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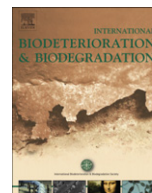

Enzymatic degradation of mould stains on paper analysed by colorimetry and DRIFT-IR spectroscopy

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ABSTRACT

Melanin is chemically and by physical characteristics very similar to lignin, a major constituent of wood, and therefore ligninolytic enzymes of white-rot fungi were tested for their ability to selectively degrade melanin. Melanin degradation was studied both in liquid suspensions of melanin and on melaninised paper samples. Liquid suspension samples were tested for changes in their chemical composition (appearance and relative representation of functional groups and chemical bonds) with FTIR spectrometry. Changes in colour of melaninised paper samples were investigated with a colorimeter. Effectiveness of the treatment (bleaching) was determined as a change in lightness (ΔL). Melanin was oxidised in the liquid suspensions, and the intensity of modification varied depending on the procedure employed. The most pronounced changes in melanin were observed in laccase-1-hydroxybenzotriazole (HBT) treatment at heightened air pressure. The most prominent discoloration of the melaninised paper samples (and no visually detectable damage to the integrity of the paper) was, like in the case of the liquid suspensions, observed after laccase-HBT treatment.

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1. Introduction

Moulds and various wood blue-staining fungi infest many art objects, especially when the artefacts are stored under high-humidity, and they produce the black pigment melanin that causes undesirable stains on or in the objects. Fungal melanins are dark-pigmented (generally black) high molecular weight phenolic polymers found in the cell walls of spores, sclerotia, mycelia, fruiting bodies, or as extracellular polymers formed in the medium around fungal cells, and are synthesised by oxidative polymerisation of phenolic and indolic monomers (Bartnicki-Garcia and Reyes, 1964; Durrell, 1964; Bell and Wheeler, 1986; Takano et al., 1997; Butler and Day, 1998b). They are thought to have a number of natural precursors including 1,8-dihydroxynaphthalene (DHN), g-glutaminyl-4-hydroxybenzene (GBH), catechol, catecholamines, and tyrosine. The most extensively analysed and possibly the most prevalent fungal melanin is often called "DHN melanin," after its immediate precursor monomer DHN (Bell and Wheeler, 1986). This precursor is produced from acetate through several intermediates in the pentaketide pathway (Tokousbalides and Sisler, 1979; Siehr, 1981; Fogarty and Tobin, 1996). These intermediates are secreted across the cell membrane in the surrounding medium, some remaining in the cell wall. Outside the cell, they oxidise or are oxidatively polymerised by phenol oxidase (Carlile and Watkinson, 1994). Synthesis, chemical structure, functionality, and distribution of various melanins in the fungal kingdom were reviewed by Butler and Day (1998b). Issues regarding melanin structure (including proposed schemata) and chemical changes that occur during its abiotic degradation have been proposed and discussed by several researchers (Blois, 1978; Bell and Wheeler, 1986; Koritowski and Sarna, 1990; Kaim and Schewederski, 1994; Jacobson, 2000).

Melanins were for a long time thought to be poorly degraded by enzymes. They were often considered as non-biodegradable polymers and defined on the basis of their supposed inertness and resistance to chemical attack (Prota, 1992). However, contradictory

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reports suggested slow fungal melanin degradation by *Aspergillus fumigatus* (Luther and Lipke, 1980), and Liu et al. (1995) showed more complete degradation of bacterial melanin by a fungus isolated from soil. Rättö et al. (2001) showed that white-rot basidiomycetes outperformed fungi from other taxa to decolorize *Aureobasidium pullulans* melanin *in vitro*. A groundbreaking work by Butler and Day (1998a) showed the ability of *Phanerochaete chrysosporium* manganese peroxidise (MnP) system to degrade melanin.

Major extracellular enzymes of white-rot fungi that can act on lignin directly or indirectly (with low molecular mass charge transfer mediators) are laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) (Kirk and Cullen, 1998). The investigations in enzymatic ligninolytic systems of white-rot fungi were in the past focused on qualitative studies of gene organization, their distribution in fungal genomes, transcription control mechanisms, and expression during fungal infestation of wood (Cullen, 1997; Kirk and Cullen, 1998; Martinez et al., 2004). Furthermore, the numerous investigations on structure and function, and potential biotechnological applications of isolated ligninolytic enzymes have been extensively reviewed (Cameron et al., 2000; Mester and Tien, 2000; Li, 2003). Extracellular ligninolytic enzymes and/or their mediators, which are excreted in the wood by the fungi during the infestation, cause one-electron oxidation of benzene rings in decaying lignin subunits, forming carbon-centred free radicals, which ultimately leads to polymer degradation (Srebotnik et al., 1997; Srebotnik and Hammel, 2000; Cantarella et al., 2003). Synthetic laccase mediators were tested for enzymatic bleaching of cellulose pulps (Bourbonnais et al., 1997). The so far best mediator tested was 1-hydroxybenzotriazole (HBT) (Call and Mücke, 1997).

