Fractionation of Yellow Thatching Grass (*Hyparrhenia filipendula)* for Sugar Production Using Combined Alkaline and Deep Eutectic Solvent Pretreatment

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Abstract

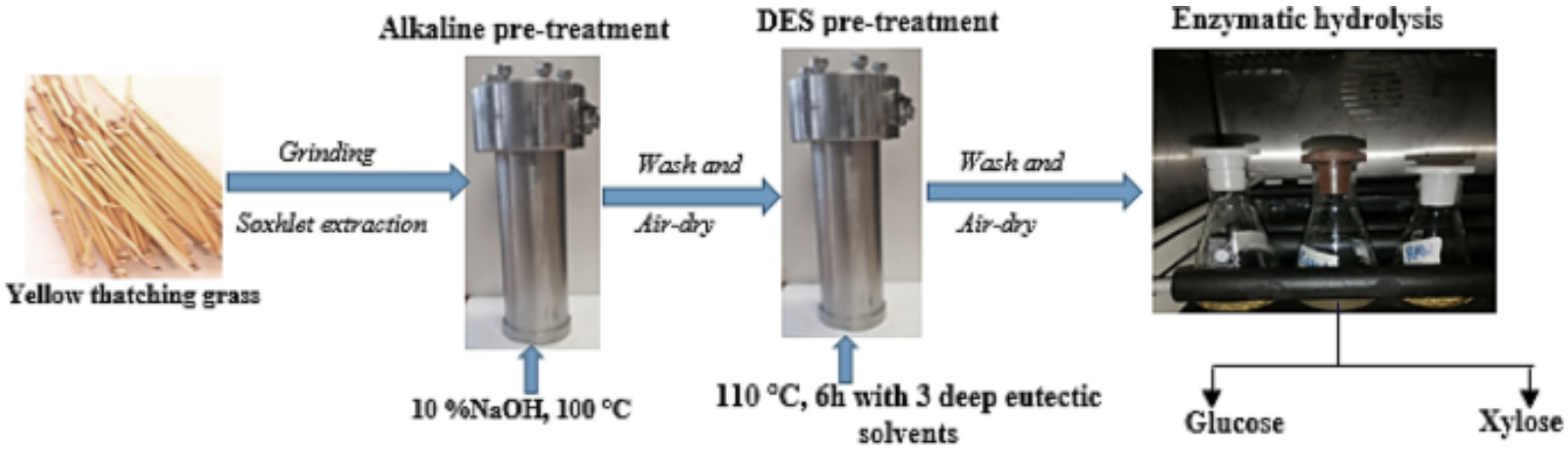
Purpose: Biomass pretreatment followed by enzymatic hydrolysis is one of the most viable ways to obtain sugars from biomass. In this work, the effect combined alkaline pretreatment and Deep Eutectic Solvent on enzyme hydrolysis of *Hyparrhenia filipendula* by cellulase is investigated. There is no previously reported literature on this substrate and the authors aim to establish baseline values for further research in the utilization of *Hyparrhenia filipendula*.

Methods: The yellow thatching grass (*Hyparrhenia filipendula)* was fractionated with a combination of alkaline and Deep Eutectic Solvent to increase sugar recovery. An alkaline solution of 10 wt % (w/v) of Sodium Hydroxide (NaOH) was used for the first stage of pretreatment at 100 °C for 4 h. Three DES, namely, Choline chloride (ChCl): urea; ChCl: glycerol; and Ethylene glycol: Citric acid at 1:2 molar ratio each, were heated to 80 °C until a clear solution was formed. The DESs were used for the second stage of pretreatment at 110 °C for 6 h in a Parr reactor. During the pretreatment, a solid: solvent ratio of 1:10 was used for the first and second stages of pre-treatment. Enzymatic hydrolysis was accomplished with a cellulase enzyme blend, Cellic CTec2, in a 50 mM sodium citrate buffer (pH 4.8) at 50 °C using a shaking incubator at a speed of 150 rpm. A solid loading of 2% and enzyme dosage of 50 g/100 g cellulose in the sample was used for all the experiments. Furthermore, samples were withdrawn every 24 h for 7 days and analyzed for glucose and xylose using High-Performance Liquid Chromatography (HPLC).

Results: A high delignification of 90% and hemicellulose removal of 70% was achieved with a combination of Alkali and ChCl: Urea pretreatment. Subsequently, the highest glucose and xylose conversion of 90% and 92% were observed, respectively, with the same sample. Additionally, the highest glucose yield achieved was 25 gL^{-1} from the combined alkaline/ChCl: Glycerol treated sample after 120 h. Moreover, the highest xylose yield was 3 gL^{-1} from the raw sample, the NaOH- pulped sample, and the ChCl: Glycerol-treated sample.

Conclusion: The results from this study demonstrated that the solvents used for fractionating biomass have a significant effect on the sugar recovery during enzymatic hydrolysis. Also, the pretreatment with a combination of NaOH and DES of ChCl: Glycerol was the most effective for the recovery of glucose and total sugar. In conclusion, yellow thatching grass is a promising

Graphical Abstract



substrate for bio-refineries. However, the ideal conditions for enzyme hydrolysis should be investigated further to promote its utilization for value-added products.

Keywords: Enzymatic hydrolysis; Pretreatment; Deep eutectic solvent; Yellow thatching grass

Statement of Novelty

The transition to greener energy sources demands the use of biomass feedstock that grows readily and does not interfere with food production. This study investigated the potential of yellow thatching grass as a feedstock for fermentation in biorefineries. During the enzymatic hydrolysis of the lignocellulosic biomass, pretreatment is critical to disrupt the cell structures of the biomass, effectively increasing enzyme access to carbohydrates. Consequently, enzymatic hydrolysis results in a high sugar yield and a more efficient biofuel production process. This study investigated the effect of a combination of pre-treatment strategies on the yield of sugars from yellow thatching grass. The results are expected to stimulate further research in the utilization of grassland biomass for biofuel production.

Introduction

Climate changes and shortage of fossil fuels have sparked a growing demand for liquid biofuels, which in turn has increased the amount of research into the production of lignocellulose-derived biofuels [1, 2]. However, effective substitution of petroleum-derived products with renewable sources requires improvement of bioconversion productivity, access to feedstock which does not compromise food supply, and optimization of land use [3, 4]. The most attractive renewable sources are fast growing perennial and annual crops, including grasses. Perennial grasses for example switch grass, *Miscanthus giganetus* JM Greef, and Napier grass have been increasingly used as energy crops because of higher cellulose and lignin contents in comparison to annual crops [5]. Grasses also have a high productivity per hectare, grow in infertile land, fast-growing, abundant, and the whole pant can be utilized [6,7,8,9]. Yellow thatching grass, *Hyparrhenia filipendula*, is a perennial grass widely distributed in East and Southern Africa [10]. The grass is mainly used for roofing houses, traditional medicine, mulching, and grazing cattle in its early stages of growth [10]. Typically, mature plants are considered weeds, and are usually burned, posing health and fire-related hazards. *Hyparrhenia filipendula* is a promising potential bioenergy crop that has not been previously thoroughly investigated for bioenergy conversion.

