Lignocellulosic bioethanol production from delignified rice straw using tailor-made crude recombinant hydrolytic enzyme cocktail and *Saccharomyces cerevisiae* MTCC170

Ph.D. Thesis

by

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SCHOOL OF ENERGY SCIENCE AND ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
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A Thesis

Submitted for partial fulfilment
of the requirements for the award of

Doctorate of Philosophy

by

Maibam Premeshworii Devi

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Prof. Arun Goyal

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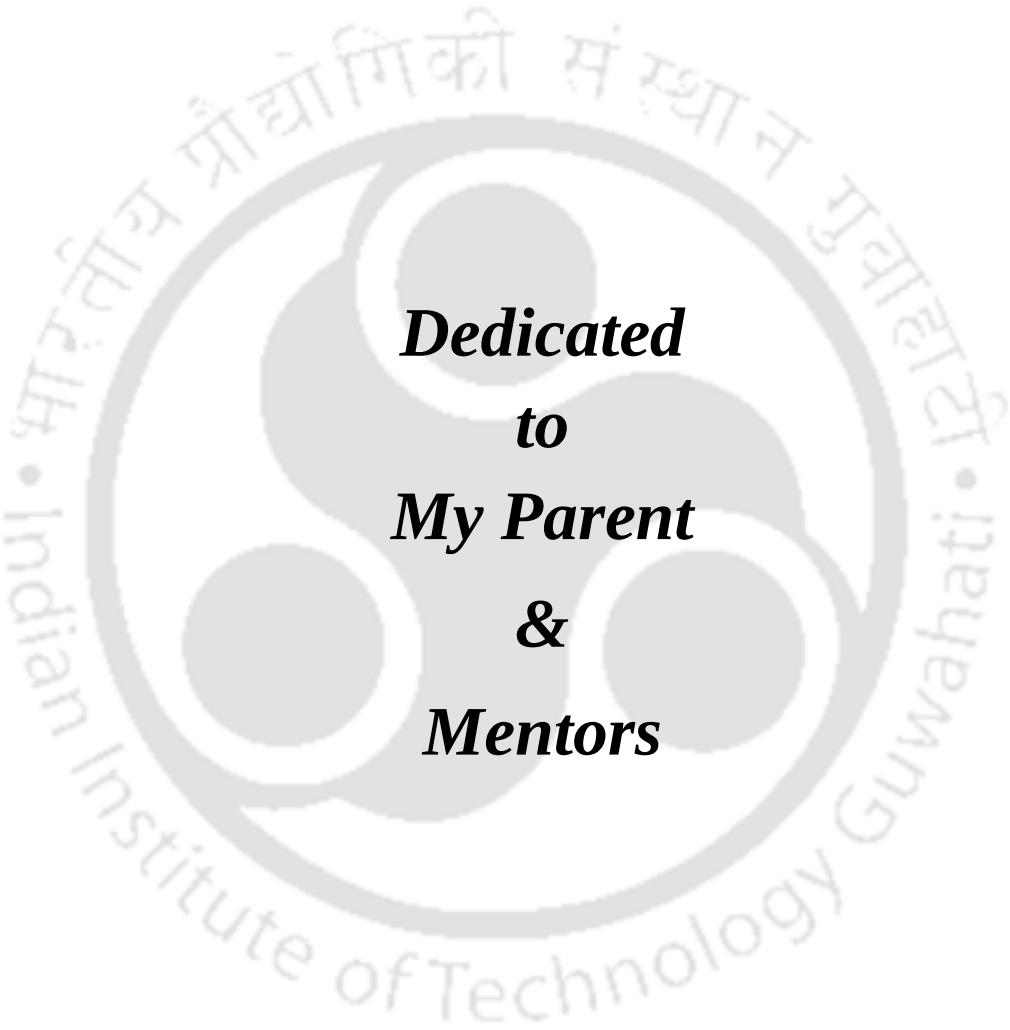

Dedicated

to

My Parent

&

Mentors

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

School of Energy Science and Engineering

STATEMENT

I do hereby declare that the content embodied in this thesis entitled "**Lignocellulosic bioethanol production from delignified rice straw using tailor-made crude recombinant hydrolytic enzyme cocktail and *Saccharomyces cerevisiae* MTCC170"** is the result of investigations carried out by me at School of Energy Science and Engineering, Indian Institute of Technology Guwahati, Guwahati, India under the guidance of **Prof. Arun Goyal**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

September, 2024 *Maibam Premeshworii Devi*

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CERTIFICATE

This is to certify that the work described in this thesis entitled "**Lignocellulosic bioethanol production from delignified rice straw using tailor-made crude recombinant hydrolytic enzyme cocktail and *Saccharomyces cerevisiae* MTCC170"** by **Ms. Maibam Premeshworii Devi** (Roll No.: 196151005) for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the School of Energy Science and Engineering, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

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Synopsis

Introduction

Energy insecurity and environmental pollution are the two major challenges mankind has ever encountered in this century. At present, bioenergy is an emerging clean, renewable and alternative energy source for economic development as well as for improving the quality of human life. The government has also imposed a blending mandate of biofuel with petroleum fuels as a step in resolving the evolved energy crisis. Bioethanol is one of the most commonly used biofuels for blending with conventional fuel. However, the first-generation ethanol is not sufficient to meet the global ethanol demand for blending with gasoline. Therefore, the utilization of lignocellulosic biomass as a raw material for the production of second-generation bioethanol has received considerable interest in recent years. Ethanol production by lignocellulosic biomass follows three successive steps *viz*. pretreatment of lignocellulosic feedstock, enzymatic hydrolysis of pretreated biomass and fermentation of reduced monosaccharides to ethanol. The economics of bioethanol production depends greatly on three factors: i) availability and transportation cost of the biomass feedstock, ii) cost of hydrolytic enzyme and iii) mode and technology of the fermentation process. In the present work, an attempt is made to develop, optimize and intensify a complete process for bioethanol production from lignocellulosic rice straw biomass comprising the three steps mentioned above.

In Asian countries, where rice is the staple food, rice straw contributes a major agricultural lignocellulosic waste. The global rice straw production is 370-520 million tons/year (International Rice Research Institute, IRRI 2019) of which 330-470 million tons are from Asia alone (Van Hung et al. 2020). This enormous amount of lignocellulosic waste can be effectively valorised to bioethanol and other chemicals as their major structural components are cellulose, hemicellulose and lignin. Considering the cellulose and hemicellulose content of rice straw, the theoretical ethanol production per tonne of rice straw would be 387-442 L. But, the reported yields of bioethanol from rice straw to date are far less than the maximum theoretical ethanol yield (Mustafa et al., 2016). Thus, to investigate intensely in uplifting the potential of rice straw as a feasible feedstock for the production of bioethanol in lignocellulose biorefinery, this study used rice straw as a potential feedstock for ethanol production.

However, the key to the success in lignocellulosic biorefineries lies in an effective pretreatment method that reduces the recalcitrance nature of biomass and modifies the polymer structure. The purpose of any pretreatment method is the removal of lignin fractions and increase the exposure of cellulosic fraction and its surface with a reduction in cellulose crystallinity (Baruah et al. 2018, Maibam et al. 2020). The use of green solvents having characteristics of biodegradability, low toxicity, recyclability and cost-effectiveness has emerged as a potential pretreatment method for biomass fractionation (Calvo-Flores et al. 2018). Deep eutectic solvents (DESs) are green solvents with low toxicity, easy synthesis process and feasibility at large scale pretreatment process (Wang et al. 2021). The mechanism of biomass fractionation by DES can be predominantly categorized as hydrolysis of hemicellulose and delignification with negligible disruption to the cellulosic fractions (Hong et al. 2020).

Choline chloride (ChCl) based DES has shown to be of great potential in the delignification of lignocellulosic biomass (Xu et al. 2020). Thus, ChCl-based DES solvent system have been explored for rice straw pretreatment in this study.

The second challenging step in biorefineries is the high conversion efficiency of cellulose and hemicellulose. The enzymatic saccharification process requires the application of multiple enzymes (cellulases and hemicellulases) with higher catalytic efficiency to hydrolyze the cellulosic and hemicellulosic fractions into monosaccharides. However, the requirement of several enzymes in the saccharification process is cost-intensive and a challenging task. The considerable high cost involved in the use of commercial enzymes in biomass-based processes has led to the development and use of on-site-produced crude hydrolytic enzyme cocktails. The development and use of recombinant multifunctional chimera that can catalyze one or more reactions can also significantly reduce the cost and simplify the process (Van Dyk et al., 2012). Therefore, the use of a crude recombinant hydrolytic enzyme cocktail was explored for rice straw biomass hydrolysis.

The final step in process development was fermentation, which was carried out in pre-saccharification and simultaneous saccharification and fermentation mode using a crude hydrolytic enzyme cocktail and *Saccharomyces cerevisiae* MTCC170 as the fermenting microbe. The efficiency of enzymatic saccharification during PSSF process depends on several factors such as i) pre-treatment conditions viz, type of solvent, time and temperature (Maibam and Goyal, 2022), ii) the enzyme properties (Rajak and Banerjee, 2020) and iii) the saccharification conditions such as biomass loading, enzyme dosage and hydrolysis time (Afedzi and Parakulsuksatid, 2023). Proper optimization of the above-mentioned significant factors could improve the overall

ethanol yield and process economics (Pratto et al., 2020). In order to boost the yield of fermentation, statistical optimization of PSSF was performed with key factors as pre-saccharification time, enzyme dosage and fermentation temperature to maximized the ethanol yield and productivity. The major findings of this study addressing different facets of the bioethanol production process have been summarized herewith.

Present work

The present work entitled "**Lignocellulosic bioethanol production from delignified rice straw using tailor-made crude recombinant hydrolytic enzyme cocktail and *Saccharomyces cerevisiae* MTCC170**" has been divided into 5 chapters.

Chapter 1 gives a general introduction to bioethanol production from lignocellulosic biomass from the viewpoint of major challenges and potential solutions. It also includes a brief review of literature focusing on governing factors and strategies for bioethanol production from lignocellulosic biomass. This chapter covers topics of rice straw as a potential feedstock for bioethanol production, brief details on the compositions of lignocellulosic biomass and the chemical interactions amongst the lignocellulosic complexes. A comparative analysis of the advantages, disadvantages and mechanisms of various pretreatment methods adopted for rice straw pretreatment has also been discussed. Subsequently, enzymatic saccharification of pretreated biomass with a focus on the applicability of crude recombinant enzymes and various fermentation strategies with a main emphasis on simultaneous saccharification and fermentation have also been discussed. Finally, the research gap in bioethanol research has been identified and justification for the present thesis work has been given.

Chapter 2 aims to screen a suitable pretreatment method for rice straw biomass from various chemical and physico-chemical pretreatment methods. Twelve different

pretreatment methods using dilute acid (H_2SO_4, HNO_3, H_3PO_4, acetic acid or oxalic acid), alkali (NaOH), dual-acid (HNO_3 + AA), organosolv (phosphoric acid- acetone), deep eutectic solvent (ChCl: AA, ChCl: OA, ChCl: glycerol) and sequential pretreatment (alkali followed by acid) have were performed under the same operating conditions for selecting the best suitable pretreatment method for RS biomass. The basis for selecting the best pretreatment method was the parameters: i) high biomass recovery with high delignification percentage, ii) maximum retainment of cellulosic fraction in the pretreated biomass and iii) high crystallinity index of the pretreated biomass. Raw RS contained 43.5±1.1% (w/w) cellulose, 17.1±0.5% (w/w) lignin. The pretreatment of RS by DES system with ChCl:AA combination showed the overall high cellulose content 65.5±0.9%, w/w, lignin content 6.2±0.2%, w/w giving delignification of 78%. The ChCl:AA pretreated RS showed high *CrI* of 48.4%. The HPLC analysis of liquid fraction obtained after the DES pretreatment process using ChCl: AA showed the least degradation of cellulosic fraction with the lowest glucose yield of 0.8 mg/g. Based on the above experimental facts i.e. high biomass recovery with high cellulose content in the pretreated biomass, high *CrI*, negligible loss of cellulosic fraction during the pretreatment process and DES solvent being green, non-toxic and reusable, ChCl:AA solvent was selected as the best suitable pretreatment method for RS biomass in further study. The next chapter focuses on the optimization of ChCl: AA DES solvent pretreatment conditions for RS biomass.

Chapter 3 presents a detailed investigation of DES with ChCl and acetic acid (AA) for the pretreatment process of rice straw (RS). Three significant pretreatment parameters; ChCl: AA molar ratio, time and temperature were scrutinized using statistical analysis to optimize the RS pretreatment process. The effect of independent

variables on lignin removal and retainment of total carbohydrate content (TCC) in the pretreatment process was evaluated by the central composite design (CCD) approach. The pretreatment temperature and molar ratio of AA to ChCl played a significant role in delignification. The optimized conditions for RS pretreatment were 1:3.59 (ChCl:AA molar ratio), 126 °C and 150 min. ChCl:AA pretreated RS (CApRS) gave 83.1% delignification, 679 mg/gCApRS TCC and 83.7% pretreatment efficiency. CApRS contained enriched cellulose content, 0.73 g/gCApRS as compared with 0.43 g/graw RS in raw RS. CApRS showed 31% higher crystallinity index, 17-fold higher surface area than raw RS. The morphological study of CApRS displayed a porous surface. The precipitation of black liquid hydrolysate obtained after the pretreatment of RS using ultrapure water and ethanol mixture resulted in the extraction of 10.4 g DES-lignin. The extraction yield of DES-lignin was 56% with the purity 86%. The FTIR study of extracted lignin confirms the presence of G and S-sub-units of lignin and aromatic skeleton in DL. Saccharification of CApRS by commercial cellulase gave a total reducing sugar of 18.8 g/L in hydrolysate with saccharification efficiency, 92.2%. The high saccharification efficiency achieved in this study denotes the efficacy of the developed pretreatment method.

Chapter 4 focuses on the enzymatic saccharification step in lignocellulosic biorefineries for bioethanol production. This chapter describes the formulation of an enzyme cocktail consisting of recombinant cellulases (bifunctional cellulolytic chimeric enzyme, *Ct*GH1-L1-*Ct*GH5-F194A and cellobiohydrolase, *Ct*CBH5A) and xylanases (endo-1,4-β-xylanase, *Ct*GH11A, and β-xylosidase, *Bo*GH43), all in crude form (unpurified enzyme present in the total cellular proteins) for producing a high yield of total reducing sugars using choline chloride: acetic acid pretreated rice-straw

(CApRS). The D-optimal mixture design approach was used for statistically designing the optimal proportion of crude recombinant enzyme cocktail for hydrolysis of delignified rice straw. The developed enzyme cocktail comprising chimera, *Ct*CBH5A, *Ct*GH11 and *Bo*GH43 in ratio 35.1:41.8:10.0:13.1 resulted in cellulolytic enzyme activity, 5.6 FPU/mL and xylanase activity, 102 U/mL. Enzymatic saccharification of 3% (w/v) delignified CApRS using the developed enzyme cocktail at 187 FPU/g_{CApRS}, pH 5.8 and 35°C, yielded total reducing sugar of 498 mg/g_{CApRS}, glucose yield (410 mg/g_{CApRS}) and xylose yield (71.3 mg/g_{CApRS}) resulting in 72% saccharification efficiency. However, the saccharification of 3% (w/v) raw RS or 3%, (w/v) partially pretreated RS by the same enzyme cocktail under the same conditions gave significantly lower TRS yields of 80 mg/g_{rawRS} and 218 mg/$g_{ptd\ RS}$, respectively, implying the effectiveness of the developed enzyme cocktail against CApRS. Thus, highlighting the importance of different enzyme proportions in the cocktail for biomass conversion and the effectiveness of the developed enzyme cocktail against CApRS biomass. The developed enzyme cocktail was compared with a commercial enzyme cocktail and prospects its applicability in lignocellulosic biorefineries. The optimum saccharification process parameters, 35ºC and pH 5.8 developed in this chapter are advantageous for its subsequent application in simultaneous saccharification and fermentation (SSF) in bioethanol-based biorefineries.

Chapter 5 presents pre-saccharification and simultaneous saccharification and fermentation (PSSF) of delignified rice straw using a crude recombinant enzyme cocktail and *Saccharomyces cerevisiae* MTCC170. The fermentation process was statistically optimized by the RSM-CCD approach. Pre-saccharification time, enzyme dosage and fermentation temperature were the variables considered for maximizing

ethanol yield and productivity. Statistical analysis of PSSF optimization inferred that low pre-saccharification time (9.6-15h) and enzyme dosage (above 60 FPU/g) display significant influence on achieving higher ethanol yield and productivity. Statistical optimization resulted in optimum conditions: 12.22h pre-saccharification time, enzyme dosage (83.78 FPU/g_{CApRS}), fermentation temperature (33.7°C) with predicted ethanol yield, 0.35 g/g and productivity, 0.63 g/L/h. The PSSF process of 10%, w/v CApRS biomass at bioreactor level with the statistically optimized condition of PS time, 12.22h using crude enzyme cocktail, 84 FPU/g_{CApRS} at a fermentation temperature of 33.7°C gave an ethanol titre of 24.8 g/L, ethanol yield of 0.37 g/g with ethanol productivity 0.82 g/L/h after 18h of fermentation. The overall yield, 182L ethanol/ tonne raw RS biomass achieved in this study prospects the feasibility of 2G ethanol production using crude recombinant enzyme cocktail which outlays an economic advantage of on-site production of crude enzyme cocktails at the fermentation plant. The overall less process time, 30.22h optimized in this study, would reduce the global processing time (72h hydrolysis + 24h fermentation) by about 68%, thus providing another aspect of the economic approach for ethanol production from CApRS

CONTENTS

Chapter 1

General Introduction

1.1 Need for non-conventional fuel

Energy insecurity and environmental pollution are the two major challenges mankind has ever encountered in this century. The dependence on fossil fuels over centuries and the rapid increase in greenhouse gases (GHG) discharged from burning fossil fuels has led to the search for alternative renewable energy sources to solve the evolved energy and environmental crisis (Abdmouleh *et al*., 2015). At present, bioenergy is an emerging and cleaner renewable alternative energy source for economic development as well as for improving the quality of human life (Siddique and Wahid, 2018). Globally, economic developments have raised the need for alternative energy resources because of the drawbacks of fossil fuels such as (1) their limited availability and supply, (2) global warming and greenhouse gasses emission and (3) their price hike

and unexpected price fluctuations. These issues have substantiated the engrossment in alternative, sustainable, renewable and economically viable fuels such as bioethanol.

The earliest report on the production of biofuel was in the 19th century with ethanol produced from corn and the use of peanut oil as fuel in Rudolf's first diesel engine (Nitske, 1965). However, with the rapid growth and development of the petroleum industry after World War II, the production of biofuel did not seek much research and development. The fast depletion of fossil fuels along with the growing concern of climate change and global warming has been responsible for rapid growth in biofuel production in the last two decades (Solarte-Toro *et al*., 2019). The depletion of oil reserves and environmental concerns have resulted in governmental actions of enforcing a blending mandate of biofuel with petroleum fuels (Hans *et al.*, 2019). The utilization of lignocellulosic biomass as a raw material for the production of biofuels and other value-added products has received considerable interest in recent years (Singh *et al*., 2016). Bioethanol and biodiesel are the most commonly used biofuels for blending with conventional fuel. Bioethanol, which has a high-octane number, serves as an oxygenated additive of gasoline. In India, according to the report of Niti Aayog report of June 2021, E10 is being used pan-India from April, 2022 for use as a protection fuel to meet the demands of existing vehicles till April 2025. The Ethanol Blending Program (EBP) committee of India suggests a gradual rollout of E20 ethanol in the country to achieve the target by 2025. However, this first-generation ethanol is not sufficient to meet the demand and supply for ethanol blending with gasoline. Thus, there is a need to focus on the non-edible part of lignocellulosic biomass as a feasible feedstock for the production of bioethanol in lignocellulose biorefinery (Antunes *et al*., 2019).

1.2 Lignocellulose biomass: An alternative source of biofuel

Lignocellulosic biomass, a renewable resource from plants, is the most extensively used raw material for the production of bioenergy and other value-added products in biorefineries (Solarte-Toro *et al*., 2019). The annual production of lignocellulosic biomass is estimated to be 181.5 billion tons worldwide (Singh *et al*., 2020). Lignocellulosic biomass is composed of an aromatic polymer (lignin) and polysaccharides (cellulose and hemicellulose), a potential source of fermentable sugars that can be directly converted to bioethanol (Antunes *et al*., 2019). Lignocellulosic biomass such as rice straw (Satlewal *et al*., 2018), wheat straw (Townsend *et al*., 2018), corn cob (Itelima *et al.*, 2013), switchgrass (Bals *et al.*, 2010), water hyacinth (Das *et al*., 2014) has been utilized as a potential feedstock for bioethanol production. A variety of farm and industrial wastes were also explored for the production of bioethanol (Ghatak, 2011).

1.2.1 Rice straw as a feedstock for bioethanol production

The lignocellulosic biomass with surplus availability and low food and fodder value, such as rice straw serves to be the most potential feedstock for bioethanol production (Chandel *et al*., 2018). The non-edible part of rice straw is either dumped into landfills or burnt. Therefore, the bioconversion of such agro-waste to bioenergy could be a plausible solution to combat the present energy crisis and environmental pollution. As per the report from the Food and Agricultural Organization, United Nations, in 2022-23, the world's rice production was 524.6 million tonnes and India alone contributed 135.7 million tonnes. The annual total rice straw production in India is 112.7 million tonnes. Rice straw is utilized for various purposes including ruminant feed, paper industries, fertilizer, heating and cooking purposes and as a feedstock for

biogas production (Chandel *et al*., 2018). Despite all these applications, more than 50% of straw is burnt in the open field and only 20% of rice straw production is utilized for bioenergy production (Oladosu *et al*., 2016, Van Nguyen *et al*., 2016). Also, considering the cellulose and hemicellulose content of rice straw, the theoretical ethanol production per ton of rice straw would be 387-442 L (Molaverdi *et al*., 2019). Thus, rice-straw can be explored as a potential feedstock for bioethanol production.

1.3 Composition of lignocellulosic material

Lignocellulosic biomass is a complex structure of carbohydrate polymers, which consist majorly of cellulose (37-50%), hemicellulose (24-32%) and lignin (16-25%) (Ho *et al*. 2019) with a small fraction of proteins, inorganic compounds and water as moisture content.

1.3.1 Cellulose

Celluloses $(C_6H_{10}O_5)_n$ are polysaccharides consisting of a linear chain of D-glucose units linked together by β-(1–4) glycosidic bond. They are also commonly referred to as a polymer of glucose units with a higher degree of polymerization (DP) (Wolfe *et al*., 2002). The DP of cellulose varies between 800–10,000 units, but can also extend up to 17,000. Each cellulose polymer chain is linked to other chains by hydrogen bonds resulting in larger units organized in crystalline structures called cellulose fibrils (Maibam and Goyal 2022). Cellulose is the major constituent in any lignocellulosic biomass and exists in crystalline and amorphous structures in the core. Around 7.5 × 1010 tonnes of cellulose is produced from woody biomass, agriculture waste, municipal solid waste, and forest residue each year, making it the most abundant organic compound on earth (Demain *et al*., 2005). The hydrolysis of the cellulosic fraction of the lignocellulosic complex depends upon the temperature. The cellulosic fraction is

resistant to hydrolysis by dilute acid solution at low temperatures (below 50°C). However, at elevated temperatures (above 100°C), cellulosic fractions are hydrolyzed in dilute acid solution to give monosaccharides as the hydrogen bonds are disrupted due to the higher energy input (Sun *et al*., 2016). However, on exposure of lignocellulosic complex to an alkaline solution, the hydrolysis of cellulosic fractions occurs even at lower temperatures that is, below 50°C (Nath *et al*., 2021). Under alkaline conditions, the solvent diffuses into the cellulosic fiber leading to its extensive swelling, resulting in the breakage of glycosidic and hydrogen bonds and subsequently resulting in low molecular mass cellulose polymers (DP < 200) and monosaccharides (Yang *et al*., 2012).

1.3.2 Hemicellulose

Hemicelluloses $(C_5H_8O_4)_n$ are heterogeneously branched biopolymers comprising pentoses, hexoses and uronic acids (Khaire *et al*., 2022). Hemicelluloses are located between the micro- and the macro-fibrils of cellulose. The major hemicelluloses present in plant biomass are xylan, arabinoxylan, glucuronoxylan, xyloglucan, mannan, glucomannan, galactomannan, galactan, arabinogalactan, and arabinan, however, xylan is the most abundant hemicellulose (Schädel *et al*., 2009). Hemicelluloses are amorphous and branched structures having relatively low molecular weight and sensitivity to pretreatment conditions, therefore readily hydrolyzed by chemical pretreatments (Maibam and Goyal, 2022). A large fraction of the hemicellulose from the lignocellulosic complex is removed during the pretreatment process which enhances the digestibility of cellulosic fraction in enzymatic saccharification (Maibam & Maiti, 2020). However, the hemicelluloses can be readily

hydrolyzed by dilute acid (Palmqvist & Hahn-Hägerdal, 2000a) or alkali solutions (Nath *et al*., 2021) at low temperatures to their respective monosaccharides.

1.3.3 Lignin

Lignin $[C_9H_{10}O_3(OCH_3)_{0.9–1.7}]_n$ is a complex organic aromatic polymer composed of cross-linked phenolic precursors (Demirbas *et al*., 2008). They are composed of a complex matrix with an amorphous structure and is a strengthening constituent. The main building blocks of lignin are hydroxyl coumaryl alcohols, coniferyl alcohol and sinapyl alcohol, with minor quantities of p-coumaryl alcohol (Maibam and Goyal, 2022). The cellulose and hemicellulose are firmly associated with lignin by hydrogen bonds and glycosidic linkages (Sun *et al*., 2016). Lignin provides structural support to the plant biomass and is mostly found in the plant cell walls (Ghatak *et al*., 2011). Lignin is soluble in solvents like lower alcohols, dioxane, acetone, pyridine, and dimethyl sulfoxide (Gall *et al*., 2017). Thermal softening of lignin occurs at higher temperatures in acidic or alkaline conditions that lead to depolymerization reactions and extensive delignification of biomass (Jazini *et al*., 2017).

The content of cellulose, hemicellulose and lignin in the lignocellulosic biomass depends on its source. Table 1.1 summarizes the composition of commonly used lignocellulosic biomass in bioethanol biorefineries.

Table 1.1. Composition of various lignocellulosic biomass.

Lignocellulose Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Hardwood stem	45.3	27.1	21.7	Sun and Cheng 2008
Softwood stem	27.2	30.4	30.5	Sun and Cheng 2008
Barley straw	33.4	22	14.1	nee'Nigam *et al*. 2009
Corn cobs	34.1	32	6.8	nee'Nigam *et al*. 2009
Corn stover	38.3	26	17.4	Li *et al*. 2010b
Cotton stalks	14.4	14.4	21.5	nee'Nigam *et al*. 2009
Wheat straw	30.2	32.1	17	Sun and Cheng 2008
Rice straw	43.5	30.3	18.5	Satlewal et al.2018
Rye straw	30.9	21.5	25	García-Cubero *et al*.2009
Oat straw	39.4	27.1	17.5	nee'Nigam *et al*. 2009
Soya stalks	35	25.1	9.8	nee'Nigam *et al*. 2009
Sunflower stalks	42.8	30.3	14.2	nee'Nigam *et al*. 2009
Switchgrass	39.5	25	21	Li *et al*. 2010a
Sugarcane bagasse	42.5	25.8	22.1	Nath *et al*. 2021
Sorghum durra stalk	31.8	23.2	5.7	Jamaldheen *et al*. 2018
Olive tree	25.0	15	18	Cara *et al*. 2008
Poplar	43.8	14.8	29.1	Kumar *et al*. 2009
Spruce	43.8	21	29	Shafiei *et al*. 2010
Oak	45.2	25	24	Shafiei *et al*. 2010

1.4 Chemical interactions among the components of lignocellulosic biomass

The lignocellulosic complex (cellulose, hemicellulose and lignin) is held together by four bonds: ether bonds, ester bonds, C-C bonds and hydrogen bonds (Shen *et al*., 2015). These four bonds act as binding linkages within the individual components of lignocellulose (intrapolymer linkages) or connect the constituting components to form the complex (interpolymer linkages).

1.4.1 Intrapolymer linkages

The glycosidic bond (ether bond) connects the glucose units thereby forming the polymeric chain of cellulose (Table 1.2). The hydrogen bond is formed between the

two hydroxyl groups of two different polymer chains, resulting in the crystalline fibrous structure of cellulose (Wolfe *et al*., 2002, Li *et al*., 2010c). The hemicellulosic molecules are mainly held together by the ether bonds (Shen *et al*., 2015). The ether bonds and C-C bonds mainly hold the building block molecules in the lignin polymer. Around 70% of the total bonds between the monomeric units of lignin are ether bonds, while the remaining 30% constitute C-C linkage (Gall *et al*., 2017). Table 1.2 summarizes the intramolecular linkage(s) between the individual polymers (cellulose, hemicellulose and lignin) and the intermolecular linkages between the polymers of the lignocellulosic complex.

1.4.2 Interpolymer linkages

The lignocellulosic complex can be broken down into individual components in order to understand the connectivity or linkage among its constituent polymers. But the resultant is the alteration of their original structure, therefore, the interpolymer linkage of the lignocellulose complex is not very well defined. However, it is reported that lignin is linked to cellulose and hemicellulose by hydrogen bond and covalent bond (Kshirsagar *et al*., 2015, Yu *et al*., 2017). Moreover, it was also shown that an ester bond links lignin with hemicellulose and an ether bond connects lignin with cellulose (Sun *et al*., 2016).

Table 1.2. Linkages between monomers of individual polymer and between the polymers of the lignocellulosic complex.

Lignocellulosic constituent	Intramolecular linkage	Intermolecular linkage
Cellulose	Ether bond, Hydrogen bond	-
Hemicellulose	Ether bond	-
Lignin	Ether bond, C-C bond	-
Cellulose-Lignin	-	Ether bond, Hydrogen bond
Hemicellulose-Lignin	-	Ester bond, Hydrogen bond
Cellulose-Hemicellulose	-	Hydrogen bond

For the efficient utilization of lignocellulosic biomass in biorefineries, the disruption of the lignocellulosic complex is necessary, which can be achieved by the cleavage of inter- and intra-polymer linkages during the pretreatment process. The disruption of these linkages occurs through solvolytic reactions under either acidic or alkaline conditions. On hydrolysis of the lignocellulosic complex under acidic conditions, the ether bond of lignin breaks and gets converted into the hydroxyl group and then to aldehyde or carboxylic acid group (He *et al*., 2008, Gu *et al*., 2015) as also shown in Fig. 1.2. In alkaline conditions, the aromatic rings in the lignin polymers are separated, thereby leading to depolymerization (Sun *et al*., 2016). The hydrogen bonds between the cellulose polymer chain and the hemicellulose polymer (Table 1.2) of the lignocellulosic complex are broken at an elevated temperature under dilute acidic or alkaline conditions (Imman *et al*., 2014).

1.5 Pretreatment of lignocellulosic biomass

The pretreatment process is an inevitable and crucial step in biomass annihilation. It loosens or breaks the inter-and intra-hydrogen bonds, ether bond, ester bond, and C-C bond between the lignin, cellulose and hemicellulose fraction of the biomass (Faulon *et al*., 1994). The pretreatment of biomass is carried out prior to enzymatic saccharification process to segregate the components of the lignocellulosic complex. The main aim of any pretreatment process is: i) to increase the porosity and surface area, ii) modification of lignin structure and also a higher extent of delignification, iii) to reduce the crystallinity of cellulose structure in the pretreated biomass, iv) partial depolymerization of hemicellulosic fraction, v) minimize the formation of unwanted inhibitors and vi) cost-effective process (Hassan *et al*., 2018). Various types of biomass pretreatment such as physical, chemical, physicochemical,

and biological pretreatments are described in subsequent sections and also illustrated in Fig. 1.1

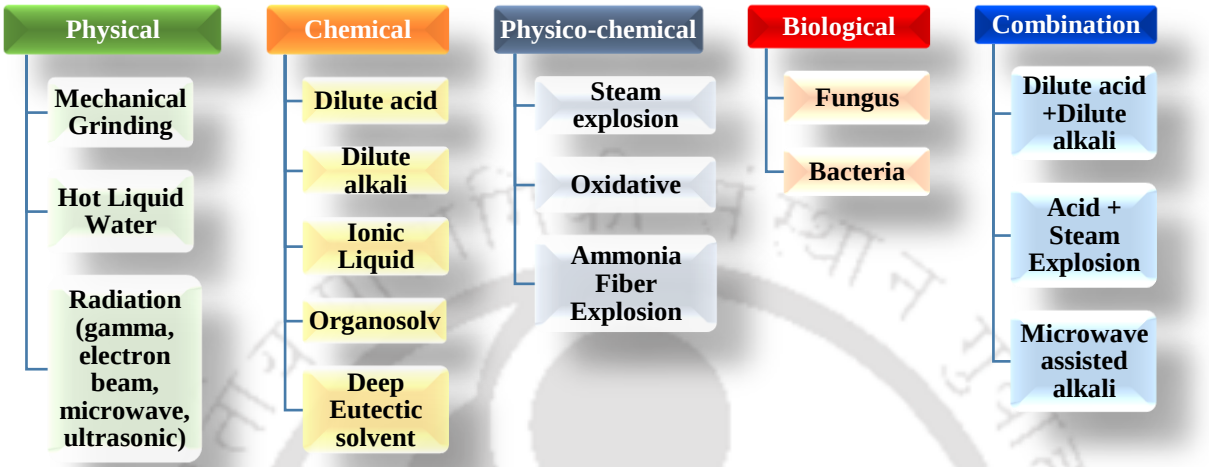

Fig. 1.1. Various pretreatment methods for lignocellulosic biomass.

1.5.1 Physical pretreatment

Physical pretreatment such as mechanical grinding, pyrolysis, steam explosion, liquid hot water treatment, and energy radiations can disrupt and soften the fiber of lignocellulosic biomass. The physical pretreatment decreases the particle size and crystallinity of fiber and increases the surface area (Harun *et al*., 2011). It results in lignin transformation and hemicellulose solubilization.

1.5.1.1 Mechanical grinding

Mechanical grinding reduces the size of the biomass and the crystallinity of the biomass fiber. With the increase in the degree of mechanical grinding, the particle's surface area increases, thereby making it suitable for enzymatic digestibility. Mechanical grinding can be achieved by chipping (10-30 mm particle size), grinding (0.2-2 mm particle size), and milling (Hideno *et al*., 2009). However, this method

requires large power input to attain the desired particle size and biomass characteristics and is a cost-intensive process (Mustafa *et al*., 2016).

1.5.1.2 Liquid hot water pretreatment

Liquid hot water pretreatment allows a relatively high hydrolysis of hemicelluloses and enhances the accessibility of cellulosic fraction by modifying the lignin structure (Prasad *et al*., 2023). This method has an advantage over others, as it does not require any chemical addition. It is generally performed at elevated pressure and temperature in the range, 150-230°C (Jiang *et al*., 2016).

1.5.1.3 Pretreatment by Radiation

Different irradiation methods such as gamma-ray, electron beam, microwave, and ultrasonication have been used for the pretreatment of lignocellulosic biomass to alter their internal structure (Yang *et al*., 2008). The cavitation damage of the cellulose structure is beneficial for enzymatic hydrolysis (Bak *et al*., 2009). However, due to the intensive energy requirement and substrate specificity, these processes are not commercially viable at the industrial level (Jacqueline *et al*., 2021).

1.5.2 Chemical pretreatment

The various reagents used in chemical pretreatment of lignocellulosic biomass are acid, alkali, oxidizing agents, surfactants, ionic liquids, organic solvents and deep eutectic solvents.

1.5.2.1 Dilute acid pretreatment

Dilute acid pre-treatment significantly changes the biodegradability of the biomass. The dilute acid hydrolyzes the hemicellulosic fraction and dissolves lignin to some extent. Dilute acid pretreatment results in an increment of crystallinity Index (*CrI*) of the pretreated biomass, which could be due to the removal of amorphous

hemicellulose and lignin (Table 1.4) (Kshirsagar *et al*., 2015). The peak intensities for hemicellulose are reduced after the dilute acid pretreatment and there is no change in the crystalline cellulose (Amnuaycheewa *et al*., 2016). However, dilute acid pretreatment does not remove silica effectively from the biomass. Inorganic acids such as sulfuric acid, hydrochloric acid, and nitric acid have been extensively used for the pretreatment of lignocellulosic biomass as they effectively remove hemicellulosic fractions and are cost-effective. However, biomass pretreatment with these strong acids leads to the corrosion of the bioreactor and other equipment, leading to various environmental issues. Therefore, organic acids such as formic acid, acetic acid, oxalic, maleic, and fumaric acid are presently being investigated as alternatives for biomass pretreatment (Amnuaycheewa *et al*., 2016).

1.5.2.2 Dilute alkali pretreatment

Alkali pretreatment loosens the ester linkage between cellulose, hemicellulose and lignin. This method is extensively used for herbaceous biomass and agriculture residues (Elumalai *et al*., 2016). Alkali pretreatment involves noncorrosive and ecofriendly chemicals such as sodium, potassium, calcium and ammonium ions of which sodium hydroxide is the most effective. It effectively breaks the side chains of the esters and glycosides causing cellulose swelling and decrystallization, slight disruption of hemicellulosic fraction and degradation of lignin (Ibrahim *et al*., 2011). In alkaline treatment, the cellulose crystallinity decreases thereby increasing the enzyme digestibility. This method operates in a milder condition with a longer incubation period and delignifies the biomass thereby enhancing the *CrI* (Table 1.4). Peak shift in FTIR analysis indicates the delignification intensity along with the

depolymerisation of carbohydrate (Table 1.4). The degree of desilication depends on the alkalinity of the pretreatment process (Yang *et al*., 2012).

1.5.2.3 Ionic liquids

The pretreatment of biomass by ionic liquids causes simultaneous dissolution of lignin and carbohydrate (Kuo & Lee, 2009). The ionic liquids containing organic salts such as 1,3-dimethyl imidazolium methyl sulfate, 1-butyl-3-methylimidazolium methyl sulfate, 1-allyl-3-methylimidazolium chloride, 1-n-butyl-3-methylimidazolium chloride, and 1-butyl-3-methylimidazolium alkylbenzenesulfonate have been extensively used for the pretreatment of raw biomass in recent years (Dadi *et al*., 2007, Kuo & Lee, 2009). The major benefit of this pretreatment method is that it results in the high recovery of amorphous cellulose (Zavrel *et al*., 2009).

1.5.2.4 Organosolv pretreatment

The use of organic solvents is one of the emerging pretreatment methods with the ability to fractionate cellulose, hemicellulose and lignin with high purity. The removal of lignin makes the cellulose easily accessible to the enzymes for its hydrolysis (Koo *et al*., 2011). Commonly used organic solvents are ethanol, acetone and tetrahydro furfuryl alcohol. Catalyst such as organic or inorganic acids and bases have been used for higher efficiency of pretreatment (Zhao *et al*., 2009). There is an advantage, of recovering the solvent used after the pretreatment by distillation process. The major disadvantages of this method include the low-boiling point of solvents, flammability and high volatility (Sun and Chen, 2008).

1.5.2.5 Deep eutectic solvent pretreatment

In the last few years, researchers have also explored the use of deep eutectic solvents for the pretreatment of lignocellulosic biomass as a green, ecological, and alternative to costly ionic liquid solvents (Kumar *et al*., 2016). Deep Eutectic Solvents (DES) comprises a hydrogen bond donor (HBD) and a hydrogen bonding acceptor (HBA) in a specific molar ratio. The HBD and HBA are held together by a strong hydrogen bond and break the lignin carbohydrate complex, thereby dissolving the hemicellulosic fraction and delignifying the biomass. Choline chloride-based solvents are commonly used in DES pretreatment of lignocellulosic biomass because of their biodegradability, low cost, and biocompatibility. The efficiency of pretreatment is greatly governed by the type of solvent and biomass (Xu *et al*., 2021). A comprehensive review of the modes of action, modifications, and delignification mechanism by various chemical pretreatment methods is summarized in Tables 1.3 and 1.4.

Table 1.3. Different pretreatment methods along with their mode of action, advantages and disadvantages using rice straw.

Pre-treatment	Process conditions	Proposed mode of action	Advantages	Disadvantages	References
Dilute acid	90-190°C, 0.1-3% acid, 5-30 min	• Breakage of glycosidic linkage and releasing of monosaccharides • Open up pores	• Low chemical cost • Required less enzyme with high recovery of monomeric hemicellulosic sugars	• Formation of inhibitors • High cost • The Need for corrosive-resistant equipment	• Agrawal *et al*. 2015 • Sun *et al*. 2016 • Kapoor *et al.* 2017 • Nedumaran *et al.* 2020
Alkali	120-150°C, 0.25-5% alkali, 10-20 min	• Reduce DP and crystallinity by cleaving glycosidic bond • Saponification of acetyl and uronic ester bonds	• More effective with agricultural residues • Higher delignification rate • Less formation of inhibitor	• Excessive removal of lignin and alter lignin structure • High alkali costs	• Imman *et al*. 2014 • Elumalai *et al*. 2016 • Jazini *et al*. 2017 • Ho *et al.* 2019 • Shukla *et al.* 2023
Steam explosion	180-250°C, 10-30 bar, 10-20 min	• Sudden pressure disrupts biomass structure and defibrillates cellulose bundles	• Chemicals are not required • High yield of hemicellulose • Low inhibitor formation	• Energy and water intensive	• Sharma *et al*. 2015, • Zhou *et al*. 2016, • Wood *et al*. 2016 • Pielhop *et al*. 2016
Organo-solv	100-190°C, 45-95% solvent, 10-90 min	• Hydrolyzed lignin and/ or hemicellulose	• Recovery of pure lignin • Low inhibitor generation • High yield of glucose	• The high cost of chemical • Required solvent recycle • High volatile and flammable	• Amiri *et al*, 2014, • Win *et al*. 2016, • Asadi & Zilouei, 2017 • Nath *et al.* 2021
Ionic liquid	70-150°C, 100% solvent, 30-1440 min	• Lignin removal • Reduce crystallinity	• Highly amorphous cellulose recovered • Saccharification required less enzyme • Mild reaction conditions	• High cost of chemical • Inhibits enzyme hydrolysis • Solvent recycling is required	• Chang *et al*. 2016, • Liu *et al*. 2016, • Chang *et al*. 2017 • Shukla *et al.* 2023
Deep eutectic	50-140°C, 50-100% solvent, 180-660 min	• Removal of lignin	• Saccharification requires less enzyme • Natural biodegradable chemicals • Cost effective • High xylose yield	• Required huge amount of water • Solvent recycling is required	• Kumar *et al*. 2016a, • Kumar *et al*. 2016b. • Tan *et al*. 2020.

Table 1.4. Physical and chemical modifications in pretreated rice straw after different chemical pre-treatment methods.

Pre-treatment	Crystallinity Index (%)	FTIR Peaks Rearrangements	Pore volume (m^2/g)	Enzymatic Saccharifi-cation (%)	References
Untreated	33.2-40.8	-	1.7-2.1	12-50	•Kshirsagar *et al*. 2015. • Wood *et al*. 2016. •Chang *et al*. 2017. •Shukla *et al*. 2023
Dilute acid	43-51.5	CH_2 bond of cellulose exposed while ester linkage between hemicellulose and lignin diminished	2.5-5	71-85	•Hsu *et al*. 2010. •Imman *et al*. 2014. •Kshirsagar *et al*. 2015 •Sun *et al*. 2016. •Nedumaran *et al.* 2020
Alkali	40.4-60.4	Carbonyl stretching of aromatic ring is absent, new peak of C-H binding in cellulose appeared	-	75.8-83.1	•He *et al*. 2008. •Gu *et al*. 2015. •Shukla *et al*. 2023
Steam	49-51	Peak intensity for C=C stretching in phenolic ring of lignin reduced	5.8-6.6	61-90	•Ibrahim *et al*. 2011. •Harun *et al*. 2013. •Gaur *et al*. 2015. •Zhou *et al*. 2016.
Organo-solv	54.5-58	Strong absorption peak of C-H binding of cellulose, ester linkage between hemicellulose and lignin decreased	-	~44-62	•Win *et al*. 2016. •Amnuaycheewa *et al*. 2016. •Nath *et al.* 2021
Ionic liquid	14-18.8	Reduction in crystalline cellulose, structural feature of hemicellulose, Absence of ester linkage between hemicellulose and lignin	-	36-93	•Liu *et al*. 2016. •Gogoi & Hazarika, 2017.
Deep eutectic	44.3-53	Reduction in peak intensity of ester linkage between hemicellulose and lignin	Increase by 5.4-fold	71-99	•Hou *et al*. 2015. •Kumar *et al*. 2016a

1.5.3 Physico-chemical pretreatment

1.5.3.1 Steam explosion

The application of steam at high temperature and pressure has an explosive action on the lignocellulosic biomass thereby fragmenting the biomass composition. Steam explosion pretreatment results in better hydrolysis of hemicellulose to its constituent monomers (Pielhop *et al*., 2016). Das *et al.* (2013 showed that pretreatment

of wild grass with a steam explosion at 121°C, 15 psi for 60 min yields significant monosaccharides and ethanol.

1.5.3.2 Ammonia Fiber Explosion

Ammonia Fiber Explosion (AFEX) is applicable to a variety of lignocellulosic biomass (Li *et al*., 2010b). The only difference of this pretreatment with the steam explosion is the use of ammonia at pH <12 instead of steam. In AFEX, the lignocellulosic biomass is treated with liquid ammonia at a 1:1 ratio at a temperature between 60-90°C, at above 3 MPa for 30-60 min. Here, the abrupt change in pressure causes explosive action on the biomass, resulting in swelling, a decrease in cellulose crystallinity and breaking of the lignin network (Uppugundla *et al*., 2014).

1.5.3.3 Oxidative pretreatment

The use of oxidizing agents such as hydrogen peroxide, ozone, or combination with alkali or ammonia significantly affects the biomass pretreatment (Ayeni *et al.*, 2013). Hydrogen peroxide break lignin-carbohydrates bonds by chemical reactions like electrophilic substitution reaction, side-chain displacement and oxidative cleavage of aromatic compounds and promote higher delignification efficiency (Alvira *et al*., 2010).

1.5.4 Biological pretreatment

The naturally occurring microorganisms and their secretions can act and break the recalcitrance of the lignocellulosic biomass. The white rot basidiomycete fungus and white rot *Irpex lacteus* can effectively hydrolyze hemicellulose and lignin (Sánchez *et al*., 2009). The commonly used white rot fungus such as *Pleurotus ostreaus* and *Cyathus stercolerus* produce peroxidases and laccases that degrade the lignin very effectively (Sánchez *et al*., 2009). The most commonly used basidiomycetes species,

which have high delignification efficiency, are *Lepista nuda* (Shi *et al*., 2008), *Trametes versicolor* (Planinić *et al*., 2016), and *Fomes fomentarius* (Kocher *et al*., 2017).

1.5.5 Combination of pretreatment methods

The single pretreatment method has some disadvantages: low, reducing sugar production, generation of inhibitors under higher concentrations of reagent used, and extreme operational conditions. Therefore, mild pretreatment methods may be combined to overcome these challenges and achieve higher saccharification efficiency and a higher ethanol yield. Some of the extensively used combined pretreatment methods are described in the sections below.

1.5.5.1 Alkaline pretreatment combined with dilute acid pretreatment

The acid pretreatment effectively hydrolyses the hemicellulosic fraction and increases the surface area of cellulosic fibers (Kshirsagar *et al*., 2015). However, only the acid pretreatment requires a higher concentration. Therefore, biomass can be first pretreated with an alkali to delignify partially the biomass (Sun *et al*., 2016). In a study, sugarcane bagasse was pretreated with 10% (w/v) NaOH at 3:1 liquid-to-solid ratio for 1.5h at 90°C in the first stage followed by para-acetic acid (PAA) with 10% (v/v) at 75°C for 2.5h. The (NaOH)-PAA pretreated sugarcane bagasse yields more reducing sugar as compared to single alkali or acid pretreatment (Zhao *et al*., 2010). Combination pretreatment of alkali and acid pretreatment reported a 92% yield of reducing sugars with cellulose loading of 15 FPU/g solid biomass at 120 h (Lu *et al*., 2009). Therefore, combined pretreatment of biomass by alkaline and dilute acid under mild conditions may result in effective lignin removal and lower carbohydrate degradation.

1.5.5.2 Dilute acid pretreatment combined with steam explosion pretreatments

Steam explosion pretreatment in presence of dilute acid can enhance the saccharification efficiency (Hsu *et al*., 2010). Dilute acid pretreatment enhances the xylose conversion and the steam explosion breaks down the lignin-hemicellulose linkage thereby modifying the lignocellulosic structure (Chen *et al*., 2011a). A study on rice straw pretreatment with 2% (v/v) H_2SO_4 at 165°C for 2 min in the first step followed by a steam explosion at 180°C for 20 min in the second step gave higher xylose yield, low inhibitor formation and a greater degree of enzymatic hydrolysis, resulting in 76% xylan conversion to xylose. The overall sugar yield was 1.5-fold higher when compared with the yield from acid-catalyzed steam explosion (Chen *et al*., 2011a).

1.5.5.3 Microwave-assisted alkali pretreatment

The synergistic effect of microwave irradiation and alkali can increase the chemical reaction rate and may lead to an explosive effect among the biomass particles. The microwave-based heating in the presence of alkali was employed instead of the conventional heating for the pretreatment of switchgrass (Li *et at.*, 2010b). Another study showed a high sugar yield of 59g/ 100g biomass obtained at 190°C with biomass content of 50 g/L, pretreated by sodium hydroxide for 30 min (Gabhane *et al*., 2011). This pretreatment method resulted in higher delignification with maximum hemicellulose degradation and a higher hydrolysis rate in lesser pretreatment time (Zhu *et al.*, 2005).

1.5.6 Analysis of lignocellulosic fractions after the pretreatment process

After the pretreatment process, the compositional analysis of the pretreated biomass is performed to check the efficiency of the pretreatment process using standard protocols. The extent of hemicellulose removal, the conversion percentage of cellulose and hemicellulose, delignification rate and the conversion percentage of inhibitors during the pretreatment process are calculated based on the following equations (Chen *et al.*, 2012):

$$\text{Hemicellulose removal (\%)} = \left(1 - \frac{\text{Hemicellulose in pretreated biomass}}{\text{Hemicellulose in raw biomass}} \times \text{solid yield}\right) \times 100 \quad \text{(Eqn. 1.1)}$$

$$\text{Delignification (\%)} = \left(1 - \frac{\text{Lignin content of pretreated biomass}}{\text{Lignin content of raw biomass}} \times \text{solid yield}\right) \times 100 \quad \text{(Eqn. 1.2)}$$

Where, solid yield = weight of pretreated biomass/ initial weight of raw biomass

$$\text{Conversion (xylose) (\%)} = \frac{\text{Xylose amount in liquid fraction} \times 0.88}{\text{Total xylan amount in raw biomass}} \times 100 \quad \text{(Eqn. 1.3)}$$

The value 0.88 is the ratio of molecular weight of $C_5H_8O_4$ over $C_5H_{10}O_5$

$$\text{Conversion (furfural) (\%)} = \frac{\text{Furfural amount in liquid fraction} \times 1.375}{\text{Total furfural amount in raw biomass}} \times 100 \quad \text{(Eqn. 1.4)}$$

The value 1.375 is the ratio of molecular weight of $C_5H_8O_4$ over $C_5H_4O_2$

$$\text{Conversion (glucose) (\%)} = \frac{\text{Glucose amount in liquid fraction} \times 0.9}{\text{Total glucose amount in raw biomass}} \times 100 \quad \text{(Eqn. 1.5)}$$

The value 0.9 is the ratio of molecular weight of $C_6H_{10}O_5$ over $C_6H_{12}O_6$

$$\text{Conversion (HMF) (\%)} = \frac{\text{HMF amount in liquid fraction} \times 1.286}{\text{Total HMF amount in raw biomass}} \times 100 \quad \text{(Eqn. 1.6)}$$

The value 1.286 is the ratio of molecular weight of $C_6H_{10}O_5$ over $C_6H_6O_3$

$C_5H_8O_4$: hemicellulose; $C_5H_{10}O_5$: xylose; $C_6H_{10}O_5$: cellulose; $C_6H_{12}O_6$: glucose; $C_5H_4O_2$: furfural; $C_6H_6O_3$: HMF (Chen *et al.*, 2012).

1.5.7 Desilication of raw biomass

The lignocellulosic biomass such as rice straw contains a high amount of silica which makes it difficult for its bioprocessing. The silica layer causes a shearing problem while grinding. Moreover, it also inhibits enzymatic and microbial actions. It prevents degradation by microbes and makes hydrolysis difficult resulting in lower saccharification and ethanol yields (Khaleghian *et al.*, 2015). Thus, silica removal from rice straw is of prime concern during the simultaneous saccharification and fermentation process. Therefore, an effective pretreatment method is required to investigate for softening and removal of silica along with a reduction in lignin content and crystallinity of rice straw. The pretreatment of silica-containing biomass by sodium compound at high temperatures leads to the formation of sodium silicate, which can be easily removed (Khaleghian *et al.*, 2017). They reported over 90% removal of silica from rice straw by sodium carbonate and sodium hydroxide treatment at high temperatures, 90-100°C for 1-3 h (Khaleghian *et al.*, 2017). They subsequently pretreated the desilicated rice straw with organosolv which gave enhanced glucose and ethanol yields of 95% and 79%, respectively, against 53% and 52% respectively, by organosolv pretreatment alone and 34% and 39% by untreated rice straw (Khaleghian *et al.*, 2017). A similar study on rice straw with sodium carbonate pretreatment for silica removal reported 100% glucose and 83% ethanol yield which were significantly higher than 35% glucose and 40% ethanol yield obtained from the control (Khaleghian *et al.*, 2015).

1.5.8 Formation of inhibitors during pretreatment of biomass

The lignocellulosic biomass pretreatment also produces certain compounds like acetic acid, formic acid, levulinic acid, furfural, 5-hydroxymethylfurfural (HMF), and

phenols that have an inhibitory effect on the fermentation process (Michelin *et al*., 2016). These compounds are generally termed fermentation inhibitors as they have a toxic effect on the fermenting microbes. The toxicity level depends on the concentration of individual inhibitors and the synergistic effect of inhibitors when they are present in large numbers (Palmqvist & Hahn-Hägerdal, 2000b). It also partly depends on fermentation variables like resistivity of fermenting microbes against the inhibitors, oxygen level, and the pH of the medium (Palmqvist & Hahn-Hägerdal, 2000b).

1.5.8.1 Origin of inhibitors and mechanisn of inhibition

Extensive hydrolysis of hemicellulose and cellulose leads to the degradation of the released pentose sugar (xylose) to furfural, and hexose sugar (glucose) to hydroxymethyl-furfural (HMF) (Kim *et al*., 2011). Both furfural and HMF are reported to affect cell growth and respiration of the fermenting microbes (Qin *et al*., 2016). Furfural is more toxic than HMF and its production is enhanced with an increase in pretreatment temperature and the reaction time (Qin *et al*., 2016). Various compounds such as aromatic, polyaromatic, phenolic, and aldehydic are also released during the pretreatment process from the hydrolysis of the lignin fraction. Phenolic compounds are more toxic (even at low concentrations) than furfural and HMF. Low molecular weight phenolic compounds such as ferulic acid and aldehydes are reported to be the most toxic amongst all the inhibitors (Michelin *et al*., 2016). Phenolic compounds cause integral cell damage, thereby reducing the fermenting microbes' cell growth, leading to mono sugars accumulation and causes feedback inhibition in the fermentation process (Michelin *et al*., 2016). The acetyl groups present in hemicellulose result in the formation of acetic acid on hydrolysis. Acetic acid inhibits the fermenting

microbe growth by lowering the pH. HMF, on further degradation, leads to the production of formic acid and levulinic acid (Palmqvist & Hahn-Hägerdal, 2000b).

Fig. 1.2 illustrates the origin and formations of various inhibitors (phenolic compounds, furans, and aliphatic carboxylic acid) during the hydrolysis of the lignocellulosic complex

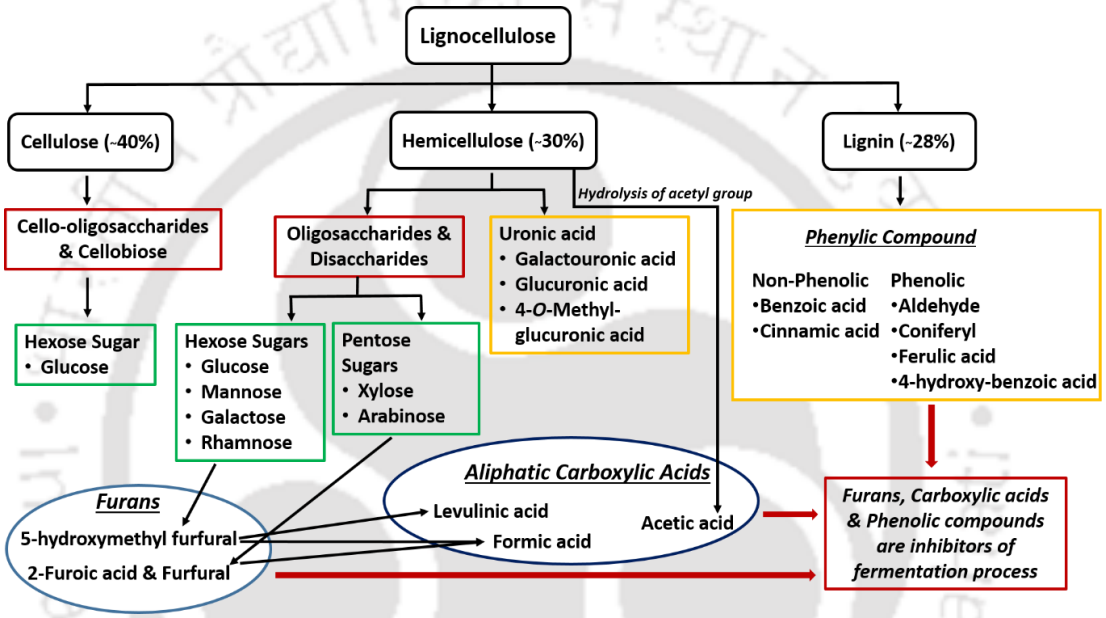

Fig. 1.2. Origin and formation of different inhibitors during hydrolysis of lignocellulosic complex (Maibam and Goyal, 2022)

1.6 Enzymatic hydrolysis of pretreated lignocellulosic biomass

The pretreated biomass that is rich in a cellulosic and hemicellulosic fraction is used as a substrate for enzymatic hydrolysis for the production of fermentable C6 and C5 reducing sugars. Therefore, cellulases and hemicellulases are required for the saccharification of pretreated biomass. The enzymes, cellulases and hemicellulases act specifically on complex cellulosic and hemicellulosic polysaccharides and hydrolyze them into fermentable monosaccharides.

