ADSORPTION OF DIFFERENT LACCASES ON CELLULOSE AND LIGNIN SURFACES

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The adsorption of *Trametes hirsuta* and *Melanocarpus albomyces* laccases on cellulose and lignin model substrates was studied by quartz crystal microbalance with dissipation, QCM-D. The laccase-treated surfaces were also analyzed by atomic force microscopy (AFM) and x-ray photoelectron spectroscopy (XPS). The laccases were found to adsorb at acidic and neutral pHs on both surfaces. The adsorbed amounts increased rather linearly with the change in dissipation when the lignin or cellulose was treated with *T. hirsuta* laccase. Higher adsorbed amounts were obtained using *M. albomyces* laccase. The adsorption of *M. albomyces* laccase on lignin was strongly dependent on pH. At low pH thin and rigid laccase layers were formed and the amount adsorbed was high, while at high pH the laccase layers formed were dissipative and loose and the amount adsorbed was low. A good correlation between the adsorbed amount of laccase and the surface nitrogen content was found. The adsorption of laccases made the surface structure of the cellulose and lignin substrates more granular.

Keywords: AFM; Cellulose; Laccase adsorption; Lignin; QCM-D; XPS

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INTRODUCTION

Wood fibers are a composite material consisting mainly of cellulose, lignin, and hemicelluloses. Cellulose is a linear polymer composed of D-glucose units joined together by β-1,4 glycosidic bonds (Sjöström and Westermark 1999). Lignin, the glue of wood, is an amorphous, branched, and cross-linked disordered aromatic polymer. It is composed of phenyl propane units linked together in irregular manner to form a lignin matrix.

Laccases (EC 1.10.3.2) are multicopper enzymes belonging to the group of blue oxidases. They are widely distributed in nature and mainly found in fungi and higher plants, but laccase activity has also been reported in some insects and bacteria (Thurston 1994; Xu 1999). Laccases catalyse the oxidation of a wide variety of organic and inorganic compounds, typically different phenols with various substitutions (Gianfreda et al. 1999). Four copper atoms are needed for the catalytic activity: the T1 copper, responsible for the blue colour, forming a mononuclear site, and one T2 copper and two T3 coppers forming a trinuclear site (Solomon et al. 1996). In oxidation the substrate loses a single electron and usually forms a free radical. Laccases are capable of

catalyzing the one-electron oxidation of phenolic hydroxyl groups while reducing O_2. This reaction leads into phenoxy radicals and water (Eq. 1, Felby et al. 1997).

$$4\ Phe\text{–}OH + O_2 \xrightarrow{laccase} 4\ Phe\text{–}O^{\bullet} + 2\ H_2O \quad (1)$$

In the laccase-catalysed oxidation of wood fibers, phenoxy radicals are formed in the lignin matrix (Felby et al. 1997). The size of laccase, *i.e.* 60-100 kDa, corresponding to 70 Å × 50 Å × 45 Å, limits the extent of oxidation in pulp applications to the surface of pulp material (Paice et al. 1995; Ducros et al. 1998; Gianfreda et al. 1999; Xu 1999). Due to the high reactivity of the product radicals, either with each other or with a secondary substrate, reactions such as polymerisation and depolymerisation can occur. This radicalisation, *i.e.* activation of fibre surfaces, can be used as the first step in fibre functionalisation aiming at an introduction of desired properties to fibres (Lund et al. 1998; Chandra and Ragauskas 2002; Chandra et al. 2004a,b; Grönqvist et al. 2006). Laccases have also been reported to improve the strength properties of the handsheets made by laccase-treated wood fibres (Mansfield 2002; Chandra et al. 2004b), to reduce the refining energy consumption (Mansfield 2002), and to decrease the surface coverage of extractives in the thermomechanical pulp (Kangas et al. 2007). The use of laccases together with a mediator in bleaching has also been widely studied. Promising results with mediated oxidation have been obtained in pulp delignification (Bourbonnais and Paice 1992; Call 1994; Call and Mücke 1997).

Traditionally, the reactivity of laccases in lignocellulosic fibres has been studied by monitoring the consumption of oxygen in the aqueous pulp suspension with an oxygen electrode and by measuring the number of radicals generated in the pulp with electron-spin resonance (EPR) spectroscopy (Felby et al. 1997; Grönqvist et al. 2003; Lund et al. 2003). Recently, the quartz crystal microbalance with dissipation (QCM-D) technique has been utilized in studying the adsorption of enzymes on solid surfaces (Marx 2003; Stengel et al. 2005; Hamlin et al. 2007; Mazur et al. 2007; Nordgren et al. 2008). QCM-D provides real-time information of bound mass, of adsorption kinetics, and of the properties of the adsorbed layer. Development of model surfaces made from wood polymers, such as cellulose (Kontturi et al. 2006) and lignin (Norgren et al. 2006; Tammelin et al. 2006a) has made it possible to follow the fundamental interactions of such materials. No earlier investigations related to enzyme adsorption on a lignin model substrate by QCM has been reported, whereas a few studies on the action of cellulases on cellulose film by QCM-D have been presented (Ahola et al. 2008; Josefsson et al. 2008; Turon et al. 2008). As mentioned above, laccases have often been applied to modify heterogeneous lignocellulosic pulp fibres. In order to fully understand the interaction mechanisms of laccases, it is very important to know in detail with which kind of component they interact and under what conditions.

