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Uncovering Challenges for Complete Carbohydrate to Bioethanol Utilization in Reductive Catalytic Fractionation Biorefinery

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KEYWORDS

Green Chemistry, Lignocellulose Biomass, Catalysis, Reductive catalytic fractionation, Fermentation, Bioethanol

ABSTRACT

The reductive catalytic fractionation (RCF) biorefinery, developed for high quality lignin oil extraction from lignocellulose biomass, concurrently produces a (hemi)cellulose pulp, suitable for bioethanol production. Depending on RCF severity and delignification, 5 to 25 % of the biomass' carbohydrates are solubilized, ending up in a distinct water fraction. With premise of full biomass exploitation, while minimizing waste and their processing costs, this work studies the integration of both pulp and water fraction from varied RCF processing parameters into separate hydrolysis and fermentation (SHF) using birch wood and a xylose-utilizing *Saccharomyces cerevisiae* strain. Besides advancing water fraction characterization revealing certain (reduced) sugar (oligomers), methyl sugars and shorter polyols, efficient saccharification of the RCF pulps, up to 98 % sugar yield, is observed. With high delignification

and carbohydrate solubilization in RCF, a significant 6.2 % ethanol titer increase can be achieved by addition of the water fraction to SHF. We identify both carbohydrate polymers and α/β-methyl carbohydrates in the water fraction as carbon source for additional bioethanol production. Introducing water fractions from alternative RCF conditions results in similar or lower ethanol yields, influencing both saccharification and yeast's xylose utilization. From the perspective of the entire, raw biomass, the maximum carbohydrate to bioethanol utilization is achieved with specific RCF parameters that focus on sufficient delignification and high carbohydrate retention, concurrently obtaining water fractions with low carbohydrate content that is better not used for bioethanol production, overall yielding 79 % sugar and 59 % bioethanol, resp. of their theoretical maximum, comparable to the reported yields for established second generation carbohydrate-centered biorefineries using hardwood.

INTRODUCTION

Reductive catalytic fractionation (RCF) is an emerging lignin-first biorefinery concept with upscaling potential[1-3] and economic feasibility[4-6] that aims to fractionate lignocellulose into two primary products: high quality lignin oil and carbohydrate pulp. A general RCF process entails a reaction at elevated temperature (180 to 260 °C) and process pressure (20 to 120 bar) with utilization of an organic solvent (typically an alcohol), an inorganic redox catalyst (e.g., Pd/C[7-12], Ru/C[2, 8, 13-15] or Ni-Al$_2$O$_3$[2, 5, 16-19]) with a hydrogen donor (*e.g.,* H$_2$ or alcohols). The solvolytic extraction capability, combined with the reductive chemical stabilization of lignin-derived intermediates, facilitated by the redox catalyst and hydrogen donor, yields a low molecular weight lignin oil containing up to 50 % of a handful of guaiacol/syringol monomers, which is close to the theoretical maximum (as based on the β-O-4 content)[8], next to guaiacol/syringol dimers and oligomers. The obtained liquid lignin oil is functional, free of contaminants such as salts and sulfur, and can serve as a resource for multiple applications generally including valorization of the guaiacol/syringol monomers to drop-in chemicals (*e.g.,* phenols[20], bisphenols[21], catechol[22], propylene[5], aromatic amines[23, 24] and biofuels[25, 26]). Also the higher molecular weight fraction (dimer and oligomers) has shown potential due to its unique functionalities, such as high aliphatic primary OH content, enabling production of polyurethane[26], epoxy resins[27-31], temperature resistant lubricants[32] or varnish and ink production[5].

Unlike carbohydrate-centered biorefineries[33-37], generally less attention is attributed to the quality of the resulting pulp and the carbohydrates that dissolve during lignin-first concepts like RCF. The latter sugar stream can account for 5 to 25 % of the initial biomass carbohydrates (mainly hemicellulose) and ultimately accumulates in a water fraction, originating from a liquid-liquid extraction during the work-up of the crude (CLO) to refined lignin oil (RLO)[4]. Due to its crystalline nature, cellulose is less

prone to being solubilized and co-extracted[38]. Depending on the solvent composition and/or the RCF process conditions, the solubilized carbohydrates can be chemically modified. Although not studied systematically, literature provides some insight into the water fraction composition. For example, in the case of RCF with pure methanol as solvent and birch wood - of which hemicellulose mainly consists of xylan - all xylose monomers observed by GC analysis are methylated[9, 13]. Using a solvent with a high water-to-alcohol ratio, not only increases solubilization of the carbohydrates, including part of the (amorphous) cellulose, but also their conversion to sugar alcohols, *e.g.*, xylitol for xylose and sorbitol for glucose[14]. Besides these solubilized carbohydrates, also sugar oligomers and smaller amounts of glycerol, ethylene glycol and propylene glycol were identified in the composition of water fraction after RCF[4, 8, 14, 39, 40]. Although research has started to show interest in this water fraction[41], exploration of its potential as a valuable source of sugar(-derived products) for use in any downstream process has not been explored.

RCF has proven successful to refine forestry waste feedstock into value-added lignin oil and a carbohydrate pulp. The carbohydrate pulps for instance perform very well as substrate for the separate enzymatic hydrolysis and fermentation (SHF) with production of bioethanol in several publications already[2, 5, 16], while also more prone to saccharification compared to pulp from a classic organosolv pretreatment[17]. However, the field of organosolv research is advancing and shows progress to both improve quality of the lignin products and the pulp's susceptibility to enzymatic hydrolysis[42, 43]. Negative effects of pulp with residual lignin on the subsequent biochemical conversion of RCF pulp, including bioethanol production with *Saccharomyces cerevisiae*, are currently debated; for example, questioning the necessity of substantial additional washing steps[41] or not[16] of the resulting RCF pulps to achieve high bioethanol yields. Although requiring more exploration, the observed discrepancies are most likely caused by process parameters such as feedstock type, solvent composition, process conditions and catalyst type that impact the pulp properties and subsequently also affect both the saccharification and fermentation. Another aspect that complicates comparison of data is the different manners of bioethanol yield calculations, either based on the carbohydrate content of the refined pulp[16] or the sugars released after saccharification[41]. In fact, the sugars solubilized during RCF are neglected in both of these yield calculations and, instead, should also be included in any report on biorefineries to depict realistic product yields, that are based on the raw biomass feedstock. This again pinpoints the issue of integrating solubilized carbohydrate streams with calculations that should be based on the entire biomass that enters the biorefinery.

Fractionation technologies play a pivotal role in the success of biorefineries and aim to concurrently achieve delignification and enhance surface area for subsequent saccharification, all while avoiding degradation of solubilized carbohydrates into inhibitors such as furfural and hydroxymethylfurfural

(HMF)[39, 44-50]. The reported ethanol yields based on crude second generation biomass vary and highly depend on the type of biomass, with a significant amount of literature focusing on agricultural residues[51]. Conversely, reports that include both full conversion of wood biomass such as forestry waste or post-consumer wood waste through pretreatment and fermentation of both xylose and glucose, while also providing sufficient information on sugar losses are scarce with reported crude-biomass-to-ethanol yields ranging from 59[52] to 81 %[53] from hardwood (Table S1).

