CHARACTERIZATION OF KEY PARAMETERS FOR BIOTECHNOLOGICAL LIGNOCELLULOSE CONVERSION ASSESSED BY FT-NIR SPECTROSCOPY. PART I: QUALITATIVE ANALYSIS OF PRETREATED STRAW

Chularat Krongtaew,[a]* Kurt Messner,[b] Thomas Ters,[c] and Karin Fackler [b]

Wheat straw (*Triticum aestivum* L.) and oat straw (*Avena sativa* L.) were subjected to acid and alkaline pre-treatments partly in combination with hydrogen peroxide. The aim was to remove lignin and increase the accessibility of the polysaccharides to enzymatic digestion. Accessibility was evaluated by digestion with a cell wall degrading enzyme complex to yield reducing sugars that may serve as precursor substrates for biofuels or building block chemicals. Changes in lignin, hemicelluloses, as well as amorphous, semi-crystalline, and crystalline regions of cellulose moieties of pretreated straw were efficiently characterized by Fourier transform near-infrared (FT-NIR) reflectance spectroscopy. These alterations of the chemical structure of straw after different pre-treatment methods were powerfully differentiated by principal component analysis (PCA). Characteristics of the different samples owing to the different pre-treatment methods could be clustered from the PCA loadings spectra.

Keywords: Lignocellulose characterization; Wheat and oat straw; Mild acidic and alkaline peroxide pre-treatments; Fourier-transform near infrared spectroscopy (FT-NIR); Principal component analysis (PCA); Multivariate data analysis; Qualitative discriminant analysis

*Contact information: a: Department of Chemical Engineering, Faculty of Engineering, Mahidol University, 25/25 Puttamonthon 4 Road, Salaya, Nakhon Pathom 73170, Thailand; b: Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/166, A-1060 Vienna, Austria; c: Department of Material Sciences and Process Engineering, BOKU - University of Natural Resources and Applied Life Sciences, Peter Jordan Strasse 82, A-1190, Vienna, Austria; *Corresponding author: egchularat@mahidol.ac.th*

INTRODUCTION

The recalcitrance of the lignocellulose complex towards enzymatic and microbial digestion is the main bottleneck in biomass utilization for value added materials and fuels (Himmel et al. 2007). At present, various (physico)chemical pre-treatment methods aiming at solubilizing hemicelluloses and/or lignin or reducing cellulose crystallinity are under investigation (Chandra et al. 2007). Pre-treatment of lignocellulosic materials under mild conditions has become more attractive as less fermentation inhibitors such as furfural and hydroxymethylfurfural are produced (Curreli et al. 1997). After lignin recovery, polysaccharide-rich substrates are efficiently saccharified. Acid and alkaline pre-treatments at atmospheric pressure are therefore promising methods, owing to the simplicity and low operational cost. Acid hydrolysis of lignocellulose appears to solubil-

ise and cleave hemicelluloses to fermentable sugars (Moiser et al. 2005; Galbe and Zacchi 2007), and lignin is partly solubilized. Alkaline pre-treatment, however, acts on ester bonds between hemicelluloses and lignin. Solubilization of lignin leads to an increase of the hydrophilicity of the material (Sjöström 1991). Swelling effects in an alkaline environment may reduce the crystallinity of cellulose (Chandra et al. 2007). Additionally, it is well known that the addition of hydrogen peroxide (H_2O_2) can have beneficial effects on biomass delignifying pre-treatment, pulping, and bleaching processes (Cara et al. 2006; Khristova et al. 2006).

Complex chemical and structural alterations of the lignocellulosic material during pre-treatment are the key for a high-yield subsequent enzymatic hydrolysis. However, current standard methods to characterise the material are tedious and time consuming and are often based on wet-laboratory analyses, during which the integrity and structure of the material is destroyed. In contrast, we present here the potential of FT-NIR spectroscopy as a non-destructive method to assess substrate characteristics of interest. FT-NIR is an alternative due to its simplicity, rapidity, and sensitivity for monitoring the chemical and physical changes of lignocellulosic materials (Ghosh and Rodgers 2001; Shenk et al. 2001). Absorption bands in the NIR region of the electromagnetic spectrum are derived from the overtones and combinations of vibrations of, for example, C-H, O-H, and C=O bonds, which have their fundamental vibrations in the mid-infrared region. Often, the descriptive information included in FT-NIR spectra is better available when the spectra are further analysed by means of multivariate statistical methods (Kelley et al. 2002; Kong et al. 2005; Fackler et al. 2007). However, recently a number of additional band assignments of great importance for the qualitative analysis of lignocellulosic materials became available, thus allowing also for the characterisation of chemically pretreated lignocellulosics (Tsuchikawa and Siesler 2003a,b; Tsuchikawa et al. 2005; Mitsui et al. 2008).

In this work we present the qualitative assessment of pre-treated straw by means of FT-NIR spectroscopy. For that purpose, 133 different pretreated oat and wheat straw samples were prepared by means of mild acidic and alkaline pre-treatments in the presence and absence of H_2O_2. Standard analytical methods served as references to assign the differences observed in the spectra to physico-chemical changes during pre-treatment.

EXPERIMENTAL

Materials

Wheat straw (*Triticum aestivum* L.) and oat straw (*Avena sativa* L.) were obtained from Wirtschaftsbetriebe Herbert Rauch Höpfner, Vienna, Austria. All chemicals for pre-treatment and analytical assays were p.a. grade and purchased from Sigma-Aldrich (www.sigmaaldrich.com).

Raw material data were as follows: wheat straw contained 63% polysaccharides, 21.5% lignin, 11.4% total extractives, and 4.2% ash, while oat straw contained 51% polysaccharides, 19.6% total lignin, 20.5% total extractives, and 8.9% ash. These data were from wet-lab analyses.

Methods

Pre-treatment of straw

Wheat and oat straw were chopped to 1-cm length. 20 g of dry straw was then treated with the following conditions: acid, acid/H_2O_2, alkaline and alkaline/H_2O_2 in 200 ml solution (solid-to-liquid ratio of 1:10). Acidic (pH 2.5 to 4.5) and alkaline (pH 9 to 12) conditions were adjusted with concentrated sulphuric acid and 1 $molL^{-1}$ sodium hydroxide. The influence of hydrogen peroxide (60 to 300 $mmolL^{-1}$, i.e. 2% to 10% based on dry straw) was investigated. The pre-treatment temperature was varied from room temperature (25°C) to 90°C, and the duration was 4 h. After pre-treatment, the straw was washed with 1 L distilled water and dried at 50°C for at least 24 h before analyses.

Enzyme hydrolysis of treated straw samples

Crude Viscozyme L (www.novozymes.com) was purified to eliminate sugars and salts with an Econo-Pac 10DG column (www.bio-rad.com), using water as eluent. After drying at 50°C for at least 24 h, 100 mg of treated straw was hydrolysed in the mixture of 1 mL purified Viscozyme L and 9 mL of 30 $mmolL^{-1}$ sodium-acetate buffer pH 4.0. The enzymatic hydrolysis was done at 40°C for 48 h in a shaking water bath. Afterwards, the mixture was centrifuged, and the supernatant analysed for reducing sugar content.

