-sterile conditions on four different feeds: corn, miscanthus, softwood and hardwood following a sequential inoculation process using colonized raw material as inoculum. Fungal pre-treatment increased the enzymatic digestibility of unpasteurized hardwoods, conifers, and miscanthus after the first generation of the process sequence, resulting in 2-, 3-, and 4.5-fold increases in glucose yield and 9-, 7- and 10-fold increases in xylose yield. However, fungal pre-treatment of unsterilized raw materials for corn storage was unsuccessful during the process and for all 2nd and 3rd generation raw materials.

Chavan and Gaikwad (2021) found that, the laccase-mediated cleavage of lignocellulosic biomass produces phenolic compounds that normally inhibit the enzymatic hydrolysis of cellulose and greatly slow down the reaction rate. Pre-treatment is necessary to overcome inhibitory effects and maximize productivity. This study suggests the extraction of phenolic compounds in the pre-treatment stage to increase the yield of glucose in the enzymatic hydrolysis of Bambusa, a lignocellulosic biomass. In addition, recovery of phenolic compounds derived from lignin is also done by absorption and then excretion. In adsorbent preparation, chemically modified cenospheres of coal fly ash and chitosan were cross-linked to form a syntactic foam adsorbent. As a result, 40.31% exfoliation was obtained when the raw material with an average particle size of 60 μm was operated in a batch reactor. Extraction of

phenolic compounds significantly increased glucose yield, with isolated and extracted biomass yielding 1.4 times more glucose than isolated biomass alone.

2.3.4 Physicochemical pre-treatment method

Steam explosion

Steam explosion at 206 °C for 30 min and 18.5 bar pressure on rice straw, created a more porous structure and increased the surface area **(Sateinbach *et al.*, 2019)**. Increase of acid-insoluble lignin due to hemicellulose decomposition, increase of water-extractable components. Heat treatment without any added chemicals was defined as another option evaluated here, but this high temperature treatment is expected to have a relatively high energy demand. In addition, pressures above 20 bar can lead to high investment costs **(Andersen *et al.*, 2022)**. However, these high requirements for treatment reactor technology are compensated by the relatively small reactor volume due to the short residence time of 15 minutes, and the biodegradability of steam explosion pre-treatment has not been observed. Although the largest up to 93% in the literature, high energy demand and expensive high-pressure reactors are limiting factors for process implementation.

Ionic liquid-Ultra Sound (IL-US)

Ziaei-Rad *et al.*, (2023), evaluated the ability of IL-US pre-treatment in biomass dissolution, a combined analysis of pre-treated and untreated straws was performed. The chemical composition of wheat straw before pre-treatment was 35.7% glucan, 29.7% hemicellulose, 18.8% lignin and 6.1% ash, which was in accordance with other reported values. The remaining mass is probably related to lipids, proteins and other compounds. Among the different pre-treatment conditions, wheat straw pre-treated at 130°C for 30min with other pre-treated samples, at 130°C for 15min, pre-treated at room temperature for 15min and 30min were compared. It showed the highest dissolution efficiency. Excellent defoliation of up to 75%, 52.2% hemicellulose removal, and 51.0% increase in glucan content were achieved, while wheat straw pre-treated for 15 min was significantly superior to pre-treated wheat straw under the same conditions, compared to the removal of unprocessed hemicellulose and lignin.

Ultrasound

Gaudino *et al.,* (2022) studied, the superiority of the physical effect of ultrasonic pre-treatment in improving the delamination of wheat straw with a solid/liquid (biomass/water) ratio of 1:20, low frequency/high power (25 kHz) has been reported. The aim of this study was to demonstrate the effectiveness of this pre-treatment method for wheat straw separation from laboratory scale to semi-industrial scale (40 kg biomass) in terms of sugar yield and energy efficiency. After the cavitation treatment of wheat straw, the amount of lignin removal was 59.2% and the subsequent enzymatic hydrolysis efficiency was 68% ± 5.7%.

2.4 Key factors for an effective pre-treatment of lignocellulosic biomass

There are a number of key factors which are considered for the low-cost and advanced pre-treatment process:

2.4.1 High yields for multiple crops

Various pre-treatment processes have been shown to be better for specific feedstocks. For example, alkaline-based pre-treatment methods can effectively reduce the lignin content of agricultural residues but less effective towards processing of recalcitrant substrate such as softwoods. Acid based pre-treatment is the process which has been shown to be effective on a wide range of lignocellulose biomass, but are relatively expensive.

2.4.2 No significant sugars degradation

High yields of fermentable cellulosic and hemi-cellulosic sugars must be obtained through pre-treatment processes.

2.4.3 Minimum number of toxic compounds

The hydrolyzate obtained from the pre-treatment should be fermentable after a low-cost and high-efficiency preparation step. Harsh conditions during pre-treatment cause partial degradation of hemicellulose and the formation of toxic compounds from sugar breakdown that can affect the ongoing hydrolysis and fermentation steps. Toxic compounds are produced and their amount depends on the raw materials and intensity of pre-treatment. Degradation products resulting from pre-treatment of lignocellulosic materials can be classified into carboxylic acids, furan derivatives and phenolic compounds. The main furan derivatives are furfural and 5-

hydroxymethylfurfural, which are obtained from the decomposition of pentoses and hexoses, respectively. Weak acids are mainly acetic acid and formic acid. Phenolic compounds include alcohols, aldehydes, ketones and acids.

2.4.5 Size reduction not necessary

Grinding or grinding raw materials to small particle sizes before pre-treatment is an energy-intensive and costly technique.

2.4.6 Operation should be reasonable

Pre-treatment reactors must be cost-effective by minimizing volume, using construction materials suitable for highly corrosive chemical environments, and maintaining appropriate operating pressures.

2.4.7 Effective at low moisture content

Using raw materials with high dry matter content reduces energy consumption during pre-treatment.

2.4.8 Obtaining high reducing sugar yield

To ensure adequate ethanol concentration and controllable recovery and other downstream costs, the combined operation of pre-treatment and enzymatic hydrolysis should achieve a sugar concentration of 10% or higher.

2.4.9 Minimum heat and power consumption

The heat and power required for pre-treatment should be low and/or compatible with thermally integrated processes **(Alvira *et al.*, 2010).**

2.5 Enzymes involved in saccharification

The plant biomass is separated after pre-treatment and thus available for enzymatic saccharification. For the production of value-added products, the important components of pre-treated biomass are cellulose and hemicellulose, which can be hydrolyzed by cellulases and hemi-cellulases, respectively. Several microorganisms are involved in the abundant production of these enzymes **(Khaire *et al.*, 2021a)**. To increase the production of these enzymes, the genes responsible for these proteins are cloned and overexpressed in suitable vectors. In addition, techniques such as direct mutagenesis **(Escovar-Kousen *et al.*, 2004)** and protein

engineering are used to improve the catalytic efficiency of enzymes **(Cao *et al.*, 2015)**. Lateral mutagenesis next to the active site of endoglucanase (CtGH5) changes the amino acid phenylalanine to alanine, resulting in a 2-fold increase in endoglucanase activity. A chimera of β-glucosidase (CtGH1) and endoglucanase (CtGH5-F194A) enzymes was shown to increase endoglucanase and β-glucosidase activities **(Nath *et al.*, 2019).**

2.5.1 Endoglucanase

Endoglucanase (EC 3.2.1.4) acts arbitrarily against amorphous cellulase and produces cello-oligosaccharides of fluctuating degrees of polymerization. Endoglucanase is available in glycoside hydrolase (GH) families 5, 8, 9, 26, 44, 48, 74 and 124. Endoglucanase is not sufficient to produce glucose from cellulose and another type of cellulase enzyme is required to complete hydrolysis of cellulose **(Khaire *et al.*, 2021a)**. Endo-β-1,4-glucanase (CtCel8A) of family 8 glycoside hydrolase and β-1,4-glucosidase (CtBgl1A) of family 1 glycoside hydrolase from *Clostridium thermocellum* were transformed in *Escherichia coli* BL21 (DE3) cells **(Jamaldheen *et al.*, 2018).**

2.5.2 Exo-glucanases

Exo-glucanases (EC 3.2.1.91 and EC 3.2.1.176), also known as cellobiohydrolases from *Chaetomium thermophilum*, act on amorphous cellulose, crystalline cellulose and the oligosaccharides. Exo-glucanases that act on the reducing end are called cellobiohydrolase-I (EC 3.2.1.176) and are found in the glycoside hydrolase (GH) families 7 and 48. The cellobiohydrolase-II (EC 3.2.1.91) severs at the non-reducing end of cellulose and is found in GH families 5, 9 and 48. The main product released by both enzymes is cellobiose, which is further broken down into two glucose molecules **(Khaire *et al.*, 2021a)**.

2.5.3 β-Glucosidase

β-Glucosidase (EC 3.2.1.21) this enzyme acts mainly on cellobiose and cleaves glucose from the non-reducing end. β-glucosidases belong to GH families 1, 2, 3, 5, 9, 30 and 39. Cellulose cannot be hydrolyzed. Therefore, all three enzymes endoglucanase, cellobiohydrolase and β-glucosidase are necessary for complete hydrolysis of lignocellulosic biomass. β-glucosidase plays an important role synergistically with cellulases in converting cellulose to glucose monomer **(Khaire *et al.*, 2021a)**.

β-glucosidases [EC 3.2.1.21] have been reported in glycosyl hydrolase (GH) families 1, 3, 5, 9, 30, and 116. These enzymes play an important role in cell signalling, host-cell

communication, because they have hydrolytic properties and synthesis of glycosidic bonds. *Hungateiclostridium thermocellum* (formerly known as *Ruminiclostridium thermocellum* or *Clostridium thermocellum*) is a gram-positive, anaerobic, thermophilic bacterium. It has the ability to hydrolyze polysaccharides in plant cells and convert them into valuable biochemical products such as biofuels, oligosaccharides, acetic acid and biohydrogen. Therefore, different enzymes produced by this microorganism can be used in different biological industries. The catalytic module, a β-1,4-glucosidase belonging to glycoside hydrolase family 1 from *Hungateiclostridium thermocellum* (HtBgl), consists of 448 amino acid residues **(Sharma *et al.*, 2019).**

Enzymatic hydrolysis of lignocellulosic biomass has become the most important process for the conversion of biomass to monomeric sugars followed by fermentation into bioethanol. The biodegradation of carbohydrates in the biomass is done using various enzymes to convert the carbohydrates in the sample into their monomeric sugars. This sugar is then fermented into bioethanol. These enzymes work together to break down substrates in a coordinated fashion, meaning that the activities of the enzymes working together are greater than the sum of their activities. initially focused on the conversion of cellulose to sugar monomers, but now focuses on the use of hexoses and pentoses in fermentation as it increases theoretical results and can improve economic replication of the process **(Merino and Cherry, 2007).**

This relates to many aspects of the process, such as the type of pretreatment used and the enzymes required for hydrolysis. It requires a variety of enzymes with different properties to break down the total amount of lignocellulose.