This study aims to examine the effect of combining the water fraction and pulp from the RCF biorefinery into a SHF set-up to maximize carbohydrate exploitation. While using identical Ni-Al$_2$O$_3$ and hydrogen donor (H$_2$), different RCF pretreatments on birch, involving both temperature (200 °C, 235 °C and 250 °C) and solvent composition (methanol and methanol/water mixture) variations, are performed to obtain pulps and water fractions with different compositions. The obtained pulps are subjected to enzymatic hydrolysis and sugar yields are compared. In-depth analysis is performed on different water fractions with identification of methylated sugars, sugar oligomers, sugar alcohols, next to sugar breakdown-products being smaller alkane diols and polyols with trace amounts of lignin monomers and deoxygenated sugars. No fermentation inhibitors, such as HMF and furfural, were detected. The obtained water fractions are then combined with the respective pulp from the RCF reactions during SHF, allowing comparison of sugar yields and ethanol titers to the control without a water fraction present. Finally, the integrated approach of RCF and SHF coupling, for maximum carbohydrate conversion towards bioethanol, is calculated with respect to the investigated RCF conditions. Overall, this study provides insight and guidance to use solubilized carbohydrate streams that originate during biorefineries and highlights challenges for its valorization by integrating them into downstream biochemical processes.

EXPERIMENTAL SECTION

A more detailed description of the experimental procedures can be found in the supporting information.

RCF experiments

RCF reactions were performed in a 2 L stainless steel reactor (Parr instruments & Co.) with compartmentalization for biomass (birch, 2 mm) and nickel catalyst pellets (Ni-Al$_2$O$_3$) and pressurized with hydrogen gas (30 bar at room temperature). The reaction mixture was stirred (750 RPM) and heated by a heating mantle that was placed around the Par reactor to reach respective temperatures of 235 °C (235°C-RCF1), 200 °C (200°C-RCF2) or 250 °C (250°C-H-RCF3) (time to reach temperature ~

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40 min/30 min/45 min) and kept constant for 3 h. Next to reaction temperatures, also solvent composition are varied and comprised either pure methanol (235°C-RCF1, 200°C-RCF2) or a mixture of methanol and water (ratio 80:20 v/v) (250°C-H-RCF3). The resulting pulp was washed with ethanol and dried at 80 °C overnight, while the solvents were evaporated resulting in a crude lignin oil (CLO). Liquid-liquid extraction of the CLO with ethyl acetate and water is used to obtain a refined lignin oil (RLO) and a water fraction containing solubilized carbohydrates and side-products. Complete compositional analysis of pulp and RLO were performed according to standardized procedures with an analytical toolbox including HPLC, GPC, GC and GC-MS.

Analysis of the water fraction

The monomeric components of the water fraction were identified and quantified *via* trimethylsilylation with myo-inositol as internal standard that was added after complete evaporation of the water and subsequently analyzed with GC and GC-MS. These were also verified by 2D HSQC NMR analysis in addition to exclude presence of furfural or hydroxymethylfurfural (HMF). Next to quantifying the total carbon content (TOC), size-exclusion chromatography (HPLC-SEC) was used to determine the degree of polymerization of remaining carbohydrate oligomers in the water fraction. Lastly, the oligomer content in the water fractions was quantified by acid hydrolysis[54] and subsequent conversion to alditol-acetates[9, 55, 56] and GC analysis, with identical procedure used to determine the carbohydrate content of the pulp.

Saccharification and separate hydrolysis and fermentation (SHF)

Saccharification and SHF were conducted in 10 mL crimp cap glass vials (Genetec) and 20 mL headspace vials (Restek), respectively, with working volumes of 3 mL and 10 mL. In both cases, pulp content was 10 % w/v in sodium citrate buffer (100 mM, pH 5 ±0.05). The enzyme cocktail Cellic® Ctec3 was used for hydrolysis of the pulp, of which the stock solution's activity was measured at 380 filter paper units (FPU) per mL with the 3,5-dinitrosalicylic acid (DNS) microplate-based protocol[57] and a protein content of 236 mg.g^{-1} or 266 mg.mL^{-1} measured with the Kjeldahl method[58]. In all cases, a final enzyme loading of 35 FPU or 27.6 mg protein/g pulp was used. Complete synthetic medium (CSM) was added to provide sufficient nutrients for fermentation during SHF. In case a water fraction was added, the water fraction was first dried by rotary evaporation and nitrogen gas flow and then redissolved in buffer containing buffer, enzyme cocktail and CSM, as described above. After 48h of saccharification at 50 °C, the slurry was cooled down and the xylose-utilizing *Saccharomyces cerevisiae* strain BMD44[59] was pitched at OD$_{600}$ 5.5, corresponding to 1 g dry weight per liter, with subsequent fermentation at 30 °C for 72 h. For both saccharification and SHF experiments, samples were taken at

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24 h intervals and analyzed on HPLC for quantification of sugar and ethanol as described in the experimental section of the supporting information.

RESULTS AND DISCUSSION

During RCF of lignocellulosic biomass, previous studies have shown that most of the carbohydrates are retained in a solid pulp, while 5 to 25 % are solubilized and accumulate in the crude lignin oil (CLO)[4]. Separation of the carbohydrate-derived products from the phenolics lignin components is achieved here by a threefold extraction using ethyl acetate and water. Phenolic lignin components are extracted in the ethyl acetate fraction, while the carbohydrate-derived products are in the water fraction. The current study explores maximizing utilization of both carbohydrate streams, the solid pulp and the water fraction, by integrating them into an SHF process. Moreover, different RCF process conditions were used (Figure 1) with the goal to vary the amount of carbohydrate solubilization and consequently the pulp and water fraction composition to investigate which RCF process conditions result in the highest overall ethanol yields.

