Effect of Soluble Phenolic Compounds From Hydrothermally Pretreated Wheat Straw on Cellulose Degrading Enzymes

Anna S. Borisova (anna.borisova@vtt.fi)
VTT Technical Research Centre of Finland https://orcid.org/0000-0002-3414-9888
Ville Pihlajaniemi
VTT Technical Research Centre of Finland Ltd: Teknologian tutkimuskeskus VTT Oy
Riin Kont
University of Tartu: Tartu Ulikool
Klaus Niemelä
VTT Technical Research Centre of Finland Ltd: Teknologian tutkimuskeskus VTT Oy
Taru Koitto
Aalto-yliopisto Kemian tekniikan korkeakoulu
Atte Mikkelson
VTT Technical Research Centre of Finland Ltd: Teknologian tutkimuskeskus VTT Oy
Priit Väljamäe
University of Tartu: Tartu Ulikool
Kristiina Kruus
Aalto-yliopisto Kemian tekniikan korkeakoulu
Kaisa Marjamaa
VTT Technical Research Centre of Finland Ltd: Teknologian tutkimuskeskus VTT Oy

Research Article

Keywords: phenolic compounds, inhibition, cellulases, cellobiohydrolases, lignin-carbohydrate complexes, XAD-7 resin, wheat straw, hydrothermal pretreatment

Posted Date: August 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-832450/v1

Version of Record: A version of this preprint was published at Biomass Conversion and Biorefinery on April 25th, 2022. See the published version at https://doi.org/10.1007/s13399-022-02674-z.

Abstract

Background: Hydrothermal methods are commonly applied in pretreatment of lignocellulose for enhanced enzymatic hydrolysis and further conversion to biofuels and chemicals. The pretreatment partially disassemble and solubilize cell wall polymers. Besides mono- and oligosaccharides, the soluble degradation products include various phenolic compounds, which may affect the efficiency of enzymatic hydrolysis.

Results: The phenolic compounds were isolated from pretreatment liquor and their effects on cellulolytic enzymes were investigated. The major enzymes in crystalline cellulose degradation, cellobiohydrolases, were inhibited by the oligophenolics and phenolic-carbohydrate conjugates the latter of which could be mitigated by other enzymes, i.e. xylanases and endoglucanases. Hydrolytic activity of a commercial enzyme cocktail containing lytic polysaccharide monooxygenase (LPMO) was enhanced in presence of low concentrations of phenolics.

Conclusions: The effects of the pretreatment liquor phenolics is dependent on enzyme concentration and type of the phenolic compounds. For optimized performance, the pretreatment conditions and enzyme cocktail composition should be designed to promote oxidative activities for enhanced hydrolysis, degradation of the oligosaccharides linked to phenolics and resistance to oligophenolic compounds.

Background

Sustainable industrial processes utilizing renewable raw materials are needed in production of fuels and chemicals, in order to meet the demands of growing human population and resolve the global environmental challenges. The use of lignocellulose feedstocks for the production of biochemicals and biofuels typically requires hydrolysis of polysaccharides to monosaccharides, which in turn are converted to products of interest. Enzymatic hydrolysis is a preferred depolymerisation method, due to high sugars yields under mild reaction conditions.

Prerequisite to an efficient enzymatic hydrolysis of lignocellulose is biomass pretreatment, which disrupts the plant tissues and cell walls and consequently improves the accessibility of the enzymes to their substrates. The most commonly applied pretreatment technologies involve heating of the wet biomass to e.g. 180–210°C with or without an added acid catalyst, leading to partial dissolution and degradation of hemicelluloses and modification of lignin [1]. A drawback in this type of technologies is the formation of pretreatment products that are inhibitory for enzymatic hydrolysis and/or for the fermenting microorganism, which impairs the process yield and cost efficiency [2]. The known pretreatment-derived inhibitors for microbes or enzymes include hemicellulose-derived mono- and oligosaccharides, acetic acid, furfural, 5-hydroxymethyfurfural (HMF), and various phenolic compounds, including modified lignin or pseudolignin [1].

The enzyme mixtures used in a lignocellulose hydrolysis typically contain a number of hydrolytic enzymes, including cellobiohydrolases (CBHs), endoglucanases (EGs), a β-glucosidase (BGL) and

different hemicellulases [3]. The state-of-the-art commercial enzyme products also contain lytic polysaccharide monooxygenases (LPMOs), which are oxidative enzymes with a capability to strongly boost overall saccharification of cellulose in presence of suitable reducing agents and oxygen or hydrogen peroxide [4–6]. Xylan- or mannan-derived oligosaccharides arising from lignocellulose pretreatment can be strong inhibitors for cellulolytic enzyme cocktails [7, 8]. Lignin adsorbs many lignocellulolytic enzymes which can lead to irreversible deactivation via denaturation [9]. The soluble phenolic fraction of the pretreatment liquors is typically very complex, and contains compounds arising both from lignin and carbohydrate degradation [10, 11]. The pretreatment liquor phenolics have negative overall effect on hydrolysis by cellulolytic enzyme mixtures [12–15], but information about effects on individual enzymes is scarce and related mostly to xylanases [16]. The effect of soluble phenolics on purified cellulases has been studied so far mainly using commercial model compounds, such as vanillin or tannic acid [17, 18].

Wheat straw is an abundant residue from agriculture, widely explored as renewable raw material in biofuel production. In previous works we have studied the inhibitory effects of hemicellulosic oligosaccharides [19] and isolated lignins [20] from the hydrothermally or steam pretreated wheat straw on purified cellulolytic enzymes. Here we have performed an optimized hydrothermal pretreatment of wheat straw, followed by fractionation of the pretreatment liquor (PL), comprehensive analysis of its composition and identification of monomeric and dimeric phenolic structures. Inhibitory effects of the soluble phenolics on purified CBHs and EGs from archetypal *Trichoderma reesei*, as well as on commercial enzyme cocktails were studied.

Methods

Enzymes and substrates

The following enzymes were used in the hydrolysis experiments with microcrystalline cellulose (Avicel) as substrate. Cellic Ctec2 (Lot # VCSI0006, protein concentration 257 g L^{-1}) was obtained from Novozymes A/S (Bagvaerd, Denmark). The enzymes TrCel7A (CBH1), TrCel6A (CBH2), TrCel7B (EG1), TrCel5A (EG2), TrXyn11 (XYN2) from *T. reesei* and *Aspergillus niger* β-glucosidase AnCel3A (BGL) were purified and assayed as described [20]. The enzymes TrCel12A (EG3) and TrCel45A (EG5) were purified from *T. reesei* culture supernatant according to a previously established protocol [21]. The protein concentrations of the purified enzymes were quantified by measuring absorbance at 280 nm and using corresponding molar extinction coefficients and masses (Supplementary information, Table S1).

Celluclast 1.5L (Lot # SLBB4803V) and Cellic CTec2 (Lot # SLBS6227) used in the experiments with radioactively labelled cellulose substrate was purchased from Sigma. The protein concentration of these blends was measured using a ninhydrin assay with acidic hydrolysis [22] and was 124 ± 10 g L^{-1} and 269 ± 7 g L^{-1} for Celluclast 1.5L and Cellic Ctec2, respectively. TrCel7A and TrCel6A used in the experiments with the 14C-BMCC substrate were purified from the culture filtrate of Tr QM 9414 as described [23].

Radioactively labelled bacterial microcrystalline cellulose (14C-BMCC) was prepared from 14C-labeled bacterial cellulose (14C-BC). Bacterial cellulose was produced by growing *Gluconobacterium xylinum* strain ATCC 53582 in the medium supplied with 14C-glucose as described [24]. 14C-BC (2 g L^{-1}) was incubated with 1.0 M HCl at 100°C for 3 h followed by extensive washing with water. The specific radioactivity of 14C-BMCC was 6.4×10^5 dpm mg^{-1}. Avicel was purchased from Serva GmbH (Heidelberg, Germany).

