

Proceedings of the 34th Risø International Symposium on Materials Science:
Processing of fibre composites – challenges for maximum materials performance
Editors: B. Madsen, H. Lilholt, Y. Kusano, S. Fæster and B. Ralph
Department of Wind Energy, Risø Campus
Technical University of Denmark, 2013

HEMP FIBRES: ENZYMATIC EFFECT OF MICROBIAL PROCESSING ON FIBRE BUNDLE STRUCTURE

A. Thygesen^{*}, M. Liu^{*}, A.S. Meyer^{*} and G. Daniel^{**}

^{*}Center for Bioprocess Engineering, Department of Chemical and
Biochemical Engineering, Technical University of Denmark,
2800 Lyngby, Denmark.

^{**}Department of Forest Products/Wood Science, Swedish University
of Agricultural Sciences, 750-07, Uppsala, Sweden

ABSTRACT

The effects of microbial pretreatment on hemp fibres were evaluated after microbial retting using the white rot fungi *Ceriporiopsis subvermispota* and *Phlebia radiata* Cel 26 and water retting. Based on chemical composition, *P. radiata* Cel 26 showed the highest selectivity for pectin and lignin degradation and lowest cellulose loss (14%) resulting in the highest cellulose content (78.4%) for the treated hemp fibres. The pectin and lignin removal after treatment with *P. radiata* Cel 26 were of the order 82% and 50%, respectively. Aligned epoxy-matrix composites were made from hemp fibres defibrated with the microbial retting to evaluate the effects on their ultrastructure. SEM microscopy of the composites showed low porosity on the fibre surfaces after defibration with *P. radiata* Cel 26 and *C. subvermispota* indicating good epoxy polymer impregnation. In contrast, fibres treated by water retting and the raw hemp fibres were badly impregnated due to porosity caused by surface impurities such as epidermis and other pectin rich plant cells. The pectin and lignin mainly located in the outer part of the fibres were assumed to be extracted and degraded by pectinase and peroxidase enzymes produced by the fungi.

1. INTRODUCTION

Hemp fibres can be used as reinforcement agents in biocomposites due to their good mechanical properties including low density and high stiffness (Thygesen, Thomsen, Daniel and Lilholt 2007). The principal constituent of hemp fibres is cellulose, which has a high theoretical strength (8 GPa) and functions as the reinforcing component in the fibres. Currently, the largest demand for cellulosic fibres is for the production of string, twine, cord and ropes (Sankari, 2000). These applications require primary processing of raw materials into yarn with a series of steps including retting, scutching, carding, cottonization and spinning. Retting is the term used for the removal of non-cellulosic components from natural fibers and separation of the fibres from the plant stem structure, to obtain cellulose-rich fibers. It is performed by microbiological

methods. Retting precedes mechanical separation (scutching) of the fibre from the stem and is essential for reduction of fibre breakage (Franck, 2005). However, recent data suggest that the gentle microbial retting process can enzymatically create defects in the fibre structure during the processing of hemp stems into single fibres. (Thygesen et al., 2007; Thygesen, Madsen, Thomsen and Lilholt, 2011).

Parenchyma cells rich in pectin and hemicellulose, which bind the hemp bast onto the stem surface, are located between the fibre bundles (Garcia-Jaldon, Dupeyre and Vignon, 1998; Franck, 2005). This binding must be degraded to obtain useful fibres for strong composites. As explained in more detail below, hemp fibres are classically separated from the plant stems by “water retting”, which in essence is a microbial process. In this process, indigenous bacteria and notably fungi present on the plant stems degrade pectin between the fibres and the stem surface at temperatures of around 15 °C to 30 °C within six to ten days (Franck, 2005; Thygesen et al., 2007). It has been shown that bacterial species of *Achromobacter*, *Clostridium* and *Pseudomonas* dominate (Rosember, 1965). Despite its long use, the process is still largely empirical, and obviously depends on the microbial flora present on the fibres. In order for the process to be successful, it is of crucial importance that the cellulose is not degraded. Knowledge about changes in hemp fibre ultrastructure and chemical composition during processing is of great importance to produce high-quality fibres.