RCF of birch wood with various process conditions

All RCF experiments in this study (Figure 1) were performed with birch biomass and Ni-Al$_2$O$_3$ catalyst pellets that were readily separated from the biomass with a catalyst basket[16] to obtain a clean pulp that does not contain any catalyst, facilitating its downstream conversion towards bioethanol. Pulp and lignin oil compositions will briefly be discussed, followed by an in-depth analysis of the water fraction compositions. Standard process conditions for the first RCF (235°C-RCF1) entails a 3-hour reaction at 235 °C using methanol as solvent and a H$_2$ pressure of 30 bar (set at room temperature).
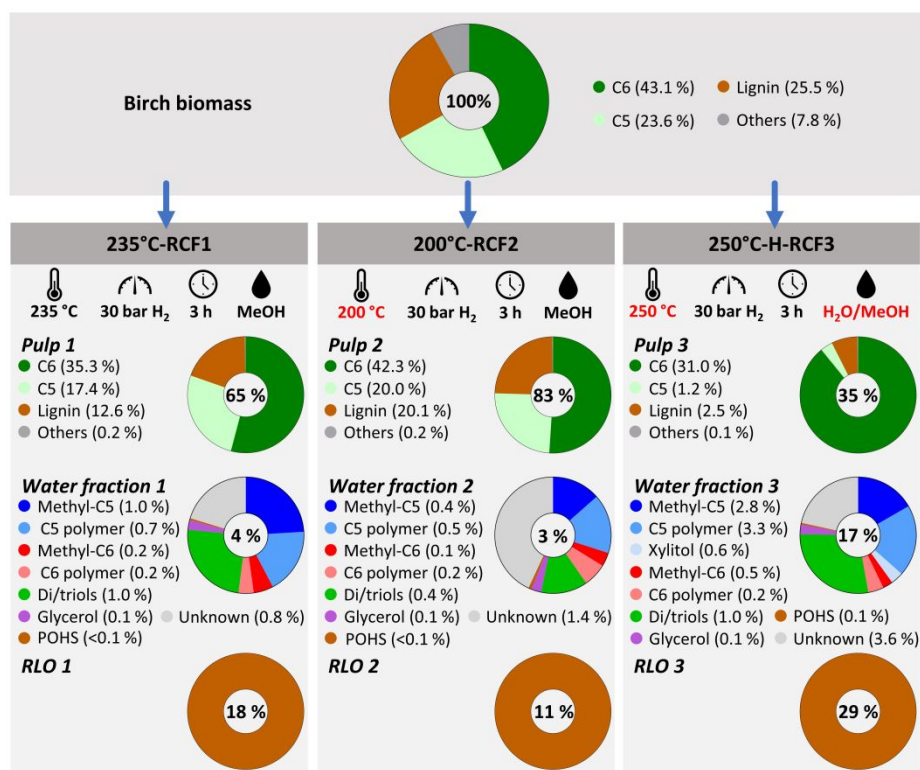
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Figure 1. Birch biomass fractionation through three different RCF process conditions using Ni-Al$_2$O$_3$ catalyst pellets with varying temperature and solvent composition: 235°C-RCF1, 200°C-RCF2, 250°C-H-RCF3. Abbreviations: RCF, reductive catalytic fractionation; MeOH, methanol; C5/C6, carbohydrates containing 5 (xylose, arabinose) or 6 (glucose, galactose, mannose) carbons; Methyl-C5/C6, methylated C5 (xylose) or C6 (glucose) carbohydrates; POHS, 4-propanolsyringol; RLO, refined lignin oil. RCF was conducted with Ni-Al$_2$O$_3$ catalyst pellets, 30 bar H$_2$ (at room temperature) and 3 h at indicated temperature for all conditions. Temperature (200, 235, 250 °C) and solvent composition (pure methanol (MeOH) or a mixture of methanol:water (20 % v/v)) were varied between the reactions, as indicated. The composition is shown graphically as wt% (based on dry matter) of each fraction and numerically as wt% of the initial biomass. Acetyl, extractives and ash content present in the biomass is represented as 'others'. Lignin oil wt% is somewhat overestimated due to the concomitant removal of extractives from the biomass during RCF. Detailed composition of birch biomass (Table S2) pulp (Table S2), water fraction (Figure S1) and lignin oil (Figure S2) can be found in supporting information.

Figure 1 shows the composition of each obtained fraction after RCF graphically, while numeric values display weight percent relative to the original biomass. Analysis of pulp 1 fraction revealed a higher retention (Table S3) for cellulose (82 %) than hemicellulose (73 %) and a lignin content of 19.4 wt% (ASL + Klason lignin content, Table S2), with a delignification of 50.4 %. Moreover, the extracted lignin oil shows a monomer content of 33.9 % (wt% of the lignin oil), predominantly composed of 4-propanolsyringol (POHS) and 4-propanolguaiacol (POHG, Figure S2) next to guaiacol/syringol dimers, trimers and oligomers (Figure S3), in agreement with Van den Bosch, 2017[16].

In comparison to 235°C-RCF1, increased carbohydrate retention (*viz.,* lower carbohydrate solubilization) was achieved by lowering the temperature from 235 to 200 °C in 200°C-RCF2, while

retaining the other RCF process parameters (Figure 1). Nearly all cellulose was retained in the pulp, next to a high retention for hemicellulose (86 %) (Table S3) with a delignification of 21 %. Although a lignin oil was obtained, the combined effect of reduced delignification and inherent removal of acetate groups and extractives during RCF resulted in a high lignin content in the birch pulp 2 of 24.4 wt%, only slightly lower than the 25.5 wt% in the original biomass (ASL + Klason lignin content, Table S2). Nevertheless, the extracted lignin molecules were successfully stabilized as evidenced by a similar monomer yield of 29.4 % (Figure 2S) and similar molecular weight distribution (Figure S3).

The last RCF was tuned to increase carbohydrate solubilization by a combination of increasing temperature to 250 °C and using a mixture of 20 % (v/v) water and methanol as solvent (250°C-H-RCF3, Figure 1). While a significant amount of cellulose was retained in the pulp 3 (72 %), only 5 % of hemicellulose remained, accompanied by a low pulp lignin content of 7.2 wt% (ASL + Klason lignin content, Table S2). Despite reaching the highest delignification of 90.2 %, compared to 235°C-RCF1 and 200°C-RCF2, the monomer content of the lignin oil was measured at 6.4 % (Figure S2) and, accordingly, contains mainly higher molecular weight lignin components (Figure S3). This indicates that lignin stabilization was less sufficient under these conditions, and instead some repolymerization of the extracted lignin molecules occurred after solvolysis during RCF. RCF with alcohol/water mixtures has been performed in the past with powdered noble metals Pd/C[60] and Ru/C[14, 15], where presence of up to 70 % water did not compromise monomer yield in comparison to RCF with pure alcohols. The nickel pellets used in this research, however, notably do not provide the same quality of metal-catalyzed stabilization of lignin molecules during RCF, indicating that developing and testing highly efficient pellets of noble metal carbon supported catalysis or hydrothermally stabilizing Ni catalysis, are important aspects to be investigated. This is work in progress, but not the purpose of this study.

This study devoted substantial efforts to thoroughly investigate the composition of water fraction, containing carbohydrate-derived molecules, with the aim of obtaining complete insight in the mass balance thereof. Prior studies showed that the RCF conditions determine the chemical modification of the dissolved carbohydrates, and hence the water fraction composition. In the case of pure methanol as solvent, methylated α/β-xylose is predominantly found[9, 13], whereas a high water to alcohol solvent ratio promotes the formation of xylitol[14].

Of the original biomass, 4.1, 3.2 and 17.0 wt% is obtained as carbon-containing molecules that end up in water fraction for 235°C-RCF1, 200°C-RCF2 and 250°C-H-RCF3, respectively (Figure 1). Thus, by varying temperature and water contents in the solvent during RCF, the amount of carbohydrates that solubilize can be tuned, which is in line with previous observations[13, 60]. Analysis of water fraction by GC-FID and GC-MS (Figure S4, Figure S1) confirmed the presence of methylated sugars, as was

expected based on prior knowledge[13] with both methyl-furanosides, and methyl-pyranosides for C5 (majority being xylose) and C6 (majority being glucose). Next to these methylated sugars, also deoxygenated (methyl) sugars were observed, and (small) quantities of di-methylated sugars, as based on GC-MS analysis. For ease of quantification and sake of discussion, they were all considered to be C5- or C6-methyl sugars (Figure 1). Due to mainly hemicellulose extraction during RCF, methyl-xylose represented most methyl-sugars in the water fraction components with 24.1, 13.5 and 16.6 % for 235°C-RCF1, 200°C-RCF2 and 250°C-H-RCF3, respectively (Figure 1, Figure S1), while methyl-C6 sugars were observed in much lower quantities.