Hydrothermal pretreatment of wheat straw

Chopped wheat straw from Finland was used as the feedstock for hydrothermal pretreatment using a 30-L pressure reactor. The composition of straw was determined according to a previously established protocol [25]. Batches of 1.71 kg (dry matter, DM) of chopped straw after pre-soaking with water to 50 % DM were heated by direct steam injection and a heating jacket to 195 ^{o}C and the temperature was maintained for 15 min. The thermal severity factor Log(R_0) of the reaction was 4.02, calculated from the reactor temperature curve accordingly [26], which was considered effective for wheat straw [27, 28]. After the reaction, the material was quickly cooled to 80 ^{o}C and the soluble fraction was recovered by displacement washing, by feeding water at 80 ^{o}C from underneath and collecting the liquid from the top of the material bed in 1–2 L fractions. The first 6 liters from each reaction were pooled together, providing the original pretreatment liquor (PL) used as the source of phenolics in this study. The DM balance was determined by oven-drying of solid and liquid samples at 105 ^{o}C. The liquor was stored frozen (–20 ˚C).

Isolation of phenolic fraction from wheat straw pretreatment liquor and preparation of Cellic Ctec2-treated pretreatment liquor

PL was fractionated on a glass column (250 mL column volume, CV) packed with water-rinsed Amberlite XAD-7 HP resin (Acros Organics, Germany). The column bed volume was 220 mL (XAD-7 pore volume 1.14 mL/g). PL (750 mL) containing 3.6 g of UV-phenolics (25 mg/g resin) was loaded into the column with flow rate of 5 mL/min (0.25 cm/min). The column was washed with 4–5 CV of water with flow rate of 10 mL/min (0.5 cm/min). Bound phenolic compounds were eluted with 100 % ethanol at flow rate of 3 mL/min (0.15 cm/min). An ethanol-eluted phenolic fraction (250 mL) was collected and analyzed for soluble phenolic compounds and carbohydrates as described below. The purification run was repeated for 6 batches. The water eluates were stored frozen (–20 ˚C) and the combined phenolic fraction (Phe-Fr) was freeze-dried. Analytical fractionation of PL was performed as described above in a single XAD-7 column chromatography run with the ethanol gradient, applied stepwise by 10 % increase of ethanol concentration (10–90 % ethanol, 250 mL each step). All the eluate fractions including water fraction were collected and freeze-dried.

Cellic Ctec2 treated PL (Ctec-PL) was prepared by incubating 12 mL PL with Cellic Ctec2 (2 mg protein) in 50 mM sodium acetate buffer, pH 5.0 at 50 ˚C, for 18 h. After incubation the enzymes were inactivated by

heating at 100°C for 20 min. Heat-inactivated enzymes were pelleted by 2 min centrifugation (10000 × g) and aliquots (1.5 mL) of supernatant were stored at − 20°C. Carbohydrates and phenolics in the Ctec-PL were analyzed as described below.

Spectrophotometric quantification of soluble phenolic compounds

The total concentration of the soluble phenolic compounds was determined with a UV-spectrophotometric assay based on the determination of acid soluble lignin in the biomass compositional analysis [25], using the absorptivities determined by method described previously [29]. UV-phenolics concentration was used further on in the paper, if not stated otherwise. Correlation factor between UV-phenolics and total solids for Phe-Fr was calculated to be 0.48 ± 0.07 (g UV-phenolics/g total solids) (Supplementary information, Figure S1).

Quantification of soluble carbohydrates and furaldehydes

Monosaccharides were analyzed by HPAEC-PAD, using CarboPac PA1 column on DIONEX ICS-5000 and 300 mM NaAc/NaOH gradient as described [30]. The linear xylooligosaccharides were analyzed using CarboPac PA1 column on DIONEX ICS-3000A as described [31]. The total amount of soluble carbohydrates was analyzed by hydrolyzing oligosaccharides with 3 % H_2SO_4 at 120°C for 1 h and analyzing the monosaccharides as described above. Furfural and 5-hydroxymethylfurfural were determined by HPLC using PerkinElmer Flexar equipment with Bio-Rad Aminex HPX-87 300×7.8 column.

Determination of phenolic fraction molecular weight distribution

The weight average molecular weight (M_W) measurements were performed by size exclusion chromatography (SEC) and analyzed as described previously [32]. For the analysis, 2 mg of lyophilized Phe-Fr was dissolved in 0.1 M NaOH and filtered (filter pore size 0.45 µm).

GC/MS analysis of the phenols

The PL and Phe-Fr were analyzed for phenolic compounds by GC/MS after trimethylsilylation. The PL in water (2 mL) was extracted twice with 5 mL of diethyl ether, after the addition of salicylic acid as the internal standard. The organic phases were combined and evaporated to dryness. The Phe-Fr in ethanol (0.3 mL) was readily evaporated to dryness, after the addition of the same salicylic acid standard. The evaporation residues were trimethylsilylated and analyzed by GC/MS as described previously[33]. The peak areas were used for the indicative concentration calculations, without any corrections.

Hydrolysis assays with Avicel as substrate

The hydrolysis assays were carried out in 50 mM sodium acetate pH 5.0 at 45°C with stirring at 1000 rpm. Enzymes concentration was 0.1 g/L (10 mg/g Avicel) for Cellic Ctec2 and purified cellulases, when assayed alone. The mixture of *T. reesei* cellulases was composed of 58.4% w/v TrCel7A, 19.5% w/v

TrCel6A, 9.8 %w/v TrCel5A and 2.6% w/v TrXyn11. The hydrolysis reactions with individual *T. reesei* cellulases and with the mixture were supplied with AnCel3A (500 nkat β-glucosidase/g Avicel). Avicel concentration in all the reactions was 10 g/L, Phe-Fr concentration in the assays with Cellic Ctec2 varied from 0.25 to 5 g/L, while the purified enzymes were assayed at 5 g/L Phe-Fr. Prior to hydrolysis the freeze-dried Phe-Fr was dissolved in 0.1 M sodium acetate at pH 5.0 by heating at 40°C for 20 min with stirring, and then incubated with Avicel for another 20 min. The hydrolysis reactions were initiated by addition of the enzyme, run in duplicates (purified enzymes) or triplicates (Cellic Ctec2) for 4, 24 or 48 h. The reactions were stopped by addition of 10 µL of 10 M NaOH and the insoluble cellulose was removed by centrifugation (5 min at 3220 x g). Soluble reducing sugars were analyzed with 4-hydroxybenzoic acid hydrazide (PAHBAH) reagent and glucose standards [34]. The method was proved to be suitable for assays including Phe-Fr within concentrations range (Supplementary information, Figure S2). Control hydrolysis reactions lacking Phe-Fr (10 g/L Avicel) and control reactions for Phe-Fr background carbohydrates (0 g/L Avicel) were carried out in the same way as described above. Tannic acid inhibition experiments were performed with purified cellulases, their mixture and Cellic Ctec2 at fixed tannic acid concentration 0.1 g/L, same way as described above for Phe-Fr. Inhibitory effects of PL analytical fractions from ethanol gradient elution were studied with Cellic CTec2 as described above, using total solids 10 g/L concentration of each fraction in Avicel hydrolysis. The released glucose was analyzed with HPLC, as described above.

Hydrolysis assays with radioactively labelled bacterial microcrystalline cellulose as substrate

All reactions were performed in 50 mM sodium acetate, pH 5 at 50°C for 18 h without stirring. Concentration of 14C-BMCC was 0.6 g/L. The reactions were stopped by adding NaOH to a final concentration of 0.1 M (in inhibition experiments the reactions were stopped by centrifugation, 2 min at 10^4 x g). Cellulose was pelleted by centrifugation (2 min at 10^4 x g) and radioactivity in the supernatants was measured using scintillation counter. The degree of cellulose degradation was found as the ratio of radioactivity in the supernatant to the total radioactivity in the mixture. Inhibition studies were carried out using protein concentrations 1.0 µM (about 0.06 g/L) for CBHs and 0.01 g/L for Celluclast 1.5L and Cellic CTec2 and different volume fractions (v/v %) of the PL, Ctec-PL and Phe-Fr. The freeze-dried Phe-Fr was dissolved for the experiments by heating at 40°C for 20 min with stirring. The concentration of PL was expressed as its volume fraction (in %). Based on the concentration of UV-phenolics in Ctec-PL and Phe-Fr their concentrations were expressed as the equivalent volume fraction of PL (100 % volume fraction in the assay corresponds to UV-phenolic concentration of PL (4.55 g/L).

