

CHARACTERIZATION OF REFINED HEMP FIBERS USING NIR FT RAMAN MICRO SPECTROSCOPY AND ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY

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The research was focused on the separation of single hemp (*Cannabis sativa* L.) fibre cells with low fineness from mechanically extracted fibre bundles of high fineness. The fiber bundles were treated with enzymes, namely panzym, pectinase, hemicellulase, and cellulase, along with a combination of panzym and ultrasonic treatments. Changes in the fiber structure were followed at molecular and microscopic levels by means of NIR FT Raman spectroscopy and Environmental Scanning Electron Microscopy (ESEM). Buffer-panzym treatments of hemp fibers had a prominent effect in loosening of the fiber cells. The best of refining was achieved when the fiber bundles were treated with buffer-panzym solution in combination with ultrasonic treatment.

Keywords: Hemp fibers; Cellulose; Enzymatic treatments; NIR FT Raman Micro Spectroscopy; Environmental Scanning Electron Microscopy (ESEM)

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INTRODUCTION

In Europe the main annual bast fiber crops are hemp (*Cannabis sativa* L.) and flax (*Linum usitatissimum* L.). The utilization of fibers from these plants is receiving renewed interest as attractive alternatives to glass fibers because of the distinct advantages arising from their high Young's moduli, low density, and biodegradability. Hence, the cellulose-based plant fibers play an important role in diversified industrial applications, especially in automotive parts, as well as in construction and food packaging materials. Nevertheless, extensive research is still required to exploit the full potential of these fibers (Kessler and Kohler 1993; Amar et al. 2003; Cappelletto et al. 2000).

The bast fibers from hemp consist of different hierarchical microstructures. The elementary units of the microstructure are the microfibrils of cellulose, which are thousands of nanometers (nm) in length and 5-50 nm in diameter. These microfibrils, impregnated with hemicelluloses, form the primary and secondary cell wall layers. Agglomerates of such microfibrils constitute single fiber cells. These single fiber cells or the elementary fibers are 10-30 μm in diameter. They are bonded together with pectins and small amounts of lignin constructing the next level of microstructure, the technical fibers, with diameters of about 50-100 μm . The technical fibers are adhered together with a pectin-lignin matrix framing the fiber bundles.

Mechanical properties of such fibers are strongly influenced by their molecular composition, as well as by the orientation of the cellulose microfibrils in the secondary cell wall layer (S2) with respect to the fiber cell axis (Thygesen 2006). The microfibrils in hemp are aligned at angles of about 0°-10° to the fiber axis. These microfibrils play a vital role in exhibiting high stiffness and thereby providing high strength to the fibers (Preston 1974). Cellulose (50-70%, w/w), hemicellulose (10-15%, w/w), lignin (1-3%), and pectins (5-10%) are the main components of hemp fibers (Thygesen 2006).

Fineness is the ratio of weight of the sample in grams to 1000 meters of yarn. There have been several attempts to improve the quality of technical fibers by decreasing their fineness. These involve surface cleaning due to the removal of residual parenchyma cells, the removal of impregnating substances like lignin, and the degradation of the middle lamella, which is associated with the decomposition of pectins and hemicelluloses. The refining of the fibers can be accomplished by different physical and chemical treatments such as chemical and enzymatic maceration, ultrasonic treatment, and combinations of these (Ademsen et al. 2002; Akin et al. 1997).

This work describes the refining of the fiber bundles of hemp by using different refining agents. Buffer solution, different enzymes of industrial and laboratory grade, ultrasonic treatments, as well as the combination of enzymes and ultrasonic treatments were applied. Changes in the fiber structure were observed at the microscopic and molecular levels by ESEM investigations and NIR FT Raman spectroscopy respectively.

EXPERIMENTAL

Plant Material and Fiber Preparation

Hemp (*Cannabis sativa* L. cv. Kompolti) was field-grown at the experimental station of Martin Luther University Halle-Wittenberg, Germany. The plants were harvested by cutting them at the ground level from a plot (2 m x 2 m) embedded in the stand. The plants were divided into three parts, equal in length.

Fibers of female plants from the upper canopy were extracted mechanically from the stems using a laboratory machine (BMBF Flaksy®). The pressure of the breaking rolls was 10 N and the velocity was 13 m/min. This extracting procedure was repeated 10 times for each part of the hemp stem.