The aim of this work was to understand the adsorption behavior of two laccases from the white rot fungus *Trametes hirsuta* and from the ascomycete fungus *Melanocarpus albomyces* on cellulose and lignin model substrates at two different pH levels (4.5 and 7.5), using the quartz crystal microbalance with dissipation (QCM-D) technique. *T. hirsuta* laccase is a high redox enzyme with a T1 copper redox potential of 780 mV (Shleev et al. 2004), while the redox potential of *M. albomyces* laccase is lower

(470 mV; Kiiskinen et al. 2004a). The two laccases also differ clearly in the optimum pH on phenolic substrates, the optimum pH being neutral for the laccase from *M. albomyces* (Kiiskinen et al. 2002) and acidic for *T. hirsuta* (Rebrikov et al. 2006). The effect of laccase treatment on the surface chemistry and surface morphology was studied with x-ray photoelectron spectroscopy (XPS) and with atomic force microscopy (AFM).

EXPERIMENTAL

Materials

Laccases

Two different purified enzymes, *T. hirsuta* laccase (ThL) and *M. albomyces* laccase (MaL) were used in the adsorption experiments. *T. hirsuta* laccase was produced according to Poppius-Levlin et al. (1999) and purified and characterised according to Rittstieg et al. (2002). *M. albomyces* laccase was produced and purified according to Kiiskinen et al. (2002). Enzyme activity was determined using 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) as a substrate (Niku-Paavola et al. 1988). The laccase activity on the QCM-D samples was monitored as a consumption of dissolved oxygen in the presence of ABTS. The measurement was done in a closed vessel with a SensorLink PCM800, using an oxygen electrode (Clark). Thereafter the oxygen consumption was converted into laccase activity. The protein concentration was determined by amino acid analysis for the *M. albomyces* laccase (Aminosyraanalyscentralen, Uppsala University, Sweden) and by using the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, USA) with bovine serum albumin as a standard for the *T. hirsuta* laccase.

Three laccase concentrations were used: 0.1, 1.0, and 5.0 μM. They corresponded to the *T. hirsuta* laccase concentrations of 5.4, 54, and 268 μg protein/ml, respectively and to the *M. albomyces* laccase concentrations of 6.2, 62, and 309 μg protein/ml, respectively. The standard buffer in the QCM-D measurements was 20 mM $NaHCO_3$ adjusted to pH 4.5 and to 7.5 with hydrochloric acid. Deionised water, which had been further purified with a Millipore Synergy UV unit, was used. The inorganic electrolytes were analytical grade.

Surfaces

The QCM-D crystals were AT-cut quartz crystals supplied by Q-Sense AB, Gothenburg, Sweden. The fundamental frequency (f_0) was 5 MHz and the sensitivity constant (C) was 0.177 mg/m^{2}Hz. The crystals were spin-coated with polystyrene by the supplier (Q-Sense, Västra Frölunda, Sweden).

Spruce milled wood lignin (MWL) was supplied by KCL Science and Consulting, Espoo, Finland. Lignin was isolated from Norway spruce (*Picea abies*), using a slight modification of the Björkman method (Björkman 1956), including an ultrasonic extraction step at 15 ºC. Lignin surfaces were prepared by spin-coating a 0.5 % solution of MWL in 1,4-dioxane on the QCM-D polystyrene crystal (Tammelin et al. 2006a). The cellulose surfaces were obtained by depositing trimethylsilyl cellulose (TMSC) onto the polystyrene crystal by using the horizontal Langmuir-Schaefer (LS) dipping technique, as described by Tammelin et al. (2006b). The lignin-coated crystals, as well as the cellulose-

coated crystals after desilylation, were allowed to swell in the buffer solution at the same pH (4.5 or 7.5) as the experiments were made for at least 12 hours before experiments.

Methods

Quartz crystal microbalance with dissipation, QCM-D

Adsorption was studied with a quartz crystal microbalance with dissipation, QCM-D E4, from Q-Sense, Västra Frölunda, Sweden. The QCM-D method allows simultaneous measurement of the adsorbed amount by detecting the changes in frequency, and of the structure and viscoelastic properties of the adsorbed layer, by monitoring the changes in dissipation. The basic principles of the instrument have been described by Rodahl et al. (1995) and Höök et al. (1998). The crystal oscillates at a resonance frequency, f_0, without adsorbate. When the material adsorbs on the surface of the crystal, the frequency decreases to f. If the adsorbed mass is evenly distributed, rigid, and small compared to the mass of the crystal, $\Delta f = (f - f_o)$ is related to the adsorbed mass via the Sauerbrey equation (Sauerbrey 1959).

$$\Delta m_{Sauerbrey} = \frac{C\Delta f}{n} \quad (2)$$

where Δm is the adsorbed mass per unit surface (mg/m^2), n is the number of the overtone used in the measurement, and C is a sensitivity constant (0.177 mg/m^{2}Hz).

After stabilization of the QCM-D instrument and the crystals in the buffer solution, the enzyme solution was added at a specific concentration at a constant and continuous rate of 100 µl/min to the QCM-D chamber at room temperature (20 ºC). The adsorption time of laccases on cellulose and lignin was four hours followed by a one hour rinsing with buffer solution. The laccase activity studies were made using a one hour adsorption time followed by 5 min rinsing with buffer solution.