Inhibition data were analyzed according to Eq. 1:

$$Relative\ activity\ (\%) = \frac{100\%}{1+\frac{[I]}{IC_{50}}} \qquad (1)$$

In Equation 1, the relative activity (%) is a ratio of the degree of cellulose conversion measured in the presence of inhibitor to that measured in the absence of inhibitor. [I] stands for the concentration of inhibitor (in v/v %) and IC50 is the inhibitor concentration that causes 50 % decrease in the degree of cellulose conversion.

Results

Hydrothermal pretreatment of wheat straw

Bench scale hydrothermal pretreatment of wheat straw was repeated 6 times to produce sufficient amount of pretreatment liquor (PL) as the source of inhibitors for the study. Solids yield of straw was 71 % (standard error 2 %), altogether 18 % of DM was recovered as solubles and the PL contained 57 % of the solubles. The major compounds of PL were carbohydrates (glucose and xylose as main sugars), phenolic compounds, acetate and furfural (Table 1). The carbohydrate part was composed of both monosaccharides (22.8 %) and oligosaccharides or other sugar conjugates (31.3 % PL DM), of which 17.2 % (of PL DM) could be identified as linear xylooligosaccharides with DP 2–6. The soluble sugar recovery corresponded to 15 % of total carbohydrate in straw, while 66% of the C5 sugars in straw were dissolved and 37 % recovered, in line with [27] and [28]. The amount of phenolics in PL was 4.55 g/L (13.2% of DM), quantified with the UV method, corresponding to 2.4 % of original straw DM.

Table 1
Composition of pretreatment liquor (PL), Ctec-hydrolysed pretreatment liquor (Ctec-PL) and phenolic fraction (Phe-Fr). All the values were calculated as % of DM.

Compound	PL	Ctec-PL	Phe-Fr
Glucose total	4.1	4.8	1.9
Glucose monomeric[1]	0.6	2.7	0.006
Xylose total	35.5	35.1	15.1
Xylose monomeric[1]	6.1	17.5	0.005
Xylooligosaccharides (DP2-6)[1]	17.2	nd[3]	1.4
Arabinose total	2.7	2.5	2.6
Arabinose monomeric[1]	nd	1.1	0
Galactose total	2.0	2	1.0
Galactose monomeric[1]	nd	0.6	0
Mannose total	1.2	1.1	0.8
Mannose monomeric[1]	nd	0.3	0
Fructose total	1.9	1.9	1.6
Fructose monomeric[1]	nd	1.8	0
Total carbohydrates	45.8	46.2	23.0
Total monosaccharides	6.7	24	0.014
Furfural	4.8	nd	nd
UV phenolics	13.2	14.6	58.3
Acetate	11.6	11.1	nd
[1]analysed from the samples directly with HPAEC-PAD			
[2]analysed after acid hydrolysis with HPAEC-PAD			
[3]nd= not detected			

Column chromatography fractionation of pretreatment liquor

Column chromatography fractionation of PL was carried out using a polyacrylic Amberlite XAD-7 resin aiming at enrichment of phenolics for the inhibition studies. Most of the carbohydrates were eluted with water, while the most of phenolic compounds (Phe-Fr) was eluted with 100 % ethanol (Fig. 1A). The recovered Phe-Fr contained 66 % of the phenolics quantified from PL, while majority of the soluble sugars was removed successfully (Fig. 1A, Table 1). The phenolic compounds composed 60 % of the Phe-Fr DM (Table 1). The amount of free monosaccharides in Phe-Fr was less than 0.1 % of the DM, but the total amount of monosaccharides, mostly xylose, released in mild acid hydrolysis of Phe-Fr contributed 23 % of the DM. The amount of linear xylooligosaccharides with DP 2–6 was, however found to be low (1.4 % DM) (Table 1). This indicates the Phe-Fr contained oligosaccharides bound to phenolics, eluted with ethanol.

Phenolic composition of pretreatment liquor and phenolic fraction

Molecular weight (M_W) distribution of Phe-Fr was determined with SEC. An average M_W was estimated to be 820 Da (Fig. 1B). Majority of the Phe-Fr compounds (60 % w/w) fell into fractions with M_W between 450 Da and 1700 Da, which corresponds to DP from 1–5 up to 8–18, as the M_W of phenolic and carbohydrate units varies approximately between 90–250 Da.

Low molecular weight phenolic compounds (LMWs), with molar mass up to 450 Da in the original PL and Phe-Fr were analyzed in detail using GC/MS (Table 2). The analysis revealed the presence of up to 130–150 different compounds (Supplementary information, Figure S3). Approximately 50 % of them turned out to be monomeric phenolic (and related) compounds, and the rest were clearly dimeric compounds, composed either of two aromatic units or one aromatic unit and one sugar unit (Table 2). Most of the main monomeric and dimeric compounds were characterized, based on our numerous earlier studies on lignin degradation products [33, 35].

Table 2

Phenolic compounds identified by GC/MS. Indicative concentrations in wheat straw pretreatment liquor (PL) and isolated phenolic fraction (Phe-Fr) are presented as mg/g UV-phenolics. Only monomeric and dimeric compounds are amenable to GC/MS.

Compound	PL	Phe-Fr
Simple monolignols		
4-Vinylphenol	1.4	0.8
4-Hydroxybenzaldehyde	2.1	1.5
4-Hydroxybenzoic acid	1.1	1.3
1-(4-Hydroxyphenyl)ethanol	4.3	2.3
3-Hydroxybenzoic acid	tr.[1]	0.7
4-Hydroxybenzoic acid	1.1	1.0
p-Coumaric acid	7.2	8.3
Guaiacyl monolignols		
Guaiacol	1.1	1.0
4-Ethylguaiacol	tr.	0.8
4-Vinylguaiacol	1.8	1.3
Eugenol	tr.	0.5
Vanillin	7.9	6.6
Acetovanillone	0.7	0.7
Guaiacylacetone	0.7	1.0
1-Guaiacylethanol	7.5	3.5
Coniferyl alcohol	0.7	2.0
1-Guaiacyl-3-hydroxyacetone	1.1	3.0
Vanillic acid	3.2	3.8
Homovanillic acid	1.4	1.3
Ferulic acid	3.6	6.6

[1] traces detected

[2] not detected

Compound	PL	Phe-Fr
Syringyl monolignols		
Syringol	2.1	1.5
Syringaldehyde	3.2	3.3
Acetosyringone	1.4	1.3
1-Hydroxy-3-syringylacetone	1.8	1.3
Syringic acid	2.9	4.8
Homosyringic acid	1.1	1.8
Benzenediols		
Hydroquinone	2.1	1.5
2-Methylhydroquinone	0.7	1.0
Catechol	2.9	2.5
3-Methylcatechol	tr.	0.5
4-Methylcatechol	0.7	0.9
Compound	**PL**	**Phe-Fr**
3,4-Dihydroxybenzaldehyde	3.2	3.3
3,4-Dihydroxyacetophenone	1.8	1.5
2,3-Dihydroxybenzoic acid	tr.	0.7
3,4-Dihydroxybenzoic acid	1.8	1.3
3-(2,3-Dihydroxyphenyl)propanoic acid	8.6	8.6
Miscellaneous/unknown monolignols		
Unknown monolignol 1 (MW/TMS. 380)	5.4	7.3
Hydroxy-methoxyacetophenone	n.d.[2]	3.3
Hydroxy-dimethoxyacetophenone	n.d.	3.9
Other monolignols (incl. unknowns)	7.2	2.0
Miscellaneous compounds		

[1] traces detected

[2] not detected

Compound	PL	Phe-Fr
Hydroxypyridines (2 isomers)	18.3	5.1
Reductic acid	n.d.	0.8
Dimeric compounds		
3,8-Dihydroxy-2-methylchromone	9.0	10.1
Stilbene derivatives	n.d.	1.3
Syringaresinol	1.1	3.5
Other dilignols (unknowns)	5.7	51.5
5-O-p-Coumaroylpentose	1.1	2.8
Other coumaric acid esters	tr.	5.5
5-O-Feruloylpentoses (arabinoses. 2)	2.6	18.1
Other feruloylpentoses (2)	tr.	8.0
Other ferulic acid esters (4)	1.1	9.7
[1] traces detected		
[2] not detected		

Majority of the identified monomeric phenolic compounds represented characteristic degradation products of lignin, i.e. 4-substituted phenols, guaiacols and syringols. In line with recent studies by [10], many of them are related to the Hibbert's ketones. Relatively high amounts of hydroquinones and catecholic compounds were also found among the monomeric compounds (Table 2). Catechols formation could arise from degradation-condensation reactions of carbohydrates, along with formation of other various phenolic compounds. It is thus possible that some simple catechols and certain unidentified phenols are at least partially also derived from such sources. A special type of phenolic compound, 3,8-dihydroxy-2-methylchromone, turned out to be one of the dominating constituents among the identified phenols. This compound belongs to the main aromatic compounds formed from xylose and hexuronic acids under mild acidic conditions [36]. A relatively abundant unknown phenolic compound with a molecular weight of 380 (as trimethylsilyl derivative) may also be derived from carbohydrates.

