Saccharification and fermentation of Finger millet straw using recombinant hydrolytic enzymes and fermentative microbe for bioethanol production

PhD Thesis

by

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CENTRE FOR ENERGY

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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Saccharification and fermentation of Finger millet straw using recombinant hydrolytic enzymes and fermentative microbe for bioethanol production

A Thesis

Submitted in partial fulfillment of the requirements for the Degree of

Doctor of Philosophy

by

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Under supervision of

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STATEMENT

I do hereby declare that the content embodied in this thesis entitled as **"Saccharification and fermentation of Finger millet straw using recombinant hydrolytic enzymes and fermentative microbe for bioethanol production"** is the result of investigations carried out by me in the Centre for Energy, Indian Institute of Technology Guwahati, Guwahati, India under the guidance of Professor Arun Goyal and Professor V. S. Moholkar.

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.

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Saccharification and fermentation of Finger millet straw using recombinant hydrolytic enzymes and fermentative microbe for bioethanol production"** by **Sumitha Banu J. (Roll No. 136151003)** 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 at the Centre for energy, Indian Institute of Technology Guwahati, Guwahati, India and this work has not been submitted elsewhere for a degree.

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Synopsis

Introduction

Initially, bioethanol was produced by fermentation of the first generation feedstocks including starchy (corn, wheat, tubers, etc.) and sugar crops (sugarcane, sugarbeet, etc.) (Nigam and Singh 2011). Since the first generation feedstocks are food crops, exploiting them for bioethanol production will directly compete and affect the world's food supply. Hence, this led to the exploration of the non-edible second generation feedstocks i.e., lignocellulosic biomass including agricultural wastes, forest wastes, grasses and weeds (Nigam and Singh 2011, Tenenbaum 2008). Lignocellulosic feedstocks are cheaper and their utilization in bioethanol production supports the enhancement of local agricultural economy too (Lange 2007). Eventhough the technology for bioethanol had been commercialized, the process is complicated due to the complex nature of lignocellulose unlike starchy feedstock. Therefore, it needs more troubleshooting in converting the lignocellulose to sugars for ethanol production (Isikgora and Becer 2015).

As the name indicates, lignocellulosic feedstocks contain cellulose, hemicellulose and lignin as the major components. Having a structural composition different from the starchy feedstocks, lignocellulosic feedstocks need an extra and very significant step called pretreatment to remove the rigid lignin exposing the cellulose and

hemicellulose for further enzyme hydrolysis. Hydrolysis with cellulase and hemicellulase results in the release of hexoses and pentoses where the sugars are consumed by fermentative microbes as the carbon source to produce bioethanol (Hagerdal et. al. 2006).

Various pretreatment methods have been tried over different types of lignocellulosic feedstock. Pretreatment methods can mainly be classified into physical, chemical, physico-chemical and biological treatments. Depending on the lignin content and biomass strength, the pretreatment method varies. But the better way is to screen out the best method among them by experimenting each of them for each feedstock (Singh et. al. 2014, Wang et. al. 2012).

Cellulases and hemicellulases are the hydrolytic enzymes that carry out hydrolysis of cellulose and hemicellulose of the pretreated biomass. Different types of cellulases exist with respect to their site of action and the main classes are exo-β-glucanase, endo-β-glucanase and β-glucosidase. Endo-β-glucanases act on internal glycosidic bonds of the cellulose chain where an exo-β-glucanase acts at the reducing or non-reducing terminal of the chain to release di- or tetra-saccharide. β-glucosidase works on the disaccharide, cellobiose to release glucose. Hemicellulases are of different types including endo-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, and acetylxylan esterase according to their specificity towards substrates. During the hydrolysis for ethanol production, the most employed hemicellulase is xylanase because xylan is the most abundantly present hemicellulose in herbs and hardwoods (Wang et. al. 2012).

Ethanol fermentation can be carried out with a variety of traditionally relevant and industrially significant fermentative microbes among which candidates such as *Saccharomyces cerevisiae, Zymomonas mobilis,* etc. utilize hexose and microbes such as *Candida shehatae* and *pichia stipitis* utilize pentose especially xylose as their carbon source (Toivola et. al. 1984, Lu et. al. 2013). The concept, biorefinery had already been investigated a lot related to biofuels. After the recovery of the desired product from a raw material, extraction of any other low/high value added chemicals from the residue or converting the final residue as the primary input for production of another product is termed as biorefinery. Coming to bioethanol fermentation, lignin is used up in the production of electricity (Tolan 2006).

This study focuses on **screening of pearl millet, finger millet and sorghum straws with respect to their structural composition, pretreatment of the selected feedstock, enzymatic hydrolysis of the pretreated biomass using cellulase and hemicellulase and fermentation of the released sugars and application of biorefinery concept to utilize the waste remaining after fermentation**.

Chapter 1 presents the general introduction and literature review on ethanol production from lignocellulosic biomass. It highlighted the advantages of utilizing lignocellulosic feedstock for ethanol production over food crops. It showed the companies that are involved in ethanol production worldwide and the obstacles in ethanol production from lignocellulose due to its complex structure. It showed the details of the components of lignocllulosic feedstock, various pretreatment methods and their effect on lignocellulosic biomass to bring the cellulose and hemicellulose available for the enzymes for sachharification. It showed the various enzymes involved in saccharification of cellulose and hemicellulose from the pretreated biomass and

fermentation classifications for the utilization of the hexose and pentose sugars by fermenting microbes for ethanol production. It highlighted the significance of utilizing the millet straws, agrowastes from less irrigated areas as feedstock for ethanol production.

Chapter 2 focuses on the comparison of the potential of three agrowastes from rainfed areas, Sorghum, Finger millet and Pearl millet straw as a feedstock for bioethanol production. The comparison was carried out with respect to the carbohydrate composition of the selected three feedstocks. All the three biomasses contained similar carbon (43% (w/w)) and hydrogen (6% (w/w)) contents. Pearl millet contained the highest nitrogen content (2.3% (w/w)) and the lowest moisture content (12.5% (w/w)). The highest holocellulose (69 wt%), cellulose (36 wt%) and hemicellulose (33.5 wt%) contents were observed in the Finger millet straw. ADL (5 wt%) contents were similar in all the three feedstocks. Therefore, with respect to the availability of higher carbohydrate content, Finger millet straw was selected to be the best among the three feedstocks for pretreatment process for ethanol production.

Chapter 3 deals with the comparative analysis of 12 pretreatment methods on Finger millet straw to find the best pretreatment method for saccharification. Among the 12 pretreatment methods on FMS involving oven heating, autoclaving, microwaving and ultra-sonication in the presence of 1% (v/v) H_2SO_4, 1% (w/v) NaOH or distilled water, NaOH treatments aided for endo-1,4-β-xylanase (*Ct*Xyn11A) involved saccharification. Among the four NaOH pretreated FMS samples, the enzyme endo-1,4-β-xylanase (*Ct*Xyn11A) was most active on the FMS treated with 1% (w/v) NaOH combined with oven heating that resulted in TRS, 32 mg/g pretreated biomass. Endo-1,4-β-glucanase (*Ct*Cel8A) gave low levels of saccharification on FMS, treated

with 1% (w/v) NaOH combined with oven heating (2.3 mg/g pretreated biomass) and with 1% (w/v) NaOH combined with autoclaving (3 mg/g pretreated biomass). The pretreament of FMS by NaOH combined with oven heating resulted in higher holocellulose (76%, w/w) and biomass yield (0.36 g/g raw biomass) as compared to the pretreatment by NaOH combined with autoclaving, that gave holocellulose 67% (w/w) and biomass yield, 0.15 g/g raw biomass. Thus, 1% (w/v) NaOH combined with oven heating at 120°C for 20 min was the best pretreatment method for FMS, as it provided better accessibility to both enzymes resulting in higher TRS yield. The FTIR spectra of pretreated biomass confirmed hemicllulose breakdown and delignification rendered by alkali treatment. The enhanced surface roughness of the pretreated Finger millet straw was clearly visible in FESEM image. Hemicellulose saccharification of the above pretreated FMS using endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) can be optimized to enhance the TRS yield.

Chapter 4 deals with the optimization of hemicellulose saccharifiction from the pretreated (1% (w/v) NaOH + Oven heating) Finger millet straw (FMS) by recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus*. Finger millet straw pretreated with 1% (w/v) NaOH combined with oven heating containing a hemicellulose content of 20.7 % (w/w) was subjected to a sequential saccharification with recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus*. Two step optimization was done using Box-behnken design, RSM. The factors for the hemicellulose hydrolysis to xylo-oligosaccharides (XOS) using endo-1,4-β-xylanase (*Ct*Xyn11A), i.e., biomass loading (A_1), xylanase loading (B_1) and hydrolysis time (C_1) were varied from 1- 7 % (w/v),

50- 600 U/g ptd biomass and 4- 72 h, respectively. The predicted optimum biomass loading (A_1), xylanase loading (B_1) and hydrolysis time (C_1) were 6 % (w/v), 559 U/g ptd biomass and 56 h, rspectively. The predicted TRS yield, $TRS_{(XOS)}$ 39.3 mg/g ptd biomass. The TRS yield from the experiment under the above conditions was 39.6 ± 0.6 mg/g ptd biomass (2.4 g/L). The parameters, xylosidase loading (A_2), XOS concentration (B_2) and hydrolysis time (C_2) for the hydrolysis of XOS using exo-1,4-β-xylosidase (*Bo*GH43A) were varied from 2- 40 U/mL, 0.52- 2.08 g/L and 5- 120 min, respectively. The predicted optimum xylosidase loading (A_2), XOS concentration (B_2) and hydrolysis time (C_2) were 40 U/mL, 2.08 g/L and 79.4 min, respectively. The predicted TRS yield from the optimum conditions was 1.84 g/g of XOS. The experimental TRS yield from the above optimum conditions was 1.77 g/g of XOS, which was equivalent to 70 mg/g ptd biomass. Saccharification of the pretreated FMS by *Ct*Xyn11A and *Bo*GH43A under optimized conditions resulted in 24.7% conversion of xylan to xylose.

Chapter 5 focusses on the detoxification of acid hydrolysate resulted from the acid pretreatment (1% (v/v) H_2SO_4 combined with autoclaving) of FMS by overliming with $Ca(OH)_2$ and ethanol production from the detoxified hydrolysate using *Pichia stipitis* NCIM-3497 and pyrolysis of the pretreated solid residue. The final xylose concentration obtained in the saccharified solution was 2 g/L. Since the xylose content in the acid hydrolysate (7.8 g/L) from the pretreatment involving 1% (v/v) H_2SO_4 combined with autoclaving of FMS (chapter 3) than in the saccharified solution (2 g/L) from chapter 4, acid hydrolysate was used as the feed for fermentation. The detoxified hydrolysate resulted from the sulphuric acid treatment along with autoclaving of FMS

contained 6 g/L of xylose and 2.3 g/L of arabinose. Fermentation of the detoxified hydrolysate using *Pichia stipitis* NCIM-3497 at 30°C, pH 5 and 150 rpm for 96 h resulted in 1.3 g/L of ethanol and 3.1 g/L of cell mass concentration. 3.3 g/L of xylose was consumed by *Pichia stipitis* leading to an ethanol yield of 0.39 g/g xylose consumed. Fermentation of the detoxified hydrolysate supplemented with synthetic fermentation medium components except xylose by *Pichia stipitis* NCIM-3497 gave the ethanol production, 2 g/L. The biomass concentration was increased to 4.4 g/L in the presence of synthetic media components. Utilization of 5.1 g/L out of 6 g/L of xylose by *Pichia stipitis* led to the ethanol yield of 0.39 g/g of xylose consumed. Arabinose remained unutilised by *Pichia stipitis* in both the fermentations. In the presence of synthetic medium components except xylose in the detoxified hydrolysate, *Pichia stipitis* NCIM-3497 was able to consume more xylose leading to 35% higher ethanol production than in the plain detoxified hydrolysate. Pyrolysis of the solid residue from sulphuric acid treatment of FMS resulted in 41.98% (w/w) and 27.2% (w/w) of bio-oil and char, respectively. The density and pH of the bio-oil were 1.1 g/mL and 3.5, respectively. The bio-oil consisted of ~50% (w/w) of aromatics, 13.7% of (w/w) heterocyclics and 12% (w/w) of non–aromatics. The bio-oil contained abundant furfural, 1-(2-hydroxy-5-methylphenyl)-ethanone and 4-allyl syringol. These valuable products can be refined from the bio-oil further for their applications in food and resin industries.

CONTENTS

Chapter 1

General Introduction

1.1. Introduction

The major concern over the continuous depletion of non-renewable fossil fuels, their negative impact on the atmosphere due to the emission of greenhouse gases and energy security had led the scientists to search for alternative and sustainable fuels from renewable resources. The International Plant Protection (IPPC) report on Global warming in 2018 warned that earth temperature will rise by 1.5°C between 2030 to 2052, if the current rate of greenhouse gas emission is not controlled (Zhang 2019). Biofuels are regarded to be neutral fuels as the CO_2 produced during their combustion is utilized during their regrowth (Lange 2007, Ruiz *et. al.* 2012). Biofuels are classified into primary biofuel (directly obtained as heat energy or electricity after combustion of the biomass) and secondary biofuel (obtained as solid, liquid or gaseous product after processing the feedstock). Bioethanol, biodiesel, biogas, biohydrogen, etc. will come under the secondary biofuel among which, bioethanol and biodiesel are the main alternative transportation fuels. Initially, bioethanol was produced by fermentation of

the first generation feedstocks including starchy crops such as wheat, corn, and tubers and sugar crops including sugarcane and sugarbeet (Nigam and Singh 2011). Since the first generation feedstocks are food crops, exploiting them for bioethanol production will directly compete and affect the world's food supply. Therefore, this led to the exploitation of the non-edible second generation feedstocks. This includes lignocellulosic biomass such as grasses, weeds, forest wastes and agricultural wastes (Nigam and Singh 2011, Tenenbaum 2008).

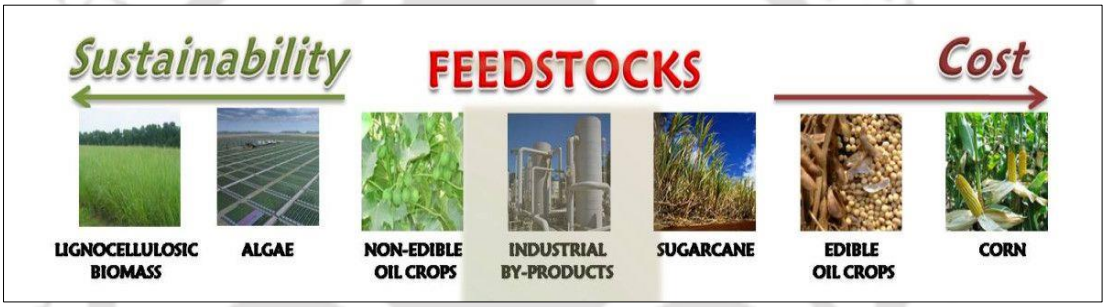

Fig. 1.1.1. Scheme for selection of feedstock for biofuel production (Adapted from Dellomonaco *et. al.* 2010)

Lignocellulosic feedstocks are cheaper as they are plant wastes. Utilization of agrowastes for bioethanol production will boost the local agricultural economy (Lange 2007). Table 1.1.2. shows the companies that produce ethanol from lignocellulosic feedstock. Eventhough the technology for bioethanol has been commercialized, the process is complicated due to the complex nature of lignocellulose unlike starchy feedstock. Therefore, it needs more troubleshooting in converting the lignocellulose to sugars for ethanol production (Isikgora and Becer 2015).

Table 1.1.1. Companies producing bioethanol from lignocellulosic biomass (Isikgora and Becer 2015).

Bioethanol company	Location	Lignocellulosic feedstock
Abengoa	USA	Corn stover, wheat straw, sorghum stubble and switch grass
Abengoa	Spain	Wheat straw
ALICO, Inc.	USA	Yard and citrus waste
American Process Inc.	USA	Woody biomass
BioEthanol Japan	Japan	Wood construction waste
BioFuels Energy Corp.	USA	Grass and tree trimmings
BlueFire Ethanol	USA	Green waste
Borregaard Industries Ltd	Norway	Wood
British Sugar	England	Sugar beet
Broin jointly with US DoE, DuPont and Novozymes	USA	Corn fiber and stover
Colusa Biomass Energy Corporation	USA	Rice straw and hulls
China Resources Alcohol Corporation	China	Corn stover
DINS Sakai	Japan	Waste construction wood
ICM Inc.	USA	Corn stover and switchgrass
Inbicon	Denmark	Wheat straw
Iogen	USA	Wheat straw, barley straw, corn stover, switchgrass and rice straw
Iogen	Canada	Wheat, oat and barley straw
Lignol	Canada	Softwood and hardwood
Mascoma	USA	Paper sludge, wood chips, switch grass and corn stover
Pacific Ethanol Inc.	USA	Wheat straw, corn cob and woody biomass
Poet	USA	Corn fiber and corn stover
Range Fuels	USA	Timber and forest residue
Sekab	Sweden	Forestry products
Tereos	France	Sugar beet, wheat and sugar cane
Verenium	USA	Sugarcane bagasse and specially bred energy cane
Western Biomass	USA	Ponderosa pine wood chips and waste

As the name suggests, lignocellulosic feedstocks contain cellulose, hemicellulose and lignin as the main components.

As the structural composition is different from the starchy feedstocks, lignocellulosic feedstocks need an extra and very significant step called pretreatment

to get rid of the rigid lignin to expose the cellulose and hemicellulose for enzymatic hydrolysis. Saccharification of cellulose and hemicellulose with cellulase and hemicellulase, respectively leads to the release of hexose and pentose sugars. These sugars will be consumed as the carbon source by fermentative microbes for bioethanol production (Hagerdal *et. al.* 2006).

Different pretreatment methods have been employed on various types of lignocellulosic feedstocks. Pretreatment methods can mainly be classified into physical, chemical, physico-chemical and biological treatments. The pretreatment method can be varied depending on the biomass strength and lignin content. But the better way is to experiment different pretreatment methods on each feedstock and screen the best method for them (Wang *et. al.* 2012, Singh *et. al.* 2014). Fig. 1.1.2. shows the effect of pretreatment on lignocellulosic biomass.

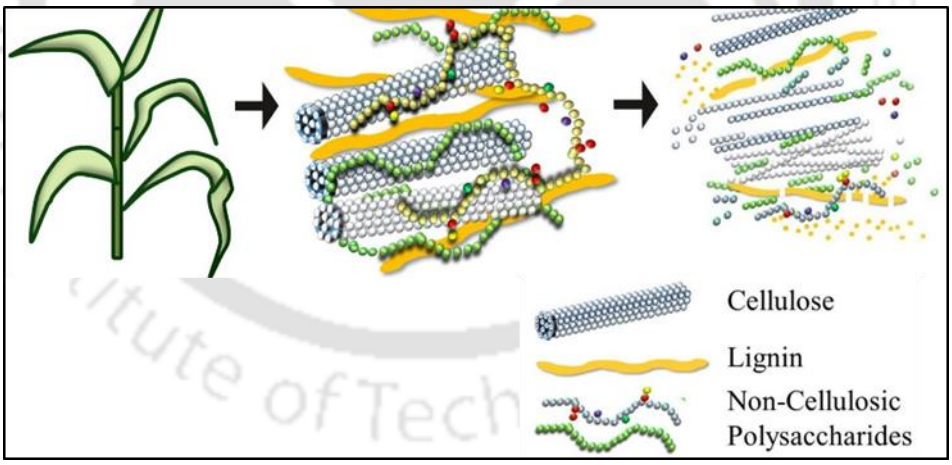

Fig. 1.1.2. General scheme for pretreatment of lignocellulosic feedstock (Adapted from Tan *et. al.* 2016)

Cellulases and hemicellulases are the hydrolytic enzymes which carry out hydrolysis of cellulose and hemicellulose, respectively of the pretreated biomass. Different types of cellulases exist with respect to their site of action and the main classes are exo-β-glucanase, endo-β-glucanase and β-glucosidase. Endo-β-glucanases

act on internal glycosidic bonds of the cellulose chain where an exo-β-glucanase acts at the reducing or non-reducing terminal of the chain to release di- or tetra-saccharide. β-glucosidase works on the disaccharide, cellobiose to release glucose. Lignocellulosic biomass contains hemicellulose, the second most abundant polysaccharide. Xylan is the most occurring component in hemicellulose. Hemicellulases are of different types including endo-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylxylan esterase, mannanase and galactanase according to their specificity towards substrates. During the hydrolysis for ethanol production, the most employed hemicellulase is xylanase because xylan is the most abundantly present hemicellulose in herbs and hardwoods (Wang *et. al.* 2012).

Ethanol fermentation can be carried out with a variety of traditionally relevant and industrially significant fermentative microbes among which candidates such as *Saccharomyces cerevisiae, Zymomonas mobilis,* etc. utilize hexose (Lu *et. al.* 2013) and microbes such as *Candida shehatae* and *pichia stipitis* utilize pentose especially xylose as their carbon source (Toivola *et. al.* 1984). Co-fermentation with these microbes from hydrolysates rather than employing single species results in higher ethanol yield than the sum of their individual yields (Toivola *et. al.* 1984, Fu *et. al.* 2009, Ndaba *et. al.* 2014).

Conventional bioconversion of lignocellulosic biomass to bioethanol through pretreatment, enzymatic hydrolysis and fermentation as individual operations is termed as Separate Hydrolysis and Fermentation (SHF). Inspite of lignocellulosic feedstocks being cheaper and abundant, involvement of more number of processing steps increases the operation cost which inturn makes bioethanol, an economically non-viable product as compared with the petroleum fuel (Hagerdal *et. al.* 2006, Lange

2007). This issue can be overcome by combining the hydrolysis and fermentation steps thereby decreasing the processing cost and operating time as well. Unlike SHF, it eliminates the formation of inhibitors after the hydrolysis as the released sugars are readily consumed by the microorganism for fermentation. This mode of fermentation is called Simultaneous Saccharification and Fermentation (SSF). When SSF is carried out with two or more fermenting microbes, it is said to be Simultaneous Saccharification and Co-Fermentation (SSCF) (Hagerdal *et. al.* 2006, Koppram *et. al.* 2013). Semi-Simultaneous Saccharification and Fermentation (SSSF) is nothing but a modified SSF process where, a pre-hydrolysis is carried out and without separating the solid mass from the hydrolysate, fermentation is continued thus gaining the benefits of both SHF and SSF (Lu *et. al.* 2013). Comparative assessment of different modes of fermentation will help in understanding the behavior of the microbial system in different environments and their synergistic effect towards efficient bioethanol production.

1.2. Review of literature

1.2.1. Feedstock selection for ethanol production

The large scale ethanol production depends on the feedstock selection, which is very important step on which the entire process depends. Wise selection of feedstock will increase the industrial as well as local agricultural economy. Table 1.2.1. shows the structural composition of various lignocellulosic feedstocks. Iogen Corporation (Ottawa, Canada) is a well-known and successful producer of bioethanol from wheat (Tolan 2006). According to Iogen, the crucial propreties that should be considered during the selection of feedstock for biothanol production are as follows:

1. The raw material should have a low market value inturn low cost and limited usage
2. Feedstock must be locally available
3. Biomass must maintain uniformity without being mixed with other materials
4. It should have low level of silica and less prone to microbial contamination
5. It should have high cellulose and hemicellulose content thus resulting in high ethanol yield
6. After pretreatment, it must undergo high efficiency of conversion.

Table 1.2.1. Feedstock composition (mg/g total solids) (Tolan 2006).

Feedstock	Cellulose	Starch	Xylan	Arabinan	Lignin	Ash	Protein
Barley straw	406	20	161	28	168	82	64
Wheat straw	455	9	165	25	204	83	64
Wheat chaff	391	14	200	36	160	121	33
Switch grass	399	3	184	38	183	48	54
Corn stover	408	3	128	35	127	60	81
Maple wood	500	4	150	5	276	6	6
Pine wood	648	1	33	14	320	0	2

1.2.2. Physical and structural properties of lignocellulosic biomass

a) Cellulose

Cellulose is linear chain polymer composed of glucose units linked by β-1,4 linkages that are held together as rigid fibrous bundles providing mechanical strength to the material. It constitutes around 40-50 wt% of the lignocellulosic biomass. Its average molecular weight is 100,000 (Lange 2007, Anwar *et. al.* 2014).

b) Hemicellulose

Hemicellulose consists of number of shorter polymers made of hexose, mannans and glucans and pentose sugars like xylose linked by β-1,4 linkages. It binds the cellulose bundles together and it accounts for about 25-35 wt% of lignocellulose. Average molecular weight of hemicellulose is less than 30,000 (Lange 2007, Anwar *et. al.* 2014).

c) Lignin

Being the most complex and smallest fraction of lignocellulose, lignin represents about 10-25 wt% of the biomass. It contains long-chain, heterogeneous polymer composed of phenyl-propane units linked by ether bonds. It acts like a glue filling the gaps in and around the cellulose and hemicellulose complexes providing rigidity to the biomass (Anwar *et. al.* 2014).

1.2.3. Pretreatment methods

The various types of well exploited pretreatments are classified under the following four major categories (Mood *et. al.* 2013):

1. Physical pretreatment: The biomass is reduced to micro particle size by milling.
2. Chemical pretreatment: Variety of chemicals such as acid, alkali, organosolvents, ionic liquids, etc. are used for delignification.

3. Physico-chemical pretreatment: The biomass size reduction and chemical reaction for delignification are carried out together with or without elevation in temperature.
4. Biological pretreatment uses microorganisms, mainly fungus to remove the lignin from the biomass.

1.2.3.1. Physical pretreatment

1.2.3.1.1. Mechanical comminution

Mechanical comminution, milling or grinding refers to the reduction of the size of the lignocellulosic biomass to increase the surface area for enzyme action during hydrolysis. It basically depolymerises the cellulose fibre and reduces its crystallinity to some extent but mechanical comminution alone will not serve as an effective pretreatment resulting in very less sugar release after enzymatic hydrolysis due to poorly exposed cellulose and hemicellulose (Singh *et. al.* 2014).

1.2.3.1.2. Extrusion

Extrusion is the process of passing and compression of the biomass through a die of desired cross section at desired temperature, pressure and rotation speed. The setup consists of a single or twin screw inside a barrel thereby applying shear force onto the biomass and once the biomass comes out of the die, it undergoes sudden expansion leading to the breakdown of the linkanges and deformation of the structural components. Low cost, better process monitoring and continuous operation make it feasible method for pretreating lignocellulosic biomass (Zheng and Rehmann 2014).

1.2.3.1.3. Irradiation

Irradiation of the lignocellulosic biomass using gamma rays, electron beam or microwaves, under controlled condition can cause the breakdown of the linkages between the cellulose and hemicellulose structures too by breaking and penetrating

through the lignin covering, resulting in the release of even cellobiose. The drawbacks of this method are high irradiation can degrade glucose molecule and it is very expensive leaving that difficult for industrial application (Taherzadeh and Karimi 2008).

1.2.3.2. Chemical pretreatment

1.2.3.2.1. Acid treatment

Dilute acid hydrolysis using H_2SO_4, HNO_3 or HCl is the most commonly used pretreatment method, because of its cost effectiveness and efficiency (Ranjan and Moholkar 2013). Concentration of reducing sugar and formation of degradation products like Furfural and 5-Hydroxy-Methyl Furfural (HMF) limits the utility of this method (Gupta *et. al.* 2013).

1.2.3.2.2. Alkali treatment

Alkali treatment is the better among the chemical pretreatments due to its effective delignification and reduction of degree of cellulose polymerization at normal laboratory conditions with better process monitoring. The method is carried out with potassium, calcium and or ammonium hydroxide but separation of the salt after the treatment is difficult and requires large quantities of water to wash it (Gupta *et. al.* 2013, Singh *et. al.* 2014).

1.2.3.2.3. Organosolv treatment

This method uses organic solvents like alcohol, acetone, formic acid, acetic acid, peracetic acid, phenol, ethylenediamine, etc. at a temperature range of 100-250^{o}C to solubilize lignin and separate it as solid during recovery. The drawbacks of this method are that organic solvents are expensive, reprecipitation of the dissolved lignin

during water wash and recovering the solvent back adds an extra step in the process thus increasing the energy consumption and operational cost (Zhao *et. al.* 2009).

1.2.3.2.4. Ionic liquid treatment

Comprising large organic cations and small inorganic anions, Ionic liquids act like a salt but exist in liquid form. The anions bind to cellulose (but not with hemicellulose or lignin) and solubilize it at high temperature range, 100-150^{o}C. Antisolvents like water, ethanol, acetone and methanol are used for the regeneration of cellulose from the ionic liquid, but residual ionic liquid in the cellulose affects in the enzyme hydrolysis. Ionic liquids are very expensive. Adding to that, hemicellulose and lignin should be recovered separately (Gupta *et. al.* 2013, Maurya *et. al.* 2015).

1.2.3.2.5. Surfactant treatment

Surfactants such as Sodium dodecyl sulfate (SDS) and Triton X 100 are also used for pretreating lignocellulosic biomass. It is shown that they can help only in breaking the lignin bonds but not in lignin removal and exposing cellulose for further enzymatic hydrolysis (Singh *et. al.* 2014).

1.2.3.3. Physico-chemical pretreatment

1.2.3.3.1. Steam explosion

Steam explosion or auto-hydrolysis means the process of using steam under pressure as the penetrating weapon through the rigid walls of the lignocellulosic biomass thereby exerting pressure on the inner layers at around 120^{o}C temperature causing them to expand and explode when the pressure is suddenly released. The simpler laboratory model of steam explosion operation is autoclaving (Ranjan and Moholkar 2013, Singh *et. al.* 2014).

1.2.3.3.2. Wet oxidation

Wet oxidation is an exothermic reaction where the biomass is treated with water and air/oxygen at temperature above 120^{o}C for 30 minutes. Due to the presence of oxygen, the reaction is faster and much heat is generated leading to the breakdown and oxidation of lignin, extensive and partial degradation of hemicellulose and cellulose (Taherzadeh and Karimi 2008).

1.2.3.3.3. Ammonia Fibre Expansion (AFEX)

AFEX is a potential pretreatment technique where the biomass is exposed to liquid ammonia at temperature ranging from 70 to 200^{o}C and pressure from 100 to 400 psi and rapid release of pressure resulting in biomass structural expansion. It decrystallizes cellulose and depolymerises lignin. Ammonia loading, temperature and pressure are the important parameters to be taken care. Operation at high pressure and recovery of the ammonia for reuse increases the energy consumption and processing cost (Bals *et. al.* 2010).

1.2.3.3.4. CO_2 explosion

Gas like mass transfer property and liquid like solvating ability give supercritical CO_2 the power to penetrate and disrupt the cellulosic biomass. High temperature and high pressure aid for the efficient treatment. Irrespective of supercritical or subcritical temperature, high pressure should be applied for the penetration of the CO_2 into the pores of lignocellulosic biomass (Taherzadeh and Karimi 2008).

1.2.3.3.5. Extrusion combined with acid/alkali treatment

The better adaptable design of the process makes extrusion to act as acid/alkali hydrolysis reactor by the addition of dilute acid or alkali in the setup and operating at

desired temperature, pressure and rotating speed of the screw thereby combining the advantages of both the extrusion and acid/alkali hydrolysis of lignocellulose (Zheng and Rehmann 2014).

1.2.3.4. Biological pretreatment

Biological pretreatment does not involve any chemical or high energy as in physical and chemical pretreatments. This makes it environmental friendly and low cost technology. Lignin can be degraded by a variety of fungi most commonly used are white rot fungus such as *Phanerochaete chrysosporium* (Shi *et. al.* 2009), *Ceriporiopsis subvermispora* (Wan and Li 2010), *Lentinula edodes*, *Pleurotus eryngii* and *Pleurotus salmoneostramineus* (Okano *et. al.* 2006). Several white rot fungi including *Pleurotus ostreatus* (oyster mushroom) have been extensively explored for their role in wood decay by producing lignolytic enzyme called laccase (Mansur *et. al.* 2003, Brijwani *et. al.* 2010). The other enzymes present in fungi, that play significant role in biological degradation of lignocellulosic biomass are xylanase, lignin peroxidase (LiP) and manganese peroxidase (MnP) (Woolridge 2014).

1.2.4. Saccharification

A wide range of bacterial and fungal species produce cellulase and hemicellulase. The most exploited cellulase producing industrially important microbes are *Clostridium thermocellum, Trichoderma reesi, Pseudomonas fluorescens, Bacillus amyloliquefaciens*, etc (Coughlan 1985). To enhance the productivity of the enzyme, the gene encoding the enzyme is expressed in fast growing model organism like *E. coli* (Coughlan 1985, Singh *et. al.* 2013). Taking the advantage of fast growth of *E. coli*, the desired gene encoding cellulase from the genome of the microorganism can be amplified by Polymerase Chain Reaction (PCR), cloned into an expression vector,

such as pET28a and transformed into competent *E. coli* cells for expression. The protein expression is induced by the addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside) (Amore et al. 2014). Different types of cellulases and hemicellulases are commercially available but in-house production of the enzymes will reduce the processing cost. In the saccharification process, the activity of the hydrolytic enzymes is measured with respect to the unit of reducing sugar released per unit of enzyme per unit time. Fig. 1.2.1. shows the mode of action of cellobiohydrolase or exo-β-glucanase (CBH), endo-β-glucanase (EG) and β-glucosidase (βG) in hydrolyzing cellulose to glucose. Endo-β-1,4-glucanase catalyzes the breakdown of internal bonds of the cellulose chain at random sites. The exo-β-1,4-glucanase, CBH I and CBH II attacks the cellulose chain at the reducing end and non-reducing end, respectively to release cellobiose. Hydrolysis of the cellobiose to release glucose monomers is catalyzed by β-glucosidase. (Wang *et. al.* 2012).