Atomic force microscopy, AFM

Atomic force microscopy was used to determine the morphology of the laccase-modified cellulose and lignin surfaces. AFM measurements were made with a Nanoscope IIIa Multimode scanning probe microscope, from Digital Instruments Inc., Santa Barbara, CA, USA. The images were scanned with tapping mode in air using silicon cantilevers. At least three areas on each sample were measured. No image processing except flattening was done.

X-ray photoelectron spectroscopy, XPS

X-ray photoelectron spectroscopy measurements were performed with a Kratos Analytical AXIS 165 electron spectrometer, using a monochromated Al Kα X-ray source. The experiments were carried out from dried samples of the QCM-D crystals on at least three different spots of each sample. All spectra were collected at an electron take-off angle of 90° from sample areas less than one mm in diameter. Elemental surface compositions were determined from low-resolution scans. The relative amounts of carbon, oxygen and nitrogen were determined from high-resolution C 1s, O 1s and N 1s spectra, using symmetric Gaussians.

RESULTS AND DISCUSSION

Adsorption of Laccases on Lignin and Cellulose

The adsorption of two laccases, *T. hirsuta* and *M. albomyces*, was studied at two pHs on cellulose and lignin (Fig. 1). After four hours adsorption, the laccase-treated substrates were rinsed for one hour with buffer solution. The laccases adsorbed on both surfaces, which can be seen as a decrease in frequency.

The laccases have been found to interact with lignin (Felby et al. 1997). The pH seemed to strongly affect the adsorption of both laccases on lignin film (Fig. 1A). At acidic pH (4.5) the changes in frequency of *T. hirsuta* and *M. albomyces* laccases were significant, whereas at neutral pH both laccases showed only slight tendency for adsorption. The adsorption rate of *M. albomyces* laccase on lignin at low pH seemed to be higher than the adsorption rates of any other systems on either surface. This further supports a high affinity between lignin and *M. albomyces* laccase at low pH.

On cellulose the changes in frequency of *M. albomyces* laccase were greater than those of *T. hirsuta* laccase, and both laccases adsorbed more extensively at lower pH (Fig. 1B). According to the literature (Kiiskinen et al. 2004b) *M. albomyces* laccase binds effectively to softwood and pure microcrystalline cellulose, whereas *T. hirsuta* laccase was not able to bind to cellulose. However, this study shows that there seems to be a slight affinity between *T. hirsuta* laccase and cellulose.

At low pH the extent of adsorption of both laccases was larger on lignin than on cellulose (note the different vertical scales in the figures). At neutral pH the differences between the laccases were minor. *T. hirsuta* laccase adsorbed similarly on both substrates at pH 7.5, but the adsorption of *M. albomyces* laccase was slightly higher on cellulose.

Rinsing with 20 mM $NaHCO_3$, adjusted to an appropriate pH, was always made after 4h laccase treatment. The frequency in Fig. 1 increased somewhat during rinsing, indicating that the crystal, including the adsorbed matter, became lighter. Either the unbound and loosely adsorbed laccases were rinsed away from the activated surface or some cellulose or lignin structures were detached with desorbed laccase. The former explanation seems to be more reasonable. However, the major part of adsorbed laccase remained on the surface after rinsing.

Conformation of the Adsorbed Layers

The adsorption process of laccases was considered by plotting the change in dissipation (ΔD) as a function of change in frequency (Δf) (called a *D-f* plot), where time as a variable was eliminated. The steepness of the slope of the *D-f* plot describes the softening or packing of the layer structure during the adsorption. Conformational changes can be seen as a curved and irregular plot during an adsorption.

All adsorption experiments were made at three different laccase concentrations. The adsorption of *T. hirsuta* laccase on lignin increased as the laccase concentration increased (Fig. 2). This was the case at both pH levels. The results are in accordance with the finding that the amount of radicals generated in the TMP pulp by *T. hirsuta* laccase increases as the enzyme dosage increases (Grönqvist et al. 2006).

