Food Chemistry 198 (2016) 132–140

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Chemical characterization of hydrothermally pretreated and enzyme-digested wheat straw: An evaluation of recalcitrance

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ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 9 July 2015
Accepted 22 July 2015
Available online 14 August 2015

Chemical compounds studied in this article:
D-Glucose (CID: 53782692)
D-Xylose (CID: 135191)
Ferulic acid (CID: 445858)
Diferulic acid (CID: 5281770)

Keywords:
Alkali extraction
Biofuels
Hydrothermal pretreatment
Enzyme saccharification
Wheat straw

ABSTRACT

There is great interest in understanding changes that occur to cell wall constituents during saccharification of pretreated lignocellulose, particularly in relation to recalcitrance of the residues. This paper reports the effects of hydrothermal pretreatment followed by enzyme hydrolysis on the extractability and properties of recalcitrant wheat straw polymers. The results show that the undigested residue had lost much of its archestructure. Compositional analysis portrayed a considerable loss of cross-linking di-ferulic acid phenolics, hemicellulosic and cellulosic sugars. The remaining cellulosic and non-cellulosic polysaccharides were much more readily extractable in alkali and molecular profiling revealed the presence of low M_w oligomers in the fractions suggesting the partial enzyme hydrolysis of hemicelluloses and cellulose. Simultaneous saccharification and fermentation of the pretreated and enzyme-digested residues surprisingly resulted in ethanol yields of up to 99% of the theoretical. This is discussed in relation to the "recalcitrant" nature of the original pretreated and enzyme digested biomass.

1. Introduction

Meeting EU targets for renewable transport fuels by 2020 will necessitate a large increase in bioenergy feedstocks (Glithero, Wilson, & Ramsden, 2013). Lignocellulosic biomass is an abundant, renewable source of cellulosic glucose and worldwide attention has focused on the major biotechnological applications of this underutilized resource, including biofuel production (Mosier et al., 2005). Despite the potential promise of cellulosic ethanol and other cellulose-derived biofuels, major obstacles need to be addressed to make the process feasible and economically viable for large scale applications (Wyman, 1996). A major problem concerns the resistance of lignocellulose to saccharification. This is due to a number of compounding factors, including steric hindrance and cellulose crystallinity. The steric hindrance to cellulolysis is associated with hemicellulosic and sometimes pectic polymers which interfere with access of hydrolytic enzymes, compounded by their interpolymeric cross linking with themselves and lignin. The latter also acts to bind to many cell-wall degrading enzymes making them inactive (Rahikainen et al., 2011; Tejirian & Xu, 2011). There is substantial evidence that the lignin polymer is cross-linked to cellulose via hemicellulose and simple phenolics such as diferulic acid (Ralph, Grabber, and Hatfield (1995). A consequence of cross-linking, despite its importance to plant growth and development is reduced digestibility of the polysaccharides (Grabber, Ralph, & Hatfield, 1998). To improve enzymatic digestibility, interactions between lignin and polysaccharide components of the cell wall are disrupted initially

Abbreviations: AIR, alcohol insoluble residue; DiFA, diferulic acid; DM, dry matter; HT, hydrothermal pretreatment; HTED, hydrothermally pretreated enzyme digested; HPSEC, high performance size exclusion chromatography; HW, hot water extraction; M_w, molecular weight; SSF, simultaneous saccharification and fermentation.

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http://dx.doi.org/10.1016/j.foodchem.2015.07.108
0308-8146/

through a combination of thermophysical/chemical pretreatments (Wyman et al., 2005; Yang & Wyman, 2008).

Some attention has been paid to understanding the effects of the pretreatments on the physicochemical characteristics of the lignocellulose. Such investigations have included assessments to varying degrees of change to the polymeric components within the cell walls (DeMartini et al., 2011; Holopainen-Mantila et al., 2013; Kabel, Bos, Zeevalking, Voragen, & Schols, 2007; Lawther, Sun, & Banks, 1996; Merali et al., 2013; Sun, Lawther, & Banks, 1996; Sun et al., 2005). However to date, there are only a few studies concerning the nature of changes that occur to the cell-wall constituents during the saccharification stage, particularly in relation to the recalcitrance or resistance to digestion of the remaining insoluble residues. Hansen et al. (2014) evaluated the extractability and digestibility of plant cell wall polysaccharides during hydrothermal and enzymatic degradation of wheat straw stems and leaves. Cell wall materials before pretreatment, after pretreatment and after saccharification were sequentially extracted using increasing strengths of alkali, and evaluated using microarray polymer profiling. The results suggested that arabinoxylans and mixed-linkage glucans were loosely bound in the cell wall, and shielded other more tightly bound non-cellulosic polysaccharides until solubilized by pretreatment. They did not, however, detect any ferulic acid moieties which have been implicated in interpolymeric cross-linking in wheat straw (Ralph et al., 1995). Also, they looked at changes resulting from only one pretreatment severity.

Previously, Merali et al. (2013) investigated the effects of two hydrothermal pretreatments on the composition and polymeric nature of cell-wall polysaccharides of wheat straw. We demonstrated that under conditions that augment enzymatic saccharification, significant changes occur in the cell wall hemicelluloses, lignin, and cross-linking phenolics. Of particular note was the reduction in the molecular weight (M_w) of the remaining arabinoxylans and the considerable reduction in ferulic and diferulic acids. In the current study, we report the carbohydrate yields and profiles of cell-wall polymers that remain after subsequent, extensive enzymatic digestion of the hydrothermally pretreated wheat straw. In order to further understand resistance to cellulases, the pretreated and enzyme-digested biomass was evaluated for changes in ultrastructure and then sequentially extracted in increasing concentrations of alkali (Redgwell & Selvendran, 1986) prior to characterization of extracted components for composition and molecular weight profiles. The recalcitrance of the pretreated and enzyme-digested material was further evaluated by subjecting it to simultaneous saccharification and fermentation (SSF).

2. Materials and methods

2.1. Raw material

Control (untreated) and HT wheat straw was provided by Biogold (wheat straw was obtained from Hõbetse farm, Pärnu, Estonia and was grown similar to all conventional cropping). For the control, wheat straw was cut into approximately 2 cm long pieces, dry milled (Krups F20342, Cedex, France) and sieved (1 mm sieve). The sieved wheat straw was oven dried at 50 °C and stored in air-tight containers at room temperature until required for analysis.

2.2. Hydrothermal pretreatment

For each pretreatment 400 g (dry weight) of knife-milled (GRINDOMIX GM 300, Tallinn, Estonia) wheat straw (particle size 0.4–10 mm) was loaded into reactor (solid-to-water-ratio 1:10) and water was pumped through the packed material bed at a circulation flow rate of 4–5 L/min. The reactor (inner diameter 106 mm, material AISI 316) had a total volume of 6 L, with an electric heater and liquid phase circulation pump (Micropump Series-2200, Tallinn, Estonia). The wheat straw was then HT at two holding temperatures (190 and 200 °C) for 15 and 20 min respectively. Following HT the solid biomass was separated from the liquor by filtration and both fractions were frozen after dry mater evaluation.