Even if mould infestation of paper is eradicated, melanin remains attached (or entangled) to or under the surfaces that were previously overgrown by the mycelium. Melanin is an extremely recalcitrant polymer and cannot be removed from the art by the means of classical conservation techniques. In previous studies on enzymatic degradation of mould stains on paper proteases, chitinases, lipases, and glucanases were tested for their ability to break down the fungal cell wall (Bloomfield and Alexander, 1967; Florian and Purinton, 1995). None of these treatments were successful in removing the pigmented fungal structures from the surface of the paper. The study by Nieto-Fernandez et al. (2003) gave the first results on the use of ligninases to digest melaninised fungal spots on paper.

In this article we report on chemical changes induced in melanin as a result of treatment with MnP or laccase-HBT system, and bleaching of fungal melanin stains on paper brought about by exposure to laccase-HBT system. The implications of these findings for applied research in art conservation science are also discussed.

2. Materials and methods

2.1. Chemicals, media and fungal strains

Chemicals were purchased in p.a. grade from Merck, Sigma, and Fluka and used without further purification. Potato dextrose agar (PDA) and Malt extract broth (MEB) were purchased from Difco. Strains of *Amorphotheca resinae* Parbery (syn. *Cladosporium resinae* Vries) and *Trametes versicolor* (L.) were obtained from the fungal collection of the Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana; strains of *Ceriporiopsis subvermispora* (Pilát) and *P. chrysosporium* Burds. from the collection of the Institute of Chemical Engineering, Vienna University of Technology.

2.2. Preparation of mould samples

2.2.1. Liquid DHN melanin suspensions

Mould *A. resinae* was cultured on PDA plates. The inoculum was transferred from the plates into 500 ml Erlenmeyer flasks with 150 ml of nutrient medium (1 l of the medium contained 30 g MEB, 5 g soy peptone and 100 μM $CuCl_2$ (end concentration)). The submerged culture was grown on a non-rotatory shaker at 100 rpm for five days. After the fermentation the extracellular DHN melanin was purified according to a modified procedure described by Liu et al. (1995). The content of Erlenmeyer flasks was filtered through a Büchner funnel (Whatman filter paper #4, Φ 125 mm). The filtrate was adjusted to pH 3 with 6 M HCl, centrifuged for 1 h (8000 rpm, $g = 11\ 500$), the supernatant was discarded, and the melanin was resuspended in distilled H_2O. The suspension was finally adjusted to pH 8 (10 M NaOH).

2.2.2. Melaninised paper

Pieces of autoclaved filter paper (Whatman filter paper #2, Φ 55 mm) were put in Petri dishes, and 5 ml of the above-mentioned (Section 2.2.1) suspension containing mould spores was sprayed onto each piece. In approximately one week the paper was overgrown with the mould (with their hyphae entangled in its structure) and "blackened". Blackened filter papers were dried in an oven for 3 days at 60 °C.

2.3. Treatment procedures

2.3.1. Liquid DHN melanin suspensions

2.3.1.1. Laccase treatments. Extracellular DHN melanin suspension (Section 2.2.1) was aliquoted in 1 ml volumes, centrifuged (30 min, 13.000 rpm, $g = 12.000$) and supernatant was discarded. A melanin pellet was resuspended in 1 ml solutions of Na-malonate (0.25 M, pH 4.5), laccase (1 U ml^{-1}, *T. versicolor*, Fluka BioChemika, lyophilised, 0.83 EU mg^{-1}), and in four instances also 1-hydroxybenzotriazole (HBT) (1 and 4 mM end concentration). One half of the samples were incubated in a pressure chamber (Kambič, Slovenia) to improve solubility of oxygen in the reaction solutions (10 bar). Solutions of only malonate buffer and buffer with adequate HBT concentrations were used as controls. All laccase treatments of melanin in suspension were performed at 30 °C.

2.3.1.2. MnP treatments. Manganese peroxidase was extracted from *C. subvermispora* (MnP activity 0.12 U ml^{-1}, no LiP activity), *P. chrysosporium* (MnP activity 0.016 U ml^{-1}, no LiP activity) and *T. versicolor* (MnP activity 0.016 U ml^{-1}, no LiP activity) cultures grown on Norway spruce (*Picea abies*) sapwood shavings. The shavings were milled in a hammer-mill (Brabender, Germany) and sieved through a variety of sieves (Fritsch, Germany) to achieve a mean particle size between 2 and 5 mm. Three grams of shavings were distributed in glass jars ($V = 320$ ml), were steam-sterilised and 3 ml of inoculum was sprayed as evenly as possible over the surface, achieving approximate wood moisture content of 150%. Activity of ligninolytic enzymes was measured between the third and the fifteenth day of growth of fungal cultures, and the parallels with the highest MnP and no LiP activity were selected for the treatment of the liquid DHN melanin. Samples were incubated for 1 h in 15 ml of extraction buffer (Na-acetate buffer, 20 mM, pH 5,0) on a non-rotatory horizontal shaker (180 rpm) in a cold room (4 °C). Following the incubation, shavings were pressed in a garlic-press to yield between 15.5 and 17 ml of extract. Upon pressing, the extract was centrifuged for 10 min (19.621 g; 4 °C) to obtain a clear solution required for spectrophotometrical measurements. The activity of laccase and MnP in the extracts was determined by measuring the kinetics of ABTS (2,2′-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid)) oxidation (96-well