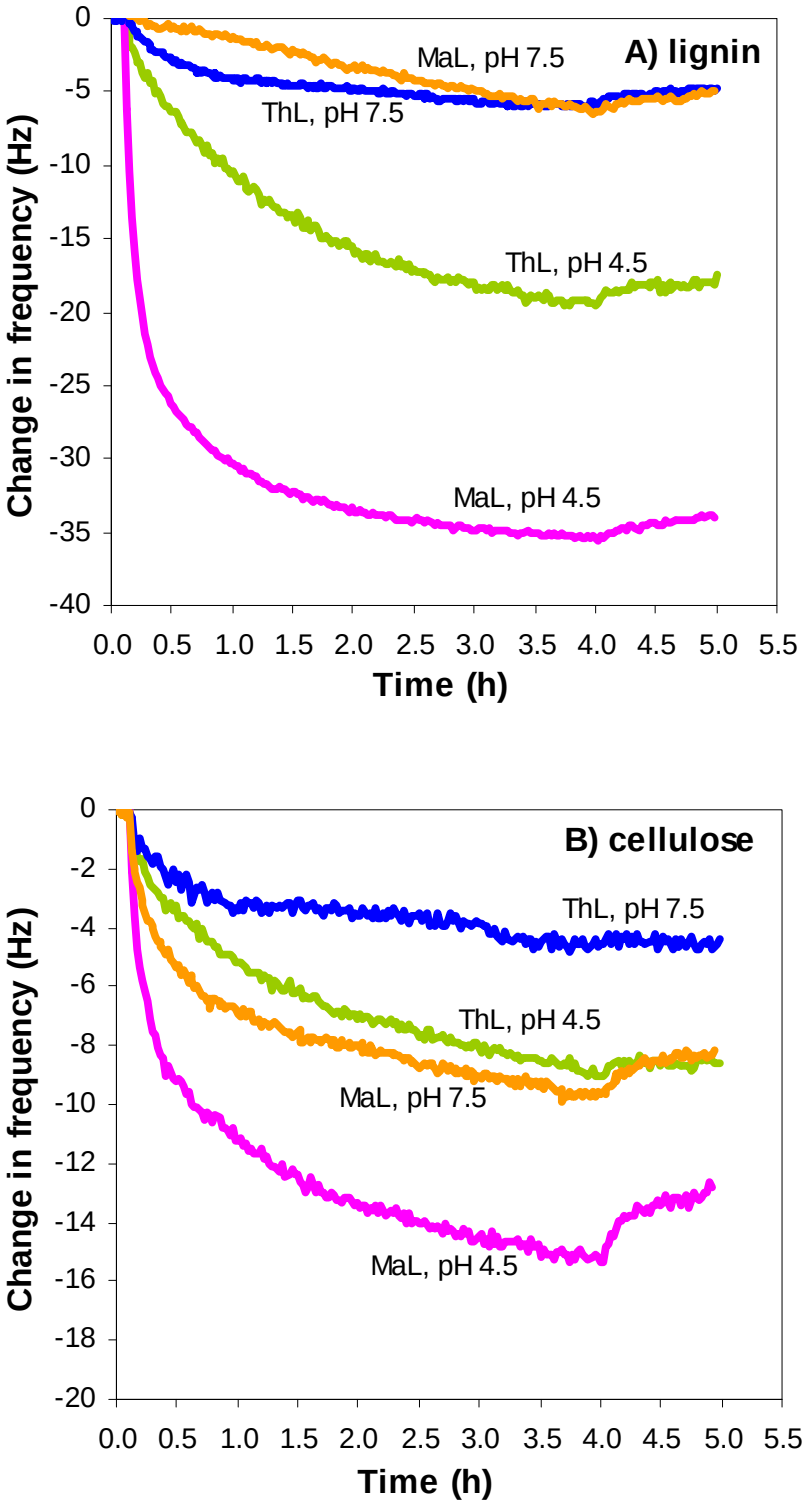

Fig. 1. Change in frequency of the normalized fifth overtone for adsorption of 1 µM *T. hirsuta* and 1 µM *M. albomyces* laccases at pH 4.5 and 7.5 in 20 mM $NaHCO_3$ on A) lignin and B) cellulose. 1 h rinsing with 20 mM $NaHCO_3$ at an appropriate pH was made after 4 h adsorption.

At the beginning of adsorption at pH 4.5, the *D-f* curves were almost linear, meaning that ΔD increased evenly with Δf at all concentrations used on the lignin surface. When the lowest laccase concentration (0.1 μM) was used, the adsorption was almost negligible, but at higher concentrations (1 and 5 μM) the change in frequency increased. As the adsorption proceeded, the laccase layer changed to some extent. The layers became either slightly looser (1 μM ThL concentration) or more compact (5 μM ThL concentration), as seen from the slopes of the curves. At neutral pH (7.5) *T. hirsuta* laccase behaved similarly as at acidic pH (Fig. 2b). The slope of the laccase curves decreased with laccase concentration, indicating more rigid layers. However, the changes in frequency and dissipation were smaller at higher pH. Probably, at low concentration the enzyme molecules that reach the surface attach to the lignin substrate at a few anchoring points, as indicated by the rather high $\Delta D/\Delta f$ ratio. At higher enzyme concentrations, the surface becomes crowded, and it is assumed that the molecules collapse and the layer becomes more rigid, which is observed as a lower $\Delta D/\Delta f$ ratio.

The *D-f* plots of *M. albomyces* laccase on lignin are shown in Fig. 3. The *M. albomyces* laccase adsorbed with a roughly linear relationship between ΔD and Δf on lignin, suggesting that the conformation of the laccase molecules on the surface did not change during the adsorption process. However, the properties of the *M. albomyces* laccase on lignin are highly dependent on the pH used. Small changes in frequency and large changes in dissipation were obtained at pH 7.5, which implies that there were dissipative and loose layers. Especially, the $\Delta D/\Delta f$ curve of 0.1 μM *M. albomyces* laccase was almost vertical, indicating that a small amount of loosely bound laccase, including coupled water inside the laccase layer, was adsorbed. Increasing the laccase concentration increased the adsorbed amount (change in frequency), which also seemed to make the laccase layers more compact. At lower pH (4.5) the behavior was the opposite: the changes in frequency were large, while the changes in dissipation were small, indicating flat and compact laccase layers. The behavior was independent of the laccase concentration used.

When comparing polymeric systems, a low $\Delta D/\Delta f$ ratio describes a compact adsorbed layer and strong interactions between the adsorbed polymer and the surface (Saarinen et al. 2009). If the interactions between the adsorbed layer and the surface are weak, a more loose structure is formed, which can be seen as a higher $\Delta D/\Delta f$ ratio. For laccases, the interpretation of QCM-D data is more complex due to the complexity of their structures. Hence, comparing the adsorption of laccase on lignin at different pH-levels, there are several factors, such as the surface charge of lignin, the charge of laccase, differences in the electrically polarized structure, binding site, etc., that can be changing. At this point we cannot separate the effects of those individual factors on our results. However, it seems to be that at low pH there is a high affinity between *M. albomyces* laccase and lignin, which leads to a rigid layer at all concentrations, whereas at higher pH the interactions are weaker. These adsorption studies are well in accordance with the previous adsorption experiments of *M. albomyces* laccase on lignin model surface (Saarinen et al. 2008).

