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Comparison of Different Pretreatment Strategies for Enzymatic Hydrolysis of Wheat and Barley Straw

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Abstract In biomass-to-ethanol processes a physico-chemical pretreatment of the lignocellulosic biomass is a critical requirement for enhancing the accessibility of the cellulose substrate to enzymatic attack. This report evaluates the efficacy on barley and wheat straw of three different pretreatment procedures: acid or water impregnation followed by steam explosion versus hot water extraction. The pretreatments were compared after enzyme treatment using a cellulase enzyme system, Celluclast 1.5 L® from *Trichoderma reesei*, and a β-glucosidase, Novozyme 188 from *Aspergillus niger*. Barley straw generally produced higher glucose concentrations after enzymatic hydrolysis than wheat straw. Acid or water impregnation followed by steam explosion of barley straw was the best pretreatment in terms of resulting glucose concentration in the liquid hydrolysate after enzymatic hydrolysis. When the glucose concentrations obtained after enzymatic hydrolyses were related to the potential glucose present in the pretreated residues, the highest yield, ~48% (g g^{-1}), was obtained with hot water extraction pretreatment of barley straw; this pretreatment also produced highest yields for wheat straw, producing a glucose yield of ~39% (g g^{-1}). Addition of extra enzyme (Celluclast 1.5 L®+Novozyme 188) during enzymatic hydrolysis resulted in the highest total glucose concentrations from barley straw, 32–39 g L^{-1}, but the relative increases in glucose yields were higher on wheat straw than on barley straw. Maldi-TOF MS analyses of supernatants of pretreated barley and wheat straw samples subjected to acid and water impregnation, respectively, and steam explosion, revealed that the water impregnated + steam-exploded samples gave a wider range of pentose oligomers than the corresponding acid-impregnated samples.

Keywords Lignocellulose · Enzymatic hydrolysis · Glucose yield · Pretreatment

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Introduction

Production of bioethanol from lignocellulosic biomass feedstocks has attracted much attention as an alternative route to expand the current starch- and sucrose-based production of ethanol for the manufacture of transport fuels to replace fossil fuels.

One of the first requirements in the utilization of lignocellulose for production of ethanol is to efficiently produce a fermentable hydrolysate rich in glucose from the cellulose present in the feedstock. Employment of enzymes for the hydrolysis of the lignocellulose is considered the prospectively most viable strategy to provide a cost-efficient, environmentally friendly process, and to avoid generation of byproducts that may inhibit the subsequent fermentation [1–3]. However, the physico-chemical and structural composition of native lignocellulose hinders direct enzymatic hydrolysis of the cellulose and hemicellulose present in lignocellulosic biomass. To increase the accessibility of the cellulose to enzymatic attack the lignocellulosic substrates therefore have to undergo a physico-chemical pretreatment before the enzymatic hydrolysis step [4].

Several different pretreatment processes are efficient in providing a relatively easily degradable substrate [4–6]. In brief, pretreatment may involve a mechanical step for reducing the substrate particle size of the native straw followed by one or more steps of heating and wetting the straw in the presence of a catalyst [4]. Acid, alkali or water itself at high temperature can be used as catalysts. With acid pretreatment, the hemicellulose present in the straw is solubilized, in effect producing a solid fraction consisting of mainly cellulose and lignin. The wet-oxidation and alkaline-based methods are generally relatively more effective at solubilizing lignin, but leaves behind much of the hemicellulose in an insoluble, polymeric form [4, 6]. If no catalyst is used, the hemicellulose is presumably solubilized as hemicellulose oligo- or polymers by the organic acids present in the native straw (i.e., by acetic acid and water itself) [7, 8].

The steam pretreatment in conjunction with an acid catalyst is known to release the hemicellulose constituents of lignocellulose as oligo- and monosaccharides; however, during the heat treatment some of the released monosaccharides may be degraded to compounds inhibitory to both cellulase enzymes and the yeast during the subsequent fermentation step [9, 10]. These inhibitory compounds include weak acids, furfural (from xylose) and 5-hydroxymethyl furfural (from C6 monosaccharides), and phenolic compounds from lignin [4, 9]. Hot liquid water extraction is a particularly attractive pretreatment process compared to steam explosion as this pretreatment involves no handling of harsh chemicals. Moreover, hot liquid water extraction is reported to produce a liquid stream, which, in contrast to acid hydrolyzed steam pretreated lignocellulose, does not inhibit the yeast during the fermentation step as hemicellulose is mainly released as oligomers [7]. Evidently, the specific pretreatment process best suited to a specific process will depend on a number of factors, including the origin of the lignocellulosic biomass—softwood, hardwood, herbaceous energy crops or other agricultural residues—and the amount and nature of inhibitory compounds. The choice of pretreatment also depends on whether the C5 monosaccharides are supposed to be utilized or not for the bioethanol production [8, 9].

