Impact of mixed feedstock of wheat straw, willow and *Miscanthus* on enzymatic hydrolysis and inhibitor production after microwave hydrothermal pre-treatment in Europe

Kamaljit Moirangthem*[a†], Darren Greetham[a‡], Jim Craigon[a], Gregory Tucker[a]

[a] School of Biosciences, University of Nottingham, Loughborough LE12 5RD, UK

* Corresponding author. kamaljit.moirangthem@hotmail.com

Abstract

Lignocellulosic feedstocks for biorefinery are likely to be seasonal and the supply of feedstock to cellulosic bio refineries remains a challenge. One way to overcome this is by utilizing a mixed feedstock which facilitates the maintenance of a year-round feedstock supply. This study investigated the impact of mixing of three industrially relevant cellulosic feedstocks - wheat straw, willow and *Miscanthus* using two major performance indicators - sugar yield and fermentation inhibitor production. A microwave hydrothermal pre-treatment regime of 200 °C for 5 minutes was applied to each feedstock individually and to 1:1 (w/w) mixes and the predicted sugar yield in the mixes was compared to the observed values. All the mixes resulted in improved sugar yields with willow + *Miscanthus* (15.4%, p = 0.015) and wheat + willow (13.6%, p = 0.010) showing a statistically significant improvement. Saccharification kinetics, inhibitor production, impact on yeast metabolic activity and growth were compared and no adverse impacts of mixing were observed. The use of mixed feedstocks in a hot water based commercial production of biofuels is unlikely to have any adverse effects on productivity and may indeed prove beneficial.

Keywords

Lignocellulosic feedstock mix, Microwave Hydrothermal Pre-treatment, Enzymatic Saccharification, Fermentation Inhibitors, Yeast response, Biorefining.

Present address

[†] Institute of Bioresources and Sustainable Development, An Autonomous Institute under Department of Biotechnology, Government of India, Imphal 795001, India

[‡] Activatec Ltd, Bio City, Nottingham NG1 1GF, United Kingdom

Introduction

Lignocellulosic biomass, such as energy crops and agricultural residues represent a potential biorefinery feedstock for the production of fuel or other chemicals. Some of the challenges to making this economical are: i) to secure a year round sustainable supply of feedstock as they are likely to be seasonal with annual variability in yields [1] and may thus have limited availability and supply [2], ii) overcoming recalcitrance of the feedstock in terms of saccharification to fermentable sugars that requires pre-treatments to disrupt the lignocellulose matrix [1], and iii) reducing inhibitory compound formation during pre-treatment which could interfere with any subsequent enzymatic hydrolysis and fermentation steps [3].

Microwave technology as a pre-treatment for lignocellulosic biorefineries is becoming popular [4]. Water is the only solvent used and the process has a faster heat transfer when compared to a conventional heating process [5]. Microwave hydrothermal pre-treatment, as utilized in this research, is generally performed by pre-treating biomass at temperatures between 140 and 220 °C under high pressure to maintain water in the liquid state [6]. This treatment is mild compared to dilute acid pre-treatment, but has similar effect in terms of the chemical and physical changes that occur in the structure of lignocellulose [7]. The reaction medium becomes acidic due to the release of acetic acid and other weak acids derived from hemicellulose and to the auto-dissociation of water at elevated temperatures. The release of acetic acid from biomass facilitates the solubilisation of hemicellulose, and the formation of monomeric sugars and sugar degradation products or inhibitors such as 5-hydroxyl methyl furfural (HMF), furfural, formic acid or levulinic acid [8]. The breakdown of lignin also releases phenolic compounds which at certain concentrations can be inhibitory to a subsequent microbial fermentation [9].

Amongst the inhibitory compounds furfural and HMF are often used as representative for the general content of inhibitory compounds [10]. They have been subjects of extensive investigation due to their negative effects on microbial physiology [11], and are considered major inhibitors in microbial conversion processes [12]. Presence of furfural or HMF inhibit the growth of yeast cells and subsequent fermentation in a dose-dependent manner [13]. Although furfural and HMF can act synergistically, yeast cells are more sensitive to growth inhibition by furfural than by HMF at the same concentration [14-16]. However, their effects on yeast are dose dependent [17]. Hence, their concentration after pre-treatment is an essential parameter to be considered.

Literature review

There have been limited studies on the effect of mixing feedstocks on process efficiency and inhibitor concentrations [18]. However, mixed feedstocks are increasingly being considered for lignocellulosic biofuel production [19] as they could potentially buffer the variation seen with a single feedstock and have been shown to provide more economic advantage [20, 21]. One study looked at the effect of mixing municipal solid waste paper and corn stover in a 1:1 ratio using an ionic liquid pre-treatment [22]. This study monitored the effect of mixing on saccharification and reported a slight improvement in glucose yield. Another study mixed eucalyptus residues, wheat straw and olive tree pruning in a 50:25:25 ratio using a hydrothermal pre-treatment and monitored sugar recovery in both the pre-treated biomass, liquor and inhibitor composition [23]. This study showed that mixtures of lignocellulosic materials can be efficiently processed by auto hydrolysis and generate a consistent product composition that was independent of the different proportions of each feedstock. Concentration of inhibitors were also reported to be low for the three different mixes (furfural 0.33 - 0.49 g/L and HMF 0.17 - 0.29 g/L). A further study examined the effect of mixing aspen, balsam and switch grass in 1:1 ratios using an acid pre-treatment and monitored saccharification and inhibitor composition and found glucose yields for the mixed feedstocks were very similar to the predicted values [24]. Oke *et al* provided a more in depth study on the prospects and challenges of mixed feed stock biorefineries [18]. While Shi *et al* obtained a 90% sugar yield after 24 h of saccharification of ionic liquid pre-treated mixed feedstocks of equal portion of switchgrass, lodgepole pine, corn stover and eucalyptus – in flour and pellet form [25]. Furthermore, Baral *et al* through supply and value chain analysis determined that a mixing ratio of corn stover, *Miscanthus*, and switchgrass of 36%, 50%, and 14%, respectively, minimized the selling price of sugar [26].

Within the context of a UK or EU biorefinery, the major feedstocks are likely to include agricultural waste (wheat straw) and dedicated energy crops such as willow and *Miscanthus*. Wheat straw is a promising feedstock for bioethanol production in Europe due to its large production and high carbohydrate content [27] and majority of agricultural straw is derived from wheat in nearly all regions of the UK, except Scotland where barley prevails [28]. For bioenergy crops in Europe, research has been focused on *Miscanthus* [29] with the sterile hybrid *Miscanthus × giganteus* currently being the main commercially exploited species of this genus for biomass purposes due to its high yield potential. Willow has many desirable characteristics for feedstock and biomass production. With its coppicing ability and vigorous juvenile growth, it can produce high biomass yields

(>11 odt (oven dried tones) ha^{-1} $year^{-1}$) on marginal land that is not suitable for conventional food crops [30]. *Miscanthus* and short rotation coppice derived from willow have been the most widely planted species in the UK.

There have been no reports in the literature regarding the potential impact of mixing wheat straw, willow and *Miscanthus* feedstocks on process efficiency nor of studying effect of mixing using a pressurized microwave hydrothermal pre-treatment. In this research, the researchers have studied the impact of wheat straw, willow and *Miscanthus* in 2 components 1:1 (w/w) mixtures on saccharification yield and inhibitor production.

