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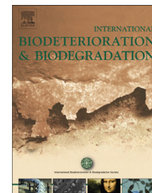

Biomimetic system for removal of fungal melanin staining on paper

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A B S T R A C T

Fungal melanin staining is a problem on many cultural objects, ranging from the French Palaeolithic cave at Lascaux to books and papers in museum collections. Melanin, because it is insoluble and resistant to bleaching, may leave behind undesirable stains long after the fungal infestation has been controlled. Research into removal of melanin stains from paper and other sensitive substrates using industrial biomimetic oxidizing systems has shown considerable success. We studied relative concentration of the bleaching reagents and the reaction kinetics both in liquid suspensions of melanin and on melanized 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 color of melanized paper samples were investigated with a CIE L*a*b system, where the effectiveness of the treatment (bleaching) was determined as a change in lightness (ΔL). Melanin was oxidized in the liquid suspensions, and the intensity of modification depended on the procedure employed. Bleaching of melanin with the biomimetic copper–pyridine complex proved to be far superior to the effect of white-rot fungal oxidizing enzymes, previously reported on by this group.

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1. Introduction

Melanin-producing fungi infest many art objects, especially when the artifacts are stored under conditions of high relative humidity. 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, and fruiting bodies, or as extracellular polymers formed in the medium around fungal cells, and they are synthesized by oxidative polymerization 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), γ-glutaminyl-4-hydroxybenzene (GBH), catechol, catecholamines, and tyrosine. The most extensively analyzed 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 into the surrounding medium, with some remaining in the cell wall. Outside the cell, they oxidize or are oxidatively polymerized by phenol oxidase (Carlile and Watkinson, 1994). Synthesis, chemical structure, functionality, and distribution of various melanins in the fungal kingdom were initially reviewed by Butler and Day (1998b), and more recently by Eisenman and Casadevall (2012). 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; Korytowski and Sarna, 1990; Kaim and Schwederski, 1994; Jacobson, 2000).

Melanins 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 reports suggested slow fungal melanin biodegradation (Luther and Lipke, 1980; Liu et al., 1995; Rättö et al., 2001). Groundbreaking work by Butler and Day (1998a) showed the ability of *Phanerochaete chrysosporium* manganese peroxidase (MnP) system to degrade melanin.

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The biomimetic copper–pyridine complex (Cu–py system) was developed for bleaching of residual lignin in cellulose pulps (Watanabe et al., 1997). In the presence of hydrogen peroxide, highly oxidative reaction intermediates, putatively copper-oxo or copper–peroxo complexes, mimic the reaction of lignin peroxidase with hydrogen peroxide and lignin (Fackler et al., 2001).

Even if fungal infestation of paper is eradicated, melanin remains attached (or entangled) to or under surfaces that were previously overgrown by the mycelia. Melanin is an extremely recalcitrant polymer and cannot be removed from the art by classical conservation techniques. In our previous study on enzymatic degradation of melanin stains on paper, laccase and MnP were tested for their ability to break down the pigmented fungal structures from the surface of the paper, with varying degrees of success (Tavzes et al., 2009). The biomimetic Cu–py–peroxide system, with its relatively small size, has a unique opportunity to deliver oxidizing potential in close proximity to an aromatic polymer, i.e., melanin, even if this polymer is embedded within cellulose fibers of the paper and/or encapsulated in proteinaceous and carbohydrate (chitin) hyphal cell walls.

This article reports on chemical changes induced in melanin as a result of treatment with the Cu–py-peroxide system, and bleaching of fungal melanin stains on paper brought about by exposure to this system. The implications of these findings for applied research in art conservation science are also discussed.

2. Materials and methods

Chemicals were purchased in p.a. grade (equivalent to reagent grade) from Acros Organics, Fluka, and Sigma–Aldrich, and used without further purification. Potato dextrose agar (PDA) was purchased from Difco, and malt extract broth (MEB) and soy peptone from Becton–Dickinson. Strains of *Amorphotheca resinae* Parbery (syn. *Cladosporium resinae* Vries) and *Sydowia polyspora* (Bref. & Tavel) E. Müll. (syn. *Sclerophoma pithyophila* (Corda) Höhn.) were obtained from the fungal collection of the Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia. In all experiments, water of MiliQ quality (miliQ H_2O, Millipore, USA) was used, with the exception of solid growth medium preparation, where distilled water (dH_2O) was used.