2.3. Enzymatic hydrolysis

Prior to hydrolysis, the solid fraction of the HT wheat straw was washed with water (55 °C, 2.1 L/kg (wet weight)) and filtered through 90 μm mesh cloth. Enzymatic hydrolysis was performed in 10 g/L substrate concentration (dry matter, DM) using mixture of Celluclast 1.5 L (10 FPU/g substrate DM) and Novozymes 188 (100 nkat β-glucosidase activity/g substrate DM) at pH 5 and 45 °C in total volume of 10 L. The hydrolysis mixtures were supplemented with 0.01% (w/v) Na-azide for prevention of microbial growth. The hydrolysis was followed by sampling (1 mL) and quantification of reducing sugars with the PAHBAH assay (Lever, 1972). The hydrolysis was allowed to proceed for 72 h after which solids and liquids were separated by centrifugation (3963×*g*, 30 min). Enzymes in the liquid fractions were inactivated by heating (95 °C, 10 min). The solids were stored frozen (−20 °C) until analyzed.

2.4. Analysis of raw and HT material

2.4.1. Dry matter (DM) determination

The DM of the samples was determined by weighing triplicate samples in an infra-red balance (Mettler PM200, Leicester, UK) heated to 100 °C.

2.4.2. Preparation of alcohol insoluble residues (AIRs)

AIRs were prepared from the control and the HT and enzyme-digested wheat straw as described in Merali et al., 2013.

2.4.3. Cell wall fractionation

Sequential extraction (in progressively stronger alkali) was conducted by a modification of the method of Redgwell and Selvendran (1986) in degassed aqueous solution and in the presence of $NaBH_4$ to minimize alkaline peeling. HT AIRs together with the control (2 g) were suspended in hot water (60 °C, 200 mL) and cell wall components extracted (shaking, 2 h, 25 °C). The extracts were centrifuged (10,000×*g*, 1 h) and the supernatant filtered through Whatman GF/C filter paper (Whatman, Maidstone, UK) and freeze-dried. The insoluble residue was further extracted with 0.5 mol/L KOH with 20 mmol L^{-1} $NaBH_4$ for 2 h (shaking, 25 °C) centrifuged (10,000×*g*, 60 min) and the supernatant filtered. The filtrate was first neutralized with acetic acid, extensively dialyzed (tubing size 30/32", Medicell International, London, UK, 7 days, changing 3× times daily) and then freeze-dried. The residue was further extracted as above in 1 mol/L and 4 mol/L KOH containing 20 mmol L^{-1} $NaBH_4$, filtered and neutralized. All the filtrates were freeze-dried as above following dialysis. The freeze-dried extracts, insoluble residues and AIRs were biochemically analyzed in duplicate.

2.4.4. Carbohydrate analysis

Sugars were released from the fractions by hydrolysis with H_2SO_4 (w = 72%) for 3 h, followed by dilution to 1 mol/L (Saemen hydrolysis). Hydrolyzed monosaccharides were analyzed as their alditol acetates by GC as described in Merali et al., 2013.

2.4.5. Molecular weight (M_w) distributions

Freeze dried alkali fractions (2 mg) were dissolved in ammonia solution (w = 30%) and heated (60 °C, 1 h) to solubilize the extracts. High performance size exclusion chromatography (HPSEC) was performed on a Dionex (Sunnyvale, USA) Ultimate 3000 UPLC system equipped with three TSKgel SuperAW columns (6.0 mm ID × 150 mm per column; 4 μm) in series (SuperAW4000, SuperAW3000, SuperAW2500; Tosoh Bioscience, Stuttgart, Germany), in combination with a guard column (Tosoh Bioscience, Stuttgart, Germany). Columns were eluted at 40 °C with 0.2 mol/L sodium nitrate at 0.6 mL/min. The eluate was monitored with a Shodex RI-101 (Kawasaki, Japan) refractive index (RI) detector. Dextran standards (Sigma–Aldrich, Poole, Dorset, UK) were used for M_w calibration.

2.4.6. Lignin and phenolic acids

Klason lignin was quantified gravimetrically as described in Merali et al. (2013) and analyzed in triplicate.

The total alkali-extractable hydroxycinnamate content of the AIRs and insoluble residues after fractionation was determined by saponification of 5 mg of sample in 4 mol/L sodium hydroxide as described in Merali et al. (2013).

2.4.7. Microscopy, SEM and AFM imaging

AIRs of samples were observed in their native state and photographed using a Wild M8 stereomicroscope. For higher magnification, samples were observed with an Olympus BX60 microscope (Olympus, Japan) interfaced with a ProRes© Capture Pro 2.1 camera and software (Jenoptik, Jena, Germany). The autofluorescence was recorded using the UV filter cube with a filter cube configuration of excitation filter band pass 330 nm to 385 nm, barrier filter 420 nm.

SEM of AIRs was conducted using Zeiss Supra 55 VP SEM (Carl Zeiss Ltd., Cambridge, UK) at 20 kV. The atomic force microscope used in this study was an MFP-3D BIO (Asylum Research, CA, USA) and it was operated in air using AC mode for imaging. The cantilevers used were Olympus AC160TS (Olympus, Japan) with a nominal spring constant of ~42 $N.m^{-1}$ oscillated at a frequency 10% below resonance (typically around 320 kHz). The damping set point for imaging was kept to the minimum value that allowed stable tracking of the sample surface in order to minimise any sample deformation. Images were acquired at a relatively slow scan rates of 0.5–1 Hz to ensure the rough topography was properly imaged. Height, amplitude and phase images were captured simultaneously.

2.5. Simultaneous saccharification and fermentation (SSF)

2.5.1. Yeast growth and cultivation

A strain of *Saccharomyces cerevisiae* used in this work was obtained from The National Collection of Yeast Cultures (NCYC strain 2826, Institute of Food Research, UK). Active cultures for inoculation were prepared by growing the organism in YM (growth medium Sigma–Aldrich, Poole, Dorset, UK) supplemented with: 0.3% Yeast Extract, 0.3% Malt Extract, 0.5% Peptone and 1% Dextrose (Difco, Lawrence, Kansas, USA) shaking (rotary shaker, 200 rpm, 18 h, 25 °C). The cells were harvested, washed with 0.9% NaCl solution to remove any residual nutrients, resuspended in 10× concentrated Yeast Nitrogen Base (YNB; Formedium, Hunstanton, UK) which contained no glucose and inoculated (10% v/v) in fermentation flasks to give initial yeast concentration of 5 g/L (dry weight).