1.6.1 Cellulases

Cellulases serves as the most vital enzyme in lignocellulosic biorefineries as it hydrolyzes the complex cellulosic fraction to glucose. Cellulases comprise three enzymes: i) endo β-(1-4)-glucanase that randomly acts on amorphous cellulose and produces cello-oligosaccharides of varying degrees of polymerization (DP) (Makroo *et al*. 2017), ii) exoglucanase acts on crystalline cellulose, amorphous cellulose and cello-oligosaccharides and produces cellobiose (Thakur *et al.*, 2020). The exoglucanase that cleaves at the reducing end is called cellobiohydrolase-I and the cellobiohydrolase-II cleaves at the non-reducing end of cellulose and iii) β-glucosidase hydrolyze cellobiose to monomeric glucose from the non-reducing end (Thakur *et al.*, 2020). The complete hydrolysis of cellulose to glucose requires the synergistic action of these three enzymes. *Trichoderma spp*. have been extensively used as a source of cellulolytic enzymes at the industrial level for more than two decades (Maibam and Maiti., 2020). Cellulase enzyme from *Trichoderma reesei* has shown its potential to hydrolyze various lignocellulosic biomasses, but the major bottleneck for the bioethanol industry is the high cost of the commercial *Trichoderma reesei* cellulolytic enzymes (Panda *et al*., 2019). So, the use of engineered enzymes with high catalytic activity paved the way for an efficient and cost-effective saccharification process. Singh *et al*. (2020) reported that UV-directed evolution in endoglucanase increased the specific activity and catalytic efficiency against carboxymethylcellulose sodium salt (CMC-Na) by 10-fold and 22-fold, respectively when compared with wild type (Singh *et al*., 2020). Another study on engineered strain of *Penicillium funiculosum* (*Pf*Mig188) reported an enzyme activity of 2.3 U/mL (exoglucanase), 28 U/mL (endoglucanase), 150 U/mL (β-glucosidase) and 76 U/mL (xylanase) (Ogunyewo *et al*., 2020). The enzymatic saccharification using 3%

(w/v) recombinant enzyme loading with 15% (w/v) biomass loading on nitric acid pretreated rice straw released 100 g/L sugars in the hydrolysate with 80% saccharification efficiency which substantiates that the *Pf*Mig188 secretome is a feasible substitute for *T. reesei*-based secretome for saccharification process (Ogunyewo *et al*., 2020). The cellulosome of *Clostridium thermocellum* showed a high rate of cellulose utilization resulting in 50-fold higher activity than that obtained by *T. reesei* system (Deka et at., 2013). For the reduction of saccharification time and production cost, Nath *et al*. (2019) developed a chimeric enzyme (*Ct*GH1-L1-CtGH5-F194A) having bifunctional activities of β-glucosidase (*Ct*GH1) and endoglucanase (*Ct*GH5-F194A) from *Clostridium thermocellum* that can catalyze two reactions during the hydrolysis of pretreated biomass (Nath *et al*., 2019).

1.6.2 Hemicellulase

Hemicellulose having a complex structure requires different enzymes for effective hydrolysis (Van Dyk *et al*., 2012). Hemicellulases can be classified into two groups; i) enzymes that break the backbone are term as core enzymes and ii) those enzymes that remove the side-chain substitution are termed as ancillary or auxillary enzymes (Sapag *et al*., 2002). These side chains often pose a steric hindrance to de-polymerizing enzymes, therefore the use of ancillary enzyme removes the side chain and thus increases the overall yield of reducing sugars during hemicellulose hydrolysis (van den Brink *et al*., 2011).

The complete hydrolysis of the complex xylan structure needs the collaborative action of a repertoire of enzymes, such as endo-β-xylanase and β-xylosidase for main chain cleavage and α-arabinofuranosidase, α-glucuronidase, p-coumaric acid esterase, acetyl xylan esterase and ferulic acid esterase for the side chain cleavage (Sharma *et*

al., 2018). Xylan backbone contains side chains of arabinose, glucuronic acid, ferulic-p-coumaric acid and acetate. These side chains interfere with the xylan hydrolysis and can be removed by ancillary enzymes such as arabinofuranosidase, acetyl xylan esterase, glucuronidase and ferulic acid esterase / p-coumaric acid esterase for effective xylan hydrolysis (Alvira *et al*., 2011). The two major enzymes involved in the hydrolysis of the xylan backbone are endo-β-1,4-xylanase and β-xylosidase. Endo-1,4-β-xylanase hydrolyzes the β-(1, 4) bonds between xylose units in the backbone of xylan and releases xylooligosaccharides (Sapag *et al*., 2002). β-Xylosidase hydrolyses xylan from the non-reducing end and also cleaves xylobiose to xylose (van den Brink *et al*., 2011).

1.6.3 Recombinant enzyme cocktail in biomass hydrolysis

The enzymatic saccharification process requires the application of multiple enzymes with higher catalytic efficiency to hydrolyze the cellulosic and hemicellulosic fractions into monosaccharides. However, the requirement of several enzymes in the saccharification process is cost-intensive and a challenging task. Therefore, the development of enzyme cocktails in vitro for efficient saccharification of the whole biomass is essential (Kallioinen *et al*., 2014). Past research on enzymatic cocktail optimization was focused only on commercially modeled cellulosic substrates like Sigmacell (Baker *et al*., 1998) and filter paper (Kim *et al*., 1998). Recently, several studies have reported the usage of enzyme cocktails for industrially relevant pretreated lignocellulosic substrates such as rice straw, sorghum stalk, sugarcane bagasse, finger millet, corn stover and wheat straw (Banerjee *et al*., 2010, Gao *et al*., 2011, Kallioinen *et al*., 2014, Chylenski *et al*., 2017). A specific enzyme cocktail is required for specific pretreated biomass based on the type of pretreatment used (Adsul *et al*.,

2020). The optimum enzyme ratio in a cocktail for efficient hydrolysis can be predicted by rational mixture design experiments based on synergistic effects of enzymes on the pretreated biomass (Bussamra *et al*., 2015). A study reported that enzymatic saccharification of alkali pretreated rice straw using commercial enzyme cocktail comprising cellulase and pectinase in an optimum ratio as calculated by response surface method (RSM) resulted in a saccharification efficiency of 94% (Takano *et al*., 2018). However, the application of commercial enzymes is cost-intensive. Therefore, the use of recombinant, engineered, chimeric enzymes and their cocktails could be a cost-effective alternative for the hydrolysis of lignocellulosic biomass (Adsul *et al*., 2020). A study on saccharification of acid and base pretreated *Sorghum durra stalk* by using crude recombinant cellulolytic enzyme cocktail with loading of 2000 U/g concluded that the enzyme ratio in a cocktail is specific for the type of pretreatment used for particular biomass (Nedumaran *et al*., 2020). Nath *et al*. (2021) reported that a recombinant cellulolytic enzyme cocktail consisting of cellobiohydrolase and chimera (β-glucosidase and endoglucanase) in the ratio 2:3 gave a total reducing sugar yield of 230 mg/g of pretreated sugarcane bagasse after the enzymatic hydrolysis (Nath and Maibam, 2021). Saccharification of pretreated finger millet straw by recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) under optimized conditions resulted in a saccharification efficiency of 25% (Jamaldheen *et al*., 2019). A study reported that the enzymatic hydrolysis of 1 g wheat arabinoxylan as a substrate by recombinant *Phanerochaete chrysosporium* xylanolytic enzyme cocktail comprising xylanase (r*Pc*XynC), arabinanase (r*Pc*Ara) and bifunctional β-xylosidase/L-arabinofuranosidase (r*Pc*Xyl) in the ratio (U) 11:3:91 resulted in 100% saccharification (Huy *et al*., 2020). They further reported that 42% saccharification efficiency could be

achieved on hydrolysis of pretreated barley straw by an enzyme cocktail of 25 U r*Pc*XynC, 5 U r*Pc*Ara, and 200 U r*Pc*Xyl (Huy *et al*., 2020). Therefore, the application of a recombinant enzyme cocktail increases the yield of fermentable sugars in the hydrolysate.

1.7. Fermentation

There are various types of fermentation processes for bioethanol production from lignocellulosic biomass. Generally, the sugar-rich hydrolysate obtained after enzymatic saccharification is used as a substrate for the fermentation process (Kuhad *et al*., 2010). The choice of the type of fermentation depends on the characteristics of biomass, the enzyme used in saccharification and the type of microorganisms used. The fermenting microbes and various fermentation strategies employed for bioethanol production are discussed below:

1.7.1 Fermenting microbes

The conventional ethanologens i.e. *Saccharomyces cerevisiae* and *Zymomonas mobilis* are glucose metabolizing microbes while *Candida shehatae* and *Pichia stipiti* are the major xylose metabolizing yeasts that have been extensively used in the fermentation process for the production of ethanol (Wirawan *et al*., 2020). *Zymomonas mobilis* gave high ethanol yield and ethanol productivity when compared to *S. cerevisiae* and also possess high ethanol-tolerance (Wirawan *et al*., 2020). *P. stipitis* has the capability of carrying out the fermentations in both aerobic and oxygen limiting conditions and is therefore commonly used for xylose production. The property of *P. stipitis* to ferment xylose to produce ethanol has been a key advantage to combat the inability to ferment pentose by *Saccharomyces cerevisiae* (Agbogbo *et al*., 2008). During the enzymatic saccharification of the pretreated lignocellulosic substrate using

cellulolytic and xylanolytic enzymes, both pentose and hexose are released in the hydrolysate. Therefore, genetically engineered recombinant fermenting strains have been developed for metabolizing both hexose and pentose during fermentation. Furthermore, various strategies have been adopted for the co-fermentation of natural variant C5 and C6 metabolizing strains. Genetically modified *S. cerevisiae* has been employed recently, but it was found that the xylose uptake rate by this modified strain is significantly lower as compared with the glucose uptake rate (Chen *et al*., 2011b). Engineered *Z. mobilis* strain showed enhancement in xylose utilization rate by 49% in the presence of glucose when compared to the wild-type thereby leading to a substantial reduction in fermentation time with concomitant utilization of glucose and xylose resulting in high ethanol production (Sarkar *et al*., 2021). The co-culturing of *Z. mobilis* and *P. stipitis* showed that xylose fermentation by *P. stipitis* is inhibited *by Z. mobilis* cells (Fu *et al*., 2009). Therefore, either designing a special fermenter for the immobilization of *Z. mobilis* strain or a sequential co-fermentation of *P. stipitis* and *Z. mobilis* could enhance the fermentation of both glucose and xylose (Fu *et al*., 2009, Singh *et al*., 2014). Thus, fermenting microbial strains are selected based on the performance parameters like microbial growth, substrate uptake rate, product yield and productivity of ethanol and operating conditions, pH and temperature.

1.7.2 Ethanol production strategies

The characteristics of the saccharifying enzymes and those of ethanologenic microbial strains are the major factors for the selection of a particular fermentation strategy. The fermentation process is classified into four types based on enzymatic saccharification and have been described in the following sub-sections.

1.7.2.1 Separate hydrolysis and fermentation

The enzyme saccharification of pretreated biomass and the fermentation of its hydrolysate for bioethanol production are separately carried out in different reactors, is termed as separate hydrolysis and fermentation (SHF). This process has an advantage over others, as the separate saccharification can be performed at higher temperatures (35-50°C) for higher TRS yield, followed by the fermentation that is preferably carried out at lower temperatures (30-37°C) for optimal performance (Saha *et al*., 2005). However, the major disadvantages associated with SHF method are contamination in the saccharification step and the feedback inhibition of hydrolytic enzymes by the produced monosaccharides (Alfred and Pramuk, 2023).

1.7.2.2 Simultaneous Saccharification and Fermentation

The simultaneous saccharification and fermentation (SSF) is the first integrated bioprocess for both biomass hydrolysis and fermentation of the released monosaccharides to produce bioethanol. The SSF is a vital process in the biofuel industry to optimize the efficiency and productivity of bioethanol. The SSF process offers numerous advantages such as reduced fermentation time, decreased capital costs, reduced extent of feedback inhibition by glucose and increased rates of hydrolysis (Alfred and Pramuk, 2023). Another merit of this method is that it gives higher bioethanol yield at lower enzyme loadings (Alfred and Pramuk, 2023). Thus, SSF process results in an overall improved productivity and ethanol yield. However, shortcomings with SSF processes include differences in optimum temperatures that are required for enzymatic activity, microbial growth and ethanol fermentation. Thus, the challenging task in the SSF method is to find compatible operating conditions such as pH, temperature and substrate concentration for both saccharification and fermentation

for maximum bioethanol production (Afedzi *et al.*, 2022). Further, an effective SSF process also requires optimization of enzymatic hydrolysis by determining the suitable enzymes and their concentrations and assessing the impact of inhibitors on enzyme activity. Over the years, several studies have been carried out with a focus on the modification of the SSF process as part of the biorefinery concept to solve the problems of temperature and pH incompatibility, increase ethanol concentration and efficiency to reduce the overall cost (Boonchuay *et al.*, 2021). Pre-saccharification and simultaneous saccharification and fermentation (PSSF) is the first modification of SSF process developed to address the problem of suboptimal temperatures for hydrolysis and fermentation (Öhgren *et al*., 2007). The PSSF is generally applied when the total solid is to be fed once at the beginning without subsequent addition. Engineered strains are used in PSSF process and it was reported that an engineered *S. cerevisiae* Y-904 strain produced an ethanol concentration of 5.7% w/w in a short time of 45h, resulting in an ethanol yield of 290 L of ethanol per ton of sugarcane straw (Pratto *et al*., 2020).

Another modification in SSF process is the application of the fed-batch to the pre-hydrolysis stage in order to increase solids loading and sugar concentration. This is known as fed-batch pre-saccharification and simultaneous saccharification and fermentation (FP-SSF). FP-SSF strategy allows the increased production in the concentration of fermentable sugars compared to the PSSF and also concurrently maintains low viscosity of the high solids, thus reducing the problems associated with viscosity and improves mass and heat transfer (Hemansi and Saini, 2023). However, prolonged PS time in FP-SSF of above 24 h, yeast cells experience osmotic stress due to high glucose concentration, which may lead to a lower ethanol yield (Li *et al*., 2014). Therefore, a short-fed-batch PS is recommended for efficient and maximum production

with no effect on substrate or product inhibition on the enzyme and fermenting organisms (Li *et al*., 2014). To improve enzyme hydrolysis in FP-SSF strategy while minimizing the enzyme dosage, supplementation with xylanases, ferulic acid esterase, acetyl xylan esterase, etc., is crucial as it augments glucose yield in acid-catalyzed hydrothermally pretreated brewers spent grain (Wilkinson *et al*., 2016). In addition, it is suggested that proper optimization be conducted to determine the appropriate time for the PS by considering the sufficient glucose concentration needed for fermentation to begin.

1.7.2.3 Simultaneous Saccharification and Co-Fermentation

In simultaneous sccharification and co-fermentation (SSCF), the reducing sugars (glucose and xylose) in the hydrolysate are fermented by ethanologenic microbes. In co-fermentation, pentose and hexose utilizing yeasts are co-cultured for economical bioconversion of lignocellulosic biomass to bioethanol. Moreover, the development of engineered yeast strains with a high capacity for simultaneous co-fermentation of hexose and pentose sugars is also a better alternative that has attracted substantial attraction in recent years. A recombinant strain of *Saccharomyces cerevisiae* was developed by evolutionary engineering methods followed by random mutagenesis and used for ethanol production from steam pretreated corn cob slurry by SSCF process (Koppram *et al*., 2013). The use of genetically engineered *S. cerevisiae* XUSEA (developed by genome editing followed by DNA transformation approach) for mixed-sugar (Saccharomate hydrolysate from lignocellulosic hydrolysate of *Miscanthus sacchariflorus Goedae-Uksae* 1) fermentation of glucose (39 g/L) and xylose (23 g/L) reported the ethanol titer, 30 g/L and ethanol yield, 0.48 g/g at 24 h, which was 2-fold higher than that of its parent strain (Tran *et al*., 2020). They further reported the

complete conversion of glucose (76 g/L) and xylose (46 g/L) to ethanol with a yield of 0.5 g/g in 72 h. The SSCF method utilizing cocktail of recombinant endoglucanase (*Ct*GH5) and arabinofurnaosidase (*Ct*GH43) both from *Clostridium thermocellum* with co-fermentation of *C. shehatae* and *S. cerevisiae* resulted in 23 g/L ethanol titer that was twice higher than that obtained with only CtGH5 and *Saccharomyces cerevisiae* with 5% (w/v) pretreated wild grass (Das *et al*., 2013). A comparative evaluation of SSCF and SHCF (Separate Hydrolysis and Co-Fermnetaion) for ethanol production from alkali pretreated sugarcane bagasse by immobilized *Zymomonas mobilis* and suspended *Pichia stipitis* resulted in 0.41 g ethanol/g cellulose by SSCF, which was higher than 0.36 g ethanol/g cellulose obtained by SHCF process (Wirawan *et al*., 2020).

1.7.2.4 Consolidated Bioprocessing

In Consolidated Bioprocessing (CBP), the process of conversion of lignocellulosic biomass to bioethanol is performed in one pot by using a single microorganism (Cardona *et al*., 2007). The same microorganism is capable of producing the cellulolytic and xylanolytic enzymes, followed by performing the enzymatic saccharification and fermenting the released monosaccharides to bioethanol (Cardona *et al*., 2007). Therefore, CBP requires relatively lower capital and operational costs than the other methods (Cardona *et al*., 2007). The most critical part for CBP is the requirement of a suitable microbe for cellulolytic and hemicellulolytic enzyme production, saccharification and fermentation. A single microbe having the potential of executing all these processes has yet to be found in nature, therefore the use of microbial consortium is the only plausible way for CBP (Zuroff *et al*., 2013).

1.8 Significance and objectives of the present study

1.8.1 Significance of Investigation

Rice straw is a potential lignocellulosic biomass for the production of biofuel. The bioconversion of rice straw to bioethanol could be a plausible solution to combat the present energy crisis and environmental pollution. The global rice straw production is 370-520 million tonnes/year (International Rice Research Institute, IRRI 2019) of which 330-470 million tonnes is prodcuded by Asia alone and it is 112.7 million tonnes by India (Van Hung *et al*. 2020). Rice straw is utilized for various purposes including ruminant feed, paper industries, fertilizer, heating and cooking purposes and as a feedstock for biogas production (Chandel *et al*., 2018). Despite all these applications, more than 50% of straw is burnt in the open field and only 20% of rice straw production is utilized for bioenergy production (Oladosu *et al*., 2016). Considering 34.2-40.6% of cellulose and 19.3-20.6% of hemicellulose present in rice straw, the theoretical maximum reducing sugars that could be produced from the entire rice straw would be 59-68% (Akhtar *et al*. 2017). And the theoretical ethanol production per tonne of rice straw would be 305-348 kg or 387-442 L. The reported yields of ethanol from rice straw are far less than the maximum theoretical ethanol yield (Molaverdi *et al*., 2019). Therefore, more efficient pretreatments, saccharification and fermentation processes need to be developed to enhance the ethanol yield, which could be nearing the theoretical yield. It is in this spirit that the current thesis work is planned and an attempt was made to develop a process for optimal bioconversion of rice straw to bioethanol at laboratory scale in a 5 L bioreactor level. All aspects of the processes: i) selection of suitable pretreatment for maximum delignification and maximum exposure for digestible cellulose, ii) development and formulation of efficient enzyme cocktail

preparation for efficient enzymatic hydrolysis process and iii) selection of suitable fermentation process for maximizing ethanol yield and productivity were stepwise addressed. The specific objectives that are formulated for the proposed work are given below.

1.8.2 Specific objectives

1) Screening and selection of best pretreatment method for rice straw biomass based on maximum delignification and retainment of cellulosic content.
2) Statistical optimization of pretreatment parameters of the selected pretreatment method and characterization of delignified pretreated rice straw.
3) Designing of crude recombinant hydrolytic enzymes cocktail for effective saccharification of delignified pretreated rice straw.
4) Statistical optimization of pre-saccharification simultaneous saccharification and fermentation of delignified pretreated rice straw using the developed crude recombinant enzyme cocktail and *Saccharomyces cerevisiae* MTCC170 for maximizing yield and productivity of bioethanol.
5) Scale-up of bioethanol production from delignified pretreated rice straw in 5L fermenter at optimized conditions of pre-saccharification simultaneous saccharification and fermentation and mass balance for the bioconversion of biomass to ethanol.

1.9 References

Abdmouleh, Z., Alammari, R. A. and Gastli, A. (2015). Review of policies encouraging renewable energy integration & best practices. Renewable and Sustainable Energy Reviews, 45, 249-262.

Adsul, M., Sandhu, S. K., Singhania, R. R., Gupta, R., Puri, S. K. and Mathur, A. (2020). Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels. Enzyme and microbial technology, 133, 109442.

Afedzi, A. E. K. and Parakulsuksatid, P. (2023). Recent advances in process modifications of simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass for bioethanol production. Biocatalysis and Agricultural Biotechnology, 102961

Afedzi, A. E. K., Rattanaporn, K. and Parakulsuksatid, P. (2022). Impeller selection for mixing high-solids lignocellulosic biomass in stirred tank bioreactor for ethanol production. Bioresource Technology Reports, 17, 100935.

Agbogbo, F. K. and Coward-Kelly, G. (2008). Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. Biotechnology Letters, 30(9), 1515-1524.

Agrawal, R., Gaur, R., Mathur, A., Kumar, R., Gupta, R. P., Tuli, D. K. and Satlewal, A. (2015). Improved saccharification of pilot-scale acid pretreated wheat straw by exploiting the synergistic behavior of lignocellulose degrading enzymes. RSC Advances, 5(87), 71462-71471.

Akhtar, N., Goyal, D. and Goyal, A. (2017). Characterization of microwave-alkali-acid pre-treated rice straw for optimization of ethanol production via simultaneous

saccharification and fermentation (SSF). Energy Conversion and Management, 141, 133-144.

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

Amiri, H., Karimi, K. and Zilouei, H. (2014). Organosolv pretreatment of rice straw for efficient acetone, butanol and ethanol production. Bioresource Technology, 152, 450-456.

Amnuaycheewa, P., Hengaroonprasan, R., Rattanaporn, K., Kirdponpattara, S., Cheenkachorn, K. and Sriariyanun, M. (2016). Enhancing enzymatic hydrolysis and biogas production from rice straw by pretreatment with organic acids. Industrial Crops and Products, 87, 247-254.

Antunes, F. A. F., Chandel, A. K., Terán-Hilares, R., Ingle, A. P., Rai, M., dos Santos Milessi, T. S. and dos Santos, J. C. (2019). Overcoming challenges in lignocellulosic biomass pretreatment for second-generation (2G) sugar production: emerging role of nano, biotechnological and promising approaches. 3 Biotech, 9(6), 230.

Asadi, N . and Zilouei, H. (2017). Optimization of organosolv pretreatment of rice straw for enhanced biohydrogen production using Enterobacter aerogenes. Bioresource Technology, 227, 335-344

Ayeni, A. O., Banerjee, S., Omoleye, J. A., Hymore, F. K., Giri, B. S., Deshmukh, S. C. and Mudliar, S. N. (2013). Optimization of pretreatment conditions using full factorial design and enzymatic convertibility of shea tree sawdust. Biomass and Bioenergy, 48, 130-138.

Bak, J. S., Ko, J. K., Han, Y. H., Lee, B. C., Choi, I. G. and Kim, K. H. (2009). Improved enzymatic hydrolysis yield of rice straw using electron beam irradiation pretreatment. Bioresource Technology, 100(3), 1285-1290.

Baker, J. O., Ehrman, C. I., Adney, W. S., Thomas, S. R. and Himmel, M. E. (1998). Hydrolysis of cellulose using ternary mixtures of purified cellulases. In Biotechnology for Fuels and Chemicals, 395-403.

Bals, B., Rogers, C., Jin, M., Balan, V. and Dale, B. (2010). Evaluation of ammonia fibre expansion (AFEX) pretreatment for enzymatic hydrolysis of switchgrass harvested in different seasons and locations. Biotechnology for Biofuels, 3(1), 1.

Banerjee, G., Scott-Craig, J. S. and Walton, J. D. (2010). Improving enzymes for biomass conversion: a basic research perspective. Bioenergy research, 3(1), 82-92.

Boonchuay, P., Techapun, C., Leksawasdi, N., Seesuriyachan, P., Hanmoungjai, P., Watanabe, M., Srisupa, S. and Chaiyaso, T. (2021). Bioethanol production from cellulose-rich corncob residue by the thermotolerant *Saccharomyces cerevisiae* TC-5. Journal of Fungi, 7(7), 547.

Cara, C., Ruiz, E., Ballesteros, M., Manzanares, P., Negro, M. J. and Castro, E. (2008). Production of fuel ethanol from steam-explosion pretreated olive tree pruning. Fuel, 87(6), 692-700.

Cardona, C. A. and Sánchez, Ó. J. (2007). Fuel ethanol production: process design trends and integration opportunities. Bioresource Technology, 98(12), 2415-2457.

Chandel, A.K., Garlapati, V.K., Singh, A.K., Antunes, F.A.F. and da Silva, S.S. (2018). The path forward for lignocellulose biorefineries: Bottlenecks, solutions and perspective on commercialization. Bioresource Technology, 264, 370-381.

Chang, K.L., Chen, X.M., Han, Y.J., Wang, X.Q., Potprommanee, L., Ning, X.A., Liu, J.Y., Sun, J., Peng, Y.P., Sun, S.Y. and Lin, Y.C. (2016). Synergistic effects of surfactant-assisted ionic liquid pretreatment rice straw. Bioresource Technology, 214, 371-375.

Chang, K.L., Chen, X.M., Wang, X.Q., Han, Y.J., Potprommanee, L., Liu, J.Y., Liao, Y.L., Ning, X.A., Sun, S.Y., and Huang, Q. (2017). Impact of surfactant type for ionic liquid pretreatment on enhancing delignification of rice straw. Bioresource Technology, 227, 388-392.

Chang, V. S. and Holtzapple, M. (2000) Fundamentals factors affecting biomass reactivity. Applied Biochemistry and Biotechnology, 84-86, 5-37.

Chen, W.H., Pen, B.L., Yu, C.T., Hwang, W.S. (2011). Pretreatment efficiency and structural characterization of rice straw by an integrated process of dilute-acid and steam explosion for bioethanol production. Bioresource Technology, 102(3), 2916-2924.

Chen, Y. (2011). Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: a systematic review. Journal of Industrial Microbiology and Biotechnology, 38(5), 581-597.

Chylenski, P., Forsberg, Z., Ståhlberg, J., Várnai, A., Lersch, M., Bengtsson, O. and Eijsink, V. G. (2017). Development of minimal enzyme cocktails for hydrolysis of sulfite-pulped lignocellulosic biomass. Journal of Biotechnology, 246, 16-23.

Claassen, P. A. M., van Lier, J. B., Lopez Contreras, A. M., van Niel, E. W. J., Sijtsma, L., Stams, A. J. M., de Vries, S. S. and Weusthuis, R. A. (1999) Utilisation of biomass for the supply of energy carriers. Applied Microbiology and Biotechnology, 52, 741-755.

Dadi, A. P., Schall, C. A. and Varanasi, S. (2007) Mitigation of cellulose recalcitrance to enzymatic hydrolyis by ionic liquid pretreatment. Applied Biochemistry and Biotechnology, 137, 407-421.

Das, S. P., Deka, D., Ghosh, A., Das, D., Jawed, M.and Goyal, A. (2013). Scale up and efficient bioethanol production involving recombinant cellulase (Glycoside hydrolase family 5) from Clostridium thermocellum. Sustainable Chemical Processes, 1(1), 19.

Das, S. P., Ghosh, A., Gupta, A., Goyal, A. and Das, D. (2013). Lignocellulosic fermentation of wild grass employing recombinant hydrolytic enzymes and fermentative microbes with effective bioethanol recovery. BioMed Research International, 2013

Das, S. P., Ravindran, R., Ghosh, A., Deka, D., Das, D., Jawed, M.and Goyal, A. (2014). Efficient pretreatment for bioethanol production from water hyacinth (*Eichhornia crassipes*) involving naturally isolated and recombinant enzymes and its recovery. Environmental Progress and Sustainable Energy, 33(4), 1396-1404.

Deka, D., Jawed, M.and Goyal, A. (2013). Purification and characterization of an alkaline cellulase produced by *Bacillus subtilis* (AS3). Preparative Biochemistry and Biotechnology, 43(3), 256-270.

Demain, A. L., Newcomb, M.and Wu, J. D. (2005). Cellulase, clostridiaand ethanol. Microbiology and Molecular Biology Reviews, 69(1), 124-154.

Demirbas, A. (2008). Heavy metal adsorption onto agro-based waste materials: a review. Journal of Hazardous Materials, 157(2-3), 220-229.

Elumalai, S., Agarwal, B.and Sangwan, R. S. (2016). Thermo-chemical pretreatment of rice straw for further processing for levulinic acid production. Bioresource Technology, 218, 232-246.

Faulon, J. L., Carlson, G. A. and Hatcher, P. G. (1994). A three-dimensional model for lignocellulose from gymnospermous wood. Organic Geochemistry, 21(12), 1169-1179.

Fu, D., Mazza, G., Tamaki, Y. (2010). Lignin extraction from straw by ionic liquids and enzymatic hydrolysis of the cellulosic residues. Journal of Agricultural and Food Chemistry, 58(5), 2915-2922.

Fu, N., Peiris, P., Markham, J. and Bavor, J. (2009). A novel co-culture process with *Zymomonas mobilis* and *Pichia stipitis* for efficient ethanol production on glucose/xylose mixtures. Enzyme and Microbial Technology, 45(3), 210-217.

Gabhane, J., Prince William, S.P.M., Vaidya, A.N., Mahapatra, K., Chakrabarti, T. (2011). Influence of heating source on the efficacy of lignocellulosic pretreatment - A cellulosic ethanol perspective. Biomass and Bioenergy, 35(1), 96-102.

Gall, D. L., Ralph, J., Donohue, T. J. and Noguera, D. R. (2017). Biochemical transformation of lignin for deriving valued commodities from lignocellulose. Current Opinion in Biotechnology, 45, 120-126.

Gao, D., Uppugundla, N., Chundawat, S. P., Yu, X., Hermanson, S., Gowda, K. and Dale, B. E. (2011). Hemicellulases and auxiliary enzymes for improved conversion of lignocellulosic biomass to monosaccharides. Biotechnology for Biofuels, 4(1), 5.

García-Cubero, M. T., González-Benito, G., Indacoechea, I., Coca, M. and Bolado, S. (2009). Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. Bioresource Technology, 100(4), 1608-1613.

Gaur, R., Agrawal, R., Kumar, R., Ramu, E., Bansal, V.R., Gupta, R.P., Kumar, R., Tuli, D.K. and Das, B. (2015). Evaluation of recalcitrant features impacting enzymatic saccharification of diverse agricultural residues treated by steam explosion and dilute acid. RSC Advances, 5(75), 60754-60762.

Gaurav, N., Sivasankari, S., Kiran, G. S., Ninawe, A.and Selvin, J. (2017). Utilization of bioresources for sustainable biofuels: a review. Renewable and Sustainable Energy Reviews, 73, 205-214.

Ghatak, H. R. (2011). Biorefineries from the perspective of sustainability: Feedstocks, productsand processes. Renewable and Sustainable Energy Reviews, 15(8), 4042-4052.

Ghosh, P. and Ghose, T. K. (2003) Bioethanol in India: recent past and emerging future. Advances in Biochemical Engineering/Biotechnology, 85, 1-27

Gogoi, G. and Hazarika, S. 2017. Coupling of ionic liquid treatment and membrane filtration for recovery of lignin from lignocellulosic biomass. Separation and Purification Technology, 173, 113-120.

Gu, Y., Zhang. and Zhou, X. (2015). Effect of Ca $(OH)_2$ pretreatment on extruded rice straw anaerobic digestion. Bioresource Technology, 196, 116-122.

Hans, M., Kumar, S., Chandel, A. K.and Polikarpov, I. (2019). A review on bioprocessing of paddy straw to ethanol using simultaneous saccharification and fermentation. Process Biochemistry, 85, 125-134.

Harun, M. Y., Radiah, A. D., Abidin, Z. Z. and Yunus, R. (2011). Effect of physical pretreatment on dilute acid hydrolysis of water hyacinth (*Eichhornia crassipes*). Bioresource technology, 102(8), 5193-5199.

Harun, S., Balan, V., Takriff, M.S., Hassan, O., Jahim, J. and Dale, B.E. (2013). Performance of AFEX™ pretreated rice straw as source of fermentable sugars: The influence of particle size. Biotechnology for Biofuels, 6(1), 123-134.

Hassan, S. S., Williams, G. A. and Jaiswal, A. K. (2018). Emerging technologies for the pretreatment of lignocellulosic biomass. Bioresource Technology, 262, 310-318.

He, Y., Pang, Y., Liu, Y., Li, X. and Wang, K. (2008). Physicochemical characterization of rice straw pretreated with sodium hydroxide in the solid state for enhancing biogas production. Energy and Fuels, 22(4), 2775-2781.

Hemansi and Saini, J. K. (2023). Enhanced cellulosic ethanol production via fed-batch simultaneous saccharification and fermentation of sequential dilute acid-alkali pretreated sugarcane bagasse. Bioresource Technology, 372, 128671.

Hideno, A., Inoue, H., Tsukahara, K., Fujimoto, S., Minowa, T., Inoue, S., Endo, T. and Sawayama, S. (2009) Wet disc milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. Bioresource Technology, 100, 2706-2711.

Ho, M. C., Ong, V. Z. and Wu, T. Y. (2019). Potential use of alkaline hydrogen peroxide in lignocellulosic biomass pretreatment and valorization–A review. Renewable and Sustainable Energy Reviews, 112, 75-86.

Hou, X.D., Xu, J., Li, N. and Zong, M.H. (2015). Effect of anion structures on cholinium ionic liquids pretreatment of rice straw and the subsequent enzymatic hydrolysis. Biotechnology and Bioengineering, 112(1), 65-73.

Hsu, T.C., Guo, G.L., Chen, W.H. and Hwang, W.S. (2010). Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresource Technology, 101(13), 4907-4913.

Huy, N. D., Saravanakumar, T., Ha, S. H. and Park, S. M. (2020). Enhanced enzymatic saccharification of wheat flour arabinoxylan and barley straw using recombinant hemicellulases. Biotechnology and Bioprocess Engineering, 25, 431-441.

Ibrahim, M.M., El-Zawawy, W.K., Abdel-Fattah, Y.R., Soliman, N.A. and Agblevor, F.A. (2011). Comparison of alkaline pulping with steam explosion for glucose production from rice straw. Carbohydrate Polymers, 83(2), 720-726.

Imman, S., Arnthong, J., Burapatana, V., Champreda, V. and Laosiripojana, N. (2014). Effects of acid and alkali promoters on compressed liquid hot water pretreatment of rice straw. Bioresource Technology, 171, 29-36.

Itelima, J., Ogbonna, A., Pandukur, S., Egbere, J. and Salami A. (2013) Simultaneous saccharification and fermentation of corn cobs to bio-ethanol by co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae*. International Journal of Environmental Science and Development, 4, 239-242.

Jacqueline, P. J., Muthuraman, V. S., Karthick, C., Alaswad, A., Velvizhi, G. and Nanthagopal, K. (2022). Catalytic microwave preheated co-pyrolysis of lignocellulosic biomasses: A study on biofuel production and its characterization. Bioresource Technology, 347, 126382.

Jamaldheen, S. B., Sharma, K., Rani, A., Moholkar, V. S. and Goyal, A. (2018). Comparative analysis of pretreatment methods on sorghum (Sorghum durra) stalk agrowaste for holocellulose content. Preparative Biochemistry and Biotechnology, 48(6), 457-464.

Jamaldheen, S. B., Thakur, A., Moholkar, V. S. and Goyal, A. (2019). Enzymatic hydrolysis of hemicellulose from pretreated Finger millet (*Eleusine coracana*) straw by recombinant endo-1, 4-β-xylanase and exo-1, 4-β-xylosidase. International Journal of Biological Macromolecules, 135, 1098-1106.

Jazini, M.H., Fereydouni, E. and Karimi, K. (2017). Microbial xanthan gum production from alkali-pretreated rice straw. RSC Advances, 7(6), 3507-3514.

Jiang, D. P., Ge, X. M., Zhang, Q. G. and Li, Y. B. (2016). Comparison of liquid hot water and alkaline pretreatments of giant reed for improved enzymatic digestibility and biogas energy production. Bioresource Technology, 216, 60-68.

Kallioinen, A., Puranen, T. and Siika-aho, M. (2014). Mixtures of thermostable enzymes show high performance in biomass saccharification. Applied biochemistry and biotechnology, 173(5), 1038-1056.

Kapoor, M., Soam, S., Agrawal, R., Gupta, R.P., Tuli, D.K. and Kumar, R. (2017). Pilot scale dilute acid pretreatment of rice straw and fermentable sugar recovery at high solid loadings. Bioresource Technology, 224, 688-693.

Khaire, K. C., Maibam, P. D., Thakur, A. and Goyal, A. (2022). Biomedical and pharmaceutical applications of xylan and its derivatives. In Brienzo M (ed) Hemicellulose biorefinery: A Sustainable Solution for Value Addition to Bio-Based Products and Bioenergy. Springer, Singapore, pp 447-465.

Khaleghian, H., Karimi, K. and Behzad, T. (2015). Ethanol production from rice straw by sodium carbonate pretreatment and *Mucor hiemalis* fermentation. Industrial Crops and Products, 76, 1079-1085.

Khaleghian, H., Molaverdi, M. and Karimi, K. (2017). Silica removal from rice straw to improve its hydrolysis and ethanol production. Industrial and Engineering Chemistry Research, 56(35), 9793-9798.

Kim, E., Irwin, D. C., Walker, L. P. and Wilson, D. B. (1998). Factorial optimization of a six-cellulase mixture. Biotechnology and Bioengineering, 58(5), 494-501.

Kocher, G. S., Kaur, P. and Taggar, M. S. (2017). An overview of pretreatment processes with special reference to biological pretreatment for rice straw delignification. Current Biochemical Engineering, 4(3), 151-163..

Koo, B.W., Kim, H.Y., Park, N., Lee, S.M., Yeo, H. and Choi, I.G. (2011). Organosolv pretreatment of Liriodendron tulipifera and simultaneous saccharification and fermentation for bioethanol production. Biomass and Bioenergy, 35(5), 1833-1840.

Koppram, R., Nielsen, F., Albers, E., Lambert, A., Wännström, S., Welin, L. and Olsson, L. (2013). Simultaneous saccharification and co-fermentation for bioethanol production using corncobs at lab, PDU and demo scales. Biotechnology for Biofuels, 6(1), 2.

Kshirsagar, S.D., Waghmare, P.R., Chandrakant Loni, P., Patil, S.A. and Govindwar, S.P. (2015). Dilute acid pretreatment of rice straw, structural characterization and optimization of enzymatic hydrolysis conditions by response surface methodology. RSC Advances, 5(58), 46525-46533.

Kumar, A.K., Parikh, B.S., Shah, E., Liu, L.Z. and Cotta, M.A. (2016). Cellulosic ethanol production from green solvent-pretreated rice straw. Biocatalysis and Agricultural Biotechnology, 7, 14-23.

Kumar, A.K., Parikh, B.S. and Pravakar, M. (2016a). Natural deep eutectic solvent mediated pretreatment of rice straw: bioanalytical characterization of lignin extract and enzymatic hydrolysis of pretreated biomass residue. Environmental Science and Pollution Research, 23(10), 9265-9275.

Kumar, A.K., Parikh, B.S., Shah, E., Liu, L.Z. and Cotta, M.A. (2016b). Cellulosic ethanol production from green solvent-pretreated rice straw. Biocatalysis and Agricultural Biotechnology, 7, 14-23.

Kumar, R., Mago, G., Balan, V. and Wyman, C. E. (2009). Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresource Technology, 100(17), 3948-3962.

Li, B. Z., Balan, V., Yuan, Y. J. and Dale, B. E. (2010a). Process optimization to convert forage and sweet sorghum bagasse to ethanol based on ammonia fiber expansion (AFEX) pretreatment. Bioresource Technology, 101(4), 1285-1292..

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H. V., Auer, M. and Singh, S. (2010b). Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. Bioresource Technology, 101(13), 4900-4906.

Li, M. F., Fan, Y. M., Xu, F., Sun, R. C. and Zhang, X. L. (2010c). Cold sodium hydroxide/urea based pretreatment of bamboo for bioethanol production: Characterization of the cellulose rich fraction. Industrial Crops and Products, 32(3), 551-559.

Li, X., Lu, J., Zhao, J. and Qu, Y. (2014). Characteristics of corn stover pretreated with liquid hot water and fed-batch semi-simultaneous saccharification and fermentation for bioethanol production. PloS One, 9(4), e95455.

Liu, C.G., Qin, J.C., Liu, L.Y., Jin, B.W. and Bai, F.W. (2016). Combination of Ionic Liquid and Instant Catapult Steam Explosion Pretreatments for Enhanced Enzymatic Digestibility of Rice Straw. ACS Sustainable Chemistry and Engineering, 4(2), 577-582.

Lu, X., Zhang, Y. and Angelidaki, I. (2009). Optimization of H_2SO_4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: Focusing on pretreatment at high solids content. Bioresource Technology, 100(12), 3048-3053.

Maibam, P.D. and Goyal, A. (2022). Pretreatment methods for overcoming biomass recalcitrance, in: Verma, P. (Eds.), Enzymes in the Valorization of Waste. CRC Press, pp. 1-21.

Maibam, P. D. and Goyal, A. (2023). Designing of recombinant hydrolytic enzymes cocktail for effective saccharification of delignified rice straw. Industrial Crops and Products, 206, 117727.

Maibam, P. D. and Maiti, S. K., (2020). A strategy for simultaneous xylose utilization and enhancement of cellulase enzyme production by *Trichoderma reesei*

cultivated on liquid hydrolysate followed by induction with feeding of solid sugarcane bagasse. Waste and Biomass Valorization. 11, 3151-3160.

Makroo, H. A., Rastogi, N. K. and Srivastava, B. (2017). Enzyme inactivation of tomato juice by ohmic heating and its effects on physico-chemical characteristics of concentrated tomato paste. Journal of Food Process Engineering, 40(3), e12464.

Molaverdi, M., Karimi, K., Mirmohamadsadeghi, S. and Galbe, M. (2019). High titer ethanol production from rice straw via solid-state simultaneous saccharification and fermentation by *Mucor indicus* at low enzyme loading. Energy Conversion and Management, 182, 520-529.

Mussatto, S. I. and Roberto, I. C. (2004). Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. Bioresource Technology, 93(1), 1-10.

Mustafa, A. M., Poulsen, T. G., Xia, Y. H. and Sheng, K. C. (2016). Combinations of fungal and milling pretreatments for enhancing rice straw biogas production during solid-state anaerobic digestion. Bioresource Technology, 224, 174-182.

Nath, P., Dhillon, A., Kumar, K., Sharma, K., Jamaldheen, S. B., Moholkar, V. S. and Goyal, A. (2019). Development of bi-functional chimeric enzyme (*Ct*GH1-L1-*Ct*GH5-F194A) from endoglucanase (*Ct*GH5) mutant F194A and β-1, 4-glucosidase (*Ct*GH1) from *Clostridium thermocellum* with enhanced activity and structural integrity. Bioresource Technology, 282, 494-501.

Nath, P., Maibam, P. D., Singh, S., Rajulapati, V. and Goyal, A. (2021). Sequential pretreatment of sugarcane bagasse by alkali and organosolv for improved delignification and cellulose saccharification by chimera and cellobiohydrolase for bioethanol production. 3 Biotech. 11, 1-16.

Nedumaran, M., Singh, S., Jamaldheen, S. B., Nath, P., Moholkar, V. S. and Goyal, A. (2020). Assessment of combination of pretreatment of *Sorghum durra stalk* and production of chimeric enzyme (β-glucosidase and endo β-1, 4 glucanase, *Ct*GH1-L1-*Ct*GH5-F194A) and cellobiohydrolase (*Ct* CBH5A) for saccharification to produce bioethanol. Preparative Biochemistry & Biotechnology, 1-14.

nee'Nigam, P.S., Gupta, N. and Anthwal, A. (2009). Pre-treatment of agro-industrial residues. in: Biotechnology for agro-industrial residues utilisation, Springer, pp. 13-33.

Nitske, W. R. (1965). Rudolf Diesel: pioneer of the age of power. Business History Review (pre-1986), 39(000002), 280.

Ogunyewo, O. A., Randhawa, A., Gupta, M., Kaladhar, V. C., Verma, P. K. and Yazdani, S. S. (2020). Synergistic action of a lytic polysaccharide monooxygenase and a cellobiohydrolase from *Penicillium funiculosum* in cellulose saccharification under high-level substrate loading. Applied and Environmental Microbiology, 86(23), e01769-20.

Öhgren, K., Vehmaanperä, J., Siika-Aho, M., Galbe, M., Viikari, L. and Zacchi, G. (2007). High temperature enzymatic prehydrolysis prior to simultaneous saccharification and fermentation of steam pretreated corn stover for ethanol production. Enzyme and Microbial Technology, 40(4), 607-613.

Oladosu, Y., Rafii, M.Y., Abdullah, N., Magaji, U., Hussin, G., Ramli, A. and Miah, G. (2016). Fermentation Quality and Additives: A Case of Rice Straw Silage. BioMed Research International, 2016.

Palmqvist, E.and Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technology, 74(1), 25-33.

Pielhop, T., Amgarten, J., Von Rohr, P.R.and Studer, M.H. (2016). Steam explosion pretreatment of softwood: The effect of the explosive decompression on enzymatic digestibility. Biotechnology for Biofuels, 9(1).

Planinić, M., Zelić, B., Čubel, I., Bucić-Kojić, A. and Tišma, M. (2016). Corn forage biological pretreatment by Trametes versicolor in a tray bioreactor. Waste Management & Research, 34(8), 802-809.

Prasad, B. R., Padhi, R. K. and Ghosh, G. (2023). A review on key pretreatment approaches for lignocellulosic biomass to produce biofuel and value-added products. International Journal of Environmental Science and Technology, 20(6), 6929-6944.

Pratto, B., dos Santos-Rocha, M. S. R., Longati, A. A., de Sousa Júnior, R. and Cruz, A. J. G. (2020). Experimental optimization and techno-economic analysis of bioethanol production by simultaneous saccharification and fermentation process using sugarcane straw. Bioresource technology, 297, 122494.

Saha, B.C., Iten, L.B., Cotta, M.A. and Wu, Y.V. (2005). Dilute acid pretreatment, enzymatic saccharificationand fermentation of rice hulls to ethanol. Biotechnology Progress, 21(3), 816-822.

Sánchez, C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnology Advances, 27(2), 185-194.

Sapag, A., Wouters, J., Lambert, C., De Ioannes, P., Eyzaguirre, J. and Depiereux, E. (2002). The endoxylanases from family 11: computer analysis of protein

sequences reveals important structural and phylogenetic relationships. Journal of Biotechnology, 95(2), 109-131.

Sarkar, P., Goswami, G., Mukherjee, M., and Das, D. (2021). Heterologous expression of xylose specific transporter improves xylose utilization by recombinant *Zymomonas mobilis* strain in presence of glucose. Process Biochemistry.

Satlewal, A., Agrawal, R., Bhagia, S., Das, P. and Ragauskas, A.J. (2018). Rice straw as a feedstock for biofuels: Availability, recalcitranceand chemical properties. Biofuels, Bioproducts and Biorefining, 12(1), 83-107.

Schädel, C., Blöchl, A., Richter, A.and Hoch, G. (2009). Short-term dynamics of nonstructural carbohydrates and hemicelluloses in young branches of temperate forest trees during bud break. Tree Physiology, 29(7), 901-911.

Shafiei, M., Karimi, K. and Taherzadeh, M. J. (2010). Pretreatment of spruce and oak by N-methylmorpholine-N-oxide (NMMO) for efficient conversion of their cellulose to ethanol. Bioresource Technology, 101(13), 4914-4918.

Sharma, K., Antunes, I. L., Rajulapati, V. and Goyal, A. (2018). Low-resolution SAXS and comparative modeling based structure analysis of endo-β-1, 4-xylanase a family 10 glycoside hydrolase from *Pseudopedobacter saltans comb. nov*. International Journal of Biological Macromolecules, 112, 1104-1114.

Sharma, S., Kumar, R., Gaur, R., Agrawal, R., Gupta, R.P., Tuli, D.K. and Das, B. (2015). Pilot scale study on steam explosion and mass balance for higher sugar recovery from rice straw. Bioresource Technology, 175, 350-357.

Shen, D., Jin, W., Hu, J., Xiao, R. and Luo, K. (2015). An overview on fast pyrolysis of the main constituents in lignocellulosic biomass to valued-added chemicals:

Structures, pathways and interactions. Renewable and Sustainable Energy Reviews, 51, 761-774.

Shi, J., Chinn, M.S. and Sharma-Shivappa, R.R. (2008). Microbial pretreatment of cotton stalks by solid state cultivation of *Phanerochaete chrysosporium*. Bioresource Technology, 99(14), 6556-6564.

Shukla, A., Kumar, D., Girdhar, M., Kumar, A., Goyal, A., Malik, T. and Mohan, A. (2023). Strategies of pretreatment of feedstocks for optimized bioethanol production: distinct and integrated approaches. Biotechnology for Biofuels and Bioproducts, 16(1), 44.

Siddique, M. N. I. and Wahid, Z. A. (2018). Achievements and perspectives of anaerobic co-digestion: a review. Journal of Cleaner Production, 194, 359-371..

Singh, L. K., Majumder, C. B. and Ghosh, S. (2014). Development of sequential-co-culture system (*Pichia stipitis* and *Zymomonas mobilis*) for bioethanol production from Kans grass biomass. Biochemical Engineering Journal, 82, 150-157.

Singh, R., Srivastava, M. and Shukla, A. (2016). Environmental sustainability of bioethanol production from rice straw in India: a review. Renewable and Sustainable Energy Reviews, 54, 202-216.

Singh, S., Rajulapati, V., Jamaldheen, S. B., Moholkar, V. S. and Goyal, A. (2020). Statistically designed cellulase mixture for saccharification of pretreated Sorghum durra stalk. Industrial Crops and Products, 154, 112678..

Solarte-Toro, J. C., Romero-García, J. M., Martínez-Patiño, J. C., Ruiz-Ramos, E., Castro-Galiano, E. and Cardona-Alzate, C. A. (2019). Acid pretreatment of lignocellulosic biomass for energy vectors production: A review focused on

operational conditions and techno-economic assessment for bioethanol production. Renewable and Sustainable Energy Reviews, 107, 587-601.

Sun, F. and Chen, H. (2008). Organosolv pretreatment by crude glycerol from oleochemicals industry for enzymatic hydrolysis of wheat straw. Bioresource Technology, 99(13), 5474-5479.

Sun, S., Chen, W., Tang, J., Wang, B., Cao, X., Sun, S. and Sun, R.C. (2016). Synergetic effect of dilute acid and alkali treatments on fractional application of rice straw. Biotechnology for Biofuels, 9(1).

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology, 83(1), 1-11.

Tan, Y. T., Chua, A. S. M. and Ngoh, G. C. (2020). Deep eutectic solvent for lignocellulosic biomass fractionation and the subsequent conversion to bio-based products–A review. Bioresource Technology, 297, 122522.

Thakur, A., Sharma, K., Khaire, K. C., Moholkar, V. S. and Goyal, A. (2020). Enzymes: Key Role in the Conversion of Waste to Bioethanol. Microbial Fermentation and Enzyme Technology.

Uppugundla, N., da Costa Sousa, L., Chundawat, S.P., Yu, X., Simmons, B., Singh, S., Gao, X., Kumar, R., Wyman, C.E. and Dale, B.E. (2014). A comparative study of ethanol production using dilute acid, ionic liquid and AFEX pretreated corn stover. Biotechnology for Biofuels, 7(1), 72.

van den Brink, J. and de Vries, R. P. (2011). Fungal enzyme sets for plant polysaccharide degradation. Applied Microbiology and Biotechnology, 91(6), 1477.

Van Dyk, J. S. and Pletschke, B. I. (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy. Biotechnology Advances, 30(6), 1458-1480.

Van Nguyen, H., Nguyen, C. D., Van Tran, T., Hau, H. D., Nguyen, N. T. and Gummert, M. (2016). Energy efficiency, greenhouse gas emission and cost of rice straw collection in the Mekong River Delta of Vietnam. Field Crops Research, 198, 16-22.

Win, N.N., Weinwurm, F. and Friedl, A. (2016). Investigation of organosolv and hot-compressed water pretreatments of rice straw. Biomass Conversion and Biorefinery, 6(3), 355-364.

Wirawan, F., Cheng, C. L., Lo, Y. C., Chen, C. Y., Chang, J. S., Leu, S. Y. and Lee, D. J. (2020). Continuous cellulosic bioethanol co-fermentation by immobilized *Zymomonas mobilis* and suspended *Pichia stipitis* in a two-stage process. Applied Energy, 266, 114871.

Wilkinson, S., Smart, K. A., James, S. and Cook, D. J. (2016). Maximising high solid loading enzymatic saccharification yield from acid-catalysed hydrothermally-pretreated brewers spent grain. Biofuel Research Journal, 3(2), 417-429.

Wolfe, B.B., Edwards, T.W., Elgood, R.J. and Beuning, K.R. (2002). Carbon and oxygen isotope analysis of lake sediment cellulose: methods and applications. in: Tracking environmental change using lake sediments, Springer, pp. 373-400.

Wood, I.P., Cao, H.G., Tran, L., Cook, N., Ryden, P., Wilson, D.R., Moates, G.K., Collins, S.R.A., Elliston, A. and Waldron, K.W. (2016). Comparison of

saccharification and fermentation of steam exploded rice straw and rice husk. Biotechnology for Biofuels, 9(1).

Xu, H., Kong, Y., Peng, J., Song, X., Liu, Y., Su, Z. and Tian, W. (2021). Comprehensive analysis of important parameters of choline chloride-based deep eutectic solvent pretreatment of lignocellulosic biomass. Bioresource Technology, 319, 124209.

Yang, C., Shen, Z., Yu, G. and Wang, J. (2008) Effect and after effect of gamma radiation pretreatment on enzymatic hydrolysis of wheat straw. Bioresource Technology, 99, 6240-6245.

Yang, L., Cao, J., Jin, Y., Chang, H. M., Jameel, H., Phillips, R. and Li, Z. (2012). Effects of sodium carbonate pretreatment on the chemical compositions and enzymatic saccharification of rice straw. Bioresource Technology, 124, 283-291.

Yu, J., Paterson, N., Blamey, J. and Millan, M. (2017). Cellulose, xylan and lignin interactions during pyrolysis of lignocellulosic biomass. Fuel, 191, 140-149.

Zavrel, M., Bross, D., Funke, M., Büchs, J.and Spiess, A.C. (2009). High-throughput screening for ionic liquids dissolving (ligno-) cellulose. Bioresource Technology, 100(9), 2580-2587.

Zhao, X., Cheng, K. and Liu, D. (2009). Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Applied Microbiology and Biotechnology, 82(5), 815-827.

Zhao, X., van der Heide, E., Zhang, T. and Liu, D. (2010). Delignification of sugarcane bagasse with alkali and peracetic acid and characterization of the pulp. BioResources, 5(3), 1565-1580.

Zhou, J., Yan, B.H., Wang, Y., Yong, X.Y., Yang, Z.H., Jia, H.H., Jiang, M. and Wei, P. (2016). Effect of steam explosion pretreatment on the anaerobic digestion of rice straw. RSC Advances, 6(91), 88417-88425.

Zhu, S., Wu, Y., Yu, Z., Liao, J. and Zhang, Y. (2005). Pretreatment by microwave/alkali of rice straw and its enzymic hydrolysis. Process Biochemistry, 40(9), 3082-3086.

Zuroff, T.R., Xiques, S. B.and Curtis, W. R. (2013). Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic *Clostridium phytofermentans*/yeast co-culture. Biotechnology for Biofuels, 6(1), 59.

Chapter 2

Screening and selection of a suitable pretreatment method for rice straw

2.1 Introduction

The utilization of lignocellulosic biomass for production of second-generation (2G) fuel, bioethanol promotes green energy. The production of 2G bioethanol involves pretreatment, saccharification (enzymatic hydrolysis), and fermentation (Afedzi and Parakulsuksatid, 2023). However, a detailed understanding of the feedstock's characteristics is essential for selecting the appropriate pretreatment and hydrolysis strategies. A comprehensive feedstock analysis involves assessing the composition of lignocellulosic material, that includes cellulose, hemicellulose and lignin content (Maibam and Goyal, 2022). Parameters such as structural complexity, heterogeneity of the constituent polymers, composition of lignin, cellulose crystallinity, surface area and pore size implied the recalcitrance nature of the lignocellulosic biomass (Pakchamni *et*

al., 2022). The pretreatment of biomass is performed prior to enzymatic saccharification to overcome its recalcitrance. Pretreatment of biomass is performed to increase the surface area and pore size, reduce the crystallinity and disrupt and remove the lignin content to maximize the cellulose accessibility to the enzymatic hydrolysis (Baruah *et al*., 2018). Various pretreatment methods such as physical, chemical, physico-chemical and biological methods are available and being practiced (Pakchamni *et al*., 2022). A detailed insights of various pretreatment methods currently being employed for rice straw pretreatment are discussed in Chapter 1, Section 1.5 (Tables 1.3 and 1.4).

The cellulosic fractions have high chemical resistance, being insoluble in most solvents due to their crystalline structure and associated hydrogen bonds (Ghatak, 2011). However, hemicelluloses are easily hydrolyzed by acids, thus more sensitive to chemical and thermal treatment than cellulose (Loow *et al*., 2015). This property of hemicellulose being readily soluble to any chemical or thermal treatment is due to the lack of crystallinity and the lower degree of polymerization, DP (up to 200). Lignin on the other hand gets only slightly affected by acids or enzymes, therefore, is the most resistant natural polymer. However, it is more sensitive to oxidation reactions and alkali agents than the polysaccharides (Alvira *et al*., 2010).

The disintegration of lignocellulosic biomass by adopting a suitable pretreatment method is necessary for the efficient utilization and conversion of these three polymers. Specific pretreatment methods resulted in specific physical and chemical properties of the polymer (Singh *et al*., 2020). Thus, it can be stated that there is no ideal pre-treatment method for a particular biomass. The best pretreatment method is selected based on the set of requirements which involves, high sugar recovery, low

formation of toxic compounds, *i.e.* fermentation and enzymatic hydrolysis inhibitors, targeted end product (lignin, cellulose rich fibre, xylan, ethanol, xylitol, etc), cost-effective, low toxicity and biodegradablility (Shastri *et al*., 2014, Singh *et al*., 2020). Previously, the interest and outlook in hemicellulosic and lignin fraction of lignocellulosic biomass complex was limited. However, to maximize the overall yield of the products obtained in a biorefinery, it is now assumed that the development of pre-treatment/fractionation methods that allow efficient recovery of both cellulose and hemicellulose-derived sugars and lignin is an absolute requirement for the economic (and environmental) sustainability of the biorefinery (Galbe and Wallberg, 2019).

Enormous progress has been made by optimizing various pretreatment methods for lignocellulosic biomasses (Tareen *et al*., 2021). These methods aim to remove or modify lignin, reduce the crystallinity of cellulose and make hemicellulose more accessible to enzymatic hydrolysis. The efficiency of enzymatic hydrolysis is entirely governed by the structural composition of pretreated biomass (Maibam and Goyal. 2022). The present study aims to screen a suitable pretreatment method for rice straw biomass from various chemical and physico-chemical pretreatment methods like dilute acid (organic and inorganic) pretreatment, alkali pretreatment, dual-acid pretreatment, organosolv pretreatment, deep eutectic solvent pretreatment and sequential pretreatment (alkali pretreatment followed by acid pretreatment). The best pretreatment method is selected based on various substrate-related factors such as: high cellulose content, low lignin content and high crystallinity index in the pretreated biomass and negligible loss of cellulosic fraction during the pretreatment process.

2.2 Materials and methods

2.2.1 Collection and Feedstock Preparation

Rice straw (RS) was collected from local fields of Barpeta town, Barpeta District, Assam, India. Initially, it was washed with running tap water to remove dirt and soil debris and was dried overnight at 80°C. The dried straw was chopped, ground and sieved through a standard test sieve of 1 mm pore size and stored in a sealed bag at room temperature for further use.

2.2.2 Pretreatment of rice straw:

Raw RS was subjected to various pretreatment methods *i.e.* acid pretreatment (organic acid and inorganic acid), alkali pretreatment, dual-acid pretreatment, organosolv pretreatment, deep eutectic solvent pretreatment and sequential pretreatment (alkali pretreatment followed by acid pretreatment). In acid pretreatment, 3% (v/v) of each H_2SO_4, HNO_3, H_3PO_4, acetic acid (AA) or oxalic acid (OA) were separately used for the pretreatment of raw biomass. For alkali pretreatment, 3% (w/v) NaOH was used. 1.5% (v/v) HNO_3 + 1.5% (v/v) AA were used in dual-acid pretreatment. 85% (v/v) phosphoric acid- with chilled acetone was used in organosolv pretreatment. For deep eutectic solvent (DES) pretreatment, choline chloride-based DES was prepared with three different hydrogen bonding donors (HBD) i.e. acetic acid, oxalic acid and glycerol in a fixed molar ratio as mentioned in Table 2.2.1. In sequential pretreatment of alkali followed by acid pretreatment, 3% NaOH was used for 1st stage pretreatment followed by 3% HNO_3 in 2nd stage pretreatment. Similar pretreatment conditions were applied to all the pretreatment methods with a biomass loading of 5% (w/v) and autoclaving at 121°C, 15 psi for 30 min. The pretreatment methods along with pretreatment conditions are shown in Table 2.2.1.