Despite the intensive experimental investigation of different pretreatment methods only very limited efforts have been devoted to systematically compare the influence of different pretreatment strategies on the enzymatic cellulose hydrolysis of wheat and barley straw substrates. In this work, we have compared the influence of three principally different pretreatment strategies on the subsequent enzymatic hydrolysis of wheat and barley straw biomass. The glucose levels and relative yields obtained were compared after cellulolytic

enzyme treatments with a commercial cellulase product Celluclast 1.5 L® from *Trichoderma reesei* supplemented with β-glucosidase, Novozyme 188, from *Aspergillus niger*.

Materials and Methods

Substrate

Barley and wheat straw were grown and harvested in 2006 on the island of Funen, Denmark. Samples of both types of straw were transported to DONG Energy (Danish Oil and Natural Gas Energy), Denmark and to the Department of Chemical Engineering, Lund University, Sweden (Professor G. Zacchi) for pretreatment. The specific conditions of the different pretreatments are specified in Table 1. In brief; the DONG Energy pretreatment method consisted of a three-stage process, which involved triple heating treatment of the straw at increasing temperatures. After the first heating step, the liquids were removed. At Lund University, the barley and wheat straw were subjected to steam explosion, both with and without the presence of an acid catalyst (in this case H_2SO_4). A major difference between the pretreatment procedures was the scale of processing: at DONG Energy the straw was fed directly to the reactor in whole bales, whereas at Lund University, the straw was chopped into pieces, approximately 2 cm long, before being fed to the reactor in batches of 400 g.

Enzymes

The enzyme system applied consisted of Celluclast 1.5 L® (Celluclast) derived from *T. reesei*, and Novozyme 188 (NS 188) derived from *Aspergillus niger*; both preparations were from Novozymes A/S (Bagsvaerd, Denmark). The activity of the Celluclast preparation was 47 FPU g^{-1} (FPU=filter paper units) as measured by the standardized filter paper assay provided by the US National Renewable Energy Laboratory [11]. The protein content of the Celluclast preparation was measured using a Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL) following the manufacturer's instructions; before the

Table 1 Overview of substrate codes, pretreatment procedures, and dry matter levels in the different barley straw and wheat straw substrates studied.

Code	Substrate	Pretreatment conditions	Dry matter % *w*/*w*
Barley 1	Acid-impregnated, steam-exploded barley straw	60% DM, 1% H_2SO_4, 1 h; 220°C, 5 min[a]	19±0.3
Barley 2	Water-impregnated, steam-exploded barley straw	70% DM, 1 h; 210°C, 5 min[a]	20±1.5
Wheat 1	Acid-impregnated, steam-exploded wheat straw	60% DM, 0.2% H_2SO_4, 1 h; 190°C, 10 min[a]	18±2.0
Wheat 2	Water-impregnated, steam-exploded wheat straw	70% DM, 1 h, 220°C, 2.5 min[a]	26±1.2
Barley 3	Hot-water-extracted barley straw	Approx. 16% DM, 60°C, 15 min; liquids removed; 180°C, 10 min; 195°C, 3 min	24±1.0
Wheat 3	Hot-water-extracted wheat straw	Approx. 16% DM, 60°C, 15 min; liquids removed; 180°C, 10 min; 195°C, 3 min	28±0.6

[a] Pretreatment was done at optimal conditions according to [10] and [13]

protein determination the Celluclast was desalted using a Micro Bio-Spin® 6 Chromatography Column (Bio-Rad, Hercules, CA) eluting with 0.05 M sodium acetate buffer pH 5. The activity of NS 188 was 260 CBU g^{-1} (Cellobiase Units) determined from glucose production on cellobiose at 40°C, pH 5 (provided by Novozymes A/S, Bagsvaerd, Denmark).

Hydrolysis Reactions

The hydrolytic enzyme treatment reactions were carried out at 5 FPU Celluclast per gram dry matter (DM) supplemented with 13 CBU of NS 188. The enzyme reactions were carried out in 100 g scale in a shaking incubator (New Brunswick, Innova 44, Edison, NJ) at 200 RPM. The various substrates were weighed and buffer (0.5 M sodium acetate, pH 5) was added to adjust the substrate DM content in the reactions to 10.0% *w*/*w* DM. Samples were drawn from the reaction at specified time points, boiled for 10 min to halt the enzyme reactions, and centrifuged for 10 min at 14,000×*g*. Each supernatant was then collected for monosaccharide analysis by high-performance anionic exchange chromatography (HPAEC), see below. The deactivation of the Celluclast and NS 188 enzyme system during reactions was tested by incubating the enzyme preparations in buffer at 50°C for 72 h corresponding to the reactions on the substrates. After the incubation, the remaining activity was measured using the standardized filter paper assay [11].

Analysis of Substrate Monosaccharides and Glucose Yields

The glucose, arabinose, and xylose (and galactose) concentrations were analyzed by HPAEC on a Dionex® BioLC system equipped with a CarboPac PA1 column (4×250 mm) (Dionex Denmark A/S, Hvidovre, DK) and a CarboPac PA1 guard column (4×50 mm). Samples were eluted isocratically with 0.01 M KOH at a flow rate of 1 ml/min and analytes were detected and quantified against standard curves by electrochemical detection in a pulsed amperiometric detection mode as described previously [12].