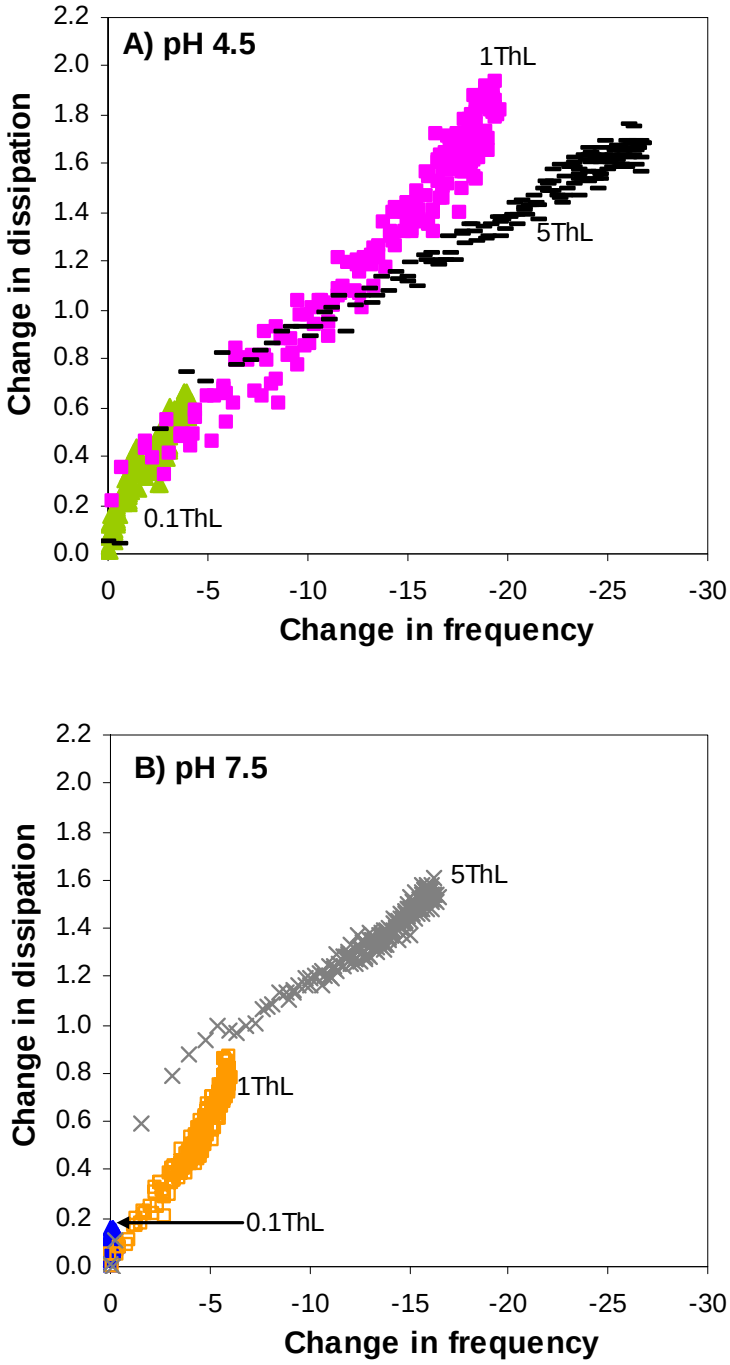

Fig. 2. Change in dissipation as a function of change in frequency of the normalized fifth overtone for adsorption of *T. hirsuta* laccase at A) pH 4.5 and B) 7.5 in 20 mM $NaHCO_3$ after 4h adsorption on lignin. The rinsing step is not shown in the curves.

Generally, the observations that laccase can strongly interact with lignin at the given conditions agrees well with the previous finding of the formation of phenoxy radicals on the substrate by laccases (Felby et al. 1997). In lignin-rich pulps laccases can

be used to activate the surface lignin (Felby et al. 1997; Lund et al. 1998, 2003; Grönqvist et al. 2003), after which specific chemical components can be bound to pulp fibres in order to tailor the wood fibre properties (Chandra and Ragauskas 2001, 2002; Chandra et al. 2004a,b; Grönqvist et al. 2003, 2006). Chemo-enzymatic functionalisation of fibres thus offers possibilities to create new fibre properties. Our recent studies showed that a high amount of ferulic acid could be adsorbed on laccase-activated lignin model surface, supporting the idea of fibre functionalisation with help of laccases (Saarinen et al. 2008). Cellulose microfibres have also been coated with laccase and cationic polyelectrolyte using a multilayer technique (Xing et al. 2007). The added high-charge polycation penetrated between the protein molecules, recharging the surface to cationic. The adsorption of the laccase in these multicomposites was clearly higher than the values obtained in this work, mainly due to the differences in the application technique used.

