Intensification of pre-treatment and fractionation of agricultural residues

Hafsat H. Ibrahim¹, Paul E. Bilsborrow², Anh N. Phan^{1*}

¹ School of Engineering, Newcastle University, UK.

² School of Natural and Environmental Sciences, Newcastle University, UK.

Newcastle upon Tyne, NE1 7RU

Corresponding author*: Email: Anh.phan@newcastle.ac.uk;

Abstract

Lignocellulose biomass is a unique renewable and sustainable source that can be used in energy generation, production of transport fuels and in chemical industries to replace petroleum-based resources. However, due to its recalcitrance to chemical and enzyme breakdown, it needs to be pre-treated prior to processing. In this study, ultrasound assisted ozone was used to pre-treat the agricultural processing residues corn cob and spelt husk. Following organosolv fractionation, About 90% and 94% of lignin with high purity (95%) were recovered for corn cob and spelt husk with guaiacyl-syringyl lignin the major fraction from corn cob and guaiacyl lignin from spelt husk respectively. In addition, cellulose pulp of 83.6% and 85.2% with 78% purity were recovered for corn cob and spelt husk respectively with a 19% decrease in cellulose crystallinity and 17% decrease in degree of polymerization (DP). Thus, the improved quality separation of biomass components will enhance downstream conversion to value added products thereby improving the sustainability and cost effectiveness of the process.

Keywords

Fractionation, pre-treatment, lignin recovery, reactivity, agricultural residues

1. Introduction

Lignocellulose materials (agricultural residues, woody materials etc.) have gained particular attention in recent years in providing sustainable, cost effective and environmentally friendly alternatives to fossil fuels [1]. Agricultural residues (corn cob, cereal straw, spelt husk, sugarcane bagasse, rice husk etc.) are currently exploited due to their high abundance/availability [2] but processing residues offer clear potential as they avoid the development of extensive collection systems for these high volumes and low-density feedstocks. Globally about 140 Gt of biomass wastes are produced annually with 66% derived from cereal crops³ but with about 517 Mt of cereal crop residue unexploited globally. Global cereal yield is predicted to increase by 0.9% per annum between 2007 and 2050 equating to a further

increase of 1 billion tonnes of residue [3]. However, using them as feedstock for chemical and biological processes needs pre-treatment to disrupt the complex structure and allow increased accessibility and easy conversion of the carbohydrate content [4,5].

Typically, lignocellulose biomass material contains mainly lignin (10-25%), hemicellulose (20-30%), cellulose (40-50%), and other components such as extractives, lipids, proteins and ash [6]. Lignin, a 3-dimensional cross-linked aromatic polymer providing rigidity and strength to plant cell walls is found intertwined with cellulose and hemicellulose hindering its biodegradation. It is made from three phenyl propane units i.e. *p*-Hydroxyphenyl (H), guaiacyl (G) and syringyl (S) alcohols in different proportions and connected by random C-C and C-O bonds [7,8]. Covalent bonds in the form of phenyl glycoside bonds, esters and benzyl ethers exist between lignin and the carbohydrate fractions (cellulose and hemicellulose) forming the lignin-carbohydrate complex (LCC linkage). These stable bonds create an obstacle to successful fractionation and selective isolation of individual components from the lignocellulose biomass making it recalcitrant to chemical/enzymatic processing [9].

Most bio-refineries concentrate on exploitation of cellulose and hemicellulose to produce advanced generation biofuels e.g. cellulosic ethanol [10] and platform chemicals such as 5-hydroxymethyl furfural (HMF) and furfural [11]. Lignin left as a residue is considered of low value and often burned as low-grade fuel or dumped as waste which causes environmental pollution [12].

Conversion of lignin into high value chemicals alongside the utilization of cellulose and hemicellulose for biofuel production is important to increase the profitability and sustainability of the bio-refinery process. However, to achieve complete utilization of all biomass components to value added products, it is important that the lignin recovered is of high purity [13] and to achieve this, it is necessary to isolate the lignin at an early stage i.e. pre-treatment to prevent irreversible adsorption of hydrolytic enzymes and lignin degradation [14].

Pre-treatment techniques using physical, chemical and biological routes have been extensively researched but they often suffer from high energy inputs, high cost, toxic waste generation, partial hemicellulose and cellulose degradation etc [15-18]. The main function of the pre-treatment step is to reduce biomass recalcitrance and enhance subsequent carbohydrate conversion. Unfortunately, the pre-treatment techniques currently available only partially satisfy these criteria [19].

Ozone assisted pre-treatment has recently gained attention due to its high reactivity at ambient temperature and pressure without decomposing the cellulose and hemicellulose fractions. In addition, ozone is generated in-situ and can be easily broken down therefore eliminating further high cost

separation/purification steps resulting in a cost effective and environmentally friendly process [20]. However, ozone cannot fractionate the biomass. In contrast, organosolv pre-treatment (heating of biomass in an aqueous organic solvent such as acetic acid, ethanol, formic acid at a temperature range of 100 – 250°C and pressure of 0.1 – 2.5 MPa) results in the separation of biomass into cellulose and lignin rich solid fractions together with an aqueous hemicellulose fraction. Organosolv offers efficient fractionation but with limited breakdown (as it only separates the biomass into components without disrupting the cellulose structure), therefore making cellulose difficult for utilization in downstream processing [19,21]. Ionic liquid, which is an alternative to organosolv fractionation, is used at elevated temperature to dissolve the biomass and components recovered by precipitation via the addition of an antisolvent. Despite its advantages in biomass fractionation, it poses problems such as: costly ionic liquids, changes in lignin structure after precipitation, high energy needed to recycle ionic liquids and high viscosity of the solution which makes it difficult to handle [2].

To date, ozone pre-treatment has largely focused on the conversion of the cellulose and hemicellulose fractions to biofuels, leaving lignin as a residue. However, only using hemicellulose/cellulose for ethanol production is not economic due to high energy cost for the production of ozone [20]. Therefore, integrating lignin valorisation with biofuel production is needed to increase profitability and sustainability of the bio-refinery process.

Corn cob represents an abundant agricultural processing residue which has attracted considerable attention in recent years for the production of cellulosic ethanol as it is obtained from the most abundant (850 million tons annually) grain crop globally [22]. It is used in this study to represent a low lignin feedstock with high moisture content while spelt (an ancient grain) is attracting considerable interest for improved human nutrition and health [23] and the residual protective husk represents a medium lignin feedstock with low moisture content.