In this study, the basidiomycete white-rot fungi *Ceriporiopsis subvermispora* (Akhtar, Attridge, Blanchette, Myers, Wall, Sykes, Koning, Burgess, Wegner and Kirk, 1992) and mutant strain (cellulase less) *Phlebia radiata* Cel 26 (Nyhlen and Nilsson, 1987), which have a limited ability to degrade cellulose were used to treat hemp stems and investigate their effect on the fibre microstructure and chemical composition compared with traditional water retting. These fungi have been used for microbial separation of woody fibres. Results were analyzed based on knowledge of the cell wall active enzymes produced by the two fungi.

2. MATERIALS AND METHODS

2.1 Raw material. The investigated hemp fibres are presented in Table 1. The H0, H1, H2, H3 fibre samples were produced from the same original hemp plants (*Cannabis Sativa* L, Felina 34) grown at Flakkebjerg Research Station, Aarhus University, Denmark (Thygesen et al., 2007). H0 fibres were manually peeled from the un-retted stems and were considered as non-processed. H1, H2 and H3 fibres were obtained from H0 by water retting as well as by fungal treatment with *C. subvermispora* (Akhtar et al., 1992) and *P. radiata* Cel 26 (Nyhlen and Nilsson, 1987), respectively. The fibre yield was determined by separation of the fibres by hand peeling followed by weighing. The yield was between 28 – 38 g fibres per 100 g plant stem and between 68 – 100 g per 100 g untreated fibres as shown in the table.

Table 1. Overview of the investigated hemp fibres.

Fibre type	Code	Treatment	Fibre yield	
			(g/100 g stem)	(g/100 g raw fibres)
Loose hemp fibre bundles	H0	Untreated	38±1	100
	H1	Water retting	27±1	68
	H2	<i>C. subvermispora</i>	29±1	75±4
	H3	<i>P. radiata</i> Cel 26	28±1	70±1

Microbial processing of hemp fibres

2.2 Chemical composition analysis. A gravimetric method was used to determine the content of wax, water-soluble components, pectin, lignin, hemicellulose and cellulose in the hemp fibres (Thygesen, Daniel, Lilholt and Thomsen, 2005). The method consists of five steps: (1) Wax was Soxhlet extracted in chloroform, (2) water soluble extractives were extracted in water, (3) pectin was extracted in 30 g/L ethylene-diamine-tetra-acetic acid (EDTA), (4) lignin was oxidized and extracted in 33 g/L NaClO₂ + 6 g/L CH₃COOH, and (5) hemicellulose was extracted in 120 g/L NaOH + 20 g/L H₃BO₃. The remaining residue, unaffected by the extraction steps was cellulose. The mineral content was determined by incineration of 0.5 g sample at 550 °C for 3 h.

2.3 Fungal treatment and water retting. Cultures of the white rot fungi *C. subvermispora* and *P. radiata* Cel 26 were applied on hemp stems as described by Thygesen et al. (2007). The fungi were kindly supplied from the Swedish Agricultural University, Dept. Forest Products. They were stored and pre-cultivated on 2% (w/v) malt agar plates at 20 °C. For inoculation, the mycelia grown on one agar plate was homogenized into 100 mL water. A solution of 1.5 g/L NH₄NO₃, 2.5 g/L KH₂PO₄, 2g/L K₂HPO₄, 1 g/L MgSO₄·7H₂O and 2.5 g/L glucose was used as growth medium in the fungal treatment experiments. The growth medium (625 mL) and hemp stem pieces (50 g) were sterilized in Erlenmeyer flasks at 120 °C for 30 min. After cooling to ambient temperature, the mycelium suspension (70 mL) was added aseptically and the fungal treatment experiments conducted at 28 °C for 14 days (72 kg hemp stem m⁻³ liquid). Following fungal treatment, the hemp stem pieces were washed in water to remove epidermal and fungal material from the stem surfaces and to separate the fibres from the woody cores of the stems. Water retting was performed with 20 kg hemp stems in 750 L water (26 kg hemp stem m⁻³ liquid) at 35 °C for 4 days. Water-retted hemp fibres became separated from the stem core during the retting process.