2.5.2. Fermentation

Fermentations were performed on HT wheat straw together with HT and enzyme hydrolyzed residues (1% w/v) in closed Universal bottles (20 mL, VWR International Ltd Leicestershire, UK) containing 9 mL of fermentation medium (YNB) without nutrients in sodium acetate buffer pH 5.0 (autoclaved, 121 °C, 90 min) and 1 mL of yeast cell suspension (10%). A cellulase load (Accellerase® 1500, Genencor, Wiltshire, UK) corresponding to 20 FPU/g dry cellulosic source supplemented with β-glucosidase (17 IU, Novozymes 188, Bagsvaerd, Denmark) was transferred to the system prior to the SSF process. The experiments were performed at pH 5 and 25 °C for 36 h (anaerobically, shaking). After the reaction time, enzyme deactivation (100 °C, 10 min) was followed by quantification using HPLC (Waters®, Waters Ltd, Hertfordshire, UK) equipped with a refractive index detector using a Bio-Rad aminex HPX-87P column (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) at 65 °C and MQ water (Milipore®, Millipore Ltd, Watford, UK) as mobile phase at a flow rate of 0.6 mL min^{-1}. Positive (copier paper) and negative (MQ water) controls were prepared using exactly the same procedure as the test samples and the ethanol peaks in the negative controls were subtracted from the test peaks prior to tabulation. Ethanol values are calculated from the carbohydrate data in Table 2 and as illustrated below.

2.6. Calculations

Calculations were performed using Microsoft Office Excel (2010; Microsoft, Berkshire, UK). The percentage carbohydrate yields were calculated from the monomeric amounts given in Table 1 and changes as a result of HT and enzyme digestion have been calculated based on the original starting material (control AIR).

The ethanol yields were calculated as follows:

Ethanol (g) = Glucose present (%) × Weight of material × 0.511 (factor for ethanol conversion) × 1.111 (water of hydrolysis).

3. Results and discussion

3.1. Chemical composition of recalcitrant residues (resistant to enzymatic hydrolysis)

Previously, it was shown that the HT based on hot pressurized water without any added catalysts efficiently fragments wheat straw structure and causes partial solubilization of predominantly hemicellulosic sugars (Merali et al., 2013). Increasing the pretreatment temperature and residence time also enhanced alkaline extractability of the remaining, insoluble carbohydrates (Holopainen-Mantila et al., 2013). In the current study, HT (190 and 200 °C, 15 and 20 min, respectively) solid fractions (100 g DM) were hydrolyzed using a mixture of commercial enzyme preparations Celluclast 1.5 L and Novozyme 188 (Supplementary Fig. S1) for 72 h. The recalcitrant residues remaining after enzymatic hydrolysis (HTED) were purified to give alcohol insoluble residues (AIRs), then fractionated in progressively stronger KOH to evaluate polymeric changes and characteristics of cell-wall components resistant to enzymatic hydrolysis.

Table 1
Compositional data of the enzymatic hydrolysis residues of the wheat straw samples hydrothermally pretreated (190 and 200 °C), and analyzed in alcohol insoluble residues (AIRs).

Component	Content (% w/w of AIR)		
	Untreated	HTED residue-190 °C	HTED residue-200 °C
DM	92.1 ± 0.22	17.0 ± 1.9	9.0 ± 2.7
Cellulose	37.1 ± 2.1	41.2 ± 2.0	41.0 ± 2.6
Hemicellulose (total)	23.5 ± 4.2	14.2 ± 0.9	11.6 ± 1.5
Phenolic acids (esters)	0.9 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
Klason lignin	15.8 ± 1.3	38.1 ± 3.3	39.9 ± 3.9
Ash	8.0 ± 1.3	9.1 ± 2.1	11.2 ± 1.2

Table 1 shows the effect of hydrothermal pretreatment and enzyme hydrolysis on the gross compositions of alcohol insoluble residues (AIRs) with detailed sugar compositions shown in Table 2. Untreated wheat straw AIR consisted of 65% carbohydrate, most of which comprised polymers of glucose, xylose and arabinose. This included 37.1% (cellulosic) glucose and 23.5% (xylan) hemicellulose and in agreement with previous reports (Kristensen, Thygesen, Felby, Jørgensen, & Elder, 2008; Merali et al., 2013). The phenolic acid content in the HTED residues was significantly reduced compared to the control and HT wheat straw (Tables 1 and 3) particularly ferulic and diferulic acids (Merali et al., 2013). The pretreatment-induced reduction in insoluble carbohydrate resulted in a concomitant increase in the level of Klason lignin, from 25% to 38% and 40%, respectively in the digested residues (Table 1). If lignin (and ash) had not been degraded or lost into the hydrolysate, they would comprise a much larger component of the whole HTED residue particularly at 200 °C. The loss of lignin is consistent with earlier reports. Yelle et al. (2013) provided 2D-NMR evidence for cleavage of lignin and xylan substituents in wheat straw as a result of hydrothermal pretreatment. HT has also been shown to release significant quantities of lignin into the pretreatment liquor (Merali et al., 2013). Furthermore, it has been reported that putative lignin droplets released from the cell wall, form on the surface wheat straw fibers during HT (Kristensen et al., 2008). Since lignin acts as a physical barrier and impedes saccharification by restricting cellulase activity, its reduction or relocation through effective pretreatment such as HT is generally thought to greatly enhance fermentation potential. Nevertheless, some reports differ. For example, in studies on poplar, DeMartini et al. (2011) suggest that it is the cross-linking of lignin with the carbohydrate moieties that creates the greatest barrier to saccharification, rather than the quantity of lignin present. Furthermore, Wood et al. (2014), in studies on oilseed rape straw, indicate that lignin can enhance saccharification of pretreated material by keeping the structure open, enabling diffusion of cell wall degrading enzymes.

3.2. Ultrastructure of the enzyme hydrolyzed residues

Enzyme hydrolysis of hydrothermally pretreated samples resulted in a considerable loss of structure (Fig. 1a in comparison with Fig. 1b and c). This was most prominent in the HTED residue that had been pretreated at 200 °C (Fig. 1c) and was due to a combination of cell wall degradation and cell separation.

Staining for lignin with phloroglucinol-HCl (Speer, 1987) (Fig. 1e and f) produced a weak coloration in the HTED residues compared with the control (Fig. 1d) indicating a reduction in the hydroxycinnamaldehyde groups or total lignin. This was also indicated by a loss of blue UV autofluorescence at pH 7 (Fig. 1g–i). Loss of lignin was also accompanied by the loss of simple cross-linking phenolics such as ferulic acid, as indicated by a loss of turquoise UV autofluorescence at pH 11 (Fig. 1j–l) (Parker & Waldron, 1995).

Ultrastructure of the HTED residues was studied using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The SEM imaging (Fig 1m–o) was consistent with considerable fragmentation following pretreatment and enzyme digestion (Fig. 2n and o). AFM imaging of the untreated wheat straw surface

Table 2
Carbohydrate composition (μg/mg dry weight) of HT (190 and 200 °C) and enzyme digested wheat straw following sequential extraction.

