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Biotechnological Wood Modification with Selective White-Rot Fungi and Its Molecular Mechanisms

Karin Fackler[1,2], Cornelia Gradinger[1,2], Marieke Schmutzer[1,2], Crtomir Tavzes[3], Ingo Burgert[4], Manfred Schwanninger[5], Barbara Hinterstoisser[4], Takashi Watanabe[6] and Kurt Messner[2]*

[1]Competence Centre for Wood Composites and Wood Chemistry (Wood *K plus*), St. Peter Straße 25, A-4021 Linz, Austria

[2]Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/166, A-1060 Vienna, Austria

[3]University of Ljubljana, Biotechnical Faculty, Department of Wood Science and Technology, Rozna dolina, Cesta VIII/34, SI-1000 Ljubljana, Slovenia

[4]Department of Material Sciences and Process Engineering, BOKU – University of Natural Resources and Applied Life Sciences, Peter Jordan Strasse 82, A-1190 Vienna, Austria

[5]Department of Chemistry, BOKU – University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria

[6]Laboratory of Biomass Conversion, Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho Uji, Kyoto 611-0011, Japan

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Summary

Microbial mechanisms of lignin degradation may be utilised for solid-state fermentations other than biopulping, during which the selective conversion of lignin is required. The current paper reviews current work into selective lignin conversion, with emphasis on the contributions made by our research group, which consists of researchers from five different laboratories. Three of them cooperate within Wood *K plus*. The recent research of this group has focussed on fermentations utilising the unique metabolism of selective white-rot fungi to modify wood surfaces during relatively short fermentation times of less than one week and on research into the molecular mechanisms causing these modifications. Lignin degradation by selective fungi (*e.g. Ceriporiopsis subvermispora* and species of the genus *Phlebia*) on the wood surfaces was significant after three days. After seven days the overall lignin content of spruce wood shavings was reduced by more than 3.5 %. Lignin loss was accompanied by an increase of extractable substances. To evaluate small changes and to trace the fungal modification processes, Fourier transform infrared spectroscopic (FTIR) techniques and electron paramagnetic resonance (EPR) spectroscopy were applied and adapted. The spectra recorded in the near infrared region (FT-NIR) turned out to be very useful for kinetic studies of the biopulping/biomodification processes and a good method to evaluate the capabilities of fungi to modify wood surfaces within this short period.

Key words: white-rot fungi, wood, lignin, biomodification, FT-NIR, EPR

*Corresponding author; Phone: ++43 1 588 011 7241; Fax: ++43 1 588 011 7299; E-mail: k.fackler@kplus-wood.at

Introduction

Natural processes that occur during fungal biodegradation of wood may be utilised for industrial purposes and have a great potential for the cellulose-producing and wood processing industries. As the value of wood and other renewable lignocellulosic materials is steadily increasing, fermentations with selective white-rot fungi have great potential for the production of biofuels or cellulose-enriched forage for ruminants (*1*). Fermentations with selective white-rot fungi would serve as a delignifying pretreatment to expose the polysaccharides to a subsequent hydrolytic digestion and would improve the efficiency of biogas or bioethanol fermentation with bacteria or yeasts (*2*,*3*). Besides fermentations of woody substrates using fungi, which degrade or modify the different wood components (cellulose, hemicelluloses and lignin), isolated oxidative enzymes from the same origin may become valuable tools for more specific and targeted chemical reactions in fibre bleaching and fibre modification and have been tested for possible applications in the pulp and paper industries (*4–6*).

In the research of our group, we have focused on selective white-rot fungi, which have been proven in extensive screening programs to be the most appropriate organisms for biopulping fermentations (*7–9*). Contrary to simultaneous white-rot fungi, which degrade all major wood constituents at similar rates and lead to an erosion of the secondary wood cell walls, selective white-rot fungi degrade only lignin and, in later stages, hemicelluloses. A cellulose-enriched fibrous material with intact cellular structures remains (*10*). Although the mechanisms of wood degradation by white-rot fungi have been extensively researched, the molecular level of lignin oxidation and degradation has not been understood in detail so far. It is generally accepted that, due to size exclusion reasons, it is not the lignin-modifying enzymes excreted by the white-rot fungi (lignin peroxidase, manganese peroxidase (MnP), versatile peroxidase, and laccase) that degrade the aromatic hetero-copolymer in sound wood (*11–13*), but rather that highly oxidative low-molecular-mass compounds lead to wood decay (*14*,*15*). It has been proposed by several authors that MnP, which is excreted by most of the white-rot fungi, plays the key role in generating these radical species from unsaturated fatty acids; these radical species, called reactive oxygen species, in turn oxidise the lignin (*16*,*17*).

