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Microwave assisted pretreatment of eucalyptus sawdust enhances enzymatic saccharification and maximizes fermentable sugar yield

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ABSTRACT

There is an urgent need of sustainable and efficient methods for the production of biofuels and chemicals from lignocellulosic feedstock. The purpose of this study was to develop a mild, cost-effective and environmentally benign pretreatment for woody lignocellulose to maximize sugar yield via enzymatic saccharification. Microwave irradiation (MW) of *Eucalyptus regnans* sawdust in water was investigated and compared directly against conventional liquid hot water (LHW) pretreatment. Following 30 min microwave irradiation at 180 °C, the sugar yield was 3.5 times higher using MW than LHW pretreatment under the same conditions. Complete release of C_5 and C_6 sugars was achieved after the two-step method of MW pretreatment followed by enzymatic hydrolysis, compared with only 4% without pretreatment and 31% after LHW. Removal of 'lignin droplets' formed on the surface of the pretreated fibers via flowing hot water showed only minor improvement in the yield of enzymatic saccharification. Our results support the hypothesis that lignin prevents access of enzymes rather than inhibits their activity. MW accelerated depolymerization of hemicellulose, opening the structure more than LHW pretreatment.

1. Introduction

The production of fuels and fine chemicals from biomass may help mitigate anthropogenic greenhouse gas emissions and fulfil our demand for energy and materials [1]. Sustainably sourced lignocellulosic biomass, composed of the aromatic polymer lignin, and polymers of C_5 and C_6 sugars, hemicellulose and cellulose, is a widely available and relatively inexpensive feedstock at $50 per ton [2–4]. Thus, the processing of lignocellulosic biomass is of benefit across multiple industries and a green alternative to greenhouse gas emitting sources. Pretreatment is essential to open up the complex lignocellulose structure and allow access to the sugar polymers for cellulolytic enzymes [5]. An impeding factor for lignocellulose pretreatment is its recalcitrance demands a high energy input. One such example is in the lignocellulosic biofuels process, where sugar release, including pretreatment and enzyme digestion, can contribute up to 45% of the total costs [6] and is one of the main factors hindering its full commercialization [7]. Agricultural residues such as corn stover, wheat straw and bagasse have been widely investigated and some are currently used as feedstocks for lignocellulosic ethanol production [8–10]. Forestry species, in particular, eucalypts, are cultivated world-wide but have not been widely considered, due in part to their recalcitrance.

While many pretreatment approaches including hydrothermal/chemical, physical and biological have been studied for biomass, a step change in energy-efficiency and green approach is needed to improve the attractiveness of woody lignocellulose as a feedstock. The more common pretreatment technologies for lignocellulosic biomass include steam explosion (acid catalyzed/uncatalyzed) and liquid hot water (LHW) [11]. While effective, these processes generally need large energy input for heating. Water is an ideal medium for lignocellulose pretreatment due to its low cost, relative simplicity of product and by-product formation and its use avoids the need for corrective action downstream. However, when water alone is used for pretreatment, as in the LHW process, it is not as effective in comparison to acid-catalyzed steam explosion [12]. Variations of the standard LHW pretreatment method have been reported to be more effective at releasing both hemicellulose and cellulose-derived sugars following enzyme treatment of woody feedstocks but these have required two sequential LHW steps [13], increasing the energy requirement.

Microwave irradiation is energy efficient as it directly heats substances via dipole polarization and ionic conduction, as opposed

Abbreviations: MW, Microwave; LHW, Liquid Hot Water; DL, Delignification; ESI, Electronic Supplementary Information.

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to convection and conduction mechanisms that transfer heat in conventional processes [14]. Industrial microwave processing has great potential and been already applied to and become successful in the food and rubber industries [15]. The main benefits of microwave processing are the rapid and uniform volumetric heating of polar materials and energy efficient processing [15,16]. In the near future, with the expected growing demand for green energy sources combined with the requirement for more energy efficient processes, alternative treatments such as microwave heating will play a crucial role. To date, most research into microwave treatment of biomass has been undertaken on agricultural by-products and grasses, converting them into polysaccharides and lignin in the presence of acids, alkali, salts, ionic liquids and other high boiling point solvents [17] as substrates for further hydrolysis and conversion into chemicals and fuels [18]. A recent publication by Aguilar-Reynosa et al. showed that the microwave pretreatment of corn residues in water resulted in marginal increase in the rate of saccharification and fermentation but achieved similar yield compared with conduction-convection heating at similar energy intensity and enzyme loadings [19]. However, woody biomass is significantly more recalcitrant due to higher lignin content and hence direct comparison of microwave pretreatment of woody biomass in water-only medium against conventional heating is necessary.

Here we report the effectiveness of pretreatment of *Eucalyptus regnans* in water only, comparing microwave irradiation with conventional heating methods under identical reaction conditions. To our knowledge, this is the first detailed study to investigate non-thermal effects of microwave pretreatment of woody biomass in water and demonstrates that microwave heating is significantly more effective than LHW. In microwave pretreatment, hemicellulose was hydrolyzed by up to 90% and lignin droplets were formed on the surface of the fibers. Pretreated biomass was further treated with flowing hot water to remove the lignin droplets, which marginally improved the enzymatic sugar yield. Following lignin droplet removal, which accounted for ~60% of the lignin, complete sugar release was achieved for the microwave pre-treated samples after enzymatic hydrolysis. The same result was achieved for microwave pre-treated samples only (without lignin droplet removal). This was over 3 times higher yield than enzymatic hydrolysis of LHW pretreated samples. These results suggest that microwave pre-treated biomass samples are sufficiently disrupted to allow enzyme access and efficient sugar release without the need for lignin droplet removal.

2. Materials and methods

2.1. Materials

Eucalyptus regnans sawdust (average $D_p = 0.5$ mm, Pollards Sawdust, Australia) was used for all experiments. The moisture content of the sawdust was determined according to standard method AS/NZS 1800; in brief, 10.0 g of the sawdust was weighed in an analytical balance and placed in an oven at 105 °C for 24 h. The sawdust was removed and immediately re-weighed, the mass loss divided by the initial sawdust weight gave a moisture content of 9.5 wt%.

