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Hydrothermal Liquefaction of Enzymatic Hydrolysis Lignin – Biomass Pretreatment Severity Affects Lignin Valorization

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Abstract

Alkaline hydrothermal liquefaction (HTL) of lignin-rich enzymatic hydrolysis residues (EnzHR) from wheat straw and *Miscanthus x giganteus* was performed at 255, 300, and 345 °C to investigate valorization of this side-stream from second-generation bioethanol production. HTL of EnzHR from biomass hydrothermally pretreated at two different levels of severity (190 °C, 10 min and 195 °C, 15 min) showed the most effective lignin depolymerization of the low severity EnzHR for both wheat straw and *Miscanthus*. The degree of depolymerization during HTL was temperature dependent and was not complete after 20 min at 255 °C, most distinctly for the *Miscanthus* EnzHR. The yields of 128 monomeric products quantified by gas chromatography – mass spectrometry were up to 15.4 wt% of dry matter. Principal component analysis of the quantified compounds showed that non-lignin HTL products are main contributors to the variance of the HTL products from the two biomasses. The chemically modified lignin polymer was found to have increased thermal stability after HTL. Analytical pyrolysis was applied to investigate the chemical composition of a larger fraction of the products. Analytical pyrolysis contributed with additional chemical information as well as confirming trends seen from quantified monomers. This work is relevant for future lignin valorization in biorefineries based on current second-generation bioethanol production.

Keywords: Base-catalyzed depolymerization, lignin depolymerization, hydrothermal pretreatment, analytical pyrolysis, principal component analysis, PARAFAC2

Introduction

Lignocellulosic side-streams from food production, paper pulp production, forestry and agriculture are good feedstock candidates for sustainable fuels and chemicals. One example is wheat, which can be utilized to produce food and straw for fuel (bioethanol)[1] and potentially chemicals.[2] Additionally, energy crops such as *Miscanthus* are candidates for production of liquid fuels and chemicals.[3] Lignocellulosic biomass primarily consists of cellulose, hemicelluloses, lignin, and extractives.[4] Lignin makes up 15–35 wt% of lignocellulosic biomass and is the largest renewable source of aromatics.[5-6] Only about 2% of industrially extracted lignins are used as chemicals, e.g., as dispersants.[7] Lignin-rich enzymatic hydrolysis residue (EnzHR) from second-generation bioethanol production is an emerging type of industrial lignin and only about 40% of this lignin side-stream is needed for internal energy use.[1,6]

Biomass in aqueous slurry can be converted into valuable products by hydrothermal liquefaction (HTL) at 200–370 ˚C and 4–20 MPa, where the changes in the physicochemical properties of water result in unique solvation and reaction conditions.[8-9] The reaction chemistry of lignin HTL is dominated by ionic rather than radical reactions.[10] The utilization of water for HTL also means that energy-intensive drying of wet biomasses is avoided. This makes HTL an ideal match with the utilization of the wet lignin residue resulting from the ethanol production, which is currently dried and burned.[1] Products from HTL of lignin have been shown to have antifungal properties[11] and can partly substitute bisphenol A in an epoxy resin.[12]

The plant species of lignocellulosic feedstocks, lignin extraction method (kraft, soda, etc.), biomass pretreatment method (hydrothermal, dilute acid, etc.), and pretreatment severity affect the structure of the lignin, and can thus be expected to affect the HTL products.[13-14] Differences in lignin HTL products due to plant species are easily evident by comparing softwood lignin (guaiacol (G) type) producing phenols/guaiacols/catechols and grass lignin (*p*-hydroxyphenyl/guaiacyl/syringyl (HGS) type) also producing syringol.[15-17] Effects of type of pretreatment on lignin HTL products have also been investigated, e.g., choice of organosolv solvent[18] and mechanical pretreatment.[19] Hydrothermal pretreatment is used in recent second generation bioethanol plants of demonstration[1] and full scale.[20] Hydrothermal pretreatment is the

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treatment of biomass with steam without additives.[1] Increasing the severity of hydrothermal pretreatment of wheat straw from a severity factor of log R_0 2.77 to 3.65 results in a large reduction of β-O-4 ether bonds and likely formation of new β-5 C-C bonds in the lignin.[21] Pretreatment of aspen wood with steam explosion in the log R_0 range 3.2–4.5 cause an almost linear decrease of β-O-4 bonds.[22] The change of lignin inter-unit bonding can be expected to affect subsequent valorization of the lignin in the EnzHR from second-generation bioethanol production. Yet, the effects of varying the severity of hydrothermal pretreatment have, to our knowledge, not received any attention in HTL literature.

In this work, we performed alkaline HTL of EnzHR from two grass species (*Miscanthus* and wheat straw) and two different and industrially relevant hydrothermal pretreatment severities (log R_0 3.65 and log R_0 3.97) after initial screening of HTL temperature (255 °C, 300 °C, and 345 °C), to investigate the effects of these parameters on HTL product composition. We characterized the composition of feedstock and HTL products, small as well as polymeric compounds, using mainly size exclusion chromatography (SEC) and gas chromatography – mass spectrometry (GC-MS) combined with pyrolysis techniques. The study is subdivided into effects of varying HTL temperature for both biomasses (Part I) and effects of varying pretreatment severity for both biomasses (Part II).

Materials and methods

Detailed descriptions of materials and experimental methods can be found in the Supporting Information. Results are reported as mean and standard deviation of data obtained from replicate HTL experiments, i.e., covering uncertainties of the entire procedure.