Table 2.2.1. Pretreatment methods and conditions used for Rice straw.

Pretreatment method	Pretreatment condition
Organic acid	3%, v/v Acetic acid
	3%, v/v Oxalic acid
Inorganic acid	3%, v/v H_3PO_4
	3%, v/v H_2SO_4
	3%, v/v HNO_3
Alkali	3%, w/v NaOH
Dual acid	1.5%, v/v HNO_3 + 1.5%, v/v CH_3COOH
Alkali followed by Acid	3%, w/v NaOH autoclaving for 30 min followed by 3%, v/v HNO_3
Organosolv	85%, v/v H_3PO_4 –Acetone at 50˚C
Deep Eutectic Solvent	ChCl:Acetic acid in molar ratio 1:2
	ChCl:Oxalic acid in molar ratio 1:1
	ChCl:Glycerol in molar ratio 1:2

2.2.3 Lignin and structural carbohydrate analysis of raw and pretreated RS

The Lignin and total carbohydrate content (TCC) in raw RS and pretreated RS were estimated by following the NREL protocol (Sluiter *et al*., 2008) followed by subsequent High-Performance Liquid Chromatography (Shimadzu Corporation, Japan) analysis. The HPLC column (Aminex HPX-87H column, Bio-Rad Laboratories, USA) was used with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min at 50°C. Monosaccharides such as glucose, xylose and arabinose were detected by refractive index (RI) detector and quantified by using a standard plot. The biomass recovery (Eqn. 2.1) and delignification percentage (Eqn. 2.2), were calculated by following the formula reported earlier (Nath *et al*. 2021),

$$\text{Biomass recovery (\%)} = \left(\frac{\text{weight of pretreated biomass}}{\text{Initial weight of raw biomass}}\right) \times 100 \quad \ldots \text{(Eqn. 2.1)}$$

$$\text{Lignin removal (\%)} = \left(1 - \frac{\text{Lignin content of pretreat biomass} * \text{mass of pretreat biomass}}{\text{Lignin content of raw biomass} * \text{mass of raw biomass}}\right) \text{x } 100 \text{ (Eqn. 2.2)}$$

2.2.4 Crystallinity index analysis of raw and pretreated rice straw by XRD

The crystallinity index (*CrI*) of raw RS and pretreated RS biomass was measured by X-Ray Diffractometer (D8 Advance, Bruker, Germany). Dried biomass samples were analyzed for crystallinity index by scanning over the range of 2θ = 10°-60° with 0.05° step size. The *CrI* of both raw and pretreated samples was calculated by using Eqn. 2.3 (Segal *et al*., 1959).

$$\text{Crystallinity index (\%)} = \left(\frac{I_{\text{crystalline}} - I_{\text{amorphous}}}{I_{\text{crystalline}}}\right) \times 100 \quad \ldots \text{(Eqn. 2.3)}$$

Where $I_{crystalline}$ is the intensity of the crystalline peak at 2θ = 22° and $I_{amorphous}$ is the intensity of the amorphous peak at 2θ = 18°.

2.2.5 Total reducing sugar and monosaccharides analysis of the liquid fraction obtained after different pretreatments of RS

The total reducing sugar (TRS) present in the liquid fraction obtained after the pretreatment processes was estimated by the method of Nelson (1944) and Somogyi (1945) for a better understanding of cellulosic and hemicellulosic fraction loss during the pretreatment process. The monosaccharides released in the liquid fraction were also analyzed by High-Performance Liquid Chromatography (Shimadzu Corporation, Japan) analysis. Aminex HPX-87H column (Bio-Rad Laboratories, USA) was used with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min at 50°C.

Monosaccharides such as glucose, xylose and arabinose were detected by a refractive index (RI) detector and quantified by using a standard plot.

2.2.6 Analytical techniques

2.2.6.1 Moisture content of biomass

The moisture content of raw and pretreated biomasses was measured for carrying out further experiments with proper accuracy in terms of weight percentage. The biomass, 2 g was placed in pre-weighted empty silica crucible and dried at 105°C in an oven for 3 h. After 3 h, the crucible was removed and put in the desiccator and allowed to cool. Then, the dried biomass was weighed and the moisture content was calculated by using the formula:

$$\text{Moisture content (\%)} = \left(\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}}\right) \times 100 \quad \ldots(\text{Eqn. 2.4})$$

2.2.6.2 Determination of holocellulose content

TAPPI protocol: Two grams of biomass (raw or pretreated RS) was mixed in 100 mL deionized water taken in a 250 mL Erlenmeyer flask. The flask was kept at 70°C in a shaking water bath and 1.5 g sodium chlorite and 5 mL of 10% (v/v) acetic acid were added to it. The mixture was stirred at every 10 min and 5 mL of 10% (v/v) acetic acid was added after 30 min. 1.5 g sodium chlorite and 5 mL acetic acid were added for 4 h at a time interval of 1h. The mixture was cooled to 10°C and then filtered through a pre-weighted sintered glass funnel. The residue was washed with water followed by acetone and air-dried to make it acetone-free and dried in a 50°C oven for 24h. The holocellulose content was calculated as a percentage based on the oven dry sample (Eqn. 2.5).

$$\text{Holocellulose content (\%)} = \left(\frac{\text{Final weight}}{\text{Initial weight}}\right) \times 100 \quad \ldots(\text{Eqn. 2.5})$$

2.2.6.3 Determination of cellulose content

The residue obtained in the previous section (Section 2.2.6.2) was used for the determination of cellulose content by TAPPI protocol. One gram of holocellulose was transferred to a 250 mL glass beaker kept at 20°C in a water bath. Different volumes of 17.5% (~ 4.4 N) NaOH solution were added periodically (7.5 mL, 5 mL, 5 mL, 17.5 mL and 5 mL at the intervals of 0 min, 1 min, 45 sec, 15 sec and 3 min, respectively) and mixed intermittently. The mixture was left at 20°C for 30 min then 50 mL of distilled water was added. After 40 min the mixture was poured on a pre-weighted sintered glass funnel for filtration using suction. The residue was washed with 12.5 mL of 8.3% (~ 2 N) NaOH solution at 20°C and transferred to a pre-weighed crucible. The residue was washed with approximately, 400 mL of distilled water at 20°C under suction. Suction was removed temporarily and the crucible was filled with 2N acetic acid for 5 min to hydrolyse and remove hemicellulosic content. Then acetic acid was removed by reapplying suction. The residue was washed with distilled water till the neutral pH was achieved. The crucible was placed in a hot air oven at 50°C and dried to constant weight. Cellulose content was calculated as a percentage based on oven dry sample.

2.2.6.4 Reagents for Total Reducing Sugar Estimation Assay

The composition of the reagents used in the **TRS estimation** assay following the reducing sugar estimation methods of (Nelson 1944) and (Somogyi 1945) is as follows:

Reagent A

Sodium carbonate (2.5 g), potassium sodium tartrate tetrahydrate (2.5g), sodium bicarbonate (2.0 g) and sodium sulphate (20.0 g) dissolved in 100 mL distilled water.

Reagent B

Copper sulphate pentahydrate (4.5 g) and conc. sulphuric acid (1-2 drops) in 30 mL of distilled water.

Reagent C

Ammonium molybdate tetrahydrate (2.5 g) and conc. sulphuric acid (2.1 mL) were added to 45 mL of distilled water. Solution of sodium arsenate heptahydrate (0.3g in 2.5 mL of distilled water was mixed to it and stored in an amber bottle at 37°C for 24 h prior to use.

Reagent D

A mixture of reagents A and B in a ratio of 25:1. Freshly prepare before use.

Estimation of reducing sugar

The TRS estimation was carried out by adding 100 µL of freshly prepared reagent D to 100 µL of pretreated hydrolysate. The solution was mixed and heated for 20 min in a boiling water bath. After 20 min cooling to room temperature (25°C), 100 µL of reagent C was added, followed by the addition of 700 µL distilled water to make the final volume of 1 mL. Finally, the absorbance at 500 nm (A_{500}) of the mixture was measured by a UV-visible spectrophotometer. D-glucose (0.01-0.1 mg/mL) or (10-100 µg/mL) was used as a standard to quantify the concentration of reducing sugar. The TRS concentration was calculated as described below,

$$\text{TRS concentration (mg/ml)} = \Delta A_{500} \times C \quad \ldots (\text{Eqn. 2.6})$$

ΔA_{500} = change in absorbance at 500 nm

C = 1 OD equivalent glucose concentration (mg/mL) from the standard plot

2.3 Results and Discussion

2.3.1 Composition analysis of raw and pretreated RS

The composition analysis of the raw and pretreated rice straw, the biomass recovery and lignin removal in the pretreated rice straw are summarized in Table 2.3.1. Raw rice straw contained 73.9 ±1.5% holocellulose (w/w), 43.5±1.1% (w/w) cellulose, 17.1±0.5% (w/w) lignin and 1.8±0.1%, w/w moisture. The biomass recovery of pretreated RS biomass varied according to the pretreatment methods and ranges from 47.5-71.8% (Table 2.3.1). The biomass recovery of organic acid pretreatment (65-72%) was highest, the DES pretreated RS resulted in biomass recovery of 59-61% under the same pretreatment conditions. Significantly lower biomass recovery was observed with HNO_3, alkali (NaOH) and sequential pretreatment giving biomass recoveries of 48%, 49% and 47%, respectively. High biomass recoveries after the pretreatment process could benefit the overall economics of the biorefinery (Afedzi and Parakulsuksatid, 2023). Different pretreatment method resulted in varied range of cellulose and lignin content in the pretreated RS (Table 2.3.1). An enhanced cellulose content of 52-70%, w/w regardless of pretreatment methods was observed in the pretreated RS compared to raw RS (43%, w/w). However, maximum cellulose content of 70%, w/w in the sequential pretreated RS was achieved followed by DES pretreated RS (61-66%, w/w) thus indicating negligible loss of cellulosic fraction during these pretreatment process as also earlier reported by Satlewal et al., (2018). Delignification of the feedstock during the pretreatment process is crucial for various reasons such as reducing the recalcitrance nature of biomass and enhancing the enzymatic saccharification efficiency by reducing the formation of inhibitors such as HMF, furfural and phenolic compounds (Singh *et al.*, 2014). The maximum lignin removal could be observed with

alkali pretreatment and DES pretreatment (75-78%, w/w), suggesting that lignin can be degraded more effectively by alkali and DES system. The DES pretreatment (ChCl:AA) of RS resulted in high biomass recovery, 60% (w/w), enhanced holocellulose and cellulose content of 80% and 65.5% (w/w), respectively with significant lignin removal percentage of 78%. This implies that hemicellulose and lignin were effectively degraded and removed by the ChCl:AA, DES solvent system, which is ideal for an effective enzymatic saccharification process as also reported earlier (Xu *et al*., 2023). High cellulose recovery in the pretreated biomass is desirable for its further enzymatic hydrolysis to yield high amount of fermentable monosaccharide sugar.

Table 2.3.1. Composition analysis of raw and pretreated rice straw.

Pretreatment	Pretreatment condition*	Biomass Recovery (%, w/w)	Holo[a] cellulose (%, w/w)	Cellulose[a] (%, w/w)	Lignin[b] (%,w/w)	Lignin removal (%)
Raw RS	-	100	73.9 ±1.5	43.5±1.1	17.1±0.5	-
Organic acid	3% Acetic acid	71.8±2.1	75.8±1.4	52.3±0.6	11.7±0.4	50.8
	3% Oxalic acid	65.4±1.6	79.6±2.1	54.2±1.7	13.4±0.8	48.7
Inorganic acid	3% H_3PO_4	55.2±1.1	76.7±0.8	54.6±0.5	13.1±0.6	57.8
	3% H_2SO_4	51.5±0.8	77.3±1.3	51.8±0.8	13.1±0.3	60.5
	3% HNO_3	48.2±1.3	82.6±0.9	53.1±0.7	10.3±0.5	70.9
Alkali	3% NaOH	48.9±1.7	87.3±0.7	60.6±0.3	8.5±0.2	75.6
Dual acid	1.5% HNO_3 + 1.5% CH_3COOH	50.3±0.9	79.7±1.2	53.2±1.4	12.2±0.3	64.1
Alkali followed by Acid	3% NaOH followed by 3% HNO_3	47.5±0.4	89.8±1.3	70.0±1.3	6.6±0.1	81.7
Organosolv	85% H_3PO_4 – Acetone at 50˚C	55.9±0.8	78.5±0.6	60.8±0.8	8.9±0.4	69.8
Deep Eutectic Solvent	ChCl:Acetic acid (molar ratio 1:2)	58.9±0.9	80.4±0.5	65.5±0.9	6.2±0.2	78.6
	ChCl:Oxalic acid (molar ratio 1:1)	59.7±2.1	82.6±0.8	60.9±0.8	6.4±0.1	77.6
	ChCl:Glycerol (molar ratio 1:2)	60.5±1.8	79.7±0.5	61.1±1.4	7.1±0.2	74.7

** Each pretreatment involved biomass loading of 5% (w/v) autoclaving at 121°C, 15 psi for 30 min; [a] TAPPI method; [b] NREL method.*

2.3.2 XRD analysis of raw and pretreated rice straw

Crystallinity index (*CrI*) measures the extent of removal amorphous components of biomass, *viz.* lignin and hemicellulose. An increase in the *CrI* is essentially an indication of the increase in cellulose content of biomass after pretreatment. The *CrI*s for biomass pretreated with different techniques have been listed in Table 2.3.2. The raw RS showed 34.7% *CrI.* However, every pretreatment method adopted in this study showed increment in *CrI.* Dilute inorganic acid pretreatment using 3%, w/v each of H_3PO_4, H_2SO_4 and HNO_3 showed crystallinity index of 39.1%, 41.3% and 40.8% respectively. Dilute organic acid (acetic acid and oxalic acid) pretreated RS resulted in *CrI* of 38.8% and 37.2%, respectively. The increment in crystallinity index after acid treatment was attributed to the hydrolysis of hemicellulose. Relatively smaller increase in *CrI* of organic acid pretreatment may be due to the low extent of hemicellulose hydrolysis by the weak organic acid causing only low removal of lignin and hemicellulose as also reported earlier (Sindhu *et al*., 2014). The NaOH, alkali followed by acid and organosolv pretreated RS resulted in higher *CrI* (~49.1, 47.9 and 48.1 respectively). The relatively higher *CrI* inferred the effectiveness of these pretreatment methods on the amorphous portion rather than the crystalline portion of the rice straw biomass. Also, high *CrI* of 48.4, 45.8 and 47.6% by ChCl- based DES pretreatment methods signify high intensity of peak denoting amorphous portion of biomass as also reported earlier (Xu *et al*., 2016). The initial rate of enzymatic hydrolysis of cellulose is governed by its crystallinity index (*CrI*), which reflects changes in the cellulose structure due to the pre-treatment. Enzyme adsorption on the surface of cellulosic fraction is greatly influenced by the cellulose crystallinity, thus having an impact on hydrolysis rates and monosaccharides yields (Fialho *et al*., 2023).

Cellulose with low crystallinity exhibits greater enzymatic saccharification as compared with the samples with high crystallinity (Yang *et al.,* 2011). Thus, it can be inferred from these results that pretreatment method such as DES, alkali and organosolv could be suitable for rice straw biomass when *CrI* is considered.

Table 2.3.2. *CrI* of raw and pretreated rice straw.

Pretreatment	**Pretreatment condition**	***CrI* (%)**
Raw	-	34.7
Organic acid	3% Acetic acid	38.8
	3% Oxalic acid	37.2
Inorganic acid	3% H_3PO_4	39.1
	3% H_2SO_4	41.3
	3% HNO_3	40.8
Alkali	3% NaOH	49.1
Dual acid	1.5% HNO_3 + 1.5% CH_3COOH	42.4
Alkali followed by Acid	3% NaOH followed by 3% HNO_3	47.9
Organosolv	85% H_3PO_4 –Acetone at 50˚C	48.1
Deep Eutectic Solvent	ChCl:Acetic acid (molar ratio 1:2)	48.4
	ChCl:Oxalic acid (molar ratio 1:1)	45.8
	ChCl:Glycerol (molar ratio 1:2)	47.6

2.3.3 Total reducing sugar and monosaccharides analysis of the liquid fraction obtained after different pretreatments of RS

The pretreatment process disrupts the hydrogen and covalent bonds among various constituents of lignocellulosic complexes thereby reducing the recalcitrance nature of biomass (Maibam and Goyal, 2022). During the pretreatment step, certain fractions of cellulosic and hemicellulosic portions are degraded and converted to monosaccharides in the liquid fraction obtained after the pretreatment process (Baruah *et al.*, 2018). The aim was to screen a pretreatment method with negligible loss cellulosic fraction or less TRS yield in the pretreated liquid hydrolysate as only the pretreated RS biomass will be used for the subsequent enzymatic saccharification process. The maximum TRS yield of 199 mg/$g_{raw\ biomass}$, xylose yield of 128.1±0.9 mg/$g_{raw\ biomass}$ and glucose yield 11.9±0.5 mg/$g_{raw\ biomass}$ was observed in the liquid

hydrolysate of HNO_3 pretreated filtrate as analysed by HPLC (Table 2.3.3). Similarly, H_2SO_4 pretreated filtrate resulted in high TRS yield of 183 $mg/g_{raw\ biomass}$, xylose yield of 116.1±0.7 $mg/g_{raw\ biomass}$ and glucose yield of 14.2±0.3 $mg/g_{raw\ biomass}$ (Table 2.3.3). The high yield of xylose resulting from the dilute strong inorganic acid pretreatment of RS implies that the major fraction of hemicellulose is degraded during the pretreatment process. The removal of hemicellulosic fraction and lignin (58-71%, Table 2.3.1) led to increase in the exposure of cellulosic fraction and its surface with a reduction in cellulose crystallinity. The increment of crystallinity Index, from 34,7% in raw RS to 40.8% and 41.3% in HNO_3 and H_2SO_4 pretreated RS filtrate (Table 2.3.2) justifies the removal of amorphous hemicellulose and lignin in the pretreatment process as also reported earlier (Kshirsagar *et al*., 2015). Dilute weak organic acid pretreatment using 3%, v/v acetic acid or oxalic acid resulted in relatively, lower TRS yields of 59.3 $mg/g_{raw\ biomass}$ and 61.6 $mg/g_{raw\ biomass}$, respectively, as compared with strong inorganic acids. Moreover, the low yields of xylose, 24.1 $mg/g_{raw\ biomass}$ and 35.9 $mg/g_{raw\ biomass}$ respectively, reflect the low impact of organic acid on the hydrolysis of a hemicellulosic fraction of the raw RS. Negligible glucose yield of 2.6 $mg/g_{raw\ biomass}$ and 2.4 $mg/g_{raw\ biomass}$ in acetic acid and oxalic acid pretreated liquid hydrolysate respectively showed the minimal loss of cellulosic fraction during the pretreatment process. NaOH pretreatment gave the lowest TRS yield of 15.7 $mg/g_{raw\ biomass}$ with a glucose yield of 4.9 $mg/g_{raw\ biomass}$ in the liquid fraction. Thus, inferring that NaOH pretreatment prevents degradation of the polysaccharides into water-soluble sugars thereby retaining maximum sugars in the solid residue after the pretreatment. However, ChCl- based DES pretreatment resulted in negligible glucose yield of 0.8, 3.9 and 1.3 $mg/g_{raw\ biomass}$ respectively, in ChCl: Acetic acid, ChCl: Oxalic acid and ChCl: Glycerol pretreated

liquid hydrolysate. The negligible degradation of cellulosic fraction during the pretreatment process (0.8 mg/$g_{raw\ biomass}$), increase in crystallinity index (48.4%, Table 2.3.2) and high delignification percentage (78%, Table 2.3.1) makes DES pretreatment, *i.e*. ChCL: AA based solvent to be an ideal method for RS pretreatment.

The higher extent of delignification, negligible degradation of cellulosic fraction, removal of amorphous hemicellulosic fraction, reduction of crystalline cellulosic portion and increment in crystallinity index in the pretreated biomass are certain crucial conditions for efficient enzymatic hydrolysis process as also reported earlier (Smith et al., 2014, Hassan *et al*., 2018).

Table 2.3.3. Analysis of TRS and monosaccharides released in liquid fraction during the pretreatment process.

Pretreatment	Pretreatment condition*	TRS [a*] (mg/g raw biomass)	Glucose [b*] (mg/g raw biomass)	Xylose [b*] (mg/g raw biomass)	Arabinose [b*] (mg/g raw biomass)
Organic acid	3% Acetic acid	59.3±0.9	2.6±0.1	24.1±0.4	4.1±0.1
	3% Oxalic acid	61.6±0.6	2.4±0.1	35.9±0.7	5.4±0.8
Inorganic acid	3% H_3PO_4	65.9±0.7	4.5±0.4	37.8±0.5	0
	3% H_2SO_4	183.2±1.4	14.2±0.3	116.1±0.7	10.6±0.4
	3% HNO_3	199.8±1.5	11.9±0.5	128.1±0.9	13.8±0.5
Alkali	3% NaOH	15.7±0.4	4.9±0.1	7.8±0.2	3.1±0.2
Dual acid	1.5% HNO_3 + 1.5% CH_3COOH	146.4±1.3	4.6±0.1	113.0±0.3	11.4±0.9
Alkali followed by Acid	3% NaOH followed by 3% HNO_3	100.4±2.5	12.8±0.2	48.3±0.8	6.6±0.1
Organosolv	85% H_3PO_4 – Acetone at 50˚C	10.9±0.8	1.5±0.6	1.8±0.3	2.9±0.8
Deep Eutectic Solvent	ChCl:Acetic acid (molar ratio 1:2)	20.9±0.5	0.8±0.1	12.9±0.1	6.9±0.2
	ChCl:Oxalic acid (molar ratio 1:1)	25.7±1.1	3.9±0.1	14.9±0.2	5.3±0.4
	ChCl:Glycerol (molar ratio 1:2)	35.5±0.6	1.3±0.1	20.4±0.1	10.8±0.5

** Each pretreatment involve: Biomass loading is 5% for all pretreatments,*
TRS: Total reducing sugar, a: TRS yield estimated by the method of Nelson (1944) and Somogyi (1945) b: HPLC analysis
All values reported are mean of duplicate experiments (± *Standard deviation, n=2)*

2.4 Conclusion

Twelve different pretreatment methods (organic acid, inorganic acid, alkali, dual acids, organosolv and ChCl-based DES solvents) were performed under the same operating conditions for selecting the best suitable pretreatment method for RS biomass. The basis for selecting the best pretreatment method was the parameters: i) high biomass recovery with high delignification percentage, ii) maximum retainment of cellulosic fraction in the pretreated biomass and iii) high crystallinity index of the pretreated biomass. These crucial factors greatly influence the effectiveness of the enzymatic saccharification process. Raw RS contained 43.5±1.1% (w/w) cellulose, 17.1±0.5% (w/w) lignin. The pretreatment of RS by DES system with ChCl:AA combination showed the overall high cellulose content 65.5±0.9%, w/w, lignin content 6.2±0.2%, w/w giving delignification of 78%. The ChCl:AA pretreated RS showed high *CrI* of 48.4%. The HPLC analysis of liquid fraction obtained after the DES pretreatment process using ChCl: AA showed the least degradation of cellulosic fraction with the lowest glucose yield of 0.8 mg/g. Based on the above experimental facts i.e. high biomass recovery with high cellulose content in the pretreated biomass, high *CrI*, negligible loss of cellulosic fraction during the pretreatment process and DES solvent being green, non-toxic and reusable, ChCl:AA solvent was selected as the best suitable pretreatment method for RS biomass in further study. The next chapter focuses on the optimization of ChCl: AA DES solvent pretreatment conditions for RS biomass.

2.5 References

Afedzi, A. E. K. and Parakulsuksatid, P. (2023). Recent advances in process modifications of simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass for bioethanol production. Biocatalysis and Agricultural Biotechnology, 102961.

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

Baruah, J., Nath, B.K., Sharma, R., Kumar, S., Deka, R.C., Baruah, D.C. and Kalita, E. (2018). Recent trends in the pretreatment of lignocellulosic biomass for value-added products. Frontiers in Energy Research, 6, 141.

Fialho, J., Moniz, P., Duarte, L. C. and Carvalheiro, F. (2023). Green fractionation approaches for the integrated upgrade of corn obs. Chemical Engineering, 7(2), 35.

Galbe, M. and Wallberg, O. (2019). Pretreatment for biorefineries: a review of common methods for efficient utilisation of lignocellulosic materials. Biotechnology for Biofuels, 12(1), 294.

Ghatak, H. R. (2011). Biorefineries from the perspective of sustainability: Feedstocks, productsand processes. Renewable and Sustainable Energy Reviews, 15(8), 4042-4052.

Hassan, S. S., Williams, G. A. and Jaiswal, A. K. (2018). Emerging technologies for the pretreatment of lignocellulosic biomass. Bioresource Technology, 262, 310-318.

Kshirsagar, S.D., Waghmare, P.R., Chandrakant Loni, P., Patil, S.A. and Govindwar, S.P. (2015). Dilute acid pretreatment of rice straw, structural characterization and optimization of enzymatic hydrolysis conditions by response surface methodology. RSC Advances, 5(58), 46525-46533.

Loow, Y. L., Wu, T. Y., Tan, K. A., Lim, Y. S., Siow, L. F., Md. Jahim, J. and Teoh, W. H. (2015). Recent advances in the application of inorganic salt pretreatment for transforming lignocellulosic biomass into reducing sugars. Journal of Agricultural and Food Chemistry, 63(38), 8349-8363.

Maibam, P.D. and Goyal, A. (2022). Pretreatment methods for overcoming biomass recalcitrance, in: Verma, P. (Eds.), Enzymes in the Valorization of Waste. CRC Press, pp. 1-21.

Nath, P., Maibam, P.D., Singh, S., Rajulapati, V. and Goyal, A. (2021). Sequential pretreatment of sugarcane bagasse by alkali and organosolv for improved delignification and cellulose saccharification by chimera and cellobiohydrolase for bioethanol production. 3 Biotech, 11(2), 1-16.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry 153(2): 375-380.

Pakchamni, P., Afedzi, A. E. K. and Parakulsuksatid, P. (2022). Optimization of alkaline-assisted organosolv pretreatment of sugarcane trash for the production of succinic acid using response surface methodology. Biocatalysis and Agricultural Biotechnology, 43, 102374.

Satlewal, A., Agrawal, R., Bhagia, S., Das, P. and Ragauskas, A.J. (2018). Rice straw as a feedstock for biofuels: Availability, recalcitrance and chemical properties. Biofuels, Bioproducts and Biorefining, 12(1), 83-107.

Segal, L.G.J.M.A., Creely, J.J., Martin Jr, A.E. and Conrad, C.M. (1959). An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. Textile Research Journal, 29(10), 786-794.

Shastri, Y. N., Miao, Z., Rodríguez, L. F., Grift, T. E., Hansen, A. C. and Ting, K. C. (2014). Determining optimal size reduction and densification for biomass feedstock using the BioFeed optimization model. Biofuels, Bioproducts and Biorefining, 8(3), 423-437.

Sindhu, R., Kuttiraja, M., Binod, P., Sukumaran, R. K. and Pandey, A. (2014). Physicochemical characterization of alkali pretreated sugarcane tops and optimization of enzymatic saccharification using response surface methodology. Renewable Energy, 62, 362-368.

Singh, R., Shukla, A., Tiwari, S. and Srivastava, M. (2014). A review on delignification of lignocellulosic biomass for enhancement of ethanol production potential. Renewable and Sustainable Energy Reviews, 32, 713-728.

Singh, S., Rajulapati, V., Jamaldheen, S. B., Moholkar, V. S. and Goyal, A. (2020). Statistically designed cellulase mixture for saccharification of pretreated Sorghum durra stalk. Industrial Crops and Products, 154, 112678.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. L. A. P. (2008). Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedure, 1617(1), 1-16.

Somogyi, M. (1945). A new reagent for the determination of sugars. Journal of

Biological Chemistry, 160(1), 61-68.

TAPPI. Technical Association of Pulp and Paper Industry, Atlanta, GA. (1992).

Tareen, A. K., Sultan, I. N., Songprom, K., Laemsak, N., Sirisansaneeyakul, S., Vanichsriratana, W. and Parakulsuksatid, P. (2021). Two-step pretreatment of oil palm trunk for ethanol production by thermotolerent Saccharomyces cerevisiae SC90. Bioresource Technology, 320, 124298.

Xu, G. C., Ding, J. C., Han, R. Z., Dong, J. J. and Ni, Y. (2016). Enhancing cellulose accessibility of corn stover by deep eutectic solvent pretreatment for butanol fermentation. Bioresource Technology, 203, 364-369.

Xu, Y., Liu, Y. H., Xu, L. H., He, Y. T., Wen, J. L. and Yuan, T. Q. (2023). Enhancing saccharification of bamboo shoot shells by rapid one-pot pretreatment of hydrated deep eutectic solvent. Bioresource Technology, 380, 129090.

Yang, B., Dai, Z., Ding, S. Y. and Wyman, C. E. (2011). Enzymatic hydrolysis of cellulosic biomass. Biofuels, 2(4), 421-449.

Chapter 3

Statistical optimization of rice straw pretreatment using deep eutectic solvent comprising choline chloride and acetic acid

3.1 Introduction

The increasing demand for crude oil and its rising price has renewed the interest in renewable energy. Bioethanol from lignocellulosic feedstocks serves as a promising renewable source of fuel (Di Marino *et al*., 2016). In Asian countries, rice straw contributes a major agriculture lignocellulosic waste. The global rice straw production is 370-520 million tons/year of which 330-470 million tons are from Asia alone (Van Hung *et al*., 2020). This enormous amount of lignocellulosic waste can be effectively valorized to bioethanol (Singh *et al*., 2016). However, the key to the success in lignocellulosic biorefineries lies in an effective pretreatment method for enhancing the efficiency of the enzymatic hydrolysis process. The purpose of any pretreatment method is to increase the exposure of cellulosic fraction and its surface with a reduction in cellulose crystallinity (Baruah *et al*., 2018, Maibam and Maiti, 2020). The

recalcitrance nature of biomass is a challenging step for any pretreatment methods. The lignocellulosic complexes are held by hydrogen and covalent bonds, that provide its recalcitrant structure. Commonly, pretreatment processes are classified as physical, chemical, physico-chemical and biological methods (Mood *et al*., 2013). Pretreatment techniques like alkali, dilute acid, steam explosion treatments have been the conventional method for biomass fractionation. However, certain conditions like the requirement of huge chemicals, high energy consumption and its associated high cost, environmental problems, etc hinder the process's feasibility (Kumar *et al*., 2020). Therefore, the use of green solvent having characteristics of biodegradability, low toxicity, recyclability and cost-effectiveness has emerged as a potential pretreatment method for biomass fractionation (Calvo-Flores *et al*., 2018).

Deep eutectic solvents (DESs) are preferred as they are green solvents because of their properties such as being low toxicity, easy synthesis process and feasibility at large scale pretreatment process (Wang *et al*., 2021). The DESs are eutectic mixtures comprising two components i.e., hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) at a fixed molar ratio, with a melting point lower than those of the individual components (Loow *et al*., 2018). Biomass pretreatment using DES leads to cleavage of the interpolymer linkage (ether bond) between lignin molecules and the intrapolymer linkage (ether and ester bond) between hemicellulose and lignin in the lignocellulosic complexes (Hong *et al*., 2020). Therefore, the mechanism of biomass fractionation by DES can be predominantly categorized as hydrolysis of hemicellulose and delignification with negligible disruption to the cellulosic fractions (Hong *et al*., 2020). This mechanism of DES pretreatment is the key requirement for the efficient valorization of the lignocellulosic complexes to a valuable product. Choline chloride

(ChCl) based DES has shown to be of great potential in the delignification of lignocellulosic biomass (Xu *et al*., 2020). Chlorine-based-DES methods delignify the biomass by forming hydrogen bonds between halogen ions (Cl^-) of DES and − OH groups in lignin (Xu *et al*., 2021). Choline chloride is the most commonly used HBA because of other benefits like biodegradability, biocompatibility and economic benefits (Xu *et al*., 2020). The features of the ChCl-based DES method can be modulated by changing HBD and its molar ratio with HBA. Various types of HBD could form DES with ChCl, such as acids, alcohols, polyols, amides and polysaccharides (Wang *et al*., 2021). The molar ratio of HBD to ChCl and the pretreatment severity had a great influence on the efficacy of delignification of lignocellulosic biomass (Xu *et al*., 2021). DES with acid-based HBD such as formic acid, oxalic acid and lactic acid was reported to be effective in lignin and xylan removal with a positive impact on enzymatic hydrolysis efficiency (Zhang *et al*., 2016, Chen *et al*., 2019).

A detailed investigation on DES with ChCl and acetic acid (AA) for the pretreatment process of rice straw (RS) has not been reported. Therefore, in this study, DES comprising ChCl and AA was studied for the pretreatment of RS. The possible combinations of ChCl/AA molar fraction were generated using statistical software to ensure the maximal delignification and retainment of the total carbohydrate content (TCC) in the pretreated RS. The pretreated RS was structurally and compositionally characterized and subjected to enzymatic saccharification. Also, DES-lignin was extracted from the black liquid hydrolysate obtained after the pretreatment of raw RS at optimum conditions and the extracted lignin was characterized. Based on statistical modeling, green and effective pretreatment method was developed with ChCl: AA system in defined proportion and excellent saccharification efficiency was achieved.

3.2 Materials and methods

3.2.1 Materials and reagents

Rice straw (RS) was obtained from local fields of Barpeta town, Barpeta District, Assam, India. The biomass was washed under tap water and dried at 80ºC in an oven till a constant weight was achieved. Subsequently, the dried biomass was grinded and subjected to sieving by using a standard sieve (pore size, 1x1 mm) and stored at 25°C for further use. Choline chloride, acetic acid, sodium chlorite, sulphuric acid, sodium azide, citric acid and sodium phosphate monobasic used were of Analytical Reagent grade and purchased from Himedia, Pvt. Ltd, India. Glucose, xylose, arabinose and commercial cellulase (*Trichoderma reesei* aqueous solution, ≥ 700 U/g) were purchased from Sigma-Aldrich Co. LLC., USA.

3.2.2 DES preparation

Fifty grams of DES was synthesized by mixing ChCl and AA in different combinations of molar ratios as shown in Tables 3.3.1 and 3.3.2. After mixing the components, each DES mixture was stirred at 80°C on a magnetic stirrer with hot plate until a homogeneous colorless viscous solution was obtained in approx. 30 min as reported earlier (Xing *et al*., 2018). The prepared DES mixtures were used for the optimization of RS pretreatment process.

3.2.3 Experimental design for optimization of RS pretreatment using ChCl:AA

The Central composite design (CCD) was employed to study the effect of independent variables on the response and mutual interactions for optimizing the pretreatment of rice straw. The independent variables selected were, the ChCl: AA molar ratio (A), treatment temperature (B) and pretreatment time (C). The factors were set at five coded levels (− α, − 1, 0, + 1 and + α) with lower/upper bounds of A, B (°C)

and C (min) at 1:2/1:4, 70/140 and 30/180, respectively (Table 3.3.2). Lignin content and total carbohydrate content (TCC) were selected as the responses to the optimization process. A statistical approach of response surface methodology (RSM) was employed to solve multivariate equations from the experimental values for the pretreatment of rice straw. The software, design expert 7.0 (Stat-Ease, Inc., USA) designed 20 experimental set-ups as listed in Table 3.3.3 for the optimization of RS pretreatment. Each experiment was conducted with 50 g DES solution at 7.5%, w/w biomass loading under the software-designed conditions. After the pretreatment process, the solid fraction was separated by filtration using a muslin cloth. The solid residue was washed with distilled water until a neutral pH was reached, thereafter it was lyophilized overnight and stored in sealed bags. Lignin content and TCC of the dried pretreated RS biomasses were estimated by using standard National Renewable Energy Laboratory (NREL) protocol (Sluiter *et al*., 2010) and fitted to the model for statistical optimization. All the experiments were conducted in triplicate sets.

3.2.4. Characterization of raw and ChCl: AA pretreated rice straw

The pretreated RS obtained from the optimized conditions of the RSM-CCD method was referred to as CApRS (choline chloride: acetic acid pretreated rice straw) and subjected to various characterization techniques and saccharification processes.

3.2.4.1 Composition analysis of raw and ChCl: AA pretreated rice straw

The holocellulose, cellulose and hemicellulose contents of raw RS and CApRS were determined by the TAPPI (Pulp *et al*., 1992). The Lignin and TCC in raw RS and CApRS were estimated by following the NREL protocol and subsequent High-Performance Liquid Chromatography (Shimadzu Corporation, Japan) analysis. The HPLC column (Aminex HPX-87H column, Bio-Rad Laboratories, USA) was used with

5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min at 50°C. Monosaccharides such as glucose, xylose and arabinose were detected by refractive index (RI) detector and quantified by using a standard plot. The biomass recovery, Eqn. 3.1, delignification percentage, Eqn. 3.2, hemicellulose removal percentage, Eqn. 3.3 (Nath *et al*., 2021). Pretreatment efficiency is defined as the percentage of lignin removed with respect to a fixed amount of cellulose present in biomass and is calculated by the formula given in Eqn. 3.4 (Tran *et al*., 2020):

$$\text{Biomass recovery (\%)} = \left(\frac{\text{weight of CApRS}}{\text{Initial weight of RS}}\right) \times 100 \quad \ldots \text{ Eqn. 3.1}$$

$$\text{Delignification (\%)} = \left(1 - \frac{\text{Lignin in CApRS} \times \text{mass of CApRS}}{\text{Lignin in RS} \times \text{mass of RS}}\right) \times 100 \quad \ldots \text{Eqn. 3.2}$$

$$\text{Hemicellulose removal (\%)} = \left(1 - \frac{\text{Hemicellulose in CApRS} \times \text{mass of CApRS}}{\text{Hemicellulose in RS} \times \text{mass of RS}}\right) \times 100 \quad \ldots \text{Eqn. 3.3}$$

$$\text{Pretreatment efficiency (\%)} = \left(1 - \frac{\text{mass ratio of } \frac{\text{lignin}}{\text{cellulose}} \text{ of CApRS}}{\text{mass ratio of } \frac{\text{lignin}}{\text{cellulose}} \text{ of RS}}\right) \times 100 \quad \ldots \text{Eqn. 3.4}$$

3.2.4.2 Analysis of raw and ChCl: AA pretreated rice straw by XRD

The crystallinity index (*CrI*) of raw RS and CApRS was measured by X-Ray Diffractometer (D8 Advance, Bruker, Germany). Dried biomass samples were analyzed for crystallinity index by scanning over the range of 2θ = 10°-60° with 0.05° step size. The *CrI* of both raw and pretreated samples was calculated by using Eqn. 3.5 (Segal *et al*., 1959).

$$\text{Crystallinity index (\%)} = \left(\frac{I_{\text{crystalline}} - I_{\text{amorphous}}}{I_{\text{crystalline}}}\right) \times 100 \quad \ldots \text{ Eqn. 3.5}$$

Where, $I_{\text{crystalline}}$ is the intensity of the crystalline peak at 2θ = 22° and $I_{\text{amorphous}}$ is the intensity of the amorphous peak at 2θ = 18°.

3.2.4.3 Surface area and pore size analyses of raw and ChCl:AA pretreated RS using BET

Brunauer-Emmet-Teller (BET) surface area and pore size of raw RS and CApRS were measured by N_2 adsorption/desorption measurements using Quantachrome (Model: Autosorb-IQ MP) performed at -196°C. The dried biomass samples were degassed at 150°C for 3 h using a vacuum prior to the measurement. About 0.05-0.07 g of samples were used for the analysis (Mohammadi *et al*., 2019). The BET surface area and pore volume were obtained from the N_2 adsorption/desorption data. The surface area indicates the amorphous nature of the sample.

3.2.4.4 Functional groups analysis of raw and ChCl: AA pretreated rice straw

The functional group distribution in the raw and CApRS was determined by Fourier Transform Infrared (FTIR) spectroscopy (Perkin Elmer, USA). The samples of dried raw RS and CApRS biomass were ground with KBr in 1:100 ratio and their pellets were made under a 15-ton hydraulic press. The pellets were subjected to scanning in the wavenumber range, 400 - 4000 cm^{-1}.

3.2.4.5 Surface morphology analysis of raw and ChCl: AA pretreated rice straw

Field Emission Scanning Electron Microscope (FESEM) (Zeiss, Gemini model) was used to study and compare the morphology of raw RS and CApRS fibers. The dried biomass samples were adhered to carbon tape and a double coating of gold film and observed the image at different magnifications at a beam voltage of 2kV.

3.2.5 Enzymatic saccharification of ChCl: AA pretreated rice straw

The pretreatment efficiency of ChCl: AA method was evaluated based on the saccharification efficiency of the CApRS. The enzymatic saccharification of 2.5%, w/v

pretreated CApRS was performed with 35 U/g_{CApRS} commercial cellulase solution from *Trichoderma ressei* containing 0.005% sodium azide in 50 mm sodium citrate buffer, pH 4.8 in 10 ml working volume in a 50 ml sealed Erlenmeyer flask. The reaction was incubated at 50°C and 150 rpm in a shaking incubator and aliquots (500 µL) were withdrawn at 24 h and 48 h. The aliquoted samples were then boiled to inactivate the enzyme, centrifuged and the amount of total reducing sugar (TRS) in the liquid hydrolysate was determined by using the methods reported by Nelson (1944) and Somogyi (1945). All the reactions were performed in a duplicate set and the saccharification process was conducted under sterile conditions. The enzymatic saccharification efficiency (Eqn. 3.6) was calculated by using the formula (Láinez *et al*., 2019)

$$\text{Saccharification efficiency (\%)} = \left(\frac{\text{TRS after enzymatic saccharification}}{\frac{1.111 \times C \times Biomass}{\text{Volume}}} \right) \times 100 \quad \text{... Eqn. 3.6}$$

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

1.111 is factor for conversion of cellulose to its equivalent glucose (Dowe *et al*., 2001);

C is the cellulose content in the CApRS (g/g_{CApRS});

Biomass is the weight of dried CApRS used in enzymatic hydrolysis (g);

Volume is the working volume of the saccharification process in (L).

3.2.6. Lignin extraction from liquid hydrolysate obtained after RS pretreatment

The liquid hydrolysate obtained after ChCl: AA pretreatment of rice straw under optimized conditions as discussed in section 3.2.3 was used to extract the lignin by precipitation. The soluble lignin present in the liquid hydrolysate was precipitated by adding 4 volumes of a mixture of ultrapure water and ethanol in the ratio 1:2 (v/v) to 1 volume of liquid hydrolysate. The resulting liquid mixture (hydrolysate + mixture of

ultrapure water and ethanol) was incubated at 4°C for 12 h and later centrifuged at 4ºC and 8000g for 30 min. The precipitated lignin named as DES-extracted lignin (DL) was further washed 4-5 times by ultrapure water to remove the impurities and dried at 40°C oven overnight. The dried DL was used for further analysis.

3.2.6.1 Characterization of DES-extracted lignin

The extracted DES-lignin (DL) was characterized for its; i) composition and purity using the NREL protocol, ii) functional group analysis using FTIR and iii) surface morphology by FESEM.

The yield of extracted DL was calculated using Eqn. 3.7

$$\text{DL yield (\%)} = \left(\frac{\text{weight of DL}}{\text{Weight of lignin in raw RS}}\right) \times 100 \quad \text{... Eqn. 3.7}$$

The lignin content and TCC in the DL were analyzed using the NREL protocol as described in section 3.2.4.1 and following the protocol described by Sluiter *et al*., 2010. The commercial lignin, Alkaline Lignin, Tokyo Chemical Industry (India) Pvt. Ltd. was used as a standard to determine the purity of DL.

Measurement of the Fourier transform infrared (FTIR) spectra of DL and commercial lignin was carried out to investigate the functional group changes and distribution by following the procedure described in section 3.2.4.4.

The FESEM imaging of the DL and commercial lignin was also performed by following the same protocol as described in section 3.2.4.5 to visualize the surface morphology.

3.3 Results and Discussion

3.3.1 Optimization of RS pretreatment using response surface methodology

Three independent variables i.e., ChCl:AA molar ratio (A), treatment temperature (B) and the pretreatment time (C) were considered as significant factors for optimization of the pretreatment process of RS by using the RSM-CCD approach. Prior to statistical optimization, the pretreatment of RS was performed by one factor at a time (OFAT) to select the significant range of each factor. The effect of factors A, B and C on the delignification percentage in the pretreated biomass was studied by the OFAT approach (Table 3.3.1). Thereafter, the selected range of each factor was used to generate software-designed experimental conditions (Table 3.3.2) for the optimization process with lignin content and TCC in the pretreated RS as responses (Table 3.3.3).

Table 3.3.1. OFAT to select the significant range of each factor; A, B and C based on the delignification percentage in the pretreated biomass

Pretreatment conditions	Biomass Recovery (%, w/w)	Lignin (%, w/w)	Delignification (%, w/w)
Raw rice straw	100	18.4	-
1:1 (ChCl:AA), 90°C, 1 h,	75.4	11.3	53.7
1:2 (ChCl:AA), 90°C, 1 h,	76.7	7.4	69.3
1:4 (ChCl:AA), 90°C, 1 h,	73.3	10.8	57.1
1:5 (ChCl:AA), 90°C, 1 h,	72.8	10.5	58.5
1:2 (ChCl:AA), 60°C, 1 h,	84.8	8.3	61.7
1:2 (ChCl:AA), 90°C, 1 h,	76.7	7.4	68.9
1:2 (ChCl:AA), 120°C, 1 h,	77.1	10.4	56.6
1:2 (ChCl:AA) 140°C, 1 h,	63.9	6.4	77.7
1:2 (ChCl:AA), 160°C, 1 h,	62.2	6.1	78.3
1:2 (ChCl:AA), 140°C, 0.5 h	63.9	7.4	74.2
1:2 (ChCl:AA), 140°C, 1 h,	61.8	6.4	78.4
1:2 (ChCl:AA), 140°C, 3 h,	59.6	6.6	78.6
1:2 (ChCl:AA), 140°C, 4 h,	52.8	7.1	79.7

Table 3.3.2 The coded value of variables for CCD-RSM design for rice straw pretreatment.

Variables	Levels				
	-α	-1	0	+1	+α
ChCl:AA molar ratio (A)	1:2	1:2.41	1:3	1:3.59	1:4
Temperature, °C (B)	70	84.19	105	125.81	140
Time, min (C)	30	60.40	105	149.60	180

Table 3.3.3 Predicted and experimental values of lignin and TCC in pretreated RS with respect to variables (ChCl: AA molar ratio, temperature and time) in RSM-CCD runs.

Run order	Variables			Predicted results (Responses)		Experimental results (Responses)	
	ChCl: AA molar ratio (A)	Temperature °C (B)	Time, min (C)	Lignin content (%, w/w)	TCC (mg/g $_{CApRS}$)	Lignin content (%, w/w)	TCC (mg/g $_{CApRS}$)
1	1:2.41	84.19	60.4	8.78	542.5	8.81	531.6
2	1:3.59	84.19	60.4	7.98	542.2	8.09	551.0
3	1:2.41	125.81	60.4	8.01	584.4	7.89	595.6
4	1:3.59	125.81	60.4	5.19	647.2	5.09	648.4
5	1:2.41	84.19	149.6	8.40	532.1	8.44	542.3
6	1:3.59	84.19	149.6	9.81	519.9	9.87	520.3
7	1:2.41	125.81	149.6	5.77	637.1	5.60	639.8
8	1:3.59	125.81	149.6	5.17	688.1	5.09	710.6
9	1:2.00	105.00	105.0	7.33	548.6	7.44	545.9
10	1:4.00	105.00	105.0	6.14	590.7	6.12	576.7
11	1:3.00	70.00	105.0	10.05	535.4	9.87	535.9
12	1:3.00	140.00	105.0	5.50	712.1	5.75	685.4
13	1:3.00	105.00	30.0	7.74	544.9	7.76	544.4
14	1:3.00	105.00	180.0	7.39	570.4	7.45	554.7
15	1:3.00	105.00	105.0	6.85	533.0	7.03	532.8
16	1:3.00	105.00	105.0	6.85	533.0	6.81	541.6
17	1:3.00	105.00	105.0	6.85	533.0	6.77	567.1
18	1:3.00	105.00	105.0	6.85	533.0	6.44	523.8
19	1:3.00	105.00	105.0	6.85	533.0	7.09	514.7
20	1:3.00	105.00	105.0	6.85	533.0	6.97	520.9

3.3.1.1 Analysis of pretreatment model

The significance of each term (A, B, C) in the quadratic regression model was estimated based on the analysis of variance (ANOVA) technique. The p-values, F-values and lack of fit were used to verify the significance of each coefficient (Table 3.3.4). The lower p-value indicates a significant interaction between the factors. The higher F-value signifies a good prediction of the experimental data. A higher lack of fit of the model represents the true shape of the surface (Dutta *et al*. 2014). From Table 3.3.4, it is clear that the model is statistically significant with the highest F-value of 82.5, very low probability value ($p < 0.0001$) and the high sum of square of 35.4 for the response, lignin content. It was also observed that linear (A and B), interaction (AB, AC and BC) and quadratic (B^2 and C^2) are significant model terms for the response, lignin content. Similarly, the TCC model was also significant as indicated by the F-value of 19.6, p-value <0.0001 and the high sum of square of 61230.2. The effects of linear (A and B), interaction (AB and BC) and quadratic (A^2 and B^2) terms contribute more significantly. Thus, it was observed that the pretreatment temperature (B) and ChCl: AA molar ratio (A) significantly affect the RS pretreatment process. In this study, the actual *vs* predicted responses for both the responses, lignin and TCC content exhibited almost a linear relationship with a high value of "R-Square" of 0.99 and 0.95, respectively (Table 3.3.4). A similar linear relationship between the actual vs. predicted responses indicating a reasonable precision of the fitted empirical model was also reported earlier (Song *et al*., 2011). Therefore, it can be concluded that the developed model is acceptable in predicting the effectiveness of ChCl: AA pretreatment method for RS.

Table 3.3.4. ANOVA table for a quadratic model of rice straw pretreatment.

Source	Lignin			TCC		
	Sum of squares	F-value	p-value	Sum of squares	F- value	p-value
Model	35.4	82.56	< 0.0001	61230.24	19.6	< 0.0001
A	1.7	35.64	0.0001	2186.08	6.3	0.0309
B	25.01	525.04	< 0.0001	37683.6	108.57	< 0.0001
C	0.14	3.04	0.1119	788.04	2.27	0.1628
AB	2.03	42.61	< 0.0001	1991.84	5.74	0.0376
AC	2.46	51.56	< 0.0001	69.43	0.2	0.6642
BC	1.71	35.98	0.0001	1998.24	5.76	0.0374
A^2	0.025	0.52	0.487	2387.47	6.88	0.0255
B^2	1.52	31.9	0.0002	14829.49	42.72	< 0.0001
C^2	0.91	19.06	0.0014	1094.56	3.15	0.1061
Lack of Fit	0.19	0.67	0.6618	1669.17	0.93	0.5324
R^2	0.9867			0.9464		
Adj R-Squared	0.9748			0.8981		
Pred R-Squared	0.9479			0.7537		

The second-order equation in the terms of actual factors generated by CCD for the response, lignin content and TCC are as follows:

a) Lignin content (%, w/w) = +12.57+ 2.19 x ChCl:AA molar ratio - 0.048 x Temperature (°C) -0.039 x Time (min) - 0.041 x ChCl:AA molar ratio x Temperature (°C) + 0.021 x ChCl:AA molar ratio x Time (min) - 4.99 E^{-004} x Temperature (°C) x Time (min) -0.12 x ChCl:AA molar ratio ^{2} +7.49 E^{-004} x Temperature (°C) ^{2} +1.26 E^{-004} x Time (min)^2

b) TCC (mg/g_{CApRS}) = + 1933.23 - 319.37 x ChCl:AA molar ratio-18.64 x Temperature (°C) -2.20 x Time (min) +1.27 x ChCl:AA molar ratio x Temperature (°C) - 0.11 x ChCl:AA molar ratio x Time (min) + 0.017 x Temperature (°C) x Time (min) + 36.41 x ChCl:AA molar ratio^2 +0.074 x Temperature (°C)^2+ 4.38 E^{-003} x Time (min)^2

3.3.1.2 Effect of parametric interactions on the responses

The interactions among the three factors (ChCl: AA molar ratio, time and temperature) involved in the process are of critical importance for multivariate optimization. Fig. 3.3.1. displays 3D response surface relationships among the factors for both the responses; lignin content and TCC. Fig. 3.3.1(a) and 3.3.1(d) represent the interaction between temperature and ChCl: AA molar ratio at the center level of the pretreatment time against the responses, lignin content and TCC, respectively. Fig. 3.3.1(b) and 3.3.1(e) represent the interaction between time and temperature at the center level of the ChCl: AA molar ratio against the responses, lignin content and TCC, respectively. Fig. 3.3.1(c) and 3.3.1(f) display 3D response surface interactions between time and ChCl: AA molar ratio at the center level of pretreatment temperature.

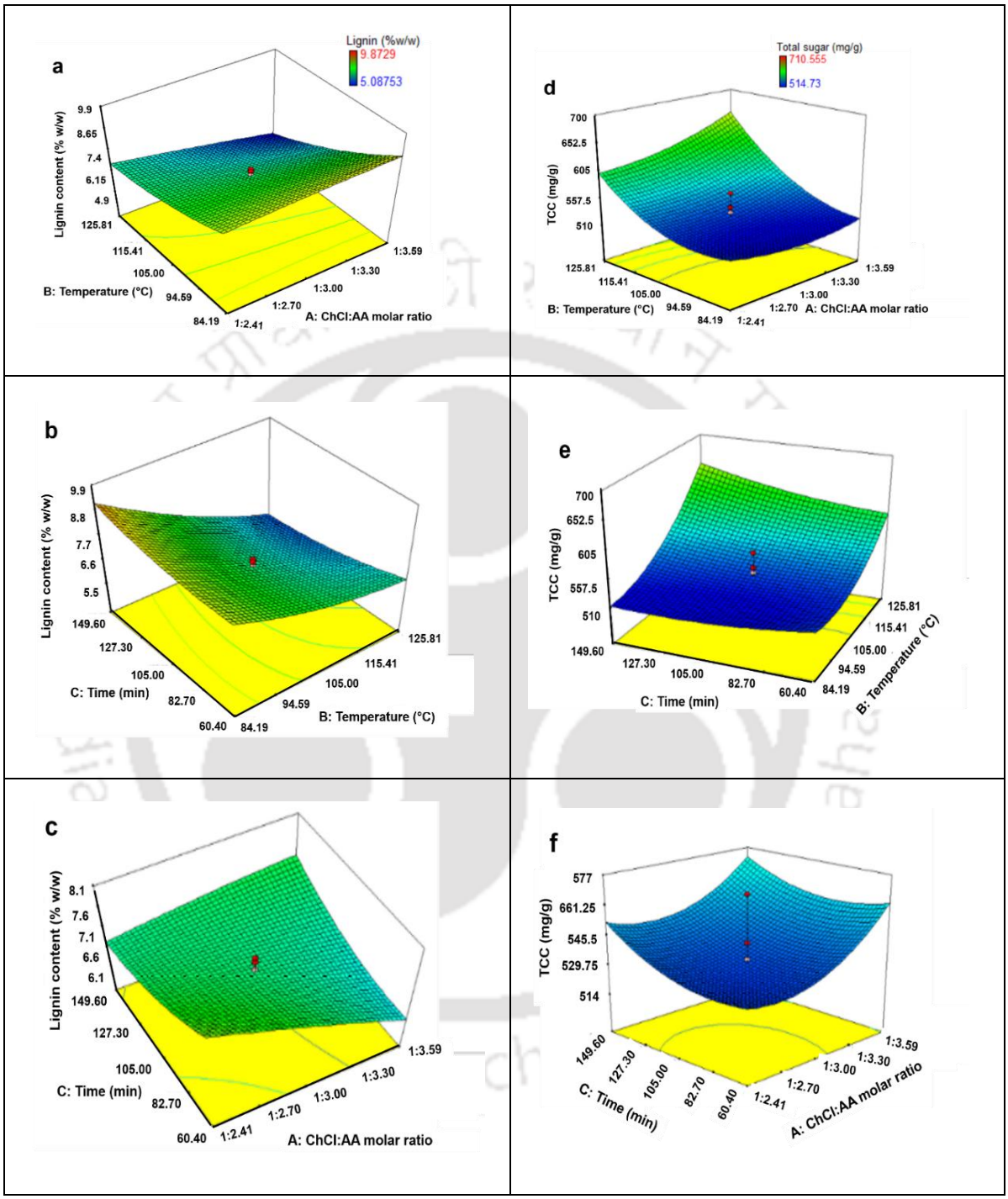

Fig. 3.3.1. 3-D surface plots (interaction among independent variables) for RSM-based pretreatment optimization of RS using ChCl: AA in terms of lignin content (%, w/w) (a, b and c) and TCC, mg/g_{CApRS} (d, e and f).
a and d) temperature vs ChCl: AA molar ratio;
b and e) time vs temperature;
c and f) time vs ChCl: AA molar ratio.

Moreover, a concomitant increase in the TCC (Fig. 3.3.1d) and the delignification percentage in the pretreated biomass was observed. Zhang *et al*., 2016 showed a collateral trend in their work on facial pretreatment of lignocellulosic biomass using DES (Zhang *et al*., 2016). It could be observed from the 3D plot (Fig 3.3.1b and 3.3.1e) that on increasing the treatment temperature both the delignification percentage and the TCC in the pretreated biomass are significantly enhanced. Similar results on RS pretreatment using DES with different constituent molar ratios were reported (Li *et al*., 2018). A higher ChCl: AA molar ratio or prolonged pretreatment time leads to a higher degree of lignin removal. Notably, at a fixed temperature, there was not any significant change in lignin content or TCC in the pretreated biomass (Fig. 3.3.1c and 3.3.1f). A possible explanation is that a higher temperature could weaken the hydrogen bonding thereby facilitating the mass transfer and biomass dissolution with the breakage of the ether and ester bonds between lignin and hemicellulosic fractions as also reported earlier (D'Agostino *et al*., 2011).

3.3.1.3 Optimization and validation of the pretreatment process

The main purpose of pretreatment optimization is to attain minimum lignin content and maximum TCC in the pretreated biomass through the optimized values of significant variables. The numerical optimization for selecting the best combinations for each factor (in range) and responses, lignin (minimized) and TCC (maximized) was performed. The optimized conditions predicted by the software were ChCl:AA molar ratio of 1:3.59, the temperature of 126°C and 149.59 min of pretreatment time. The model was validated by performing the ChCl-AA pretreatment of RS for 50 g DES (ChCl:AA) at 7.5% raw biomass loading at the above-mentioned optimized conditions. The experimental responses i.e., lignin content and TCC in the pretreated biomass

obtained were 5.09 (% w/w) and 679 mg/g_{CApRS}, respectively, which are close to the predicted values (5.16, % w/w lignin and 688 g/g_{CApRS} TCC, respectively).

3.3.2 Composition analyses of raw and ChCl: AA pretreated rice straw

The raw RS contained 73.9 ±1.5% holocellulose (w/w), 43.5±1.1% (w/w) cellulose, 30.3±1.2% (w/w) hemicellulose and 18.5±0.5% (w/w) lignin (Table 3.3.5). The raw RS biomass on pretreatment with ChCl: AA under optimized conditions resulted in increased content of holocellulose, 82.6±0.6% (w/w) and cellulose, 73.5±0.7% (w/w) with a significant reduction in hemicellulose, 9.1±0.5% (w/w) and lignin, 5.09±0.2% (w/w) content. The probable reason for the significant reduction in hemicellulose and lignin content could be due to the breakage of ether and ester bonds between hemicellulose and lignin molecules as also reported earlier (Xu *et al*., 2021). Moreover, this could also be due to the potential of acetic acid to solubilize the hemicellulosic fraction as mentioned earlier (Zhao *et al*., 2010). CApRS resulted in biomass recovery (57.9%), hemicellulose removal, 82% and delignification percentage, 83% (w/w) (Table 3.3.5). In addition to the high delignification percentage, a significant pretreatment efficiency of 83.7% (Equation 3.4) was achieved using the ChCl: AA pretreatment method which is essential for effective enzymatic hydrolysis of pretreated biomass to monosaccharides.