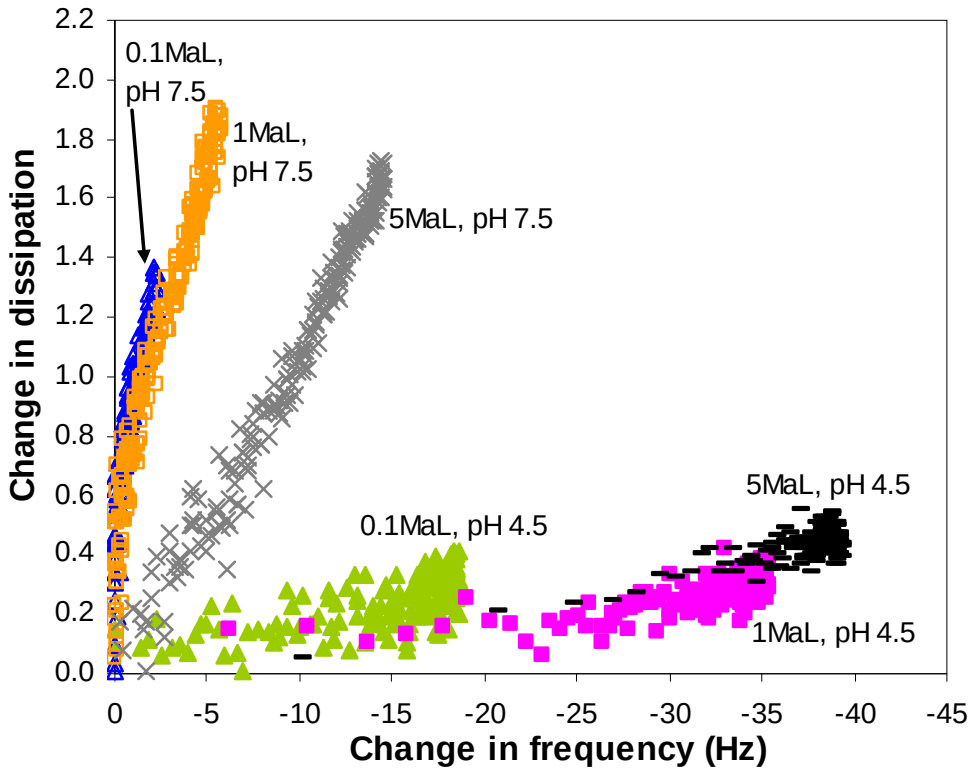

Fig. 3. Change in dissipation as a function of change in frequency of the normalized fifth overtone for adsorption of *M. albomyces* laccase at pH 4.5 and 7.5 in 20 mM $NaHCO_3$ after 4h adsorption on lignin. The rinsing step is not shown in the curves.

On cellulose, the adsorption behavior of *T. hirsuta* laccase was roughly similar to the behavior on lignin, but the changes in frequency and dissipation were smaller (data not shown). The layer structure of *T. hirsuta* laccase was almost unchanged, irrespective of the laccase concentrations. At neutral pH, however, the layers were slightly more compact than at acidic pH.

The *D-f* plot for the adsorption of *M. albomyces* laccase on cellulose is shown in Fig. 4. In this case the adsorption behavior was not linear as it was on lignin. At the

beginning, the *Δf* increased more in relation to *ΔD* at pH 4.5. This implies flat and stiff laccase layers. The slope significantly increased when the laccase treatment proceeded, suggesting a thicker and looser layer and a lower affinity between the laccase and cellulose. At pH 7.5, the *Δf* and *ΔD* were lower than that at pH 4.5 (note the different scale in the figures), and the laccase did not adsorb on cellulose at the lowest laccase concentration (0.1 μM). Increasing the laccase concentration to 1μM increased both *Δf* and *ΔD*, i.e. the curve was more or less linear. At the highest laccase concentration (5 μM) the behavior resembled the behavior of the *M. albomyces* laccase at low pH: first the change in frequency increased more with relation to the *ΔD,* but as the adsorption proceeded to higher values, only the change in dissipation continued to increase, which is seen as a vertical line in the curve.

Comparing the layer structures formed by *M. albomyces* laccase on different lignocellulosic surfaces, the layers seemed to be more dissipative and loose on cellulose than on lignin at low pH (Figs. 3 and 4). At high pH, the concentration of laccase more strongly influenced the layer structure, and the behavior on cellulose was opposite to the behavior on lignin: on cellulose the layers became looser when the laccase concentration increased, whereas on lignin the layers were more rigid at the highest laccase concentration.

In polyelectrolyte adsorptions, the adsorbed layers become more extended as the polyelectrolyte concentration increases (Stemme and Ödberg 1999; Plunkett et al. 2002; Saarinen et al. 2009). The conformation of polyelectrolytes has also been found to change during the adsorption process. It has been suggested that the last polyelectrolytes reaching the surface adsorb in a more extended conformation compared to the first molecules (Plunkett et al. 2002; Saarinen et al. 2009).

Morphological Studies with AFM

All samples measured by QCM-D were also analysed by AFM. The morphological changes were minor, if the laccase-treated substrates were compared to the non-treated cellulose and lignin substrates. Probably the small size of the laccase makes it difficult to distinguish the adsorbed laccases from the surface features of granular cellulose and lignin substrates. In addition, the laccases probably settle, conforming to the uneven cellulose and lignin surface, which makes the detection even more difficult. However, when analysing the lignin samples by AFM, the *M. albomyces* laccase adsorption at pH 4.5 seemed to increase the size of the granules and the rms roughness of the surfaces increased from 0.4 to 0.9 nm. When comparing it to the QCM-D results, the highest adsorption was achieved by adding this laccase at acidic pH to lignin surface. Similar observations have been reported by Mazur et al. (2007), where aggregated laccase (*Cerrena unicolor*) units have been noticed on a smooth gold surface by AFM.