Wet laboratory analyses

The weight loss of pretreated straw was determined after drying at 50°C for 24 h. This relatively low temperature was chosen to avoid chemical alterations during drying that might have influenced the NIR spectra. The amount of reducing sugars in the supernatant after enzymatic hydrolysis was determined by the dinitrosalicylic acid (DNS) method (Miller 1959). Reducing sugar yield (mg sugar g^{-1} pretreated straw) was calculated based on the glucose equivalent from the calibration curve. Released pentoses mainly from arabinoglucuronoxylan thus were overestimated (f = 1.2). Dry straw sample was then milled to 80-µm particles using an ultracentrifugal mill (Retsch ZM 1000, Germany) for analyses of extractives, Klason lignin, acid-soluble lignin, and FT-NIR spectroscopy. For extractive determination, 3.5 g milled sample was sequentially extracted using 1) cyclohexane-ethanol (2:1 v/v) for 6 h, 2) ethanol (95% v/v) for 1 h, and 3) water for 24 h according to TAPPI T 264 om-88. Klason lignin was determined after acid hydrolysis (72% H_2SO_4, 20°C, 2 h and 3% H_2SO_4, 100°C, 4 h) (Schwanniger and Hinterstoisser 2002). Acid-soluble lignin in 3% H_2SO_4 supernatant was measured by spectrophotometry at 205 nm according to TAPPI T250 and (Schöning and Johansson 1965). Total lignin content refers to the sum of Klason and acid-soluble lignin content based on extractives-free straw. Ash content was the residue after ignition of a known dry weight sample at 550±50°C for 2 h and the disappearance of so-called volatile solids. Total polysaccharide content is calculated by subtraction of weight loss, total lignin, extractives, and ash contents from 100% untreated straw.

Fourier transform near infrared spectroscopy and multivariate analyses

NIR spectroscopy of milled samples (80 µm particles) was carried out in terms of apparent absorbance, log [1/Reflectance], by Fourier transform near-infrared (FT-NIR) spectrometer using a fibre optic probe (Equinox 55, Bruker Optics Inc., Germany).

Spectroscopy was performed at 8 cm^{-1} resolution in the wavenumber range from 10000 to 4000 cm^{-1} and ratioed against a Spectralon reference spectrum. To increase signal to noise ratio, 100 scans were averaged for each FT-NIR spectrum (Chen et al. 1986). The average spectra of four replicates were calculated and used for subsequent mathematical treatment performed by OPUS software, version 6.0 (Bruker, Germany). Second derivative spectra (21 smoothing points, 2nd order polynomial fit) were calculated after unit vector normalization with the Savitzky and Golay algorithm (1964).

Qualitative discriminant analysis was conducted by the software OPUS IDENT. The algorithm chosen in this study was factorization, which represents spectra as linear combinations of so-called factor spectra or loadings as shown in Equation (1). This algorithm is based on principal component analysis (PCA) (www.brukeroptics.com). The eigenvector *a* was computed from the spectrum *a* and the factor spectra *f*. *T* indicates the coefficients (scores) required to reconstruct the original *a* spectrum.

$$a = T_{1a} \cdot f_1 + T_{2a} \cdot f_2 + T_{3a} \cdot f_3 + \ldots \quad (1)$$

Prior to cluster analysis, 164 spectra of untreated wheat straw (4 spectra) and alkaline treated wheat straw (20 spectra), alkaline/H_2O_2 (68 spectra), acid (4 spectra), and acid/H_2O_2 (68 spectra) pre-treatments were pre-processed between 7200 and 5500 cm^{-1} by vector normalization and sequential differentiation to second derivative (21 smoothing points). The factor spectra from factorization algorithm were calculated, and the proper number of factor spectra was chosen from the calculated eigenvector and separation. The threshold was determined from the fixed algorithm using the default value 0.25. Plots of principal component scores and PC loading vectors were used to examine not only the possibility for grouping of samples but also the influential variables on clustering.

RESULTS AND DISCUSSION

Acid and Alkaline Pre-Treatment and Influence of Hydrogen Peroxide

During acid pre-treatment of wheat and oat straw, a small amount of lignin moieties is solubilized. Only 6.1% and 10.9% lignin removal (20.2% and 17.4% total residual lignin) at 10.5% and 13.5% weight loss were obtained for wheat and oat straw respectively (Tables 1 and 2). The dilute acid pre-treatment acts on outlying layers of non-crystalline cell wall components; therefore, mainly hemicelluloses are hydrolysed to soluble mono- and oligomers, while cellulose remains widely intact. During this process, the digestibility of cellulose increases (Kumar et al. 2009), as indicated by hydrolysable reducing sugar yield in this study.

Reducing sugar yields after enzyme hydrolysis were considerably lower from straw treated by acid and acid/H_2O_2 at ambient temperature than those treated by alkaline and alkaline/H_2O_2 at elevated temperature for both wheat and oat straw. Small amounts of reducing sugars were released from solid straw after acid pre-treatment at room temperature, suggesting that polysaccharides were only slightly attacked. Similar results have been observed when wheat straw was treated at dilute sulphuric acid at 34°C (González et al. 1986).

Table 1. Native and Treated Wheat Straw Composition

Chemical composition	Total poly-saccharides (% w/w)	Total lignin (% w/w)	Reducing sugars (mg g^{-1} straw)	Extractives (% w/w)	Ash (% w/w)	Volatile solids (% w/w)	Weight loss (%)
Native wheat straw	62.9 (0.7)	21.5 (0.0)	128.9 (1.1)	11.4 (0.5)	4.2 (0.2)	95.8 (0.2)	0%
[1]Acid pretreat-ment	67.3 (0.4)	20.2 (0.0)	172.0 (2.4)	9.2 (0.1)	3.3 (0.3)	96.7 (0.3)	10.5%
[2]Acid/H_2O_2 pretreatment	70.4 (0.4)	18.4 (0.4)	44.7 (0.5)	8.2 (0.0)	3.1 (0.0)	97.0 (0.0)	11.5%
[3]Alkali pretreatment	74.0 (0.6)	15.1 (0.3)	247.8 (2.8)	8.6 (0.3)	2.3 (0.0)	97.7 (0.0)	15.0%
[4]Alkali/H_2O_2 pretreatment	74.0 (1.5)	13.3 (0.9)	294.8 (0.9)	10.8 (0.6)	2.0 (0.0)	98.0 (0.0)	23.5%

Note: The values in parentheses are the standard deviations.
[1] Dilute-acid pretreatment at pH 3, 25°C for 4 h
[2] Dilute-acid/H_2O_2 pretreatment at pH 3, 10.2% w/w H_2O_2, 25°C, 4 h
[3] Alkali pretreatment at pH 11.5, 90°C, 4 h
[4] Alkali/H_2O_2 pretreatment at pH 11.5, 10.2% w/w H_2O_2, 90°C, 4 h

Table 2. Native and Treated Oat Straw Composition

Chemical composition	Total polysac-charides (% w/w)	Total lignin (% w/w)	Reducing sugars (mg g^{-1} straw)	Extractives (% w/w)	Ash (% w/w)	Volatile solids (% w/w)	Weight loss (%)
Native oat straw	51.0 (0.2)	19.6 (0.0)	153.5 (11.3)	20.5 (0.1)	8.9 (0.1)	91.1 (0.1)	0%
[1]Acid pretreatment	71.5 (0.1)	17.4 (0.0)	128.9 (5.0)	8.2 (0.1)	2.8 (0.0)	97.2 (0.0)	13.5%
[2]Acid/H_2O_2 pretreatment	71.6 (0.2)	17.4 (0.1)	141.0 (1.4)	8.2 (0.1)	2.8 (0.0)	97.2 (0.0)	17.5%
[3]Alkali pretreatment	74.0 (1.6)	13.2 (0.1)	364.6 (24.4)	10.5 (1.5)	2.4 (0.0)	97.6 (0.0)	21.5%
[4]Alkali/H_2O_2 pretreatment	86.0 (0.7)	5.4 (0.3)	467.8 (4.4)	7.1 (0.4)	1.5 (0.0)	98.5 (0.0)	37.0%