2.1. Preparation of experimental samples

2.1.1. Liquid DHN melanin suspensions

The melanin-producing fungus *S. polyspora* 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 $CuCl_2$ [final concentration]). The submerged culture was grown on a non-rotary shaker at 100 shakes per minute for 8 days (28 °C). After the fermentation the extracellular DHN melanin was purified according to a modified procedure described by Liu et al. (1995). The content of the 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 and centrifuged for 1 h (8000 rpm, $g = 11500$), the supernatant was discarded, and the melanin was re-suspended in miliQ H_2O. The suspension was finally adjusted to pH 8 (10 M NaOH).

2.1.2. Melanized paper

Pieces of autoclaved filter paper (Whatman filter paper #2, Φ 55 mm) were put in Petri dishes, and each piece was inoculated evenly with 1 ml of the suspension containing spores of *A. resinae*, prepared by the procedure described above (Section 2.1.1). In approximately two weeks, the paper was overgrown with the melanin-producing fungus (with its hyphae entangled in its structure) and "blackened". Blackened filter papers were dried in an oven for 3 days at 60 °C.

2.2. Treatment procedures

2.2.1. Liquid DHN melanin suspensions

The samples were prepared with the aim of comparing the influence on melanin decolorization of the biomimetic system and its respective components, and different hydrogen peroxide concentrations.

Each of the experimental samples (initial total volume 1 ml) contained 300 μl of the extracellular melanin suspension (Section 2.1.1.). The samples were supplemented by either water (700 μl miliQ H_2O), copper (10 μl 50 mM $Cu(II)Cl_2$) and water (690 μl miliQ H_2O), pyridine and water (100 and 600 μl, respectively), or copper, pyridine and water (10, 100 and 590 μl, respectively) – the complete Cu–py system. Hydrogen peroxide solutions (0.5, 1, or 3%) were continuously administered to the samples with a Cole-Palmer 74900 syringe pump (24 h; 4.17 μl h^{-1}). Residual peroxide in the reaction was measured with QUANTOFIX® Peroxide 100 (Macherey–Nagel) test strips at each measurement point.

Aside from the experimental samples, control (I) samples represented each combination of components, but instead of continuously adding hydrogen peroxide, 100 ml of miliQ H_2O was added once every 24 h. Control (I) samples were used to eliminate the influence of dilution on the results, due to the constant addition of H_2O_2.

Control (II) samples were prepared representing the same combinations of the system components, but without melanin. They were used to determine the contribution of the system and its components to absorbance in the UV–VIS range. The hydrogen peroxide was added to these samples in the same manner as it was to the experimental samples.

2.2.2. Melanized paper

Melanized and control filter papers were cut into squares (10 × 10 mm) and were scanned prior to the decolorization process, to obtain the initial color value of the samples. They were put into Eppendorf tubes (five samples each) containing 1 ml of the biomimetic system or its respective components (either water [1000 μl miliQ H_2O], copper [10 μl 50 mM $Cu(II)Cl_2$] and water [990 μl miliQ H_2O], pyridine and water [100 and 900 μl, respectively], or copper, pyridine and water [10, 100 and 890 μl, respectively]). Hydrogen peroxide solutions (3, 15, or 30%) were continuously administered to the treatment solutions (24 h; 4.17 μl h^{-1}). Residual peroxide in the reaction was measured with QUANTOFIX® Peroxide 100 (Macherey–Nagel) test strips at each measurement point. After the treatment, samples were removed from the Eppendorf tubes, carefully washed in distilled water, and oven-dried (3 days at 60 °C).