The effect of ozone on pre-treated agricultural residues was examined, the pre-treated residues were subjected to organosolv fractionation into individual components with their recovery yield as well as purity assessed. The structure and composition of the resultant lignin and cellulose were evaluated for its potential use in downstream chemical production.

2. Materials and methods

2.1 Materials

Corn cobs were provided by Barfoots of Botley Ltd, UK and were harvested at stage R3 (milk stage) of maturity across a range of countries i.e. United States of America, South Africa, United Kingdom,

France, Spain, Morocco, Germany, Greece and Senegal (thereby representing a typical agricultural processing residue) followed by storage at 0-5°C for 1–25 days. Waste corn cobs were sent to Newcastle University and stored in a cold room at 6°C prior to use. The cobs were cut into pieces < 5mm and blended (HGBTWTS3 laboratory blender 8010 ES) for 3 minutes to obtain a particle size of ~2.0mm (which was the minimum particle size achievable due to the high moisture content of fresh cobs (i.e. 72%).

Spelt husks obtained from Gilchesters Organics (Stamfordham, United Kingdom) were ground (HGBTWTS3 laboratory blender), sieved to a particle size of 2.0mm and stored at room temperature prior to use.

2.2 Feedstock analysis

Lignin analysis

Acid insoluble lignin (AIL) was determined whereby approximately 1g of extracted sample was weighed and treated with 10mL 72% (w/v) sulphuric acid for 2h at 30°C with constant stirring. After complete disintegration, the solution was transferred to a 1L round bottom flask, diluted with 3% sulphuric acid solution and refluxed for 4h. The acid insoluble lignin was filtered and washed to neutrality (pH 7) with hot water, dried at 105°C and gravimetrically determined, prior to ignition at 850°C for 45 minutes to obtain lignin ash. The lignin ash was subtracted from the lignin to give the % of ash-free lignin according to the method developed for the pulp and paper industry (TAI T-222) while the acid soluble lignin (ASL) was determined from the filtrate according to TAPPI method UM 250 [24], 1mL of filtrate (from sulfuric acid hydrolysis) was placed in a UV-vis spectrophotometer to measure absorbance at 205nm. Acid soluble lignin was calculated using the following formula.

$$ASL = \frac{Abs \times volume \times Dilution}{a \times W \times pathlength} \times 1000 \text{ mg/g.}$$

Where Abs = absorbance at 205nm,

volume = volume of filtrate (l)

a= molar absorptivity of lignin at 205 nm (110 Lg⁻¹cm⁻¹),

W= weight of sample (g)

Pathlength (cm)

Holocellulose (hemicellulose and cellulose) determination

Around 5g of the ethanol/toluene (2:1) extracted sample was suspended in 150mL of distilled water at 75°C at a stirring speed of 170 rpm followed by the addition of 10 drops of glacial acetic acid and 1.5g of sodium chloride. After 1h, the same amount of reagent was again added, and the reaction was continued for a further 3h. The solution was left to cool, filtered and washed with distilled water until neutrality (pH 7), washed with acetone and dried at 105°C to a constant weight. The holocellulose was estimated gravimetrically.

Cellulose content

Cellulose content was estimated gravimetrically after drying at 105°C to constant weight following extraction with 17.5 % sodium hydroxide according to the method of Wise *et. al.* [25]. The composition of the agricultural residues used are presented in Table 1; the moisture content of the corn cob and spelt husk was 72.1% and 11.2% respectively with other components i.e. extractives, lipids and proteins contributing to the rest of the mass.

Table 1. Chemical composition (%) of raw corn cob and spelt husk feedstocks expressed on a dry weight basis

PROPERTIES	CORN COB	SPELT HUSK
Acid insoluble lignin (AIL)	5.1 ± 0.3	16.03 ± 0.1
Acid soluble lignin (ASL)	2.98 ± 0.49	3.27 ± 0.16
Cellulose	43.2 ± 0.2	31.8 ± 0.5
Hemicellulose	37.8 ± 0.2	29.1 ± 0.1
Ash	3.2 ± 0.01	8.4 ± 0.04

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proved to be sufficient for optimum lignin separation from biomass [26 - 29]. Moisture content of corn cob was varied from 72. % to 42.9% by drying down at the feedstock for different times. Whereas moisture content for spelt husk was varied from 11% to 100% (wet basis) by adding water.

The pre-treated materials were dried in the oven at 105°C to a constant weight prior to compositional analysis.

2.4 Biomass Fractionation

About 30g of feedstock (raw or pre-treated) was weighed into 250mL round bottom flask and 50mL of toluene added and refluxed for 2h to remove fatty acids. The mixture was cooled, filtered and the residue dried overnight. The residue (toluene extracted) was subjected to mild (70-80°C) ethanol extraction by weighing 12.5g of dried residue into a 250mL round bottom flask, 100mL of ethanol:water (60:40) was added to obtain a solid-liquid ratio of 1:8 which corresponds to optimum conditions needed for efficient carbohydrate and lignin recovery [30]. Thereafter 2mL of 37% HCl was added to enhance the hydrolysis of hemicellulose. The mixture was refluxed for 1- 4h, cooled, filtered and washed 4 times with 25mL ethanol to remove any lignin present. The residue (cellulose pulp) was dried while the solvent from the filtrate (lignin and hemicellulose) was evaporated at 40°C and 150 mbar using a rotary evaporator. 15mL of acetone was added to dissolve the resultant solid and lignin precipitated by adding 100mL of water leaving mainly the hydrolysed hemicellulose (xylose and arabinose) and lignin degradation products as water soluble fractions. The mixture was filtered, washed 4 times with 25mL of water and the lignin residue dried [31].

2.5 Analytical methods

The fractionated cellulose, hemicellulose, lignin was characterised, and the purity of extracted lignin was determined as described previously. Water soluble fractions were measured from the lignin hydrolysate (filtrate) by HPLC using an Aminex HPX-87H column at 65°C with a RI detector at 0.6mL/min flow rate for 30min and 0.005M H₂SO₄ as mobile phase. Ash content was determined gravimetrically by heating the biomass at 550°C for 3h.

The morphology of untreated and pre-treated feedstocks was examined via Scanning electron microscope (SEM) using a TM3030 Hitachi microscope. A sample was mounted on a conductive carbon tape prior to imaging. X-ray diffraction (XRD) analysis was carried out using an 18 KW D/Max-2500PC diffractometer at a 2 θ range of 5°- 45° with a scan speed of 0.6° 2 θ per minute. The crystallinity index (CI) was calculated using peak height [32].

¹H NMR spectra of lignin was generated using a Bruker Avance – 300 spectrometer operating at a frequency of 300 MHz using 50mg of fractionated lignin dissolved in dimethyl sulfoxide (DMSO) d₆ (0.5mL).

