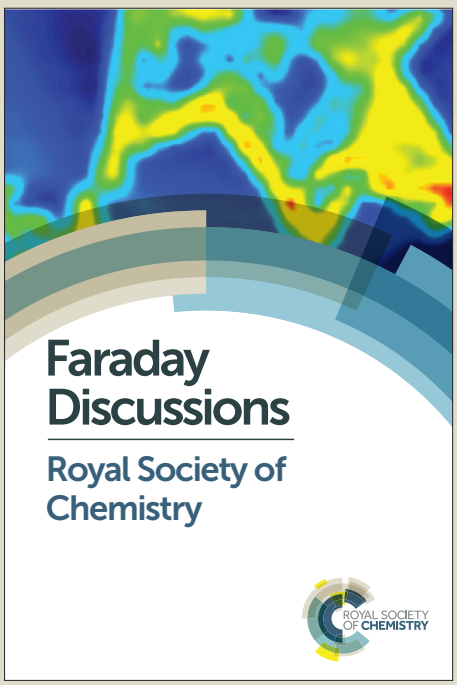
Faraday Discussions

Accepted Manuscript



This article can be cited before page numbers have been issued, to do this please use: L. Zhou, F. Santomauro, J. Fan, D. J. Macquarrie, J. Clark, C. J. Chuck and V. L. Budarin, *Faraday Discuss.*, 2017, DOI: 10.1039/C7FD00102A.

View Article Online
DOI: 10.1039/C7FD00102A

Faraday Discussions

ARTICLE

Fast microwave-assisted acidolysis, a new biorefinery approach for a zero-waste utilisation of lignocellulosic biomass to produce high quality lignin and fermentable saccharides

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Long Zhou,[a] Fabio Santomauro,[b] Jiajun Fan,[a] Duncan Macquarrie,[a] James Clark,[a] Christopher J. Chuck,[*b] Vitaliy Budarin[*a]

Generally biorefineries convert lignocellulosic biomass into a range of biofuels and further value added chemicals. However, conventional biorefinery processes focus mainly on the cellulose and hemicellulose fractions and therefore produce only low quality lignin, which is commonly burnt to provide process heat. To make full use of the biomass, more attention needs to be focussed on novel separation techniques, where the lignin can be isolated in a high quality suitable for further valorisation into aromatic chemicals and fuel components. In this paper, three types of lignocellulosic biomass (softwood, hardwood and herbaceous biomass) were processed by microwave-assisted acidolysis, to produce high quality lignin. The lignin from the softwood was isolated largely intact in the solid residue after acidolysis. For example, a 10 min treatment, microwave-assisted acidolysis produced a lignin with a purity of 93% and yield of 82%, superior to other conventional separation methods reported. Furthermore, the py-GC/MS analysis proved that the isolated lignin retained the original structure as native lignin in the feedstock without severe chemical modification. This is a large advantage, and the purified lignin is suitable for further chemical processing. To assess the suitability of this methodology as part of a biorefinery system, the aqueous phase, produced after acidolysis of the softwood, was characterised and assessed for its suitability for fermentation. The broth contained some mono- and disaccharides but mainly organic acids, oligosaccharides and furans. While this is unsuitable for *S. cerevisiae* and other common ethanol producing yeasts, two oleaginous yeasts with known inhibitor tolerances were selected; *Cryptococcus curvatus* and *Metschnikowia pulcherrima*. Both yeasts could grow on the broth, demonstrating suitable catabolism of the oligosaccharides and inhibitors over 7 days. In addition, both yeasts were shown to be able to produce an oil with a similar composition to palm oil. This preliminary work demonstrates new protocols of microwave-assisted acidolysis and therefore offers an effective approach to produce high purity lignin and fermentable chemicals, a key step towards a zero-waste lignocellulosic biorefinery.

Introduction

Due to fluctuations in crude oil prices, the finite nature of the resource and need for national energy reserves, there has been an increasing interest in developing lignocellulosic biorefineries for fuels and chemicals in the last few decades.[1,2,3] Lignocellulose is mainly constituted of cellulose, hemicellulose and lignin, and can be converted both chemically or biologically into a range of value added chemicals and fuels.[4,5,6] For example, in conventional biorefinery processes, cellulose and hemicellulose can be depolymerised to produce mono- and di-saccharides such as cellobiose, glucose, xylose and furanose, which can be fermented to alcohols or other microbial products or chemically converted into furans.[7,8] However, the current utilization of lignocellulosic biomass is still relatively inefficient.[9] In industrial processes, such as paper pulping, commonly only one product is targeted at the expense of large waste streams.[10,11] Side products such as lignin are commonly discarded or used to provide low value energy for the process, and not valorised into suitable phenolic components,[12] as these processes are designed only to achieve high quality cellulose. The cellulose purification protocols damage the lignin structure and produce lignin with significant polysaccharide contamination. However, pure lignin, with little degradation is a valuable source of aromatic functionality and could be converted into high value fuel additives or chemicals.[13] Therefore, a more selective pre-treatment to cleave the bonds connecting polysaccharide and lignin efficiently is of interest. Classical lignin isolation protocols, such as the Klason method or milled wood lignin (MWL), take hours or even days according to the intensity, and involve a range of additional acids and chemicals. As such, conventional

[a] Green Chemistry Centre of Excellence, Department of Chemistry, University of York, York, YO10 5DD, UK. Corresponding author email: vitaliy.budarin@york.ac.uk
[b] Department of Chemical Engineering, University of Bath, Claverton Down, Bath, United Kingdom BA2 7AY. Corresponding author email: c.chuck@bath.ac.uk

Electronic Supplementary Information (ESI) available: [proximate, ultimate and ICP analysis of feedstock; TAPPI T222 method]. See: DOI: 10.1039/x0xx00000x

ARTICLE View Article Online Journal Name DOI: 10.1039/C7FD00102A

separation methods cannot effectively isolate lignin with both a high yield and low structure modification at the same time.

Compared with conventional thermal treatment, microwave-assisted treatment has the characteristics of high efficiency and selectivity, making it an efficient tool in biomass processing and solid waste recycling. Microwave heating has been widely used in pyrolysis, gasification as well as delignification of biomass.[14,15,16] Microwave heating has a dramatic effect on the reaction kinetics[17] and reduces overall reaction times substantially.[18] Cellulose and hemicellulose are more polar than lignin,[19,20] therefore they receive more energy input on microwave irradiation. Based on this selective heating, a highly efficient separation could potentially be achieved through microwave treatment, with far shorter reaction times.

However, to further develop the zero waste biorefinery concept, various products must be produced alongside the lignin[21,22,23,24] To this end, a new protocol of rapid microwave-assisted acidolysis was examined to separate lignin effectively in three types of lignocellulosic biomass. The saccharide-rich aqueous phase was then investigated as a fermentation source for two oleaginous, inhibitor-tolerant yeast.

Experimental

Materials

Three lignocellulosic biomass were investigated in this research: mixed softwood pellets (MSP,UK Biochar Research Centre, School of Geosciences, University of Edinburgh), willow edging (WE, Gardman Ltd) and wheat straw (WS, Biorenewables Development Centre, University of York), representing softwood, hardwood and herbaceous biomass respectively. The elemental and ICP analysis are given in ESI Table 1-2†.