Mechanisms of Fungal Wood Degradation

Mechanisms of lignin degradation by selective white-rot fungi

Lignin-degrading basidiomycetes excrete a comprehensive and unique set of oxidative enzymes (*18*) such as laccases, and various peroxidases that are able to oxidise or degrade lignin *in vitro*, as well as oxidases, which may provide hydrogen peroxide to the peroxidases (*19*). Of the numerous pathways suggested, the peroxidation of unsaturated lipids (*17*) is the one which is most widely accepted as the lignin depolymerisation mechanism of white-rot fungi: Mn^{2+}, when it is chelated by the white-rot metabolite oxalic acid, is oxidised by manganese peroxidase and H_2O to Mn^{3+} (*20*), which is able to abstract hydrogen from unsaturated lipids to form acyl radicals (*16*). These initial radicals then propagate lipid peroxidation and the formation of peroxyl radicals. Hammel *et al.* (*14*) proposed that, contrary to the unselective hydroxyl radicals, peroxyl radicals abstract electrons only from activated carbons leading to lignin oxidation in preference to cellulose degradation.

Oxidative degradation of polysaccharides by wood rotting basidiomycetes and possible inhibition mechanisms used by selective white-rot fungi

Despite their close phylogenetic relationship (most wood rotting basidiomycetes belong to the Aphyllophorales), brown-rot fungi lack the oxidative enzymes (laccase and peroxidase activities) of white-rot fungi and are supposed to use a catechol- or hydroquinone-driven Fenton reaction to degrade the wood polysaccharides selectively and to modify the lignin (*21–23*). However, hydroxyl radicals were found not only in cultures of brown-rot fungi (*24*), but also in cultures of white-rot fungi (*25*). All prerequisites for the formation of free hydroxyl radicals *via* a Fenton mechanism, namely oxidases that form H_2O_2 from oxygen, iron, and iron reducing agents (*26*), are also present in cultures of selective white-rot fungi, albeit less pronounced than in brown-rot cultures (*27*). The extremely high redox potential of free hydroxyl radicals prevents chemical reactions other than diffusion controlled ones (*28*). As a consequence, they react at the site of their generation, exhibiting similar rate constants with all organic molecules. This mechanism is not consistent with the wood decay pattern caused by selective white-rot fungi.

Lignin biodegradation proceeds *via* a free radical process in the presence of molecular oxygen and transition metals. Reductive radical intermediates such as the semiquinone radical reduce molecular oxygen to produce superoxide, which in turn reduces Fe^{3+} or disproportionates into H_2O_2 and molecular oxygen. Fe^{3+} is also directly reduced by lignin-derived phenols such as guaiacol and catechol. Thus, if the wood-decaying systems of selective white-rot fungi did not have systems to inhibit the iron redox reactions, the formation of the hydroxyl radical, which is a cellulolytic oxidant, would be inevitable. This suggests that the selective white-rot fungi possess extracellular systems that attenuate the production of hydroxyl radicals. Watanabe *et al. (29*) reported that selective white-rot fungi excrete metabolites that are able to inhibit the Fenton reaction (Fig. 1). They isolated alkylitaconic acids (ceriporic acids) in wood cultures of *Ceriporiopsis subvermispora* and demonstrated that the metabolites suppressed production of hydroxyl radicals by the Fenton reaction even in the presence of reductants for Fe^{3+} (*29–32*). Moreover, the metabolites inhibited cellulose depolymerization by the Fenton system at the physiological pH of the fungus (*33*). Gutiérrez *et al.* (*30*) found the same compounds by GC-MS analysis of crude extracts from eucalyptus wood decayed by *C. subvermispora*, *Phlebia radiata*, *Pleurotus pulmonarius*, and *Bjerkandera adusta*. These metabolites are excreted by *C. subvermispora* in the mg/g scale but the production by other fungi is very low.