Surface Chemical Analysis

Pure cellulose and lignin consist only of carbon and oxygen, whereas the laccases also have nitrogen in their molecule structure. The nitrogen in the laccase structure enables the study of the adsorption of laccases by XPS.

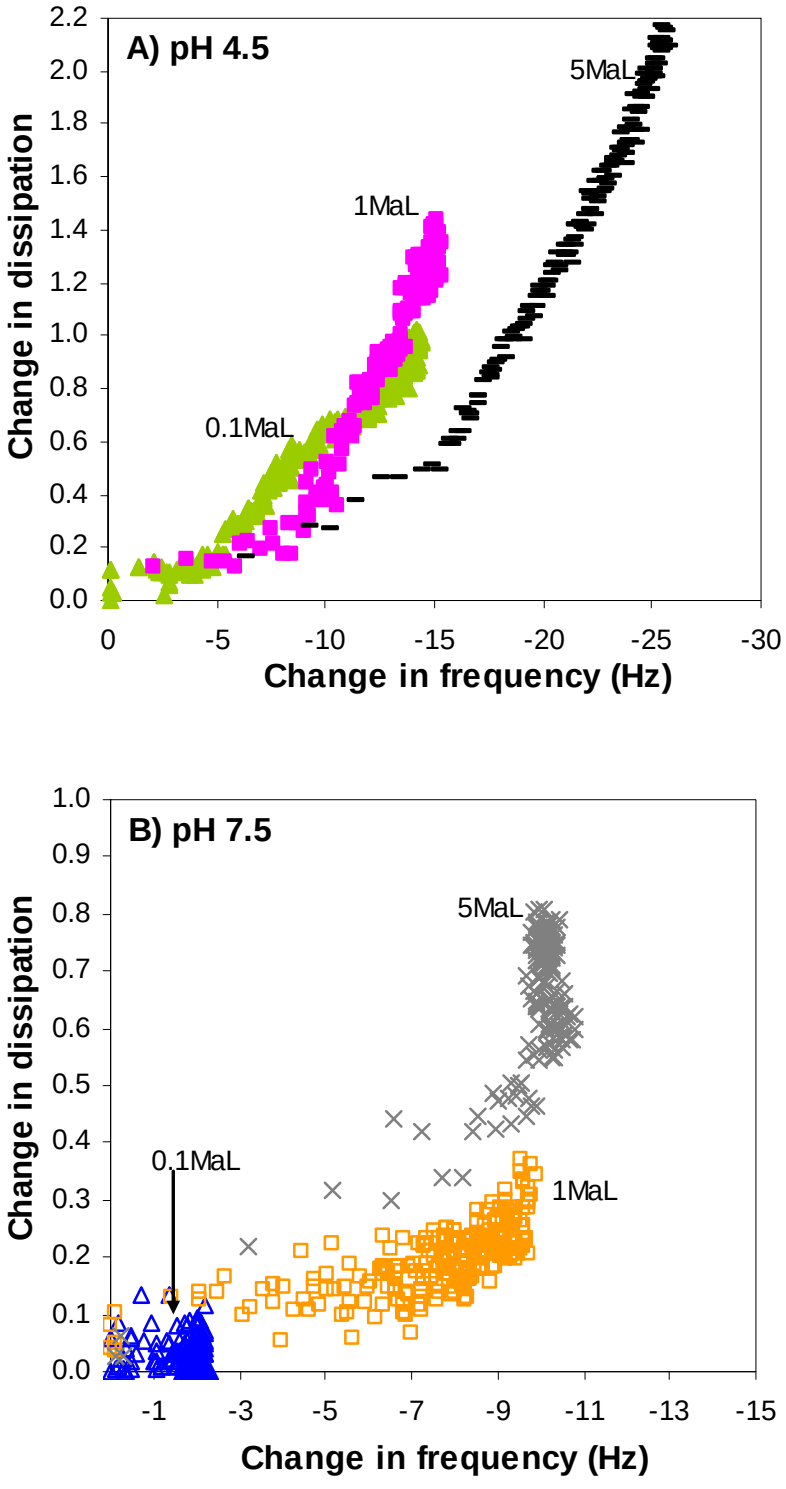

Fig. 4. Change in dissipation as a function of change in frequency of the normalized fifth overtone for adsorption of *M. albomyces* laccase at A) pH 4.5 and B) 7.5 in 20 mM $NaHCO_3$ after 4h adsorption on cellulose. The rinsing step is not shown in the curves.

The nitrogen content analysed by XPS from the dried cellulose and lignin substrates and the adsorbed amount calculated using the Sauerbrey equation (Eq. 2) are shown in Table 1. The Sauerbrey relation between Δf and Δm assumes that the adsorbed layer is rigid and follows the oscillation of the crystal. This means that at high ΔD values, the Sauerbrey equation underestimates the adsorbed mass. In this study, the changes in dissipation of all 1 µM laccase-treated substrates were relatively low ($0.1\text{-}1.7\times10^{-6}$). Therefore, the adsorbed amounts were determined by the Sauerbrey equation.