Sulfuric acid and sodium hydroxide were purchased from Fisher Chemicals. Creosol, vanillin, 2-methoxy-phenol, glucose, fructose, rhamnose, formic acid and furfural were purchased from Sigma-Aldrich. E-Isoeugenol was purchased from Acros Organic. Levoglucosan and 5-hydroxymethylfurfural (HMF) were purchased from Carbosynth. Xylose was purchased from VWR. Cellobiose was purchased from Fluorochem. Lactic acid was purchased from Wardle. Acetic acid was purchased from Alfa Aesar. Levoglucosenone was purchased from Dextra.

Metschnikowia pulcherrima was isolated from the local area (named strain I1) and *Cryptococcus curvatus* (strain DSM-70022) was purchased from the German Collection of Microorganisms and Cell Cultures.

Microwave-assisted acidolysis method

All biomass was milled to 60-mesh powders using a cutting mill (Retsch SM300, Germany) in Biorenewables Development Center (BDC), University of York. The microwave treatment was performed in a Discovery SP microwave reactor (CEM Corporation, USA) in capped vessels. Maximum power (300 W) was applied in all the experiments in order that the holding temperature was achieved as quickly as possible. Acid solutions of different types and concentrations were applied for isolation. Processing temperatures from 170 °C to 210 °C at intervals of 10 °C were used for isolation. The holding time was 10 min. During microwave treatment, the biomass feedstock and acid solvent were heated in a capped vessel with stirring in the ratios of 0.08 g : 6 ml, 0.2 g : 6 ml, 0.4 g : 6 ml and 0.6 g : 6 ml (13, 33, 67, 100g/L). After microwave treatment, the residue was recovered by filtration. Then the residue was washed several times with deionized water until the rinsed water was neutral. The residue was dried (105 °C, 24 h) and then weighed. All the experiments were repeated 3 times.

Analysis of acidolysis residue

The purity and yield were calculated by the TAPPI T222 method.[25] The method is shown schematically in ESI Fig. 1†. In this method, approximately 0.1 g dewaxed sample was treated with 10 g sulfuric acid (72 wt%) at 20 °C for 2 h. The solution was then diluted with deionised water to 3 wt% sulfuric acid and refluxed for 4h. The insoluble residue (lignin) was isolated by filtration. After washing with hot water, the residue was dried at 105 °C for 24 h. This dried residue is Klason lignin (KL). The purity and yield were calculated according to the equation in Table 1. The purity result was adjusted by subtracting the ash content measured by TG analysis.

Elemental analysis and ICP analysis data were obtained from the analytical service offered by Department of Chemistry, University of York.

Thermogravimetric (TG) analysis was performed using a Netzsch STA 409 analyser (Germany). The following parameters were applied: temperature ramp rate 20 K/min, final temperature 600 °C, carrier gas 50 ml / min pure nitrogen. To measure ash content, the following parameters were applied: temperature ramp rate 20 K/min, final temperature 625 °C holding for 1h, carrier gas 50 ml/min N_2 and 100 ml/min O_2. The final mass % was used as the ash content.

FTIR data was obtained using a Perkin Elmer FTIR/FTNIR Spectrum 400 analyser (USA). The spectra were acquired between 700 cm-1 and 4000 cm^{-1} with resolution of 2 cm^{-1} and scan time of 64s.

Solid State ^{13}C NMR spectroscopy (SSNMR) results were obtained at the EPSRC UK National Solid-state NMR Service at University of Durham. The spectra were obtained at 100.562 MHz. The chemical shift range from 0 ppm to 240 ppm was recorded.

Py-GC/MS results were obtained from BDC, University of York. CDS Analytical 5250-T Trapping Pyrolysis Autosampler (UK) was used as the pyrolysis unit, Agilent Technologies 7890B GC System (USA) as gas chromatography unit and Agilent Technologies 5977A MSD (USA) as mass spectrometer. The sample was loaded into the pyrolysis unit and pyrolyzed at 600 °C for 10 s. The volatile materials released were carried into the GC/MS unit by nitrogen for analysis. The following GC/MS parameters were applied: GC inlet temperature at 350 °C, initial temperature at 40 °C for 2 min, ramp rate at 10 K/min till 300 °C, holding at 300 °C for 30 min, split ratio with 50:1. Volatile compounds were identified by comparing the mass spectra with NIST Lab database. A standard sample mixture of four compounds, creosol / vanillin / 2-

View Article Online
ARTICLE
DOI: 10.1039/C7FD00102A

methoxyphenol (guaiacol) / E-isoeugenol, was also subjected to pyrolysis and GC/MS in order to verify the mass spectral identities.

Yeast cultures

Inoculum cultures for both yeasts were prepared by culturing in 10 ml SPME media (soy peptone: 30 g/L; malt extract: 25 g/L) and 10 ml YPD media (yeast extract: 10 g/L; peptone: 20 g/L; glucose: 20 g/L) and incubated at 20°C and 25°C for *M. pulcherrima* and *C. curvatus* respectively, with an agitation of 180rpm for 24h.

The hydrolysates prepared from the microwave processing stage were filtered, adjusted to pH 4 with 2 molar NaOH solution, then diluted 1:2 with a salt solution. For *M. pulcherrima*, the salt solution was composed of the following: KH_2PO_4 2 g/L; $MgSO_4$ * $7H_2O$ 0.376 g/L; $MgCl_2$ * $6H_2O$ 2.16 g/L; $ZnSO_4$* $7H_2O$ 0.04 g/L, $(NH_4)_2SO_4$ 0.126 g/L; NH_4Cl 0.708 g/L; yeast extract 2.00 g/L; $CaCl_2$*$2H_2O$ 0.300 g/L, tartaric acid 30.0 g/L. The pH was adjusted to 4 before dilution. For *C. curvatus*, the concentrated salt solution was composed as follow: KH_2PO_4 14.0 g/L; Na_2HPO_4 5.00 g/L; $MgSO_4$*$7H_2O$ 3.00 g/L; $FeCl_3$*$6H_2O$ 0.300 g/L; $ZnSO_4$* $7H_2O$ 0.040 g/L; $MnSO_4$* $1H_2O$ 0.120 g/L; $CaCl_2$*$2H_2O$ 0.300 g/L; $(NH_4)_2SO_4$ 1.00 g/L; yeast extract 2.00 g/L. The pH was adjusted to 6.5 before diluting.

Cultures of 1ml volume were inoculated with 25 µL of inoculum in 24-well plates and incubated at 180rpm at 20°C and 25°C for *M.pulcherrima* and *C.curvatus* respectively for 168h. Throughout the fermentation the growth of the cultures was estimated for absorbance at 600nm and the biomass was recovered by centrifugation in 1.5 eppendorf tubes (10,000 rpm, 5min). The supernatant and the pellets were stored separately at -20°C prior to further analysis

For the characterization of the fatty acid profile, an adapted literature method was used,[26] where the biomass pellets were suspended in methanol/H_2SO_4 1% v/v and heated at 90 °C for 3h in sealed pressure tubes. The resulting fatty acid methyl esters were extracted with hexane, washed with water and analysed through GC-MS.

The DP1 and DP2 saccharides were analysed using an Agilent 1260 Infinity HPLC (USA) equipped with an Agilent Hi-Plex H (300 x 7.7mm, 8um particle size) column. For levoglucosan, glucose, fructose, xylose, cellobiose, rhamnose and organic acids (lactic, formic, acetic acids), the mobile phase of 0.005M H2S04, isocratic (no gradient), flow-rate of 0.4 ml/min, column temperature of 60°C, refractive index detector of 55°C, total run time of 35 minutes, injection volume of 5ul was used. For inhibitor analysis (furfural, levoglucosenone and 5-HMF), the following parameters were used: column of ACE C18 (250 x 4.6mm, 5um particle size), mobile phase of Acetonitrile : Water (25/75), isocratic (no gradient), flow-rate of 0.8 ml/min, column temperature of 30°C, DAD detector of 220nm, total run time of 22 minutes, injection volume of 5ul. For both experiments, five samples of mixed standard chemicals (0.5, 0.75, 1.0, 1.5, 2.0 mg/ml) were used for the calibration.

