Chemistry Europe
European Chemical Societies Publishing

WILEY VCH

Excellence in Chemistry Research

Announcing our new flagship journal

- Gold Open Access
- Publishing charges waived
- Preprints welcome
- Edited by active scientists

Meet the Editors of *ChemistryEurope*

Luisa De Cola
Università degli Studi di Milano Statale, Italy

Ive Hermans
University of Wisconsin-Madison, USA

Ken Tanaka
Tokyo Institute of Technology, Japan

ChemCatChem

The European Society Journal for Catalysis

Chemistry Europe

European Chemical Societies Publishing

Supported by

GECATS GERMAN CATALYSIS SOCIETY

Accepted Article

Title: Melanin, a fungal photosensitizer for cellulose oxidizing AA9-LPMO enzymes

Authors: Antonielle Monclaro, Thiago Gonçalves, Silvia Magri, Justin Ovaert, Davide Decembrino, Damien P. Debecker, Marco Kadowaki, Thomas Doneux, Gaël De Leener, Marco Zarattini, Michel Luhmer, and David Cannella

To be cited as: *ChemCatChem* **2023**, e202300602

Link to VoR: https://doi.org/10.1002/cctc.202300602

WILEY VCH

10.1002/cctc.202300602

WILEY-VCH

RESEARCH ARTICLE

Melanin, a fungal photosensitizer for cellulose oxidizing AA9-LPMO enzymes

Antonielle Monclaro[a]¥, Thiago Gonçalves[a]¥, Silvia Magri[a], Justin Ovaert[a,c], Davide Decembrino[a], Damien P. Debecker[c], Marco Kadowaki[a], Thomas Doneux[d], Gaël De Leener[b], Marco Zarattini[a], Michel Luhmer[b] and David Cannella[a]*

[a] Dr. A. Monclaro, Dr. T. Gonçalves, S. Magri, Dr. M. Kadowaki, J. Ovaert Dr. M. Zarattini, Dr. D. Decembrino, Prof. D. Cannella
Photo-Biocatalysis Unit – CPBL, Biomass transformation Lab, Brussels Bioengineering School, Université libre de Bruxelles, Av. FD Roosevelt 50, 1050 Brussels (Belgium)
* Corresponding author, e-mail: david.cannella@ulb.be
¥ Equally contributed
[b] Dr. G. De Leener, Prof. M. Luhmer
Laboratoire de Résonance Magnétique Nucléaire Haute Résolution (RMN-HR), Centre d'Instrumentation en REsonance Magnétique (CIREM)
Université libre de Bruxelles – ULB, 1050 Brussels (Belgium)
[c] Prof. D.P. Debecker
Institute of Condensed Matter and Nanoscience (IMCN), UCLouvain, Place Louis Pasteur 1, 1348 Louvain-La-Neuve (Belgium)
[d] Prof. T. Doneux
Chemistry of Surfaces, Interfaces and Nanomaterials (ChemSIN) - Université libre de Bruxelles – ULB, 1050 Brussels (Belgium)

Supporting information for this article is given via a link at the end of the document.

Abstract: Melanin is a class of hetero-polymer pigments commonly found in Nature and widely in fungi. Often referred to as the "animal lignin", melanin is a very abundant bioresource and features many catalytically interesting properties. We conceived that, upon light absorbance, the polymer could promote long-distance electron donation to fuel redox enzymatic catalysis or controlled in-situ generation of H_2O_2. Here, we report on a fungal photo-biocatalytic system extracted from the commercially relevant *A. nidulans*, where photoactivated melanin acts as an electron donor for the cellulose-degrading *An*AA9A and *Tt*AA9E metalloenzymes. Furthermore, there was a stable and significant accumulation of H_2O_2 when melanin was irradiated by visible light; having the peroxide functioning as a co-substrate for the AA9 LPMO enzymes. Oxidized cellulose-derived oligosaccharides were detected in the dark and under light conditions, confirming the potential of melanin to reduce AA9s. When placed under light conditions, they provided hydrogen peroxide as a co-substrate for AA9s. The use of light to tune the in-situ generation of H_2O_2 by natural pigments might be pivotal to enable also another peroxide-dependent enzymatic catalysis.