Materials and Methods

Feedstock

Wheat straw – Revelation – was obtained from the Sutton Bonington farm (University of Nottingham, UK). Willow - tora and *Miscanthus* - goliath were kindly provided by Prof Iain Donnison (Aberystwyth University, UK). Wheat straw was air-dried, while willow and *Miscanthus* were oven dried at 60 °C until dry weight was constant, prior to milling. The three feedstocks were milled separately to 2 mm mesh size using a FRITSCH Pulverisette 19 knife mill and stored at 4 °C in bags with airtight seals.

Composition analysis

Sugar Analysis

Sugar composition was measured following Saeman hydrolysis [31]. 1 mL of 12M H_2SO_4 was added to 30 mg of sample at 37 °C for 1 h. This was followed by addition of 11 mL of MilliQ water (reducing the molarity to 1M) and incubation at 100 °C for 2 h. The hydrolysate was analyzed using High Performance Anion Exchange Chromatography (HPAEC).

HPAEC – High Performance Anion Exchange Chromatography

Analysis of sugars was performed on a Dionex ICS-3000 comprised of a high-pressure GD 50 gradient pump, a guard column (Carbopac PA1, 4 mm × 50 mm), an analytical column (Carbopac PA20, 4 mm × 250 mm) and a pulsed amperometric detector (PAD). 10 mM NaOH was used as the mobile phase and the column was

flushed with 200 mM NaOH between runs. All chromatographic analyses were carried out at 30 °C with a flow rate of 1.0 mL min^{-1}. Samples were diluted 1:100 in 10 mM NaOH and centrifuged at 4472 x G for 10 min before loading to Dionex vials. Sugar standards ranged from 0.250 to 2 g L^{-1} of arabinose, galactose, glucose and xylose, respectively.

Microwave Hydrothermal Pre-treatment and Enzymatic Saccharification

Microwave Hydrothermal Pre-treatment

Pre-treatment was conducted in a Monowave 300 microwave generator (Anton Paar, Germany). 1 g of sample was mixed with 10 mL of MilliQ water, left at room temperature for 2 min and then heated at different combinations of temperature and holding times ranging from 180 - 220 °C in steps of 20 °C and 5-20 min in steps of 5 min, respectively. The resultant pre-treated mixture was centrifuged for 10 min at 4472 x G to decant the hydrolysate. The hydrolysate composition was analyzed using HPAEC and after measuring its pH, was stored at -20° C. The residue was air dried for 72 h, weighed and stored at 4 °C for further analysis.

Pre-treatment severity (log R_0) was calculated using equation 1 [32]:

$R_0 = t.e\hat{}(\frac{T-100}{14.75})$---------- (1),

Where, *t* is the time (minute) and T the temperature (° C).

Enzyme Saccharification

Pre-treated biomass residue, produced as described above, was hydrolyzed using Cellic® CTec3 (kindly provided by Novozyme A/S, Demark). This was carried out using a slight modification to the method described by the National Research Energy Laboratory (NREL) [33]. Enzyme solution was made up using 5 mL of Cellic® CTec3 in 1 L of 50 mM Sodium citrate buffer at pH 3.76. 40 mL of this solution was added to 200 mg of sample and incubated at 50 °C for 72 h at 150rpm (MaxQ 4358 shaking incubator, Thermo Scientific, UK). This level of enzyme loading was used to avoid enzyme being a limiting factor in the digestion. Aliquots were taken at 6 different time points – 15 min, 3 h, 6 h, 24 h, 33 h and 72 h and monomeric sugar composition assessed using HPAEC. The sugar concentration present in the enzyme solution was also analyzed and subtracted to allow accurate calculation of % glucose released.

Kinetics analysis of the enzyme hydrolysis was carried out assuming the reaction followed first order behaviour. This was found to be valid for the data sets of this study, thus avoiding the need for more complex kinetic algorithms, and allowing simple comparison of kinetic parameters [34]. A non-linear optimization method was used to fit a simulated curve to the experimental points using the Solver function in Microsoft Excel 2016. A function was used describing a single first-order exponential growth process, according to equation (2), where S is the glucose yield at time t, x is the rate constant and A is the final equilibrium glucose yield.

S = $A(1 - e^{-xt})$ ---------- (2)

Inhibitor Analysis

Microorganism

Saccharomyces cerevisiae strain NCYC2592 (National Collection of Yeast Cultures, UK) was incubated at 30 °C for ~48 h in yeast peptone broth (YPD- 20 g/L glucose, 20 g/L bacto peptone, 10 g/L yeast extract). Cell density was adjusted using a Transmittance meter to reduce 100% transmittance of MilliQ water to ~65%. 1 mL of this was mixed with IFY buffer™ (Biolog) to make the working cell suspension (~5 x 10^4 cells/mL).

Yeast metabolic activity measurement

Yeast metabolism was measured using a Phenotypic Microarray Omnilog reader (Biolog, USA) [35]. A redox reporter within the assay (dye D) permits analysis of metabolic activity defined as redox signal intensity. The composition of each well was as follows: 20.8 µL of hydrolysate, 9 µL of glucose (80% stock), 0.2 µL of dye D (Biolog, Hayward, CA, USA) and 90 µL of cell suspension. Control consisted of 2.8 µL YNB (28%), 18 µL MilliQ water, 9 µL glucose (80% stock), 0.2 µL of dye D and 90 µL of cell suspension. The plates were then placed in the Omnilog reader and incubated for 48 h at 30 °C with readings every 15 min.

Yeast metabolism was represented as % inhibition according to equation [3]

Metabolic inhibition = $\int_0^t \frac{Redox\ intensity\ of\ sample}{Redox\ intensity\ of\ control} X100$---------- [3]; t = 48 h.

Yeast growth measurement

Yeast growth was measured using a FLUOstar OPTIMA (BMG LABTECH, Germany) under identical growth conditions as for Phenotypic Microarray assays and monitored for 48 h at 30 °C with a readings every 15 min.

Inhibitor composition

Inhibitors furfural and 5- hydroxyl methyl furfural in the hydrolysate were measured by HPLC using UV detection at 280 nm (2695 HPLC system and 996 Photodiode Array Detector, Waters, USA) with UV spectra for secondary confirmation [36]. The chromatographic conditions were as follows: Techsphere ODS C18 column (5 µm, 4.6 mm × 250 mm; HPLC Technologies, UK) at ambient temperature. The mobile phase was a binary mixture of 1% acetic acid (solvent A) and methanol (solvent B) with an overall flow rate of 1.0 $mL.min^{-1}$ and compounds were detected at 280nm. The system was operated in gradient mode, ramping from 20% to 50% methanol over 30 min with a 100% methanol column cleaning phase and a 9-min re-equilibration period. The sample injection volume was 10 µL. Data were recorded using Millennium Chromatography software (Waters, USA). Quantification was performed by comparison of peak areas of authentic standards (0.025–0.4 g/L concentration range) of HMF and Furfural.

Experimental design and Statistical analysis

For the laboratory analysis, samples of each milled feedstock were placed in three separate test tubes. Similarly each 50:50 mixture of feedstocks was also made up in three separate test tubes. Thereafter, the material from each single feedstock or mixture tube was processed, measured and recorded separate and independent from the material in the other tubes. There were thus 18 tubes of material representing a completely randomised design with 6 treatments [the 3 single and 3 mixed feedstocks] each with 3 replicates.

The data were analysed by one-way Analysis of Variance (ANOVA) using Genstat 18.1 (VSN International Ltd). For the comparison of observed and expected values, three predefined contrasts were included in the ANOVA to directly compare each mixed feedstock to the average of its two components when they were used alone. A significant effect would indicate that the components were responding differently when mixed with the other feedstock.