The samples were also analysed for their oligosaccharides profile using a Dionex ICS-5000 HPLC (Thermo Scientific, USA) with a Dionex CarboPac SA10 column (4×250 mm) and guard column on 1.5 ml/min of flow rate using pulsed amperometric detection. The separation was performed at 25°C for 10 min using gradient elution with 1 mM sodium hydroxide (NaOH) at 1.5 ml/min and 10 µl injection volume. The samples were appropriately diluted in Milli-Q water and filtered through a 0.22 µm of syringe filter prior to analysis.

Results and Discussion

Analysis of acidolysis residue

Fig. 1 shows the influence of temperature on microwave lignin isolation (Fig. 1a, b, c). Clearly, for MSP and WE the residual mass decreased sharply from 170 °C to 180 °C. For WE, residual mass kept decreasing until 190 °C and remained at 20 % at higher holding temperatures. In contrast to woody biomass, WS had a relatively low residual mass even at 170 °C. The C/H results show similar trends. For MSP and WE, C/H content was relatively stable when $\geqslant$ 190 °C holding temperature was applied; while for WS, carbon content stabilised at 180 °C but hydrogen content kept decreasing with increasing temperature. For all three feedstocks, the residue colour kept getting darker when holding temperature increased. It is important to note that after 200 °C treatment, samples became totally black, suggesting that these temperatures may be causing the destruction of the lignin, and may therefore be too high to be of value. Microwave-assisted acidolyses were also performed with different types of acid and solution concentrations (Fig. 1d). It was found that the concentration had a significant influence on the acidolysis process. When concentrated H_2SO_4 (1mol/L) was applied, the residual mass was much less than that of diluted H_2SO_4, showing a higher concentration of solution

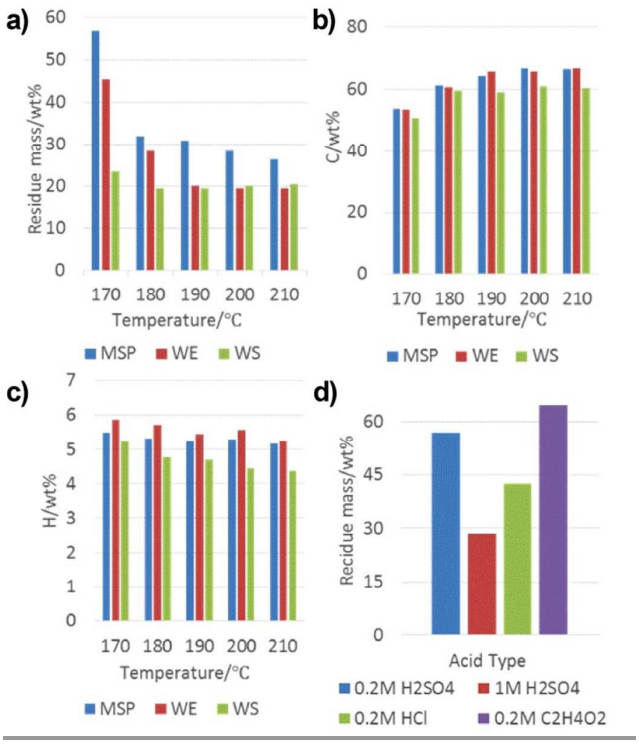


Fig. 1 Influence of temperature preparation on a) residue yield, b) carbon and c) hydrogen content; d) Nature of acid influence on residue mass yield. Conditions: holding time 10 min, dynamic mode, 0.2M acid solutions.

ARTICLE
View Article Online
Journal Name
DOI: 10.1039/C7FD00102A

Table 1. Purity and yield of residue lignin (wt%) obtained from different biomasses. Conditions: 0.2M H_2SO_4 solution, 190°C

	Lignin content in feedstock (M_{a1}/M_d)	Purity (M_{a2}/M_d)	Yield (M_{a2}/M_{a1})
MSP	39.03	92.85	82.43
WE	40.55	87.27	30.30
WS	33.83	63.12	31.23

Note: All the results are based on extractive-basis.
M_0, M_d, M_{a1}, M_i, M_{di}, M_{a2}, see ESI Fig. 1

could accelerate the separation even at relatively low temperature. The mass of residue formed by acetic acid (0.2mol/L) was only slightly higher than that by sulphuric acid. This suggested there was potential to perform acidolysis by acetic acid. Acetic acid can be produced from biomass, which is a more benign and potentially more attractive option than sulphuric acid. Among the three residues treated by diluted acid solution (0.2mol/L), the one by hydrochloric acid has the lowest residual mass, indicating hydrochloric acid can cause a higher extent of solubilisation of biomass in acidolysis.

By the method showed in ESI Fig. 1, the purities and yields were calculated in Table 1. In terms of purity and yield, MSP was a very suitable feedstock for the microwave-assisted acidolysis at 190 °C (10 min holding time, 0.2M sulphuric acid), but for WE and WS the protocol still needs improvement. Compared with hardwood and herbaceous biomass, it was reported softwood has the lowest content of acid-soluble lignin, only 0.2-0.5% of total lignin.[25] For MSP, 82 wt% of acid-insoluble lignin could be retained in the residue and, after the removal of soluble matter, the purity of the lignin residue could be as high as 93 wt%. Compared to MSP, the yield of WE was much lower at 30 %, but the purity was still relatively high at 87%. Wu and Argyropoulos[27] produced MWL with a 14-day milling process on black spruce (softwood) and poplar (hardwood). The yields were 28.5wt%/28.7wt% and purities were 88.3wt%/84.7wt%. Compared with these data, the microwave lignin isolation could compete with MWL protocol in terms of both the yield and purity, especially when the feedstock was softwood where both high yield and purity was achieved. More importantly, the microwave lignin isolation only took 10min, which was far more efficient than the MWL method.

The purity of WS was 63%, lower than that of MSP and WE. However, the low purity was caused not only by sugar contamination, but also ash content. The ash content extractive-free basis) of 190 °C WS residue was as high as 25wt% while the ash contents of MSP and WE residues are both < 3 wt%. ESI Table.1 shows the WS had an ash content of 14wt%. This indicates the ash content of the feedstock could (greatly affect the quality of residual lignin. Most herbaceous energy crops contain high amount of ash,[28,29] suggesting an ash-wash is necessary for effective lignin isolation from herbaceous biomass. Fig. 2a-f showed the mass and DTG curves of three feedstocks and their isolated residues at 170 / 190 °C. The TG curves of the three feedstocks and treated residues shared some similar trends: 1) for all the feedstocks,