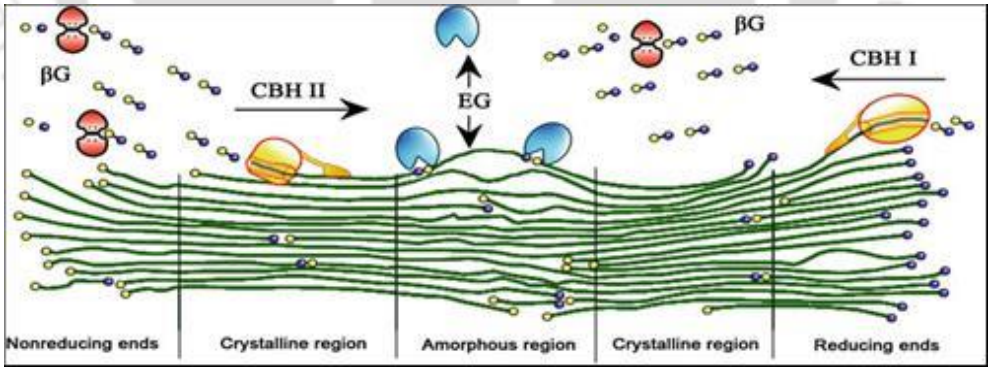

Fig. 1.2.1. Mechanism of cellulose biodegradation (Wang *et. al.* 2012).

The mode of action of different hemicellulases such as endo-β-xylanase, β-D-xylosidase, α-L-arabinofuranosidase, feruloyl esterase, acetylxylan esterase, α-D-galactosidase and α-D-glucuronidase are shown in Fig. 1.2.2. Endo-β-1,4- xylanase attacks the main chain of xylan backbone at random sites. β-D-xylosidase cleaves the

xylobiose to release D-xylose monomeric units. α-L-arabinofuranosidase, α-D-glucuronidase, feruloyl esterase, acetylxylan esterase and α-D-galactosidase catalyzes the removal of the terminal L-arabinose, 4-O-methyl-D-glucuronic acid, ferulic acid, acetyl groups and α-galactose, respectively from the xylan side chains (Adebayo and Martínez-Carrera 2015).

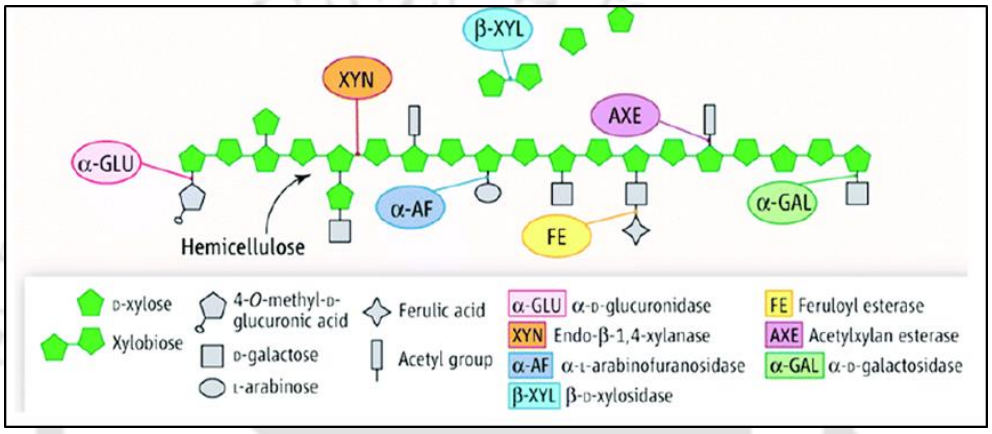

Fig. 1.2.2. Mechanism of hemicellulose biodegradation (Adebayo and Martínez-Carrera 2015).

1.2.5. Fermentation

Comparison of the two modes of fermentation, Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF) with using different lignocellulosic feedstocks of wheat straw and cornstover has showed that SSF contributes for higher ethanol productivity and yield with less time consumption than SHF (Alfani *et. al.* 2000). No inhibitory compound formed and affected SSF as in SHF (Ohgren *et. al.* 2007). By feeding intermittently, SSF can withstand high solid loading (Koppram and Olsson 2014). A mixture of cotton gin waste and recycled paper sludge, when subjected to SHF, SSF and SSSF (Semi-Simultaneous Saccharification and Fermentation), the best performance was resulted from SSSF (Shen and Agblevor 2011). Employing two or more fermentative microbes

for the complete fermentation of the sugars to bioethanol is depicted in the Fig. 1.2.3. Immobilization of atleast one strain helps in the species to species competition towards oxygen thus dominant species inhibiting the growth of the recessive (Fu *et. al.* 2009).

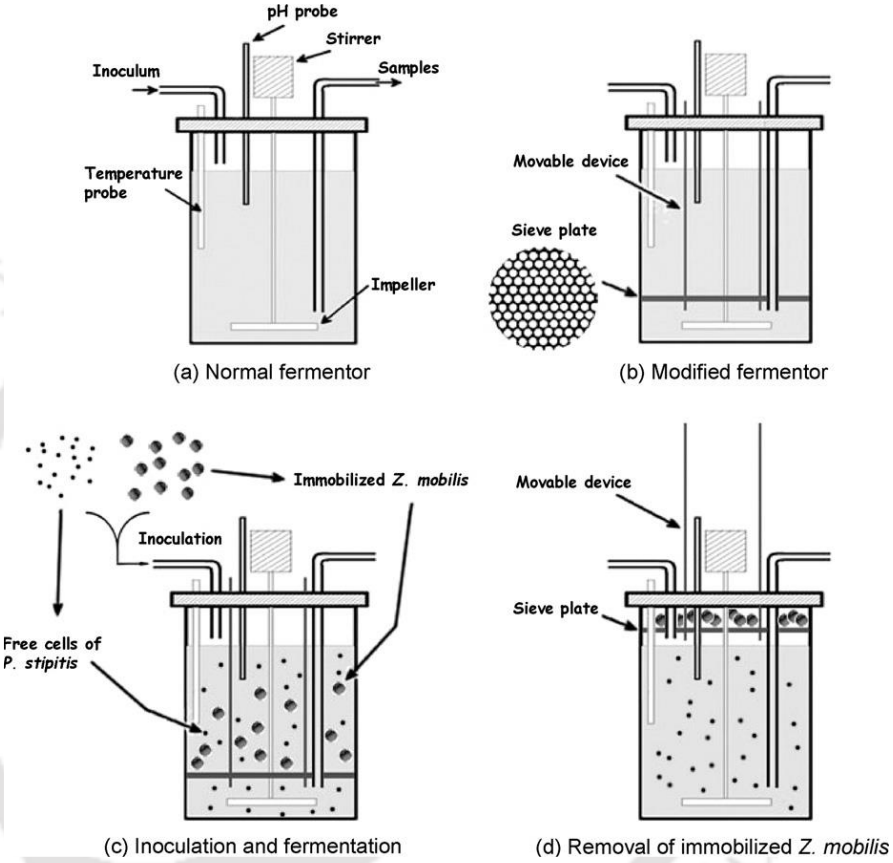

Fig. 1.2.3. Schematic demonstration of the modified fermentor. (a) A normal fermentor; (b) addition of a sieve plate and a moving device; (c) inoculation of immobilized *Z. mobilis* and free cells of *P. stipitis*; and (d) removal of the immobilized *Z. mobilis* beads from the fermentation medium without disturbing the on-going xylose fermentation (Fu *et. al.* 2009).

1.2.6. Biorefinery

The biorefinery concept has been investigated related to several biofuels. After the recovery of the desired product from a raw material, extraction of any other low/high value added chemicals from the residue is termed as biorefinery. In other terms, biorefinery is converting the final waste residue from any process as the primary

input for production of another product. In case of bioethanol fermentation, lignin is used in the production of electricity (Tolan 2006). Lignin separated from the hydrolysate after the enzymatic hydrolysis from Iogen process shown in the Fig. 1.2.4. is burnt to provide energy for the entire process (Tolan 2006).

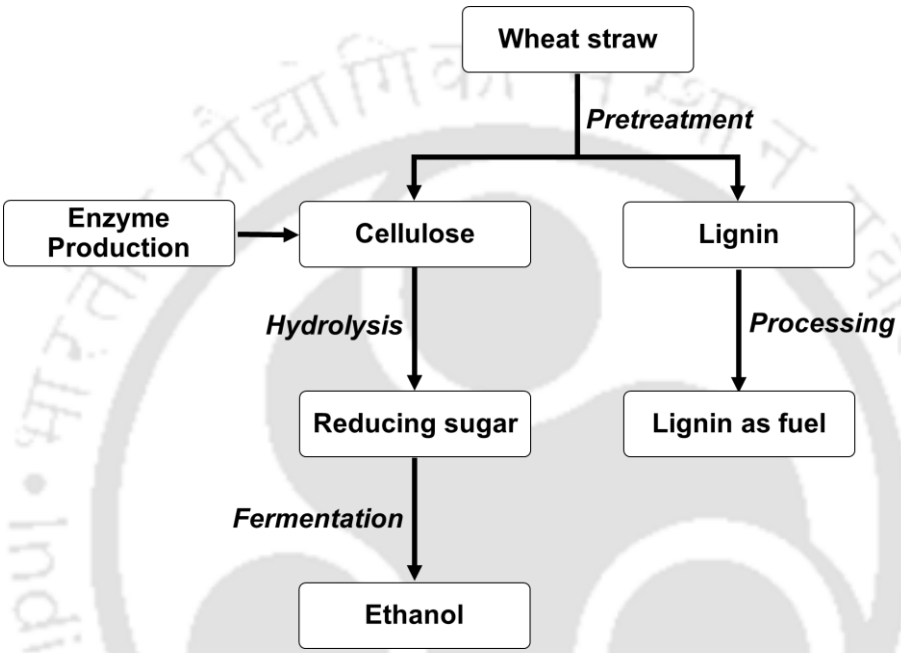

Fig. 1.2.4. Iogen's process for converting wheat straw to ethanol (Tolan 2006).

The residue coming after the SSF process may contain a variety of compounds in addition to lignin. For the complete utilization of the residue for any other process, data of its components must be acquired. Autolysed Ethanol Fermentation Waste (EFW) was characterized and used as the nutrient source for lactic acid production by *Lactobacillus paracasei* (Moon *et. al.* 2013).

Significance of investigation

As per the study report of National Rainfed Area Authority (NRAA), India (2012), 55% of the net sown area of the country are rainfed areas (areas with <30% irrigation). The major crops grown in these areas are cereals and millets as they require very less water compared with rice and wheat. About 44% of the total grains, 75% of the total pulses and 90% of the total millets and groundnut of the country are contributed by these rainfed areas only. These areas provide livelihood for about 50% of the rural workforce (NRAA 2012). Millet grains are consumed as food and the stalks of the crops are used partially as fodder for cattle and partially as left-over serving as nutrient supply for the soil. Millet crops also belong to grass family and hence may have the similar structural properties of rice and wheat straw. Exploring the structural cellulose and hemicellulose compositions of the stalks of major millets such pearl millet, finger millet and sorghum will pave the route for their utilization as the feedstock for bioethanol production. Hence, this study concentrates on the screening of pearl millet, finger millet and sorghum straws with respect to their structural composition, pretreatment of the selected feedstock, enzymatic hydrolysis of the pretreated biomass using cellulase and hemicellulase and fermentation of the released sugars targeting high ethanol production and yield.

Specific Objectives

1. Selection of lignocellulosic biomass and composition analysis.
2. Identification of the best pretreatment method for the selected lignocellulosic feedstock.
3. Production and purification of recombinant bacterial hydrolytic enzymes and their activity analysis against pretreated biomass.
4. Fermentation of the sugars produced from the enzymatic hydrolysis using fermentative microbes.
5. Utilization of the waste residue remaining after the bioethanol production for the production of any other value added product by applying biorefinery concept.

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Chapter 2

Screening of potential feedstock among Finger millet, Pearl millet and Sorghum straws for bioethanol production

2.1. Introduction

The rainfed area is 55% out of the total net sown area in India, which contributes for more than 75% of the country's pulses and millet production. Being cultivated in rainfed area, millet crops require less water supply as compared with rice and wheat (NRAA, 2012). Rice and wheat straws have commercial value in mushroom cultivation and fiberboard production (Zhang *et. al.*, 2002; Halvarsson *et. al.*, 2010). Like rice and wheat straw, millet straw does not have any other reported commercial value. Therefore, during the unavailability of rice or wheat straw in dry season, millet straw can contribute to the continuous supply of lignocellulosic biomass for bioethanol production. Research work carried out with Sorghum, finger millet or pearl millet straw used as feedstock for bioethanol production is scanty and countable (Rodhe *et. al.*, 2011; Ndaba *et. al.*, 2014; Jamaldheen *et. al.*, 2018; Jamaldheen *et. al.*, 2019).

2.1.1. Millets

Millets belong to the grass family, *Gramineae/Paniceae*. They are referred to as coarse cereals, variety of small edible grain bearing grasses. These are distributed in about 10 genera and 20 species (Lupien, 1990). These small seeded annual grasses are cultivated as grain crops on the marginal lands in dry areas of temperate, subtropical and tropical regions, mainly in Asia and Africa (Baker, 1996). The millets include five genera of the *Paniceae* family (*Panicum, Setaria, Echinochloa, Pennesetum and Eleusine*) (Singh and Raghuvanshi, 2012). The cultural practices followed for the major millet crops are shown in Table 2.1.1.

2.1.1.1. Pearl millet

Pearl millet (*Pennisetum glaucum*) is a highly cross-pollinated crop. Free-tillered pearl millet grows robustly to a height of 3 meters, whereas the most productive hybrids varieties are often shorter than the wild variety. The stems of Pearl millet are 10-20 mm thick with flat and green leaves up to 8 cm wide. The grain-bearing head (ear) of the plant forms a compact, cylindrical, terminal, spike-like panicle. As the name shows, the seeds are small, wedge-shaped to spherical (http://exploreit.icrisat.org/profile/Pearl%20Millet/178).

2.1.1.2. Finger millet

Finger millet (*Eleusine coracana*) is one of the major millet crops cultivated in Asia and Africa (Tekaligne et al., 2015). Finger millet crop is a grass that can grow up to a height of 2 m. The expert system for Finger millet (http://agritech.tnau.ac.in/expert_system/ragi/index.html), an Indian Council of

Agricultural Research (ICAR) funded project reported that the straw yield from Finger millet crop varies between 3-10 tonnes/ha.

2.1.1.3. Sorghum

Sorghum is the 5th most consumed cereal worldwide and it serves as a major contributor of agricultural residues in tropical regions. Nigeria alone produces 2–3 million metric tons of agrowaste from sorghum crop. 40% is used as livestock feed and fencing, whereas 60% of the biomass is burnt in open fields to get rid of the waste. This creates environmental pollution and health risks (Nasidi *et. al.*, 2016). Sorghum, also referred as the Great millet, is one of the majorly grown millets in India and its production was 10.62 MT in 2013-2014. Sorghum grain is used as food and their straws are partially used as fodder and rest is the left-over (Zalkuwi *et. al.*, 2014). Sorghum Juice and bagasse were reported as a resource for ethanol production (Capecchi *et. al.*, 2017). The sweet sorghum (*Sorghum bicolor*) bagasse is getting attention of scientists for its use in ethanol production (Choudhary *et. al.*, 2013; Ndaba *et. al.*, 2014). Another variety of sorghum (*Sorghum durra*) has not been reported for its use in ethanol production. Exploring the structural characteristics of the above millet straws will pave the route for their utilization as the lignocellulosic feedstock for bioethanol production. Therefore, this study focuses on the comparison of the structural composition of Sorghum, Finger millet and pearl millet straw to find out the potential of the biomass for bioethanol production.

2.2. Materials and Methods

2.2.1. Feedstock collection and processing

Three millet crops *viz*. Pearl millet (Fig. 2.1A1), Sorghum (Fig. 2.1B1) and Finger millet (Fig. 2.1C1**)** were selected for present study. The three lignocellulosic biomasses (crop residue after harvesting), Pearl millet (Fig. 2.1A2) and Sorghum straws (Fig. 2.1B2) were collected from Kappalpatti, Dindigul district, Tamil Nadu and Finger Millet straw (Fig. 2.1C2) was collected from Thenur, Madurai district, Tamil Nadu, India.

A1 **B1** **C1**

A2 **B2** **C2**

Fig. 2.1 (A1) Pearl millet plant, (A2) Pearl millet straw, (B1) Sorghum plant, (B2) Sorghum straw, (C1) Finger millet plant and (C2) Finger millet straw. (A1, B1, C1, Adapted from Google Images).

All the three straws were chopped with cutter, washed twice with tap water in trays and rinsed with distilled water, dried in hot air oven at 70°C for 24 h, then ground to powder and passed through a 0.6 mm sieve.

2.2.2. Characterization of feedstock

2.2.2.1. Moisture and ultimate analysis

One gram of raw unground biomass was taken and dried in hot-air oven at 105°C for 24 h. The biomass was weighed after drying to determine the moisture content and the analysis was carried out in triplicate. The Carbon, Hydrogen, Nitrogen and Sulfur (CHNS) content analysis of the powdered biomass was done by using CHNS analyzer (EuroEA Elemental Analyzer, Eurovector EA3000, Italy).

2.2.2.2. Structure composition analysis of biomass

0.5 g of raw biomass was used for holocellulose, α-cellulose and hemicellulose determination and 1 g of raw biomass was used for acid insoluble/detergent lignin (ADL) determination. Holocellulose content was determined by the method described by Browning (1968). α-Cellulose, hemicellulose and ADL contents were determined by following the standard TAPPI protocols (TAPPI, 1992). Each analysis was done in triplicate. Care was taken for achieving maximum accuracy and minimal wastage during the analysis in order to reduce the internal error.

2.2.2.2.1. Holocellulose content analysis

1 g of powdered biomass was suspended in 50 mL distilled water in a 100 mL conical flask. 0.75 g sodium chlorite and 2.5 mL of 10% acetic acid were added to the above biomass, mixed and kept in a water bath at 70°C for 30 min (Browning, 1968). After that 2.5 mL of 10% acetic acid was added to above flask and incubated at 70°C for 1 h. Then, to this flask, 0.75 g of sodium chlorite and 2.5 mL of 10% acetic acid were added at every 1 h for 4 h while incubating at 70°C. Then the flask was cooled to 10°C and the sample was filtered through sintered funnel. The residue was washed

with water and then with acetone. The funnel with residue was dried at 105°C and weighed to determine the holocellulose content gravimetrically.

2.2.2.2.2. α-Cellulose content analysis

500 mg of holocellulose was taken in 100 mL Erlenmeyer flask and kept in a water bath at 20°C. Then 3.75 mL of 17.5% NaOH was added to above flask containing holocellulose followed by 2.5 mL of 7.5% NaOH after 1 min. Thereafter, 2.5 mL (17.5% NaOH) was added after 45 s, 8.75 mL after 15 s and 2.5 mL after 3 min mixed and kept at 20°C for 30 min. After that, 25 mL distilled water was added to the mixture and kept at 20°C for 40 min. The mixture was filtered through sintered funnel and the residue was washed with distilled water at 20°C. The funnel was filled with 2N acetic acid, left for 5 min and filtered. The residue was washed with distilled water until it reaches neutral pH. The funnel containing the residue was dried at 50°C and weighed to determine the α-Cellulose content.

2.2.2.2.3. Hemicellulose content analysis

Hemicellulose content is the measure of the difference between the Neutral detergent fibre (NDF) and acid detergent fibre (ADF).

Hemicellulose content = NDF – ADF.

Reagents

Neutral detergent solution

Neutral detergent solution was made by dissolving 18.61 g of disodium ethylene diamine tetra acetate dihydrate (EDTA) and 6.81 g of sodium borate decahydrate in 800 mL distilled water in a 2 L beaker by heating. 30 g of Sodium lauryl sulphate, 4.5 g disodium hydrogen phosphate and 10 mL of 2-ethoxy ethanol were

added to the above solution. The pH was adjusted to 7.0 and the volume was made up to 1 L to make Neutral detergent solution.

Acid detergent solution

20 g of cetyl trimethyl ammonium bromide (CTAB) was dissolved in 1 L of 1 N H_2SO_4.

2.2.2.2.4. NDF determination

1 g of powdered biomass was taken in a 250 mL round bottom flask connected to reflux condenser. 100 mL of Neutral detergent solution (cold), 2 mL decahydronaphthalene and 0.5 g sodium sulphite were added. The mixture was refluxed with condenser for 1 h and filtered through a sintered glass funnel. The residue was washed with hot water and then with acetone. The content was dried at 100°C and weighed to determine the NDF content in the biomass.

2.2.2.2.5. ADF determination

100 mL cold acid detergent solution with 1 g of powdered biomass was taken in 250 mL round bottom flask connected to a reflux condenser. 2 mL of decahydronaphthalene was added to the flask and refluxed for 1 h. The content was filtered through a sintered glass funnel, washed with hot water and then with acetone. The residue was dried at 100°C and weighed to determine the ADF content.

2.2.2.2.6. Acid-insoluble/Detergent lignin (ADL) content analysis

The sintered glass funnel containing the residue from ADF analysis was filled with 72% sulphuric acid. Once the acid drained, the funnel was refilled with 72% sulphuric acid and left for 3 h. The acid was filtered and removed and the content was washed with hot water until neutral pH. The residue was dried at 105°C, ignited at

500°C (TGA, STA7200, Hitachi, Japan) to get the ash content. ADL content was calculated as follows:

$$\text{ADL} = \frac{(\text{wt. of funnel at } 105°\text{C}) - (\text{wt. of funnel after ignition at } 500°\text{C})}{\text{Initial wt. of biomass taken for ADF determination}}$$

2.3. Results and Discussion

2.3.1. Characterization of feedstocks

2.3.1.1. Moisture and ultimate analysis

Table 2.3.1 shows the moisture content and Carbon, hydrogen, nitrogen and sulfur (CHNS) contents of Finger millet, Pearl millet and Sorghum straw.

Table 2.3.1. Moisture content and ultimate analysis of selected feedstocks.

Biomass (straw)	Moisture content (wt%)*	Carbon (wt%)	Hydrogen (wt%)	Nitrogen (wt%)	Sulfur (wt%)
Finger millet	16.7± 0.8	43.9	5.7	1.7	Nil
Pearl millet	12.5±0.9	42.5	5.4	2.3	Nil
Sorghum	17.0±0.6	44.2	5.7	1.6	Nil

***Mean+SD (n=3).**

The moisture content of the selected millet crops ranged from 12 to 17% (w/w). Finger millet and Sorghum straw contained higher moisture content than the pearl millet straw. Carbon content was slightly higher in Sorghum straw than the other two feedstocks. Hydrogen was present in a similar amount in all the three biomasses. Nitrogen present 35% and 40% more in the pearl millet straw than the finger millet and sorghum straw, respectively. Sulphur was absent in all the three feedstocks.

2.3.1.2. Structural composition analysis

The holocellulose, α-cellulose, hemicellulose and ADL contents of the raw ground Finger millet, Pearl millet and Sorghum straw are shown in the Table 2.3.2. The maximum holocellulose content was found in Finger millet straw (69 wt%) followed by Pearl millet straw (66 wt%) and Sorghum straw (55 wt%) (Table 2.3.2). Finger millet straw contained the highest α-cellulose (36 wt%) and hemicellulose (33.5 wt%) contents as compared with Pearl millet or Sorghum straw. Each, the α-cellulose

and hemicellulose content, shared approximately, 50% of the holocellulose content present in Finger millet straw. The hemicellulose content was found to be similar (23 wt%) in Pearl millet and Sorghum straw. This showed that Finger millet straw contained approximately, 43% higher hemicellulose content than the other two feedstocks. Acid insoluble lignin (ADL) was approximately 5% (w/w) in all the three biomasses.

Table 2.3.2. Composition of Finger millet, Pearl millet and Sorghum straws.

Biomass (straw)	Holocellulose (wt%)*	α-Cellulose (wt%)*	Hemicellulose (wt%)*	Acid insoluble Lignin (ADL) (wt%)*
Finger millet	69.3±1.3	35.8±0.1	33.5±0.4	5.1±0.3
Pearl millet	65.8±1.5	33.4±1.7	23.6±1.4	5.6±0.7
Sorghum	55.0±1.8	31.8±1.2	23.2±0.6	5.7±0.2

***Mean+SD (n=3).**

Table 2.3.3. shows the reported cellulose and hemicellulose contents of different agrowaste straws that come under grass family. The average cellulose content in the three biomasses, Finger millet, Pearl millet and Sorghum straw (Table 2.3.2) are similar to the values reported for cellulose contents of the other biomasses (Table 2.3.3). The hemicellulose content observed in the three selected millet straws was 23-33% (w/w) (Table 2.3.2) which is similar to the hemicellulose content of other feedstocks as reported earlier (Table 2.3.3).

Table 2.3.3. Composition of lignocellulosic biomass from grass family.
(Adapted from Tayyab *et. al*., 2017)

Agrowaste (straw)	Cellulose (wt%)	Hemicellulose (wt%)	Reference
Wheat straw	33-38	26-32	Rabemanolontsoa and Saka, 2013; Saini *et. al.* 2015
Rice straw	28-36	23-28	Qu *et. al.*, 2011; Saini *et. al.*, 2015
Barley straw	31-45	27-38	Saini *et. al.*, 2015
Rye straw	33-35	27-30	Sanchez, 2009
Oat straw	31-37	27-38	Sanchez, 2009

Higher the cellulose content, higher the glucose content, which can be utilised by C6 fermenting microbe like *Saccharomyces cereviceae* for ethanol prduction. If the hemicellulose content is higher, the availability of C5 sugars will be more, which can also be fermented to ethanol. However, holocellulose content, which is the sum of total cellulose and hemicellulose contents in the biomass is significant in mixed fermentation of C6 and C5 sugars. Lower ADL content is important, as lignin inhibits the enzyme action on the biomass. As the ADL and cellulose contents were similar in all the three feedstocks, holocellulose and hemicellulose contents were taken as the determining factors for screening the potential feedstock among the three for ethanol production. Finger millet straw was selected as the best candidate for ethanol production, because it contained the highest holocellulose content along with the highest cellulose and hemicellulose contents among the three feedstocks.

Conclusion

Three biomasses, Finger millet, Pearl millet and Sorghum straw were evaluated for their potential as feedstock with respect to their carbohydrate compositions for the purpose of ethanol production. Carbon and hydrogen content were approximately 43% (w/w) and 6% (w/w), respectively in all three biomasses. The highest nitrogen content, 2.3% (w/w) and the lowest moisture content of 12.5% (w/w) was found in Pearl millet straw. No sulfur was present in any of the above three feedstocks. The highest holocellulose (69 wt%), cellulose (36 wt%) and hemicellulose (33.5 wt%) contents were observed in the Finger millet straw. The α-cellulose (32- 33 wt%) content was similar in Pearl millet and Sorghum straw. ADL (5 wt%) contents were similar in all the three feedstocks. Therefore, with respect to the availability of higher carbohydrate content, Finger millet straw was selected to be the best among the three feedstocks for pretreatment process for ethanol production.

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Chapter 3

Comparative analysis of pretreatment methods on Finger millet straw for saccharification of cellulose and hemicellulose

3.1. Introduction

Pretreatment of the lignocellulosic biomass is the foremost step in ethanol production (Binod *et. al.,* 2010). The pretreatment process helps in removing the unwanted compounds and concentrating the polysaccharides such as cellulose and hemicellulose (Kumar *et. al.,* 2009; Maurya *et. al.,* 2015). Various pretreatment methods, such as physical pretreatment, chemical pretreatment, thermal pretreatment and pretreatment involving organosolvents or ionic liquids can be used. Among chemical pretreatment methods, the acid pretreatment method using dilute sulphuric acid (H_2SO_4) or hydrochloric acid (HCl) or alkali method using dilute sodium hydroxide (NaOH) or potassium hydroxide (KOH) are cheaper (Badiei *et. al.,* 2014; Kumar and Sharma, 2017). Any particular pretreatment method is not ideal for all lignocellulosic feedstocks, as each feedstock has different structure composition with variations in rigidity. Hence a variety of pretreatment methods are needed to be

explored, compared and an effective pretreatment method for each selected feedstock has to be employed (Singh et al., 2014b). Combining multiple pretreatment methods or applying them in a step-wise manner have also been shown to be effective on some lignocellulosic feedstocks (Gabhane *et. al.*, 2014; Zhu et al., 2015). The conventional and easier way to compare the efficiency of pretreatments is to calculate the total reducing sugar (TRS) released during each pretreatment (Wi *et. al.*, 2013; Chaturvedi and Verma, 2013). In addition to the TRS analysis, the efficiency of a given pretreatment method can also be analysed directly and accurately by comparing the structure composition of the untreated and pretreated biomass (Chen *et. al.*, 2012) and also by enzymatic hydrolysis of the pretreated biomass resulting in TRS yield. This study presents the comparative analysis of 12 different pretreatment methods on Finger millet straw to screen the best pretreatment method for ethanol production.

3.2. Materials and Methods

3.2.1. Pretreatment of Finger millet straw

Different pretreatment methods applied on the Finger millet straw were thermal pretreatment, microwave irradiation and ultrasonication in the presence or absence of dilute acid (1% (v/v) H_2SO_4) or dilute alkali (1% (w/v) NaOH). To analyze the impact of the chemical coupled with each method, uniform concentration of the chemical (1% (v/v) H_2SO_4 or 1% (w/v) NaOH) was used. Each pretreatment was carried out using 5% (w/v) of powdered Finger millet straw (as described in section 2.2.1) soaked in 200 mL aqueous solution. The conditions applied for each pretreatment are as follows:

Thermal pretreatment: Two kinds of thermal treatments were carried out as follows:

i) Dry heat: This treatment was carried out at 120°C for 20 min I n hot-air oven.

ii) Autoclaving: In this treatment, the conditions applied were 121°C at 15 psi for 20 min. The thermal pretreatment methods used in this study were adopted from Singh *et. al.*, 2014b.

Microwave Irradiation: This was carried out by using the microwave oven (Samsung, South Korea) at 180 W for 3 min. The microwave pretreatment followed was adopted from Singh *et. al.*, 2014c.

Ultrasonication: This treatment was done by keeping the sample in an ultrasonication bath (PCi analytics, India) at 33 kHz for 10 min. The ultrasonication method followed was from Singh *et. al.*, 2014a.

Each pretreatment was carried out in three individual aqueous systems such as distilled water, 1% (v/v) H_2SO_4 and 1% (w/v) NaOH thereby leading to a total number of 12 pretreatment methods. After each pretreatment, the sample was filtered through

muslin cloth and the filtrate was used for reducing sugar and inhibitor analyses. The solid residue was washed with distilled water till the neutral pH and dried at 70°C.

3.2.2. Carbohydrate and Lignin analysis of untreated and pretreated FMS

Holocellulose content of untreated and each pretreated FMS (initial biomass, 0.5 g) was determined by following Browning method (Schuerch, 1968). Standard TAPPI protocols (TAPPI, 1992) were used for the determination of hemicellulose and acid insoluble lignin (ADL) contents (initial biomass, 1 g). The analysis was carried out with more precision and negligible wastage of biomass to achieve less internal error. Approximate cellulose content was determined by subtracting the hemicellulose from holocellulose content of each sample.

3.2.3. Biomass yield after pretreatment method

The biomass residue remaining after each pretreatment was dried in an oven at 105°C to remove the moisture. Pretreated biomass yield from each pretreatment method was determined by weighing the respective dried solid residue and expressed as biomass yield (g/g raw biomass).

3.2.4. Crystallinity index (*CrI*) of untreated and pretreated FMS

The crystallinity indices of untreated and each pretreated samples were analysed by X-ray diffractometer (D8 Advance, Bruker, Germany). The samples were scanned over a range of 2θ= 10° to 30°, where the step size used was 0.05°. The formula for calculating the crystallinity index as described by Segal et al. (1962) is given below:

$$Crystallinity\ index\ (CrI\%) = \frac{I_{crystalline} - I_{amorphous}}{I_{crystalline}} \times 100 \qquad (1)$$

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

3.2.5. Reducing sugar analysis in pretreated hydrolysates by high performance liquid chromatography (HPLC)

50 mL of filtrate from each pretreatment was centrifuged at 6000 rpm and 25°C for 15 min and the supernatant was filtered through a 25 mm polyvinylidene fluoride (PVDF) membrane with 0.45 µm pore size. 1 mL of the final filtrate was used for the determination of glucose, xylose, arabinose and cellobiose contents. The analysis was carried out by using HPLC system (Shimadzu corporation, LC-20AD, Japan) coupled with an autosampler (Shimadzu corporation, SIL-20AHT, Japan) and RI detector (Shimadzu corporation, RID-10A, Japan). Standard glucose, xylose, arabinose and cellobiose procured from Sigma Aldrich, USA were used at concentrations, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL. The HPLC column (Phenomenex Rezex ROA (H+) organic acid and monosaccharide column, 300 mm x 7.8 mm) coupled with a guard column (50 mm x 7.8 mm) was used. A mobile phase of 0.005 N H_2SO_4 was run at a flow rate of 0.5 mL/min through the column. The concentration of each sugar in the sample was calculated with respect to the area of the respective peak.