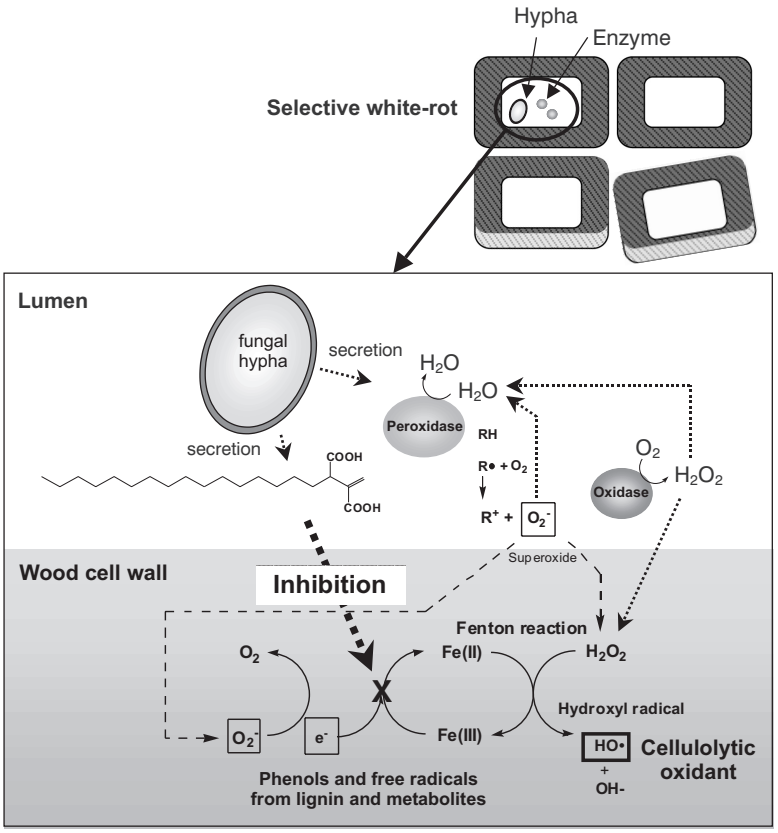

Fig. 1. Proposed mechanism for the inhibition of cellulose degradation during selective lignin oxidation by *C. subvermispora*

An alternative pathway of producing Fenton's reagent Fe^{2+} in wood-degrading fungal cultures is the reduction of Fe^{3+} by cellobiose dehydrogenase (*34*). Interestingly, *C. subvermispora* is not reported to excrete cellobiose dehydrogenase activity, and the selective *Phlebia* strains produce only low levels in solid-state fermentations (*35*,*36*).

FT-IR Techniques to Determine Changes of Wood During Fungal Decay

Fourier transform near infrared spectroscopic (FT--NIR) methods were able to serve as good tools to monitor the small changes in wood chemistry that occur after short biotreatment with wood rotting basidiomycetes, including brown-rot, selective white-rot and simultaneous white-rot fungi, and to predict the lignin content of wood meals and surfaces of solid wood samples (*37–39*). NIR radiation is located between the visible and the mid infrared region (MIR) of the electromagnetic spectrum. Absorbance bands in this spectral region are derived from overtones and combinations of molecular vibrations, which have their fundamental vibrations in the mid infrared region. One of the characteristics of NIR spectra is their relatively unstructured shape caused by highly overlapping bands, at least when compared to MIR spectra. This inconvenience can be overcome by the use of chemometric methods such as principal component analysis (PCA) or partial least squares regression (PLSR). To accentuate the spectral differences, which are hardly visible with the naked eye in the log reflectance (log 1/R) spectra, NIR spectra in this paper are plotted in the second derivative mode. When compared to MIR absorptions, NIR signals are weak. Thus reflectance spectra can be recorded directly from wood surfaces with, for example, fibre optic probes and serve as alternatives to the attenuated total reflectance (ATR) and diffuse reflectance (DRIFT) techniques, which are recorded in the mid infrared region.