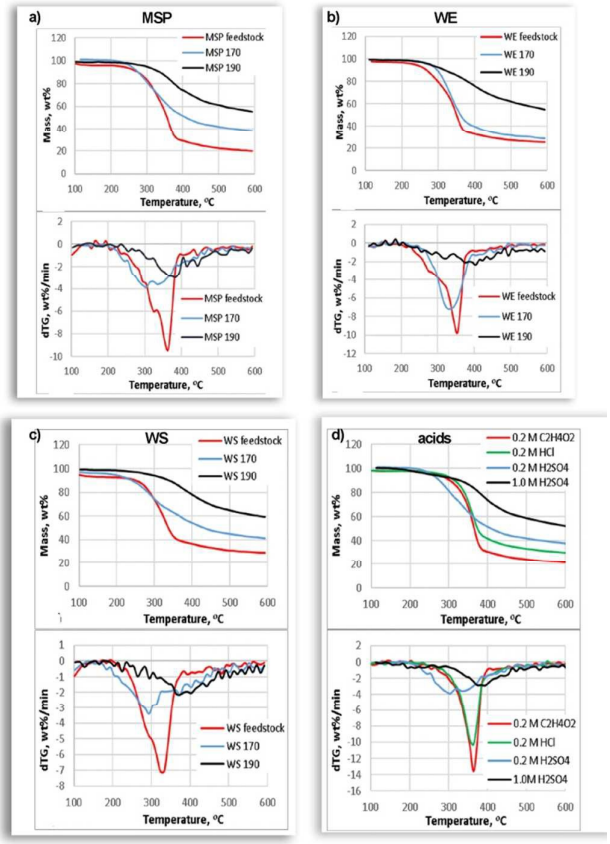


Fig. 2 Comparison of TG and dTG curves for a) MSP, b) WE, c) WS; d) influence of acid nature on properties of MSP. Conditions for a)-c): holding time 10 min, dynamic mode, 300W, treatment temperatures at 170/190 °C. Conditions for d: treatment temperatures at 170 °C.

the maximum DTG peaks occurred in the temerature range of 330-360 °C. According to the literature[30,31], the strong peaks are attributed to the pyrolysis of cellulose. There were also well-pronounced shoulders at 280-330°C, related to the pyrolysis of hemicellulose;[30,31] After microwave treatment, the residues were more recalcitrant to pyrolysis. After acidolysis, polysaccharides were removed and lignin was retained in the residues. The 190 °C residues had higher final mass than those produced at 170 °C, showing higher temperature benefited the acidolysis. 190 °C WS residue had the highest final residual mass and the lowest maximum mass loss rate, which was perhaps caused by the much higher ash content in straw (ESI Table 1). For 170 °C residues, the peaks at 330-360 °C were weakened significantly, but the peaks between 280-330 °C were still sharp, indicating a microwave-acidolysis at 170 °C could remove cellulose to a certain level, but was not able to solubilize hemicellulose effectively. This was most obvious for 170 °C WE residue, showing it had the most polysaccharide among the three 170 °C residues; for the three 190 °C residues, the TG and DTG curves were very similar. The DTG curves only showed weak peaks around 370-410 °C, which were caused by the pyrolysis of lignin. This demonstrated that the microwave acidolysis at 190 °C could solubilize the saccharide component and lignin was predominant in the

Journal Name

View Article Online
ARTICLE
DOI: 10.1039/C7FD00102A

residues. This provided evidence that a relatively thorough separation between lignin and polysaccharides could be achieved by acidolysis at 190 °C using microwave assisted heating.

Fig. 2g-h shows TG curves of four 170 °C residues processed by different acid solution. The peak of DTG curve of 1M H_2SO_4 was at 393.86 °C , showing it was highly pure lignin. So its final residual mass was highest at 49.85%. It illustrated the lignin could be efficiently isolated at relatively low temperature with concentrated sulphuric acid. In Fig. 2h, the DTG curves of HCl and acetic acid residues showed sharp peaks at 350 ° C, while the curve in H_2SO_4 residue only had two broad peaks at 294.23 °C and 333.99 °C. Furthermore, in Fig. 2g, the final residual mass of the H_2SO_4 residue was lower than that of the HCl and acetic acid residues. The higher thermal stability of the H_2SO_4 residue suggesting that it contained more lignin. This indicated sulphuric acid was more effective in lignin isolation. Although dilute HCl and acetic acid could dissolve similar amounts of biomass as diluted H_2SO_4 (as shown in Fig. 1), the fact that their dTG curves are somewhat similar to that of original feedstock, suggesting that a selective dissolution has not been achieved. This demonstrates that further optimisation is needed fopr the use of HCl or acetic acid in microwave acidolysis.

Fig. 3a shows the FT-IR spectra of MSP and its residual lignin at different temperatures. For the three biomasses, as the holding temperature rose, the bands at 1601 cm^{-1}, 1508 cm^{-1}, 1451 cm^{-1} and 1424 cm^{-1} (assigned as aromatic skeleton bands [32,33,34]) intensified. These strong peaks suggested high aromatic content in the residues after treatment. Similarly, the peak at 1208-1215 cm^{-1} that was ascribed as OH deformation of phenol was strengthened as processing temperature rose, indicating more phenolic units in higher temperature residues. The peak at 1030 cm^{-1}, assigned as primary alcohol from polysugars,[34] weakened as holding temperature increased, suggesting better removal of polysaccharide at higher temperatures. At temperatures higher than 190 ° C, the weakening of this peak became less obvious, showing the residues were relatively stable after processing by microwave acidolysis at 190 ° C or higher temperatures.

Besides the general trends discussed above, there were also some minor differences among the three residue lignin (Fig. 2b), such as 1250-1270 cm^{-1} (C-O stretching of guaiacyl unit[33,34]) and 1100-1125 cm^{-1} (C-H in-plane deformation of syringyl-unit[33,34]). This is caused by the variety of lignin, because softwood lignin contains only guaiacol units, while hardwood and herbaceous lignin was constituted of both guaiacyl and syringyl units in different proportions. Based on this, lignin from softwood, hardwood and herbaceous biomass could be used for the production of different chemicals after further activation. For example, softwood lignin could produce more vanillin; while if the target product is acetosyringone or phenol, lignin from hardwood and straw would make better raw materials.

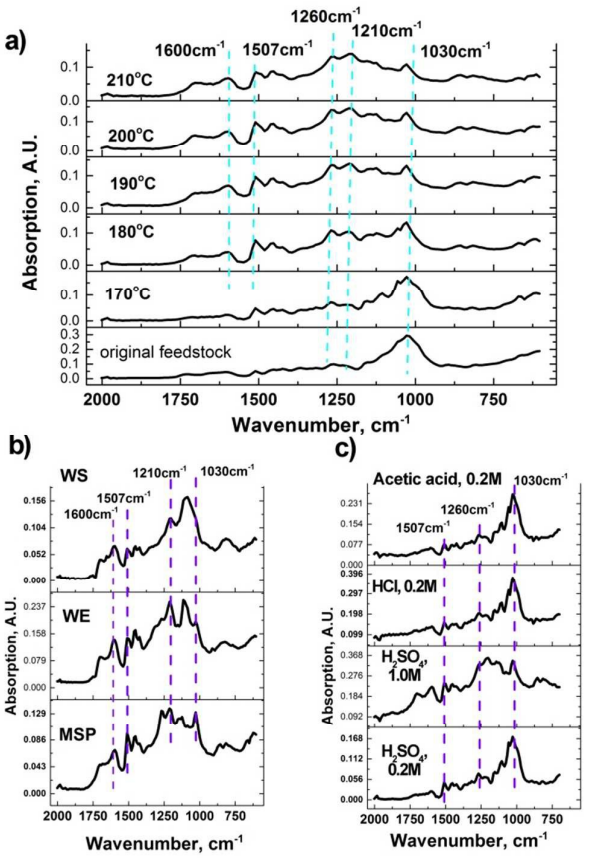


Fig. 3 FTIR analysis of feedstocks and the processed residues. a) Temperature influence on spectra of MW treated MSP residue. The conditions are: holding time 10 min, dynamic mode, 300W, MSP, 0.2M H2SO4 solution; b) Influence of feedstock nature on MW residues spectra obtained at 190 oC . The conditions are: holding time 10 min, dynamic mode, 300W, MSP, 0.2M H2SO4 solution c) Influence of acid nature on MW residues spectra obtained at 170 oC are: The conditions are: holding time 10 min, dynamic mode, 300W, MSP.