2.4 Staining pectin and use of light microscopy. Sections of hemp stem were stained with 1 g/L ruthenium red (Sigma-Aldrich #R2751; ammoniated ruthenium oxychloride [Linear formula: [(NH₃)₃RuORu(NH₃)₄ORu(NH₃)₅]Cl₆]). Transverse sections of hemp stem (30 µm thickness) were viewed using light microscopy at 100 times magnification (Strivastava, 1996; Thygesen et al., 2005).

2.5 Epoxy composites and scanning electron microscopy (SEM). The hemp fibres were wetted in water in order to keep them aligned followed by vacuum drying. The hemp fibres were embedded in epoxy polymer (SPX 6872 + SPX 6873; Ekompelit, Denmark) to form composites by press consolidation. A low viscosity epoxy resin (SPX 6872) and a hardener (SPX 6873) were mixed in the ratio of 100 g resin to 36 g hardener. The final composites contained 20 – 40 weight-% fibres corresponding to 80 – 60 weight-% epoxy. The lay-up was pre-cured at 40 °C for 16 h and thereafter cured at 120 °C for 6 h. Composite pieces were polished perpendicular to the fibre axis using wetted silicon carbide paper (Thygesen et al., 2007). Platinum coated samples were observed using a Philips XL30 ESEM scanning electron microscope.

3. RESULTS AND DISCUSSION

3.1 Chemical composition of the fibres. Pectin is the main component that closely binds the fibres to the other constituents of the hemp stem. Reducing the content of pectin and hemicellulose helps to expose the highly ordered crystalline structure of cellulose and facilitates separation of the flexible fibres for subsequent mechanical processing. The effects of biological treatments on the chemical composition of the investigated hemp fibres are shown in Table 2. All the fibre components were to some extent degraded with the investigated treatments and pectin to the highest extent.

The pectin loss in hemp fibres caused by fungal treatment with *C. subvermispora*, *P. radiata* Cel and or by water retting was 57%, 82% and 77%, respectively. The weight loss of hemicellulose in samples treated with water retting (44%) and *P. radiata* Cel 26 (36%) were not significantly different. *C. subvermispora* caused the lowest hemicellulose degradation (25%), while water retting caused a significant loss in cellulose with 22%. However, *P. radiata* Cel 26 and *C. subvermispora* treatment caused only 14% and 12% weight loss in cellulose, respectively.

The results showed that *P. radiata* Cel 26 had the highest selectivity for pectin degradation. The selectivity value is defined as the ratio of pectin degradation to cellulose degradation and is used to describe the depectinization efficiency. According to the definition, a higher selectivity value reflects improved preferential depectinization. On the contrary, a lower selectivity value means relatively high amount of cellulose degradation during the biological treatment resulting in a loss. As shown in Table 2, *P. radiata* Cel 26 demonstrated higher selectivity (6.0) than water retting (3.5) and *C. subvermispora* (4.6). Besides best depectinization, *P. radiata* Cel 26 also caused the highest lignin (50%) and hemicellulose degradation (36%) confirming its limited cellulase activity and that the strain can produce pectinase and hemicellulase enzymes (Nyhlén, Nilsson, T. 1987; Daniel, Volc and Niku-Paavola, 2004). The highest (50%) weight loss in lignin resulting from the fungal treatment with *P. radiata* Cel 26 on the stem indicates the highest activity of Pyranose oxidase (POD) and Mn-dependent peroxidases (MnP). This was suggested due to the presence of much higher concentration of H₂O₂ (1-5 mg/L) in the fungal treatment broth, since H₂O₂ produced by POD has a crucial role in the ligninolytic systems and is necessary for the ligninolytic peroxidases (Daniel et al, 2004; Kantelinen, Hatakka and Viikari, 1989).

Table 2. Process results obtained by fungal treatment and water retting of hemp.