Introduction

Melanin is a natural pigment produced by a wide array of fungi, bacteria, and helminths that cause disease in humans and plants. Melanotic fungi have the particularity of being able to inhabit inhospitable places and harsh-living environments such as dry and hot deserts, earth poles (Arctic and Antarctic), extra-terrestrial conditions (i.e., International Space Station), environments polluted with metals, and areas with high radiation (i.e., Chernobyl), among others.[1,2] Melanins are found in contact with the fungal cell wall, either internally or externally, mostly in granule form. Their presence influences the structure, architecture, and porosity of the cell wall, thus assisting the fungi with environmental stress and, in some cases, increasing the virulence of some fungi.[3]

Fungi are receiving increased attention in the industrial biotechnology sector as potential workhorses through increasing exploration and expansion of the nascent mycotechnology sector.[4,5] Added to the growing demand for industrial enzymes, with more than half of them of fungal origin (valued at $8.63 billion in 2019), a remarkable increase in the intellectual properties related to fungi arise[6,7], with Novozymes, AB enzymes, Dupont, DSM, Codexis and Toray Industries being key players for the development of new technologies. The fungi commonly used for these platforms encompass melanin producers (e.g., *Aspergillus* spp.). The melanin synthesized by these fungi is commonly either secreted or attached to the cell wall, making it a significant but often overlooked by-product; and it is likely to be released into wastewater. Recognizing the numerous applications of melanin [8], it presents itself as an abundant biological resource molecule with the potential for use in the circular economy.

Fungi have two melanin biosynthesis pathways: the 1,8-dihydroxy naphthalene (DHN) pathway, where acetyl-CoA or malonyl-CoA are precursors and which most melanotic fungi possess, and the L-3,4-dihydroxyphenylalanine (L-DOPA) pathway, where tyrosine or L-DOPA is the precursors (Fig. 1). Both paths involve several enzymatic steps. [3] Melanin is made up of covalently polymerized indole and phenol-type compounds forming building blocks. Those polymerize to form a negatively charged hydrophobic pigment with a high molecular mass.[3] To date, the resolution of the polymeric network structure is a challenge. X-ray analyses suggest that this polymeric network is composed of planar structures that may differ in stacking distances and also exhibit electronic properties typical of natural photosensitizers due to the presence of π-electrons.[9] The DHN and L-DOPA melanin come from different building blocks, and shared physical properties suggest a common structural core. But studies showed that, after the excitation states of the electrons, the excited-state deactivation can have different mechanisms.[10] Thanks to these characteristics, melanin has the ability for energy transduction, becoming an excellent photoprotective molecule. One of its characteristics is to shield the organism against UV irradiation, and as reported more recently, it also responds to

radiation.[11] The response to radiation was evidenced by three biological actions: i) radiotropism (the ability of fungi to grow towards radioactivity), ii) enhanced growth triggered by exposure to high energy levels, and iii) metabolic changes. These observations arise from an *in vitro* observation that irradiated melanin was able to reduce NAD to NADH, suggesting a possible link between the conversion of electromagnetic energy to chemical energy that could have utility in biological processes.[12]

Within the 300 species of Aspergillus genus, the soil fungi *Aspergillus nidulans* is recognized as a eukaryotic model for fungal genetics and biology.[13] *A. nidulans* mainly produce L-DOPA melanin, which can be affected by pH, temperature, and the availability of carbon sources. Indeed, for *A. nidulans*, the high level of melanization indicates stress.[14] The genome of *A. nidulans* contains several genes encoding for lytic polysaccharide monooxygenases (LPMOs)[15]. Specifically, the AA9 LPMO family is represented by nine different enzymes with distinct regioselectivity and affinity for substrates[15]. LPMOs are critical enzymes involved in lignocellulose conversion. The secretion of the arsenal of *An*LPMOs is subjected to the saprophytic lifestyle of the fungi, which aims to degrade a complex lignocellulosic substrate, thus being industrially relevant biocatalytic targets.[16] LPMOs have a single copper active site in a highly-conserved structure populated by two histidine forming the peculiar T-shape arrangement (His-brace). The precise mechanism of action of LPMOs is still widely debated yet is currently accepted to describe it by a dual mechanism: one following the monooxygenase reaction involving molecular oxygen, and the second instead via peroxygenase reactivity using hydrogen peroxide as co-substrate. It was lately found that the peroxygenase activity takes place through the auto-production of H_2O_2 by the LPMO via the activated Cu[I] (different from its resting state, Cu[II]). These reactive species facilitate the hydroxylation of substrates, leading to the breakdown of glycosidic bonds. To reduce the Cu[II] to Cu[I], so to activate the catalytic cycle, LPMOs require external electrons, and a wide range of molecules and proteins had been described serving as electron donors. [17]