Single fiber filaments were then extracted by hand from the fibers obtained by the above extraction process. Splices and splinters were removed from these single filaments and they were cut to 10 cm in length. 20 of such single fibers were used for each treatment.

Chemical Fiber Treatments:

The untreated fibers were subjected to treatments with the following aqueous solutions:

- (i) Solution B or Buffer: *citrate – phosphate buffer* was made from 22.7 ml of 0.2 M disodium hydrogen phosphate (Carl Roth GmbH, Karlsruhe, Germany) and 27.2 ml of 0.1 M citric acid (Chemapol, Praha, Czech Republic) mixed with deionised water to make up a solution of 100ml. The pH was adjusted to 4.5.

- (ii) Solution BPa: 100 ml of solution B (pH 4.5) + 8 vol-% of Panzym (SIHA-Panzym[®]DF, technical grade, Langenlonsheim, Germany) were mixed and pH adjusted to 4.5.
- (iii) Solution BC: 10 ml of solution B (pH 5.0) + 100 mg and 1000 mg of cellulase from *Aspergillus niger* (1.3 U mg⁻¹, laboratory grade; Fluka Chemie AG, Buchs, Switzerland) were mixed and pH adjusted to 5.0.
- (iv) Solution BH: 10 ml of solution B (pH 4.5) + 100 mg of hemicellulase from *Aspergillus niger* (1.5 U mg⁻¹, laboratory grade; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) were mixed and pH adjusted to 4.5.
- (v) Solution BPe: 10 ml of solution B (pH 4.1) + 100 mg of pectinase from *Aspergillus niger* (1.6 U mg⁻¹, laboratory grade; Fluka Chemie AG, Buchs, Switzerland) were mixed and pH adjusted to 4.1.

All fibers were infiltrated under low pressure for 30 minutes. Afterwards, each fiber was transferred into a test tube with 10 ml of fresh solution of each of the solutions (i)-(v). Fibers treated with solutions B, and BPa were incubated at 25°C for 24 hours. Fibers with solutions BC and BH were incubated at 35°C for 24 hours. Only the sample with a cellulase concentration of 1000 mg/10 ml buffer was incubated at 35°C for 7 days. For treatments using solution BPe, the fibers were incubated at 50°C for 24 hours. 20 fibers were incubated with solution BPa followed by an ultrasonic treatment for 1 min. After the treatments, all fibers were dried at room temperature.

NIR FT Raman Micro Spectroscopy

FT Raman microscopic measurements were carried out using the RFS/100 S spectrometer coupled directly to the microscope NIKON, Eclipse E400. The microscope was equipped with a video device, two objective lenses of 10x and 50x magnification, a rotatable half-wave plate, and a mapping table. Both the incident laser beam and Raman scattering were collected through the Nikon microscope.

All micro-spectra were measured in the frequency range of 3500 – 100 cm⁻¹ with a spectral resolution of 4 cm⁻¹. The single fibers were fixed on a metal plate, which was placed on the mapping table. The fiber axis was oriented parallel to the electric field-vector of the exciting laser beam. Laser spot size was focused to 10–20 μm diameter on the fiber using the 50x objective magnification. Spectra were recorded at three different positions on a single fiber. At each measuring position, 600 scans were accumulated using 400 mW of laser power output. Then, an average of the three spectra was procured for each fiber. The spectrum from the metal plate was subtracted from the average spectrum obtained. Finally, the spectra were normalized using vector mode, and baseline correction was done using the rubber band mode with 64 baseline points used with OPUS software 4.2 (Bruker, Germany).

Statistical Calculations

For statistical calculations, the software SAS 9.2 (SAS Institute Inc., USA) was used. Raman intensity ratios were subjected to Tukey's grouping using the GLM procedure. Means with the same letter indicate that there was no significant difference according to this statistical method of calculation.

The alpha value (percent error allowed in the measured values) was set to 30% to account for the high variability of biological samples.

Environmental Scanning Electron Microscopy (ESEM)

ESEM micrographs of the fibers were recorded with different magnifications by means of an XL30, ESEM-FEG microscope (Philips). The ESEM was equipped with environmental SE-detectors (ESD or GSED). Fiber micrographs were recorded at highest resolution (2-3 nm) in a gaseous environment of water vapor at a pressure of 0.13 KPa and an accelerating voltage of 15.0 KeV. Fibers were recorded in wet mode i.e., the fibers were not coated with gold-palladium to be conductive.