Dynex Technologies plate-reader, USA; absorption at 420 nm), with a unit defined as formation of 1 μmol $ABTS^{\cdot+}$ per min, and the activity of LiP by veratryl alcohol (VA) oxidation measurement (Shimadzu UV-2101PC, Japan; absorption at 310 nm) (modified procedures of Böhmer et al., 1998). All spectrophotometric measurements were performed at room temperature (25 °C). Lignin degradation products (mono- and oligomers) would distort LiP activity measurement (absorb at similar wavelength as veratraldehyde, the VA oxidation product), therefore the extracts were centrifuged (20 min, 12.092 g) through Centricon (Centricon, USA) membrane filters (cut off 10 kDa) and resuspended to the initial volume before the measurement.

Similar to the experiment with laccase (Section 2.3.1.1), melanin pellets were resuspended in 1 ml solutions of the extracts (800 μl) (or water for the control), Na-malonate (100 μl, 0.25 M, pH 4.5), and $MnSO_4$ (100 μl, 2 mM). Hydrogen peroxide solutions (0.1 and 0.2 M – for extracts of *C. subvermispora*; 0.01 and 0.02 M – for extracts of *P. chrysosporium* and *T. versicolor*) were continuously administered with Cole-Palmer 74900 syringe pump (24 h; 4.17 μl h^{-1}). The molar ratio of H_2O_2 against the units of MnP activity was therefore approximately 1:5 and 1:10.

2.3.2. Melaninised paper

2.3.2.1. Laccase treatment at normal air pressure. Ten melaninised paper samples were put in each of five Petri dishes (∅ 120 mm), into 20 ml of reaction solution (Na-malonate buffer (0.25 M, pH 4.5), laccase (1 EU ml^{-1}, *T. versicolor*, Fluka BioChemika, lyophilised, 0.83 EU mg^{-1}), and HBT (1 mM end concentration)). Samples were removed from one of the Petri dishes in 24 h intervals, carefully washed in distilled water and oven-dried (3 days at 60 °C). Solutions of only malonate buffer and buffer with or without HBT were used as controls. All laccase treatments of melaninised paper samples were performed at 30 °C.

2.3.2.2. Laccase treatment at elevated air pressure. Five Petri dishes (∅ 120 mm) with 20 ml of different reaction solutions (Na-malonate buffer (0.25 M, pH 4.5) (control); buffer + HBT (5 mM); buffer + laccase (1 EU ml^{-1}); buffer + laccase (1 EU ml^{-1}) + HBT (1 mM); buffer + laccase (1) + HBT (5 mM)) were prepared for this experiment. Ten melaninised paper samples were put in each of five Petri dishes, and they were then placed in a pressure chamber (Kambič, Slovenia) to improve solubility of oxygen in the reaction solutions (72 h, 10 bar, 30 °C). After the treatment, samples were removed from Petri dishes, carefully washed in distilled water and oven-dried (3 days at 60 °C).

2.4. Chemical change measurements

Liquid suspension samples were tested for any changes in their chemical composition (appearance and relative representation of functional groups and chemical bonds) with FTIR spectrometry. After the treatment with either laccase or MnP (Section 2.3.1), the solutions were again centrifuged (30 min, 13.000 rpm, $g = 12.000$), the pellet of treated melanin was transferred onto a diamond-coated stick (Perkin Elmer, USA) and thoroughly dried with a fan at room temperature.

Diffuse Reflectance FTIR spectroscopy (DRIFT) measurements of the melanin concentrated on diamond-coated sticks were performed with a Spectrum One (Perkin Elmer, USA) spectrometer using a TGS detector at a spectral resolution of 4 cm^{-1} and expressed in Kubelka-Munk units ([K-M]).

Each diamond-coated stick was measured in fifteen different positions (8 scans per position) and an average spectrum was created using Spectrum ONE (www.PerkinElmer.com) software. These average spectra were unit vector normalized using the Unscrambler software (version 9.5, www.camo.com) in the spectral region between 3800 and 930 cm^{-1} and a linear baseline was drawn between 1900 and 930 cm^{-1}. The degree of melanin modification was estimated by comparing the relative intensity of relevant absorption bands in the fingerprint region of the investigated spectrum. The assignment of bands observed in the spectra to structural components (as determined by other researchers) is provided in Table 1.