Enzymatic hydrolysis residues

EnzHR used as feedstocks for HTL were prepared by subjecting *Miscanthus x giganteus* stalks and wheat straw to hydrothermal pretreatment (steam pretreatment with autohydrolysis) at severity log R_0 3.65 (190 °C, 10 min) or log R_0 3.97 (195 °C, 15 min) as detailed elsewhere.[23] The pretreated biomasses were then cut to <3 cm, knife-milling to <300 µm, and subjected to enzymatic hydrolysis and protease treatment according to a published procedure.[24] The resulting solids were the *Miscanthus* (M3.65 and M3.97) and wheat straw (W3.65 and W3.97) enzymatic hydrolysis residues (EnzHR). The compositions of the EnzHR are shown in Table S1. The severity factor log R_0 is calculated according to Overend and Chornet:[25]

$$\log R_0 = \log\left[t(\mathrm{min}) \cdot \exp\left(\frac{T(^\circ\mathrm{C}) - 100}{14.75} \right) \right] \quad (1)$$

Hydrothermal liquefaction of enzymatic hydrolysis residues

Lignin-rich EnzHR containing 1 g dry matter (DM) was added to a 9 g aqueous solution of KOH giving a 10 g slurry of 10 wt% DM EnzHR and 5.6 wt% KOH. The slurry was transferred to a 20 mL stainless steel (SAE316) batch reactor and heated in an Omega Engineering FSB-4 fluidized sand bath. The reaction time was 20 min after heating for 4 min. The temperature for the start of reaction was defined as $T_{Rstart} = T_I + 0.92(T_{Rmax} - T_I)$, where T_I is the initial temperature of the slurry (ca. 25 °C) and T_{Rmax} is maximum reaction temperature. The reactor content was cooled to below 50 °C in less than 2 min by submersion in a water bath. The gas yield was low (<4 wt%). HTL experiments were generally performed in duplicate.

Product fractionation

The liquid and solid products were separated using the procedure outlined in Figure 1. The product fractionation has been shown to aid in interpretation of the complex product mixture from HTL of lignin.[15]

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This is achieved by fractionation into a low molecular weight fraction (denoted liquid product, LP) and a high molecular weight fraction (denoted chemically modified lignin, CML). The product solution was acidified with 4M HCl to pH 2. The CO_2 evolved during acidification from dissolved carbonates was not included in the mass balance. Acidification resulted in precipitation, and solids were separated from the water phase (WP) by filtration. The solids were washed with diethyl ether (Et_2O) until the filtrate appeared colorless. The WP was liquid-liquid extracted 3 times with Et_2O (~1:1 ratio). The Et_2O extracts were dried over Na_2SO_4. The liquid product (LP) was obtained as a dark orange/brown viscous oil after evaporation of the Et_2O at reduced pressure. Chemically modified lignin (CML) was obtained as the tetrahydrofuran (THF) soluble solids by washing the reactor and filter retentate with THF until the filtrate appeared colorless. The CML was a dark brown/black brittle solid fraction after evaporation of the THF under reduced pressure. The solid residue (SR) remaining on the filters was oven dried at 105 °C overnight. SR caught in the sintered glass filter gave additional 0.7 wt% yield in the W3.97-300-2 experiment.

The yields of the dried/evaporated product fractions were calculated as DM corrected wt% using Equation (2). Monomer yields are reported on both DM (Equation (2)) and lignin basis (Equation (3)).

$$Y_{product} = \frac{m_{product}}{m_{EnzHR} \cdot DM_{EnzHR}} \cdot 100\% \quad (2)$$

$$Y_{aromatic\,monomer} = \frac{m_{product}}{m_{EnzHR} \cdot DM_{EnzHR} \cdot (AIL_{EnzHR} + ASL_{EnzHR})} \cdot 100\% \quad (3)$$

where $m_{product}$ is the mass of the product fraction or monomer, m_{EnzHR} is the mass of added EnzHR, and DM_{EnzHR}, AIL_{EnzHR}, and ASL_{EnzHR} is the fraction of dry matter, acid insoluble lignin, and acid soluble lignin in the EnzHR, respectively.

The yield of the CML fraction was corrected for the 2,6-di-tert-butyl-4-methylphenol (BHT) introduced into this fraction by the use of BHT-stabilized THF.

The effects of slurry preparation and fractionation procedure on the biomass were accounted for by control experiments using M3.65 and W3.65 EnzHR, where the slurry was transferred to the reactor and kept at room temperature for 24 min before going through the same fractionation procedure as the HTL products.

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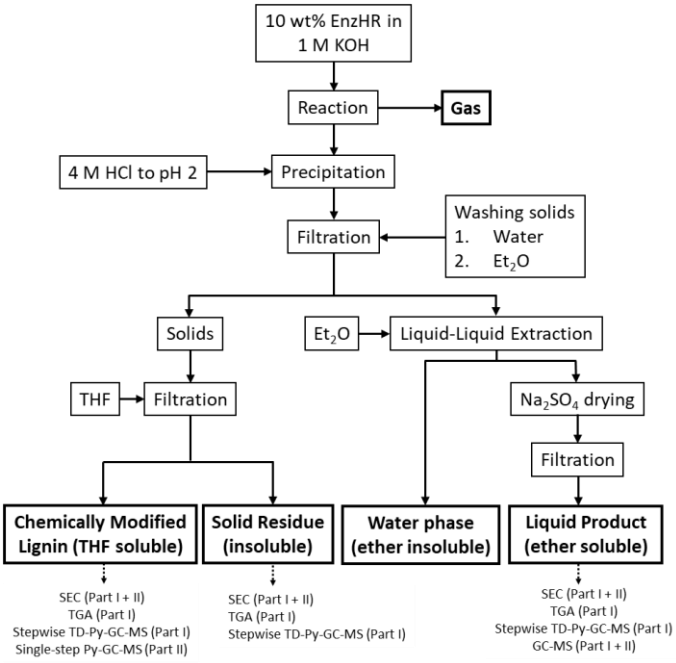

Figure 1. Procedure for hydrothermal liquefaction of enzymatic hydrolysis residues (EnzHR) and subsequent product fractionation into liquid product (LP), chemically modified lignin (CML), and solid residue (SR).

Size exclusion chromatography

The molar mass distribution of samples was determined using size exclusion chromatography (SEC) with UV detection at 280 nm. Compounds were separated on a Polymer Standards Service PolarSil column (300 x 8 mm, 5 µm, 100 Å) at 70 °C in a 9:1 (v/v) dimethyl sulfoxide/water eluent with 0.05 M LiBr. The molar mass scale was calibrated using the retention times of 5 phenolic compounds in the range 152–1701 g/mol and extrapolated using a linear regression curve of time (min) versus log(Mw).