Table 3.3.5. Composition analysis of raw and ChCl: AA pretreated rice straw.

Biomass	Biomass Recovery, % w/w	Holo-cellulose, % w/w	Cellulose, % w/w	Hemi-cellulose, % w/w	Lignin, % w/w	Hemi-Cellulose removal %,w/w	Delignification % w/w	Crystallinity index (*CrI*) %
Raw RS	100	73.9±1.5	43.5±1.1	30.3±1.2	18.5±0.5	-	-	34.7%
CApRS	57.9±0.9	82.6±0.8	73.5±0.7	9.1±0.5	5.09±0.2	82.1	83	45.4%

Hemicellulose removal and delignification was calculated per 100 g of raw RS.
± Standard deviation (n=3).

A detailed comparison of RS delignification in this study with other reports

earlier was analyzed (Table 3.3.6). RS pretreatment using ChCl: lactic acid in molar ratio 1:3 at 120°C for 3 h reported a higher delignification percentage, 82.5% (Huang *et al*., 2020). However, RS pretreated in a molar ratio of 1:5 (ChCl: lactic acid) at 60°C for 12 h resulted in lower, 60% delignification (Kumar *et al*., 2016). A study on Bambara groundnut haulm biomass pretreated with ChCl: AA in molar ratio 1:2 at 100°C for 3 h reported delignification of 58% (Okuofu *et al*., 2020). Xing *et al*., 2018 reported a delignification of 44% on RS using ChCl: formic acid: acetic acid at molar ratio 1:1:1 at 120°C for 8 h (Xing *et al*., 2018). Thus, when compared with RS pretreatment with other DES systems or ChCl:AA pretreatment method on other biomass, the current study gave a higher delignification percentage

Table 3.3.6. Comparison of lignocellulosic feedstock pretreatment using different DES combinations in terms of delignification and saccharification efficiency.

Feedstock	DES type/ operating condition	Delignification (%)	Enzyme type/ hydrolysis conditions	Saccharifiaction efficiency (%)	References
Bambara groundnut haulm	ChCl:Acetic acid = 1:2, 100°C, 3 h	57.6	Accellerase: 30 mg/ g biomass, 50°C, 48 h	79.4	(Okuofu *et al*., 2020)
Rice straw	ChCl:Lactic acid =1:3, 120°C, 3 h	82.5	Cellulase:40 FPU /g biomass, 50°C, 144 h	75.7	(Huang *et al*., 2020)
Rice straw	ChCl:Lactic acid = 1:5, 60°C, 12 h	59.9	Cellulase: 9 FPU /g biomass, 42°C, 24 h	TSY= 333 mg/g	(Kumar *et al*., 2016)
Rice straw	ChCl:Oxalic acid, 100°C,1h – ChCl : Urea, 100°C, 2 h	65	Cellulase: 17.5 U/mL, 50°C, 24 h	92	(Hou *et al*., 2017)
Rice straw	ChCl:Formic acid:Acetic acid = 1:1:1, 120°C, 8 h	43.6	Cellulase: 50 FPU/g biomass, 50°C, 48 h	TSC= 21.5 g/L	(Xing *et al*., 2018)
Rice straw	ChCl:Acetic acid= 1:3.59, 126°C, 2.5h	84	Cellulase: 35 U/g biomass, 50°C, 48 h	92.2 TSY=746 mg/g TSC= 18.8 g/L	This study

TSY= Total sugar yield, TSC= Total sugar concentration

3.3.3 XRD analysis of raw and ChCl: AA pretreated rice straw

The cellulose crystallinity and crystallinity index of the lignocellulosic biomass play an important role in susceptibility to enzymatic hydrolysis (Le Tan *et al*., 2021). The impact of pretreatment processes on the crystalline structure of cellulose would affect the hydrolysis of cellulose in the enzymatic saccharification process (Procentese *et al*., 2015, Rocha *et al*., 2020). To explore the effect of the ChCl: AA pretreatment method on the structure and enzymatic digestibility of pretreated biomass, raw RS and CApRS samples were subjected to X-ray diffraction analysis. The raw RS and CApRS powder displayed 34.7% and 45.4% crystallinity index (*CrI*), respectively. The *CrI* of CApRS was 31% higher than the raw RS. A study on RS pretreatment with ChCl: AA + formic acid in a fixed molar ratio of 1:2:1 reported a 16% increment in *CrI* as compared with the raw RS (Xing *et al*., 2018). Thus, this study suggested the effective removal of lignin and hemicellulosic fractions from RS leading to the exposure of the cellulosic fraction in the pretreated solid residue.

3.3.4 BET of raw and ChCl: AA pretreated rice straw

The specific surface area of the sample does not only determines the nature of the interaction between the biomass and the hydrolytic enzymes but also indicates the amorphous nature of the sample. Raw RS and CApRS samples were subjected to BET-specific surface and pore size analysis. The CApRS showed a surface area of 17.56 m^2/g which was 17 fold higher than that of raw RS. The raw RS showed a total pore volume of $1.07x10^{-2}$ cc/g, whereas CApRS resulted in a higher total pore volume of $5.42x10^{-2}$ cc/g. The specific surface area obtained by ChCl: AA pretreatment in this study is higher than pretreatment of RS with other methods like integrated pretreatment of acid and steam explosion, 6.63 m^2/g (Chen *et al*., 2011) and ionic liquid pretreatment,

9.36 m^2/g (Mohammadi *et al*., 2019). The increase in the surface area is due to the delignification and removal of hemicellulosic fraction from the RS during the pretreatment process which ultimately leads to efficient enzymatic saccharification as also reported earlier (Wi *et al*., 2013, Shen *et al*., 2019).

3.3.5 Functional groups analysis of raw and ChCl: AA pretreated rice straw

The FT-IR spectroscopic analysis of raw RS and CApRS was carried out for functional group analysis. A peak at wavenumber 1640 cm^{-1} signifies lignin and its aromatic ring (Xu *et al*., 2013). The wavenumber range, 1328 cm^{-1}-1433 cm^{-1} represents OH vibrations due to intermolecular and intramolecular hydrogen bonds in cellulose (Mohammadi *et al*., 2019). The CApRS spectra show a significant reduction in the peak intensity of wavenumber 1640 cm^{-1} (Fig. 3.3.2) indicating efficient lignin removal as deciphered earlier (Liu *et al*., 2018). A prominent reduction in peak intensity between 1328 cm^{-1} and 1433 cm^{-1} indicates the loosening of cellulose structure and reduction of cellulose crystallinity in CApRS biomass, thereby increasing the surface area of the pretreated biomass as reported earlier (Jamaldheen *et al*., 2018). A higher CH_2 peak stretching from wavenumber 2857 cm^{-1} to 2911 cm^{-1} in CApRS sample specifies the exposure of cellulosic fiber as reported earlier (Jamaldheen *et al*., 2018). Therefore, the spectral analysis of CApRS sample clearly showed the effectiveness of the pretreatment process.

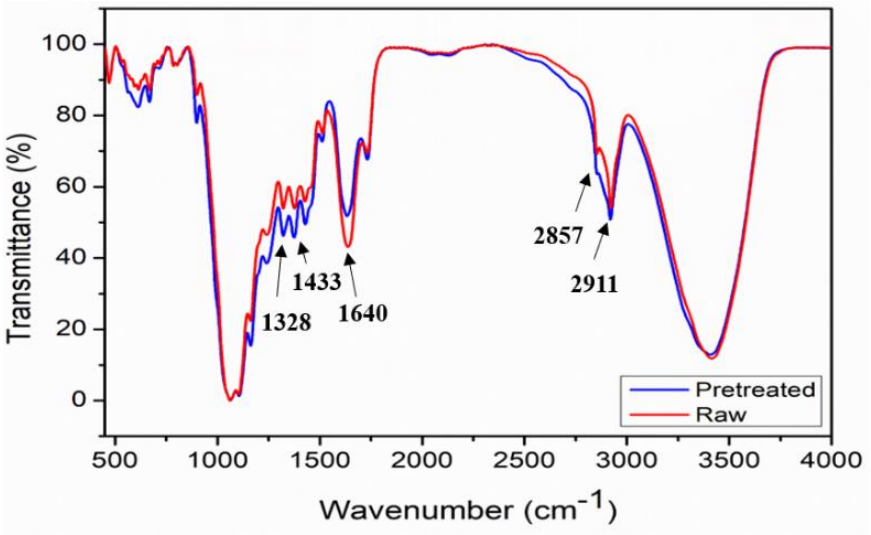

Fig. 3.3.2 FT-IR spectra of raw RS and CApRS biomass.

3.3.6 Surface morphology analysis of raw and ChCl: AA pretreated rice straw by FESEM

The surface topology analysis of raw RS and CApRS samples by FESEM was performed. The rigid, rough and non-porous surface of raw RS depicted the intact structural integrity of cellulose, hemicelluloses and lignin (Fig. 3.3.3). CApRS showed a surface with visible pores or voids and fibrous structures, unlike the raw RS. The ChCl: AA pretreatment resulted in alteration of biomass morphology from organized structure to smooth fibrous structures with cellulosic fibers exposure, increased surface area and pore volume that is in line with the findings of earlier section 3.3.4, 3.3.5 and also other researchers (Mohammadi *et al*., 2019, Nath *et al*., 2021).

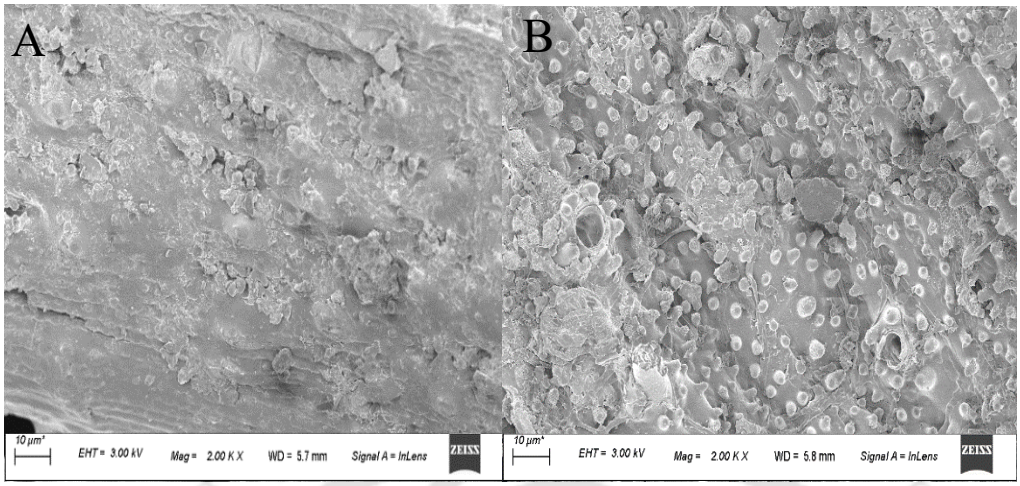

Fig. 3.3.3. (A) FE-SEM image of raw RS and (B) CApRS biomass.

3.3.7 Enzymatic saccharification of raw and ChCl: AA pretreated rice straw

Maximum delignification of RS during the pretreatment process is essential for the conversion of cellulose to glucose and its subsequent application. To check the efficacy of the pretreatment process, the enzymatic saccharification of raw RS and CApRS was performed by using the commercial cellulase from *T. ressei*, at a loading of 35 $U/g_{CApRS/raw\ RS}$ with 2.5% (w/v) CApRS/ raw RS loading in 10 ml working volume at 50°C for 48 h. The raw RS and CApRS resulted in TRS of 5.1 g/l and 18.8 g/l, respectively after 48 h of saccharification. The ChCl: AA pretreatment remarkably improved the biomass digestibility in the subsequent enzymatic hydrolysis by giving a saccharification efficiency of 92.2%. A detailed comparison of saccharification efficiency of rice straw and other biomass pretreated by the DES method is illustrated in Table 3.3.6.

3.3.8 Extraction and characterization of lignin by precipitation

100 g raw RS was pretreated using 1333 mL (biomass loading at 7.5%, w/v) of ChCl: AA DES solvent under the optimized conditions as described in section 3.3.1.3. After the pretreatment process, around 1000 mL of lignin-rich liquid hydrolysate was

obtained, which was used for extraction of DES-lignin (DL) by precipitating using a mixture of ultrapure water and ethanol. Around 10.4 g of DL was recovered (Fig. 3.3.6) from 1000 mL of liquid fraction with 100 g initial biomass resulting in an overall DL extraction yield of 56% (Eqn. 3.7) based on total lignin present in the raw RS biomass (18.5 g). The lignin extracted by precipitation using a mixture of water and ethanol (1:2 volume ratio) from ChCl: lactic acid and ChCl: citric acid pretreated oil palm empty fruit bunch resulted in a lignin extraction yield of 34% and 21%, respectively (Tan et al., 2019).

3.3.8.1 Composition and purity analysis of extracted DES-lignin

The commercial lignin and DL were subjected to a double acid hydrolysis method of NREL protocol for lignin and TCC estimation. The TCC (glucose, xylose and arabinose) in the DL was analyzed using HPLC as described in 3.2.4.1. The commercial lignin showed 98%, w/w lignin content (acid soluble + acid insoluble lignin) and 1.2%, w/v ash content. The HPLC analysis of commercial lignin showed the presence of trace amounts of 0.1%, w/w glucose and 0.3%, w/w xylose thus confirming its high purity as there was negligible contamination of glucan and xylan content. However, the DL showed 86% lignin content, 8% ash content, 4% glucose and 2% xylose based on dry weight basis. The presence of glucose and xylose indicated the extracted lignin, DL needs further purification.

3.3.8.2 Functional group analysis of extracted DES-lignin

The FTIR spectra of commercial lignin and DL (Fig. 3A) were studied to compare the change in peaks and functional groups. The main peaks obtained from FTIR spectra of DL were assigned based on various literature (Constant et al., 2016;

Lynam et al., 2017). The DL and commercial lignin spectra showed presence of both guiacyl, G (1269, 850 cm^{-1}) and syringyl, S (1328, 1120 cm^{-1}) sub-units of lignin. The peak at 1269 cm^{-1} and 850 cm^{-1} denotes the C-H stretching out of plane symmetry (feature of the G sub-unit) and the peak at 1328 cm^{-1} and 1120 cm^{-1} denotes the C-O and Ar-CH group in the S sub-unit (Prado et al., 2016). The C=O bond stretching of unconjugated carbonyl, ester and ketone groups at 1718 cm^{-1}, the C-C and C=C bonds stretching at 1515 cm^{-1} and 1610 cm^{-1}, respectively confirmed the aromatic skeleton of DL as also mentioned earlier by Nan et al., 2020. The high-intensity peak in DL at 2938 cm^{-1} indicated the stretching of C-H band vibration in $-CH_2$, $-CH_3O$, and $-CH_3$ groups. The C-H stretching at 2843 cm^{-1} in $-OCH_3$ group could be observed in both DL and commercial lignin spectra. A very similar predominant absorption peak at 3430 cm^{-1} in both DL and commercial lignin spectra confirms the presence of aliphatic and aromatic -OH bands stretching in the DES-extracted lignin (DL) as also earlier reported by Mousavioun et al., 2012. Thus, the similarity in lignin characteristic functional groups of DL with the previously reported lignin and commercial lignin also confirms the purity of DL.

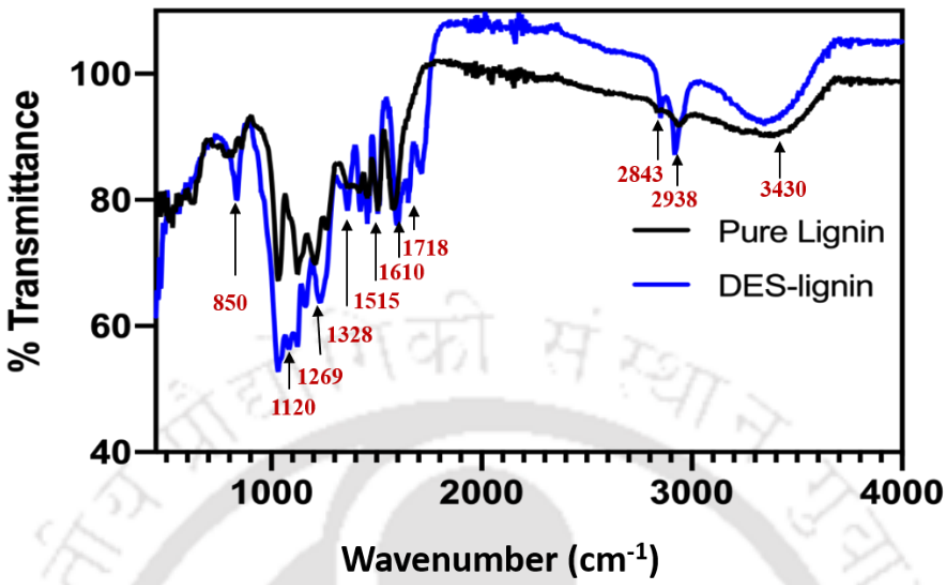

Fig. 3.3.4 FTIR spectra of extracted lignin (blue) and commercial lignin (black).

3.3.8.3 Surface morphology of extracted DES-lignin

The digital images of extracted DES-lignin and commercial lignin are shown in Fig. 3.3.5 A and B. Both the lignin, commercial and DL showed similar physical appearance with fine particle texture. The dark color of commercial lignin may be due to refining and polishing processes. The commercial lignin and DL were analyzed for surface morphology by FESEM (Fig. 3.3.5 C and D). The electron micrographs of both commercial and extracted DES-lignin exhibited similar spherical structures on the surface which signifies the precipitation of the polymeric particle.

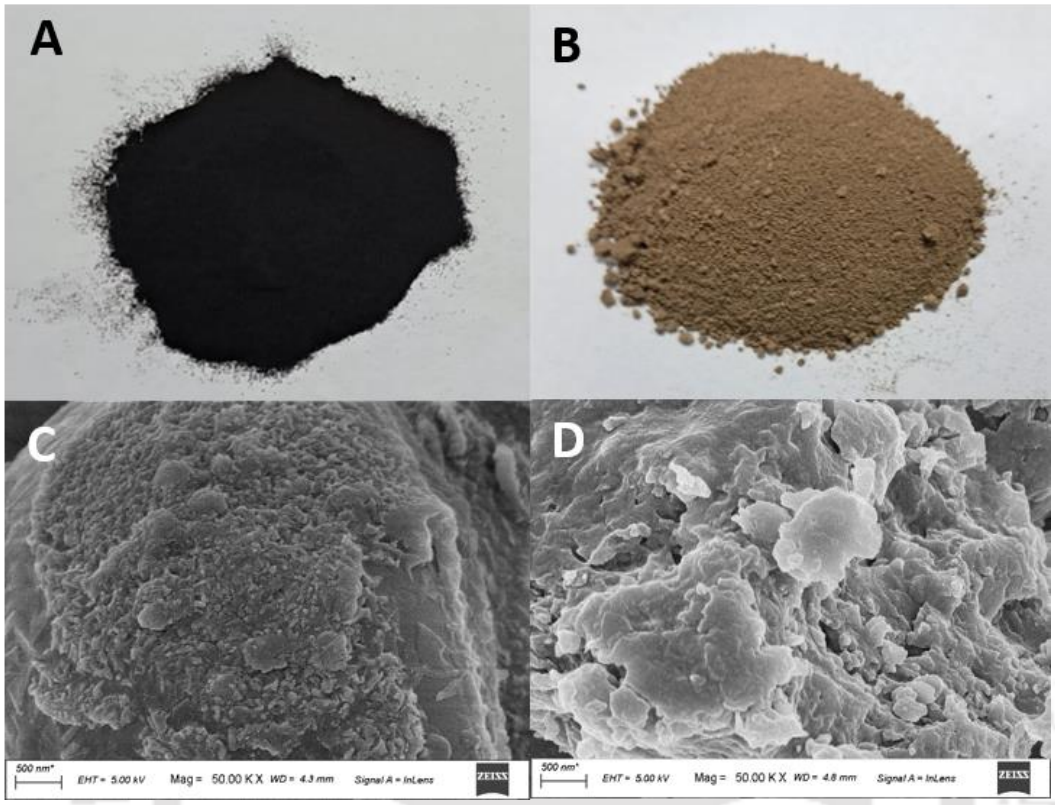

Fig. 3.3.5. Digital images of A) commercial lignin and B) extracted DL; FESEM images of C) commercial lignin and D) extracted DL at 50 KX magnification.

3.3.9 Mass balance

The mass balance of the bioconversion process of 100 g raw RS to soluble monosaccharides under optimized conditions of pretreatment followed by its subsequent saccharification was analyzed and shown in Fig. 3.3.6. Upon optimized DES pretreatment of 100 g raw RS with 7.5%,w/v biomass loading, 57.9 g of CApRS was recovered and 1000 mL of lignin-rich liquid hydrolysate was obtained. The cellulose, hemicellulose and lignin content of CApRS biomass was 73.9%, 9.1% and 5.09% (w/w) which implies 57.9 g of CApRS contained 42.5 g cellulose, 5.3 g hemicellulose and 3 g lignin thereby resulting in a pretreatment efficiency of 83.7%.

From 1000 mL liquid hydrolysate obtained after RS pretreatment, 10.4 g of DES-lignin was extracted resulting in an overall DES-lignin extraction yield of 56%. The enzymatic saccharification of 57.9 g CApRS biomass by using commercial cellulase under the condition described in section 2.5 gave a TRS amount of 46.2 g in the hydrolysate accounting for the saccharification efficiency of 92.2%. Thus, 100 g raw RS after pretreatment with ChCl: AA method under the statistically optimized conditions and subsequent enzymatic saccharification yielded a total reducing sugar, 46 g.

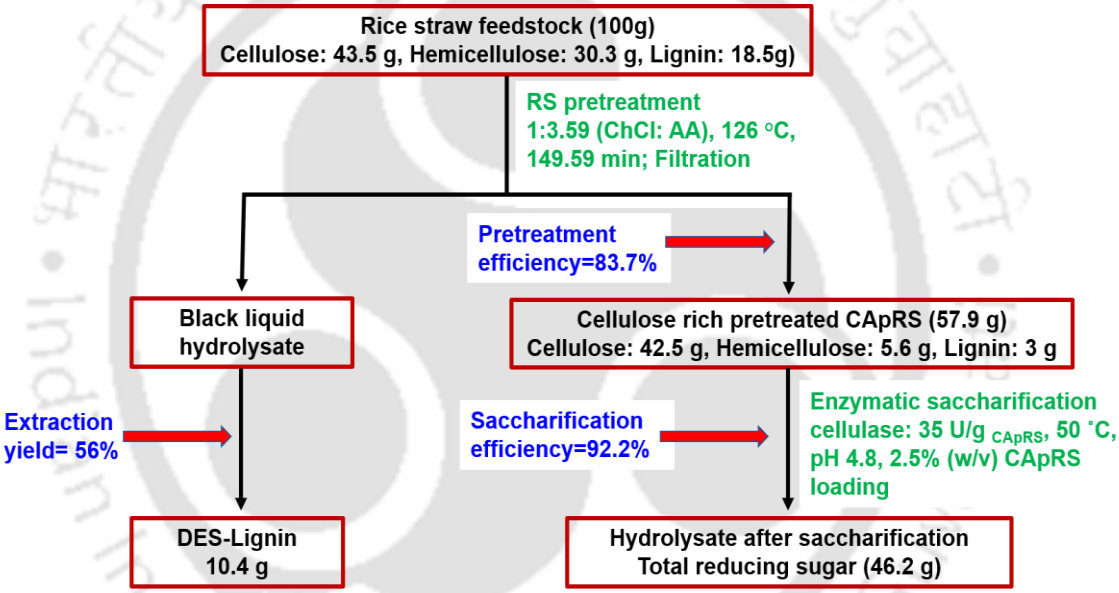

Fig. 3.3.6 Mass balance for bioconversion of raw RS to total reducing sugar, the feedstock for bioethanol production.

3.4. Conclusion

A deep eutectic solvent comprising choline chloride (ChCl) and acetic acid (AA) was used for rice straw (RS) pretreatment. Three significant pretreatment parameters; ChCl: AA molar ratio, time and temperature were scrutinized using statistical analysis. The effect of independent variables on lignin removal and retainment of total carbohydrate content (TCC) in the pretreatment process was evaluated by the central composite design (CCD) approach. The pretreatment temperature and molar ratio of AA to ChCl played a significant role in delignification. The optimized conditions for RS pretreatment were 1:3.59 (ChCl:AA molar ratio), 126 °C and 150 min. ChCl:AA pretreated RS (CApRS) gave 83.1% delignification, 679 mg/g_{CApRS} TCC and 83.7% pretreatment efficiency. CApRS contained enriched cellulose content, 0.73 g/gCApRS as compared with 0.43 g/g_{raw} RS in raw RS. CApRS showed 31% higher crystallinity index, a 17-fold higher surface area than raw RS. The morphological study of CApRS displayed a porous surface. The precipitation of black liquid hydrolysate obtained after the pretreatment of RS using ultrapure water and ethanol mixture resulted in the extraction of 10.4 g DES-lignin. The extraction yield of DES-lignin was 56% with the purity of 86%. The FTIR study of extracted lignin confirmed the presence of G and S-sub-units of lignin and aromatic skeleton in DL. Saccharification of CApRS by commercial cellulase gave a total reducing sugar of 18.8 g/L in hydrolysate with saccharification efficiency, 92.2%. The high saccharification efficiency achieved in this study denotes the efficacy of the developed pretreatment method.

3.5 References

Baruah, J., Nath, B.K., Sharma, R., Kumar, S., Deka, R.C., Baruah, D.C. and Kalita, E. (2018). Recent trends in the pretreatment of lignocellulosic biomass for value-added products. Frontiers in Energy Research, 6, 141.

Calvo-Flores, F.G., Monteagudo-Arrebola, M.J., Dobado, J.A. and Isac-García, J. (2018). Green and bio-based solvents. Topics in Current Chemistry, 376 (3),1-40.

Chen, W.H., Pen, B.L., Yu, C.T. and Hwang, W.S. (2011). Pretreatment efficiency and structural characterization of rice straw by an integrated process of dilute-acid and steam explosion for bioethanol production. Bioresource Technology, 102(3), 2916-2924.

Chen, Y. and Mu, T. (2019). Application of deep eutectic solvents in biomass pretreatment and conversion. Green Energy & Environment, 4(2), 95-115.

Constant, S., Wienk, H.L., Frissen, A.E., De Peinder, P., Boelens, R., Van Es, D.S., Grisel, R.J., Weckhuysen, B.M., Huijgen, W.J., Gosselink, R.J. and Bruijnincx, P.C. (2016). New insights into the structure and composition of technical lignins: a comparative characterisation study. Green Chemistry, 18(9), 2651-2665.

D'Agostino, C., Harris, R.C., Abbott, A.P., Gladden, L.F. and Mantle, M.D. (2011). Molecular motion and ion diffusion in choline chloride based deep eutectic solvents studied by 1 H pulsed field gradient NMR spectroscopy. Physical Chemistry Chemical Physics, 13(48), 21383-21391.

Di Marino, D., Stöckmann, D., Kriescher, S., Stiefel, S. and Wessling, M. (2016). Electrochemical depolymerisation of lignin in a deep eutectic solvent. Green Chemistry, 18(22), 6021-6028.

Dowe, N. and McMillan, J. (2001). SSF experimental protocols: lignocellulosic biomass hydrolysis and fermentation. National Renewable Energy Laboratory (NREL) Analytical Procedures: 510-42630.

Dutta, S.K., Halder, G. and Mandal, M.K. (2014). Modeling and optimization of bi-directional delignification of rice straw for production of bio-fuel feedstock using central composite design approach. Energy, 71, 579-587.

Hong, S., Shen, X.J., Pang, B., Xue, Z., Cao, X.F., Wen, J.L., Sun, Z.H., Lam, S.S., Yuan, T.Q. and Sun, R.C. (2020). In-depth interpretation of the structural changes of lignin and formation of diketones during acidic deep eutectic solvent pretreatment. Green Chemistry, 22(6), 1851-1858.

Hou, X.D., Feng, G.J., Ye, M., Huang, C.M. and Zhang, Y. (2017). Significantly enhanced enzymatic hydrolysis of rice straw via a high-performance two-stage deep eutectic solvents synergistic pretreatment. Bioresource Technology, 238, 139-146.

Huang, Z.J., Feng, G.J., Lin, K.P., Pu, F.L., Tan, Y.M., Tu, W.C., Han, Y.L., Hou, X.D., Zhang, H.M. and Zhang, Y. (2020). Significant boost in xylose yield and enhanced economic value with one-pot process using deep eutectic solvent for the pretreatment and saccharification of rice straw. Industrial Crops and Products, 152, 112515.

Jamaldheen, S.B., Sharma, K., Rani, A., Moholkar, V.S. and Goyal, A. (2018). Comparative analysis of pretreatment methods on sorghum (Sorghum durra)

stalk agrowaste for holocellulose content. Preparative Biochemistry and Biotechnology, 48(6), 457-464.

Kumar, A.K., Parikh, B.S. and Pravakar, M. (2016). Natural deep eutectic solvent mediated pretreatment of rice straw: bioanalytical characterization of lignin extract and enzymatic hydrolysis of pretreated biomass residue. Environmental Science and Pollution Research, 23(10), 9265-9275.

Kumar, B., Bhardwaj, N., Agrawal, K., Chaturvedi, V. and Verma, P. (2020). Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept. Fuel Processing Technology, 199, 106244.

Láinez, M., Ruiz, H.A., Arellano-Plaza, M. and Martínez-Hernández, S. (2019). Bioethanol production from enzymatic hydrolysates of Agave salmiana leaves comparing S. cerevisiae and K. marxianus. Renewable Energy, 138, 1127-1133.

Le Tan, N.T., Dam, Q.P., Mai, T.P. and Nguyen, D.Q. (2021). The Combination of Acidic and Alkaline Pretreatment for a Lignocellulose Material in Simultaneous Saccharification and Fermentation (SSF) Process. Chemical Engineering Transactions, 89, 43-48.

Li, A.L., Hou, X.D., Lin, K.P., Zhang, X. and Fu, M.H. (2018). Rice straw pretreatment using deep eutectic solvents with different constituents molar ratios: Biomass fractionation, polysaccharides enzymatic digestion and solvent reuse. Journal of Bioscience and Bioengineering, 126(3), 346-354.

Liu, D., Yan, X., Zhuo, S., Si, M., Liu, M., Wang, S., Ren, L., Chai, L. and Shi, Y. (2018). Pandoraea sp. B-6 assists the deep eutectic solvent pretreatment of rice straw via promoting lignin depolymerization. Bioresource Technology, 257, 62-68.

Loow, Y.L., Wu, T.Y., Yang, G.H., Ang, L.Y., New, E.K., Siow, L.F., Jahim, J.M., Mohammad, A.W. and Teoh, W.H. (2018). Deep eutectic solvent and inorganic salt pretreatment of lignocellulosic biomass for improving xylose recovery. Bioresource Technology, 249, 818-825.

Lynam, J.G., Kumar, N. and Wong, M.J. (2017). Deep eutectic solvents' ability to solubilize lignin, cellulose, and hemicellulose; thermal stability; and density. Bioresource Technology, 238, 684-689.

Maibam, P.D. and Maiti, S.K. (2020). A strategy for simultaneous xylose utilization and enhancement of cellulase enzyme production by Trichoderma reesei cultivated on liquid hydrolysate followed by induction with feeding of solid sugarcane bagasse. Waste and Biomass Valorization, 11(7), 3151-3160.

Mohammadi, M., Shafiei, M., Abdolmaleki, A., Karimi, K., Mikkola, J.P. and Larsson, C. (2019). A morpholinium ionic liquid for rice straw pretreatment to enhance ethanol production. Industrial Crops and Products, 139, 111494.

Mood, S. H., Golfeshan, A. H., Tabatabaei, M., Jouzani, G. S., Najafi, G. H., Gholami, M. and Ardjmand, M. (2013). Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renewable and Sustainable Energy Reviews, 27, 77-93.

Mousavioun, P., George, G. A. and Doherty, W. O. (2012). Environmental degradation of lignin/poly (hydroxybutyrate) blends. Polymer Degradation and Stability, 97(7), 1114-1122.

Nan, H., Zhao, L., Yang, F., Liu, Y., Xiao, Z., Cao, X. and Qiu, H. (2020). Different alkaline minerals interacted with biomass carbon during pyrolysis: Which one

improved biochar carbon sequestration?. Journal of Cleaner Production, 255, 120162.

Nath, P., Maibam, P.D., Singh, S., Rajulapati, V. and Goyal, A. (2021). Sequential pretreatment of sugarcane bagasse by alkali and organosolv for improved delignification and cellulose saccharification by chimera and cellobiohydrolase for bioethanol production. 3 Biotech, 11(2), 1-16.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry 153(2): 375-380.

Okuofu, S.I., Gerrano, A.S., Singh, S. and Pillai, S. (2020). Deep eutectic solvent pretreatment of Bambara groundnut haulm for enhanced saccharification and bioethanol production. Biomass Conversion and Biorefinery, 1-9.

Prado, R., Erdocia, X. and Labidi, J. (2016). Study of the influence of reutilization ionic liquid on lignin extraction. Journal of Cleaner Production, 111, 125-132.

Procentese, A., Johnson, E., Orr, V., Campanile, A.G., Wood, J.A., Marzocchella, A. and Rehmann, L. (2015). Deep eutectic solvent pretreatment and subsequent saccharification of corncob. Bioresource Technology, 192, 31-36.

Rocha, G.J.M., Andrade, L.P., Martin, C., Araujo, G.T., Mouchrek Filho, V.E. and Curvelo, A.A.S. (2020). Simultaneous obtaining of oxidized lignin and cellulosic pulp from steam-exploded sugarcane bagasse. Industrial Crops and Products, 147, 112227.

Segal, L.G.J.M.A., Creely, J.J., Martin Jr, A.E. and Conrad, C.M. (1959). An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. Textile Research Journal, 29(10), 786-794.

Shen, Z., Hou, D., Jin, F., Shi, J., Fan, X., Tsang, D.C. and Alessi, D.S. (2019). Effect of production temperature on lead removal mechanisms by rice straw biochars. Science of the Total Environment, 655, 751-758.

Singh, R., Srivastava, M. and Shukla, A. (2016). Environmental sustainability of bioethanol production from rice straw in India: a review. Renewable and Sustainable Energy Reviews, 54, 202-216.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. and Crocker, D.L.A.P. (2010). Determination of structural carbohydrates and lignin in biomass. Laboratory Analytical Procedure, (TP-510-42618).

Somogyi, M. (1945). A new reagent for the determination of sugars. Journal of Biological Chemistry, 160(1), 61-68.

Song, Y., Du, B., Zhou, T., Han, B., Yu, F., Yang, R., Hu, X., Ni, Y. and Li, Q. (2011). Optimization of extraction process by response surface methodology and preliminary structural analysis of polysaccharides from defatted peanut (Arachis hypogaea) cakes. Carbohydrate Research, 346(2), 305-310.

Tan, Y. T., Ngoh, G. C. and Chua, A. S. M. (2019). Effect of functional groups in acid constituent of deep eutectic solvent for extraction of reactive lignin. Bioresource Technology, 281, 359-366.

TAPPI. Technical Association of Pulp and Paper Industry, Atlanta, GA. (1992).

Tran, A.T., Cao, N.H., Le, P.T.K., Mai, P.T. and Nguyen, Q.D. (2020). Reusing alkaline solution in lignocellulose pretreatment to save consumable chemicals without losing efficiency. Chemical Engineering Transactions, 78, 307-312.

Van Hung, N., Maguyon-Detras, M.C., Migo, M.V., Quilloy, R., Balingbing, C., Chivenge, P. and Gummert, M. (2020). Rice straw overview: availability, properties, and management practices. Sustainable Rice Straw Management, 1.

Wang, W. and Lee, D.J. (2021). Lignocellulosic biomass pretreatment by deep eutectic solvents on lignin extraction and saccharification enhancement: A review. Bioresource Technology, 339, 125587.

Wi, S.G., Choi, I.S., Kim, K.H., Kim, H.M. and Bae, H.J. (2013). Bioethanol production from rice straw by popping pretreatment. Biotechnology for Biofuels, 6(1), 1-7.

Xing, W., Xu, G., Dong, J., Han, R. and Ni, Y. (2018). Novel dihydrogen-bonding deep eutectic solvents: Pretreatment of rice straw for butanol fermentation featuring enzyme recycling and high solvent yield. Chemical Engineering Journal, 333, 712-720.

Xu, F., Yu, J., Tesso, T., Dowell, F. and Wang, D. (2013). Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: a mini-review. Applied Energy, 104, 801-809.

Xu, H., Kong, Y., Peng, J., Song, X., Liu, Y., Su, Z., Li, B., Gao, C. and Tian, W. (2021). Comprehensive analysis of important parameters of choline chloride-based deep eutectic solvent pretreatment of lignocellulosic biomass. Bioresource Technology, 319, 124209.

Xu, H., Peng, J., Kong, Y., Liu, Y., Su, Z., Li, B., Song, X., Liu, S. and Tian, W. (2020). Key process parameters for deep eutectic solvents pretreatment of lignocellulosic biomass materials: A review. Bioresource Technology, 310, 123416.

Zhang, C.W., Xia, S.Q. and Ma, P.S. (2016). Facile pretreatment of lignocellulosic biomass using deep eutectic solvents. Bioresource Technology, 219, 1-5.

Zhao, X. and Liu, D. (2010). Chemical and thermal characteristics of lignins isolated from Siam weed stem by acetic acid and formic acid delignification. Industrial Crops and Products, 32(3), 284-291.

Chapter 4

Designing of recombinant hydrolytic enzymes cocktail for effective saccharification of delignified pretreated rice straw

4.1 Introduction

The lignocellulosic biomass which is abundant and has low food and fodder value serves to be a potentially renewable resource for bioethanol production (Abdmouleh *et al*., 2015; Zhang *et al*., 2023a). In lignocellulosic biorefinery, three key processes are: i) biomass pretreatment ii) enzymatic conversion of complex polymer to reduced sugars and iii) fermentation of reduced sugar to bioethanol (Rajak *et al*., 2018, Guo *et al*., 2018). However, the challenging step in biorefineries is the conversion efficiency of the cellulosic and hemicellulosic fractions of lignocellulosic biomass to C6 and C5 sugars, respectively (Guo *et al*., 2018). Lignocellulosic biomass constitutes approximately, 45%-75% holocellulose that can be hydrolyzed into fermentable sugars (Maibam and Goyal, 2022a). However, for the complete degradation of the cellulosic and hemicellulosic fraction into monosaccharides, enzymatic hydrolysis is necessary

(Thakur *et al*., 2020b). Therefore, the enzymatic saccharification process requires the repertoire of cellulases and hemicellulases to hydrolyze both fractions and reduce to fermentable monosaccharides (Saritha *et al*., 2013).

Bioethanol industries and recent research showed the application of commercial enzymes such as Celluclast 1.5 L and Cellic Ctec2 from Novozymes (Franklinton, USA); Multifect xylanase from Genencor International Inc. (NY, USA) for the hydrolysis of lignocellulosic biomass and could achieve ~60%- 80% saccharification efficiency (Samayam *et al*., 2010, Lan *et al*., 2013, Méndez-Líter *et al*., 2021, Zhang *et al*., 2023b). However, the drawback of using commercial enzymes is the high production cost and the limited selection of enzymes. Cellulases and xylanases are also produced by wild-type or genetically-modified microbial strains (Wang *et al*., 2021). *Trichoderma ressei* and some strains from the fungal genera *Penicillium* and *Talaromyces* are the predominant producers of cellulase and xylanase for biomass hydrolysis (Méndez-Líter *et al*., 2021, Maibam and Maiti, 2020). The drawback of using these in-house produced fungal enzymes is the inefficient saccharification efficiency (Maibam and Maiti, 2020). Recently, research has been focused on the use of recombinant enzymes with enhanced activity to improve hydrolytic efficiencies (Zhang *et al*., 2021, Velasco *et al*., 2021, Huang *et al*., 2022). The recombinant hydrolytic enzymes such as endoglucanase (Nath *et al*. 2019), exoglucanase (Rajak *et al*. 2020), β-glucosidase (Nath *et al*., 2019), endoxylanase and β-xylosidase (Huy *et al*., 2020, Thakur *et al*., 2020a) exhibiting high activity have been expressed and produced by using *E. coli*. However, due to the sensitivity of each hydrolytic enzyme to pH and temperature, there have been several trials to model the enzyme cocktails and optimize their operating conditions for an efficient saccharification process.

In order to address these challenges of the saccharification process, this study aims to design a crude (unpurified) enzyme cocktail comprising recombinant cellulases and xylanases to enhance the total reducing sugar yield as compared with the earlier reports. Extensive work on enzymatic saccharification of various substrates by using in-house purified enzymes (recombinant, wild-type, or mutant variants) and commercial enzymes has been reported. However, only a few studies have investigated the potential of crude recombinant hydrolytic enzymes, which should offer an economic advantage in the overall process. Therefore, in the present study, the crude form (unpurified) of four efficient recombinant hydrolytic enzymes; i) cellulolytic chimeric enzyme (*Ct*GH1-L1-*Ct*GH5-F194A) with a bi-functional activity of β-1,4-endoglucanase and β-1,4-glucosidase, ii) cellobiohydrolase (*Ct*CBH5A), iii) endo-1,4-β-xylanase (*Ct*Xyn11A) and iv) exo-1,4-β-xylosidase (*Bo*GH43A) are used for statistically designing an enzyme cocktail with optimal proportion of each constituting enzyme for the saccharification of choline chloride: acetic acid pretreated rice straw (CApRS) biomass. The effectiveness of the developed enzyme cocktail was compared with the commercial enzymes, cellulase (source: *Trichoderma ressei*) and xylanase (source: *Aspergillus niger*) based on the saccharification efficiency.

4.2 Materials and methods

4.2.1 Chemicals and reagents

The analytical reagent grade chemicals, including choline chloride, acetic acid, Luria Bertani, isopropyl -D-1-thiogalactopyranoside (IPTG), kanamycin, ampicillin and sodium azide were obtained from Himedia Pvt. Ltd., India. Substrates such as glucose, xylose, cellobiose, carboxymethyl cellulose sodium salt (CMC-Na), birchwood xylan, bovine serum albumin and commercial cellulase, enzyme blend (Cat. No. SAE 0020, synonymous to industrially used Cellic CTec2) from *Trichoderma reesei* were from Sigma-Aldrich Co. LLC, USA. The commercial xylanase from *Aspergillus niger* used in the study was purchased from Sisco Research Laboratories Pvt. Ltd., India.

4.2.2 Feedstock for enzymatic hydrolysis

The raw rice straw (RS) containing 43.5% (w/w) cellulose, 30.3% (w/w) hemicellulose and 18.5% (w/w) lignin was pretreated with choline chloride (ChCl) and acetic acid (AA) based deep eutectic solvent under previously optimized condition of ChCl: AA in the molar ratio 1:3.59 at 126°C for 150 min as reported earlier (Maibam and Goyal, 2022b). The structure and composition analysis of choline chloride-acetic acid pretreated RS (CApRS) showed cellulose 73.5% (w/w), hemicellulose 9.1% (w/w), lignin 5.1% (w/w). The CApRS showed 83% delignification and enriched total carbohydrate content of 688 mg/g_{CApRS} as reported earlier in our previous study (Maibam and Goyal, 2022b). The pretreated biomass, CApRS was further used as a substrate for designing enzyme cocktails for the saccharification process.

4.2.3 Production and enzyme activity estimation of recombinant hydrolytic enzymes

Recombinant cellulases (cellobiohydrolase, *Ct*CBH5A and chimera, *Ct*GH1-L1-*Ct*GH5-F194A) and xylanases (endo-1,4-β-xylanase, *Ct*GH11A and β-Xylosidase, *Bo*GH43) were used for the enzymatic saccharification of CApRS biomass. The chimera (*Ct*GH1-L1-*Ct*GH5-F194A) cloned and expressed earlier was used (Nath *et al*., 2019). Cellobiohydrolase (*Ct*CBH5A) and endo-1,4-β-xylanase (*Ct*Xyn11A) cloned from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) cloned from *Bacteroides ovatus* were gift from NZYTech Pvt. Ltd., Portugal. Around 1% (v/v) of *E. coli* BL21 (DE3) cells harboring pET28a(+) vector containing gene encoding chimera (*Ct*GH1-L1-CtGH5-F194A), *Ct*GH11A or *Bo*GH43 were inoculated in 800 mL Luria-Bertani medium supplemented with kanamycin (50 µg/mL). The only difference in *Ct*GH11 is the supplementation with ampicillin (100 µg/mL) as its gene was cloned using the pET21a(+) vector. The culture was incubated at 37°C, 180 rpm for 4 h, then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and incubated for 16 h at 24°C, 180 rpm. The culture was centrifuged at 6000g for 10 min at 4°C. The supernatant was discarded and the cell pellet containing the intracellular enzyme was re-suspended in 10 mL of 0.05 M sodium phosphate buffer (pH 5.8). This was subjected to ultra-sonication for 15 min (5s on/10s off pulse at 33% amplitude, Sonics, Vibra cell). The cell lysate obtained was centrifuged at 13,000xg at 4°C for 1 h. The cell debris was discarded and the supernatant (~10 mL) containing crude (unpurified enzyme present in the total cellular proteins) recombinant enzyme was analyzed for protein content and enzyme activity for further use. The enzyme activity of all the four enzymes in crude form was determined by the quantification of reducing

sugars using the methods of Nelson (1944) and Somogyi (1945) under optimized conditions as reported earlier (Nath *et al*., 2019, Jamaldheen *et al*., 2019 and Nedumaran *et al*., 2020) and β-glucosidase activity of chimera was estimated by GOD-POD method as reported earlier (Nath *et al*., 2019). The enzymatic reaction for the endoglucanase activity of chimera was conducted for 4 min at 60°C with a substrate concentration of 1%, w/v carboxymethyl cellulose sodium salt (CMC-Na). For the β-glucosidase activity of chimera, the enzymatic reaction was conducted at 70°C for 10 min at 1%, w/v cellobiose (Nath *et al*., 2019). The enzymatic reaction for the cellobiohydrolase activity of *Ct*CBH5A was conducted for 5 min at 65°C with a substrate concentration of 1%, w/v carboxymethyl cellulose sodium salt (CMC-Na). The enzymatic reactions for endoxylanase activity of *Ct*GH11 and β-xylosidase activity of *Bo*GH43 were conducted at 65°C for 1 min and 37°C for 5 min, respectively, at 1% (w/v) birchwood xylan. The enzyme activity of all the four enzymes was conducted using a common buffer, 0.05 M sodium phosphate buffer (pH 5.8). The enzyme activity of the crude enzymes is shown in (Table 4.3.1). The total protein concentration of individual crude enzymes was determined by using Folin-Lowry's method with bovine serum albumin as a standard protein. All the enzyme activities were expressed in international units (U). One unit of activity corresponds to the quantity of enzyme that releases 1 µmol of reducing sugars (in glucose/ xylose equivalents) per minute.

4.2.4. Determination of optimum pH and temperature for saccharification

Chimera showed 100% activity at 0.05 M citrate-phosphate buffer, a pH range of 4.0 to 5.5 and a temperature range of 60-70°C (Nath *et al*., 2019). *Ct*CBH5A shows its optimum activity at 0.05 M sodium-phosphate buffer in the pH range of 6.2-6.6 and temperature range of 30-65°C (Nedumaran *et al*., 2020). *Ct*GH11 gave optimum

activity in 0.05 M sodium-phosphate buffer in the pH range of 7.0-7.5 at 65°C and *Bo*GH43 at 0.05 M sodium-phosphate buffer, pH 7 at 37°C (Jamaldheen *et al*., 2019). However, for the operation of the saccharification process using the aforementioned four hydrolytic enzymes, a common stable pH, buffer concentration and temperature at which all four enzymes retain their maximum residual activity needs to be determined. So, the enzyme activity of all four enzymes at three different pHs: 5, 5.8 and 6.2 with a concentration of 0.05M, citrate-phosphate and sodium-phosphate buffer was estimated at three different temperatures: 30°C, 35°C and 40°C. Also, to understand the stability of each enzyme, each set of enzymes (50µL, 20 mg/mL) was incubated till 96 h and the enzyme activity at 24 h intervals of incubation was estimated by following individual assay methods and the relative residual enzyme activity (%) was calculated and reported in Table 4.3.2.

4.2.5 Experimental design for formulating enzyme cocktail for CApRS saccharification

The Design-Expert (7.0.0) software (Stat-Ease, Inc., USA) was employed to determine the optimum enzyme ratios for the formulation of a hydrolytic enzyme cocktail for CApRS saccharification. A D-optimal design constraints was Chimera (A) + *Ct*CBH5A (B) + *Ct*GH11 (C) + *Bo*GH43 (D)=100% as expressed in Table 4.3.3. The enzyme component ranges (in terms of percentage) were as follows: 20 < Chimera< 50, 30< *Ct*CBH5A < 60, 10 < *Ct*GH11 < 40, 10 < *Bo*GH43< 20 and the total enzyme unit in each run was 25 U/mL of crude (unpurified enzyme present in the total cellular proteins) enzymes. The yield (mg/g) of total reducing sugar (TRS) in the saccharification process was set to the response for the optimization process. The software generates 20 runs of which 10 runs were model points, 5 runs to determine

lack of fits and 5 runs were replicates. Each experiment was conducted using 0.05 M sodium-phosphate buffer, pH 5.8 at 35°C, 150 rpm in an incubator shaker (Orbitek, Scigenics Biotech, India) in a reaction volume of 3 mL with 3%, w/w substrate loading under software designed conditions for 48 h. 0.05% (w/v) of sodium azide was added to avoid microbial contamination. After 48 h of saccharification, the total reducing sugars were determined and fitted to the model for statistical optimization.

The quadratic model representing the relationship between factors and response is shown in Eqn. 4.1:

$$Y= \beta_1A+\beta_2B+\beta_3C+ \beta_4D+ \beta_{12}AB+ \beta_{13}AC+ \beta_{14}AD+ \beta_{23}BC+ \beta_{24}BD+ \beta_{34}CD \quad \text{(Eqn. 4.1)}$$

where, Y represents the response and A, B, C and D are indicative of the factors. β_1, β_2, β_3 and β_4 denote the coefficients of individual factors. β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} represent the coefficients of two interacting factors.

To analyze the models, analysis of variance (ANOVA) and diagnostics were conducted. Additionally, optimization was carried out on the 3D response surfaces of the model's interaction variables to identify the regions with the highest desirability (Gunst *et al*., 1996). By employing this optimization process, software-based optimal parameters for the saccharification process were established. These parameters were determined by evaluating the impact of enzyme ratios (considered as factors) within the enzyme cocktail on the total reducing sugars (TRS) and the yields of glucose and xylose during the saccharification process.

4.2.6 Saccharification of rice straw using developed and commercial enzymes cocktail

The enzyme cocktail formulated at the optimized proportion of Chimera, *Ct*CBH5A, *Ct*GH11 and *Bo*GH43 contains all the cellulases (endoglucanase,

cellobiohydrolase and β-glucosidase) and core xylanase enzyme (endoxylanase and β-xylosidase). Thus, cellulase activity and xylanase activity of the developed cocktail are estimated and expressed in terms of FPU/mL against Whatman No. 1 filter paper and U/mL against 1% (w/v) birchwood xylan, respectively. The filter paper activity (FPA) of the developed crude enzyme cocktail was estimated by using the procedure developed by Ghose (1987). The xylanolytic activity of the cocktail was measured by using 1% (w/v) birchwood xylan as substrate and by following the method described earlier in section 4.2.3. The enzyme activity was expressed as U/mL and defined as the amount of reducing sugar released in µmole of the enzyme cocktail. The total protein concentration of the developed crude (unpurified) enzyme cocktail was also measured following the procedure described in section 4.2.3.

Subsequently, the saccharification efficiency of the developed crude enzyme cocktail was compared against commercially available cellulase and xylanase preparation under optimized conditions. Equal proportions of commercial cellulase (source *Trichoderma reesei*) and xylanase (source: *Aspergillus niger*) in terms of FPU/mL and U/mL, respectively as that of the proportion present in the developed crude enzyme cocktail was used and the saccharification was performed for 3 mL reaction volume with 3%, w/w CApRS biomass loading at 35°C and pH 5.8 for 48 h. The effectiveness of the developed crude enzyme cocktail against CApRS saccharification was compared with raw RS and partially pretreated RS (using 3%, v/v acetic acid at 120°C for 30 min, containing 52.3% cellulose, 23.5% hemicellulose and 11.7% lignin) was performed using the same hydrolysis conditions as mentioned above and the TRS yield was compared. The saccharification efficiency was calculated by Eqn. 4.2 given below:

$$\text{Saccharification efficiency (\%)} = \left(\frac{\text{Total reduced sugar released } (\frac{\text{mg}}{\text{g}})}{\text{Total Carbohydrate content in CApRS biomass } (\frac{\text{mg}}{\text{g}})} \right) \times 100 \ldots Eqn.\ 4.2$$

4.2.7 Analytical procedures

4.2.7.1. Quantitative analysis of monosaccharides

The hydrolysate after the enzymatic saccharification was analyzed for the monosaccharide content. The glucose and xylose concentration in the saccharified hydrolysate was determined using High-Performance Liquid Chromatography (Shimadzu Corporation, Japan). Aminex HPX-87H column (Bio-Rad Laboratories, USA) with a mobile phase, 5 mM H_2SO_4, flow rate of 0.6 mL/min, oven temperature at 50°C and injection volume of sample, 20 µL was used.

4.2.7.2. Composition analysis of CApRS biomass

Structural carbohydrate analysis and lignin content of CApRS biomass were done using NREL protocol as described and reported earlier (Maibam and Goyal, 2022b). Cellulose and hemicellulose contents were estimated by using TAPPI method (TAPPI, 1992).

4.2.7.3. Statistical analysis

The experimental results were presented as the mean value along with the standard deviation (n=3). To assess statistical significance, the student's t-test was employed, and a *p*-value of less than 0.05 was considered to indicate statistical significance. The statistical values assigned to the response were determined using the Design-Expert software.

4.3. Results and discussion

4.3.1 Production of recombinant cellulolytic and xylanolytic enzymes

The recombinant cellulases (chimera and *Ct*CBH5A) and xylanases (*Ct*GH11A and *Bo*GH43) are thermostable enzymes (Table 4.1). From each 800 mL culture of *E. coli* BL21 (DE3) cells expressing enzymes viz. *Ct*GH1-L1-*Ct*GH5- F194A, *Ct*CBH5A, *Ct*GH11A and *Bo*GH43 after sonication, 10 ml of supernatant containing crude recombinant enzyme was obtained. The enzyme concentration and the activity of each crude enzyme were estimated at respective optimum pH and temperature by the procedure described in section 4.2.3 and are presented in Table 4.3.1. The total cell protein concentration in the supernatant containing crude chimera was 29.8 mg/ mL and the endoglucanase activity against 1% (w/v) CMC-Na was 17.7 U/ mL. The β-glucosidase activity of chimera against 1% (w/v) cellobiose was 9.4 U/ mL. The total protein concentration in *Ct*CBH5A was 24.7 mg/ mL and a cellobiohydrolase activity against 1% (w/v) CMC-Na was 10.3 U/ mL. *Ct*Xyn11 showed endoxylanase activity of 97.3 U/ mL against 1% (w/v) birchwood xylan and a total protein concentration in crude enzyme preparation was 26.2 mg/ mL. The β-xylosidase activity of *Bo*GH43 against 1% (w/v) birchwood xylan was 8.7 U/mL and a total protein concentration was 28.9 mg/mL. The mixture of all four enzymes could serve as a potential enzyme cocktail for the saccharification of CApRS biomass that contains 82%, w/w holocellulose.

Table 4.3.1. Cellulase and xylanase production and activity.

Enzyme name (Acronym)	Bacterial Source	Optimum condition		Total crude enzyme volume (mL)	#Crude enzyme activity (U/mL)	Total enzyme Unit (U)	¥Crude protein conc. (mg/mL)
		pH	Temp °C				
chimera *Ct*GH1-L1-*Ct*GH5-F194A	*Clostridium thermocellum*	5	60	10	17.7±1.1[a]	177	29.8±0.6
Cellobiohydrolase *Ct*CBH5A	*Clostridium thermocellum*	6.4	65	10	10.3±0.4[b]	103	24.7±0.4
Endo-1,4-β-xylanase *Ct*GH11A	*Clostridium thermocellum*	7.5	65	10	97.3±0.9[c]	973	26.2±0.7
β-Xylosidase *Bo*GH43	*Bacteroides ovatus*	7.0	37	10	8.7±0.3[d]	87	28.9±1.2

[a] 1% (w/v) CMC-Na salt at 60°C for 4 min, [b] 1% (w/v) CMC-Na salt at 65°C for 5 min.
[c] 1% (w/v) birchwood xylan at 65°C/1 min, [d] 1% (w/v) birchwood xylan at 37°C/ 5 min.
Enzyme activity was measured by Nelson (1944) and Somogyi (1945) methods.
¥Total protein concentration was estimated by the Folin-Lowry (1951) method.

4.3.2. Determination of optimum pH and temperature for saccharification

The determination of common pH, buffer concentration and operating temperature is the most crucial step in the designing of an enzyme cocktail for the saccharification process (Lee *et al*., 2017). All four hydrolytic enzymes (chimera, *Ct*CBH5A, *Ct*GH11 and *Bo*GH43) used in this study possess different optimum pH and temperatures for their maximum activity. Therefore, for the application of these hydrolytic enzymes in the saccharification of CApRS biomass, common operating conditions were evaluated by performing the experiments mentioned in section 4.2.4. Table 4.3.2 displays the residual enzyme activities (in terms of %) of chimera, *Ct*CBH5A, *Ct*GH11and *Bo*GH43 at different pH and temperature. It can be seen from Table 4.3.2 that at pH 5.8 and 35°C, the chimera showed a maximum residual activity of 80% after 48h of incubation. *Ct*CBH5A showed a maximum of 87% residual enzyme

activity at both pH 5.8 and 6.2 and 35°C after 48h of incubation. Similarly, endo-1,4-β-xylanase, *Ct*GH11 showed the highest residual activity of 80% at pH 5.8 and an incubating temperature of 35°C. Also, *Bo*GH43 retained a maximum residual activity of 82% at pH 5.8 and 35°C. Thus, all four enzymes (chimera, *Ct*CBH5A, *Ct*GH11and *Bo*GH43) showed 80 -85% residual enzyme activity after 48h of incubation at pH 5.8 and 35°C. Therefore, the common buffer, 0.05 M sodium-phosphate buffer was used for designing of enzyme cocktail comprising the above four hydrolytic enzymes at the operating conditions of pH 5.8 and temperature 35°C. Subsequently, different combinations of chimera, *Ct*CBH5A, *Ct*GH11A and *Bo*GH43 were used to generate a specific enzyme cocktail for the saccharification of CApRS operated using 0.05 M sodium-phosphate buffer, pH 5.8 at 35°C.

Table 4.3.2. Determination of common buffer pH and temperature for a cocktail of crude recombinant hydrolytic enzymes.