2.2. Microwave and liquid hot water pretreatment of biomass

Eucalyptus sawdust (0.4 g) and 10 ml of Milli-Q water were added to a Teflon microwave reactor vessel with 100 ml capacity and heated using a QLab Pro Microwave Digester (Questron Technology Corp, Canada) at 500 W until the desired temperature is reached. Microwave frequency of 2.45 GHz is commonly used, as it was in this study, to prevent interference with telecommunication, wireless networks and cellular phone frequencies [20]. The set temperature for the experiments was 180 °C and the average time taken to heat the sample to this temperature was 17 min. Once the temperature was reached, the sample was maintained at 180 °C for periods between 5 and 60 min. On completion of the microwave irradiation, the vessel was cooled to room temperature, the contents were filtered using Filtech filter paper CAT NO 1830-055 and washed with water to recover two fractions: unreacted solid residue and liquid supernatant. The solid residue was air dried and stored in a sealed tube. Microwave reactions were carried out in duplicate.

For LHW pretreatment, 0.4 g of eucalyptus sawdust and 10 mL of Milli-Q water were introduced into a Swagelok stainless steel single-ended 50 mL sample cylinder equipped with a thermocouple. The cylinder was capped and placed in an oil bath and heated with approximately 90% of the sample cylinder submerged into the oil bath and covered with aluminum foil. The average time taken to reach 180 °C in the biomass solution was 15 min. Once the intended temperature was reached, samples were maintained at the set temperature for 15 or 30 min with the temperature being controlled within ±5 °C. On completion of the experiment, the vessel was cooled in a room temperature oil bath, the contents removed and centrifuged to recover the unreacted solid residue and supernatant.

2.3. Delignification by removal of lignin droplets on biomass using flowing hot water

The microwave and LHW pre-treated fibers were washed with flowing liquid hot water in a pressurized fixed bed reactor as shown in Fig. 1. The flowing hot water (1.25 ml/min for 2 h) at 120 °C and 2 bar was used to remove the lignin droplets deposited onto the surface of the pretreated samples. The wash-water containing the lignin droplets was collected periodically from the vapor-liquid phase separator and filtered using Whatman™ qualitative filter paper, Grade 1 with 11 μm pore size. The mass fraction of the solid lignin droplets was calculated according to equation (1) to give mg of lignin droplet per gram of dry biomass.

$$\text{Mass Fraction Removed Lignin } (mg/g) = \frac{\text{dry mass lignin droplet}}{\text{dry mass of original sawdust}} \quad (1)$$

2.4. Analysis of supernatant and solid residue from delignification of pretreated biomass

The supernatant liquid fraction from the delignification process were centrifuged and analyzed on a TOC-L Shimadzu Total Organic

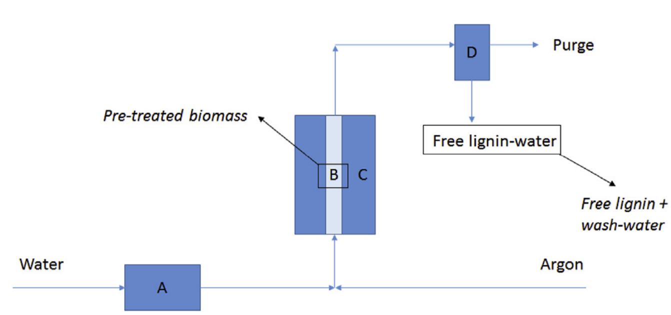

Fig. 1. Scheme of a pressurized fixed bed reactor used to remove lignin droplets on pretreated biomass (A) HPLC pump, (B) fixed bed reactor, (C) furnace and (D) vapor-liquid phase separator.

Carbon Analyzer to quantify the carbon compounds present. A 0.2 μm syringe filter was used to remove suspended particles in the solution. Of the 150 ml collected, 5 ml of sample was transferred to a TOC vial and diluted 8-fold prior to analysis.

2.5. Hydrolysis of xylo-oligomers to xylose monosaccharides in the pretreatment supernatants

Following the 15 and 30 min microwave and LHW pretreatments, 1 ml of the supernatant was combined with 20 μl of endo-1,4-β-xylanase M6 (rumen microorganism, Megazyme E-XYRU6) and incubated for 20 h at 40 °C without shaking. HPLC analysis of the supernatant before and after xylanase digestion was performed to determine the extent of xylo-oligomers in the supernatant and to allow determination of the sugar concentration of those oligomers as monomeric sugars following enzymatic hydrolysis.

2.6. Enzyme -catalyzed hydrolysis of cellulose from biomass

Enzymatic hydrolysis of pretreated and raw biomass was performed with cellulase mixtures from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich, >=700 UI/g) supplemented with cellobiase from *Aspergillus niger* (Sigma-Aldrich, ≥250 UI/g) at two levels of application; high enzyme load of 40 FPU/g(Filter paper units per gram of biomass) which is an overload to reflect industry practice to release the most sugars and low enzyme load of 10 FPU/g which was intentionally done to study the effectiveness of microwave exposure time and the effect of delignification. The biomass samples were prepared by mixing 30 mg of dried, pretreated or raw biomass, 1 ml 50 mM sodium citrate buffer pH 4.7 and the requisite volume of enzyme solution to achieve the required loading, followed by incubation for 48 h at 50 °C. Following digestion, supernatant sugar concentrations were determined by HPLC as described below and by the DNS assay [21]. For the DNS assay, glucose standards were prepared by dilution of a stock solution of anhydrous glucose (10 mg/ml) and DNS reagent was added to sample and standard solutions and incubated at 90 °C for 10 min. The absorbance of suitably diluted standards and samples was determined at 540 nm using a Shimadzu UV-2450 UV–Vis spectrophotometer. The concentration of reducing sugars in the samples was determined from the glucose standard curve. All experiments were carried out in duplicate and the results presented as the average value. There was close agreement between the sugar concentrations obtained by HPLC analysis and the DNS assay with ≤6% standard deviation.