Delignification of wood surfaces and wood shavings

With NIR, fungal lignin degradation could be monitored on the surface of spruce wood blocks or veneers that had been treated with *C. subvermispora*. Delignification of the surface (0.5–1.0 % decrease) was significant already after three days. Lower lignin contents are reflected in a lower amplitude minimum of the second derivative of the NIR spectrum near 5980 cm^{-1} (*40*). The depth of the fungal wood modification was between 0.6 and 0.8 mm (Fig. 2). The same method was used to assess the modification of wood with several white- and brown-rot fungi, and to follow the kinetics of white-rot degradation of spruce wood shavings. Small differences in lignin content between consecutive cultivation days were found with this method (*39*).

Changes in the water adsorption behaviour of white-rot treated spruce shavings

Hunt *et al.* (*41*) found that, apart from the degradation of lignin, acid groups are introduced into the fibres during biopulping. The acid groups are derived from oxalic acid esters and it was concluded by the authors

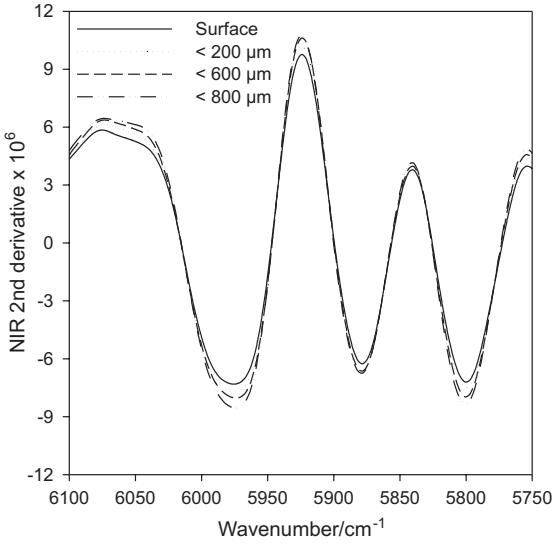

Fig. 2. Second derivative of the NIR spectrum of a spruce wood surface exposed to *C. subvermispora* for three days. The depth of the fungal wood modification does not exceed 800 µm

that these functionalities on cellulose are decisive for the softening of the fibre matrix. This means that the biotreatment leads to energy saving and improved paper quality. Furthermore, they found an increase of the fibre saturation point of fungus-treated wood. An earlier reference reported swelling of the wood cell wall caused by the lytic system of selective white-rot fungi (*13*). During our own studies, we found that two strains of *C. subvermispora* change the sorption behaviour of water to wood. Although milled samples had been dried for several weeks at 50 °C, an NIR band derived from interactions between cellulose and water (near 5550 cm^{-1}) never regained the intensity of the band of native dried wood (*40*). Fig. 3 indicates that these interactions between water and cellulose hydroxyl groups are changed during selective white-rot. However, further research is required to investigate if there is a connection between the findings of Hunt *et al.* (*41*) and the NIR signal derived from adsorbed water found in our studies.

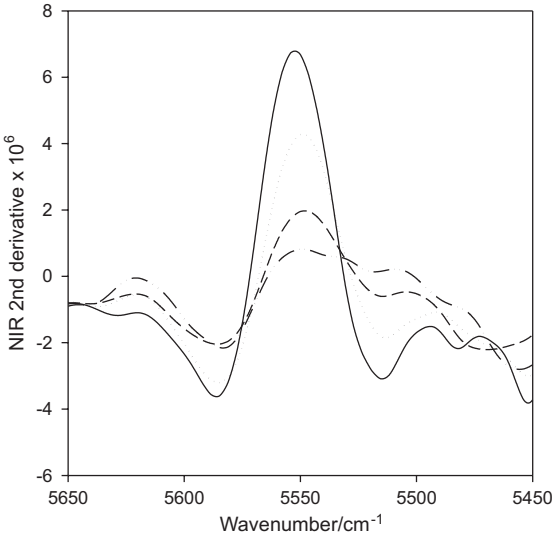

Fig. 3. Second derivative of the NIR reflectance spectrum of spruce wood meal, showing the band that indicates the changing water to cellulose interactions after two weeks of biotreatment with three strains of *C. subvermispora*: CBS 347.42 – solid line, FPL 90.031 – dotted line, FPL 105.752 – dashed line, untreated spruce – dash-dotted line