RESULTS AND DISCUSSION

Analysis of the Microstructure of Hemp Fibers

Electron microscopic investigations

Surface morphologies of hemp fiber bundles were studied by electron microscopy (ESEM). Typical differences between untreated and macerated fiber bundles can be identified, as shown in Figs. 1 (a) - (e). The bundles of the untreated plant fibers are covered by surface impurities and waxy substances see Fig. 1(a).

The surface impurities were removed from the fiber surfaces when the hemp fibers were treated with buffer solution. A certain level of surface refining and partial inter-fibrillar disintegration of the fiber bundle were achieved by means of this chemical fiber treatment. However, the elementary fibers were still covered and adhered by non-cellulosic components. This is represented in Fig. 1(b).

However, buffer-panzym treated fibers exhibited a smoother surface and better inter-fibrillar disintegration of the bundle, as shown in Fig. 1(c). The ESEM micrograph with a different magnification was used in order to show the effect of inter-fibrillar disintegration more clearly. This effect is attributed to the enzymatic activity of panzym on the pectic substances that are located in the region of the middle lamella (Saleem et al. 2007).

Hemp fiber bundles treated with buffer-panzym solution in combination with ultrasonic treatment revealed a predominant effect in separating the microfibrils of the fibers.

As shown in Fig. 1(d), smooth fiber surfaces without any cracks along the cell wall layers were observed. These results demonstrate that the mechanical shear force of ultrasonic treatment was intense enough to remove adhesive substances confined in between the fiber bundles. This signifies that chemo-enzymatic treatment along with ultrasonic treatment enhanced the fiber separation to a greater extent than the chemo-enzymatic treatment alone.

Cracks across the cell wall layers were observed in the fibers after cellulase treatment, as shown in Fig. 1(e). This indicates the degeneration of the micro-structure of the fiber cellulose.