Quantification by GC-MS

Aliquots of LP were dissolved in ethyl acetate containing 4-bromotoluene and 4-bromophenol as internal standards and were derivatized to their trimethylsilyl (TMS) derivatives. The GC-MS analyses were performed using an Agilent system (7890B GC, 5977A MSD) equipped with an Agilent VF-5ms (60 m, 0.25 mm, 0.25 µm) column. Twenty-three compounds were quantified using authentic standards. Additional 105 compounds were semi-quantified using standards of a similar compound. Peak areas below the signal detection limit were excluded. Deconvolution of peaks was performed with the PARAFAC2 based Deconvolution and Identification System (PARADISe) ver. 1.1.1.[26] The deconvoluted mass spectra were identified using the NIST11 library and a personal library of phenolic compounds.

Principal component analysis

Principal component analysis (PCA) including data preprocessing were performed using PLS_toolbox 8.2 in MatLab R2017a. The deconvoluted TIC peak areas without a lower area threshold were used as input and were mean centered and Pareto scaled.

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Thermogravimetric analysis

The weight loss from volatilization and pyrolysis of the LP, CML and SR was measured using thermogravimetric analysis (TGA) on a Mettler Toledo TGA/SDTA 851e. The TGA program was 25–700 °C (10 °C min^{-1}) under a nitrogen flow of 90 mL min^{-1}.

Stepwise thermal desorption and pyrolysis of Part I samples

Samples of LP, CML, and SR from the study of biomass and HTL temperature (Part I) were subjected to stepwise thermal desorption and pyrolysis on a CDS Pyroprobe 1000 prior to GC-MS analysis (TD-Py-GC-MS). Thermal desorption and pyrolysis using solid phase microextraction (SPME) sampling of desorbed compounds were performed using a procedure modified from a previous study.[27] Briefly, samples were heated in steps to 200, 280, 350, 420, and 500 °C at a heating rate of 0.01 °C ms^{-1} under nitrogen flow. The temperature of each step was held for 5 min. Pyrolysates were sampled on a carboxen/polydimethylsiloxane SPME fiber (Supelco), and subjected to on-fiber derivatization using a procedure modified from a previous study.[28] The SPME fiber was exposed for 15 min in the headspace of a vial containing TMS-derivatization reagents and desorbed in the GC-MS inlet (Agilent 6851 GC, 5668 MSD) at 300 °C. Desorbed compounds were separated on an Agilent HP-5ms (30 m, 0.25 mm, 0.25 µm) column. The data showed repeatability poorer than the 10–30% relative standard deviation previously reported for quantitative analysis.[27,29] The stepwise TD-Py-GC-MS data were thus only used qualitatively.

Single-step pyrolysis of Part II samples

Single-step Py-GC-MS was performed using an Agilent GC-MS system (7890B GC, 5977A MSD) equipped with a Gerstel automated pyrolysis setup as described elsewhere.[15] CML samples from the study of biomass pretreatment severity (Part II) were analyzed in duplicate. Flash pyrolysis was performed under helium flow at 500 °C. Peak areas were obtained by deconvolution of the TIC using PARADISe and normalized to sample mass (corrected for BHT content). Peak identification was performed using authentic standards, published mass spectra, or the NIST11 libraries.[30] A solution of a homologous series of straight-chain alkanes was analyzed to provide Kovats retention index (RI) in the C_7-C_{34} range. Aliphatic compounds and structural isomers of aromatic compounds were identified using the NIST RI library where possible. The RI for each identified compound is reported in Table S8 and can be used for future studies to avoid incorrect identifications from relying purely on mass spectral libraries.

Results and discussion

Product fractionation

Initial experiments were performed to study the effect of HTL temperature (255, 300, and 345 °C) on products from *Miscanthus* and wheat straw EnzHR. The product solution was fractionated into a low molecular weight fraction (LP); a high molecular weight, THF soluble fraction (CML); and an insoluble residue (SR) in order to aid interpretation of the HTL process. The yields of the product fractions from HTL of the M3.65 and W3.65 EnzHRs (Table 1) showed increased liquefaction at higher temperature (higher LP and lower CML yields). The LP, CML, and SR fraction yields suggest that liquefaction of M3.65 and W3.65 was not complete after 20 min at 255 °C.

The yields of the product fractions from HTL of the EnzHRs from *Miscanthus* and wheat straw pretreated at two different severity levels (M3.65-300, M3.97-300, W3.65-300, and W3.97-300, Table 1) showed few differences. The CML yield of W3.65 (16 wt%) was lower than W3.97 (22 wt%). Plotting the fraction yields against the lignin content in the EnzHR (Figure S1) showed that the LP yields correlated with the lignin content (r^2 = 0.87, n = 9). The HTL of EnzHR resulted in low yields of SR (Table 1) due to the concentration of

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alkaline base, extensive washing with THF, losses in the filter, and likely the type of lignin.[19,31-32] This type of product fractionation has previously resulted in mass balances of 88–92% in the solid and extracted fractions after HTL of high-purity organosolv and soda lignins.[15,33] Here, the LP, CML, and SR account for only 55–74% of the feedstock EnzHR. The unaccounted mass from lignin HTL is likely trapped CO_2 and highly volatile and water-soluble HTL products.[34] The mass balances correlate well with the content of carbohydrate content of the EnzHR (Table S1). CO_2 is the main product from HTL of cellulose.[35] The KOH used here would be able to trap CO_2 as carbonate in the aqueous product solution having pH > 10. This CO_2 was not quantified. The control samples have mass balances of 91% and 106% showing that trapped CO_2 and losses of highly volatile and water-soluble HTL products are the likely causes of the lack of mass closure.

Table 1. Average yields and associated standard deviations of the product fractions from the HTL and control experiments (20 °C) of the EnzHR.

Fraction yields (% w/w DM)										
EnzHR[1]	M3.65				M3.97	W3.65				W3.97
$T_{reaction}$	20 °C	255 °C	300 °C	345 °C	300 °C	20 °C	255 °C	300 °C	345 °C	300 °C
LP	2.0	39.1	42.8 ± 0.1	44.9 ± 0.3	48.9 ± 2.6	1.0	47.7	52.4 ± 1.3	53.5 ± 0.5	52.1 ± 0.2
CML	37.0	24.6	18.6 ± 9.3	8.7 ± 0.5	21.6 ± 1.2	68.0	24.3	15.7 ± 1.5	13.8 ± 0.3	21.9 ± 1.1
SR	52.0	6.2	1.2 ± 0.7	1.0 ± 0.2	0.5 ± 0.1	36.7	1.4	1.0 ± 0.7	1.7 ± 0.4	0.3 ± 0.2
$n_{replicates}$	1	1	2	2	2	1	1	3	2	2

[1]M: *Miscanthus* × *giganteus* stalks; W: wheat straw. The numbers denote severity factor used during biomass pretreatment: 3.65 - 190 °C 10 min; 3.97 - 195 °C 15 min.