As expected from the QCM-D measurements, the surface nitrogen contents were clearly lower for cellulose than for lignin (Table 1). The differences seen in the nitrogen contents between the two laccases were minor on the cellulose surface. The only difference in the nitrogen content of the cellulose sample was the slightly lower values at higher pH. On the lignin surface, a clear correlation was seen between the adsorbed amount and nitrogen content. A high adsorbed amount and a high nitrogen content was obtained, especially at acidic pH (4.5). It has to be pointed out that the structural differences between the two laccases that were studied influence not only the amount adsorbed (change in frequency), but also the conformation of the adsorbed laccase layer and, hence, the amount of coupled water. Furthermore, differences in the nitrogen content between these two laccases influence the ratio between the adsorbed amount and nitrogen content. The nitrogen content of the pulp has also been found to increase when bleached TMP was treated with *T. hirsuta* laccase (Grönqvist et al. 2006).

Table 1. Nitrogen Content Analysed by XPS.

Laccase	pH	Adsorbed amount (mg/m^2)		Nitrogen content (%)	
		cellulose	lignin	cellulose	lignin
-	4.5	-	-	0	0.13
-	7.5	-	-	0.11	0.15
T. hirsuta	4.5	1.52	3.22	0.72	3.11
T. hirsuta	7.5	0.81	0.84	0.41	0.46
M. albomyces	4.5	2.30	6.02	0.65	7.41
M. albomyces	7.5	1.45	1.96	0.37	1.96

The amount adsorbed was calculated from Eq. 2 for adsorption of 1 µM *T. hirsuta* and 1 µM *M. albomyces* laccases at pH 4.5 and 7.5 in 20 mM $NaHCO_3$ on cellulose and lignin. The adsorption time of laccases was 4 h followed by 1 h rinsing with 20 mM $NaHCO_3$ at an appropriate pH.

The reason for the different adsorption behavior between the two laccases at different pH-levels on cellulose and lignin is not clear. Electrostatic and hydrophobic interactions between protein and surface are believed to play a major role in the protein adsorption phenomena (Holmberg 2002). The cellulose substrate has been found to be uncharged at pH 4.5, while at neutral pH the cellulose becomes charged due to an increased number of dissociated carboxylic groups (Österberg and Claesson 2000). For lignin film the pH variations between 4.5 and 7.5 has not been found to affect the charge density (Notley and Norgren 2006). On the other hand, lignin is more hydrophobic than cellulose. The theoretical isoelectric points for *M. albomyces* and *T. hirsuta* laccases are 4.9 and 4.7, respectively (ExPASy ProtParam tool, Gasteiger et al. 2005), meaning that the laccases are negatively charged at pH 7.5. Based on consideration of the hydrophobic interactions, the maximum adsorption would occur around the isoelectric point of the protein, and an increase in pH would weaken the hydrophobic interactions (a lower

adsorption). The electrostatic repulsion becomes stronger at higher pH, leading into a lower adsorption. Thus, both the electrostatic and hydophobic interactions would explain the higher adsorption of both laccases at pH 4.5 and the differences obtained between the lignin and cellulose surface.

The differences in the specific activity of the laccases and in the redox potential might play a role in their different modes of action. The about five times higher specific activity of *T. hirsuta* laccase than *M. albomyces* laccase and significantly higher redox potential of *T. hirsuta* laccase (780 mV for ThL and 470 mV for MaL) might affect the different adsorption behavior. This study also confirmed that the laccases are highly surface-specific and the interactions are different, depending on the substrate. These findings are important for clarifying the attachment of laccases on solid substrates and for utilizing the information on enzyme adsorption in various industrial applications. Not only does the amount adsorbed, but also the layer structure of the laccases on lignocellulosic surfaces influence the activation and further the possible chemo-enzymatic bonding of different compounds on pulp fibres (Grönqvist et al. 2006; Saarinen et al. 2008). The combination of QCM-D, AFM, and XPS offer a great potential to clarify the adsorption of laccases on model lignocellulosic surfaces. Further research is, however, needed to explore the effect of the structural differences of the enzymes on their attachment to model lignocellulosic substrates and further, to wood fibres.

CONCLUSIONS

1. Both laccases, *T. hirsuta* and *M. albomyces*, adsorbed at acidic and neutral pH-levels on both cellulose and lignin model surfaces.
2. Generally the adsorbed amounts were larger using *M. albomyces* laccase, lower pH (4.5), and lignin as a substrate.
3. The properties of *M. albomyces* laccase on lignin were highly dependent on the pH used. At neutral pH the laccase layers were loose, while at lower pH thin and rigid layers were obtained.
4. There was a good correlation between the adsorbed amount of laccase and the surface nitrogen content.

ACKNOWLEDGMENTS

This work was funded by Tekes (Finnish Funding Agency for Technology and Innovation), Hansaprint Oy, Tervakoski Oy, Ciba Specialty Chemicals Oy, Eagle Filters Oy, and Starcke Oy Securities. Financial support was also received from the Commission of the European Communities for the project BIOSYNERGY (038994 – SES6) and EU-NILE, New Improvements for Lignocellulosic Ethanol (Contract number 019882). The authors would like to thank Monika Österberg, Ph.D., for valuable discussions and comments on the manuscript and Ms. Ritva Kivelä, Ms. Heini Virtanen, and Ms. Nina Vihersola for excellent laboratory assistance.

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Article submitted: Oct. 13, 2008; Peer review completed: Nov. 8, 2008; Revised version received and accepted: Nov. 17, 2008; Published: Nov. 20, 2008.