Results and Discussion

In the first instance, a screen was carried out to assess the impact of various hydrothermal pre-treatment conditions on the saccharification of wheat straw, willow and *Miscanthus* individually (Additional file **1**: **Fig. S1-S3**). The impact of the various pre-treatments on the sugar composition of the residue, and release of sugars into the hydrolysate liquor was similar in all three feedstocks. As expected, there was an increased loss of xylose into the liquor as severity of treatment increased and, in all cases, there was little impact on glucose retention in the residue. Severity factors for this scoping work are shown in Additional file 1: Table S1 and ranged from 3.05 - 4.84. Although pre-treatment at 180 °C for 20 min had a higher severity factor (3.66) than at 200 °C for 5 min (3.64), the latter resulted in higher saccharification. The same was true for 200 °C for 20 min (4.25) compared to 220 °C for 5 min (4.23). This indicates that the effect of temperature was more important than residence time for reduced recalcitrance, which was also suggested previously by Alvira et al using wheat straw [27].

From this scoping experiment a pre-treatment regime of 200 °C for 5 min was selected and this was applied to each feedstock individually and to 1:1 (w/w) mixtures. This was a severity that resulted in around 50 - 60% glucose digestibility in all three feed stocks and as such would allow any synergistic enhancement of digestibility by mixing to be detected.

Effect of mixed feedstocks on sugar composition of pre-treated residue

Sugar composition of the residues from individual pre-treated feedstocks were measured (Fig.1). These values were then used to calculate expected compositions from 1:1 mixes and these compared to those obtained experimentally (Fig.1). Values of the individual feedstock shown in the

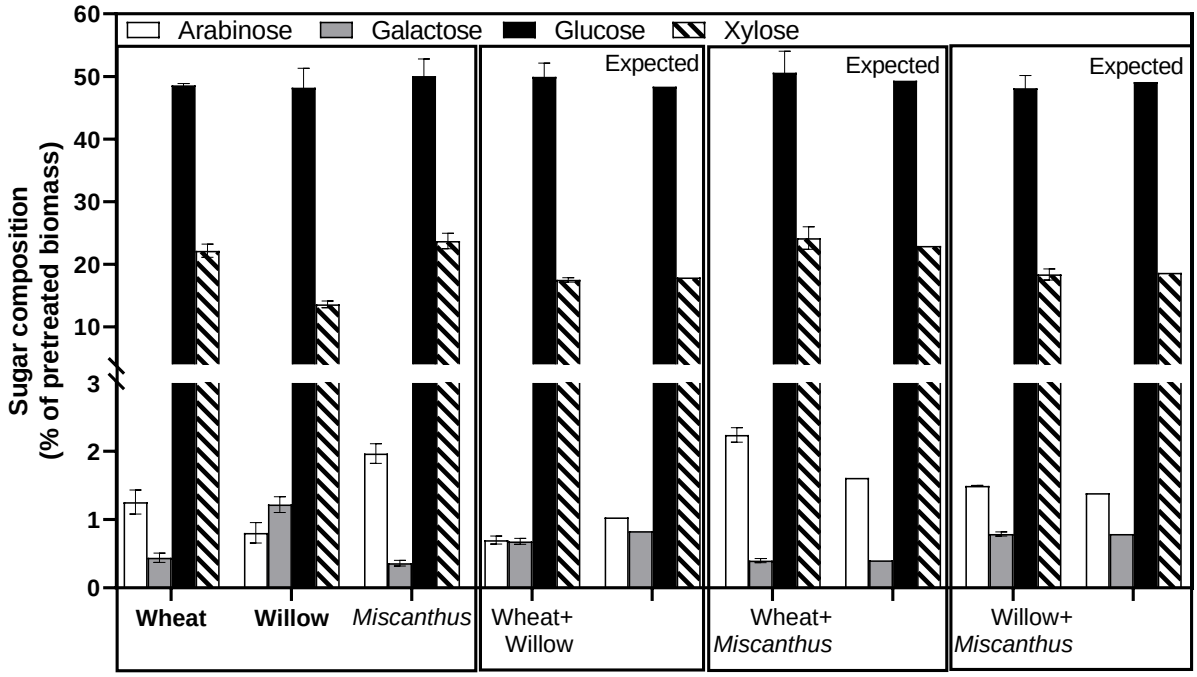

Fig.1 Effect of mixed feedstocks on sugar composition of pre-treatment residues.

Individual feedstocks and 1:1 mixtures were subjected to a standard pre-treatment (microwave 200°C for 5 min) and the resultant residues analysed for sugar composition. Expected values are the theoretical composition of the 1:1 mixes calculated from the experimental values of the individual pre-treated feedstock used in the respective mix. Values shown for individual feedstock are the experimental values. Results are expressed as mean ± SD (n = 3).

The sugar composition of the residue obtained after pre-treatment did not alter significantly when compared to their expected values, and glucose compositions of all the 3 mixes were at a similar level. Silva-Fernandes *et al.,* reported similar findings: mixtures of lignocellulosic materials processed by auto hydrolysis generated consistent product composition independently of the different proportions of each feedstock [23].

Enzyme saccharification

For each case, the impact on saccharification of the resultant residue was determined by monitoring glucose and xylose release (Fig. 2). Enzyme saccharification focussed on the yield of major sugars (glucose and xylose) as the impact on yields would be more apparent for the major sugars. Experimental glucose and xylose yields of the mixes were then compared against theoretical expected values (Table 1). For the individual feedstocks, it can be seen that wheat straw was the least recalcitrant followed by willow and then *Miscanthus*.

ANOVA test between the 3 individual feedstocks showed that their responses to the pre-treatment were statistically different for wheat vs willow (p = 0.0013) and wheat vs *Miscanthus* (p = 0.0005) but, not for willow vs *Miscanthus* (p = 0.0763), respectively.

Table 1 Comparison of observed and predicted glucose and xylose yields at 72 h.

Categories of Performance Indicators	**Yields calculated after 72 hours of enzymatic saccharification**					
	Individual Feedstock			**Mixed Feedstock**		
	Wheat	**Willow**	***Miscanthus***	[a] **Wheat+ Willow**	[a] **Wheat+ *Miscanthus***	[a] **Willow+ *Miscanthus***
Glucose yield (g/g original biomass)	0.178 ± 0.008	0.132 ± 0.006	0.123 ± 0.004	0.176 ± 0.012**	0.165 ± 0.018	0.147 ± 0.004**
Theoretical	n.a	n.a	n.a	0.155	0.151	0.127
% yield gain	n.a	n.a	n.a	+13.6%	+9.4%	+15.5%
Xylose yield (g/g original biomass)	0.144 ± 0.005	0.082 ± .002	0.116 ± 0.001	0.118 ± 0.008	0.133 ± 0.010	0.111 ± 0.006**
Theoretical	n.a	n.a	n.a	0.113	0.130	0.099
% yield gain	n.a	n.a	n.a	+4.6%	+2%	+11.8%

[a] The performance of the individual feedstocks was used to calculate an expected yield of glucose and xylose in the 1:1 mixed experiments. These were then compared to the observed yields. Results are expressed as g/g of original biomass and are the mean ± SD (n = 3). P > 0.05 are not shown as they were not deemed significant; * = P ≤ 0.05; ** = P ≤ 0.01; *** = P ≤ 0.001.