Stable Radicals and Colour Changes on White-Rot Treated Wood

Formation of free radicals on wood

The awareness that lignin is oxidatively degraded *via* free radical intermediates dates from the 1980s (*42*). Contrary to lignin degradation, the formation of free radicals can be achieved by isolated fungal phenoloxidases (laccases or peroxidases) on wood fibres (*4,43*). The radicals are immobilised on the lignocellulosic matrix and thus have a relatively long life-time of days to weeks when compared to chemically similar but dissolved radicals, because radical-radical coupling is prevented by their restricted mobility. However, when fibres or wood surfaces are brought into close contact as, for example, when they are pressed to fibre boards, radical coupling between the fibres occurs, and strength properties of the boards increase (*43*). We followed the formation of the same radical species after fungal incubation using electron paramagnetic resonance (EPR) spectroscopy and found that the time course of the relative intensities of the signal was consistent with the growth of the fungus on wood, with the excretion of ligninolytic and hemicellulytic activites and with the decrease of the lignin content (Fig. 4).

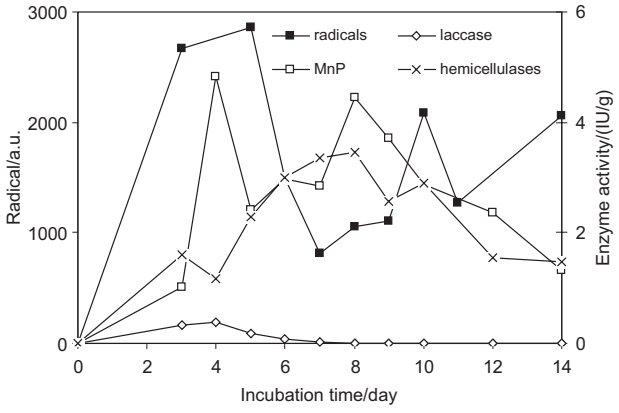

Fig. 4. Excretion of ligninolytic and hemicellulolytic (xylanase + mannanase) enzyme activities compared to the relative intensity of free radicals during biotreatment with *C. subvermispora*

Colour changes of short-time-treated wood

The formation of free phenoxy radicals and the consequent formation of quinoid structures leads to chemical changes that are reflected in the visible reflectance spectra of the wood surfaces. Fig. 5 shows the colour changes of *C. subvermispora*-treated spruce wood samples during the first ten days of biotreatment plotted as coordinate values of the *L*a*b** colour space, where *L** represents the lightness of a colour, *a** represents its redness (positive values), and *b** represents its yellowness

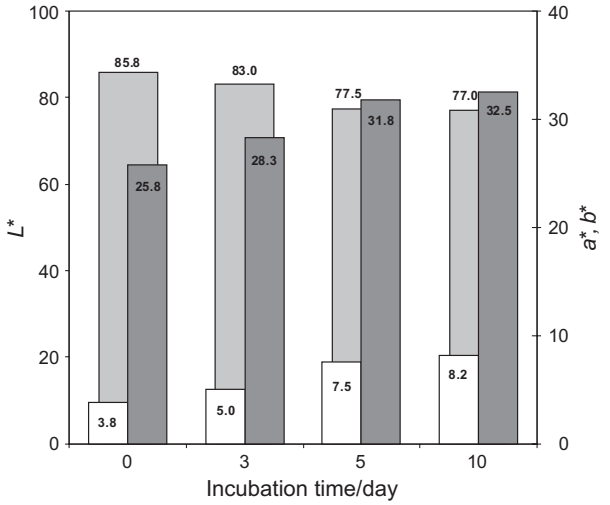

Fig. 5. Changes of the visible reflectance spectrum during biotreatment with *C. subvermispora* expressed as coordinate values of the *L*a*b** colour space

(positive values). The fungus significantly reduces the lightness, and increases the redness and yellowness of the wood surface. Differences are significant after three days; the colour change is completed after five days. Similar reduction of the lightness but lower increase of the redness and yellowness were observed when wood surfaces had been biotreated for ten days with *Phanerochaete chrysosporium* or *Trametes versicolor* (not shown). The colour change is attributed to the formation of free lignin radicals and their decay products (*44*).