Enzyme	Temperature °C	Residual enzyme activity (%) pH 5			Residual enzyme activity (%) pH 5.8			Residual enzyme activity (%) pH 6.2		
		0 h	48 h	96 h	0 h	48 h	96 h	0 h	48 h	96 h
Chimera	30	100 ±2.6	75±0.4	64±3.9	100 ±1.2	78±2.1	70±3.1	100 ±0.6	74±1.6	68±0.7
*Ct*CBH5A		100 ±1.9	82±1.2	76±1.4	100 ±0.6	87±3.3	81±1.2	100 ±0.9	85±2.1	70±1.2
*Ct*GH11		100 ±3.1	69±4.1	54±2.1	100 ±1.6	75±1.5	61±3.6	100 ±1.6	74±2.4	69±2.5
*Bo*GH43		100 ±1.9	77±3.6	66±2.7	100 ±2.9	81±4.2	74±0.8	100 ±3.4	84±3.5	74±3.1
Chimera	35	100 ±2.9	76±2.7	72±1.7	100 ±1.1	**80±1.4**	75±2.4	100 ±1.1	72±0.8	61±4.6
*Ct*CBH5A		100 ±4.1	83±1.9	75±3.9	100 ±3.5	**85±2.1**	76±3.1	100 ±2.4	87±1.2	79±2.5
*Ct*GH11		100 ±0.5	71±0.8	63±0.5	100 ±4.1	**80±3.4**	66±0.9	100 ±1.5	76±1.4	68±0.5
*Bo*GH43		100 ±3.1	75±2.1	66±4.1	100 ±0.9	**82±1.6**	74±0.6	100 ±0.9	86±4.6	74±1.2
Chimera	40	100 ±3.9	77±3.6	74±2.9	100 ±1.5	75±0.9	63±2.1	100 ±1.8	65±3.1	59±0.9
*Ct*CBH5A		100 ±2.7	74±1.1	52±2.6	100 ±1.9	75±2.3	56±1.8	100 ±1.4	79±1.5	61±1.1
*Ct*GH11		100 ±4.2	56±1.7	52±1.9	100 ±2.1	60±3.1	54±2.4	100 ±0.9	62±3.2	54±2.4
*Bo*GH43		100 ±0.9	73±2.6	61±1.2	100 ±3.5	75±4.7	71±3.1	100 ±3.1	71±2.7	69±3.1

4.3.3 Model development, Analysis of Variance (ANOVA) and Diagnosis

The empirical model employed in this study aimed to assess the interactions and impacts of different components (Chimaera, *Ct*CBH5A, *Ct*Xyn11, and *Bo*GH43) within the enzyme mixture on the yield of total reducing sugars (TRS). To evaluate the reliability of the proposed model, the standard error of design was analyzed and depicted in Fig. 4.3.1. The model's predictions were observed to be reasonable and accurate, as the overall errors are in the range of 0.5 to 0.8σ across almost all experimental runs (Jeirani *et al.*, 2012). The red dots plotted on the standard error graphs represent the design points of the conducted experiments.

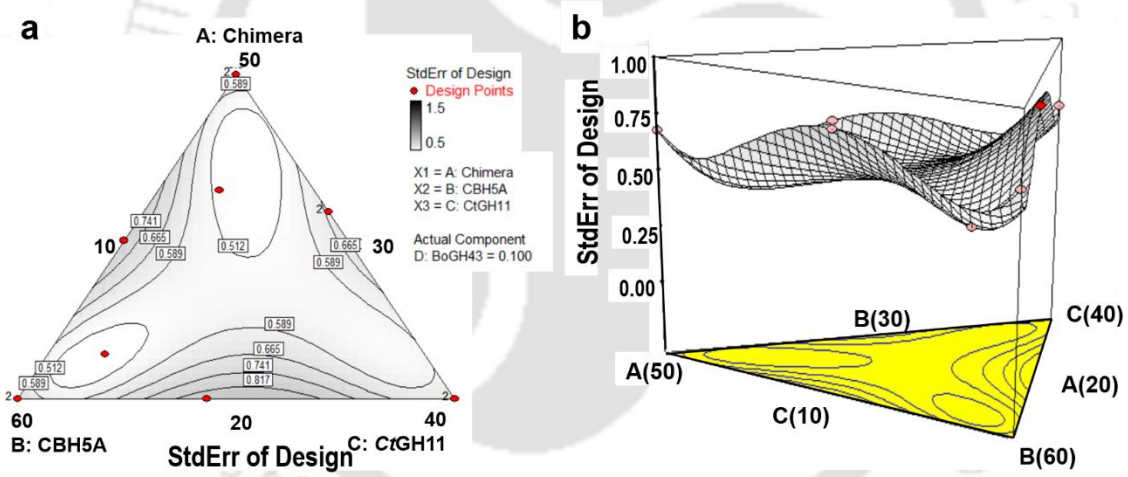

Fig. 4.3.1. (a) 2D contour plot and (b) 3D surface plot of the standard error of design at different points in the design space.

The experimental results and the predicted results of yield of TRS (mg/g) for each model-designed run for formulating the optimum ratio of enzymes in the cocktail are shown in Table 4.3.3.

Table 4.3.3. Predicted and experimental values of TRS with respect to variables in cocktail ratio optimization of RS saccharification by D-optimal mixture design approach.

Run order	Variables				Predicted results	Experimental results
	Chimera (A) (%)	*Ct*CBH5A (B) (%)	*Ct*GH11 (C) (%)	*Bo*GH43 (D) (%)	TRS (mg/g)	TRS (mg/g)
1	34.6	45.4	10.0	10.0	505.60	526.65
2	21.1	48.2	10.7	20.0	341.98	315.90
3	20.0	47.0	23.0	10.0	315.52	326.50
4	20.0	60.0	10.0	10.0	434.31	429.98
5	50.0	30.0	10.0	10.0	397.73	406.58
6	37.3	30.0	22.7	10.0	407.99	415.35
7	20.0	30.0	40.0	10.0	250.62	245.70
8	21.4	31.6	27.0	20.0	439.15	432.13
9	20.0	38.9	26.0	15.1	382.93	400.73
10	43.8	30.0	10.0	16.2	487.00	494.33
11	29.7	35.9	15.6	18.8	469.05	464.13
12	39.2	36.5	14.2	10.0	456.45	459.98
13	27.6	43.2	17.3	11.9	446.89	403.65
14	24.1	51.8	13.9	10.2	432.28	425.43
15	20.6	30.2	33.7	15.4	419.79	424.13
16	21.1	48.2	10.7	20.0	341.98	371.68
17	20.0	30.0	40.0	10.0	250.62	248.63
18	37.3	30.0	22.7	10.0	407.99	412.43
19	50.0	30.0	10.0	10.0	397.73	377.33
20	20.0	60.0	10.0	10.0	434.31	438.75

The results were statistically analyzed using Analysis of Variance (ANOVA) and the quadratic model was developed to examine the effects of all the four enzymes in the hydrolysis of biomass (CApRS) based on the yield of TRS released during the process. The quadratic equation representing the correlation between the response (TRS yield) and the variables in terms of coded factors was presented as follows:

$$TRS = +397.38\,A + 434.38\,B + 249.91\,C - 604.98\,D + 357.01\,AB + 297.72\,AC + 1809.49\,AD - 159.15\,BC + 1063.26\,BD + 2153.07\,CD \quad \text{...(Eqn. 3.3)}$$

where A, B, C and D represent factors i.e., Chimera, *Ct*CBH5A, *Ct*Xyn11 and *Bo*GH43, respectively.

The significance of the constructed models and their variables was assessed using ANOVA, as presented in Table 4.3.4 for the cocktail design. The high F-value and low *p*-value (<0.0001) indicate the significant interaction of process-based parameters and the statistical significance of the model at a 95% confidence level (Zafar *et al*., 2017, Pattanaik *et al*., 2018). Table 4.3.4 reveals significant *p*-values for the linear mixture and all interacting terms, except for BC and BD. The high value of R^2 (0.94) and adjusted R^2 (0.90) indicate its acceptability (Pillai *et al*., 2017).

Table 4.3.4. ANOVA table for D-optimal quadratic model for cocktail design.

Source	Sum of Squares	df	Mean Square	F Value	*p*-value
Model	93096.02	9	10344	20.08	< 0.0001
Linear Mixture	37497.09	3	12499.03	24.26	< 0.0001
AB	9124.878	1	9124.878	17.71	0.0018
AC	7631.622	1	7631.622	14.81	0.0032
AD	4154.44	1	4154.44	8.06	0.0176
BC	1623.804	1	1623.804	3.15	0.1062
BD	1104.909	1	1104.909	2.14	0.1738
CD	5205.863	1	5205.863	10.10	0.0098
Residual	5152.585	10	515.2585		
Lack of Fit	3122.323	5	624.4646	1.537891	0.3241
Pure Error	2030.263	5	406.0525		
Cor Total	98248.6	19			
R^2	0.9476				
Adj R-Squared	0.9004		**Adeq. precision**	15.885	
Pred R-Squared	0.7635		**C.V.%**	5.66	

To examine whether the errors are uniform and independent, diagnostics were performed (Anderson and Whitcomb, 2016). Graphical analysis of the model through the plots of residuals vs. predicted response plots, the normal plot of residuals, leverage plot and cook's distance were carried out. Fig. 4.3.2a illustrates the model's residual in the normal plot which closely resembles the normal distribution thereby indicating the adequacy of the model with no transformation of the response being needed (Mitchell, 2000). The externally studentized residuals detect the outliers and help in assessing the

equal variance assumption (Anderson and Whitcomb, 2017). Also, Cook's distance was used to spot the outliers. Fig. 4.3.2b and 4.3.2c showed the externally studentized residuals and Cook's distance, respectively suggesting uniform data distribution with no outliers (Varanda *et al.*, 2017). The analysis of leverage (Fig. 4.3.2d) and residualsvs.s predicted (Fig. 4.3.2e) plots demonstrate that there is the absence of any particular trend and that the experimental runs have a consistent impact on modeled responses (Jeirani *et al*., 2012). The model effectively represents the experimental data and allows for exploration within the desired range. The findings from the experimental data (Table 4.3.3) and the ANOVA (Table 4.3.4) align with the observations presented in Fig. 4.3.2. To examine how the combination of all four enzymes influences the production of TRS (m/g) and to analyze their synergistic effects, 3D surface plots were generated.

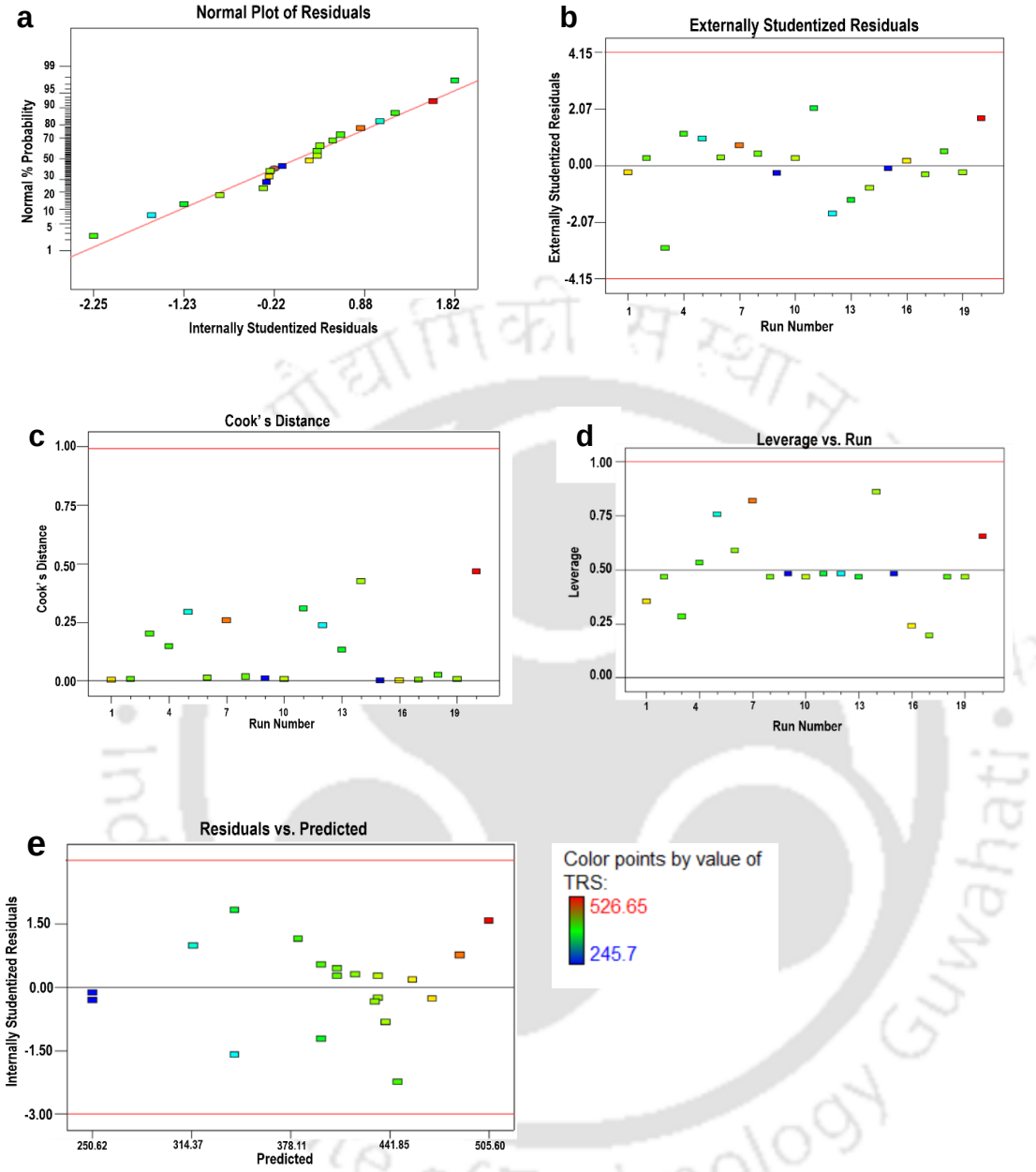

Fig 4.3.2. Diagnostics of TRS model by (a) normal plot of residuals, (b) externally studentized residuals, (c) cook's distance, (d) leverage plot and (e) residuals vs predicted plot for the cocktail design.

4.3.4 Effect of cocktail enzymes on TRS yield and their synergistic effect on hydrolysis

The impact of mixture components on TRS yield could be analyzed from the overview of the response surface plot (Fig. 4.3.3), software-designed experiments (Table 4.3.3) and ANOVA (Table 4.3.4). Fig. 4.3.3 depicts the impact of mixture components ABC, BDC and ABD, respectively on the yield of TRS (mg/g). At a constant proportion of *Bo*GH43 (13.1%) (Fig. 4.3.3a and 4.3.3b), maximum TRS was achieved at the extreme edge of the contour with *Ct*GH11 at its lowest proportion (10%), *Ct*CBH5A at a middle range (40-50%) of its limit and Chimera at an upper limit of its range (35-45%). Upon limiting the proportion of Chimera in its lower limit (20%) (Fig. 4.3.3a) and increasing the proportion of *Ct*GH11 (B) and *Ct*CBH5A (C) from lower to higher limit (10- ~36%) and (30- ~56%), the TRS yield is lowest throughout, thereby indicating the significance of Chimera's proportion, non-significance of the interacting variable BC and negative proportionality of BC with the TRS yield.

The enzymes, *Ct*GH11 and *Bo*GH43 when present in a proportion of any range from the set limit with Chimera at a fixed proportion of 35.1% and *Ct*CBH5A in a range of 30-45%, resulted in an overall high TRS yield as shown in the contour (Fig. 4.3.3c and 4.3.3d). This signifies that the hydrolysis process is mainly governed by the appropriate proportion of Chimera and *Ct*CBH5A and the range chosen for *Ct*GH11 and *Bo*GH43 is appropriate for the mixture design. The enzyme cocktail with *Ct*GH11 at its lowest limit of 10% and upon varying the proportion from the lower to the upper limit of the other three enzymes, the highest TRS yield of 516 mg/g could be achieved in the central region of the contour (Fig. 4.3.3e and 4.3.3f). This implies the high sensitivity of the components A (Chimera), B (*Ct*CBH5A) and D (*Bo*GH43).

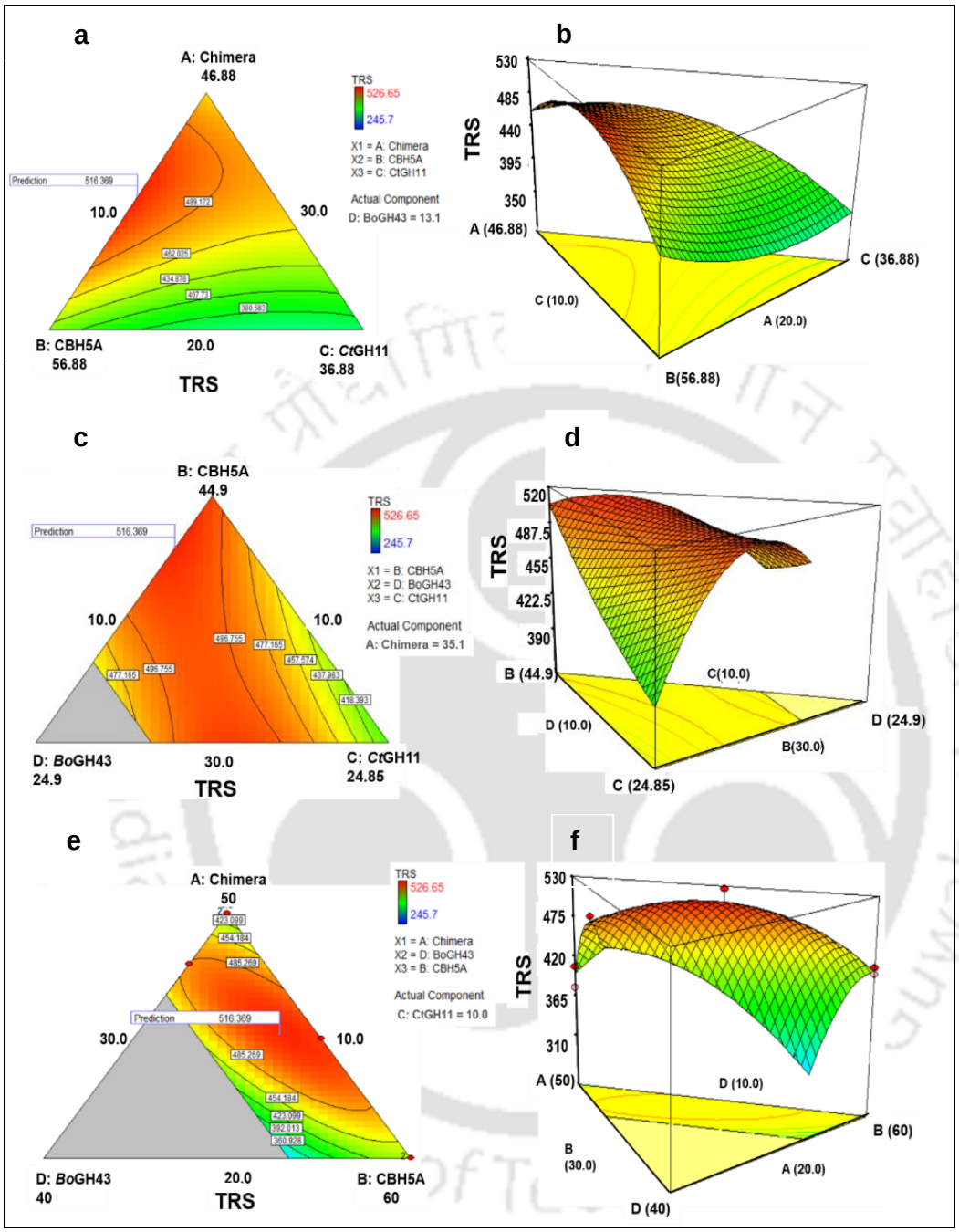

Fig. 4.3.3. (a), (c), (e) represent 2D contour and their corresponding 3D plot (b), (d), and (f) generated from a quadratic model developed for crude recombinant hydrolytic enzyme cocktail formulation. The red regions indicate the highest TRS.

*Ct*CBH5A at the highest proportion (60%) and varying the proportion of *Bo*GH43 from low to high (10-20%) in a cocktail with a fixed proportion of Chimera, 20% gives no significant change in the TRS yield (Fig. 4.3.3f) thereby inferring the non-significance interacting variable of quadratic function BD. The enzyme cocktail with chimera at the lowest proportion (20%) and *Ct*CBH5A with the highest proportion resulted in the lowest TRS yield of 380 mg/g (Fig. 4.3.3e) thereby signifying that the synergistic action of endoglucanase and β-glucosidase with cellobiohydrolase is essential for the efficient hydrolysis of CApRS. In essence, the 3D and 2D response surface plots in Fig. 4.3.3 could identify the optimal proportion of all four enzymes for efficient saccharification of CApRS.

4.3.5. Quantification of monosaccharides after saccharification of CApRS by enzyme cocktail.

After the enzymatic saccharification of each 20 sets of experiments generated by D-optimal design for the optimization of cocktail ratios, the enzymatic hydrolysates of CApRS were analyzed for the quantification of monosaccharides (Table 4.3.5). The titre of individual monosaccharides, glucose and xylose released after the saccharification by enzyme cocktail in each software design experimental run was analyzed by using HPLC. This monosaccharides titre analysis is necessary for its application in the further conversion process in biorefineries. The CApRS biomass contains low, 9.01% (w/v) hemicellulose content. Therefore, the maximum possible theoretical titre of xylose (g/L) from hemicellulosic fraction present in the CApRS biomass is 2.4 g/L (Maibam and Goyal, 2022a). It can be seen from Table 4.3.5, that the concentration of xylose released in all the experimental runs ranges between 1.61 g/L – 2.13 g/L, thereby indicating an effective hydrolysis of hemicellulosic fraction

with the conversion efficiency ranging between 75% – 90%. These results inferred that the ranges of proportion and the enzyme units for xylanases (*Ct*GH11 and *Bo*GH43) selected for the cocktail formulation are appropriate and efficient for maximum hydrolysis of hemicellulosic fractions. Similarly, the cellulosic content in CApRS biomass is 73% (w/v) and the maximum theoretical glucose titre that can be achieved is 19.8 g/L. When the chimera is present at a minimum proportion (20%) irrespective of the proportion of *Ct*CBH5A in the cocktail, the glucose concentration released during the saccharification is low, 4.32 g/L - 6.21 g/L. The low titre of glucose could be due to the low proportion and enzyme unit of β-glucosidase. When the chimera's proportion in the cocktail is increased to 30-40%, the glucose concentration increases to 10.49-12.76 g/L. The increase in glucose titre might be due to a high proportion of active β-glucosidase. The presence of a high proportion of *Ct*CBH5A in the cocktail does not give any positive effect on the glucose titre, however, the presence of chimera and *Ct*CBH5A in the ratio (35:45) results in a maximum glucose titre of 12.76 g/L thereby indicating an effective hydrolysis of cellulosic fraction with the conversion efficiency ranging between 30% - 65%. This signifies the synergistic effect of cellobiohydrolase and β-glucosidase in the conversion of cellulose to glucose.

4.3.6. Mixture proportion optimization, desirability function and validation

The primary objective of this study was to optimize the enzyme ratios in the cocktail for efficient saccharification of CApRS. The formulated optimization targets high TRS yield and equal weights were assigned to all the variables (four factors and one response). The highest desirability of 0.96 was achieved at an optimized proportion of Chimera (35.1%), *Ct*CBH5A (41.8%), *Ct*GH11 (10%) and *Bo*GH43 (13.1%) in the enzyme cocktail. The predicted TRS yield by the software under optimized conditions

was 516 mg/g_{CApRS}. The predicted yield was further validated by performing enzymatic saccharification of CApRS using a recombinant enzyme cocktail formulated under optimized proportion. The enzymatic saccharification process was carried out at 3%, w/v substrate loading in 100 mL reaction volume using 0.05 M sodium-phosphate buffer, pH 5.8 at 35°C and resulted in a TRS yield of 498 mg/$g_{CApRS\ biomass}$ at 48h giving a saccharification efficiency of 72%.

Table 4.3.5. Quantification of monosaccharides in the enzyme saccharified hydrolysate of each experiment for optimizing the enzyme ratio in the cocktail.

Run order	Variables (%)				**Monosaccharide (g/L)**		
	Chimera	*Ct*CBH5A	*Ct*GH11	*Bo*GH43	**Glucose**	**Xylose**	**Total**
1	34.6	45.4	10.0	10.0	12.76	2.08	14.84
2	21.1	48.2	10.7	20.0	6.89	1.89	8.78
3	20.0	47.0	23.0	10.0	5.15	2.00	7.15
4	20.0	60.0	10.0	10.0	6.18	1.98	8.16
5	50.0	30.0	10.0	10.0	9.97	1.86	11.83
6	37.3	30.0	22.7	10.0	10.23	1.79	11.84
7	20.0	30.0	40.0	10.0	4.32	2.09	6.41
8	21.4	31.6	27.0	20.0	6.98	2.14	9.12
9	20.0	38.9	26.0	15.1	6.21	1.98	8.19
10	43.8	30.0	10.0	16.2	11.06	1.97	13.03
11	29.7	35.9	15.6	18.8	10.49	2.01	12.5
12	39.2	36.5	14.2	10.0	9.86	1.82	11.68
13	27.6	43.2	17.3	11.9	9.48	1.79	11.27
14	24.1	51.8	13.9	10.2	8.03	2.09	10.12
15	20.6	30.2	33.7	15.4	5.87	1.98	7.85
16	21.1	48.2	10.7	20.0	7.01	1.90	8.91
17	20.0	30.0	40.0	10.0	4.29	2.13	6.42
18	37.3	30.0	22.7	10.0	10.09	1.84	11.93
19	50.0	30.0	10.0	10.0	9.56	2.01	11.57
20	20.0	60.0	10.0	10.0	5.98	2.01	7.99

4.3.7. Overall yields of monosaccharides and TRS using developed enzyme cocktail and commercial enzyme cocktail against CApRS biomass

The developed crude enzyme cocktail showed a cellulase activity of 21.24 U/ mL against 1% (w/v) CMC-Na and 5.62 FPU/ mL against Whatman No. 1 filter paper and a xylanase activity of 102 U/mL against 1% (w/v) birchwood xylan at the optimum conditions. The total cell protein concentration of the developed crude (unpurified enzyme present in the total cellular proteins) enzyme cocktail was 27.1 mg/mL thus giving activity of 208 FPU/$g_{\text{total cell protein}}$. The enzymatic hydrolysis of 3% (w/v) CApRS biomass was performed under optimum conditions of the saccharification process (0.05 M sodium-phosphate buffer, pH 5.8 at 35°C) by using i) a developed crude enzyme cocktail and ii) commercial cellulase and xylanase cocktail. The enzyme dosage of cellulase 5.6 FPU/mL and xylanase 102 U/mL activities were used in each case. The enzymatic hydrolysis using a commercial cocktail resulted in a TRS yield of 553 mg/$g_{\text{CApRS biomass}}$, glucose 480 mg/$g_{\text{CApRS biomass}}$ and xylose 67 mg/ $g_{\text{CApRS biomass}}$ with the saccharification efficiency of 80% (Eqn. 4.2) after 48 h (Table 4.3.6). However, under the same operating conditions of the saccharification process, the developed crude enzyme cocktail resulted in a TRS yield of 498 mg/$g_{\text{CApRS biomass}}$, glucose 410 mg/$g_{\text{CApRS biomass}}$ and xylose 71.3 mg/$g_{\text{CApRS biomass}}$ with saccharification efficiency of 72% after 48 h. The commercial enzyme cocktail gave 11% and 17% higher TRS and glucose yields, respectively as compared with the developed cocktail. However, the xylose titre was low by 6% in the saccharified hydrolysate from the commercial enzyme cocktail. The commercial enzyme cocktail gave 8% higher saccharification efficiency than the developed crude enzyme cocktail. The enzymatic saccharification of raw RS and partially pretreated RS (acetic acid pretreated RS) using a developed enzyme cocktail

under the same saccharification conditions and enzyme dosage as described above resulted in a lower TRS yield of 80 mg/$g_{raw\ RS}$ and 218 mg/$g_{ptd\ RS}$, respectively compared to CApRS biomass. The justification for high TRS yield could be due to the exposure of soluble and digestible cellulose (Zhang *et al*., 2023) present in the CApRS biomass which is generated from the strong, effective and green choline chloride: acetic acid pretreatment method conducted in the previous study (Maibam and Goyal, 2022b). Thus, it can be inferred that the developed crude hydrolytic recombinant enzyme cocktail is efficient and specific for CApRS biomass.

4.3.8. Comparative analysis of developed enzyme cocktail for biomass saccharification

A study on statistical designing of enzyme cocktail using core cellulases and accessory enzymes by Karnaouri *et al*., 2016 reported that the purified recombinant cellulases, *Mt*CBH7, *Mt*CBH6, *Mt*EG5 and *Mt*EG7 from *Myceliophthora thermophila* in the ratio, 38.9:20.0:20:21.1 with 3% *Mt*BGL3 (β-glucosidase) resulted in 26% saccharification efficiency using microwave-assisted pretreated wheat straw under the saccharification conditions described in Table 4.3.6 and also (Karnaouri *et al*., 2016). They further reported that the addition of accessory enzymes *Mt*Man26a (mannanase), *Mt*GH61 (lytic polysaccharide monooxygenase), *Mt*Fae1a (xylanases) could enhance the saccharification efficiency to 38%. Another study by Singh *et al*. (2020) reported the use of a crude recombinant cellulase enzyme cocktail for the hydrolysis of base pretreated 2% (w/v) *Sorghum durra* stalk resulting in a TRS yield of 211 mg/ $g_{pretreated\ biomass}$ (Table 4.3.6). The effect of an enzyme cocktail comprising natural cellulase (source: *Aspergillus oryzae*) and natural xylanase (*Schizophyllum commune*) in crude and purified form for the saccharification of 10% (w/v) laccase delignified rice straw

was illustrated (Bharadwaj *et al*., 2020). They reported that enzymatic saccharification of laccase delignified rice straw using crude cellulase and xylanase cocktail in the ratio 1:1 gives a TRS yield of 107 mg/$g_{\text{pretreated biomass}}$. However, the use of purified cellulase and xylanase cocktail in the same ratio and enzyme dosage under similar operating conditions of saccharification gave a higher, 181 mg/$g_{\text{pretreated biomass}}$ TRS yield (Table 4.3.6). The effect of a purified enzyme cocktail comprising cellulases and xylanases on the saccharification efficiency of pretreated sugarcane bagasse was evaluated (Karp *et al*., 2021). They reported that the combination of cellulases, 5 mg/$g_{\text{substrate}}$ and xylanases, 2 mg/$g_{\text{substrate}}$ for the saccharification of 10% (w/v) alkali pretreated sugarcane bagasse gave TRS yield of 570 mg/$g_{\text{pretreated biomass}}$ (Table 4.3.6). Nath *et al* (2020) reported a TRS and glucose yield of 230 mg/$g_{\text{pretreated biomass}}$ and 137 mg/$g_{\text{pretreated biomass}}$, respectively, after the enzymatic saccharification of 2% (w/v) pretreated sugarcane bagasse using purified recombinant cellulase cocktail comprising chimera and *Ct*CBH5A at ratio 2:3 with total enzyme dose, 600 U/$g_{\text{pretreated biomass}}$. Whereas, Nedumaran *et al*. (2020) showed a higher TRS and glucose yield of 417 mg/g pretreated biomass and 285 mg/g pretreated biomass, respectively, with 350 U/g using the same purified enzyme cocktail (chimera and *Ct*CBH5A at a ratio 2:3) at 3% (w/v) pretreated *Sorghum durra* stalk (Table 4.3.6). However, the current study involving the same cellulase enzymes (chimera and *Ct*CBH5A) along with xylanases (*Ct*GH11 and *Bo*GH43) all in crude form (unpurified enzyme present in the total cellular proteins) resulted in higher TRS and glucose yield as compared with previous studies of Nath *et al* (2020) and Nedumaran *et al* (2020). This comparative analysis highlights the importance of enzyme components and their proportions in the cocktail, structural composition of the pretreated biomass and the hydrolysis process parameters on the

saccharification efficiency. The plausible reason for the high yield of TRS and glucose in the current study could be due to i) higher delignification and hemicellulose removal in the pretreated biomass due to the strong pretreatment method (ChCl:AA) used (Maibam and Goyal, 2022b) and ii) core xylanase enzymes completely hydrolyze the xylan fractions thereby enhancing the exposure of cellulose and escalating its accessibility to cellulases and iii) statistically optimized proportions of enzyme in the cocktail based on the carbohydrate content in the pretreated biomass. Thus, from this study, it is noteworthy that i) the presence of both cellulases and hemicellulases in the enzyme cocktail significantly affects the saccharification efficiency, ii) the proportion of hydrolytic enzymes in the cocktail is specific for a particular biomass based on the carbohydrate content in the pretreated substrate, iii) the content of cellulosic and hemicellulosic fractions and the extent of their exposure, the operating parameters and conditions such as biomass loading, temperature and enzyme dosages, all factors account for an effective saccharification process.

Table 4.3.6. Comparison of various enzyme cocktail formulations for biomass hydrolysis in terms of TRS yield and saccharification efficiency

Enzymes cocktail (enzyme component ratio)	Source of microorganism	Saccharification conditions	Biomass	Total reducing sugar (mg/g)	Saccharification efficiency	Reference
Recombinant cellulases (*Mt*CBH7, *Mt*CBH6, *Mt*EG5, *Mt*EG7 and *Mt*BGL3) (38.9:20.0:20:21.1)	*Myceliophthora thermophila*	pH 5, 50ºC, 1200 rpm, biomass: 2.5% (w/v), Purified enzyme: 1mg/$g_{substrate}$	Wheat straw	118	26	Karnaouri *et al*. 2016
Recombinant cellulase (*Ba*GH5-UV2, *Ct*CBH5A and *Ct*GH1) (80:10:10)	*Bacillus amyloliquefaciens Clostridium thermocellum*	pH 6.5, 40ºC, 150 rpm, biomass: 2% (w/v), Crude enzyme: 2000 U/$g_{ptd\ biomass}$	*Sorghum durra* stalk	211	32	Singh *et al*. 2020
Natural variant cellulase and xylanase (1:1)	*Schizophyllum commune* *A. oryzae*	pH 5, 50ºC, 120 rpm, biomass: 10% (w/v), Purified enzyme: 200 U/$g_{ptd\ biomass}$	Rice straw	181	35	Bharadwaj *et al*.2020
Recombinant cellulolytic chimera and *Ct*CBH5A (2:3)	*Clostridium thermocellum*	pH 6.4, 30ºC, 150 rpm, biomass: 3% (w/v), Purified enzyme: 350U/g_{ptd}	*Sorghum durra* stalk	417	58	Nedumaran *et al*.2020
Recombinant cellulases (CBH+EG+BG=7:2:1) and xylanases (Xyl+BX=1:1)	*P. verruculosum* *A. niger* *P. canescens* *A. japonicus*	pH 5, 45ºC, 900 rpm, biomass: 10% (w/v), Purified enzyme: 7 mg/$g_{substrate}$	Sugarcane bagasse	570	80	Karp *et al*. 2021
Recombinant cellulolytic chimera and *Ct*CBH5A (2:3)	*Clostridium thermocellum*	pH 6.4, 30ºC, 150 rpm, biomass: 2% (w/v), Purified enzyme: 600U/g_{ptd}	Sugarcane bagasse	230	35	Nath *et al*. 2021
Recombinant cellulase (celluloytic chimera+ *Ct*CBH5A) and xylanases (*Ct*GH11 + *Bo*GH43) (35.1:41.8:10.0:13.1)	*Clostridium thermocellum* *Bacteroides ovatus*	pH 5.8, 35ºC, 150 rpm, biomass: 3% (w/v), Crude enzyme: 150U/$g_{ptd\ biomass}$	Rice straw	498	72	This study
Commercial cellulase (*T. ressei*) and xylanase (*A. niger*)	*T. ressei* *A. niger*	pH 5.8, 35ºC, 150 rpm, biomass: 3% (w/v), Purified enzyme: 150U/g_{ptd}	Rice straw	553	80	This study

4.4. Conclusions

Enzymatic saccharification is the most crucial and challenging step in lignocellulosic biorefineries for bioethanol production. The enzymatic saccharification requires a cocktail of hydrolytic enzymes, cellulases and hemicellulases for effective degradation of biomass. However, the complete bioconversion of biomass is governed by the proportion and synergistic action of hydrolytic enzymes present in the cocktail. This chapter aims to formulate an enzyme cocktail consisting of recombinant cellulases (bifunctional cellulolytic chimeric enzyme, *Ct*GH1-L1-*Ct*GH5-F194A and cellobiohydrolase, *Ct*CBH5A) and xylanases (endo-1,4-β-xylanase, *Ct*GH11A, and β-xylosidase, *Bo*GH43), all in crude form (unpurified enzyme present in the total cellular proteins) for producing a high yield of total reducing sugars using choline chloride:acetic acid pretreated rice-straw (CApRS). The d-optimal mixture design approach was used for statistically designing the optimal proportion of crude recombinant enzyme cocktail for hydrolysis of delignified rice straw. The developed enzyme cocktail comprising chimera, *Ct*CBH5A, *Ct*GH11 and *Bo*GH43 in ratio 35.1:41.8:10.0:13.1 resulted in cellulolytic enzyme activity, 5.6 FPU/mL and xylanase activity, 102 U/mL. Enzymatic saccharification of 3% (w/v) delignified CApRS using the developed enzyme cocktail at 187 FPU/g_{CApRS}, pH 5.8 and 35°C, yielded total reducing sugar of 498 mg/g_{CApRS}, glucose yield (410 mg/g_{CApRS}) and xylose yield (71.3 mg/g_{CApRS}) resulting in 72% saccharification efficiency. However, the saccharification of 3% (w/v) of raw RS or 3%, (w/v) partially pretreated RS by the same enzyme cocktail under the same conditions gave significantly lower TRS yields of 80 mg/g_{rawRS} and 218 mg/$g_{ptd\ RS}$, respectively, implying the effectiveness of the developed enzyme cocktail against CApRS. Thus, highlighting the importance of different enzyme proportions in

the cocktail for biomass conversion and the effectiveness of the developed enzyme cocktail against CApRS biomass. The developed enzyme cocktail was compared with a commercial enzyme cocktail and prospects its applicability in lignocellulosic biorefineries. Further, the dosage of the optimized enzyme cocktail has to be optimized based on the percent of biomass loading for the saccharification process. The optimum saccharification process parameters, 35ºC and pH 5.8 developed in this chapter are advantageous for its application in subsequent processes like simultaneous saccharification and fermentation (SSF) in bioethanol-based biorefineries

4.5 References

Abdmouleh, Z., Alammari, R.A., Gastli, A. (2015). Review of policies encouraging renewable energy integration & best practices. Renewable and Sustainable Energy Reviews. 45, 249-62.

Anderson, M. J., Whitcomb, P. J. (2016). RSM simplified: optimizing processes using response surface methods for design of experiments. second ed., Productivity press, New York.

Anderson, M. J., Whitcomb, P. J. (2017). DOE simplified: practical tools for effective experimentation, Boca Raton, CRC press.

Bhardwaj, N., Kumar, B., Agrawal, K., Verma, P. (2020). Bioconversion of rice straw by synergistic effect of in-house produced ligno-hemicellulolytic enzymes for enhanced bioethanol production. Bioresource Technology Reports. 10:100352.

Classics Lowry, O., Rosebrough, N., Farr, A., Bandall, R. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 193(1), 265-75.

Ghose, T. K. (1987). Measurement of cellulase activities. Pure and Applied Chemistry. 59(2), 257-268.

Gunst, R.F. (1996). Response surface methodology: process and product optimization using designed experiments. Technometrics. 38, 285.

Guo, H., Chang, Y., Lee, D.J. (2018). Enzymatic saccharification of lignocellulosic biorefinery: research focuses. Bioresource Technology. 252, 198-215.

Huang, C., Jiang, X., Shen, X., Hu, J., Tang, W., Wu, X., Yong, Q. (2022). Lignin-enzyme interaction: A roadblock for efficient enzymatic hydrolysis of lignocellulosics. Renewable and Sustainable Energy Reviews. 154, 111822.

Huy, N. D., Saravanakumar, T., Ha, S. H., Park, S. M. (2020). Enhanced enzymatic saccharification of wheat flour arabinoxylan and barley straw using recombinant hemicellulases. Biotechnology and Bioprocess Engineering. 25(3), 431-441.

Jamaldheen, S. B., Thakur, A., Moholkar, V. S., Goyal, A. (2019). Enzymatic hydrolysis of hemicellulose from pretreated Finger millet (*Eleusine coracana*) straw by recombinant endo-1, 4-β-xylanase and exo-1, 4-β-xylosidase. International Journal of Biological Macromolecules. 135, 1098-1106.

Jeirani, Z., Jan, B. M., Ali, B. S., Noor, I. M., Hwa, S. C., Saphanuchart, W. (2012). The optimal mixture design of experiments: Alternative method in optimizing the aqueous phase composition of a microemulsion. Chemometrics and Intelligent Laboratory Systems. 112, 1-7.

Karnaouri, A., Matsakas, L., Topakas, E., Rova, U., Christakopoulos, P. (2016). Development of thermophilic tailor-made enzyme mixtures for the bioconversion of agricultural and forest residues. Frontiers in Microbiology, 7, 177.

Karp, S.G., Rozhkova, A.M., Semenova, M.V., Osipov, D.O., de pauli, S.T., Sinitsyna, O.A., Zorov, I.N., de Souza Vandenberghe, L.P., Soccol, C.R., Sinitsyn, A.P. (2021). Designing enzyme cocktails from Penicillium and Aspergillus species

for the enhanced saccharification of agro-industrial wastes. Bioresource Technology. 330:124888.

Lan, T. Q., Lou, H., Zhu, J. Y. (2013). Enzymatic saccharification of lignocelluloses should be conducted at elevated pH 5.2–6.2. Bioenergy Research. 6, 476-485.

Lee, H., Lee, Y. M., Heo, Y. M., Lee, J., Kim, J. S., Kang, K. Y., Kim, J. J. (2017). Utilization of agricultural residues for enhancement of cellulolytic enzyme production and enzymatic saccharification by *Trichoderma harzianum* KUC1716. Industrial Crops and Products. 109, 185-191.

Maibam, P.D., Goyal, A. (2022a). Pretreatment methods for overcoming biomass recalcitrance, in: Verma, P. (Eds.), Enzymes in the Valorization of Waste. CRC Press, pp. 1-21.

Maibam, P. D., Goyal, A. (2022b). Approach to an efficient pretreatment method for rice straw by deep eutectic solvent for high saccharification efficiency. Bioresource Technology. 351, 127057.

Maibam, P. D., Maiti, S. K. (2020). A strategy for simultaneous xylose utilization and enhancement of cellulase enzyme production by *Trichoderma reesei* cultivated on liquid hydrolysate followed by induction with feeding of solid sugarcane bagasse. Waste and Biomass Valorization. 11, 3151-3160.

Méndez-Líter, J. A., de Eugenio, L. I., Nieto-Domínguez, M., Prieto, A., Martínez, M. J. (2021). Hemicellulases from Penicillium and Talaromyces for lignocellulosic biomass valorization: A review. Bioresource Technology. 324, 124623.

Mitchell, T. J. (2000). An algorithm for the construction of “D-optimal” experimental

designs. Technometrics. 42(1), 48-54.

Nath, P., Dhillon, A., Kumar, K., Sharma, K., Jamaldheen, S. B., Moholkar, V. S., Goyal, A. (2019). Development of bi-functional chimeric enzyme (CtGH1-L1-CtGH5-F194A) from endoglucanase (*Ct*GH5) mutant F194A and β-1, 4-glucosidase (CtGH1) from *Clostridium thermocellum* with enhanced activity and structural integrity. Bioresource Technology. 282, 494-501.

Nath, P., Maibam, P. D., Singh, S., Rajulapati, V., Goyal, A. (2021). Sequential pretreatment of sugarcane bagasse by alkali and organosolv for improved delignification and cellulose saccharification by chimera and cellobiohydrolase for bioethanol production. 3 Biotech. 11, 1-16.

Nedumaran, M., Singh, S., Jamaldheen, S. B., Nath, P., Moholkar, V. S., Goyal, A. (2020). Assessment of combination of pretreatment of Sorghum durra stalk and production of chimeric enzyme (β-glucosidase and endo β-1, 4 glucanase, *Ct* GH1-L1-*Ct* GH5-F194A) and cellobiohydrolase (*Ct* CTCBH5A) for saccharification to produce bioethanol. Preparative Biochemistry & Biotechnology. 50(9), 883-896.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry. 153(2), 375-380.

Pattanaik, A., Rayasam, V. (2018). Analysis of reverse cationic iron ore fines flotation using RSM-D-optimal design–An approach towards sustainability. Advanced Powder Technology. 29(12), 3404-3414.

Pillai, N. S., Kannan, P. S., Vettivel, S. C., Suresh, S. (2017). Optimization of

transesterification of biodiesel using green catalyst derived from Albizia Lebbeck Pods by mixture design. Renewable Energy. 104, 185-196.

Rajak, R. C., Banerjee, R. (2020). An innovative approach of mixed enzymatic venture for 2G ethanol production from lignocellulosic feedstock. Energy Conversion and Management. 207, 112504.

Rajak, R.C., Banerjee, R. (2018). An eco-friendly process integration for second-generation bioethanol production from laccase delignified Kans grass. Energy Conversion and Management. 157, 364-71.

Samayam, I. P., Schall, C. A. (2010). Saccharification of ionic liquid pretreated biomass with commercial enzyme mixtures. Bioresource Technology. 101(10), 3561-3566.

Saritha, M., Arora, A., Singh, S., Nain, L. (2013). *Streptomyces griseorubens* mediated delignification of paddy straw for improved enzymatic saccharification yields. Bioresource Technology. 135:12-7.

Singh, S., Rajulapati, V., Jamaldheen, S. B., Moholkar, V. S., Goyal, A. (2020). Statistically designed cellulase mixture for saccharification of pretreated *Sorghum durra stalk*. Industrial Crops and Products. 154, 112678.

Somogyi, M. (1945). A new reagent for the determination of sugars. Journal of Biological Chemistry. 160(1), 61-68.

Technical Association of the Pulp, Paper Industry. TAPPI test methods. TAPPI; (1992).

Thakur, A., Sharma, K., Jamaldheen, S. B., Goyal, A. (2020a). Molecular Characterization, Regioselective and Synergistic Action of First Recombinant

Type III α-L-arabinofuranosidase of Family 43 Glycoside Hydrolase (PsGH43_12) from *Pseudopedobacter saltans*. Molecular Biotechnology. 62(9), 443-455.

Thakur, A., Sharma, K., Khaire, K. C., Moholkar, V. S., Goyal, A. (2020b). Enzymes: key role in the conversion of waste to bioethanol, in: Thatoi, H. (Eds.), Microbial Fermentation and Enzyme Technology. CRC Press, pp. 257-268.

Varanda, C., Portugal, I., Ribeiro, J., Silva, A. M., Silva, C. M. (2017). Optimization of bitumen formulations using mixture design of experiments (MDOE). Construction and Building Materials. 156, 611-620.

Velasco, J., Pellegrini, V. D. O. A., Sepulchro, A. G. V., Kadowaki, M. A. S., Santo, M. C. E., Polikarpov, I., Segato, F. (2021). Comparative analysis of two recombinant LPMOs from *Aspergillus fumigatus* and their effects on sugarcane bagasse saccharification. Enzyme and Microbial Technology. 144, 109746.

Wang, Y., Liu, P., Zhang, G., Yang, Q., Lu, J., Xia, T., Peng, L., Wang, Y. (2021). Cascading of engineered bioenergy plants and fungi sustainable for low-cost bioethanol and high-value biomaterials under green-like biomass processing. Renewable and Sustainable Energy Reviews. 137, 110586.

Zafar, M., Van Vinh, N., Behera, S. K., Park, H. S. (2017). Ethanol mediated As (III) adsorption onto Zn-loaded pinecone hybrid artificial neural network-genetic algorithm approach. Journal of Environmental Sciences. 54, 114-125.

Zhang, H., Han, L., Dong, H. (2021). An insight to pretreatment, enzyme adsorption and enzymatic hydrolysis of lignocellulosic biomass: Experimental and modeling studies. Renewable and Sustainable Energy Reviews. 140, 110758.

Zhang, R., Gao, H., Wang, Y., He, B., Lu, J., Zhu, W., Peng, L., Wang, Y. (2023a). Challenges and perspectives of green-like lignocellulose pretreatments selectable for low-cost biofuels and high-value bioproduction. Bioresource Technology. 369, 128315.

Zhang, R., Hu, Z., Wang, Y., Hu, H., Li, F., Li, M., Ragauskas, A., Xia, A., Han, H., Tang, J., Yu, H., Xu, B., Peng, L. (2023b). Single-molecular insights into the breakpoint of cellulose nanofibers assembly during saccharification. Nature Communications. 14(1), 1100.

Chapter 5

Optimization of pre-saccharification simultaneous saccharification and fermentation of delignified pretreated rice straw using a novel crude recombinant hydrolytic enzyme cocktail for bioethanol production

5.1 Introduction

The second-generation (2G) bioethanol produced from waste lignocellulosic biomass promotes green and sustainable energy (Siddique and Wahid, 2018). Rice straw (RS), an agriculture lignocellulosic waste, serves as a promising feedstock for 2G ethanol and various bioethanol biorefineries use rice straw as a potential substrate (Van Hung et al., 2020). The bioethanol production involves feedstock pretreatment, enzymatic saccharification and fermentation of reduced monosaccharides. The selection of suitable pretreatment methods is necessary for the success of any bioethanol production plant. The pretreatment of the biomass causes structure

modification and reduction of the crystallinity of cellulose making it accessible to enzymes for hydrolysis process (Maibam and Goyal, 2022a).

The simultaneous saccharification and fermentation (SSF) process is an integrated process designed to enhance the productivity and economics of the bioethanol plant (Paschos et al., 2020). The advantages the SSF process offers over SHF (separate hydrolysis and fermentation) are i) a decrease in fermentation time, ii) reduced process economics and iii) a high rate of hydrolysis process thus, improving overall ethanol yield and productivity (Afedzi and Parakulsuksatid, 2023). However, SSF processes have certain shortcomings, the most crucial ones being the differences in optimum temperatures for enzymatic hydrolysis and microbial growth for fermentation (Mazaheri et al., 2021). Recently, to overcome the shortcomings of difference in optimum temperature, focus has been made on the modification of SSF process. Pre-saccharification and simultaneous saccharification and fermentation (PSSF) is one such modification that can resolve the problems of compromising the optimum temperature in saccharification and fermentation (Afedzi and Parakulsuksatid, 2023). Further, an effective SSF process also requires optimization of enzymatic hydrolysis by selecting suitable enzymes and its concentrations.

The development of enzyme cocktails comprising efficient cellulases and hemicellulases is essential for effective SSF strategies (Alvira et al, 2010). For an economical SSF process, reducing the enzyme dosage by improving the catalytic activity can accelerate the hydrolysis performance. Methods such as systems biology strategies, cloning, mutagenesis and protein engineering have been successfully applied to enhance the production of cellulases (Nath et al., 2019; Sharma et al., 2022; Raheja et al., 2024). Formulating stable enzyme cocktails for synergistic effects in biomass

hydrolysis is crucial in order to achieve maximum efficiency in lignocellulosic polysaccharides breakdown and conversion (Raheja et al., 2022; Maibam and Goyal, 2023). The efficiency of enzymatic saccharification during SSF process depends on several factors such as i) pre-treatment conditions viz, type of solvent, time and temperature (Maibam and Goyal, 2022), ii) the enzyme properties (Rajak and Banerjee, 2020) and iii) the saccharification conditions such as biomass loading, enzyme dosage and hydrolysis time (Afedzi and Parakulsuksatid, 2023). Proper optimization of the above-mentioned significant factors could improve the overall product yield and process economics (Pratto et al., 2020). A high enzyme dosage in the process produces more fermentable sugars and hence higher saccharification efficiency. Thus, an enzyme cocktail should involve enzymes with high activity for efficient hydrolysis at low dosages and in a shorter duration. In this regard, on-site production of efficient cellulases and the use of these enzymes in their crude form for the SSF modification strategies (Maibam and Maiti, 2020; Panda and Maiti, 2024) could be an economical approach.

The present study focuses on the statistical optimization of the PSSF process of delignified rice straw biomass for bioethanol production considering important factors like PS time, enzyme dosage and fermentation temperature as a significant variable. This study also aims to exploit the potential of tailor-made crude recombinant bacterial hydrolytic enzyme cocktail formulation (Maibam and Goyal, 2023) for its application in bioethanol production from pretreated rice straw. The desirability function approach was used to maximize the multi-responses such as ethanol yield and ethanol productivity in order to achieve global efficient overall performance.

5.2 Materials and Methods

5.2.1. Feedstock and pretreatment

Choline chloride: acetic acid-based deep eutectic solvent pretreated rice straw (CApRS) biomass produced after optimization, as reported earlier in our previous study (Maibam and Goyal, 2022b), was used as a potential substrate for bioethanol production. The delignified CApRS consists of 73% (w/w) cellulose, 9% (w/v) hemicellulose and lignin of 5.1% (w/w) on a dry biomass basis analyzed by TAPPI (TAPPI, 1992) and NREL method.

5.2.2. Enzyme cocktail for feedstock hydrolysis

A novel tailor-made bacterial crude recombinant hydrolytic enzyme cocktail as reported earlier in our previous study (Maibam and Goyal, 2023) was used for the hydrolysis process. The enzyme cocktail consisted of cellulases (chimera enzyme with β-glucosidase and endoglucanase activity: cellobiohydrolase) and xylanases (endo-xylanase: xylosidase) in an optimized ratio 35.1:41.8:10.0:13.1. The concentrated crude enzyme preparation possesses an activity of 208 FPU/$g_{\text{total cellular proteins}}$ and a protein concentration of 27.1 mg/mL (Lowry et al., 1951). Filter paper activity was measured by the protocol of IUPAC (International Union of Pure and Applied Chemistry) (Ghose, 1987).

5.2.3. Microorganism and fermentation medium

The yeast, *Saccharomyces cerevisiae* MTCC170 was procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The yeast culture was maintained as MGYP agar slant and the growth medium consisted of (g/L): glucose 20; yeast extract 10; $MgCl_2$ 1; KH_2PO_4 1; $(NH_4)_2SO_4$ 1. The growth medium pH was

adjusted to 5.5 ± 0.2 and the fermentation with 5%, v/v inoculum was carried out in 100 ml volume in 250 ml Erlenmeyer flasks incubated at 30°C, 150 rpm for 24h.

5.2.3.1. Effect of glucose on the growth of *Saccharomyces cerevisiae* MTCC170

The effect of substrate (glucose) on the growth and ethanol titre produced by *S. cerevisiae* MTCC170 was studied by varying the initial concentrations of glucose ranging from 80 g/L to 250 g/L. The fermentation media consisted of (g/L): yeast extract 10; $MgCl_2$ 1; KH_2PO_4 1; $(NH_4)_2SO_4$ 1 and the medium pH was adjusted to 5.5 ± 0.2. The fermentation was carried out in 100 ml volume in 250 ml Erlenmeyer flasks incubated in shaking incubator at 30°C, 150 rpm. 5%, w/v yeast culture with cell OD_{600nm} of 8 was used as an inoculum and fermentation was carried out for 48h. At regular intervals of 6h, 2 mL samples were withdrawn and analyzed for glucose and ethanol concentration using HPLC. The protocol for HPLC analysis for determining the glucose and ethanol concentration are described in detail in section 5.2.9.

5.2.3.2. Effect of exogenous ethanol on the growth of *S. cerevisiae* MTCC170

Exogenous ethanol concentration at varied concentrations from 10 g/L to 80 g/L was added to the fermentation medium. The initial glucose concentration of 25 g/L, other medium composition and the conditions were kept same as described in section 5.2.3.1. Ethanol production and glucose consumption were estimated based on HPLC analysis as described in section 5.2.9. The cell OD_{600nm} was checked for each experiment with 24h samples to find the exogenous ethanol tolerance limit of yeast *S. cerevisiae* MTCC170 used in fermentation.

5.2.4 Pre-saccharification and simultaneous saccharification and fermentation

The short pre-hydrolysis step prior to SSF generally referred to as the pre-saccharification (PS) process has been shown to enhance the saccharification efficiency

and reduce the viscosity of the feedstock in the fermentation process (Afedzi and Parakulsuksatid, 2023). A PS experiments were performed with 10%, w/v delignified CApRS biomasses at software-generated conditions using the crude hydrolytic enzyme preparation in 0.05M sodium phosphate buffer, pH 5.8 at a working volume of 50 mL in 250 mL Erlenmeyer flask at 35°C, 200 rpm under sterile condition. The PS was followed by SSF by supplementing the reaction mixture with (g/L): yeast extract 3, $MgCl_2$ 1; KH_2PO_4 1; $(NH_4)_2SO_4$ 1. 5%, w/v yeast culture with cell OD_{600nm} of 8 was used as an inoculum for the PSSF process carried out at software-generated fermentation conditions at a working volume of 50 mL for 24h.

5.2.5 Experimental design

The efficiency of the enzymatic saccharification process, particularly during the PS steps is governed by various factors such as the type of enzyme, saccharification conditions like biomass loading, enzyme loading, hydrolysis time and operating temperature. For the optimization of PSSF process, central composite design (CCD) was employed to evaluate the influence of three significant operational variables: PS time (A, h), enzyme dosage (B, FPU/g_{CApRS}) and fermentation temperature (C, °C). The factors were set at five coded levels ($-\alpha$, -1, 0, $+1$ and $+\alpha$) and the upper and lower limits of the variables: A (6-24h), B (20-100 FPU/g) and C (28-40°C) as shown in Table 5.2.1, were set based on prior experiments performed and as per related literature (Cavalaglio et al., 2016; de Barros et al., 2017; Pratto et al., 2020). The two selected responses for the optimization process were ethanol yield and ethanol productivity. The software, Design Expert 7.0 (Stat-Ease, Inc., USA) generates 20 experimental setups as listed in Table 2 for the optimization of the PSSF process. Each experimental run was conducted as described in section 5.2.4 in triplicate sets, under software-generated

conditions. After 24h of PSSF, 2 mL of samples were withdrawn, centrifuged at 8000 g for 15 min and the supernatant was analysed for ethanol concentration by using HPLC. The responses, ethanol yield and ethanol productivity were fitted to the model for statistical optimization.

Table 5.2.1. **The coded value of variables for CCD-RSM design for PSSF process.**

Variables	Levels				
	-α	-1	0	+1	+α
PS time, h (A)	6	9.65	15	20.35	24
Enzyme loading, FPU/g_{CApRS} (B)	20	36.22	60	83.78	100
Temperature, °C (C)	28	30.43	34	37.57	40

5.2.6 Statistical analysis

Software design expert 7.0 (Stat-Ease, Inc., USA) was used for statistical data analysis through the analysis of variance (ANOVA). The effectiveness of the fitted model was expressed by the R^2 coefficient and the statistical significance was evaluated by F-test and *p*-value. A second-order polynomial regression equation was proposed to correlate the relations of each independent variable with the response Eqn. 5.1:

$$Y_i = a_0 + \sum_{i=1}^{n} a_i . X_i + \sum_{i \neq j}^{n} a_{ij} . X_i X_j + \sum_{j=1}^{n} a_{jj} . X_j^2 \quad \text{…Eqn.5.1}$$

where Y_i: experimental response; a_0: constant; X: set of normalized operating parameters; a_i's: set of coefficients of linear terms; a_{ij}'s: set of coefficients of interacting variables; a_{jj}'s: set of coefficients of quadratic term and n: number of independent variables.

5.2.7 Statistical optimization and validation of PSSF process

The statistical optimization of both responses, ethanol yield and productivity were performed based on the desirability function (D). Both the responses were simultaneously maximized to achieve the optimized condition and the response surface plot generates the interactive effects of the independent variables on the responses.

The predicted optimum value of the factors i.e. PS time of 12.22h, enzyme dosage of 83.78 FPU/g $_{CApRS}$ and fermentation temperature of 33.7°C for the PSSF process suggested by the software based on the desirability function was validated for both the responses, ethanol yield and ethanol productivity. The validation PSSF experiment was carried out for 10%, w/v delignified CApRS biomasses using crude hydrolytic enzyme cocktail preparation (pH 5.8) in a fermentation medium described in section 5.2.4 at a working volume of 100 mL in 250 mL Erlenmeyer flask in triplicate under the software predicted optimum conditions. 2 mL samples were withdrawn at an interval of 6h from PSSF flask process and analyzed for glucose and ethanol concentration.