Analysis of Oligomer Profiles by Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was performed on supernatant fractions of pretreated straw in the positive ion mode on a Voyager DE STR mass spectrometer (Perseptive Biosystems, Framingham, MA, USA). Analyses were carried out in the reflector mode at a mass range of 500–4,000 *m*/*z* with an accelerating voltage of 20 kV and a delay time of 400 ns. A low mass gate value of *m*/*z* 500 was selected for analysis to avoid saturation of the detector; 20 mg ml^{-1} DHB in 70% ACN/0.1% TFA (*w*/*w*) was used as matrix. The samples were subjected to ion exchange (AG 50W-x12 analytical grade cation exchange resin 100–200 mesh hydrogen, Bio-Rad [Hercules, CA]) before being applied to the matrix.

Results

Glucose Potential After Pretreatment

To assess the glucose potential and evaluate the hemicellulose monosaccharides in the differently pretreated batches of barley and wheat straw, the monosaccharide composition in

each of the substrates was analyzed before enzymatic treatment. Based on the measured levels of arabinose and xylose (only negligible amounts of galactose were detected; Table 2) the hemicellulose was estimated to make up approximately 5–12% of the dry matter—and, as expected, to be mostly contributed by xylan. The glucose potential was defined as the amount of glucose available after acid hydrolysis of the pretreated lignocellulose (Table 2). Except for the hot-water-extracted samples (Barley 3 and Wheat 3), the pretreated barley straw samples had a higher glucose potential than the pretreated wheat straw. The glucose potentials of the acid- or water-impregnated, steam-exploded barley straw samples ranged from 57 to 61% of the DM, whereas the equivalent values for the wheat straw samples were 50–55% (Table 2). For both wheat and barley straw, the DONG Energy hot water extraction process resulted in a lower glucose potential (41–48% of the DM) than the steam explosion pretreatment (Table 2).

The pretreatment conditions used for the steam explosion pretreatments were carefully selected as optimal for each of the substrates [10, 13]. Hence, the acid-impregnated, steam-exploded barley straw pretreatment was somewhat harsher than the equivalent treatment for the wheat straw with respect to the amount of H_2SO_4 and the temperature during treatment (Table 1). The observed higher glucose potential in the barley straw compared to the wheat straw could be caused by the slightly harsher pretreatment conditions employed for the barley straw. Alternatively, the levels could be a result of barley straw simply being richer than wheat straw in cellulose per unit dry matter. The composition of straw is dependent on the straw variety and the climatic factors during the growth season. Whereas the hemicellulose levels have been found to be approximately similar, the cellulose content has consistently been found to be a little higher in barley straw than in wheat straw—wheat straw is thus slightly more lignified and also contains more silica than barley straw [14].

Enzymatic Glucose Release

The enzymatic hydrolysis reactions were run for 72 h; samples were taken at time points 2, 4, 6, 24, 48, and 72 h. However, as the structure of the substrate was not degraded to any substantial degree until after 24 h of reaction—as evaluated from the glucose levels released—only the results originating from samples taken at 24 and 72 h are considered in the following. The pretreatment processes evaluated in this study could be ranked according to severity; H_2SO_4 impregnation followed by steam explosion was considered the harshest pretreatment in terms of breaking the lignocellulose structure. In the next place came the water impregnation with steam explosion and finally the liquid hot water extraction, which was considered the least harsh pretreatment method. According to the severity of the pretreatment, the released glucose levels, in the case of barley straw, were highest with the most severe pretreatment and lowest with the liquid hot water extraction (Fig. 1).

Table 2 Carbohydrate composition of straw substrates: values are given in percent of total dry matter.

	Arabinose	Xylose	Glucose
Barley 1	5.4±0.1	5.5±0.4	57±7.4
Barley 2	1.7±0.2	3.4±0.3	61±1.7
Barley 3	2.4±0.5	10±2.0	41±2.4
Wheat 1	4.4±1.5	5.3±0.8	50±0.3
Wheat 2	2.2±0.1	5.8±0.1	55±7.1
Wheat 3	5.8±1.0	7.1±0.6	48±7.8

However, the final glucose levels obtained from the barley straw subjected to steam explosion pretreatment with either water or H_2SO_4 impregnation did not differ substantially after 72 h of reaction (Fig. 1, Barley 1 versus 2). The enzymatic treatment of the hot-water-pretreated barley straw (Barley 3) resulted in a glucose concentration that tended to be lower than that obtained by steam explosion, suggesting that the steam explosion pretreatment rendered the barley straw more easily degradable. However, when comparing the glucose concentrations in relation to the amount of potential glucose in the residues (yield percent: [g glucose × g potential glucose^{-1}] × 100%), the hot-water-extracted straw appeared to be more efficiently enzymatically hydrolyzed than the steam pretreated substrates (Fig. 1).