Note: The values in parentheses are the standard deviations
[1] Dilute-acid pretreatment at pH 4.5, 25°C for 4 h
[2] Dilute-acid/H_2O_2 pretreatment at pH 4.5, 6.8% w/w H_2O_2, 25°C, 4 h
[3] Alkali pretreatment at pH 11.5, 90°C, 4 h
[4] Alkali/H_2O_2 pretreatment at pH 11.5, 6.8% w/w H_2O_2, 90°C, 4 h

Alkaline pre-treatment at 90° C gave a better effect on lignin degradation and carbohydrate susceptibility compared to those from acid pre-treatment. Relatively high lignin removals of alkaline treated wheat and oat straw were obtained: 29.5% and 32.6% (15.1% and 13.2% total residual lignin) at 15.0% and 21.5% weight loss. The amounts of enzymatically released reducing sugar were 248 and 365 mg g^{-1} pretreated straw, respectively (approx. 40% and 72% theoretical sugar yield based on total polysaccharides in untreated straw), as shown in Tables 1 and 2. During alkaline pre-treatment lignin is extracted mainly after saponification of lignin-carbohydrate ester bonds between ferulic acid residues from lignin and arabinose sidechains of xylan. Acetyl groups from hemicellulose (predominantly from O-acetyl-glucuronoarabinoxylan) are also hydrolyzed. Delignification is desired because lignin provides non-productive adsorption sites for hydrolytic enzymes and thus may retard enzymatic hydrolysis (Sjöström 1991; Kumar 2009). Hemicelluloses, after that, are released into liquid during pre-treatment. In

addition, alkali increases the fibre swelling, produces a more open structure of plant cell wall, and makes cellulose more accessible for cellulase enzymes (Sjöström 1991). Consequently, the enzymatic hydrolysis of alkaline treated lignocellulosic material was improved.

The addition of H_2O_2 for alkaline pre-treatment at elevated temperature enhanced the delignification efficiency and hydrolysis yield in terms of reducing sugars, as shown in Tables 1 and 2. The amount of reducing sugar after enzymatic hydrolysis was increased from 248 to 295 mg g^{-1} (39.3 to 46.8% theoretical yield) and from 365 to 468 mg g^{-1} (71.5 to 91.7% theoretical yield) after adding H_2O_2 for the alkaline pre-treatment of wheat and oat straw, respectively. Total residual lignin contents in wheat and oat straw were 13.3% and 5.4% (37.9 and 72.3% lignin removal) after alkaline/H_2O_2 pre-treatment. These results also showed that oat straw was apparently easier to degrade than wheat straw.

Highly active radical species generated from hydrogen peroxide are well known to play a role in oxidizing aromatic lignin compounds of plant cell walls in nature and during alkaline pulp bleaching (Joseleau et al. 1994; Goodell et al. 1997). Transition metal ions in native straw are believed to participate in hydroxyl radical (·OH) generation in the presence of H_2O_2 via Fenton chemistry. Although formed at different rates, the hydroperoxy anion (HOO^-) and hydroxyl radical (·OH) are the active species present in both acid and alkaline conditions in the presence of H_2O_2 (Hobbs and Abbot 1994). In an alkaline environment, the proposed reaction mechanisms are 1) dissociation of H_2O_2 to hydroperoxy anion (HOO^-) and 2) the reaction of hydroperoxy anion with hydrogen peroxide led to generate hydroxyl radical (HO·) (Fang et al. 1999).

In contrast to the alkaline pre-treatment, no beneficial effect of H_2O_2 in an acid environment was observed on both delignification efficiency and enzyme accessibility of straw. Only a small amount of lignin was solubilized, and reducing sugar yields were lower even compared to untreated straw (Tables 1 and 2), although an oxidative Fenton-based reaction in acidic environment has been proposed to take place during fungal wood decay in nature (Goodell et al. 1997). One possible reason was presumably the deformation of the carbohydrate matrix by hydroxyl radicals generated via Fenton chemistry in acidic condition, which caused changes in the cellulose network and led to suppression or inhibition of cellulolytic enzyme activities (Joseleau et al. 1994).

The sum of hydrophilic and lipophilic extractives, so-called total extractives, was found to decrease after all delignifying pre-treatments in both acid and alkaline conditions relative to untreated straw; however no correlation (R=0.2) between the reduction of extractives and the residual lignin content was observed (data not shown). Oat straw contains more extractives (20.5% w/w) than wheat straw (11.4% w/w). All pre-treatments resulted in a lower amount of ash and more volatile solids, indicating the extraction of minerals. Weight loss was found to increase especially when H_2O_2 was added for both acid and alkaline pre-treatment, owing to the more severe attack by oxidants. The increase in weight loss could be attributed to losses of salts, extractives, and matrix substances (lignin and carbohydrates).

Characterization of Chemical Structures of Delignified Straw by FT-NIR

To accentuate differences between FT-NIR spectra and facilitate qualitative interpretation, all spectra were plotted in second derivative mode. This kind of data pre-processing has no influence on the position of the original bands. What has to be considered, however, is that absorbance maxima of reflectance spectra appear as amplitude minima in this mode.

Lignin

Lignin not only influences the accessibility of polysaccharides to hydrolytic enzymes but also may lead to non-productive binding between lignocellulosic substrates and cellulase enzymes (Kumar et al. 2009). Several bands in NIR are characteristic for lignin structures (Table 3). The most prominent lignin band is the first overtone of the C-H stretching vibration of aromatics near 5980 cm^{-1}. In native wheat straw, this band overlaps with the first overtone of C-H bonds of acetyl groups covalently bound to xylan (5990 cm^{-1} and 5955 cm^{-1}), resulting in a local maximum at 5980 cm^{-1}. The lignin band decreased after alkaline and alkaline/H_2O_2 pre-treatments, respectively, relative to untreated straw (Fig. 1). This was mainly because of the removal of lignin from the substrate or the oxidative cleavage of C-H bonds due to initial electrophilic reaction by reactive oxygen species, i.e. HO·, HOO·, and nucleophilic attack by peroxide anions, hydroperoxide ions, and hydronium ions in acidic media in the presence of H_2O_2 either to attack phenolic hydroxyl groups and phenolate ions of lignin moieties or to open the aromatic ring, the main structure of lignin subunits (Gellerstedt 2007).

Figure 2 shows 2nd derivative spectra of wheat and oat straw samples that had been subjected to alkaline peroxide pre-treatments at different severities and thus showing different residual lignin contents. The delignification during the pre-treatment is clearly visible by the changes of the local amplitude minimum, which were much less pronounced or even disappeared when the lignin content of the pre-treated straw was low.