Fibre code	H ₂ O ₂ (mg/L)	Selectivity value*	Weight loss (%)			
			Cellulose	Hemicellulose	Lignin	Pectin
H1	n.a.	3.5	22	44	0	77
H2	0-1	4.6±2	12±5	25±5	-12±12	57±5
H3	1-5	6.0±1	14±2	36±1	50±22	82±2

* Selectivity value = Pectin loss / Cellulose loss.

Table 3. Chemical composition and structural properties of the investigated hemp fibres.

Fibre code	Chemical composition					Structure
	Cellulose (% w/w)	Hemicellulose (% w/w)	Lignin (% w/w)	Pectin (% w/w)	Residuals (% w/w)	Transverse section (µm ²)
H0	64.4±0.8	14.4±0.1	3.4±0.2	7.3±0.5	10.5	160000
H1	73.8	11.8	5.0	2.5	6.9	10000
H2	72.4±1.4	13.8±0.8	4.9±0.4	4.0±0.6	4.9±0.2	50000
H3	78.4±1.0	13.0±0.1	2.4±1.0	1.9±0.3	4.3±0.2	3000

Table 4. ANOVA table for cellulose content of the investigated hemp fibres.

Source of variation	Sums of squares	DF	Mean square	F	P value
Treatments	19.9	2	9.929	6.336	0.136
Error	3.13	2	1.567		
Total	23.03	4			

Microbial processing of hemp fibres

The defibration of hemp treated by water retting and cultivation of *C. subvermispora* and *P. radiata* Cel 26 resulted in hemicellulose, pectin and lignin degradation (Table 2). The cellulose content increased compared to un-retted hemp fibres (H0) though there were slight cellulose losses caused by these treatments. Overall, the principle constituent of the four samples was cellulose, at 64 – 78% of total weight. The remainder consisted of 11 – 14% hemicellulose, 2 – 5 % lignin, 2 – 7% pectin and 4 – 11% residuals. *P. radiata* Cel 26 treatment resulted in the highest cellulose (78.4%) and lowest contents of lignin and pectin due to the preferential attack of *P. radiata* Cel 26 for pectin and lignin (Tables 2, 3). However, according to Table 4, the content of cellulose in samples pretreated by cultivation with *C. subvermispora* (72.4%), *P. radiata* Cel 26 (78.4%) or by water retting (73.8%) was not statistically significant different ($P=0.136>0.05$ ANOVA, F (2, 2)). Moreover, smaller hemp fibre bundles were obtained with *P. radiata* Cel 26 ($3000 \mu\text{m}^2$) (H3) than by water retting ($10000 \mu\text{m}^2$) (H1) and with *C. subvermispora* ($50000 \mu\text{m}^2$) (H2), due to the greater degradation of the lignin and pectin rich middle lamellae between the fibres. Based on the transverse section data the fibres treated with *C. subvermispora* (H2) were best despite that *P. radiata* Cel 26 (H3) has the highest selectivity for pectin and lignin degradation.