When the observed glucose and xylose yields after 72h of the 3 different mixes were compared with their respective theoretical yields (Table 1), the observed yields were found to be higher than expected yields in all the cases. Since the results in Fig.1 showed very little differences in the sugar composition of the pre-treated residues, it is unlikely that this is the reason for the observed improvements in saccharification. The cause may therefore be due to a reduction in recalcitrance possibly due to improved response to pre-treatment brought

about by the mixed nature of the feedstock. Microwave radiation has been known to result in a decrease of cellulose crystals size enhancing lignocellulose hydrolysis [4].

The *Miscanthus* + willow with an expected 0.127 g glucose /g original biomass and an observed 0.147 g glucose /g original biomass; and wheat + willow mixes with an expected 0.155 g glucose /g original biomass and an observed 0.176 g glucose /g original biomass, demonstrated the largest enhancement between expected and observed values these being 15.4% and 13.6%, respectively - both being statistically significant at the 5% probability level (p = 0.015** and 0.010**, respectively). For the wheat + *Miscanthus* mix while the actual glucose yield of 0.165 g glucose/g original biomass was still higher than the expected 0.151 g glucose/g original biomass, there was no evidence of an effect (p = 0.065).

The observed xylose yields from all three mixes were again higher than expected however, in the case of the two mixtures- *Miscanthus* + wheat and willow + wheat, there was no evidence of an effect (p = 0.499, 0.255, respectively). Only the mixed *Miscanthus* and willow feedstock showed any significant enhancement in xylose yield (11.8 %, p = 0.019**).

Previous studies reported glucose yields of 84% from municipal solid waste paper and corn stover mix after ionic liquid pre-treatment compared to a 82.2% expected, with a xylose yield of only 40% compared to an expected value of 59.3 % [22]. Another study on mixes of Aspen and Balsam reported a glucose yield of 27.34% compared to the expected value of 24.5%, and a xylose yield of 8.16% compared to 2.2% expected [24]. However, Aspen and switch grass in the same study gave a glucose yield of 52.51% compared to an expected value of 53%, with an observed xylose yield of 3.81% compared to the expected 4.8%.

Saccharification kinetics

Data shown in Fig.2 was used to calculate rate constants describing the kinetics of glucose release for either individual feedstocks or their 1:1 mix (Table 2).

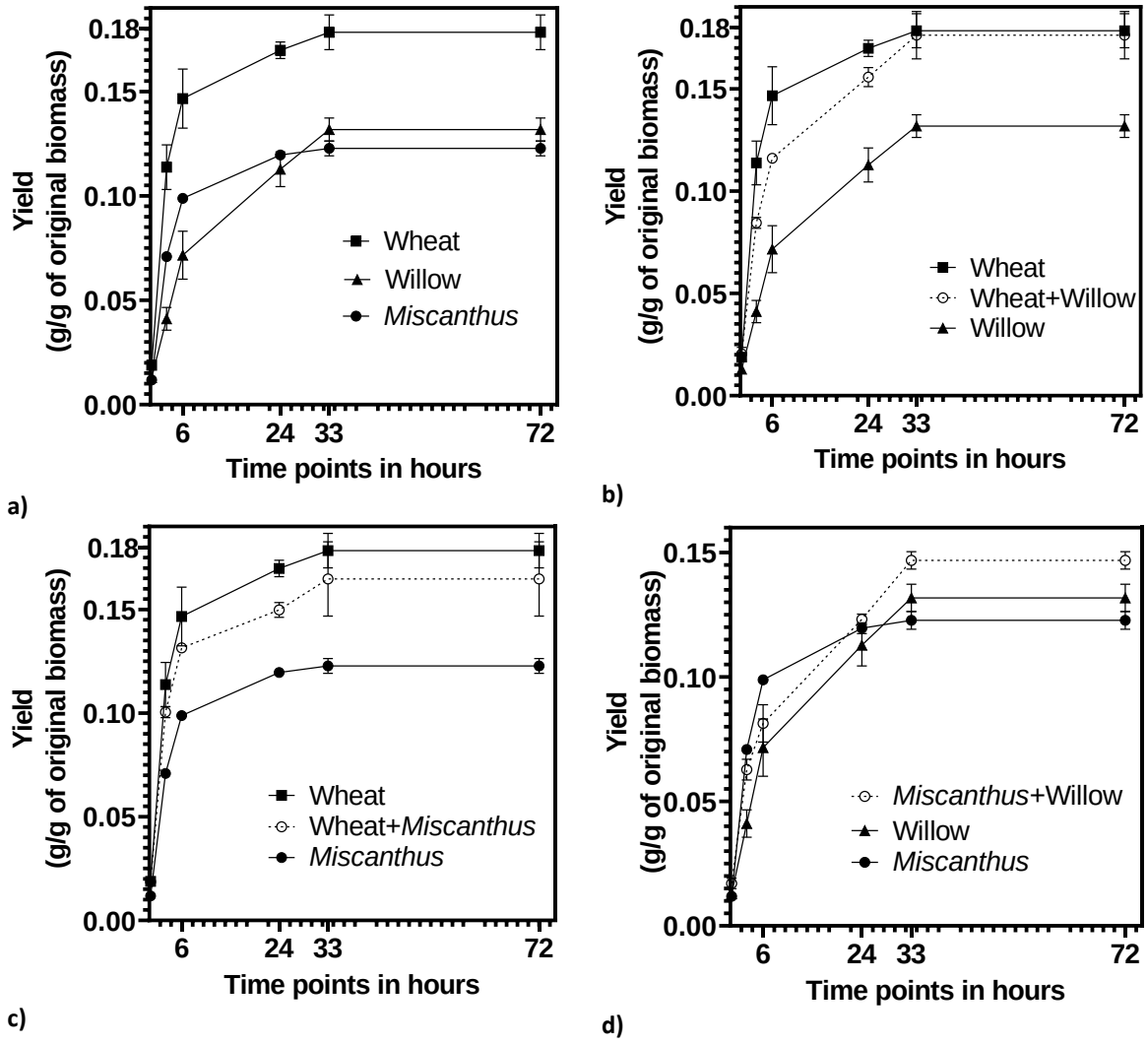

Fig.2

Glucose saccharification yields of pre-treated feedstocks.

Individual feedstocks (a) and 1:1 mixture (b,c,d) were all subjected to a standard pre-treatment of 200 °C hot water for 5 min, and saccharification potential assessed by following glucose release during digestion of the dried residue with Cellic® CTec3 enzyme cocktail. Results are expressed as g/g of original biomass and are the mean ± SD (n = 3).

Table 2 Impact of mixed feedstocks on saccharification kinetics, yeast metabolism and growth and inhibitor composition.

Categories of Performance Indicators	Individual Feedstock			Mixed Feedstock		
	Wheat	**Willow**	***Miscanthus***	**Wheat+ Willow**	**Wheat+ *Miscanthus***	**Willow+ *Miscanthus***
Rate Constant (h^{-1})[a]	0.34± 0.03	0.13± 0.03	0.29± 0.01	0.21± 0.03	0.32± 0.07	0.14±0.02
Yeast metabolism (% of control)[b]	84.05 ±1.49	111.03 ±5.46	69.86±3.59	109.34 ±0.90	70.91 ±6.45	68.29 ±5.35
Yeast growth (% of control)[c]	33.06 ± 3.22	41.12 ± 1.07	37.9± 1.7	35.63 ± 0.35	31.88 ± 2.96	44.05 ± 3.38
HMF (mg/L)[d]	13.55 ±0.96	167.63 ±1.6	42.44 ±1.73	55.51 ±0.90	18.40 ± 0.89	84.56 ± 11.45
Furfural (mg/L)[e]	59.57 ±.03	105.16 ±8.08	134.54 ±3.41	118.29 ±6.72	109.77 ±4.21	131.29 ±11.93

[a] Rate constant (h^{-1}) was calculated from g/g glucose release from original biomass at the 6 time points in enzyme hydrolysis (final time point at 72 h).