	*Yield (g)	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	GlcA	Total
Ctrl AIR	2.60	1.82 ± 0.17	0.52 ± 0.06	25.39 ± 1.55	213.84 ± 10.91	6.51 ± 0.97	6.13 ± 0.22	370.88 ± 9.30	23.15 ± 0.96	648.80 ± 6.56
Ctrl HW	0.03	5.12 ± 0.53	0.74 ± 0.08	15.38 ± 2.11	27.03 ± 1.12	1.31 ± 0.09	3.20 ± 0.10	41.94 ± 2.13	14.10 ± 1.88	108.80 ± 1.61
Ctrl 0.5 M	0.26	nd	nd	42.55 ± 2.22	289.00 ± 5.16	3.96 ± 0.16	6.87 ± 1.40	19.26 ± 1.43	15.95 ± 1.54	377.90 ± 2.19
Ctrl 1 M	0.20	nd	nd	68.00 ± 1.38	429.60 ± 6.67	3.80 ± 0.06	6.10 ± 1.06	22.30 ± 1.02	19.09 ± 1.16	548.90 ± 4.24
Ctrl 4 M	0.26	nd	nd	51.36 ± 2.32	399.21 ± 5.11	9.79 ± 1.18	6.81 ± 1.08	29.10 ± 1.62	18.39 ± 1.18	514.70 ± 4.56
Ctrl residue	1.53	nd	nd	22.94 ± 1.97	191.19 ± 3.43	4.28 ± 0.20	6.90 ± 1.21	435.03 ± 5.66	20.69 ± 1.33	730.80 ± 6.33
HTED 190-AIR	2.10	nd	nd	12.28 ± 2.15	129.73 ± 3.16	6.38 ± 1.38	6.61 ± 1.15	412.00 ± 3.10	18.65 ± 1.13	587.90 ± 5.21
HTED 190-HW	0.05	nd	nd	5.80 ± 1.41	37.40 ± 1.12	6.02 ± 1.17	2.11 ± 0.10	91.97 ± 1.23	12.30 ± 0.52	155.80 ± 1.66
HTED 190-0.5 mol/L	0.30	nd	nd	18.30 ± 4.32	364.12 ± 3.30	4.92 ± 0.22	1.10 ± 0.30	28.97 ± 1.31	17.06 ± 0.56	434.50 ± 4.02
HTED 190-1 mol/L	0.27	nd	nd	14.78 ± 2.99	339.90 ± 4.52	1.86 ± 0.01	1.63 ± 0.14	46.90 ± 2.11	17.11 ± 0.53	432.20 ± 4.11
HTED 190-4 mol/L	0.30	nd	nd	18.31 ± 3.12	290.26 ± 2.21	6.38 ± 1.79	1.78 ± 0.09	41.94 ± 2.30	17.86 ± 0.61	376.50 ± 3.31
HTED 190-residue	0.86	nd	nd	5.79 ± 1.19	84.30 ± 1.14	3.67 ± 0.14	0.94 ± 0.01	421.00 ± 6.03	16.84 ± 0.55	533.10 ± 4.29
HTED 200-AIR	2.20	nd	nd	10.01 ± 3.33	105.73 ± 2.55	4.54 ± 1.04	2.85 ± 0.71	411.68 ± 3.04	20.63 ± 1.01	555.40 ± 5.71
HTED 200-HW	0.12	nd	nd	3.87 ± 0.92	40.82 ± 0.99	10.75 ± 1.03	14.84 ± 1.41	92.00 ± 1.17	12.13 ± 0.16	174.50 ± 1.23
HTED 200-0.5 mol/L	0.33	nd	nd	47.04 ± 5.41	384.30 ± 2.09	6.34 ± 0.55	1.04 ± 0.36	112.34 ± 2.31	12.56 ± 0.30	563.60 ± 5.21
HTED 200-1 mol/L	0.37	nd	nd	45.70 ± 4.06	299.23 ± 1.78	2.65 ± 0.12	1.33 ± 0.29	89.20 ± 3.11	12.28 ± 0.46	450.60 ± 4.22
HTED 200-4 mol/L	0.35	nd	nd	44.16 ± 5.18	238.21 ± 1.69	5.69 ± 0.52	1.73 ± 0.40	43.50 ± 1.15	12.41 ± 0.33	355.70 ± 4.19
HTED 200-residue	0.61	nd	nd	1.92 ± 0.19	62.14 ± 1.72	2.94 ± 0.14	1.26 ± 0.09	417.10 ± 5.23	17.44 ± 0.40	498.00 ± 5.02

Ctrl, control; HTED, hydrothermal pretreated and enzyme digested; nd, none detected.
* Yield is the dry weight of fraction recovered after sequential extraction of the AIR in hot water (HW) and progressively stronger alkali (0.5, 1 and 4 mol/L KOH); Residue represents the insoluble material after fractionation. Values are expressed as means of duplicate analysis ± SD.

Table 3
Phenolic acid composition (μg/mg) of hydrothermally pretreated and enzyme digested wheat straw (AIRs) and the insoluble residues following alkaline sequential extraction.

	p-Hydroxy benzaldehyde	Vanillin	*p*-Coumaric acid	Ferulic acid	8,8′-DiFA	8,5′-DiFA	5,5′-DiFA	8-*O*-4′-DiFA
Ctrl AIR	0.052 ± 0.03	0.204 ± 0.13	3.708 ± 0.11	2.979 ± 0.53	0.027 ± 0.01	0.391 ± 0.13	0.187 ± 0.07	0.497 ± 0.15
190 °C AIR	0.043 ± 0.02	0.331 ± 0.06	1.428 ± 0.30	0.810 ± 0.07	nd	0.069 ± 0.02	0.040 ± 0.03	nd
200 °C AIR	0.028 ± 0.02	0.277 ± 0.04	1.361 ± 0.16	0.903 ± 0.08	nd	0.039 ± 0.01	0.035 ± 0.02	nd
Ctrl residue	0.037 ± 0.01	0.207 ± 0.03	1.928 ± 0.12	0.344 ± 0.13	0.145 ± 0.09	nd	nd	nd
190 °C residue	0.072 ± 0.02	0.307 ± 0.03	0.989 ± 0.08	0.283 ± 0.22	0.174 ± 0.05	nd	nd	nd
200 °C residue	0.103 ± 0.05	0.199 ± 0.05	0.782 ± 0.07	0.269 ± 0.14	0.036 ± 0.01	nd	nd	nd

Ctrl, untreated control; DiFA, diferulic acid; nd represents none detected; Values are expressed as means of duplicate analysis ± SD.