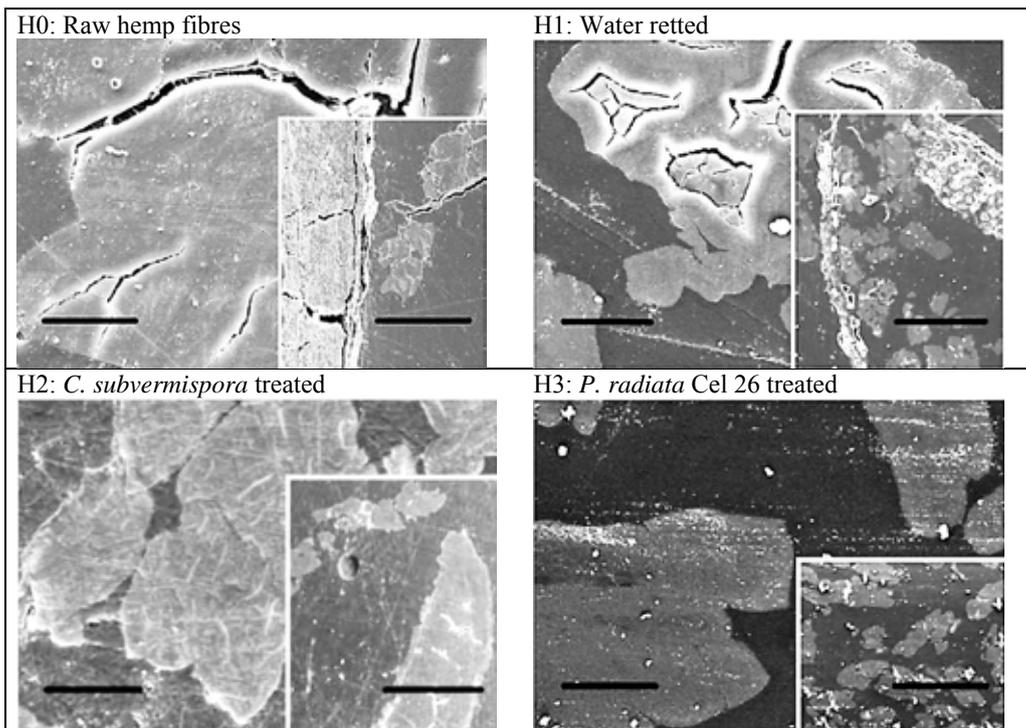


Figure 1. SEM images of transverse composite sections recorded at low and high magnification with the investigated hemp fibres. Inset images have scale bars of $200 \mu\text{m}$ and the background images $20 \mu\text{m}$ (Thygesen et al., 2007).

3.2 Microstructure of fibre bundles and epoxy impregnation. In lignocellulosic materials, hemicellulose and lignin are considered to be amorphous components, while cellulose is partly crystalline (Gharpuray, Lee and Fan, 1983). Since there was significant lignin and hemicellulose degradation during the treatments, it was of interest to investigate the effect of different treatments on the microstructure of treated hemp fibres. The microstructure was observed in transverse cross sections using SEM (Figure 1). The figure shows the fibres (light grey areas), the matrix (dark grey areas) and the air filled voids described as porosity areas (black areas).

Increased porosities and cracks appeared inside the epidermis on the fibre bundle surfaces of raw hemp fibres (H0) and water retted (H1) hemp fibres (Figure 1). This resulted in incomplete impregnation of the fibre bundles forming gaps between the matrix and the fibres. This increase in porosity appeared to the highest extent in composites reinforced with raw hemp bast and water-retted hemp fibres due to surface impurities such as epidermis and other pectin rich plant cells. In contrast, hemp fibres defibrated by cultivation of *C. subvermispota* (H2) and *P. radiata* Cel 26 (H3) were well impregnated in epoxy since no porosity was observed on the surfaces of the fibres due to fewer impurities (Figure 1). It was in accordance with the chemical composition analysis in Table 3 showing that residuals were higher in the raw fibres (10.5%) and water retted fibres (6.9%) as compared to the fibers retted with *P. radiata* Cel 26 (4.9%) and *C. subvermispota* (4.3%).

3.3 Pectin distribution in the fibres. Pectin was stained using ruthenium red by reaction with carboxylic acid side groups and shown as a red colour in Figure 2. Both the parenchyma cells and the single fibre compound middle lamellae contained pectin, while the secondary cell wall appeared to lack pectin. It shows that the pectin rich parenchyma cells in the cross section were degraded resulting in smaller fibre bundles. Parenchyma cells have large lumina and will in a composite give high porosity content. The treatment with *P. radiata* Cel 26 resulted in complete detachment of the fibres from the stem surface so fabrication of cross sections was not possible. Therefore an example of stained whole fibres is shown instead.