2.3. UV–VIS spectrometry of extracellular melanin

UV–VIS absorption measurements of experimental samples, controls (I), and controls (II) were made with a Perkin Elmer - Lambda 2 spectrophotometer (data interval 1 nm, measurement speed 120 nm/min, measurement range 250–800 nm) and data were gathered and processed with "Lambda 2" software (Perkin Elmer, USA). Quartz spectroscopy cells (0.7 ml, Perkin Elmer) were used for the measurements (reference and samples).

Measured absorbance of experimental samples was corrected for the effects of dilution (due to the continuous addition of H_2O_2) using the results of control (I), and the contribution of the system or its components to the absorbance [control (II)] was subtracted.

2.4. Chemical measurements

Liquid suspension samples were tested for any changes in their chemical composition (appearance and relative representation of functional groups and chemical bonds) with diffuse reflectance FTIR spectroscopy (DRIFT). Before and after the treatment with the biomimetic system or its components, 50 μl of the experimental samples, controls (I), and controls (II) solutions were transferred onto separate reflective-flake-covered abrasive pads (Perkin Elmer, USA) and thoroughly dried with a fan at room temperature.

DRIFT measurements of the dried melanin were performed with a Spectrum One spectrometer (Perkin Elmer, USA) using a TGS detector at a spectral resolution of 4 cm^{-1} and expressed as apparent absorbance $log_{10}[1/R]$.

Each reflective-flake-covered abrasive pad was measured in 16 scans (400–4000 cm^{-1}) and an average spectrum was created using Spectrum ONE software (www.PerkinElmer.com). These average spectra were unit vector normalized using Opus software (version 6.0, www.brukeroptics.com) in the spectral region between 1850 and 750 cm^{-1} and baseline-correction using a rubber-band method was drawn in the respective region. The amount 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. Color changes in melanized paper samples

Changes in color of melanized paper samples were investigated with a scanner (HP Scanjet G4050), using Corel Photo-Paint software (Corel Corporation, version 8.232). 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 color value on red-green axis; b* – a color value on yellow–blue axis).

Each melanized paper sample was measured for its color before and after the treatment, and the difference in L values indicated the effectiveness of the particular treatment. For better graphical presentation, L (lightness) values were recalculated into "darkness" values [darkness = 100 – (L/99.6 × 100) % – the value 99.6 presents maximal measured L value (non-melanized, control filter paper samples)].

Table 1
Infrared (FTIR) band identities of DHN melanin spectra. 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– Korytowski and Sarna (1990).

3. Results

3.1. Liquid DHN melanin suspensions

Melanin was oxidized in the liquid suspensions, and the intensity of modification varied on the procedure employed. The most pronounced changes in melanin were observed in the treatment with the complete copper–pyridine system with the continuous administration of 3% hydrogen peroxide.

The first support for this statement is given in Fig. 1, where it is shown that treatments with both pyridine-only and the complete copper–pyridine system yielded a very good decrease of absorption at 450 nm (UV–VIS measurements).

The DRIFT spectrum of isolated melanin from *S. polyspora* (Fig. 2) 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, prominent bands are due to C=O stretching (1738 cm^{-1} due to carbonyls and 1600 cm^{-1} due to amides – Amide I and/or aromatic C=C double bonds). A weaker Amide II band (shoulder) 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 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).

Figs. 2–5 show the chemical changes (measured by DRIFT) that occurred in the melanin during the various treatments. In Fig. 2, the relative decrease of the absorption bands, assigned to O–H, N–H, and C–H bonds, can be observed in the overall mid-IR absorption spectra. The higher the concentration of the continuously administered H_2O_2, the more pronounced differences in the spectra were observed.

Fig. 3 shows the same spectra, but only in the so-called fingerprint region (1800–800 cm^{-1}). It can easily be discerned that the bands in the melanin spectra, assigned to less-oxidized bonds (the region between 1450 and 1350 cm^{-1} – C–H; the region around 1050 cm^{-1} – O–H of primary alcohols), become much less prominent after the treatment with the higher (1% and even more 3%) concentration of the H_2O_2 solution. Additionally, the band assigned to the aromatic ring-conjugated carbonyls (1700 cm^{-1}) is replaced with that