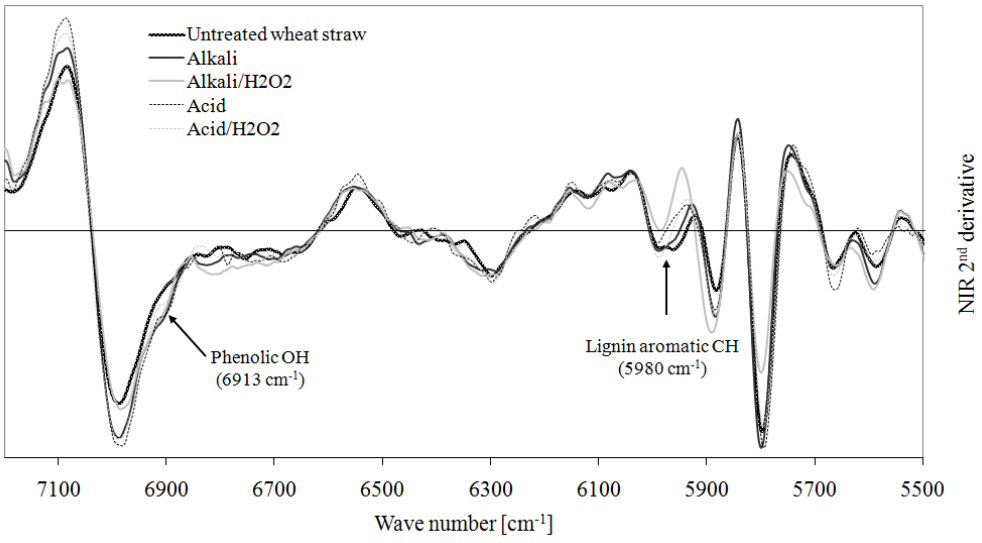

Fig. 1. Second derivatives of the NIR spectra (7200 to 5500 cm^{-1}) of untreated wheat straw and wheat straw pre-treated by acid, acid/H_2O_2, alkali and alkali/H_2O_2

Table 3. Wavenumbers NIR Bands and their Assignments

Wavenumber	Type	Structure	References
Lignin:			
6913	O-H stretch 1st overtone	Phenolic hydroxyl group	Mitsui et al. 2008
6916	2xCH-stretch + C-H deformation	Aromatic	
5980	C-H stretch 1st overtone	Aromatic	Shenk et al. 2001
5800*	C-H stretch 1st overtone	CH_2	Shenk et al. 2001
Polysaccharides:			
7100-6200	O-H stretch 1st overtone	Alcoholic	Shenk et al. 2001
7000	O-H stretch 1st overtone	Amorphous Polysaccharides Free OH groups or weakly H-bonded OH	Tsuchikawa and Siesler 2003a,b; Watanabe et al. 2006; Mitsui et al. 2008
6970 - 6800		OH groups with H-bonds of intermediate strength	Watanabe et al. 2006
6757	O-H stretch 1st overtone	Crystalline cellulose	Yonenobu et al. 2009
~ 6722	O-H stretch 1st overtone	Semi-crystalline cellulose	Tsuchikawa and Siesler 2003a, b
6622		O6-H6···O3″ interchain H-bonds	Watanabe et al. 2006
6464		O3-H3···O5″ intrachain H-bonds	Watanabe et al. 2006
6460	O-H stretch 1st overtone	Crystalline cellulose C_I	Tsuchikawa and Siesler 2003a, b
6281	O-H stretch 1st overtone	Crystalline cellulose C_{II}	Tsuchikawa and Siesler 2003a, b
6286		H-bonds in the cellulose I_β-allomorph	Watanabe et al. 2006
5990 and 5960**	C-H stretch 1st overtone	CH_3, acetyl	Fackler et al. 2007b
5800	C-H stretch 1st overtone	CH, furanose or pyranose due to hemicellulose, furanose	Tsuchikawa et al. 2005; Mitsui et al. 2008
5618	C-H stretch + H-O-H deformation combination	Cellulose + Water	Shenk et al. 2001
5208***	O-H stretch, OH-bend	Polysaccharides	Shenk et al. 2001
~ 4785	O-H combination	Polysaccharides	Shenk et al. 2001
4405	O-H stretch/ C-O stretch combination	Polysaccharides	Shenk et al. 2001
4295	C-H stretch/ C-H deformation comb.	Polysaccharides	Shenk et al. 2001

* Overlaps with xylan band, ** Second derivative, and *** Overlaps with H_2O

Another characteristic lignin band is located near 6913 cm^{-1}; this is assigned to phenolic hydroxyl group originated from lignin (Mitsui et al. 2008). Although this band overlaps with the O-H stretch 1st overtone of amorphous polysaccharides near 7000 cm^{-1}, a shoulder at alkaline and alkaline peroxide pretreated samples is clearly visible (Fig. 1). This appearance indicates that pre-treatment led to an increase of phenolic hydroxyl groups, supposedly caused by either degradation of β-O-4 ether linkages between lignin units or hydrolysis of methoxyl groups (Nada et al. 1998). It could be postulated that an increase of phenolic hydrolxyl groups of lignin was likely due to the nucleophilic attack by hydroxide ions (HO^-) from alkaline pre-treatment or hydroperoxide (HOO^-) ions from alkaline peroxide pre-treatment. However, this shoulder was not visible any more in highly delignified samples with lignin contents lower than 10%.

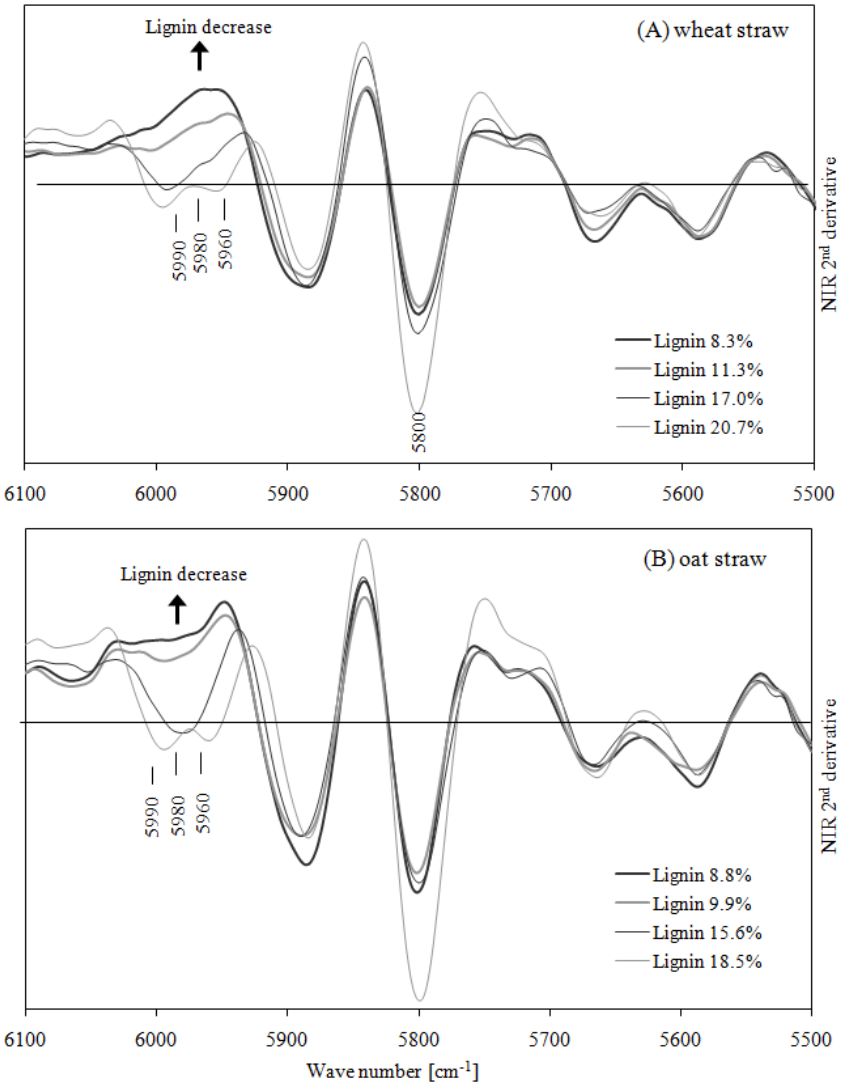

Fig. 2. Second derivatives of the NIR spectra of native and delignified wheat straw (A) and oat straw (B) samples respectively in the spectral region assigned to carbohydrate and lignin moieties (6100 to 5500 cm^{-1}).