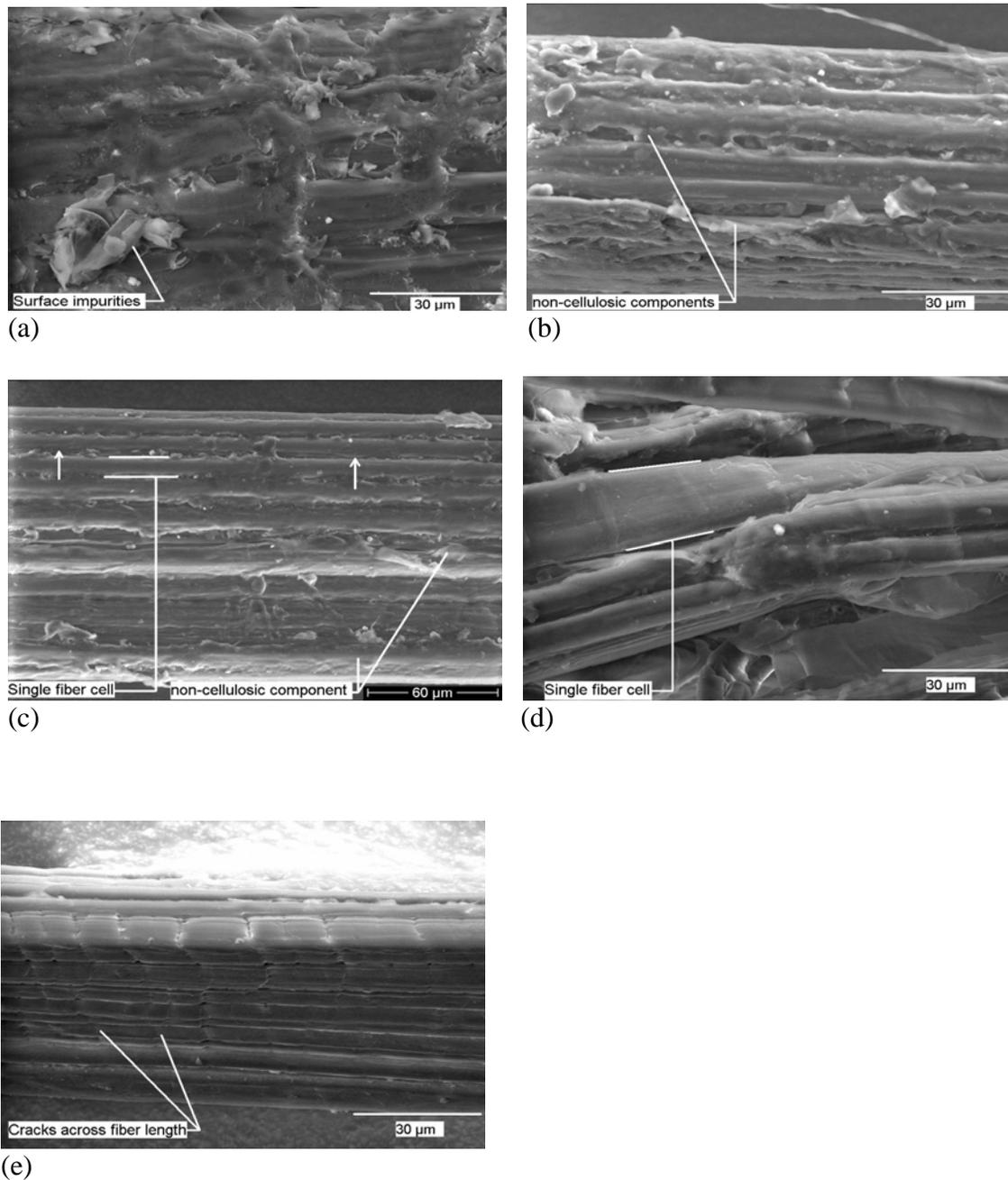


Fig. 1. ESEM images of hemp fibers: (a) untreated; (b) treated with solution B; (c) solution BPa; (d) solution BPa + ultrasonic treatment and (e) cellulase treated.

Analysis of the Molecular Structure of Hemp Fibers

Orientation dependent micro FT Raman spectra

For uniaxial oriented polymers like cellulose, changes in their vibrational spectra can be observed depending on the orientation of the macromolecule with respect to the *E*-vector of the excitation energy (Siesler et al. 1980; Atalla et al. 1980). Cellulose consists

of anhydroglucopyranose units, which are connected by β -1,4-glycosidic linkages. These anhydroglucopyranose units and their (COC) linkages are oriented linearly and nearly parallel to the axis of the cellulose-based plant fibers. By contrast, the methine groups (CH) of the glucopyranose units are oriented nearly perpendicular to the cellulose skeletons.

Thus, the signal of the $\nu_s(\text{COC})$ mode of the glycosidic linkages occurs with higher intensity in the Raman spectrum at 1096 cm^{-1} in parallel orientation mode, wherein the fiber and the E-vector of the exciting laser beam are parallelly oriented, compare Fig. 2. In contrast, the signal of the $\nu(\text{CH})$ modes of the methine groups appears with a lower intensity in that spectrum at 2900 cm^{-1} . However, the signals appear with complementary intensities in the perpendicular polarization mode, see Fig. 2. Therefore, the micro-FT Raman spectra of cellulose-based plant fibers always have to be discussed with reference to the orientation of the plant fiber to the E-vector of the exciting laser beam.

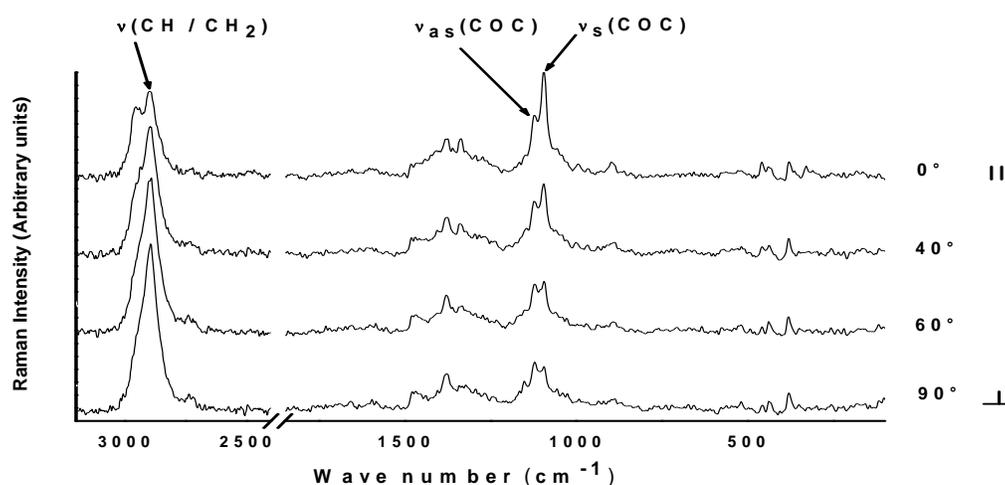


Fig. 2. An orientation-dependent micro-FT Raman spectrum of a single hemp fiber is represented. Polarisation angles of the exciting laser were from zero to 90° . Fibers measured were oriented parallel to the E-vector of the laser beam.