With regard to glucose levels obtained, the trends for wheat were opposite those of barley. Hence, for the wheat straw the highest glucose levels were achieved with the least severe pretreatment, namely, the hot water extraction pretreatment—the glucose concentrations obtained on the pretreated wheat samples did not, however, differ significantly after the comparative enzymatic hydrolysis (Fig. 2). However, the yield obtained in the enzymatic hydrolysis of Wheat 3 (after 72 h) was 10–15 % higher than the yields obtained in the other enzymatic hydrolysis reactions with Wheat 1 and 2 (Fig. 2). In all three enzymatic hydrolysis reactions with wheat straw, the glucose concentrations and the yields were lower than those obtained with barley straw (Fig. 1 versus 2). These results might be related to the interplay between the differences in straw structure of barley and wheat and the difference in pretreatment conditions as the least severe pretreatment condition gave the highest glucose concentration and yield for wheat straw, whereas the opposite was true for barley straw. Taken together, the data were in accordance, but expanded, to previous findings [10], indicating that barley and wheat straw differ with respect to the conditions needed for efficient opening of the lignocellulose structure for enzymatic hydrolysis.

Supplementation of Enzymes During Reaction

Cellulases acting on pretreated straw may lose activity if adsorbed to the lignin or the crystalline cellulose in the substrate; alternatively, the enzymes may be thermally inactivated during the reaction or inhibited by substances resulting from the pretreatment. In all cases, the enzymatic hydrolysis, and in turn the glucose release, will be lower than optimal. To assess whether any of these effects could be overcome in practice by addition of more enzymes during hydrolysis, an experiment was carried out in which extra enzymes (in the form of Celluclast + NS 188) were added to the hydrolysis reactions after 24 h of reaction. The released glucose levels obtained with this addition of extra enzymes after 72 h were highest for the hydrolysis of barley straw and especially high for the barley straw subjected to acid

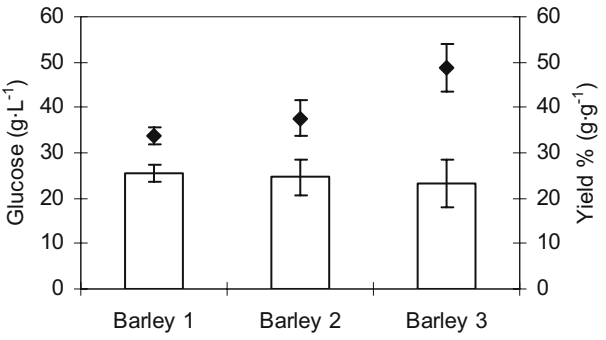

Fig. 1 Glucose concentration (*columns*) and yield (*black diamonds*) (based on the potential glucose in the substrate as determined by acid hydrolysis) after 72 h hydrolysis of pretreated barley straw at 10% *w*/*w* dry matter. Legends refer to pretreatment conditions shown in Table 1

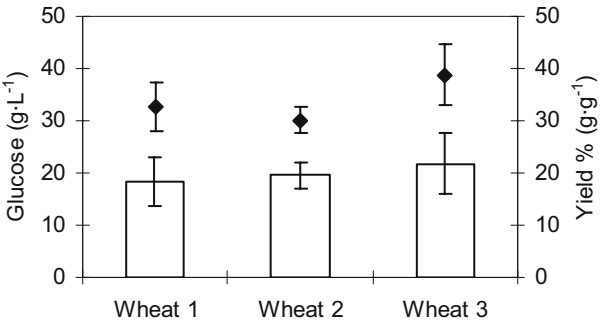

Fig. 2 Glucose concentration (*columns*) and yield (*black diamonds*) (based on the potential glucose in the substrate as determined by acid hydrolysis) after 72 h hydrolysis of pretreated wheat straw at 10% *w/w* dry matter. Legends refer to pretreatment conditions shown in Table 1

pretreatment, Barley 1, which gave a maximal concentration of released glucose of 39 g L^{-1} after 72 h (Table 3). The reaction included 48 h of reaction with additional enzymes as the extra enzymes were added at 24 h. After a total reaction time of 72 h the boosting effect was equivalent to a relative glucose increase with extra enzymes of ~54% as compared to the nonsupplemented reaction (([39.2–25.4]×100)/25.4] ≈ 54%, Table 3) and a relative increase in glucose concentration of 89% from 24–72 h with the extra enzymes added (Table 3). This relative increase should be compared to a relative increase in glucose concentration from 24–72 h of ~23% during the regular enzymatic hydrolysis of Barley 1 (Table 3).

The highest *relative* increases in released glucose levels by addition of extra enzymes were obtained for the pretreated wheat samples. For Wheat 1 and Wheat 2 the extra enzyme addition boosted the relative increase in glucose concentrations by ~180–200% during the reaction from 24 to 72 h (Table 3). Addition of extra enzymes after 24 h of reaction thus significantly increased the yields with all the four tested pretreated substrates (Table 3).