The complex structure of lignocellulose biomass makes it difficult to be fractionated, thereby limiting its conversion into valuable products like bioethanol, biomethane and organic acids [11, 12]. Biomass pretreatment, followed by enzymatic hydrolysis, has been viewed as a viable way to obtain sugars from biomass due to its fractionation effect and the high selectivity of enzymes for the hydrolysis of polysaccharides (cellulose and hemicellulose) to sugars (glucose and xylose) [3, 13]. This pathway has been intensively studied for years, for a variety of lignocellulosic biomass, but the extent of hydrolysis is mainly a function of the biomass composition, pretreatment method and type of enzyme used [3, 14, 15].

Alkaline pretreatment using sodium hydroxide (NaOH) is considered effective for delignification and can increase cellulose accessibility during enzymatic hydrolysis [16,17,18]. Jung et al. [19] obtained glucan and xylan conversion of up to 85% and 63%, respectively

during enzymatic hydrolysis of Switchgrass pretreated with NaOH. Obeng et al. [20] also reported a five-fold increase in glucose production with *Chloris barbata* pretreated with NaOH. Furthermore, Liu et al. [21] achieved 71% delignification of corn stover with NaOH pretreatment, and 83% conversion of glucan with subsequent enzymatic hydrolysis. Wang et al. [22] similarly reported a glucan and xylan conversion of 90% and 65%, respectively, with coastal Bermuda grass pretreated with NaOH.

Deep eutectic solvents (DESs) are also considered one of the most popular green solvents of the twenty-first century. DESs have attracted research attention because they are of low cost; biodegradable; biocompatible; non-flammable; easy to recycle; highly soluble and environmentally friendly among others [23,24,25]. Pretreatment with DESs increases the surface area and porosity of cellulose macromolecular structure, thereby improving its reactivity [26]. The DES also removes hemicellulose by breaking its glycosidic bonds (C–O–C) to form monosaccharides [26]. Additionally, DES selectively dissolves high amounts of lignin by predominantly cleaving the aryl ether linkage (β–O–4) linkages in biomass [27, 28]. Effective DES pretreatment is based on the DES ability to dissociate carbon–carbon bonds and aryl ether bonds inside the biomass structures [26]. A comprehensive review of the use of DES for fractionation and pretreatment of lignocellulosic biomass has been published [24, 25, 29,30,31].

Several studies have suggested that the combination of two or more pretreatment methods can yield greater delignification and higher sugar yields than a single pretreatment method [32,33,34,35]. In this work, the effect of combined alkaline pretreatment and Deep eutectic solvent [1] pretreatment on enzymatic hydrolysis of yellow thatching grass (*Hyparrhenia filipendula)* is investigated. There is no previously reported literature on sugar production from this substrate and the authors aim to establish its potential for bioenergy applications.

Materials and Methods

Materials

Sampling of the Grass

The *Hyparrhenia filipendula* grass was obtained from Palabek Kal, Lamwo district, Uganda, at 3°25′51"N 32°33′51"E and 964 m above sea level. The grass was harvested in January 2021 and sun-dried for two weeks or until the blades and sheaths were dry. A knife was used to remove the dried blades and sheaths from the stems. The stems were then cut into small pieces with lengths of about 3–4 cm and milled using a Retsch PM 100 ball mill for 3 h with 10 mm diameter stainless steel grinding balls at 300 rpm. The resulting sample was then passed through a 38 µm sieve and stored in an air -tight container to prevent reaction with moisture in the air. Soxhlet extraction, using acetone for 4 h at 100 °C, was used to remove the extractives. The sample was washed with de-ionized water, air-dried at room temperature and stowed. The samples in this study were all air-dried to prevent hornification of the lignocellulosic biomass.

Chemicals and Enzyme

The chemicals, Cellulase enzyme blend (Cellic CTec2), sodium hydroxide pellets (≥ 98%), citric acid monohydrate (99.8%), urea crystals (99.8%), choline chloride (≥ 98%), glycerol (99.5%), acetone (99.99%), citric acid, and ethylene glycol (99, 9%), were purchased from Sigma-Aldrich, South Africa, and used without further purification.

Two- stage pretreatment of yellow thatching grass

A summary of the pre-treatment of the yellow thatching grass is shown in Fig. 1. In the first stage, the yellow thatching grass was pulped with 10 wt % NaOH at 100 °C for 4 h in a Parr reactor using a solid/solvent ratio of 1:10 (w/v). The pulping conditions were optimized in our unpublished preliminary research activities. The sample was washed with de-ionized water to a neutral pH and air-dried for 12 h. For the second stage, the alkaline- pulped sample was further pretreated with three DES solutions in a Parr reactor at 110 °C for 6 h using a solid/solvent ratio of 1:10 (w/v).