Table 2: Enzymes required for hydrolysis of lignocellulosic biomass

Lignin	**Laccase and Lignin peroxidase**
Pectin	Pectin methyl esterase, pectate lyase, polygalacturonase, rhamnogalacturonan lyase
Hemicellulose	Endo-xylanase, acetyl xylan esterase, β-xylosidase, endo-mannanase, β-mannosidase, α-galactosidase, p-coumaric acid esterase
Cellulose	Cellobiohydrolase, endoglucanase, β-glucosidase

It is generally accepted that three types of enzymes are required for the hydrolysis of cellulose to sugar monomers: exo-1,4-β-glucanases, EC 3.2.1.91 and EC 3.2.1.176 (cellobiohydrolase), endo-1,4-β-glucanases, EC 3.2.1.4 and β-glucosidase, EC 3.2.1.21 (cellobiases) **(Van Dyk *et al.*, 2012)**. Cellobiohydrolases kill the ends of the cellulose chains, while endoglucanases remove the middle cellulose chains, reducing the degree of polymerization. Cellobiohydrolases can preferentially attack the reducing or non-reducing ends of cellulose chains **(Teeri, 1997)**. Due to the urgent need for alternative energy sources, there is interest in biocatalytic methods for advanced biofuel projects, but this is still a challenging target. The effectiveness of this biocatalytic method depends on the activity of different cellulases like endoglucanase (EG), cellobiohydrolase (CBH) and β-glucosidase (BG) involved in the saccharification of the lignocellulosic biomass (LBM). Hydrolysis is possible. These enzymes form the main product of cellulases, which are better produced by a type of bacteria called a base cellulase preparation. Depending on the type and amount of enzymes present in the enzyme preparation base, the addition of other microbial enzyme sources would be considered to improve it. The cellulase blend can be difficult to develop, but is essential for the proper and commercial conversion of LBM into oil or other valuable products **(Fernandes *et al.*, 2022)**.

2.6 Enzymatic Saccharification

Tsegaye *et al.*, (2019) showed that at the starting point, minimal sugar was released in all pretreated straws, as the bacteria adapted to the hydrolysis and prepared the enzymes. The amount of reducing sugars obtained increased with hydrolysis time, with maximum sugar release observed on day 13 for 3% treated straw and 7% on day 15 for treated straw. The maximum sugar released from polysaccharides was 0.753 g/100 ml for 3% pretreated rice straw and 0.741 g/100 ml for 7% pretreated rice straw. After 15 days of hydrolysis, it was observed that 27.88% of sugars were released from 3% pretreated rice straw and 88.40% from 7% pretreated rice straw on the 13th day. This is the maximum percentage released after simultaneous treatment of rice straw pretreated with xylan and cellulolytic bacteria (*Bacillus sp.* BMP01).

Enzymatic hydrolysis of raw sugarcane tops (SCT) using endoglucanase (CtCel8A), cellobiohydrolase (CtCBH5A), and β-glucosidase (HtBgl) yielded a total reducing sugar (TRS) yield of 2.83 mg/g crude SCT, while separated cellulose yielded a TRS of 188 mg/g separated cellulose. GOD-POD analysis of enzymatically hydrolyzed separated cellulose yielded

glucose, 115 mg/g separated cellulose **(Khaire *et al.,* 2021b)**. TRS (188 mg/g of separated cellulose) released from 1 g of separated cellulose gave a 21% yield of glucose determined by HPLC analysis, from the total 91% of glucose present in the separated cellulose.

Removal of water-soluble extracts before alkaline pre-treatment of SCT biomass increased the cellulose content in alkaline pre-treated sugarcane solids (apSCT), which shows superiority over other conventional pre-treatment methods **(Khaire *et al.,* 2021)**. Optimization of apSCT glycation based on the response surface method resulted in a yield of 265 mg/g TRS (214 mg/g glucose). This was in close agreement with the predicted TRS yield of 276.6 mg/g. Therefore, SCT can be used as a potential substrate for bioethanol production. Fermentation of glucose released by *S. cerevisiae* yielded 0.19 g per g of glucose, bioethanol with a fermentation efficiency of 37.7%.

MATERIAL AND METHODS

3.1 Collection and processing of rice straw

1. Rice straw was collected from nearby villages of Patiala, Punjab and stored in air tight containers at room temperature.
2. The biomass was grinded with the help of a mixer grinder.
3. After grinding, biomass sieved with a sieve having pore size of 0.5mm and the powder was collected.
4. The leftover remained on the sieve (trash) and was also collected and used for studies.

3.2 Pre-treatment of rice straw

3.2.1 Delignification (Khaire *et al.*, 2021b)

1. 10g of the powdered biomass (rice straw) was suspended in 200mL distilled water.
2. This was followed by addition of 4 g sodium chlorite ($NaClO_2$) and 0.5mL glacial acetic acid and mixed thoroughly.
3. The suspension was heated and stirred on a hot plate at 1200 rpm at 70°C for 3 hrs.
4. The hydrolysate was then removed by filtration through muslin cloth and biomass were neutralized by washing with distilled water.
5. The delignified biomass was dried at 80°C for 24 hrs.

3.2.2 Separation of cellulose from delignified rice straw (Khaire *et al.*, 2021b)

1. The alkaline separation method was used for the removal of hemicellulose which is required to obtain pure cellulose from rice straw.
2. Collected de-lignified rice straw was mixed with 70mL of 10% (w/v) of sodium hydroxide (NaOH) and 1% (w/v) of boric acid (H_3BO_3).
3. The suspension was stirred on a hot plate at 1200 rpm, 60°C for 3 hrs and filtered through muslin cloth.
4. The separated solid residues, cellulosic fibers were neutralized by washing under tap water and final washing with distilled water.
5. The separated cellulosic fibers were dried at 80°C for 24 hrs.
6. The dry weight of separated biomass was noted for determining the cellulosic yield.

3.3 Chemical analysis of biomass

3.3.1 Holocellulose estimation (Ioelovich, 2015)

1. From pre-treated extract of rice straw, 0.5g biomass sample was placed into a 50mL glass reagent bottle.
2. And then 40mL distilled water, 0.5g of sodium chlorite ($NaClO_2$) and glacial acetic acid 1mL added to the bottle and mixed thoroughly.
3. Covered the bottle with their respective caps was put into a water bath having temperature 90°C and treated for 45min, shaked manually at certain time intervals.
4. Then to the bottle an additional portion of 0.5g of sodium chlorite ($NaClO_2$) was added and the treatment was again continued for 45min.
5. After cooling it at room temperature, the dispersion of holocellulose was poured in a 50mL falcon tube and centrifuged at RCF of 5000g for 5min.
6. The sediment biomass of holocellulose was washed with hot water and finally washed with distilled water till pH reached to 7.
7. The washed holocellulose biomass was dried in a petri plate at 105°C till the weight was constant.
8. The percentage of holocellulose (HC) in the extracted biomass was calculated by:

$$\textbf{HC}\ (\%) = 100\%\ (P - P_t)/P_s$$

where, P = weight of dried holocellulose with petri dish;

P_t = weight of empty petri dish;

P_s = initial weight of extracted biomass.

3.3.2 Cellulose estimation (Ioelovich, 2015)

1. To find the content of cellulose in pre-treated rice straw, the acquired holocellulose was weighed and hydrolysed with acid for the removal of holocellulose.
2. The dried holocellulose biomass sample was mixed with 45mL of 2% (v/v) hydrochloric acid (HCl) in a 50mL glass reagent bottle.
3. The sample was treated with dilute acid at boiling temperature for 2hrs at hot plate.
4. After cooling it at room temperature, an acid dispersion was poured in a 50mL falcon tube and centrifuged at 5000g for 5min.
5. The sediment biomass of cellulose was washed with hot water followed by 1% sodium bicarbonate ($NaHCO_3$) and finally washed by distilled water till the pH reached to 7.
6. The washed biomass of cellulose was dried at 105°C till the weight was constant.

7. The percentage of cellulose (C) and hemicellulose (H) in the extracted biomass was calculated by:

$$\mathbf{C}\ (\%) = HC\ (P - P_t)/P_{HC}$$

$$\mathbf{H}\ (\%) = HC - C$$

where, HC = percentage of holocellulose;

P = weight of dried cellulose with petri dish;

P_t = weight of empty petri dish;

P_{HC} = dried weight of holocellulose.

3.3.3 Lignin estimation

1. To estimate the lignin content, 10mL of 72% (v/v) sulphuric acid (H_2SO_4) was added to 1g of dried pre-treated and raw biomass in a beaker, thoroughly mixed using a glass rod.
2. Beakers were kept in a water bath at 30°C for 2hrs and stirred after every 15-20 min.
3. After completion of 2hrs, beakers were removed and sample was diluted to 4% by adding 170mL of distilled water.
4. Then the preparations were then autoclaved at 121°C & 15psi for 1hrs. After cooling, the sample was filtered.
5. The residue and filtrate are both used for estimation for acid soluble lignin (ASL) and acid insoluble lignin (AIL), respectively.
6. For determination of acid in-soluble lignin, the residue was washed with hot distilled water and dried till the constant weight at 105°C and then weighed.
7. And for determination of acid soluble lignin, the filtrate optical density was measured at 320nm after diluted to bring the absorbance to 0.5-1.0 and dilution factor was recorded.
8. Sulphuric acid 4% (v/v) used as blank and also for dilution of samples.
9. The percentage of acid insoluble lignin (AIL) and acid soluble lignin (ASL) in the extracted biomass was calculated by:

$$\mathbf{AIL}\ (\%) = 100\%\ (P - P_t)/P_s$$

where, P = weight of dried acid insoluble lignin with petri dish;

P_t = weight of empty petri dish;

P_s = initial weight of extracted biomass.

$$\textbf{ASL}\ (\%) = \frac{\text{UV absorbance} \times \text{volume of filtrate} \times \text{DF}}{\varepsilon \times B \times a}$$

where, ε = extinction coefficient;

B = initial biomass (g);

a = length of the cuvette (1cm).

The efficiency percentage was calculated on the bases on compositional analysis,

1. For delignification efficiency (%): $\left(\frac{m_1 - m_2}{m_1}\right) \times 100$ **(Mukherjee *et al.*, 2018)**

   where, m_1 = lignin content in native rice straw;

   m_2 = lignin content in different forms of pre-treated rice straw.

2. For pre-treatment efficiency (%):

$$\left(1 - \frac{\text{mass ratio of } \frac{\text{lignin}}{\text{cellulose}} \text{ of CApRS}}{\text{mass ratio of } \frac{\text{lignin}}{\text{cellulose}} \text{ of RS}}\right) \times 100$$ **(Maibam & Goyal, 2022)**

3. Cellulose yield (%): $\left(\frac{\text{Dried weight of cellulose}}{\text{Total cellulose present in native RS}}\right) \times 100$ **(Khaire *et al.*, 2021b)**

3.4 Production of hydrolytic recombinant cellulase enzyme

3.4.1 Enzyme cocktails

Bacterial stains producing recombinant cellulases were procured from IIT Guwahati. The enzyme cocktails are designed to enhance the hydrolysis efficiencies in lignocellulosic biorefinery. Enzyme cocktail involved CtCel8A, endo-b-1,4-glucanase of family 8 glycoside hydrolase from *Clostridium thermocellum*. CtCBH 5A, cellobiohydrolase of family 5 glycoside hydrolase from *Chaetomium thermophilum*. HtBGI: ß-1,4-glucosidase of family 1 glycoside hydrolase from *Hungateiclostridium thermocellum.* CtCel8A, CtCBH5A and HtBgl were transformed in *Escherichia coli* cells, grown, expressed and extracted.