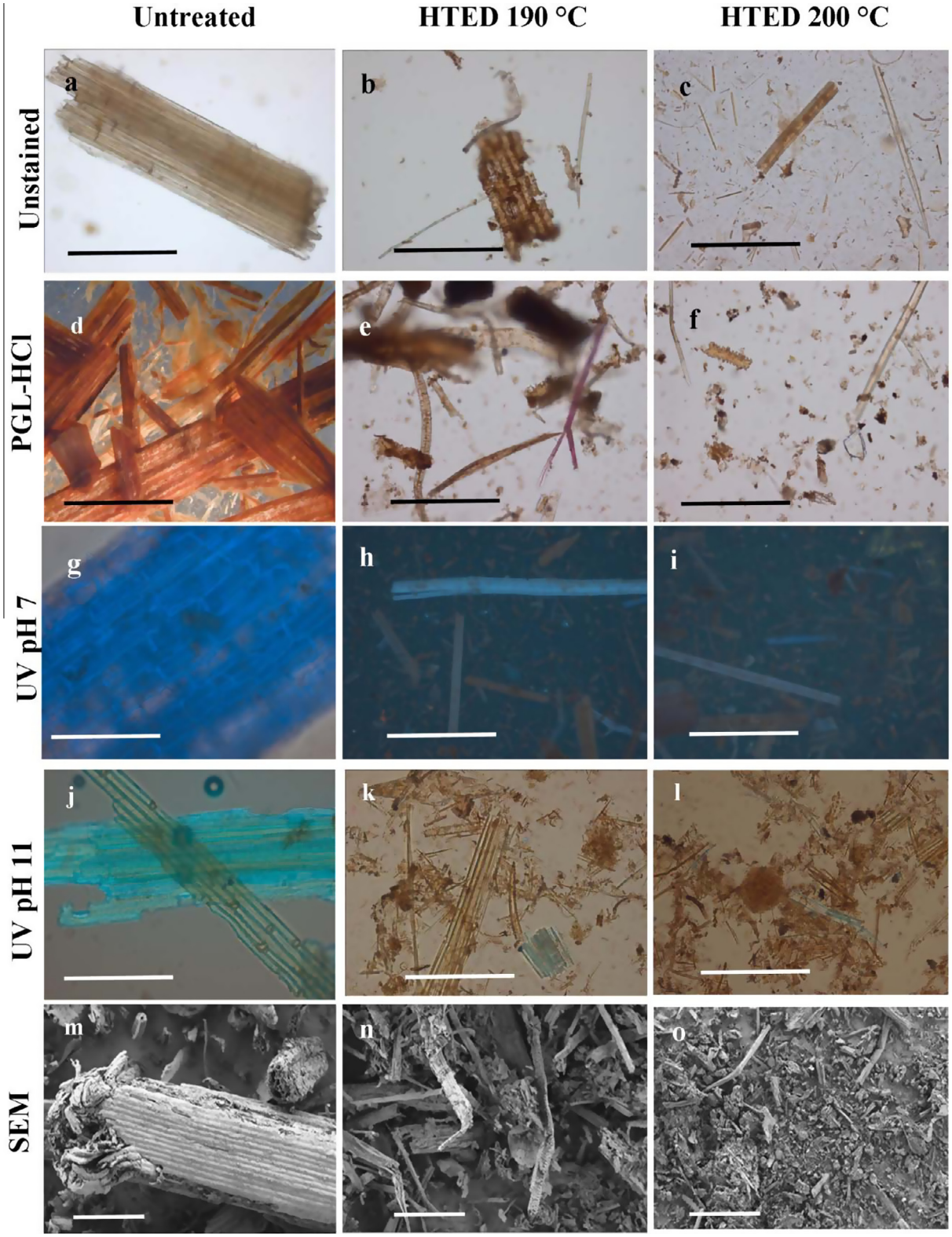

Fig. 1. Light, autofluorescence and SEM images of untreated straw together with hydrothermally pretreated and enzyme-digested (HTED) wheat straw. (a–c) Unstained, native micrographs depicting a loss of structure in b and c; (d–f) as above but stained with phloroglucinol-HCl whereby presence of lignin is indicated by a reddish/pink coloration; (g–i) under UV, lignified tissues autofluoresce blue at neutral pH; (j–l) phenolics and related moieties autofluorescence turquoise/ green at pH 11; (m–o) SEM photomicrographs of control wheat straw (m) together with the digested samples (n and o) supporting fragmentation and loss of structure observed in b, c above as a result of pretreatment and enzyme digestion. Bars: 500 μm (a–c); 1 mm (d–f); 100 μm (g–o).

clearly showed the highly ordered arrays of cellulosic microfibrils on the cell surface (Supplementary Fig. 2a and b). However, after HT and enzymatic digestion, the recalcitrant residue exhibited reduced structure, highlighting the loss of the cellulosic components (Supplementary Fig. 2c and d). The remaining material appeared to consist of globules of varying sizes which is characteristic of lignin deposits reported in literature as a result of pretreatment of wheat straw (Kristensen et al., 2008).