2.5. Measurements of colour changes in melaninised paper samples

Changes in colour of melaninised paper samples were investigated with a colorimeter (Microcolor Data Station, Dr. LANGE). Effectiveness of the treatment (bleaching) was determined as a change in lightness (ΔL) in the CIE L^*a^*b system (L^* – lightness values from 0 (absolute black) to 100 (absolute white); a^* – a colour value on red-green axis; b^* – a colour value on yellow-blue axis).

Each melaninised paper sample was measured for its colour before and after the treatment, and the difference in ΔL values indicated the effectiveness of the particular treatment. The lightening of the samples results was expressed in percent of change in ΔL values.

3. Results

3.1. Liquid DHN melanin suspensions

Melanin was oxidised in the liquid suspensions, and the intensity of modification varied depending on the procedure employed. The most pronounced changes in melanin were observed in laccase-HBT treatment at elevated air pressure.

The DRIFT-IR spectrum of isolated melanin from *A. resinae* (Fig. 1) shows a broad absorption band with a maximum between 3460 and 3300 cm^{-1}, where bands assigned to the stretching vibrations of free O–H and N–H occur. Near 2920 cm^{-1} a more differentiated region of stretching vibrations of various C–H groups is situated. In the fingerprint region, the most prominent bands are due to C═O stretching (1738 cm^{-1} due to carbonyls and a strong absorption at 1657 cm^{-1} due to amides – Amide I and/or aromatic C═C double bonds). A weaker Amide II band is found at 1550 cm^{-1}. An Amide III band, expected as a very weak absorption between 1430 cm^{-1} and 1395 cm^{-1} was not found, due to the stronger absorption of various C–H vibrations at 1400 (methylene scissoring) and 1455 cm^{-1} (aliphatic C–H deformations). Phenolic

Table 1
Infrared (FTIR) band identities of DHN melanin spectra. The assignment of bands observed in the spectra to structural components.

Wavenumber (cm^{-1})	Assignment	Source[a]
3440	O–H stretch	2, 3
3300	N–H stretching	2
~2900	C–H stretching in methyl and methylene groups	2, 3
1800–1740	C═O stretching in free carboxylic acids	4
1740–1730	C═O stretching in aliphatic aldehydes, ketones, and carboxyls not conjugated with benzene ring	2, 4
1670–1650	Amide I: C═O stretching in amides Aldehydes, ketones, and carboxyls conjugated with C═C or benzene ring, conjugated quinone structures	2, 4, 5, 6
1550	Amide II: C–N and N–H deformation in amides	2, 4
1480–1350	C–H deformation vibrations of CH_3 and CH_2	2, 4
1240	Phenolic C–O	1
1100–1050	Alcoholic C–O	1

[a] 1 – Bilińska (1996); 2 – Polanc and Stanovnik (1993); 3 – Zink and Fengel (1990); 4 – Gottwald and Wachter (1997); 5 – Bode and Zeeck (2000); 6 – Koritowski and Sarna (1990).

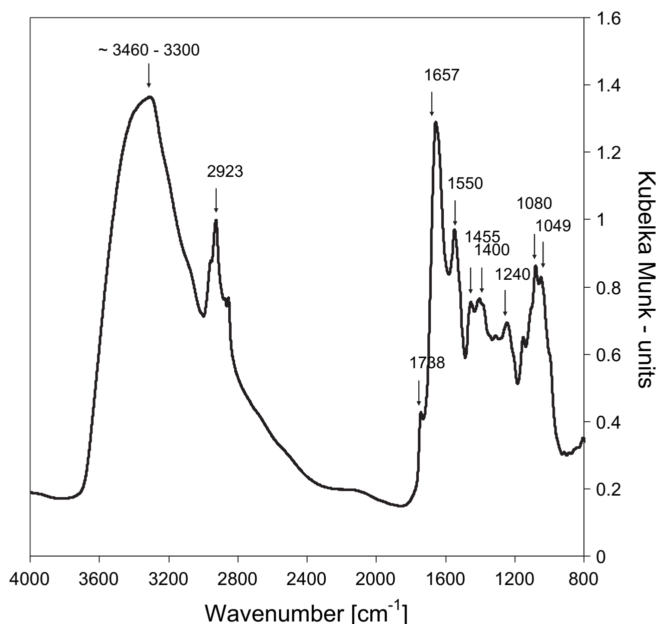

Fig. 1. DRIFT-IR spectrum of isolated melanin from *A. resinae*; assignment of the bands given in Table 1.

groups (C–O) of DHN melanin absorb near 1240 cm^{-1}. Finally a strong region of bands due to C–O deformation vibrations of aliphatic alcohols was found between 1100 and 1050 cm^{-1} (Table 1).