It is well documented that adsorption of enzymes to both crystalline cellulose and lignin may significantly decrease the rate and extent of cellulolytic degradation of cellulose in lignocellulosic substrates [15, 16]. Moreover, inhibitors generated during steam explosion pretreatment may exert a negative impact on the enzymatic hydrolysis of the straw [17]. In the pretreated Barley 1 and 2 and in the Wheat 1 and 2 samples, both furfural and 5-

Table 3 Released glucose levels (g L^{-1}) and relative increases in percent after 24 and 72 h of enzyme treatment (Celluclast + Novozyme 188) without and with supplementation of extra Celluclast + Novozyme 188 after 24 h of reaction.

	Glucose release (g L^{-1})					
	24 h	72 h	72 h extra enzyme[a]	24–72 h % increase	24–72 h % increase extra enzyme[b]	72 h % relative improvement with extra enzyme[c]
Barley 1	20.7±4	25.4±2	39.2±3	22.7	89.4	54.3
Barley 2	15.9±2	24.6±3	32.0±1	54.7	101	30.1
Wheat 1	9.6±4	18.5±4	26.9±4	92.7	180	45.4
Wheat 2	9.8±1	19.5±4	29.3±4	99.0	199	50.3

[a] Extra dose of Celluclast and Novozym 188 added after 24 hours of reaction

[b] Each value calculated as the difference in percent between the glucose concentration after 24 h and 72 h of enzyme reaction

[c] Each value calculated from relative difference in the glucose release (g L^{-1}) at 72 h between samples with extra enzyme added and samples without extra enzyme added

hydroxymethyl furfural were identified by HPLC analysis of the (non-enzyme treated) supernatants (data not shown)—but the differences in the levels of these substances could not explain the different yields obtained after enzymatic hydrolysis. Also other mechanisms, such as the cellulases getting "stuck" within the cellulose, may slow down the enzymatic hydrolysis during cellulolytic hydrolysis reactions [18]. Another limiting factor may be that the enzymes are simply heat inactivated during the prolonged hydrolysis reaction at 50°C. To test this latter hypothesis, we measured the remaining FPU activity of the enzymes (Celluclast and NS188 incubated together) after 72 h of incubation in buffer at pH 5, 50°C. This resulted in a loss of approximately 80% of the initial enzyme activity (FPU) after 72 h of reaction (data not shown). Hence, although the presence of substrate may partly stabilize the enzymes, the gradual slowing down of the hydrolysis rates observed during prolonged enzymatic treatment of lignocellulosic biomass may partly be a result of gradual heat inactivation of the added cellulases—in addition to adsorption of enzymes to lignin and cellulose. This finding provides a strong stimulus to develop more heat-stable cellulases for hydrolysis of lignocellulosic biomass.

Oligomer Profiles of Supernatants

To fully evaluate the differences between the differently pretreated biomass samples, the oligomeric profiles of the supernatants of the pretreated Barley 1, Barley 2, Wheat 1, and Wheat 2 substrates were examined by Maldi-TOF MS analysis. As it was not possible to remove any liquids from Barley 3 and Wheat 3, the liquid fractions in this material were not analyzed. The oligomeric profiles obtained by the MS analysis differed significantly in accordance with the type of straw and in response to the use of acid or water impregnation before the steam explosion pretreatment. The supernatant of the acid-impregnated and steam-exploded barley and wheat straw substrates, Barley 1 and Wheat 1, both mainly contained hexose oligomers ranging from 5 C_6 (HO5) up to 16 C_6 (HO16) (Fig. 3a,c). In addition, the Barley 1 and Wheat 1 supernatants also harbored a few, relatively short pentose oligomers: In the Barley 1 supernatant, the analyzed pentose oligomers were a mixture of hexa-, hepta-, and octamers (PO6-PO8, Fig. 3a), whereas in the Wheat 1 supernatant, the pentose oligomers were penta- and hexa-oligomers, and included acetylated pentamer species (PO5, PO6, PO5AC, Fig. 3c). In comparison, the water-impregnated supernatants from Barley 2 and Wheat 2 both contained a much wider profile of pentose oligomers containing from three to four and up to 13 C_5 monomers (Fig. 3b,d). In both of these supernatants, the pentose oligomers were found to occur with and without acetic and ferulic acid substitutions. The water-impregnated and steam-exploded barley supernatant, Barley 2, moreover appeared to contain a wide range of hexose oligomers (HO5–HO14, Fig. 3b). These were not found in the corresponding water-impregnated supernatant from wheat straw, Wheat 2, which appeared to only harbor pentose oligomers (Fig. 3d).