Thermogravimetric (TGA) analysis was done using Netzsch TG 209 thermogravimetric analyser with ~10mg of sample heated from 25°C to 800°C at a rate of 10°C min⁻¹ in an atmosphere of helium (flow rate 20 mL min⁻¹).

Degree of polymerisation of untreated and pre-treated cellulose was analysed using Matrix- assisted laser desorption/ionization time of flight (MALDI-TOF) according to methods described by Kosyakova *et. al.*, [33] using DMF as solvent and α -cyano-4-hydroxycinnamic acid as matrix.

3. Results and Discussion

3.1 Effect of pre-treatment on biomass properties and lignin recovery

Including ultrasound in the ozone pre-treatment of corn cobs enhanced the mixing between biomass and ozone, providing good interactions therefore resulting in an increase in ozone consumption from 0.24g O₃ g⁻¹ corn cob to 0.39g O₃ g⁻¹ with a 38.5% decrease in the acid insoluble lignin content from 52 to 32 mg g⁻¹ compared to using ozone only. Ultrasound increased the diffusion of ozone into the feedstock, most likely via the creation of a pressure difference resulting in pressure waves passing through the liquid leading to formation of bubbles. The bubbles grow and then collapse resulting in the re-distribution of molecules around the bubbles to other parts of the feedstock, this results in formation of microjets that penetrates, break cell walls and increase surface area of the substrate [34]. The results in this study are similar to other studies [35 -37] in which the lowest acid insoluble lignin content was achieved by increasing contact between ozone and the feedstock using fixed bed or bubble column reactors. To the best of our knowledge, this is the first study where ultrasound is incorporated with ozone pre-treatment of biomass in a batch reactor to improve surface contact between ozone and the substrate.

From SEM images, it can be seen that untreated corn cob exhibited compact fibrillary morphology (Fig 2i) with intertwined fibres covering the cellulose while ozone pre-treatment alone (Fig 2ii) showed a looser fibrous network and disorganised morphology due to disruption of the cell wall structure. The fibre network following ozone with ultrasound pre-treatment (Fig 2iii) was more collapsed and separated with greater exposure of the inner part (cellulose) thereby accounting for the lower lignin

content. Similar structural changes were observed when sugarcane bagasse was treated with ozone [38,39] and an oxygen-alkali mix [40].

Fig 2. SEM images of corn cob; i) untreated, ii) pre-treated with ozone and iii) pre-treated with ultrasound and ozone. The ozone concentration was 2% (by weight) for 30 min with a 250mL min⁻¹ oxygen flow rate.

Pre-treatment with both ozone and ultrasound for > 30mins had a negligible effect on total lignin (acid insoluble lignin + acid soluble lignin) recovery. From the reaction mechanism, electron deficient ozone can access the electron rich aromatic bonds of lignin as long as ozone can penetrate the biomass which in this case was aided by the use of ultrasound. After this initial reaction the available aromatic bonds decline leading to a small and non-significant decrease in the acid insoluble lignin and increase in acid soluble lignin content. Incorporation of ultrasound in the pre-treatment led to a shorter peak reaction time (30min) compared with 360min for poplar sawdust [36], 150 min for wheat & rye straw [26] and 120 min for sugarcane bagasse [39] when using ozone alone in a fixed bed reactor.

Feedstock moisture content was reported as the most important ozone process parameter [20,26,35,41] because it serves as a medium to aid ozone movement within the feedstock as well as affecting the concentration of ozone and of generated radicals. However, optimum moisture value is material specific. Feedstocks of varying moisture content (corn cobs 42.9%-100% and spelt husk 11%-100%) were pre-treated and showed that acid insoluble lignin decreased (up to 66% for corn cob and 40% for spelt husk) with moisture content but then increased thereafter (Table 2). The acid soluble lignin (ASL) content reported in this study is within range as compared to literature e.g. 3.1-3.2% ASL corn cob [42,43]. Acid soluble lignin (ASL) increased following reaction with ozone likely due to the

insertion of hydrophilic groups from the lignin-carbohydrate complex (LCC) converting acid insoluble lignin (AIL) to acid soluble lignin [26,44]

The highest acid soluble lignin of 44.6mg g⁻¹ and 42.1mg g⁻¹ was achieved at 66% and 40% moisture content for corn cob and spelt husk corresponding to a total lignin recovery of 50.4 and 73.6% respectively (Table 2), the loss in total mass of lignin during pre-treatment is likely due to its oxidation to low molecular weight compounds such as phenolics and carboxylic acids such as acetic acid, oxalic acid, formic acid and glycolic acid (lignin degradation products) [39]. At a moisture content below the saturation point of the biomass (66% and 40% for corn cob and spelt husk respectively), there is a limited mass transfer of ozone which results in reduced reactivity with the substrate. However, at moisture contents above the saturation point, the biomass pores are blocked by a thick film of water leading to large residence time of ozone, therefore more ozone is decomposed without any positive contribution to the pre-treatment [36,41]. The optimum moisture content for increasing the ozone reactivity was feedstock specific, e.g. 40-50% for Japanese Cedar sawdust [45], 160% for Aspen sawdust [46] and 90% for cotton stalk [47]. Therefore, saturation point of biomass/feedstock should be identified when using ozone assisted pre-treatment.

Table 2. Lignin fractions following ultrasound/ozone pre-treatment of spelt husk and corn cob with varying moisture content at an oxygen flow rate of 250mL min⁻¹ and ozone concentration of 2% for 30 mins

Feedstock	Moisture content (%)	Acid insoluble lignin (mg g ⁻¹)	Acid soluble lignin (mg g ⁻¹)	Total lignin (mg g ⁻¹)	Lignin recovery (%)	Glucose (mg g ⁻¹)
Spelt husk	0	160.3 ± 0.1	32.7 ± 0.16	193.0	-	-
	11	84.0 ± 0.44	34.3 ± 0.05	118.3	61.1	1.23
	30	60.2 ± 0.28	38.9 ± 0.78	99.2	51.4	0.85
	40	55.2 ± 0.71	42.1 ± 0.54	97.3	50.4	1.41
	50	58.2 ± 0.88	39.5 ± 0.34	97.7	50.6	1.52
	70	61.6 ± 0.49	37.8 ± 0.29	99.4	51.5	1.17
	80	64.4 ± 0.13	36.9 ± 0.05	101.3	52.5	1.09
	100	70.0 ± 0.32	35.0 ± 0.34	105.0	54.4	1.00