[b] Impact of pre-treatment liquor on yeast metabolism observed for 48 h. Results are presented as the % compared to a control 100%.

[c] Impact of pre-treatment liquor on yeast growth observed for 48 h. Results are presented as the % compared to a control 100%.

[d,e] Inhibitor composition of the liquor. All results are expressed as mean ± SD (n = 3).

For the individual feedstocks, wheat straw and *Miscanthus* showed the fastest saccharification rates (p = 0.0549). In contrast, willow showed a significantly slower rate of glucose release than either of the other two feedstocks [wheat vs willow (p = 0.001) and willow vs *Miscanthus* (p = 0.0006)]. This could be attributed to the differences in their cell wall composition, where willow has a higher hemicellulose and lignin content compared with wheat straw and *Miscanthus* [28] or higher bulk density of willow (210 kg/m^3) compared to *Miscanthus* (180 kg/m^3) and wheat straw (20 kg/m^3) [37]. Mixing wheat and *Miscanthus* showed no evidence of an effect on the rate constant for glucose release. The rate of release for the wheat and willow mix was intermediate between that for the two feedstocks individually while, the rate for the willow and *Miscanthus* was similar to

that for the willow alone. Thus, the enhancement in glucose yields seen above (Table 1) when willow was mixed with either of the two “grasses”, is not associated with an increased rate of initial release.

Effect of mixed feedstocks on Inhibitor production

The impact of the pre-treatment liquors (hydrolysates) on the metabolic activity and growth of yeast were assessed. Concentrations of two major inhibitors furfural and HMF in the pre-treated liquor were also measured (Table 2). Studying both yeast metabolism and growth is crucial as inhibitory compounds could result in either i) a reduction in the conversion of sugars into biomass (growth) or ii) reduced metabolic output, or both simultaneously. It has also been shown that yeast metabolism does not always correlate with growth [35].

For the individual feedstocks, the inhibitory impact on metabolism ranged from about 30% inhibition for *Miscanthus* to an actual apparent stimulation in the case of willow (Table 2). This observed stimulation may be related to an increased metabolic response to stress. In terms of the mixed feedstocks the observed inhibitory responses were in the same range as for the individual feedstocks.

The impact on yeast growth is probably a better indicator on the potential impact of the liquor on yeast performance than metabolism (Table 2). In this case, wheat demonstrated the greatest inhibition and willow again had the least effect. In contrast to the effect on metabolic rate, the impact on growth was quite substantial ranging from 70 to 60% inhibition of growth as compared to the control. The range of the inhibitory effect of the liquors from the mixed feedstocks was very similar to that for the individual feedstocks.

The concentrations of two major inhibitory compounds- furfural and HMF in the liquors were analyzed (Table 2). *Miscanthus* had the highest level of furfural and wheat the lowest while willow had the highest level of HMF and wheat again the lowest. Amongst the individual feedstocks, willow showed the highest overall inhibitor content. This could be attribute to the higher hemicellulose content of willow cell wall [28], which resulted in a larger amount of xylose/galactose sugars for the production of these inhibitors during pre-treatment at 200 °C for 5 min. The only major difference exhibited between the observed and expected levels of these inhibitors in the mixed feedstocks was for the level of furfural in the wheat + willow mix which was higher than expected. The highest furfural concentration recorded was 0.135 g/L. Given that the reported

toxicity threshold for yeast is 2.4 g/L of furfural [38], it is unlikely that the inhibitory effects on growth seen in the present experiment are directly due to furfural but are more likely to have arisen from additional or synergistic impacts of a wider range of inhibitory compounds.

Synergistic effect of inhibitors such as HMF and vanillin or HMF and furfural have been reported on yeast performance where it stopped or slowed yeast activity [39]. Additionally, presence of low concentrations of acetic acid has been shown to have beneficial effect on fermentations and improved yeast tolerance to other inhibitory compounds. However, at higher concentrations of acetic acid (>25 mM) a synergistic effect on yeast metabolism is observed with other inhibitory compounds (furfural, vanillin, HMF etc) [39]. This could contribute to the higher yeast metabolism seen with the willow hydrolysate. In a study with aspen, switch grass and balsam, furfural concentrations at the optimal pre-treatment conditions were 0.36 g/L for the aspen: balsam blend (0.33 g/L expected), and 0.67 for the aspen: switch- grass blend (1.125 g/L expected) [24]. While the study with wheat straw, olive pruning and Eucalyptus residues, furfural levels were 0.33 - 0.44 g/L and HMF levels were 0.17 to 0.29 g/L depending on the mixes [23]. The inhibitors levels produced in this study are much lower when compared to previous studies. This may reflect the different solid loadings and pre-treatment regimes used in each study, or, directly as a result of the different feedstocks used. Hydrothermal pre-treatment used in this study is known to solubilize hemicellulose mainly as oligomers and not monomers, which reduces the risk of degradation of the soluble hemicellulose fraction [40] and the resultant inhibitors.

Conclusion

Mixing feedstocks of wheat straw, willow and *Miscanthus* does not appear to have any adverse effects on the effectiveness of hot water pre-treatment. In fact, all the mixes gave higher than expected sugar yields with wheat + willow and willow + *Miscanthus* mixes in particular showing statistically significant improvement. Hence, it could be possible to reduced reliance on single biomass and overcome potential feedstock bottle neck in a biorefinery by utilizing mixed lignocellulosic biomass while achieving significant improvement in sugar yield. The biochemical or biological basis for this enhanced sugar release or synergistic effect is unclear. The impact of mixing on inhibitor productions seems to be minimal and does not adversely further impact on either metabolic activity or growth of yeast. Mixing feedstocks may prove technically difficult on a commercial scale and that wheat as a sole biomass inputted into the system appears to be the best option. However, if willow and

Miscanthus are the only available feedstocks, then mixing either these two together or willow with some wheat could provide significant improvements in yields. This warrants a further investigation to ascertain whether or not these benefits are seen on a larger scale and with feedstocks grown under a range of environmental conditions.

Acknowledgements

We would like to thank the University of Nottingham's Bioenergy and Brewing Sciences (BABS) technical team for the use of analytical facilities such as HPLC, the Microwave reactor and the Phenotypic Microarray Omnilog reader. We are grateful to Prof Iain Donnison from Aberystwyth University, UK, for kindly providing the Willow and *Miscanthus* samples; Dr Kirsty Jewell, from the University of Nottingham, UK, for assistance with the yeast growth measurement; and Novozyme A/S, Denmark for kindly providing the Cellic CTec3 enzyme.

Disclosures

The authors indicate no potential conflicts of interest.