Reducing sugars, hemicelluloses and cellulose

The polysaccharides composition influences not only the enzymatic hydrolysis of the pretreated material; in addition, the composition of sugar monomers yielded has a great impact on further process steps (e.g. alcohol fermentation). Arabinoglucuronoxylan is the main hemicellulose found in wheat straw, while galactoglucomannan is observed only in smaller amounts, along with rhamnose, which is also present as a minor sugar component (Sun et al. 1998).

Characteristic bands of polysaccharides are found among the 1st overtones of the O-H stretching vibration between 7100 and 6100 cm^{-1} and among the C-H combination vibrations as well as the 2nd overtone of the CH_2 bending vibration near 4250 cm^{-1}. Characteristic xylan bands are due to the 1st overtone C-H stretching vibration of acetyl

groups. Pyranoses and furanoses of hemicelluloses strongly absorb near 5800 cm^{-1} (Table 3).

The alkaline and alkaline/H_2O_2 pre-treatment led to a relative increase of the polysaccharides content, which is visible at the minima near 6300, 5208, 4813, 4405, and 4295 cm^{-1} attributed to the CH combination and the CH 1st overtone of saccharides (Ghosh and Rodgers 2001). The amplitude minimum at 5800 cm^{-1}, however, indicates that hemicelluloses (xylan) were reduced, particularly after alkaline/H_2O_2 pre-treatment (Fig. 1). Furthermore, acidic, and alkaline peroxide pre-treatments led to a reduction of the acetyl group content near 5990 and 5960 cm^{-1} (Shenk et al. 2001; Fackler et al. 2007). After more severe alkaline/H_2O_2 pre-treatment, these two peaks disappeared completely due to the extraction, degradation, or deacetylation of xylan, as shown in Fig. 2.

Figure 1 shows the largest loss of amorphous polysaccharides by alkaline/H_2O_2 pre-treatment near 7000 cm^{-1}, a band due to the O-H stretching 1st overtone of amorphous polysaccharides (Tsuchikawa and Siesler 2003a,b; Mitsui et al. 2008). In contrast, other pre-treatments gave higher contents of amorphous polysaccharides relative to all other components. Pre-treatment in alkaline media resulted in a reduction of the minimum near 6280 cm^{-1} attributed to crystalline cellulose C_{II} (Tsuchikawa and Siesler 2003a,b) or H-bonds in the β-allomorph phase of cellulose II (Watanabe et al. 2006). Minima decrease of the band assigned to cellulose intrachain bonds (Watanabe et al. 2006) or crystalline cellulose C_I between 6450 and 6500 cm^{-1} (Tsuchikawa and Siesler 2003a,b) was also found. Semi-crystalline regions of cellulose shown as an amplitude minimum at 6722 cm^{-1} (Tsuchikawa and Siesler 2003a,b) and intermediately strong H-bonded OH groups (Watanabe et al. 2006) did not show a corresponding increase after alkaline/H_2O_2 pre-treatment. Acid and acid/H_2O_2 pre-treatments showed less modification in all amorphous, semi-crystalline, and crystalline polysaccharides.

Figure 3 exemplifies the NIR spectra ranging from 7200 to 6000 cm^{-1} containing the absorption bands of OH groups (1st overtone of OH stretching vibration) of intra-molecular bonds in cellulose of treated straw by alkaline/H_2O_2 at different hydrogen peroxide concentrations. The band absorption ranges of 7200-6950, 6720±20, 6480±20, and 6290±20 cm^{-1} corresponded to amorphous (Am), semi-crystalline (Sc) and crystalline cellulose (C_I and C_{II}) regions respectively (Tsuchikawa and Siesler 2003a, Yanenobu et al. 2009).

It was found that the amorphous region (Am) was apparently degraded to a higher extent when hydrogen peroxide concentration was increased. The reason was supposedly related to extraction or degradation by higher concentration of hydroxyl radicals (HO·) or the hydroperoxide anion (HOO^-). The reduction of C_I and C_{II} bands could not be observed clearly at this degree of delignification. Xylan extraction and amplitude minima only slightly decreased. However, the increase of semi-crystalline cellulose or intermediately strong H-bonded OH groups is apparent in these spectra. This finding suggests the conversion of the crystalline cellulose regions to semi-crystalline ones. Moreover, the crystalline region near 6460 cm^{-1} (C_I) was seemingly more reactive than the crystalline region at 6281 cm^{-1} (C_{II}). Comparable results have been obtained during acetylation of hydroxyl groups of cellulose, where the crystalline structure near to 6460 cm^{-1} was more reactive than that near 6280 cm^{-1} (Mitsui et al. 2008).

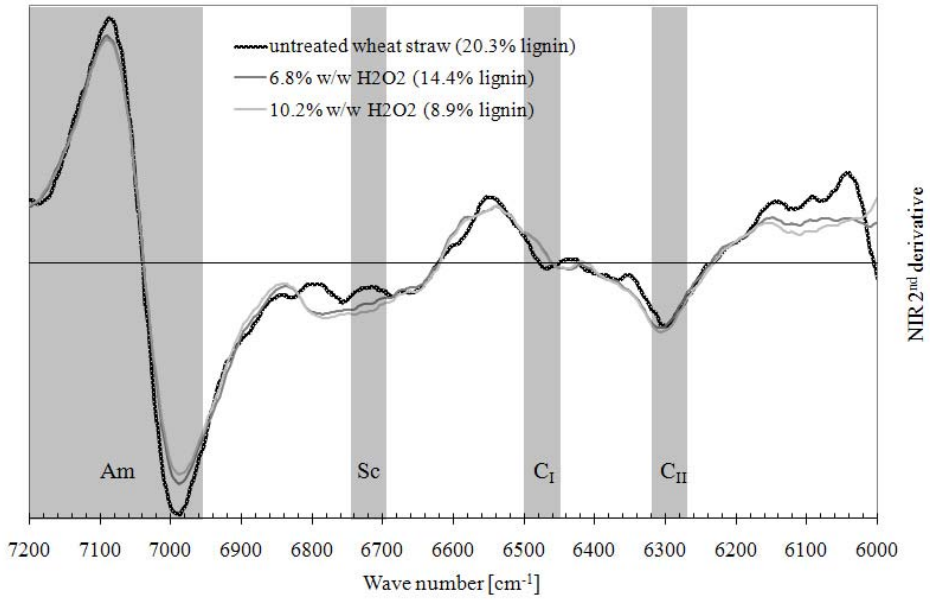

Fig. 3. Second derivatives of the NIR spectra of alkali/H_2O_2 (pH 11.5, 90°C, 4h) delignified wheat straw samples and untreated wheat straw in the spectral region between 7200 and 6000 cm^{-1}, assigned to the polysaccharides absorption including amorphous polysaccharides (Am), semi-crystalline cellulose (Sc), and crystalline cellulose (C_I and C_{II}).