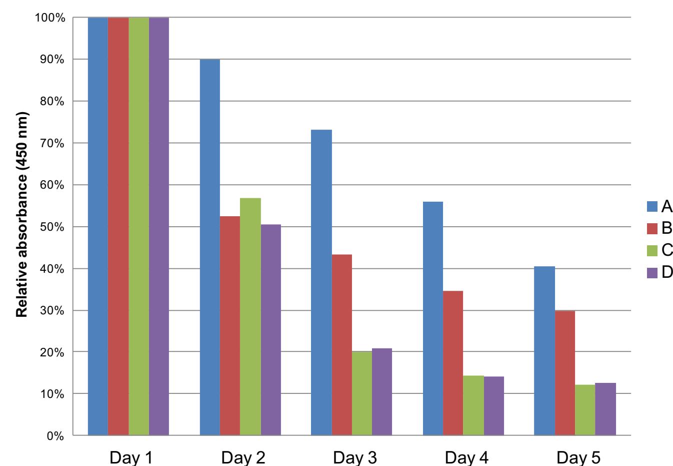

Fig. 1. Relative absorbance (at 450 nm) of extracellular melanin after treatment with (3% solution, 4.17 μl h^{-1}) H_2O_2 only (A), H_2O_2 and copper (B), H_2O_2 and pyridine (C), and H_2O_2 and the complete copper–pyridine system (D).

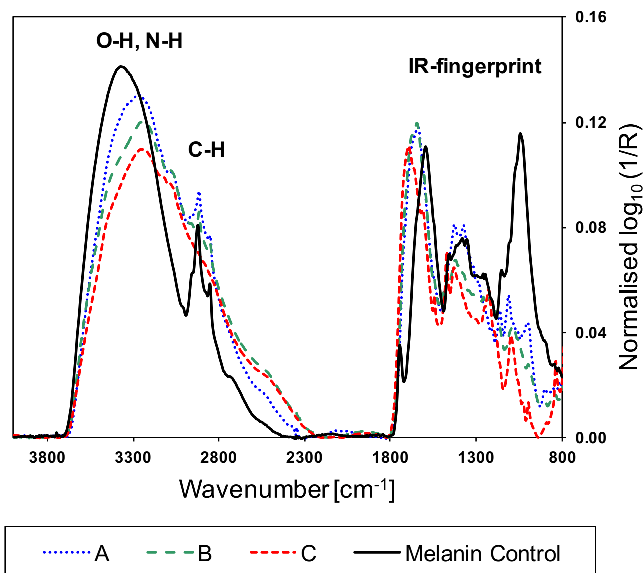

Fig. 2. FTIR (DRIFT) spectra of melanin treated for five days with the complete copper–pyridine system and increasing concentrations of H_2O_2 (0.5% – A, 1% – B, and 3% – C).

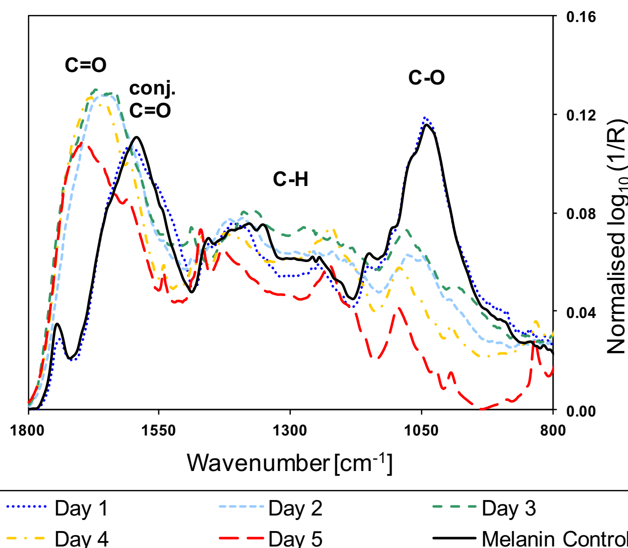

Fig. 4. FTIR (DRIFT) spectra of melanin (fingerprint region), treated with the complete copper–pyridine system and 3% H_2O_2 solution for different time periods (1–5 days).

assigned to non-conjugated carbonyls (1730–1770 cm^{-1}). All these differences in the spectra indicate that a strong oxidation was taking place on the melanin molecules during treatment with the Cu–py biomimetic system and the continuously administered H_2O_2.