Fig. 3c shows the spectra of residues when different acid types and acid concentrations were applied at 170 °C. The spectrum of residue treated by concentrated sulphuric acid showed notable differences compared with those treated by dilute acid. The sugar peak (1030 cm^{-1}) was much weaker compared to the other three spectra. On the other hand, aromatic peaks (1601 cm^{-1}, 1508 cm^{-1}, 1451 cm^{-1}, 1424 cm^{-1}, 1213 cm^{-1}) were very sharp. These signals showed the processed residue was highly pure lignin, which proved concentrated acid could accelerate the separation between lignin and sugar at relatively low temperature. This result is consistent with TG analysis, showing lignin was dominant in residue processed by 1M sulphuric acid.

ARTICLE

Solid state NMR (SSNMR) spectra are shown in Fig. 4. Obviously, after microwave acidolysis, the peaks between 109-162 ppm were much stronger. According to Jindong Mao et al[35], the peaks in the range between 60-108 ppm can be ascribed as aliphatic carbon mainly from carbohydrate (a portion of side-chain of lignin also contributes to these peaks); while peaks between 109-162 ppm are aromatic. Thus the spectra clearly show the existence of lignin as a major component of the material.[35,36] This demonstrated that lignin was well isolated from the biomass, especially at 190 °C holding temperature. Comparing the SSNMR spectra of 190 °C residues with 170 °C residues, the aromatic peaks were stronger, while the carbohydrate peaks became weaker,

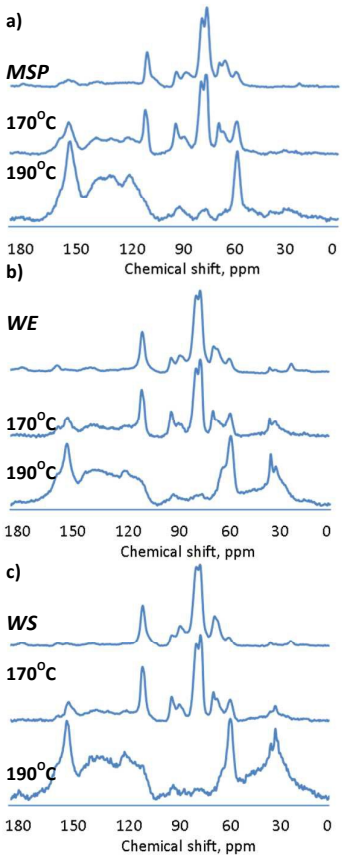


Fig. 4 Solid State NMR spectra of three feedstocks and their processed residues. The conditions are: holding time 10 min, dynamic mode, 300W, MSP, 0.2M H_2SO_4 solution a) spectra of MSP and its processed residue; b) spectra of WE and its processed residue; c) spectra of WS and its processed residue.

proving that the isolation of lignin by microwave-assisted acidolysis is affected greatly by holding temperature. At high temperature (190 °C), microwave acidolysis could dissolve most cellulose and hemicellulose, while at relatively low temperature (170 °C), the treated residues still contained severe sugar contamination. The peak at 55 ppm was attributed to the methoxy groups of lignin[35,37]. The change of this peak at 55 ppm proved lignin was well isolated at 190 °C

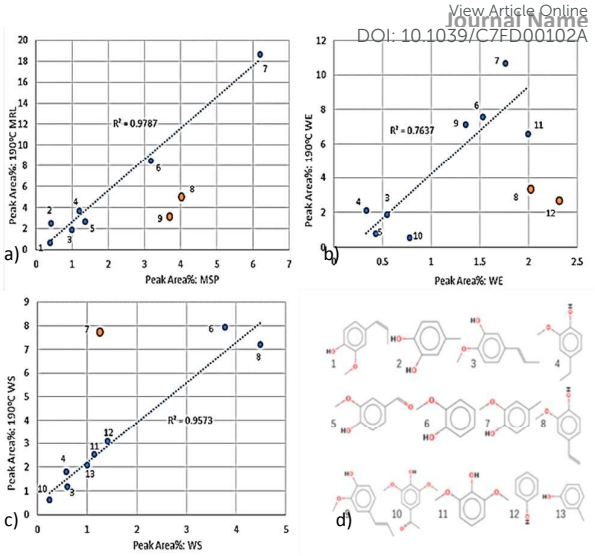


Fig. 5 Py-GC/MS analysis. Comparisons of GC peak areas of phenolic compounds between feedstock and 190 °C reside. Conditions: holding time 10 min, dynamic mode, 300W, MSP, 0.2M H_2SO_4 solution, 190 °C. a) MSP samples; b) WE samples; c) WS samples; d) structures of the standard compounds in figure. 1) cis-Isoeugenol; 2) 4-Methylcatechol; 3) Isochavibetol; 4) 4-Ethylguaiacol; 5) Vanillin; 6) Guaiacol; 7) 2-Methoxy-4-methylphenol; 8) 4-Vinylguaiacol; 9) trans-Isoeugenol; 10) Acetosyringone; 11) Syringol; 12) phenol; 13) m-Cresol (compounds showed by red dots are out of trend line.)

holding temperature.

The SSNMR spectra of the three types of biomass also showed some differences besides the general trend above. One of the most obvious differences was the peaks located in the range from 50-15 ppm that was attributed to aliphatic carbon[35,38]. Compared with MSP residues, these peaks in spectra of WE and WS residues were much stronger. As shown in Table 1, MSP residue was purest among three isolated residues. It had the least sugar contamination, therefore, this peak very weak in MSP residue spectra. Difference was also found at the peak at ~105 ppm for three 190 °C residue spectra. The 190 °C residues of hardwood and straw had well-pronounced shoulders at 105 ppm which that of softwood did not have. This peak was assigned as C2 and C6 carbon in syringyl unit[38]. Because softwood lignin only has G units, this peak was weak on spectrum of 190 °C MSP residue.

Fig. 5 shows the contents of pyrolytic compounds (phenolic) of MSP, WE and WS and their 190 ° C residual lignin in py-GC/MS analysis. After microwave acidolysis, the content of phenolic compounds increased significantly as indicated in the Fig. 5. For feedstocks, the compounds from polysaccharides, such as furfural and mannose, were dominant. On the other hand, for residue lignins, phenolic compounds were the main pyrolytic products. As expected, the three types of residual lignin produced pyrolytic products by different distribution, due to softwood, hardwood and herbaceous lignin having different monomers. Softwood was mainly composed by G-unit, hardwood by G-unit and S-unit, and herbaceous lignin had all three units (G/S/H-units). Thus, 190 °C MSP residual lignin had more vanillin (G-unit pyrolytic product), while the residual lignin of WE and WS had more phenol (H-unit pyrolytic product) and acetosyringone (S-unit pyrolytic product).