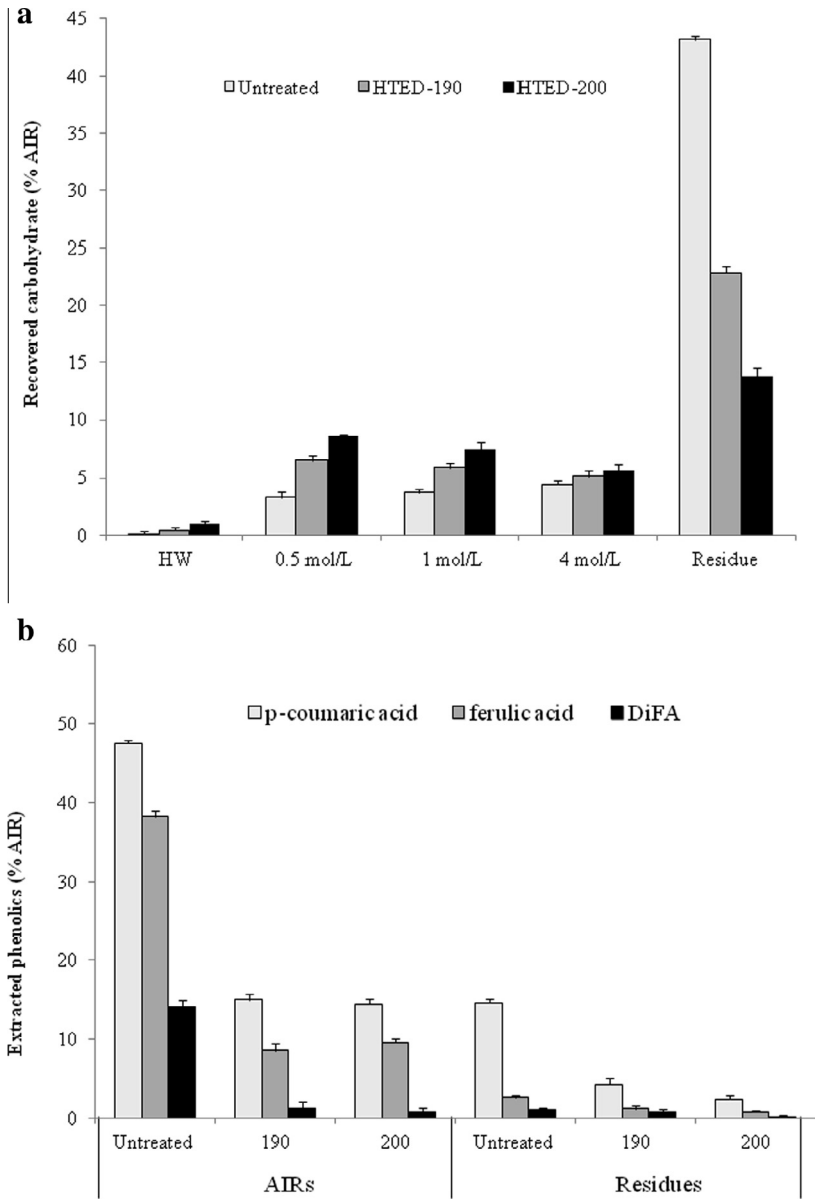

Fig. 2. Comparison of the control and HT (190 and 200 °C) enzyme-digested wheat straw (a) the total recovered carbohydrate in alkali-fractions and residue and (b) the total recovered phenolic acids in AIRs and residues following sequential extraction in alkali. HW, hot water extract; DiFA, diferulic acids.

3.3. Sequential extraction and characterization of recalcitrant residues

Sequential extraction in progressively stronger alkali provides a basis for comparing changes in cell walls during plant growth and development as well as physicochemical/thermal processing (Brett & Waldron, 1996). PTED AIRs were sequentially extracted in hot water followed by progressively stronger alkali (in degassed aqueous solution and in the presence of $NaBH_4$ to minimize alkaline peeling). The yields of extracted carbohydrate as a percentage of the AIR are shown in Fig. 2a. The results illustrate that the polysaccharides remaining in the recalcitrant residues were much more readily extracted in alkali compared with the untreated straw. Furthermore, the results indicate that hydrothermal pretreatment influenced this solubility, being significantly greater at 200 °C. This suggests that the alkaline solubility of the residual polymers was affected by both the HT conditions together with the digestion process, presumably due to complementary depolymerization and cleavage of inter- and intra-polymeric cross-links. Of particular note was the observation that larger quantities of polysaccharide were extracted from 190 °C and 200 °C HTED residues in 0.5 mol/L KOH as compared with the amount extracted from HT material (Merali et al., 2013) since smaller quantities were left in the unextracted residues. The alkali-extracted polymers from the HTED residues were rich in arabinoglucuronoxylans. In addition, they contained increased glucose levels compared to the extracts from the control AIR (Table 2). This may have arisen from the depolymerisation and enhanced solubility of cellulose. Indeed, it has been previously reported that cellulase treatment of dissolving pulps increases solubility of cellulose to alkali by decreasing degree of polymerization (Cao & Tan, 2006; Rahkamo et al., 1996). However it is possible that some of the solubilized glucose could have originated from mixed linkage glucan or xyloglucan identified by Hansen et al. (2014), although in their study, mixed-linkage glucans became water soluble rather than being released in alkali, and their study lacked quantification of extracted polymers. The unextracted residues from all the HTED samples were enriched

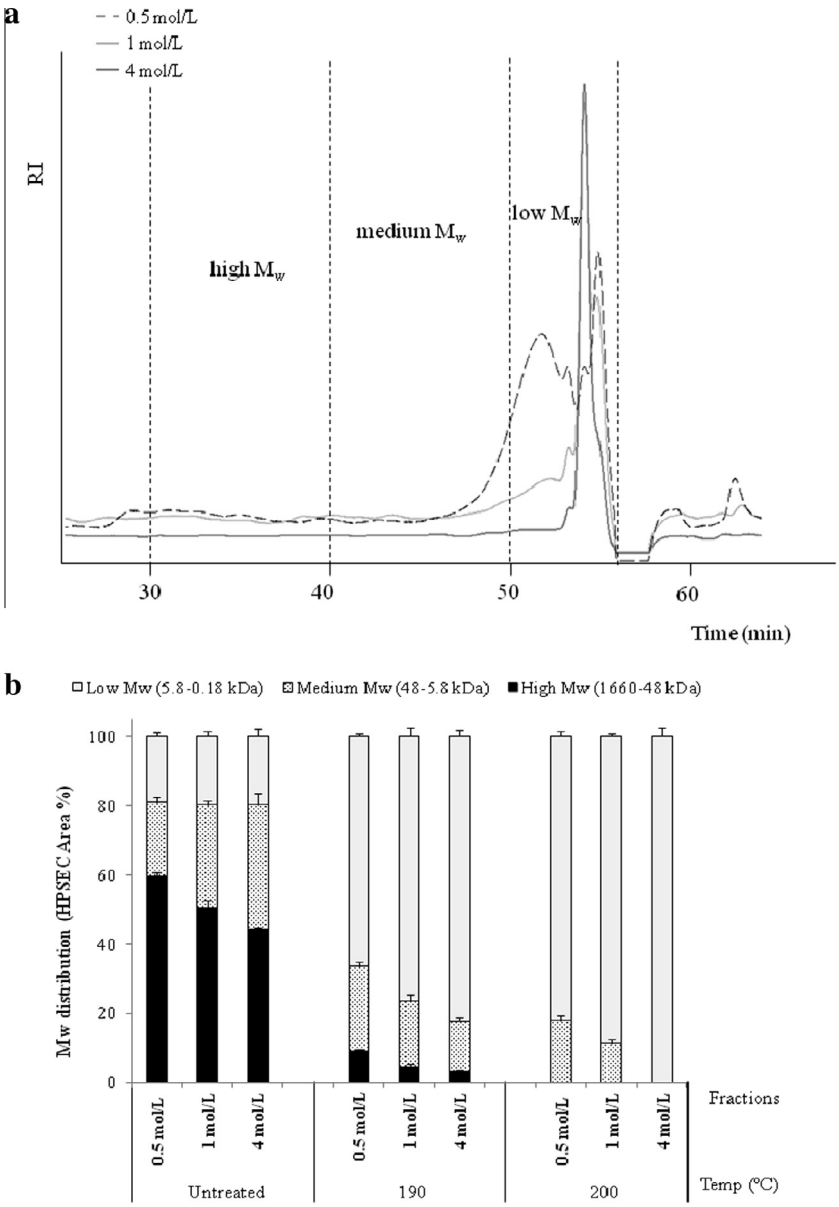

Fig. 3. Molecular weight (M_w) distribution of the KOH-extracted carbohydrate present in the control, HT (190 and 200 °C) and enzyme-digested wheat straw. (a) Depicting representative spectra of alkali-extracts from HT (200 °C) and digested straw, obtained following HPSEC and (b) HPSEC area percentages based on peak areas calculated using dextran standards.

in cellulosic glucose. However a significant reduction in residual xylose was noted in HTED residues (Table 2) reflecting the solubilization and loss of xylose during the HT process (Merali et al., 2013) resulting in a greater ease of digestion during the saccharification step.