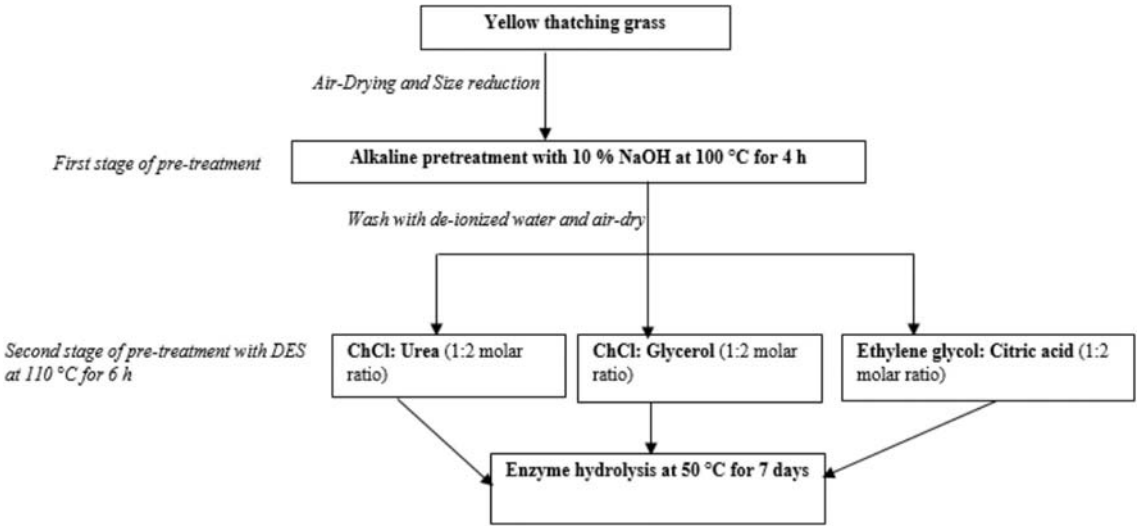


Fig. 1. Illustration of two-stage pre-treatment of yellow thatching grass

The DES were Choline chloride (ChCl): Urea; ChCl: Glycerol; and Ethylene Glycol: Citric acid (EG: CA). They were all prepared using a molar ratio of 1:2. The mixtures were heated to 80 °C, with constant stirring using a magnetic stirrer at 150 rpm, until a clear solution was formed. The DESs were then cooled to room temperature and stored in desiccator until a constant weight of the DES was obtained. The physicochemical properties of the DES are summarized in Table 1. The density was measured using an Attension Sigma force tensiometer (Sigma 700, Biolin Scientific USA). The conductivity was measured with a conductivity meter (Jenco instruments 3020 M) and the dynamic viscosity with an RV-viscometer (NDJ-8S, W&J instrument Co. Ltd China).

Table 1. Physicochemical properties of Deep Eutectic solvents

Solvent	Density(g/mL)	Viscosity (mPa.s)	Conductivity (µS/cm)
Choline chloride: urea (1:2)	1.24 ± 0.03	616.23 ± 0.06	333.5 ± 0.04
Choline chloride: glycerol (1:2)	1.19 ± 0.01	430.03 ± 0.11	1048 ± 0.7
Ethylene Glycol: citric acid (1:2)	1.50 ± 0.02	7532 ± 0.04	1334 ± 0.05

Enzymatic Hydrolysis

Enzymatic hydrolysis was accomplished with a cellulase enzyme blend, Cellic CTec2, in a 50 mM sodium citrate buffer (pH 4.8) at 50 °C using a shaking incubator at a speed of 150 rpm.

The substrate used to determine the enzymatic activity of cellulase was 5 mg of Whatman No. 1 filter paper strips (1.0 × 6.0 cm). The enzymatic activity was determined as 60.66 filter paper units (FPU)/mL. A solid loading of 2% of untreated and pretreated biomass, and enzyme dosage of 50 g/100 g cellulose was used for all the experiments. Furthermore, a hydrolysate was withdrawn every 24 h for 7 days and analyzed for glucose and xylose using a High-Performance Liquid Chromatography (HPLC).

Yield of Sugars

A high-performance liquid chromatograph (Agilent 1260 Infinity, Germany) equipped with a refractive index detector and UV–visible Spectrophotometer (Hitachi U-3900 Spectrophotometer) was used to determine the carbohydrates and lignin in the sample using the procedure described by Sluiter et al. [36]. The Bio-Rad Aminex HPX-87C column (300 mm × 7.8 mm) was operated at 60 °C, with a flow rate of 0.60 mL/min and 0.005 M H_2SO_4 eluent. The anhydro corrected yield of glucose and xylose were calculated using the Eqs. (1) and (2) [18, 37].

$$\text{Glucose yield} = \frac{\text{Total Glucose released} \times 0.9}{\text{Initial glucan loading}} \times 100\% \quad (1)$$

$$\text{Xylose yield} = \frac{\text{Total xylose released} \times 0.88}{\text{Initial xylan loading}} \times 100\% \quad (2)$$

Physico-Chemical Characterization

The morphology and elemental composition of the samples was examined using a Zeiss Ultra plus FEG Scanning electron microscope equipped with an energy dispersive X-ray spectroscope (EDS). The samples were coated with carbon using a Quorum Q150T ES sputter coater to enhance image resolution.

Functional groups in the samples were determined by Fourier Transform Infrared Spectroscopy (FT-IR) in the medium infrared region of 4000 – 400 cm^{-1} with a Bruker Alpha FTIR spectroscope. X-ray diffraction (XRD) analysis of the samples was conducted on a PANalytical X'Pert Pro powder diffractometer with Fe filtered Co-Kα radiation (λ = 1.789 Å). The crystallinity index, I_C, was obtained from Eq. 3.

$$I_C = \frac{I_{002} - I_{am}}{I_{002}} \quad (3)$$

where I_{002} is the diffraction intensity close to 2θ = 22–24° and represents a crystalline and amorphous component of the material; I_{am} is the diffraction intensity close to 2θ = 16–18° and refers to amorphous component of cellulosic fibers [38].

Results and Discussion

Chemical Composition of the Samples

The composition of the raw yellow thatching grass, as shown in Table 2 was 33% cellulose, 25% hemicellulose and 24% lignin, comparable to other grass species reported in literature [39]. After pulping with NaOH, the cellulose composition increased by 85% and a

Table 2. Chemical composition of *Hyparrhenia filipendula*

Sample	Composition, %					Hemicellulose removal, %	Lignin removal %
	Cellulose	Hemicellulose	Lignin	Acid insoluble ash	Others*		
Untreated	32.87 ± 2.63^{a}	24.51 ± 5.27^{b}	23.53 ± 0.00^{a}	2.4 ± 0.00	16.69 ± 0.00	–	–
Pulped	60.63 ± 2.35^{a}	9.96 ± 2.05^{a}	14.12 ± 0.00^{b}	0.17 ± 0.00	15.12 ± 0.00	59.36	40
ChCl: Urea	35.36 ± 0.99^{b}	7.15 ± 2.32^{a}	3.33 ± 0.00^{c}	–	54.16 ± 0.00	70.83	90
ChCl: Glycerol	80.12 ± 2.98^{b}	12.36 ± 3.19^{a}	5.51 ± 0.01^{d}	0.03 ± 0.00	1.98 ± 0.00	49.57	83
Ethylene glycol: Citric acid	56.20 ± 2.81^{c}	8.50 ± 0.85^{a}	6.67 ± 0.00^{e}	–	28.63 ± 0.00	65.34	79

*others may contain organic compounds (proteins, resins, and gums), waxes, fats and monosaccharides lost in aliquot

delignification of 59% delignification was achieved. Notably, a delignification of 81% was achieved by Haldar and Purkait [40] after pulping elephant grass with 3% (w/v) NaOH, using a solid: solvent ratio of 1:10 at 121 °C for 1 h. Similarly, delignification of Napier grass with 2% (w/v) NaOH, using a solid: solvent ratio of 1:6, at 121 °C for 1 h achieved a delignification of 84% and glucan recovery of 71% [37]. Based on our preliminary investigations, pulping the yellow thatching grass with a 2% (w/v) NaOH resulted in a higher delignification of 60%, and lower glucan recovery of 58%. A higher concentration of NaOH was therefore used based on previous studies, but the solid: solvent ratio and temperature used for pulping should be optimized to achieve a higher delignification and xylan solubility.