3.2.6. Inhibitor analysis in pretreated hydrolysates by HPLC

Inhibitors such as furfural, hydroxymethylfurfural (HMF) and acetic acid in the hydrolysates of pretreated samples were analysed by HPLC. Standard furfural and HMF (HiMedia Pvt. Ltd., India) were used at concentrations, 1, 2, 3, 4, 5, 6 and 7 mg/L and standard acetic acid (Sigma Aldrich, USA) was used at 5, 10, 15, 20, 25, 30 and 35 mg/mL concentrations. Each sample was filtered using PVDF membrane (0.45 µm pore size). Each sample along with the mobile phase, 80% acetonitrile was run through a 5 µm C18 column, shim-pack GIST C18 (250 mm x 4.6 mm) coupled with a guard

column (4.0 mm x 20 mm) at a flow rate of 1 mL/min and detected using UV detector (Shimadzu corporation, SPD-20A, Japan) at 210 nm.

3.2.7. Production of recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) and endo-1,4-β-glucanase (*Ct*Cel8A) from *Clostridium thermocellum*

Endo-1,4-β-xylanase (*Ct*Xyn11A) of family 11 glycoside hydrolase from *Clostridium thermocellum* was a generous gift from Prof. Carlos Fontes of NZYTech Pvt. Ltd. Portugal. Endo-1,4-β-glucanase (*Ct*Cel8A) of family 8 glycoside hydrolase from *Clostridium thermocellum* was cloned and expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) cells in the laboratory. 1% (v/v) of *E. coli* BL21 (DE3) cells harbouring the pET21a(+) vector containing gene encoding an endo-1,4-β-xylanase (*Ct*Xyn11A) was inoculated in 800 mL Luria-Bertani medium (HiMedia, Pvt. Ltd., India) supplemented with ampicillin (100 µg/mL). The 800 mL culture was incubated initially at 37°C, 180 rpm for 4 h and then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and incubated at 24°C, 180 rpm for 16 h. The *E. coli* cells were centrifuged at 5000*g* at 4°C for 10 min and the cell pellet was used for enzyme purification. The production of enzyme, endo-1,4-β-glucanase (*Ct*Cel8A) was carried out in the same way as *Ct*Xyn11A except that the antibiotic used was kanamycin (50 µg/mL), because the pET28a(+) vector contains kanamycin resistant gene.

3.2.8. Purification of endo-1,4-β-xylanase (*Ct*Xyn11A) and endo-1,4-β-glucanase (*Ct*Cel8A)

The cell pellet containing *Ct*Xyn11A or *Ct*Cel8A was re-suspended in 10 mL of sodium phosphate buffer (50 mM, pH 7.4), kept on ice and lysed by ultra-sonication for 15 min (5s on/10s off pulse at 33% amplitude, Sonics, Vibra cell). The cell lysate was centrifuged at 16,000g for 1 h and the supernatant containing enzyme (*Ct*Xyn11A

with N-terminal hexa-His tag) was purified by immobilized metal-ion affinity chromatography (IMAC) using 2x5 mL Sepharose column (HiTrap Chelating, GE Healthcare, US). The protein was purified by following protocol described in the Handbook on Affinity Chromatography, GE Healthcare. The columns were incubated with 10 mL of 1M NaOH, washed with water to remove any impurity and charged with 0.1 M $NiSO_4$. The charged column was then passed with equilibration buffer (50 mM sodium phosphate, pH 7.4, 300 mM NaCl and 60 mM imidazole). The supernatant containing desired enzyme was passed through the column and eluted with the aid of elution buffer (50 mM sodium phosphate, pH 7.4, 300 mM NaCl and 300 mM imidazole). The fractions containing the enzyme were collected, pooled, dialysed against 50 mM sodium phosphate buffer (pH 7.4) and concentrated using centricon with molecular weight cut-off of 10 kDa (Merck Millipore, USA).

3.2.9. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Each enzyme (*Ct*Xyn11A or *Ct*Cel8A) was further analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), to check the purity of the enzyme. The SDS-PAGE was carried out in the vertical slab mini gel unit (Mini-PROTEAN®Tetra cell, BioRad, USA) with a 1.5 mm thick gel casting mold. The compositions of 12% (w/v) acrylamide resolving gel and 4% (w/v) acrylamide stacking gel are given in Table 3.2.1 and Table 3.2.2, respectively.

Table 3.2.1. Composition of 12% (w/v) resolving gel.

Component	Volume (mL)
Acrylamide-bisacrylamide solution (30%, w/v)	4.00
SDS solution (10%, w/v)	1.00
1.5 M Tris (pH 8.8)	3.30
APS (10%, w/v)	0.10
TEMED	0.01
Deionised water	1.70
Total volume	10.00

Table 3.2.2. Composition of 4% (w/v) stacking gel.

Component	Volume (mL)
Acrylamide-bisacrylamide solution (30%, w/v)	0.70
SDS solution (10%, w/v)	0.50
0.5 M Tris (pH 6.8)	1.00
APS (10%, w/v)	0.05
TEMED	0.005
Deionised water	2.8
Total volume	5.00

3.2.9.1. Reagents and buffers for SDS-PAGE

3.2.9.1.1. Acrylamide solution (30%, w/v)

29.2 g of acrylamide and 0.8 g of bis acrylamide were dissolved in 100 mL distilled water and the solution was filtered using Whatman No. 1 filter paper under dark condition. The solution was stored in an amber colour bottle at 4°C.

3.2.9.1.2. Tris-HCl (1.5 M, pH 8.8)

54.45 g of Tris base (121.14 g/mol) was dissolved in 150 mL of distilled water. The pH of the solution was adjusted to 8.8 and then the volume was made up to 300 mL and stored at 4°C.

3.2.9.1.3. Tris-HCl (0.5 M, pH 6.8)

6 g of Tris base (121.14 g/mol) was dissolved in 60 mL of distilled water. The

pH of the solution was adjusted to 6.8 and then the volume was made up to 100 mL and stored at 4°C.

3.2.9.1.4. SDS solution (10%, w/v)

10 g SDS was dissolved in 100 mL distilled water.

3.2.9.1.5. Loading buffer

The sample loading buffer (5x) was prepared by mixing the components given in the Table 3.2.3. The 5x loading buffer stock solution diluted to 1x by adding 4 volumes of protein sample before loading on to the gel.

Table 3.2.3. Composition of 5x sample loading buffer.

Component	Final concentration
Tris HCl (pH 6.8)	62.5 mM
Glycerol	20% (v/v)
SDS	2% (v/v)
Bromophenol blue	0.025% (w/v)
β-mercaptoethanol	5% (v/v)

3.2.9.1.6. Running buffer

The 5x running buffer was prepared as given in the Table 3.2.4, filtered (Whatman, Filter No. 1) and stored at 4°C. The running buffer, 1x Tris-glycine was prepared by diluting the 5x Tris glycine buffer stock solution with deionised water before running the electrophoresis.

Table 3.2.4. Composition of 5x running buffer.

Component	Final concentration
Tris base	0.125 M
Glycine	1.25 M
SDS	0.5% (w/v)

3.2.9.1.7. Staining and destaining solutions

250 mg of Coomassie brilliant blue (CBB) R-250 dye was dissolved in 50 mL of distilled water and the solution was filtered through Whatman, Filter No. 1. To the filtrate, 40 mL of methanol and 10 mL of glacial acetic acid were added, made up to 100 mL with distilled water and the solution was stored in amber colour bottle. Hot distilled water was used as the destaining solution.

3.2.10. Bradford Assay of endo-1,4-β-xylanase (*Ct*Xyn11A) and endo-1,4-β glucanase (*Ct*Cel8A)

The concentration of enzyme, *Ct*Xyn11A or *Ct*Cel8A was determined by Bradford (1976) method. 10 µl of purified *Ct*Xyn11A or *Ct*Cel8A was taken in a 1.5 mL and 90 µl of 50 mM sodium phosphate buffer (pH 7.4) was added. Immediately, 1 mL of Bradford reagent was added to the mixture, vortexed and incubated in dark for 15 min. The protein concentration was determined by measuring the absorbance of the mixture at 595 nm in UV-Visible spectrophotometer (GeneQuant 1300, GE Healthcare, UK). Bovine serum albumin (BSA) was used as protein standard. The amount of recombinant protein was estimated using the following equation,

$$[\text{Protein}]\ (\text{mg/mL}) = \frac{\Delta A_{595} \times V \times C}{v}$$

Where,

A_{595} = change in absorbance of the sample

V = volume of the protein-buffer mixture (mL)

C = 1 OD equivalent of BSA from standard plot (mg/mL)

v = volume of the enzyme used for assay (mL).

3.2.10.1. Bradford's reagent

100 mg 0.01% (w/v) Coomassie Brilliant Blue G-250 was in 50 mL of 95% ethanol in an amber colour bottle. 100 mL of 85% (w/v) phosphoric acid was added and stirred. The solution was diluted to 1 L with deionized water, filtered with Whatman, No. 1 filter paper and stored at 4°C.

3.2.11. Activity assay of endo-1,4-β-xylanase (*Ct*Xyn11A) and endo-1,4-β glucanase (*Ct*Cel8A)

To determine *Ct*Xyn11A activity, 10 µL of enzyme (5 µg/mL) was incubated with 1% (w/v) birchwood xylan in a 100 µL reaction in 50 mM sodium phosphate buffer, pH 7.5 at 65°C for 1 min. To determine the enzyme activity of *Ct*Cel8A, 10 µL of *Ct*Cel8A (10 µg/mL) was incubated with 1% (w/v) carboxymethyl cellulose in a total reaction volume of 100 µL (50 mM sodium phosphate buffer, pH 5.8) at 60°C for 5 min. The activities of *Ct*Xyn11A and *Ct*Cel8A after the reactions were calculated by estimating the reducing sugar released by following the method of Nelson (1944) and Somogyi (1945).

The activity assay was carried out by adding 100 µl of reagent D to 100 µl of the reaction mixture containing the reducing sugar. The solution was mixed and heated for 20 min in the boiling water bath. After 20 min of boiling, 100 µl of reagent C was added. 700 µl of water was added to the mixture to make up the volume to 1 mL and the absorbance at 500 nm (A500) was measured by UV-Visible spectrophotometer (GeneQuant 1300, GE Healthcare, UK). D-xylose and D-glucose were used as standards to quantify the reducing sugar released by *Ct*Xyn11A and *Ct*Cel8A, respectively in the reaction mixture. The activity of the enzyme was expressed as U/mL and the specific activity as U/mg of protein. One unit (U) of enzyme activity is defined

as the amount of enzyme that liberates 1 µmole of reducing sugar per min. The enzyme activity was calculated as described below,

$$\text{Enzyme activity (U/mL), } ((\mu\text{mol/min})/\text{mL}) = \frac{\Delta A_{500} \times C \times V}{\text{M.W.} \times t \times v}$$

Where,

ΔA_{500} = change in absorbance of the sample at 500 nm
C = 1 OD equivalent of D-glucose or D-xylose from standard plot (mg/mL)
V = volume of the reaction mixture (mL)
M.W. = Molecular weight of D-glucose (180 g/mol) or D-xylose (150 g/mol)
t = time of reaction (min)
v = volume of the enzyme taken in assay (mL) for reducing sugar estimation.

3.2.11.1. Reagents for enzyme activity assay

3.2.11.1.1. Reagent A

2.5 g of sodium carbonate, 2.5 g of potassium sodium tartrate tetrahydrate, 2 g of sodium bicarbonate and 20 g of sodium sulphate were dissolved in 100 mL distilled water.

3.2.11.1.2. Reagent B

4.5 g of copper sulphate pentahydrate was dissolved in 30 mL of distilled water and then 1-2 drops of conc. sulphuric acid was added.

3.2.11.1.3. Reagent C

2.5 g of ammonium molybdate tetrahydrate and 2.1 mL of conc. sulphuric acid were added to 45 mL of distilled water. 2.5 mL of solution containing 0.3 g of sodium arsenate heptahydrate dissolved in distilled water was mixed to the former solution. It was made up to 50 mL and stored in an amber bottle at 37ºC for 24 h prior to use.

3.2.11.1.4. Reagent D

Reagent D is the mixture of reagents A and B in a ratio of 25:1.

3.2.12. Enzymatic hydrolysis of untreated and each pretreated FMS

The enzyme, endo-1,4-β-xylanase (*Ct*Xyn11A, 0.2 mg/mL, 3900 U/mg) hydrolysis of 1% (w/v) untreated and 1% (w/v) each pretreated FMS biomass sample in 1.0 mL 50 mM sodium phosphate buffer (pH 7.5) was carried out by incubating the reaction mixture at 65°C and 150 rpm in a shaking incubator for 1 h. Similarly, 1% (w/v) untreated and 1% (w/v) each pretreated FMS biomass sample was incubated with endo-1,4-β-glucanase (*Ct*Cel8A, 0.2 mg/mL, 80 U/mg) in a 1 mL reaction volume at pH 6 (50 mM sodium phosphate buffer), 60°C and 150 rpm for 1 h. Each reaction was stopped by boiling the reaction mixture in a boiling water bath for 10 min. The control without the enzyme for each sample was included to eliminate any reducing sugar released from the biomass during boiling. 0.05% (w/v) sodium azide solution was added to the reaction mixture to prevent contamination. The samples were centrifuged at 12,600g for 10 min and the supernatants were analysed for total reducing sugar (TRS) by following the method of Nelson (1944) and Somogyi (1945).

3.2.13. FTIR and FESEM analyses of untreated and the best pretreated (1% (w/v) NaOH + oven heating) FMS

The untreated FMS sample and the pretreated FMS from the best pretreatment (1% (w/v) NaOH + oven heating) screened from the enzymatic hydrolysis of section 3.2.12 were used for Fourier-transform infrared **(**FTIR) and Field emission scanning electron microscopic (FESEM) analyses. The samples were separately mixed with KBr (200 mg) in the ratio of 100:1 (KBr: Biomass), ground using mortar and pestle and pelleted using a press. The pellets were scanned by Fourier-transform infrared **(**FTIR**)** spectroscope (Spectrum Two, Perkin-Elmer, Waltham, MA) within the wavenumber range, 4000- 450 cm^{-1}. Field emission scanning electron microscopic (FESEM)

analysis of the above samples were carried out by placing the biomasses on a carbon tape fixed to a stub, coating them with gold and scanning by FESEM (Zeiss, Sigma, Germany).

3.3. Results and Discussion

3.3.1. Carbohydrate and Lignin analysis of untreated and pretreated FMS

The untreated FMS contained 69.3% holocellulose, 33.5% hemicellulose, 35.8% cellulose and 5.1% acid insoluble lignin (ADL) as mentioned in the section 2.3.1.2. Table 3.3.1 shows the structural carbohydrate and lignin composition of each pretreated FMS. The impact of the selected 12 pretreatments on FMS are discussed in the following sections.

3.3.2. Impact of pretreatment on holocellulose

FMS samples treated with the selected 12 pretreatments contained more than 75% holocellulose content except the samples treated with autoclaving along with either H_2SO_4 or NaOH (Table 3.3.1). Oven heating in the presence of every selected aqueous system resulted in more than 76% holocellulose. Autoclaving along with H_2SO_4 completely removed the hemicellulose from the biomass. Pedersen et al. (2011) has also reported the removal of almost all the hemicellulose portion of wheat straw by dilute acid treatment. Moreover, autoclaving with NaOH also resulted in more than 50% reduction in the hemicellulose content (Table 3.3.1). Therefore, all the selected pretreatments on FMS except the autoclaving method involving acid or alkali were gentle enough to retain the hemicellulose content in the pretreated FMS samples.

3.3.3. Impact of pretreatment on ADL

FMS treated by autoclaving combined with NaOH contained the lowest ADL content (3.0%) among all the other pretreated samples (Table 3.3.1). This proves the

efficiency of alkali method in delignification of lignocellulosic biomass as also reported earlier (Singh *et. al.*, 2014a). ADL content (5.6%) was lesser in the biomass treated by oven heating along with NaOH than the oven heating with only water (6.5%) or H_2SO_4 (7.3%) (Table 3.3.1). This must be because the dilute acid hydrolysis of lignocellulosic biomass is capable of hydrolysing hemicellulose but does not remove the lignin and cellulose (Binod *et. al.*, 2010; Chaturvedi and Verma, 2013). Autoclaving along with 1% H_2SO_4 showed an inverse impact on the ADL content causing highest ADL content (15%) and thereby making the pretreated biomass unsuitable for bioethanol production (Table 3.3.1). All the pretreatments involving microwave or sonication resulted in slight increase of the ADL content (6-7%) as compared with the untreated FMS (5%). With respect to ADL content, NaOH combined with oven heating or autoclaving treatments were found to be better for FMS among all the pretreatments owing to their higher delignification efficiency.

3.3.4. Impact of pretreatment on biomass yield

Among all the pretreatment methods carried out on FMS, microwaving with water at 180 W for 3 min yielded the maximum amount of pretreated biomass (0.84 g/g raw biomass) (Table 3.3.1). Being mild treatment and for short time duration, it did not result in the loss of cellulose and hemicellulose from the FMS. The lowest pretreated biomass yield of 0.15 g/g raw biomass obtained with autoclaving with NaOH as compared with the other treatments (Table 3.3.1). Therefore, autoclaving with NaOH turned out to be the most inefficient method as it caused extensive biomass wastage (Table 3.3.1).

Table 3.3.1. Structure composition, biomass yield analysis and crystallinity index of untreated and pretreated FMS samples.

Pretreatment	Aqueous system	Holocellulose* (wt% of ptd. B.)	Hemicellulose* (wt% of ptd. B.)	Cellulose* (wt% of ptd. B.)	ADL* (wt% of ptd. B.)	Pretreated biomass yield[#] (g /g raw biomass)	*CrI* (%)
Raw FMS	--	69.3±1.3	33.5±0.4	35.8±0.1	5.1±0.3	1	55.6
Oven heating at	D.W.	77.5±0.3	32.2±0.6	45.3±0.8	6.5±0.5	0.63	41.7
120°C for 20 min	1% H_2SO_4	77.1±0.3	34.1±0.4	43.1±0.6	7.3±0.4	0.66	30.9
	1% NaOH	76.4±0.2	20.7±0.5	55.7±0.4	5.6±0.5	0.36	55.2
Autoclaving at	D.W.	76.7±0.2	33.4±0.4	43.3±0.2	6.2±0.3	0.54	59.5
121°C, 15 psi for 20 min	1% H_2SO_4	68.5±0.3	0.00	68.5±0.3	15.6±0.2	0.26	60.9
	1% NaOH	67.2±0.4	15.4±0.9	51.8±1.2	3.1±0.4	0.15	68.6
Microwaving	D.W.	75.6±0.4	31.9±1.4	43.7±1.1	6.4±0.6	0.84	51.7
at 180 W for 3 min	1% H_2SO_4	79.5±0.4	31.8±0.8	47.7±0.4	7.4±0.3	0.47	9.5
	1% NaOH	77.5±0.3	23.0±0.3	54.5±0.3	7.3±0.5	0.36	21.7
Ultra-sonication	D.W.	75.9±0.8	32.3±0.3	43.5±0.6	6.6±0.2	0.56	28.3
at 33 kHz for 10 min	1% H_2SO_4	79.7±0.2	32.2±0.6	47.4±0.5	7.4±0.4	0.53	39.0
	1% NaOH	79.1±0.3	25.4±0.2	53.6±0.2	6.6±0.3	0.33	37.8

D.W. = Distilled water; ptd. B. = pretreated biomass; ADL= Acid insoluble lignin; Cellulose = (Holocellulose-hemicellulose); *CrI*= Crystallinity index; *mean±SD (n=3); [#]mean±SD (n=3) where, SD< 0

3.3.5. Reducing sugar and Crystallinity index (*CrI*) analyses of pretreated FMS

After the pretreatment of FMS, the reducing sugar content of the filtrate and the crystallinity index (CrI) of the solid biomass residue were determined. FMS on treatment by autoclaving with H_2SO_4 gave the maximum TRS yield of 222.8 mg/g raw biomass (Table 3.3.2). The acid treatments combined with all four physical treatments released significantly higher cellobiose than the respective alkali or water treatments. This showed the efficiency of dilute acid in decrystallization of cellulose in lignocellulosic biomass as also reported earlier (Kim et al., 2013).

Table 3.3.2. Reducing sugar analysis in the filtrate after each pretreatment.

Pretreatment	Aqueous system	Glucose[#] (g/L)	Xylose[#] (g/L)	Arabinose[#] (g/L)	Cello-biose[#] (g/L)	TRS yield (mg/g raw biomass)*
Oven heating at 120°C for 20 min	D.W.	0.02	0.03	0.01	0.04	2.0
	1% H_2SO_4	0.01	0.04	0.05	0.14	4.8
	1% NaOH	0.10	0.02	0.02	0.03	3.4
Autoclaving at 121°C, 15 psi for 20 min	D.W.	0.22	0.03	0.02	0.04	6.2
	1% H_2SO_4	0.37	7.79	2.90	0.08	222.8
	1% NaOH	0.47	0.03	0.03	0.03	11.2
Microwaving at 180 W for 3 min	D.W.	0.03	0.02	0.01	0.04	2.0
	1% H_2SO_4	0.02	0.02	0.03	0.16	4.6
	1% NaOH	0.02	0.02	0.02	0.05	2.2
Ultra-sonication at 33 kHz for 10 min	D.W.	0.02	0.03	0.01	0.04	2.0
	1% H_2SO_4	0.01	0.02	0.003	0.14	3.46
	1% NaOH	0.01	0.02	0.02	0.04	1.8

D.W. = Distilled water; TRS = Total Reducing Sugar; [#]mean±SD (n=3) where, SD< 0.01; *TRS yield (mg/g raw biomass) = (glucose + xylose + arabinose + cellobiose) / 0.05 where, biomass used in each pretreatment was 5% (w/v).

H_2SO_4 combined with autoclaving released maximum xylose and arabinose concentrations (7.8 and 2.9 g/L, respectively) in the filtrate (Table 3.3.2) which is also clear from the complete loss of hemicellulose content (Table 3.3.1). Moreover, the increase in *CrI* to 60.9% from 55.6% of untreated biomass also explains that the cellulose is exposed due to the removal of the hemicellulose content (Table 3.3.1). The next TRS value (11.2 mg/g raw biomass) resulted was very low by autoclaving with

NaOH (Table 3.3.2), which gave the maximum *CrI* of 68.6%. The maximum *CrI* of 68.8% was due to the maximum delignification (Table 3.3.1) by NaOH, exposing the crystalline cellulose. The TRS and *CrI* analyses showed that the pretreatment of FMS by autoclaving with H_2SO_4 or NaOH are better methods among 12 pretreatment methods for exposing crystalline cellulose.

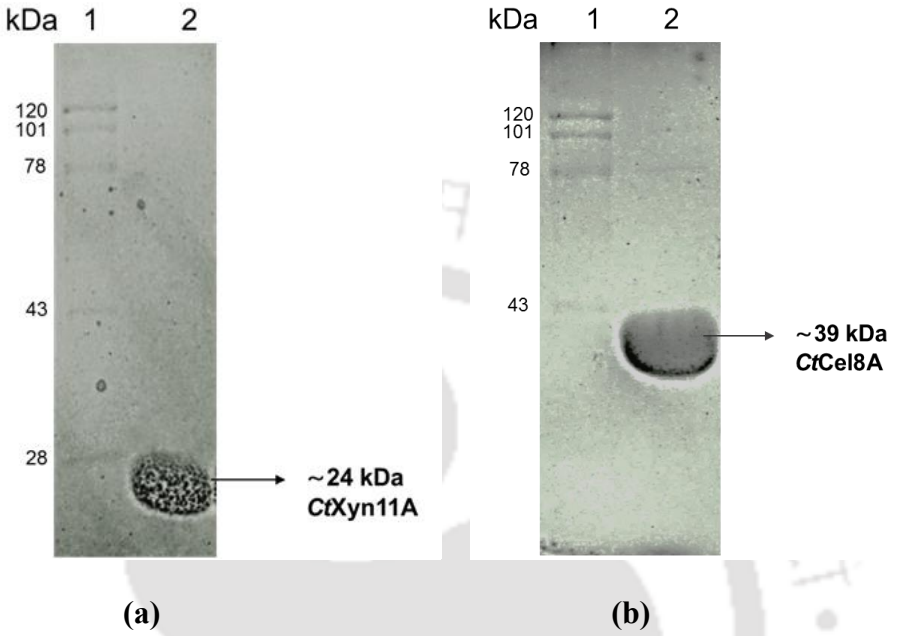
3.3.6. Inhibitor analysis in the hydrolysates of pretreated FMS

The inhibitors, furfural and hydroxymethyl furfural are derived from pentose sugars and hexose sugars, respectively and are released during acid pretreatment. Acetic acid is formed during the alkaline pretreatment of lignocellulosic biomass with KOH or NaOH (Wikandari *et. al.*, 2010; Jonsson and Martin, 2016). In this study, furfural found in the filtrate was 35.8 mg/L from FMS pretreated by H_2SO_4 combined with autoclaving. This correlates well with the reducing sugar data where autoclaving along with H_2SO_4 yielded the maximum concentration of xylose (7.8 g/L) and arabinose (2.9 g/L) in the hydrolysate among all the chosen pretreatment methods (Table 3.3.2). Other pretreatment methods did not release any of these inhibitory compounds.

3.3.7. Production and purification of endo-1,4-β-xylanase (*Ct*Xyn11A) and endo-1,4-β-glucanase (*Ct*Cel8A) from *Clostridium thermocellum*

The purified recombinant xylanase, *Ct*Xyn11A (Fig. 3.3.1a) showed a single band of 24 kDa soluble protein and a yield of 3.7 mg/mL from 800 mL culture. It showed a specific activity of 3900 U/mg on 1% (w/v) birchwood xylan at 65°C and pH 7.5 (50 mM sodium phosphate buffer) (Table 3.3.3). The purified endoglucanase, *Ct*Cel8A (Fig. 3.3.1b) showed a single band of 39 kDa soluble protein. Its concentration was determined to be 2.5 mg/mL from 800 mL culture, showing a

specific activity of 80 U/mg against 1% (w/v) CMC at 60°C and pH 5.8 (50 mM sodium phosphate buffer) (Table 3.3.3).



(a) **(b)**

Fig. 3.3.1. (a) Lane 1-Marker (Biobharti) and lane 2-Purified fraction of *Ct*Xyn11A; (b) Lane 1-Marker and lane 2-Purified fraction of *Ct*Cel8A.

Table 3.3.3. Purified enzyme yields and activities of endo-1,4-β-xylanase (*Ct*Xyn11A) and endo-1,4-β-glucanase (*Ct*Cel8A).

Enzyme	Volume (mL)	Protein concentration (mg/mL)	Purified protein (mg)	Enzyme activity (U/mL)	Specific activity (U/mg)
Endo-1,4-β-xylanase, *Ct*Xyn11A	5	3.7	18.5	19.8	3900
Endo-1,4-β-glucanase, *Ct*Cel8A	4	2.5	10	0.8	80

3.3.8. Enzymatic hydrolysis of untreated and each pretreated FMS with endo-1,4-β-xylanase (*Ct*Xyn11A) and endo-1,4-β-glucanase (*Ct*Cel8A)

*Ct*Xyn11A was active against only the NaOH pretreated FMS samples. The maximum TRS yield observed was 31.5 mg/g pretreated biomass upon saccharification with *Ct*Xyn11A after the treatment with NaOH combined with oven heating (Fig. 3.3.2). This was followed by TRS yield of 28.6 mg/g pretreated biomass obtained by *Ct*Xyn11A saccharification of FMS pretreated with NaOH combined with autoclaving. Enzymatic hydrolysis of FMS pretreated with NaOH combined with microwaving or sonication by *Ct*Xyn11A resulted TRS yield of 18.3 or 12.7 mg/g pretreated biomass, respectively. This showed that the alkali treatment releases hemicellulose, thereby paving the way for xylanase action as also reported earlier (Barman *et. al.*, 2012). Endo-1,4-β-glucanase (*Ct*Cel8A) showed very low activity with 1% (w/v) NaOH combined with oven heating or autoclaving treated FMS. This showed that none of the 12 pretreatments on FMS led to the de-crystallization of cellulose. Enzymatic hydrolysis using *Ct*Xyn11A and *Ct*Cel8A on pretreated FMS samples revealed that 1% (w/v) NaOH combined with oven heating and 1% (w/v) NaOH combined with autoclaving were better treatments among the 12 pretreatment methods on FMS.

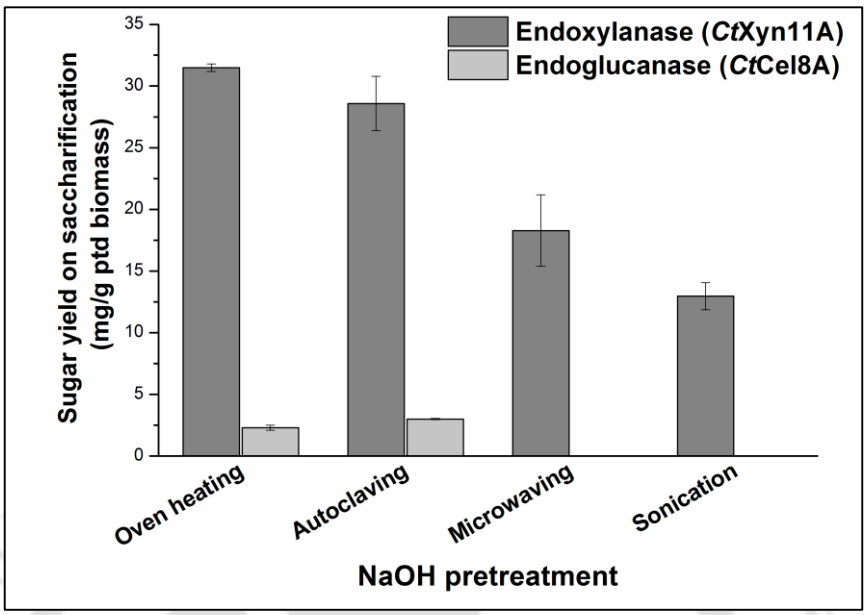


Fig. 3.3.2. Comparison of enzymatic hydrolysis of untreated and pretreated FMS samples by endo-1,4-β-xylanase (*Ct*Xyn11A) and endo-1,4-β-glucanase (*Ct*Cel8A).

Among the FMS samples pretreated by 1% (w/v) NaOH combined with oven heating and 1% (w/v) NaOH combined with autoclaving, holocellulose content (76% (w/w)) and biomass yield (0.36 g/g raw biomass) of the FMS treated by 1% (w/v) NaOH combined with oven heating were higher than the holocellulose content (67% (w/w)) and biomass yield (0.15 g/g raw biomass) of the FMS treated by 1% (w/v) NaOH combined with autoclaving. Therefore, enzymatic hydrolysis along with composition analysis revealed that 1% (w/v) NaOH combined with oven heating serves as the best pretreatment among the 12 methods. The conventional pretreatment screening method involves the comparison of TRS yield in the filtrate from each pretreatment and considers the highest TRS releasing pretreatment as the best method (Wi *et. al.*, 2013; Chaturvedi and Verma, 2013; Singh *et. al.*, 2014c). In this study, even though the TRS yield was highest obtained from H_2SO_4 + autoclaving treatment, the enzyme, endo-1,4-β-xylanase (*Ct*Xyn11A) did not give any activity on this specific

pretreated FMS. Therefore, it is also important to evaluate the activity of the desired enzyme on each pretreated biomass sample to determine the best pretreatment method for any given biomass.

3.3.9. FTIR and FESEM analyses of untreated and pretreated FMS

The FTIR and FESEM analyses of untreated and pretreated (NaOH + oven heating) FMS are shown in Fig. 3.3.3. The FTIR spectrum showing peak between 3600-3000 cm^{-1} is related to O-H stretching of cellulose in the lignocellulosic biomass as reported earlier (Agrawal *et. al.*, 2011). The peak at 1732 cm^{-1} was assigned to the ester linkage between hemicellulose and lignin. The peaks at 1640 cm^{-1} and 1512 cm^{-1} are related to the carbonyl stretching of aromatic rings and C=C stretching vibration in phenol rings in lignin, respectively. 1249 cm^{-1} is related to the C-O stretching of acetyl groups of xylan (Pandey, 1999; Ren *et. al.*, 2016). The peaks at 1513 cm^{-1} and 1247 cm^{-1} were absent in the FMS sample pretreated by NaOH + oven heating when compared with the untreated FMS (Fig. 3.3.3a and 3.3.3b). This showed that the pretreatment involving NaOH combined with oven heating helps in delignification and hemicellulose breakdown in the FMS. The FESEM images of untreated and pretreated FMS showed that NaOH + oven heating pretreatment method caused significant structure disruption, leading to the roughness on the surface (Fig. 3.3.3d) as compared with the untreated biomass (Fig. 3.3.3c) displaying the effectiveness of the pretreatment.