View Article Online
ARTICLE
DOI: 10.1039/C7FD00102A

In each of Fig. 5a-c, a trend line was drawn to show the linear relation of contents of phenolic compounds between feedstock and residual lignin. The gradient of the line is approximately the mass ratio of the biomass to the residue. Thus, if the phenolic compounds are stable during microwave acidolysis, they should be concentrated in the residues to the extent that the mass is reduced, and thus they should sit on the line; if not, it suggests structure modification happened during the treatment. So the coefficient of determination (R2) of trend line can be used as an index to show the extent of lignin structure modification during isolation. Obviously, most phenolic compounds obeyed the trend except only one or two compounds in each figure, showing generally lignin structure was well maintained during microwave acidolysis. Comparing the three trend lines, the R^2 in Fig. 5b was the lowest (0.76), indicating WE lignin experienced relatively more modification during microwave treatment, while for MSP and WS, the R^2 values were higher at 0.97 and 0.95 respectively. This suggests the microwave acidolysis protocol can preserve lignin structure very well for softwood and herbaceous biomass, but might damage hardwood lignin structure to some extent. It is worth noting that in the purity calculation section, for WS microwave isolation protocol produced lignin with much lower purity and yield than WE. However, from the results of py-GC/MS, it seems that structure of WS lignin structure was better kept than WE lignin, though WS lignin experienced more dissolution during microwave treatment.

Combining the residue analysis above, it was found MSP was the most suitable material for microwave-acidolysis among the three feedstocks, and produced lignin with the highest yield, and the least ash and sugar contamination. Lignin and polysaccharide of softwood biomass could be separated thoroughly by a 10-min microwave treatment while lignin structure was well preserved in residue, making softwood the most potentially useful feedstock for a zero-waste microwave biorefinery. Therefore the aqueous phase after acidolysis of MSP was used for fermentation.

Fermentation of the aqueous phase

A proportion of the saccharide feedstock partitions into the aqueous phase, this is a dilute solution containing some monosaccharide and disaccharide sugars (DP1 and DP2), organic acids, furans, a small amount of lower molecular weight oligosaccharides (DP3-DP5) and larger oligosaccharides (DP6+). Conventional yeast based processes, such as the production of bioethanol using *S. cerevisiae*, require inhibitor concentrations below 10mM and high hexose sugar concentrations.[39] However, several oleaginous yeasts are known to be able to survive in highly inhibitory conditions and can even metabolise some of these types of carbon sources. For example, *Cryptococcus curvatus* has been demonstrated to have a high threshold for furfural inhibitors,[40] while being able to metabolise acetate and some oligosaccharides.[41] Similarly we recently reported on the yeast *Metschnikowia pulcherrima*, that is able to grow in extremely acidic conditions,[42] and has been shown to produce a range of cellulases.[43]

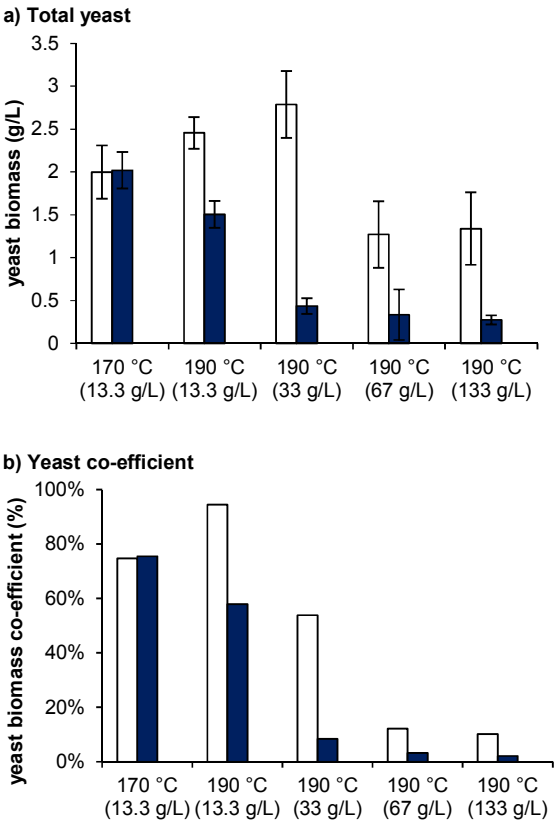


Fig. 6. Yeast biomass produced from the various hydrolysate samples, given as the initial loading of wood biomass at the temperature depolymerised, for C. curvatus (white bars, pH 6.5, 180 rpm, 168h) and M. pulcherrima (blue bars, pH 4, 180 rpm, 168h), where a) is the total yeast biomass b) is the yeast biomass coefficient, based as a proportion of the yeast grown compared to the initial level of saccharides (DP1-DP5), organic acids and furan inhibitors.

Yeast derived lipids have generated an increasing amount of interest in recent years, and have been demonstrated to be suitable for a range of applications including as a feedstock for biodiesel,[44] advanced biofuels[45] and alternative chemical transformations.[46] However, one of the most sought after applications could be in the replacement of terrestrial crops, such as palm oil, that lead to wide spread deforestation. For this application the product must be highly saturated, with the majority of the rest of the lipid being composed of oleic acid.[47] To determine the suitability of the solubilised fraction for fermentation, C. curavtus (Cc) and M. pulcherrima (Mp) were cultured on the solubilised fraction produced from the microwave processing (Fig. 6). Both yeasts grew reasonably well on the material depolymerised at 170 °C with 2 g/L of yeast biomass produced for both species. Under these conditions, 1.5 g/L mono- and disaccharides were available for

ARTICLE View Article Online Journal Name DOI: 10.1039/C7FD00102A

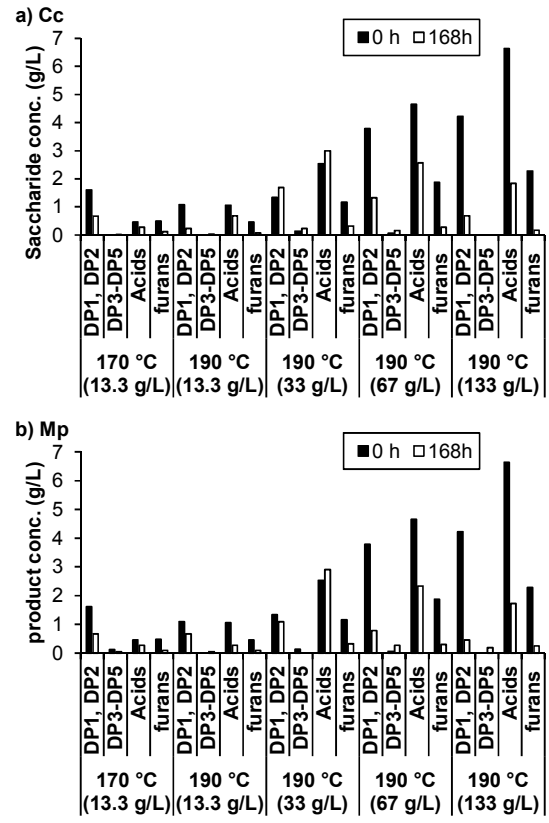


Fig 7. The quantification of saccharides (DP1-DP5), acids (formic, lactic and acetic), furans (5-HMF and furfural) before and after fermentation over the range of hydrolysates for a) *C. curvatus* (pH 6.5, 180 rpm, 168h) and b) *M. pulcherrima* (pH 4, 180 rpm, 168), c) Qualitative oligosaccharide chromatograph demonstrating DP6 and larger oligosaccharides for the original hydrolysate (depolymerised at 170 °C, 13.3 g/L wood biomass) and those left after fermentation with *M. pulcherrima* (pH 4, 180 rpm, 168h) and *C. curvatus* (pH 6.5, 180 rpm, 168h).