3.1.1. Laccase treatments

At normal air pressure, the most pronounced changes in the FTIR spectra of treated extracellular DHN melanin, compared to the untreated melanin spectrum (Fig. 1), were found in the absorption region around 1755 cm^{-1} at the samples that had been treated with a combination of laccase and HBT (spectra not shown). The highest absorption change was found in the spectra of samples treated with laccase and HBT particularly at the lower concentration of HBT. Control spectra (laccase alone, and buffer-HBT) exhibited their absorbance maximum at wavenumbers near 1740 cm^{-1}. Compared to the melanin spectrum (Fig. 1), the absence of the Amide bands near 1660 and 1550 cm^{-1} was most pronounced. A band, which is not visible in the melanin spectrum, was observed in all samples including the controls (laccase alone, and buffer-HBT) at 1670 cm^{-1}. In the samples treated with laccase and the lower concentration of HBT an additional band at 1645 cm^{-1} was observed. Absorption bands at 1450 cm^{-1} and 1400 were significantly lowest after treatments with any combination of laccase/HBT system, although also the controls showed lower absorption in this spectral region.

At elevated air pressure (further indicated as "O_2") the absorbance changes were even higher: a very pronounced absorption band with its maximum near 1755 cm^{-1} was observed in FTIR spectra of laccase-HBT treated melanin (Fig. 2A–d and e). The controls (laccase-O_2, and buffer-4 mM HBT) exhibited their absorbance maximum near 1740 cm^{-1} (Fig. 2A–b and c). Due to absence of the strong Amide I absorption, a band at 1670 cm^{-1} was visible in all treated samples, a band at 1645 cm^{-1} was only observed in the spectra of samples treated with laccase and HBT. Bands between 1450 cm^{-1} and 1400 cm^{-1} were reduced. Like at normal pressure, the changes caused by the laccase-HBT system were higher at the lower concentrations of the mediator HBT.

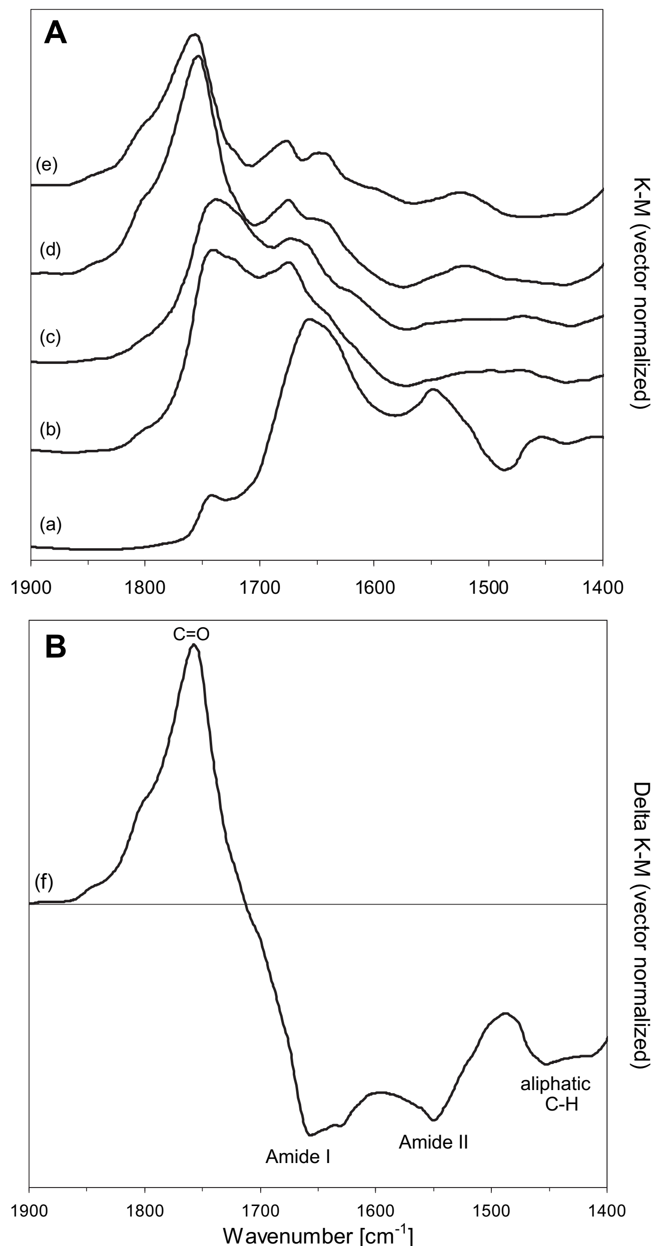

Fig. 2. (A) Vector normalized DRIFT-IR spectra of *A. resinae* melanin (a), melanin treated with buffer-4 mM HBT-O_2 (b), with laccase-O_2 (c) laccase-1 mM HBT-O_2 (d), and laccase-4 mM HBT-O_2 (e) (B) Difference spectrum (e) – (a) to accentuate increases and decreases of IR absorption bands after melanin treatment with the laccase-HBT-O_2 system; O_2 = elevated air pressure (10 bar).