Corn cob	0	51.0 ± 0.3	29.8 ± 0.49	80.8	-	-
	42.9	31.5 ± 0.08	34.8 ± 0.05	66.3	82.1	¹ ND
	51.7	22.2 ± 0.12	38.6 ± 0.41	60.8	75.2	ND
	57.98	15.3 ± 0.67	42.7 ± 0.12	58.0	71.8	ND
	66.02	14.9 ± 0.95	44.6 ± 0.17	59.5	73.6	<0.1
	72.02	16.7 ± 0.10	42.5 ± 0.54	59.2	73.3	ND
	82	21.3 ± 0.16	38.8 ± 0.77	60.1	74.4	ND
	100	35.3 ± 0.3	32.0 ± 0.42	67.3	83.3	ND

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270 Table 2 also shows a low concentration of glucose (<2%) remaining in the wash water with no traces
271 of xylose, HMF and furfural detected indicating that only a small amount of cellulose was hydrolysed
272 during pre-treatment. Therefore, it can be concluded that ozone assisted pre-treatment provides an
273 approach where the cellulose and hemicellulose remain relatively intact [48]. This is one of the unique
274 advantages of ozone pre-treatment over commercial pre-treatment alternatives such as steam
275 explosion and dilute acid (H₂SO₄) where substantial amounts of cellulose and hemicellulose are
276 lost/solubilized in the process due to high temperature and pressure producing inhibitory products
277 such as HMF and furfural which hinders subsequent hydrolysis [49].

278 The SEM images of raw spelt husk (Fig 3Ai) show a more densely packed structure than that of raw
279 corn cob (Fig 3Bi) which has a more loosely packed fibrous structure likely due to a higher lignin
280 content. Following pre-treatment, SEM images of both feedstocks showed a more porous structure
281 with an increased surface area resulting from the breakage of lignin bonds. The images with the widest
282 pores ~15mm (Fig 3A & B (iii)) correspond to the lowest acid insoluble lignin content showing a more
283 open porous structure. A similar behaviour was also shown by Barros [50] for ozone pre-treated
284 sugarcane bagasse and straw.

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¹ ND means not detected

Fig 3. SEM images of; A) spelt husk and B) corn cob following ozone/ultrasound pre-treatment for 30 min at an ozone concentration of 2% mol mol⁻¹ with varying feedstock moisture content: i) raw, ii) 11%, iii) 40% and iv) 50% for spelt husk and i) raw, ii) 42.91%, iii) 66.02% and iv) 100% for corn cob

Usually, after pre-treatment, the pre-treated solid is subjected directly to enzymatic or catalyst hydrolysis for further downstream processing generating a low-quality lignin residue (due to presence of irreversible adsorption of enzymes and fermentation components) separated at the end of these processes [13]. Recovering high quality lignin and converting it into high value chemicals alongside the utilization of cellulose and hemicellulose for biofuel and platform chemical production is of importance from an economic and sustainable perspective. Lignin, composing of aromatic compounds, can be used to produce low cost carbon fibre which can replace steel in the body of automobiles, used as a substitute for polymeric materials such as polyurethane foams, phenolic powder resins and a precursor for green diesel production through hydrogenation [12]. However, to achieve complete utilization of all three structural components of the biomass to value added products, it is necessary to fractionate high quality lignin prior to the hydrolysis stage to prevent irreversible adsorption of hydrolytic enzymes and the breakdown of lignin [14].

Ozone pre-treated feedstock at saturation moisture content (40% for spelt husk and 66.06% for corn cob) containing 55.2 mg g⁻¹ and 14.9mg g⁻¹ of acid insoluble lignin respectively were fractionated with ethanol:water (60:40) at 80°C and yielded 54.1mg g⁻¹ (94%) and 13.2mg g⁻¹ (90%) acid insoluble

lignin and 852mg g⁻¹ and 836mg g⁻¹ cellulose pulp respectively . A small amount of water-soluble hemicellulose (xylose and arabinose) of 1.13% from spelt husk and 1.04% from corn cob was also obtained. The high lignin recovery of >90% for spelt husk and corn cob were higher than the 85% achieved using the ethanol/ organosolv pre-treatment alone for raw corn cob and spelt husk. This higher lignin recovery is likely as a result of increased disruption and opening of the lignocellulose matrix following ozone pre-treatment as the lignin-hemicellulose bonds are already disrupted making it easier to solubilize in the organic solvent at mild temperatures. In addition, the hemicellulose water soluble fraction of ozone pre-treated feedstock was much lower (<2.0%) than that obtained using conventional organosolv for raw corn cob and spelt husk (7-8%). High hemicellulose water soluble fractions (7-10%) as reported with the use of conventional organosolv are likely due to increased hydrolysis of the hemicellulose which occurs at the higher temperatures of 150 – 200°C and pressures in the presence of an acid needed to chemically break the ether bonds in the lignin [19,21]. In the current study increased lignin and cellulose recovery were both obtained at a relatively mild temperature of 80°C.

The purity of the extracted lignin was determined by measuring the amount of sugars arising from cellulose and hemicellulose hydrolysis as well as ash content of the lignin. This is important as purity is the major factor that determines the use of lignin for advanced applications [19]. About 95% pure lignin was achieved for both ozone pre-treated spelt husk and corn cob with ash content and total carbohydrate impurities both below 2%.

3.2 Fractionated lignin

The lignin fractionated from raw spelt husk showed small micron particles agglomerated to form clusters while the lignin following pre-treatment showed a smooth and increased surface area due to collapse of the internal structure as well as breakage of chemical bonds such as β -O-4 and 4-O-5 (Fig S1). Similar changes were observed on switch grass (*Panicum Virgatum*) [51] with a smooth surface for lignin treated with hot compressed water at 180°C compared to loose agglomerated particles of lignin in the raw feedstock. Stewart *et. al.*, [52] also observed small and dense micron particles when lignin from *Salix purpurea* was precipitated using water at 40°C. Fractionated lignin from raw corn cobs showed a loosely packed agglomeration of micron particles compared to that of raw spelt husk; likely due to the lower lignin content of corn cob. Furthermore, decreased ¹H NMR spectral intensity in the aromatic region of ozone pre-treated lignin (Fig 4) was observed compared to lignin in the untreated feedstock, suggesting increased reactivity of ozone with the aromatic components of lignin and is in agreement with other studies [27,53]. ¹H NMR spectra (Fig 4) of corn cob showed that the

lignin consists of H, G and S units of monomers while spelt husk spectra is made up of H and G monomers with no peak in the S region of the spectra. Hence, the type of lignin obtained from corn cob is classified as guaiacyl-syringyl while that from spelt husk is guaiacyl lignin. This is in agreement with Sun⁷ that the unit of monomers that make up a lignin polymer differ in different feedstocks and this influences its suitability for value added product and platform chemical production.