The extractives in the yellow thatching grass is approximately 17%, comparable to values reported by other authors for similar species [39]. However, this study has a significant limitation in that the NREL/TP-510–42,619 procedure [41] was not strictly followed to determine extractives in the yellow thatching grass. Additionally, the values in the "others" column presumably represents the sugars in the aliquot of the pre-treated samples. Another shortcoming of the study is that the cellulose and hemicellulose lost in the form of monosaccharides in the aliquot were not determined. Moreover, the pre-treated samples had a lower concentration of acid-insoluble ash. The acid-insoluble ash is correlated to the amount of Silica in the sample. According to Table 2 the concentration of Silica in the samples decreased at every pretreatment stage.

The two-stage pre-treated samples achieved a higher delignification of 90% for the ChCl: Urea; 84% for ChCl: Glycerol and 80% for ethylene glycol: citric acid. The combination of pre-treatment with DES therefore leads to a more efficient delignification of the biomass, making the cellulose more available for enzyme hydrolysis.

Furthermore, the cellulose content of the sample pretreated with ChCl: Glycerol was improved by 32%. However, a sugar loss of 42% and 7% was observed after pretreating the samples with alkali/ ChCl: Urea and alkali/ ethylene glycol: citric acid, respectively. Zhang et al. [42] stated that DESs do not weaken the interactions between anions and cations in hydrogen bonds in cellulose but form hydrogen bonds with cellulose, thereby dissolving it. One urea molecule, which has one carbonyl group and two amine groups, can form eight hydrogen bonds. Also, one glycerol molecule, with three hydroxyl groups can form three hydrogen bonds and one citric acid molecule (with four hydroxyl groups and three carbonyl groups) can form 10 hydrogen bonds [42]. The choline chloride can only accept three (3) hydrogen atoms, therefore; urea can form four hydrogen bonds with cellulose; and glycerol can form three hydrogen bonds with cellulose and three hydrogen bonds with ChCl. Similarly, Ethylene glycol can only accept two hydrogen atoms, therefore citric acid can form eight hydrogen bonds with cellulose. Glycerol/ChCl, therefore forms a lesser number of hydrogen bonds with cellulose, reducing the solubility of cellulose in the DES, hence no sugar loss observed as shown in Table 1. Moreover, ChCl/Urea forms four hydrogen bonds with cellulose, increasing the solubility of cellulose in the solution, thus a sugar loss of 42%. However, temperature is also a key factor in the solubility of cellulose in DES. Evidence shows that as the temperature increases, the solubility of cellulose in the DES increases as well [42]. Therefore to reduce the sugar loss, the pretreatment can be done at a temperature lower than 80 °C so that the change in Gibbs free energy of the mixture is less than zero, making it difficult for the DESs to form new molecular hydrogen bonds with cellulose [42].

The hemicellulose content of the yellow thatching grass also decreased as follows after combined alkali/DES pre-treatment; 71% for ChCl: Urea; 50% for ChCl: Glycerol and 65%

for EG:CA. Hemicellulose dissolves well in the DESs due to the formation of hydrogen bonds between the DES and polysaccharide, which in turn destroys the intermolecular hydrogen bond of the solute [43]. Yu et al. [44] achieved 100% xylan solubility with a DES of ChCl: glycolic acid (1:6 molar ratio) at 120 °C for Akebia herbal residues. Additionally, Hou et al. [45] also observed that the acidity and alkalinity of the DESs are closely related to the lignin and hemicellulose removal capabilities, and ultimately influence enzymatic polysaccharide digestion. Therefore, prior to using the combined pretreatment, a comprehensive evaluation of the influence of DES properties on xylan removal and delignification of yellow thatching grass is required.

A one-way ANOVA was performed to compare the effect of the treatments on the chemical composition of the yellow thatching grass. There was a statistically significant difference in the hemicellulose composition between the treatments [$F(4, 5) = (10.12)$, $p = 0.013$]. Tukey's HSD test revealed that there was no significant difference in the mean value of cellulose composition between the untreated and pulped samples at 95% confidence interval. Also, there was no significant difference between the cellulose composition between the ChCl: Urea and ChCl: Glycerol samples. Further still, there was a significant difference between the hemicellulose composition in the untreated sample and all the samples that were subjected to thermochemical treatment. The lignin composition between all samples was statistically different.

Enzymatic Digestibility

The results from the enzyme saccharification are shown in Fig. 2.

The glucose and xylose concentrations increased rapidly within 24 h for all the samples, agreeable with the observations of other authors [37]. The highest yield of glucose was 25 gL^{-1} obtained from the pretreated sample with alkaline/ChCl: Glycerol after 120 h of hydrolysis. The maximum glucan conversion to glucose was 100% for ChCl: Urea; 90% for NaOH-pulped sample; 88% for the raw sample; 77% for ChCl: Glycerol; and 62% for EG: CA. The ChCl: Urea pre-treated sample had the least lignin composition as shown in Table 1, thus increasing the enzyme digestibility. The glucose conversion of the raw sample can be attributed to the particle size of the sample and cellulolytic system of the enzyme [46]. While the glucose conversion reported in this study are similar to those in open literature, the glucose yield is much lower than those of biomass like wheat straw, rice straw, and corn straw as highlighted in Table 3. The pre-treatment conditions for the yellow thatching grass therefore need to be optimized to improve the glucose yield during enzymatic hydrolysis.

The enzyme blend contains Xylanase and therefore residual Xylans are degraded during enzyme saccharification [37, 47]. The highest xylose yield was 3 gL^{-1} obtained from the raw sample, NaOH pulped sample, and ChCl: Glycerol treated sample. The maximum xylan conversion was 92% for ChCl: Urea; 88% for pulped sample; 73% for EG: CA; 66% for ChCl: Glycerol; and 51% for raw sample. The Alkali/ChCl: Urea treated sample had the highest hemicellulose removal as shown in Table 1, thus, reducing the biomass recalcitrance during enzymatic hydrolysis.