2.7. HPLC analysis of sugars and chemicals from treated biomass

Samples obtained from microwave and LHW pretreated supernatants, the wash-water from the delignification experiments and the supernatants from enzyme treatment of biomass were analyzed by HPLC using an Agilent Technologies 1220 Infinity instrument equipped with REZEX monosaccharide RHM column using diode array (DAD) and refractive index (RID) detectors. The flow rate for the mobile phase (water) was set at 0.5 ml/min and a sample injection volume of 10 μl. The temperature of the column was set at 65 °C and temperature of the RID at 45 °C. A standard curve was created for the sugars, furfural and 5-hydroxymethylfurfural each with 4 data points.

2.8. Calculation of sugar and chemical yields

The composition of the *Eucalyptus regnans* used in the experiments, as shown in Fig. 2, was determined according to the NREL

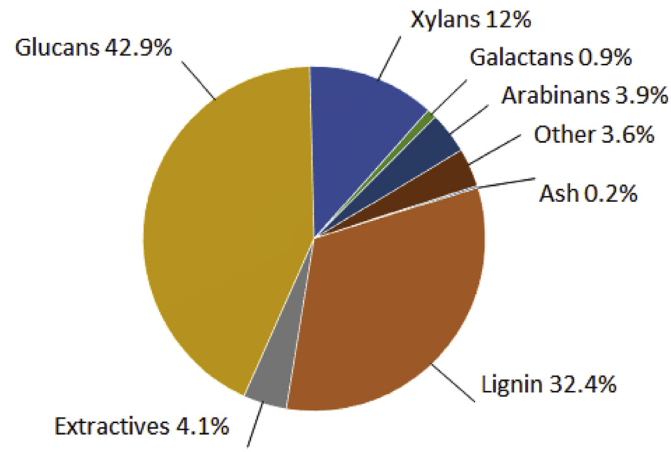

Fig. 2. Chemical composition of *Eucalyptus regnans* (Melbourne, Australia).

method at Queensland University of Technology in Brisbane, Australia. The molar yield of sugars was calculated by dividing the experimental number of moles of sugar by the actual number of moles present in biomass.

The yield of sugars on the mass and mole basis were calculated according to equations (2) and (3), respectively. The percentage molar yield of furfural (FUR) and 5-hydromethylfurfural (5-HMF) are described in equations (4) and (5), respectively. There have been reports of other reaction pathways for the decomposition of glucose, one such pathway is the isomerization of glucose to fructose followed by the decomposition to furfural [22]. Therefore, the molar yield of furfural takes into account both carbon five and six sugars.

$$Mass\ Yield\ of\ Sugars\ (mg/g) = \frac{mass\ of\ sugar\ released}{mass\ of\ sawdust\ on\ dry\ basis} \tag{2}$$

$$Molar\ Yield\ of\ Sugars\ (\%) = \frac{Moles\ of\ sugar\ released}{initial\ moles\ of\ sugar\ in\ sawdust} \times 100 \tag{3}$$

$$Molar\ yield\ (FUR)\ (\%) = \frac{moles\ FUR\ released}{initial\ moles\ of\ glucose + xylose} \times 100 \tag{4}$$

$$Molar\ yield\ (5-HMF)\ (\%) = \frac{moles\ 5-HMF\ released}{initial\ moles\ of\ glucose} \times 100 \tag{5}$$

2.9. Biomass particle and lignin droplet size measurement

The average diameters of the untreated and pretreated biomass samples were measured on a Malvern Instruments Master Sizer 3000 using the dry method. In brief, dried pretreated biomass was placed in a compartment and air used to carry the sample through a cell. The diameter (mm) of the detected particles were recorded with respect to the percentage volume distribution and the samples assessed in triplicate experiments.

Dynamic light scattering (DLS) measurements were performed using the Malvern Instruments Nano series Zetasizer to detect the size of the colloidal lignin droplets found in the supernatant after delignification (ESI). The supernatant liquid was ultrasonicated using a 700 W probe for 2 min at 20 MHz to break up aggregates and 1 ml of the sample was diluted in 10 ml water and placed in a cuvette for analysis by the % volume method.

2.10. Analysis of lignin droplets by FT-IR

An Agilent Technologies Cary 630 FTIR Spectrometer with diamond crystal was used to analyze the solid fraction removed from the delignification process. A small fraction of the air-dried sample was placed on the crystal and pressed down. The spectra obtained was scanned across 750 to 3800 cm^{-1}.

2.11. Microstructure analysis of pretreated biomass

SEM images were taken using a FEI Nova Nano SEM 450 FEG SEM and prepared by placing on a carbon tape attached to stubs and coated with approximately 1 nm of iridium. Samples of raw, microwave, LHW, the respective delignified fibers and lignin droplets were air dried and directly imaged.

3. Results and discussion

3.1. Microwave pretreatment fully hydrolyzes hemicellulose

Eucalyptus regnans sawdust samples in aqueous medium were exposed to microwave irradiation or LHW heating at 180 °C, the temperature selected was based on the reported thermal softening temperature of hard and softwoods [23]. Microwave treatment selectively hydrolyzed hemicellulose from the biomass without significantly hydrolyzing the cellulose network as the main identified sugar was xylose (Fig. 3A). This result is expected since hemicellulose polymers in hardwood are comprised mainly of xylans that have a backbone of β-1, 4-D-xylopyranose [24,25]. Hydrolysis of the glycosidic linkages within hemicellulose was likely catalyzed by the hydronium ions generated in water at high temperatures [17,26]. In addition, the depolymerization of hemicellulose releases acetyl and uronic acids bound to the xylan backbone in eucalyptus, which creates an acidic environment that promotes further hemicellulose hydrolysis and sugar dehydration into furfural [26,27]. The optimal exposure time was 30 min at which xylose yield peaked at 88.7 mol%; increasing the heating time resulted in dehydration of xylose to furfural with 15 mol% yield at 60 min (Fig. 3A). The branching characteristic of hemicellulose polymers coupled with the low degree of polymerization, allows them to be more readily hydrolyzed than cellulose [28].