Delignification and the Formation of Extractable Wood Components Go Hand-in-Hand

Lignin degradation in incipient stages of colonisation with *C. subvermispora* was found to be initiated by α–β cleavage of the lignin, leading to a depolymerisation of the lignin macromolecule (*45*). Although a set of hemicellulolytic activities was found in the fungal cultures, the degradation of the wood polyoses was not significant in this early stage since the presence of lignin meant that hemicelluloses were not accessible to the enzymes (*46*). In the first three weeks of white-rot delignification, the mass loss of the decaying wood was low or even negligible (*47*). However, lignin loss of spruce wood during the same time determined by wet laboratory methods and by FT-NIR spectroscopy exceeded 7 % after two weeks (*39,40*), indicating that only a small part of the modified lignin had been mineralised. During the short time treatments with different white-rot species, we found that soluble components were formed during biotreatment and that their amount was correlated to the decrease of the total lignin content (Fig. 6). These findings suggest that lignin depolymerisation (*45*) and solubilisation precede lignin mineralisation.

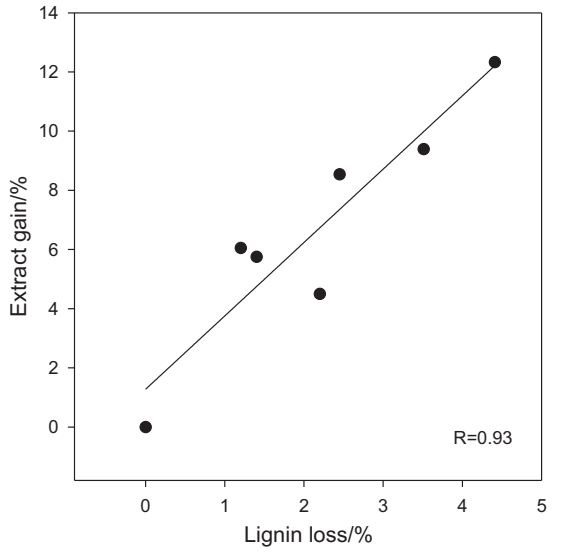

Fig. 6. Gain of total extractives *vs*. loss of lignin after a ten-day treatment with selective white-rot fungi

Fungal Wood Surface Modification Improves the Binding Properties with Aminoplastic Resins

The whole set of white-rot modifications, namely the reduction of the lignin content and its oxidative modification and thus the exposure of wood polysaccharides on the wood surface, as well as the formation of long-lived free radicals, and the increase of the fibre saturation point, are expected to lead to a higher affinity of the wood surfaces to aminoplastic resins. When two small areas of 4 cm² of white-rot modified spruce veneer strips were glued together with resin and exposed to a shearing force in a small scale assay, the shearing force that could be applied was higher when the spruce wood surfaces had been biomodified with the selective white-rot fungi *C. subvermispora* (+17 %), *Phlebia tremellosa* (+21 %), *P. radiata* (+16 %) or *Dichomitus squalens* (+20 %) (Fig. 7). Scanning electron microscopy (SEM) of the surface areas exposed to the shearing force revealed that in the sample biomodified with *C. subvermispora* breaking of the wood cells occurred (Fig. 8A), unlike in the control, where the

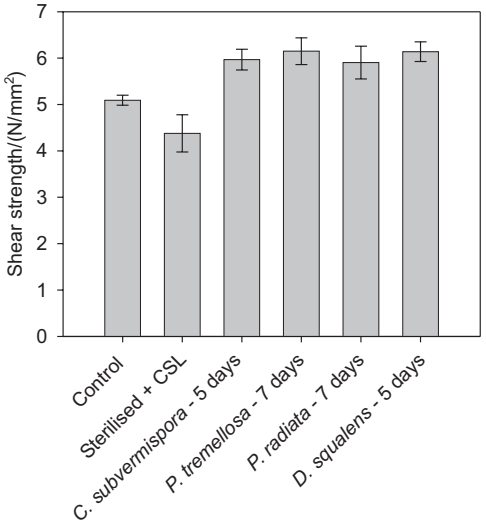

Fig. 7. Shear strength of white-rot modified spruce veneer strips (*C. subvermispora, P. tremellosa, P. radiata, D. squalens*) glued together with urea-formaldehyde resin compared to untreated and sterilised control strips