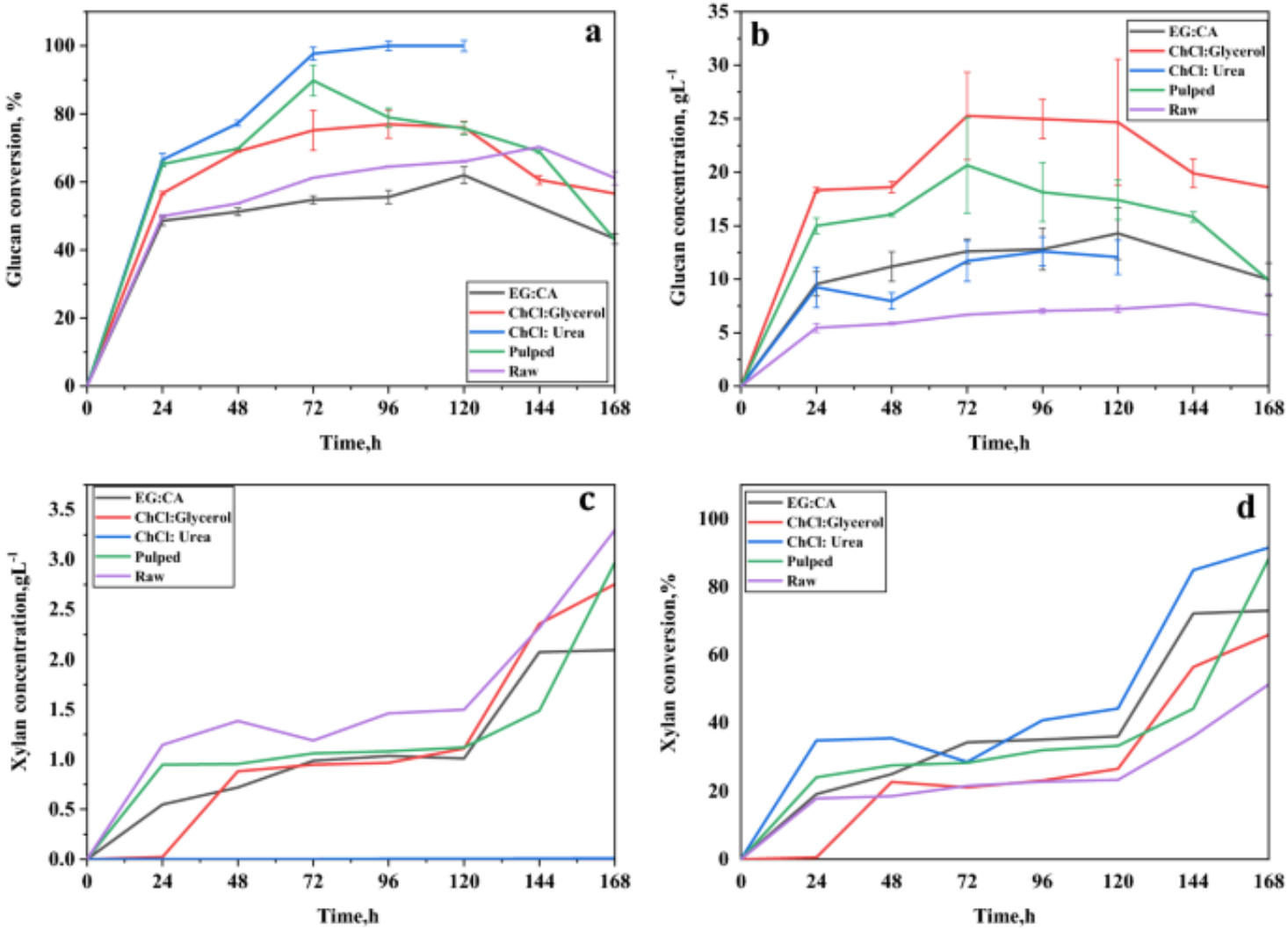


Fig. 2. Enzymatic hydrolysis of grass; **a** Glucan conversion, **b** glucan release, **c** xylan conversion, **d** xylan release, for untreated and treated biomass

Table 3 Glucose and xylose yield from enzymatic hydrolysis of biomass

Biomass	Pre-treatment conditions	Enzymatic hydrolysis	Glucose yield	Xylose yield	Reference
Wheat straw	1st stage: Steam-explosion at 198 °C for 10 min 2nd stage: 4% (v/v) H_2O_2 and 1% NaOH (w/v) with a solvent: solid ratio of 1:10 at 25 °C for 120 h	Sodium acetate buffer (100 mM, pH 4.5), at 50 °C with gentle agitation in water bath for 72 h, *Trichoderma viride* cellulase (110 FPU/mL) loading of 30 FPU/ (g substrate), solid loading of 16.7% (w/v)	110.9 gL^{-1}	–	[48]
-Rice straw (RS) -Sugarcane bagasse (SB) -Napier grass (NP)	1st stage: Hydrothermal pre-treatment at 121 °C for 40 min 2nd stage: DES (ChCl: Lactic acid) at 130 °C for 1.5 h, solid loading 10% (*w*/w)	50 mM citrate buffer (pH 4.8) at 50 °C in an orbital shaker at a speed of 150 rpm, solid loading of 2.5% using CelluClast 1.5 L (20 FPU/g) and 40 µL sodium azide for 72 h	RS 91% SB 91% NP 91%	–	[49]
Rice straw	1st stage: DES consisting of Formic acid, Acetic acid and ChCl (1:1:1 molar ratio) at 140 °C, 1:10 ratio for 2 h 2nd stage: 1% (w/v) Na_2CO_3 for 1 h at 1:10 ratio and 140 °C	Citrate buffer (50 mM, pH 4.8), 100 µL ampicillin (1 $g{\cdot}L^{-1}$) and 50 FPU cellulase (142 ± 1.1 FPU/g) incubated in a water bath at 50 °C and 150 rpm for 48 h	37.3 $g{\cdot}L^{-1}$ Total sugar 42.7 $g{\cdot}L^{-1}$	–	[50]
Bambara groundnut haulm	The following DES were used at 100 °C for 3 h using a solid: liquid ratio of 1:10 (w/w); -ChCl: Acetic acid (1:2 molar ratio) -ChCl: glycerol (1:2 molar ratio) -ChCl: urea (1:2 molar ratio) -ChCl: lactic acid (1:2 molar ratio) -ChCl: formic acid (1:2 molar ratio)	10% solid loading, citrate buffer (50 mM, pH 4.8), 0.01 wt.% NaN_3 and the enzyme cocktails, Accellerase 1500 and Accellerase XC loaded at a ratio of 1:1 with an enzyme load of 30 mg protein/g biomass and incubated at 50 °C in a water bath, at 150 rpm for 48 h	Total sugar yield of − 85% for ChCl: urea − 72% for ChCl: glycerol 79% for ChCl: Acetic acid 98% for ChCl: Lactic acid 86% for ChCl: formic acid		[51]
Corn straw	Extrusion of corn stover added to 23% (w/w) distilled water, 2% (w/w) glycerol and 1 g/L $NaHCO_3$. A screw speed of 100 rpm, mass barrel temperature was 120 °C and the feeding rate of 2 kg/h was used	Sodium acetate buffer (pH = 4.8), 0.02% (w/v) sodium azide, enzyme loading of 15 FPU/g of dry matter, at 50 °C and 150 rpm for 72 h in a shaking incubator. Enzymes used were Hemicellulose (Cellic HTec, 10,000 U/g) and Cellulase (Cellic CTec2, 10,000 U/g)	50 gL^{-1}	43 gL^{-1}	[52]
Yellow thatching grass	1st stage: 10% NaOH at 100 °C for 4 h 2nd stage: DES (ChCl: Glycerol) at 110 °C for 6 h	Enzymatic hydrolysis using cellulase enzyme blend Cellic CTec2 (60.66 FPU/mL), in a 50 mM sodium citrate buffer (pH 4.8) at 50 °C using a shaking incubator at a speed of 150 rpm; solid loading of 2%, and enzyme dosage of 50 g/100 g cellulose in substrate for 7 days	25 gL^{-1}	3 gL^{-1}	This study