The main dimeric phenolic compounds could be identified [10] as different 4-hydroxycinnamic and ferulic acid esters with pentoses. Interestingly, the presence of several other hydroxycinnamic and ferulic acids esters was also recognized. One of the most characteristic features in the mass spectra of their trimethylsilylated derivatives was the presence of intense (typically 50–70 %) ions corresponding to the molecular ion peaks of trimethylsilylated 4-hydroxycinnamic acid (m/z 308) and ferulic acid (338) (Supplementary information, Figure S3). Their more detailed identification is currently not possible,

although in most cases their apparent molecular weights could also be established. Of the dilignols, only syringaresinol could be fully identified in the analysis. Most of the other dilignols are characterized by the intense benzylic ions in their mass spectra, representing both guaiacyl (m/z 209, 223, 297) and syringyl (239, 253, 327) structures (Supplementary information, Figure S3).

Screening for inhibitory effects of phenolic fraction

The effect of isolated Phe-Fr was first tested with microcrystalline cellulosic substrate (Avicel) and a commercial enzyme preparation Cellic Ctec2. The hydrolysis of Avicel by Cellic Ctec2 was inhibited by Phe-Fr when phenolic concentration was above 1.25 g/L (Fig. 2A). The Cellic CTec2 also hydrolysed carbohydrates in Phe-Fr (Fig. 2C). The background arising from Phe-Fr hydrolysis (Supplementary information, Figure S4) was subtracted from the progress curves of Avicel hydrolysis. Interestingly, higher amount of reducing sugars was released by Cellic Ctec2 in presence of the lowest phenolic concentrations (0.25–0.5 g/L) compared to the Avicel hydrolysis alone (Fig. 2A). However, this increase was detectable only in the final 48h time-point. Analytical fractionation of original PL on XAD-7 column with ethanol gradient showed clear dependence of phenolics-carbohydrate ratio in different fractions and their inhibitory effect on Cellic Ctec2 (Fig. 2D). Phenolic compounds eluted with higher concentration of ethanol contained less carbohydrates and were more inhibitory for the Cellic Ctec2.

Relative activities of purified *T. reesei* cellulases (TrCel7A, TrCel6A, TrCel7B, TrCel5A, TrCel12A) and their mixture with xylanase TrXyn11 (all supplemented with β-glucosidase) remained in Avicel hydrolysis in presence of Phe-Fr (5 g/L) are shown in Fig. 2B. The CBHs TrCel7A and TrCel6A were clearly inhibited by Phe-Fr: their activity dropped by 70–75 % in comparison to the reference hydrolysis without Phe-Fr. EGs were also inhibited by Phe-Fr (Fig. 2B), but differently from CBHs, they were also able to release reducing sugars from Phe-Fr (Fig. 2C). In latter context, the most efficient enzyme at 4 h was xylanase TrXyn11, followed by EGs TrCel7B and TrCel12A, while after 24 h TrCel7B released the highest amount of reducing sugars among these enzymes (Fig. 2C). The mixture of purified *T. reesei* enzymes was inhibited by the Phe-Fr to similar extent as the individual CBHs (Fig. 2B). To estimate the impact of high molecular weight phenolic compounds (HMWs) onto inhibition of purified enzymes, their mixture and Cellic Ctec2, a model polyphenol tannic acid (M_W 1701 Da) was used in Avicel hydrolysis at concentration mimicking the amount of HMWs in the Phe-Fr (0.1 g/L, i.e. 10 % of total phenolics, see Fig. 1B). The inhibitory effect of tannic acid at this concentration was negligible for EGs, about 20% decrease in hydrolytic activity was observed for CBHs, enzyme mixture, and Cellic Ctec2 at 24 h time point (Supplementary information, Figure S5).

Inhibition of cellulases acting on radioactively labelled bacterial microcrystalline cellulose

Here we studied the inhibition of two CBHs (TrCel7A and TrCel6A) as well as two commercial cellulolytic preparations (Celluclast 1.5L and Cellic Ctec2) using 14C-labeled BMCC as substrate. In order to avoid well known product inhibition of CBHs [37] we used low concentration of BMCC (0.6 g/L). To ensure significant degree of cellulose conversion (around 50 %) in inhibition experiments, BMCC was first

incubated with enzymes at different concentration (Fig. 3A). Based on these results we chose the protein concentration of 0.01 g/L and 0.06 g/L for commercial enzyme mixtures and purified CBHs, respectively, for inhibition studies (Fig. 3B-D).

The most sensitive enzymes towards inhibition by PL were CBHs followed by Celluclast 1.5L and Cellic Ctec2 (Fig. 3B). Inhibition by isolated Phe-Fr followed the same trends, being strongest for CBHs and weakest for Cellic Ctec2, however for all enzymes the inhibition was about 3–4 fold weaker than that by PL (Fig. 3C). The weaker inhibition by Phe-Fr was possibly caused by the removal of free oligosaccharides during separation of Phe-Fr (Fig. 1A, Table 1). However, significant amount of oligosaccharides were bound to phenolics and eluted with ethanol during fractionation of PL on the XAD resin (Fig. 1A, Table 1). Since Cellic Ctec2 was efficient in releasing reducing sugars from Phe-Fr (Fig. 2C), we also used pretreatment of PL with Cellic Ctec2 as an alternative strategy to remove oligosaccharides (both free and phenolics associated). About 50 % of the oligosaccharides present in PL were hydrolyzed by Cellic Ctec2 as evidenced by the increase in the amount of monosaccharides (Table 1). Pre-treatment of PL with Cellic Ctec2 significantly reduced its inhibitory power (Fig. 3D). The effect was most prominent with CBHs, inhibition of which was relieved by the factor of 50–100.

Another difference between the inhibition of CBHs by Ctec-PL and PL/Phe-Fr was that the residual activity decreased nearly linearly with concentration of Ctec-PL (Fig. 3D). This is not consistent with Eq. 1 (the IC50 values were found using linear regression analysis), which suggests different mechanism of inhibition. Collectively these results suggest that inhibition of CBHs by Ctec-PL mostly represents inhibition by phenolic compounds whereas inhibition by PL and Phe-Fr is additionally governed by free- and phenolics-bound oligosaccharides.

It is interesting to note that, with all fractions tested, there was a slight activating effect on hydrolysis of BMCC by Cellic Ctec2 at the lowest inhibitor concentration tested (Fig. 3B-D). A small increase in degradation of Avicel by Cellic Ctec2 was also observed when Phe-Fr was present at low concentrations (Fig. 2A). Notably, similar activating effect was not observed in the case of individual CBHs and Celluclast 1.5 L.

Discussion

Commercial cellulolytic enzyme mixtures, Celluclast 1-5L and Cellic Ctc2, were inhibited by the wheat straw PL and its's fractions produced via XAD fractionation and enzymatic pre-hydrolysis. Comparison of different types of cellulases here showed that the CBHs in particular were sensitive to the inhibition. CBHs are key enzymes in the hydrolysis of crystalline cellulose [38], and thus their inhibition is expected to have major overall net effect on the cellulose saccharification by the cellulolytic enzyme mixtures.