Fig 4. ¹H NMR spectra of ethanol fractionated lignin from a) ozone pre-treated spelt husk, b) untreated spelt husk, c) untreated corn cob and d) ozone pre-treated corn cob.

3.3 Fractionated Cellulose

The raw corn cob and spelt husk feedstocks showed a compact organised and rigid structure of cellulose likely due to strong hydrogen bonding between the glucose monomers [2] (Fig A2). However, the ozone pre-treated cellulose showed a more disorganised surface morphology with a loosening of the structure likely due to partial oxidation/depolymerisation of cellulose as suggested by Mamleeva [41]. This more disorganised structure will likely enhance the solubility of cellulose and aid its conversion into platform chemicals and biofuels [27].

X-ray diffraction (XRD) spectra of the cellulose showed signal peaks at 2θ of 16.5° , 22.1° and 34.4° (Fig 5) corresponding to the lattice plane of cellulose I crystals [54,55]. The cellulose crystallinity index (CI) of raw corn cob (45.5%) was higher than spelt husk (30.2%) due to lower lignin content of corn

cob (5.1%). Ozone pre-treatment led to a decrease in the CI values of corn cob and spelt husk from 45.5% to 36.8% and 30.2% to 28.3% respectively due to increased disorder and amorphization of the cellulose (Fig 5). A similar decrease in the CI of aspen sawdust cellulose was reported as a result of loosened structure and reduced crystal size caused by partial oxidative depolymerization of cellulose by ozone [27].

The thermal stability of the fractionated cellulose from untreated and ozone pre-treated feedstock (Fig 5) showed a rapid loss of mass at a temperature of 350°C likely as a result of cellulose degradation [55,56]. Ozone pre-treated cellulose showed a higher decomposition rate compared to untreated feedstock. A slow degradation of cellulose in the raw feedstocks at temperatures > 300°C could be due to stronger cellulose intermolecular forces, together with the presence of lignin and ash impurities. The peak degradation temperature (350°C) remained the same for both untreated and pre-treated feedstocks which shows that ozone pre-treatment did not affect the thermal stability of cellulose.

Fig 5 (I) XRD and (II) TG and DTG curves of recovered cellulose from untreated and ozone pre-treated a) corn cob and b) spelt husk.

Subsequently, the effect of ozone pre-treatment on degree of polymerization (DP) of cellulose was determined using matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) analysis. The difference in mass between adjacent signals in the spectra was 162 m/z corresponding to one glucosyl unit (C₆H₁₀O₅) [57]. The poly-glucose ions observed in untreated spelt husk and corn cob were up to a m/z of 2812 containing glucosyl monomer with one potassium adduct (Fig A3). In addition, a decrease in intensity was observed at higher m/z, this is because cationisation from adduct formation of bigger molecules are unfavourable making it difficult to reach the mass spectrometer and generate a signal [58]. However, the DP of spelt husk remained unchanged after ozone pre-treatment while that of corn cob decreased by 17% (m/z 2309.712) (Fig S3). This decrease in DP of ozone pre-treated corn cob can be due to the lower content of lignin in the raw feedstock enabling partial oxidation of cellulose. Hubbell *et. al.* [59] also determined the DP of Avicel PH-101 cellulose with varying amounts of lignin after acid chlorite pre-treatment and observed a significant reduction in degree of polymerization of cellulose at lower lignin levels.

3.4 Mechanism of organosolv and ozone reactions

3.4.1 Organosolv fractionation

Conventional organosolv fractionation of biomass occurs by breakdown of ether bonds in lignin to fragments small enough to dissolve in the organic solvent. α-ether bonds are the main linkages broken as these are weaker than the β-ether linkages. β-ether linkages cleavage only occurs in a highly acidic medium (>1M conc) and at high temperatures (>150°C) [60].

Two consecutive processes occur during organosolv fractionation, the first process involves splitting of lignin to smaller fragments. This can occur through hydrolysis at the benzylic position of α-ether bonds in the presence of an acid through the formation of an intermediate (quinone methide) to form an S_N2 nucleophilic substituted compound (R1) or direct splitting of the ether bonds forming a reactive stabilized carbocation (R2) which can undergo unwanted condensation reactions with other molecules to form compounds containing a single carbon bond.

(R1)

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(R2)

418 The second process involves hydrolysis of lignin-carbohydrate ether linkages (glucosidic bonds) by
 419 acid to separate the lignin and carbohydrate fractions (R 3). This fractionated lignin solubilises in the
 420 solvent/mixture of solvents, yielding a cellulose rich solid fraction and a liquid fraction containing lignin
 421 fragments and hydrolysed carbohydrates [60].

(R3)

426 3.4.2 Ozone treatment mechanism

427 Ozone reacts with biomass in 3 consecutive stages; firstly, ozone solubilization in water where ozone
 428 bonds with water in the biomass. The formation of reactive species mainly ozone and hydroxyl radicals
 429 occur depending on the reaction conditions, but other radicals such as hydrogen peroxide can also be
 430 generated. Secondly, ozone selectively reacts with aromatic bonds (carbon double bonds) due to their
 431 high electron density leading to opening of the lignin structure. Ozone reacts with aromatic bonds
 432 through hydroxylation of the aromatic ring after an initial electrophilic attack. Followed by 1,3-
 433 cycloaddition to open olefinic double bonds (R4) by the Criegee mechanism [61].

434 Finally, ozone reacts with the carbon double bonds through 1,3-dipolar cycloaddition to produce the
 435 1,2,3-trioxolane intermediate (R4) which then decomposes to *syn* and *anti* isomers of carbonyl oxides
 436 (Zwitterions) and a carbonyl compound (R5).

437

(R4)

438

(R5)

Depending on reaction conditions, a number of different products can be formed. The *syn* and *anti* isomers of zwitterions can combine with carbonyl compounds to form ozonide (R6). Alternatively, the *syn* and *anti* isomers of zwitterions can react with solvents such as water to form other carbonyl compounds liberating hydrogen peroxide (R7) or can polymerize to form diperoxides or polymeric peroxides (R8).