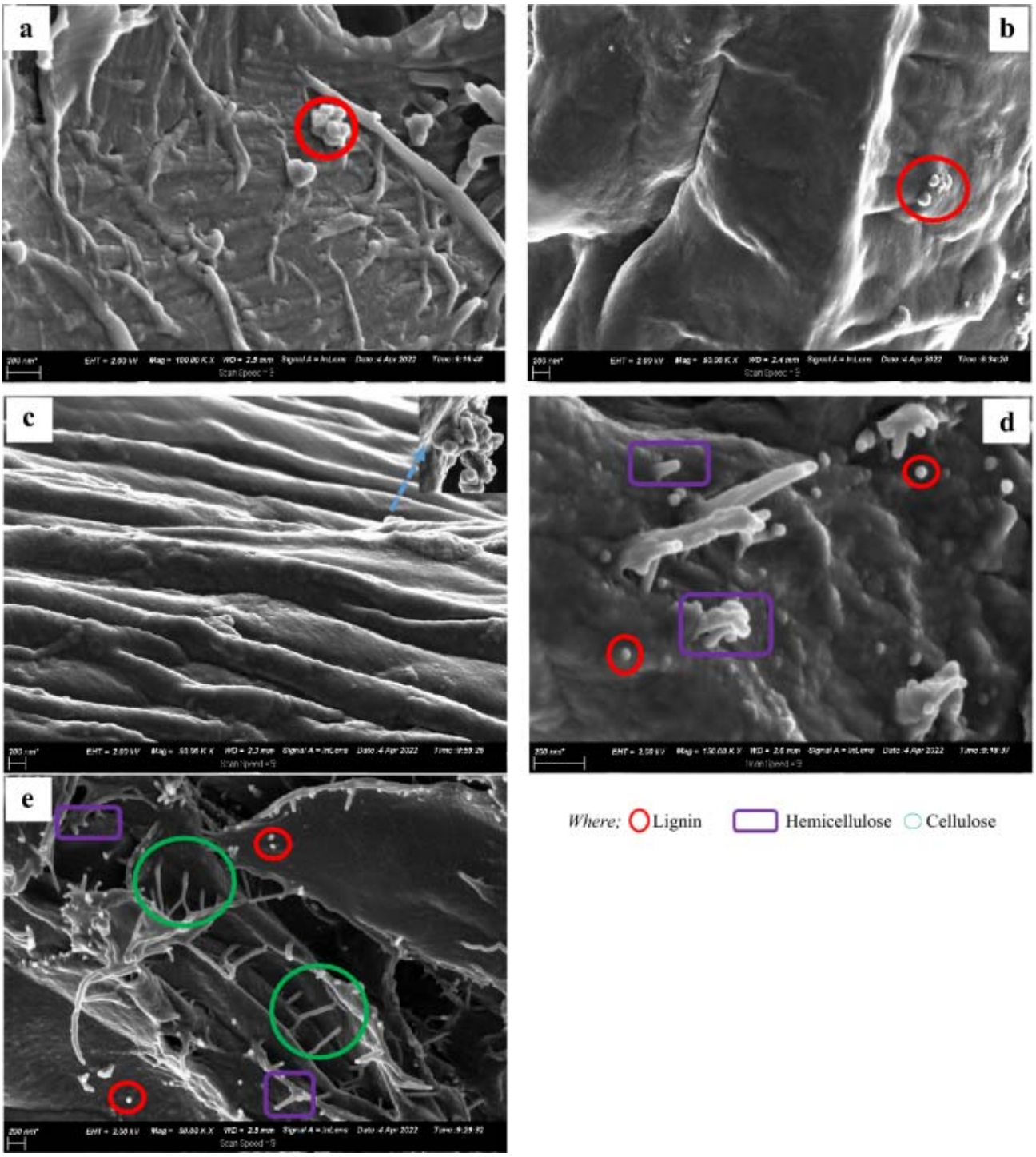


Fig. 3. SEM for yellow thatching grass; **a** for untreated sample; **b** for pulped sample; **c** combined alkali and ChCl: Urea; **d** for combined alkali and glycerol; **e** for combined alkali and EG: CA, respectively

Morphology

The morphological changes during pretreatment is shown in Fig. 3 For the raw sample, the lignin is arranged in aggregates and tightly adhered to the cellulose fibers (Fig. 2a). The lignin

Table 4 EDX analysis of samples

Sample	Element, wt %								
	O	Si	P	Cl	K	Ca	S	Na	Mg
Untreated	59.08 ± 7.49	27.33 ± 15.74	1.22 ± 0.91	1.65 ± 1.19	9.70 ± 6.17	0.52 ± 0.69	0.25 ± 0.26	–	0.22 ± 0.50
Pulped	86.67 ± 5.92	4.40 ± 0.92	–	–	–	4.74 ± 4.62	–	1.58 ± 1.84	2.61 ± 1.98
ChCl: urea	68.89 ± 7.16	2.98 ± 0.75	–	26.52 ± 6.14		–	–	–	1.93 ± 0.41
ChCl: glycerol	94.83 ± 1.63	3.02 ± 0.26	–	–	–	0.59 ± 0.52	–	–	2.10 ± 1.09
EG:CA	94.88 ± 1.67	5.12 ± 1.67	–	–		–	–	–	–

is deposited as droplets on the alkaline treated surface wall, and the cellulose appears corrugated. The lignin is re-deposited as droplets on the surface of cell walls when the melting temperature of the lignin is exceeded during the pulping process [53]. The surface of the sample pretreated with alkali and ChCl: Urea is glossy and serrated with few aggregated lignin deposits observed at high magnification. Typical short chains of hemicellulose and droplets of lignin are also observed on the surface of the sample pretreated with alkali and ChCl: glycerol DES. Furthermore, the surface of the sample pretreated with the combination of alkali and EG: CA has exposed short chains of hemicellulose, interconnected cellulose chains, and lignin droplets on the surface. The combination of Alkaline and DES pretreatment methods results in visible exposure of the carbohydrates, especially for ChCl: glycerol and EG: CA (Fig. 2d, e), thereby increasing the adsorption of enzymes [32].