Water adsorption

The second derivative NIR spectra showed a broad peak near 5208 to 5219 cm^{-1} (Fig. 4) and 5592 cm^{-1} (Fig. 1) allocated to the O-H stretching and O-H bending of water

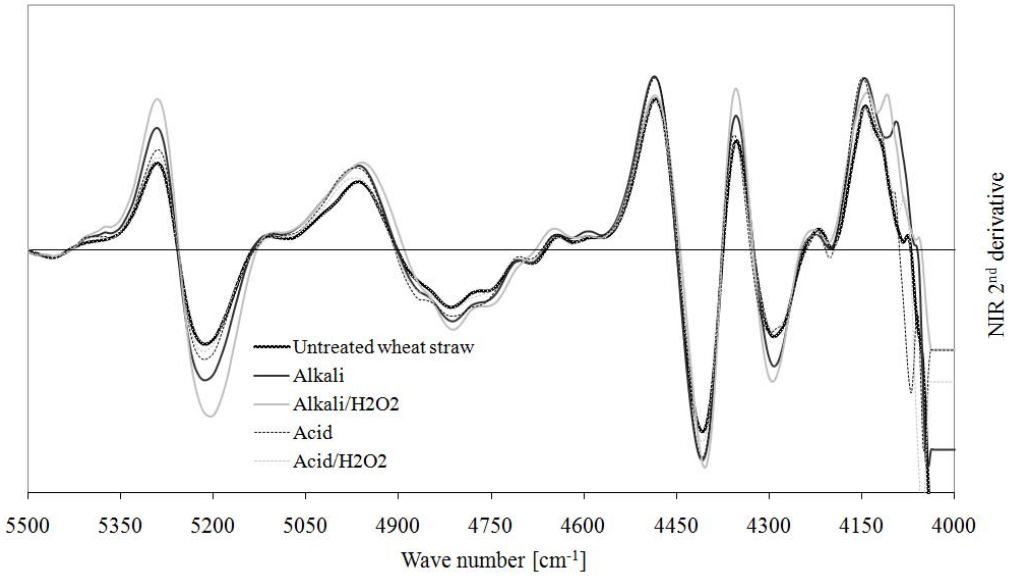

Fig. 4. Second derivatives of the NIR spectra (5500 to 4000 cm^{-1}) of untreated wheat straw and wheat straw pretreated by acid, acid/H_2O_2, alkali, or alkali/H_2O_2.

(Mitsui et al. 2008). Although samples had been dried for several days before spectra were recorded, from the NIR absorption, it was found that the molecular water adsorption of straw was enhanced after all pre-treatments shown here, particularly after those carried out in alkaline media. This may be caused by the increased hydrophilicity due to delignification, cleavage of acetyl groups, to some extent to the cellulose swelling effect, or even to the low drying temperature. Higher affinity to water appears to be beneficial for enzymatic hydrolysis or simultaneous saccharification and fermentation.

PCA Loadings and Qualitative Discriminant Analysis

The identification setup of NIR spectral data after vector-normalization and differentiation to second derivative (21 smoothing points) was performed for the spectral region ranging from 5500 to 7200 cm^{-1}. This spectral region contains qualitative information on crucial substrate parameters such as lignin and xylan content, degree of acetylation of the hemicelluloses, and polysaccharides crystallinity, as described above (Table 3). By means of the factorization algorithm also known as principal component analysis (PCA), the multivariate spectral data space (164 samples 212 variables i.e. data points of the FT-NIR spectra), in which variables are often correlated to each other, was reduced to only four but orthogonal latent factors - the principal components (PCs). The contribution of each of the original variables to each factor is plotted in the loadings spectra (Fig. 5). The contribution of each factor to each spectrum can be plotted in scores plots (Equation 1 and Fig. 6).

Figure 5 shows the PC loadings for the first, second, third, and forth principal components. They contain the descriptive information of chemical differences that are responsible for clustering by means of PCA. The first PC (Fig. 5 (PC1)) does not explain a significant distinction among all groups as it resembles to mean spectrum of all samples after vector normalized and differentiation to second derivative. Therefore the second, third and forth principal components were here considered in more detail. The characteristics of PC2, PC3 and PC4 illustrated in Fig. 5 were distinct compared to PC1. Band assignments of the significant loading vectors which gave high influence on PCA are described in Table 3.

As shown in Fig. 5, the loading spectrum of PC2 was most significant between 6000 and 5700 cm^{-1}. The highest absolute values appeared near 5800 cm^{-1} attributed to overlapping bands of CH_2 structure of lignin and CH stretch 1^{st} overtone of pyranoses and furanoses of hemicelluloses (Table 3). The second highest value was found near 5960 cm^{-1} assigned to acetyl group of xylan (CH_3). A broad shoulder at 5980 cm^{-1} indicates also a large contribution of lignin. Differences in the spectral region near 5800 and 5960 cm^{-1}, influenced all principal components discussed here (PC2, PC3 and PC4). Other influential band allocations for PC2, PC3 and PC4 shown in Fig. 5 were near 6280, 6800 to 6900 and ~7000 cm^{-1}, the spectral region of OH groups with H-bonds of intermediate strength of crystalline, semi-crystalline, and amorphous cellulose bands. Consequently, samples are clustered according not only to different contents of lignin, hemicelluloses, and cellulose but also to differences in cellulose crystallinity. PC2 is most important for the qualitative discriminant analysis, as it includes the highest proportion of variation within the data set, whereas the absolute contributions of PC3 and particularly PC4 were much lower.