5.2.8 Scale-up of PSSF process under the optimized condition at bioreactor level

The PSSF process was carried out at a working volume of 1L with 10%, w/v CApRS biomass in a 4L fermenter system (Minifors, Switzerland). Pre-saccharification (PS) process was carried out using crude hydrolytic enzyme cocktail at an optimum dosage of 83.78 FPU/g_{CApRS} for a PS time of 12.22h at 35°C. The PS process was converted to PSSF process by supplementing the PS reaction mixture with a medium component (mentioned in section 5.2.4) and 5%, v/v yeast inoculum (cell OD_{600nm} of 8). The PSSF fermentation was carried out at an optimum temperature of 33.7°C for 18h. The stirring speed and aeration rate were 900 rpm and 1 vvm, respectively, throughout the experiment. 5 mL sample was withdrawn after 18h of fermentation and analysed for ethanol concentration.

5.2.9 Glucose and ethanol estimation

Glucose and ethanol concentrations were quantified using High-Performance Liquid Chromatography (Shimadzu Corporation, Japan), equipped with a refractive index detector (RID-10A, Shimadzu). Aminex HPX-87H column (Bio-Rad Laboratories, USA) was used with a mobile phase, 5 mM H_2SO_4 (0.6 mL/min) and at 50°C oven temperature.

The ethanol yield and ethanol productivity were calculated according to Eqn. (5.2) and (5.3), respectively.

$$\text{Ethanol Yield } \left(\frac{g}{g}\right) = \frac{[Ethanol]}{[Biomass] \times c \times 0.9} \quad \text{... Eqn. 5.2}$$

The term "[Biomass] x c x 0.9" represents theoretical glucose concentration,

[Biomass]: concentration of dry CApRS biomass (g/L) in PSSF process;

c: cellulose fraction in the CApRS biomass (0.73 g/g, mentioned in section 2.1.);

0.9: conversion factor of cellulose to equivalent glucose;

$$\text{Ethanol Productivity (g/L/h)} = \frac{[Ethanol]}{PS\ time + SSF\ time} \quad \text{... Eqn. 5.3}$$

5.3. Results and Discussion

5.3.1 Effect of glucose on the growth of *Saccharomyces cerevisiae* MTCC170

Fermentation of yeast, *S. cerevisiae* MTCC170 was carried out at different initial glucose concentrations of 80 g/L, 120 g/L, 160 g/L and 200 g/L in fermentation medium under conditions as described in section 5.2.3.1. At initial glucose concentration of 80 g/L, the resulting ethanol titre was 38 g/L giving an ethanol yield of 0.48 g/g and the final cell OD at 600 nm was 18 (Fig. 5.3.1A). Increasing the initial glucose concentration to 120 g/L (Fig. 5.3.1B) in the fermentation medium, resulted in ethanol production of 51.6 g/L with cell OD of 18.5 at 600nm but the ethanol yield drop to 0.43 g/g. Further increment in initial glucose concentration to 160 g/L (Fig. 5.3.1C) in the fermentation medium increased the ethanol titre to 64 g/L and a cell OD to 31, however, there was reduction in ethanol yield, 0.40 g/g. The increase in cell OD to 31 (at initial glucose concentration of 160 g/L) as compared with cell OD of 18.5 at 120 g/L glucose indicated that there is no inhibition in the growth of yeast cells at 160 g/L glucose in the fermenting medium. At a higher initial glucose concentration, 200 g/L in the fermenting media, there was a slow uptake of glucose (Fig. 5.3.1D). The maximum ethanol titre of 60 g/L with yield 0.3 g/g was achieved at 24h of fermentation and no further increment was observed as fermentation proceeded. The maximum cell OD of 32 was achieved at 48h of fermentation. A significantly low ethanol yield of 0.3 g/g at glucose concentration of 200 g/L as compared with 0.48 g/g (initial glucose 80 g/L) in the fermentation medium showing the stagnation of the cell growth with reducing ethanol yield. The results indicated that *S. cerevisiae* MTCC170 can tolerate glucose upto 160 g/L in the fermenting medium without affecting its cell growth. However, for

a better performance of yeast, *S. cerevisiae* MTCC170 to achieve high ethanol yield, a maximum initial glucose concentration of 120 g/L is favourable.

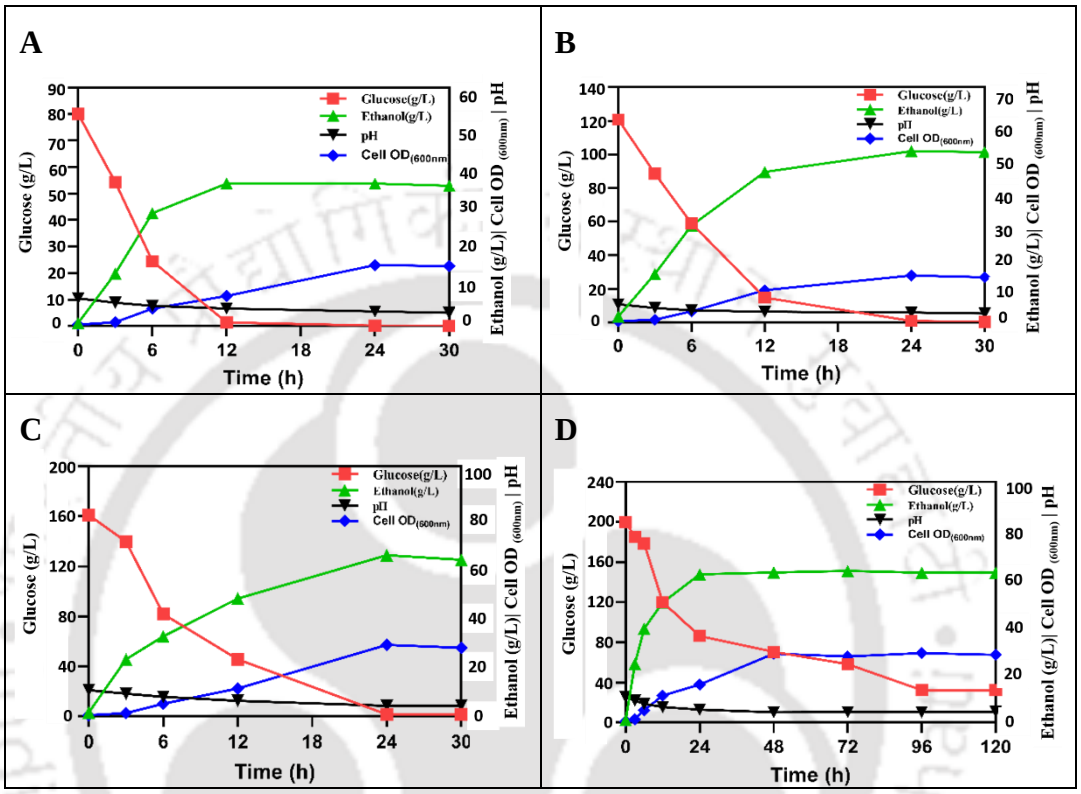

Fig. 5.3.1. Fermentation profile of *S. cerevisiae* MTCC170 at different initial glucose concentrations (A) 80 g/L, (B) 120 g/L, (C) 160 g/L and (D) 200 g/L operated at 30°C, 150 rpm with 5%, w/v yeast culture.

5.3.2 Ethanol tolerance of *S. cerevisiae* MTCC170

Ethanol tolerance of *S. cerevisiae* MTCC 170 was evaluated by supplementing exogenous ethanol at increasing concentration (10 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L) to the fermentation medium under the conditions described in sections 5.2.3.1 and 5.2.3.2. The ethanol production, cell growth (OD) and glucose consumption at 24h of fermentation (Fig. 5.3.2) were checked at each exogenous ethanol concentration to find the tolerance limit of the yeast strain used in fermentation. In Fig. 5.3.2, ethanol

concentration represents only the ethanol produced during the fermentation i.e., exogenous ethanol concentration was subtracted from final ethanol concentration.

In the control experiment with no addition of exogenous ethanol, there was complete consumption of glucose (25 g/L) by the yeast resulting in the production of 11.8 g/L ethanol. Supplementation of exogenous ethanol in the initial fermentation medium up to 40 g/L showed a fair tolerance by the yeast strain as 10.4 g/L ethanol was produced by utilizing 24 g/L glucose (96% consumption). A gradual decrease in cell growth and glucose consumption was observed upon increasing exogenous ethanol concentration from 40 g/L to 80 g/L thereby reducing ethanol production (Fig. 5.3.2). At 60 g/L initial exogenous ethanol concentration in fermentation medium reduces the ethanol production to 9 g/L by utilizing 20 g/L glucose (80% consumption). At 80 g/L initial exogenous ethanol concentration in the fermentation medium, the ethanol production was reduced significantly, 5.9 g/L. Thus, it can be inferred that *S. cerevisiae* MTCC170 showed ethanol tolerance up to 60 g/L.

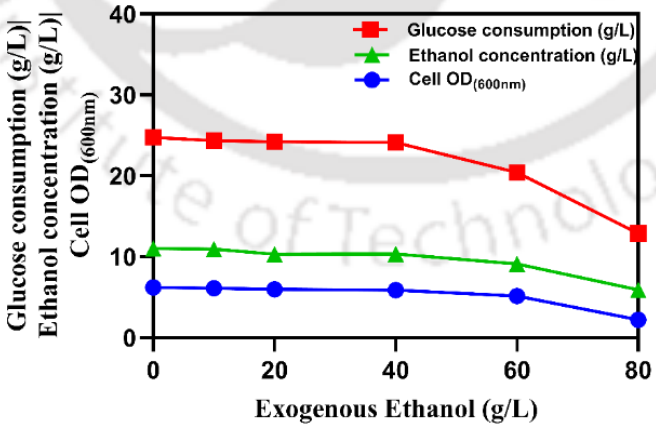

Fig. 5.3.2. Determination of exogenous ethanol tolerance of *S. cerevisiae* MTCC170 with different exogenous ethanol concentrations in the fermentation medium operated at 30°C, 150 rpm with 5%, w/v yeast culture.

5.3.3 Experimental design for optimization of PSSF process

The predicted and the experimental results for ethanol yield and productivity obtained from the execution of the PSSF process of 10%, w/v CApRS biomass using crude recombinant hydrolytic enzyme cocktail (pH 5.8) at a working volume of 50 mL in 250 mL Erlenmeyer, 200 rpm at sterile condition under the software design conditions are listed in Table 5.3.1. The experimental ethanol yield and ethanol productivity ranged from 0.20 g/g to 0.36 g/g and from 0.30 to 0.63 g/L/h, respectively. The experimental results showed that the highest ethanol yield of 0.36 g/g was achieved with PS time 15h at enzyme dose of 60 FPU/g_{CApRS} and fermentation temperature of 34°C and highest productivity of 0.63 g/L/h was obtained at 9.65h PS time, 83.8 FPU/g_{CApRS} at 37.6°C (Table 5.3.1).

Table 5.3.1. Predicted and experimental values of ethanol yield and productivity of PSSF process using CApRS biomass in RSM-CCD runs.

	Variables			Predicted Responses		Experimental Responses	
Run order	PS time h (A)	Enzyme loading FPU/g_{CApRS} (B)	Temperature °C (C)	Ethanol yield (g/g)	Ethanol productivity (g/L/h)	Ethanol yield (g/g)	Ethanol productivity (g/L/h)
1	9.65	36.22	30.43	0.18	0.38	0.197	0.388
2	20.35	36.22	30.43	0.22	0.33	0.212	0.317
3	9.65	83.78	30.43	0.29	0.59	0.289	0.569
4	20.35	83.78	30.43	0.30	0.49	0.306	0.467
5	9.65	36.22	37.57	0.29	0.55	0.289	0.573
6	20.35	36.22	37.57	0.22	0.32	0.226	0.338
7	9.65	83.78	37.57	0.31	0.61	0.319	0.627
8	20.35	83.78	37.57	0.22	0.32	0.207	0.309
9	6.00	60.00	34.00	0.26	0.57	0.249	0.550
10	24.00	60.00	34.00	0.21	0.29	0.219	0.302
11	15.00	20.00	34.00	0.26	0.40	0.252	0.377
12	15.00	100.00	34.00	0.35	0.57	0.348	0.584
13	15.00	60.00	28.00	0.23	0.45	0.231	0.476
14	15.00	60.00	40.00	0.25	0.45	0.252	0.423
15	15.00	60.00	34.00	0.34	0.57	0.357	0.610
16	15.00	60.00	34.00	0.34	0.57	0.342	0.564
17	15.00	60.00	34.00	0.34	0.57	0.275	0.467
18	15.00	60.00	34.00	0.34	0.57	0.346	0.587
19	15.00	60.00	34.00	0.34	0.57	0.352	0.609
20	15.00	60.00	34.00	0.34	0.57	0.339	0.567

5.3.4 Analysis of the statistical model of PSSF process

The analysis of variance (ANOVA) was performed to determine the significance of each term in the quadratic regression model and the results for both the responses was presented in Table 5.3.1. The high F-values, 11.4 and 13.10, respectively for both the responses, ethanol yield and ethanol productivity represent significant interaction of the parameters and the statistical significance of the model at a 95% confidence level. The low p-values (p-value <0.0005) signify that the regression models are highly significant, indicating that the second-order polynomial models are valid and reliable (Gunst, 1996). The insignificant p-values (p-value > 0.05) of lack of fit for both the responses, ethanol yield and ethanol productivity indicated a satisfactory correlation between the predicted and experimental data. Moreover, the high values of R^2, 0.91 and 0.92, for both the responses, ethanol yield and ethanol productivity respectively also validated the linear relationship between actual and predicted responses. The second-order polynomial equation representing the interaction among the variables for the responses, ethanol yield and ethanol productivity are shown in Eqn. 5.4 and Eqn. 5.5, respectively.

$$\text{Ethanol yield} = +0.34 - 0.014\,A + 0.026\,B + 5.396 \times 10^{-3}\,C - 5.984 \times 10^{-3}\,AB - 0.026\,AC - 0.022\,BC - 0.035\,A^2 - 0.012\,B^2 - 0.033\,C^2 \quad \text{...Eqn. 5.4}$$

$$\text{Ethanol productivity} = +0.57 - 0.084\,A + 0.052\,B + 1.298 \times 10^{-3}\,C - 0.014\,AB - 0.048\,AC - 0.038\,BC - 0.049\,A^2 - 0.030\,B^2 - 0.041\,C^2 \quad \text{…Eqn. 5.5}$$

It was observed from the ANOVA table that the linear term (A and B), the interacting terms (AC and BC) and all the quadratic terms (A^2, B^2 and C^2) showed significant influence (p-value < 0.05) to the quadratic model for both the responses, ethanol yield and ethanol productivity. The enzyme dosage (B) acts as the most

significant variable (p-value, 0.0017) followed by PS time (A) with p-value, 0.0427 for maximizing ethanol yield (Table 5.3.2). However, the PS time (A) has the higher influence (p-value < 0.0001) in maximizing ethanol productivity followed by enzyme dosage (B) with p-value, 0.0014. The positive sign assigned to the coefficients of linear variables, B and C of both the responses in Eq. 5.3 and 5.4 indicates the direct proportionality of independent variables to the responses. This inferred that increasing enzyme dosage has a positive impact on ethanol yield and ethanol productivity. However, the negative sign assigned to the coefficients of linear variable, A indicates a reciprocal relation to the responses thus, signifying that low PS time can significantly enhance the ethanol yield and ethanol productivity. The interpretation of the quadratic equation of both responses is in line with the results of the ANOVA table. Tareen et al. (2021) reported a comparable result verifying that the short pre-saccharification time of 6h plays a crucial role in enhancing the ethanol yield and ethanol productivity in the fed-batch PSSF process for ethanol production using alkaline hydrogen peroxide pretreated oil palm trunk fibers. Pratto et al, 2020 reported a similar finding that pre-saccharification times were the most significant (p < 0.0001) parameters for maximal ethanol yield (0.36 g/g) on the optimization of PSSF of hydrothermally pretreated sugarcane straw. Another study by Lopez-Linares et al. 2014 reported on SSF of hydrothermally pretreated rapeseed straw that enzyme concentration (40 FPU/g) and fermentation time (71h) have a significant positive impact on ethanol yield (0.35 g/g).

Table 5.3.2. ANOVA table for a quadratic model of PS-SSF process.

Source	Ethanol yield			Ethanol productivity		
	Sum of squares	F-value	p-value	Sum of squares	F- value	p-value
Model	0.053	11.44	0.0004	0.22	13.10	0.0002
A	0.0027	5.39	0.0427	0.096	50.44	< 0.0001
B	0.0099	18.17	0.0017	0.036	19.03	0.0014
C	0.0003	0.78	0.3988	2.302E-005	0.012	0.9146
AB	0.0002	0.56	0.4715	0.001	0.85	0.3777
AC	0.0053	10.42	0.0091	0.018	9.54	0.0115
BC	0.0038	7.52	0.0208	0.012	6.18	0.0323
A^2	0.018	34.97	0.0001	0.035	18.31	0.0016
B^2	0.002	4.06	0.0715	0.013	6.80	0.0261
C^2	0.015	29.98	0.0003	0.024	12.63	0.0052
Lack of Fit	0.00064	0.14	0.9731	0.005	0.37	0.8524
R^2	0.9114			0.9218		
Adj R-Squared	0.8318			0.8515		
Pred R-Squared	0.7949			0.7562		

5.3.5 Effect of interacting variables on the responses

The operation of PSSF at a condition when the independent variables are under optimum conditions is crucial for achieving maximum ethanol yield and productivity making it also cost-effective. For better insights into the interactions among the variables for maximizing the responses, the response surface plots of the second-order polynomial models were graphically analysed. Fig. 5.3.3 (a-c) and Fig. 5.3.3 (d-e) display the 3D contour of the effect of the independent variable on ethanol yield and ethanol productivity, respectively. Fig. 5.3.3 a shows the interaction of PS time (A) and enzyme loading (B) at a fixed fermentation temperature, C=33.7°C. Optimal region (red zone) of ethanol yield of 0.36 g/g could be achieved in a wide range of enzyme dosage higher than 60 FPU/g_{CApRS} with PS time ranging between 9.65h to 17.65h. Prolonged PS time was observed to show a negative impact on ethanol yield. Fig. 5.3.3b

represent the interacting effect of PS time (A) and Temperature (C) on ethanol yield at a fixed enzyme dosage of 83.78 FPU/g_{CApRS}. It was seen that temperature or PS time of upper or lower limit negatively affect the ethanol yield. The central values of fermenting temperature (32-35°C) in combination with PS time ranging from 12.32-17.68h resulted in maximal range of ethanol yield. At low temperature, 30°C, the catalytic efficiency of enzyme cocktail reduces thus leading to low rate of enzymatic saccharification hence low production of ethanol (Mazaheri et al., 2021). At high temperatures (above 40°C), there is a probability of lysis of yeast cells thus reducing ethanol production (Yingling et al., 2011). Fig. 5.3.3c represents the effect of interaction of enzyme loading (B) and fermenting temperature (C) on ethanol yield at fixed PS time, 12.22h. The contour inferred that high enzyme dosage with fermentation temperature ranging from 32-35°C resulted in high ethanol yield. This observation is in line with the interpretation of Fig. 5.3.3a and 5.3.3b, thus concluding that less PS time, enzyme dosage above 60 FPU/g_{CApRS} and fermenting temperature ranging from 32-35°C lead to maximal ethanol yield.

Fig. 5.3.3d represents the effect of PS time and enzyme loading interaction at fixed temperature of 33.7°C against ethanol productivity. Fig. 5.3.3e depicts the contour of PS time interaction with temperature at a fixed enzyme loading of 83.78 FPU/g_{CApRS}. Fig. 5.3.3f shows the combination effect of enzyme dosage and temperature on ethanol productivity at a fixed PS time of 12.22h. From Fig. 5.3.3d, e and f, it was observed that ethanol productivity is greatly influenced by PS time. The highest ethanol productivity of 0.64 g/L/h was achieved at a low PS time range of 9.65-12.32h and high enzyme dosage above 60 FPU/g_{CApRS} (Fig. 5.3.3d). Similarly, the red region of high ethanol productivity, 0.62 g/L/h was achieved at fermentation temperature performing

between 32-35°C with low PS time of 9.65-15h (Fig. 5.3.3e). At a fixed PS time of 12.22h, SSF process operating at high enzyme dosage above 60 FPU/g_{CApRS} and temperature at 32-35°C resulted in high ethanol productivity (0.62-0.64 g/L/h), thus conferring the significance of PS time and the combined effect of operating parameters, enzyme dosage and fermentation temperature on achieving high ethanol productivity.

A high ethanol productivity of 1.36 g/L/h from 22.5%, w/v molasses + hydrothermally pretreated paddy straw was reported at PS time of 12h (Pandey et al., 2022). Another study on PSSF fermentation of 15%, w/v sequential dilute acid-alkali pretreated cotton stalk with less PS time of 12h resulted higher ethanol yield of 0.44 g/g (Hemansi et al., 2022). Further, a very low PS time of 6h was reported with ethanol productivity of 0.66 g/L/h using 10%, w/v alkaline extracted oil palm trunk (Tareen et al., 2021).

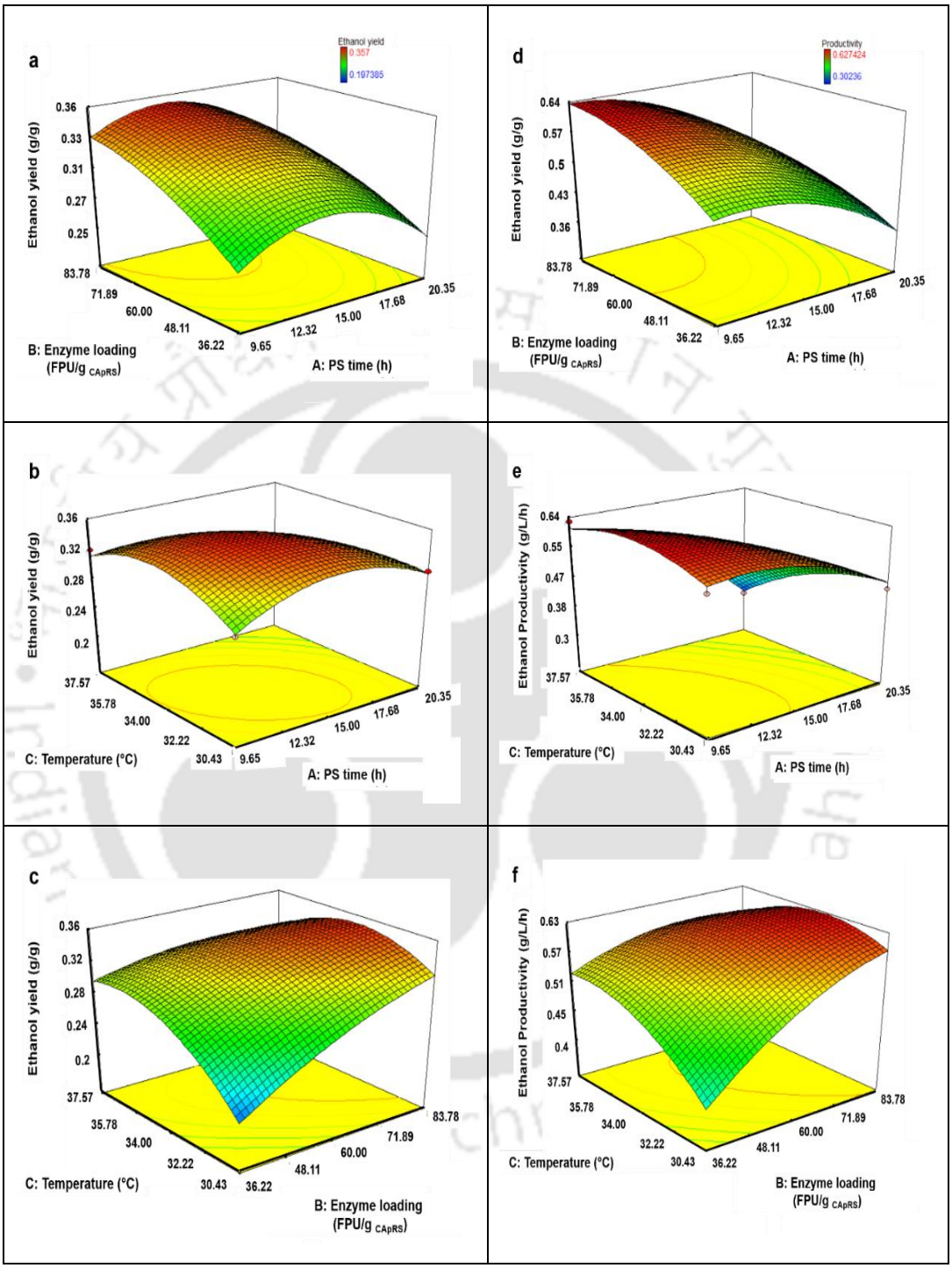

Fig. 5.3.3. Response surface plots (interaction among independent variables) of ethanol yield, g/g (a, b and c) and ethanol productivity, g/L/h (d, e and f). (a and d) PS time vs enzyme loading; (b and e) PS time vs temperature; (c and f) temperature vs enzyme loading.

5.3.6 Optimization and validation of PSSF process of CApRS biomass

The operation of the PSSF process at optimum conditions and parameters is crucial for achieving high ethanol yield and productivity. The parameters such as PS time, enzyme dosage and fermentation temperature cumulatively impact the ethanol yield and productivity. The statistical optimization of the PSSF process was set to maximize both the responses, ethanol yield and ethanol productivity based on the desirability function. A high overall desirability of 0.97 was predicted by the software indicating 97% fulfillment of the simultaneous optimization of the response variables (Anderson and Whitcomb, 2016). The software predicted optimum operating conditions were: PS time (12.22h), enzyme loading (83.78 FPU/g CApRS), fermentation temperature (33.7°C) and the responses, ethanol yield of 0.35 g/g and ethanol productivity, 0.63 g/L/h. The validation of PSSF experiment at 100 mL volume was performed under the optimum parameters as mentioned above using the protocol described in section 5.2.4. Fig. 5.3.4 displays the glucose and ethanol profile of the PSSF validation test performed using 10%, w/v CApRS biomass. The final ethanol titer of 23.7±0.09 g/L was obtained during the process, thus giving an ethanol yield, 0.36±0.01 g/g and ethanol productivity of 0.66±0.02 g/L/h considering (12.22h PS time+24h fermentation time). However, after 18h of fermentation, glucose was completely utilized and no increment in ethanol concentration was observed (Fig. 5.3.4). This gave an effective ethanol productivity of 0.79±0.01 g/L/h (12.2h PS time + 18h fermentation time).

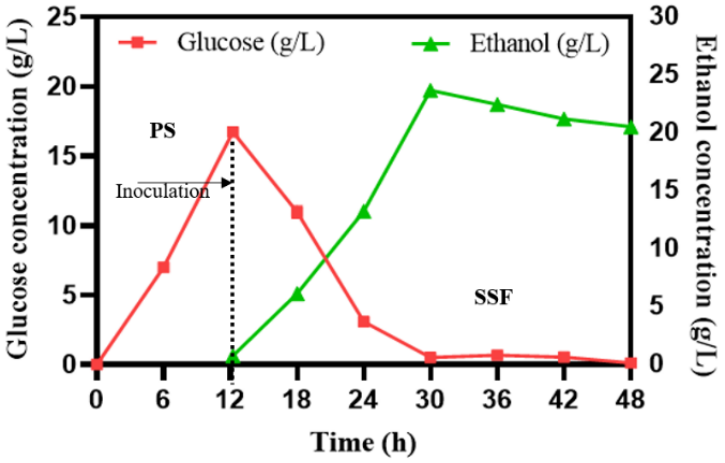

Fig. 5.3.4. PSSF process indicating the PS (operated at 35°C) and SSF (operated at 33.7°C) phases along with glucose and ethanol profiles from the validation test using crude hydrolytic enzyme cocktail and *S. cerevisiae* MTCC 170 .

5.3.7 PSSF process of CApRS biomass at bioreactor level

The scale-up of PSSF process of 10%, w/v CApRS biomass performed at an optimum condition of 12.22h PS time using crude recombinant hydrolytic enzyme cocktail at a dosage of 83.78 FPU/g_{CApRS} and a fermentation temperature of 33.7°C as described in section 5.2.8 in 1L reaction volume using a 4L capacity fermenter resulted in an ethanol tire of 24.8 g/L after 18h of fermentation or 30.22h of total PSSF process. The corresponding ethanol yield was 0.37 g/g and ethanol productivity was 0.82 g/L/h thus giving 72.5% of the theoretical ethanol yield.

5.3.8 Overall yield and mass balance of ethanol yield at the bioreactor level

To gain insight into the mass changes in rice straw its biomass pretreatment to bioethanol production, the mass balance of the scale-up PSSF process using 10% CApRS was carried out and illustrated in Fig. 5.3.5. As shown in Fig. 5.3.5, 100 g raw rice straw gave 58 g CApRS (pretreated rice straw) biomass using optimized

pretreatment conditions as reported earlier in Maibam and Goyal, 2022b. The PSSF process of 58 g CApRS biomass at optimized conditions (Fig. 5.3.5) resulted in 15 g ethanol which can be represented as 314 L ethanol/ tonne of CApRS biomass or 182 L ethanol/ tonne of raw RS biomass.

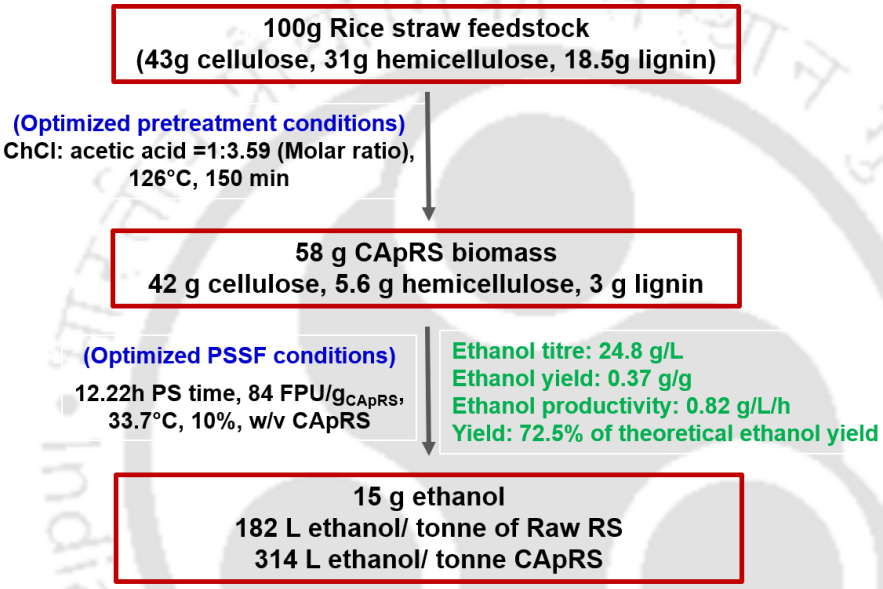

Fig. 5.3.5. Conversion of raw RS to ethanol under optimized conditions of PSSF process using crude hydrolytic enzyme cocktail and *S. cerevisiae* MTCC170.

5.3.9 Comparative analysis of PSSF process

A comparative analysis of the findings of the current study with previously reported literature is listed in Table 5.3.3. A study on the PSSF process with PS time of 33h using commercial cellulase, Cellic Ctec2 of enzyme dosage 14.5 FPU/g at a biomass loading of 19.3%, w/v hydrothermally pretreated sugarcane straw resulted in ethanol titre of 44 g/L giving 71% of the theoretical ethanol yield at a total processing time of 45h (Pratto et al., 2020). They reported an ethanol yield of 151 L/ tonne of raw

sugarcane straw. The lower ethanol yield obtained in their study as compared with the present study may be due to various reasons: i) low enzyme dosage, ii) high biomass loading, which reduces the efficiency of enzymatic hydrolysis as a result of high viscosity and low rate of heat transfer and hence lesser ethanol yield and iii) low biomass recovery, 52% and cellulose content, 55%, w/v in the pretreated sugarcane straw of their study may affect the overall yield of the process.

A similar study by Jin et al (2021) reported the potential of crude fungal, *A. fumigatus* enzyme in fed-batch semi-simultaneous saccharification and fermentation (FB-S-SSF) process using sodium carbonate-pretreated rice straw. Their study reported that feeding of both enzyme (total dosage, 50 FPU/$g_{cellulose}$) and substrate (final biomass dosage, 25%, w/v) could result in a maximum ethanol yield of 0.46 g/g (373 L/ tonne raw rice straw biomass) with low PS time of 12h at 50°C followed by fermentation at 37°C for 36h (Jin et al., 2021). The probable reason for the high ethanol yield in their study could be: i) use of wild-type efficient crude fungal enzyme cocktail constituting cellulases, xylanases, manganese-dependent peroxidase and laccase which act synergistically and are stable during the entire hydrolysis process (Gilbert and Hazlewood, 1993), ii) the feeding strategy adopted in their study and iii) high biomass recovery, 63% and difference in pretreatment method may lead to different structural modification in the pretreated biomass which greatly affects the enzymatic hydrolysis efficiency (Sun et al., 2016). Chilari et al. (2017) performed non-isothermal PSSF process using alkaline-pretreated cotton stalks (20% w/v), 44 FPU/g enzyme dosage (Cellic Ctec2) at PS time 14h and a prolonged SSF of 66h resulting in an ethanol yield of 0.28 g/g (overall ethanol yield of 142 L ethanol/ tonne of raw biomass). They reported a higher ethanol titre of 35 g/L which was due to high solid loading of 20%,

w/v. However, a very low ethanol productivity of 0.43 g/L/h was obtained as compared with the present study, which may be due to prolonged overall SSF process, 80h (Chilari et al., 2017). Another study by Mesa et al. (2017) on the PSSF process using 8%, w/v dilute-acid pretreated sugarcane straw, 30 FPU/g enzyme dosage (Cellic Ctec2) and a PS time of 24h reported a cellulose-to-ethanol yield of 187 L ethanol/ tonne of sugarcane straw (0.37 g/g). The overall ethanol yield of their study was high, however, the ethanol titre was low 15 g/L which may be due to low 8% (w/v) biomass loading. de Barros et al. (2017), reported a high ethanol titre of 59 g/L with a low overall ethanol productivity of 35L ethanol/ tonne raw biomass from acid followed by alkali pretreated cashew apple bagasse (20% w/v) in the PSSF process. The low ethanol productivity could be due to the double pretreatment method adopted in their study. The double pretreatment method leads to a very low percentage of biomass recovery (below 40%, 10% solid recovery in their case) and the cellulosic and hemicellulosic fractions are lost during the pretreatment process.

Table. 5.3.3. Comparative analysis of PSSF process in terms of overall yield of bioethanol

Biomass type	Biomass recovery,% Cellulose content, %	Solids loading % (w/v)	PS time (h)	Enzyme type, Enzyme loading	Time of maximum ethanol conc. (h)	Ethanol titre, yield & productivity	Overall yield L/ tonne of Raw biomass	Reference
Dilute-acid pretreated sugarcane straw	55 NA	8	14	Cellic CTec2, 30 FPU/g	24	15 g/L 0.37 g/g 0.63 g/L/h	187	Mesa et al., 2017
Alkaline-pretreated cotton stalks	64 55	20	14	Cellic CTec2, 44 FPU/g	80	35 g/L 0.28 g/g 0.44 g/L/h	142	Chilari et al., 2017
Alkali pretreated cashew apple bagasse	10 74.7	15	12	Celluclast: 30FPU/g β-glucosidase 60U/g	48	59 g/L 0.41 g/g 1.2 g/L/h	35	de Barros et al., 2017
Cellulose-rich corncob	64 74	7.5	24	AC cellulase 22.04 FPU/g	144	20.8 g/L 0.41 g/g 0.15 g/L/h	224	Boonchuay et al., 2021
Alkali treated Barley straw	63 66	20	16	Cellic CTec2, 40 FPU/g	72	46.6 g/L 0.31 g/g 0.65 g/L/h	188	Paschos et al., 2020
Hydrothermally treated Sugarcane straw	52 55	19.3	33	Cellic CTec2, 14.5 FPU/g	45	44 g/L 0.36 g/g0.98 g/L/h	151	Pratto et al., 2020
DES (ChCl: AA) treated Rice straw	58 73	10	12.2	Crude recombinant, 84 FPU/g	30.2	24.8 g/L 0.38 g/g0.82 g/L/h	182	This study

From the analysis of comparative study with various literature report, few notable observations can be drawn are as follows: i) use of high biomass loading in the PSSF process would result in high ethanol titre but not necessarily the overall ethanol yield/ ton of raw biomass, ii) low PS time and fermentation time leads to higher ethanol productivity and hence more economical, iii) the enzyme efficiency and substrate viscosity greatly influence the enzyme hydrolysis rate in the SSF process, iv) from industrial perspective, high ethanol titre above 40g/L is considered benchmark for a process to be labelled as economical as reported by (Chandel et al., 2010; Aui et al., 2021), however achieving such high ethanol titre could only be possible at high substrate loading, a challenging task for efficient enzymatic hydrolysis, v) the overall ethanol yield is greatly dependent on the biomass recovery percentage and the content of the cellulosic fraction in the pretreated biomass obtained after a particular pretreatment process and vi) the overall ethanol yield is always compromised at high solid loading processes as the major fraction of the pretreated biomass remains unhydrolyzed at the end of the process.

5.4. Conclusion

Pre-saccharification and simultaneous saccharification and fermentation (PSSF) of delignified rice-straw using crude recombinant enzyme cocktail and *Saccharomyces cerevisiae* MTCC170 were statistically optimized by RSM-CCD approach. Pre-saccharification time, enzyme dosage and fermentation temperature were the variables considered for maximizing ethanol yield and productivity. Statistical analysis of PSSF optimization inferred that low pre-saccharification time (9.6-15h) and enzyme dosage (above 60 FPU/g) display significant influence on achieving higher ethanol yield and productivity. Statistical optimization resulted in optimum conditions: 12.22h pre-saccharification time, enzyme dosage (83.78 FPU/g_{CApRS}), fermentation temperature (33.7°C) with predicted ethanol yield, 0.35 g/g and productivity, 0.63 g/L/h. The PSSF process of 10%, w/v CApRS biomass at bioreactor level with statistically optimized condition of PS time, 12.22h using crude enzyme cocktail, 84 FPU/g_{CApRS} at a fermentation temperature of 33.7°C gave an ethanol titre of 24.8 g/L, ethanol yield of 0.37 g/g with ethanol productivity 0.82 g/L/h after 18h of fermentation. The overall yield, 182L ethanol/ tonne raw RS biomass achieved in this study prospects the feasibility of 2G ethanol production using crude recombinant enzyme cocktail which outlays an economic advantage of on-site production of crude enzyme cocktails at the fermentation plant. The overall less process time, 30.22h optimized in this study, would reduce the global processing time (72h hydrolysis + 24h fermentation) by about 68%, thus providing another aspect on economic approach for ethanol production from CApRS.

5.5. References

Afedzi, A. E. K. and Parakulsuksatid, P. (2023). Recent advances in process modifications of simultaneous saccharification and fermentation (SSF) of lignocellulosic biomass for bioethanol production. Biocatalysis and Agricultural Biotechnology, 102961.

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

Anderson, M. J., Whitcomb, P. J. (2016). RSM simplified: optimizing processes using response surface methods for design of experiments. second ed., Productivity press, New York.

Aui, A., Wang, Y. and Mba-Wright, M. (2021). Evaluating the economic feasibility of cellulosic ethanol: A meta-analysis of techno-economic analysis studies. Renewable and Sustainable Energy Reviews, 145, 111098.

Boonchuay, P., Techapun, C., Leksawasdi, N., Seesuriyachan, P., Hanmoungjai, P., Watanabe, M., Srisupa, S. and Chaiyaso, T. (2021). Bioethanol production from cellulose-rich corncob residue by the thermotolerant *Saccharomyces cerevisiae* TC-5. Journal of Fungi, 7(7), 547.

Cavalaglio, G., Gelosia, M., Ingles, D., Pompili, E., D'Antonio, S. and Cotana, F. (2016). Response surface methodology for the optimization of cellulosic ethanol production from *Phragmites australis* through pre-saccharification and simultaneous saccharification and fermentation. Industrial Crops and Products, 83, 431-437.

Chandel, A. K., Singh, O. V., Chandrasekhar, G., Rao, L. V. and Narasu, M. L. (2010).

Key drivers influencing the commercialization of ethanol-based biorefineries. Journal of Commercial Biotechnology, 16, 239-257.

Chilari, D., Dimos, K., Georgoula, G., Paschos, T., Mamma, D., Louloudi, A., Papayannakos, N. and Kekos, D. (2017). Bioethanol production from alkali-treated cotton stalks at high solids loading applying non-isothermal simultaneous saccharification and fermentation. Waste and Biomass Valorization, 8, 1919-1929.

Classics Lowry, O., Rosebrough, N., Farr, A. and Bandall, R. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 193(1), 265-75.

de Barros, E. M., Carvalho, V. M., Rodrigues, T. H. S., Rocha, M. V. P. and Gonçalves, L. R. B. (2017). Comparison of strategies for the simultaneous saccharification and fermentation of cashew apple bagasse using a thermotolerant *Kluyveromyces marxianus* to enhance cellulosic ethanol production. Chemical Engineering Journal, 307, 939-947.

Ghose, T. K. (1987). Measurement of cellulase activities. Pure and Applied Chemistry, 59(2), 257-268.

Gilbert, H. J. and Hazlewood, G. P. (1993). Bacterial cellulases and xylanases. Microbiology, 139(2), 187-194.

Gunst, R.F. (1996). Response surface methodology: process and product optimization using designed experiments. Technometrics. 38, 285.

Hemansi, Kaushik, A., Yadav, G. and Saini, J. K. (2022). Simultaneous saccharification and fermentation of sequential dilute acid-alkali pretreated cotton (*Gossypium hirsutum L.*) stalk for cellulosic ethanol production. Journal of Chemical

Technology and Biotechnology, 97(2), 534-542.

Jin, X., Ma, J., Song, J. and Liu, G. Q. (2021). Promoted bioethanol production through fed-batch semisimultaneous saccharification and fermentation at a high biomass load of sodium carbonate-pretreated rice straw. Energy, 226, 120353.

López-Linares, J. C., Romero, I., Cara, C., Ruiz, E., Castro, E. and Moya, M. (2014). Experimental study on ethanol production from hydrothermal pretreated rapeseed straw by simultaneous saccharification and fermentation. Journal of Chemical Technology & Biotechnology, 89(1), 104-110.

Maibam, P. D. and Goyal, A. (2023). Designing of recombinant hydrolytic enzymes cocktail for effective saccharification of delignified rice straw. Industrial Crops and Products, 206, 117727.

Maibam, P. D., Goyal, A. (2022b). Approach to an efficient pretreatment method for rice straw by deep eutectic solvent for high saccharification efficiency. Bioresource Technology. 351, 127057.

Maibam, P. D., Maiti, S. K. (2020). A strategy for simultaneous xylose utilization and enhancement of cellulase enzyme production by *Trichoderma reesei* cultivated on liquid hydrolysate followed by induction with feeding of solid sugarcane bagasse. Waste and Biomass Valorization. 11, 3151-3160.

Maibam, P.D., Goyal, A. (2022a). Pretreatment methods for overcoming biomass recalcitrance, in: Verma, P. (Eds.), Enzymes in the Valorization of Waste. CRC Press, pp. 1-21.

Mazaheri, D., Orooji, Y., Mazaheri, M., Moghaddam, M. S. and Karimi-Maleh, H. (2021). Bioethanol production from pomegranate peel by simultaneous saccharification and fermentation process. Biomass Conversion and

Biorefinery, 1-9.

Mesa, L., Martínez, Y., Barrio, E. and González, E. (2017). Desirability function for optimization of Dilute Acid pretreatment of sugarcane straw for ethanol production and preliminary economic analysis based in three fermentation configurations. Applied Energy, 198, 299-311.

Nath, P., Dhillon, A., Kumar, K., Sharma, K., Jamaldheen, S. B., Moholkar, V. S., Goyal, A. (2019). Development of bi-functional chimeric enzyme (*Ct*GH1-L1-*Ct*GH5-F194A) from endoglucanase (*Ct*GH5) mutant F194A and β-1, 4-glucosidase (*Ct*GH1) from *Clostridium thermocellum* with enhanced activity and structural integrity. Bioresource Technology. 282, 494-501.

Panda, S. K. and Maiti, S. K. (2024). Novel cyclic shifting of temperature strategy for simultaneous saccharification and fermentation for lignocellulosic bioethanol production. Bioresource Technology, 391, 129975.

Pandey, A. K., Kumar, M., Kumari, S. and Gaur, N. A. (2022). Integration of acid pre-treated paddy straw hydrolysate to molasses as a diluent enhances ethanol production using a robust *Saccharomyces cerevisiae* NGY10 strain. Renewable Energy, 186, 790-801.

Paschos, T., Louloudi, A., Papayannakos, N., Kekos, D. and Mamma, D. (2020). Potential of barley straw for high titer bioethanol production applying pre-hydrolysis and simultaneous saccharification and fermentation at high solid loading. Biofuels 13, 467–473.

Pratto, B., dos Santos-Rocha, M. S. R., Longati, A. A., de Sousa Júnior, R. and Cruz, A. J. G. (2020). Experimental optimization and techno-economic analysis of bioethanol production by simultaneous saccharification and fermentation

process using sugarcane straw. Bioresource Technology, 297, 122494.

Raheja, Y., Singh, V., Kaur, B., Basotra, N., Di Falco, M., Tsang, A. and Chadha, B. S. (2022). Combination of system biology and classical approaches for developing biorefinery relevant lignocellulolytic *Rasamsonia emersonii* strain. Bioresource Technology, 351, 127039.

Raheja, Y., Singh, V., Sharma, G., Tsang, A. and Chadha, B. S. (2024). A thermostable and inhibitor resistant β-glucosidase from *Rasamsonia emersonii* for efficient hydrolysis of lignocellulosics biomass. Bioprocess and Biosystems Engineering, 1-16.

Rajak, R. C., Banerjee, R. (2020). An innovative approach of mixed enzymatic venture for 2G ethanol production from lignocellulosic feedstock. Energy Conversion and Management. 207, 112504.

Sharma, G., Kaur, B., Raheja, Y., Agrawal, D., Basotra, N., Di Falco, M., Tsang, A. and Chadha, B.S. (2022). Lignocellulolytic enzymes from *Aspergillus* allahabadii for efficient bioconversion of rice straw into fermentable sugars and biogas. Bioresource Technology, 360, 127507.

Siddique, M. N. I. and Wahid, Z. A. (2018). Achievements and perspectives of anaerobic co-digestion: a review. Journal of Cleaner Production, 194, 359-371.

Sun, S., Sun, S., Cao, X. and Sun, R. (2016). The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. Bioresource Technology, 199, 49-58.

Tareen, A. K., Punsuvon, V., Sultan, I. N., Khan, M. W. and Parakulsuksatid, P. (2021). Cellulase addition and pre-hydrolysis effect of high solid fed-batch simultaneous saccharification and ethanol fermentation from a combined

pretreated oil palm trunk. ACS Omega, 6(40), 26119-26129.

Technical Association of the Pulp, Paper Industry. TAPPI test methods. TAPPI; (1992).

Van Hung, N., Maguyon-Detras, M.C., Migo, M.V., Quilloy, R., Balingbing, C., Chivenge, P. and Gummert, M. (2020). Rice straw overview: availability, properties, and management practices. Sustainable Rice Straw Management, 1.

Yingling, B., Zongcheng, Y., Honglin, W. and Li, C. (2011). Optimization of bioethanol production during simultaneous saccharification and fermentation in very high-gravity cassava mash. Antonie van Leeuwenhoek, 99, 329-339.

Future Prospects

The present thesis established the applicability of crude recombinant enzyme cocktail preparation and the modeled process (PSSF) as a promising and cost-effective industrial approach for bioethanol production using rice straw at the laboratory level. However, there are some suggestions for future work in this regard as follows:

1. The reusability of DES for rice straw pretreatment can be evaluated for maximum reuse of solvent to reduce the cost of the pretreatment process.
2. The crude enzymes can be produced using minimal media to reduce the cost of enzyme production.
3. The addition of additives and enhancers (Tween 80, Triton X-100, PEG 4000, etc.) during the hydrolysis process or in the PSSF process can be investigated for achieving high product yield. However, the capital cost for these inputs and the revenue generated for the final product needs to be calculated.
4. In the present thesis the fermentation was carried out in batch mode. Further, improvement to either fed-batch or continuous modes could be a future scope for an economic and viable operation at large-scale processes.
5. The lignin extracted from liquid hydrolysate can be further purified, characterized and evaluated for its market value.

Journal Publications

From the Thesis

1. **Premeshworii D. Maibam** and Arun Goyal. "Approach to an efficient pretreatment method for rice straw by deep eutectic solvent for high saccharification efficiency." Bioresource Technology 351 (2022): 127057. (JIF: 11.4).
2. **Premeshworii D. Maibam**, and Arun Goyal. "Designing of recombinant hydrolytic enzymes cocktail for effective saccharification of delignified rice straw." Industrial Crops and Products 206 (2023): 117727. (JIF: 5.9).
3. **Premeshworii D. Maibam**, and Arun Goyal. " Bioethanol production by statistical optimization of pre-saccharification simultaneous saccharification and fermentation of delignified rice straw using a novel crude recombinant hydrolytic enzyme cocktail." (Under review: RENE-D-24-02739).

Publication from other collaboration

1. Nath, Priyanka, **Premeshworii D. Maibam**, Shweta Singh, Vikky Rajulapati, and Arun Goyal. "Sequential pretreatment of sugarcane bagasse by alkali and organosolv for improved delignification and cellulose saccharification by chimera and cellobiohydrolase for bioethanol production." 3 Biotech 11 (2021): 1-16. (JIF: 2.8).
2. Aishwarya, Aishwarya, **Premeshworii D. Maibam** and Arun Goyal. "Statistical optimization of thermo-chemical pretreatment of Elephant grass for efficient delignification and enzymatic saccharification". Biomass Conversion and Biorefinery (2024). https://doi.org/10.1007/s13399-024-05650-x (JIF: 4.0)

Book Chapters

1. **Premeshworii D. Maibam**, and Arun Goyal. "Pretreatment Methods for Overcoming Biomass Recalcitrance." In: Verma, P. (Eds.), Enzymes in the Valorization of Waste, (Ed) CRC Press, (2022), 1-21.
2. Khaire, Kaustubh C., **Premeshworii D. Maibam**, Abhijeet Thakur, and Arun Goyal. "Biomedical and pharmaceutical applications of xylan and its derivatives." In: Brienzo, M. (Eds), Hemicellulose Biorefinery: A Sustainable Solution for Value Addition to Bio-Based Products and Bioenergy. Clean Energy Production Technologies. Springer, Singapore. (2022): 447-465.
3. Shrivastava, Madhulika, **Premeshworii D. Maibam**, Aishwarya, Aishwarya and Arun Goyal. “Hemicellulases and auxiliary activities for biomass hydrolysis.” In Bisaria, V. S. (Eds), Handbook of Biorefinery Research and Technology. Springer, Singapore (2023) (In Press).

Awards and Honours

1. **Best Paper Award** at 4th International Conference on Environmental Science and Applications, 4-6th Dec 2023, Lisbon, Portugal.
2. **Awarded with SERB-International Travel Scheme from DST** for presenting oral presentation in 4th International Conference on Environmental Science and Applications, 4-6th Dec 2023, Lisbon, Portugal.
3. **Awarded with Student Travel Assistant Fund (STAF) from IITG** for attending 4th International Conference on Environmental Science and Applications, 4-6th Dec 2023, Lisbon, Portugal.
4. **1st Prize for Best Oral Presentation** by Research and Industrial Conclave, 20-23rd Jan 2022, IIT Guwahati, Assam.
5. **Young Scientist Award** at International Conference on Advances in Chemistry and Biology of Carbohydrates (CARBO XXXV) jointly organized by Forest Research Institute, Dehradun & Association of Carbohydrate Chemists and Technologists India (ACCTI), 4-5th Dec 2021, FRI, Dehradun.

List of conference papers

1. **Premeshworii Devi Maibam** and Arun Goyal **(2023)**. Green bioethanol production from delignified rice straw using statistically designed crude recombinant enzyme cocktail. 4th International Conference on Environmental Science and Applications, **Dec 4-6,** 2023, Lisbon, Portugal.
2. Oinam Riya Devi, Akshay Kumar, **Premeshworii Devi Maibam**, Aishwarya and Arun Goyal **(2023)** Bioethanol production from wheat straw using commercial cellulase (Sacchari SEB C6L) and Saccharomyces cerevisiae NCIM 3215. Biotech Research Society India- International Conference on New Horizons in Biotechnology INDIA (BRSI-NHBT 2023), **Nov 26-29**, 2023, Thiruvananthapuram, Kerala, India.
3. **Premeshworii D Maibam** and Arun Goyal **(2023)**. An approach to green and cost effective bioethanol production from delignified rice straw using crude recombinant enzyme cocktail. Research and Industrial Conclave 2023, **May 14-16**, 2023, IIT Guwahati, Assam, India.
4. Aishwarya, **Premeshworii Devi Maibam** and Arun Goyal **(2022)**. Delignification of elephant grass by alkaline hydrogen peroxide for its application in biorefineries. Biotech Research Society of India (BRSI), International Conference on Biotechnology for Sustainable Bioresources and Bioeconomy (BSBB-2022), **Dec 8-11**, 2022, IIT Guwahati, India.
5. **Premeshworii Devi Maibam** and Arun Goyal **(2022)**. Designing of recombinant clostridial enzymes cocktail statistically for the saccharification of delignified rice-straw: An economic biorefinery approach. International

conference on advances in chemistry and biology of carbohydrates (CARBO XXXVI) **Dec 5-7,** 2022, IIT Bombay, India.

6. **Premeshworii Devi Maibam** and Arun Goyal **(2022).** Strategic biorefinery approach for bioethanol production from rice-straw using green pretreatment method and recombinant enzyme cocktail for high saccharification efficiency. North-East Research Conclave Sustainable Science and Technology (**NERC**) **May 20-22**, 2022, IIT Guwahati, India.
7. **Premeshworii Devi Maibam** and Arun Goyal **(2022).** Optimization of saccharification for enhancing the production of monosaccharides for biorefineries approach using delignified rice-straw". Research and Industrial Conclave 2022: An amalgamation of Academia, Industry & Start-up (RIC) **Jan 20-23**, 2022, IIT Guwahati, India. (**Best Paper Award**).
8. **Premeshworii Devi Maibam** and Arun Goyal **(2021).** Modelling and optimization of rice-straw delignification by deep eutectic solvent for the production of bio-fuel feedstock using RSM-CCD approach. "Sixth Sustainable Energy and Environmental Challenges (**VI-SEEC, 2021**) **Dec 27- 29**, 2021, Lucknow, India.
9. **Premeshworii Devi Maibam**, Bipasha Choudhury and Arun Goyal **(2021)**. Delignification of rice straw by deep eutectic solvent for enhancing saccharification of biofuel feedstock and extraction of lignin using RSM-CCD approach. International conference on advances in chemistry and biology of carbohydrates (CARBO XXXV) **Dec 4 -5,** 2021, Dehradun, India **(Best Paper Award).**
10. **Premeshworii Devi Maibam** and Arun Goyal **(2021)**. Effect of clostridial recombinant enzyme cocktail on saccharifications of rice straw pretreated by different methods International Conference Bioengineering Solutions for Healthcare, Food, Energy, and Environment (BSHFEE) **Apr 9-10**, 2021, IIT Jodhpur.
11. **Premeshworii Devi Maibam**, Priyanka Nath, Shweta Singh, Vikky Rajulapati and Arun Goyal **(2019)** Comparative pretreatment using mild alkali and organosolv method for improving enzymatic digestibility of sugarcane bagasse using cocktail of Chimera (*Ct*GH1-L1-CtGH5-F194A) and Cellobiohydrolase (*Ct*CBH5A) for bioethanol production. 60th Annual Conference of AMI & International Symposium on Microbial Technologies in Sustainable Development of Energy, Environment, Agriculture and Health, **Nov 15-18**, 2019, Central University of Haryana, Mahendra Garh.

VITAE

Ms Maibam Premeshworii Devi was born on May 4, 1993 in Imphal, Manipur, India. She passed the Secondary School Examination (10th Standard) conducted by Board of Secondary Education Manipur (BOSEM) in 2009 and Higher Secondary Education (12th Standard) conducted by Central Board of Secondary Education (CBSE) in 2011. She completed B.Tech. Biotechnology from Lovely Professional University, Punjab, India in 2015. She qualified Graduate Aptitude Test in Engineering (GATE) 2016 and did M.Tech. Biotechnology from Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India in 2018. She worked as Junior Research Fellow in a twinning project jointly operated by Manipur University, Manipur and Dr. B.R. Ambedkar Centre for Biomedical Research, New Delhi, India for 8 months (October, 2018- May, 2019).

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Approach to an efficient pretreatment method for rice straw by deep eutectic solvent for high saccharification efficiency

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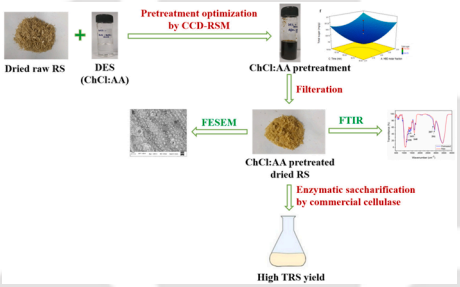
HIGHLIGHTS:

- ChCl:AA pretreatement method gave effective, 83% delignification of rice straw.
- ChCl:AA pretreated rice straw gave higher cellulose content, 0.73 $g/g_{ptd\ rice\ straw}$.
- Pretreatment efficiency of 84% was achieved for rice straw by ChCl:AA method.
- Total carbohydrate content after ChCl: AA pretreatment was 679 $mg/g_{ptd\ rice\ straw}$.
- Enzyme hydrolysis of ChCl:AA pretreated rice straw gave 18.8 g/L total reducing sugar.

GRAPHICAL ABSTRACT



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ABSTRACT

Deep eutectic solvent comprising choline chloride (ChCl) and acetic acid (AA) was used for rice straw (RS) pretreatment. Effect of ChCl: AA molar ratio, time and temperature on lignin removal and retainment of total carbohydrate content (TCC) in pretreatment process were evaluated by central composite design (CCD) approach. The pretreatment temperature and molar ratio of AA to ChCl played a significant role in delignification. The optimized conditions for RS pretreatment were 1:3.59 (ChCl:AA molar ratio), 126 °C and 150 min. ChCl:AA pretreated RS (CApRS) gave 83.1% delignification, 679 mg/g_{CApRS} TCC and 83.7% pretreatment efficiency. CApRS contained enriched cellulose content, 0.73 g/g_{CApRS} as compared with 0.43 $g/g_{raw\ RS}$ in raw RS. CApRS showed 31% higher crystallinity index, 17-fold higher surface area than raw RS. The morphological study of CApRS displayed porous surface. Saccharification of CApRS by commercial cellulase gave total reducing sugar of 18.8 g/L in hydrolysate with saccharification efficiency, 92.2%.

1. Introduction

The increasing demand for crude oil and its rising price has renewed the interest in renewable energy. Bioethanol from lignocellulosic feedstocks serves as a promising renewable source of fuel (Di Marino et al. 2016). In Asian countries, rice straw contributes a major agriculture lignocellulosic waste. The global rice straw production is 370–520 million tons/year of which 330–470 million tons are from Asia alone

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(Van Hung et al. 2020). This enormous amount of lignocellulosic waste can be effectively valorized to bioethanol (Singh et al. 2016). However, the key to the success in lignocellulosic biorefineries lies in an effective pretreatment method for enhancing the efficiency of the enzymatic hydrolysis process. The purpose of any pretreatment method is the removal of hemicellulosic and lignin fractions that increases the exposure of cellulosic fraction and its surface with the reduction in cellulose crystallinity (Baruah et al., 2018; Maibam and Maiti, 2020). The use of green solvent having characteristics of biodegradability, low toxicity, recyclability and cost-effectiveness has emerged as a potential pretreatment method for biomass fractionation (Calvo-Flores et al. 2018).