3.1.2. MnP treatments

After treatment of DHN melanin with MnP, contained in extracts of white-rot fungi, in the presence of the high peroxide concentration, the C=O band near 1738 cm^{-1} showed a relative increase in absorbance and became a strong shoulder in the FT-IR spectra, whereas the absorbance maxima at 1080 and 1049 cm^{-1} (C–O of aliphatic alcohols) decreased markedly (Fig. 3A). This change was most pronounced in the *P. chrysosporium* extract (Fig. 3A–e and difference spectrum Fig. 3B) followed by extracts of *C. subvermispora* (Fig. 3A–d) and *T. versicolor* (Fig. 3A–c). The control test, which was conducted at the highest H_2O_2 concentration (Fig. 3A–b), led to only a slight increase of this band. Fungal extracts in the presence of low amounts of peroxide changed this band to

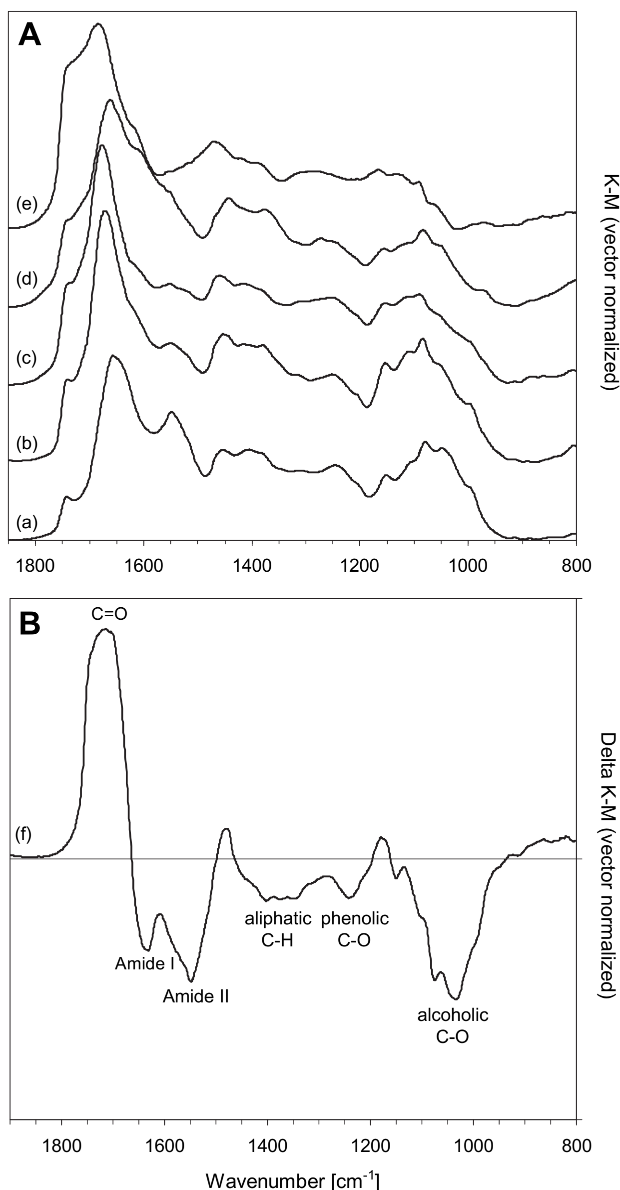

Fig. 3. (A) Vector normalized DRIFT-IR spectra of *A. resinae* melanin (a), of melanin treated with the highest amount of H_2O_2 in aqueous solution (b), and of melanin treated with MnP containing extracts of (c) *T. versicolor*, (d) *C. subvermispora*, and (e) *P. chrysosporium* and H_2O_2. (B) Difference spectrum (e) – (a) to accentuate increases and decreases of IR absorption bands after melanin treatment with MnP containing extract of *P. chrysosporium.*

only a minor extent (not shown). Amide bands (at 1657 and 1550 cm^{-1}) and the absorbance in the aliphatic C–H region between 1450 and 1400 cm^{-1} were also lower in all MnP containing samples, as was the phenolic C–O band near 1240 cm^{-1}. The relative extent of all these changes was in the same order (*P. chrysosporium* > *C. subvermispora* > *T. versicolor*) as described for the band at 1738 cm^{-1}.

3.2. Melaninised paper

The most prominent decolourisation of the melaninised paper samples (and no visually detectible damage to the integrity of the paper) was, like with the treatment of liquid extracellular melanin suspensions, observed after laccase/HBT treatment.

3.2.1. Laccase treatment at normal air pressure

Regardless of the length of exposure, laccase/HBT treatment increased the lightness of the melaninised paper samples. The increase in lightness (decrease of blackness) of melaninised paper samples was 3.39% after 24 h treatment with laccase and HBT. It was raised to 3.78% after 48 h of treatment. The highest increase in lightness of melaninised paper samples (5.10%) was measured after 72 h treatment. At the next two measurements, after 96 and 120 h, the trend of progressive lightening of the samples was reversed. The samples were lightened to 4.76 and 3.56%, respectively (Fig. 4).