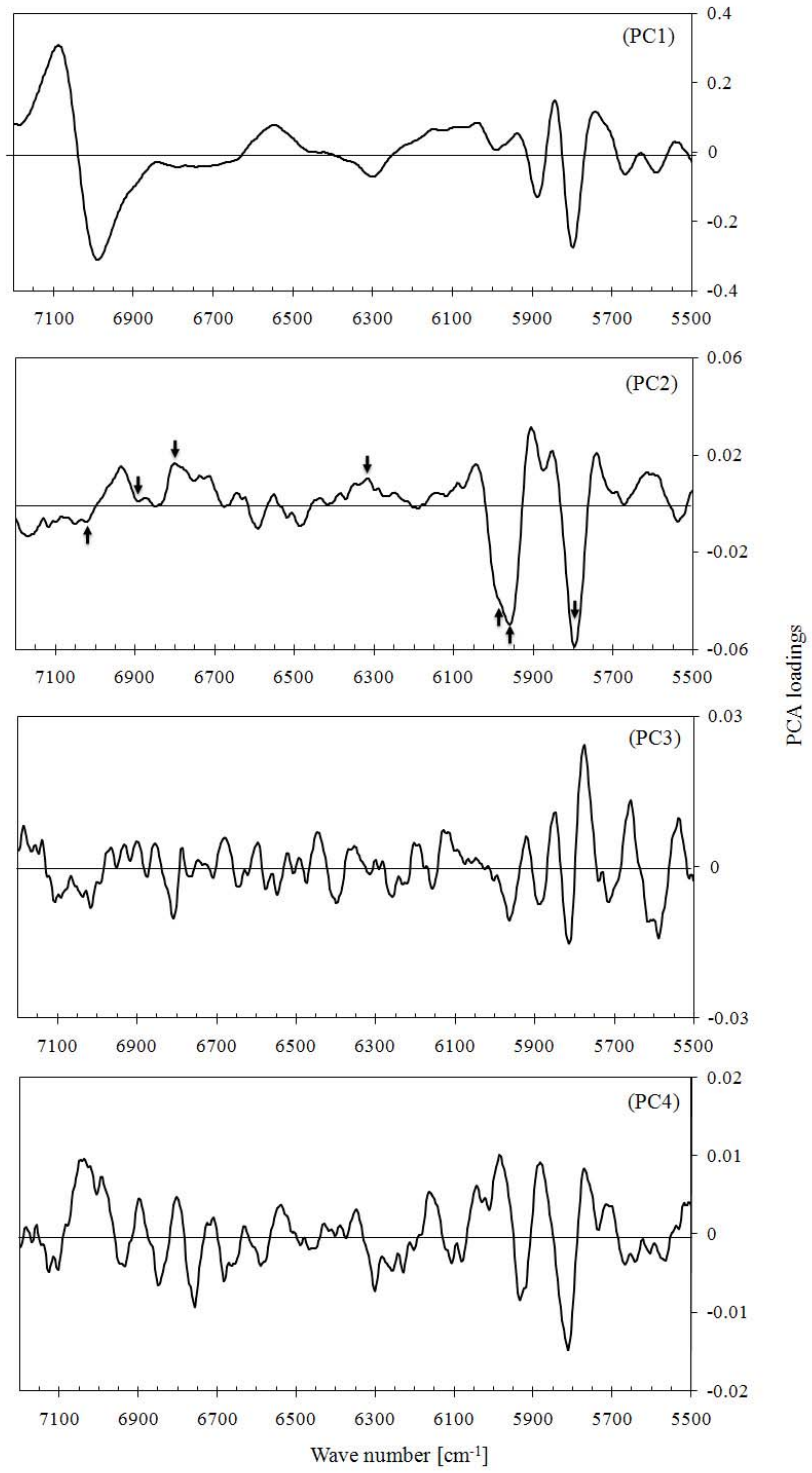

Fig. 5. PCA loading vectors of the FT-NIR spectra of untreated and acid, acid/H_2O_2, alkali, alkali/H_2O_2 pretreated wheat straw: PC1, PC2, PC3 and PC4

For example, samples scoring positively on PC2 (Fig. 6) – i.e. the acidic treated and untreated samples – were higher in lignin and hemicelluloses (Fig. 5 (PC2)) than the average of all samples, because the loading bands derived from these compounds – like in the second derivative spectra – minima in the loadings spectra. Bands near 6913, 6800 and 6280 cm^{-1}, however, show maxima in the loadings spectra, indicating that the same samples show lower contents of cellulose and less phenolic hydroxyl or free phenolic groups on the lignin moieties. Samples scoring negatively on PC2 (Fig. 6) – i.e. the alkaline treated straw – thus have lower lignin and hemicelluloses contents and consequently relatively higher cellulose and phenol contents.

The minima (~7000, 5960, 5800 cm^{-1}) of PC3 indicate a further separation according to the hemicellulose content. The band near 5600 cm^{-1} could refer to a higher hydrophilicity of the positively scoring samples (Table 3). Acid and Acid/H_2O_2 pre-treatment, with samples scoring similarly on PC2 are separated on PC3 (Fig. 6), because of the lower hemicellulose content after acid pre-treatment compared to acid/H_2O_2 pre-treatment.

Finally, in PC4 the small spectral differences between untreated straw samples and acid/H_2O_2 pretreated ones are expressed: the latter show slightly lower lignin contents indicated by their positive score on PC4 (Fig. 6) and the maximum of the loading vector near 5980 cm^{-1} (Fig. 5 (PC4)). The cellulose content of these samples appeared to be higher indicated by rather noisy minima of the loading vector near 6757 cm^{-1} (Yanenobu et al. 2009), 6598 cm^{-1} (Mitsui et al. 2008), 6480, and 6280 cm^{-1}. The maximum near 7000 cm^{-1} attributed to amorphous polysaccharides indicates the slightly lower hemicellulose content of the acid/H_2O_2 pretreated samples compared to the untreated ones. A small minimum near 6846 cm^{-1} attributed to symmetrical N-H stretch 1st overtone near which could be due to the presence of protein (Shenk et al. 2001).

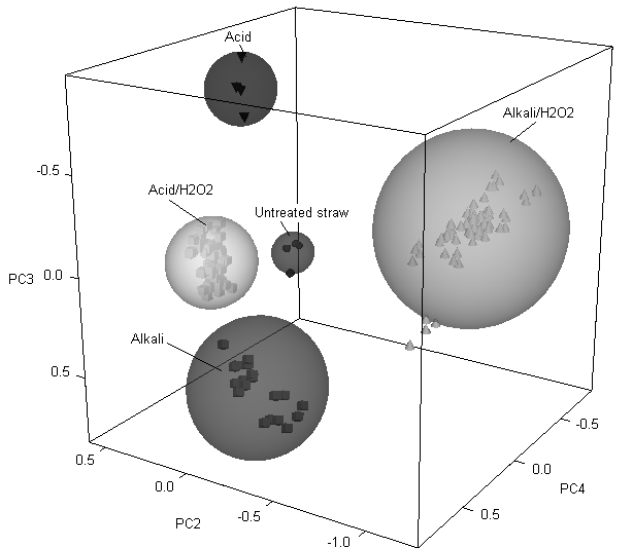

Fig. 6. Clustering of untreated wheat straw samples (●; 4 samples) and samples treated with acid (▼; 4 samples), alkali (◆; 20 samples), acid/H_2O_2 (■; 68 samples), and alkali/H_2O_2 (▲; 68 samples). The spheres indicate the supreme spectral distances from the average spectra of straw samples from each cluster represented as the coordinate origins.

Figure 6 shows the three-dimensional projection of PC2, PC3, and PC4. Each point in the plot represents one spectrum of the identity test using a factorization algorithm with two factors (OPUS software 6.0, Germany). PC2 influenced the separation of all clusters that represent the different pre-treatment methods mostly. The spectra from untreated straw, straw treated by acid, alkaline, acid/H_2O_2, and alkaline/H_2O_2 pre-treatments were efficiently discriminated from each other and no overlapping area was found. However, four samples from alkaline/H_2O_2 pre-treatment were outliners from the cluster sphere. These four samples were treated by the same condition (6% w/w H_2O_2 at pH 11.5, 90°C for 4 h) and contained a moderate amount of hydrolysable reducing sugar (445 mg g^{-1}) but rather low residual lignin content (8.7%) compared to other samples containing the same amount of reducing sugar.

CONCLUSIONS

Near-infrared spectroscopy combined with multivariate data analyses has potential as an analytical tool for qualitatively characterizing straw samples after different pre-treatments. Key properties influencing the enzymatic hydrolysis yield and rates, such as lignin content, hemicellulose content, and cellulose crystallinity, which are primarily influenced by pre-treatment processes, are well resolved in FT-NIR spectra. These differences can be evaluated not only from characteristic NIR absorption bands of the material, but can also be discriminated and explained using principal component analysis. This qualitative discriminant analysis can be used as a potential tool to evaluate a large number of samples simultaneously and systematically for their structural and chemical alterations after different pre-treatments, which offers a great advantage for further assessment of biomass properties.

Thus, FT-NIR spectroscopy is a powerful tool to assess biomass digestibility, with a potential to be used for process control for biomass utilization and biomass-to-energy conversion. However, for that purpose additional quantitative analyses are required.

ACKNOWLEDGMENTS

The authors are grateful to the financial support from the Austrian Research Promotion Agency (FFG project number 819549/14233SCK/SAI). K.F. acknowledges the support of FWF the Austrian Science Fund (FWF project number V117-N17).

REFERENCES CITED

Cara, C., Ruiz, E., Ballesteros, I., Negro, M. J., and Castro, E. (2006). "Enhanced enzymatic hydrolysis of olive tree wood by steam explosion and alkaline peroxide delignification," *Process Biochemistry* 41(2), 423-429.