glue line failed during the mechanical strain (Fig. 8B). Both findings, namely that the shearing strength increases and that breaking does not occur anymore at the glue line but in adjacent wood cell layers, indicate the enhanced interaction between wood surfaces and glue.

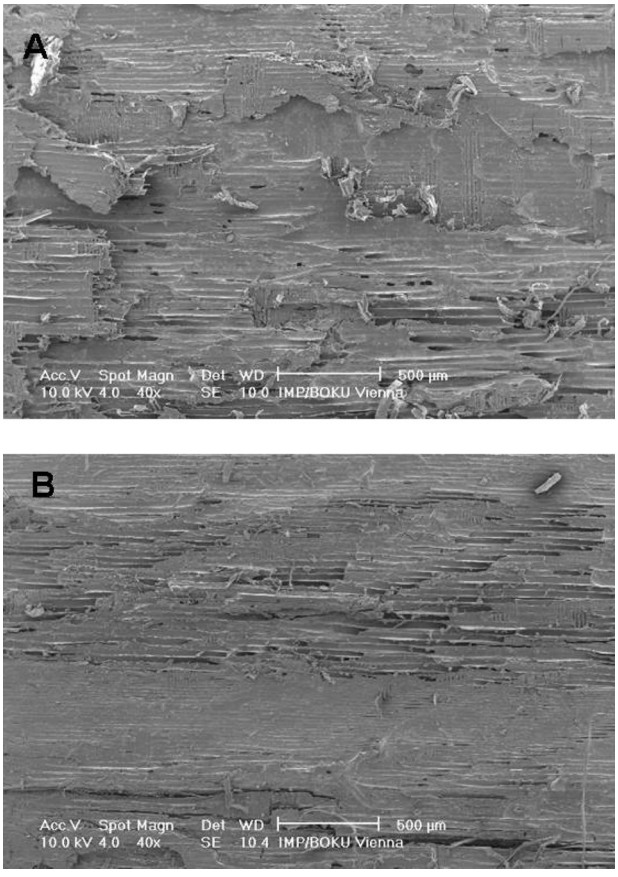

Fig. 8. SEM images of spruce veneer surfaces after exposition to a small scale shearing test: A – *C. subvermispora* modified surface, B – non-treated surface

Overall Evaluation

The molecular mechanisms of selective white-rot fungi offer a series of applications in the field of biotechnology of renewable resources. We focussed on short--term biomodification of wood surfaces to generate a modified raw material for the wood processing industries. During the first week of cultivation with selective white-rot fungi, remarkable changes occur in the wood structure, particularly on the surface. After three days, the colour of the pale spruce surface becomes darker and browner. At the same time, the excretion of ligninolytic and hemicellulolytic enzymes starts and stable radicals are formed in wood (*47*). Delignification of the wood surface becomes significant, and cellulose-water interactions change (*40*). After one week, the total lignin content of spruce wood shavings is reduced significantly by 3.5 % points (*39*), and extractable components are formed at a rate correlated with that of the delignification. Depolymerisation of the wood polysaccharides is not significant in this early cultivation stage (*45*). Hemicelluloly-tic enzymes (xylanase and mannanase) that are excreted in this early phase are not able to access their substrate. The formation of non-selective oxidative low molecular mass agents, such as free hydroxyl radicals generated in transition metal catalysed reactions may be inhibited by the redox silencing effects of alkylitaconic acids excreted by *C. subvermispora* (*29,33*). The whole set of modifications found on spruce wood surfaces treated for five days with *C. subvermispora* or *P. tremellosa* improved the binding capacities to aminoplastic resins, as was demonstrated in laboratory scale shearing tests, and it expands the spectrum of possible industrial applications of these fungi.

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