An earlier study by Mihiretu et al. [29] examined microwave pretreatment of woody biomass in water only and found two thirds of the xylan hydrolyzed in oligomer form from aspen wood heated at 195 °C by microwave, whereas in this study more than 90% of the xylan was released, all in monomeric form, at a lower temperature. Similarly, Xu et al. [30]. examined the pretreatment of eucalyptus biomass in water plus acidic ionic liquid [bmim]HSO4 as a catalyst. The maximum xylose yield achieved by Xu et al. was 49.6 mg/g after treatment at 190 °C, whereas 107.1 mg/g was achieved in this study at 180 °C in water only. In Xu et al., the addition of acidic ionic liquid to the microwave pretreatment led to significant formation of acetic acid, furfural and lactic acid thereby reducing the yield of sugar [30]; these products can negatively affect downstream fermentation. In our case, no solvent removal was required after treatment and production of dehydrated sugar products was minimal up to 30 min microwave exposure time. Compared with microwave pretreatment, LHW pretreatment at identical conditions resulted in lower yields of all the products, indicating lesser extent of deploymerization (Fig. 3B).

LHW pretreatment released 3–3.5 times lower xylose, the yield of xylo-oligosaccharides was higher and increased with treatment time in the LHW samples (Table 1). Since xylo-oligomers remaining in hemicellulose can interfere with the action of cellulolytic enzymes, it is desirable to remove as much of the hemicellulose fraction as possible prior to enzyme application [31].

Product calculation is based on a bone dry biomass basis. The yields of sugars for each treatment includes the products that have undergone dehydration at 180 °C. Note: Furfural can derive from both C_5 and C_6 sugars. # Calculated by difference between the after- and before-xylanase treatment.

Xylan depolymerization into xylose was accelerated in microwave pretreatment, with more than 80% of the xylan hydrolyzed to xylose and the remainder as soluble xylo-oligomers after 30 min pretreatment. By contrast, 31% of xylan was hydrolyzed into xylose after 30 min LHW pretreatment, and ~60% in xylo-oligomer form. Our LHW results were similar to those obtained by Yu et al. on eucalyptus wood chips treated in a batch stirred reactor at 180 °C for 20 min which gave a total xylose recovery of 84.4% of which most of the xylose was recovered as oligosaccharides (46.8%) [32]. Unlike conventional heating, microwave irradiation can disrupt the polymeric sugar structure due to both thermal and non-thermal effects [33]. Furthermore, it can accelerate the rate of chemical reactions through ionic conduction and dipolar polarization and reduce overall reaction time [14,34]. The current work demonstrates that microwave irradiation is a faster and efficient form of pretreatment compared with liquid hot water, accelerating the hydrolysis of xylan and xylo-oligomers into xylose [31,35].

Raw eucalyptus sawdust used in this study had a particle size distribution of 0.3–2.2 mm with a peak size at approximately 1 mm (ESI) and following 30 min microwave pretreatment in water, the residual solids were reanalyzed for particle size (ESI). The results showed a minor reduction in the intensity of the peak, around 0.5–0.9 mm, and a new peak at 0.09–0.25 mm, which accounted for 1.2% volume distribution of the whole sample. This result clearly demonstrates that the biomass is not changing substantially during

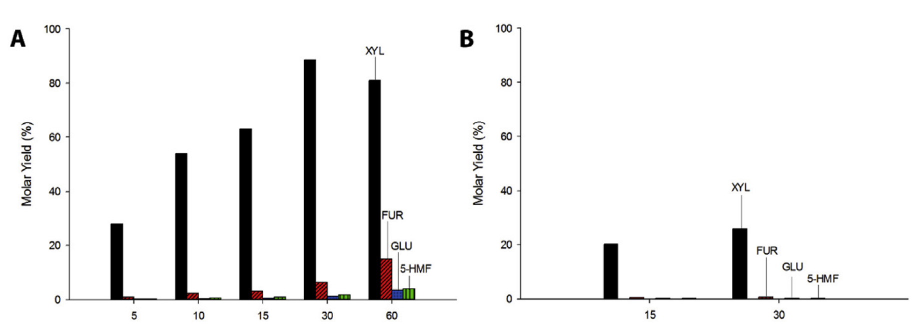

Fig. 3. Molar yields of products detected in the supernatants of pretreated *Eucalyptus regnans* sawdust in water only at 180 °C versus exposure time. (A) Microwave-irradiated and (B) Liquid hot water. Reaction conditions: 4 wt% eucalyptus sawdust loading. Xylose (XYL), furfural (FUR), glucose (GLU) and 5-hydroxymethylfurfural (5-HMF).

Table 1
Yields of dehydrated sugar products and monomeric sugar release following *Eucalyptus* biomass pretreatments at 180 °C.

Pretreatment Duration (min)	Mass Yield (mg/g_{db})				
	Xylose	Water soluble C_5 oligomers[#]	Glucose	Furfural	5-HMF
Microwave					
15	75.9 ± 2.7	27.0 ± 0.1	2.2 ± 0.0	10.9 ± 0.6	3.0 ± 0.5
30	107.1 ± 2.2	22.7 ± 7.5	5.0 ± 0.2	21.8 ± 1.8	6.1 ± 0.9
Liquid hot water					
15	24.2 ± 21.7	34.2 ± 0.9	0.9 ± 0.4	1.7 ± 1.5	0.6 ± 0.4
30	31.4 ± 13.9	67.0 ± 0.0	0.9 ± 0.1	2.5 ± 1.3	1.0 ± 0.4

microwave treatment and that some smaller particles in the starting material are breaking down during microwave pretreatment.

3.2. Pretreated biomass shows “lignin droplet” deposits

SEM images of the samples before and after pretreatment showed that samples pretreated for 5 min or longer had spherical complexes deposited on the surface of the fibers (Fig. 4) and their number and size increased with pretreatment exposure time. Previous studies of pretreated biomass have also described the appearance of these spherical complexes after acid-catalyzed or hydrothermal pretreatment and termed them “lignin droplets” or “pseudo lignin”. The mechanism of the formation of lignin droplets observed on corn stover has been hypothesized to be due to the coalescence of lignin during the pretreatment in aqueous conditions at temperatures above the lignin melting temperature [36]. The coalescence is thought to happen within the cell wall and migrate to the surface after thermal expansion [36]. Pseudo-lignin, by contrast, are droplets that form on biomass treated under aqueous acid conditions at moderate temperatures [37], and have a similar appearance to lignin droplets. The observation of lignin droplets on woody biomass under microwave treatment in this study (Fig. 4) is important as their presence has been considered to be inhibitory for the enzymatic hydrolysis of cellulose [38] therefore an effective and efficient method of removal of these droplets may be needed for optimal sugar release from pretreated biomass.