HTL temperature study of EnzHR conversion (Part I)

Molar mass distributions of the products determined by SEC (Table S3) showed that HTL of EnzHR lignin lower the molar mass and give a more narrow molar mass distribution of the products based on comparing the average molar masses of the LP and CML product fractions with the control experiments. This is in contrast to previous results from HTL of Protobind 1000 soda lignin.[15] HTL results in both depolymerization and repolymerization of aromatics, and the relative importance of these reactions are apparently different for the two types of lignin.

Characterization of LP (Part I)Figure 2**Errore. L'origine riferimento non è stata trovata.** shows TGA-curves for LP samples. Separate TGA-curves and differential TGA-curves are included in the electronic Supporting Information (Figure S3). The TGA data revealed substantially different curves for the two replicates of M3.65-345 LP, which could be due to sample heterogeneity. Consequently, only large differences in mass losses were interpreted for the LP samples. The LP fractions from HTL at 255 °C had a lower mass loss of volatiles during TGA than the other LP, which is consistent with the quantification of monomers. This supports that depolymerization of the bulk biomass is not complete after 20 min at 255 °C. Note that HTL of lignin in low concentration and volumes has shown depolymerization to be complete within seconds.[36] Interestingly, introducing the on-fiber silylation to the stepwise TD-Py-GC-MS procedure resulted in detection of a hump of decreasingly volatile compounds continuing into the dimeric range in the pyrolysis steps of the LP (Figure S4), which indicates that volatilization of heavy compounds occurs alongside pyrolysis. This observation shows that monomers and dimers potentially can be fractionated by distillation.

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The TGA suggested that the LP consist of about 35–48% mass, which can be detected by the GC-MS method (b.p. 120–300 °C). The 5–8% of the mass evaporating at temperatures below 120 °C likely consists of residual solvent, moisture, and products with low boiling points. The total (semi-)quantified compounds corresponds to 24–29% of the mass of the LP. Thus, it appears that GC-MS with TMS derivatization is suitable for quantification of the volatile products from HTL of lignin-rich feedstocks. The 10–20% of volatiles not quantified is likely due to a large number of compounds at low concentrations. Oligomeric products appear to be the main group of compounds comprising the remaining 45–60% mass of the LP, as based on SEC relative areas of UV detectable compounds (Figure S1). The complexity of this mass fraction appear high, based on the stepwise TD-Py-GC-MS results, and characterization of oligomer products could benefit from additional, dedicated studies.

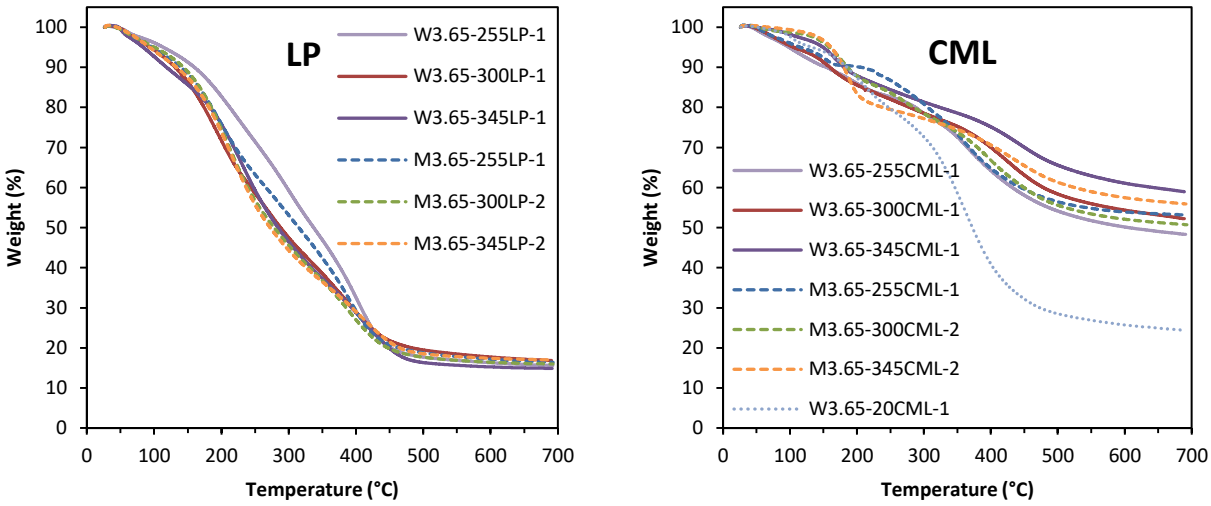

Figure 2. Thermogravimetric curves for LP and CML fractions.

PCA (Figure 3 and 4) was used to visualize and explore the GC-MS results of the LP fraction prior to quantitative analysis based on groups of compounds (Figure 5). Three principal components (PC) were found to describe the model based on the scree plot of eigenvalues. Three PCs explained 93% of the total variance of the LP. The explorative PCA showed a clear grouping on principal component (PC) 1 and PC 2 according to the latent variables of biomass and HTL temperature (Figure 3, left). PC 1 explained 51% of the total variance and separated samples according to HTL temperature. PC 2 explaining 21% of the total variance and primarily spanned the binary choice of biomasses. The loadings plot (Figure 3, right) shows the variables that contributed most to the separation. The compounds are colored according to main functional group. High loadings for hydroxy acids (mainly lactic acid and 2-hydroxybutanoic acid) and phenol on PC2 separate *Miscanthus* from wheat straw, while catechol, an unknown catechol/benzenetriol, fatty acids and acetosyringone separate wheat straw from *Miscanthus*. The higher yield of phenol from *Miscanthus* can be partly caused by the higher carbohydrate content (Table S1), since phenol is a product of both cellulose and lignin.[37] The above shows that the non-lignin HTL products, and thus non-lignin biomass content, are main contributors to the variance of the products. A HTL plant producing lignin-derived aromatic chemicals can thus expect a different product if changing from *Miscanthus* to wheat straw EnzHR as feedstock. However, the product change would appear to be mostly caused by differences in non-lignin components in the feedstocks and to a lesser degree by differences in lignin structure.