It is important to note that measurements of excess H_2O_2 (data not shown) showed that the continuous administration of 3% H_2O_2 solution was optimal, as there was no excess H_2O_2 accumulation measured, and the Cu–py system was fully functional. Overall, even after five days of continuous administration of H_2O_2, the highest concentration of residual H_2O_2 measured was 0.01%, with the typical concentration being 0.003%.

Very similar differences in the treated melanin spectra were observed if the variable in the treatment was not the concentration of continuously administered H_2O_2, but the duration of addition of 3% H_2O_2 (Fig. 4). Again, it is clear that the prominence of the differences in the spectra is correlated to the amount of added H_2O_2.

Fig. 5 shows that when the melanin was treated with continuously administered H_2O_2 and the full biomimetic system, and not with the pyridine alone, the differences in the spectra were much more pronounced. This shows that although the treatment with continuously administered H_2O_2 and pyridine only does cause substantial oxidation (and thus discoloration of the melanin), these changes were much greater when the whole biomimetic system was used (Fig. 5).

3.2. Melanized paper

The superior efficacy of the complete biomimetic system can also be seen in Fig. 6, where the treatment effect on lightening of the melanized paper samples is shown. Compared to treatment in the presence of only pyridine, bleaching of melanin staining appeared after fewer additions of hydrogen peroxide (expressed in days of continuous administration); this happened also when the treatment

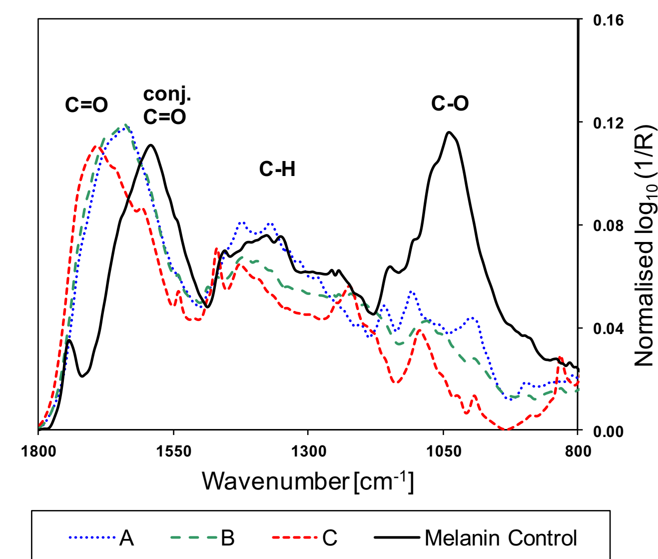

Fig. 3. FTIR (DRIFT) spectra of melanin (fingerprint region), treated for five days with the complete copper–pyridine system and increasing concentrations of H_2O_2 (0.5% – A, 1% – B, and 3% – C).

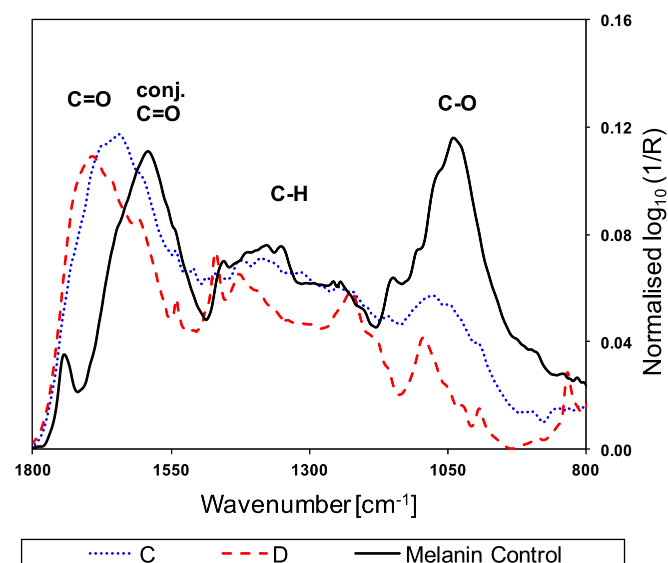

Fig. 5. FTIR (DRIFT) spectra of melanin (fingerprint region), treated with 3% H_2O_2 solution and – pyridine only (C) or the complete copper–pyridine system (D).