Deep eutectic solvents (DESs) are preferred as they are green solvents because of their properties such as being low toxicity, easy synthesis process and feasibility at large scale pretreatment process (Wang and Lee, 2021). The DESs are eutectic mixtures comprising two components i.e., hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) at a fixed molar ratio, with a melting point lower than those of the individual components (Loow et al. 2018). The mechanism of biomass fractionation by DES can be predominantly categorized as hydrolysis of hemicellulose and delignification with negligible disruption to the cellulosic fractions (Hong et al. 2020). Choline chloride (ChCl) based DES has shown to be of great potential in the delignification of lignocellulosic biomass (Xu et al. 2020). Chlorine-based-DES methods delignifies the biomass by forming hydrogen bonds between halogen ions (Cl^-) of DES and − OH groups in lignin (Xu et al. 2021). The features of ChCl-based DES method can be modulated by changing HBD and its molar ratio with HBA. Various types of HBD could form DES with ChCl, such as acids, alcohols, polyols, amides and polysaccharides (Wang and Lee, 2021). The molar ratio of HBD to ChCl and the pretreatment severity had a great influence on the efficacy of delignification of lignocellulosic biomass (Xu et al. 2021). DES with acid-based HBD such as formic acid, oxalic acid, lactic acid were reported to be effective in lignin and xylan removal with a positive impact on enzymatic hydrolysis efficiency (Zhang et al., 2016; Hou et al., 2017; Chen and Mu, 2019).

A detailed investigation on DES with ChCl and acetic acid (AA) for the pretreatment process of rice straw (RS) has not been reported. Therefore, in this work, DES comprising ChCl and AA was studied for the pretreatment of RS. The possible combinations of ChCl/AA molar fraction were generated using statistical software to ensure the maximal delignification and retainment of the total carbohydrate content (TCC) in the pretreated RS. The pretreated RS was structurally and compositionally characterized and subjected to enzymatic saccharification. Based on statistical modeling, green and effective pretreatment method was developed with ChCl: AA system in defined proportion and an excellent saccharification efficiency was achieved.

2. Materials and methods

2.1. Materials and reagents

Rice straw (RS) was obtained from local fields of Barpeta District, Assam, India. The biomass was washed under tap water and dried at 80 °C in an oven till a constant weight was achieved. Subsequently, the dried biomass was grinded and subjected to sieving by using a standard sieve (pore size, 1x1 mm) and stored at 25 °C for further use. Choline chloride, acetic acid, sodium chlorite, sulphuric acid, sodium azide, citric acid and sodium phosphate monobasic used were of Analytical Reagent grade and purchased from Himedia, Pvt. Ltd, India. Glucose, xylose, arabinose and commercial cellulase (*Trichoderma reesei* aqueous solution, ≥ 700 U/g) were purchased from Sigma-Aldrich Co. LLC., USA.

2.2. DES preparation

Fifty grams of DES was synthesized by mixing ChCl and AA in different combinations of molar ratios as shown in Table 1. After mixing the components, each DES mixture was stirred at 80 °C on a magnetic stirrer with hot plate until a homogeneous colorless viscous solution was obtained in approx. 30 min as reported earlier (Xing et al. 2018). The prepared DES mixtures were used for the optimization of RS pretreatment process.

Table 1
The coded value of variables for CCD-RSM design for rice straw pretreatment.

Variables	Levels				
	-α	−1	0	+1	+α
ChCl:AA molar ratio (A)	1:2	1:2.41	1:3	1:3.59	1:4
Temperature, °C (B)	70	84.19	105	125.81	140
Time, min (C)	30	60.40	105	149.60	180

2.3. Experimental design for optimization of rice straw pretreatment using ChCl: AA

The Central composite design (CCD) was employed to study the effect of independent variables on the response and mutual interactions for optimizing the pretreatment of rice straw. The independent variables selected were, the ChCl: AA molar ratio (A), treatment temperature (B) and pretreatment time (C). The factors were set at five coded levels (−α, − 1, 0, + 1 and + α) with lower/upper bounds of A, B (°C) and C (min) at 1:2/1:4, 70/140 and 30/180, respectively (Table 1). Lignin content and total carbohydrate content (TCC) were selected as the responses to the optimization process. A statistical approach of response surface methodology (RSM) was employed to solve multivariate equations from the experimental values for the pretreatment of rice straw. The software, design expert 7.0 (Stat-Ease, Inc., USA) designed 20 experimental setups as listed in Table 2 for the optimization of RS pretreatment. Each experiment was conducted with 50 g DES solution at 7.5%, w/w biomass loading under the software designed conditions. After the pretreatment process, the solid fraction was separated by filtration using a muslin cloth. The solid residue was washed with distilled water until a neutral pH was reached, thereafter it was lyophilized overnight and stored in sealed bags. Lignin content and TCC of the dried pretreated RS biomasses were estimated by using standard National Renewable Energy Laboratory (NREL) protocol (Sluiter et al. 2010) and fitted to the model for statistical optimization. All the experiments were conducted in triplicate sets.

2.4. Characterization of raw and ChCl: AA pretreated rice straw

The pretreated RS obtained from the optimized conditions of the RSM-CCD method was referred to as CApRS (choline chloride: acetic acid pretreated rice straw) and subjected to various characterization techniques and saccharification processes.

2.4.1. Composition analysis of raw and ChCl: AA pretreated rice straw

The holocellulose, cellulose and hemicellulose contents of raw RS and CApRS were determined by the TAPPI (TAPPI, 1992). The Lignin and TCC in raw RS and CApRS were estimated by following the NREL protocol and subsequent High-Performance Liquid Chromatography (Shimadzu Corporation, Japan) analysis. The HPLC column (Aminex HPX-87H column, Bio-Rad Laboratories, USA) was used with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min at 50 °C. Monosaccharides such as glucose, xylose and arabinose were detected by refractive index (RI) detector and quantified by using a standard plot. The biomass recovery, Eqn. 1, delignification percentage, Eqn. 2, hemicellulose removal percentage, Eqn. 3 (Nath et al. 2021) and the pretreatment efficiency, Eqn. 4 (Tran et al. 2020) of CApRS were calculated by using the following formulae:

$$\text{Biomass recovery } (\%) = \left(\frac{\text{weight of CApRS}}{\text{Initial weight of RS}}\right) \times 100 \quad (1)$$

Table 2
Predicted and experimental values of lignin and TCC in pretreated RS with respect to variables (ChCl: AA molar ratio, temperature and time) in RSM-CCD runs.

Run order	Variables			Predicted results (Responses)		Experimental results (Responses)	
	ChCl: AA molar ratio (A)	Temperature, °C (B)	Time,min (C)	Lignin content (%, w/w)	TCC (mg/g $_{CApRS}$)	Lignin content (%, w/w)	TCC (mg/g $_{CApRS}$)
1	1:2.41	84.19	60.4	8.78	542.5	8.81	531.6
2	1:3.59	84.19	60.4	7.98	542.2	8.09	551.0
3	1:2.41	125.81	60.4	8.01	584.4	7.89	595.6
4	1:3.59	125.81	60.4	5.19	647.2	5.09	648.4
5	1:2.41	84.19	149.6	8.40	532.1	8.44	542.3
6	1:3.59	84.19	149.6	9.81	519.9	9.87	520.3
7	1:2.41	125.81	149.6	5.77	637.1	5.60	639.8
8	1:3.59	125.81	149.6	5.17	688.1	5.09	710.6
9	1:2.00	105.00	105.0	7.33	548.6	7.44	545.9
10	1:4.00	105.00	105.0	6.14	590.7	6.12	576.7
11	1:3.00	70.00	105.0	10.05	535.4	9.87	535.9
12	1:3.00	140.00	105.0	5.50	712.1	5.75	685.4
13	1:3.00	105.00	30.0	7.74	544.9	7.76	544.4
14	1:3.00	105.00	180.0	7.39	570.4	7.45	554.7
15	1:3.00	105.00	105.0	6.85	533.0	7.03	532.8
16	1:3.00	105.00	105.0	6.85	533.0	6.81	541.6
17	1:3.00	105.00	105.0	6.85	533.0	6.77	567.1
18	1:3.00	105.00	105.0	6.85	533.0	6.44	523.8
19	1:3.00	105.00	105.0	6.85	533.0	7.09	514.7
20	1:3.00	105.00	105.0	6.85	533.0	6.97	520.9

$$\text{Delignification } (\%) = \left(1 - \frac{\text{Lignin in CApRS} \times \text{mass of CApRS}}{\text{Lignin in RS} \times \text{mass of RS}}\right) \times 100 \quad (1)$$

$$\text{Hemicellulose removal}\,(\%) = \left(1 - \frac{\text{Hemicellulose in CApRS} \times \text{mass of CApRS}}{\text{Hemicellulose in RS} \times \text{mass of RS}}\right) \times 100 \quad (3)$$

$$\text{Pretreatment efficiency } (\%) = \left(1 - \frac{\text{mass ratio of } \frac{\text{lignin}}{\text{cellulose}} \text{ of CApRS}}{\text{mass ratio of } \frac{\text{lignin}}{\text{cellulose}} \text{ of RS}}\right) \times 100 \quad (4)$$

2.4.2. Analysis of raw and ChCl: AA pretreated rice straw by XRD

The crystallinity index (*CrI*) of raw RS and CApRS was measured by X-Ray Diffractometer (D8 Advance, Bruker, Germany). Dried biomass samples were analyzed for crystallinity index by scanning over the range of $2\theta = 10°$-$60°$ with 0.05° step size. The *CrI* of both raw and pretreated samples was calculated by using Eqn. 5 (Segal et al. 1959).

$$\text{Crystallinity index } (\%) = \left(\frac{I_{crystalline} - I_{amorphous}}{I_{crystalline}}\right) \times 100 \quad (5)$$

Where, $I_{crystalline}$ is the intensity of the crystalline peak at $2\theta = 22°$ and $I_{amorphous}$ is the intensity of the amorphous peak at $2\theta = 18°$.

2.4.3. Surface area and pore size analyses of raw and ChCl:AA pretreated RS using BET

Brunauer–Emmet–Teller (BET) surface area and pore size of raw RS and CApRS were measured by N_2 adsorption/desorption measurements using Quantachrome (Model: Autosorb-IQ MP) performed at −196 °C. The dried biomass samples were degassed at 150 °C for 3 h using a vacuum prior to the measurement. About 0.05–0.07 g of samples were used for the analysis (Mohammadi et al. 2019). The BET surface area and pore volume were obtained from the N_2 adsorption/desorption data. The surface area indicates the amorphous nature of the sample.

2.4.4. Functional groups analysis of raw and ChCl: AA pretreated rice straw

The functional group distribution in the raw and CApRS was determined by Fourier Transform Infrared (FTIR) spectroscopy (Perkin Elmer, USA). The samples of dried raw RS and CApRS biomass were ground with KBr in 1:100 ratio and their pellets were made under a 15-ton hydraulic press. The pellets were subjected to scanning in the wavenumber range, 400–4000 cm^{-1}.

2.4.5. Surface morphology analysis of raw and ChCl: AA pretreated rice straw

Field Emission Scanning Electron Microscope (FESEM) (Zeiss, Gemini model) was used to study and compare the morphology of raw RS and CApRS fibers. The dried biomass samples were adhered to carbon tape and a double coating of gold film and observed the image at different magnifications at a beam voltage of 2 kV.

2.5. Enzymatic saccharification of ChCl: AA pretreated rice straw

The pretreatment efficiency of ChCl: AA method was evaluated based on the saccharification efficiency of the CApRS. The enzymatic saccharification of 2.5 %, w/v pretreated CApRS was performed with 35 U/g_{CApRS} commercial cellulase solution from *Trichoderma ressei* containing 0.005% sodium azide in 50 mm sodium citrate buffer, pH 4.8 in 10 ml working volume in a 50 ml sealed Erlenmeyer flask. The reaction was incubated at 50 °C and 150 rpm in a shaking incubator and aliquots (500 μL) were withdrawn at 24 h and 48 h. The aliquoted samples were then boiled to inactivate the enzyme, centrifuged and the amount of total reducing sugar (TRS) in the liquid hydrolysate was determined by using the methods reported by Nelson (1944) and Somogyi (1945). All the reactions were performed in a duplicate set and the saccharification process was conducted under sterile conditions. The enzymatic saccharification efficiency (Eqn. 6) was calculated by using the formula (Láinez et al. 2019).

$$\text{Saccharification efficiency}\,(\%) = \left(\frac{\text{TRS after enzymatic saccharification}}{\frac{1.111 \times C \times \text{Biomass}}{\text{Volume}}}\right) \times 100 \quad (6)$$

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

1.111 is the factor for conversion of cellulose to its equivalent glucose (Dowe and McMillan, 2001);

C is the cellulose content in the CApRS (g/g_{CApRS});

Biomass is the weight of dried CApRS used in enzymatic hydrolysis (g);

Volume is the working volume of the saccharification process in (L).

3. Results and discussion

3.1. Optimization of RS pretreatment using response surface methodology

Three independent variables i.e., ChCl:AA molar ratio (A), treatment temperature (B) and the pretreatment time (C) were considered as significant factors for optimization of the pretreatment process of RS by using the RSM-CCD approach (Table 1). Prior to statistical optimization, the pretreatment of RS was performed by one factor at a time (OFAT) to select the significant range of each factor. The effect of factors A, B and C on the delignification percentage in the pretreated biomass was studied by the OFAT approach (see supplementary material). Thereafter, the selected range of each factor was used to generate software-designed experimental conditions for the optimization process with lignin content and TCC in the pretreated RS as responses (Table 2).

3.1.1. Analysis of pretreatment model

The significance of each term (A, B, C) in the quadratic regression model was estimated based on the analysis of variance (ANOVA) technique. The p-values, F-values and lack of fit were used to verify the significance of each coefficient (Table 3). The lower p-value indicates a significant interaction between the factors. The higher F-value signifies a good prediction of the experimental data. A higher lack of fit of the model represents the true shape of the surface (Dutta et al. 2014). From Table 3, it is clear that the model is statistically significant with the highest F-value of 82.5, very low probability value ($p < 0.0001$) and the high sum of square of 35.4 for the response, lignin content. It was also observed that linear (A and B), interaction (AB, AC and BC) and quadratic (B^2 and C^2) are significant model terms for the response, lignin content (Table 3). Similarly, the TCC model was also significant as indicated by the F-value of 19.6, p-value < 0.0001 and the high sum of square of 61230.2. The effects of linear (A and B), interaction (AB and BC) and quadratic (A^2 and B^2) terms contribute more significantly. Thus, it was observed that the pretreatment temperature (B) and ChCl: AA molar ratio (A) significantly affect the RS pretreatment process. In this study, the actual vs predicted responses for both the responses, lignin and TCC content exhibited almost a linear relationship with a high value of "R-Square" of 0.99 and 0.95, respectively (Table 2). A similar linear relationship between the actual vs. predicted responses indicating a reasonable precision of the fitted empirical model was also reported earlier (Song et al. 2011). Therefore, it can be concluded that the developed model is acceptable in predicting the effectiveness of ChCl: AA pretreatment method for RS. The second-order equation in the terms of actual factors generated by CCD for the response, lignin content and TCC are as follows:

a) Lignin content (%, w/w) = +12.57 + 2.19 × ChCl:AA molar ratio − 0.048 × Temperature (°C) −0.039 × Time (min) − 0.041 × ChCl:AA molar ratio × Temperature (°C) + 0.021 × ChCl:AA molar ratio × Time (min) − 4.99 E^{-004} × Temperature (°C) × Time (min) −0.12 × ChCl:AA molar ratio ^{2} + 7.49 E^{-004} × Temperature (°C) ^{2} + 1.26 E^{-004} × Time (min) ^{2}

b) TCC (mg/g_{CApRS}) = + 1933.23–319.37 × ChCl:AA molar ratio-18.64 × Temperature (°C) −2.20 × Time (min) + 1.27 × ChCl:AA molar ratio × Temperature (°C) − 0.11 × ChCl:AA molar ratio × Time (min) + 0.017 × Temperature (°C) × Time (min) + 36.41 × ChCl:AA molar ratio^2 + 0.074 × Temperature (°C)^2 + 4.38 E^{-003} × Time (min)^2

Table 3
ANOVA table for a quadratic model of rice straw pretreatment.

Source	Lignin Sum of squares	F-value	p-value	TCC Sum of squares	F-value	p-value
Model	35.4	82.56	< 0.0001	61230.24	19.6	< 0.0001
A	1.7	35.64	0.0001	2186.08	6.3	0.0309
B	25.01	525.04	< 0.0001	37683.6	108.57	< 0.0001
C	0.14	3.04	0.1119	788.04	2.27	0.1628
AB	2.03	42.61	< 0.0001	1991.84	5.74	0.0376
AC	2.46	51.56	< 0.0001	69.43	0.2	0.6642
BC	1.71	35.98	0.0001	1998.24	5.76	0.0374
A^2	0.025	0.52	0.487	2387.47	6.88	0.0255
B^2	1.52	31.9	0.0002	14829.49	42.72	< 0.0001
C^2	0.91	19.06	0.0014	1094.56	3.15	0.1061
Lack of Fit	0.19	0.67	0.6618	1669.17	0.93	0.5324
R^2	0.9867			0.9464		
Adj R-Squared	0.9748			0.8981		
Pred R-Squared	0.9479			0.7537		

3.1.2. Effect of parametric interactions on the responses

The interactions among the three factors (ChCl: AA molar ratio, time and temperature) involved in the process are of critical importance for multivariate optimization. Fig. 1. displays three-dimensional response surface relationships among the factors for both the responses; lignin content and TCC. Fig. 1(a) and 1(d) represent the interaction between temperature and ChCl: AA molar ratio at the center level of the pretreatment time against the responses, lignin content and TCC, respectively. The lignin content in the pretreated biomass (Fig. 1a) decreased with the increment of the molar ratio of AA to ChCl due to the delignification caused by the higher acidic solvent with stronger pretreatment severity. Moreover, a concomitant increase in the TCC (Fig. 1d) and the delignification percentage in the pretreated biomass was observed. Zhang et al. 2016 showed a collateral trend in their work on facial pretreatment of lignocellulosic biomass using DES (Zhang et al. 2016). Fig. 1(b) and 1(e) represent the interaction between time and temperature at the center level of the ChCl: AA molar ratio against the responses, lignin content and TCC, respectively. It could be observed from the 3D plot (Fig. 1b and 1e) that on increasing the treatment temperature both the delignification percentage and the TCC in the pretreated biomass are significantly enhanced. Similar results on RS pretreatment using DES with different constituent molar ratios were reported (Li et al. 2018). Fig. 1(c) and 1(f) display 3D response surface interactions between time and ChCl: AA molar ratio at the center level of pretreatment temperature. A higher ChCl: AA molar ratio or prolonged pretreatment time leads to a higher degree of lignin removal. Notably, at a fixed temperature, there was not any significant change in lignin content or TCC in the pretreated biomass (Fig. 1c and 1f). A possible explanation is that a higher temperature could weaken the hydrogen bonding thereby facilitating the mass transfer and biomass dissolution with the breakage of the ether and ester bonds between lignin and hemicellulosic fractions as also reported earlier (D'Agostino et al., 2011).

3.1.3. Optimization and validation of the pretreatment process

The main purpose of pretreatment optimization is to attain minimum lignin content and maximum TCC in the pretreated biomass through the optimized values of significant variables. The numerical optimization for selecting the best combinations for each factor (in range) and responses, lignin (minimized) and TCC (maximized) was performed. The optimized conditions predicted by the software were ChCl:AA molar

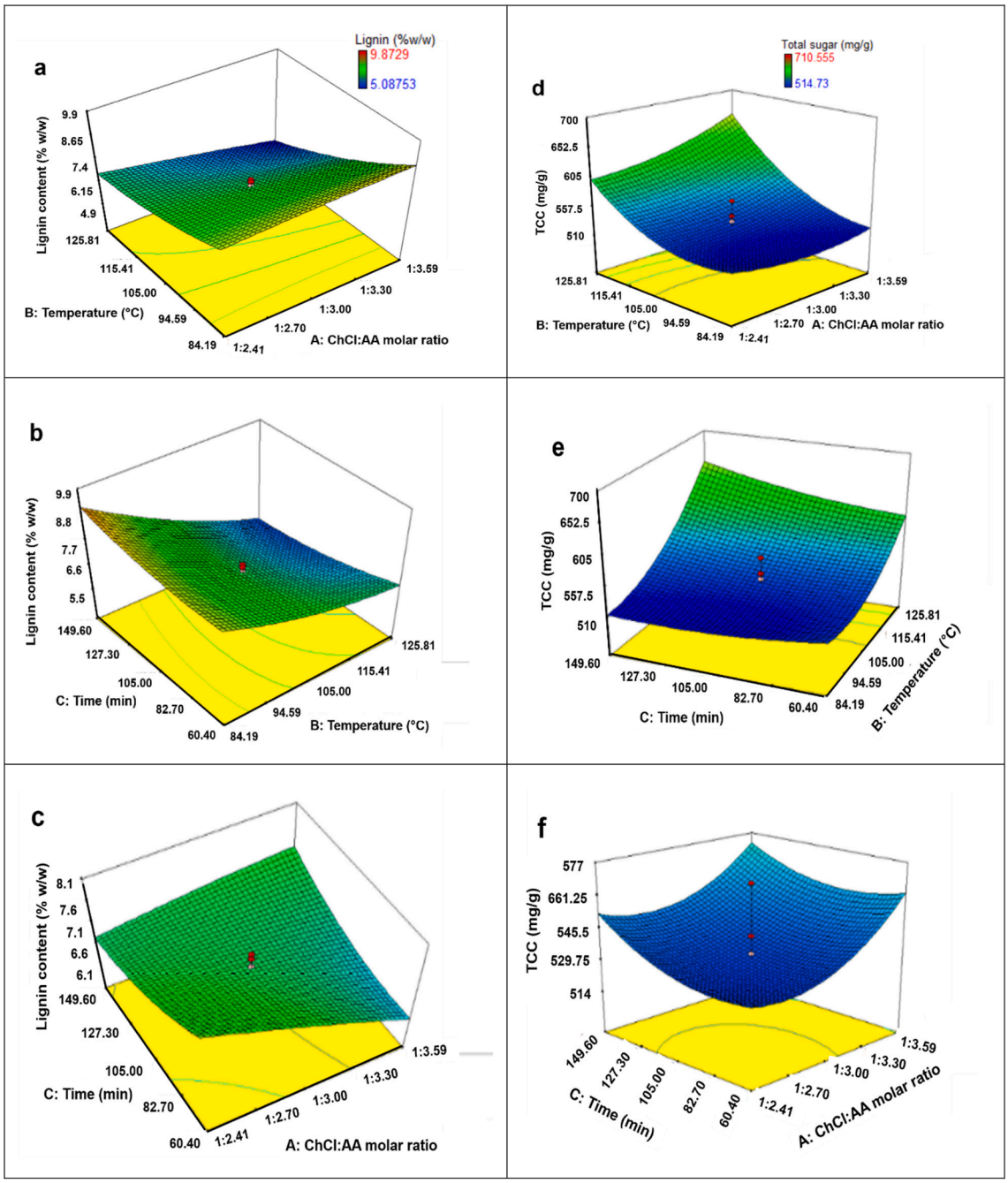

Fig. 1. 3-D surface plots (interaction among independent variables) for RSM-based pretreatment optimization of RS using ChCl: AA in terms of lignin content (%, w/w) (1a, 1b and 1c) and TCC, mg/g_{CApRS} (1d, 1e and 1f). a) temperature vs ChCl: AA molar ratio, b) time vs temperature, c) time vs ChCl: AA molar ratio, d) temperature vs ChCl: AA molar ratio, e) time vs temperature, f) time vs ChCl: AA molar ratio.

ratio of 1:3.59, the temperature of 126 °C and 149.59 min of pretreatment time. The model was validated by performing the ChCl-AA pretreatment of RS for 50 g DES (ChCl:AA) at 7.5% raw biomass loading at the above-mentioned optimized conditions. The experimental responses i.e., lignin content and TCC in the pretreated biomass obtained were 5.09 (% w/w) and 679 mg/g_{CApRS}, respectively, which are close to the predicted values (5.16, % w/w lignin and 688 g/g_{CApRS} TCC, respectively).

3.2. Composition analyses of raw and ChCl: AA pretreated rice straw

The raw RS contained 73.9 ± 1.5% holocellulose (w/w), 43.5 ± 1.1% (w/w) cellulose, 30.3 ± 1.2% (w/w) hemicellulose and 18.5 ± 0.5% (w/w) lignin. The raw RS biomass on pretreatment with ChCl: AA under optimized conditions resulted in increased content of holocellulose, 82.6 ± 0.6% (w/w) and cellulose, 73.5 ± 0.7% (w/w) with a significant reduction in hemicellulose, 9.1 ± 0.5% (w/w) and lignin, 5.09 ± 0.2% (w/w) content. The probable reason for the significant reduction in hemicellulose and lignin content could be due to the breakage of ether and ester bonds between hemicellulose and lignin molecules as also reported earlier (Xu et al. 2021). Moreover, this could also be due to the potential of acetic acid to solubilize the hemicellulosic fraction as mentioned earlier (Zhao and Liu, 2010). CApRS resulted in biomass recovery (61.4%), hemicellulose removal, 82.1% and

delignification percentage, 83.1% (w/w) (Table 4). In addition to the high delignification percentage, a significant pretreatment efficiency of 83.7% (Equation 4) was achieved using the ChCl: AA pretreatment method which is essential for effective enzymatic hydrolysis of pretreated biomass to monosaccharides. A detailed comparison of RS delignification in this study with other reports earlier was analyzed (see supplementary material). RS pretreatment using ChCl: lactic acid in molar ratio 1:3 at 120 °C for 3 h reported a higher delignification percentage, 82.5% (Huang et al. 2020). However, RS pretreated in a molar ratio of 1:5 (ChCl: lactic acid) at 60 °C for 12 h resulted in lower, 60% delignification (Kumar et al. 2016). A study on Bambara groundnut haulm biomass pretreated with ChCl: AA in molar ratio 1:2 at 100 °C for 3 h reported delignification of 58% (Okuofu et al. 2020). Xing et al. 2018 reported a delignification of 44% on RS using ChCl: formic acid: acetic acid at molar ratio 1:1:1 at 120 °C for 8 h (Xing et al. 2018). Thus, when compared with RS pretreatment with other DES systems or ChCl:AA pretreatment method on other biomass, the current study gave a higher delignification percentage.

3.3. XRD analysis of raw and ChCl: AA pretreated rice straw

The cellulose crystallinity and crystallinity index of the lignocellulosic biomass play an important role in susceptibility to enzymatic hydrolysis (Le Tan et al. 2021). The impact of pretreatment processes on the crystalline structure of cellulose would affect the hydrolysis of cellulose in the enzymatic saccharification process (Procentese et al. 2015, Rocha et al. 2020). To explore the effect of the ChCl: AA pretreatment method on the structure and enzymatic digestibility of pretreated biomass, raw RS and CApRS samples were subjected to X-ray diffraction analysis. The raw RS and CApRS powder displayed 34.7% and 45.4% crystallinity index (*CrI*), respectively. The *CrI* of CApRS was 31% higher than the raw RS. A study on RS pretreatment with ChCl: AA + formic acid in a fixed molar ratio of 1:2:1 reported a 16% increment in *CrI* as compared with the raw RS (Xing et al. 2018). Thus, this study suggested the effective removal of lignin and hemicellulosic fractions from RS leading to the exposure of the cellulosic fraction in the pretreated solid residue.

3.4. BET of raw and ChCl: AA pretreated rice straw

The specific surface area of the sample does not only determines the nature of the interaction between the biomass and the hydrolytic enzymes but also indicates the amorphous nature of the sample. Raw RS and CApRS samples were subjected to BET-specific surface and pore size analysis. The CApRS showed a surface area of 17.56 m^2/g which was 17 fold higher than that of raw RS. The raw RS showed a total pore volume of $1.07x10^{-2}$ cc/g, whereas CApRS resulted in a higher total pore volume of $5.42x10^{-2}$ cc/g. The specific surface area obtained by ChCl: AA pretreatment in this study is higher than pretreatment of RS with other methods like integrated pretreatment of acid and steam explosion, 6.63 m^2/g (Chen et al., 2011) and ionic liquid pretreatment, 9.36 m^2/g (Mohammadi et al. 2019). The increase in the surface area is due to the delignification and removal of hemicellulosic fraction from the RS during the pretreatment process which ultimately leads to efficient enzymatic saccharification as also reported earlier (Wi et al. 2013, Shen et al. 2019).

3.5. Functional groups analysis of raw and ChCl: AA pretreated rice straw

The FT-IR spectroscopic analysis of raw RS and CApRS was carried out for functional group analysis. A peak at wavenumber 1640 cm^{-1} signifies lignin and its aromatic ring (Xu et al. 2013). The wavenumber range, 1328 cm^{-1}-1433 cm^{-1} represents OH vibrations due to intermolecular and intramolecular hydrogen bonds in cellulose (Mohammadi et al. 2019). The CApRS spectra show a significant reduction in the peak intensity of wavenumber 1640 cm^{-1}, indicating efficient lignin removal as deciphered earlier (Liu et al. 2018). A prominent reduction in peak intensity between 1328 cm^{-1} and 1433 cm^{-1} indicates the loosening of cellulose structure and reduction of cellulose crystallinity in CApRS biomass, thereby increasing the surface area of the pretreated biomass as reported earlier (Jamaldheen et al. 2018). A higher CH_2 peak stretching from wavenumber 2857 cm^{-1} to 2911 cm^{-1} in CApRS sample specifies the exposure of cellulosic fiber as reported earlier (Jamaldheen et al. 2018). Therefore, the spectral analysis of CApRS sample clearly showed the effectiveness of the pretreatment process.

3.6. Surface morphology analysis of raw and ChCl: AA pretreated rice straw by FESEM

The surface topology analysis of raw RS and CApRS samples by FESEM was performed. The rigid, rough and non-porous surface of raw RS depicted the intact structural integrity of cellulose, hemicelluloses and lignin. CApRS showed a surface with visible pores or voids and fibrous structures, unlike the raw RS. The ChCl: AA pretreatment resulted in alteration of biomass morphology from organized structure to smooth fibrous structures with cellulosic fibers exposure, increased surface area and pore volume that is in line with the findings of earlier section 3.4, 3.5 and also other researchers (Mohammadi et al. 2019, Nath et al. 2021).

3.7. Enzymatic saccharification of raw and ChCl: AA pretreated rice straw

Maximum delignification of RS during the pretreatment process is essential for the conversion of cellulose to glucose and its subsequent application. To check the efficacy of the pretreatment process, the enzymatic saccharification of raw RS and CApRS was performed by using the commercial cellulase from *T. ressei*, at a loading of 35 U/$g_{CApRS/raw\ RS}$ with 2.5% (w/v) CApRS/ raw RS loading in 10 ml working volume at 50 °C for 48 h. The raw RS and CApRS resulted in TRS of 5.1 g/l and 18.8 g/l, respectively after 48 h of saccharification. The ChCl: AA pretreatment remarkably improved the biomass digestibility in the subsequent enzymatic hydrolysis by giving a saccharification efficiency of 92.2%. A detailed comparison of saccharification efficiency of RS and other biomass pretreated by the DES method is illustrated (see supplementary material).

3.8. Mass balance

The mass balance of the bioconversion process of 100 g raw RS to soluble monosaccharides under optimized conditions of pretreatment followed by its subsequent saccharification was analyzed and shown in Fig. 2. Upon optimized DES pretreatment of 100 g raw RS, 61.4 g of

Table 4
Composition analysis of raw and DES (ChCl: AA) pretreated rice straw.

Biomass	Biomass Recovery, % w/w	Holo-cellulose, % w/w	Cellulose, % w/w	Hemicellulose, % w/w	Lignin, % w/w	Hemicellulose Removal %, w/w	Delignification % w/w	Crystallinity index (*CrI*) %
Raw RS	100	73.9 ± 1.5	43.5 ± 1.1	30.3 ± 1.2	18.5 ± 0.5	–	–	34.7%
CApRS	61.4 ± 0.9	82.6 ± 0.8	73.5 ± 0.7	9.1 ± 0.5	5.09 ± 0.2	82.1	83.1	45.4%

± Standard deviation (n = 2). Hemicellulose removal and delignification was calculated per 100 g of raw RS.

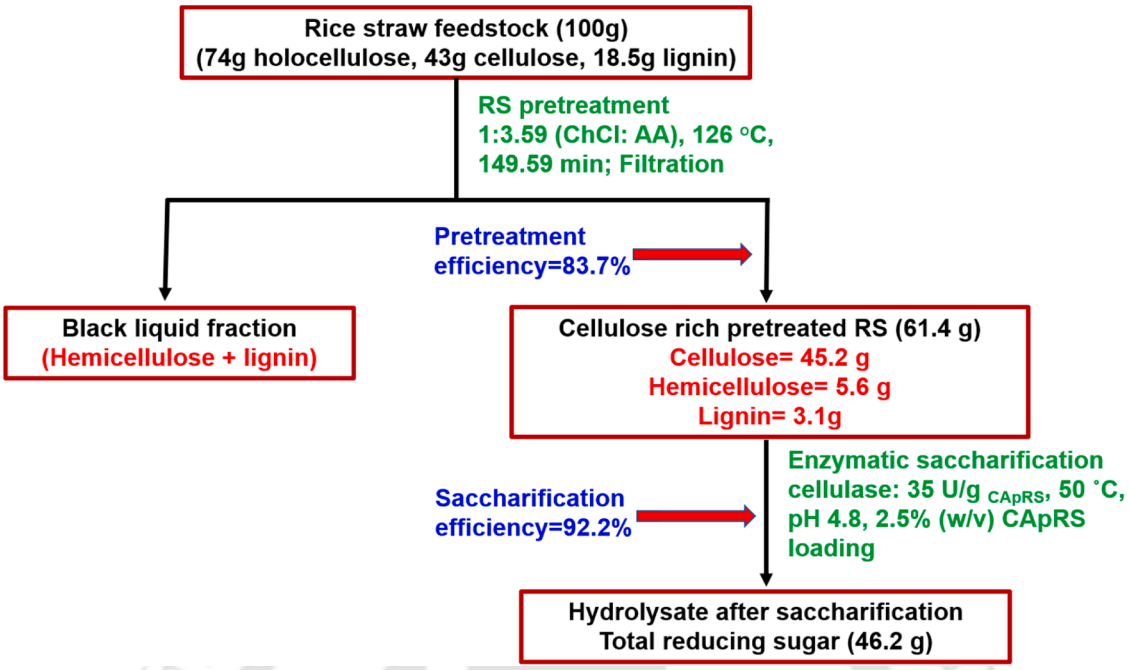

Fig. 2. Mass balance for bioconversion of raw RS to total reducing sugar, the feedstock for bioethanol production.

CApRS was recovered and its holocellulose content was 73.9% (w/w). This implies that 61.4 g of CApRS contained 50.7 g hollocellulose, of which 42.5 g was cellulose, 5.6 g was hemicellulose and 3.1 g was lignin thereby resulting in a pretreatment efficiency of 83.7%. The enzymatic saccharification of 61.4 g CApRS biomass by using commercial cellulase under the condition described in section 2.5 gave a TRS amount of 46.2 g in the hydrolysate accounting for the saccharification efficiency of 92.2%. Thus, 100 g raw RS after pretreatment with ChCl: AA method under the statistically optimized conditions and subsequent enzymatic saccharification yield a total reducing sugar, 46 g.

4. Conclusions

This study highlights the DES pretreatment method of RS using ChCl and acetic acid. Three significant pretreatment parameters; ChCl: AA molar ratio, time and temperature were scrutinized using statistical analysis. The result inferred that ChCl: AA molar ratio and temperature are significant variables for a delignification with concomitant retainment of maximum TCC in the pretreated RS. ChCl: AA pretreatment under optimized conditions resulted in a cellulose-rich pretreated RS and subsequent saccharification yielded a high concentration of total reducing sugar in the hydrolysate. The high saccharification efficiency achieved in this study denotes the efficacy of the developed pretreatment method.

CRediT authorship contribution statement

Premeshworii Devi Maibam: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Arun Goyal:** Supervision, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127057.

References:

Baruah, J., Nath, B.K., Sharma, R., Kumar, S., Deka, R.C., Baruah, D.C., Kalita, E., 2018. Recent trends in the pretreatment of lignocellulosic biomass for value-added products. Frontiers in Energy Research 6, 141.

Calvo-Flores, F.G., Monteagudo-Arrebola, M.J., Dobado, J.A., Isac-García, J., 2018. Green and bio-based solvents. Topics in Current Chemistry 376 (3), 1–40.

Chen, W.H., Pen, B.L., Yu, C.T., Hwang, W.S., 2011. Pretreatment efficiency and structural characterization of rice straw by an integrated process of dilute-acid and steam explosion for bioethanol production. Bioresource Technology 102 (3), 2916–2924.

Chen, Y., Mu, T., 2019. Application of deep eutectic solvents in biomass pretreatment and conversion. Green Energy & Environment 4 (2), 95–115.

D'Agostino, C., Harris, R.C., Abbott, A.P., Gladden, L.F., Mantle, M.D., 2011. Molecular motion and ion diffusion in choline chloride based deep eutectic solvents studied by 1 H pulsed field gradient NMR spectroscopy. Physical Chemistry Chemical Physics 13 (48), 21383–21391.

Di Marino, D., Stöckmann, D., Kriescher, S., Stiefel, S., Wessling, M., 2016. Electrochemical depolymerisation of lignin in a deep eutectic solvent. Green Chemistry 18 (22), 6021–6028.

Dowe, N., McMillan, J., 2001. SSF experimental protocols: lignocellulosic biomass hydrolysis and fermentation. National Renewable Energy Laboratory (NREL) Analytical Procedures 510–42630.

Dutta, S.K., Halder, G., Mandal, M.K., 2014. Modeling and optimization of bi-directional delignification of rice straw for production of bio-fuel feedstock using central composite design approach. Energy 71, 579–587.

Hong, S., Shen, X.J., Pang, B., Xue, Z., Cao, X.F., Wen, J.L., Sun, Z.H., Lam, S.S., Yuan, T. Q., Sun, R.C., 2020. In-depth interpretation of the structural changes of lignin and formation of diketones during acidic deep eutectic solvent pretreatment. Green Chemistry 22 (6), 1851–1858.
Hou, X.D., Feng, G.J., Ye, M., Huang, C.M., Zhang, Y., 2017. Significantly enhanced enzymatic hydrolysis of rice straw via a high-performance two-stage deep eutectic solvents synergistic pretreatment. Bioresource Technology 238, 139–146.
Huang, Z.J., Feng, G.J., Lin, K.P., Pu, F.L., Tan, Y.M., Tu, W.C., Han, Y.L., Hou, X.D., Zhang, H.M., Zhang, Y., 2020. Significant boost in xylose yield and enhanced economic value with one-pot process using deep eutectic solvent for the pretreatment and saccharification of rice straw. Industrial Crops and Products 152, 112515.
Jamaldheen, S.B., Sharma, K., Rani, A., Moholkar, V.S., Goyal, A., 2018. Comparative analysis of pretreatment methods on sorghum (Sorghum durra) stalk agrowaste for holocellulose content. Preparative Biochemistry and Biotechnology 48 (6), 457–464.
Kumar, A.K., Parikh, B.S., Pravakar, M., 2016. Natural deep eutectic solvent mediated pretreatment of rice straw: bioanalytical characterization of lignin extract and enzymatic hydrolysis of pretreated biomass residue. Environmental Science and Pollution Research 23 (10), 9265–9275.
Láinez, M., Ruiz, H.A., Arellano-Plaza, M., Martínez-Hernández, S., 2019. Bioethanol production from enzymatic hydrolysates of Agave salmiana leaves comparing S. cerevisiae and K. marxianus. Renewable Energy 138, 1127–1133.
Le Tan, N.T., Dam, Q.P., Mai, T.P., Nguyen, D.Q., 2021. The Combination of Acidic and Alkaline Pretreatment for a Lignocellulose Material in Simultaneous Saccharification and Fermentation (SSF) Process. Chemical Engineering Transactions 89, 43–48.
Li, A.L., Hou, X.D., Lin, K.P., Zhang, X., Fu, M.H., 2018. Rice straw pretreatment using deep eutectic solvents with different constituents molar ratios: Biomass fractionation, polysaccharides enzymatic digestion and solvent reuse. Journal of Bioscience and Bioengineering 126 (3), 346–354.
Liu, D., Yan, X., Zhuo, S., Si, M., Liu, M., Wang, S., Ren, L., Chai, L., Shi, Y., 2018. Pandoraea sp. B-6 assists the deep eutectic solvent pretreatment of rice straw via promoting lignin depolymerization. Bioresource Technology 257, 62–68.
Loow, Y.L., Wu, T.Y., Yang, G.H., Ang, L.Y., New, E.K., Siow, L.F., Jahim, J.M., Mohammad, A.W., Teoh, W.H., 2018. Deep eutectic solvent and inorganic salt pretreatment of lignocellulosic biomass for improving xylose recovery. Bioresource Technology 249, 818–825.
Maibam, P.D., Maiti, S.K., 2020. A strategy for simultaneous xylose utilization and enhancement of cellulase enzyme production by Trichoderma reesei cultivated on liquid hydrolysate followed by induction with feeding of solid sugarcane bagasse. Waste and Biomass Valorization 11 (7), 3151–3160.
Mohammadi, M., Shafiei, M., Abdolmaleki, A., Karimi, K., Mikkola, J.P., Larsson, C., 2019. A morpholinium ionic liquid for rice straw pretreatment to enhance ethanol production. Industrial Crops and Products 139, 111494.
Nath, P., Maibam, P.D., Singh, S., Rajulapati, V., Goyal, A., 2021. Sequential pretreatment of sugarcane bagasse by alkali and organosolv for improved delignification and cellulose saccharification by chimera and cellobiohydrolase for bioethanol production. 3. Biotech 11 (2), 1–16.
Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry 153 (2), 375–380.
Okuofu, S.I., Gerrano, A.S., Singh, S., Pillai, S., 2020. Deep eutectic solvent pretreatment of Bambara groundnut haulm for enhanced saccharification and bioethanol production. Biomass Conversion and Biorefinery 1–9.
Procentese, A., Johnson, E., Orr, V., Campanile, A.G., Wood, J.A., Marzocchella, A., Rehmann, L., 2015. Deep eutectic solvent pretreatment and subsequent saccharification of corncob. Bioresource Technology 192, 31–36.
Rocha, G.J.M., Andrade, L.P., Martin, C., Araujo, G.T., Mouchrek Filho, V.E., Curvelo, A. A.S., 2020. Simultaneous obtaining of oxidized lignin and cellulosic pulp from steam-exploded sugarcane bagasse. Industrial Crops and Products 147, 112227.
Segal, L.G.J.M.A., Creely, J.J., Martin Jr, A.E., Conrad, C.M., 1959. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. Textile Research Journal 29 (10), 786–794.
Shen, Z., Hou, D., Jin, F., Shi, J., Fan, X., Tsang, D.C., Alessi, D.S., 2019. Effect of production temperature on lead removal mechanisms by rice straw biochars. Science of the Total Environment 655, 751–758.
Singh, R., Srivastava, M., Shukla, A., 2016. Environmental sustainability of bioethanol production from rice straw in India: a review. Renewable and Sustainable Energy Reviews 54, 202–216.
Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D.L.A.P., 2010. Determination of structural carbohydrates and lignin in biomass. Laboratory Analytical Procedure.
Somogyi, M., 1945. A new reagent for the determination of sugars. Journal of Biological Chemistry 160 (1), 61–68.
Song, Y., Du, B., Zhou, T., Han, B., Yu, F., Yang, R., Hu, X., Ni, Y., Li, Q., 2011. Optimization of extraction process by response surface methodology and preliminary structural analysis of polysaccharides from defatted peanut (Arachis hypogaea) cakes. Carbohydrate Research 346 (2), 305–310.
TAPPI. Technical Association of Pulp and Paper Industry, Atlanta, GA, 1992.
Tran, A.T., Cao, N.H., Le, P.T.K., Mai, P.T., Nguyen, Q.D., 2020. Reusing alkaline solution in lignocellulose pretreatment to save consumable chemicals without losing efficiency. Chemical Engineering Transactions 78, 307–312.
Van Hung, N., Maguyon-Detras, M.C., Migo, M.V., Quilloy, R., Balingbing, C., Chivenge, P., Gummert, M., 2020. Rice straw overview: availability, properties, and management practices. Sustainable Rice Straw Management 1.
Wang, W., Lee, D.J., 2021. Lignocellulosic biomass pretreatment by deep eutectic solvents on lignin extraction and saccharification enhancement: A review. Bioresource Technology 339, 125587.
Wi, S.G., Choi, I.S., Kim, K.H., Kim, H.M., Bae, H.J., 2013. Bioethanol production from rice straw by popping pretreatment. Biotechnology for Biofuels 6 (1), 1–7.
Xing, W., Xu, G., Dong, J., Han, R., Ni, Y., 2018. Novel dihydrogen-bonding deep eutectic solvents: Pretreatment of rice straw for butanol fermentation featuring enzyme recycling and high solvent yield. Chemical Engineering Journal 333, 712–720.
Xu, F., Yu, J., Tesso, T., Dowell, F., Wang, D., 2013. Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: a mini-review. Applied Energy 104, 801–809.
Xu, H., Kong, Y., Peng, J., Song, X., Liu, Y., Su, Z., Li, B., Gao, C., Tian, W., 2021. Comprehensive analysis of important parameters of choline chloride-based deep eutectic solvent pretreatment of lignocellulosic biomass. Bioresource Technology 319, 124209.
Xu, H., Peng, J., Kong, Y., Liu, Y., Su, Z., Li, B., Song, X., Liu, S., Tian, W., 2020. Key process parameters for deep eutectic solvents pretreatment of lignocellulosic biomass materials: A review. Bioresource Technology 310, 123416.
Zhang, C.W., Xia, S.Q., Ma, P.S., 2016. Facile pretreatment of lignocellulosic biomass using deep eutectic solvents. Bioresource Technology 219, 1–5.
Zhao, X., Liu, D., 2010. Chemical and thermal characteristics of lignins isolated from Siam weed stem by acetic acid and formic acid delignification. Industrial Crops and Products 32 (3), 284–291.

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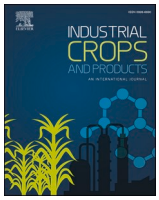

Designing of recombinant hydrolytic enzymes cocktail for effective saccharification of delignified rice straw

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ABSTRACT

Enzymatic saccharification is the most crucial and challenging step in lignocellulosic biorefineries for bioethanol production. The enzymatic saccharification requires a cocktail of hydrolytic enzymes, cellulases and hemicellulases for effective degradation of biomass. However, the complete bioconversion of biomass is governed by the proportion and synergistic action of hydrolytic enzymes present in the cocktail. Thus, this study aims to formulate an enzyme cocktail consisting of recombinant cellulases (bifunctional cellulolytic chimeric enzyme, *Ct*GH1-L1-*Ct*GH5-F194A and cellobiohydrolase, *Ct*CBH5A) and xylanases (endo-1,4-β-xylanase, *Ct*GH11A, and β-xylosidase, *Bo*GH43), all in crude form (unpurified enzyme present in the total cellular proteins) for producing high yield of total reducing sugars using choline chloride:acetic acid pretreated rice-straw (CApRS). D-optimal mixture design approach was used for statistically designing the optimal proportion of crude recombinant enzyme cocktail for hydrolysis of delignified rice straw. The developed enzyme cocktail comprising chimera, *Ct*CBH5A, *Ct*GH11 and *Bo*GH43 in ratio 35.1:41.8:10.0:13.1 resulted in cellulolytic enzyme activity, 5.6 FPU/mL and xylanase activity, 102 U/mL. Enzymatic saccharification of 3% (w/v) delignified CApRS using developed enzyme cocktail at 187 FPU/g_{CApRS}, pH 5.8 and 35 °C, yielded total reducing sugar of 498 mg/$g_{biomass}$, glucose yield (410 mg/g) and xylose yield (71.3 mg/g) resulting in 72% saccharification efficiency. Whereas, significantly low total reducing sugar yield of 80 mg/g and 218 mg/g were released from raw RS and partially pretreated RS, respectively, by the developed cocktail under the same hydrolytic conditions, thereby inferring the effectiveness of the enzyme cocktail against CApRS biomass. The developed enzyme cocktail was compared with a commercial enzyme cocktail and prospects its applicability in lignocellulosic biorefineries.

1. Introduction

The energy crisis and environmental pollution are the two main challenges mankind has encountered in this century. The rapid rise in greenhouse gases discharged from the combustion of fossil fuels has persuaded to search for a substitute renewable energy source to resolve the evolved energy and environmental problems (Abdmouleh et al., 2015). The lignocellulosic biomass which is abundant and has low food and fodder value serves to be a potentially renewable resource for bioethanol production (Zhang et al., 2023a). In lignocellulosic biorefinery, three key processes are: i) biomass pretreatment ii) enzymatic conversion of complex polymer to reduced sugars and iii) fermentation of reduced sugar to bioethanol (Rajak et al., 2018, Guo et al., 2018). However, the challenging step in biorefineries is the conversion efficiency of the cellulosic and hemicellulosic fractions of lignocellulosic biomass to C6 and C5 sugars, respectively (Guo et al., 2018). Lignocellulosic biomass constitutes approximately, 45%– 75% holocellulose that can be hydrolyzed into fermentable sugars (Maibam and Goyal, 2022a). However, for the complete degradation of the cellulosic and hemicellulosic fraction into monosaccharides, enzymatic hydrolysis is necessary (Thakur et al., 2020b). Therefore, the enzymatic saccharification process requires the repertoire of cellulases and hemicellulases to hydrolyze both fractions and reduce to fermentable monosaccharides (Saritha et al., 2013).

Bioethanol industries and recent research showed the application of commercial enzymes such as Celluclast 1.5 L and Cellic Ctec2 from Novozymes (Franklinton, USA); Multifect xylanase from Genencor International Inc. (NY, USA) for the hydrolysis of lignocellulosic biomass

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and could achieve ~60–80% saccharification efficiency (Samayam et al., 2010, Lan et al., 2013, Méndez-Líter et al., 2021, Zhang et al., 2023b). However, the drawback of using commercial enzymes is the high production cost and the limited selection of enzymes. Cellulases and xylanases are also produced by wild-type or genetically-modified microbial strains (Wang et al., 2021). *Trichoderma ressei* and some strains from the fungal genera *Penicillium* and *Talaromyces* are the predominant producers of cellulase and xylanase for biomass hydrolysis (Méndez-Líter et al., 2021; Maibam and Maiti, 2020). The drawback of using these in-house produced fungal enzymes is the inefficient saccharification efficiency (Maibam and Maiti, 2020). Recently, research has been focused on the use of recombinant enzymes with enhanced activity to improve hydrolytic efficiencies (Zhang et al., 2021; Velasco et al., 2021; Huang et al., 2022). The recombinant hydrolytic enzymes such as endoglucanase (Nath et al., 2019), exoglucanase (Rajak et al., 2020), β-glucosidase (Nath et al., 2019), endoxylanase and β-xylosidase (Huy et al., 2020; Thakur et al., 2020a) exhibiting high activity have been expressed and produced by using *E. coli*. However, due to the sensitivity of each hydrolytic enzyme to pH and temperature, there have been several trials to model the enzyme cocktails and optimize their operating conditions for an efficient saccharification process.

In order to address these challenges of the saccharification process, this study aims to design a crude (unpurified) enzyme cocktail comprising recombinant cellulases and xylanases to enhance the total reducing sugar yield as compared with the earlier reports. Extensive work on enzymatic saccharification of various substrates by using in-house purified enzymes (recombinant, wild-type, or mutant variants) and commercial enzymes has been reported. However, only a few studies have investigated the potential of crude recombinant hydrolytic enzymes, which should offer an economic advantage in the overall process. Therefore, in the present study, the crude form (unpurified) of four efficient recombinant hydrolytic enzymes; i) cellulolytic chimeric enzyme (*Ct*GH1-L1-*Ct*GH5-F194A) with a bi-functional activity of β-1,4-endoglucanase and β-1,4-glucosidase, ii) cellobiohydrolase (*Ct*CBH5A), iii) endo-1,4-β-xylanase (*Ct*Xyn11A) and iv) exo-1,4-β-xylosidase (*Bo*GH43A) are used for statistically designing an enzyme cocktail with optimal proportion of each constituting enzyme for the saccharification of choline chloride: acetic acid pretreated rice straw (CApRS) biomass. The effectiveness of the developed enzyme cocktail was compared with the commercial enzymes, cellulase (source: *Trichoderma ressei*) and xylanase (source: *Aspergillus niger*) based on the saccharification efficiency.

2. Materials and methods

2.1. Chemicals and reagents

The analytical reagent grade chemicals, including choline chloride, acetic acid, Luria Bertani, isopropyl -D-1-thiogalactopyranoside (IPTG), kanamycin, ampicillin and sodium azide were obtained from Himedia Pvt. Ltd., India. Substrates such as glucose, xylose, cellobiose, carboxymethyl cellulose sodium salt (CMC-Na), birchwood xylan, bovine serum albumin and commercial cellulase, enzyme blend (Cat. No. SAE 0020, synonymous to industrially used Cellic CTec2) from *Trichoderma reesei* were from Sigma-Aldrich Co. LLC, USA. The commercial xylanase from *Aspergillus niger* used in the study was purchased from Sisco Research Laboratories Pvt. Ltd., India.

2.2. Feedstock for enzymatic hydrolysis

The raw rice straw (RS) containing 43.5% (w/w) cellulose, 30.3% (w/w) hemicellulose and 18.5% (w/w) lignin was pretreated with choline chloride (ChCl) and acetic acid (AA) based deep eutectic solvent under previously optimized condition of ChCl: AA in the molar ratio 1:3.59 at 126 °C for 150 min as reported earlier (Maibam and Goyal, 2022b). The structure and composition analysis of choline chloride-acetic acid pretreated RS (CApRS) showed cellulose 73.5% (w/w), hemicellulose 9.1% (w/w), lignin 5.1% (w/w). The CApRS showed 83% delignification and enriched total carbohydrate content of 688 mg/g_{CApRS} as reported earlier in our previous study (Maibam and Goyal, 2022b). The pretreated biomass, CApRS was further used as a substrate for designing enzyme cocktails for the saccharification process.

2.3. Production and enzyme activity estimation of recombinant hydrolytic enzymes

Recombinant cellulases (cellobiohydrolase, *Ct*CBH5A and chimera, *Ct*GH1-L1-*Ct*GH5-F194A) and xylanases (endo-1,4-β-xylanase, *Ct*GH11A and β-Xylosidase, *Bo*GH43) were used for the enzymatic saccharification of CApRS biomass. The chimera (*Ct*GH1-L1-*Ct*GH5-F194A) cloned and expressed earlier was used (Nath et al., 2019). Cellobiohydrolase (*Ct*CBH5A) and endo-1,4-β-xylanase (*Ct*Xyn11A) cloned from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) cloned from *Bacteroides ovatus* were gift from NZYTech Pvt. Ltd., Portugal. Around 1% (v/v) of *E. coli* BL21 (DE3) cells harboring pET28a(+) vector containing gene encoding chimera (*Ct*GH1-L1-CtGH5-F194A), *Ct*GH11A or *Bo*GH43 were inoculated in 800 mL Luria-Bertani medium supplemented with kanamycin (50 μg/mL). The only difference in *Ct*GH11 is the supplementation with ampicillin (100 μg/mL) as its gene was cloned using the pET21a(+) vector. The culture was incubated at 37 °C, 180 rpm for 4 h, then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and incubated for 16 h at 24 °C, 180 rpm. The culture was centrifuged at 6000 g for 10 min at 4 °C. The supernatant was discarded and the cell pellet containing the intracellular enzyme was re-suspended in 10 mL of 0.05 M sodium phosphate buffer (pH 5.8). This was subjected to ultra-sonication for 15 min (5 s on/10 s off pulse at 33% amplitude, Sonics, Vibra cell). The cell lysate obtained was centrifuged at 13,000xg at 4 °C for 1 h. The cell debris was discarded and the supernatant (~10 mL) containing crude (unpurified enzyme present in the total cellular proteins) recombinant enzyme was analyzed for protein content and enzyme activity for further use. The enzyme activity of all the four enzymes in crude form was determined by the quantification of reducing sugars using the methods of Nelson (1944) and Somogyi (1945) under optimized conditions as reported earlier (Nath et al., 2019; Jamaldheen, and Nedumaran et al., 2019, 2020) and β-glucosidase activity of chimera was estimated by GOD-POD method as reported earlier (Nath et al., 2019). The enzymatic reaction for the endoglucanase activity of chimera was conducted for 4 min at 60 °C with a substrate concentration of 1%, w/v carboxymethyl cellulose sodium salt (CMC-Na). For the β-glucosidase activity of chimera, the enzymatic reaction was conducted at 70 °C for 5 min at 1%, w/v cellobiose (Nath et al., 2019). The enzymatic reaction for the cellobiohydrolase activity of *Ct*CBH5A was conducted for 5 min at 65 °C with a substrate concentration of 1%, w/v carboxymethyl cellulose sodium salt (CMC-Na). The enzymatic reactions for endoxylanase activity of *Ct*GH11 and β-xylosidase activity of *Bo*GH43 were conducted at 65 °C for 1 min and 37 °C for 5 min, respectively, at 1% (w/v) birchwood xylan. The enzyme activity of all the four enzymes was conducted using a common buffer, 0.05 M sodium phosphate buffer (pH 5.8). The enzyme activity of the crude enzymes is shown in (Table 1). The total protein concentration of individual crude enzymes was determined by using Folin-Lowry's method with bovine serum albumin as a standard protein (Lowry et al., 1951). All the enzyme activities were expressed in international units (U). One unit of activity corresponds to the quantity of enzyme that releases 1 μmol of reducing sugars (in glucose/ xylose equivalents) per minute.

2.4. Determination of optimum pH and temperature for saccharification

Chimera showed 100% activity at 0.05 M citrate-phosphate buffer, a pH range of 4.0–5.5 and a temperature range of 60–70 °C (Nath et al.,

Table 1
Cellulase and xylanase production and activity.

Enzyme name (Acronym)	Bacterial Source	Optimum condition		Total crude enzyme volume (mL)	#Crude enzyme activity (U/mL)	Total enzyme Unit (U)	¥Crude protein conc. (mg/mL)
		pH	Temp, °C				
chimera *Ct*GH1-L1-*Ct*GH5-F194A	*Clostridium thermocellum*	5	60	10	17.7 ± 1.1[a]	117	29.8 ± 0.6
Cellobiohydrolase *Ct*CBH5A	*Clostridium thermocellum*	6.4	65	10	10.3 ± 0.4[b]	103	24.7 ± 0.4
Endo-1,4-β-xylanase *Ct*GH11A	*Clostridium thermocellum*	7.5	65	10	97.3 ± 0.9[c]	973	26.2 ± 0.7
β-Xylosidase *Bo*GH43	*Bacteroides ovatus*	7.0	37	10	8.7 ± 0.3[d]	87	28.9 ± 1.2

[a] 1% (w/v) CMC-Na salt at 60 °C for 4 min, [b] 1% (w/v) CMC-Na salt at 65 °C for 5 min
[c] 1% (w/v) birchwood xylan at 65 °C for 1 min, [d] 1% (w/v) birchwood xylan at 37 °C for 5 min
Enzyme activity was measured by Nelson (1944) and Somogyi (1945) methods.
¥Total protein concentration was estimated by the Folin-Lowry (1951) method.

2019). *Ct*CBH5A shows its optimum activity at 0.05 M sodium-phosphate buffer in the pH range of 6.2–6.6 and temperature range of 30–65 °C (Nedumaran et al., 2020). *Ct*GH11 gave optimum activity in 0.05 M sodium-phosphate buffer in the pH range of 7.0–7.5 at 65 °C and *Bo*GH43 at 0.05 M sodium-phosphate buffer, pH 7 at 37 °C (Jamaldheen et al., 2019). However, for the operation of the saccharification process using the aforesaid four hydrolytic enzymes, a common stable pH, buffer concentration and temperature at which all four enzymes retain their maximum residual activity needs to be determined. So, the enzyme activity of all four enzymes at three different pHs: 5, 5.8 and 6.2 with a concentration of 0.05 M, sodium-phosphate buffer was estimated at three different temperatures: 30 °C, 35 °C and 40 °C. Also, to understand the stability of each enzyme, each set of enzymes (50 μL, 20 mg/mL) was incubated till 96 h and the enzyme activity at 24 h intervals of incubation was estimated by following individual assay methods and the relative residual enzyme activity (%) was calculated and reported in Table 2.