3.2.2. Laccase treatment at elevated air pressure

Lightening of melaninised paper samples was achieved regardless of the reaction solution used in this experiment. Treatment with malonate buffer and malonate buffer with HBT (5 mM) lightened the samples for 0.49 and 1.30%, respectively. Laccase only (without the mediator HBT) treatment resulted in lightening of 1.97%, and laccase treatment with HBT (1 and 5 mM) lightened paper samples for 2.69% and 6.65%, respectively (Fig. 5).

4. Discussion

4.1. Liquid DHN melanin suspensions

The DRIFT-IR spectrum of pure melanin is comparable to IR spectra of isolated microbial melanins before acid hydrolysis (Zink and Fengel, 1990). Contrary to synthetic melanins and isolates after acid hydrolysis, the *A. resinae* pigment exhibits strong IR absorptions at bands assigned to amide bound nitrogen, which is not incorporated in pure DHN melanin. Therefore, the isolated pigment of *A. resinae* contains not only DHN melanin but also significant amounts of protein which confirms that fungal melanins are associated with proteins.

4.1.1. Laccase treatments

Laccase-HBT treatments resulted in the most prominent changes of melanin amongst all other treatment procedures in this experiment. This can be due to both high concentration/activity of laccase used in the experiment (compared to the activities of MnP in different MnP treatments of melanin) and efficacy of HBT as laccase mediator (LMS) (Thurston, 1994; Yaropolov et al., 1994; Call and Mücke, 1997; Böhmer et al., 1998; Cantarella et al., 2003). Further optimisation of the procedure was achieved with

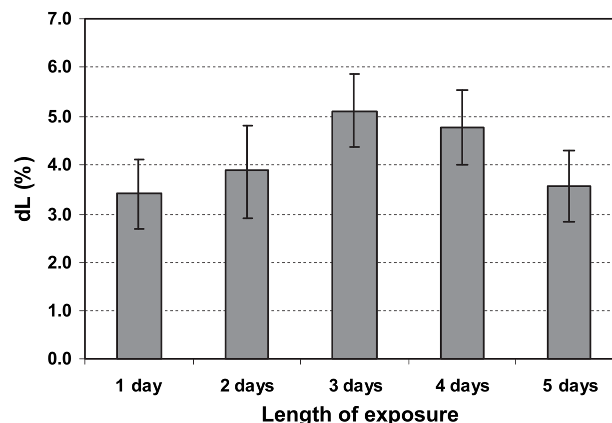

Fig. 4. Laccase treatment of melaninised paper samples at normal air pressure (dL – a change of lightness; error bars indicate standard deviation of ten parallel samples).

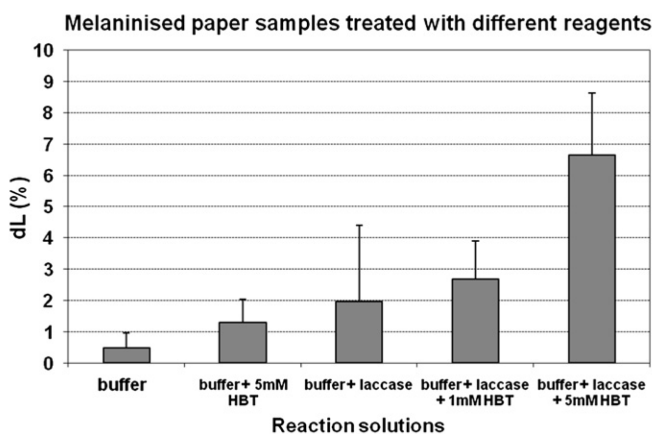

Fig. 5. Treatment of melaninised paper samples with different reaction solutions at elevated air pressure (dL – a change of lightness; error bars indicate standard deviation of ten parallel samples).

heightened air pressure that enabled better dissolution of oxygen in water (oxygen is a co-substrate of laccase).

At normal air pressure, treatment with laccase and the lower concentration of HBT resulted in significant chemical changes of melanin. The changes were not so pronounced if laccase only, or only HBT were used.

Treatment with laccase and HBT caused melanin oxidation: Besides a general absorbance increase of the C=O band, the absorbance maximum was shifted to above 1755 cm^{-1} indicating the increased presence of free carboxylic acid groups. Additionally, the unconjugated carbonylic groups increased as a consequence of the oxidative nature of the laccase-HBT system. At the same time aliphatic structures expressed as aliphatic C–H bands were reduced. However, this could also be due to the reduction of protein content which was also caused by the laccase/HBT treatment. In the absence of the strong Amide I absorption assigned to melanin attached proteins (Zink and Fengel, 1990) absorption bands assigned to carbonyls and/or quinones conjugated to aromatic rings or C=C double bonds at 1670 and 1645 cm^{-1} become visible, which also supports melanin oxidation. These bands are also strongest in the laccase-HBT treatments.