References

1. Yue, D., You, F., & Snyder, S. W. (2014). Biomass-to-bioenergy and biofuel supply chain optimization: Overview, key issues and challenges. *Computers and Chemical Engineering*, *66*, 36–56. doi:10.1016/j.compchemeng.2013.11.016
2. Balat, M. (2011). Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. *Energy Conversion and Management*, *52*(2), 858–875. doi:10.1016/j.enconman.2010.08.013
3. Ko, J. K., Um, Y., Park, Y., Seo, J., & Kim, K. H. (2015). Compounds inhibiting the bioconversion of hydrothermally pretreated lignocellulose. *Applied Microbiology and Biotechnology*, *99*(10), 4201–4212. doi:10.1007/s00253-015-6595-0
4. Kucharska, K., Rybarczyk, P., Hołowacz, I., Łukajtis, R., Glinka, M., & Kamiński, M. (2018). Pretreatment of Lignocellulosic Materials as Substrates for Fermentation Processes. *Molecules*, *23*(11), 2937–32. doi:10.3390/molecules23112937
5. Chiaramonti, D., Prussi, M., Ferrero, S., Oriani, L., Ottonello, P., Torre, P., & Cherchi, F. (2012). Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method. *Biomass and Bioenergy*, *46*, 25–35.
6. Kim, Y., Kreke, T., Ko, J. K., & Ladisch, M. R. (2015). Hydrolysis-determining substrate characteristics in liquid hot water pretreated hardwood. *Biotechnology and Bioengineering*, *112*(4), 677–687. doi:10.1002/bit.25465
7. Pu, Y., Hu, F., Huang, F., Davison, B. H., & Ragauskas, A. J. (2013). Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. *Biotechnology for Biofuels*, *6*(1), 1–13. doi:10.1186/1754-6834-6-15
8. Gurram, R. N., Datta, S., Lin, Y. J., Snyder, S. W., & Menkhaus, T. J. (2011). Removal of enzymatic and fermentation inhibitory compounds from biomass slurries for enhanced biorefinery process efficiencies. *Bioresource Technology*, *102*(17), 7850–7859. doi:10.1016/j.biortech.2011.05.043

9. Palmqvist, E., & Hähn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technology*, *74*(1), 25–33. doi:10.1016/S0960-8524(99)00161-3
10. Galbe, M., & Wallberg, O. (2019). Pretreatment for biorefineries: a review of common methods for efficient utilisation of lignocellulosic materials. *Biotechnology for Biofuels*, 1–26. doi:10.1186/s13068-019-1634-1
11. Almeida, J. R. M., Bertilsson, M., Gorwa-Grauslund, M. F., Gorsich, S., & Lidén, G. (2009). Metabolic effects of furaldehydes and impacts on biotechnological processes. *Applied Microbiology and Biotechnology*, *82*(4), 625–638.
12. Guarnieri, M. T., Franden, M. A., Johnson, C. W., & Beckham, G. T. (2017). Conversion and assimilation of furfural and 5-(hydroxymethyl)furfural by Pseudomonas putida KT2440. *Metabolic Engineering Communications*, *4*, 22–28. doi:10.1016/j.meteno.2017.02.001
13. Almeida, J. R., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., & Gorwa-Grauslund, M. F. (2007). Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates bySaccharomyces cerevisiae. *J Chem Technol Biotechnol*, *82*(4), 340–349. doi:10.1002/jctb.1676
14. Sanchez, B., & Bautista, J. (1988). Effects of furfural and 5-hydroxymethylfurfural on the fermentation of Saccharomyces cerevisiae and biomass production from Candida guilliermondii. *Enzyme and Microbial Technology*, *10*(5), 315–318.
15. Taherzadeh, M. J., Gustafsson, L., Niklasson, C., & Liden, G. (2000). Physiological effects of 5-hydroxymethylfurfural on Saccharomyces cerevisiae. *Applied Microbiology and Biotechnology*, *53*(6), 701–708.
16. Liu, Z. L., Slininger, P. J., Dien, B. S., Berhow, M. A., Kurtzman, C. P., & Gorsich, S. W. (2004). Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. *Journal of Industrial Microbiology & Biotechnology*, *31*(8), 345–352.
17. Liu, Z. L., Slininger, P. J., & Gorsich, S. W. (2005). Enhanced Biotransformation of Furfural and Hydroxymethylfurfural by Newly Developed Ethanologenic Yeast Strains. In B. H. Davison, B. R. Evans, M. Finkelstein, & J. D. McMillan (Eds.), *Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals* (pp. 451–460). Totowa, NJ: Humana Press. doi:10.1007/978-1-59259-991-2_39
18. Oke, M. A., Annuar, M. S. M., & Simarani, K. (2017). Mixed Feedstock Approach to Lignocellulosic Ethanol Production—Prospects and Limitations. *BioEnergy Research*, 1–15. doi:10.1007/s12155-016-9765-8
19. Althuri, A., Gujjala, L. K. S., & Banerjee, R. (2017). Partially consolidated bioprocessing of mixed lignocellulosic feedstocks for ethanol production. *Bioresource Technology*, *245*, 530–539.
20. Michelin, M., & Teixeira, J. A. (2016). Liquid hot water pretreatment of multi feedstocks and enzymatic hydrolysis of solids obtained thereof. *Bioresource Technology*, *216*, 862–869.
21. Ashraf, M. T., & Schmidt, J. E. (2018). Process simulation and economic assessment of hydrothermal pretreatment and enzymatic hydrolysis of multi-feedstock lignocellulose – Separate vs combined processing. *Bioresource Technology*, *249*, 835–843.
22. Sun, N., Xu, F., Sathitsuksanoh, N., Thompson, V. S., Cafferty, K., Li, C., et al. (2015). Blending municipal solid waste with corn stover for sugar production using ionic liquid process. *Bioresource Technology*, *186*(C), 200–206. doi:10.1016/j.biortech.2015.02.087
23. Silva-Fernandes, T., Duarte, L. C., Carvalheiro, F., Loureiro-Dias, M. C., Fonseca, C., & Gírio, F. (2015). Hydrothermal pretreatment of several lignocellulosic mixtures containing wheat straw and two hardwood residues available in Southern Europe. *Bioresource Technology*, *183*(C), 213–220. doi:10.1016/j.biortech.2015.01.059
24. Brodeur-Campbell, M., Klinger, J., & Shonnard, D. (2012). Feedstock mixture effects on sugar monomer recovery following dilute acid pretreatment and enzymatic hydrolysis. *Bioresource Technology*, *116*(C), 320–326. doi:10.1016/j.biortech.2012.03.090
25. Shi, J., Thompson, V. S., Yancey, N. A., Stavila, V., Simmons, B. A., & Singh, S. (2014). Impact of mixed feedstocks and feedstock densification on ionic liquid pretreatment efficiency. *Biofuels*, *4*(1), 63–72. doi:10.4155/bfs.12.82
26. Baral, N. R., Davis, R., & Bradley, T. H. (2018). Supply and value chain analysis of mixed biomass feedstock supply system for lignocellulosic sugar production. *Biofuels, Bioproducts and Biorefining*, *13*(3), 635–659. doi:10.1002/bbb.1975