2.5. Experimental design for formulating enzyme cocktail for CApRS saccharification

The Design-Expert (7.0.0) software (Stat-Ease, Inc., USA) was employed to determine the optimum enzyme ratios for the formulation of a hydrolytic enzyme cocktail for CApRS saccharification. A D-optimal design constraints was Chimera (A) + *Ct*CBH5A (B) + *Ct*GH11 (C) + *Bo*GH43 (D)= 100% as expressed in Table 3. The enzyme component ranges (in terms of percentage) were as follows: 20 < Chimera< 50, 30 < *Ct*CBH5A < 60, 10 < *Ct*GH11 < 40, 10 < *Bo*GH43 < 20 and the total enzyme unit in each run was 25 U/mL of crude (unpurified enzyme present in the total cellular proteins) enzymes. The yield (mg/g) of total reducing sugar (TRS) in the saccharification process was set to the response for the optimization process. The software generates 20 runs of which 10 runs were model points, 5 runs to determine lack of fits and 5 runs were replicates. Each experiment was conducted using 0.05 M sodium-phosphate buffer, pH 5.8 at 35 °C, 150 rpm in an incubator shaker (Orbitek, Scigenics Biotech, India) in a reaction volume of 3 mL with 3%, w/w substrate loading under software designed conditions for 48 h. 0.05% (w/v) of sodium azide was added to avoid microbial contamination. After 48 h of saccharification, the total reducing sugars were determined and fitted to the model for statistical optimization.

The quadratic model representing the relationship between factors and response is shown in Eq. (1):

$$Y = \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} B(1)$$
$$+ \beta_{24} BD + \beta_{34} CD$$

where, Y represents the response and A, B, C and D are indicative of the factors. β_1, β_2, β_3 and β_4 denote the coefficients of individual factors. β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} represent the coefficients of two interacting factors.

To analyze the models, analysis of variance (ANOVA) and diagnostics were conducted. Additionally, optimization was carried out on the 3D response surfaces of the model's interaction variables to identify the regions with the highest desirability (Gunst et al., 1996). By employing this optimization process, software-based optimal parameters for the saccharification process were established. These parameters were determined by evaluating the impact of enzyme ratios (considered as factors) within the enzyme cocktail on the total reducing sugars (TRS)

Table 2
Determination of common buffer pH and temperature for a cocktail of crude recombinant hydrolytic enzymes.

Enzyme	Temperature °C	Residual enzyme activity (%) pH 5			Residual enzyme activity (%) pH 5.8			Residual enzyme activity (%) pH 6.2		
		0 h	48 h	96 h	0 h	48 h	96 h	0 h	48 h	96 h
Chimera	30	100 ± 2.6	75 ± 0.4	64 ± 3.9	100 ± 1.2	78 ± 2.1	70 ± 3.1	100 ± 0.6	74 ± 1.6	68 ± 0.7
*Ct*CBH5A		100 ± 1.9	82 ± 1.2	76 ± 1.4	100 ± 0.6	87 ± 3.3	81 ± 1.2	100 ± 0.9	85 ± 2.1	70 ± 1.2
*Ct*GH11		100 ± 3.1	69 ± 4.1	54 ± 2.1	100 ± 1.6	75 ± 1.5	61 ± 3.6	100 ± 1.6	74 ± 2.4	69 ± 2.5
*Bo*GH43		100 ± 1.9	77 ± 3.6	66 ± 2.7	100 ± 2.9	81 ± 4.2	74 ± 0.8	100 ± 3.4	84 ± 3.5	74 ± 3.1
Chimera	35	100 ± 2.9	76 ± 2.7	72 ± 1.7	100 ± 1.1	**80 ± 1.4**	75 ± 2.4	100 ± 1.1	72 ± 0.8	61 ± 4.6
*Ct*CBH5A		100 ± 4.1	83 ± 1.9	75 ± 3.9	100 ± 3.5	**85 ± 2.1**	76 ± 3.1	100 ± 2.4	87 ± 1.2	79 ± 2.5
*Ct*GH11		100 ± 0.5	71 ± 0.8	63 ± 0.5	100 ± 4.1	**80 ± 3.4**	66 ± 0.9	100 ± 1.5	76 ± 1.4	68 ± 0.5
*Bo*GH43		100 ± 3.1	75 ± 2.1	66 ± 4.1	100 ± 0.9	**82 ± 1.6**	74 ± 0.6	100 ± 0.9	86 ± 4.6	74 ± 1.2
Chimera	40	100 ± 3.9	77 ± 3.6	74 ± 2.9	100 ± 1.5	75 ± 0.9	63 ± 2.1	100 ± 1.8	65 ± 3.1	59 ± 0.9
*Ct*CBH5A		100 ± 2.7	74 ± 1.1	52 ± 2.6	100 ± 1.9	75 ± 2.3	56 ± 1.8	100 ± 1.4	79 ± 1.5	61 ± 1.1
*Ct*GH11		100 ± 4.2	56 ± 1.7	52 ± 1.9	100 ± 2.1	60 ± 3.1	54 ± 2.4	100 ± 0.9	62 ± 3.2	54 ± 2.4
*Bo*GH43		100 ± 0.9	73 ± 2.6	61 ± 1.2	100 ± 3.5	75 ± 4.7	71 ± 3.1	100 ± 3.1	71 ± 2.7	69 ± 3.1

Table 3
Predicted and experimental values of TRS with respect to variables in cocktail ratio optimization of RS saccharification by D-optimal mixture design approach.

Run order	Variables				Predicted results	Experimental results
	Chimera (A) (%)	*Ct*CBH5A (B) (%)	*Ct*GH11 (C) (%)	*Bo*GH43 (D) (%)	TRS (mg/g)	TRS (mg/g)
1	34.6	45.4	10.0	10.0	505.60	526.65
2	21.1	48.2	10.7	20.0	341.98	315.90
3	20.0	47.0	23.0	10.0	315.52	326.50
4	20.0	60.0	10.0	10.0	434.31	429.98
5	50.0	30.0	10.0	10.0	397.73	406.58
6	37.3	30.0	22.7	10.0	407.99	415.35
7	20.0	30.0	40.0	10.0	250.62	245.70
8	21.4	31.6	27.0	20.0	439.15	432.13
9	20.0	38.9	26.0	15.1	382.93	400.73
10	43.8	30.0	10.0	16.2	487.00	494.33
11	29.7	35.9	15.6	18.8	469.05	464.13
12	39.2	36.5	14.2	10.0	456.45	459.98
13	27.6	43.2	17.3	11.9	446.89	403.65
14	24.1	51.8	13.9	10.2	432.28	425.43
15	20.6	30.2	33.7	15.4	419.79	424.13
16	21.1	48.2	10.7	20.0	341.98	371.68
17	20.0	30.0	40.0	10.0	250.62	248.63
18	37.3	30.0	22.7	10.0	407.99	412.43
19	50.0	30.0	10.0	10.0	397.73	377.33
20	20.0	60.0	10.0	10.0	434.31	438.75

and the yields of glucose and xylose during the saccharification process.

2.6. Saccharification of rice straw using developed and commercial enzymes cocktail

The enzyme cocktail formulated at the optimized proportion of Chimera, *Ct*CBH5A, *Ct*GH11 and *Bo*GH43 contains all the cellulases (endoglucanase, cellobiohydrolase and β-glucosidase) and core xylanase enzyme (endoxylanase and β-xylosidase). Thus, cellulase activity and xylanase activity of the developed cocktail are estimated and expressed in terms of FPU/mL against Whatman No. 1 filter paper and U/mL against 1% (w/v) birchwood xylan, respectively. The filter paper activity (FPA) of the developed crude enzyme cocktail was estimated by using the procedure developed by Ghose (1987). The xylanolytic activity of the cocktail was measured by using 1% (w/v) birchwood xylan as substrate and by following the method described earlier in Section 2.3. The enzyme activity was expressed as U/mL and defined as the amount of reducing sugar released in μmole of the enzyme cocktail. The total protein concentration of the developed crude (unpurified) enzyme cocktail was also measured following the procedure described in Section 2.3.

Subsequently, the saccharification efficiency of the developed crude enzyme cocktail was compared against commercially available cellulase and xylanase preparation under optimized conditions. Equal proportions of commercial cellulase (source *Trichoderma reesei*) and xylanase (source: *Aspergillus niger*) in terms of FPU/mL and U/mL, respectively as that of the proportion present in the developed crude enzyme cocktail was used and the saccharification was performed for 3 mL reaction volume with 3%, w/w CApRS biomass loading at 35 °C and pH 5.8 for 48 h. The effectiveness of the developed crude enzyme cocktail against CApRS saccharification was compared with raw RS and partially pretreated RS (using 3%, v/v acetic acid at 120 °C for 30 min, containing 52.3% cellulose, 23.5% hemicellulose and 11.7% lignin) was performed using the same hydrolysis conditions as mentioned above and the TRS yield was compared. The saccharification efficiency was calculated by Eq. 2 given below:

$$\text{Saccharification efficiency}(\%) = \left(\frac{\text{Total reduced sugar released}(\frac{\text{mg}}{\text{g}})}{\text{Total Carbohydrate content in CApRS biomass}(\frac{\text{mg}}{\text{g}})} \right) \times 100 \quad (2)$$

2.7. Analytical procedures

2.7.1. Quantitative analysis of monosaccharides

The hydrolysate after the enzymatic saccharification was analyzed for the monosaccharide content. The glucose and xylose concentration in the saccharified hydrolysate was determined using High-Performance Liquid Chromatography (Shimadzu Corporation, Japan). Aminex HPX-87 H column (Bio-Rad Laboratories, USA) with a mobile phase, 5 mM H_2SO_4, flow rate of 0.6 mL/min, oven temperature at 50 °C and injection volume of sample, 20 μL was used.

2.7.2. Composition analysis of CApRS biomass

The structural carbohydrate analysis and lignin content of CApRS biomass were done using the NREL protocol as described and reported in our previous study (Maibam and Goyal, 2022b). Cellulose and hemicellulose contents were estimated by using the TAPPI method (TAPPI, 1992).

2.7.3. Statistical analysis

The experimental results were presented as the mean value along with the standard deviation (n = 3). To assess statistical significance, the student's t-test was employed, and a *p*-value of less than 0.05 was considered to indicate statistical significance. The statistical values assigned to the response were determined using the Design-Expert software.

3. Results and discussion

3.1. Production of recombinant cellulolytic and xylanolytic enzymes

The recombinant cellulases (chimera and *Ct*CBH5A) and xylanases (*Ct*GH11A and *Bo*GH43) are thermostable enzymes (Table 1). From each 800 mL culture of *E. coli* BL21 (DE3) cells expressing enzymes viz. *Ct*GH1-L1-*Ct*GH5- F194A, *Ct*CBH5A, *Ct*GH11A and *Bo*GH43 after sonication, 10 mL of supernatant containing crude recombinant enzyme was obtained. The enzyme concentration and the activity of each crude

enzyme were estimated at respective optimum pH and temperature by the procedure described in Section 2.3 and are presented in Table 1. The total cell protein concentration in the supernatant containing crude chimera was 29.8 mg/ mL and the endoglucanase activity against 1% (w/v) CMC-Na was 17.7 U/ mL. The β-glucosidase activity of chimera against 1% (w/v) cellobiose was 9.4 U/ mL. The total protein concentration in *Ct*CBH5A was 24.7 mg/ mL and a cellobiohydrolase activity against 1% (w/v) CMC-Na was 10.3 U/ mL. *Ct*Xyn11 showed endoxylanase activity of 97.3 U/ mL against 1% (w/v) birchwood xylan and a total protein concentration in crude enzyme preparation was 26.2 mg/ mL. The β-xylosidase activity of *Bo*GH43 against 1% (w/v) birchwood xylan was 8.7 U/mL and a total protein concentration was 28.9 mg/mL. The mixture of all four enzymes could serve as a potential enzyme cocktail for the saccharification of CApRS biomass that contains 82%, w/w holocellulose.

3.2. Determination of optimum pH and temperature for saccharification

The determination of common pH, buffer concentration and operating temperature is the most crucial step in the designing of an enzyme cocktail for the saccharification process (Lee et al., 2017). All four hydrolytic enzymes (chimera, *Ct*CBH5A, *Ct*GH11 and *Bo*GH43) used in this study possess different optimum pH and temperatures for their maximum activity. Therefore, for the application of these hydrolytic enzymes in the saccharification of CApRS biomass, common operating conditions were evaluated by performing the experiments mentioned in Section 2.4. Table 2 displays the residual enzyme activities (in terms of %) of chimera, *Ct*CBH5A, *Ct*GH11and *Bo*GH43 at different pH and temperature. It can be seen from Table 2 that at pH 5.8 and 35 °C, the chimera showed a maximum residual activity of 80% after 48 h of incubation. *Ct*CBH5A showed a maximum of 87% residual enzyme activity at both pH 5.8 and 6.2 and 35 °C after 48 h of incubation. Similarly, endo-1,4-β-xylanase, *Ct*GH11 showed the highest residual activity of 80% at pH 5.8 and an incubating temperature of 35 °C. Also, *Bo*GH43 retained a maximum residual activity of 82% at pH 5.8 and 35 °C. Thus, all four enzymes (chimera, *Ct*CBH5A, *Ct*GH11and *Bo*GH43) showed 80 – 85% residual enzyme activity after 48 h of incubation at pH 5.8 and 35 °C. Therefore, the common buffer, 0.05 M sodium-phosphate buffer was used for designing of enzyme cocktail comprising the above four hydrolytic enzymes at the operating conditions of pH 5.8 and temperature 35 °C. Subsequently, different combinations of chimera, *Ct*CBH5A, *Ct*GH11A and *Bo*GH43 were used to generate a specific enzyme cocktail for the saccharification of CApRS operated using 0.05 M sodium-phosphate buffer, pH 5.8 at 35 °C.

3.3. Model development, Analysis of Variance (ANOVA) and Diagnosis

The empirical model employed in this study aimed to assess the interactions and impacts of different components (Chimaera, *Ct*CBH5A, *Ct*Xyn11, and *Bo*GH43) within the enzyme mixture on the yield of total reducing sugars (TRS). To evaluate the reliability of the proposed model, the standard error of design was analyzed and depicted in Fig. 1. The model's predictions were observed to be reasonable and accurate, as the overall errors are in the range of 0.5–0.8σ across almost all experimental runs (Jeirani et al., 2012). The red dots plotted on the standard error graphs represent the design points of the conducted experiments.

The experimental results and the predicted results of yield of TRS (mg/g) for each model-designed run for formulating the optimum ratio of enzymes in the cocktail are shown in Table 3.

The results were statistically analyzed using Analysis of Variance (ANOVA) and the quadratic model was developed to examine the effects of all the four enzymes in the hydrolysis of biomass (CApRS) based on the yield of TRS released during the process. The quadratic equation representing the correlation between the response (TRS yield) and the variables in terms of coded factors was presented as follows:

$$TRS = +397{\cdot}38\ A + 434{\cdot}38\ B + 249{\cdot}91\ C - 604{\cdot}98\ D + 357{\cdot}01\ AB + 297{\cdot}72\ AC + 1809{\cdot}49\ AD - 159{\cdot}15\ BC + 1063{\cdot}26\ BD + 2153{\cdot}07\ CD \quad (3)$$

where A, B, C and D represent factors i.e., Chimera, *Ct*CBH5A, *Ct*Xyn11 and *Bo*GH43, respectively.

The significance of the constructed models and their variables was assessed using ANOVA, as presented in Table 4 for the cocktail design. The high F-value and low *p*-value (<0.0001) indicate the significant interaction of process-based parameters and the statistical significance of the model at a 95% confidence level (Zafar et al., 2017, Pattanaik et al., 2018). Table 4 reveals significant *p*-values for the linear mixture and all interacting terms, except for BC and BD. The high value of R^2 (0.94) and adjusted R^2 (0.90) indicate its acceptability (Pillai et al., 2017).

To examine whether the errors are uniform and independent, diagnostics were performed (Anderson and Whitcomb, 2016). Graphical analysis of the model through the plots of residuals vs. predicted response plots, the normal plot of residuals, leverage plot and cook's distance were carried out. Fig. 2a illustrates the model's residual in the normal plot which closely resembles the normal distribution thereby indicating the adequacy of the model with no transformation of the response being needed (Mitchell, 2000). The externally studentized residuals detect the outliers and help in assessing the equal variance assumption (Anderson and Whitcomb, 2017). Also, Cook's distance was used to spot the outliers. Figs. 2b and 2c showed the externally studentized residuals and Cook's distance, respectively suggesting uniform

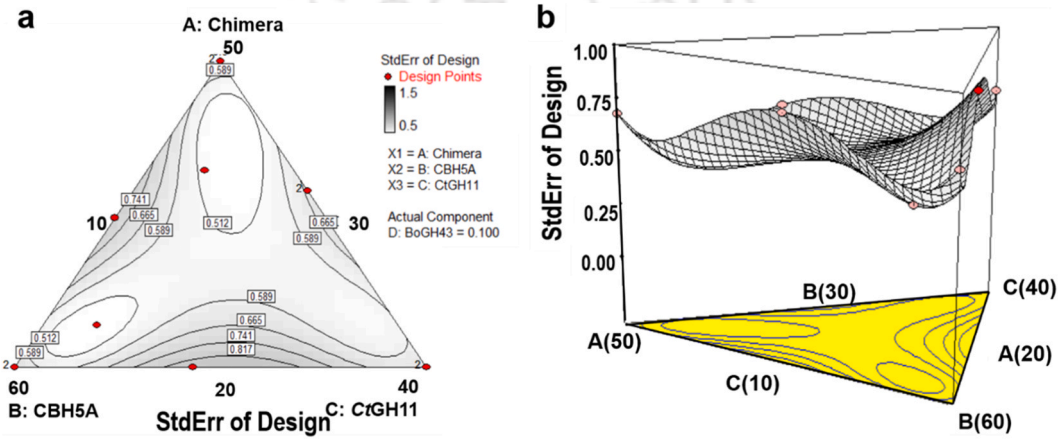

Fig. 1. (a) 2D contour plot and (b) 3D surface plot of the standard error of design at different points in the design space.

Table 4
ANOVA table for D-optimal quadratic model for cocktail design.

Source	Sum of Squares	df	Mean Square	F Value	*p*-value
Model	93096.02	9	10344	20.08	< 0.0001
Linear Mixture	37497.09	3	12499.03	24.26	< 0.0001
AB	9124.878	1	9124.878	17.71	0.0018
AC	7631.622	1	7631.622	14.81	0.0032
AD	4154.44	1	4154.44	8.06	0.0176
BC	1623.804	1	1623.804	3.15	0.1062
BD	1104.909	1	1104.909	2.14	0.1738
CD	5205.863	1	5205.863	10.10	0.0098
Residual	5152.585	10	515.2585		
Lack of Fit	3122.323	5	624.4646	1.537891	0.3241
Pure Error	2030.263	5	406.0525		
Cor Total	98248.6	19			
R^2	0.9476				
Adj R-Squared	0.9004		**Adeq. precision**	15.885	
Pred R-Squared	0.7635		**C.V.%**	5.66	

data distribution with no outliers (Varanda et al., 2017). The analysis of leverage (Fig. 2d) and residualsvs.s predicted (Fig. 2e) plots demonstrate that there is the absence of any particular trend and that the experimental runs have a consistent impact on modeled responses (Jeirani et al., 2012).

The model effectively represents the experimental data and allows for exploration within the desired range. The findings from the experimental data (Table 3) and the ANOVA (Table 4) align with the observations presented in Fig. 2. To examine how the combination of all four enzymes influences the production of TRS (m/g) and to analyze their synergistic effects, 3D surface plots were generated.

3.4. Effect of cocktail enzymes on TRS yield and their synergistic effect on hydrolysis

The impact of mixture components on TRS yield could be analyzed from the overview of the response surface plot (Fig. 3), software-designed experiments (Table 3) and ANOVA (Table 4). Fig. 3 depicts the impact of mixture components ABC, BDC and ABD, respectively on the yield of TRS (mg/g). At a constant proportion of *Bo*GH43 (13.1%) (Figs. 3a and 3b), maximum TRS was achieved at the extreme edge of the contour with *Ct*GH11 at its lowest proportion (10%), *Ct*CBH5A at a middle range (40–50%) of its limit and Chimera at an upper limit of its range (35–45%). Upon limiting the proportion of Chimera in its lower limit (20%) (Fig. 3a) and increasing the proportion of *Ct*GH11 (B) and *Ct*CBH5A (C) from lower to higher limit (10- ~36%) and (30- ~56%), the TRS yield is lowest throughout, thereby indicating the significance of Chimera's proportion, non-significance of the interacting variable BC and negative proportionality of BC with the TRS yield.

The enzymes, *Ct*GH11 and *Bo*GH43 when present in a proportion of any range from the set limit with Chimera at a fixed proportion of 35.1% and *Ct*CBH5A in a range of 30–45%, resulted in an overall high TRS yield as shown in the contour (Figs. 3c and 3d). This signifies that the hydrolysis process is mainly governed by the appropriate proportion of Chimera and *Ct*CBH5A and the range chosen for *Ct*GH11 and *Bo*GH43 is appropriate for the mixture design. The enzyme cocktail with *Ct*GH11 at its lowest limit of 10% and upon varying the proportion from the lower to the upper limit of the other three enzymes, the highest TRS yield of 516 mg/g could be achieved in the central region of the contour (Figs. 3e and 3f). This implies the high sensitivity of the components A (Chimera), B (*Ct*CBH5A) and D (*Bo*GH43).

*Ct*CBH5A at the highest proportion (60%) and varying the proportion of *Bo*GH43 from low to high (10–20%) in a cocktail with a fixed proportion of Chimera, 20% gives no significant change in the TRS yield (Fig. 3f) thereby inferring the non-significance interacting variable of quadratic function BD. The enzyme cocktail with chimera at the lowest proportion (20%) and *Ct*CBH5A with the highest proportion resulted in the lowest TRS yield of 380 mg/g (Fig. 3e) thereby signifying that the synergistic action of endoglucanase and β-glucosidase with cellobiohydrolase is essential for the efficient hydrolysis of CApRS. In essence, the 3D and 2D response surface plots in Fig. 3 could identify the optimal proportion of all four enzymes for efficient saccharification of CApRS.

3.5. Quantification of monosaccharides after saccharification of CApRS by enzyme cocktail

After the enzymatic saccharification of each 20 sets of experiments generated by D-optimal design for the optimization of cocktail ratios, the enzymatic hydrolysates of CApRS were analyzed for the quantification of monosaccharides (Table 5). The titre of individual monosaccharides, glucose and xylose released after the saccharification by enzyme cocktail in each software design experimental run was analyzed by using HPLC. This monosaccharides titre analysis is necessary for its application in the further conversion process in biorefineries. The CApRS biomass contains low, 9.01% (w/v) hemicellulose content. Therefore, the maximum possible theoretical titre of xylose (g/L) from hemicellulosic fraction present in the CApRS biomass is 2.4 g/L (Maibam and Goyal, 2022a). It can be seen from Table 5, that the concentration of xylose released in all the experimental runs ranges between 1.61 g/L – 2.13 g/L, thereby indicating an effective hydrolysis of hemicellulosic fraction with the conversion efficiency ranging between 75% and 90%. These results inferred that the ranges of proportion and the enzyme units for xylanases (*Ct*GH11 and *Bo*GH43) selected for the cocktail formulation are appropriate and efficient for maximum hydrolysis of hemicellulosic fractions. Similarly, the cellulosic content in CApRS biomass is 73% (w/v) and the maximum theoretical glucose titre that can be achieved is 19.8 g/L. When the chimera is present at a minimum proportion (20%) irrespective of the proportion of *Ct*CBH5A in the cocktail, the glucose concentration released during the saccharification is low, 4.32–6.21 g/L. The low titre of glucose could be due to the low proportion and enzyme unit of β-glucosidase. When the chimera's proportion in the cocktail is increased to 30–40%, the glucose concentration increases to 10.49 – 12.76 g/L. The increase in glucose titre might be due to a high proportion of active β-glucosidase. The presence of a high proportion of *Ct*CBH5A in the cocktail does not give any positive effect on the glucose titre, however, the presence of chimera and *Ct*CBH5A in the ratio (35:45) results in a maximum glucose titre of 12.76 g/L thereby indicating an effective hydrolysis of cellulosic fraction with the conversion efficiency ranging between 30% and 65%. This signifies the synergistic effect of cellobiohydrolase and β-glucosidase in the conversion of cellulose to glucose.

3.6. Mixture proportion optimization, desirability function and validation

The primary objective of this study was to optimize the enzyme ratios in the cocktail for efficient saccharification of CApRS. The formulated optimization targets high TRS yield and equal weights were assigned to all the variables (four factors and one response). The highest desirability of 0.96 was achieved at an optimized proportion of Chimera (35.1%), *Ct*CBH5A (41.8%), *Ct*GH11 (10%) and *Bo*GH43 (13.1%) in the enzyme cocktail. The predicted TRS yield by the software under optimized conditions was 516 mg/g_{CApRS}. The predicted yield was further validated by performing enzymatic saccharification of CApRS using a recombinant enzyme cocktail formulated under optimized proportion. The enzymatic saccharification process was carried out at 3%, w/v substrate loading in 100 mL reaction volume using 0.05 M sodium-phosphate buffer, pH 5.8 at 35 °C and resulted in a TRS yield of 498 mg/$g_{CApRS\ biomass}$ at 48 h giving a saccharification efficiency of 72%.

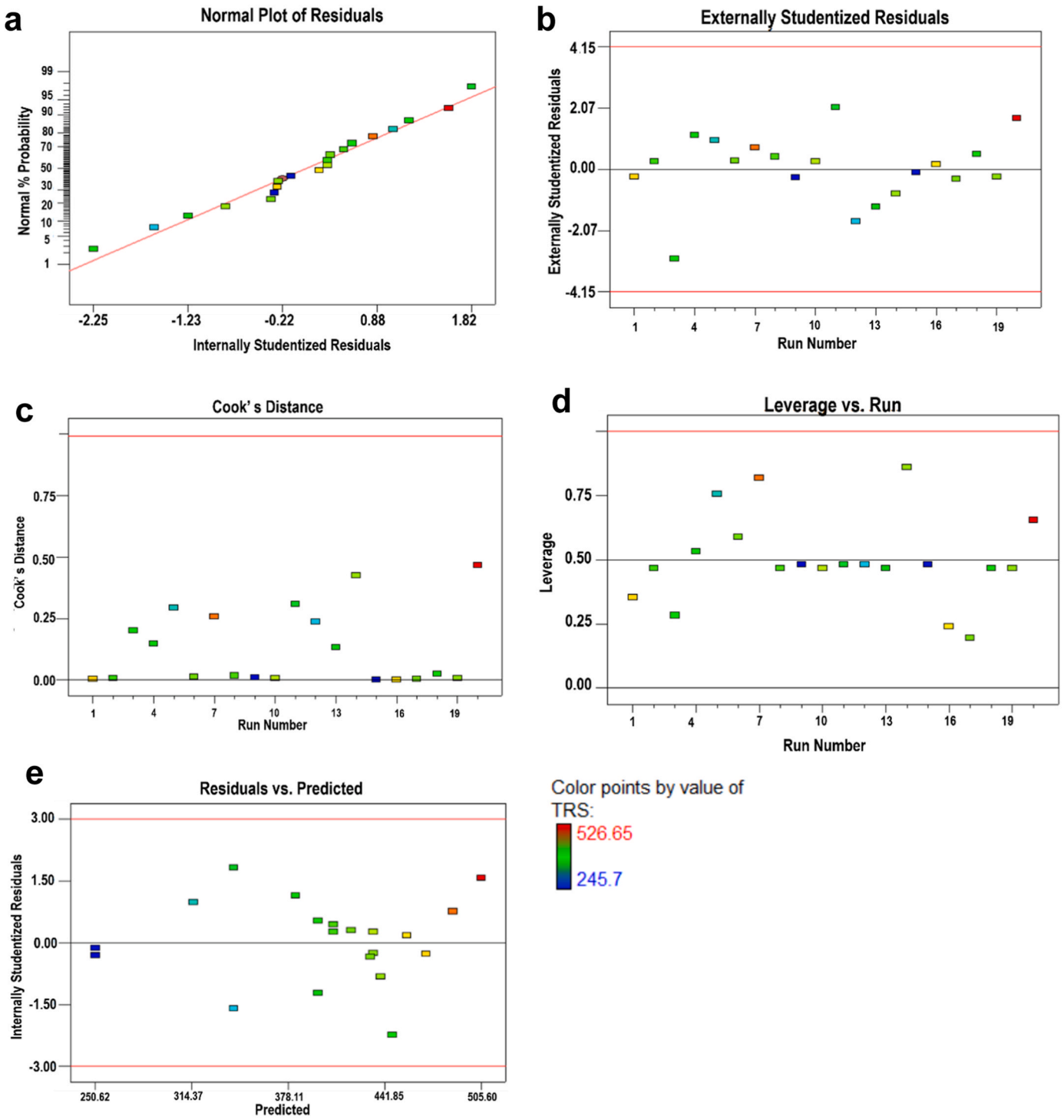

Fig. 2. Diagnostics of TRS model by (a) normal plot of residuals, (b) externally studentized residuals, (c) cook's distance, (d) leverage plot and (e) residuals vs predicted plot for the cocktail design.

3.7. Overall yields of monosaccharides and TRS using developed enzyme cocktail and commercial enzyme cocktail against CApRS biomass

The developed crude enzyme cocktail showed a cellulase activity of 21.24 U/ mL against 1% (w/v) CMC-Na and 5.62 FPU/ mL against Whatman No. 1 filter paper and a xylanase activity of 102 U/mL against 1% (w/v) birchwood xylan at the optimum conditions as mentioned in Section 2.6. The total cell protein concentration of the developed crude (unpurified enzyme present in the total cellular proteins) enzyme cocktail was 27.1 mg/mL thus giving activity of 208 FPU/g $_{\text{total cell protein}}$ and xylanase activity 239 U/g $_{\text{total cell protein}}$.

The enzymatic hydrolysis of 3% (w/v) CApRS biomass was performed under optimum conditions of the saccharification process (0.05 M sodium-phosphate buffer, pH 5.8 at 35 °C) by using i) a developed crude enzyme cocktail and ii) commercial cellulase and xylanase cocktail. The enzyme dosage of cellulase is 187 FPU/g$_{\cdot\text{CApRS}}$ (or 5.6 FPU/mL) and xylanase 172 U/g$_{\cdot\text{CApRS}}$ (or 102 U/mL) activities were used in each case. The enzymatic hydrolysis using a commercial cocktail resulted in a TRS yield of 553 mg/g$_{\text{CApRS biomass}}$, glucose 480 mg/g$_{\text{CApRS biomass}}$ and xylose 67 mg/ g$_{\text{CApRS biomass}}$ with the saccharification

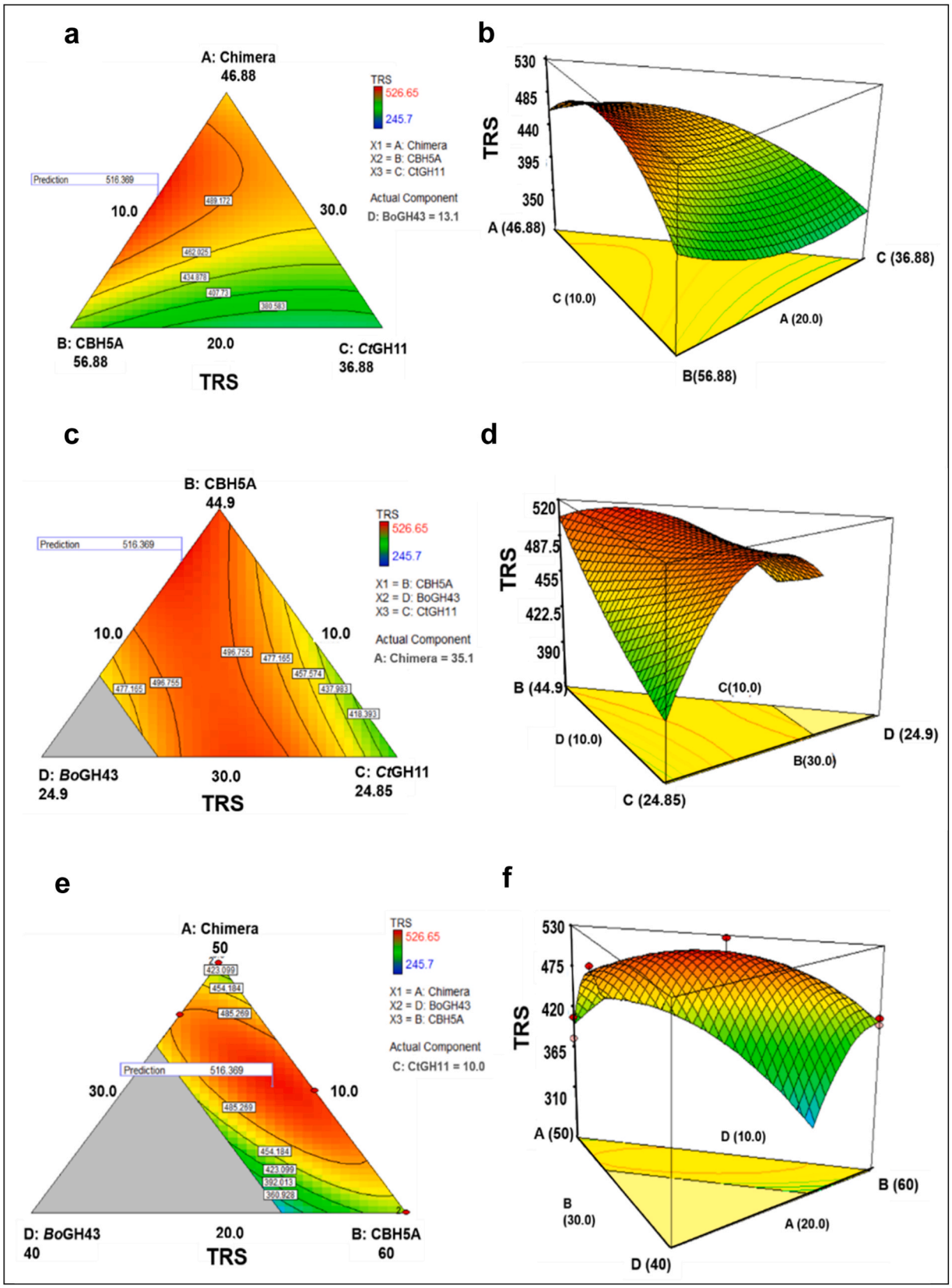

Fig. 3. (a), (c), (e) represent 2D contour and their corresponding 3D plot (b), (d), and (f) generated from a quadratic model developed for crude recombinant hydrolytic enzyme cocktail formulation. The red regions indicate the highest TRS.

Table 5
Quantification of monosaccharides in the enzyme saccharified hydrolysate of each experiment for optimizing the enzyme ratio in the cocktail.

Run order	Variables (%)				Monosaccharide (g/L)		
	Chimera	*Ct*CBH5A	*Ct*GH11	*Bo*GH43	Glucose	Xylose	Total
1	34.6	45.4	10.0	10.0	12.76	2.08	14.84
2	21.1	48.2	10.7	20.0	6.89	1.89	8.78
3	20.0	47.0	23.0	10.0	5.15	2.00	7.15
4	20.0	60.0	10.0	10.0	6.18	1.98	8.16
5	50.0	30.0	10.0	10.0	9.97	1.86	11.83
6	37.3	30.0	22.7	10.0	10.23	1.79	11.84
7	20.0	30.0	40.0	10.0	4.32	2.09	6.41
8	21.4	31.6	27.0	20.0	6.98	2.14	9.12
9	20.0	38.9	26.0	15.1	6.21	1.98	8.19
10	43.8	30.0	10.0	16.2	11.06	1.97	13.03
11	29.7	35.9	15.6	18.8	10.49	2.01	12.5
12	39.2	36.5	14.2	10.0	9.86	1.82	11.68
13	27.6	43.2	17.3	11.9	9.48	1.79	11.27
14	24.1	51.8	13.9	10.2	8.03	2.09	10.12
15	20.6	30.2	33.7	15.4	5.87	1.98	7.85
16	21.1	48.2	10.7	20.0	7.01	1.90	8.91
17	20.0	30.0	40.0	10.0	4.29	2.13	6.42
18	37.3	30.0	22.7	10.0	10.09	1.84	11.93
19	50.0	30.0	10.0	10.0	9.56	2.01	11.57
20	20.0	60.0	10.0	10.0	5.98	2.01	7.99

efficiency of 80% (Eq. 2) after 48 h (Table 6). However, under the same operating conditions of the saccharification process, the developed crude enzyme cocktail resulted in a TRS yield of 498 mg/$g_{CApRS\ biomass}$, glucose 410 mg/$g_{CApRS\ biomass}$ and xylose 71.3 mg/$g_{CApRS\ biomass}$ with saccharification efficiency of 72% after 48 h. The commercial enzyme cocktail gave 11% and 17% higher TRS and glucose yields, respectively as compared with the developed cocktail. However, the xylose titre was low by 6% in the saccharified hydrolysate from the commercial enzyme cocktail. The commercial enzyme cocktail gave 8% higher saccharification efficiency than the developed crude enzyme cocktail. The

Table 6
Comparison of various enzyme cocktail formulations for biomass hydrolysis in terms of TRS yield and saccharification efficiency.

Enzymes cocktail (enzyme component ratio)	Source of microorganism	Saccharification conditions	Pretreatment method	Biomass	Total reducing sugar (mg/g)	Saccharification efficiency	Reference
Recombinant cellulases (*Mt*CBH7, *Mt*CBH6, *Mt*EG5, *Mt*EG7 and *Mt*BGL3) 38.9:20.0:20:21.1	*Myceliophthora thermophila*	pH 5, 50°C, 1200 rpm, biomass: 2.5% (w/v), Purified enzyme: 1 mg/$g_{substrate}$	Microwave-assisted hydrothermal treatment	Wheat straw	118	26	Karnaouri et al. (2016)
Recombinant endoglucanase (*Ba*GH5-UV2), Cellobiohydrolase (*Ct*CBH5A) and β-glucosidase (*Ct*GH1) (80:10:10)	*Bacillus amyloliquefaciens* *Clostridium thermocellum*	pH 6.5, 40°C, 150 rpm, biomass: 2% (w/v), Crude enzyme: 2000 U/$g_{ptd\ biomass}$	1% (w/v) NaOH	*Sorghum durra* stalk	211	32	Singh et al. (2020)
Natural variant cellulase and xylanase (1:1)	*Schizophyllum commune* *Aspergillus oryzae*	pH 5, 50°C, 120 rpm, biomass: 10% (w/v), Purified enzyme: 200 U/$g_{ptd\ biomass}$	Laccase from *M. verrucaria* ITCC-8447	Rice straw	181	35	Bharadwaj et al.2020
Recombinant cellulolytic chimera and *Ct*CBH5A (2:3)	*Clostridium thermocellum*	pH 6.4, 30°C, 150 rpm, biomass: 3% (w/v), Purified enzyme: 350 U/$g_{ptd\ biomass}$	1% (w/v) NaOH followed by dilute 1.5% (w/v) H_2SO_4	*Sorghum durra* stalk	417	58	Nedumaran et al. (2020)
Recombinant cellulases (CBH+EG+BG=7:2:1) and xylanases (Xyl+BX=1:1)	(CBH & EG) *Penicillium verruculosum* (EG) *Aspergillus niger* (Xyl) *Penicillium canescens* (BX) *Aspergillus japonicus*	pH 5, 45°C, 900 rpm, biomass: 10% (w/v), Purified enzyme: 7 mg/$g_{substrate}$	2% (w/v) NaOH	Sugarcane bagasse	570	80	Karp et al. (2021)
Recombinant cellulolytic chimera and *Ct*CBH5A (2:3)	*Clostridium thermocellum*	pH 6.4, 30°C, 150 rpm, biomass: 2% (w/v), Purified enzyme: 600 U/$g_{ptd\ biomass}$	1% (w/v) NaOH followed by 2% (w/v) organosolv	Sugarcane bagasse	230	35	Nath et al. (2021)
Recombinant cellulase (celluloytic chimera+ *Ct*CBH5A) and xylanases (*Ct*GH11 + *Bo*GH43) (35.1:41.8:10.0:13.1)	*Clostridium thermocellum* *Bacteroides ovatus*	pH 5.8, 35°C, 150 rpm, biomass: 3% (w/v), Crude enzyme: 187 FPU/$g_{ptd\ biomass}$	Choline chloride: acetic acid molar ratio (1:3.59)	Rice straw	498	72	This study
Commercial cellulase (*T. ressei*) and xylanase (*A. niger*)	*Trichoderma. ressei* *Aspergillus niger*	pH 5.8, 35°C, 150 rpm, biomass: 3% (w/v), Purified enzyme: 187 FPU/$g_{ptd\ biomass}$	Choline chloride: acetic acid molar ratio (1:3.59)	Rice straw	553	80	This study

enzymatic saccharification of raw RS and partially pretreated RS (acetic acid pretreated RS) using a developed enzyme cocktail under the same saccharification conditions and enzyme dosage as described above resulted in a lower TRS yield of 80 mg/$g_{raw\ RS}$ and 218 mg/$g_{ptd\ RS}$, respectively compared to CApRS biomass. The justification for high TRS yield could be due to the exposure of soluble and digestible cellulose (Zhang et al., 2023) present in the CApRS biomass which is generated from the strong, effective and green choline chloride: acetic acid pretreatment method conducted in the previous study (Maibam and Goyal, 2022b). Thus, it can be inferred that the developed crude hydrolytic recombinant enzyme cocktail is efficient and specific for CApRS biomass.

3.8. Comparative analysis of developed enzyme cocktail for biomass saccharification

A study on statistical designing of enzyme cocktail using core cellulases and accessory enzymes by Karnaouri et al., 2016 reported that the purified recombinant cellulases, *Mt*CBH7, *Mt*CBH6, *Mt*EG5 and *Mt*EG7 from *Myceliophthora thermophila* in the ratio, 38.9:20.0:20:21.1 with 3% *Mt*BGL3 (β-glucosidase) resulted in 26% saccharification efficiency using microwave-assisted pretreated wheat straw under the saccharification conditions described in Table 6 and also (Karnaouri et al., 2016). They further reported that the addition of accessory enzymes *Mt*Man26a (mannanase), *Mt*GH61 (lytic polysaccharide monooxygenase), *Mt*Fae1a (xylanases) could enhance the saccharification efficiency to 38%. Another study by Singh et al. (2020) reported the use of a crude recombinant cellulase enzyme cocktail for the hydrolysis of base pretreated 2% (w/v) *Sorghum durra* stalk resulting in a TRS yield of 211 mg/ $g_{pretreated\ biomass}$ (Table 6). The effect of an enzyme cocktail comprising natural cellulase (source: *Aspergillus oryzae*) and natural xylanase (*Schizophyllum commune*) in crude and purified form for the saccharification of 10% (w/v) laccase delignified rice straw was illustrated (Bhardwaj et al., 2020). They reported that enzymatic saccharification of laccase delignified rice straw using crude cellulase and xylanase cocktail in the ratio 1:1 gives a TRS yield of 107 mg/$g_{pretreated\ biomass}$. However, the use of purified cellulase and xylanase cocktail in the same ratio and enzyme dosage under similar operating conditions of saccharification gave a higher, 181 mg/$g_{pretreated\ biomass}$ TRS yield (Table 6). The effect of a purified enzyme cocktail comprising cellulases and xylanases on the saccharification efficiency of pretreated sugarcane bagasse was evaluated (Karp et al., 2021). They reported that the combination of cellulases, 5 mg/$g_{substrate}$ and xylanases, 2 mg/$g_{substrate}$ for the saccharification of 10% (w/v) alkali pretreated sugarcane bagasse gave TRS yield of 570 mg/$g_{pretreated\ biomass}$ (Table 6). Nath et al (2020) reported a TRS and glucose yield of 230 mg/$g_{pretreated\ biomass}$ and 137 mg/$g_{pretreated\ biomass}$, respectively, after the enzymatic saccharification of 2% (w/v) pretreated sugarcane bagasse using purified recombinant cellulase cocktail comprising chimera and *Ct*CBH5A at ratio 2:3 with total enzyme dose, 600 U/$g_{pretreated\ biomass}$. Whereas, Nedumaran et al. (2020) showed a higher TRS and glucose yield of 417 mg/g pretreated biomass and 285 mg/g pretreated biomass, respectively, with 350 U/g using the same purified enzyme cocktail (chimera and *Ct*CBH5A at a ratio 2:3) at 3% (w/v) pretreated *Sorghum durra* stalk (Table 6). However, the current study involving the same cellulase enzymes (chimera and *Ct*CBH5A) along with xylanases (*Ct*GH11 and *Bo*GH43) all in crude form (unpurified enzyme present in the total cellular proteins) resulted in higher TRS and glucose yield as compared with previous studies of Nath et al (2020) and Nedumaran et al. (2020). This comparative analysis highlights the importance of enzyme components and their proportions in the cocktail, structural composition of the pretreated biomass and the hydrolysis process parameters on the saccharification efficiency. The plausible reason for the high yield of TRS and glucose in the current study could be due to i) higher delignification and hemicellulose removal in the pretreated biomass due to the strong pretreatment method (ChCl:AA) used (Maibam and Goyal, 2022b) and ii) core xylanase enzymes completely hydrolyze the xylan fractions thereby enhancing the exposure of cellulose and escalating its accessibility to cellulases and iii) statistically optimized proportions of enzyme in the cocktail based on the carbohydrate content in the pretreated biomass. Thus, from this study, it is noteworthy that i) the presence of both cellulases and hemicellulases in the enzyme cocktail significantly affects the saccharification efficiency, ii) the proportion of hydrolytic enzymes in the cocktail is specific for a particular biomass based on the carbohydrate content in the pretreated substrate, iii) the content of cellulosic and hemicellulosic fractions and the extent of their exposure, the operating parameters and conditions such as biomass loading, temperature and enzyme dosages, all factors account for an effective saccharification process.

4. Conclusions

The optimum temperature and pH for enzymatic saccharification of choline chloride: acetic acid pretreated rice straw (CApRS) using crude recombinant cellulases (chimera and cellobiohydrolase, *Ct*CBH5A) and xylanase (endo-xylanase, *Ct*GH11 and β-xylosidase, *Bo*GH43) were 35 °C, pH 5.8, respectively. The statistical approach using a D-optimal mixture design gave chimera, *Ct*CBH5A, *Ct*GH11 and *Bo*GH43 in the ratio of 35.1:41.8:10.0:13.1, as an optimum proportion in the developed enzyme cocktail for CApRS hydrolysis. The enzymatic saccharification of 3%, w/v CApRS biomass by the developed enzyme cocktail under optimum conditions yielded TRS, 498 mg/g_{CApRS} with glucose and xylose yield of 410 mg/$g_{CApRS\ biomass}$ and 71.3 mg/$g_{CApRS\ biomass}$, respectively at a saccharification efficiency of 72%. However, the saccharification of 3% (w/v) of raw RS or 3%, (w/v) partially pretreated RS by the same enzyme cocktail under the same conditions gave significantly lower TRS yields of 80 mg/g_{rawRS} and 218 mg/$g_{ptd\ RS}$, respectively, implying the effectiveness of the developed enzyme cocktail against CApRS. The present study highlights the importance of different enzyme proportions in the cocktail for biomass conversion. However, the dosage of the optimized enzyme cocktail has to be further optimized based on the percent of biomass loading for the saccharification process. The optimum saccharification process parameters at 35°C and pH 5.8 developed in this study are advantageous for its application in subsequent processes like simultaneous saccharification and fermentation (SSF) in bioethanol-based biorefineries.

CRediT authorship contribution statement

Premeshworii Devi Maibam: Conceptualization; Data curation; Formal analysis; Methodology; Writing- original draft, review & editing, revised. **Arun Goyal**: Funding acquisition; Supervision; Resources; Conceptualization; Data analysis, Validation; review & editing, revised.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Arun Goyal reports financial support was provided by India Ministry of Science & Technology Department of Biotechnology.

Data Availability

Data will be made available on request.

Acknowledgments

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References

Abdmouleh, Z., Alammari, R.A., Gastli, A., 2015. Review of policies encouraging renewable energy integration & best practices. Renew. Sustain. Energy Rev. 45, 249–262. https://doi.org/10.1016/j.rser.2015.01.035.

Anderson, M.J., Whitcomb, P.J., 2016. RSM simplified: optimizing processes using response surface methods for design of experiments. second ed., Productivity press, New York.

Anderson, M.J., Whitcomb, P.J., 2017. DOE simplified: practical tools for effective experimentation, Boca Raton, CRC press.

Bhardwaj, N., Kumar, B., Agrawal, K., Verma, P., 2020. Bioconversion of rice straw by synergistic effect of in-house produced ligno-hemicellulolytic enzymes for enhanced bioethanol production. Bioresour. Technol. Rep. 10, 100352 https://doi.org/10.1016/j.biteb.2019.100352.

Classics Lowry, O., Rosebrough, N., Farr, A., Bandall, R., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 (1), 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59 (2), 257–268. https://doi.org/10.1351/pac198759020257.

Gunst, R.F., 1996. Response surface methodology: process and product optimization using designed experiments. Technometrics 38, 285. https://doi.org/10.1080/00401706.1996.10484509.

Guo, H., Chang, Y., Lee, D.J., 2018. Enzymatic saccharification of lignocellulosic biorefinery: research focuses. Bioresour. Technol. 252, 198–215. https://doi.org/10.1016/j.biortech.2017.12.062.

Huang, C., Jiang, X., Shen, X., Hu, J., Tang, W., Wu, X., Yong, Q., 2022. Lignin-enzyme interaction: a roadblock for efficient enzymatic hydrolysis of lignocellulosics. Renew. Sustain. Energy Rev. 154, 111822 https://doi.org/10.1016/j.rser.2021.111822.

Huy, N.D., Saravanakumar, T., Ha, S.H., Park, S.M., 2020. Enhanced enzymatic saccharification of wheat flour arabinoxylan and barley straw using recombinant hemicellulases. Biotechnol. Bioprocess Eng. 25 (3), 431–441.

Jamaldheen, S.B., Thakur, A., Moholkar, V.S., Goyal, A., 2019. Enzymatic hydrolysis of hemicellulose from pretreated Finger millet (*Eleusine coracana*) straw by recombinant endo-1, 4-β-xylanase and exo-1, 4-β-xylosidase. Int. J. Biol. Macromol. 135, 1098–1106. https://doi.org/10.1016/j.ijbiomac.2019.06.010.

Jeirani, Z., Jan, B.M., Ali, B.S., Noor, I.M., Hwa, S.C., Saphanuchart, W., 2012. The optimal mixture design of experiments: alternative method in optimizing the aqueous phase composition of a microemulsion. Chemom. Intell. Lab. Syst. 112, 1–7. https://doi.org/10.1016/j.chemolab.2011.10.008.

Karnaouri, A., Matsakas, L., Topakas, E., Rova, U., Christakopoulos, P., 2016. Development of thermophilic tailor-made enzyme mixtures for the bioconversion of agricultural and forest residues. Front. Microbiol. 7, 177. https://doi.org/10.3389/fmicb.2016.00177.

Karp, S.G., Rozhkova, A.M., Semenova, M.V., Osipov, D.O., de pauli, S.T., Sinitsyna, O. A., Zorov, I.N., de Souza Vandenberghe, L.P., Soccol, C.R., Sinitsyn, A.P., 2021. Designing enzyme cocktails from Penicillium and Aspergillus species for the enhanced saccharification of agro-industrial wastes. Bioresour. Technol. 330, 124888 https://doi.org/10.1016/j.biortech.2021.124888.

Lan, T.Q., Lou, H., Zhu, J.Y., 2013. Enzymatic saccharification of lignocelluloses should be conducted at elevated pH 5.2–6.2. Bioenergy Res. 6, 476–485.

Lee, H., Lee, Y.M., Heo, Y.M., Lee, J., Kim, J.S., Kang, K.Y., Kim, J.J., 2017. Utilization of agricultural residues for enhancement of cellulolytic enzyme production and enzymatic saccharification by *Trichoderma harzianum* KUC1716. Ind. Crops Prod. 109, 185–191. https://doi.org/10.1016/j.indcrop.2017.08.042.

Maibam, P.D., Goyal, A., 2022a. Pretreatment methods for overcoming biomass recalcitrance, in: Verma, P. (Eds.), Enzymes in the Valorization of Waste. CRC Press, pp. 1–21.

Maibam, P.D., Goyal, A., 2022b. Approach to an efficient pretreatment method for rice straw by deep eutectic solvent for high saccharification efficiency. Bioresour. Technol. 351, 127057 https://doi.org/10.1016/j.biortech.2022.127057.

Maibam, P.D., Maiti, S.K., 2020. A strategy for simultaneous xylose utilization and enhancement of cellulase enzyme production by *Trichoderma reesei* cultivated on liquid hydrolysate followed by induction with feeding of solid sugarcane bagasse. Waste Biomass-.-. Valoriz. 11, 3151–3160.

Méndez-Líter, J.A., de Eugenio, L.I., Nieto-Domínguez, M., Prieto, A., Martínez, M.J., 2021. Hemicellulases from Penicillium and Talaromyces for lignocellulosic biomass valorization: A review. Bioresour. Technol. 324, 124623 https://doi.org/10.1016/j.biortech.2020.124623.

Mitchell, T.J., 2000. An algorithm for the construction of "D-optimal" experimental designs. Technometrics 42 (1), 48–54. https://doi.org/10.1080/00401706.2000.10485978.

Nath, P., Dhillon, A., Kumar, K., Sharma, K., Jamaldheen, S.B., Moholkar, V.S., Goyal, A., 2019. Development of bi-functional chimeric enzyme (CtGH1-L1-CtGH5-F194A) from endoglucanase (*Ct*GH5) mutant F194A and β-1, 4-glucosidase (CtGH1) from *Clostridium thermocellum* with enhanced activity and structural integrity. Bioresour. Technol. 282, 494–501. https://doi.org/10.1016/j.biortech.2019.03.051.

Nath, P., Maibam, P.D., Singh, S., Rajulapati, V., Goyal, A., 2021. Sequential pretreatment of sugarcane bagasse by alkali and organosolv for improved delignification and cellulose saccharification by chimera and cellobiohydrolase for bioethanol production. 3 Biotech 11, 1–16.

Nedumaran, M., Singh, S., Jamaldheen, S.B., Nath, P., Moholkar, V.S., Goyal, A., 2020. Assessment of combination of pretreatment of Sorghum durra stalk and production of chimeric enzyme (β-glucosidase and endo β-1, 4 glucanase, *Ct* GH1-L1-*Ct* GH5-F194A) and cellobiohydrolase (*Ct* CTCBH5A) for saccharification to produce bioethanol. Prep. Biochem. Biotechnol. 50 (9), 883–896. https://doi.org/10.1080/10826068.2020.1762214.

Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153 (2), 375–380.

Pattanaik, A., Rayasam, V., 2018. Analysis of reverse cationic iron ore fines flotation using RSM-D-optimal design–An approach towards sustainability. Adv. Powder Technol. 29 (12), 3404–3414. https://doi.org/10.1016/j.apt.2018.09.021.

Pillai, N.S., Kannan, P.S., Vettivel, S.C., Suresh, S., 2017. Optimization of transesterification of biodiesel using green catalyst derived from Albizia lebbeck Pods by mixture design. Renew. Energy 104, 185–196. https://doi.org/10.1016/j.renene.2016.12.035.

Rajak, R.C., Banerjee, R., 2018. An eco-friendly process integration for second generation bioethanol production from laccase delignified Kans grass. Energy Convers. Manag. 157, 364–371. https://doi.org/10.1016/j.enconman.2017.11.060.

Rajak, R.C., Banerjee, R., 2020. An innovative approach of mixed enzymatic venture for 2G ethanol production from lignocellulosic feedstock. Energy Convers. Manag. 207, 112504 https://doi.org/10.1016/j.enconman.2020.112504.

Samayam, I.P., Schall, C.A., 2010. Saccharification of ionic liquid pretreated biomass with commercial enzyme mixtures. Bioresour. Technol. 101 (10), 3561–3566. https://doi.org/10.1016/j.biortech.2009.12.066.

Saritha, M., Arora, A., Singh, S., Nain, L., 2013. Streptomyces griseorubens mediated delignification of paddy straw for improved enzymatic saccharification yields. Bioresour. Technol. 135, 12–17. https://doi.org/10.1016/j.biortech.2012.11.040.

Singh, S., Rajulapati, V., Jamaldheen, S.B., Moholkar, V.S., Goyal, A., 2020. Statistically designed cellulase mixture for saccharification of pretreated Sorghum durra stalk. Ind. Crops Prod. 154, 112678 https://doi.org/10.1016/j.indcrop.2020.112678.

Somogyi, M., 1945. A new reagent for the determination of sugars. J. Biol. Chem. 160 (1), 61–68.

TAPPI, 1992. Technical Association of the Pulp, Paper Industry, TAPPI test methods.

Thakur, A., Sharma, K., Jamaldheen, S.B., Goyal, A., 2020a. Molecular characterization, regioselective and synergistic action of first recombinant type iii α-l-arabinofuranosidase of family 43 glycoside hydrolase (PsGH43_12) from *Pseudopedobacter saltans*. Mol. Biotechnol. 62 (9), 443–455.

Thakur, A., Sharma, K., Khaire, K.C., Moholkar, V.S., Goyal, A., 2020b. Enzymes: key role in the conversion of waste to bioethanol. In: Thatoi, H. (Ed.), Microbial Fermentation and Enzyme Technology. CRC Press, pp. 257–268.

Varanda, C., Portugal, I., Ribeiro, J., Silva, A.M., Silva, C.M., 2017. Optimization of bitumen formulations using mixture design of experiments (MDOE). Constr. Build. Mater. 156, 611–620. https://doi.org/10.1016/j.conbuildmat.2017.08.146.

Velasco, J., Pellegrini, V.D.O.A., Sepulchro, A.G.V., Kadowaki, M.A.S., Santo, M.C.E., Polikarpov, I., Segato, F., 2021. Comparative analysis of two recombinant LPMOs from Aspergillus fumigatus and their effects on sugarcane bagasse saccharification. Enzym. Microb. Technol. 144, 109746 https://doi.org/10.1016/j.enzmictec.2021.109746.

Wang, Y., Liu, P., Zhang, G., Yang, Q., Lu, J., Xia, T., Peng, L., Wang, Y., 2021. Cascading of engineered bioenergy plants and fungi sustainable for low-cost bioethanol and high-value biomaterials under green-like biomass processing. Renew. Sustain. Energy Rev. 137, 110586 https://doi.org/10.1016/j.rser.2020.110586.

Zafar, M., Van Vinh, N., Behera, S.K., Park, H.S., 2017. Ethanol mediated As (III) adsorption onto Zn-loaded pinecone biochar: experimental investigation, modeling, and optimization using hybrid artificial neural network-genetic algorithm approach. J. Environ. Sci. 54, 114–125. https://doi.org/10.1016/j.jes.2016.06.008.

Zhang, H., Han, L., Dong, H., 2021. An insight to pretreatment, enzyme adsorption and enzymatic hydrolysis of lignocellulosic biomass: Experimental and modeling studies. Renew. Sustain. Energy Rev. 140, 110758 https://doi.org/10.1016/j.rser.2021.110758.

Zhang, R., Gao, H., Wang, Y., He, B., Lu, J., Zhu, W., Peng, L., Wang, Y., 2023a. Challenges and perspectives of green-like lignocellulose pretreatments selectable for low-cost biofuels and high-value bioproduction. Bioresour. Technol. 369, 128315 https://doi.org/10.1016/j.biortech.2022.128315.

Zhang, R., Hu, Z., Wang, Y., Hu, H., Li, F., Li, M., Ragauskas, A., Xia, A., Han, H., Tang, J., Yu, H., Xu, B., Peng, L., 2023b. Single-molecular insights into the breakpoint of cellulose nanofibers assembly during saccharification. Nat. Commun. 14 (1), 1100 https://doi.org/10.1038/s41467-023-36856-8.