Depending on reaction conditions, resulting radicals (such as OH, H), peroxide and ozone may react with lignin degradation products to form low molecular weight fragments such as HCOOH, although the reaction mechanism between ozone and lignin is not completely understood [62]. Apart from reacting with the carbon double bonds of the lignin aromatics, ozone undergoes reactions with carbon-hydrogen and carbon - oxygen bonds leading to cleavage of β -O-4 bonds and methoxyl groups of the lignin polymer [63]. The reaction between ozone and carbon double bonds occurs at a very fast rate leading to faster lignin degradation and high initial ozone consumption. Carbohydrates (carbon single bonds) are more resistant to ozone and their reaction is 10^6 times slower than lignin [20]. Ozone reacts directly with carbohydrates to form carboxyl groups or reacts with water to generate hydroxyl radicals, which then react with the carbohydrate components leading to unsystematic cleavage of the glycosidic bonds with the production of acetic, formic, oxalic, glycolic and other dicarboxylic acids.

Therefore, ozone pre-treatment prior to organosolv pre-treatment leads to disruption and opening of the biomass structure which makes it easier for the hydrolysis and breakage of the lignin-carbohydrates bonds. These bonds weaken during pre-treatment and increased solubilisation of lignin

at mild temperatures and atmospheric pressure leading to a high percentage of fractionated lignin, cellulose and hemicellulose recovery while for organosolv alone, higher extraction temperatures (120°C - 180°C) and pressure of about 2MPa are needed to accelerate lignin-lignin bond (ether bond) as well as lignin-carbohydrate bond (glycosidic bonds) breakage and solubilisation. These conditions (high temperature and pressure) cause increased hydrolysis of the hemicellulose and cellulose fractions leading to a reduced recovery.

Overall, it is known that the inclusion of ultrasound in a process is energy intensive, however in this study the benefits it provides such as faster pre-treatment time, improved separation of lignin and ozone consumption is worth considering as well as high quality product streams obtained from this study will lead to complete utilization of all biomass components to value added product minimizing waste from the process thus increasing the profitability and sustainability of the process. In addition, an energy efficient cold plasma ozone generator was used for ozone generation in this study, its combination with ultrasound will significantly reduce the overall cost and energy needed for the process.

4. Conclusion

Incorporation of ultrasound with ozone pre-treatment of biomass improves the mass transfer of ozone leading to faster ozonation and improved consumption. Ultrasound assisted ozone pre-treatment also led to a decrease in crystallinity, degree of polymerization and a loosened/collapsed cellulose structure. This enhances the reactivity and solubility of the cellulose for chemical/enzymatic hydrolysis to produce cellulosic ethanol or useful platform chemicals. Overall, ozone and ultrasound pre-treatment increased the recovery of both lignin and cellulose with the potential to increase the efficiency and economics of platform chemical (furfural, HMF) and cellulosic ethanol production.

“E-supplementary data of this work can be found in online version of the paper”

Conflict of interest

There are no conflicts to declare.

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References

- [1] J.J. Bozell, Connecting Biomass and Petroleum Processing with a Chemical Bridge. *Sci.* 329(5991) (2010) 522-523.
- [2] S. Kumar, K. Paritosh, N. Pareek, A. Chawade and V. Vivekanand, De-construction of major Indian cereal crop residues through chemical pretreatment for improved biogas production: An overview. *Renew. and Sust. Energy Rev.* 90 (2018) 160-170.
- [3] N. Tripathi, C.D. Hills, R.S. Singh and C.J. Atkinson, Biomass waste utilisation in low-carbon products: harnessing a major potential resource. *J. Clim. and Atm. Sci.* 2(1) (2019) 35.
- [4] B. Satari, K. Karimi and R. Kumar, Cellulose solvent based pre-treatment for enhanced second generation biofuel production: a review. *Sust. Energy & fuels*, 3 (2019) 11-62.
- [5] S. Haghighi Mood, A. Hossein Golfeshan, M. Tabatabaei, G. Salehi Jouzani, G.H. Najafi, M. Gholami and M. Ardjmand, Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renew. and Sustainable Energy Rev.* 27 (2013) 77-93.
- [6] U. Brémond, R. de Buyer, J.P. Steyer, N. Bernet and H. Carrere, Biological pretreatments of biomass for improving biogas production: an overview from lab scale to full-scale. *Renew. and Sust. Energy Rev.* 90 (2018) 583-604.
- [7] M. B. Figueiredo, H. J. Heeres and P.J. Deuss, Ozone mediated depolymerization and solvolysis of technical lignin under ambient conditions in ethanol. *Sust. Energy & Fuels* 4 (2020) 265 - 276.
- [8] X. Zhao, Z. Huang, Y. Zhang, M. Yang, D.Chen, K. Huang, H. Hu, A. Huang, X. Qin and Z. Feng, Efficient solid-phase synthesis of acetylated lignin and a comparison of the properties of different modified lignins. *J. of A. Polymer Sci.* 134(1) (2017) 44276.
- [9] M. Balakshin, E. Capanema, H. Gracz, H. M. Chang and H. Jameel, Quantification of lignin–carbohydrate linkages with high-resolution NMR spectroscopy. *Planta.* 233(6) (2011) 1097-1110.
- [10] A.G. Vishtal and A. Kraslawski, Challenges in industrial applications of technical lignins. *Bioresour. Technol.* 6(3) (2011) 3547 – 3568.
- [11] L. Zhang, G. Xi, Z. Chen, Z. Qi and Wang, Enhanced formation of 5-HMF from glucose using a highly selective and stable SAPO-34 catalyst. *Chem. Eng. J.* 307 (2017) 877-883.