Untreated and treated hemp fibers showed similar vibrational patterns in their micro-FT Raman spectra, which are represented in Fig. 3. However, varying intensities of several Raman signals were observed. This is attributable to the molecular constitution of the plant fibers. The cell tissue of the bast fibers consists of hydrocarbons of several molecular structures like pectins, hemicelluloses, and cellulose, which all exhibit a similar vibrational behaviour. Therefore, Raman spectra of cellulose-based plant fibers always have to be considered as superpositions of both the molecular vibrations of cellulose and non-cellulosic components.

Due to this fact, intensity ratios of characteristic Raman signals were used for identifying changes in the molecular fiber composition with respect to the different fiber treatments (Edwards et al. 1997; Jaehn et al. 2002).

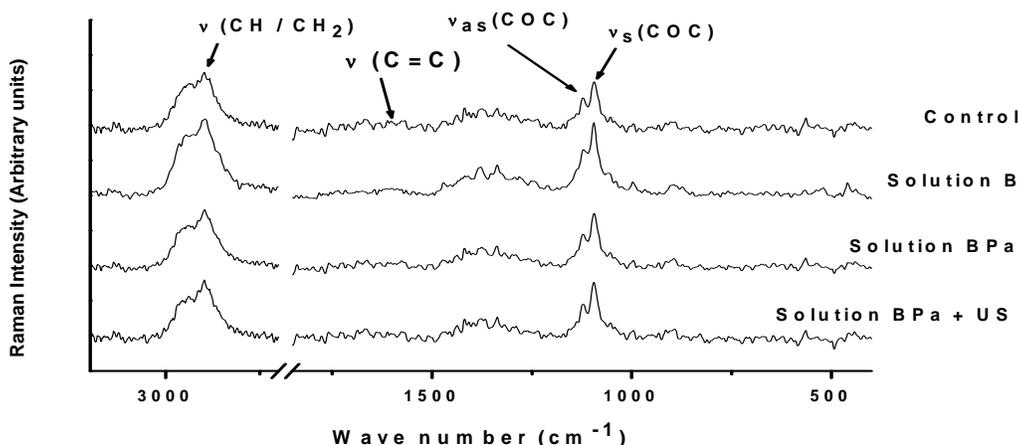


Fig. 3. FT Raman micro spectra of hemp fibers treated with following solutions: B, BPa, and BPa plus ultrasonic treatment. Fibers oriented parallel to the *E*-vector of the laser beam.

The following Raman intensity ratios were considered:

$$R1 = I^{\nu_{as}(COC)} / I^{\nu_s(COC)} \quad (1)$$

$$R2 = I^{\nu(C=C)} / I^{\nu_s(COC)} \quad (2)$$

$$R3 = I^{\nu_s(COC)} / I^{\nu(CH)} \quad (3)$$

R1 represents the ratio of the Raman signal intensities of the anti-symmetric $\nu_{as}(COC)$ to the symmetric stretching mode $\nu_s(COC)$. The modes $\nu_{as}(COC)$ at 1120 cm^{-1} and $\nu_s(COC)$ at 1096 cm^{-1} are mainly attributed to the β -1,4-glycosidic linkages of the anhydroglucopyranose units of cellulose (Wiley and Atalla 1987; Schenzel and Fischer 2001). However, the intensity of the peak at 1120 cm^{-1} is also influenced by other non-cellulosic carbon-hydrogen groups such as degrading phenolic groups and the coniferyl aldehyde groups from lignin (Agarwal et al. 1995; Edwards et al. 1997). Consequentially, the ratio R1 describes the cleanness of the cellulose structures of the hemp fibers.

R2 represents the ratio of the Raman intensity of the vibrational mode $\nu(C=C)$ appearing at 1604 cm^{-1} , which is assigned to the $C=C$ conjugated ring structures of lignin, with respect to the intensity of $\nu_s(COC)$ of the glycosidic linkages of cellulose. Thus, the lignin content is determined relatively with respect to the cellulose portion of the fibers. However, a distinct signal for lignin could not be detected with this micro FT-Raman mode of measurement, as the lignin content in the fiber was too low to be detected by an NIR excitation. Therefore, discussion about lignin content and its intensity ratio is excluded.