3.4.2 Media Preparation

1. Luria broth (LB) was prepared (20g/L) and was dispensed in glass tubes and flasks respectively. Then, the broth was autoclaved at 121°C, 15psi for 20min. After the autoclave process, they were used for inoculation only after 1-2 days, so as to ensure a contamination free status.

3.4.3 Primary culture

1. Took 6 LB tubes each contained 10mL media.
2. Added 10µL kanamycin from stock solution (stock-100mg/mL) in each tube and 100µL of culture from glycerol stock of CtCel8A, CtCBH5A and HtBGI in replicates, media only contained kanamycin served as blank.
3. Incubated at 37°C for 12-16hrs, in an incubator shaker.

3.4.4 Secondary culture

1. For preparation of secondary culture 100µL of culture from glycerol stock of CtCel8A, CtCBH5A and HtBGI in the same tubes again.
2. Again, incubated at 37°C for 12-16hrs, in an incubator shaker.
3. After incubation hours, took the absorbance at 600nm to check the growth.

3.4.5 Expression

1. Took 6 LB flasks each containing 150mL media.
2. Added 70µL kanamycin from the stock solution (1mg/mL) in each flask.
3. Took approx. 10mL fully grown secondary culture and added into the respective flask in replicates, media only containing kanamycin served as blank.
4. Incubated at 37°C for 4-5hrs till absorbance ranges from 0.8 to 1.0 at 600nm.
5. Added 100µL isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction of protein expression, then incubated the flask at 24°C for 16-18hrs in an incubator shaker.
6. After incubation hrs completed, centrifuged at 6000rpm for 10min.
7. Took the pallet and stored it at -20°C for further uses.

3.4.6 Extraction

1. For extraction of enzymes, the pellet was dissolved in phosphate buffer {pH 7.5} and vortexed.
2. Sonicated at 33% amplitude, 9sec on and 9sec off for 1hrs.
3. Then, centrifuged the mixture at 8000g for 10min at 4°C.
4. And the supernatant was collected, stored at -20°C for further used.
5. And the concentration and enzyme activity of enzyme solution was analysed.

3.5 Bradford Assay

1. Assay reagent: Dissolved 10 mg of Coomassie Blue G-250 in 5 mL of 95% ethanol. The solution was then mixed with 10 mL of 85% phosphoric acid and made up the volume to 500mL with distilled water. The reagent was filtered through Whatman No. 1 filter paper and then stored in an amber bottle at room temperature.
2. Protein standard: Bovine serum albumin (BSA) at a concentration of 1mg/mL (1000µg/mL for the micro-assay) in distilled water was used as a stock solution. This should be stored frozen in small aliquots at −20°C.
3. For sample:100µL of enzyme solution was taken to make up the volume to 500µL in duplicates.
4. Standard Assay Method:
   - i. Prepared the stock solution of BSA for the standard curve.
   - ii. For the standard curve, pipet volumes of 20µg/mL, 40µg/mL, 60µg/mL, 80µg/mL, 100µg/mL, 120µg/mL and 150µg/mL from 1mg/mL BSA standard solution into test tubes, and made up the volume to 500µL with distilled water.
   - iii. For sample analysis, 100µL crude enzyme solution was taken and made up the volume 500µL with distilled water.
   - iv. Pipetted 500µL of distilled water into a further tube to provide the reagent blank.
   - v. Added 1 mL of the assay reagent (Bradford reagent) to each tube of standard and sample, the tubes were gently vortexed. Foaming was avoided, which leads to poor reproducibility.
   - vi. Measure the Absorbance at 595nm of the sample and the protein standards against the reagent blank after 10-15min.
   - vii. The absorbance was noted and a graph was prepared, absorbance vs concentration.
   - viii. The crude enzyme glucose concentration was determined by the equation generated by the standard graph.

3.6 Enzyme activity determination of CtCel8A (endoglucanase) and CtCBH5A (cellobiohydrolase) enzyme

1. 10µL, 20µL and 30µL of crude enzyme solution was taken from CtCel8A and CtCBH5A in eppendorf.
2. Added 100µL of 1% CMC in CtCel8A and CtCBH5A as substrate and only substrate was served as blank.

3. Incubated the solution in a water bath, for CtCel8A the incubation condition was 70°C for 1min and for CtCBH5A the incubation condition was 60°C for 2min.
4. Then the sample was cooled down, the enzyme activity was calculated by determining the released total reducing sugar concentration by the DNS Assay method.

3.7 Determination of total reducing sugar (TRS) by Dinitro salicylic acid assay (DNSA)

1. Assay reagent: Dissolve 30g of Potassium sodium tartrate tetrahydrate in 20mL distilled water. Prepared 2N sodium hydroxide solution 20mL. Dissolved 1g of 3,5-dinitrosalicylic acid in 50mL of distilled water while the solution was mixed by magnetic stirrer with hot plate at 90-95°C. Added gradually the solution of potassium sodium tartrate tetrahydrate prepared in previous step to 3,5-dinitrosalicylic acid solution while the solution was mixed by magnetic stirrer with hot plate at 90-95°C. Added slowly, 2N sodium hydroxide solution prepared earlier to the same solution of 3,5-dinitrosalicylic again. After all the components were completely dissolved, filtered the final solution by filter paper. Transferred the solution in a clear amber bottle and stored at ambient temperature.
2. Substrate preparation: 1% of carboxymethylcellulose (CMC) and 1% of cellobiose in sodium phosphate buffer.
3. Assay method:
   i. Prepared the stock solution of glucose for the standard curve.
   ii. For the standard curve, pipet volumes of 0.1mg/mL, 0.3mg/mL, 0.5mg/mL, 0.7mg/mL, 1mg/mL, 2mg/mL and 3mg/mL of glucose was added in fresh tubes and made up to 100µL with distilled water.
   iii. Only distilled water was served as blank.
   iv. And for sample analysis, after incubation time completed, took 100µL sample (from step 3.7) from eppendorf transferred to fresh tubes and only substrate was served as blank.
   v. Added 100µL of DNSA in all tubes including the blank and boiled it in a water bath for 10min.
   vi. Note the absorbance at 540nm against blank.

3.8 Enzyme activity determination of recombinant hydrolytic HtBgI (β-glucosidase) enzyme

1. 10µL, 20µL and 30µL of crude enzyme solution was taken from HtBgI in eppendorf.
2. Added 100µL of 5mM p-nitrophenyl in HtBGI as substrate and only substrate was served as blank.
3. Incubated the solution in a water bath at 65°C for 5min.
4. Boiled for 10min in a water bath to stop the reaction.
5. Then the sample was cooled down, the enzyme activity was calculated by determining the released total reducing sugar concentration by Nelson and Somogyi method.

3.9 Determination of reducing sugar using Nelson-Somogyi method

1. Nelson's reagent A: Dissolved the 2.5g of Na_2CO_3 (anhydrous), 2.5g potassium sodium tartrate, 2g sodium bicarbonate and 2g sodium sulphate (anhydrous) in 70mL water and made up the volume up to 100mL. The sulphate should be added in small amounts.
2. Nelson's reagent B: Dissolved 7.5g $CuSO_4.5H_2O$ in 50ml water and added 1 drop of conc. H_2SO_4.
3. Nelson's reagent C: 25mL of Nelson's A and 1mL of Nelson's B mixed together. Prepared fresh every day.
4. Nelson's color reagent (Arseno-molybdate reagent): Dissolved 5g ammonium molybdate in 80mL water and added 4.2mL of Conc.H_2SO_4. Dissolved 0.6g sodium arsenate in 5mL water and added to above acid molybdate. Make it up to 100mL and store it in a brown bottle for 24hrs at 37°C. Reagent should be yellow without green tinge.
5. Glucose stock solution (1mg/mL): Dissolved the 10mg of glucose in 10mL distilled water in the beaker.
6. Assay method:
   i. Prepared the stock solution of glucose for the standard curve.
   ii. For the standard curve, pipet volumes of 20µg/mL, 40µg/mL, 60µg/mL, 80µg/mL and 100µg/mL of glucose were added in fresh tubes and made up to 1 mL with distilled water.
   iii. Only distilled water was served as blank.
   iv. For samples, after incubation time completed, took 100µL sample (from step 3.8) from eppendorf transferred to fresh tubes, only the substrate was severed as blank.

v. Added 1ml of Nelson's reagent C in all tubes boiled all the tubes for 20min at a hot plate.

vi. Cooled down the solutions and added 1mL of Nelson's color reagent D in each tube, vortexed.

vii. Noted the absorbance at 540nm against blanks.

3.10 Enzymatic saccharification of biomass

1. The enzymatic saccharification was done on pre-treated grinded and pre-treated non-grinded biomass of rice straw.
2. The hydrolysis of pre-treated grinded and pre-treated non-grinded was carried out by taking 2% (w/v) biomass in a 5mL 50mM sodium phosphate buffer of pH 7.5 in a 15mL centrifuge tube. The experiment was performed in triplicate sets.
3. The recombinant enzymes, endoglucanase (CtCel8A), cellobiohydrolase (CtCBH5A) and β-glucosidase (HtBgl) in different combinations were tested.
4. Total six combinations were tested (300 U/g) on the bases of their enzyme activity.
5. The combinations of recombinant enzymes, CtCel8A, CtCBH5A and HtBgl were 250:20:30 U/g, 230:30:40 U/g, 200:50:50 U/g, 200:40:60 U/g, 210:50:40 U/g and 250:30:20 U/g.
6. The combination was added to each of the reaction tubes and followed by Sodium azide 0.03% (w/v) was added to each reaction mixture containing tube to prevent the contamination.
7. The reaction mixture was incubated at 50°C in a shaker incubator at 180rpm.
8. And total reducing sugar was tested at 12hrs interval till 3 days.
9. The reaction was stopped by boiling the reaction mixture for 5min in a boiling water bath and then centrifuged at 10,000 g at 25°C for 5min, to collect the supernatant containing released sugar.
10. TRS released was estimated by the method of DNS assay and the saccharification efficiency **(Maibam & Goyal, 2022)** was calculated by using formula:

$$\text{Saccharification efficiency (\%)} = \left(\frac{\text{TRS after enzymatic saccharification}}{\frac{1.111 \times C \times \text{Biomass}}{\text{Volume}}} \right) \times 100$$

where, TRS is total reducing sugar (g/L) release after saccharification;

1.111 = factor for conversion of cellulose to its equivalent glucose;

C = Cellulose content in the RS (g/g_{RS});

Biomass = Weight of dried RS used in enzymatic hydrolysis (g);

Volume = Working volume of the saccharification process in (L).

RESULTS AND DISCUSSION

4.1 Processing of rice straw

The rice straw was chopped with the help of a mixer grinder and sieved. The powder of size 0.5mm was collected referred to as grinded biomass. The leftover remains after sieving process particle size more than 0.5 mm, called as trash. And the native rice straw was referred as non-grinded rice straw. These forms of rice straw, named as grinded (particle size >0.5mm), trash (particle size <0.5mm) and non-grinded (native) rice straw was used in further studies.