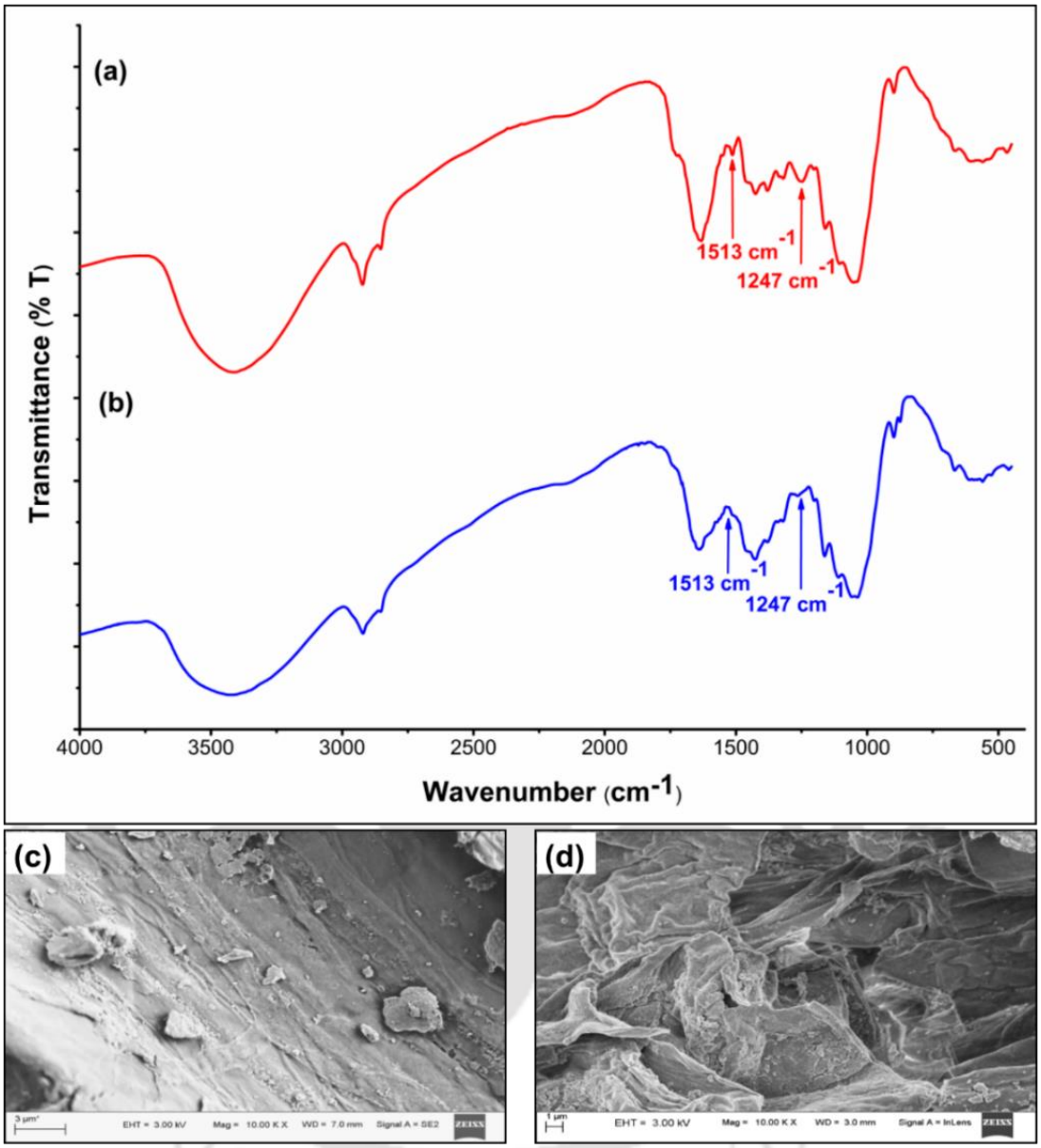


Fig. 3.3.3. FTIR spectra of (a) untreated and (b) pretreated (1%, w/v NaOH combined with oven heating) FMS, corresponding FESEM images of (c) untreated and (d) pretreated (1%, w/v NaOH combined with oven heating) FMS.

Conclusions

Among the 12 pretreatment methods on FMS involving oven heating, autoclaving, microwaving and ultra-sonication in the presence of 1% (v/v) H_2SO_4, 1% (w/v) NaOH or distilled water, NaOH treatments aided for endo-1,4-β-xylanase (*Ct*Xyn11A) involved saccharification. Among the four NaOH pretreated FMS samples, the enzyme endo-1,4-β-xylanase (*Ct*Xyn11A) was most active on the FMS treated with 1% (w/v) NaOH combined with oven heating that resulted in TRS, 32 mg/g pretreated biomass. Endo-1,4-β-glucanase (*Ct*Cel8A) gave low levels of saccharification on FMS, treated with 1% (w/v) NaOH combined with oven heating (2.3 mg/g pretreated biomass) and with 1% (w/v) NaOH combined with autoclaving (3 mg/g pretreated biomass). The pretreament of FMS by NaOH combined with oven heating resulted in higher holocellulose (76%, w/w) and biomass yield (0.36 g/g raw biomass) as compared to the pretreatment by NaOH combined with autoclaving, that gave holocellulose 67% (w/w) and biomass yield, 0.15 g/g raw biomass. Thus, 1% (w/v) NaOH combined with oven heating at 120°C for 20 min was the best pretreatment method for FMS, as it provided better accessibility to both enzymes resulting in higher TRS yield. The FTIR spectra of pretreated biomass confirmed hemicllulose breakdown and delignification rendered by alkali treatment. The enhanced surface roughness of the pretreated Finger millet straw was clearly visible in FESEM image. Hemicellulose saccharification of the above pretreated FMS using endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) can be optimized to enhance the TRS yield.

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Chapter 4

Enzymatic hydrolysis of hemicellulose from pretreated Finger millet straw by recombinant endo-1,4-β-xylanase and exo-1,4-β-xylosidase

4.1 Introduction

In ethanol production, the next step after pretreatment is saccharification of the pretreated biomass. Hemicelluloses are complex mixtures of heterogeneous polysaccharides such as xylan, xyloglucan, glucomannan, galactoglucomannan, arabinogalactans, etc. (Scheller and Ulvskov, 2010). The major hemicellulosic components occur in the cell walls of grasses are xylans (Faik, 2010). Xylan accounts for approximately one-third of the renewable organic carbon on Earth. Xylanases are enzymes that catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages of xylan (Collins et al., 20005). Xylanases have vast applications in food and textile industries, waste treatment, bioethanol production, bleaching of pulp in the paper industry (Verma and Satyanarayana, 2012; Roncero *et. al.*, 2005) and deinking newsprint (Kalpana and Rajeswari, 2015). Xylanase hydrolyzes the xylan to xylo-oligosaccharides and xylosidase catalyzes the conversion of xylo-oligosaccharides to xylose (Bhalla *et. al.*,

2014; Bhalla *et. al.*, 2015). Xylanase and xylosidase work together and effectively convert the xylan to xylose, substrate required for pentose fermentation to ethanol (Qing and Wyman, 2011). Endo-1,4-β glucanase (*Ct*Cel8A) gave TRS of approximately 3 mg/g pretreated biomass on saccharification of FMS treated with 1% (w/v) NaOH combined with oven heating or 1% (w/v) NaOH combined with autoclaving. As it was too low, cellulose saccharification was not considered for further study. Hence, the further work was focussed on hemicellulose saccharification of pretreated FMS using recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A). In this study, the optimization of hemicellulose saccharification from the pretreated (1% (w/v) NaOH + Oven heating) Finger millet straw (FMS) by recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus* was carried out and conversion of the xylan to xylose during the process was determined.

4.2. Materials and Methods

4.2.1. Production of recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus*

Endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* was produced as described in the section 3.2.7. Its specific activity was determined to be 3900 U/mg on 1% (w/v) Birchwood xylan. Exo-1,4-β-xylosidase (*Bo*GH43A) was also a generous gift from Prof. Carlos Fontes of NZYTech Pvt. Ltd. Portugal. The inoculum, 1% (v/v) of *Escherichia coli* (*E. coli*) BL21 (DE3) cells harbouring the pET28a(+) vector containing gene encoding an exo-1,4-β-xylosidase (*Bo*GH43A), a family 43 Glycoside Hydrolase from *Bacteroides ovatus* was inoculated in 800 mL Luria-Bertani medium (HiMedia, Pvt. Ltd., India) supplemented with kanamycin (50 µg/mL). The 800 mL culture was incubated initially at 37°C, 180 rpm for 4 h and then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and incubated at 24°C, 180 rpm for 16 h. The *E. coli* cells were centrifuged at 5000g at 4°C for 10 min and the cell pellet was further used for enzyme purification.

4.2.2. Purification of recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus*

Endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* was purified as described in the section 3.2.8. The cell pellet obtained was re-suspended in 10 mL of sodium phosphate buffer (50 mM, pH 7.4), kept on ice and lysed by ultra-sonication for 15 min (5s on/10s off pulse at 33% amplitude, Sonics, Vibra cell). The cell lysate was centrifuged at 16,000g for 1 h and the supernatant containing enzyme (*Bo*GH43A with N-terminal hexa-His tag) was purified by immobilized metal-ion affinity chromatography (IMAC) using 2x5 mL Sepharose column (HiTrap Chelating,

GE Healthcare, US). The protein was purified by following protocol described in the Handbook on Affinity Chromatography, GE Healthcare. The columns were incubated with 10 mL of 1M NaOH, washed with water to remove any impurity and charged with 0.1 M $NiSO_4$. The charged column was then passed with equilibration buffer (50 mM sodium phosphate, pH 7.4, 300 mM NaCl and 60 mM imidazole). The supernatant containing desired enzyme was passed through the column and eluted with the aid of elution buffer (50 mM sodium phosphate, pH 7.4, 300 mM NaCl and 300 mM imidazole). The fractions containing the enzyme were collected, pooled, dialysed against 50 mM sodium phosphate buffer (pH 7.4) and concentrated using centricon with molecular weight cut-off of 10 kDa (Merck Millipore, USA). The enzyme (*Bo*GH43A) was further analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), to check the purity of the enzyme following the procedure described in section 3.2.9. The concentration of enzyme was determined by Bradford (1976) method using BSA as standard as described in section 3.2.10.

4.2.3. Assay of exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus*

To determine the enzyme activity, 10 µL of *Bo*GH43A (50 µg/mL) was incubated with 1% (w/v) birchwood xylan in a total reaction volume of 100 µL (50 mM sodium phosphate buffer, pH 7.0) at 37°C for 5 min. The activity *Bo*GH43A after the reaction was calculated by estimating the reducing sugar released by following the method of Nelson (1944) and Somogyi (1945) as described in section 3.2.11.

4.2.4. Optimization of enzymatic hydrolysis of hemicellulose from pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A)

The enzymatic hydrolysis of pretreated (NaOH + oven heating) FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) was carried out in 1 mL volume to produce xylo-oligosaccharides (XOS). The biomass and endo-1,4-β-xylanase (*Ct*Xyn11A) loading and the hydrolysis time were optimized at 55°C, pH 7.5 (50 mM sodium phosphate buffer) and 150 rpm by using Box-Behnken design, Response surface methodology (Design-Expert 7.0 software). The range for the factors were provided manually (Table 4.2.1) with respect to the preliminary experiments performed. The response, TRS yield$_{(XOS)}$ from each experiment was determined by Nelson (1944) and Somogyi (1945) method.

Table 4.2.1 Process variables for the optimization of hemicellulose hydrolysis by endo-1,4-β-xylanase (*Ct*Xyn11A).

Factor	Factor name	Level	
		Low (-1)	High (+1)
A_1	Biomass loading (%, w/v)	1	7
B_1	Xylanase loading (U/g ptd biomass)	50	600
C_1	Time (h)	4	72

4.2.5. Optimization of hydrolysis of xylo-oligosaccharides by exo-1,4-β-xylosidase (*Bo*GH43A)

The xylo-oligosaccharides (XOS) were produced from 6% (w/v) pretreated FMS suspended in 50 mL of 50 mM sodium phosphate buffer, pH 7.5 by treating with endo-1,4-β-xylanase (*Ct*Xyn11A) at loading of 559 U/g ptd biomass and incubating the reaction mixture at 55°C and 150 rpm for 56 h. The reaction was stopped by boiling the reaction mixture for 10 min and the XOS was stored at 4°C. Further hydrolysis of xylo-oligosaccharides was carried out by exo-1,4-β-xylosidase (*Bo*GH43A) in 1 mL

reaction volume. For optimization of hydrolysis of xylo-oligosaccharides, the XOS concentration, xylosidase loading and hydrolysis time were optimized at 37°C under static condition using Box-behnken design. The range for the factors were provided manually (Table 4.2.2) with respect to the preliminary experiments.

Table 4.2.2. Process variables for the optimization of xylo-oligosaccharide (XOS) hydrolysis by exo-1,4-β-xylosidase (*Bo*GH43A).

Factor	Factor name	Level	
		Low (-1)	High (+1)
A_2	Xylosidase loading (U/mL)	2	40
B_2	XOS Concentration (g/L)	0.52	2.08
C_2	Time (min)	5	120

The TRS release per unit XOS (response) from each experiment was determined by Nelson (1944) and Somogyi (1945) method. The equivalent TRS release per unit of pretreated biomass was calculated by using the formula as follows:

$$\text{Final TRS}\left(\frac{\text{mg}}{\text{g ptd biomass}}\right) = \text{TRS yield}_{(\text{XOS})} \times \text{TRS yield} \qquad (1)$$

where," $\text{TRS yield}_{(\text{XOS})}$" is the Total Reducing Sugar yield as xylo-oligosaccharides (mg XOS/g ptd biomass) from the hydrolysis with endo-1,4-β-xylanase (*Ct*Xyn11A) and "TRS yield" is the Total Reducing Sugar yield (g/g XOS) from the hydrolysis with exo-1,4-β-xylosidase (*Bo*GH43A).

4.2.6. Thin layer chromatography (TLC) analysis of hydrolysed products of pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A)

The hydrolysed samples from the enzymatic hydrolysis of pretreated FMS under optimized conditions by *Ct*Xyn11A alone and *Ct*Xyn11A followed by *Bo*GH43A were analysed by TLC. In one set, 6% (w/v) pretreated FMS suspended in 1 mL final volume of 50 mM sodium phosphate buffer, pH 7.5 with enzyme, *Ct*Xyn11A loading at 559 U/g ptd biomass was incubated at 55°C and 150 rpm for 56 h. In another set, 1 mL of 2.08 g/L of XOS produced from the first set having 50 mM sodium phosphate buffer pH 7.5 was treated with 40 U/mL final loading of xylosidase and incubated at 37°C for the optimized time period, 79.4 min. The samples from both the sets were filtered through 0.45 µm membrane (PVDF). 0.5 µL of each sample and standard xylose (4 mg/mL) were loaded on the TLC plate (13 cm x 3 cm, TLC Silica gel 60 F254, Merck, Germany) and dried. A mixture containing acetonitrile and water (80:20) was used as the mobile phase (Gauch *et. al.*, 1979). The TLC plate was kept inside the developing chamber and run for 30 min. After the run, the plate was immersed in the visualizing solution containing sulphuric acid: methanol (5:95, v/v) and 5.0% (w/v) α-naphthol and then dried at 80°C to visualize the hydrolysed products.

4.2.7. Estimation of xylan content in hemicellulose in pretreated biomass by HPLC

The 1% (w/v) pretreated FMS (NaOH + oven heating) was further treated with 2M Trifluoroacetic acid (TFA) at 100°C for 2 h. The total xylose concentration in the TFA treated sample was taken as the total xylan available for the enzymes (*Ct*Xyn11A and *Bo*GH43A) for the saccharification process under optimized conditions. The xylan to xylose conversion during saccharification was calculated by determining the xylose

in the TFA treated sample (total xylan present) and the saccharified sample (hydrolysed xylose). The xylose concentration was determined by HPLC method by following the method described in the section 3.2.5. The samples were filtered through a 25 mm polyvinylidene fluoride (PVDF) membrane with 0.45 µm pore size. The analysis was carried out by using HPLC system (Shimadzu corporation, LC-20AD, Japan) coupled with an autosampler (Shimadzu corporation, SIL-20AHT, Japan) and RI detector (Shimadzu corporation, RID-10A, Japan). Xylose procured from Sigma Aldrich, USA was used as the standard. The HPLC column (Phenomenex Rezex ROA (H+) organic acid and monosaccharide column, 300 mm x 7.8 mm) coupled with a guard column (50 mm x 7.8 mm) was used. A mobile phase of 0.005 N H_2SO_4 was run at a flow rate of 0.5 mL/min through the column. The formula used for the calculation of xylan to xylose conversion is as follows:

$$\text{Xylan to xylose conversion (\%)} = \frac{X_1}{X_2} \times 100 \qquad (2)$$

where, X_1 is the xylose (g/L) released during the saccharifiaction of alkali treated FMS and X_2 is the xylose (g/L) released from the non-saccharified alkali treated FMS on TFA treatment.

4.3. Results and Discussion

4.3.1. Optimization of hemicellulose hydrolysis from pretreated FMS to xylo-oligosaccharides by endo-1,4-β-xylanase (*Ct*Xyn11A)

The purified recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) had a concentration of 3.7 mg/mL from 800 mL culture. The total amount of protein was 18.5 mg and showed a specific activity of 3900 U/mg on 1% (w/v) birchwood xylan as described in the section 3.3.7. The Box-Behnken design for the optimization of hydrolysis of hemicellulose from pretreated (NaOH + oven heating) FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) and the response, TRS yield are shown in the Table 4.3.1.

Table 4.3.1. Box-Behnken design and responses for the hemicellulose hydrolysis from pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A).

Run order	Biomass loading (%, w/v) (A_1)	Xylanase loading (U/g ptd biomass) (B_1)	Time (h) (C_1)	Total Reducing Sugar, $TRS_{(XOS)}$ (mg/g ptd biomass)	
				Predicted	Experimental[#]
1	7	325	4	17.93	17.98
2	4	325	38	29.79	30.22
3	4	50	4	7.84	8.9
4	4	600	4	19.54	18.94
5	7	325	72	33.41	33.91
6	1	600	38	29.57	30.67
7	4	600	72	38.13	37.07
8	7	600	38	37.20	37.75
9	1	50	38	16.53	15.97
10	1	325	4	12.12	11.62
11	1	325	72	27.55	27.5
12	4	325	38	29.79	29.36
13	4	325	38	29.79	29.81
14	4	325	38	29.79	29.22
15	7	50	38	20.57	19.47
16	4	325	38	29.79	30.36
17	4	50	72	20.16	20.76

[#]**Mean value (n=3).**

The second-order quadratic equation for the hydrolysis process by xylanase is as follows:

$$\text{TRS yield}_{(XOS)}\ (\text{mg/g ptd biomass}) = -1.06 + 1.72A_1 + 0.04B_1 + 0.55C_1 + 0.002A_1B_1 + 0.0001A_1C_1 + 0.0002B_1C_1 - 0.14{A_1}^2 - 0.00003{B_1}^2 - 0.005{C_1}^2 \quad (3)$$

The quadratic model fits well with the experimental values of TRS yield (Table 4.3.2). The F-value of 122.7 from ANOVA showed that the model was significant as the p-value was less than 0.05. The p-values of A_1, B_1, C_1, B_1C_1, ${A_1}^2$, ${B_1}^2$ and ${C_1}^2$ being less than 0.05 implied they were significant terms. "Pred-R^2" of 0.9115 of shows the predictability of the model to predict responses for new observations The coefficient of determination (R^2) of the model was 0.9937, which indicated that 99.37% of the variability in the response could be explained by the model. The "Pred-R^2" of 0.9115 was in reasonable agreement with R^2, 0.9937. The coefficient of variation (C.V.) shows how much volatility is there while using the model. Lower the C.V., higher the reliability. The C.V. in this study was 4.1%, which showed that the model was reliable.

Fig. 4.3.1a showed that the $TRS_{(XOS)}$ yield increased with increase in the xylanase loading (B_1) from 50 to 600 U/g ptd biomass. When the biomass loading (A_1) increased, from 1 to 7 % (w/v), a gradual increase in the $TRS_{(XOS)}$ yield was observed. Fig. 4.3.1b showed that the $TRS_{(XOS)}$ yield increased as time (C_1) increased from 4 to 72 h. It also showed a gradual increase in the $TRS_{(XOS)}$ yield with increase in the biomass loading from 1 to 7 % (w/v). This showed that higher biomass loading can be considered as it can increase the $TRS_{(XOS)}$ concentration in the reaction without

compromising the $TRS_{(XOS)}$ yield. Fig. 4.3.1c showed that the increase in xylanase loading and time together increased the $TRS_{(XOS)}$ yield.

Table 4.3.2. ANOVA for the quadratic model of hemicellulose hydrolysis by endo-1,4-β-xylanase (*Ct*Xyn11A).

Source	SS	Df	Co-efficient	F-Value	p-value
Quadratic model	1187.58	9		122.70	< 0.0001
A_1-Substrate loading	68.15	1	2.92	63.37	< 0.0001
B_1-Xylanase loading	440.01	1	7.42	409.14	< 0.0001
C_1-Time	477.41	1	7.73	443.92	< 0.0001
A_1B_1	3.20	1	0.89	2.98	0.128
A_1C_1	0.0006	1	0.012	0.00	0.9814
B_1C_1	9.83	1	1.57	9.14	0.0193
A_1^2	6.55	1	-1.25	6.09	0.043
B_1^2	28.07	1	-2.58	26.10	0.0014
C_1^2	141.37	1	-5.79	131.46	< 0.0001
Intercept			29.79		
Residual	7.53	7	1.08		
Lack of Fit	6.51	3	2.17	8.51	0.03
Pure Error	1.02	4	0.25		
Total	1195.11	16			
Model statistics					
S.D.	1.04		R^2	0.9937	
Mean	25.27		Adj-R^2	0.9856	
C.V. %	4.10		Pred-R^2	0.9115	

Df - degrees of freedom; SS - sum of squares; C.V.% - Coefficient of variation; Adj-R^2 - Adjusted R^2; Pred-R^2 - Predicted R^2

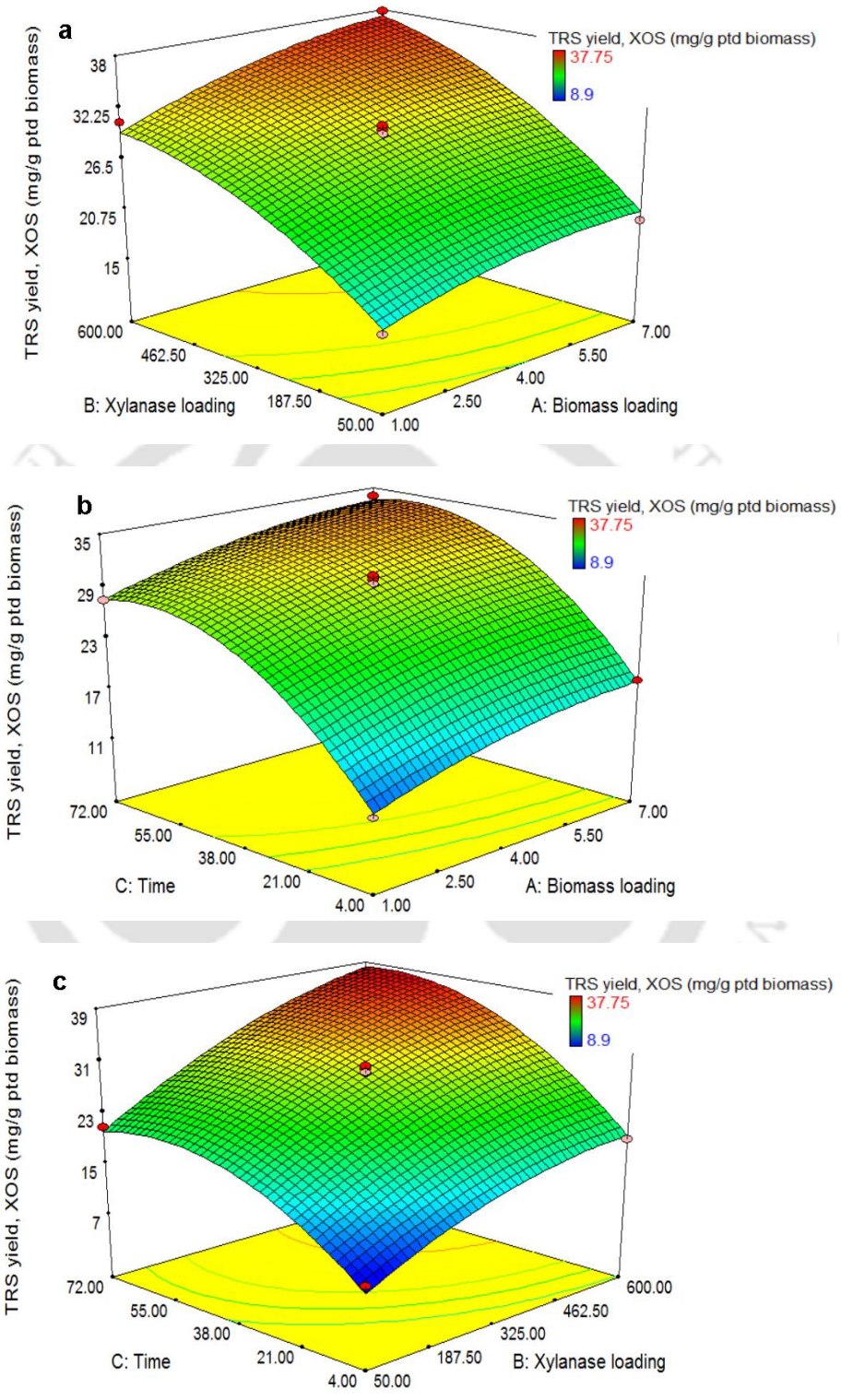

Fig. 4.3.1. 3-D response surface plots for the interaction between the independent variables involved in the optimization of hemicellulose hydrolysis by endo-1,4-β-xylanase (*Ct*Xyn11A). (a) biomass loading (%, w/v) and xylanase loading (U/g biomass); (b) biomass loading (%, w/v) and time (h); and (c) xylanase loading (U/g biomass) and time (h).

The predicted optimum biomass loading (A_1), xylanase loading (B_1) and hydrolysis time (C_1) from the model were 6 % (w/v), 559 U/g ptd biomass and 56 h (Table 4.3.3). The model predicted TRS yield from the above conditions was 39.3 mg/g ptd biomass. It was validated by carrying out an experiment (triplicate) in 1 mL reaction volume at the predicted optimum conditions and the observed TRS yield was 39.6 ± 0.6 mg/g ptd biomass, which was equivalent to 2.4 g/L. The TRS titre at the flask level of 50 mL volume was determined to be 2.6 g/L.

Table 4.3.3. Validation of predicted optimum parameters for hemicellulose hydrolysis by xylanase (*Ct*Xyn11A)

Factor name	Predicted optimum condition	Total Reducing Sugar, $TRS_{(XOS)}$ (mg/g ptd biomass)	
		Predicted	Experimental[#]
Biomass loading (%, w/v)	6	39.3	39.6
Xylanase loading (U/g ptd biomass)	559		
Time (h)	56		

[#]**Mean value (n=3).**

4.3.2. Purification of exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus*

The purified recombinant exo-1,4-β-xylosidase, *Bo*GH43A (Fig. 4.3.2) showed a single homogenous and soluble protein band of ~40 kDa molecular size (Fig. 4.3.2). The protein concentration of 2.8 mg/mL from 800 mL culture was obtained. The total amount of protein obtained was 11.2 mg with a specific activity of 59 U/mg against 1% (w/v) birchwood xylan.

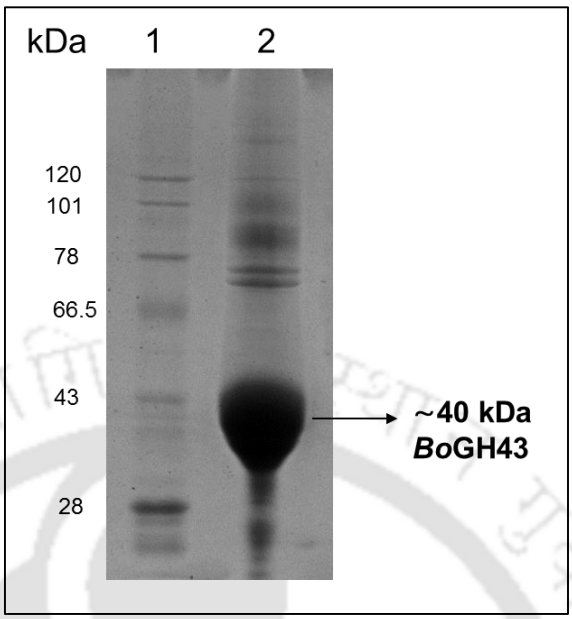

Fig. 4.3.2. Lane 1- Marker (Biobharti), lane 2- Purified fraction of *Bo*GH43.

4.3.3. Optimization of hydrolysis of xylo-oligosaccharides (XOS) by exo-1,4-β-xylosidase (*Bo*GH43A)

17 experiments designed by the Box-Behnken design for the hydrolysis of xylo-oligosaccharides by xylosidase, the predicted and the observed response (TRS yield) are shown in the Table 4.3.4. The second-order quadratic equation for the XOS hydrolysis involving xylosidase is as follows:

$$\text{TRS yield } (\text{g/g XOS}) = 1.77 + 0.002A_2 - 0.24B_2 + 0.004C_2 + 0.0002A_2B_2 + 0.000005A_2C_2 + 0.0008B_2C_2 + 0.000004{A_2}^2 + 0.05{B_2}^2 - 0.00004{C_2}^2 \quad (4)$$

Table 4.3.4. Box-Behnken design and responses for the xylo-oligosaccharide (XOS) hydrolysis by exo-1,4-β-xylosidase (*Bo*GH43A).

Run order	Xylosidase loading (U/mL) (A_2)	XOS concentration (g/L) (B_2)	Time (min) (C_2)	Total Reducing Sugar, TRS (g/g XOS)	
				Predicted	Experimental[#]
1	21	1.30	62.5	1.79	1.79
2	40	2.08	62.5	1.83	1.83
3	21	1.30	62.5	1.79	1.79
4	21	1.30	62.5	1.79	1.8
5	40	1.30	120	1.75	1.76
6	21	1.30	62.5	1.79	1.79
7	21	2.08	5	1.57	1.58
8	21	1.30	62.5	1.79	1.79
9	2	1.30	120	1.635	1.64
10	2	1.30	5	1.58	1.57
11	2	0.52	62.5	1.82	1.82
12	21	2.08	120	1.71	1.71
13	40	1.30	5	1.675	1.67
14	40	0.52	62.5	1.92	1.92
15	2	2.08	62.5	1.72	1.72
16	21	0.52	5	1.74	1.74
17	21	0.52	120	1.73	1.72

[#]Mean value (n=3).

ANOVA shows the F-value was 224.5 and hence the model was significant with a determination co-efficient (R^2) of 0.9965 (Table 4.3.5). p-values of A_2, B_2, C_2, B_2C_2, B_2^2 and C_2^2 were less than 0.05, making them significant terms. The predictability of the model to predict new responses for future observations (Pred-R^2) was 0.9535. The determination coefficient (R^2) of 0.9965 indicated that 99.65% of the variability in the response could be explained by the model. The "Pred-R^2" of 0.9535 was close to the R^2, 0.9965. The model was highly reliable with a much lower coefficient of variation (C.V.) of 0.46%.

Fig. 4.3.3a shows the 3-D plot of TRS yield with respect to Xylosidase loading (U/mL) (A_2) and XOS Concentration (g/L) (B_2). As the xylosidase loading increased from 2 to 40 U/mL, the TRS yield also increased. The TRS yield decreased with the increase in XOS loading from 0.52 to 2.08 g/L. Fig. 4.3.3b shows the TRS yield plot

with respect to time (C_2) and xylosidase loading (A_2). When time was varied from 5 to 120 min, the TRS yield increased till approximately 80 min and then started decreasing. TRS yield gradually increased with increase in xylosidase loading as in Fig. 4.3.3a.

Table 4.3.5. ANOVA for the quadratic model of Xylo-oligosaccharide (XOS) hydrolysis by exo-1,4-β-xylosidase (*Bo*GH43A).