Similarly, R3 is depicted as the ratio of the Raman intensity of the stretching vibrations $\nu_s(COC)$ of the glycosidic linkages of cellulose to the intensity of the signal at 2900 cm^{-1} , which is assigned to methine stretching vibrations $\nu(CH)$. By means of this ratio, the proportion between C-H bonds of non-cellulosic materials or degenerated cellulose is determined with respect to the cellulose portion of the fibers.

In Table 1, Raman intensity ratios R1 and R3 of macerated and untreated fibers are shown. As expected, the intensity ratio R1 showed decreasing values for macerated fibers in comparison to the control. The decrement is associated with the decrease in

intensity of the signal at 1120 cm^{-1} with respect to the cellulose signal at 1096 cm^{-1} indicating the loss of non-cellulosic fiber components (Agarwal et al. 1995). Thus, finally after the refining process, the cellulose content of the fiber increased in relative terms.

Table 1. Micro-FT Raman intensity Ratios R1 and R3 of Single Hemp Fibers Treated with Different Solutions. (Letters describe statistical differences among the treatments with respect to the control.)

Treatment	FT Raman intensity ratios	
	R1	R3
Untreated (control)	0.64 a	0.80 a
Solution B	0.61 ab	0.88 ab
Solution BPa	0.60 b	0.88 ab
Solution BPa + US	0.60 b	0.88 ab

The lowest R1 values were obtained when the fibers were treated with buffer-panzym solution as well as with BPa solution in combination with an ultrasonic treatment. This result indicates that enzymatic treatments yield highly refined fibers. The effect is primarily attributed to the enzymatic activity of the panzym in loosening of the pectin lamellae (Saleem et al. 2007). The ultrasonic treatment suggests only the removal of adhered surface impurities and a better physical separation of the fiber cells as it is shown in the ESEM micrographs of Figs. 1(c) and 1 (d). No change in molecular composition was obtained.

Increasing values of R3 indicate the elimination of non-cellulosic hydrocarbons by all of the treatments carried out. This is illustrated by the decrease in intensity of the Raman signal at 2900 cm^{-1} with respect to the cellulose signal at 1096 cm^{-1} . This is in accordance with the R1 ratios. However, the R3 values were equal for all the different treated fibers. Here, an incomplete removal of non-cellulosic hydrocarbons has to be expected.

Generally, fibers treated with buffer-panzym solutions (BPa) showed different properties than untreated fibers and fibers treated with buffer only. This was also confirmed by statistical analysis of the Raman data and ESEM, compare Fig. 1(c). Fiber treatments using buffer solution and panzym in combination with ultrasonic (BPa + US) caused a comparably high impact in loosening the fiber cells. This shows that a mechanical force is required for removal of adhered substances, which are difficult to remove through any of the chemo-enzymatical methods.

Furthermore, isolated fiber bundles were treated with cellulase, hemicellulase, and pectinase of varying concentrations in different reaction conditions to detect the influence of the single enzymes on the fiber refining process. Effects achieved through fiber refining are reflected by the Raman ratios R1 and R3, which are depicted in Table 2.

Table 2. Micro FT Raman Intensity Ratios R1 and R3 of Single Hemp Fibers Treated with Different Enzymes (pectinase, hemicellulase, and cellulase) of Different Concentration.

Treatment	FT Raman intensity ratios	
	R1	R3
Untreated (control)	0.64 bc	0.80 c
Solution BPe : 100 mg /10 ml	0.66 c	1.00 b
Solution BH : 100 mg /10 ml	0.68 b	0.90 c
Solution BC : 100 mg /10 ml	0.63 c	1.38 a
Solution BC : 1000 mg /10 ml	1.00 a	0.12 d

An increase in the R1 ratios was observed except for the fibers treated with cellulase with an enzyme concentration of 100 mg/10 ml buffer solution. This is attributable to the intensity enhancement of the signal at 1120 cm^{-1} with respect to the cellulose signal at 1096 cm^{-1} . Therewith, the presence of residual lignin due to specific enzyme activities of pectinase and hemicellulase is expected. Also a partial degradation of the cellulose cannot be ruled out, as it happens directly by the cellulase treatment of the fibers resulting in a lower value of R1, see Table 2.