A significant reduction in the major wall-bound phenolics occurred as a result of HT followed by enzyme hydrolysis (Fig. 2b and Table 3) and a further reduction was noted (up to 83%) as a result of alkali extraction which destabilizes the labile ester bond between the phenolics and arabinose (Fig. 2b, residues). The most prominent phenolics were ferulic acid, followed by *p*-coumaric acid and dehydrodimers of ferulic acid. In the HTED AIRs the reduction in phenolics correlated with increasing severity of temperature and in terms of the remaining phenolics present in the residues, mainly *p*-coumaric acid (which is thought to be ether-linked with the matrix polymers and/or with lignin) was detected with small amounts of ferulic acid (thought to be involved in cell-to-cell adhesion; Parker and Waldron, 1995). Similar reductions in ferulate, coumarate and diferulate moieties have been noted as a direct consequence of HT in wheat straw (Holopainen-Mantila et al., 2013; Merali et al., 2013). Furthermore, hydrolysis of ester bonds between phenolic acids and carbohydrates is catalyzed by ferulic/*p*-coumaric acid esterases (Crepin, Faulds, & Connerton, 2004). Ferulic acid esterase (FAE) activity is found from the commercial (hemi) cellulase preparations especially Novozyme 188 (Saha & Cotta, 2007) and it has been reported that *Aspergillus Niger*, which is the source of Novozyme 188 produces FAE which is able to release diferulates (Garcia-Conesa et al., 1999). This indicates that the a low degree of phenolic cross-linking, consistent with the a reduction in arabinoxylans as observed in HTED samples is likely to, in part, affect the ease of accessibility of the cellulases into the polymeric structure. This may also explain the increase in carbohydrate extractability of the recalcitrant polymers following enzyme hydrolysis.

3.4. Molecular weight (M_w) distribution of solubilized xylan following fractionation

Changes in molecular weight of alkali-extracted polysaccharides due to pretreatment and digestion indicated depolymerisation were investigated by HPSEC (Fig. 3a and b). The representative spectra in Fig. 3a show the profiles of polymers from the AIR of the 200 °C pretreated and enzyme digested sample. To simplify the comparison, the relative distributions of the polymers were designated high, medium and low M_w on the basis of areas under the respective peaks and between boundaries used previously (Kabel et al., 2007; Merali et al., 2013). Pretreatment followed by enzyme digestion resulted in a large increase in the low M_w material and this was significantly greater after HT at 200 °C compared with 190 °C (Fig. 3b). Hence, the residual hemicelluloses remaining in the recalcitrant residues after enzymatic digestion of HT material were in the form of low M_w oligomers (<6 kDa). It is interesting that such low M_w oligomers were retained in the cell wall during the HT process; however, it is probable that linearization or de-branching of xylan by pretreatment and enzymatic hydrolysis may decrease the solubility of the low M_w oligomers. The degree of arabinose substitution in the hot water fraction (HTED 200-HW) was low (1:13; Table 2) in concordance with a decrease in water solubility. Although the levels of phenolic acids remaining in unextracted residues were low (0.2%; Table 1), it is possible that these recalcitrant saccharides are closely associated with phenolic moieties, such as *p* hyroxybenzaldehyde, vanillin and/or *p*-coumarate. It has been suggested that temperature has a larger impact on phenolic solubility in pretreated liquors of wheat straw, than reaction times or alkalinity (McIntosh & Vancov, 2011). This may also reflect the effectiveness of hydrothermal pretreatments on the physicochemical properties of a variety of biomass types.

3.5. SSF of HT and enzyme digested wheat straw

The digestibility of pretreated and enzyme digested wheat straw was evaluated by SSF. The values are expressed as a percentage of the theoretical ethanol yield and shown in Supplementary Fig. S3. After 36 h, the untreated control produced a very low yield. However, HT 190 and 200 °C and HTED 190 and 200 °C resulted in overall ethanol yields of 37%, 63%, 86% and 99% of theoretical respectively. Hence, the previous lack of total digestion in HTED samples was not due to inherent indigestibility of the material, but other factors for example the loss of key hydrolytic enzymes through their binding to the lignin fraction, and the total removal of any fermentation inhibitors by the exhaustive extraction process (Waldron, 2010).

The SSF-HT data presented here shows that pretreatment at 190 °C is less effective than pretreatment at 200 °C consistent with other studies (Ballesteros et al., 2006; McIntosh & Vancov, 2011). The yields here are lower for each temperature. However, factors such as the differing pretreatment conditions and longer incubation period, as well as potential varietal differences (Collins et al., 2014) may be contributory factors. The cooler fermentation temperature used in this study (25 °C) may also result in a slightly lower yield.

4. Conclusions

Cellulosic and non-cellulosic polysaccharides remaining in recalcitrant wheat straw after pre-treatment and enzyme digestion were much more readily extractable in alkali demonstrating their partial degradation without dissolution during the process. HPSEC revealed the presence of low M_w oligomers in the alkali-soluble extracts suggesting that they had been released by saponification of phenolic esters, and hydrogen bonds. Comparative simultaneous saccharification and fermentation resulted in ethanol yields of 37% and 63% theoretical were from HT straw (190 and 200 °C, respectively) and up to 99% of the theoretical, from HTED straw. This suggests that the lack of initial digestion is not due to the indigestibility of the residual cellulose but other factors.

Conflict of interest statement

The authors confirm that they have no conflicts of interest.

Acknowledgements

We acknowledge the European Commission Seventh Framework Programme, EU-Disco project (Targeted DISCOvery of novel cellulases and hemicellulases and their reaction mechanisms for hydrolysis of lignocellulosic biomass Grant agreement: 211863) and the UK Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme 'Food and Health' (Grant number BB/J004545/1) for funding this collaborative project. We are grateful to Dr. Mary Parker and Kathryn Cross for microscopy and SEM imaging; Mr. David Wilson for preparing yeast culture; Dr. Adam Elliston and Mr. Sam Collins for maintaining and running samples on HPLC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.07.108.

References

Ballesteros, I., Negro, M. J., Olivia, J. M., Cabanas, A., Manzanares, P., & Ballesteros, M. (2006). Ethanol production from steam-explosion pretreated wheat straw. *Applied Biochemistry and Biotechnology, 12*, 496–508.

Brett, C. T., & Waldron, K. W. (1996). Cell Wall Structure and the skeletal functions of the wall. In C. T. Brett & K. W. Waldron (Eds.), *Physiology and biochemistry of plant cell walls* (2nd ed., pp. 2–24). London: Chapman and Hall.