Source	SS	Df	Co-efficient	F-Value	p-value
Quadratic model	0.131333	9		224.50	< 0.0001
A_2-Xylosidase loading	0.023113	1	0.05375	355.58	< 0.0001
B_2-XOS concentration	0.0162	1	-0.045	249.23	< 0.0001
C_2-Time	0.009113	1	0.03375	140.19	< 0.0001
A_2B_2	0.00002	1	0.0025	0.38	0.5548
A_2C_2	0.0001	1	0.005	1.54	0.2548
B_2C_2	0.005625	1	0.0375	86.54	< 0.0001
A_2^2	0.000009	1	0.0015	0.15	0.714
B_2^2	0.003541	1	0.029	54.48	0.0002
C_2^2	0.075041	1	-0.1335	1154.48	< 0.0001
Intercept			1.79		
Residual	0.000455	7	0.00006		
Lack of Fit	0.000375	3	0.000125	6.25	0.0544
Pure Error	0.00008	4	0.00002		
Total	0.131788	16			
Model statistics					
S.D.	0.0081		R^2	0.9965	
Mean	1.74		Adj-R^2	0.9921	
C.V. %	0.46		Pred-R^2	0.9535	

Df - degrees of freedom; SS - sum of squares; C.V.% - Coefficient of variation; Adj-R^2 - Adjusted R^2; Pred-R^2 - Predicted R^2

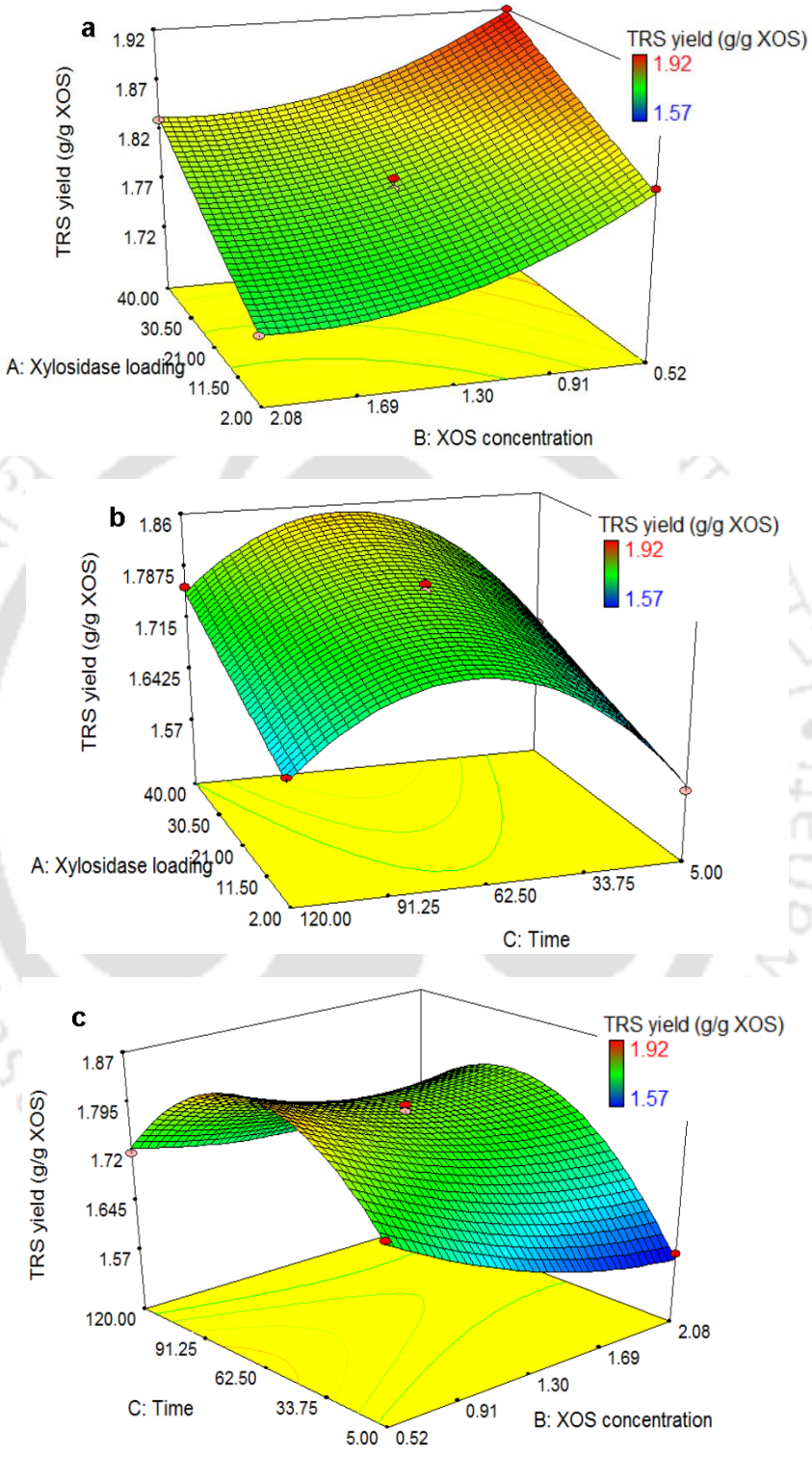

Fig. 4.3.3. 3-D surface response plots for the interaction between the independent variables involved in the optimization of xylo-oligosaccharide (XOS) hydrolysis by exo-1,4-β-xylosidase (*Bo*GH43A) (a) xylosidase loading (U/mL) and XOS concentration (g/L); (b) xylosidase loading (U/mL) and time (min); and (c) XOS concentration (g/L) and time (min).

Fig. 4.3.3c shows the 3-D plot of TRS yield with respect to time (C_2) and XOS concentration (B_2). It also showed that time is very significant factor for XOS hydrolysis with *Bo*GH43A. The difference in the TRS yield at different XOS loading was insignificant (Fig. 4.3.3a and 4.3.3c). Hence, higher XOS loading can be considered to get high TRS concentration.

The predicted optimum xylosidase loading (A_2), XOS concentration (B_2) and hydrolysis time (C_2) obtained from the quadratic model of RSM were 40 U/mL, 2.08 g/L and 79.4 min, respectively (Table 4.3.6). The predicted TRS yield from the above conditions was 1.84 g/g of XOS, whereas, the experimentally observed TRS yield was 1.77 g/g of XOS which was equivalent to 70 mg/g ptd biomass.

Table 4.3.6. Validation of predicted optimum parameters for Xylo-oligosaccharide (XOS) hydrolysis by xylosidase (*Bo*GH43A).

Factor name	Predicted optimum condition	Total Reducing Sugar, TRS (g/g XOS)	
		Predicted	Experimental[#]
Xylosidase loading (U/mL)	40	1.84	1.77
XOS Concentration (g/L)	2.08		
Time (min)	79.4		

[#]**Mean value (n=3).**

4.3.4. Thin layer chromatography (TLC) analysis of hydrolysed products of the pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A)

The spots of hydrolysed products from the enzymatic hydrolysis of pretreated FMS by *Ct*Xyn11A and *Bo*GH43A were visualized on the TLC plate (Fig. 4.3.4).

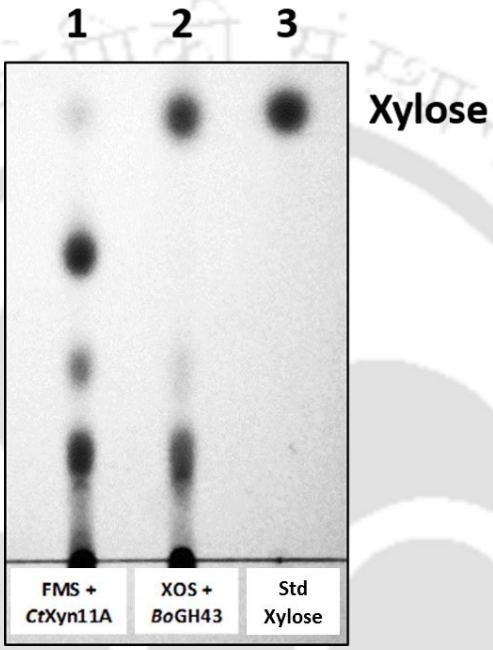

Fig. 4.3.4. Thin layer chromatography (TLC) of the hydrolysed products from pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) under optimised conditions. Lane 1- Xylo-oligosaccharides (XOS) production from the enzymatic hydrolysis of pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A); Lane 2- Xylose production from the enzymatic hydrolysis of xylo-oligosaccharides by exo-1,4-β-xylosidase (*Bo*GH43A); and Lane 3- Xylose standard.

Lane 1 shows the xylo-oligosaccharides and only a faint spot of xylose produced by endo-1,4-β-xylanase (*Ct*Xyn11A) at 55°C. Lane 2 shows the hydrolysis of xylo-oligosaccharides by exo-1,4-β-xylosidase (*Bo*GH43A) at 37°C producing intense spot of xylose. The results showed that the enzymes *Ct*Xyn11A and *Bo*GH43A rendered efficient consecutive action on the hemicellulose portion of pretreated FMS under the mentioned optimised conditions to release xylose. The lane 2 also shows the

un-hydrolysed oligomers from the xylosidase action. These might be the oligomers composed of residues other than xylose, cross-linked with the xylan. The application of *Ct*Xyn11A and *Bo*GH43A along with other hemicellulases and cellulases can be exploited to enhance the saccharification yield.

4.3.5. Determination of percentage of xylan to xylose conversion by HPLC

The HPLC analysis showed that the TFA treated FMS sample, which was pretreated by 1% (w/v) NaOH combined with oven heating contained 169 mg xylose/g ptd biomass. Therefore, the xylan content in the pretreated FMS was considered to be 169 mg/g ptd biomass. The final TRS yield from enzymatic hydrolysis of the pretreated FMS with *Ct*Xyn11A and *Bo*GH43A under optimum conditions was 70 mg/g ptd biomass (section 4.3.3). This was the overall reducing sugar resulted from the above saccharification. The HPLC analysis showed that particularly the xylose yield from the above enzymatic hydrolysis was 41.7 mg xylose/g ptd biomass. This showed that 41.7 mg/g ptd biomass out of the final TRS yield of 70 mg/g ptd biomass was xylose. Hence, there was a difference of 28 mg/g ptd biomass between the final TRS and xylose yield. This must be other oligosaccharides resulted from endo-xylanase hydrolysis. The xylan conversion from pretreated FMS to xylose was 24.7%. FMS has not been reported earlier as a bioresource for bioethanol production. Hence, the comparison of xylan saccharification from FMS was made against other lignocellulosic biomasses from earlier studies (Table 4.3.7). Peng *et. al.*, (2015) reported 59% xylan conversion to xylose from pretreated corn stover using crude enzyme from *Caldicellulosiruptor owensensis*, a xylanolytic bacterium, but the initial xylan present in the biomass was very less (2%). Few other reports have focussed on the synergistic effect of xylanases along with the cellulases. Amore et al. (2014)

reported 44% xylan conversion from ammonia treated Brewers spent grain on enzymatic hydrolysis with the native xylanase from *Bacillus amyloliquefaciens* along with commercial β-xylosidase, cellulase and cellobiase. The higher xylan conversion from above report was due to the synergistic effects of other enzymes present in the cocktail. Maitan-Alfenas et al. (2016) reported 19.6% xylan conversion from NaOH treated sugarcane bagasse using recombinant xylanase from *Asperginllus nidulans* along with commercial accellerase. The enzymatic hydrolysis of biomass, when compared with the similar pretreatment method (1% (w/v) NaOH), the xylan conversion by *Ct*Xyn11A used in this study was higher than that reported by Maitan-Alfenas et al. (2016). However, each lignocellulosic biomass differs in its structural composition and organization (Sorek N. *et. al.*, 2014). This study will serve as the primary report on the xylan saccharification of alkali-treated FMS using endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) for future studies on the biomass.

Table 4.3.7. Comparison of xylan saccharification by different xylanases from other studies.

Biomass	Pretreatment	Enzyme(s)	Source organism	Specific activity (U/mg)	Xylan (%, w/w)	Xylan to xylose conversion (%, w/w)	Reference
Corn stover	Steam explosion	Exrta-enzyme (native) + Intra-enzyme (native)	*C. owensensis*	4.72 1.57	2.2	59.1	Peng *et. al.*, 2015
Brewer's spent grain	5% (v/v) aqueous ammonia solution at 70°C for 22 h	Xylanase (native) + commercial cellulase, cellobiase and β-xylosidase	*B. amylolique-faciens*	NA	23.3	43.7	Amore *et. al.*, 2014
Sugarcane bagasse	Autoclaving with 1.0% (w/v) NaOH at 120°C for 60 min	Xylanase (XlnC, recombinant) + accellerase	*A. nidulans*	105.13	NA	19.6	Maitan-Alfenas *et. al.*, 2016
Finger millet straw	1% NaOH + Oven heating at 120°C for 20 min	Endo-1,4-β-xylanase (*Ct*Xyn11A, recombinant) followed by exo-1,4-β-xylosidase (*Bo*GH43, recombinant)	*C. thermocellum* *B. ovatus*	3900 59	16.9	24.7	This study

NA-*not available*

Conclusions

Finger millet straw pretreated with 1% (w/v) NaOH combined with oven heating containing a hemicellulose content of 20.7 % (w/w) was subjected to a sequential saccharification with recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus*. Two step optimization was done using Box-behnken design, RSM. The factors for the hemicellulose hydrolysis to xylo-oligosaccharides (XOS) using endo-1,4-β-xylanase (*Ct*Xyn11A), i.e., biomass loading (A_1), xylanase loading (B_1) and hydrolysis time (C_1) were varied from 1- 7 % (w/v), 50- 600 U/g ptd biomass and 4- 72 h, respectively. The predicted optimum biomass loading (A_1), xylanase loading (B_1) and hydrolysis time (C_1) were 6 % (w/v), 559 U/g ptd biomass and 56 h, rspectively. The predicted TRS yield, $TRS_{(XOS)}$ 39.3 mg/g ptd biomass. The TRS yield from the experiment under the above conditions was 39.6 ± 0.6 mg/g ptd biomass (2.4 g/L). The parameters, xylosidase loading (A_2), XOS concentration (B_2) and hydrolysis time (C_2) for the hydrolysis of XOS using exo-1,4-β-xylosidase (*Bo*GH43A) were varied from 2- 40 U/mL, 0.52- 2.08 g/L and 5- 120 min, respectively. The predicted optimum xylosidase loading (A_2), XOS concentration (B_2) and hydrolysis time (C_2) were 40 U/mL, 2.08 g/L and 79.4 min, respectively. The predicted TRS yield from the optimum conditions was 1.84 g/g of XOS. The experimental TRS yield from the above optimum conditions was 1.77 g/g of XOS, which was equivalent to 70 mg/g ptd biomass. Saccharification of the pretreated FMS by *Ct*Xyn11A and *Bo*GH43A under optimized conditions resulted in 24.7% conversion of xylan to xylose.

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Chapter 5

Fermentation of xylose of hydrolysate from acid treated FMS and pyrolysis of solid residue: A biorefinery approach

5.1. Introduction

The xylose concentration resulted from the optimization of enzymatic hydrolysis of hemicellulose from the pretreated (1% (w/v) NaOH combined with oven heating) FMS using recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) from *Clostridium thermocellum* and exo-1,4-β-xylosidase (*Bo*GH43A) from *Bacteroides ovatus* was 2 g/L (Chapter 4). It was lower than the xylose concentration in the acid hydrolysate (7.8 g/L) from the pretreatment involving 1% (v/v) H_2SO_4 combined with autoclaving on FMS (Chapter 3, Section 3.3.5, Table 3.3.2). If the xylose from the latter method can be used as the feed for fermentation, the cost and time on the enzyme production and saccharification can be eliminated, making the ethanol production process cost effective. To utilize the hydrolysate obtained from the acid treatment of any lignocellulosic biomass, it needs to be detoxified. Biological, physical, and chemical methods have been employed for detoxification. Biological detoxification involves

utilization of laccase to get rid of small phenolic compounds resulting from acid treatment. Physical detoxification involves employment of evaporation techniques to remove the volatile inhibitors from the hydrolysate such as furfural and acetic acid. Chemical detoxification involves the use of chemicals like Calcium hydroxide, $Ca(OH)_2$ and sodium sulphite, Na_2SO_3 to reduce the concentrations of hydroxymethylfurfural (HMF) and furfural. Among above mentioned detoxification methods, chemical detoxification serves as the most efficient detoxification method (Palmqvist and Hahn-Hagerdal 2000). Chemical detoxification methods include neutralization of acidic hydrolysate with base, NH_4OH (Agbogbo and Wenger 2007) or overliming to higher pH up to 10-11 with $Ca(OH)_2$ and then bringing down the pH that is favourable for fermentation with sulphuric acid (Ranatunga *et. al.* 2000). *Pichia stipitis, Candida shehatae and Pachysolen tannophilus* are naturally-occurring microorganisms that can ferment pentose sugars (Agbogbo and Coward-Kelly 2008). *Pichia stipitis* is an active and industrially promising C5 fermenting yeast in anaerobic conditions, resulting high ethanol yield (Diaz *et. al.* 2009, Shrivastava *et. al.* 2014).

Biorefinery approach can be applied to utilize the solid residue obtained from the pretreatment of biomass, while utilizing the acid hydrolysate for fermentation. For the complete utilization of the solid residue, pyrolysis can be carried out aiming to produce various value added products. A variety of solid wastes from agrowaste to sewage sludge have been pyrolysed to produce bio-oil and bio-char in earlier reports (Sanna *et. al.* 2011, Callegari and Capodaglio 2018). In this study, detoxification of acid hydrolysate resulting from the acid pretreatment (1% (v/v) H_2SO_4 combined with autoclaving) of FMS by overliming with $Ca(OH)_2$ was carried out. Further the ethanol

production from the detoxified hydrolysate was carried out by using *Pichia stipitis* NCIM-3497. The pyrolysis of the solid residue obtained from the pretreatment of FMS for the production of Bio-char and Bio-oil and their characterization was carried out.

5.2. Materials and Methods

5.2.1. Detoxification of acid hydrolysate

The acid pretreatment of FMS was carried out by taking 5% (w/v) of powdered biomass soaked in 2 L of 1% (v/v) H_2SO_4 and autoclaved at 121°C, 15 psi for 20 min as described in section 3.2.1. The hydrolysed biomass was filtered by using muslin cloth and the remaining solid residue was washed with distilled water till the neutral pH was achieved and then dried at 70°C. The filtrate containing acid hydrolysate (1 L) from the above pretreatment was heated up to 100°C for 15 min to remove the volatile compounds. The solution was then overlimed by adding $Ca(OH)_2$ while stirring until pH 10.0 was achieved. The overlimed hydrolysate was centrifuged at 5000 rpm for 15 min and the pellet was discarded. The supernatant was reacidified to pH 6.0 by adding 1N H_2SO_4 and filtered through Whatman No. 1 filter paper and stored at 4°C for fermentation (Kumar *et. al.* 2019).

5.2.2. Microorganism and maintenance

The pentose fermenting yeast, *Pichia stipitis* NCIM-3497 was procured from National Collection of Industrial Microorganisms (NCIM), CSIR- National Chemical Laboratory (NCL), Pune, India. The yeast was grown in the malt extract medium containing 30 g/L malt extract, pH 5.5 at 30°C and 150 rpm for 24 h and then aliquoted as glycerol stocks and maintained at -20°C. The inoculum for fermentation was prepared in the YPMX medium containing the following media components in g/L: 20.0 D-xylose, 1.5 yeast extract, 3.0 malt extract and 5.0 peptone, pH 5.0 (Magdum *et. al.* 2009).

5.2.3. Pentose fermentation for ethanol production by *Pichia stipitis* NCIM-3497

Two fermentation media for pentose fermentation to produce ethanol were compared by using *Pichia stipitis* NCIM-3497.

i) The detoxified hydrolysate at pH 5 was used as the fermentation medium.

ii) The detoxified hydrolysate supplemented with the following additional medium components at pH 5 (Kumar *et. al.* 2009):

Component	Concentration (g/L)
Yeast extract	1
KH_2PO_4	2
$(NH_4)SO_4$	1
$MgSO_4.\ 7H_2O$	0.5
Trace element solution	1 mL/L

Trace element solution:

Trace element	Concentration (g/L)
$CuSO_4.\ 5H_2O$	2.5
$FeCl_3.\ 6H_2O$	2.7
$MnSO_4.\ H_2O$	1.7
$Na_2Mo2O_4.\ 2H_2O$	2.42
$ZnSO_4.\ 7H_2O$	2.87
$CaCl_2.\ 6H_2O$	2.4

5% (v/v) inoculum by *Pichia stipitis* NCIM-3497 having 0.6 OD was added to 50 mL of either fermentation media in a 100 mL screw cap bottle in order to maintain anaerobic condition. The fermentation was carried out at 30°C, pH 5 and 150 rpm in an incubation shaker for 4 days. The experiments were carried out in duplicate and sampling was done at a regular interval of 12 h to determine the cell mass, xylose, arabinose and ethanol concentration.

5.2.4. Cell mass (*Pichia stipitis* NCIM-3497) estimation

At every 12 h interval, 1 mL of the culture in a pre-weighed microfuge tube was centrifuged at 5000 rpm for 10 min and the supernatant was removed. The cell pellet was dried at 60°C and weighed until no change in weightwas observed to determine the dry cell weight.

5.2.5. HPLC analysis of xylose, arabinose and ethanol from fermentation

The supernatant samples from both fermentations (0 h to 96 h) were filtered through a 25 mm polyvinylidene fluoride (PVDF) membrane filter with 0.45 µm pore size. The xylose, arabinose and ethanol contents in the filtrate were determined. The analysis was carried out by using HPLC system (Shimadzu corporation, LC-20AD, Japan) coupled with an autosampler (Shimadzu corporation, SIL-20AHT, Japan) and RI detector (Shimadzu corporation, RID-10A, Japan). The HPLC column, Phenomenex Rezex ROA (H+) organic acid and monosaccharide column (300 mm x 7.8 mm) coupled with a guard column (50 mm x 7.8 mm) was used. A mobile phase of 0.005 N H_2SO_4 was run at a flow rate of 0.6 mL/min through the column. The concentration of each sugar and ethanol in the sample was calculated with respect to the area of the respective peak.

5.2.6. Pyrolysis of the solid residue from the acid pretreatment of FMS

The pretreated (1% (v/v) H_2SO_4 combined with autoclaving) FMS was pyrolysed. The pyrolysis setup consists an inert gas system, heating unit, batch pyrolysis reactor vessel, bio-oil vapour condensation system and collection system. 99.9% pure nitrogen gas was used as the inert gas. Pyrolysis reactor vessel which is made of Stainless Steel 316 Grade is highly resistant to corrosion. This is due to the

acidic nature of the bio-oil. The reactor has a capacity of 600 ml. The pyrolysis reactor vessel is placed inside an electrical furnace which is capable of heating up to 900 °C. The approximate heating rate of the furnace is 25 °C/min and can reach the maximum temperature of 900 °C within 45 min. The temperature inside the reactor was monitored by a sensitive thermocouple. The temperature of the electrical furnace is controlled using a PID controller. The condensers are made of stainless steel to achieve faster heat transfer rates which assist in the condensation. Cold water at temperature around 10 °C was circulated outside the bio-oil outlet vapour flow line for condensation. The bio-oil collection system consists of separating funnels where the condensed vapours are collected. Funnels are used so that the bio-oil can be tapped out from time-to-time for analysis. 30 g of acid treated FMS was taken in the reactor and the furnace was set at 600 °C. The resulting bio-oil was analysed for chemical composition by using gas chromatography and mass spectroscopy. The residual char obtained after pyrolysis was weighed.

5.2.7. GC-MS analysis of the oil produced from pyrolysis

The pyrolysed oil obtained from the pyrolysis of acid treated FMS was analysed for its chemical composition by using a Clarus 680-GC (Gas Chromatography) and Clarus 600C-MS (Mass Spectroscopy) using a 60 m X 250 µm VF–5MS column (inert 5% phenyl–methyl column). 99.9% Helium gas was used as the carrier gas. 1.5 µL was the injection volume. Injection point temperature was set at 280 °C to vaporize the acetone solubilized bio-oil. The instrument was operated with a split ratio of 1:10. The GC oven temperature was kept at 60 °C for 2 min followed by a heating rate of 5 °C/min up to 140 °C and followed by a holding time at 140 °C for 5 min. The column was further heated at a heating rate of 5 °C/min till 300 °C and

held for 5 min. The pyrolysis oil was diluted with acetone (HPLC grade, Merck, India) in 1:200 ratio and was filtered to screen out large particles through a hydrophilic Polytetrafluoroethylene (PTFE) filter (Millipore, USA) (0.2 µm pore size) before sample injection.

5.3. Results and Discussion

5.3.1. Fermentation of the detoxified hydrolysate

The detoxified hydrolysate contained 6 g/L of xylose and 2.3 g/L of arabinose at 0^{th} h. Fermentation of the detoxified hydrolysate using *Pichia stipitis* NCIM-3497 was carried out at 30°C, pH 5 and 150 rpm in shaking incubator for 96 h. The ethanol produced in the detoxified hydrolysate at the end of 96 h fermentation was 1.3 g/L (Fig 5.3.1).

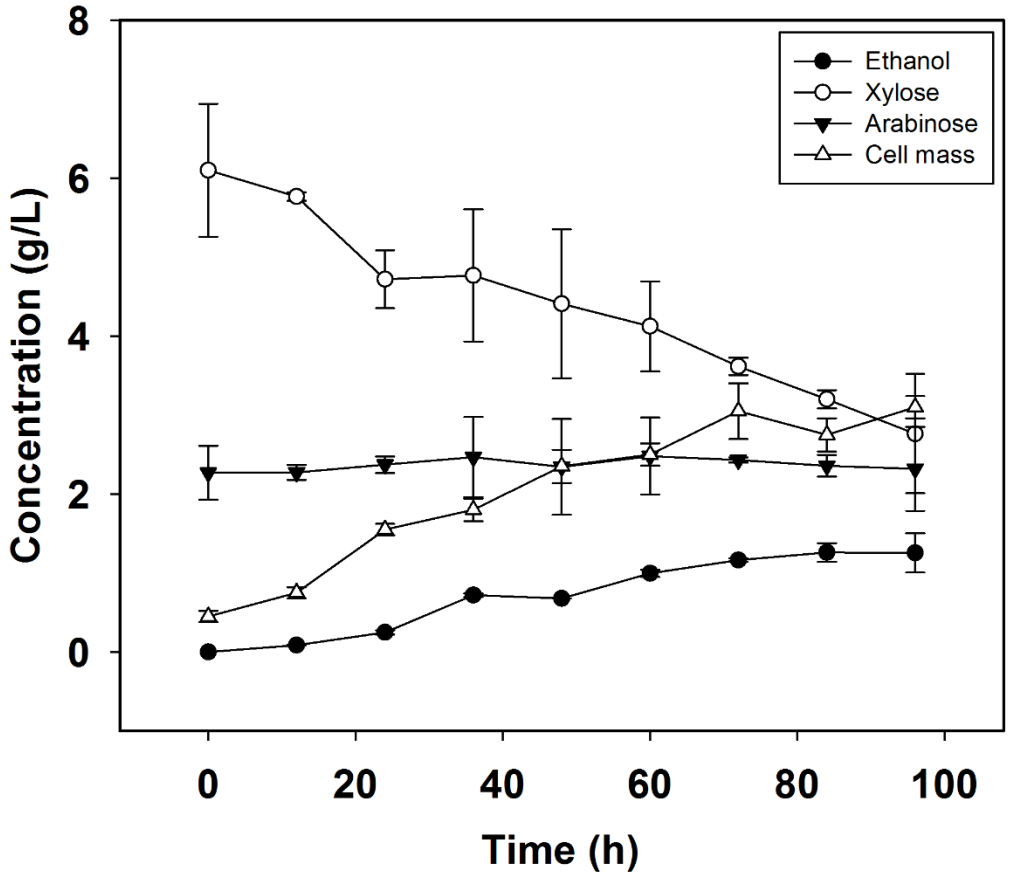

Fig 5.3.1. Fermentation profile of detoxified hydrolysate from acid treated FMS by *Pichia stipitis* NCIM-3497.

Xylose consumption during the fermentation was 3.3 g/L at 96 h. Arabinose was not utilized by the yeast, *Pichia stipitis* NCIM-3497. Therefore, the ethanol yield from the fermentation of xylose from the detoxified hydrolysate was 0.39 g/g xylose consumed. 2.7 g/L of xylose remained unutilized by the microorganism. The biomass growth during the fermentation resulted in 3.1 g/L of cell mass concentration at 96 h.

When the detoxified hydrolysate was supplemented with synthetic fermentation media components except xylose, the ethanol concentration increased to 2 g/L from 1.3 g/L at 96 h (Fig 5.3.2).

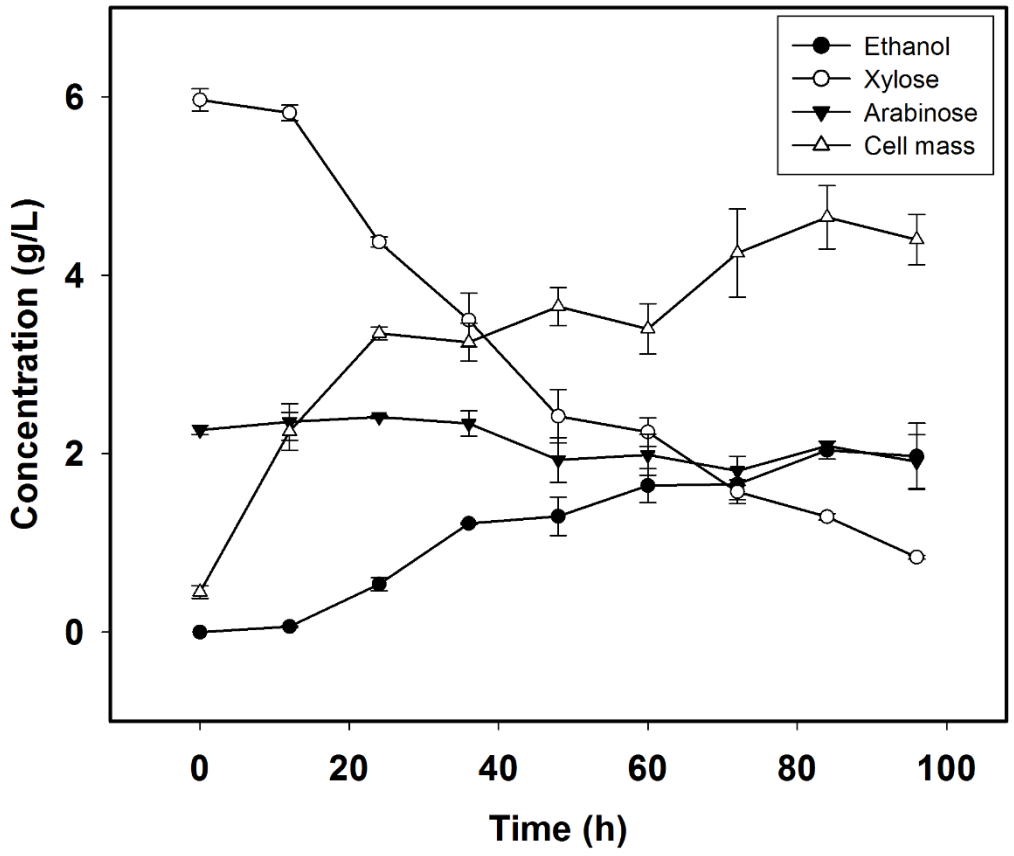

Fig 5.3.2. Fermentation profile of detoxified hydrolysate from acid treated FMS supplemented with medium components except xylose by *Pichia stipitis* NCIM-3497.

The xylose consumption was also increased from 3.3 g/L to 5.1 g/L. Only 0.9 g/L of xylose was observed to be unutilized. Irrespective of the presence of synthetic media components, arabinose remained unutilized by *Pichia stipitis* NCIM-3497. This showed that xylose was more favourable substrate than arabinose for the yeast, *Pichia stipitis* NCIM-3497. The ethanol yield was found to be 0.39 g/g xylose consumed, which is similar to the ethanol yield from the plain detoxified media without any addition of synthetic media components. The biomass concentration was found to be 4.4 g/L at 96 h in the presence of synthetic media components. This clearly showed that the presence of other synthetic media components favoured both cell growth and ethanol production by the cells of *Pichia stipitis* NCIM-3497.

The results showed that the detoxified hydrolysate resulted from the acid treatment along with autoclaving of FMS served as a good fermentation medium for ethanol production by *Pichia stipitis* NCIM-3497. Supplementation of additional medium components except xylose led to enhanced ethanol production. The low concentration of ethanol (2 g/L) from the fermentation of detoxified hydrolysate supplemented with additional medium components in the present study was due to the low concentration of xylose (6 g/L) available in the hydrolysate. For the level of xylose present in the above medium, the yeast, *Pichia stipitis* NCIM-3497 performed well by utilizing 85% of xylose to attain an ethanol yield of 0.39 g/g xylose utilised. The ethanol yield presented in this study is in agreement with the earlier reported ethanol yields from the fermentation of detoxified hydrolysates of different lignocellulosic feedstock using other *Pichia stipitis* strains (Nigam 2001a and Huang *et. al.* 2009) as shown in Table 5.3.1.