The elemental compositions of the samples are shown in Table 4. The elemental analysis shown was taken at 10 positions for all the samples. The results show a significant reduction in silica during pretreatment, though not totally removed. Silica removal is important during pretreatment so that enzymes can access the hemicellulose and cellulose [54]. The Sodium (Na) detected in the pulped sample is primary due to inadequate washing of the sample after pulping with NaOH.

Surface Chemistry Via FTIR

The FTIR spectra of the samples shown in Fig. 4 explains the structural modification of the yellow thatching grass after pretreatment. The peak at 3338 cm^{-1} represents the stretching vibration of O–H hydrogen bonds in cellulose [40]. Stretching vibration of C–H groups due to the presence of cellulose or lignin components of biomass is confirmed by the peak at 2895 cm^{-1} [40]. Enhanced intensity of this peak in the NaOH -pulped sample could be attributed to increased exposure of cellulose fraction of *Hyparrhenia filipendula*. The peak intensity at 1730 cm^{-1} is attributed to the linkages of ester bonds between lignin and hemicellulose[40]. This peak is absent in three samples; pre-treated sample with pulping, sample pretreated using a combination of NaOH and DES (ChCl: Urea), ChCl: glycerol; indicating effective disruption of the ester bond. The peak at 1235 cm^{-1} shows the presence of ether bond. High intensity of this peak for untreated biomass represents an intact presence of lignin, while the intensity of the signal is decreased with pretreated samples[40]. Additionally, the presence of a peak at 1605 cm^{-1} or the untreated sample only shows that the pretreatment methods were effective for delignification [40]. The intensity of the peak at 1160 cm^{-1} attributed to the presence of β-1–4 glycosidic linkages, is lowest for the untreated sample but highest for the pulped sample[40]. The high intensity band at 1032 cm^{-1} is due to C–C, C–OH, and C–H ring and side group stretching vibrations assigned to cellulose [40]. The intensity of this band is lowest for the untreated sample, which contains the least cellulose as shown in Table 1.

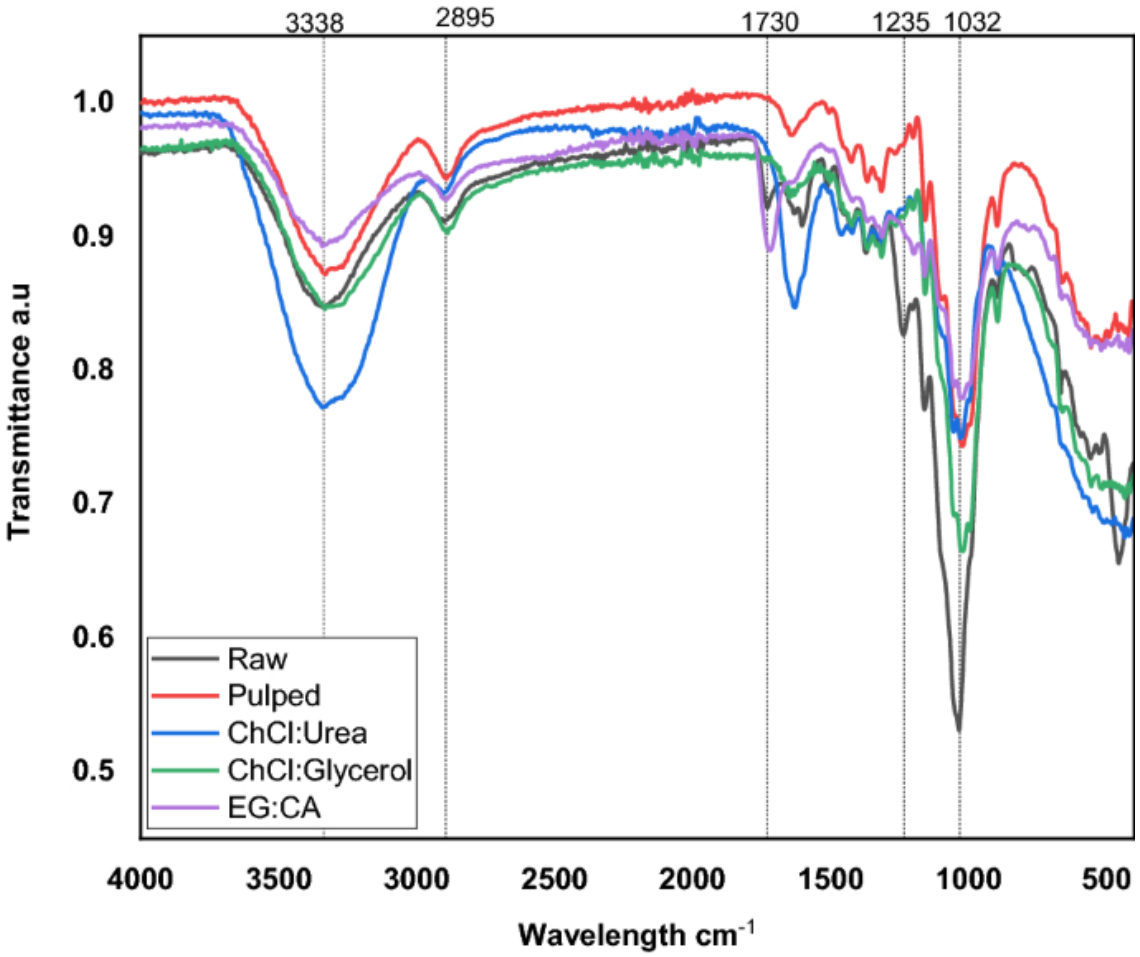


Fig. 4. FTIR Spectra of samples

Crystallinity

The XRD patterns of the samples are shown in Fig. 5. The raw sample has three distinct peaks at 18°, 26°and 40°, characteristic of cellulose I. The peak at 18° flattens out in the pretreated samples, indicating reduction in crystalline level and transformation of cellulose I to II [55]. Cellulose II allomorph is more stable than cellulose I due to mercerization [56]. The intensity of the peaks also reduced for the pretreated samples.