3.3. Flowing compressed hot water removes lignin droplets from pretreated biomass

Lignin droplets from microwave and LHW pretreated samples were removed, and the resulting free lignin was characterized. SEM images of the pretreated biomass samples show that microwave and LHW treated eucalyptus wood had similar coverage of lignin droplets on the surface of biomass (Fig. 5A and D, respectively). The lignin droplets were successfully removed from the biomass surfaces via flowing compressed hot water (Fig. 5B and E) yielding free insoluble lignin (Fig. 5C and F) which were collected from washwater by filtration. Fig. 5B and E suggest that the pressurized hot

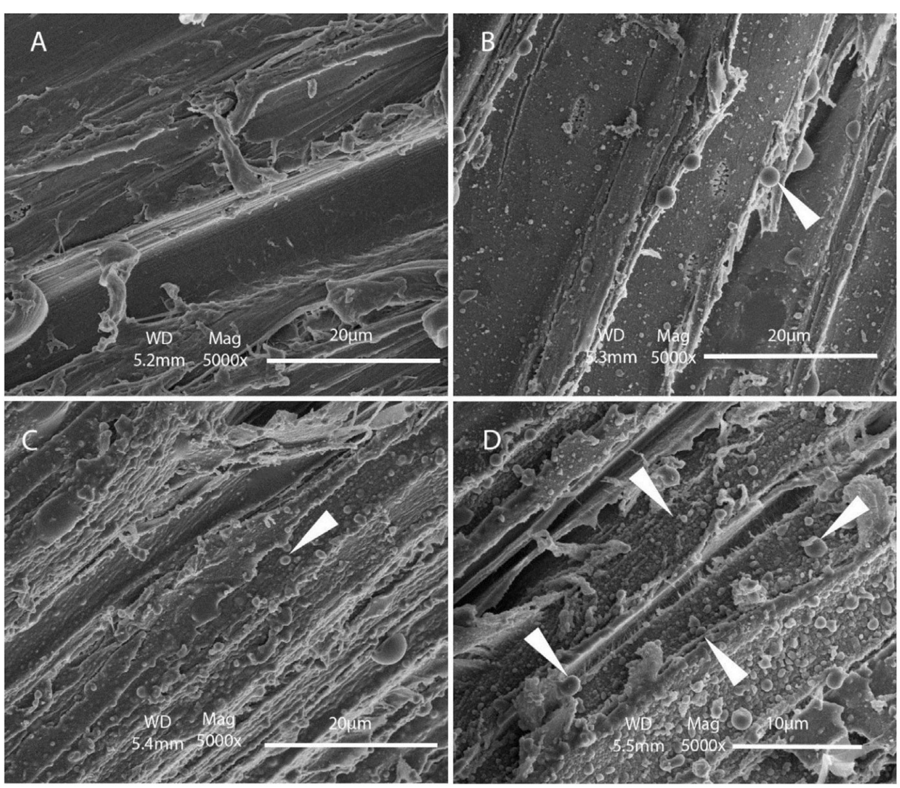

Fig. 4. SEM micrographs showing the morphological structure of *Eucalyptus regnans* sawdust and the effect of microwave heating in water (A) untreated eucalyptus sawdust, and heated for (B) 15, (C) 30 and (D) 60 min at 180 °C.

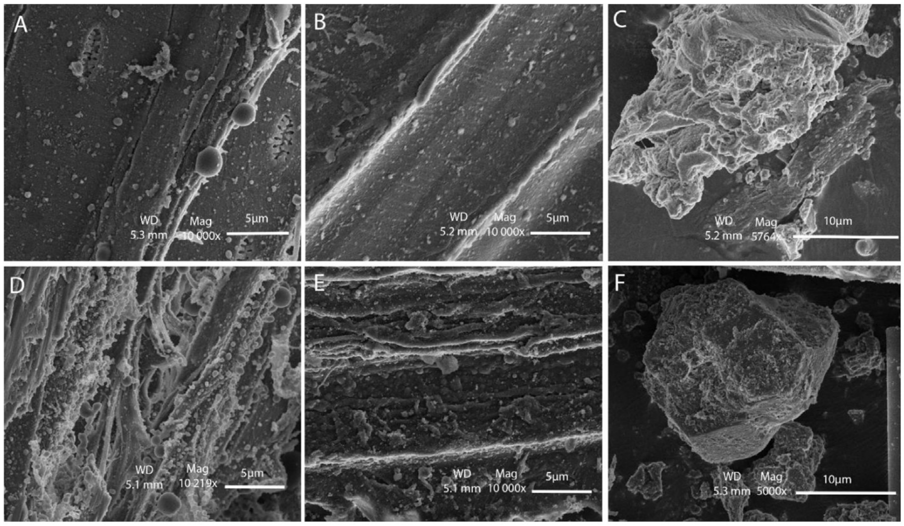

Fig. 5. Representative SEM micrographs of pretreated, delignified eucalyptus sawdust and lignin-carbohydrate complexes removed from biomass using compressed hot water at 120 °C for 2 h. A. Microwave treated biomass 15 min 180 °C. B. delignified, microwave treated biomass C. recovered lignin droplets D. Liquid Hot Water pretreated biomass 15 min, 180° E. delignified, LHW treated biomass F. recovered lignin droplets.

water process was effective for removing the larger lignin droplets, however, some smaller droplets can be observed on the surface. The maximum amount of lignin droplet removed during the delignification step was similar for both microwave and LHW treated samples at approximately 180 mg/g, which corresponds to roughly 60% of lignin in the raw biomass sample.

There has been an ongoing debate about the role of lignin and its effect on enzymatic hydrolysis. Lignin is an intricate network of aromatic polymers connected via carbon to carbon or carbon to oxygen bonds; with aryl ether bonds being their most significant internal linkage [26]. During pretreatment, these linkages are broken and the lignin either breaks down to fragments or forms lignin globules [26]. Previous studies have proposed that there is a correlation between the enzyme loading and presence of lignin as the enzymes are either adsorbed or associated with the lignin, inhibiting their activity [39,40].