Strong inhibition of CBHs by hemicellulosic oligosaccharides is well known [19] and observed also in this study. This is caused by binding of the xylooligosaccharides the tunnel-shaped active site of CBHs [19]. However, this type of inhibition can be relieved by oligosaccharide-degrading enzymes present in cellulolytic cocktails. Interestingly, we observed that a substantial fraction of xylooligosaccharides was

bound to the hydrophobic XAD resin, suggesting that they were interlinked with phenolics. The Phe-Fr fraction, containing these phenolics-associated oligosaccharides, was clearly more inhibitory to the CBHs than Ctec-PL fraction, where PL was pretreatead and partially hydrolysed by Cellic Ctec 2 enzymes.

XAD-resins have been previously used in binding of lignin-fragments and other aromatic compounds from lignocellulose extracts and hydrolysates [39, 40]. Fractions isolated with XAD resins from pretreated hardwood, softwood and sugarcane baggase have also been reported to contain lignin-carbohydrate complexes (LCCs) [39, 41]. On the other hand, wheat straw LCCs are known to contain lignin bound to glucan and xylan moieties via ferulic acid and phenyl glycosidic linkages, respectively [42] and feruloylated oligosaccharides have also previously been identified in wheat straw hydrothermal pretreatment liquor [14]. The LCC type of compounds can thus be the source of oligosaccharides in the XAD bound fraction of wheat straw PL. This was supported by the GC-MS analysis, which showed that 4-hydroxycinnamic and ferulic acid esters with pentoses were present in the XAD bound Phe-Fr.

The effect of LCC type of compounds on cellulolytic enzymes is not well known, though the inhibition of cellulases by softwood LCCs was reported [43]. The study showed clear difference in inhibition by ethanol organosolv dissolved lignin and its intensively hydrolysed fraction (enzymatic residual lignin), which correlates well with difference of Phe-Fr and Ctec-PL inhibition in our study. Proposed inhibition mechanism involved interactions of enzymes with the minor solubilized low-molecular lignin component, along with binding to lignin [43].

Inhibition of CBHs was relieved by degradation of oligosaccharides by other enzymes present in cellulolytic cocktails. Endoglucanases and xylanases from *T. reesei* were also able to release sugars from the oligosaccharides in Phe-Fr, suggesting that they can contribute to relieving the inhibitory effect of LCCs. Ferulic acid derivatives have been reported to bind in xylanase active site [44]. One can speculate that the open-cleft architecture in the active site of EGs allows similar binding of these type of compounds. EG TrCel7B is known to be able to hydrolyse a wide range of substrates, including celluloses, xyloglucan, arylated disaccharides and xylans [45]. The TrCel12A also hydrolyses, besides cellulose at least, β-glucan, glucomannan and xyloglucan [21, 46]. However, it is noteworthy that despite high enzyme load (16.5 mg protein/g non-monomeric xylan), all the bound saccharides in PL were not hydrolysed to monosaccharides during pretreatment by Cellic Ctec2.

The inhibition of enzymes by Ctec-PL, i.e. PL that was pre-hydrolysed by Cellic Ctec2, represents an inhibitory power of PL that cannot be mitigated by the enzymes present in this state-of-the-art enzyme cocktail. Combating with this remaining inhibitory power, may pave a way for mitigating the enzymes inhibition and improving their performance on lignocellulose degradation.

The composition of the soluble Phe-Fr in the wheat straw PL was found to be very complex. Numerous monomeric and dimeric compounds were detected using the GC-MS analysis, but their proportion of the total amount of phenolics was low (0.4 g/L of the 4.5 g/L). The effects of pure phenolic monomers on enzymes have been widely studied [18, 47]. The reported inhibitory/deactivating concentrations of the phenolic monomers are typically in the order of 1–10 g/L, which is much higher than the concentrations

detected in the PL studied in present work. This suggests that the phenolic monomers may not necessarily be the major drivers of the inhibition of enzymes.

A vast majority of the phenolic compounds in the PL of wheat straw had M_w characteristic to oligophenolic compounds (between 450 and 1700 Da). It was proposed that wheat straw derived oligophenolic compounds are responsible for phenolic inhibition of commercial enzyme cocktail, Cellic Ctec3 via non-specific binding to the enzymes [14]. The wheat straw LCCs should also fall into this M_w fraction. However, the analytical fractionation of the PL showed that the carbohydrates bound to phenolics were not the only cause of enzyme inhibition, since purer phenolic fractions, eluted with higher ethanol concentrations, were more inhibitory to the enzymatic cellulose hydrolysis, than carbohydrate containing fractions. Tannic acid was used as model compound to represent HMW phenolics in the inhibition study. Tannic acid was reported to be a strong inhibitor for *T. reesei* CBHs (TrCel7A and TrCel6A) and EGs (TrCel7B and TrCel5A) [17]. However, here the inhibition by tannic acid had only a moderate effect on purified enzymes and Cellic CTec2 in concentration mimicking the amount of HMWs in the Phe-Fr.

An activating effect of phenolic compounds was steadily observed with Cellic Ctec2 in hydrolysis of BMCC and Avicel, while not present with Celluclast 1.5L. Our recent study has revealed that the phenolic compounds in PL of wheat straw can support LPMOs with both electrons and H_2O_2 co-substrate [48]. The major difference between Celluclast 1.5L and Cellic Ctec2 is that the latter is rich in LPMOs [4]. Thus, the overall effect of PL/Phe-Fr/Ctec-PL on cellulose degradation by Cellic Ctec2 may reflect the balance between LPMO supporting and enzymes inhibiting effects.

Conclusions

The soluble phenolics and phenol-carbohydrate complexes (or LCCs) in the hydrothermal PL of wheat straw have variable effects on commercial cellulolytic mixtures and purified cellulases. The results suggested the key enzymes of cellulolytic cocktails (CBHs) were sensitive to inhibition driven by xylooligosaccharides interlinked to phenolics. Supplementation of cellulolytic enzyme cocktails with LCC cleaving enzymes have thus potential in mitigating the inhibition. The purer HMW oligophenols were also found to be inhibitory, but their relative amount in the pretreatment liquor was low. However, development of oligophenol-tolerant CBHs could be another route to improve the overall hydrolysis. On the other hand, at low concentration the phenolic compounds stimulate hydrolysis by activation of LPMOs, which should be considered in design of pretreatment and hydrolysis processes.

Declarations

Supplementary Information

The online version contains supplementary material available

Ethical approval and consent to participate

Not applicable.

Consent for publication Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have or could be perceived to have influenced the work reported in this article.

Funding

This work was funded by the INNO INDIGO Partnership Programme Biobased Energy (grant # 311971) and by the Estonian Research Council (grant, PRG1236). We are also grateful for the support by the FinnCERES Bioeconomy Ecosystem (Academy of Finland).

Authors' contributions

KK and KM coordinated and supervised the research. PV supervised and advised on kinetic studies. ASB, VP, RK, KN, PV and KM designed experiments; ASB, VP, RK, KN, TK; AM performed experiments and analysed the data; ASB, PV and KM wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Riitta Alander is acknowledged for technical assistance in enzyme activity experiments, Ulla Vornamo and Marita Ikonen are thanked for technical assistance in carbohydrate analysis.

References

1. Sannigrahi, P., Kim, D.H., Jung, S., Ragauskas, A. Pseudo-lignin and pretreatment chemistry. Energy and Environmental Science, 2011; 4(4):1306–1310. doi: 10.1039/c0ee00378f.
2. Jönsson, L. J. and Martín, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. Bioresource Technology, 2016; 199:103–112. doi: 10.1016/j.biortech.2015.10.009.
3. Van Dyk, J.S. and Pletschke, B.I. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-Factors affecting enzymes, conversion and synergy. Biotechnology Advances, 2012; 30(6):1458–1480. doi: 10.1016/j.biotechadv.2012.03.002.
4. Müller, G., Várnai, A., Johansen, K.S., Eijsink, V.G.H., Horn, S.J. (2015) Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions. Biotechnology for Biofuels; 2015; 8(1):1–9. doi: 10.1186/s13068-015-0376-y.
5. Bissaro, B., Røhr, Å.K., Müller, G., Chylenski, P., Skaugen, M., Forsberg, Z., Horn, S.J.,Vaaje-Kolstad, G., Eijsink, V.G.H. Oxidative cleavage of polysaccharides by monocopper enzymes depends on H_2O_2.

Nature Chemical Biology, 2017; 13(10):1123–1128. doi: 10.1038/nchembio.2470.