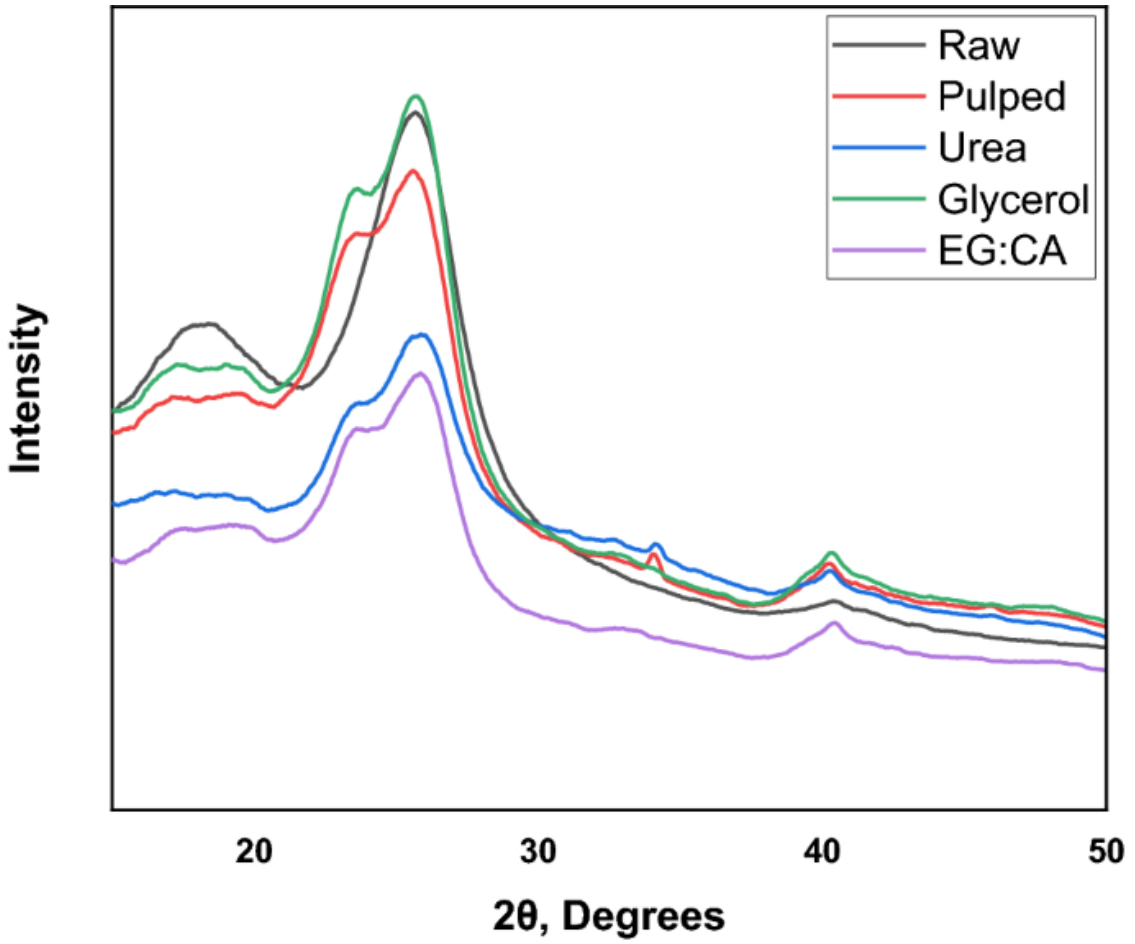


Fig. 5. XRD analysis of samples

The crystallinity of the sample also reduced with pretreatment. The raw sample has a crystallinity of 73%, while the pulped sample has a crystallinity of 70%. The samples that underwent combined pretreatment had a crystallinity of 37% for ChCl: urea, 72% for EG: CA and 64% for ChCl: glycerol. The crystallinity index is correlated to the extent of transition from cellulose I to cellulose II[57]. In the raw sample, there is a greater concentration of cellulose I, leading to a higher crystallinity index. The alkali and ChCl: urea treated sample has the highest concentration of cellulose II, leading to the lowest crystallinity index. A low degree of crystallinity increases the efficiency of enzyme hydrolysis, hence more sugar production as shown in Fig. 2 [58].

Conclusion and Future Perspectives

Pre-treatment is a critical step in the conversion of lignocellulosic biomass into value-added products like biomethane and bioethanol. Over the years, various pre-treatment processes have been researched for a variety of biomass in order to maximize fermentable sugar yields during enzymatic hydrolysis. Due to the heterogeneity and complexity of lignocellulosic biomass, there is no single effective pretreatment method reported yet for all biomass. In this study, *Hyparrhenia filipendula* was pretreated with NaOH and three deep eutectic solvents; ChCl: Glycerol; ChCl: Urea and Ethylene Glycol: Citric acid. The selection of sodium hydroxide and deep eutectic solvents was primarily due to their benefits, such as inexpensive neutralization and environmental friendliness. Enzyme hydrolysis was carried out under the same conditions

for all samples to determine which pretreatment method would be most effective for bio-fuel production.

The pre-treatment with Alkali and a combination of Alkali and DES was effective for delignification and de-polymerization of hemicellulose. The sample treated with NaOH and ChCl: Urea resulted in a delignification of up to 90% and hemicellulose removal of up to 71%. Although the cellulose concentration increased in the alkaline and DES pre-treated samples, it was not consistent as expected. This could be attributed to sugar loss during the first and second pretreatment stages, which needs to be investigated further. In addition, the sugar levels in the aliquot were not quantified during this study, affecting the material balance. The loss of sugars should be accounted for in future studies, in order to accurately determine the optimal conditions for pretreatment.

Furthermore, enzyme digestibility is improved with samples fractionated with the Alkaline and a combination of Alkaline and DES pretreatment. The highest yield of glucose was 25 gL^{-1} obtained from the combined alkaline/ ChCl: Glycerol treated sample after 120 h. The highest xylose yield was 3 gL^{-1} obtained from the raw sample, NaOH pulped sample, and ChCl: Glycerol treated samples. The maximum glucan and xylan conversion of 100% and 92% respectively was achieved for the sample treated with combined NaOH and ChCl: Urea. The total sugar yield from the enzymatic saccharification was low in comparison to previously investigated agricultural residues. Further research is required to optimize the time, solid loading of the substrate, particle size, and enzyme blend and loading to increase the total sugar yield. Fungi can also be added to improve the enzymatic hydrolysis rate and reduce the production of inhibitors during the pretreatment phase, thereby reducing the energy consumption of the entire process.

Overall, the potential of *Hyparrhenia filipendula* as a source of raw material for bio-refineries was demonstrated in this study. Future work will focus on optimizing the pretreatment process parameters and enzyme hydrolysis conditions.

Data Availability

The datasets generated during and/or analysed during the study are not publicly available due to Copyright regulations by Makerere University and University of Pretoria, but are available from the corresponding author on reasonable request.

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Contributions

All authors contributed to the successful completion of this study. NMF, AF, OCM and SAI conceptualized the project.MOD, JBK, SAI, AF and OC Mary reviewed the first draft. MOD and JBK were responsible for funding acquisition and project administration.

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