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PC 3 explained 20% of the total variance and in itself separates the 300 °C samples from the rest. The compounds that cause this separation are mainly hydroxy acids, catechol and 3-methoxycatechol that have maximum yields at 300 °C regardless of biomass. Plotting the scores of PC 1 against PC 3 (Figure 4, left) resulted in grouping according to HTL temperature, with little separation according to biomass. The corresponding loadings plot (Figure 4, right) visualizes guaiacol, syringol and acetosyringone that are highly correlated to HTL at 255 °C, and 3-methylcatechol, alkylcatechols and propanoic acid that are highly correlated to HTL at 345 °C regardless of biomass.

The overall picture from PC1, PC2 and PC3 was that the monomeric aromatics had the most lignin-like functionalities from HTL of EnzHR lignin at 255 °C, though with syringol more correlated with *Miscanthus* than wheat straw, and acetosyringone and 3-methoxycatechol produced in higher amounts from wheat straw. The 345 °C HTL resulted in a higher abundance of most catechols, with the notable exception of catechol, the single most abundant compound, which is found at highest concentration after HTL at 300 °C. Additionally, catechol and 4-methylcatechol are produced in higher abundance from wheat straw than *Miscanthus* on DM basis. The trends of the PCA model were verified by the quantitative results (Table S4). The PCA suggests that, although some information is lost, compounds can generally be grouped according to functional groups, as the overall temperature trends are similar within each class of compounds. However for studying reaction mechanisms, some exceptions, e.g., guaiacol, syringol, catechol, and 3-methoxycatechol, should be interpreted separately.

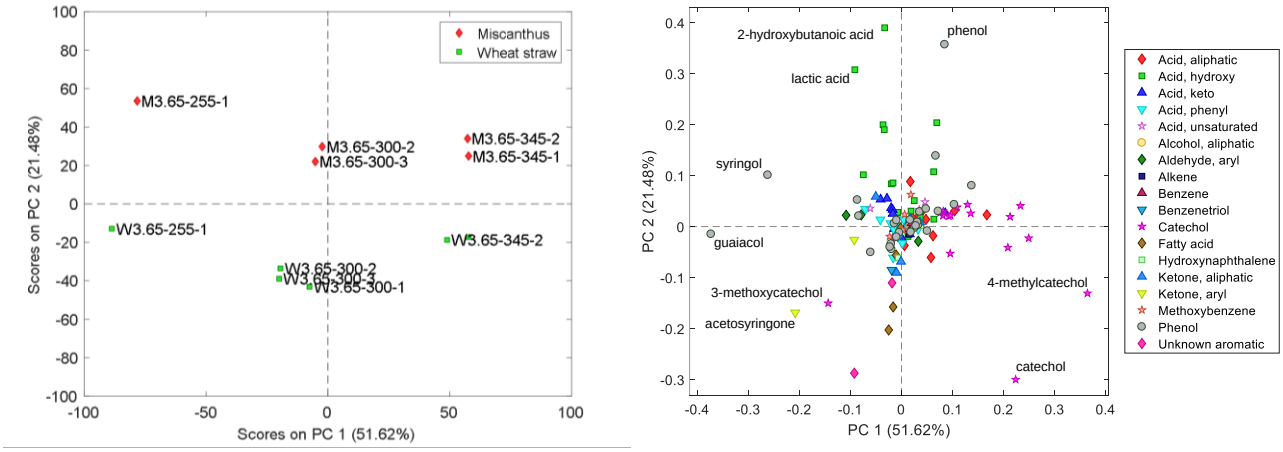

Figure 3. Scores (left) and loadings (right) plots for PC 1 and PC 2 of PCA model for the EnzHR HTL temperature study.

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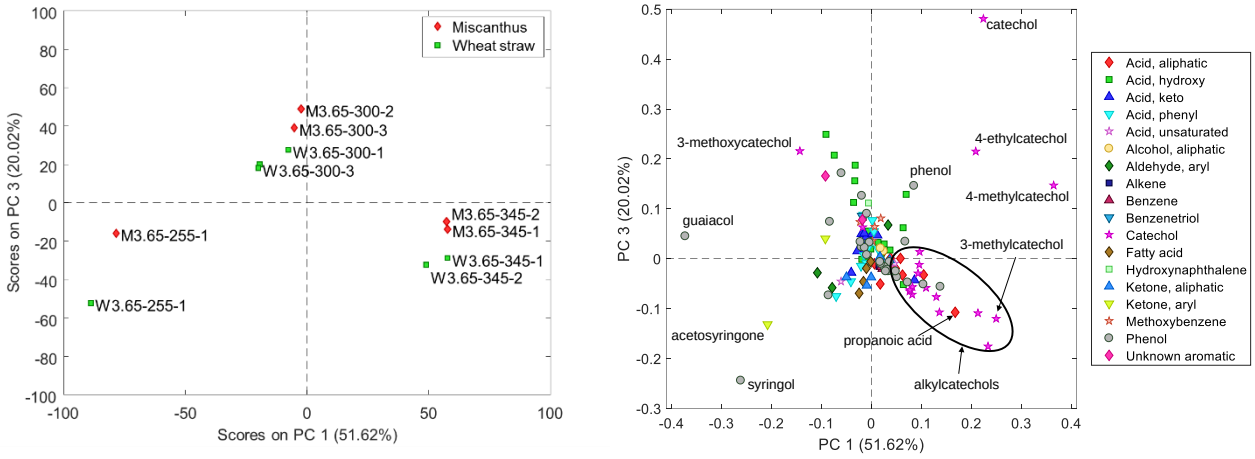

Figure 4. Scores (left) and loadings (right) plots for PC 1 and PC 3 of PCA model for the EnzHR HTL temperature study.