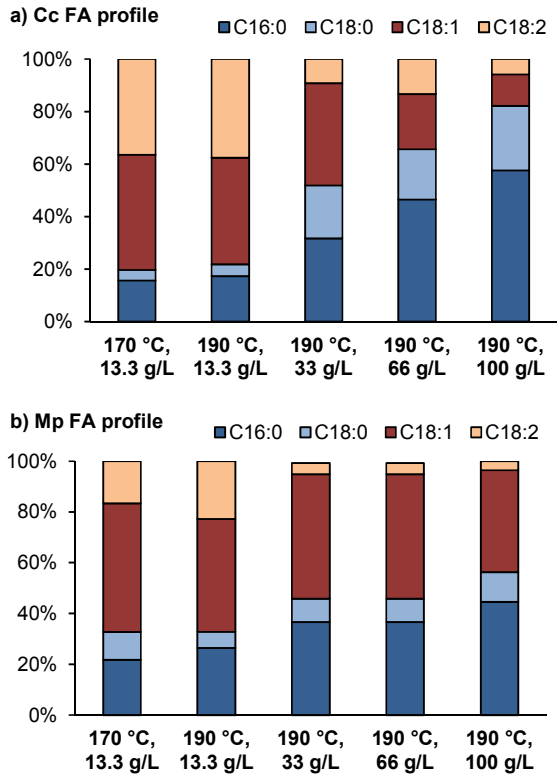


Fig. 8. Fatty acid profile of the lipid extracted from the yeasts *C. curvatus* (pH 6.5, 180 rpm, 168h) and *M. pulcherrima* (pH 6.5, 180 rpm, 168h)

fermentation but less than 0.5 g/L acids and furfurals were produced. The high yeast co-efficients strongly suggest that both yeasts are using the larger oligosaccharides in the broth as well. On increasing the processing temperature to 190 °C, the acid and furfural concentration were increased at the expense of the monosaccharide concentration. *C. curvatus* was able to metabolise some of this feedstock, though the impact on *M. pulcherrima* was more pronounced. On increasing the loading of initial wood biomass to 33 g/L, the mono- and disaccharide concentration was only increased partially, though over 2.5 g/L of organic acids were now present in the hydrolysate. Presumably as a result, the growth of *M. pulcherrima* was reduced substantially, though *C. curvatus* continued to thrive on this feedstock producing 2.8 g/L yeast biomass. A co-efficient of 0.54 on the available acids, furans and saccharides (DP1-DP5) in the media. While higher loadings of biomass promoted higher sugar concentrations, the furfural and acids produced increased substantially also. With initial loadings of 67 g/L and 100 g/L wood biomass, little yeast biomass was produced from either yeast. Both yeasts metabolise the majority of the mono and di-saccharides over the 7 days, though *M. pulcherrima* was more capable of metabolising the small amount of DP3-DP5 oligosaccharides in the broth than *C. curvatus* (Fig. 7a & b). Both yeasts demonstrated some ability to breakdown some of the larger oligosaccharide feedstock too (Fig. 7c). *M. pulcherrima* appeared to reduce some of the larger oligosaccharides, leaving a different oligosaccharide profile in the broth after the fermentation was complete. More strikingly, *C. curvatus* seemed able to convert a proportion of the larger oligosaccahrides presumably producing saccharides in the DP6 and smaller range in the process. This ability supports the

View Article Online
ARTICLE
DOI: 10.1039/C7FD00102A

higher levels of yeast biomass observed with *C. curvatus*, and demonstrates why the yeast could grow so well on the hydrolysed feedstock.

Interestingly both yeasts seem to have similar mechanisms for dealing with the inhibitory compounds. At 33 g/L loading neither *M. pulcherrima* or *C. curvatus* convert the acids in the hydrolysate, and the majority present at the start of the fermentation remain in the broth. However, at higher concentrations, where both yeasts grew poorly, the acids were seemingly metabolised. The furfural concentration in the mixtures were found to be below detectable limits for both yeasts, under all conditions, while the 5-HMF concentration was also severely reduced. This suggests that the yeasts were converting the furan compounds producing a less toxic substrate, a mechanism commonly seen with these types of yeasts.[48]

Both yeasts are oleaginous, and as such the lipid produced was investigated as a possible palm oil substitute (fig. 8). Despite both yeasts having been reported as effective producers of saturated lipid, less than 20% cell weight was recovered under any of the conditions analysed. In addition, the lipid profile was found to be highly dependent on the feedstock processing temperature. Under these conditions *C. curvatus* produced a lipid more similar to rapeseed oil, at low loadings of initial biomass, but the saturation increased substantially on increasing the initial biomass loading. Similarly, *M. pulcherrima* produced a lipid analogous to palm at the increased loadings of biomass. This suggests that highly stressful fermentation conditions can contribute to increasing saturation. This opens the possibility of being able to stress these yeast into producing desirable lipid profiles for industrial production, though more work would be necessary to increase the lipid content up to suitable levels.

Conclusions

A new method of rapid microwave-assisted acidolysis for a potential biomass biorefinery is proposed to achieve a zero-waste utilization of lignocellulosic biomass. Three types of lignocellulosic biomass were investigated for the separation of lignin and polysaccharides. Compared with WE and WS, MSP was the most suitable feedstock for microwave acidolysis, and produced high quality lignin with high yield (82%) and purity (93%). According to the py-GC/MS result, it was found the lignin structure could be well preserved during acidolysis, making this unmodified lignin suitable for phenolic compound production. Sulphuric acid could isolate lignin with the highest quality. If concentrated sulphuric acid was used in acidolysis, lignin could be isolated at a relatively low temperature than is required for dilute acid, though it is likely that the use of this strong acid caused extensive degradation of the saccharide feedstock. To convert the inhibitor rich aqueous phase two yeasts known for their inhibitor tolerance were selected (*Cryptococcus curvatus* and *Metschnikowia pulcherrima*). Both yeasts could grow on the saccharide feedstock metabolising a range of acids, furans, sugars and oligosaccharides. Though at high inhibitor loadings the yeast biomass was severely reduced. Both yeasts are known to be oleaginous and produced an oil akin to palm oil from this feedstock.

Based on these results microwave-assisted acidolysis offers a potentially powerful tool for lignocellulosic biomass utilization, especially for softwoods. Due to the efficiency and selectivity of microwave heating, 10 mins is enough time to produce a high quality lignin and fermentable aqueous phase.

Acknowledgements

This work was supported by EPSRC (EP/K014773/1) and Industrial Biotechnology Catalyst (Innovate UK, BBSRC, EPSRC) to support the translation, development and commercialisation of innovative Industrial Biotechnology processes (EP/N013522/1). Solid-state NMR spectra were obtained at the EPSRC UK National Solid-state NMR Service at Durham. Thanks to Biorenewables Development Centre (Department of Chemistry, University of York) for help with the sample milling and Py-GCMS experiments. We thank Dr. Christopher Ibenegbu for the oligosaccharide analysis and Dr Hannah Briers for the mono-, disaccharide and inhibitors analysis.