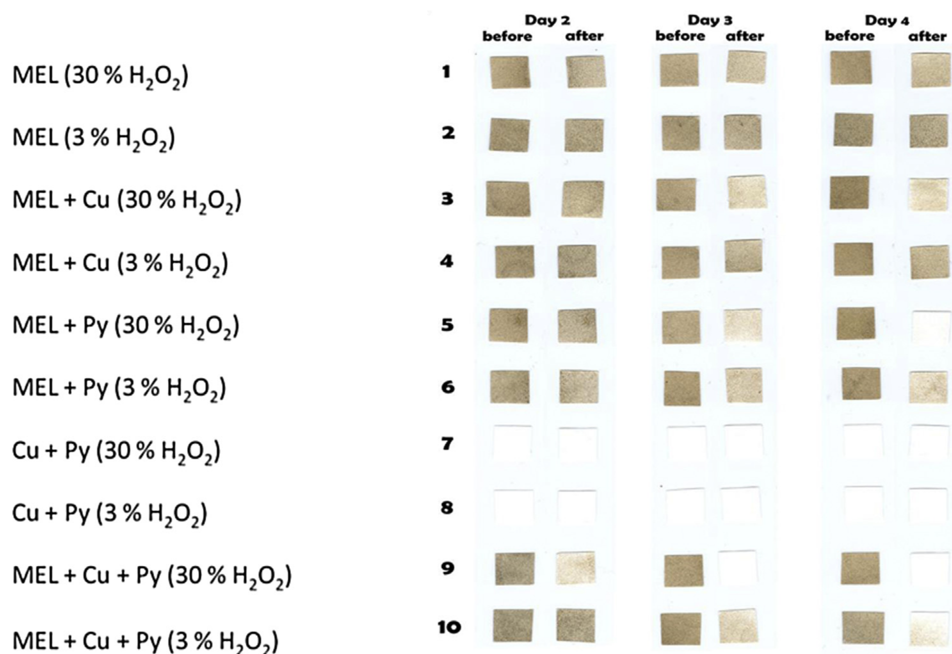

Fig. 6. Melanized paper samples, treated with continuously administered H_2O_2 (3 or 30%) alone (MEL), or in the presence of only copper (MEL + Cu), only pyridine (MEL + Py), or the complete biomimetic system (MEL + Cu + Py); non-melanized paper samples were treated in the presence of the biomimetic system (Cu + Py) as control.

was with a lower concentration of H_2O_2, and was generally much more pronounced. In other samples, the bleaching occurred only at the higher concentration of the oxidant, was much less intense, and took longer to appear (Fig. 6). The treatment did not have any influence on the color of control, non-melanized paper samples.

Melanized paper samples were also measured for color in the CIELAB system, before and after treatment. Lightening of the samples (degradation of melanin staining) was expressed as the percentage decrease of the “darkness” value (the higher the L value, the lighter the sample, the lower the “darkness” value). In Figs. 7–9, it can be seen that the lower concentration (3%) of continuously administered H_2O_2 did not have the desired effect (thorough bleaching of the melanized samples) (Fig. 7). The highest concentration (30%) achieved complete bleaching (Fig. 9), but so did the middle concentration (15%) of continuously administered H_2O_2 (Fig. 8). However, in the latter case, the treatment was more targeted, as pyridine alone achieved less bleaching than the complete biomimetic system. Therefore, treatment with the middle concentration (15%) of continuously administered H_2O_2 in the presence of the complete biomimetic system was chosen as most appropriate for the removal of melanin staining on paper substrates (Fig. 8).