Inter-study comparisons of monomer yields are difficult because GC-MS results are commonly reported as relative peak areas, and when quantification of monomers is performed, it is often limited to selected compounds that vary from study to study. Here we present a procedure for quantification of a feasible number of compounds combined with a procedure for semi-quantification of a large number of products. We find that this procedure has a suitable balance between cost of chemical analysis and benefit from accuracy of quantification. Twenty-three compounds in the LP were quantified and 105 compounds were semi-quantified using GC-MS (Table S4 on DM and S5 on lignin basis). The results are summarized in Figure 5 as grouped yields on DM basis in order to include the yields of non-lignin products (yields of aromatic compounds are reported on the more relevant lignin basis below). The maximum yield of 71 quantified aromatic monomers found here was 14.4 wt% on basis of lignin content. This is lower than the 21.5 wt% (kraft lignin from Norway spruce) and 17.9 wt% (concentrated sulfuric acid hydrolysis lignin from oak) of 13 aromatics,[38] yet, higher than the 8.4 wt% of 9 aromatics (Indulin AT kraft lignin from mixed softwood),[34] and higher than the 4.6–5.2 wt% of 22 aromatics from HTL of three different organosolv lignins from olive tree pruning.[18] Despite differences in analytical procedures and HTL parameters, it would appear that the type of lignin and plant species are major contributors to the differences in yield of aromatic monomers. A recent summary of base-catalyzed depolymerization of lignin can be found elsewhere.[38]

Wheat straw and *Miscanthus* gave equal yields of total aromatics at 300 °C, yet quite different yields at 255 °C and 345 °C. M3.65-255 showed uniquely low total yield. This suggests that the two biomasses have different optimal temperature for depolymerization of the lignin. The optimal HTL temperature might not be 300 °C for either biomass and requires further investigations as it cannot be inferred from this work.

Vanillin is a widely used aroma compound and can be synthesized from guaiacol and catechol.[39] Vanillin was produced in low yield (<0.6 wt%), though in highest yield at 255 °C. The sum of vanillin, guaiacol, and catechol was largest for wheat straw at 300 °C (5.1 wt%). Syringaldehyde is a precursor for the synthesis of 3,4,5-trimethoxybenzaldehyde for the pharmaceutical industry.[40] Yet, syringaldehyde was also produced in low yield in this study (<0.5 wt%).

HTL of veratraldehyde and vanillin is suggested to proceed through a set of reactions with catechol as final product and e.g., vanillic acid, guaiacol, and 1,2-dimethoxybenzene as intermediate products, with all

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intermediates present after 6 min reaction time at 350 °C.[10] This suggests that the observed trends in temperature-dependent concentrations of aryl aldehydes, methoxyphenols, methoxybenzenes, and catechols (Figure 5) are results of reaction kinetics rather than change in depolymerization mechanisms. The trends of lignin depolymerization into methoxyphenols and catechols observed here are in agreement with a kinetic study by Forchheim et al.[41]

Lui et al.[10] demonstrated that alkaline HTL of a β-*O*-4 model compound resulted in a phenol and an aryl ketone. In the present work, the quantity of aryl ketones was found to decrease with temperature, proportional to aryl aldehydes (*Miscanthus* r^2 = 0.96, n = 5; wheat straw r^2 = 0.99, n = 6). This indicates a common reaction mechanism for aryl aldehydes and aryl ketones. A common mechanism for removal of the aryl carbonyls during HTL has not yet been identified.[10,33,42-43]

However, aryl ketones have been found likely to engage in repolymerization, and aryl carbonyls in general can dimerize through aldol addition, reducing their monomer concentration.[15,33,43] A lower yield of total quantified monomer aromatics is observed at 345 °C compared to 300 °C, supporting repolymerization and dimerization as explanations for lower levels of aryl ketones and aryl carbonyls at higher temperatures. Note that the yields of LP increase and CML decrease from 300 °C to 345 °C, which appear to be due to increased dimerization (Figure S2) while depolymerization of the lignin/CML continues.

Isomerization of alkylphenols and alkylcatechols was observed at 345 °C, resulting in a more diverse and complex LP composition. Increased rate of alkylation reactions was seen at 345 °C from the large increase of "other catechols" (Table S5), which are C_2-catechols (3/5-ethyl and dimethyl) and C_3-catechols (3/4/5-propyl and combinations of methyls and ethyls). However, assigning isomer identification to individual alkylcatechol peaks was not possible due to very similar mass spectra. Multiple alkylated phenols and catechols were also reported from HTL of *Miscanthus* at 338 °C.[46] In addition, extensive demethylation/demethoxylation of aromatic methoxy groups was observed at 345 °C, as seen from the near-zero yields of methoxybenzenes, guaiacols, syringol and 3-methoxycatechol.

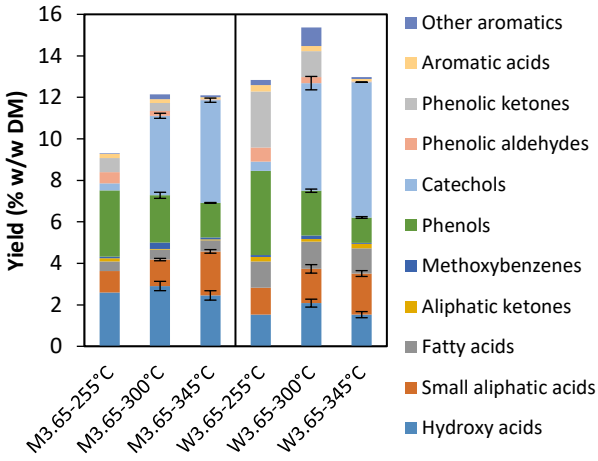

Figure 5. Yield (% w/w dry matter of EnzHR) of groups of monomeric compounds in the HTL temperature study. Standard deviations are only shown for selected groups for clarity.

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Characterization of high molar mass fractions (Part I)

The weight loss from volatilization and pyrolysis of the CML and SR was investigated using TGA. Additionally, chemical features of the volatile and thermally degradable organics of the HTL fractions were investigated by stepwise TD-Py-GC-MS. Figure 2 shows TGA-curves for a CML sample for each biomass and HTL temperature. Additionally, all CML and SR TGA-curves and differential TGA-curves are included in the electronic Supporting Information (Figure S5 and S6).

The pyrolytic mass loss of the CML from the control experiment W3.65-20 was significantly larger and initiated at a lower temperature than for the CMLs after HTL. Additionally, thermal stability of the CML appears to be higher after HTL at 345 °C than the lower HTL temperatures. The increased thermal stability after HTL is likely a result of a more condensed structure. Lignin requires thermal stabilization prior to pyrolytic conversion into carbon fibers.[6] The lignin HTL products could be candidates for feedstock for carbon fiber production. Additionally, increased thermal stability could be beneficial for using the lignin HTL products for adhesives in materials where pyrolytic conditions can occur during a fire.