Interestingly, a significant amount of smaller C2-C4 sugar-derived alkane polyols were also present with the majority being ethylene glycol in all water fractions (Figure S1). Conversion of sugars to ethylene, propylene glycol, and glycerol occurs through a combination of isomerization, retro-aldol reaction, hydrogenolysis and hydrogenation reactions[61] and their presence has previously been reported in water fraction after RCF wood processing under similar conditions[8]. Next to alkane polyols, the longer 1,2,5-pentenetriol was also observed, and comprises 16.8 % of the quantified polyols in water fraction 2. Combined, the aliphatic di- and triols accounted for 24.5, 12.9 and 28.4 % for water fraction 1, water fraction 2 and water fraction 3, respectively.

Dehydration of sugars during common lignocellulose pretreatment methodologies often occurs, with the formation of furfural or HMF that are considered inhibitors due to their negative effect on the subsequent fermentation process[62-64]. However, we do not observe these products in the initial GC-FID/MS analysis of any obtained water fractions (Figure S4). To rule out the formation of these inhibitors, water fraction 3 was further characterized by NMR analysis (see supporting text ST1). The presence of water in 250°C-H-RCF3 was thought to act as a mild acid under these high temperature conditions, which supports the acid-catalyzed dehydration of glucose or xylose to form furfural or HMF, with subsequent condensation reactions to form the highly polymerized molecules referred to as humins[65, 66]. Although the macromolecular structure of humins does not allow complete solubilization in water or organic solvents, and thus likely precipitate on pulp or any heterogeneous catalyst present in the reaction, a limited part of humin precursors (*viz.,* HMF and levulinic acid) or oligomers is said to dissolve[65] and should be detectable in case humin formation occurs. NMR analysis supports those from GC: no furans or humin precursors are detected (see supporting text ST1). Hence, we can conclude that the presence of hydrogen during the RCF reaction competitively prevents sugar dehydration and the formation of furfural or HMF as inhibitory compounds for fermentation.

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Also, the oligomeric carbohydrate content was analyzed by using identical methodologies as were used for the substrate and pulp composition (see experimental procedures in supporting information for details). Due to the highest amounts of sugars being hydrolyzed in 250°C-H-RCF3, compared to the other reactions, we expected the lowest amounts of polymers to be present in the associated water fraction 3. Conversely, water fraction 3 contained the highest amounts of carbohydrate oligomers with 19.8 %, as opposed to 18.3 % and 12.4 % for water fraction 1 and 2, respectively (Figure S1). Additionally, HPLC-SEC analysis of the three water fractions shows that the oligomeric sugars have a degree of polymerization up to 5 (Figure S10), and a relatively lower amount of even higher polymerization as shown by the smaller peaks that elute earlier (at 19.2 min in Figure S10). These observations indicate that the hydrolysis and breakdown of polymers and sugar products did not occur instantaneously during RCF processing. Instead, it appears to be continuous and relatively slow. Next to carbohydrates, presence of the water-soluble POHS lignin monomer was also observed in the different water fractions (Figure S1), albeit in <1 % of all water fraction components.

Combined, significant advances are made to identify water fraction components with a total mass balance of 80 %, 53.5 % and 78.7 % for water fraction 1, water fraction 2 and water fraction 3, respectively (Figure S1). One may speculate that the remaining components could potentially be carbohydrate polymers, due to estimations of higher polymer content in the water fraction reported previously[14], and less likely some humins, although often formed as side-product during lignocellulose pretreatment (see above). However, identical methodologies were used to quantify the carbohydrate content of the wood/pulp substrates with a combined mass balance of +95 %, indicating that insufficient hydrolysis of polymers in the water fraction is highly unlikely, while no humins (precursors) were detected with NMR (see supporting text ST1). Therefore, it is not entirely clear which (type of) molecules are not identified, and supplementary analytical research will be necessary to complete the composition and mass balance of the water fractions. However, the improved compositional analysis of the water fraction conducted in this study provides sufficient insights for its intended purpose, revealing the potential value as an additional carbohydrate source for fermentation.

Investigating the benefit of water fraction to SHF addition by growth test and saccharification

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Based on the compositional analysis of the obtained water fractions, we investigated whether *S. cerevisiae,* the organism of choice for fermentation, can metabolize the characterized monomeric carbohydrates (*viz.,* methylated glucose, methylated xylose and xylitol) and thus constitute a carbon source for additional ethanol production if added during SHF. In addition to the controls with glucose and xylose, the growth tests with the xylose-utilizing *S. cerevisiae* strain BMD44 showed growth on methyl α-D-glucose, but not on methyl β-D-glucose, methyl α/β-D-xylose or xylitol (Figure 2).
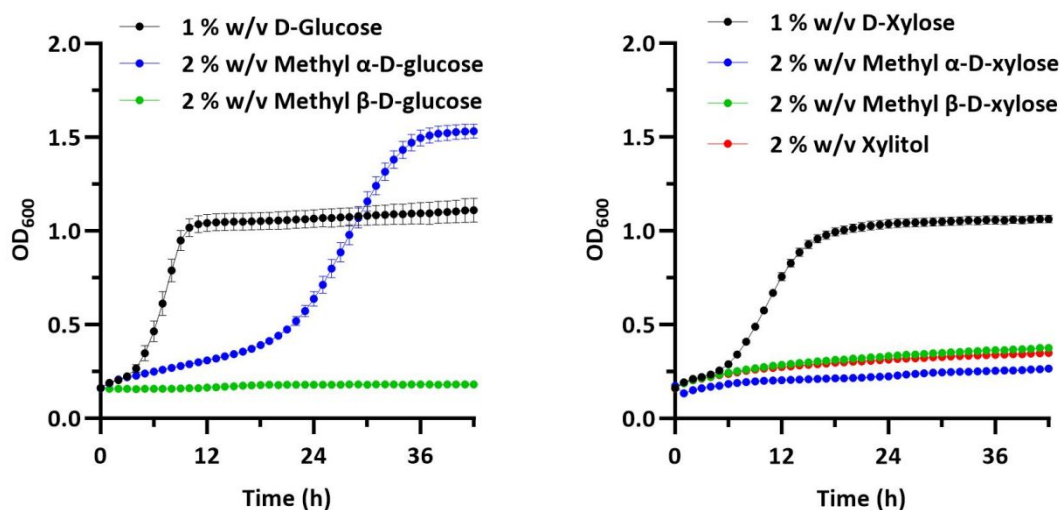

Figure 2. Growth test of S. cerevisiae strain BMD44 on various carbon sources that are present in the water fraction after RCF of lignocellulose biomass. BMD44 was pitched in complete synthetic medium (CSM) containing the carbon sources at indicated concentrations. Sugar consumptions after the growth test as percentage of the initial concentrations can be found in Figure S11 (n=5).

Xylitol is known as an accumulating intermediate in the native metabolic pathway of industrial *S. cerevisiae* strains, resulting from a redox imbalance caused by the two-step conversion of xylose to xylulose[67]. Isolation and characterization of xylitol-assimilating *S. cerevisiae* strains has resulted in the identification of the genes related to xylitol transport[68, 69] and full xylitol utilization under aerobic conditions. Under anaerobic conditions, however, the redox imbalance caused less than 30 % of the xylitol to be consumed over a period of 100 hours, which shows additional challenges. An interesting aspect to consider is the higher theoretical ethanol yields associated with reduced sugars, such as sorbitol and xylitol, compared to their non-reduced forms, owing to the additional supplementation of electrons. This phenomenon has been reported in genetically modified *S. cerevisiae* strains capable of fermenting sorbitol under anaerobic conditions[70]. While analogous strategies for xylitol are not (yet) documented, they could potentially favor the strategy of solubilizing xylose and reducing it to xylitol during RCF, given the higher theoretical ethanol yields.