6. Kont, R., Bissaro, B., Eijsink, V. G. H., and Väljamäe, P. Kinetic insights into the peroxygenase activity of cellulose-active lytic polysaccharide monooxygenases (LPMOs). *Nat. Commun.,* 2020*;* **11**:5786. doi: 10.1038/s41467-020-19561-8.
7. Qing, Q., Yang, B. and Wyman, C.E. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresource Technology, 2010; 101(24):9624–9630. doi: 10.1016/j.biortech.2010.06.137.
8. Kumar, R. and Wyman, C.E. Strong cellulase inhibition by Mannan polysaccharides in cellulose conversion to sugars. Biotechnology and Bioengineering, 2014; 111(7):1341–1353. doi: 10.1002/bit.25218.
9. Rahikainen, J., Mikander, S., Marjamaa, K., Tamminen, T., Lappas, A., Viikari, L., Kruus, K. (2011) Inhibition of enzymatic hydrolysis by residual lignins from softwood-study of enzyme binding and inactivation on lignin-rich surface. Biotechnology and Bioengineering, 2011; 108(12): 2823-2834. doi: 10.1002/bit.23242.
10. Mitchell, V.D., Taylor, C.M. and Bauer, S. (2014) Comprehensive Analysis of Monomeric Phenolics in Dilute Acid Plant Hydrolysates. BioEnergy Research, 2014; 7(2):654–669. doi: 10.1007/s12155-013-9392-6.
11. Rasmussen, H., Sørensen, H.R. and Meyer, A.S. Formation of degradation compounds from lignocellulosic biomass in the biorefinery: sugar reaction mechanisms. Carbohydrate Research, 2014; 385:45–57. doi: 10.1016/j.carres.2013.08.029.
12. Djioleu, A. and Carrier, D. J. Statistical Approach for the Identification of Cellulolytic Enzyme Inhibitors Using Switchgrass Dilute Acid Prehydrolyzates as a Model System. ACS Sustainable Chemistry & Engineering, 2018; 6(3): 3443–3452.
13. Rasmussen, H., Tanner, D., Sørensen, H. R., Meyer, A. S. New degradation compounds from lignocellulosic biomass pretreatment: routes for formation of potent oligophenolic enzyme inhibitors. Green Chem. Royal Society of Chemistry, 2017; 19(2):464–473. doi: 10.1039/C6GC01809B.
14. Rasmussen, H., Sørensen, H.R., Tanner, D., Meyer, A.S. New pentose dimers with bicyclic moieties from pretreated biomass. RSC Adv. Royal Society of Chemistry, 2017; 7(9):5206–5213. doi: 10.1039/C6RA25432B.
15. Kim, Y., Ximenes, E., Mosier, N.S., Ladisch, M.R. (2011) Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. Enzyme and Microbial Technology, 2011; 48:408–415. doi: 10.1016/j.enzmictec.2011.01.007.
16. Mathibe, B.N., Malgas, S., Radosavljevic, L., Kumar, V., Shukla, P., Pletschke, B.I. (2020) Lignocellulosic pretreatment-mediated phenolic by-products generation and their effect on the inhibition of an endo-1,4-β-xylanase from Thermomyces lanuginosus VAPS-24. 3 Biotech. Springer International Publishing, 2020; 10(8):1–11. doi: 10.1007/s13205-020-02343-w
17. Tejirian, A. and Xu, F. Inhibition of enzymatic cellulolysis by phenolic compounds. Enzyme and Microbial Technology, 2011; 48(3):239–247. doi: 10.1016/j.enzmictec.2010.11.004.

18. Qin, L., Li, W-Ch., Liu, L., Zhu, J-q., Li, X., Li, B-zh., Yuan, Y-j. (2016) Inhibition of lignin-derived phenolic compounds to cellulase. Biotechnology for Biofuels, 2016; 9(1):70. doi: 10.1186/s13068-016-0485-2.
19. Kont, R., Kurašin, M., Teugjas, H., Väljamäe, P. (2013) Strong cellulase inhibitors from the hydrothermal pretreatment of wheat straw. Biotechnology for Biofuels, 2013; 6(1):1–14. doi: 10.1186/1754-6834-6-135.
20. Kellock, M., Rahikainen, J., Marjamaa, K., Kruus, K. Lignin-derived inhibition of monocomponent cellulases and a xylanase in the hydrolysis of lignocellulosics. Bioresource Technology, 2017; 232:183–191. doi: 10.1016/j.biortech.2017.01.072.
21. Karlsson, J., Siika-Aho, M., Tenkanen, M.,Tjerneld, F.b (2002) Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. Journal of Biotechnology, 2002; 99(1):63–78. doi: 10.1016/S0168-1656(02)00156-6.
22. Haven, M. Ø. and Jørgensen, H. The challenging measurement of protein in complex biomass-derived samples. Applied Biochemistry and Biotechnology, 2014; 172(1):87–101. doi: 10.1007/s12010-013-0466-x.
23. Bhikhabhai, R., Johansson, G. and Pettersson, G. Isolation of cellulolytic enzymes from *Trichoderma reesei* QM 9414. Journal of Applied Biochemistry, 1984; 6(5–6):336–345.
24. Velleste, R., Teugjas, H. and Väljamäe, P. Reducing end-specific fluorescence labeled celluloses for cellulase mode of action. Cellulose, 2010; 17(1):125–138. doi: 10.1007/s10570-009-9356-3.
25. Sluiter, A., Hames, B., Ruiz, R.O., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP), Technical Report NREL/TP-510-42618. National Renewable Energy Laboratory, U.S. Department of Energy, 2011.
26. Overend, R.P. and Chornet, E. Fractionation of lignocellulosics by steam-aqueous pretreatments. Phil. Trans. R. Soc. Lond. A, 1987; 321:23–536.
27. Carvalheiro, F., Silva-Fernandes, T., Duarte, L.C., Gírio, F.M. Wheat straw autohydrolysis: Process optimization and products characterization. Applied Biochemistry and Biotechnology, 2009; 153(1–3): 84–93. doi: 10.1007/s12010-008-8448-0.
28. Petersen, M.Ø., Larsen, J. and Thomsen, M.H. (2009) Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals. Biomass and Bioenergy, 2009; 33(5):834–840. doi: 10.1016/j.biombioe.2009.01.004.
29. Goldschmid, O. Ultraviolet spectra. In Sarkanen, K. V. and Ludwig, C. H. (eds) Lignins: Occurrence, Formation, Structure and Reactions. John Wiley & Sons Inc., 1971; pp. 241–298.
30. Tenkanen, M. and Siika-aho, M. An α-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. Journal of Biotechnology, 2000; 78(2):149–161. doi: 10.1016/S0168-1656(99)00240-0.
31. Tenkanen, M., Makkonen, M., Perttula, M., Viikari, L., Teleman, A. Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. Journal of Biotechnology, 1997; 57(1–3):191–204. doi: 10.1016/S0168-1656(97)00099-0.