Access Statement

All data used in the preparation of this manuscript for the sections funded by the EPSRC grants EP/K014773/1 and EP/N013522/1 is contained within this document, the electronic supplementary information, or available on request from DOI:

Notes and references

1 G. Altinay and E. Karagol, *Energy Econ.*, 2004, **26**, 985–994.
2 A. B. Awan and Z. A. Khan, *Renew. Sustain. Energy Rev.*, 2014, **33**, 236–253.
3 J. Goldemberg and S. Teixeira Coelho, *Energy Policy*, 2004, **32**, 711–714.
4 P. Kumar, P. Kumar, D. M. Barrett, D. M. Barrett, M. J. Delwiche, M. J. Delwiche, P. Stroeve and P. Stroeve, *Ind. Eng. Chem. (Analytical Ed.*, 2009, **48**, 3713–3729.
5 J. J. Bozell and G. R. Petersen, *Green Chem.*, 2010, **12**, 539.
6 F. Cherubini, *Energy Convers. Manag.*, 2010, **51**, 1412–1421.
7 B. Kamm and M. Kamm, *Appl. Microbiol. Biotechnol.*, 2004, 64, 137–145.
8 P. Kaparaju, M. Serrano, A. B. Thomsen, P. Kongjan and I. Angelidaki, *Bioresour. Technol.*, 2009, **100**, 2562–2568.
9 M. Ni, D. Y. C. Leung, M. K. H. Leung and K. Sumathy, *Fuel Process. Technol.*, 2006, 87, 461–472.
10 A. Toledano, A. García, I. Mondragon and J. Labidi, *Sep. Purif. Technol.*, 2010, **71**, 38–43.
11 J. J. Bozell, *Clean - Soil, Air, Water*, 2008, 36, 641–647.
12 D. D. Laskar, M. P. Tucker, X. Chen, G. L. Helms and B. Yang, *Green Chem.*, 2014, **16**, 897.

ARTICLE
View Article Online
Journal Name
DOI: 10.1039/C7FD00102A

13 T. Q. Yuan, F. Xu and R. C. Sun, *J. Chem. Technol. Biotechnol.*, 2013, 88, 346–352.
14 Y. Wan, P. Chen, B. Zhang, C. Yang, Y. Liu, X. Lin and R. Ruan, *J. Anal. Appl. Pyrolysis*, 2009, **86**, 161–167.
15 R. Singh, A. Shukla, S. Tiwari and M. Srivastava, *Renew. Sustain. Energy Rev.*, 2014, 32, 713–728.
16 Q. Xie, F. C. Borges, Y. Cheng, Y. Wan, Y. Li, X. Lin, Y. Liu, F. Hussain, P. Chen and R. Ruan, *Bioresour. Technol.*, 2014, **156**, 291–296.
17 J. H. Clark, V. Budarin, F. E. I. Deswarte, J. J. E. Hardy, F. M. Kerton, A. J. Hunt, R. Luque, D. J. Macquarrie, K. Milkowski, A. Rodriguez, O. Samuel, S. J. Tavener, R. J. White and A. J. Wilson, *Green Chem.*, 2006, **8**, 853–860.
18 H. Li, Y. Qu, Y. Yang, S. Chang and J. Xu, *Bioresour. Technol.*, 2016, 199, 34–41.
19 W. Gindl-Altmutter, M. Obersriebnig, S. Veigel and F. Liebner, *ChemSusChem*, 2015, **8**, 87–91.
20 E. Rojo, M. S. Peresin, W. W. Sampson, I. C. Hoeger, J. Vartiainen, J. Laine and O. J. Rojas, *Green Chem.*, 2015, **17**, 1853–1866.
21 V. Budarin, a. B. Ross, P. Biller, R. Riley, J. H. Clark, J. M. Jones, D. J. Gilmour and W. Zimmerman, *Green Chem.*, 2012, 3251–3254.
22 Y. Yuan and D. J. Macquarrie, *Bioresour. Technol.*, 2015, **198**, 819–827.
23 D. Hernández, M. Solana, B. Riaño, M. C. García-González and A. Bertucco, *Bioresour. Technol.*, 2014, **170**, 370–378.
24 Q. Bu, H. Lei, S. Ren, L. Wang, Q. Zhang, J. Tang and R. Ruan, *Bioresour. Technol.*, 2012, **108**, 274–279.
25 T. Tappi, *2002–2003 TAPPI Test Methods*, 2002.
26 W. W. Christie, *Adv. lipid Methodol.*, 1993, **2**, e111.
27 S. Wu and D. . Argyropoulos, *J. pulp Pap. Sci.*, 2003, 29, 235–240.
28 H. Kludze, B. Deen and A. Dutta, *Fuel Process. Technol.*, 2013, **109**, 96–102.
29 D. Porbatzki, M. Stemmler and M. Müller, *Biomass and Bioenergy*, 2011, **35**.
30 G. Wang, W. Li, B. Li and H. Chen, *Fuel*, 2008, **87**, 552–558.
31 E. Biagini, F. Barontini and L. Tognotti, *Ind. Eng. Chem. Res.*, 2006, **45**, 4486–4493.
32 J.-Y. Chen, Y. Shimizu, M. Takai and J. Hayashi, *Wood Sci. Technol.*, 1995, **29**, 295–306.
33 Y. Huang, L. Wang, Y. Chao, D. S. Nawawi, T. Akiyama, T. Yokoyama and Y. Matsumoto, *J. Wood Chem. Technol.*, 2012, **32**, 294–303.
34 L. M. Kline, D. G. Hayes, A. R. Womac and N. Labbé, *BioResources*, 2010, **5**, 1366–1383.
35 M. Jingdong, K. M. Holtman, J. T. Scott, J. F. Kadla and K. Schmidt-Rohr, *J. Agric. Food Chem.*, 2006, **54**, 9677–9686.
36 G. R. Hatfield, G. E. Maciel, O. Erbatur and G. Erbatur, *Anal. Chem.;(United States)*, 1987, **59**.
37 M. Bardet, M. F. Foray and Q. K. Trân, *Anal. Chem.*, 2002, **74**, 4386–4390.
38 A. T. Martínez, G. Almendros, F. J. González-Vila and R. Fründ, *Solid State Nucl. Magn. Reson.*, 1999, **15**, 41–48.
39 C. J. Chuck, F. Santomauro, L. A. Sargeant, F. Whiffin, T. Chantasuban, N. R. A. Ghaffar, J. L. Wagner and R. J. Scott, *Biofuels*, 2014, **5**, 293–311.
40 X. Yu, Y. Zheng, K. M. Dorgan and S. Chen, *Bioresour. Technol.*, 2011, **102**, 6134–6140.
41 Z. Gong, H. Shen, X. Yang, Q. Wang, H. Xie and Z. K. Zhao, *Biotechnol. Biofuels*, 2014, **7**, 158.
42 F. Santomauro, F. M. Whiffin, R. J. Scott and C. J. Chuck, *Biotechnol. Biofuels*, 2014, **7**, 42.
43 M. L. A. Strauss, N. P. Jolly, M. G. Lambrechts and P. Van Rensburg, *J. Appl. Microbiol.*, 2001, **91**, 182–190.
44 L. A. Sargeant, C. J. Chuck, J. Donnelly, C. D. Bannister and R. J. Scott, *Biofuels*, 2014, **5**, 33–43.
45 J. L. Wagner, V. P. Ting and C. J. Chuck, *Fuel*, 2014, **130**, 315–323.
46 R. W. Jenkins, L. A. Sargeant, F. M. Whiffin, F. Santomauro, D. Kaloudis, P. Mozzanega, C. D. Bannister, S. Baena and C. J. Chuck, *ACS Sustain. Chem. Eng.*, 2015, **3**, 1526–1535.
47 F. Whiffin, F. Santomauro and C. J. Chuck, *Biofuels, Bioprod. Biorefining*, 2016, **10**, 316–334.
48 J. R. M. Almeida, T. Modig, A. Petersson, B. Hähn-Hägerdal, G. Lidén and M. F. Gorwa-Grauslund, *J. Chem. Technol. Biotechnol.*, 2007, **82**, 340–349.