TGA of SR was only possible for M3.65-255 and W3.65-20 due to low SR yield in the other experiments in Part I. The differential TGA-curves showed a simple mass loss pattern with a large mass loss within a narrow temperature interval with a maximum at 345 °C and a less distinct mass loss over a wider temperature interval with maximum at 410 °C. Similar mass loss characteristics are observed for extracted α-cellulose and Klason lignin, respectively.[47] The presence of a characteristic cellulose mass loss in one or more fractions of the control experiment was expected due to the glucan content of the EnzHR. It is more surprising that cellulose appears not to have depolymerized completely during HTL at 255 °C, since carbohydrates are generally hydrolyzed above 140 °C at alkaline conditions.[48] Stepwise TD-Py-GC-MS found aromatic pyrolysates alongside a dominating levoglucosan (cellulose pyrolysate) presence in the SR of both M3.65-255 and W3.65-20. It would consequently appear that the SR fraction from HTL of EnzHR at 255 °C mainly contains unconverted or partly converted cellulose with a trace of lignin-like polymers.

Effects of biomass pretreatment severity on HTL of EnzHR (Part II)

The analytical approach for studying the effects of biomass pretreatment severity was focused on fewer methods based on the observations from Part I. Quantification of monomers was performed using GC-MS, molar mass distributions were determined by SEC, and chemical features of the CML fraction were investigated by single-step Py-GC-MS. HTL at 300 °C was chosen for the study of potential effects of biomass pretreatment severity on the HTL products, based on the maximum yield of total monomeric aromatics and the bifunctional catechol. Also, optimization of yields of high-value compounds (e.g. vanillin and syringaldehyde) were not pursued due to the lack of selectivity within the parameter space of Part I.

Quantification of monomers in LP (Part II)

The yields of the 128 quantified compounds are listed in Table S6 and S7 on DM and lignin basis, respectively. The higher severity of biomass pretreatment resulted in lower abundance of hydroxy acids and higher abundance of fatty acids and aromatics in the HTL product. The hydroxy acids likely derived from carbohydrates since the yields correlate with cellulose and hemicellulose content of the EnzHR (Figure S8, $r^2 = 0.82$, n = 9 and $r^2 = 0.94$, n = 9, respectively). The higher abundance of fatty acids is likely a result of an increase in relative abundance of extractives in the EnzHR due to lower residual carbohydrate content at higher pretreatment severity. The higher yield of aromatics on DM basis at higher pretreatment severity will at least partly be a result of the higher lignin content of the EnzHR. Consequently, the yields of

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aromatics will in the following be discussed on lignin basis in order to explore the effects of biomass pretreatment severity on HTL of the lignin. The yields of aromatics are summarized in Figure 6 as grouped yields on lignin basis. Regardless of pretreatment severity, *Miscanthus* EnzHR produced more phenol, *p*-cresol and 4-ethylphenol than wheat straw, which in turn produced more acetosyringone than *Miscanthus*. The data from single-step Py-GC-MS (see below) indicate that it is at least partly a result of differences in H and S monolignol content. Catechols (except 3-methoxycatechol) and methoxybenzenes were found in higher yields at the low severity. The phenolic aryl carbonyls, other aromatics, and likely also phenols were not sensitive to pretreatment severity.

Figure 6 shows that HTL of wheat straw and *Miscanthus* EnzHRs hydrothermally pretreated at log R_0 3.65 resulted in higher yield of monomeric aromatics on lignin basis, and hence, more effective lignin depolymerization, than pretreated at log R_0 3.97. The less effective depolymerization at log R_0 3.97 is likely a result of fewer ether and more C-C bonds in the more severely pretreated lignin. This shows that lignin depolymerization is affected by changing pretreatment severity within even a narrow range. Choosing pretreatment severity would thus become a matter of whether the most effective lignin depolymerization (log R_0 3.65) or a more concentrated aromatic product fraction (log R_0 3.97) is best for the direct use (e.g. resin) or cost of purification of the HTL product. The aromatic monomer yield trend is in contrast to the observed lowest glucose yields by enzymatic hydrolysis for the log R_0 3.65 pretreated biomasses of this study.[23] Furthermore, conversion of wheat-straw cellulose into ethanol increases with increased severity in the log R_0 3.28–3.88 range.[49]

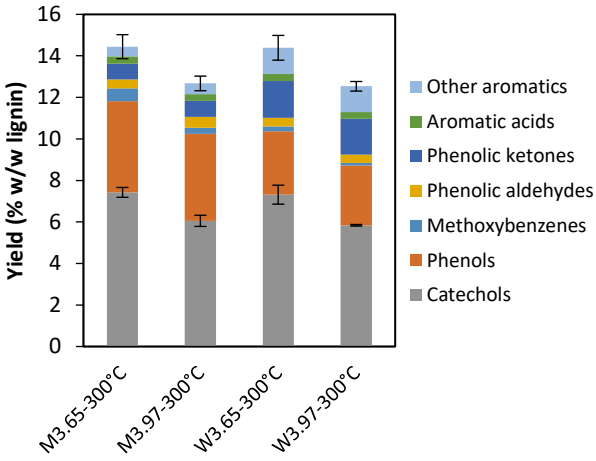

Figure 6. Yield (% w/w lignin (AIL+ASL) of EnzHR) of compounds in the HTL study of biomass pretreatment effects. Standard deviations are shown for catechols and the total sum.

Characterization of CML fraction (Part II)

Single-step Py-GC-MS of the high molecular weight fraction, CML, (Table 2) gave additional information and confirmed trends seen from GC-MS of the LP. The higher severity of biomass pretreatment resulted in higher abundance of extractives in the HTL product, supporting the higher fatty acid content found in the LP from HTL of the log R_0 3.97 pretreated biomasses. The lower yield of monomeric aromatics on lignin basis at higher pretreatment severity (Figure 6) was mirrored by a seemingly higher abundance of aromatic

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pyrolysates in the corresponding CML fraction. However, the aromatic results are inconclusive due to the larger uncertainty of the Py-GC-MS results.