As in the liquid melanin suspensions, measurements of excess H_2O_2 (data not shown) showed that the continuous administration of 15% H_2O_2 was optimal, as there was no excess H_2O_2 accumulation and the Cu–py system was fully functional. The highest concentration of residual H_2O_2 measured in any of the treatment solutions was 0.01%.

4. Discussion

4.1. Liquid DHN melanin suspensions

The prominent decrease of UV–VIS absorbance of the melanin-containing reaction solutions to just above 10% of the initial value indicated that Cu–py system/H_2O_2 treatment caused strong chemical change and/or degradation of the extracellular melanin polymer.

The DRIFT spectrum of *S. polyspora* melanin is comparable to IR spectra of isolated microbial melanins before acid hydrolysis (Zink and Fengel, 1990) and to *A. resinae* pigment (Tavzes et al., 2009). However, it exhibits weaker IR absorptions at bands assigned to amide-bound nitrogen, which is not incorporated in pure DHN melanin, than the melanin of *A. resinae*. Nevertheless, this was sufficient to reconfirm that fungi contain not only DHN melanin but also significant amounts of protein, or that proteins are associated with fungal melanins.

The intensity of the chemical change in the melanin due to the activity of the Cu–py–H_2O_2 system was much higher in the present study than previously reported by this group for enzymatic treatment of this fungal pigment (Tavzes et al., 2009), although a very high efficacy of HBT as laccase mediator (LMS) is known (Thurston, 1994; Yaropolov et al., 1994; Call and Mücke, 1997; Böhmer et al.,

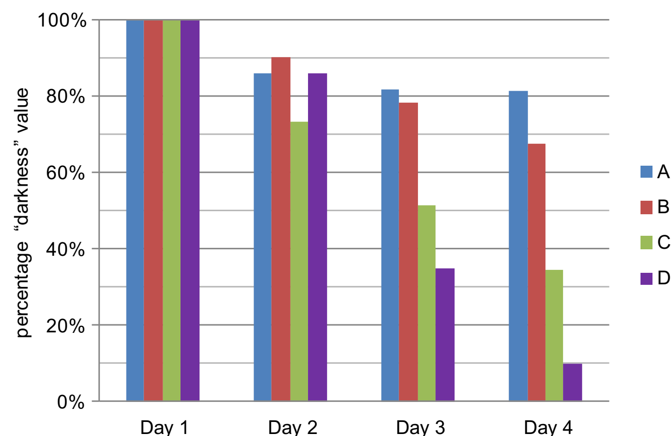

Fig. 7. Percentage decrease of the “darkness” value of melanized paper samples; the samples were treated with continuously administered 3% H_2O_2 solution alone (A), or in the presence of only copper (B), only pyridine (C), or the complete biomimetic system (D).

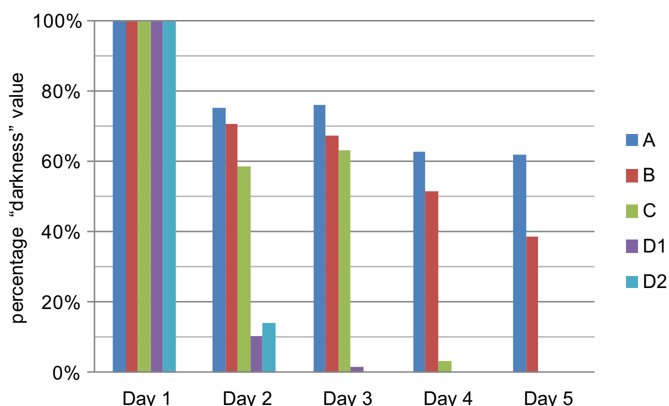

Fig. 8. Percentage decrease of the "darkness" value of melanized paper samples; the samples were treated with continuously administered 15% H_2O_2 solution alone (A), or in the presence of only copper (B), only pyridine (C), or the complete biomimetic system (D1 and D2).

1998; Cantarella et al., 2003). This is true for laccase/HBT treatments (which resulted in more prominent changes of melanin than all other enzymatic treatments), as well as for different MnP melanin treatments.