32. Liang, K. Y., Chiu, Y. F. and Beaty, T. H. A robust identity-by-descent procedure using affected sib pairs: multipoint mapping for complex diseases. Human heredity, 2001; 51(1–2):64–78. doi: 10.1159/000022961.
33. Borrega, M., Niemelä, K. and Sixta, H. Effect of hydrothermal treatment intensity on the formation of degradation products from birchwood. Holzforschung, 2013; 67(8): 871–879. doi: 10.1515/hf-2013-0019.
34. Lever, M. A new reaction for colorimetric determination of carbohydrates. Analytical Biochemistry, 1972; 47(1):273–279. doi: 10.1016/0003-2697(72)90301-6.
35. Niemela, K. Unusial Propiophenones Formed During Hydrolyis of Birch Lignin. Holzforschung, 1990; 45(3):233–235. doi: https://doi.org/10.1515/hfsg.1991.45.3.233.
36. Popoff, T. and Theander, O. Formation of aromatic compounds from carbohydrates: part 1. Reaction of D-glucuronic acid, D-glacturonic acid, D-xylose, and L-arabinose in slightly acidic, aqueous solution. Carbohydrate Research, 1972; 21(1):135–149.
37. Teugjas, H. and Väljamäe, P. Product inhibition of cellulases studied with 14C-labeled cellulose substrates. Biotechnology for Biofuels, 2013; 6(1):1–14. doi: 10.1186/1754-6834-6-104.
38. Payne, C.M., Knott, B.C., Mayes, H.B., Hansson, H., Himmel, M.E., Sandgren, M., Ståhlberg, J., Beckham, G.T. Fungal Cellulases. Chemical Reviews, 2015; 115(3):1308–1448. doi: 10.1021/cr500351c.
39. Narron, R.H., Chang, H.-m., Jameel, H., Park, S. Soluble Lignin Recovered from Biorefinery Pretreatment Hydrolyzate Characterized by Lignin–Carbohydrate Complexes. ACS Sustainable Chemistry & Engineering, 2017; 5(11):10763–10771. doi: 10.1021/acssuschemeng.7b02716.
40. Sumerskiy, I., Pranovich, A., Holmbom, B., Willför, S. Lignin and Other Aromatic Substances Released from Spruce Wood During Pressurized Hot-Water Extraction, Part 1: Extraction, Fractionation and Physico-Chemical Characterization', Journal of Wood Chemistry and Technology, 2015; 35(6):387–397. doi: 10.1080/02773813.2014.965331.
41. Zhang, Y., Wang, S., Xu, W., Cheng, F., Pranovich, A., Smeds, A., Willför, S., Xu, C. Valorization of Lignin-Carbohydrate Complexes from Hydrolysates of Norway Spruce: Efficient Separation, Structural Characterization, and Antioxidant Activity. ACS Sustainable Chemistry and Engineering, 2019; 7(1):1447–1456. doi: 10.1021/acssuschemeng.8b05142.
42. Tarasov, D., Leitch, M. and Fatehi, P. Lignin-carbohydrate complexes: Properties, applications, analyses, and methods of extraction: A review. Biotechnology for Biofuels, 2018; 11(1):1–28. doi: 10.1186/s13068-018-1262-1.
43. Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., Saddler, J. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. Journal of Biotechnology, 2006; 125(2):198–209. doi: 10.1016/j.jbiotec.2006.02.021.
44. Vardakou, M., Flint, J., Christakopoulos, P., Lewis, R.J., Gilbert, H.J.,Murray, J.W. A family 10 *Thermoascus aurantiacus* xylanase utilizes arabinose decorations of xylan as significant substrate

specificity determinants. Journal of Molecular Biology, 2005; 352(5):1060–1067. doi: 10.1016/j.jmb.2005.07.051.

45. Bailey, M., Buchert, J. and Viikari, L. Effect of pH on production of xylanase by *Trichoderma reesei* on xylan- and cellulose-based media. Applied Microbiology and Biotechnology, 1993; 40(2–3):224–229. doi: 10.1007/BF00170371.
46. Nakazawa, H., Okada, K., Kobayashi, R., Kubota, T., Onodera, T.,Ochiai, N., Omata, N., Ogasawara, W., Okada, H., Morikawa, Y. Characterization of the catalytic domains of *Trichoderma reesei* endoglucanase I, II, and III, expressed in Escherichia coli. Applied Microbiology and Biotechnology, 2008; 81(4):681–689. doi: 10.1007/s00253-008-1667-z.
47. Ximenes, E., Kim, Y., Mosier, N., Dien, B., Ladisch, M. Inhibition of cellulases by phenols. Enzyme and Microbial Technology, 2010; 46(3–4):170–176. doi: 10.1016/j.enzmictec.2009.11.001.
48. Kont, R., Pihlajaniemi, V., Borisova, A.S., Aro, N., Marjamaa, K., Loogen, J., Büchs, J., Eijsink, V.G.H., Kruus, K.,Väljamaë, P. The liquid fraction from hydrothermal pretreatment of wheat straw provides lytic polysaccharide monooxygenases with both electrons and H_2O_2 co-substrate. Biotechnology for Biofuels, 2019; 12(1):235. doi: 10.1186/s13068-019-1578-5.

Figures

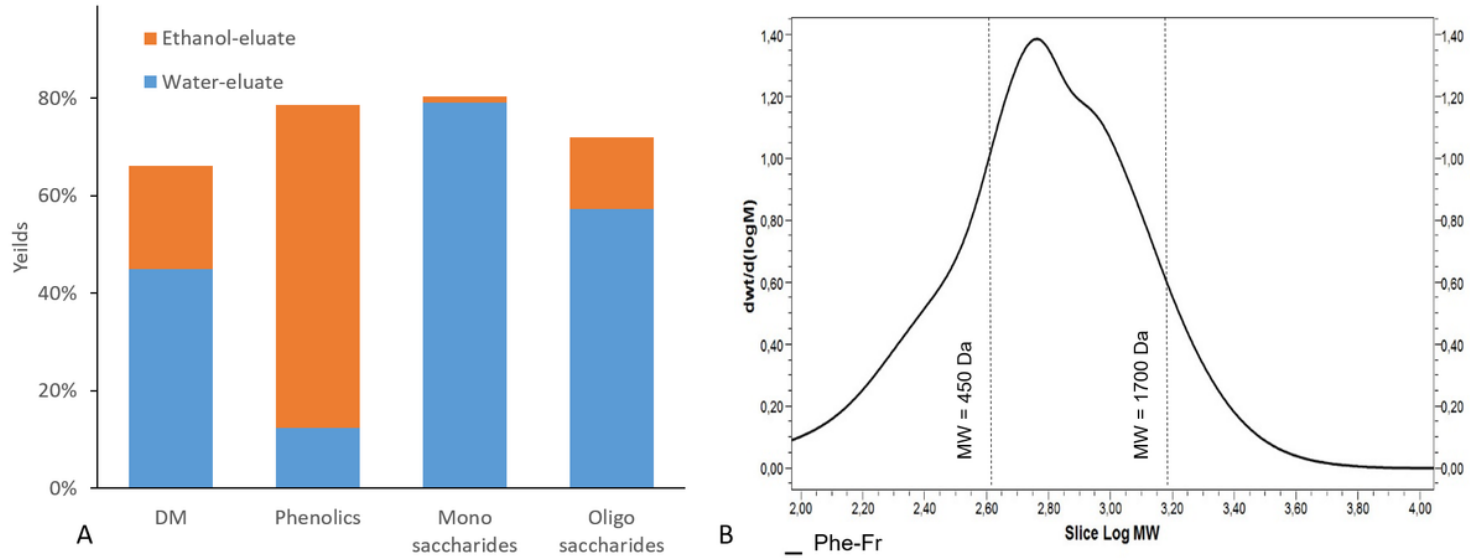

Figure 1

Isolation of Phe-Fr on a column packed with Amberlite XAD-7 resin. A - recovery of DM, carbohydrates and phenolics in fractionations eluted with water and ethanol (Phe-Fr). B - MW distribution in isolated Phe-Fr. The carbohydrates were analyzed after acid hydrolysis with HPAEC-PAD and phenolics were quantified with the UV-method. MW distribution was determined with SEC connected to UV detector.