The wash-water obtained from the delignification step was also light red in color. Dynamic Light Scattering analysis showed that the wash-water contained colloidal particles of average 351 ± 42.8 nm in size which may have been further lignin droplets too small to be filtered from the solution (ESI). Total Organic Carbon analysis showed all water samples contained carbon with the highest value being ~630 mg/L for the delignified 15 min microwave-treated biomass sample. HPLC analyses also showed trace amounts of sugar in all the samples plus a significant peak of an unknown compound. LCMS analysis of the unknown peak suggested a dimeric chemical generated by the interaction of water and a polar aromatic component of the wood resin (ESI), this suggests that the compound could be of lignin or extractives origin.

The ATR spectrum of the free lignin droplets shows several peaks common to functional groups present in lignin and carbohydrates (Fig. 6). The peak at 1327 cm^{-1} is C=O bond stretching of syringyl groups known to be found in hardwoods and the peak at 1267 cm^{-1} represents C–O bond stretching present in guaiacyl unit [41]. The intense peak at 829 cm^{-1} is associated with the out of plane deformation band for 1,3,5-tri substituted benzene rings and the peak at 1086 cm^{-1} represents C–O bond functional groups of ring alcohols in the carbohydrate moieties [36]. Intense alkyl aromatic ethers (Ar–O) stretch is found at 1236 cm^{-1} and the carbon to carbon (C–C) stretching for the ring is represented by the peak at 1554 cm^{-1}.

3.4. *Enzymatic hydrolysis of microwave pretreated samples yielded complete sugar release*

The absence of significant glucose in supernatants from microwave and LHW-pretreated samples and the low levels of 5-HMF suggests that cellulose hydrolysis was minor under these conditions. Cellulases were applied to the pretreated wood samples at high enzyme loading which released 100% of the cellulose-derived sugars in microwave-treated samples (Table 2). Overall, out of the total sugars in biomass, c. a. 13% was released during the pretreatment step and the remainder during the enzymatic treatment. In comparison, without the pretreatment step, the eucalyptus sample released only 4% of the total sugars during the enzymatic hydrolysis.

Table 2 shows enzymatic digestion of the pretreated only and pretreated and delignified samples at a high enzyme loading (40 FPU/g). Excess enzyme loading can increase the hydrolysis rate to a

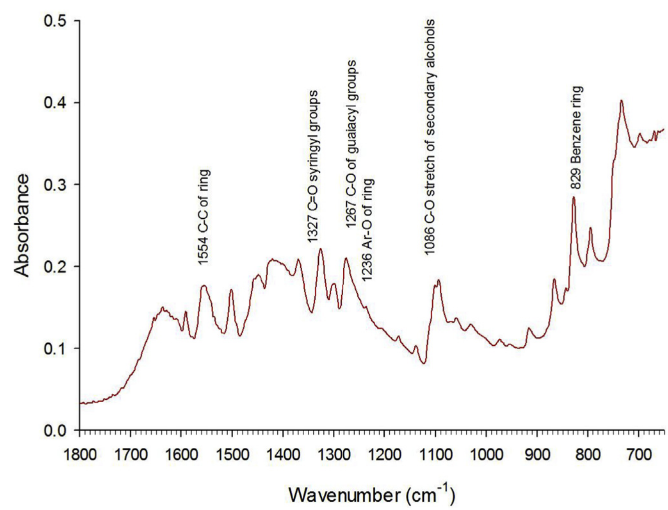

Fig. 6. FTIR Spectrum of the recovered solid free lignin from Eucalyptus sawdust pretreated at 15 min at 180 °C in water, then delignified with flowing compressed hot water at 120 °C for 2 h.

Table 2
Effectiveness of microwave pretreatment with or without a further delignification step with flowing hot water on release of sugar from *Eucalyptus regnans* biomass treated with cellulases at high loading.

Sample	Pretreatment Mass Yield[a] (mg/g_{db})	Enzymatic Hydrolysis Mass Yield[a] (mg/g_{db})	Total Sugars Mass Yield[b] (mg/g_{db})	Total Sugars % Yield[c]
	Xylose + Glucose	Reducing Sugars		
Raw	–	22.2 ± 0.8	22.2 ± 0.8	4 ± 0.1
Pretreated only/min				
MW 15	78.1 ± 1.0	523.2 ± 4.8	601.3 ± 2.1	102 ± 0.4
LHW 15	25.1 ± 8.1	102.1 ± 21.5	127.2 ± 0.6	22 ± 2.8
MW 30	112.1 ± 0.9	496.8 ± 103.7	608.9 ± 105.6	104 ± 18.0
LHW 30	32.3 ± 0.5	112.6 ± 20.0	144.9 ± 34.0	25 ± 5.8
Pretreated and Delignified/min				
MW/15	78.1 ± 1.0	497.3 ± 20.1	575.4 ± 17.4	98 ± 3.0
LHW/15	25.1 ± 8.1	96.5 ± 29.4	121.6 ± 51.5	21 ± 8.8
MW/30	112.1 ± 0.9	430.9 ± 120.4	543.9 ± 118.5	93 ± 20.2
LHW/30	32.3 ± 0.5	148.9 ± 55.3	181.2 ± 69.3	31 ± 11.8

[a] Mass yield of sugars after pre-treatment and enzymatic hydrolysis are expressed as mass of the product obtained per unit mass dry biomass (mg/g_{db}). Sugars yield from pre-treatment include xylose and glucose.
[b] Total Sugars Mass Yield include C_5 and C_6 Sugars yield from pre-treatment and reducing sugars yield from enzymatic hydrolysis.
[c] Total Sugars % Yield is the percentage of available C_5 and C_6 sugars produced on dry biomass mass basis.

degree, but in turn increase the process cost [42]. The typical amount of cellulase used in laboratory is 10 FPU/g as it gives a reasonable profile within a practical timeframe [43]. Table 3 shows enzymatic digestion results for the same pretreated biomass samples using a low enzyme loading (10 FPU/g).