However, the treatment with laccase and the higher concentration of HBT caused much less melanin oxidation at normal air pressure and to some extent also at elevated air pressure. The reason could be on the one hand a too-low oxygen concentration in the reaction solution (oxygen is a co-substrate of laccase) and laccase was able to oxidise only a fraction of the available HBT. Supposedly also unoxidised HBT and HBT oxidation products were in competition with the melanin in redox reactions with the oxidised form of HBT, thus inhibiting the oxidation of melanin.

Dissolution of oxygen in water is much better under elevated air pressure, resulting in higher oxygen concentration in the solution and therefore higher laccase efficiency (Yaropolov et al., 1994; Thurston, 1994). In this case laccase was able to oxidise sufficient quantity of HBT to prevent inhibitive competition of HBT, and the oxidation of melanin was more successful. Oxidation of melanin (as described above for the treatment with laccase and the lower concentration of HBT at normal air pressure) was very pronounced in the treatments of melanin with laccase and both concentrations of HBT used in the experiment with elevated air pressure.

4.1.2. MnP treatments

Perceived lightening of treated solutions suggested chemical changes, as previously reported by Butler and Day (1998a). Indeed, treatment of the extracellular DHN melanin with MnP containing fungal extracts resulted in several chemical changes of the polymer. Although the MnP activity in the extracts of *C. subvermispora* was tenfold, compared to the extracts of *P. chrysosporium* and *T. versicolor*, and the concentration of continuously administered hydrogen peroxide was ten times greater, accordingly, the highest changes were found after treatment with MnP from *P. chrysosporium*. The increase of the absorption band at 1660 cm^{-1}, assigned to carbonyl groups conjugated with an aromatic ring, which was clearly visible only after treatment with the *P. chrysosporium* extract and the high amount of peroxide – in all other treatment overlaps with the protein Amide I band occurred – also pointed to oxidative modification of phenol substituted benzene rings of DHN melanin. The intensity of the absorption bands at 1738 cm^{-1} (assigned to carbonyl groups not conjugated with aromatic ring) also increased. However, unlike in laccase-HBT system, the band shift to higher wavenumbers did not occur, indicating less intensive formation of free carboxylic acids. As a consequence, oxidation resulted in a reduction of bands attributed to aliphatic structures, and alcoholic and phenolic groups. Particularly the decrease of the latter is an indication that the phenolic groups of DHN melanin were oxidised, which led to the formation of carbonylic and quinoid structures.

4.2. Melaninised paper

The treatments of extracellular DHN melanin in reaction solutions showed that laccase/HBT system caused the most prominent changes in melanin composition compared to other treatment procedures in the experiment. Therefore it was decided to test for discoloration of the melaninised paper samples with this system only. Visual inspection of the treated samples indicated lightening, and this indication was confirmed with colorimetry measurements. Therefore it was proven that even an isolated laccase (with the mediator HBT) is able to change the appearance of melaninised paper samples in the desired direction via degradation and oxidation of DHN melanin, the responsible agent (pigment) in aesthetic devaluation of paper. A thorough visual inspection of treated melaninised paper samples did not reveal any significant damage to the integrity of paper that might have happened as a consequence of the treatment, regardless of the procedure employed.

The first set of experiments was performed to establish a meaningful duration of melaninised paper exposure to the action of laccase/HBT system. The highest increase in lightness of melaninised paper samples was measured after three days of treatment, and afterwards the trend of progressive lightening of the samples was reversed. It is believed that laccase was performing optimally in the first three days of the experiment, but it was slowly being inactivated. Laccase is also known to catalyse polymerising reactions of dissolved phenolic compounds in different conditions, and not only depolymerising reactions (Chen et al., 2000; Chandra and Ragauskas, 2002). That could explain loss of lightening in already lightened melaninised paper samples.

The second experiment was an optimisation of the treatment procedure for lightning of melaninised paper using elevated air pressure, resulting in higher oxygen concentration in the solution and therefore higher laccase efficiency. Several reaction mixtures were tested for the ability to lighten melaninised paper. As expected from the results on degradation of extracellular DHN melanin in suspensions, laccase/HBT system in 1:5 molar ratio of HBT against laccase activity proved to be the most effective. The concentration of oxygen diluted in the reaction solution was apparently high enough that laccase was able to oxidise the majority of the available HBT (inhibitive competition of reduced forms of HBT was prevented), and the oxidation of melanin was more successful than in any of the other combinations of reagents.

These procedures look very promising, but they need to be optimised and the integrity (or possible damage) of paper samples after the procedure should be carefully evaluated. After scientifically verifying that the melanin degradation procedure(s) is (are) not causing damage to the paper and that the procedure(s) fulfils paper conservators requirements, the procedure would be available to join the panoply of conservation treatments.

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