Table 5.3.1: Ethanol yield from fermentation of the detoxified acid hydrolysates from various lignocellulosic feedstock

Feedstock	Pretreatment	Fermenting yeast strain (*P. stipitis*)	Ethanol Yield ($Y_{p/s}$)	Reference
Wheat straw	1.85% (w/v) H_2SO_4 at 90°C, 18 h.	NRRL 7154	0.35	Nigam 2001a
Red-oak wood chips	0.5% (v/v) H_2SO_4 for 4 h, 1212 kPa steam, 4 min.	NRRL Y-7124	0.25	Nigam 2001b
Rice straw	1–3% (w/w) of H_2SO_4, 130°C with low pressure steam, 15 min.	BCRC 21777	0.37	Huang *et. al.* 2009
Water hyacinth	2% (v/v) H_2SO_4, refluxing at room temperature, 7 h, 250 rpm.	NCIM 3497	0.43	Kumar *et. al.* 2009
Water hyacinth	1% (v/v) H_2SO_4, refluxing for 8 h, 250 rpm.	NCIM 3497	0.45	Magdum *et. al.* 2009
Sorghum stalk	0.2 M H_2SO_4 at 121 °C, 2 h.	NCIM 3498	0.46	Deshavath *et. al.* 2017
Finger millet straw	1% (v/v) H_2SO_4, autoclaving at 121°C, 15 psi, 20 min.	NCIM 3497	0.39	This study

Kumar *et. al.* 2009, Magdum *et. al.* 2009 and Deshavath *et. al.* 2017 applied vacuum using rotary evaporator to concentrate the available xylose in the detoxified hydrolysates from water hyacinth and sorghum stalk, respectively. The vacuum evaporation method can be applied for our substrate and the media components can be optimised for enhanced ethanol production in future.

5.3.2. Pyrolysis of the solid residue from the acid pretreatment of FMS

Pyrolysis of the acid treated FMS resulted in 41.98% (w/w) yield of bio-oil and 27.2% (w/w) char. The bio-oil had a density of 1.09 g/mL with a pH of 3.49. Fig 5.3.3 shows the chromatogram from the GC-MS analysis of the bio-oil from the pyrolysis of solid residue resulted after the acid treatment of FMS. The prominent peaks were analysed with respect to the GC-MS library and earlier literature (Branca *et. al.* 2003). The chemical composition of the bio-oil obtained from GC–MS analysis is given in Table 5.3.2.

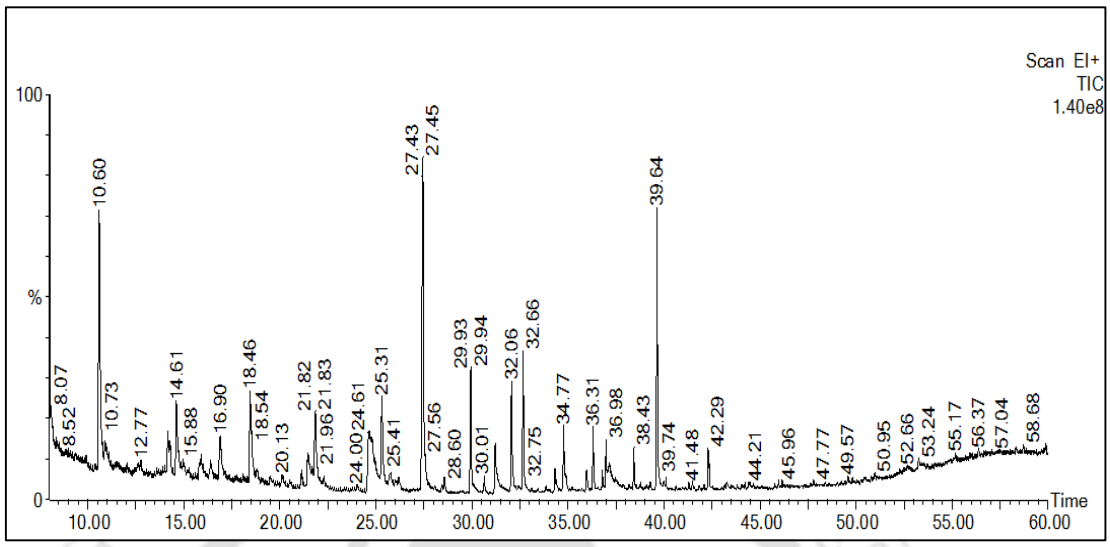

Fig. 5.3.3. GC-MS chromatogram of the bio-oil obtained after pyrolysis of acid pretreated (1% (v/v) H_2SO_4 combined with autoclaving) finger millet straw.

Table 5.3.2: GC-MS analysis of bio-oil.

Retention Time (min)	Name of the compound	% Composition
10.60	Furfural	10.3
14.16	N-butyl ether	1.4
14.26	1-(acetyloxy)-2-butanone	1.1
14.61	5-methyl-2-furaldehyde	3.4
16.90	2-hydroxy-3-methyl-2-cyclopenten-1-one	2.0
18.46	1-(2-methyl-1-cyclopenten-1-yl)-Ethanone	4.4
21.45	2-ethylphenol	2.0
21.82	Creosol	3.8
24.61	2,3-dihydrobenzofuran	7.1
25.31	4-ethylguaiacol	2.7
27.45	1-(2-hydroxy-5-methylphenyl)-Ethanone	12.4
29.93	Syringol	3.4
31.21	1-ethyl-1-methoxy-1-silacyclohexane	2.5
32.06	Eugenol	3.0
32.66	1,2,4-trimethoxybenzene	3.4
34.77	1,2,3-Trimethoxy-5-methyl-benzene	1.5
36.31	1-(3,4-Dimethoxyphenyl)-Ethanone	1.5
37.15	D-Allose	2.0
39.64	4-allylsyringol	8.4
42.29	1,1'-butylidenebis-Benzene	0.8

The classification of compounds was done based on the work of Stas *et. al.* (2014) (Table 5.3.3). This analysis shows that ~ 50% (w/w) oil comprises aromatics, 13.7% (w/w) heterocyclics and 12% (w/w) of non–aromatics. Table 5.3.3 shows the sub-classification of the components present in the bio-oil. A major part of the aromatic compounds is classified under methoxyphenol derivatives which are attributed to the thermal cracking of lignin (Branca *et. al.* 2003). The compounds which comes under the methoxyphenol derivatives category are syringol, creosol, eugenol and a few derivatives of syringol. 12% of bio-oil components being heterocyclic in nature are purely attributed to the presence of furans which are obtained as a result of the pyrolysis of cellulose (Lua *et. al.* 2011). 2% D-allose was detected in the bio-oil which is also one of the products of the pyrolysis of cellulose.

Table 5.3.3: Relative distribution of compounds in bio-oil resulting from pyrolysis of acid treated FMS

Classification	Compounds	% Composition
Heterocyclics	Furans	13.7
Aromatics	Phenols	14.4
	Metoxy-, dimethoxyphenol derivatives	22.8
	Aromatic Compounds (Oxygenates)	12.1
	Aromatic Compounds (Non-oxygenates)	0.8
Non-Aromatics	Non-Aromatic Ketones	7.5
	Other Non-Aromatic Compounds	5.9

The identified bio-oil products ranged between C6 and C15 with a distribution of about 60.6% of the total 75.8% identified peaks in the C6 - C9 range. Thermal cracking of the biomass also results in the formation of C1-C4 compounds which are accounted in the vapour steam which accounts to 30.82%. Batch pyrolysis reactors are

characterized by high vapour residence time compared to continuous reactors. This results in further cracking of products formed initially by pyrolysis which results in the formation of lower carbon chain compounds (Czernik and Bridgwater 2004).

The char can be utilised as a combustion fuel for industrial boilers and as a soil amendment to improve the soil quality for agriculture (Abnisa *et. al.* 2013, Liang *et. al.* 2015). The abundance of value added chemicals such as furfural, 1-(2-hydroxy-5-methylphenyl)-ethanone and 4-allyl syringol in the bio-oil was high (Table 5.3.2). The bio-oil can be refined to separate the above value added chemicals for their applications in food and other industries. Furfural is also a flavouring agent. Its derivatives have applications in producing adhesives and resins and as adjuvant for pesiticides (Eseyin and Steele 2015). 1-(2-hydroxy-5-methylphenyl)-ethanone is a flavouring agent in coffee (Gilbert 2004). Syringol and its derivative like 4-allyl syringol have application in food industry as aromatic agent and in making phenolic resins and adhesive production (Mohan *et. al.* 2006, Holladay *et. al.* 2007, Effendi *et. al.* 2008).

Conclusions

The detoxified hydrolysate resulted from the sulphuric acid treatment along with autoclaving of FMS contained 6 g/L of xylose and 2.3 g/L of arabinose. Fermentation of the detoxified hydrolysate using *Pichia stipitis* NCIM-3497 at 30°C, pH 5 and 150 rpm for 96 h resulted in 1.3 g/L of ethanol and 3.1 g/L of cell mass concentration. 3.3 g/L of xylose was consumed by *Pichia stipitis* leading to an ethanol yield of 0.39 g/g xylose consumed. Fermentation of the detoxified hydrolysate supplemented with synthetic fermentation medium components except xylose by *Pichia stipitis* NCIM-3497 gave the ethanol production, 2 g/L. The biomass concentration was increased to 4.4 g/L in the presence of synthetic media components. Utilization of 5.1 g/L out of 6 g/L of xylose by *Pichia stipitis* led to the ethanol yield of 0.39 g/g of xylose consumed. Arabinose remained unutilised by *Pichia stipitis* in both the fermentations. In the presence of synthetic medium components except xylose in the detoxified hydrolysate, *Pichia stipitis* NCIM-3497 was able to consume more xylose leading to 35% higher ethanol production than in the plain detoxified hydrolysate. Pyrolysis of the solid residue from sulphuric acid treatment of FMS resulted in 41.98% (w/w) and 27.2% (w/w) of bio-oil and char, respectively. The density and pH of the bio-oil were 1.1 g/mL and 3.5, respectively. The bio-oil consisted of ~ 50% (w/w) of aromatics, 13.7% of (w/w) heterocyclics and 12% (w/w) of non–aromatics. The bio-oil contained abundant furfural, 1-(2-hydroxy-5-methylphenyl)-ethanone and 4-allyl syringol. These valuable products can be refined from the bio-oil further for their applications in food and resin industries.

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FUTURE PROSPECTS

This thesis has presented the potential of Finger millet straw as a feedstock for bioethanol production. Being an agrowaste coming from less irrigated areas, it will serve as the feedstock for ethanol production during the unavailability of irrigated crop wastes like rice and wheat straw. More research can help in complete utilization of the lignocellulosic biomass, Finger millet straw for the production of other valuable products along with ethanol. The future scope of this work are as follows:

1. The xylo-oligosaccharides produced from the enzymatic hydrolysis of alkali treated FMS with endo-1,4-β-xylanase (*Ct*Xyn11A) can be characterized for their role in food applications.
2. Concentration of the xylose in the detoxified hydrolysate from acid pretreatment of FMS for ethanol production can be focussed.
3. Optimization of the supplemented fermentation medium components in the detoxified hydrolysate can be carried out for enhanced ethanol production.
4. Pearl millet straw and Sorghum straw can also be exploited for their capabilities to act as a potential feedstock for ethanol production.

Journal publications
From Thesis:

1. **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.

2. **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.

3. **Jamaldheen S. B.**, Saynik P. B., Moholkar V. S., Goyal A. Fermentation of xylose of hydrolysate from acid treated Finger millet (*Eleusine coracana*) straw and pyrolysis of solid residue: A biorefinery approach. (To be submitted).

Other Publications:

1. 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.

2. N M., Singh S., Nath P., **Jamaldheen S. B.**, Moholkar V. S., Goyal A. Pretreatment of *Sorghum durra* stalk and saccharification by chimera (β-glucosidase and endo β-1,4 glucanase) and cellobiohydrolase for bioethanol production (Submitted).

Conferences

1. Presented a poster entitled "Comparative study of pretreatment methods for agrowaste pearl millet (*Pennisetum glaucum*) stalk for bioethanol production" at 9th NABS, School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai, India on Aug 11-12, 2016.

2. Presented a poster entitled "Comparative evaluation of pretreatment methods on agrowaste *Sorghum bicolor* stalk for bioethanol production" at BRSI, VIT University, Vellore, Tamil Nadu, India on Dec 8-10, 2016.

3. Presented a poster entitled "Pretreatment and clostridial enzymes hydrolysis of finger millet stalk for biofuel production" at DBT National Workshop on Bioenergy, IIT Roorkee, Uttarakhand, India on July 6-7, 2018.

4. Presented a poster entitled "Saccharification of hemicellulose from pretreated finger millet straw by β-1,4-endoxylanase for bioethanol production" at AMI, University of Hyderabad, India on Dec 9-12, 2018.

5. Presented an oral presentation entitled "Hemicellulose saccharification from pretreated finger millet straw by recombinant hemicellulases for bioethanol production" at IBA-IFIBiop 2019, Imperial Hotel, Miri, Sarawak, Malaysia on May 1-5, 2019.

6. Attended the 10th World Renewable Energy Technology Congress-2019 (WRETC) as a delegate at Convention Centre-NDCC, New Delhi, India on Aug 21-23, 2019.

Awards

1. Best poster award on the work entitled "Comparative study of pretreatment methods for agrowaste pearl millet (*Pennisetum glaucum*) stalk for bioethanol production" at 9th NABS, School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai, India on Aug 11-12, 2016.
2. Awarded with DBT-CTEP travel grant (Travel) from Department of Biotechnology, India for presenting (oral presentation) the work at IBA-IFIBiop 2019, Imperial Hotel, Miri, Sarawak, Malaysia on May 1-5, 2019.
3. Awarded with Student Travel Assistance Fund (STAF) fund (Registration) from IIT Guwahati, India for presenting (oral presentation) the work at IBA-IFIBiop 2019, Imperial Hotel, Miri, Sarawak, Malaysia on May 1-5, 2019.

VITAE

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Comparative analysis of pretreatment methods on sorghum (*Sorghum durra*) stalk agrowaste for holocellulose content

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ABSTRACT

This study compares different types of pretreatment methods, such as thermal pretreatment at 120 °C, autoclaving, microwaving and ultrasonication in the presence of water, dilute acid (1% H_2SO_4) or dilute alkali (1% NaOH) on Sorghum stalk with respect to the holocellulose and Acid Detergent/Insoluble Lignin content. Among all the methods, pretreatment with 1% NaOH along with autoclaving at 121 °C and 15 psi for 30 min was the most effective method for Sorghum stalk. Fourier Transform Infra-Red spectroscopy analysis of this pretreated biomass showed the removal of lignin and Field Emission Scanning Electron Microscope analysis displayed enhanced surface roughness. The enzymatic hydrolysis of raw and best pretreated Sorghum stalk using recombinant endo-β-1,4-glucanase (*Ct*Cel8A) and β-1,4-glucosidase (*Ct*Bgl1A) both from *Clostridium thermocellum* gave glucose yields, 22.4 mg/g raw biomass and 34 mg/g pretreated biomass, respectively, resulting in 1.5-fold increase of glucose yield after the pretreatment.

KEYWORDS

Acid insoluble lignin; enzymatic hydrolysis; FESEM; holocellulose; pretreatment; Sorghum stalk

Introduction

Non-renewable fossil fuels are continuously being depleted leading the scientific community to search for alternative fuels from renewable sources.[1,2] First generation bioethanol is produced by fermentation of the primary food stock including starchy (corn, wheat and tubers) and sugar crops such as sugarcane and sugarbeet.[3] Exploiting these food crops for bioethanol production competes with human food and affects the food supply. Hence, this led to the second generation bioethanol production from non-edible feedstocks such as lignocellulosic biomass including agricultural wastes, forest wastes, grasses, and weeds.[3,4] Exploration of various other lignocellulosic feedstocks such as forest wastes, grasses, and weeds besides the bulk seasonal feedstocks such as rice and wheat straw will help in identifying better candidates for running an alcoholic biofuel plant continuously.

Lignocellulosic feedstocks are cheaper and their utilization in bioethanol production supports the enhancement of local agricultural economy too.[1] As the name indicates, a lignocellulosic feedstock contains cellulose, hemicellulose, and lignin as the major components. Lignocellulosic feedstocks have a structural composition different from the starchy feedstocks. Hence, they require an extra pretreatment step to remove the rigid lignin exposing the cellulose and hemicellulose, for further enzyme hydrolysis. Hydrolysis with cellulase and hemicellulase results in the release of hexoses and pentoses, which are consumed as the carbon source by fermentative microbes to produce bioethanol.[5]

An efficient pretreatment method should concentrate the holocellulosic portion along with the delignification. The major pretreatment methods exploited on lignocellulosic biomass can be classified into physical, chemical, physico-chemical, thermal, and biological pretreatment.[6] Physical pretreatment reduces the particle size of the biomass through ball milling, extrusion, and so on.[7] Chemical treatment uses a variety of chemicals such as acid, alkali, organosolvents, ionic liquids, and so on[8,9] for delignification and decrystallization of biomass. Physico-chemical pretreatment is a combination of physical and chemical pretreatments. Pilot plants of Iogen Corporation, Canada and FibreETOH project, under 7th Framework Program of the European Commission use mild acid hydrolysis combined with steam explosion at high temperature and pressure for pretreating lignocellulosic biomass before enzymatic hydrolysis and fermentation.[10] In biological pretreatment, microorganisms, especially fungus is used to delignify the biomass with the help of enzymes they produce.[11,12] Other pretreatment methods which are less exploited are irradiation and ultrasonication.[13,14] Various pretreatment methods have been tried over different types of lignocellulosic feedstock. Depending on the lignin content and biomass strength, the pretreatment method varies. The better way is to screen the best method among them by experimenting each of them for each feedstock.[15,16]

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Out of the total net sown area in India, 55% is rainfed area, which contributes for more than 75% of the country's pulses and millet production. Sorghum (Great millet), is one of the majorly grown millets in India and its production was 10.62 MT in 2013–2014.[17] Sorghum grain is used as food and their stalks are partially used as fodder and rest is the left-over. Being a crop cultivated in rainfed area, Sorghum requires less water supply as compared with rice and wheat.[18] Rice and wheat straws have commercial value in mushroom cultivation and fiberboard production.[19,20] Unlike rice and wheat straw, Sorghum stalk does not have any other reported commercial value. Therefore, during the unavailability of rice or wheat straw in dry season, Sorghum stalk can contribute to continuous supply of lignocellulosic biomass for bioethanol production. Research work carried out with Sorghum stalk used as feedstock for bioethanol production is scanty and countable.[21–23] Exploring the structural characteristics of Sorghum stalk will pave the route for its utilization as the lignocellulosic feedstock for bioethanol production. Therefore, this study focuses on the structural composition analysis, comparison of various pretreatment methods on Sorghum stalk for increasing the accessible area for the hydrolytic enzymes targeting bioethanol production.

Experimental

Feedstock collection and processing

Sorghum (*Sorghum durra*) stalks were collected from Kappalpatti, Dindigul District, Tamil Nadu, India. The stalks were chopped, washed twice with tap water and rinsed with distilled water, dried in oven at 70 °C for 24 hr, ground to powder and passed through a 0.6 mm sieve.

Characterization of raw sorghum stalk

One gram of raw unground sorghum stalk was taken and dried in hot-air oven at 105 °C for 24 hr. The biomass was weighed after drying to determine the moisture content and the analysis was carried out in triplicate. The Carbon, Hydrogen, Nitrogen and Sulfur (CHNS) analyses of the powdered biomass was done using CHNS analyzer (EuroEA3000 Elemental Analyzer, Euro Vector, Italy). 0.5 g of raw biomass was used for holocellulose, α-cellulose, and hemicellulose determination and 1 g of raw biomass was used for acid insoluble lignin (ADL) determination. Holocellulose content was determined by the method described by Browning (1968).[24] α-Cellulose, hemicellulose, and ADL contents were determined by following the standard TAPPI protocols.[25] The biomass was taken in less quantity (0.5–1 g) for composition analysis and care was taken for achieving maximum accuracy and minimal wastage during the analysis in order to reduce the internal error.

Pretreatment of sorghum stalk biomass

Different pretreatment methods applied on the biomass were thermal pretreatment, microwave irradiation and ultrasonication in the presence or absence of dilute acid (1% H_2SO_4) or dilute alkali (1% NaOH). To analyze the impact of the chemical coupled with each method, uniform concentration of the chemical (1% H_2SO_4 or 1% NaOH) was used. Each pretreatment was carried out using 5% (w/v) of powdered biomass soaked in 200 mL aqueous solution. The conditions applied for each pretreatment are as follows:

Thermal pretreatment

Two kinds of thermal treatments were carried out as follows: (i) Dry heat: This treatment was carried out at 120 °C for 30 min in hot-air oven. (ii) Autoclaving: In this treatment, the conditions applied were 121 °C at 15 psi for 20 min. The thermal pretreatment methods used in this study were adopted from Singh et al.[15]

Microwave irradiation

This was carried out by using the microwave oven (Samsung, South Korea) at 180 W for 3 min. The microwave pretreatment followed was adopted from Singh et al.[13]

Ultrasonication

This treatment was done by keeping the sample in an ultrasonication bath (PCi analytics, India) at 33 kHz for 10 min. The ultrasonication method followed was from Singh et al.[26]

Time optimization of pretreatment by 1% NaOH and autoclaving

About 5% (*w/v*) of powdered raw biomass was pretreated by autoclaving at 121 °C and 15 psi with 1% NaOH for 10, 20, and 30 min. The determination of holocellulose content was carried out by following the method of Browning (1968)[24] and ADL content was determined by standard TAPPI method.[25]

Analytical methods

After each pretreatment, the biomass was filtered through a muslin cloth and the filtrate was separated from the solid part. The solid part was washed until the neutral pH was achieved. It was then dried at 70 °C for 24 hr for further studies, such as composition analysis, X-ray diffraction (XRD), Fourier transform infra-red spectroscopy (FTIR), and Field emission scanning electron microscope (FESEM).

Reducing sugars analyses of pretreated sorghum stalk by HPLC

The filtrate collected after each pretreatment was again filtered through poly(vinylidene fluoride) (PVDF) membrane of pore size, 0.45 µm using a membrane filter for reducing sugars analysis. The concentrations of glucose, xylose, and arabinose in the filtrate of each sample were determined by high performance liquid chromatography (HPLC, Agilent

technologies, 1220 Infinite LC, Santa Clara, CA) coupled with RI detector. The standard sugars, glucose, xylose, and arabinose (procured from Sigma Aldrich, St. Louis, MO) at the concentrations, 0.01, 0.02, 0.1, 0.2, 1, 2, 10, and 20 mg/mL were used. The HPLC column used was Phenomenex Rezex ROA (H+) (300 × 7.8 mm) along with a guard column (50 × 7.8 mm) meant for organic acid and monosaccharide analysis. The mobile phase, 0.005 N H_2SO_4 at a flow rate of 0.5 mL/min was used.

Structure composition analysis of pretreated sorghum stalk

Holocellulose and lignin content of the pretreated Sorghum stalk were determined gravimetrically by method described by Browning 1968[24] and standard TAPPI method,[25] respectively.

XRD analysis of untreated and pretreated sorghum stalk

The dried untreated Sorghum stalk and pretreated solid residue after each pretreatment (approximately, 1 g of each) were analyzed for their crystallinity indices (CrI) using X-ray diffractrometer (D8 Advance, Bruker, Germany). Each sample was scanned over a range of $2\theta = 10°–30°$ with a step size of 0.05°. The crystallinity index was calculated using the formula as described by Segal et al.[27]:

$$\text{CrI } (\%) = \frac{I_{\text{crystalline}} - I_{\text{amorphous}}}{I_{\text{crystalline}}} \times 100$$

where $I_{\text{crystalline}}$ is the intensity at $2\theta = 22°$ and $I_{\text{amorphous}}$ is the intensity at $2\theta = 18°$.

FTIR analysis

FTIR analysis of the dried untreated Sorghum stalk and the biomass from the effective pretreatment (1% NaOH + autoclaving at 121 °C and 15 psi for 30 min) was carried out. The pellets were prepared by mixing and grinding each sample (2 mg) and KBr in the ratio 1:100 (*w/w*). Each pellet was scanned in the wavenumber range 4000–400 cm^{-1} and the spectrum was recorded using FTIR spectrometer (Spectrum Two, PerkinElmer, Waltham, MA).

FESEM

A pinch of dried raw Sorghum stalk and the effectively pretreated (1% NaOH + autoclaving at 121 °C and 15 psi for 30 min) biomass samples were spread over a carbon tape and placed over the surface of a stub. The samples were gold coated and placed in vacuum chamber before imaging using FESEM (Zeiss, Sigma, Germany).

Production of recombinant enzymes for hydrolysis of sorghum stalk biomass

Endo-β-1,4-glucanase (*Ct*Cel8A) of family 8 glycoside hydrolase and β-1,4-glucosidase (*Ct*Bgl1A) of family 1 glycoside hydrolase from *Clostridium thermocellum* were transformed in *Escherichia coli* BL21 (DE3) cells. 50 µL from glycerol stock of each was inoculated in 5 mL Luria-Bertani (LB) medium supplemented with kanamycin (50 µg/mL) and incubated at 37 °C, 16 hr at 180 rpm. The 1% (v/v) inoculum of *E. coli* cells harboring genes encoding *Ct*Cel8A or *Ct*Bgl1A were inoculated each in 2 × 200 mL auto-induction medium (Himedia, Pvt. Ltd., India). It contains 1% Tryptone, 0.5% yeast extract, 171 mM NaCl, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 20 µM $FeCl_3$, 0.5% glycerol, 0.05% D-glucose, 0.2% lactose, pH 7.0). The medium supplemented with kanamycin (50 µg/mL) contained in 1 L Erlenmeyer flasks and incubated at 37 °C, 200 rpm initially for 4 hr. After that the cultures were incubated at 24 °C, 180 rpm for 24 hr for enzyme production. The *E. coli* cell biomass was collected by centrifugation at 8000*g* at 4 °C for 10 min. The pelleted biomass was then, resuspended in 10 mL lysis buffer containing 50 mM Tris–HCl buffer, pH 7.5, 1 mM phenyl-methylsulfonyl fluoride, 300 mM NaCl, and 50 mM imidazole. Resuspended cell biomass was lysed by ultra-sonication on ice for 30 min (5 s on/15 s off pulse, 33% amplitude, Sonics, Vibra cell) and cell free extract was obtained by centrifugation at 22,000*g* at 4 °C for 50 min. The N-terminal hexa-His tag containing *Ct*Cel8A and *Ct*Bgl1A enzymes were purified by immobilized metal-ion affinity chromatography (IMAC) using a 5 mL Sepharose column (HiTrap Chelating, GE Healthcare). The column was pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 300 mM NaCl and 50 mM imidazole. Both enzymes were eluted with elution buffer having composition, 50 mM Tris–HCl (pH 7.5), 300 M NaCl and 300 mM Imidazole. Five aliquots of eluent (1 mL each) were collected. Purified *Ct*Cel8A and *Ct*Bgl1A enzymes were dialyzed against 50 mM Tris–HCl buffer (pH 7.5). The concentration of enzymes was determined by UV absorption at A_{280} on a UV spectrophotometer (Varian, Cary 100) using molar extinction coefficients (ε) 98,120 $M^{-1} cm^{-1}$ for *Ct*Cel8A and 100,730 $M^{-1} cm^{-1}$ for *Ct*Bgl1.

Activity assay of endo-β-1,4-glucanase (CtCel8A) and β-1,4-glucosidase (CtBgl1A)

The *Ct*Cel8A activity was determined by incubating 10 µL (35 µg/mL) of enzyme with 1% (w/v) final concentration of carboxymethyl cellulose in 50 mM citrate phosphate buffer, pH 5.8 in a 0.1 mL reaction mixture at 60 °C for 5 min. The activity was determined by estimating the release of reduced sugar by the Nelson, 1944 and Somogyi, 1945 method. The amount of reducing sugar was determined by the standard curve of D-Glucose. The amount of *Ct*Cel8A requires for producing 1 µmole of reducing sugar (D-glucose) per min under optimum condition was defined as 1 unit (U) of enzyme activity.

The *Ct*Bgl1A activity was determined by using *p*-nitrophenyl-β-D-glucopyranoside (pNP Glucose). The enzyme reaction was performed in 1.0 mL reaction mixture containing 250 µM *p*-nitrophenyl-β-D-glucopyranoside in 50 mM citrate phosphate buffer, pH 5.8, 10 µL (80 µg/mL of stock)

of the enzyme, incubated at 60 °C for 5 min. The enzyme activity was measured by estimating the release of 4-nitrophenol (*p*NP), by taking absorbance at 405 nm (A_{405}) on a UV–Vis spectrophotometer (Varian, Cary 100 Bio) as described by Ahmed et al.[28] One unit of enzyme activity for *p*-nitrophenyl-β-D-glucopyranoside was defined as the amount of *Ct*Bgl1A required for liberating 1 µmole of *p*-nitrophenol per min.

Enzymatic hydrolysis of untreated and pretreated sorghum stalk biomass

The enzymatic hydrolysis of untreated and pretreated Sorghum stalk was carried out by taking 2.5% (*w/v*) substrates in 20 mL 50 mM citrate phosphate buffer, pH 5.8 in 100 mL Erlenmeyer flasks in triplicate sets. 200 µL of endoglucanase (*Ct*Cel8A, 2.5 mg/mL, 80.0 U/mg) and 350 µL of β-glucosidase (*Ct*Bgl1A, 1.8 mg/mL, 33.0 U/mg) were added to each of the flask. 0.03% (*w/v*) sodium azide solution was added to the reaction mixture to prevent the contamination. The reaction mixture was incubated at 60 °C in a shaker incubator orbital shaking at 180 rpm and for 24 hr. The reaction was stopped by boiling the reaction mixture for 5 min. It was then centrifuged at 14,000*g* at 25 °C for 10 min, to collect the supernatant containing released sugar. The supernatant of each sample was filtered through PVDF membrane filter, 0.45 µm pore size, for glucose estimation by HPLC analysis.

Analysis of enzyme hydrolyzed samples by TLC and HPLC

About 0.4 µL of each untreated and pretreated Sorghum stalk after hydrolysis with endo-β-1,4-glucanase (*Ct*Cel8A) and β-1,4-glucosidase (*Ct*Bgl1A) as mentioned in previous section and 0.4 µL of standard solution containing mixture of glucose (1.0 mg/mL) and cellobiose (1.0 mg/mL) were loaded on a TLC plate (6 × 10 cm, TLC Silica gel 60 F254, Merck, Germany). The plate was kept in the developing chamber saturated with developing solution (mobile phase), containing *n*-butanol, acetic acid, and water in the ratio of 2:1:1.[29] At the end of the run, migrated sugars were visualized by immersing the TLC plate in a visualizing solution (5:95, *v/v* sulfuric acid: methanol; 5.0%, *w/v* α-naphthol). The plates were dried at 80 °C for 20 min and the migrated reaction products (sugars) were visualized as spots on the TLC plate. The concentrations of glucose in the enzyme hydrolyzed samples were then determined using HPLC following the procedure described in one of the earlier sections.

Results and discussion

Characterization of sorghum stalk biomass

The moisture content of Sorghum stalk was 17.0% ± 0.6%. The element composition analysis of powdered Sorghum stalk revealed the carbon, hydrogen, and nitrogen contents to be 44.2%, 5.7%, and 1.6% of biomass, respectively. Sulphur was absent in the biomass. The holocellulose content of biomass was 55.0% ±1.8% (Table 1) as analyzed by the method of Browning.[24] The biomass contained, 31.8% ± 1.2% α-cellulose, 23.2% ± 0.6% hemicellulose, and 5.7% ± 0.2% ADL as determined by TAPPI methods.[25]

Pretreatment of biomass

Structural composition and HPLC analysis of pretreated sorghum stalk

Several researchers have used total reducing sugar (TRS) content in the filtrate after pretreatment as an indirect factor to compare the efficiency of different pretreatment methods or for optimization of certain pretreatment method.[13,14,26] Structural composition analysis of the pretreated biomass samples will give a direct insight into the effect of each pretreatment on the biomass than the indirect reducing sugar assay except the fact that the former method is laborious and time consuming. The effective pretreatment on Sorghum stalk among a set of 12 pretreatment methods with respect to the holocellulose and ADL contents of the pretreated biomass samples were employed. The highest holocellulose (83.9% ± 0.7%) and lowest ADL (2.3% ± 0.6%)

Table 1. Carbohydrate composition, TRS, and CrI of untreated and pretreated Sorghum stalk.

Pretreatment	Condition	Holo-cellulose (%)[a]	ADL (%)[a]	Glucose (g/L)[b]	Xylose (g/L)[b]	Arabinose (g/L)[c]	TRS (mg/g raw biomass)[d]	CrI (%)
Raw Sorghum stalk	–	55.0 ± 1.8	5.7 ± 0.2	–	–	–	––	18.3
Oven heating	120 °C for 30 min	51.4 ± 0.2	6.6 ± 0.1	2.2 ± 0.1	2.8 ± 0.1	–	100.0	28.4
1% H_2SO_4 + oven heating		65.4 ± 1.1	7.4 ± 0.3	2.0 ± 0.1	3.4 ± 0.1	–	108.0	55.2
1% NaOH + oven heating		68.2 ± 0.6	6.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.3	32.0	40.9
Autoclaving	121 °C and 15 psi for 20 min	60.8 ± 0.6	8.4 ± 0.1	2.2 ± 0.1	2.8 ± 0.1	–	100.0	23.7
1% H_2SO_4 + autoclaving		67.6 ± 0.6	13.2 ± 0.1	4.2 ± 0.2	7.4 ± 0.1	0.5	242.0	48.5
1% NaOH + autoclaving		83.9 ± 0.7	2.3 ± 0.1	1.6 ± 0.1	0.3 ± 0.1	0.3	44.0	58.5
Microwaving	180 W for 3 min	63.8 ± 0.4	4.8 ± 0.1	1.8 ± 0.1	2.7 ± 0.1	–	90.0	51.8
1% H_2SO_4 + microwaving		61.3 ± 1.0	7.4 ± 0.1	2.0 ± 0.1	3.2 ± 0.1	–	104.0	13.7
1% NaOH + microwaving		63.1 ± 1.0	8.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	0.1	70.0	46.0
Ultrasonication	10 min in ultrasonication bath	70.8 ± 0.4	5.8 ± 0.1	1.4 ± 0.1	2.2 ± 0.1	–	72.0	56
1% H_2SO_4 + ultrasonication		63.2 ± 0.3	6.5 ± 0.1	1.8 ± 0.1	2.9 ± 0.1	–	94.0	32.8
1% NaOH + ultrasonication		68.0 ± 0.2	6.4 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.1	34.0	60

[a]Mean ± SD ($n = 2$).
[b]Mean ± SD ($n = 3$).
[c]Mean ± SD ($n = 3$) where, SD < 0.01.
[d]Sum of reducing sugars [(glucose + xylose + arabinose)/0.05], where biomass used was 5% (*w/v*) in each pretreatment.