During the enzymatic hydrolysis step, LHW pretreated samples released one-fifth of the sugars compared to the microwave-pretreated samples (Table 2). Furthermore, the total sugar yield (pretreatment + enzymatic hydrolysis) from LHW-treated samples was approximately one quarter of the equivalent release from microwave pretreated samples. The presence of residual xylan and acetyl groups bound to the xylan polymer are likely to have contributed to the slower enzymatic hydrolysis of LHW samples [26,44].

Overall, the microwave pretreated biomass yielded >90% xylose release from the pretreatment step and complete sugar release from cellulose digestion, resulting in a far greater sugar release compared with the LHW pretreatment. Complete release of sugars has been previously been reported with *E. grandis* wood chips in water but it required two sequential LHW steps at 180 and 200 °C with vigorous stirring, followed by enzyme digestion of the biomass to achieve this [13]. The direct heating of microwave irradiation excites the water and polar species in the dissociated biomass causing friction which dissipates as heat [14]. In addition, the depolymerized monosaccharides contribute to the polar state over time and assist in the rapid heating. Therefore, microwave treatment on its own was sufficient to open up access to the cellulose and improve enzymatic sugar yield.

The cellulosic sugar release figures in Table 2 were obtained using excess enzyme and thus it was not possible to determine if enzyme adsorption onto the "lignin droplets" was occurring. As shown in Table 3, removal of lignin droplets with flowing hot water followed by hydrolysis with a low enzyme loading led to a small improvement in sugar yield for the 30 min pretreated biomass but no difference for the shorter pretreatment. A study by Yang and Wyman compared batch and flow-through pre-treatment of corn stover using water and 0.1 wt% sulfuric acid. It was reported that the catalyzed flow through pretreatment removes the lignin either through forming insoluble species with the acid or the lignin dissolving and flowing through the system, which increases the enzymatic digestibility [45]. In addition, they reported that the enzymatic digestibility of the corn stover increased with increasing severity of these parameters [45]. Our results indicate that longer exposure to microwave irradiation increases xylose release and lignin droplet deposition which opens up access to the cellulose fibers. Removal of the lignin droplets uncovers the cellulose fibers still further, allowing access to the enzymes. As such, enzyme activity may be reduced due to steric hindrance only in the presence of lignin and so the presence of lignin droplets do not seem to inhibit the enzyme activity. Therefore, lignin droplet removal may not be crucial for a high saccharification result as much as the disruption in the biomass matrix and opening the access to the cellulose fibers.

Table 3
Effectiveness of microwave pre-treatment with or without a further delignification step with flowing hot water on release of sugar from *Eucalyptus regnans* biomass treated with cellulases at low loading.

Sample	Pre-Treatment Mass Yield[a] (mg/g_{db})	Enzymatic Hydrolysis Mass Yield[a] (mg/g_{db})	Total Sugars Mass Yield[b] (mg/g_{db})	Total Sugars % Yield[c]
	Xylose + Glucose	Reducing Sugars		
Raw	–	10.1 ± 2.0	10.1 ± 2.0	2 ± 0.3
Pretreated only/min				
15	78.1 ± 1.0	13.4 ± 1.8	91.5 ± 1.8	16 ± 0.3
30	112.1 ± 0.9	64.3 ± 6.7	176.5 ± 6.7	30 ± 1.1
Pretreated and delignified/min				
15	78.1 ± 1.0	14.7 ± 1.1	92.8 ± 1.1	16 ± 0.2
30	112.1 ± 0.9	104.4 ± 6.3	216.5 ± 6.3	37 ± 1.1

[a] Mass yield of sugars after pre-treatment and enzymatic hydrolysis are expressed as mass of the product obtained per unit mass dry biomass (mg/g_{db}). Sugars yield from pre-treatment include xylose and glucose.
[b] Total Sugars Mass Yield include C_5 and C_6 sugars yield from pre-treatment and reducing sugars yield from enzymatic hydrolysis.
[c] Total Sugars % Yield is the percentage of available C_5 and C_6 sugars produced on dry biomass mass basis.

4. Conclusions

Microwave pretreatment resulted in coalesced lignin droplets on the biomass surface and hydrolyzed up to 88 mol% yield of xylose monosaccharides which was 3.5 times higher than LHW pretreatment. Greater access for enzymatic hydrolysis was achieved after microwave pre-treatment of woody biomass, resulting in 100% saccharification compared to only 31% after LHW pre-treated fibers from the two-step method. Removal of the lignin droplets, which accounted for over half of the lignin, prior to saccharification did not improve enzyme release of sugars compared with biomass without lignin droplet removal, which also gave 100% sugar release after enzymatic hydrolysis. These results suggests that lignin in biomass limits cellulose hydrolysis via steric hindrance rather than enzyme inhibition. Microwave pretreatment in water-only media may provide a viable option to use woody biomass as a feedstock for biofuels and chemicals production.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.renene.2018.05.001.