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To the best of our knowledge, there is no report on the utilization and growth of industrial *S. cerevisiae* strains using the methyl-xylose anomers as a carbon source. This lack of knowledge is not surprising, considering that methyl-xylose is not naturally occurring nor is it formed as a byproduct in conventional lignocellulose fractionations. Although *S. cerevisiae* cannot grow (Figure 2) or consume (Figure S11) either methyl-xylose anomers under the tested conditions, it is unknown whether this is due to lack of substrate transport or metabolism thereof. In the fungus *Aspergillus tamarii*[71, 72], however, methylated glucose and xylose are reported as non-metabolizable inducers for increased production of xylanases and shows potential for further elucidation.

In contrast to the other water fraction-related carbon sources that were investigated, growth of the BMD44 strain was observed on methyl α-D-glucose, albeit with a significant lag-phase compared to glucose (Figure 2). This observation may be in line with literature where the Agt1 transporter enables uptake of multiple α-glucosides including maltose, maltotriose, sucrose and methyl α-D-glucose[73, 74] (Figure S12). Once internalized, intracellular α-glucosidase cleaves the glycosidic bond, resulting in the hydrolysis of maltose to release the monomeric glucose molecules. Based on the observations in Figure 2, it is thus likely that a similar hydrolysis of methyl α-D-glucose can take place resulting in an intracellular demethylation forming glucose. Similarly, the observed lag phase can then be attributed to the required metabolic shift to express sufficient α-glucosidases to support growth. Note that such internal mechanism is presumably not at hand for the methylated xylose anomers as no growth was observed (Figure 2).

In addition to the methylated monomeric sugars and sugar alcohols, the water fractions also contain a significant quantity of sugar oligomers, reaching up to 4.1 % of the initial carbohydrates for 250°C-H-RCF3 (Figure 1). These oligomers can be an additional source for bioethanol production after hydrolysis to sugar monomers during the SHF step. To estimate the potential ethanol production gain during SHF with the addition of a water fraction, we measured the increase in glucose and xylose concentrations obtained from the saccharification of water fraction solubles alone. This was done using an equivalent concentration of solubles when introduced in the SHF process, discussed below (see experimental procedures in supporting information). After saccharification, water fraction 3 exhibits the highest monomeric sugar concentration increase of 6.7 g/L, followed by water fraction 1 and water fraction 2 with 6.2 g/L and 4.4 g/L, respectively.
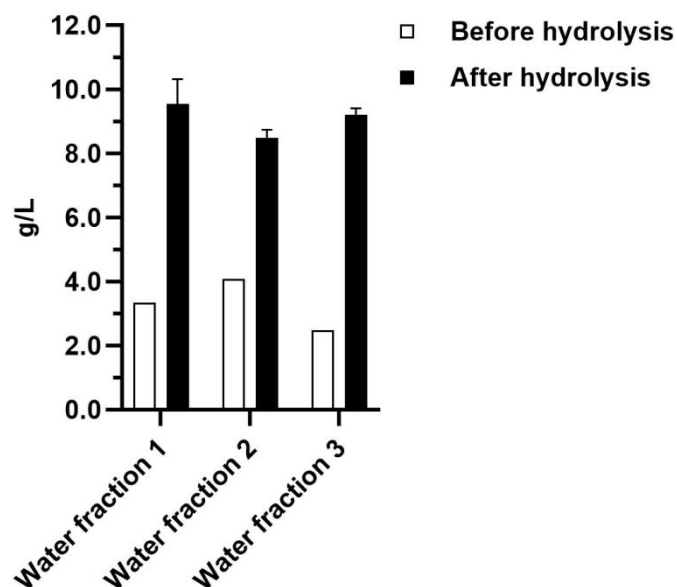
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Figure 3. The combined concentration of glucose and xylose before and after saccharification of the water fractions obtained after 235°C-RCF1, 200°C-RCF2 and 250°C-H-RCF3. *Saccharification was performed at 50 °C with water fraction solubles after removing water by rotary evaporation and redissolving in a 100 mM citrate buffer at pH 5 containing 1 % w/v Cellic® Ctec3, sampled after 72h (n=3). Note that some of the components in the water fractions have identical retention times, and thus quantified as unmodified monomeric carbohydrates, resulting in the observed concentrations before hydrolysis (n=3).*

Interestingly, GC-FID analysis comparing water fractions before and after saccharification revealed that among the methylated glucose and xylose anomers present in the raw water fraction, only the α-methylated anomer of both sugars remained unconverted and visible in the hydrolyzed samples after saccharification (Figures S13 − S15). The Cellic® Ctec3 enzyme cocktail that was used during saccharification contains both β-glucosidases and β-xylosidases, required for hydrolysis of lignocellulose biomass[75]. In this case, we showed that it also catalyzes the demethylation of the β-methylated sugars, but not the α-anomer (Figures S13 – S15), and thus forming additional glucose and xylose monomers which will be available for subsequent fermentation.

In conclusion, α-methyl glucose (consumed directly by *S. cerevisiae*), β-methyl and oligomeric carbohydrates (by demethylation during saccharification) are expected to add value to SHF by addition of water fraction that increased the concentration of fermentable carbohydrates. Consequently, a higher ethanol yield can be anticipated compared to scenarios where no water fraction is added, provided that no unexpected inhibition occurs.

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Saccharification of RCF pulps

Saccharification of RCF pulp is required to be a usable substrate for biochemical fermentation processes. Therefore, the susceptibility of the three different pulps obtained after RCF was shown by enzymatic hydrolysis using the Cellic® Ctec3 enzyme cocktail at a loading of 35 FPU/g pulp (27.6 mg protein/g pulp). A very similarly high sugar yield was achieved with pulp 3 and pulp 1, reaching 97.7 % and 97 %, respectively (Figure 4). Saccharification of pulp 3 is most efficient reaching the maximum ethanol yield value after 48h (compared to 72h for pulp 1). Although still achieving a high sugar yield of 89.5 %, pulp 2 was least susceptible to enzymatic hydrolysis with a clear attenuation of sugar release under the tested conditions (Figure 4). It is well-established that the presence of lignin hampers enzyme activity during saccharification[76]. The observed negative correlation of increasing sugar yields obtained during saccharification with decreasing lignin content (pulp 2 < pulp 1 < pulp 3) supports the necessity to perform not only adequate pretreatment but also adequate delignification.
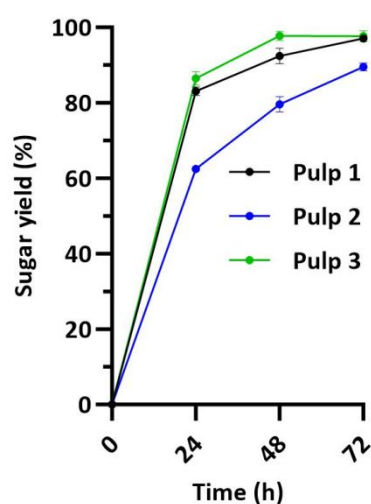

Figure 4. Sugar yields expressed as percentage of the theoretical maximum after saccharification of RCF pulps obtained in this study. Saccharification performed at 50 °C with 10 % w/v RCF pulps in 100 mM citrate buffer pH 5 containing 35 FPU/g pulp (27.6 mg protein/g pulp) Cellic® Ctec3, sampled after 24h up to 72 h (n=3).