Figure 6: A. Rice straw, **B.** Without chopping rice straw (non-grinded or native)

C. Rice straw after grinding and sieving (Grinded, particle size <0.5mm) **D.** The leftover part of rice straw (Trash, particle size >0.5mm)

4.2 Pretreatment of rice straw

Rice straw was pre-treated as per optimized protocol already established by the previous researcher (as discussed in 3.2). The objective of pre-treatment was to separate the three main constituents of lignocellulosic biomass that is cellulose, hemicellulose and lignin and convert

to these fractions into value-added products **(Singh *et al.*, 2021)**. Delignification process was performed by acid chlorite pre-treatment, using a mixed solution of acetic acid and sodium chlorite which is milder and is considered to be one of the most well-known methods of lignin removal. Acidic solutions of sodium chlorite rapidly decompose into highly reactive chloride anions (ClO2-) and chloride anions (Cl-) and selectively remove lignin. The cellulose degradation during acid chlorite pre-treatment may have two possibilities, including acid cleavage of glycosidic bonds or oxidative degradation of polysaccharides **(Nan *et al.*, 2018)**. The first acid treatment of 10g of rice straw gave and 6.2g of dried biomass in grinded RS, 6.8g of dried biomass in trash RS and in non-grinded RS 6.9g dried biomass was recovered. Recovery of delignified biomass more than 60% from processing 10g of rice straw and highest was in non-grinded biomass. (Table 3, Figure 7).

Table 3: Delignified biomass recovered after acid pre-treatment of 10 gm of rice straw

Rice straw	Biomass recovered (g)	Biomass recovered (%)
Grinded (Particle size >0.5mm)	6.20 ±0.02	62
Trash (Particle size <0.5mm)	6.86 ±0.02	68.6
Non-grinded (native)	6.92 ±0.01	69.2

Figure 7: Rice straw after acid treatment process (delignification)

After delignification, the dried biomass was subjected to aqueous alkali pre-treatment for cellulose separation. The alkaline separation method used for the removal of hemicellulose is

required to achieve pure cellulose from rice straw. The process was performed using boric acid and sodium hydroxide, where boric acid acts as a monobasic acid that removes OH groups and causes separation of xylan from cellulose **(Khaire *et al.*, 2021)** and sodium hydroxide can selectively dissolve hemicellulose, from lignocellulosic biomass. Removal of hemicellulose reduces the complexity of biomass and facilitates the enzymatic hydrolysis of cellulose. Alkaline reagents selectively dissolve the ester bonds between hemicellulose and lignin, leaving the cellulose largely unchanged **(Farhat *et al.*, 2017).** So, the NaOH and H_3BO_3 treatment gave 34mL of supernatant and 3.1g of dry biomass in grinded RS, 27.1mL of supernatant and 3.1g of dry biomass in trash RS and 55mL supernatant and 2.7g dry biomass in non-grinded RS (Table 4, Figure 8). 50% cellulose rich biomass was recovered in case of non-grinded rice straw.

Table 4: Cellulose rich biomass recovered after alkali pre-treatment of delignified rice straw

Rice straw	Biomass recovered (g)	Biomass recovered (%)
Grinded (Particle size >0.5mm)	3.12 ±0.14	50.3
Trash (Particle size <0.5mm)	3.14 ±0.037	45.8
Non-grinded (native)	2.76 ±0.040	40

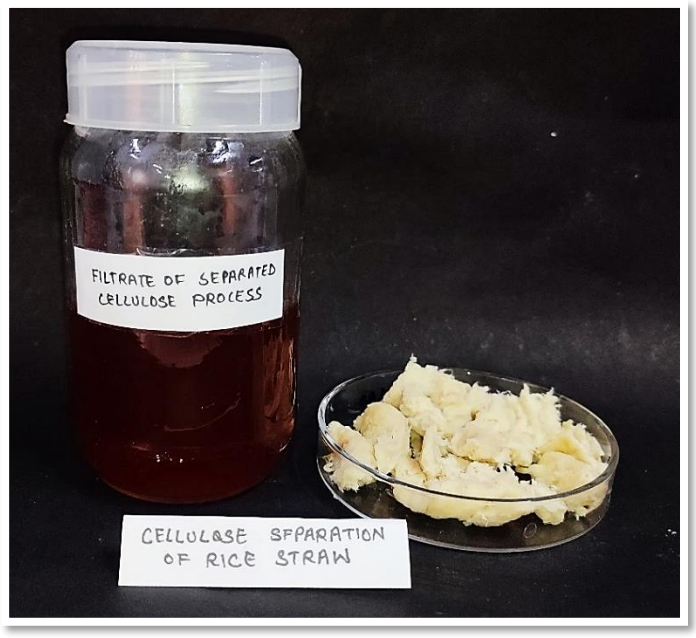

Figure 8: Rice straw after alkali treatment process (cellulose separation)

4.3 Chemical analysis of biomass

The lignocellulosic biomass, mainly composed of 18.30% hemicellulose, 38.02% cellulose, 21.6% lignin, 8.46% moisture and traces of pectin, nitrogen compounds, and inorganic ingredients. These polymers can be cross-linked to build a complex and recalcitrant polymer network that can naturally resist biological attack and provide support to plants **(Tsegaye *et al.*, 2019).** Rice straw chemical analysis was done as per optimized protocol already established by the previous researcher (discussed in 3.3) and percentage was calculated by the formula as mentioned in protocol. Using the proposed DE methods for estimation of AIL, cellulose and hemicellulose, furthermore conventional NREL methods for determination of ASL. Full analysis of chemical composition of the investigated rice straw biomass was performed and mentioned (Table 5 and Figure 9, 10, 11 and 12).

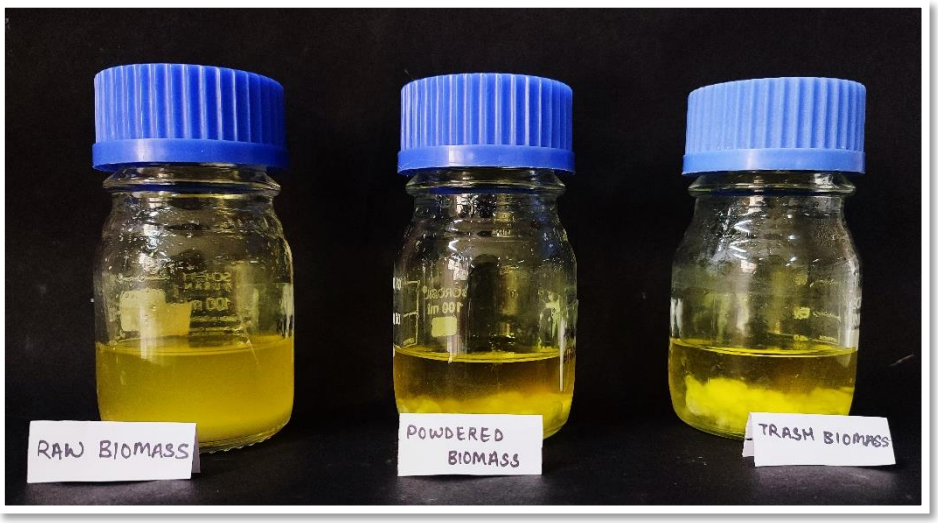

Figure 9: Analysis of hemicellulose in rice straw

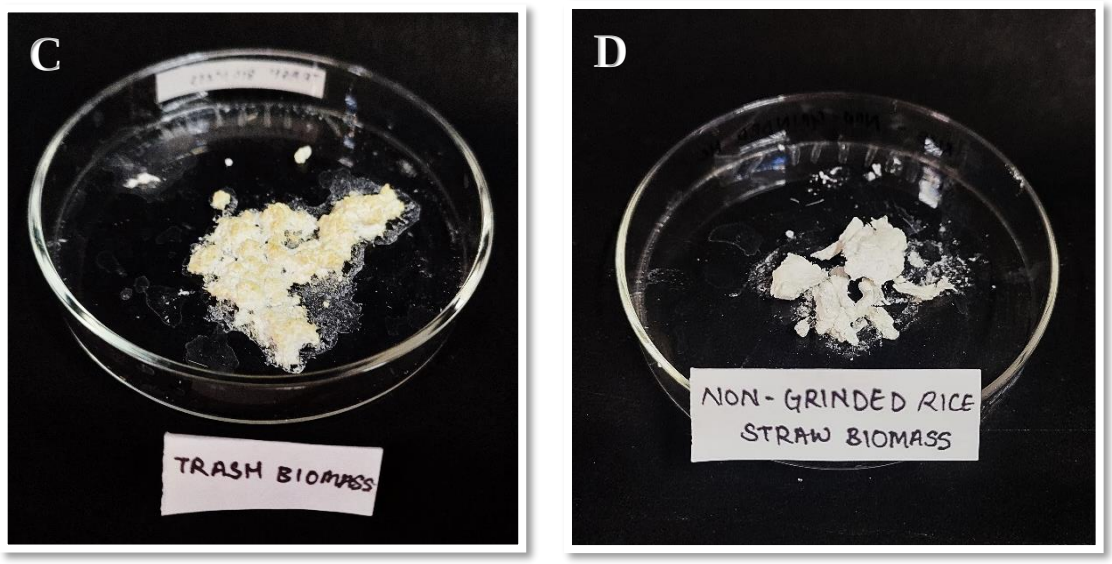

Figure 10: Analysis of cellulose in rice straw **A.** Raw (native) rice straw, **B.** Grinded straw **C.** Trash straw **D.** Non-grinded straw

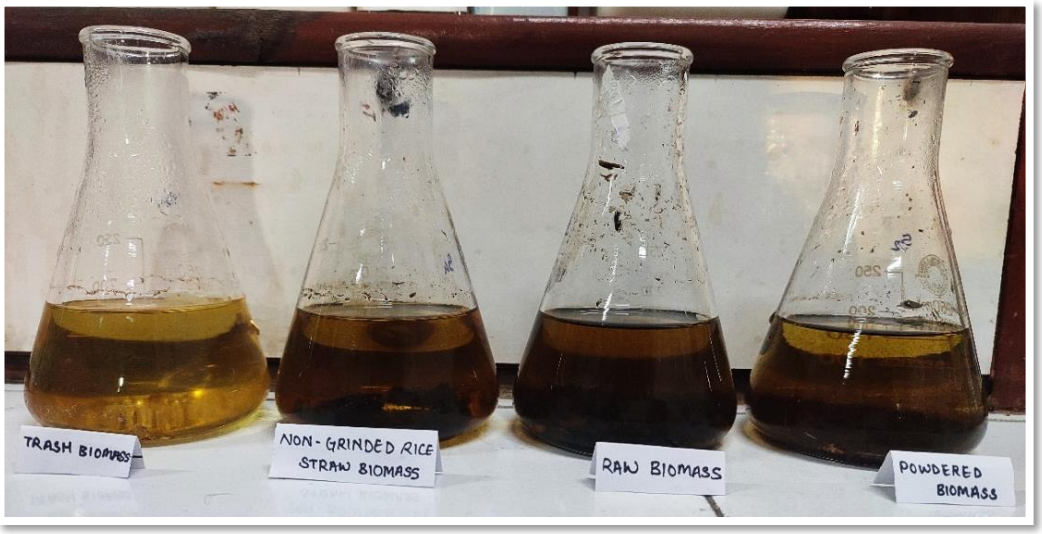

Figure 11: Analysis of lignin in rice straw

Table 5: Compositional analysis of native and pre-treated rice straw

Rice straw	Holo-cellulose (%)	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)
Native	55.5	35.5	20	22.26
Pre-treated grinded (Particle size >0.5mm)	77	54.4	22.6	7.3
Pre-treated Trash (Particle size <0.5mm)	80.8	62.55	18.24	3.24
Pre-treated Non-grinded	82.4	68	16.33	1.2