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contents were found in the sample pretreated with dilute alkali (1% NaOH) combined with autoclaving at 121 °C and 15 psi for 20 min among all the pretreatments carried out on the powdered raw Sorghum stalk as shown in the Table 1. This proves that the effective delignification was rendered by the combination of alkali treatment and autoclaving.

Oven heating at 120 °C for 30 min resulted in the lowest holocellulose content of 51.4% ± 0.2%, which showed vulnerability of the biomass for losing its cellulose and hemicellulose in presence of dry heat. Dilute acid treatment combined with either oven heating, autoclaving, microwaving or ultrasonication resulted in similar or slightly enhanced holocellulose content and higher ADL content of pretreated biomass. 1% H_2SO_4 along with autoclaving showed ADL content of 13.2% ± 0.1% which must be due to the inefficiency of dilute acid in the removal of lignin content associated with the biomass. This is being supported by the highest levels of glucose (4.2 ± 0.2 g/L), xylose (7.4 ± 0.1 g/L), and arabinose (0.5 g/L) in the hydrolysate of acid treated sample (1% H_2SO_4 + autoclaving) as shown in Table 1. Presence of arabinose in all the alkali treated filtrates shows that 1% NaOH acts on the hemicellulose part of the biomass also during delignification process resulting in the release of pentose sugars in addition to glucose.

X-ray diffraction analysis of untreated and pretreated samples

The XRD analysis showed that all the pretreatments carried out have contributed to the exposing of the crystalline cellulose from the lignin–hemicellulose cover. The powdered raw biomass had a crystallinity index (CrI) of 18.3% as shown in the Table 1. The highest CrI of 60% was achieved through the 1% NaOH along with ultrasonication treatment of the biomass. 1% NaOH and autoclaving treatment gave a CrI of 58.5%. Increase in crystallinity index in the latter case might be due to the delignification effect of alkali and swelling of fibers during autoclaving, thereby resulting in the exposure of the crystalline cellulose part of the biomass. With respect to the structural composition analysis and crystallinity index data, dilute alkali (1% NaOH) along with autoclaving was found to be the most efficient pretreatment for Sorghum stalk.

Time optimization of pretreatment by 1% NaOH along with autoclaving

Figure 1 shows the effect of time period on the pretreatment process by 1% NaOH along with autoclaving of Sorghum stalk at a temperature and pressure of 121 °C and 15 psi, respectively. McIntosh and Vancov[30] reported alkali treatment combined with autoclaving of Sorghum straw for the time period range 30–90 min. However, they did not study the effect of temperatures lower than 30 min. Moreover, they optimized the process from the viewpoint of delignification without considering the carbohydrate content of biomass. This study compared the treatment at three different time durations, that is, 10, 20, and 30 min. The holocellulose and ADL contents of the resulting biomass were considered as yardsticks. Among the chosen treatment time durations, 30 min treatment was found to provide the highest holocellulose content of 85.7% ± 0.4% and the lowest ADL content of 2.1% ± 0.5%. Hence, 1% NaOH combined with autoclaving at 121 °C and 15 psi for 30 min was chosen for further analysis and saccharification.

Analysis of untreated and pretreated samples by FTIR and FESEM

The comparison of the FTIR spectra of untreated and pretreated (1% NaOH + autoclaving at 121 °C and 15 psi for 30 min) samples showed that the peaks related to the presence of lignin are absent in the pretreated sample (Figure 2). The peak positions related to different functional groups in the lignocellulosic biomass are shown in Table 2. The peak related to the ester linkage between hemicellulose and lignin 1732 cm^{-1} and the peak meant for C=C stretching vibration in phenol rings of lignin at 1515 cm^{-1} are absent in the pretreated sample (Figure 2). C–O stretching of acetyl groups from xylan at 1246 cm^{-1} is faintly present in the pretreated sample comparing with the untreated sample. This explains

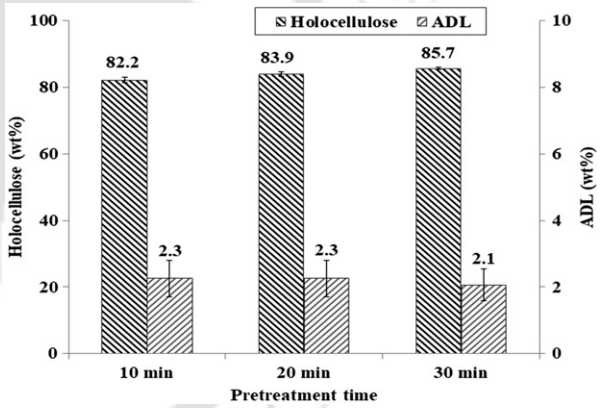

Figure 1. Time optimization of pretreatment (1% NaOH combined with autoclaving at 121 °C and 15 psi) of Sorghum stalk with respect to holocellulose and acid insoluble lignin (ADL) content.

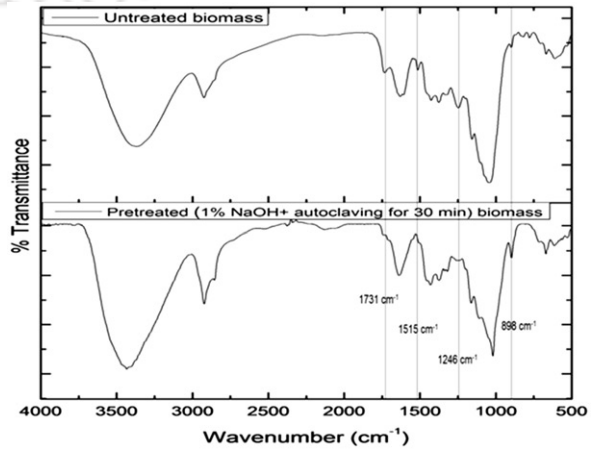

Figure 2. FTIR spectra of untreated and pretreated (1% NaOH combined with autoclaving at 121 °C and 15 psi for 30 min) Sorghum stalks.

Table 2. Peak assignment for different functional groups of lignocellulosic biomass in the FTIR spectrum.

Wavenumber (cm^{-1})	Band assignment	Reference(s)
3600–3000	O–H stretching of cellulose	Agarwal et al.[31]
2902	CH and CH_2 stretching of cellulose	Ren et al.,[32] Pandey[33]
1732	Ester linkage between hemicellulose and lignin	Ren et al.[32]
1640	Carbonyl stretching of aromatic rings	Pandey[33]
1512	C=C stretching vibration in phenol rings of lignin	Ren et al.[32]
1457	C–H deformation in aliphatic part (methyl and methylene group) of lignin	Pandey, [33] Sim et al.[34]
1370	C–H bending in cellulose	Fan et al.[35]
1320	C–C and C–O skeletal vibrations of cellulose	Ren et al.[32]
1249	C–O stretching of acetyl groups from xylan	Ren et al.[32]
1098	Crystalline cellulose	Pandey[33]
1059	C–O vibration in amorphous cellulose	Fan et al.[35]
898	Amorphous cellulose	Fan et al.[35], Pandey[33]
832	Stretching of β-glucosidic linkage	Liu et al.[36]
669	O–H out-of-plane bending in amorphous cellulose	Fan et al.[35]

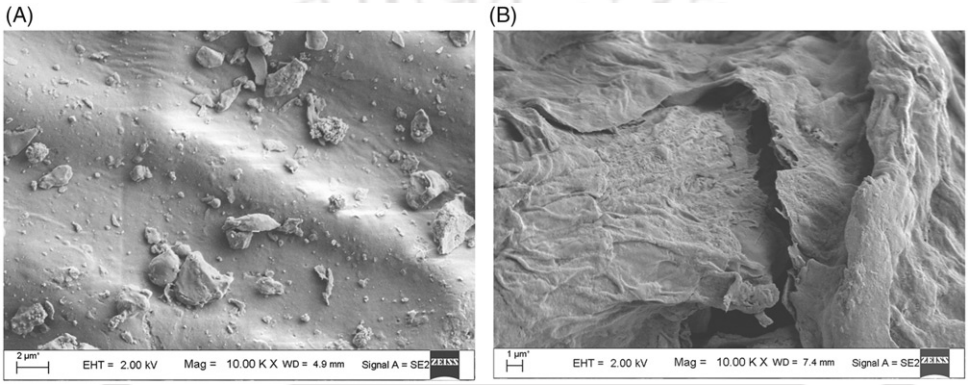

Figure 3. FESEM image of (A) untreated Sorghum stalk and (B) Sorghum stalk pretreated with 1% NaOH combined with autoclaving at 121 °C and 15 psi for 30 min.

the action of alkali on the hemicellulose part of biomass. Peak at 898 cm^{-1} related to amorphous cellulose is clearly visible in the pretreated sample, whereas it is faintly present in the untreated sample. This shows that the crystalline part of the cellulose was converted into amorphous cellulose. FESEM image of the pretreated biomass sample showed enhanced roughness as compared to the untreated sample (Figure 3) portraying how the alkali delignification led to the exposure of holocellulose. Therefore, the structural composition of untreated and effectively pretreated Sorghum stalk is validated well by their respective FTIR spectrum and FESEM image.

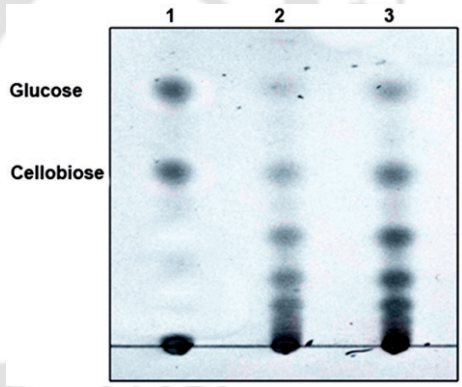

Figure 4. Thin layer chromatographic (TLC) analysis of enzymatically hydrolyzed products of untreated and pretreated Sorghum stalk. Lane 1, Standard containing glucose and cellobiose; Lane 2, untreated Sorghum stalk biomass after enzymatic hydrolysis with endo-β-1,4-glucanase (*Ct*Cel8A) and β-1,4-glucosidase (*Ct*Bgl1A) and Lane 3, pretreated (1% NaOH + autoclaving at 121 °C and 15 psi for 30 min) Sorghum stalk biomass after enzymatic hydrolysis endo-β-1,4-glucanase (*Ct*Cel8A) and β-1,4-glucosidase (*Ct*Bgl1A).

Production of enzymes and hydrolysis of untreated and pretreated sorghum stalk

The recombinant endo-β-1,4-glucanase (*Ct*Cel8A) and β-1,4-glucosidase (*Ct*Bgl1A) containing the N-terminal hexa-histidine tags were purified by IMAC. The recombinant enzymes *Ct*Cel8A and *Ct*Bgl1A were expressed as soluble proteins. The concentration of purified enzymes was determined by UV spectroscopic method and found to be 2.5 mg/mL for *Ct*Cel8A (80 U/mg) and 1.8 mg/mL for *Ct*Bgl1A (33 U/mg). The thin layer chromatogram shows the hydrolyzed products including glucose and cellobiose present in the hydrolysates of the untreated and pretreated (1% NaOH + autoclaving at 121 °C and 15 psi for 30 min) Sorghum stalk samples after enzymatic hydrolysis with endo-β-1,4-glucanase (*Ct*Cel8A) and β-1,4-glucosidase (*Ct*Bgl1A) incubated at 60 °C and 180 rpm for 24 hr (Figure 4). It was reported that in-house production and usage of cellulase had an advantage of eliminating the cost for preservatives and stabilizers that are mandatory to store the commercial enzyme until it is used.[37] Hence, in this study, the recombinant enzymes *viz.* endo-β-1,4-glucanase (*Ct*Cel8A) and β-1,4-glucosidase (*Ct*Bgl1A), expressed and purified in the lab were used for saccharification. HPLC analysis showed that enzymatic hydrolysis of untreated biomass

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yielded 22.4 ± 1.8 mg glucose/g raw biomass, whereas hydrolysis of pretreated biomass yielded 34.2 ± 1.7 mg glucose/g pretreated biomass. This shows that the chosen pretreatment aids in the enhancement of saccharification of Sorghum stalk. However, Chen et al.[38] reported a much higher glucose yield of 420 mg/g pretreated biomass by using commercial enzymes on pretreated Sorghum bagasse. This could be attributed to type of Sorghum species and the type of the pretreatment and also the commercial enzymes used. Rodhe et al.[21] reported that the process parameters such as incubation time duration, temperature, enzyme, and substrate loading play crucial role in enzymatic hydrolysis of pretreated biomass of sweet Sorghum bagasse using cellulase from *Trichoderma reesei*. Hence, saccharification of pretreated biomass with recombinant enzymes *Ct*Cel8A and *Ct*Bgl1A can be further enhanced by optimization of process parameters.

Conclusions

The agro-waste, Sorghum stalk was characterized and found to contain 55% holocellulose content, which is the significant component that can be converted into reducing sugars and finally into bioethanol. Comparison of various pretreatment methods aiming at increasing the holocellulose content and reducing the lignin content of the Sorghum stalk showed that 1% NaOH combined with autoclaving at 121 °C and 15 psi for 30 min was the most effective pretreatment method. It helped in removing the insignificant components along with lignin (ADL), thereby increasing the holocellulose content by 1.6 times (85.7%) of the raw Sorghum stalk (55%). The FTIR spectra of pretreated biomass confirmed lignin removal. The enhanced surface roughness of the pretreated Sorghum stalk was clearly visible in FESEM image. The enzymatic saccharification of pretreated Sorghum stalk using recombinant endo-β-1,4-glucanase (*Ct*Cel8A) and β-1,4-glucosidase (*Ct*Bgl1A) from *C. thermocellum* resulted in 1.5-fold higher glucose yield (34 mg/g pretreated biomass) as compared with the untreated biomass (22.4 mg/g raw biomass). However, the enzymatic hydrolysis using these enzymes can be optimized for further enhancement of the saccharification efficiency and yield.

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Enzymatic hydrolysis of hemicellulose from pretreated Finger millet (*Eleusine coracana*) straw by recombinant endo-1,4-β-xylanase and exo-1,4-β-xylosidase

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ABSTRACT

This study focuses on enzymatic saccharification of hemicellulose part of the pretreated Finger millet straw (FMS) for production of xylose. The variation in the carbohydrate composition of FMS was analysed when subjected to different pretreatments. The recombinant endo-1,4-β-xylanase (*Ct*Xyn11A) was most active on the FMS pretreated with 1% (w/v) NaOH combined with oven heating at 120 °C for 20 min, resulting in a total reducing sugar yield (TRS) of 32 mg/g pretreated biomass. The pretreatment aided in concentrating the holocellulose content from 69.3% of raw powdered FMS to 76.4%. The post-treatment solid biomass yield was 0.36 g/g raw biomass. The two-step optimization of hemicellulose saccharification from the above pretreated FMS with i) endo-1,4-β-xylanase (*Ct*Xyn11A) at 55 °C and ii) exo-1,4-β-xylosidase (*Bo*GH43A) at 37 °C, both at pH 7.5 by Box-Behnken design yielded the TRS of 70 mg/g pretreated biomass. The percentage conversion of xylan to xylose by *Ct*Xyn11A and *Bo*GH43A was 24.7%.

1. Introduction

Biofuels have immense significance owing to their renewable and sustainable resources unlike petroleum fuels. Lignocellulosic biomass from agrowastes, forest residues and weeds serve as the feedstocks for second generation biofuel [1,2]. Bioethanol production from lignocellulosic biomass includes pretreatment of the biomass, enzymatic saccharification of the pretreated biomass and fermentation of the monosugars into bioethanol [3]. The pretreatment process helps in removing the unwanted compounds and concentrating the polysaccharides such as cellulose and hemicellulose [4,5]. Various pretreatment methods such as physical pretreatment, chemical pretreatment, thermal pretreatment and pretreatment involving organosolvents or ionic liquids can be used. Among chemical pretreatment methods, the acid pretreatment method using dilute sulphuric acid (H_2SO_4) or hydrochloric acid (HCl) or alkali method using dilute sodium hydroxide (NaOH) or potassium hydroxide (KOH) are cheaper [6,7]. Any particular pretreatment method is not ideal for all lignocellulosic feedstocks, as each feedstock has different structure composition with variation in rigidity. Hence a variety of pretreatment methods are needed to be explored, compared and an effective pretreatment method for each selected feedstock has to be employed [8]. Combining multiple pretreatment methods or applying them in a step-wise manner has also been shown to be effective on some lignocellulosic feedstocks [9,10]. Multiple lignocellulosic feedstocks have also been mixed and pretreated for biofuel production [11]. This strategy may help in maintaining the continuous biofuel production by making up the constant biomass quantity with one feedstock when the other is available in lesser quantity in different seasons. Hence, finding out the effective pretreatment method for each lignocellulosic biomass or combining multiple biomasses that respond similar to a particular pretreatment method is relevant in order to make the continuous supply of the biomass. The conventional and easier way to compare the effect of various pretreatments is to calculate the total reducing sugar (TRS) released during each pretreatment [12,13]. In addition to the TRS analysis, the efficiency of a given pretreatment method can be analysed directly and accurately by comparing the structure composition of the untreated and pretreated biomass [14] and enzymatic hydrolysis of the pretreated biomass. The next step after pretreatment is saccharification of the pretreated biomass. In order to make the bioethanol production process more efficient, along with the cellulose saccharification, the enzymatic hydrolysis of hemicellulosic portion of the pretreated biomass also needs to be focussed.

Rice and wheat are seasonal crops, that need well irrigated system to provide continuous supply of water for growth. Finger millet is a

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drought tolerant crop, which is cultivated in rainfed areas of India [15]. Expert system for Finger millet [16], an Indian Council of Agricultural Research (ICAR) funded project reported that Finger millet is a 2 m long grass and the straw yield ranges between 3 and 10 t/ha. Exploration of various agrowastes from rainfed areas is important to maintain the continuous operation and economy of the bioethanol plant during the unavailability of the irrigation dependent feedstocks. Therefore, this study focuses on comparison of 12 pretreatments on Finger millet (*Eleusine coracana*) straw (FMS) to select the efficient method based on the structure composition and hemicellulose saccharification of the pretreated biomass. There is no report available on the utilization of FMS as biomass for saccharification and fermentation for biofuel production. This study is an attempt to understand the characteristics of FMS and to explore its capability to serve as a potential bioenergy resource.

2. Materials and methods

2.1. Feedstock processing

Finger millet (*Eleusine coracana*) straw was collected from Thenur, Madurai district, Tamil Nadu, India. The feedstock was washed and dried at 70 °C for 24 h. The dried biomass was ground and separated using a 600 μm sieve.

2.2. Moisture content and elemental analysis of raw FMS

The unground FMS (1 g) was dried overnight at 105 °C in a hot-air oven and weighed to determine the moisture content of the biomass. Carbon, Hydrogen, Nitrogen and Sulphur (CHNS) content in the powdered raw biomass was determined by using Elemental Analyser (EuroEA3000, Euro Vector, Italy).

2.3. Pretreatment of FMS

5% (w/v) powdered raw FMS suspended in 200 mL aqueous solution was used for each pretreatment. Four different pretreatment methods were selected as follows:

(a) Oven heating: Hot-air oven at 120 °C for 20 min [8].
(b) Autoclaving: Autoclave at 121 °C, 15 psi for 20 min [8].
(c) Microwaving: Microwave oven (Samsung, USA) at 180 W for 3 min [17].
(d) Ultra-sonication: Ultra-sonication bath (PCi analytics) at 33 kHz for 10 min [18].

Each pretreatment was carried out in three individual aqueous systems such as distilled water, 1% (v/v) H_2SO_4 and 1% (w/v) NaOH thereby leading to a total number of 12 pretreatment methods. After each pretreatment, the sample was filtered through muslin cloth and the filtrate was used for reducing sugar and inhibitor analyses. The solid residue was washed with distilled water till the neutral pH and dried at 70 °C.

2.4. Structural carbohydrate and Lignin analysis of untreated and pretreated FMS

Holocellulose content of untreated and each pretreated FMS (initial biomass, 0.5 g) was determined by following Browning method [19]. Standard TAPPI protocols [20] were used for the determination of hemicellulose and acid insoluble lignin (ADL) contents (initial biomass, 1 g). The analysis was carried out with more precision and negligible wastage of biomass to achieve less internal error. Approximate cellulose content was determined by subtracting the hemicellulose from holocellulose content of each sample.

2.5. Biomass yield after pretreatment method

The biomass residue remaining after each pretreatment was dried in an oven at 105 °C to remove the moisture. Pretreated biomass yield from each pretreatment method was determined by weighing the respective dried solid residue and expressed as biomass yield (g/g raw biomass).

2.6. Crystallinity index (CrI) of untreated and pretreated FMS

The crystallinity indices of untreated and each pretreated samples were analysed by X-ray diffractometer (D8 Advance, Bruker, Germany). The samples were scanned over a range of $2\theta = 10°$ to 30°, where the step size used was 0.05°. The formula for calculating the crystallinity index as described by Segal et al. [21] is given below:

$$\text{Crystallinity index } (CrI\%) = \frac{I_{crystalline} - I_{amorphous}}{I_{crystalline}} \times 100 \quad (1)$$

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

2.7. Reducing sugar analysis in pretreated hydrolysates by high performance liquid chromatography (HPLC)

50 mL of filtrate from each pretreatment was centrifuged at 6000 rpm for 15 min and the supernatant was filtered again through a 25 mm polyvinylidene fluoride (PVDF) membrane filter with 0.45 μm pore size. 1 mL of the final filtrate was used for the determination of glucose, xylose, arabinose and cellobiose contents. The analysis was carried out by using HPLC system (Shimadzu corporation, LC-20AD, Japan) coupled with an autosampler (Shimadzu corporation, SIL-20AHT, Japan) and RI detector (Shimadzu corporation, RID-10A, Japan). Standard glucose, xylose, arabinose and cellobiose procured from Sigma Aldrich, USA were used at concentrations, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL. The HPLC column (Phenomenex Rezex ROA (H+) organic acid and monosaccharide column, 300 mm × 7.8 mm) coupled with a guard column (50 mm × 7.8 mm) was used. A mobile phase of 0.005 N H_2SO_4 was run at a flow rate of 0.5 mL/min through the column. The concentration of each sugar in the sample was calculated with respect to the area of the respective peak.

2.8. Inhibitor analysis in pretreated hydrolysates by HPLC

Inhibitors such as furfural, hydroxymethylfurfural (HMF) and acetic acid in the hydrolysates of pretreated samples were analysed by HPLC. Standard furfural and HMF (HiMedia Pvt. Ltd., India) were used at concentrations, 1, 2, 3, 4, 5, 6 and 7 mg/L and standard acetic acid (Sigma Aldrich, USA) was used at 5, 10, 15, 20, 25, 30 and 35 mg/mL concentrations. Each sample was filtered using PVDF membrane (0.45 μm pore size). Each sample along with the mobile phase, 80% acetonitrile was run through a 5 μm C18 column, shim-pack GIST C18 (250 mm × 4.6 mm) coupled with a guard column (4.0 mm × 20 mm) at a flow rate of 1 mL/min and detected using UV detector (Shimadzu corporation, SPD-20A, Japan) at 210 nm.

2.9. Production, purification and activity assay of recombinant endo-1,4-β-xylanase (CtXyn11A) from Clostridium thermocellum and exo-1,4-β-xylosidase (BoGH43A) from Bacteroides ovatus

Endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) were generous gift from Prof. Carlos Fontes of NZYTech Pvt. Ltd. Portugal. 1% (v/v) of *Escherichia coli* (*E. coli*) BL21 (DE3) cells harbouring the pET21a(+) vector containing gene encoding an endo-1,4-β-xylanase (*Ct*Xyn11A), a family 11 glycoside hydrolase from *Clostridium thermocellum* was inoculated in 800 mL Luria-Bertani medium

(HiMedia, Pvt. Ltd., India) supplemented with ampicillin (100 μg/mL). The 800 mL culture was incubated initially at 37 °C, 180 rpm for 4 h and then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and incubated at 24 °C, 180 rpm for 16 h. The *E. coli* cells were centrifuged at 5000*g* at 4 °C for 10 min. The cell pellet obtained was re-suspended in 10 mL of sodium phosphate buffer (50 mM, pH 7.4), kept on ice and lysed by ultra-sonication for 15 min (5 s on/10s off pulse at 33% amplitude, Sonics, Vibra cell). The cell lysate was centrifuged at 20,000*g* for 1 h and the supernatant containing enzyme (*Ct*Xyn11A with N-terminal hexa-His tag) was purified by immobilized metal-ion affinity chromatography (IMAC) using 2 × 5 mL Sepharose column (HiTrap Chelating, GE Healthcare, US). The columns were incubated with 10 mL of 1 M NaOH, washed with water to remove any impurity and charged with 0.1 M $NiSO_4$. The charged column was then passed with equilibration buffer (50 mM sodium phosphate, pH 7.4, 300 mM NaCl and 60 mM imidazole). The supernatant containing desired enzyme was passed through the column and eluted with the aid of elution buffer (50 mM sodium phosphate, pH 7.4, 300 mM NaCl and 300 mM imidazole). The fractions containing the enzyme were collected, pooled, dialysed against 50 mM sodium phosphate buffer (pH 7.4) and concentrated using centricon with molecular weight cut-off of 10 kDa (Merck Millipore, USA). The concentration of enzyme was determined by Bradford method [22] using BSA as standard. To determine *Ct*Xyn11A activity, 10 μL of enzyme (5 μg/mL) was incubated with 1% (w/v) birchwood xylan in a 100 μL reaction in 50 mM sodium phosphate buffer, pH 7.5 at 65 °C for 1 min.

E. coli BL21 (DE3) cells harbouring the gene encoding exo-1,4-β-xylosidase (*Bo*GH43A), a family 43 Glycoside Hydrolase from *Bacteroides ovatus* were used. The production and purification of enzyme was carried out in the same way as *Ct*Xyn11A except that the antibiotic used was kanamycin (50 μg/mL), because the pET28a(+) vector contains kanamycin resistant gene. To determine the enzyme activity, 10 μL of *Bo*GH43A (50 μg/mL) was incubated with 1% (w/v) birchwood xylan in a total reaction volume of 100 μL (50 mM sodium phosphate buffer, pH 7.0) at 37 °C for 5 min. The activities of *Ct*Xyn11A and *Bo*GH43A after the reaction were calculated by estimating the reducing sugar released by following the method of Nelson and Somogyi [23,24].

2.10. Enzymatic hydrolysis of untreated and each pretreated FMS

The enzyme, endo-1,4-β-xylanase (*Ct*Xyn11A, 0.2 mg/mL, 3900 U/mg) hydrolysis of 1% (w/v) untreated and 1% (w/v) each pretreated FMS biomass sample in 1.0 mL 50 mM sodium phosphate buffer (pH 7.5) was carried out by incubating the reaction at 65 °C and 150 rpm in a shaking incubator for 1 h. Each reaction was stopped by boiling the reaction mixture in a boiling water bath for 10 min. The control without the enzyme for each sample was included to eliminate any reducing sugar released from the biomass during boiling. The samples were centrifuged at 12,600*g* for 10 min and the supernatants were analysed for total reducing sugar (TRS) by following the method of Nelson and Somogyi [23,24].

2.11. FTIR and FESEM analyses of untreated and pretreated FMS

The untreated FMS sample and the biomass pretreated by NaOH + oven heating were separately mixed with KBr (200 mg) in the ratio of 100:1 (KBr: Biomass), ground using mortar and pestle and pelleted using a press. The pellets were scanned by Fourier-transform infrared (FTIR) spectroscope (Spectrum Two, Perkin-Elmer, Waltham, MA) within the wavenumber range, 4000–450 cm^{-1}. Field emission scanning electron microscopic (FESEM) analysis of the above samples were carried out by placing the biomasses on a carbon tape fixed to a stub, coating them with gold and scanning by FESEM (Zeiss, Sigma, Germany).

2.12. Optimization of enzymatic hydrolysis of hemicellulose from pretreated FMS by endo-1,4-β-xylanase (CtXyn11A)

The enzymatic hydrolysis of pretreated (NaOH + oven heating) FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) was carried out in 1 mL volume to produce xylo-oligosaccharides (XOS). The biomass and endo-1,4-β-xylanase (*Ct*Xyn11A) loading and the hydrolysis time were optimized at 55 °C, pH 7.5 (50 mM sodium phosphate buffer) and 150 rpm by using Box-Behnken design, Response surface methodology (Design-Expert 7.0 software). The range for the factors were provided manually (Table S1) with respect to the preliminary experiments performed. The response, TRS $yield_{(XOS)}$ from each experiment was determined by Nelson and Somogyi method [23,24].

2.13. Optimization of xylose production from xylo-oligosaccharides by exo-1,4-β-xylosidase (BoGH43A)

The xylo-oligosaccharides (XOS) were produced from 6% (w/v) pretreated FMS suspended in 50 mL of 50 mM sodium phosphate buffer, pH 7.5 by treating with endo-1,4-β-xylanase, *Ct*Xyn11A at loading of 559 U/g ptd biomass and incubating the reaction mixture at 55 °C and 150 rpm for 56 h. The reaction was stopped by boiling the mixture and TRS $yield_{(XOS)}$ of XOS was determined to be 2.6 g/L and was stored at 4 °C. Further hydrolysis of xylo-oligosaccharides to produce xylose was carried out by exo-1,4-β-xylosidase (*Bo*GH43A) in 1 mL reaction volume. For optimization of xylose production, the XOS concentration, xylosidase loading and hydrolysis time were optimized at 37 °C in static condition using Box-behnken design. The ranges for the factors were provided manually (Table S4) with respect to the preliminary experiments. The TRS release per unit XOS (response) from each experiment was determined by Nelson and Somogyi method. The equivalent TRS release per unit of pretreated biomass was calculated by using the formula as follows:

$$Final\ TRS\left(\frac{mg}{g\ ptd\ biomass}\right) = TRS\ yield_{(XOS)} \times TRS\ yield \quad (2)$$

where, "$TRS\ yield_{(XOS)}$" is the Total Reducing Sugar yield as xylo-oligosaccharides (mg XOS/g ptd biomass) from the hydrolysis with endo-1,4-β-xylanase (*Ct*Xyn11A) and "*TRS yield*" is the Total Reducing Sugar yield (g/g XOS) from the hydrolysis with exo-1,4-β-xylosidase (*Bo*GH43A).

2.14. Thin layer chromatography (TLC) analysis of hydrolysed products of pretreated FMS by endo-1,4-β-xylanase (CtXyn11A) and exo-1,4-β-xylosidase (BoGH43A)

The hydrolysed samples from the enzymatic hydrolysis of pretreated FMS under optimized conditions by *Ct*Xyn11A alone and *Ct*Xyn11A followed by *Bo*GH43A were analysed by TLC. In one set, 6% (w/v) pretreated FMS suspended in 1 mL final volume of 50 mM sodium phosphate buffer, pH 7.5 with enzyme, *Ct*Xyn11A loading at 559 U/g ptd biomass was incubated at 55 °C and 150 rpm for 56 h. In another set, 1 mL of 2.08 g/L of XOS produced from the first set having 50 mM sodium phosphate buffer pH 7.5 was treated with 40 U/mL final loading of xylosidase and incubated at 37 °C for the optimized time period, 79.4 min. The samples from both the sets were filtered through 0.45 μm membrane (PVDF). 0.5 μL of each sample and standard xylose (4 mg/mL) were loaded on the TLC plate (3 cm × 13 cm, TLC Silica gel 60 F254, Merck, Germany) and dried. A mixture containing acetonitrile and water (80:20) was used as the mobile phase [25]. The TLC plate was kept inside the developing chamber and run for 30 min. After the run, the plate was immersed in the visualizing solution containing sulphuric acid: methanol (5:95, v/v) and 5.0%, w/v α-naphthol and then dried at 80 °C to visualize the hydrolysed products.