The Maldi-TOF MS chromatograms thus revealed significant differences of the impact of the acid vs. water impregnation pretreatment on the oligomer profiles on the differently pretreated barley and wheat straw. These differences between acid and water impregnations before the steam explosion confirmed that the hemicellulose is almost completely solubilized to monomeric species (not detected in the MS analysis) in acid-impregnated samples, whereas the hemicellulose is apparently solubilized in the form of a wider spectrum of oligomers with water impregnation and steam explosion. The results also indicated significant differences in the hexose oligomer structures between barley and wheat straw after pretreatments employing water impregnation and steam explosion. The currently

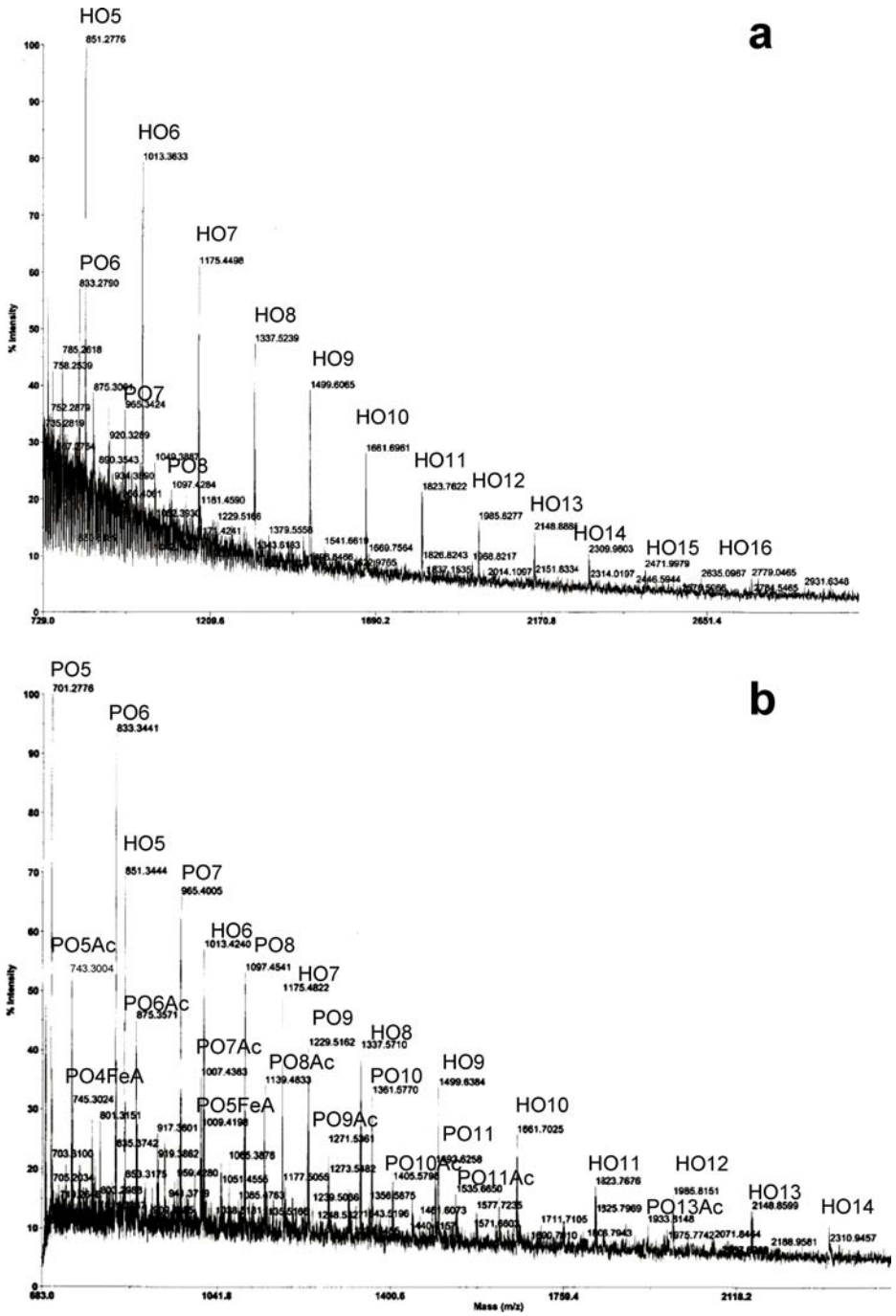

Fig. 3 MALDI-TOF MS spectra (sodium adducts) of the liquid fractions from Barley 1 (**a**), Barley 2 (**b**), Wheat 1 (**c**), and Wheat 2 (**d**), respectively. *HO* denotes hexose oligomer, *PO* denotes pentose oligomer, *FeA* denotes ferulic acid and *Ac* denotes acetic acid. *Numbers* refer to the number of monosaccharides in the oligomer