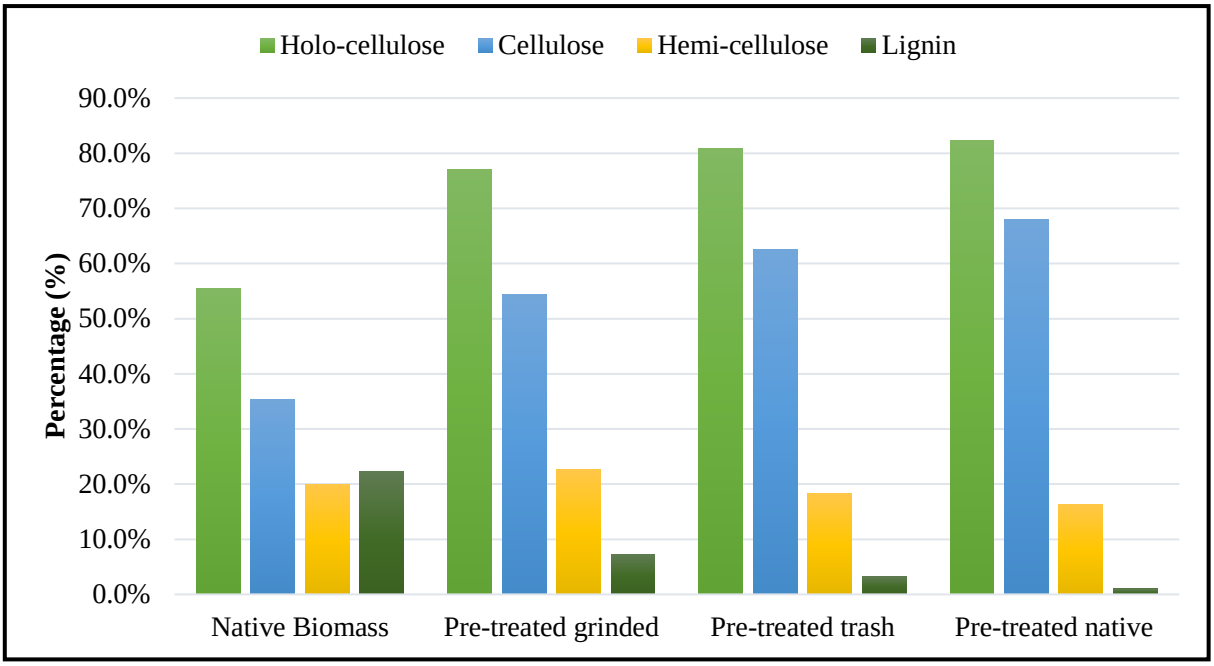

Figure 12: Percentage of cellulose, hemicellulose and lignin content in rice straw

After the pre-treatment using the method of acid & alkali treatment and chemical analysis of rice straw, particle size reduction by grinding and sieving lead to lowering of yield of cellulose. It was found, without grinding the biomass the cellulosic content is higher as compared to after grinding & sieving. So, for large scale production of value-added products from lignocellulosic biomass only particle size can be reduced by mechanical methods but no sieving is required. It is necessary and important to reduce one step of processing and also for higher yield of the final substrate for the biorefining process.

Table 6: Percentage of delignification efficiency, pre-treatment efficiency and cellulose yield

Rice straw	Delignification efficiency (%)	Pre-treatment efficiency (%)	Cellulose yield (%)
Grinded (Particle size >0.5mm)	67.2	78.6	90
Trash (Particle size <0.5mm)	85.4	91.7	-
Non-grinded (native)	94.6	97.3	-

4.4 Production of recombinant hydrolytic cellulase enzyme

Enzyme cocktail involved CtCel8A, endo-b-1,4-glucanase of family 8 glycoside hydrolase from Clostridium thermocellum. HtBGI: ß-1,4-glucosidase of family 1 glycoside hydrolase from Hungateiclostridium thermocellum **(Jamaldheen *et al.*, 2018) (Sharma *et al.*, 2019).** CtCBH5A, cellobiohydrolase of family 5 glycoside hydrolase from Chaetomium thermophilum. CtCel8A, CtCBH5A and HtBgl were transformed into *Escherichia coli* cells. In production, the aminoglycoside antibiotic kanamycin can inhibit E. coli peptide synthesis by blocking the translocation process **(Liu *et al.*, 2020)**, and IPTG plays an important role in the induction process. This compound is a molecular mimic of allolactose, a lactose metabolite that induces transcription of the lac operon and is therefore used to induce the expression of E. coli proteins whose genes are under the control of the lac operon. Like allolactose, IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby enabling the transcription of genes in the lac operon. The growth of culture can be compared with blank which contains only medium along with antibiotic and IPTG (Figure 13).

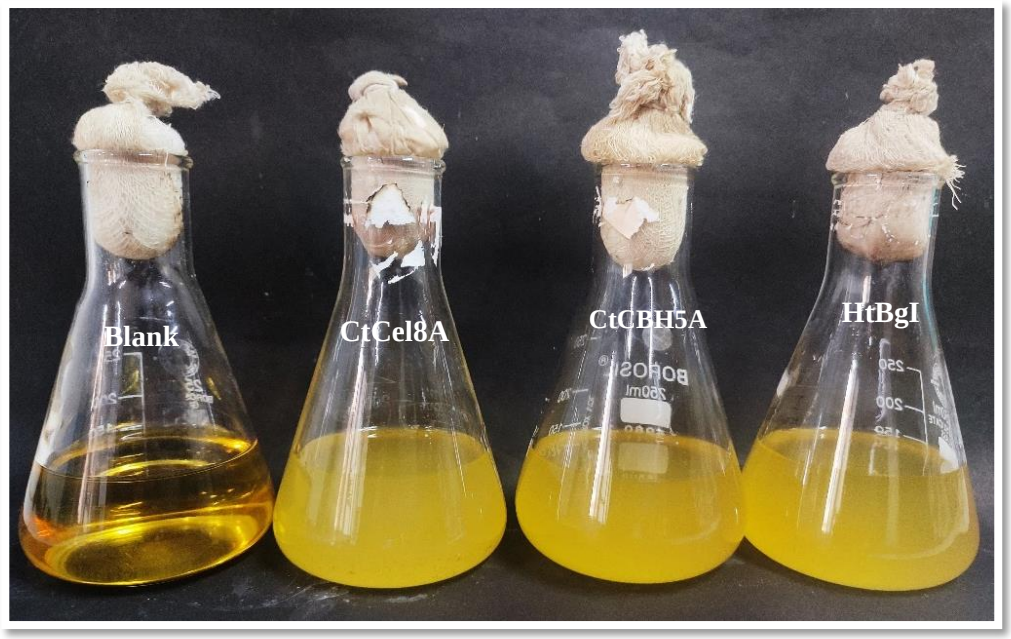

Figure 13: *E. coli* culture after growth

4.5 Enzyme protein concentration analysis

Protein concentration was determined by Bradford method **(Bradford, 1976)**. The Bradford method is a fast and relatively sensitive method for measuring protein concentration. This is

based on the shift in maximum absorbance of Coomassie Brilliant Blue G-250 dye from 465 to 595 nm upon binding to denatured proteins in solution **(Kielkopf *et al.*, 2020)**. The standard graph was obtained by using BSA of conc. 1mg/mL (Table 7 and Figure 14, Table 8 and 15).

Table 7: Absorbance for standard curve of Bovine Serum Albumin (BSA)

Bovine Serum Albumin (BSA) (µg/mL)	**Absorbance (595nm)**
20	0.0851 ±0.03
40	0.2083 ±0.0008
60	0.359 ±0.10
80	0.4731 ±0.006
100	0.5231 ±0.09
120	0.6537 ±0.003
150	0.7109 ±0.23

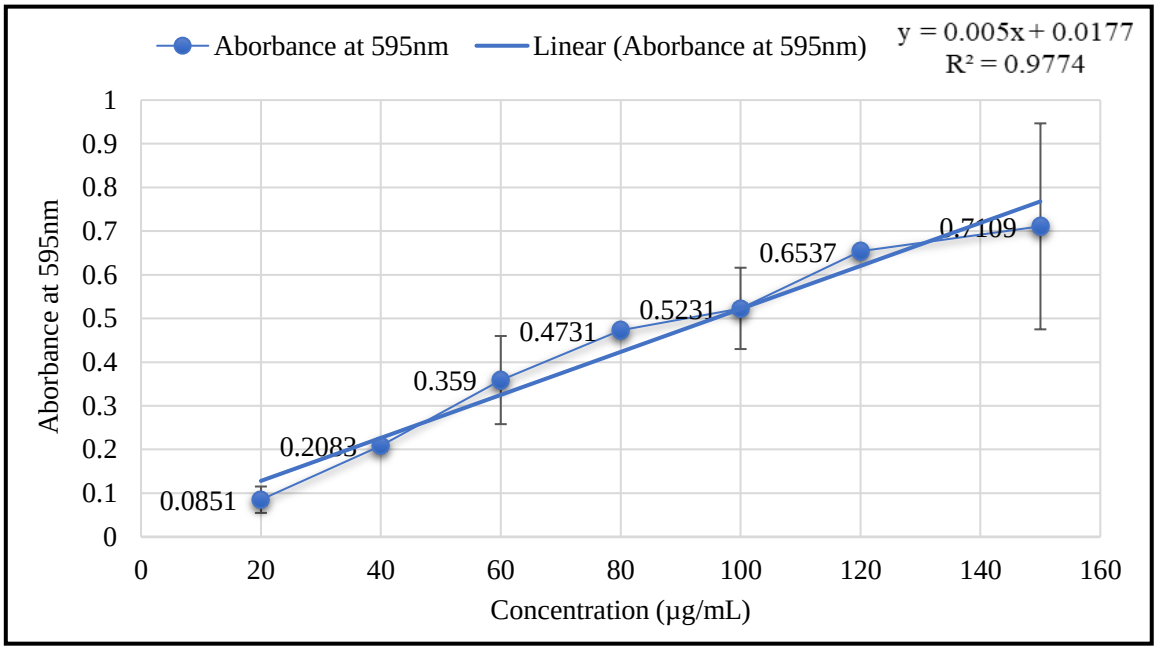

Figure 14: Standard curve of Bovine Serum Albumin (BSA) by Bradford method

The protein concentration of enzymes was determined by the using equation of BSA standard graph obtain above.