27. Alvira, P., Negro, M. J., Ballesteros, I., González, A., & Ballesteros, M. (2016). Steam Explosion for Wheat Straw Pretreatment for Sugars Production. *Bioethanol*, *2*(1), 1–10. doi:10.1515/bioeth-2016-0003
28. Di Maio, D., & Turley, D. (2014). *Lignocellulosic feedstock in the UK*. *NNFC The Bioeconomy Consultants* (pp. 1–45). Retrieved from http://lb-net.net/wp-content/uploads/2015/04/LBNet-Lignocellulosic-feedstock-in-the-UK.pdf
29. Lewandowski, I., Clifton-Brown, J. C., Scurlock, J. M. O., & Huisman, W. (2000). Miscanthus: European experience with a novel energy crop. *Biomass and Bioenergy*, *19*(4), 209–227. doi:10.1016/S0961-9534(00)00032-5
30. Volk, T. A., Abrahamson, L. P., Cameron, K. D., Castellano, P., Corbin, T., Fabio, E., et al. (2011). Yields of willow biomass crops across a range of sites in North America. *Aspects of Applied Biology*, *112*, 67–74.
31. Saeman, J. F., Bubl, J. L., & Harris, E. E. (1945). Quantitative saccharification of wood and cellulose. *Industrial Engineering Chemistry Analytical Edition*, *17*(1), 35–37.
32. Overend, R. P., & Chornet, E. (1987). Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments [and Discussion]. *Philosophical Transactions of the Royal Society A*, *321*(1561), 523–563.
33. Selig, M., Weiss, N., & (null), Y., Ji. (2008). *Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP)* (pp. 1–8). NREL- National Renewable Energy Laboratory.
34. Ibbett, R., Gaddipati, S., Hill, S., & Tucker, G. (2013). Structural reorganisation of cellulose fibrils in hydrothermally deconstructed lignocellulosic biomass and relationships with enzyme digestibility. *Biotechnology for Biofuels*, *6*(1), 33–15. doi:10.1186/1754-6834-6-33
35. Greetham, D., Wimalasena, T., Kerruish, D. W. M., Brindley, S., Ibbett, R. N., Linforth, R. L., et al. (2014). Development of a phenotypic assay for characterisation of ethanologenic yeast strain sensitivity to inhibitors released from lignocellulosic feedstocks. *Journal of Industrial Microbiology & Biotechnology*, *41*(6), 931–945. doi:10.1007/s10295-014-1431-6
36. Wilkinson, S., Smart, K. A., & Cook, D. J. (2014). Optimisation of alkaline reagent based chemical pre-treatment of Brewers spent grains for bioethanol production. *Industrial Crops & Products*, *62*, 219–227. doi:10.1016/j.indcrop.2014.08.036
37. Carroll, J. P., & Finnan, J. (2010). The relationship between bulk density and energy input, in biomass pellet production. *Advances in Animal Biosciences*, *1*(1), 331–331. doi: https://doi.org/10.1017/S2040470010004747
38. Allen, S. A., Clark, W., McCaffery, J. M., Cai, Z., Lanctot, A., Slininger, P. J., et al. (2010). Furfural induces reactive oxygen species accumulation and cellular damage in Saccharomyces cerevisiae. *Biotechnology for Biofuels*, *3*(1), 2–10. doi:10.1186/1754-6834-3-2
39. Greetham, D., Hart, A. J., & Tucker, G. A. (2016). Presence of low concentrations of acetic acid improves yeast tolerance to hydroxymethylfurfural (HMF) and furfural. *Biomass and Bioenergy*, *85*(C), 53–60. doi:10.1016/j.biombioe.2015.11.026
40. Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., & Ladisch, M. R. (2005). Optimization of pH controlled liquid hot water pretreatment of corn stover. *Bioresource Technology*, *96*(18), 1986–1993. doi:10.1016/j.biortech.2005.01.013

Supplementary Files

Table S1
Pretreatment severity of the different pretreatment regimes (temperature °C, time min)

	180-5	180-10	180-20	200-5	200-10	200-20	220-5	220-10	220-20
Severity Log R_o	3.05	3.36	3.66	3.64	3.94	4.25	4.23	4.53	4.83

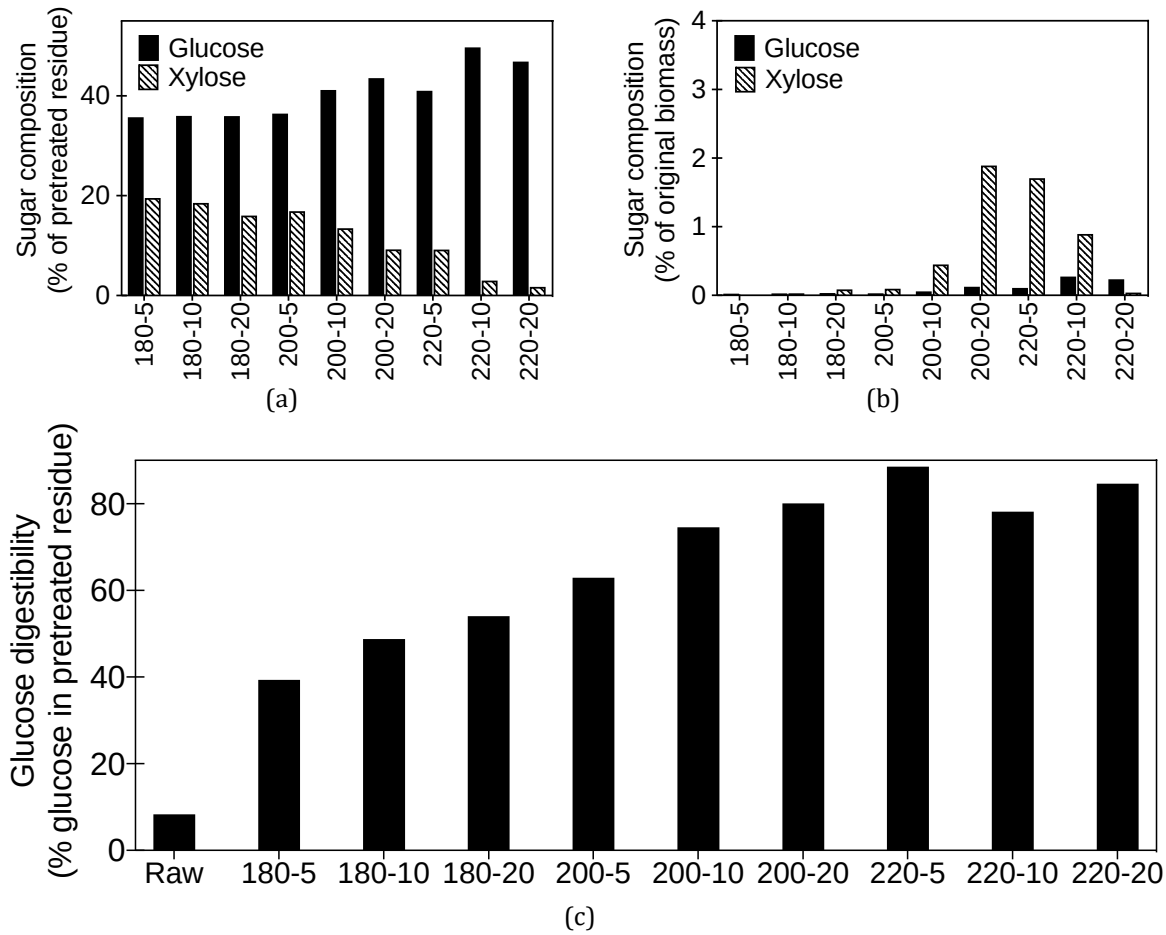

Fig.S1. Effect of varying temperature/time on pre-treatment of wheat straw. (a) Sugar composition of pre-treated residue, (b) Sugar composition of hydrolysate liquor after pre-treatment and (c) Digestibility after 72 hours expressed as % of available glucose (n=1).