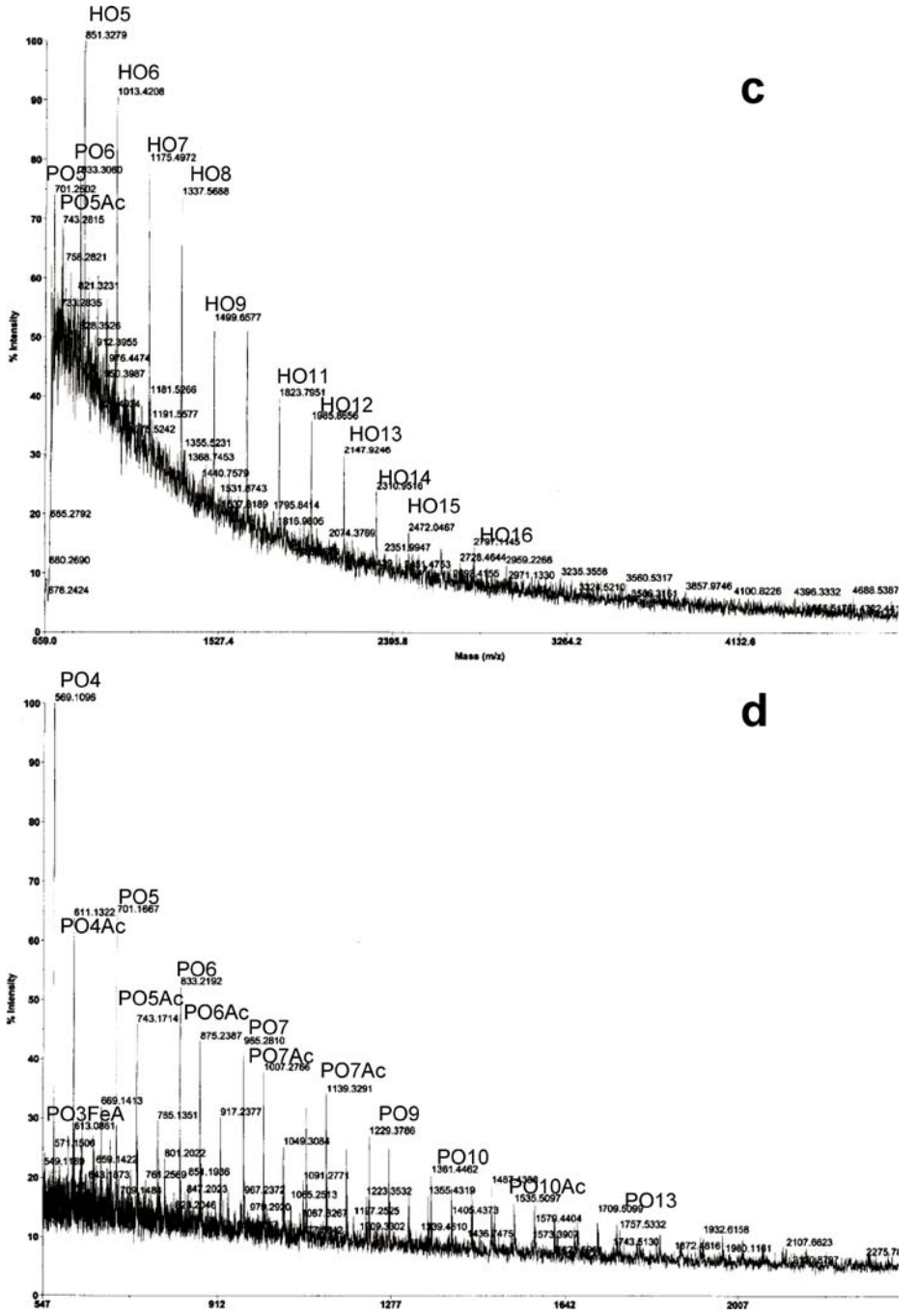

Fig. 3 (continued)

available knowledge does not provide a clear explanation for this difference—other than the biological difference between barley and wheat straw. The wheat straw pretreatment conditions included treatment at a slightly higher temperature by only 10°C, but on the other hand, a shorter treatment time of only 2.5 min (Table 1). As the Maldi-TOF MS chromatograms give only qualitative, and not quantitative, oligomeric profiles, the potential glucose and xylose yields contributed by the oligomers in the supernatants could only be compared after enzymatic hydrolysis of these four supernatants. The glucose concentration, including the net gain contributed by enzymatic hydrolysis of the oligomers, was highest for both of the barley supernatants (Fig. 4). However, hydrolysis of the hexose oligomers in the supernatant(s) contributed a gain of only maximum ~2 g glucose L^{-1} (Fig. 4). The lowest contribution, found with Wheat 2, was only marginal: ~0.5 g glucose L^{-1} (Fig. 4). This latter finding was in accordance with the MS profile, which showed that the Wheat 2 supernatant did not contain detectable amounts of hexose oligomers. On the other hand, the supernatant of the latter harbored the highest pentose (xylose) potential: thus, the xylose released by enzymatic hydrolysis, the pentose oligomers brought about by the Celluclast+NS 188 enzyme treatment of the Wheat 2 supernatant, was ~7 g xylose L^{-1} (Fig. 4). The net xylose yields from the other supernatants varied from 2 to 6 g xylose L^{-1}, and was lowest for the Barley 1 supernatant (Fig. 4), which, on the other hand, had the highest xylose content before the addition of enzymes. These results confirmed that the acid impregnation and steam explosion directly released most of the xylose from both the barley and wheat straw substrates.