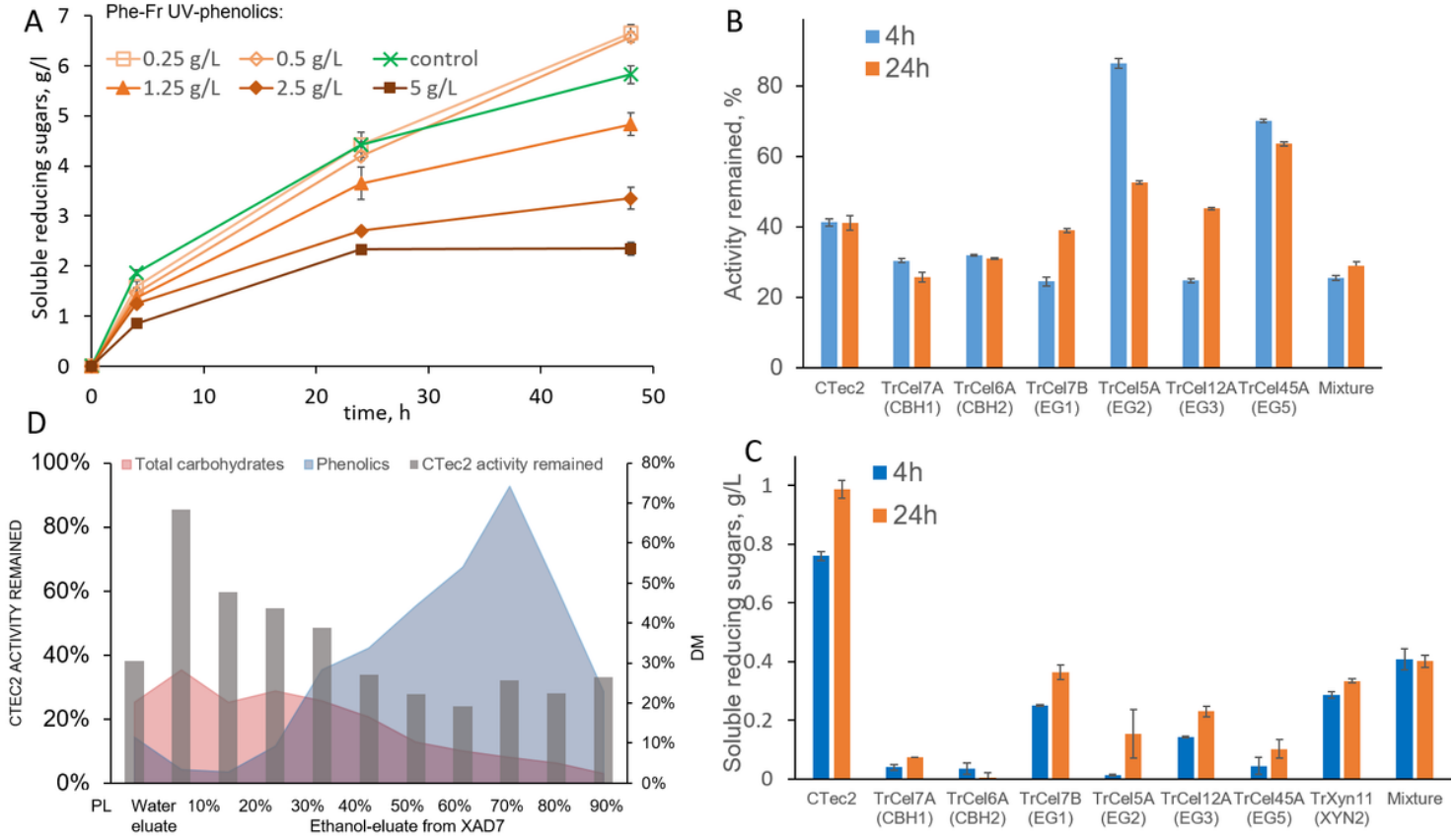

Figure 2

Screening for inhibitory effect of Phe-Fr. All hydrolysis reactions were performed at 45°C in 50 mM sodium acetate pH 5.0 with Avicel concentration 10 g/L (A,B,D) and enzyme dosage 0.1 g/L. A - Progress curves for Cellic CTec2 in presence of Phe-Fr (0-5 g/L); B - Remained activities in presence of Phe-Fr (5 g/L) of purified cellulases (TrCel7A, TrCe6A, TrCel5A, TrCel12A, TrCel45A) and their mixture with TrXyn11, all supplemented with AnCel3A (5000 nkat/L). C - Hydrolysis of carbohydrates in Phe-Fr (5 g/L) by Cellic CTec2 and purified enzymes from T. reesei.; D – Analytical fractionation of PL with ethanol gradient and inhibitory effect of different fractions (10 g/L total solids) on Cellic CTec2 at 24 h time point. Soluble reducing sugars were determined with PAHBAH (A-C) and with HPLC (D).

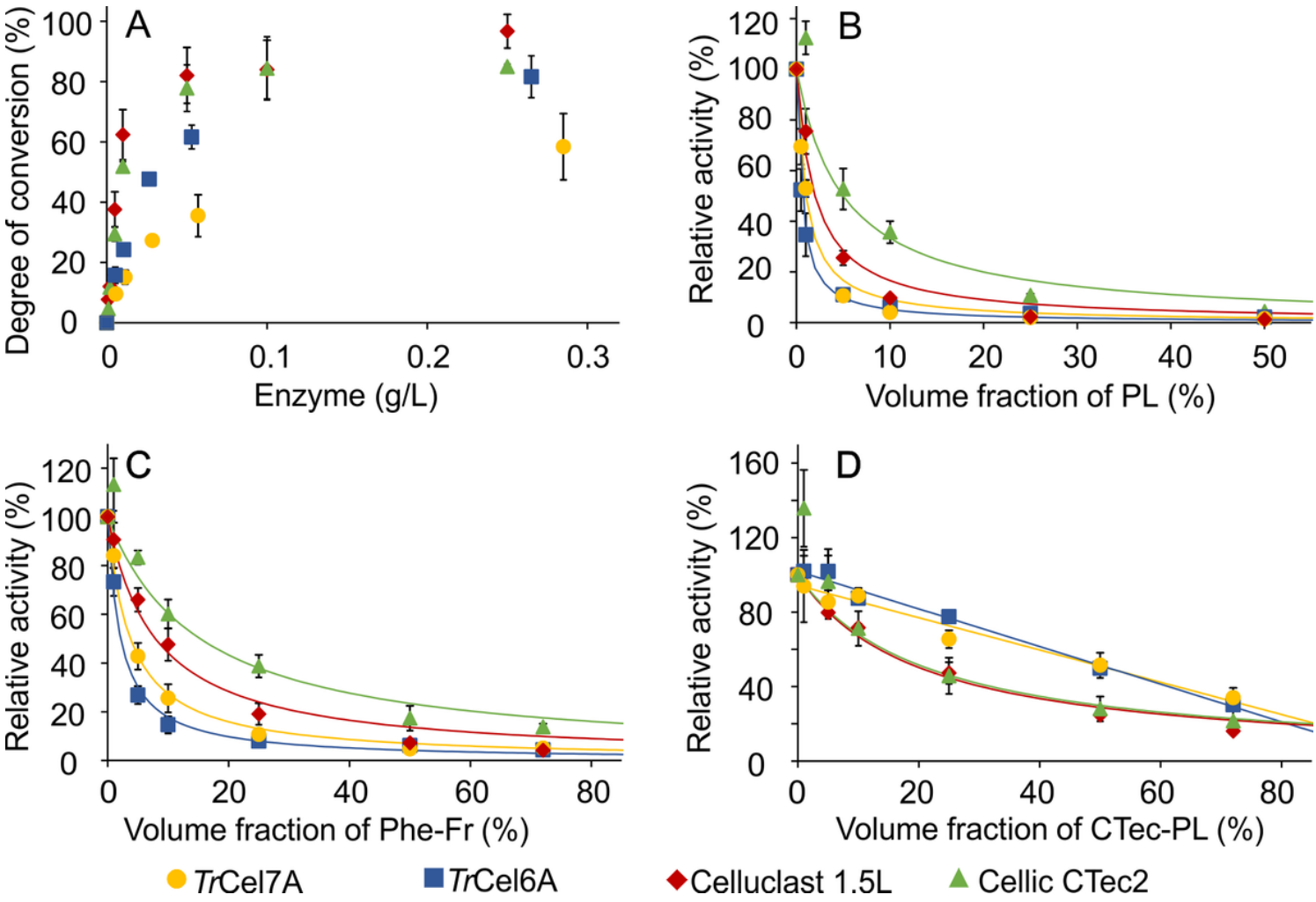

Figure 3

Inhibition of cellulases studied with 14C-BMCC model substrate. All reactions were made in 50 mM sodium acetate pH 5.0 at 50°C for 18 h with 14C-BMCC concentration of 0.6 g/L. A - the effect of enzyme dosage on the degree of degradation of 14C-BMCC. Inhibition of cellulases by PL (B), Phe-Fr (C), and Ctec-PL (C). For B-D, the concentration of TrCel7A and TrCel6A was 1.0 μM (about 0.06 g/L) and the concentration of Celluclast 1.5 L and Cellic Ctec2 was 0.01 g/L. The concentration of inhibitors is expressed as their volume fraction in the reaction mixture (in %). The Phe-Fr and Ctec-PL were dosed based on their content of phenolics taking the concentration of phenolics in PL (4.55 g/L) equal to 100 % volume fraction.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinfo19082021.docx