Pioneering the implementation of the water fraction into a SHF process

Implementing the RCF water fraction in a downstream processes rather than disposing it may offer two main advantages. Firstly, it allows a holistic use of biomass, while minimizing carbon loss of carbohydrate-derived products that are otherwise not used. Secondly, it has the potential of lowering the continuous need for fresh process water and the necessity for an additional, costly[77, 78] water treatment step. Based on previous sections we showed that the water fraction can provide additional

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monomeric sugars as substrate. As an example, we therefore investigate the impact of combining pulp and WF, that both originate from the RCF, into a SHF process (Figure 5).

As initial screenings showed that the presence of ethyl acetate in the water fraction, being a potential remnant of the work-up from CLO to RLO during liquid-liquid extraction (see experimental procedures in supporting information), severely hindered the yeast's growth (at concentrations of 87 g/L, data not shown), it is removed prior to integration into a SHF process. As a scalable and less intensive alternative, distillation has been proposed to lower or completely remove ethyl acetate in industrial set-ups as well[4], and thus the inhibitory effect of ethyl acetate does not imply the inability to utilize water fraction as envisioned. For the small-scale SHF set-up used in this study, the water fractions were dried using rotary evaporation and re-dissolved in a mixture suitable for subsequent saccharification and fermentation. This mixture comprises a 100 mM citrate buffer (pH 5) and complete synthetic medium (CSM) together with an enzyme loading of 35 FPU/g pulp (27.6 mg protein/g pulp) Cellic® Ctec3 with 10 % w/v pulp loading used in all conditions. The presence of CSM does not influence the saccharification process (Figure S16) and supports sufficient nitrogen being present for the yeast strain during SHF.

In absence of water fraction during SHF, pulp 3 exhibited the highest sugar yield during the saccharification phase (0h to 48 h), being 86.0 % (Figure 5B), followed by pulp 1 and pulp 2 with 79.1 and 45.1 %, respectively (Figure 5B). Notably, sugar yields obtained during SHF were lower than expected based on the saccharification experiment alone (Figure 4) and is further discussed in supporting text ST2. Although pulp 2 shows low sugar yield after the saccharification during SHF (Figure 5), it cannot be dismissed compared to pulp 1 and 3, as it was shown to perform well in the saccharification experiment alone, albeit still with lower sugar yield (Figure 4). Nevertheless, despite this discrepancy of sugar yields between experiments, we can still investigate the effect of water fraction addition to SHF.

After initiating fermentation by pitching the xylose-utilizing yeast strain BMD44, all sugars were consumed within 48 h when no water fraction was present (time point 96 h, Figure 5A left).
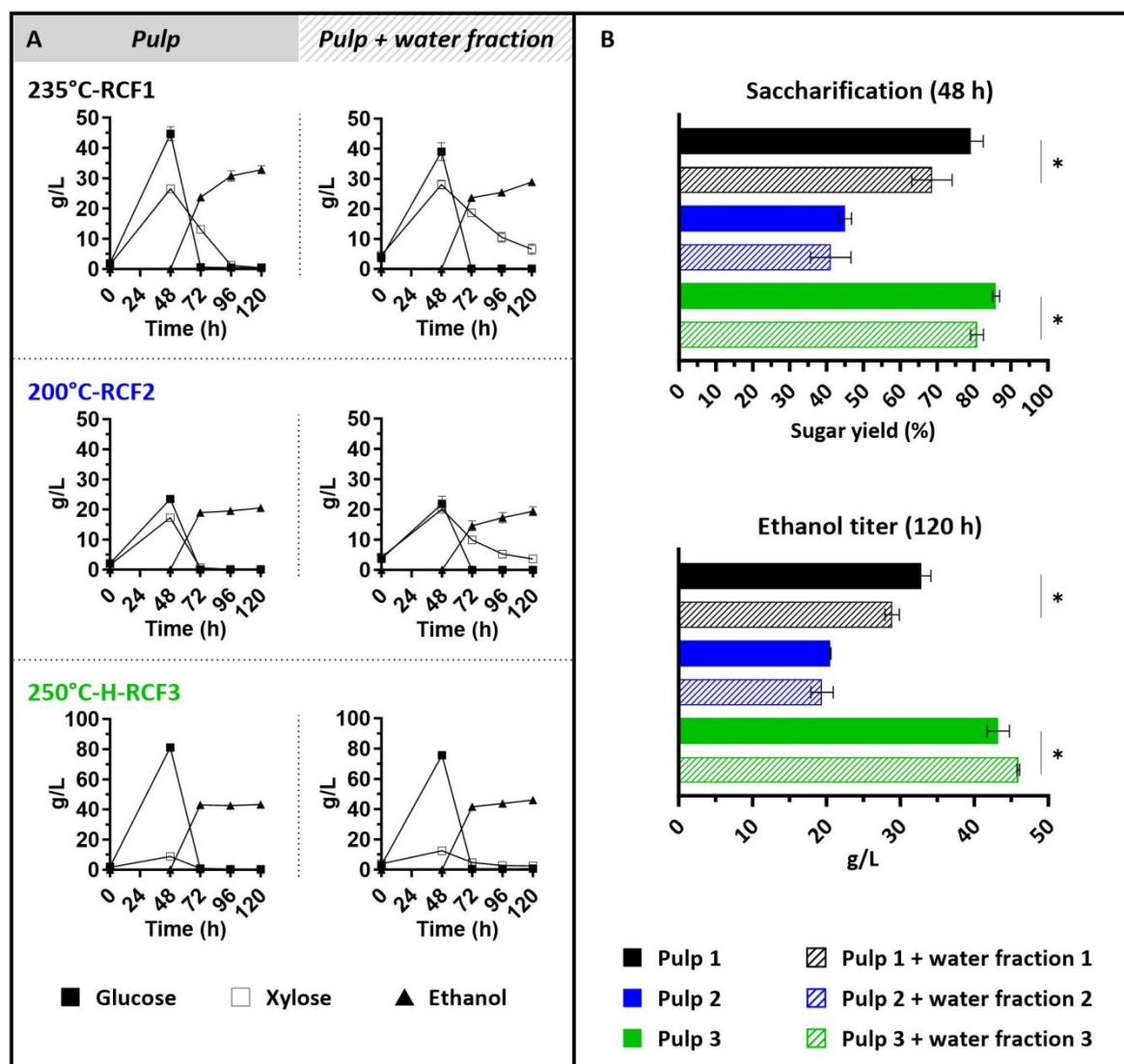
Figure 5. SHF of RCF pulp without or with water fraction added. A *) Saccharification conditions are the following: 10 % w/v pulp, 35 FPU/g pulp (27.6 mg protein/g pulp) Cellic® Ctec3, 100 mM citrate buffer pH 5 with CSM at 50 °C for 48 h after which the S. cerevisiae strain BMD44 was pitched (1 g DW/L) and fermented at 30 °C for 72 h (n=3).* ***B)*** *Comparison of sugar yields expressed as percentage of the theoretical maximum after saccharification during SHF at 48 h (top) and ethanol titer at the end of the SHF (bottom) for all conditions shown in A. Note that pulp composition (Table S2) and, as a consequence, the total amount of carbohydrates that are present in the SHF differs, despite pulp loading being 10 % w/v, for pulp 1, 2 and 3. Unpaired t-test comparing the sugar yield or ethanol titer for SHF without or with water fraction added: Top; pulp 1 + water fraction 1 *p=0.0463, pulp 3 + water fraction 3 *p=0.0111, Bottom; pulp 1 + water fraction 1 *p=0.0147, pulp 3 + water fraction 3 *p=0.0365.*