HTL resulted in a clear decrease in the abundance of pyrolysates produced from native biomass molecules (carbohydrates, lignols and hydroxycinnamic acids). An almost complete disappearance of native lignin structure and hydroxycinnamic acids after HTL has been reported for Protobind 1000 and Lignoboost lignins.[15,50] An increased abundancy of aromatic nitrogen pyrolysates was observed after HTL, which requires further investigation, yet, could be a result of reaction between protein and lignocellulose.[51]

HTL could result in isomerization of aliphatic substituents on the phenyl ring of the macromolecular HTL products due to repolymerization, in addition to the isomerization of monomeric aromatics observed in Part I. A previous Py-GC-MS study in our group found that *ortho*-directed reactions are important for repolymerization during HTL.[15] Here HTL appears to increase the relative abundance of *o*-isomers and decrease the *p*-isomers in CML when including all types of aliphatic substituents (*ortho*-Ph% and *para*-Ph%). However, this calculation contains a much larger number of *p*-compounds than *o*-compounds due the native structure of lignin. The results are ambiguous when calculating the *o*/*p*-ratio using only the *o*-alkyl phenolics and their corresponding *p*-isomer (*o*/*p*-subgroup). The near equal *o*/*p*-subgroup ratios after HTL could be a result of loss of alkyl substituents followed by both *p*- and *o*-directed repolymerization after HTL.[51]

HTL of EnzHR resulted in higher H and lower S abundance of the CML fraction compared to the control CML. This could be due to more repolymerization of H units compared to G and S, as well as more efficient depolymerization of the S units that have fewer interunit C-C bonds in the native lignin.[15,19] *Miscanthus* CML was found to have higher H monomer content than wheat straw, both before and after HTL. Pretreatment severity did not affect the post-HTL H/G/S ratios significantly in the CML fraction.

Implications for biorefining

The results of this study show that plant species and pretreatment severity will affect the HTL product composition and lignin depolymerization. These parameters are important choices for processes upstream of HTL, and should consequently be considered carefully for a biorefinery concept based on lignin valorization of enzymatic hydrolysis residue by HTL. The trends seen by varying HTL temperature were generally in agreement with previous results from HTL of other biorefinery lignins.[19] Additional information was found on temperature dependency of non-lignin products and alkylation of aromatics. *Miscanthus* and wheat straw showed differences in temperature-dependent behavior. *Miscanthus* and wheat straw yielded on lignin basis equal amounts, yet different distributions, of monomeric aromatics at 300 °C. Lignin depolymerization during HTL was more effective for both biomasses when pretreated at log R_0 3.65 than 3.97. This shows that biomass pretreatment severity must be taken into account for the optimization of lignin depolymerization. Additionally, it suggests that pretreatment severity for optimal lignin depolymerization has the opposite trend compared to cellulose conversion into bioethanol. Consequently, it will be necessary in a future biorefinery to identify the overall optimal pretreatment conditions for obtaining maximum value of the entire biomass by balancing yields of ethanol and aromatics.

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Table 2. Summary of results (peak areas) from single-step Py-GC-MS of CML fractions from HTL and control experiments.

	M3.65-20°C	W3.65-20°C	M3.65-300°C	M3.97-300°C	W3.65-300°C	W3.97-300°C
Pyrolysates						
Carbohydrate	+	+	-	-	-	-
Lignols	+++	+++	+	+	+	+
Hydroxycinnamic acids	++++	++++	+	+	+	+
Aromatic N	+	+	+++	+++	+++	+++
Extractives	++	++++	+++	++++	+	++
Catechols	++	++	+(+)	++	+(+)	++
Phenols	++++	++++	+(+)	++	+(+)	++
Methoxybenzenes	++	+	+++(+)	++++	++(+)	+++
Ph-aryl carbonyls	++++	++++	+	+	+	+
Phenolic R-isomers						
meta-Ph%	1.8 ± 0.1	2.4 ± 0.2	1.8 ± 0.01	1.9 ± 0.2	2.1 ± 0.2	2.1 ± 0.3
ortho-Ph%	2.4 ± 0.4	2.9 ± 0.7	16.2 ± 0.2	19.1 ± 0.8	16.7 ± 1.0	17.1 ± 0.5
para-Ph%	95.7 ± 0.5	94.7 ± 1.0	82.0 ± 0.2	79.1 ± 0.7	81.2 ± 1.1	80.9 ± 0.5
o/*p*-subgroup*	0.29 ± 0.004	0.43 ± 0.001	0.35 ± 0.004	0.38 ± 0.012	0.34 ± 0.023	0.35 ± 0.011
Monomer ratios						
H%	16.6 ± 1.7	11.8 ± 2.3	52.6 ± 1.5	52.8 ± 2.8	46.2 ± 2.1	42.6 ± 1.9
G%	51.6 ± 0.03	52.1 ± 0.5	44.5 ± 1.3	44.9 ± 2.5	49.3 ± 1.9	52.8 ± 0.7
S%	31.9 ± 1.7	36.1 ± 2.9	2.9 ± 0.2	2.3 ± 0.4	4.6 ± 0.6	4.7 ± 1.4

The symbolic scale (number of +) is based on pyrolysate peak areas and is relative within each row, i.e., ++ in phenols and catechols are not equal. Parentheses emphasize uncertainty.

*Calculated as the ratio between the *o*-phenolic pyrolysates and their corresponding *p*-isomer, $n_o = 4$, $n_p = 4$.

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Supporting Information

Detailed experimental procedures. BHT content of CML fractions. SEC results. Yields of quantified monomeric compounds on both DM and lignin basis. TGA and differential TGA curves, including a figure of combining TGA and stepwise TD-Py-GC-MS results. Linear regressions of fraction and compound yields as functions of biopolymer contents. Results of single-step Py-GC-MS, including pyrolysate categorization and RI for improved identification of pyrolysates.

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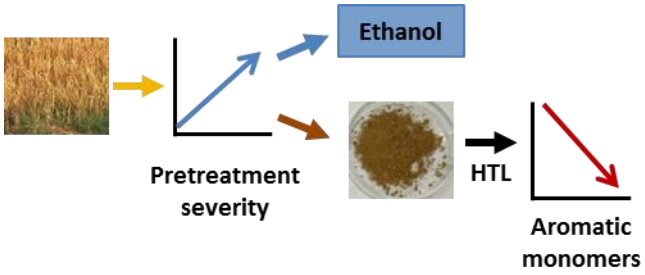

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