References

[1] M.E. Himmel, S.K. Picataggio, Our challenge is to acquire deeper understanding of biomass recalcitrance and conversion, Biomass recalcitrance: Deconstructing the plant cell wall for bioenergy (2009) 1–6.
[2] W.B.D.R.K. Betts, A.S. Ball, S.L. Pedlar, Biodegradation: Natural and Synthetic Materials, 1991, pp. 139–155.
[3] P. Bhaumik, P.L. Dhepe, Conversion of biomass into sugars, RSC Green Chemistry (2015).
[4] Alibaba-Group. 1999-2017 4/7/2017]; Available from: <http://www.alibaba.-com/Products?tracelog¼beaconcate_140704>.
[5] S.M. Kim, B.S. Dien, V. Singh, Promise of combined hydrothermal/chemical and mechanical refining for pretreatment of woody and herbaceous biomass, Biotechnol. Biofuels 9 (1) (2016) 1.
[6] S. Banerjee, et al., Commercializing lignocellulosic bioethanol: technology bottlenecks and possible remedies. Biofuels, Bioproducts and Biorefining 4 (1) (2010) 77–93.
[7] L.R. Lynd, et al., How biotech can transform biofuels, Nat. Biotechnol. 26 (2) (2008) 169–172.
[8] V. Kumar, et al., Bioconversion of lignocellulosic biomass for bioethanol production, Biofuels Production (2013) 85–118.
[9] P. Binod, et al., Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse, Renew. Energy 37 (1) (2012) 109–116.
[10] C.-M. Liu, S.-Y. Wu, From biomass waste to biofuels and biomaterial building blocks, Renew. Energy 96 (2016) 1056–1062.
[11] S.P. Chundawat, et al., Deconstruction of lignocellulosic biomass to fuels and chemicals, Annual review of chemical and biomolecular engineering 2 (2011) 121–145.
[12] D.K. Johnson, R.T. Elander, Pretreatments for Enhanced Digestibility of Feedstocks. Biomass Recalcitrance: Deconstructing the Plant Cell Wall for Bioenergy, 2008, pp. 436–453.
[13] Q. Yu, et al., Two-step liquid hot water pretreatment of Eucalyptus grandis to enhance sugar recovery and enzymatic digestibility of cellulose, Bioresour. Technol. 101 (13) (2010) 4895–4899.
[14] S.A. Galema, Microwave chemistry, Chem. Soc. Rev. 26 (3) (1997) 233–238.
[15] A.a. Metaxas, R.J. Meredith, Industrial Microwave Heating, IET, 1983.
[16] E. Thostenson, T.-W. Chou, Microwave processing: fundamentals and applications, Compos. Appl. Sci. Manuf. 30 (9) (1999) 1055–1071.
[17] P. Alvira, et al., Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review, Bioresour. Technol. 101 (13) (2010) 4851–4861.
[18] J.S. Kim, Y. Lee, T.H. Kim, A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass, Bioresour. Technol. 199 (2016) 42–48.
[19] A. Aguilar-Reynosa, et al., Comparison of microwave and conduction-convection heating autohydrolysis pretreatment for bioethanol production, Bioresour. Technol. (2017).
[20] C.O. Kappe, A. Stadler, D. Dallinger, Microwaves in Organic and Medicinal Chemistry, John Wiley & Sons, 2012.
[21] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (3) (1959) 426–428.
[22] B.M. Kabyemela, et al., Glucose and fructose decomposition in subcritical and supercritical water: detailed reaction pathway, mechanisms, and kinetics, Ind. Eng. Chem. Res. 38 (8) (1999) 2888–2895.
[23] S.-Z. Chow, K. Pickles, Thermal softening and degradation of wood and bark, Wood Fiber Sci. 3 (3) (2007) 166–178.
[24] N. Zhang, et al., Cellulose-hemicellulose interaction in wood secondary cell-wall, Model. Simulat. Mater. Sci. Eng. 23 (8) (2015).
[25] D. Fengel, G. Wegener, Wood: Chemistry, Ultrastructure, Reactions, Walter de Gruyter, 1983.
[26] Y. Pu, et al., Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments, Biotechnol. Biofuels 6 (1) (2013) 1.
[27] H. Inoue, et al., Combining hot-compressed water and ball milling pretreatments to improve the efficiency of the enzymatic hydrolysis of eucalyptus, Biotechnol. Biofuels 1 (2) (2008) 1–9.
[28] H. Chen, Biotechnology of Lignocellulose: Theory and Practice, Springer, 2014.
[29] G.T. Mihiretu, et al., Single-step microwave-assisted hot water Extraction of Hemicelluloses from selected lignocellulosic materials—a biorefinery approach, Bioresour. Technol. (2017).
[30] H. Li, Y. Qu, J. Xu, Microwave-assisted conversion of lignin, in: Production of Biofuels and Chemicals with Microwave, Springer, 2015, pp. 61–82.
[31] Q. Qing, B. Yang, C.E. Wyman, Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes, Bioresour. Technol. 101 (24) (2010) 9624–9630.
[32] Q. Yu, et al., Hydrolysis of sweet sorghum bagasse and eucalyptus wood chips with liquid hot water, Bioresour. Technol. 116 (2012) 220–225.
[33] D.R. Keshwani, J.J. Cheng, Microwave-based alkali pretreatment of switchgrass and coastal bermudagrass for bioethanol production, Biotechnol. Prog. 26 (3) (2010) 644–652.
[34] D. Beneroso, et al., Microwave pyrolysis of organic wastes for syngas-derived biopolymers production, in: Production of Biofuels and Chemicals with Microwave, Springe, 2015, pp. 99–127.
[35] X. Zhuang, et al., Liquid hot water pretreatment of lignocellulosic biomass for bioethanol production accompanying with high valuable products, Bioresour. Technol. 199 (2016) 68–75.
[36] B.S. Donohoe, et al., Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment, Biotechnol. Bioeng. 101 (5) (2008) 913–925.
[37] P. Sannigrahi, et al., Pseudo-lignin and pretreatment chemistry, Energy Environ. Sci. 4 (4) (2011) 1306–1310.
[38] M.J. Selig, et al., Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose, Biotechnol. Prog. 23 (6) (2007) 1333–1339.
[39] T.D. Bernardez, et al., Adsorption of Clostridium thermocellum cellulases onto pretreated mixed hardwood, Avicel, and lignin, Biotechnol. Bioeng. 42 (7) (1993) 899–907.
[40] V.M. Chernoglazov, O.V. Ermolova, A.A. Klyosov, Adsorption of high-purity endo-1, 4-β-glucanases from Trichoderma reesei on components of lignocellulosic materials: cellulose, lignin, and xylan, Enzym. Microb. Technol. 10 (8) (1988) 503–507.
[41] L.M. Kline, et al., Simplified determination of lignin content in hard and soft woods via UV-spectrophotometric analysis of biomass dissolved in ionic liquids, BioResources 5 (3) (2010) 1366–1383.
[42] Y. Sun, J. Cheng, Hydrolysis of lignocellulosic materials for ethanol production: a review, Bioresour. Technol. 83 (1) (2002) 1–11.
[43] D.J. Gregg, J.N. Saddler, Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process, Biotechnol. Bioeng. 51 (4) (1996) 375–383.
[44] M.J. Selig, et al., The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes, Cellulose 16 (4) (2009) 711–722.
[45] B. Yang, C.E. Wyman, Effect of xylan and lignin removal by batch and flow-through pretreatment on the enzymatic digestibility of corn stover cellulose, Biotechnol. Bioeng. 86 (1) (2004) 88–98.