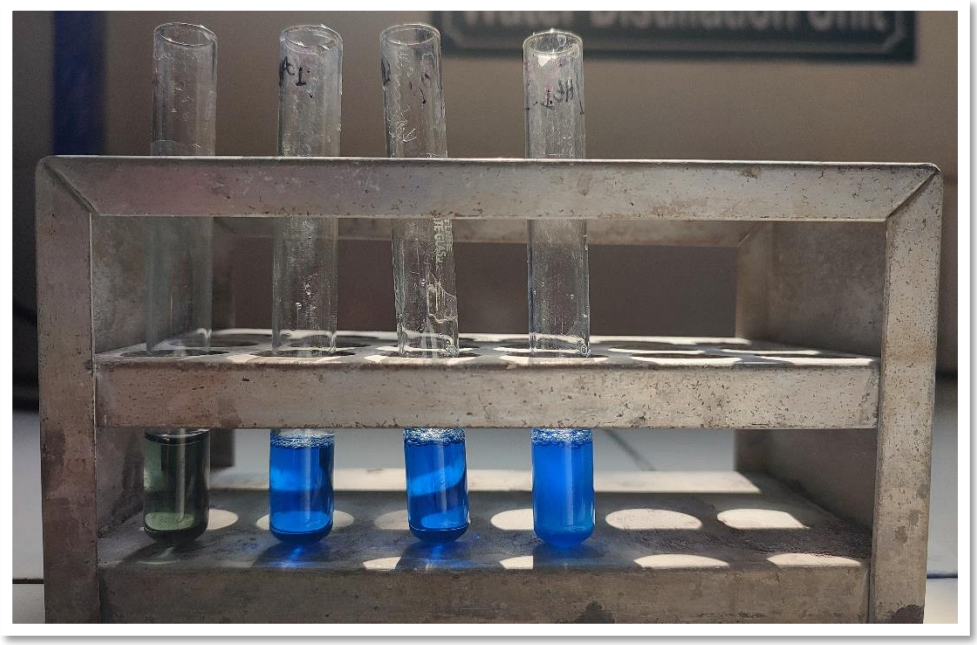

Figure 15: Colour appeared in protein concentration estimation of recombinant enzymes

Table 8: Protein concentration of recombinant enzymes

Recombinant enzymes	Absorbance (595nm)	Enzyme Concentration (mg/mL)
CtCel 8A	1.450	1.432 ±0.007
CtCBH 5A	1.533	1.515 ±0.0009
HtBGI	1.779	1.762 ±0.099

4.6 Determination of enzymatic activity recombinant hydrolytic enzyme

Enzymes have additional chemical moiety called cofactors which directly involved in catalysis. Cofactors or coenzymes are organic molecules such as vitamins or inorganic metal ions. When cofactors are tightly bound, they are called prosthetic groups. It prevents the natural breakdown of complex molecules such as proteins and nucleic acids and is essential for sustaining life. However, if metabolic changes in the cell are desired, some of these complex molecules must be broken down to overcome this energy barrier. Heat can provide the additional required energy called activation energy. This is the role of enzymes. These react with substrates to form

intermediate complexes. This reduces the energy required for the reaction to proceed. Sensitive intermediates are rapidly degraded to form reaction products, and the enzyme is free to react with other substrate molecules unchanged. Only a specific region of the enzyme called the active site binds to the substrate. The active site is a groove or pocket formed by the folding pattern of the protein. This three-dimensional structure, together with the chemical and electrical properties of the amino acids and cofactors in the active site, allows only certain substrates to bind to that site, thus determining the specificity of the enzyme.

The enzyme activity of CtCel8A and CtCBH5A was determined by the method DNS assay. When enzymes came in contact with substrate (1% CMC) by giving them mild conditions the product was formed and that product (reducing sugar) was determined by the DNS method **(Miller, 1959)**. The DNS assay is the most widely used method for the estimation of reducing sugars, it is easy to perform and allows rapid quantification of more samples in less time. The 3,5-dinitrosalicylic acid (DNS) colorimetric method is widely used to measure sugars with reducing properties produced by the presence of potential aldehyde or keto groups. This method is based on simultaneous oxidation of functional groups of sugar and reduction of DNS to 3-amino-5-nitrosalicylic acid by applying heat and alkaline conditions of light absorption at 540 nm wavelength. DNS is susceptible to reduction to the corresponding 3-amino-5-nitrosalicylic acid, which oxidizes its carbonyl centre to a carboxylic acid. So, this is actually a redox reaction with a color change from yellow to brick red. However, it is the reduced form of DNS that is responsible for the color change regardless of the origin of the carbonyl group **(Deshavath *et al.*, 2020; Teixeira *et al.*, 2012)**.

Firstly, the standard graph was plotted by using different concentrations of glucose and absorbances were noted. With the help of the glucose standard curve equation, the activity of CtCel8A and CtCBH5A was determined (Table 9, Figure 16, Table 10 and Table 11).

Table 9: Standard curve of glucose by DNS Assay

Glucose (mg/mL)	Absorbance (540nm)
0.1	0.0855
0.3	0.3166
0.5	0.5239
0.7	0.9229
1	1.2041
2	2.5386
3	3.3031

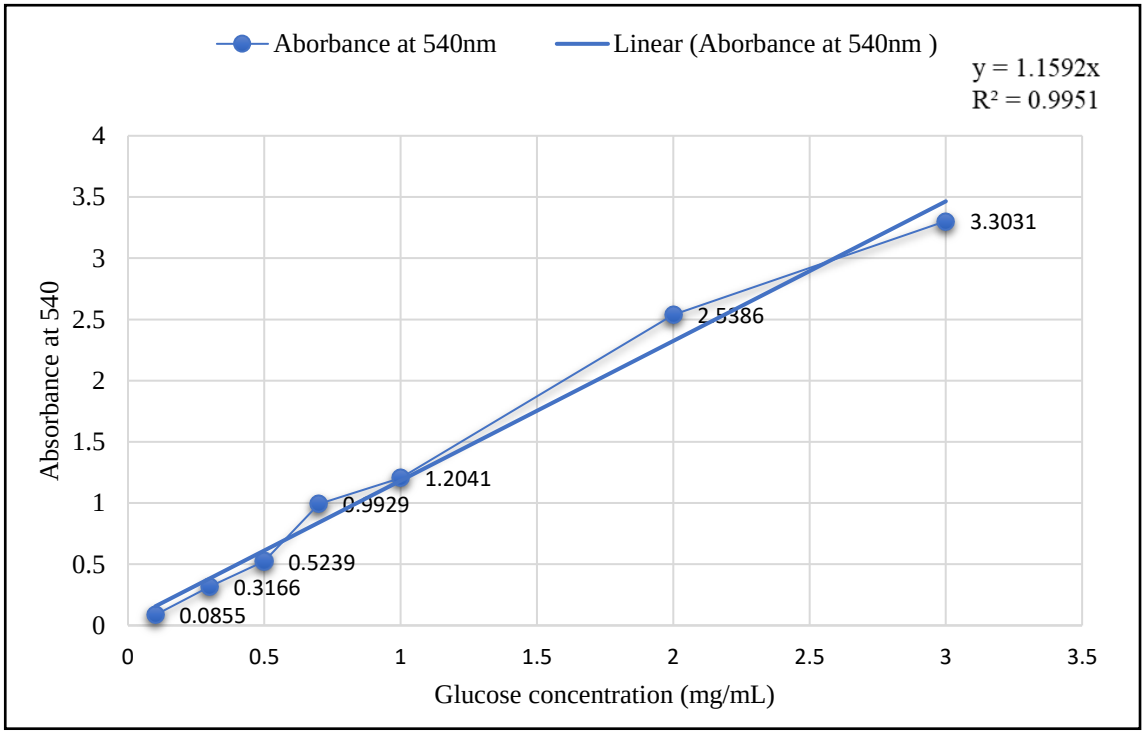

Figure 16: Standard curve of glucose by DNS Assay method

Table 10: Enzymatic activity of recombinant enzyme CtCel8A (endoglucanase)

Enzyme	Enzyme (µL)	Absorbance (540nm)	Glucose (mg/mL)	Enzyme activity (U/mL)
CtCel8A	5	0.1971	0.1700 ±0.04	19.81
	10	0.5531	0.4773 ±0.16	29.14
	20	0.6662	0.5747 ±0.11	19.14
	30	0.7527	0.6493 ±0.10	15.61

Table 11: Enzymatic activity of recombinant enzyme CtCel5A (cellobiohydrolase)

Enzyme	Enzyme (µL)	Absorbance (540nm)	Glucose (mg/mL)	Enzyme activity (U/mL)
CtCel5A	5	0.0538	0.0464 ±0.026	2.70
	10	0.0924	0.0797 ±0.005	2.23
	20	0.1354	0.1168 ±0.031	1.94
	30	0.1550	0.1337 ±0.028	1.60

Enzymatic hydrolysis of cellulose is a complex reaction that depends on the synergistic action of different cellulases like 1,4-β-D-glucan 4-ghicanohydrolase acting on different substrates. Traditionally, the activity of various cellulases has been correlated with D-glucose released when crude culture filtrates of various cellulolytic microorganisms are incubated with relatively unspecified substrates such as CMC. This is expressed as a number of equivalents. Apart from the inaccuracy associated with different cellulase components acting on the same substrate, and the synergistic effect that the addition of one enzyme component has on another

cellulase assay, the lack of specificity and sensitivity of the method used has several problems to analyze the hydrolysis products of cellulose.

One of the most common methods, which is relatively quick and convenient, is Miller's alkaline 3,5-dinitrosalicylic acid method. However, this method has major disadvantages that it is prone to interference of different materials and is not very accurate, especially at low substrate concentrations. It has been shown that the nature of the buffer used to dilute the enzyme can reduce the sensitivity of the DNS assay, while the addition of certain metals can increase the sensitivity. It has also been shown that when amylases or cellulases are used to hydrolyze their specific substrates, the amounts of reducing sugars measured are not proportional to the amount of enzyme added. The copper method of Nelson and Somogyi is not as convenient as the DNS method, but it is reported to be more reliable and sensitive **(Breuil *et al.*, 1985)**. Firstly, the standard curve of glucose was prepared by using Nelson- Somogyi method and with the help of that standard curve, enzyme activity of HtBGI was calculated (Table 12 and Figure 17, Table 13 and figure 18).