- 524 [12] T.Q. Yuan, F. Xu, and R. C. Sun, Role of lignin in a biorefinery: separation characterization
525 and valorization. *J. of Chem. Technol. & Biotechnol.* 88(3) (2013) 346-352.
- 526 [13] A. J. Ragauskas, G.T. Beckham, M.J. Biddy, R. Chandra, F. Chen, M.F. Davis, B.H. Davison,
527 R.A. Dixon, P. Gilna, M. Keller, P. Langan, A.K. Naskar, J.N. Saddler, T. J. Tschaplinski, G.A
528 Tuskan and C.E. Wyman, Lignin valorization: Improving lignin processing in the biorefinery.
529 *Sci.* 344(6185) (2014) 1246843.
- 530 [14] J.K. Saini, A.K. Patel, M. Adsul and R.R. Singhanian, Cellulase adsorption on lignin: A
531 roadblock for economic hydrolysis of biomass. *Renew. Energy.* 98 (2016) 29-42.
- 532 [15] R.P. Chandra, R. Bura, W.E. Mabey, A. Berlin, X. Pan and J.N. Saddler, Substrate
533 pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics. *Biotechnol.*
534 *Bioeng.* 108 (2007) 67-93.
- 535 [16] S. Behera, R. Arora, N. Nandhagopal and S. Kumar, Importance of chemical pretreatment
536 for bioconversion of lignocellulosic biomass. *Renew. and Sust. Energy Rev.* 36 (2014) 91-
537 106.
- 538 [17] Y. Zheng, J. Zhao, F. Xu and Y. Li, Pretreatment of lignocellulosic biomass for enhanced
539 biogas production. *Prog. in Energy and Combust. Sci.* 42(1) (2014) 35-53.
- 540 [18] A.A. Elgharbawy, M.Z. Alam, M. Moniruzzaman, and M. Goto, Ionic liquid pretreatment as
541 emerging approaches for enhanced enzymatic hydrolysis of lignocellulosic biomass. *J. Biochem.*
542 *Eng.* 109 (2016) 252-267.
- 543 [19] L. Matsakas, C. Nitsos, V. Raghavendran, O. Yakimenko, G. Persson, E. Olsson, U. Rova,
544 L. Olsson and P. Christakopoulos, A novel hybrid organosolv: steam explosion method for
545 the efficient fractionation and pretreatment of birch biomass. *Biotechnol. for Biofuels*, 11(1)
546 (2018) 160.
- 547 [20] R. Travaini, J. Martín-Juárez, A. Lorenzo-Hernando and S. Bolado-Rodríguez, Ozonolysis:
548 An advantageous pretreatment for lignocellulosic biomass revisited. *Bioresour. Technol.* 199
549 (2016) 2-12.
- 550 [21] S.J.B. Duff and W.D. Murray, Bioconversion of forest products industry waste cellulose to
551 fuel ethanol: A review. *Bioresour. Technol.* 55(1) (1996) 1-33.

552 [22] S. Wang, W. Gao, L. Xiao, J. Shi, R. Sun and G. Song, Hydrogenolysis of biorefinery corn
553 cob lignin into aromatic phenols over activated carbon-suported nickel. *Sust. Energy & Fuels*.
554 3 (2019) 401 - 408.

555 [23] G. Bonafaccia, V. Galli, R. Francisci, V. Mair, V. Skrabanja and I. Kreft, Characteristics of
556 spelt wheat products and nutritional value of spelt wheat-based bread. *Food Chem.* 68(4)
557 (2000) 437-441.

558 [24] M.Saha and V.S. Moholkar, Dioxane-based extraction process for production of high quality
559 lignin. *Bioresour. Technol. Reports* 5 (2019) 206 – 211.

560 [25] L.E. Wise, M. Murphy and A.A. D’addieco, TAPPI. *Paper trade j.* (1946)11-19.

561 [26] M.T. García-Cubero, G. González-Benito, I. Indacoechea, M. Coca and S. Bolado, Effect of
562 ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. *Bioresour. Technol.*
563 100(4) (2009) 1608-1613.

564 [27] E.M. Ben’ko, O.R. Manisova, G.P. Murav’eva and V.V. Lunin, Structural changes in wood
565 during ozonation. *J. of Phy. Chem.* 87(7) (2013) 1097-1101.

566 [28] A. Panneerselvam, R.R. Sharma-Shivaa, P. Kolar, T. Ranney and S. Peretti, Potential of
567 ozonolysis as a pretreatment for energy grasses. *Bioresour. Technol.* 148 (2013) 242-248.

568 [29] C. Li, L. Wang, Z. Chen, Y. Li, R. Wang, X. Luo, G. Cai, Y. Li, Q. Yu and J. Lu, Ozonolysis
569 pretreatment of maize stover: The interactive effect of sample particle size and moisture on
570 ozonolysis process. *Bioresour. Technol.* 183 (2015) 240-247.

571 [30] K. Zhang, Z. Pei and D. Wang, Organic solvent pretreatment of lignocellulosic biomass for
572 biofuels and biochemicals: A review. *Bioresour. Technol.* 199 (2016) 21-33.

573 [31] D.S. Au - Zijlstra, A. de Santi, B. Oldenburger, J. de Vries, K. Barta and P.J. Deuss,
574 Extraction of Lignin with High β -O-4 Content by Mild Ethanol Extraction and Its Effect on the
575 Depolymerization Yield. *J. Vis. Exp.* 143 (2019) 58575.

576 [32] L. Segal, J.J. Creely, A.E. Martin and C.M. Conrad, An Empirical Method for Estimating the
577 Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. *Text. Res. J.*
578 29(10) (1959) 786-794.

579 [33] D.S. Kosyakov, N. V. Ul’yanovskii, E.A. Sorokina and N.S. Gorbova, Optimization of sample
580 preparation conditions in the study of lignin by maldi mass spectrometry. *J. Analy. Chem.* 69
581 (2014) 1344-1350.

- 582 [34] M.J. Bussemaker and D. Zhang, Effect of ultrasound on Lignocellulosic Biomass as a
583 Pretreatment for Biorefinery and Biofuel Applications. *Industrial & Eng. Chem. Res.* 52(10)
584 (2013) 3563-3580.
- 585 [35] W.C. Neely, Factors affecting the pretreatment of biomass with gaseous ozone. *Biotechnol.*
586 *and Bioeng.* 26(1) (1984) 59-65.
- 587 [36] P.F. Vidal, and J. Molinier, Ozonolysis of lignin - Improvement of in vitro digestibility of poplar
588 sawdust. *Biomass.* 16(1) (1988) 1-17.
- 589 [37] A. Cesaro and V. Belgiorno, Sonolysis and ozonation as pretreatment for anaerobic digestion
590 of solid organic waste. *Ultrasonics Sonochem.* 20(3) (2013) 931-936.
- 591 [38] J.A. Souza-Corrêa, C. Oliveira, L.D. Wolf, V.M. Nascimento, G.J.M. Rocha and J. Amorim,
592 Atmospheric pressure plasma pretreatment of sugarcane bagasse: The influence of moisture
593 in the ozonation process. *A. Biochem. and Biotechnol.* 171(1) (2013a) 104-116.
- 594 [39] R. Travaini, M.D.M. Otero, M. Coca, R. Da-Silva and S. Bolado, Sugarcane bagasse
595 ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation.
596 *Bioresour. Technol.* 133 (2013) 332-339.
- 597 [40] S. Bi, L. Peng, K. Chen and Z. Zhu, Enhanced enzymatic saccharification of sugarcane
598 bagasse pretreated by combining O₂ and NaOH. *Bioresour. Technol.* 214 (2016) 692-699.
- 599 [41] N.A. Mamleeva, S.A. Autlov, N.G. Bazarnova and V.V. Lunin, Delignification of softwood by
600 ozonation. *Pure and A. Chem.* 81(11) (2009) 2081-2091.
- 601 [42] F. Yao, F. Shen, X. Wan and C. Hu, High yield and high concentration glucose production
602 from corn cob residues after tetrahydrofuran + H₂O co-solvent pre-treatment and followed by
603 enzymatic hydrolysis. *Renew. and sust. energy reviews*, 132(2020) 1101107.
- 604 [43] A. Procentese, E. Johnson, V. Orr, A. G. Campanile, J. A. Wood, A. Marzocchella and L.
605 Rehmann, Deep eutectic solvent pre-treatment and subsequent saccharification of corn cob.
606 *Bioresour. Technol.* 192 (2015) 31-36.
- 607 [44] Z. Yu, H. Jameel, H.M. Chang and S. Park, The effect of delignification of forest biomass on
608 enzymatic hydrolysis. *Bioresour. Technol.* 102(19) (2011) 9083-9089.
- 609 [45] T. Sugimoto, K. Magara, S. Hosoya, S. Oosawa, T. Shimoda and K. Nishibori, Ozone
610 pretreatment of lignocellulosic materials for ethanol production: Improvement of enzymatic
611 susceptibility of softwood. *Holzforschung.* 63(5) (2009) 537-543.