Chandra, R. P., Bura, R., Mabee, W., Pan, X., Berlin, A., and Saddler, J. N. (2007). "Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics?" *Adv. Biochem. Eng./Biotechnol*. 108, 67-93.

Chen, L., Cottrell, C. E., and Marshell, A. G. (1986). "Effect of signal-to-noise ratio and number of data points upon precision in measurement of peak amplitude, position and width in Fourier transform spectrometry," *Chemom Intell Lab Syst* 1(1), 51-58.

Curreli, N., Fadda, M. B., Rescigno, A., Rinaldi, A. C., Soddu, G., Sollai, F., Vaccargiu, S., Sanjust, E., and Rinaldi, A. (1997). "Mild alkaline/oxidative pretreatment of wheat straw," *Process Biochemistry* 32(8), 665-670.

Fackler, K., Schmutzer, M., Manoch, L., Schwanninger, M., Hinterstoisser, B., Ters, T., Messner, K., and Gradinger, C. (2007a). "Evaluation of the selectivity of white rot isolates using near infrared spectroscopic techniques," *Enzyme and Microbial Technology* 41, 881-887.

Fackler, K., Schwanninger, M., Gradinger, C., Hinterstoisser, B., and Messner, K. (2007b). "Qualitative and quantitative changes of beech wood degraded by wood-rotting basidiomycetes monitored by Fourier transform infrared spectroscopic methods and multivariate data analysis," *FEMS Microbiol. Lett.* 271(2), 162-169.

Fang, J. M., Sun, R. C., Salisbury, D., Fowler, P., and Tomkinson, J. (1999). "Comparative study of hemicelluloses from wheat straw by alkali and hydrogen peroxide extractions," *Polymer Degradation and Stability* 66, 423-432.

Galbe, M., and Zacchi, G. (2007). "Pretreatment of lignocellulosic materials for efficient bioethanol production," *Advances in Biochemical Engineering/Biotechnology* 108, 41-65.

Gellerstedt, G. (2007). "Chemistry of bleaching of chemical pulp," In: Gellerstedt, G. (ed.), *Fibre and Polymer Technology*, Ljungberg Textbook, Stockholm, KTH, Book 2.

Ghosh, S., and Rodgers, J. (2001). "NIR analysis of textiles," In: Burns, D. A., and Ciurczak, E. W. (eds.), *Handbook of Near-Infrared Analysis*, Marcel Dekker, Inc., New York, 573-607.

González, G., López-Santín, J., Caminal, G., and Solà, C. (1986). "Dilute acid hydrolysis of wheat straw hemicellulose at moderate temperature: A simplified kinetic model," *Biotechnology and Bioengineering* 28, 288-293.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., Krishnamurthy, S., Jun, L., and Xu, G. (1997). "Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood," *Journal of Biotechnology* 53, 133-162.

Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Numlos, M. R., Brady, J. W., and Foust, T. D. (2007). "Biomass recalcitrance: Engineering plants and enzymes for biofuels production," *Science* 315, 804-807.

Hobbs, G. C., and Abbot, J. (1999). "The role of the hydroxyl radical in peroxide bleaching processes," *Journal of Wood Chemistry and Technology* 14(2), 195-225.

Joseleau, J. P., Gharibian, S., Comtat, J., Lefebvre, A., and Ruel, K. (1994). "Indirect involvement of ligninolytic enzyme systems in cell wall degradation," *FEMS Microbiology Reviews* 13(2-3), 255-263.

Kelley, S. S., Jellison, J., and Goodell, B. (2002). "Use of NIR and pyrolysis-MBMS coupled with multivariate analysis for detecting the chemical changes associated with brown-rot biodegradation of spruce wood," *FEMS Microbiology Letters* 209, 107-111.

Khristova, P., Kordsachia, O., Patt, R., and Khider, T. (2006). "Environmentally friendly pulping and bleaching of bagasse," *Industrial Crops and Products* 23(2), 131-139.

Kong, X., Xie, J., Wu, Y., Huang, Y., and Bao, J. (2005). "Rapid prediction of acid detergent fiber, neutral detergent fiber, and acid detergent lignin of rice materials by near-infrared spectroscopy," *J. Agric. Food Chem*. 53, 2843-2848.

Kumar, P., Barrett, D. M., Delwiche, M. J., and Stroeve, P. (2009). "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production," *Ind. Eng. Chem. Res*. 48, 3713-3729.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry* 31(3), 426-428.

Mitsui, K., Inagaki, T., and Tsuchikawa, S. (2008). "Monitoring of hydroxyl groups in wood during heat treatment using NIR spectroscopy," *Biomacromolecules* 9, 286-288.

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

Nada, A.-A. M. A., Yousef, M. A., Shaffei, K. A., and Salah, A. M. (1998). "Infrared spectroscopy of some treated lignin," *Polymer Degradation and Stability* 62, 157-163.

Savitzky, A., and Golay, M. J. E. (1964). "Smoothing and differentiation of data by simplified least squares procedures," *Anal Chem* 36(8), 1627-1639.

Schöning, A. G., and Johansson, G. (1965). "Absorptiometric determination of acid-soluble lignin in semi-chemical bisulfite pulps and in some woods and plants," *Svensk Papperstidn.* 18, 607-613.

Schwanninger, M., and Hinterstoisser, B. (2002). "Klason lignin: Modification to improve the precision of the standardized determination," *Holzforschung* 56(2), 161-166.

Shenk, J. S., Workman, J. J., and Westerhaus, M. O. (2001). "Application of NIR spectroscopy to agricultural products," In: Burns, D. A., and Ciurczak, E. W. (eds.), *Handbook of Near-Infrared Analysis*, Marcel Dekker Inc., New York, 419-474.

Sjöström, E. (1991). "Carbohydrate degradation products from alkaline treatment of biomass," *Biomass and Bioenergy* 1(1), 61-64.

Sun, R. C., Fang, J. M., Rowlands, P., and Bolton, J. (1998). "Physicochemical and thermal characterization of wheat straw hemicelluloses and cellulose," *J. Agric. Food Chem*. 46(7), 2804-2809.

TAPPI Test Method T264 om-88. "Preparation of wood for chemical analysis," In: *Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

TAPPI Test Method T250. "Acid-soluble lignin in wood and pulp," *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

Tsuchikawa, S., and Siesler, H. W. (2003). "Near-infrared spectroscopic monitoring of the diffusion process of deuterium-labeled molecules in wood. Part II: Hardwood," *Appl. Spectrosc*. 57, 675-681.

Tsuchikawa, S., and Siesler, H. W. (2003). "Near-infrared spectroscopic monitoring of the diffusion process of deuterium-labeled molecules in wood. Part I: Softwood," *Appl. Spectrosc*. 57, 667-674.

Tsuchikawa, S., Yonenobu, H., and Siesler, H. W. (2005). "Near-infrared spectroscopic observation of the ageing process in archaeological wood using a deuterium exchange method," *Analyst* 130, 379-384.

Watanabe, A., Morita, S., and Ozaki, Y. (2006). "Temperature-dependent structural changes in hydrogen bonds in microcrystalline cellulose studied by infrared and near-infrared spectroscopy with perturbation-correlation moving-window two-dimensional correlation analysis," *Appl. Spectrosc*. 60(6), 611-618.

Yonenobu, H., Tsuchikawa, S., and Sato, K. (2009). "Near-infrared spectroscopic analysis of aging degradation in antique washi paper using a deuterium exchange method," *Vibrational Spectroscopy* 51, 100-104.

Article submitted: June 8, 2010; Peer review completed: July 27, 2010; Revised version received and accepted: August 5, 2010; Published: August 7, 2010.