Pectin is commonly regarded as intercellular glue having important functions in cell growth and differentiation. However, in order to separate fibres from a hemp stem, it is essential to remove pectic contents by selective attack by bacteria and/or fungi. As shown in Figure 2, compared to the raw hemp fibres (Figure 2a), significant amounts of pectin were degraded by water retting (Figure 2b) as well as by cultivation of *C. subvermispota* (Figure 2c) and *P. radiata* Cel 26 (Figure 2d). To be more specific, large amounts of dark red staining materials surrounding white areas are shown in Figure 2a. This indicated that a lot of pectin existed in the raw hemp fibres. A large amount of the same dark red area outside the cell wall existed in Figure 2c, but the vast majority of the dark red area between fibres has been removed compared to Figure 2a. This indicated that a large amount of pectin was degraded by cultivation of *C. subvermispota*.

In Figure 2b, there was also a small area of dark red on the fibre surface. However, there were almost no dark areas remaining around individual fibres while small dark areas appeared on the surface of the fibres (Figure 2d). This result suggests that most pectin was degraded by treatment with *P. radiata* Cel 26, which corresponds to the results discussed in Section 3.1 that the largest pectin loss (82%) was caused by *P. radiata* Cel 26 compared to water retting (77%) and *C. subvermispota* (57%).

Microbial processing of hemp fibres

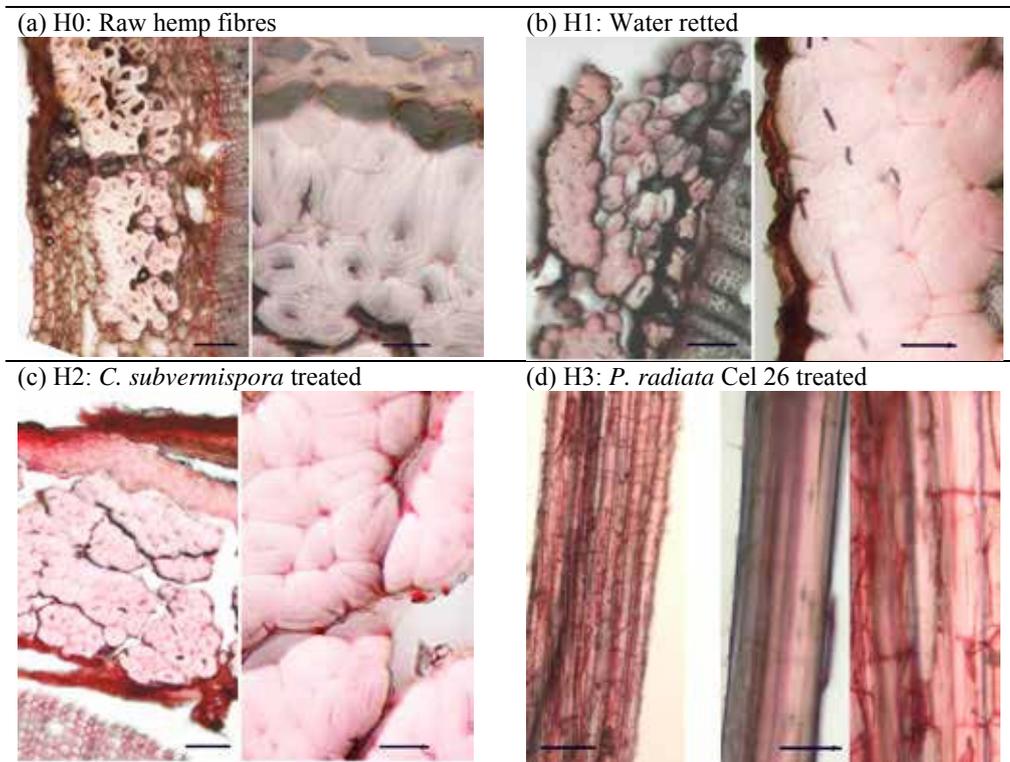


Figure 2. Pectin stained in hemp transverse sections and hemp fibre surfaces using ruthenium red. Scale bars: left side: 200 µm and right side: 50 µm.