Cao, Y., & Tan, H. (2006). Improvement of alkali solubility of cellulose with enzymatic treatment. *Applied Microbiology and Biotechnology, 70*, 176–182.

Collins, S. R. A., Wellner, N., Martinez Bordonado, I., Harper, A. L., Miller, C. N., Bancroft, I., & Waldron, K. W. (2014). Variation in the chemical composition of wheat straw: The role of tissue ratio and composition. *Biotechnology for Biofuels, 7*, 121.

Crepin, V. F., Faulds, C. B., & Connerton, I. F. (2004). Functional classification of the microbial feruloyl esterases. *Applied Microbiology and Biotechnology, 63*, 647–652.

DeMartini, J. D., Pattathil, S., Avci, U., Szekalski, K., Mazumder, K., Hahn, M. G., & Wyman, C. E. (2011). Application of monoclonal antibodies to investigate plant cell wall deconstruction for biofuels production. *Energy and Environmental Science, 4*, 4332–4339.

Garcia-Conesa, M. T., Kroon, P. A., Ralph, J., Mellon, F. A., Colquhoun, I. J., Saulnier, L., ... Williamson, G. (1999). A cinnamoyl esterase from *Aspergillus niger* can break plant cell wall cross-links without release of free diferulic acids. *European Journal of Biochemistry, 266*, 644–652.

Glithero, N. J., Wilson, P., & Ramsden, S. J. (2013). Straw use and availability for second generation biofuels in England. *Biomass and Bioenergy, 55*, 311–321.

Grabber, J. H., Ralph, J., & Hatfield, R. D. (1998). Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. *Journal of Agricultural and Food Chemistry, 46*, 2609–2614.

Hansen, M. A. T., Ahl, L. I., Pedersen, H. L., Westereng, B., Willats, W. G. T., Jorgensen, H., & Felby, C. (2014). Extractability and digestibility of plant cell wall polysaccharides during hydrothermal and enzymatic degradation of wheat straw (*Triticum aestivum* L.). *Industrial Crops and Products, 55*, 63–69.

Holopainen-Mantila, U., Merali, Z., Marjamaa, K., Käsper, A., De Bot, P., Jääskeläinen, A. S., ... Tamminen, T. (2013). Impact of hydrothermal pretreatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers. *Bioresource Technology, 138*, 156–162.

Kabel, M. A., Bos, G., Zeevalking, J., Voragen, A. G., & Schols, H. A. (2007). Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. *Bioresource Technology, 98*, 2034–2042.

Kristensen, J. B., Thygesen, L. G., Felby, C., Jørgensen, H., & Elder, T. (2008). Cell-wall structural changes in wheat straw pretreated for bioethanol production. *Biotechnology for Biofuels, 1*, 5.

Lawther, J. M., Sun, R. C., & Banks, W. B. (1996). Effects of extraction conditions and alkali type on yield and composition of wheat straw hemicellulose. *Journal of Applied Polymer Science, 60*, 1827–1837.

Lever, M. (1972). A new reaction for colorimetric determination of carbohydrates. *Analytical Biochemistry, 47*, 276–279.

Merali, Z., Ho, J. D., Collins, S. R. A., Le Gall, G., Elliston, A., Käsper, A., & Waldron, K. W. (2013). Characterization of cell wall components of wheat straw following hydrothermal pretreatment and fractionation. *Bioresource Technology, 131*, 226–234.

McIntosh, S., & Vancov, T. (2011). Optimisation of dilute alkaline pretreatment for enzymatic saccharification of wheat straw. *Biomass and Bioenergy, 35*, 3094–3103.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology, 96*, 673–686.

Parker, M. L., & Waldron, K. W. (1995). Texture of Chinese water chestnut – Involvement of cell wall phenolics. *Journal of the Science of Food and Agriculture, 68*, 337–346.

Rahkamo, L., Siika-Aho, M., Vehviläinen, M., Dolk, M., Viikari, L., Nousiainen, P., & Buchert, J. (1996). Modification of hardwood dissolving pulp with purified *Trichoderma reesei* cellulases. *Cellulose, 3*, 153–163.

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, 108*, 2823–2834.

Ralph, J., Grabber, J. H., & Hatfield, R. D. (1995). Lignin-ferulate cross-links in grasses - Active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydrate Research, 275*, 167–178.

Redgwell, R. J., & Selvendran, R. R. (1986). Structural features of cell-wall polysaccharides of onion *Allium-cepa*. *Carbohydrate Research, 157*, 183–199.

Saha, B. C., & Cotta, M. A. (2007). Enzymatic hydrolysis and fermentation of lime pretreated wheat straw to ethanol. *Journal of Chemical Technology and Biotechnology, 82*, 913–919.

Speer, E. O. (1987). A method for retaining phloroglucinol proof of lignin. *Stain Technology, 62*, 279–280.

Sun, R. C., Lawther, J. M., & Banks, W. B. (1996). Fractional and structural characterization of wheat straw hemicelluloses. *Carbohydrate Polymers, 29*, 325–331.

Sun, X. F., Xu, F., Sun, R. C., Geng, Z. C., Fowler, P., & Baird, M. S. (2005). Characteristics of degraded hemicellulosic polymers obtained from steam exploded wheat straw. *Carbohydrate Polymers, 60*, 15–26.

Tejirian, A., & Xu, F. (2011). Inhibition of enzymatic cellulolysis by phenolic compounds. *Enzyme and Microbial Technology, 48*, 239–247.

Waldron, K. W. (2010). Bioalcohol production. In K. W. Waldron (Ed.). *Biochemical conversion of lignocellulosic biomass* (Vol. 3, pp. 3–23). Woodhead Publishing Series in Energy.

Wood, I. P., Elliston, A., Collins, S. R. A., Wilson, D., Bancroft, I., & Waldron, K. W. (2014). Steam explosion of oilseed rape straw: Extablishing key determinants of saccharification efficiency. *Bioresource Technology, 162*, 175–183.

Wyman, C. E. (1996). Ethanol production from lignocellulosic biomass: Overview. In C. E. Wyman (Ed.), *Handbook on Bioethanol: Production and Utilization* (pp. 1–18). Washington: Taylor & Francis.

Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R., & Lee, Y. Y. (2005). Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology, 96*, 1959–1966.

Yang, B., & Wyman, C. E. (2008). Pretreatment: The key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts & Biorefining, 2*, 26–40.

Yelle, D. J., Kaparaju, P., Hunt, C. G., Hirth, K., Kim, H., Ralph, J., & Felby, C. (2013). Two-dimensional NMR evidence for cleavage of lignin and xylan substituents in wheat straw through hydrothermal pretreatment and enzymatic hydrolysis. *Bioenergy Research, 6*, 211–221.