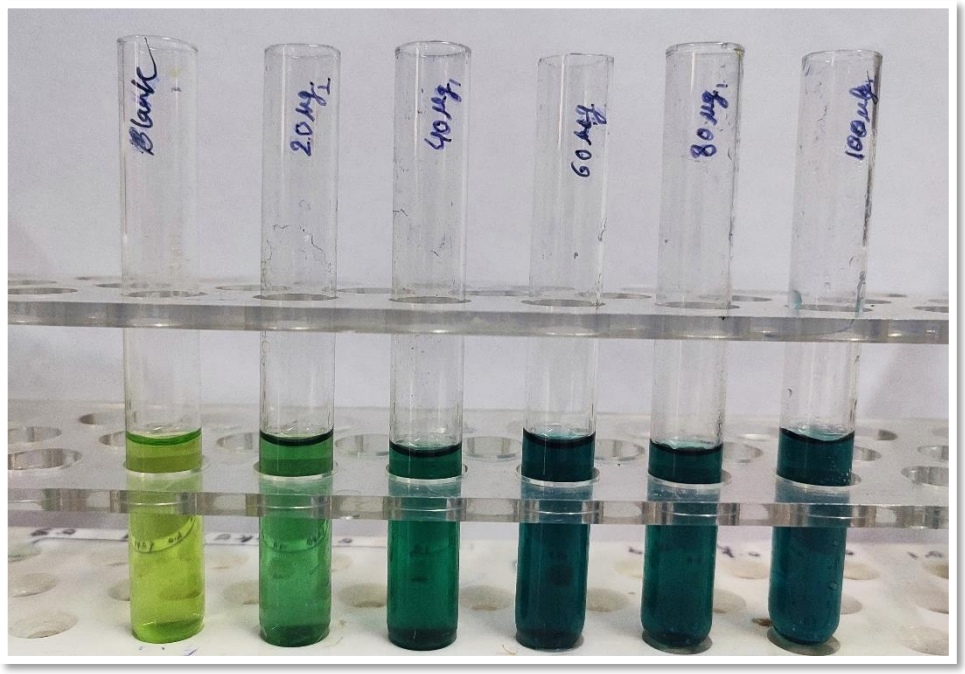

Figure 17: Colour appeared in different concentration of glucose Nelson-Somogyi method

Table 12: Standard curve of glucose by Nelson-Somogyi method

Glucose (µg/mL)	Absorbance (540nm)
20	0.15773 ±0.09
40	0.308067 ±0.09
60	0.5178 ±0.13
80	0.6084 ±0.08
100	0.6799 ±0.13

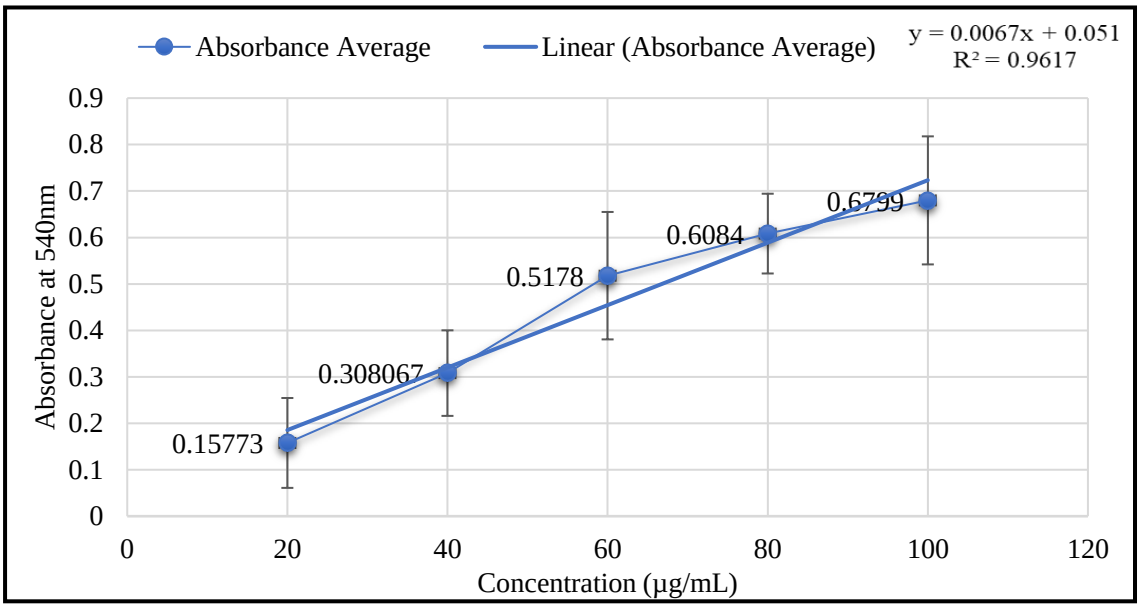

Figure 18: Standard curve of glucose by Nelson- Somogyi method

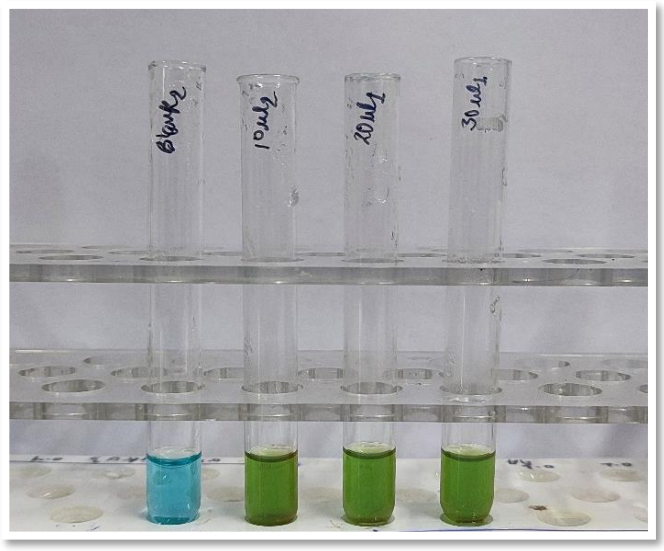

Figure 19: Colour appeared in different concentration of recombinant enzyme HtBgI (β-glucosidase)

Table 13: Enzymatic activity of recombinant enzyme HtBgI (β-glucosidase)

Enzyme	Enzyme (µL)	Absorbance (540nm)	Glucose (mg/mL)	Enzyme activity (U/mL)
HtBgI	5	0.346	0.0440 ±0.02	1.02
	10	1.9145	0.2784 ±0.005	3.4
	20	2.2438	0.3702 ±0.03	2.46
	30	2.4017	0.3507 ±0.02	1.68

If the substrate concentration remains constant, the amount of enzyme can affect product formation. Generally, increasing the amount of enzyme in the reaction increases the rate of product formation up to a point. However, above a certain amount of enzyme, the rate of product formation may not increase significantly or plateau. This phenomenon is often called enzyme saturation. If the substrate concentration remains constant, increasing the amount of enzyme in the reaction can affect product formation. Increasing enzyme concentration means increasing the amount of enzyme increases the concentration of enzyme molecules in the reaction mixture. A higher concentration of enzyme available to interact with the substrate may increase the reaction rate. This means that more substrate molecules are converted to product and more product is produced per unit of time. However, the effect of increasing enzyme concentration is limited. As the enzyme concentration increases, the limiting point of the substrate concentration can occur. Enzyme saturation occurs when all available enzyme active sites are occupied by substrate molecules. In this case, further increase in enzyme concentration does not increase product production because there is no additional substrate for conversion. The reaction rate plateaus and remains constant.

The relationship between enzyme abundance and product formation rate is often described by the Michaelis-Menten equation and provides insight into enzyme kinetics. According to this equation, with increasing enzyme concentration, the initial speed of the reaction increases until it reaches the maximum (Vmax). The relationship between enzyme concentration and product

formation is not necessarily linear. From that point on, further increases in enzyme concentration have no effect on the rate of product formation **(Robinson, 2015)**.

The protein concentration of crude endo-b-1,4-glucanase (CtCel8A), cellobiohydrolase (CtCBH5A) and b-1,4-glucosidase (HtBgl) enzymes was 1.43 mg/mL, 1.51 mg/mL and 1.76 mg/mL, respectively. The crude CtCel8A and CtCBH5A enzymes showed endoglucanase activity, 19.81 U/mL at 5 µL, 29.41 U/mg at 10 µL, 19.14 U/mL at 20 µL and 15.61 U/mL at 30 µL whereas, cellobiohydrolase activity, 2.70 U/mg at 5 µL, 2.23 U/mL at 10 µL, 1.94 U/mL at 20 µL and 1.60 U/mL at 30 µL respectively, against CMC as substrate. The crude HtBgl showed β-glucosidase activity, 1.02 U/mg at 5 µL, 3.4 U/mL at 10 µL, 2.46 U/mL at 20 µL and 1.68 U/mL at 30 µL against p-nitrophenyl as substrate.

4.7 Enzymatic Saccharification

Saccharification is basically the process in which complex carbohydrates are broken down into reducing sugars. This process involves chemicals or enzymes. Each method has its own advantages as well as disadvantages. Very low temperatures are used for enzymatic hydrolysis, which reduces the risk of degradation. Enzymes such as cellulase, pectinase and amylase are used for saccharification. Saccharification is usually performed to obtain fermentable sugars from cellulosic or hemicellulosic components. Finally, microbial fermentation, which uses fermentable sugars as carbon and energy sources, produces fuel, materials, and chemicals of interest as metabolites. Total six different combinations of were endoglucanase (CtCel8A), cellobiohydrolase (CtCBH5A) and β-glucosidase (HtBgl) tested. The absorbance was noted every 12hrs interval and TRS were estimated with the help of DNS assay method. The enzymatic hydrolysis of pre-treated grinded and native (non-grinded) RS by using recombinant hydrolytic enzymes gave the different TRS yield (Table 14 and Figure 20; Table 15 and Figure 21).

Table 14: Total Reducing Sugar release during enzymatic hydrolysis of pre-treated grinded rice straw (Combo I: 250:20:30; Combo II: 230:30:40; Combo III: 200:50:50; Combo IV: 200:40:60; Combo V: 210:50:40; Combo VI: 250:30:20)

Time interval (hrs)	Total reducing sugar (mg/mL)	Total reducing sugar (mg/g)
	Combo I {250:20:30}	

12	0.7293 ±0.038	35 ±0.038
24	0.3907 ±0.039	19.5 ±0.039
36	0.254 ±0.004	12.7 ±0.004
48	0.3163 ±0.018	15.8 ±0.018
60	0.3014 ±0.016	15 ±0.016
72	0.4414 ±0.016	22 ±0.016
Combo II {230:30:40}		
12	0.7582 ±0.017	37.9 ±0.017
24	0.5431 ±0.004	27.1 ±0.004
36	0.2427 ±0.019	12.1 ±0.019
48	0.2913 ±0.023	14.5 ±0.023
60	0.2155 ±0.029	10.7 ±0.029
72	0.2735 ±0.004	13.6 ±0.004
Combo III {200:50:50}		
12	0.6746 ±0.013	33.7 ±0.013
24	0.5373 ±0.013	26.8 ±0.013
36	0.2369 ±0.010	11.8 ±0.010
48	0.0975 ±0.003	4.8 ±0.003
60	0.0819 ±0.004	4 ±0.004
72	0.0635 ±0.007	3.1 ±0.007
Combo IV {200:40:60}		
12	0.6940 ±0.004	32 ±0.004
24	0.5552 ±0.016	27.7 ±0.016
36	0.3002 ±0.008	15 ±0.008
48	0.0840 ±0.001	4.2 ±0.001
60	0.0641 ±0.002	3.2 ±0.002
72	0.0251 ±0.0002	1.2 ±0.0002
Combo V {210:50:40}		
12	0.4205 ±0.003	21 ±0.003
24	0.3103 ±0.006	15.5 ±0.006
36	0.2003 ±0.013	10 ±0.013
48	0.0561 ±0.004	2.8 ±0.004