There are also qualitative differences among the three (laccase, MnP, and Cu–py–H_2O_2) melanin oxidizing systems. Treatment with laccase/HBT caused a general absorbance increase of the C=O attributed band, but the absorbance maximum was shifted to above 1755 cm^{-1}, indicating the increased presence of free carboxylic acid groups. However, unlike in the laccase/HBT system, the band shift to higher wavenumbers did not occur at either MnP (Tavzes et al., 2009) or in the present experiment with the Cu–py–H_2O_2 system, indicating less intensive formation of free carboxylic acids. As a consequence, oxidation by these two systems resulted in a reduction of bands attributed to aliphatic structures, and alcoholic and phenolic groups. This similarity between the MnP and Cu–py–H_2O_2 system in melanin oxidation further indicates that highly oxidative reaction intermediates of the reaction of the Cu–py complex with H_2O_2 mimic the oxidative intermediates of fungal peroxidases (Fackler et al., 2001).

4.2. Melanized paper

As with degradation of the extracellular melanin, the biomimetic system had a much greater effect on melanized paper

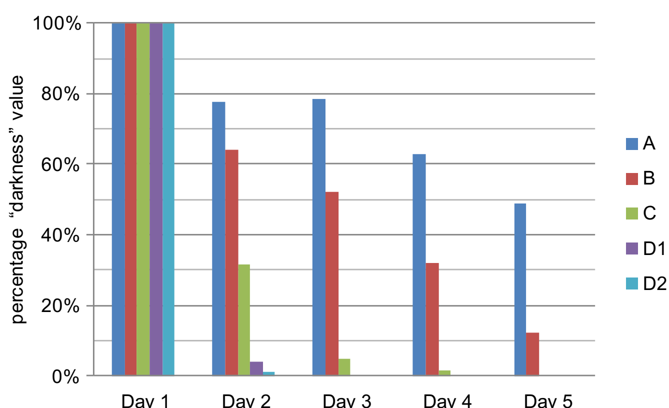

Fig. 9. Percentage decrease of the "darkness" value of melanized paper samples; the samples were treated with continuously administered 30% H_2O_2 solution alone (A), or in the presence of only copper (B), only pyridine (C), or the complete biomimetic system (D1 and D2).

bleaching than did isolated laccase (even with the mediator HBT). Although laccase could change the appearance of melanized paper in the desired direction via degradation and oxidation of DHN melanin, colorimetric measurements with the CIELAB system showed that this change was not higher than 10% of the L value (Tavzes et al., 2009). Conversely, Cu–py–H_2O_2 system treatment easily achieved total lightening of the samples. Furthermore, even separate components of the system accelerated H_2O_2 degradation of the melanin, albeit not as successfully as in the treatment with the complete system.

It is also very encouraging that a thorough visual inspection of treated melanized paper samples did not reveal any significant damage to the integrity of the paper as a consequence of the treatment, regardless of the procedure employed. It was reported previously that the oxidative reaction intermediates are highly selective for aromatic structures, oxidizing aromatic lignin structures selectively and not affecting cellulose (Rahmawati et al., 2005). The near-neutral conditions (pH ca. 8.0) and ambient temperature used in this experiment also prevent swelling of the cellulose and end-group oxidation [peeling reactions (Fackler et al., 2001)]. However, even in conditions similar to those used in paper pulp processing, the biomimetic copper–pyridine complex did not damage the cellulose molecules as significantly as other processes (Watanabe et al., 1997).

5. Conclusions

The Cu–py–H_2O_2 treatment procedure looks very promising for the removal of fungal melanin staining on paper, but it needs to be optimized and paper integrity, possible oxidative damage to paper samples, and degree of polymerization of cellulose (and other indicators of possible degradation) after the procedure should be carefully evaluated. After scientifically verifying that this melanin degradation procedure does not cause damage to the paper and that it fulfils paper conservators' requirements, the procedure will be further developed. These procedures could be readily tested also for applications on other materials such as stone (no dissolution of stone expected due to near neutral pH of the reaction solutions), wood, and textiles, all of which may be negatively affected by classical conservation procedures for melanin staining removal.

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