4. CONCLUSIONS

1. Compared to water retting and fungal treatment with *C. subvermispora*, fungal treatment with *P. radiata* Cel 26 was most preferable with highest selectivity for pectin degradation. This resulting in 36% hemicellulose, 50% lignin and 82% pectin degradation and a minimal loss of cellulose (14%).
2. SEM microscopy of hemp fibre reinforced composites showed good epoxy impregnation after fungal treatment with *P. radiata* Cel 26 and *C. subvermispora*. In contrast, hemp samples treated by water retting and the raw hemp fibres were badly impregnated due to porosity caused by surface impurities. The surface impurities were in accord with the chemical composition showing that residuals were higher in the raw and water retted fibres as compared to the fibers retted in a controlled fashion with *P. radiata* Cel 26 and *C. subvermispora*.

ACKNOWLEDGEMENTS

Tomas Fernqvist (DTU Chemical Engineering) is thanked for technical support. Dr. Bo Madsen and Dr. Hans Lilholt (DTU Wind Energy) are acknowledged for composite materials related support. The Danish Council for Independent Research is acknowledged for supporting the CelFiMat project (IP No. 12-127446: “High quality cellulosic fibres for strong biocomposite materials”).

REFERENCES

- Akhtar, M., Attridge, M.C., Blanchette, R.A., Myers, G.C., Wall, M.B., Sykes, M.S., Koning, J.W., Burgess, R.R., Wegner, T.H., Kirk, T.K. (1992). The white-rot fungus *Ceriporiopsis subvermispora* saves electrical energy and improves strength properties during biomechanical pulping of wood. In: Kuwahara, M., Shimada, M. (Ed.), *Biotechnology in pulp and paper industry*, Uni Publishers CO., Tokyo, Japan. 5, pp 3-8.
- Daniel, G., Volc, J., Niku-Paavola, M.L. (2004). Cryo-FE-SEM & TEM immuno-techniques reveal new details for understanding white-rot decay of lignocellulose. *C. R. Biol.* 9-10, 861-871.
- Franck, R.R. (2005). *Bast and other plant fibres* (CRC Press, New York) p.185-186.
- Garcia-Jaldon, C., Dupeyre, D., Vignon, M.R. (1998). Fibres from semi-retted hemp bundles by steam explosion treatment. *Biomass Bioenerg.* 14, 251-260.
- Gharpuray, M., Lee, Y.H., Fan, L. (1983). Structural modification of lignocellulosics by pretreatments to enhance enzymatic hydrolysis. *Biotechnol. Bioeng.* 25, 157-172.
- Kantelinen, A., Hatakka, A., Viikari, L. (1989). Production of lignin peroxidase and laccase by *Phlebia radiata*. *Appl. Microbiol. Biotechnol.* 31, 234-239.
- Nyhlen, L., Nilsson, T. (1987). Combined T.E.M. and UV-microscopy on delignification of pine wood by *Phlebia radiata* and four other white rotters. In: Odier, E. (Ed.), *Proc. of Lignin Enzymic and Microbial Degradation Symposium*, INRA Publications, pp. 277-282.
- Rosember, J.A. (1965). Bacteria responsible for retting of Brazilian flax. *Appl. Microbiol.* 13, 991-992.
- Sankari, H.S. (2000). Comparison of bast fibre yield and mechanical fibre properties of hemp (*Cannabis sativa* L.) cultivars. *Ind. Crops Prod.* 11, 73-84.
- Strivastava, L. (1996). Histochemical studies on lignin. *Tappi.* 49, 173-183.
- Thygesen, A., Daniel, G., Lilholt, H., Thomsen, A.B. (2005). Hemp fibre microstructure and use of fungal defibration to obtain fibres for composite materials. *J. Nat. Fibres* 2-4, 19-37.
- Thygesen, A., Thomsen, A.B., Daniel, G., Lilholt, H. (2007). Comparison of composites made from fungal defibrated hemp with composites of traditional hemp yarn. *Ind. Crops Prod.* 25, 147-159.
- Thygesen A, Madsen B, Thomsen AB, Lilholt H (2011). Cellulosic fibres: Effect of processing on fibre bundle strength. *J Nat Fibres*, 8, 161-175.