2.15. Estimation of xylan content in hemicellulose in pretreated biomass by HPLC

The 1% (w/v) pretreated FMS (NaOH + oven heating) was further treated with 2 M Trifluoroacetic acid (TFA) at 100 °C for 2 h. The total xylose concentration in the TFA treated sample was taken as the total xylan available for the enzymes (*Ct*Xyn11A and *Bo*GH43A) during the saccharification process under optimized conditions. The xylan to xylose conversion during saccharification was calculated by determining the xylose in the TFA treated sample (total xylan present) and the saccharified sample (hydrolysed xylose). The xylose concentration was determined by HPLC method as described in Section 2.7.

3. Results and discussion

3.1. Moisture content and elemental analysis of raw FMS

The unground raw FMS contained 16.7 ± 0.8% (w/w) moisture content. The powdered raw FMS contained 43.9% carbon, 5.7% hydrogen and 1.7% nitrogen. CHNS analysis showed no sulphur in the raw biomass.

3.2. Structural carbohydrate and Lignin analysis of untreated and pretreated FMS

Powdered raw FMS contained 69.3% holocellulose, 33.5% hemicellulose, 35.8% cellulose and 5.1% acid insoluble lignin (ADL) (Table 1). Table 1 shows the structural carbohydrate and lignin composition of each pretreated FMS. The impact of the selected pretreatments on FMS is discussed in the following sections.

3.2.1. Impact of pretreatment on holocellulose

Biomass samples treated with the selected pretreatments contained >75% holocellulose content except the samples treated with autoclaving along with either H_2SO_4 or NaOH (Table 1). Oven heating in the presence of every selected aqueous system resulted in >76% holocellulose. Autoclaving along with H_2SO_4 completely removed the hemicellulose from the biomass. Pedersen et al. [26] has also reported the removal of almost all the hemicellulose portion of wheat straw by dilute acid treatment. Moreover, autoclaving with NaOH also resulted in >50% reduction in the hemicellulose content (Table 1). Therefore, autoclaving method involving acid or alkali appeared to be an inefficient method for FMS in terms of hemicellulose loss.

3.2.2. Impact of pretreatment on ADL

FMS treated by autoclaving combined with NaOH contained the lowest ADL content (3.0%) among all the other pretreated samples. This proves the efficiency of alkali method in delignification of lignocellulosic biomass as also reported earlier [18]. ADL content (5.6%) was lesser in the biomass treated by oven heating along with NaOH than the oven heating with only water (6.5%) and H_2SO_4, (7.3%) (Table 1). Autoclaving along with 1% H_2SO_4 had an inverse impact on the ADL content leading to 15% ADL and thereby making the pretreated biomass unsuitable for bioethanol production. All the pretreatments involving microwaving or sonication resulted slight increase in the ADL content (6–7%) as compared with the untreated FMS (5%). With respect to ADL content, NaOH combined with oven heating or autoclaving were found to be better for FMS among all the pretreatments owing to their higher delignification efficiency.

3.3. Impact of pretreatment on biomass yield

Among all the pretreatment methods carried out on FMS, microwaving with water at 180 W for 3 min yielded the maximum amount of pretreated biomass (0.84 g/g raw biomass) (Table 1). Being mild treatment and for short time duration, it did not result in the loss of cellulose and hemicellulose from the FMS. The lowest pretreated biomass yield of 0.15 g/g raw biomass obtained with autoclaving with NaOH as compared with the other treatments (Table 1). Therefore, autoclaving with NaOH turned out to be the most inefficient method as it caused extensive biomass wastage (Table 1).

3.4. Reducing sugar and Crystallinity index (CrI) analyses of pretreated FMS

After the pretreatment of FMS, the reducing sugar content of the filtrate and the crystallinity index (CrI) of the solid biomass residue were determined. FMS on treatment by autoclaving with H_2SO_4 gave the maximum TRS yield of 222.8 mg/g raw biomass (Table 2). The acid treatments combined with all four physical treatments released significantly higher cellobiose than the respective alkali or water treatments. This showed the efficiency of dilute acid in decrystallization of cellulose in lignocellulosic biomass as also reported earlier [27]. H_2SO_4 combined with autoclaving released maximum xylose and arabinose concentrations (7.8 and 2.9 g/L, respectively) in the filtrate (Table 2) which also explains the complete loss of hemicellulose content (Table 1). Moreover, the increase in *CrI* to 60.9% from 55.6% of untreated biomass also explains that the cellulose is exposed due to the removal of the hemicellulose content (Table 1). The next higher TRS value (11.2 mg/g raw biomass) was resulted by autoclaving with NaOH (Table 2), which also gave the maximum *CrI* of 68.6%. The maximum *CrI* of 68.8% was due to the maximum delignification (Table 1) exposing the crystalline cellulose. The TRS and *CrI* analyses showed that the pretreatment of FMS by autoclaving with H_2SO_4 or NaOH are better methods among 12 pretreatment methods for exposing crystalline cellulose.

Table 1
Structure composition, biomass yield analysis and crystallinity index of untreated and pretreated FMS samples.

Pretreatment	Aqueous system	Holocellulose[a] (wt% of ptd. B.)	Hemicellulose[a] (wt% of ptd. B.)	Cellulose[a] (wt% of ptd. B.)	ADL[a] (wt% of ptd. B.)	Pretreated biomass yield[b] (g/g raw biomass)	*CrI* (%)
Raw FMS	–	69.3 ± 1.3	33.5 ± 0.4	35.8 ± 0.1	5.1 ± 0.3	1	55.6
Oven heating at 120 °C for 20 min	D.W.	77.5 ± 0.3	32.2 ± 0.6	45.3 ± 0.8	6.5 ± 0.5	0.63	41.7
	1% H_2SO_4	77.1 ± 0.3	34.1 ± 0.4	43.1 ± 0.6	7.3 ± 0.4	0.66	30.9
	1% NaOH	76.4 ± 0.2	20.7 ± 0.5	55.7 ± 0.4	5.6 ± 0.5	0.36	55.2
Autoclaving at 121 °C, 15 psi for 20 min	D.W.	76.7 ± 0.2	33.4 ± 0.4	43.3 ± 0.2	6.2 ± 0.3	0.54	59.5
	1% H_2SO_4	68.5 ± 0.3	0.00	68.5 ± 0.3	15.6 ± 0.2	0.26	60.9
	1% NaOH	67.2 ± 0.4	15.4 ± 0.9	51.8 ± 1.2	3.1 ± 0.4	0.15	68.6
Microwaving at 180 W for 3 min	D.W.	75.6 ± 0.4	31.9 ± 1.4	43.7 ± 1.1	6.4 ± 0.6	0.84	51.7
	1% H_2SO_4	79.5 ± 0.4	31.8 ± 0.8	47.7 ± 0.4	7.4 ± 0.3	0.47	9.5
	1% NaOH	77.5 ± 0.3	23.0 ± 0.3	54.5 ± 0.3	7.3 ± 0.5	0.36	21.7
Ultra-sonication at 33 kHz for 10 min	D.W.	75.9 ± 0.8	32.3 ± 0.3	43.5 ± 0.6	6.6 ± 0.2	0.56	28.3
	1% H_2SO_4	79.7 ± 0.2	32.2 ± 0.6	47.4 ± 0.5	7.4 ± 0.4	0.53	39.0
	1% NaOH	79.1 ± 0.3	25.4 ± 0.2	53.6 ± 0.2	6.6 ± 0.3	0.33	37.8

D.W. = distilled water; ptd. B. = pretreated biomass; ADL = acid insoluble lignin; Cellulose = (Holocellulose-hemicellulose); *CrI* = crystallinity index;
[a] Mean ± SD (n = 3).
[b] Mean ± SD (n = 3) where, SD < 0.

Table 2
Reducing sugar analysis in the filtrate after each pretreatment.

Pretreatment	Aqueous system	Glucose[a] (g/L)	Xylose[a] (g/L)	Arabinose[a] (g/L)	Cello-biose[a] (g/L)	TRS yield (mg/g raw biomass)[b]
Oven heating at 120 °C for 20 min	D.W.	0.02	0.03	0.01	0.04	2.0
	1% H_2SO_4	0.01	0.04	0.05	0.14	4.8
	1% NaOH	0.10	0.02	0.02	0.03	3.4
Autoclaving at 121 °C, 15 psi for 20 min	D.W.	0.22	0.03	0.02	0.04	6.2
	1% H_2SO_4	0.37	7.79	2.90	0.08	222.8
	1% NaOH	0.47	0.03	0.03	0.03	11.2
Microwaving at 180 W for 3 min	D.W.	0.03	0.02	0.01	0.04	2.0
	1% H_2SO_4	0.02	0.02	0.03	0.16	4.6
	1% NaOH	0.02	0.02	0.02	0.05	2.2
Ultra-sonication at 33 kHz for 10 min	D.W.	0.02	0.03	0.01	0.04	2.0
	1% H_2SO_4	0.01	0.02	0.003	0.14	3.46
	1% NaOH	0.01	0.02	0.02	0.04	1.8

D.W. = distilled water; TRS = total reducing sugar.
[a] Mean ± SD (n = 3) where, SD < 0.01.
[b] TRS yield (mg/g raw biomass) = (glucose + xylose + arabinose + cellobiose) / 0.05 where, biomass used in each pretreatment was 5% (w/v).

3.5. Inhibitor analysis in the hydrolysates of pretreated FMS

The inhibitors, furfural and hydroxymethyl furfural are derived from pentose sugars and hexose sugars, respectively and are released during acid pretreatment. Acetic acid is formed during the alkaline pretreatment of lignocellulosic biomass with KOH or NaOH [28,29]. In this study, furfural found was 35.8 mg/L in the filtrate of FMS pretreated by H_2SO_4 combined with autoclaving. This correlates well with the reducing sugar data where autoclaving along with H_2SO_4 yielded the maximum concentration of xylose (7.8 g/L) and arabinose (2.9 g/L) in the hydrolysate among all the chosen pretreatment methods (Table 2). Other pretreatment methods did not release any of these inhibitory compounds.

3.6. Enzymatic hydrolysis of untreated and each pretreated FMS with recombinant endo-1,4-β-xylanase (CtXyn11A)

The purified recombinant xylanase, *Ct*Xyn11A showed a concentration of 3.7 mg/mL and a specific activity of 3900 U/mg on 1% (w/v) birchwood xylan. *Ct*Xyn11A was active against only on all the NaOH pretreated FMS samples. The maximum TRS yield observed was 31.5 mg/g pretreated biomass upon saccharification with *Ct*Xyn11A after the treatment with NaOH combined with oven heating (Fig. S1). This was followed by TRS yield of 28.6 mg/g pretreated biomass obtained by *Ct*Xyn11A saccharification of FMS pretreated with NaOH combined with autoclaving. Enzymatic hydrolysis of FMS pretreated with NaOH combined with microwaving or sonication by *Ct*Xyn11A resulted TRS yield of 18.3 or 12.7 mg/g pretreated biomass, respectively. This showed that the alkali treatment releases hemicellulose, thereby paving the way for xylanase action as also reported earlier [30]. Enzymatic hydrolysis of pretreated FMS samples by endo-1,4-β-xylanase (*Ct*Xyn11A) revealed that NaOH + oven heating serves as the best pretreatment among 12 methods. The conventional pretreatment screening method involves the comparison of TRS yield in the filtrate from each pretreatment and considers the highest TRS releasing pretreatment as the best method [12,13,17]. In this study, even though the TRS yield was highest obtained from H_2SO_4 + autoclaving treatment, the enzyme, endo-1,4-β-xylanase (*Ct*Xyn11A) did not give any activity on this specific pretreated FMS. Therefore, it is also important to evaluate the activity of the desired enzyme on each pretreated biomass sample to determine the best pretreatment method for any given biomass.

3.7. FTIR and FESEM analyses of untreated and pretreated FMS

The FTIR and FESEM analyses of untreated and pretreated (NaOH + oven heating) FMS are shown in Fig. 1. The FTIR spectrum ranging between 3600 and 3000 cm^{-1} is related to O—H stretching of cellulose in the lignocellulosic biomass [31]. The peak at 1732 cm^{-1} is assigned to the ester linkage between hemicellulose and lignin. The peaks at 1640 cm^{-1} and 1512 cm^{-1} are related to the carbonyl stretching of aromatic rings and C=C stretching vibration in phenol rings in lignin, respectively. 1249 cm^{-1} is related to the C—O stretching of acetyl groups of xylan [32,33]. The peaks at 1513 cm^{-1} and 1247 cm^{-1} were absent in the FMS sample pretreated by NaOH + oven heating when compared with the untreated FMS (Fig. 1a and b). This showed that the pretreatment involving NaOH combined with oven heating helps in delignification and hemicellulose breakdown in the FMS. The FESEM images of untreated and pretreated FMS showed that NaOH + oven heating pretreatment method caused significant structure disruption, leading to the roughness on the surface (Fig. 1d) as compared with the untreated biomass (Fig. 1c) displaying the effectiveness of the pretreatment.

3.8. Optimization of hemicellulose hydrolysis from pretreated FMS to xylo-oligosaccharides by endo-1,4-β-xylanase (CtXyn11A)

The Box-Behnken design for the optimization of hydrolysis of hemicellulose from pretreated (NaOH + oven heating) FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) and the response, TRS yield are shown in the Table S2. The second-order quadratic equation for the hydrolysis process by xylanase is as follows:

$$TRS\ yield_{(XOS)}\ (mg/g\ ptd\ biomass) = -1.06 + 1.72A_1 + 0.04B_1 + 0.55C_1 + 0.002A_1B_1 + 0.0001A_1C_1 + 0.0002B_1C_1 - 0.14A_1^2 - 0.00003B_1^2 - 0.005C_1^2 \quad (3)$$

The quadratic model fits well with the experimental values of TRS yield (Table S2). The F-value of 122.7 from ANOVA showed that the model was significant. The p-value being <0.05 implied that A_1, B_1, C_1, B_1C_1, A_1^2, B_1^2 and C_1^2 were significant terms. The regression co-efficient (R^2) was 0.9937, which shows the accuracy of the model (Table S3). The $TRS_{(XOS)}$ yield increased with increase in the biomass loading and xylanase loading (Fig. 2a, b and c). As time increased, a gradual increase in the $TRS_{(XOS)}$ yield was observed (Fig. 2b). The predicted optimum biomass loading (A_1), xylanase loading (B_1) and hydrolysis time (C_1) were 6% (w/v), 559 U/g ptd biomass and 56 h. The predicted TRS yield from the above conditions was 39.3 mg/g ptd biomass. It was validated by carrying out an experiment (triplicate) in 1 mL reaction volume at the predicted optimum conditions and the observed TRS yield was 39.6 ± 0.6 mg/g ptd biomass (2.4 g/L). The TRS titre at the flask level was determined to be 2.6 g/L.

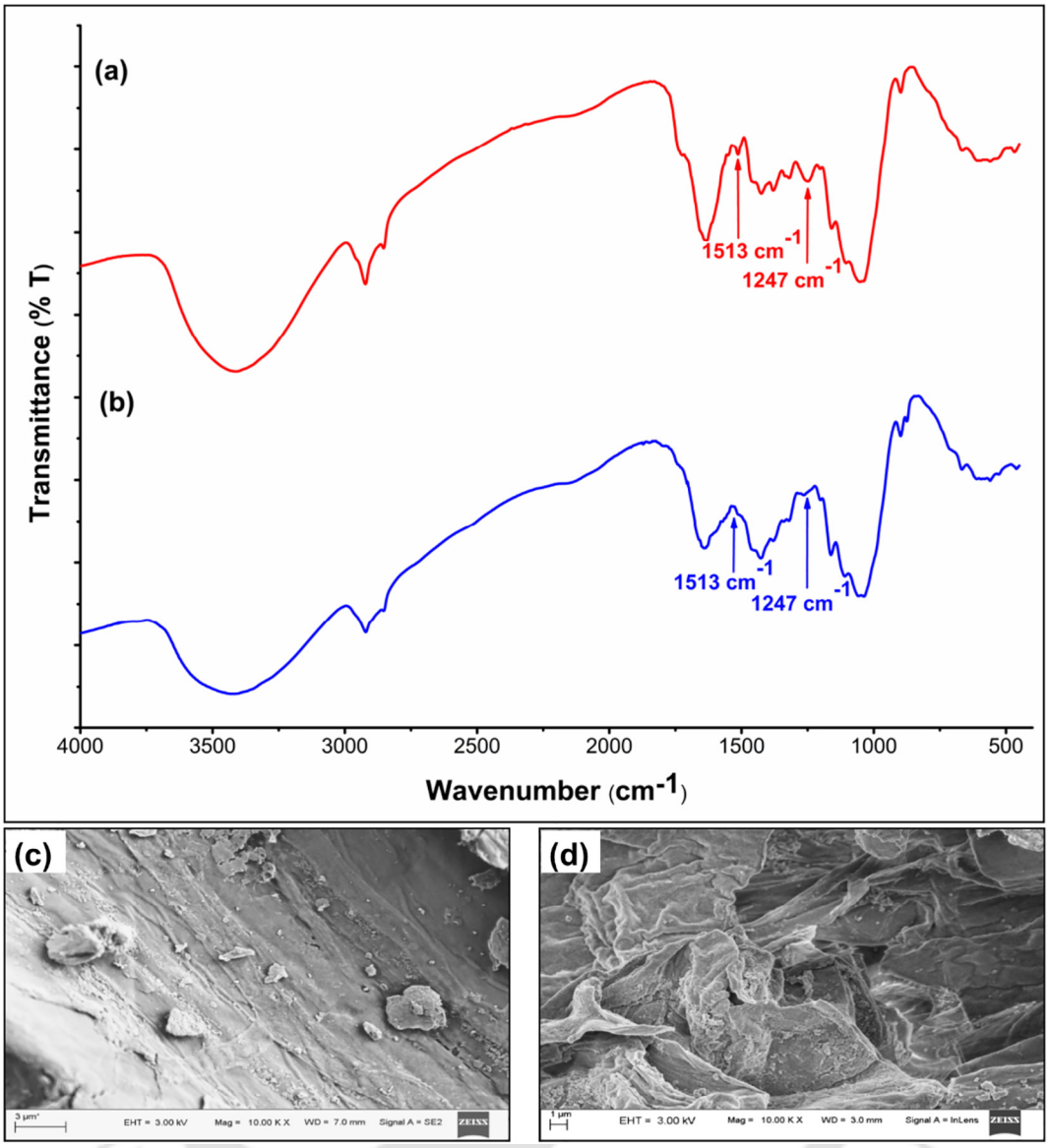

Fig. 1. FTIR plots of (a) untreated and (b) pretreated (1%, w/v NaOH combined with oven heating) FMS, corresponding FESEM images of (c) untreated and (d) pretreated (1%, w/v NaOH combined with oven heating) FMS.

3.9. Optimization of xylose production from xylo-oligosaccharides (XOS) by exo-1,4-β-xylosidase (BoGH43A)

The purified recombinant exo-1,4-β-xylosidase, *Bo*GH43A showed a concentration of 2.8 mg/mL and a specific activity of 59 U/mg on 1% (w/v) birchwood xylan. 17 experiments designed by the Box-Behnken design for the hydrolysis of xylo-oligosaccharides by xylosidase, the predicted and observed response (TRS yield) are shown in the Table S5. The second-order quadratic equation for the hydrolysis involving xylosidase is as follows:

$$\begin{aligned} \text{TRS yield } (g/g\ XOS) = {} & 1.77 + 0.002A_2 - 0.24B_2 + 0.004C_2 \\ & + 0.0002A_2B_2 + 0.000005A_2C_2 + 0.0008B_2C_2 \\ & + 0.000004{A_2}^2 + 0.05{B_2}^2 - 0.00004{C_2}^2 \end{aligned} \quad (4)$$

ANOVA shows the F-value was 224.5 and hence the model was significant with a regression co-efficient (R^2) of 0.9965 (Table S6). p-Values of A_2, B_2, C_2, B_2C_2, B_2^2 and C_2^2 were <0.05, making them significant terms. As the xylosidase loading increased, the TRS yield also increased (Fig. 3a). The TRS yield decreased with the increase in XOS loading but the difference in the TRS yield at different XOS loading was insignificant (Fig. 3a and c). The TRS yield increased up to 80 min and then started decreasing (Fig. 3b and c). The predicted optimum xylosidase loading (A_2), XOS concentration (B_2) and hydrolysis time (C_2) were 40 U/mL, 2.08 g/L and 79.4 min, respectively. The predicted TRS yield from the above conditions was 1.84 g/g of XOS, whereas, the experimentally observed TRS yield was 1.77 g/g of XOS which was equivalent to 70 mg/g ptd biomass.

3.10. Thin layer chromatography (TLC) analysis of hydrolysed products of the pretreated FMS by endo-1,4-β-xylanase (CtXyn11A) and exo-1,4-β-xylosidase (BoGH43A)

The spots of hydrolysed products from the enzymatic hydrolysis of pretreated FMS by *Ct*Xyn11A and *Bo*GH43A were visualized on the TLC plate (Fig. 4). Lane 1 shows the xylo-oligosaccharides and only a faint spot of xylose produced by endo-1,4-β-xylanase (*Ct*Xyn11A). Lane 2 shows the hydrolysis of xylo-oligosaccharides by exo-1,4-β-xylosidase (*Bo*GH43A) to produce intense spot of xylose. The results showed that the enzymes *Ct*Xyn11A and *Bo*GH43A rendered efficient

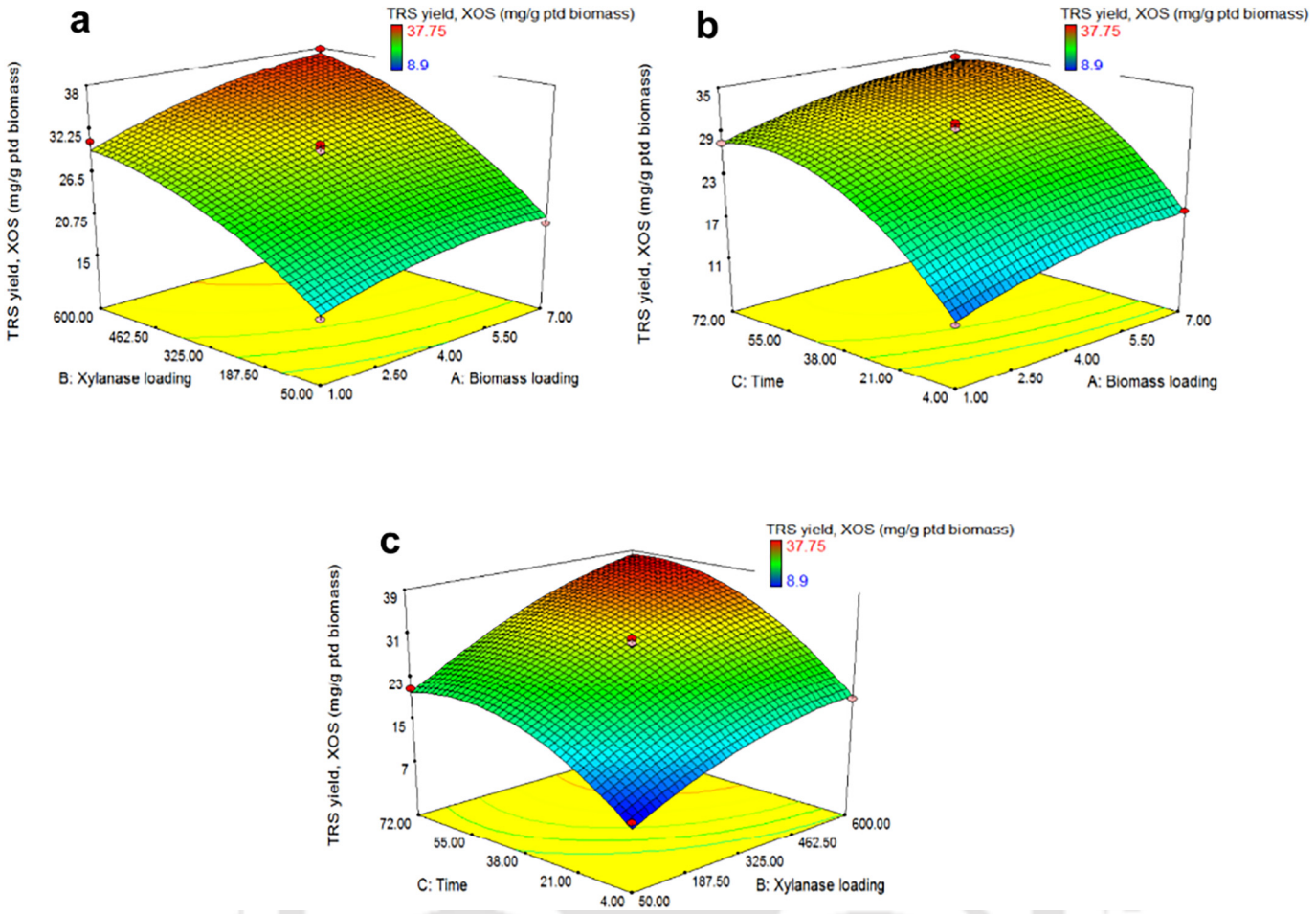

Fig. 2. 3-D response surface plots for the interaction between the independent variables involved in the optimization of hemicellulose hydrolysis by endo-1,4-β-xylanase (*Ct*Xyn11A). (a) biomass loading (%, w/v) and xylanase loading (U/g biomass); (b) biomass loading (%, w/v) and time (h); and (c) xylanase loading (U/g biomass) and time (h).

consecutive action on the hemicellulose portion of pretreated FMS under the mentioned optimized conditions to release xylose. The lane 2 also shows the un-hydrolysed oligomers from the xylosidase action. These might be the oligomers composed of residues other than xylose, cross-linked with the xylan. The application of *Ct*Xyn11A and *Bo*GH43A along with other hemicellulases and cellulases can be exploited to enhance the saccharification yield.

3.11. Determination of percentage of xylan to xylose conversion by HPLC

The TFA treated sample (pretreated FMS) contained 169 mg xylose/g ptd biomass. The xylose yield from enzymatic hydrolysis of the pretreated FMS with *Ct*Xyn11A and *Bo*GH43A at optimum conditions was 41.7 mg xylose/g ptd biomass. The remaining TRS of 28 mg/g ptd biomass from the previous section must be other oligosaccharides resulted from endo-xylanase hydrolysis. The xylan conversion from pretreated FMS to xylose was 24.7%. FMS has not been reported earlier as a bioresource for bioethanol production. Hence, the comparison of xylan saccharification from FMS was made against other lignocellulosic biomasses from earlier studies (Table S7). Peng et al. [34] reported 59% xylan conversion from pretreated corn stover using crude enzyme from *Caldicellulosiruptor owensensis*, a xylanolytic bacterium, but the initial xylan present in the biomass was very less (2%). Few other reports have focussed on the synergistic effect of xylanases along with the cellulases. Amore et al. [35] reported 44% xylan conversion from ammonia treated Brewers spent grain on enzymatic hydrolysis with the native xylanase from *Bacillus amyloliquefaciens* along with commercial β-xylosidase, cellulase and cellobiase. The higher xylan conversion from above report was due to the synergistic effects of other enzymes present in the cocktail. Maitan-Alfenas et al. [36] reported 19.6% xylan conversion from NaOH treated sugarcane bagasse using recombinant xylanase from *Aspergillus nidulans* along with accellerase. The enzymatic hydrolysis of biomasses, when compared with the similar pretreatment method (1% NaOH), the xylan conversion by *Ct*Xyn11A used in this study was higher than that reported by Maitan-Alfenas et al. [36]. However, each lignocellulosic biomass differs in its structural composition and organization [37]. This study will serve as the primary report on the xylan saccharification from alkali-treated FMS using endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) for future studies on the biomass.

4. Conclusions

Among 12 pretreated FMS samples, endo-1,4-β-xylanase (*Ct*Xyn11A) was most active on the biomass treated with 1% (w/v) NaOH combined with oven heating at 120 °C for 20 min resulting in TRS, 32 mg/g pretreated biomass. The pretreatment caused enhancement in the holocellulose content from 69% to 76%. Saccharification of the pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) under optimized conditions resulted in 24.7% conversion of xylan to xylose. The xylose can be used for ethanol fermentation and the left biomass can be further pretreated by the acid to make its cellulose content accessible for cellulose hydrolysis.

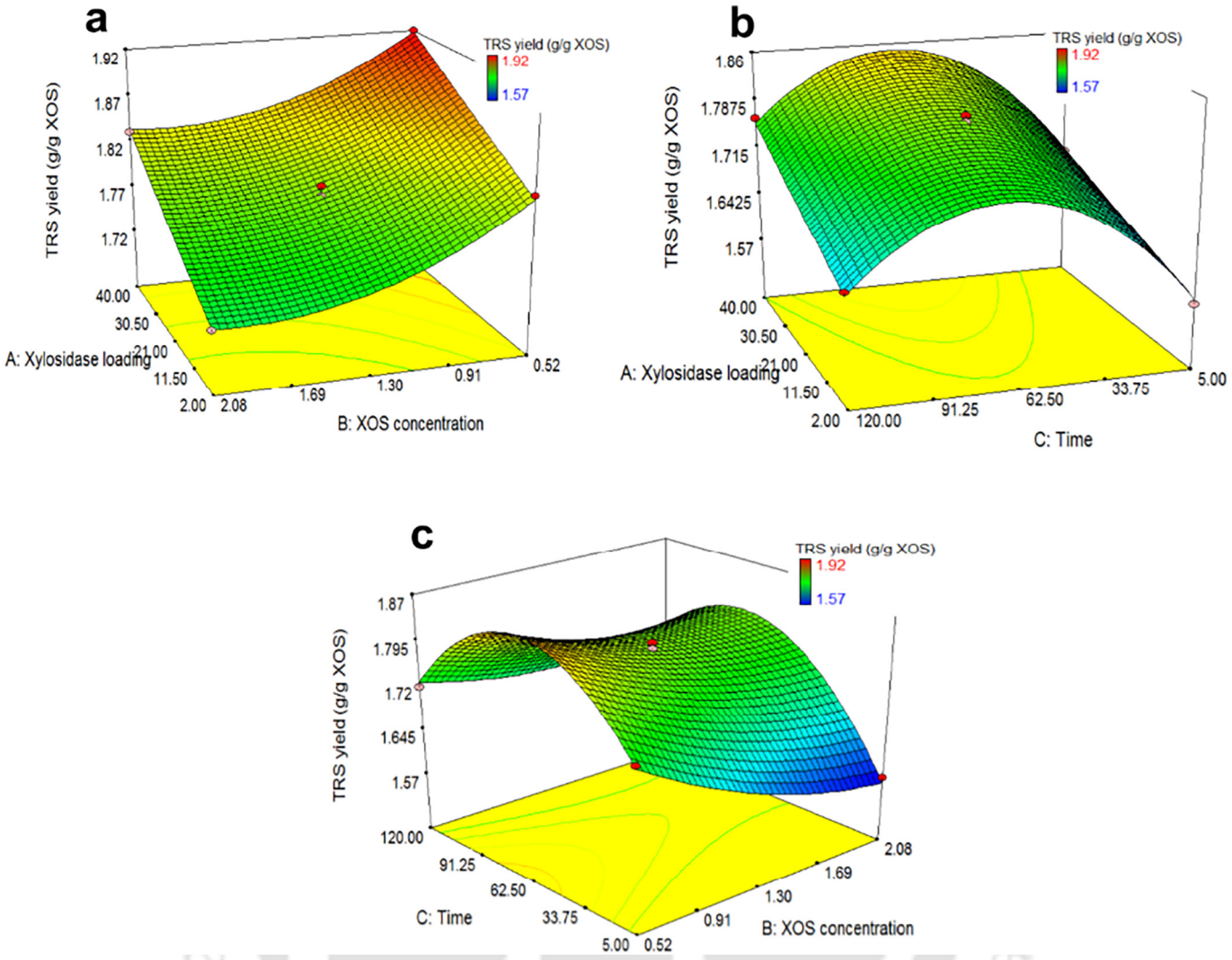

Fig. 3. 3-D surface response plots for the interaction between the independent variables involved in the optimization of xylo-oligosaccharide (XOS) hydrolysis by exo-1,4-β-xylosidase (*Bo*GH43A) (a) xylosidase loading (U/mL) and XOS concentration (g/L); (b) xylosidase loading (U/mL) and time (min); and (c) XOS concentration (g/L) and time (min).

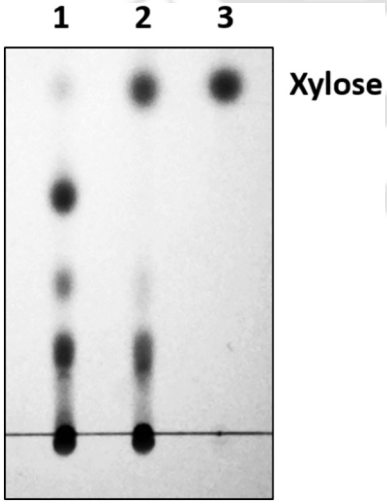

Fig. 4. Thin layer chromatogram (TLC) of the hydrolysed products from pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A) and exo-1,4-β-xylosidase (*Bo*GH43A) at optimized conditions. Lane 1- Xylo-oligosaccharides (XOS) production from the enzymatic hydrolysis of pretreated FMS by endo-1,4-β-xylanase (*Ct*Xyn11A); Lane 2- Xylose production from the enzymatic hydrolysis of xylo-oligosaccharides by exo-1,4-β-xylosidase (*Bo*GH43A); and Lane 3- Xylose standard.

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Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.06.010.

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