60	0.1340 ±0.171	6.7 ±0.171
72	0.1191 ±0.012	5.1 ±0.012
	Combo VI {250:30:20}	
12	0.5339 ±0.005	26.6 ±0.005
24	0.3625 ±0.007	18.1 ±0.007
36	0.0839 ±0.001	4.1 ±0.001
48	0.0307 ±0.001	1.5 ±0.001
60	0.0177 ±0.0004	0.8 ±0.0004
72	0.0097 ±0.0003	0.4 ±0.0003

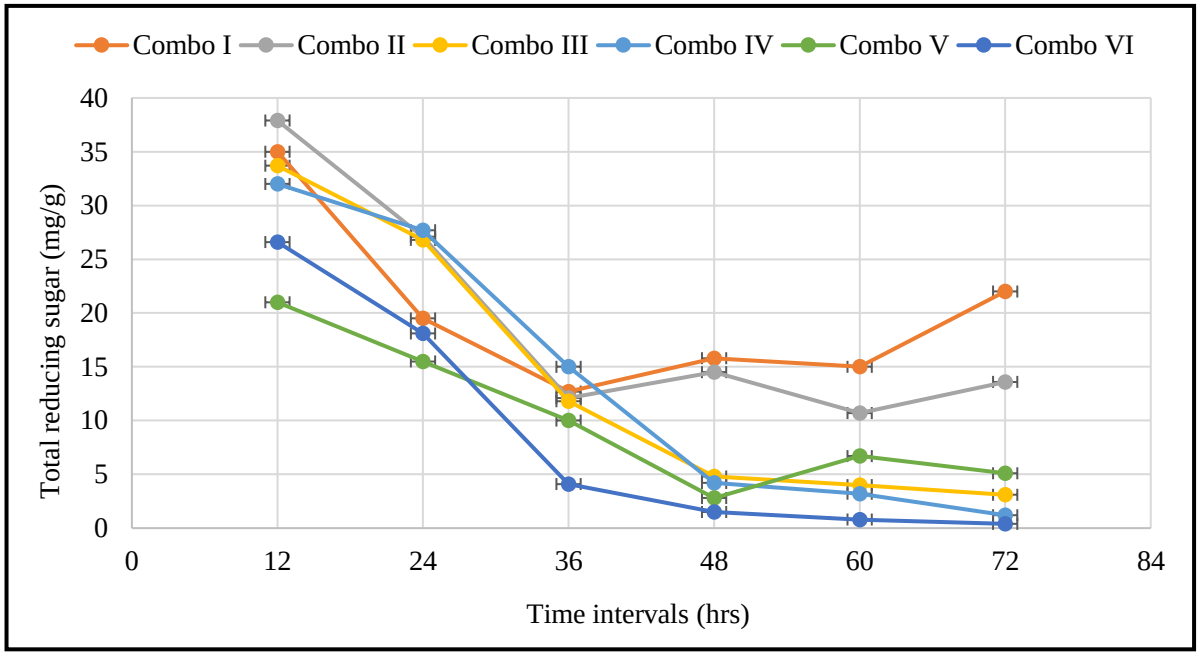

Figure 20: Total Reducing Sugar release during enzymatic hydrolysis of pre-treated grinded rice straw (Combo I: 250:20:30; Combo II: 230:30:40; Combo III: 200:50:50; Combo IV: 200:40:60; Combo V: 210:50:40; Combo VI: 250:30:20)

Table 15: Total Reducing Sugar release during enzymatic hydrolysis of pre-treated non-grinded rice straw (Combo I: 250:20:30; Combo II: 230:30:40; Combo III: 200:50:50; Combo IV: 200:40:60; Combo V: 210:50:40; Combo VI: 250:30:20)

Time intervals (hrs)	**Total reducing sugar (mg/mL)**	**Total reducing sugar (mg/g)**
	Combo I {250:20:30}	

12	1.5820 ±0.016	79 ±0.016
24	0.9272 ±0.021	46.3 ±0.021
36	0.7002 ±0.021	35 ±0.021
48	0.3151 ±0.028	15.7 ±0.028
60	0.2277 ±0.021	11.3 ±0.021
72	0.4488 ±0.013	22.4 ±0.013
Combo II {230:30:40}		
12	1.1348 ±0.204	56.7 ±0.204
24	0.7154 ±0.022	35.7 ±0.022
36	0.5778 ±0.027	28.8 ±0.027
48	0.3119 ±0.014	15.5 ±0.014
60	0.4528 ±0.023	22.6 ±0.023
72	0.4375 ±0.006	21.8 ±0.006
Combo III {200:50:50}		
12	1.4264 ±0.017	71.3 ±0.017
24	1.1199 ±0.079	55.9 ±0.079
36	0.5478 ±0.013	27.3 ±0.013
48	0.3414 ±0.0004	17 ±0.004
60	0.2743 ±0.017	13.7 ±0.017
72	0.1679 ±0.004	8.3 ±0.004
Combo IV {200:40:60}		
12	1.3010 ±0.007	65 ±0.007
24	0.8980 ±0.048	44.9 ±0.048
36	0.5383 ±0.008	26.9 ±0.008
48	0.4685 ±0.012	23.4 ±0.012
60	0.2891 ±0.027	14.4 ±0.027
72	0.1635 ±0.006	8.1 ±0.006
Combo V {210:50:40}		
12	0.5427 ±0.007	27.1 ±0.007
24	0.5010 ±0.013	25 ±0.013
36	0.2827 ±0.005	14.1 ±0.005
48	0.1998 ±0.005	9.9 ±0.005

60	0.0355 ±0.001	1.7 ±0.001
72	0.1111 ±0.006	5.5 ±0.006
	Combo VI {250:30:20}	
12	0.7828 ±0.009	39.1 ±0.009
24	1.3052 ±0.012	65.2 ±0.012
36	0.6801 ±0.003	34 ±0.003
48	0.6364 ±0.015	31.8 ±0.015
60	0.3453 ±0.0008	17.2 ±0.008
72	0.0435 ±0.0007	2.1 ±0.007

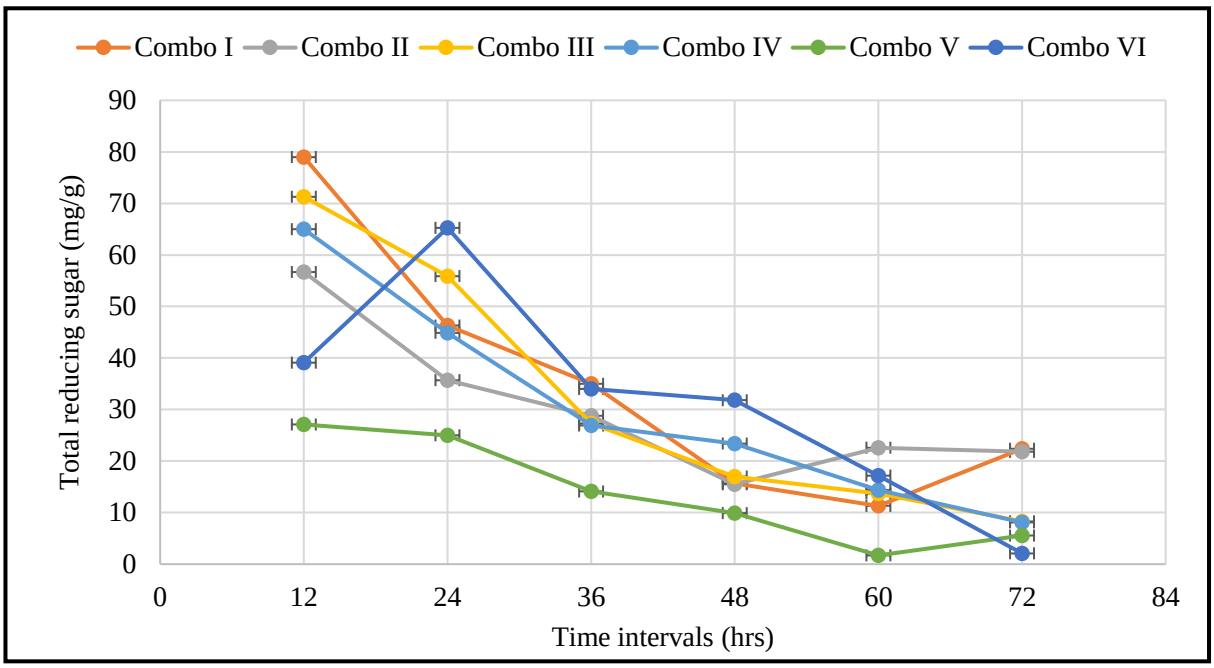

Figure 21: Total Reducing Sugar release during enzymatic hydrolysis of pre-treated non-grinded rice straw (Combo I: 250:20:30; Combo II: 230:30:40; Combo III: 200:50:50; Combo IV: 200:40:60; Combo V: 210:50:40; Combo VI: 250:30:20)

The enzymatic hydrolysis of pre-treated grinded by using endoglucanase (CtCel8A), cellobiohydrolase (CtCBH5A) and β-glucosidase (HtBgl) gave the Total Reducing Sugar (TRS) yield, 37.9 mg/g, from the combination II of 8A:5A:Ht (230:30:40 U/g) whereas, of separated cellulose from non-grinded yielded TRS, 79 mg/g released from the combination I of 8A:5A:Ht (250:20:30 U/g) by using 0.1 g of pre-treated non-grinded rice straw.

CONCLUSION

1. In this study, we investigated the saccharification of pre-treated rice straw using recombinant cellulases to assess their efficacy in converting cellulose into fermentable sugars.
2. Compositional analysis of the native biomass of rice straw revealed presence of 35.5% cellulose, 20% hemicellulose, and 22.2% lignin.
3. The pre-treatment of non-grinded, grinding (particle size >0.5mm) and trash (particle size <0.5mm) rice straw was done using a combination of chemical processes to enhance the accessibility of cellulose for enzymatic hydrolysis. Compositional analysis of different forms of pre-treated rice straw showed that non-grinded rice straw had the highest cellulose content of 68% in comparison to grinded having 54.4% cellulose and 62.5% in trash fraction.
4. Delignification and pre-treatment efficiency of pre-treated non-grinded biomass was found higher than grinded and trash biomass, suggesting no grinding and sieving is necessary.
5. Enzymatic saccharification of 0.1g of pre-treated non-grinded rice straw biomass by recombinant endo-b-1,4-glucanase (CtCel8A), cellobiohydrolase (CtCBH5A) and β-1,4-glucosidase (HtBg1) yielded 79 mg/g total reducing sugar from the combination I of 8A:5A:Ht (250:20:30 U/g) after 12hrs at 50°C, which was found better than the yield of 37.9 mg/g from pre-treated grinded biomass.
6. Overall, this study highlights the potential of recombinant cellulases for the efficient saccharification of pre-treated rice straw. The acid and alkali pre-treatment, including delignification, significantly improved the accessibility of cellulose, resulting in enhanced saccharification efficiency.
7. Rice straw can serve as a valuable feedstock for the production of fermentable sugars and subsequent bioethanol production.

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