When a water fraction was introduced into the SHF process, significantly lower sugar yields were observed with water fraction 1 and 3, but not with water fraction 2 (mostly due to high standard deviation when water fraction 2 was present) when compared to the respective controls without water fraction (Figure 5B). Although trace amounts of lignin (*viz.* POHS) were present in water fractions, these were deemed negligible (<0.03 % w/v final concentration in SHF, Figure 1S), compared

to the lignin that was present in the pulp, and likely not causing the lower sugar yield. During fermentation, glucose consumption was rapid and alike control conditions without water fraction (Figure 5A). However, in all cases, the xylose consumption was attenuated ('+ water fraction 1') or incomplete ('+ water fraction 2', '+ water fraction 3'), even after 72 h of fermentation (*viz.,* time point 120 h in figure 5A), being less pronounced for pulp 3 that contained low amounts of xylan (3.4 wt%, Table S2). Overall, this resulted in a significantly lower ethanol titer for pulp 1 with water fraction 1 added, compared to pulp 1 alone, with 28.9 g/L and 32.8 g/L, respectively (Figure 5B, bottom). Interestingly, however, although the presence of water fraction 3 resulted in a lower sugar yield after 48 h saccharification (Figure 5B, Top), a 6.2 % ethanol titer increase is observed after 120 h fermentation, with 45.9 g/L, compared to pulp 3 alone with 43.2 g/L (Figure 5B, bottom). These results indicate that high content of the supplemented carbohydrates present in water fraction 3 (Figure S1), including polymers, β-anomers of methylated glucose and xylose, and methyl α-glucose, are respectively subject to hydrolysis, demethylation, or consumption by BMD44 during fermentation in SHF, resulting in an increase in ethanol titer. However, saccharification appears to be slower as lower sugar yields were observed at 48h compared to the control, but the Cellic® Ctec3 enzyme cocktail is still active even at lower temperatures during the fermentation phase (48 h – 120 h, Figure 5) and ultimately resulting in higher concentrations of fermentable monomeric sugars. When water fraction 3 alone was saccharified (at an identical water fraction soluble concentration of 5.3 % w/v, Figure 3), a 6.7 g/L increased concentration of monomeric sugars was quantified. The increase of 2.7 g/L ethanol in SHF when water fraction 3 was supplemented compared to the control without supplementation is only slightly lower than expected (with theoretical maximum of 0.51 gram ethanol per gram carbohydrates).

Based on the observations of an increased ethanol titer when water fraction 3 is supplemented in SHF and knowing that its soluble concentration is 5.3 % w/v (Figure S1), we can calculate that, on average, an additional 0.05 g ethanol/g solubles in the water fraction can be produced (defined as ethanol yield from water fraction, $Y_{water\ fraction}$, Table S5). Comparatively, water fraction 1 had similar potential to provide additional monomeric sugars for fermentation, but it is apparent that it contains an (unexpected) inhibitor affecting saccharification, fermentation, or a combination of both, ultimately leading to lower ethanol titers. Similarly, as described for water fraction 3 above, we calculate that both water fractions 1 and 2 result in an average decrease in ethanol titer of -0.11 and -0.02 g ethanol/g soluble for each water fraction, respectively ($Y_{water\ fraction}$, Table S5). In addition to calculating the average yield of ethanol from the water fraction, we also compute these values for the monomeric carbohydrates in pulp 1, 2, and 3 (Y_{pulp}, table S5). These numbers can be used to calculate the overall ethanol yield based on the initial biomass, while also

considering the carbohydrate-derived soluble fraction (*viz.*, the water fraction) formed during RCF, in addition to those retained in the pulp, as further described in the next section.

To investigate which component in the water fractions caused the observed decreased xylose fermentation rate, a growth test was performed in presence of the identified carbohydrate-derived chemicals that are most prominent in the water fractions, *viz*. ethylene glycol and methylated glucose/xylose, next to 2-deoxyglucose (DOG), and compared to a control without supplementation (see supporting text ST3 and Figure S17). Furthermore, methanol is generated through the demethylation of the β-methyl carbohydrate monomers, of which it's presence was also investigated during a growth test (Figure S18). While the addition of most supplemented chemicals did not affect growth, the presence of DOG inhibited growth on both glucose and xylose, which is in sharp contrast to the observed attenuation of xylose consumption in the presence of a water fraction during SHF. Therefore, it is unlikely that DOG alone is responsible for the observed effect during SHF, requiring further investigation.

In conclusion, the solubilized carbohydrates in water fraction that are formed during RCF can, depending on the RCF conditions, lead to an increased ethanol titer and improve valorization of the substrate's carbohydrates when introduced in a SHF set-up. Additional research is necessary to investigate which water fraction soluble compounds, identified or not, adversely affect saccharification and fermentation and how these effects can be mitigated.

Maximum carbohydrate to bioethanol conversion by a combination of RCF and SHF

Obtaining a high-quality lignin oil by RCF is important, but it is essential to recognize that the carbohydrate fraction generally represents approximately 70 % of the lignocellulose biomass, forming the bulk primary product for valorization. Variations in RCF process parameters steers delignification and carbohydrate retention, while also affecting the composition of the water fraction and the susceptibility of the pulp to enzymatic hydrolysis, as evidenced by differences in obtained fractions from 235°C-RCF1, 200°C-RCF2 and 250°C-H-RCF3. Considering both pulp and water fraction is crucial to obtain a holistic approach to biorefinery strategies and defining the most suitable process parameters that foresee maximal product yields. Therefore, the ethanol titers should be discussed in relation to the maximum theoretical yield based on the initial biomass and the total amount of carbohydrates entering the biorefinery, envisioning a valorization route for birch biomass through a combination of RCF and SHF towards bioethanol. This allows a comparison of the three different RCF processes and two SHF conditions (*viz.,* with or without water fraction added) that were investigated.

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Based on the results in previous section, we defined the ethanol yields from either the pulp (Y_{pulp}) or the water fraction ($Y_{water\ fraction}$) as listed in table S5. In addition, we can also calculate the carbohydrate retention in the pulp after processing of the birch biomass through RCF (R) or solubilized and ending up in the water fraction after work-up of the CLO to RLO (1-R). With these values, we can calculate the overall ethanol yield from the raw biomass for each RCF variation investigated in this study (table S5, Figure 6).