- [46] L. Matsakas, V. Raghavendran, O. Yakimenko, G. Persson, E. Olsson, U. Rova, L. Olsson and P. Christakopoulos, Lignin-first biomass fractionation using a hybrid organosolv – Steam explosion pretreatment technology improves the saccharification and fermentability of spruce biomass. *Bioresour. Technol.* 273 (2019) 521-528.
- [47] R.A. Silverstein, Y. Chen, R.R. Sharma-Shivaa, M.D. Boyette and J.A. Osborne, comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresour. Technol.* 98(16) (2007) 3000-3011.
- [48] N. Schultz – jensen, A. Thygesen, F. Leipold, T.S. Thomsen, C. Roslander, H. Lilholt and A.B. Bjerre, Pretreatment of macroalgae chaetomorpha linum for the production of bioethanol – comparison of five pretreatment technologies. *Bioresour. Technol.* 140 (2013) 36 -42.
- [49] I.A. Nges, C. Li, B. Wang, L. Xiao, Z. Yi and J. Liu, Physio-chemical pretreatments for improved methane potential of *Miscanthus lutarioriparius*. *Fuel*. 166 (2016) 29-35.
- [50] R. Barros, S. Paredes, T. Endo, S. Bon and S.H. Lee, Association of wet disk milling and ozonolysis as pretreatment for enzymatic saccharification of sugarcane bagasse and straw. *Bioresour. Technol.* 136, (2013) 288-294.
- [51] J. Long, Y. Xu, T. Wang, R. Shu, Q. Zhang, X. Zhang, J. Fu and L. Ma, *Hydrothermal Depolymerization of Lignin: Understanding the Structural Evolution*. *Bioresour. Technol.* 9(4) (2014) 7162-7175.
- [52] H. Stewart, M. Golding, L. Matia-Merino, R. Archer and C. Davies, Manufacture of lignin microparticles by anti-solvent precipitation: Effect of preparation temperature and presence of sodium dodecyl sulfate. *Food Res. International*. 66 (2014) 93-99.
- [53] M.V. Bule, A.H. Gao, B. Hiscox and S. Chen, Structural modification of lignin and characterization of pretreated wheat straw by ozonation. *J. Agric. and Food Chem.* 61(16) (2013) 3916-3925.
- [54] S. Park, J.O. Baker, M.E. Himmel, P.A. Parilla and D.K. Johnson, Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotech. for Biofuels*, 3(1) (2010) 10.
- [55] Y. Liu, J. Xie, N. Wu, Y. Ma, C. Menon and J. Tong, Characterization of natural cellulose fiber from corn stalk waste subjected to different surface treatments. *Cellulose*. 26(8) (2019) 4707-4719.

- 642 [56] K. Cheng, W. T. Winter and A.J. Stipanovic, A modulated-TGA approach to the kinetics of
 643 lignocellulosic biomass pyrolysis/combustion. *Polym. Degrad. and Stab.* 97(9) (2012) 1606-
 644 1615.
- 645 [57] L. Yin, H. Lin and Z. Xiao., Purification and characterization of a cellulase from bacillus subtilis
 646 YJ1. *J. Marine Sci. and Technol.* 18(3) (2010) 466-471.
- 647 [58] C. Brasseur, J. Bauwens, C. Tarayre, C. Matteotti, P. Thonart, J. Destain, F. Francis, E.
 648 Haubruge, D. Portetelle, M. Vandenboi, J. Focant and E. Pauw, Maldi-tof MS analysis of
 649 cellodextrins and xylo-oligosaccharides produced by hindgut homogenates of reticulitermes
 650 santonesis. *Molecules.* 19(4) (2014) 4578 – 4594.
- 651 [59] C.A. Hubbell and J. A. Ragauskas, Effect of acid-chlorite delignification on cellulose degree
 652 of polymerization. *Bioresour. Technol.* 101(19) (2010) 7410 – 7415.
- 653 [60] T.J. McDonough, The chemistry of organosolv delignification. *Inst. Of paper sci. and*
 654 *technol. Atlanta* 1992, 1-15.
- 655 [61] R. Criegee, Mechanism of Ozonolysis. *Angewandte Chemie International Edition in English.*
 656 14(11) (1975) 745-752.
- 657 [62] J.A. Souza-Corrêa, M.A. Ridenti, C. Oliveira, S.R. Araújo and J. Amorim, Decomposition of
 658 lignin from sugar cane bagasse during ozonation process monitored by optical and mass
 659 spectrometries. *J. Phy. Chem.* 117(11) (2013b) 3110-3119.
- 660 [63] M. Ragnar, T. Eriksson and T. Reitberger, Radical Formation in Ozone Reactions with Lignin
 661 and Carbohydrate Model Compounds. *Holzforschung.* 53 (1999) 292 3. Available at:
 662 <https://www.degruyter.com/view/j/hfsg.1999.53.issue-3/hf.1999.049/hf.1999.049.xml>
 663 (Accessed: 2019-11-05)

Supplementary data

Fig A1. SEM images of fractionated lignin at 80°C with 1% HCl catalyst for 120min for: a) untreated, b) ozone pre-treated, spelt husk c) untreated and d) ozone pre-treated corn cob.

Fig A2. SEM of fractionated cellulose at 80°C, with 1% HCl for 120min from: a) untreated b) ozone pre-treated corn cob c) untreated spelt husk d) ozone pre-treated spelt husk.