Fibers treated with highly concentrated cellulase-buffer solution (1000 mg/10 ml) confirmed a strong molecular degradation of the cellulose backbone. This can be clearly observed in the FT Raman spectra in Fig. 4. The intensity of the signals of ν_s and $\nu_{as}(\text{COC})$ modes decreased dramatically, and the highest R1 value was obtained, see Table 2. Distinct statistical significance was determined for all ratios R1 and R3 for this treatment.

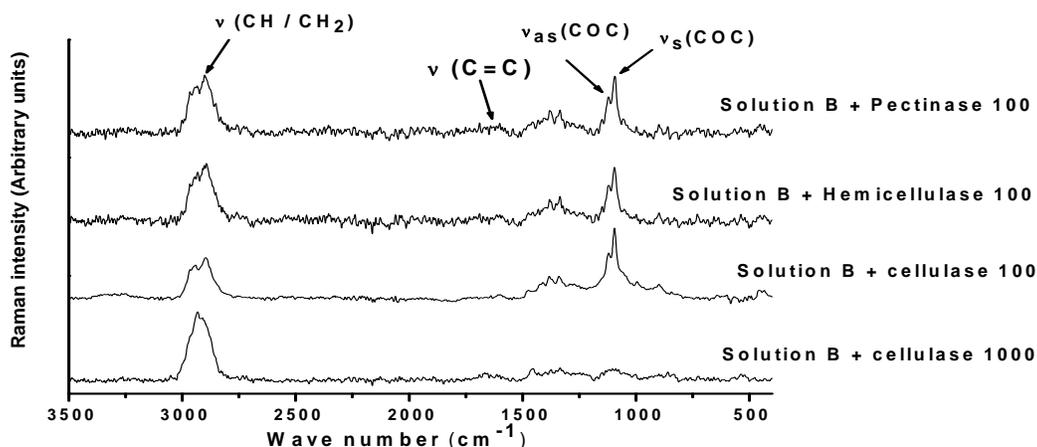


Fig. 4. Micro FT Raman spectra of single hemp fibers treated with different enzymes (pectinase, hemicellulase and cellulase) and different concentrations. Fibers were oriented parallel to the *E*-vector of the laser beam.

An increase in the Raman intensity ratio R3 is expected for enzymatically treated fibers with respect to the untreated control, conforming to the removal of other cell wall compounds than cellulose. This was observed for fibers treated with BPe, BH, and BC of

low concentrations. However, fibers treated with cellulase under strong reaction conditions such as long treatment period and high concentration caused a decrease in R3, compare Table 3. Such a low value confirms the degradation of cellulose as illustrated by the intensity decrease of the Raman signal at 1096 cm^{-1} , which is attributable to the hydrolysis of the glycosidic linkages. A relative increase in signal intensity at 2900 cm^{-1} was observed upon treatment with $1000\text{mg}/10\text{ml}$ concentration of cellulase, owing to new developing $\nu(\text{CH})$ modes, which evolve from the decomposition products of cellulose (Edwards et al. 1997). A decrease in the degree of polymerization of cellulose has to be assumed. This is in contrast to the other enzymatic fiber treatments.

According to the statistical analysis, significant results of the R1 and R3 ratios were determined only for hemp fibers treated with cellulase concentration of $1000\text{ mg}/10\text{ ml}$ buffer. Therewith, real differences in their molecular constitution were confirmed with respect to untreated and otherwise treated fibers. This is attributed to the strong reaction conditions, such as long treatment period and high enzyme concentration. Generally, fiber treatments with low enzyme concentrations had low impact in the fiber refining process. However, the usage of high enzyme concentrations causes the destruction of the cellulose chains, which is detrimental to the development of strong fiber properties. Therefore, moderate conditions are recommended for the fiber refining process. Further research is also recommended focusing on methods for extraction of nano fibers without damaging the cellulose backbone.

CONCLUSIONS

1. A two step procedure of hemp fiber refining was demonstrated. In the first step, when the fibres were treated with enzyme buffer solution (BPa), degradation of middle lamella along with loosening and partial removal of adhesive substances occurred. In the next step ultrasonic treatment, which was powerful enough to remove the loosened adhesive substances and non-cellulosic components from the fiber surface, led to the best fiber separation possible.
2. Enzymatic concentrations that are not excessively high are optimal for the refining of hemp fibers in order to minimise damage to the cellulosic structure.
3. NIR FT Raman microscopy and electron microscopy (ESEM) have shown their capability for monitoring molecular differences of cellulose-based plant fibers.

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