Effect of Substrate Loading Level

The effect of substrate loading was of interest, as it has previously been shown that increasing the substrate concentration results in higher glucose concentration but lower overall yields [19, 20]. The substrate that resulted in the highest glucose concentration after enzymatic hydrolysis was the acid treated, steam exploded barley straw, Barley 1 (Table 3). Hence, the effect of lowering the substrate concentration was evaluated for this substrate to assess if it was possible to obtain a higher yield ([g glucose × g potential glucose^{-1}] × 100%) with lower substrate loading. As expected, a reduction of the substrate loading from 10% *w/w* to 2.5% *w/w* increased the final extent of conversion as evaluated from the yields of glucose, but resulted in a lowered released glucose concentration (Fig. 5). A compromise between maximum yield versus maximum glucose concentration after enzymatic hydrolysis was found at the substrate dry matter concentration of 5.0% *w/w* (Fig. 5). This substrate loading resulted in a glucose concentration of approximately 20 g L^{-1} and a yield of ~67% of the available glucan in the straw (Fig. 5). The occurrence of a clear *positive* relation between the final glucose

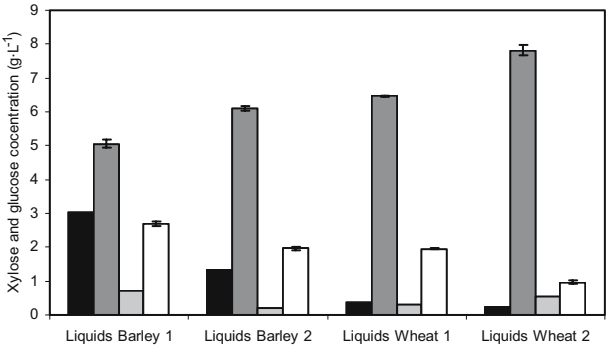

Fig. 4 Xylose and glucose concentrations measured in the liquid fractions of the pretreated straw samples. Black and dark gray correspond to xylose concentration before and after incubation with Celluclast + NS188, respectively. Light gray and white correspond to glucose before and after addition of Celluclast + NS 188, respectively

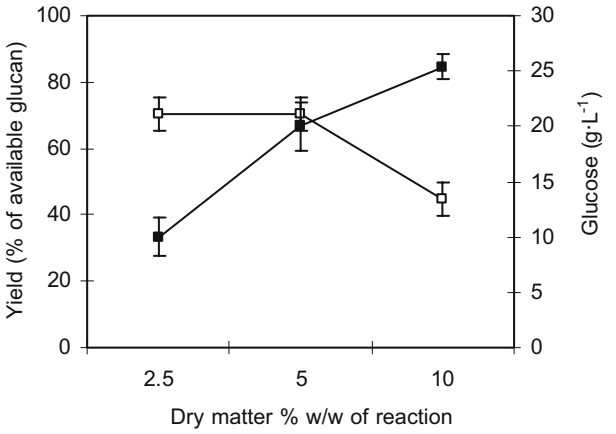

Fig. 5 Glucose concentrations (*filled squares*) and yields in percent of substrate glucan (*open squares*) from Barley 1 after 72 h of enzymatic treatment (Celluclast + NS 188) as a function of dry matter. The yields were calculated as percent of glucose obtained in relation to the potential glucose release possible from the native barley straw ([g glucose×g potential glucose^{-1}]×100%)

concentration and the DM substrate level, and a *negative* relation between glucose yields and substrate DM concentration were in complete accordance with previous data [19, 20].

Conclusions

The highest glucose release was found after enzymatic hydrolysis of barley straw, which had been pretreated using H_2SO_4 impregnation and steam explosion. Ranked after this was the barley straw subjected to water impregnation and steam explosion pretreatment. The lowest enzyme-catalyzed glucose release from barley straw was obtained for the substrate having been subjected to hot water extraction pretreatment. An opposite trend of pretreatment efficacy was found for wheat straw. These results indicate that the optimal pretreatment conditions differ substantially for wheat and barley straw with respect to the resulting cellulose substrate accessibility and in turn with respect to glucose concentration to be obtained by enzymatic hydrolysis. It was also shown that addition of extra cellulolytic enzymes to the hydrolysis reaction had a significantly positive impact on glucose release when the enzymes were added after 24 h reaction. Addition of the extra enzymes gave a maximal glucose release of 39 g L^{-1} for the barley straw, which had been acid-impregnated before steam explosion, but the relative increase in glucose liberation by addition of more enzymes during the reaction versus the regularly enzyme-treated samples were largest for the wheat samples irrespective of their pretreatment. It was also shown that the cellulolytic enzymes (Celluclast + NS188) lost significant activity as a result of thermal inactivation during prolonged incubation at 50°C. These results indicated that some of the problems of decreased glucose liberation during prolonged enzymatic lignocellulose hydrolysis might be overcome by simply adding more enzyme during the reaction. The cost–benefit analysis between the cost investment effectuated by addition of extra enzymes to achieve higher glucose levels and higher ethanol yields versus the revenues obtained from the extra ethanol yields awaits further study.

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