The highest ethanol yield of 59 % was obtained with pulp resulting from 235°C-RCF1, that combined respectable delignification (50 %) and carbohydrate retention (79 %, Table S3), without addition of water fraction (Figure 6). Next to delignification, the carbohydrate retention has been a marker to assess RCF, where both need to be well-balanced[13], and used to determine optimal process parameters to maximize full biomass valorization. The process parameters used in 200°C-RCF2 are exemplary of an RCF reaction where this balance is not maintained, and although maximized for the carbohydrate retention (94 %, Table S3), it also caused a low delignification (21 %). The associated pulp 2 exhibited varying sugar yields during saccharification in different set-ups, resulting in an overall low 46 % ethanol yield from the raw biomass (Figure 6). As discussed in supporting text ST2, this was reasoned by a combined effect of high lignin content and sub-optimal setup (*viz.,* interaction of agitation, solid loading and high pulp lignin content) during SHF, given that a higher sugar yield was obtained during saccharification alone. Compared to 200°C-RCF2, the primary advantage of 235°C-RCF1 may thus not be in the additional ethanol produced, but rather in the amount of extracted, valuable lignin oil (800 – 1000 €/ton[4]), influencing the choice between the two options. Nevertheless, the enzymatic hydrolysis of pulp 2 is evidently more sensitive to the downstream processing conditions and require additional fine-tuning when envisioning upscaling.

On the other hand, the concomitant delignification and carbohydrate solubilization achieved with more severe process conditions in 250°C-H-RCF3 resulted in pulp 3 with low lignin and high cellulose content (Table S2), and high sugar yields after saccharification. Furthermore, the addition of water fraction 3 resulted in a 6.2 % increase in ethanol titer, relative to when no water fraction was added (Figure 5) with an overall ethanol yield of 46 % and 41 %, respectively (Figure 6). Despite the observed increase in ethanol titer with the addition of water fraction 3 to SHF, it does not outweigh the higher ethanol yields obtained solely from pulps obtained in 235°C-RCF1 or 200°C-RCF2. Another aspect to consider, however, is the increased amount of valuable lignin oil extracted with 250°C-H-RCF3 (Figure 1). Even considering the lignin oil's higher molecular weight, it still holds value in various application fields[26, 79]. In addition, a purer cellulose pulp is obtained that contains less lignin (6 wt%) and hemicellulose (4 wt%) resembling a dissolving pulp with higher value[80] (*viz.,* above 800 €/ton).

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Combined, process economics might still favor 250°C-H-RCF3 and again highlight the intricate considerations concerning RCF process parameters.
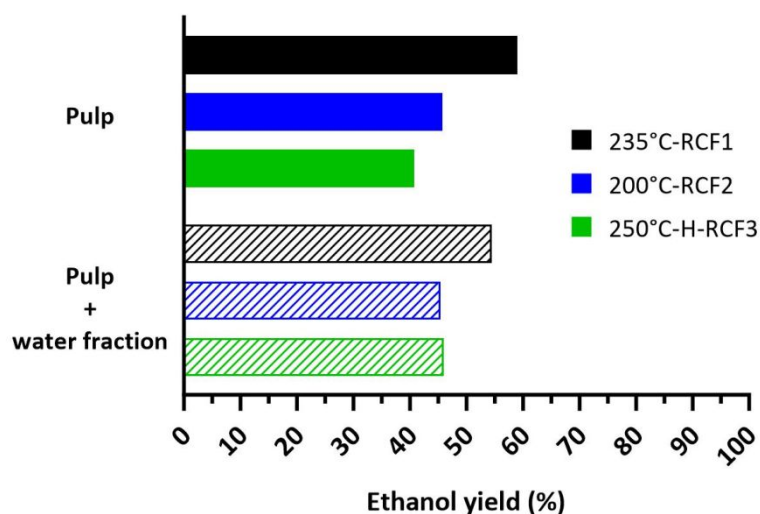

Figure 6. Ethanol yield expressed as percentage of the theoretical maximum relative to the raw birch biomass as processed through a combination RCF and SHF investigated in this study. Data obtained from the SHF experiment represented in figure 5 and detailed calculations shown in table S5.

These insights reveal two important novelties. Firstly, neither high carbohydrate retention (200°C-RCF2), nor high solubilization (250°C-H-RCF3) during RCF is ideal for processing of birch wood biomass regarding maximizing ethanol production from pulp (with or without water fraction). Instead, a balance of sufficient delignification/pretreatment (235°C-RCF1) is necessary to result in an overall higher ethanol yield. Secondly, the RCF process conditions have a significant effect on water fraction and pulp composition, which, in turn, either hampers or benefits ethanol yields when combined in a SHF process with *S. cerevisiae*. Chemo-catalytic processes are generally known to be less susceptible to perturbations caused by impurities, with light naphtha production being a possible option to include both pulp and water fraction obtained from RCF[81, 82].

CONCLUSIONS

This study enhances our understanding of maximizing carbohydrate utilization from lignocellulose through RCF and SHF processing. The RCF biorefinery parameters (235°C-RCF1, 200°C-RCF2, 250°C-H-RCF3) determine whether the carbohydrates are retained in the pulp or co-extracted with lignin, and separated post-processing in a water fraction. Implementation of this water fraction in a downstream process is explored for the first time. This water fraction contains α and β anomeric forms of methylated carbohydrates, xylitol diols, triols, and trace amounts of water-soluble lignin and

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deoxygenated carbohydrates, while lacking common pretreatment byproducts like furfural or HMF. Although the pulps are susceptible to enzymatic hydrolysis, with sugar yields ranging from 89 to 98 %, utilizing the carbohydrate-derived solubles in the water fraction presents challenges. Its presence in SHF affects the xylose fermentation capacity of *S. cerevisiae* but can also provide additional carbohydrates with a 6.2 % increase in ethanol titer. Further research is necessary to elucidate the water fraction's effect during SHF. Overall, birch biomass processing through 235°C-RCF1, that combines decent delignification (50 %) and pulp carbohydrate retention (82 % C6 and 73 % C5 sugars), and subsequent SHF resulted in the highest ethanol yield of 59 % from pulp without addition of a water fraction. This result is in line with established bioethanol production yields that are reported from hardwood using the more established carbohydrate-centered biorefinery.

CONFLICTS OF INTEREST

There are no conflicts to declare.

Supporting information

Elaborate description of experimental procedures; supporting texts; figures and tables with RCF fraction analysis; growth tests with *S. cerevisiae* and saccharification; calculations of bioethanol yields in reported and current study.

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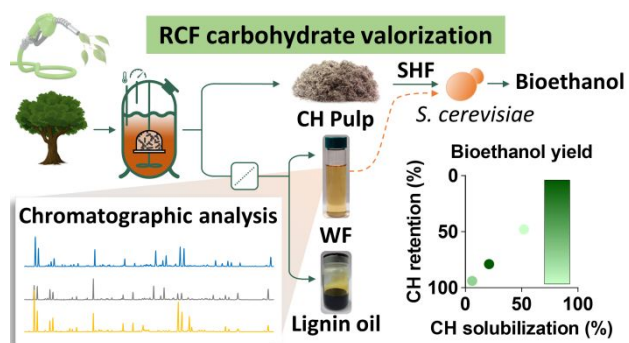
Synopsis

Wood-to-chemical transformation: smart wood processing enhances ethanol yield and offers a promising solution for sustainable biofuel.