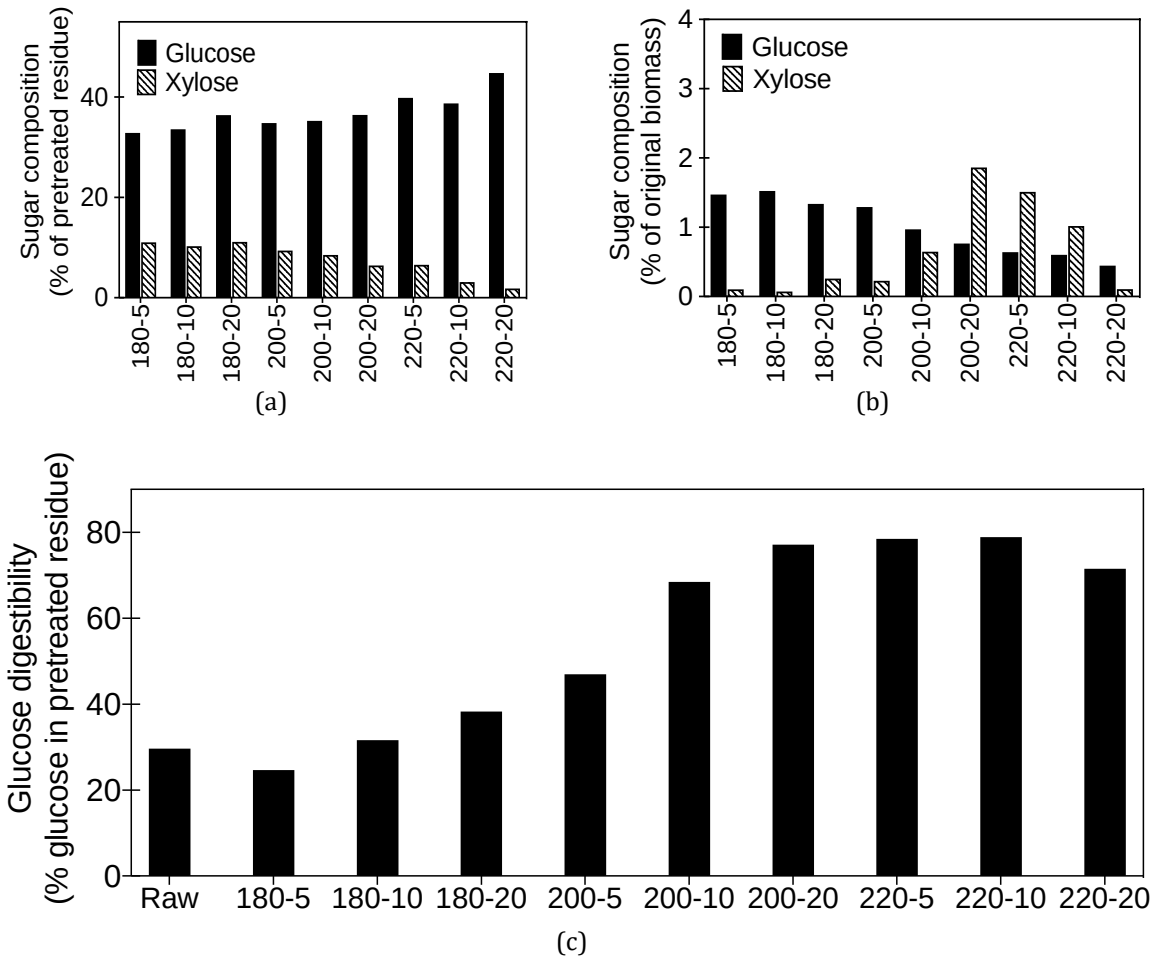

Fig.S2. Effect of varying temperature/time on pre-treatment of willow. (a) Sugar composition of pre-treated residue, (b) Sugar composition of hydrolysate liquor after pre-treatment and (c) Digestibility after 72 hours expressed as % of available glucose (n=1).

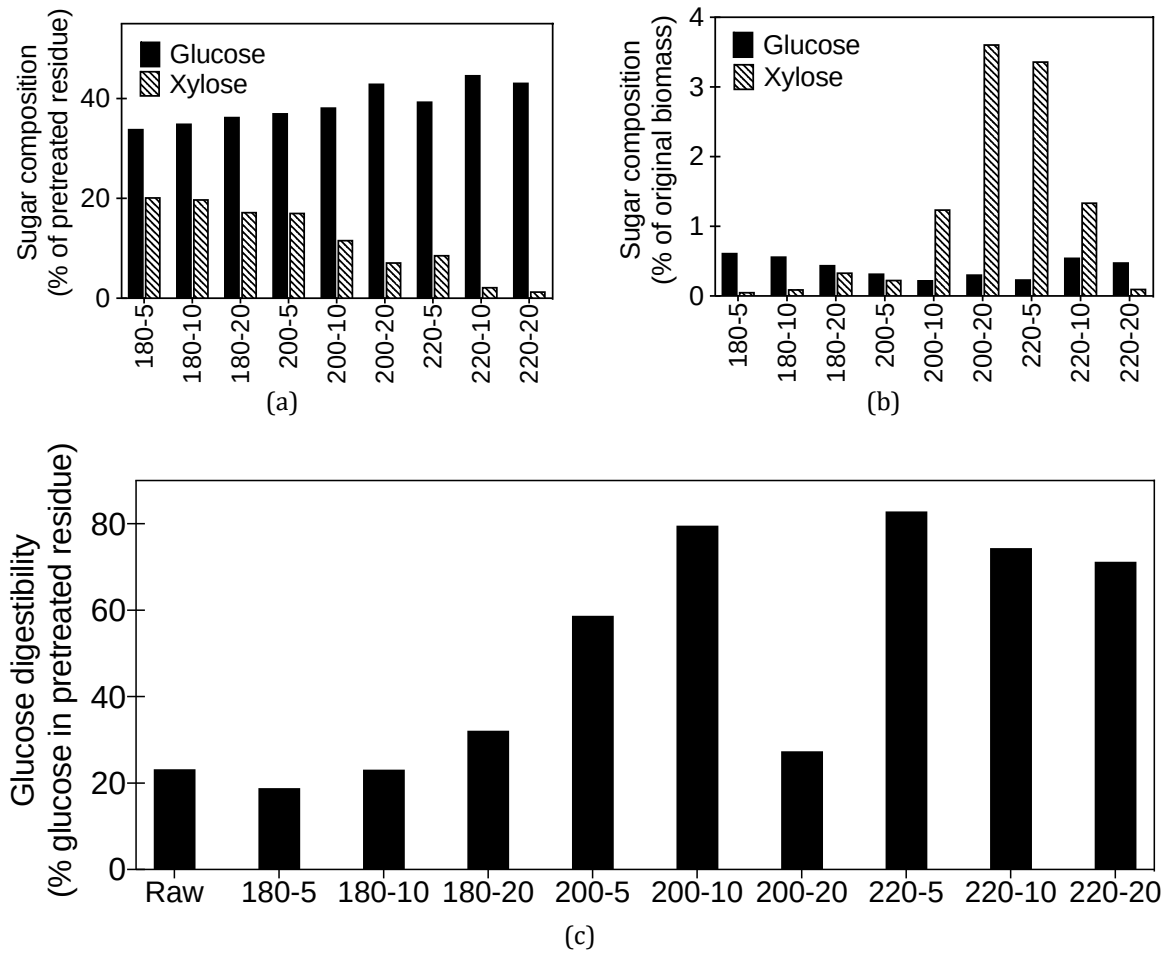

Fig.S3. Effect of varying temperature/time on pre-treatment of Miscanthus. (a) Sugar composition of pre-treated residue, (b) Sugar composition of hydrolysate liquor after pre-treatment and (c) Digestibility after 72 hours expressed as % of available glucose (n=1).