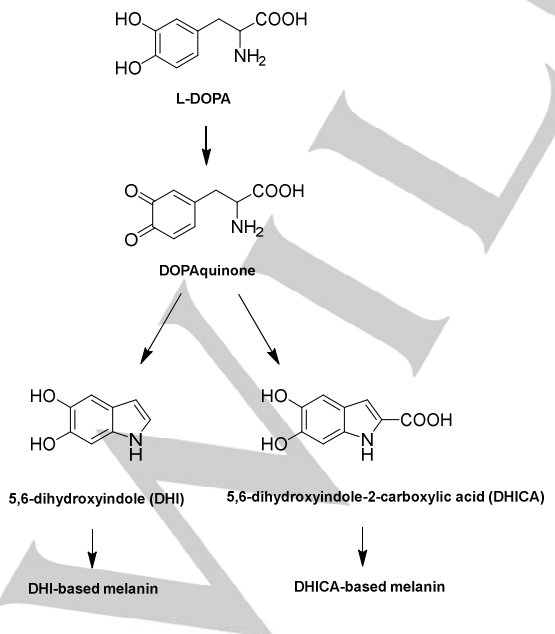


Figure 1. Summary of L-DOPA melanin biosynthesis. Scheme based on Meyer and co-workers.[18]

Broadening the spectrum of biocatalysis applications, one recent field is emerging: photo-biocatalysis. In this domain, the enzymatic catalyst is propelled by electrons donated from a sacrificial molecule upon photoexcitation of the molecule itself or an intermediate pigment molecule. One of the earliest successful applications targeting the LPMO had seen the uses of chlorophyllin as a mediator pigment and lignin as a sacrificial molecule.[19] Later studies also confirmed the ability of lignin[20] and hematite[21] to donate photoexcited electrons. Moreover, together with the work from Bissaro and co-workers.[22], these works showed the involvement of the production of H_2O_2 from the pigment itself. Recent studies have described the functional characterization of different LPMOs through photo-biocatalysis[23–27], indicating its potential as a trending hot topic.

Considering the intrinsic physicochemical characteristics of melanin and its biological role in fungi, in this work, we tested whether melanin would be a suitable reducing agent for AA9 LPMOs, especially in photo-inducing conditions. We show that the L-DOPA melanin extracted from *A. nidulans* was able to act as an electron donor for fungal AA9 LPMOs, especially when irradiated by visible light. These findings shed light on a new perspective on the biological role of L-DOPA melanin and AA9 enzymes.

Results and Discussion

Melanin production and characterization

It is known that under starvation or carbon depletion stress, *A. nidulans* secret melanin. Melanization plays a role in protecting cells from exogenous stress factors and protects living cells from the autolysis process.[14] In our experiments, we induced melanization of the medium using a low glucose concentration (0.5%). Over time, *A. nidulans* liquid culture medium became darker. Twentieth days after, the dark pigment was extracted by acid precipitation and characterized with NMR and UV-Vis spectroscopy (Fig. 2 and Fig. S1, respectively). The melanin extracted from the *A. nidulans* growth was marginally soluble in water and citrate-phosphate buffer at pH 6.0, leaving a two-phase separation. The precipitate contained insoluble dark melanin, while the water phase contained certain amount of soluble melanin molecules that we hypothesized being <1% yet enough for turning the solution pale-yellowish. Yet, a complete solubilization was achieved with 1 mM NaOH and 10 % DMSO at 80 °C, allowing further characterization. We used the commercial melanin L-DOPA as a standard to quantify the melanin extracted from the *A. nidulans* culture, corresponding to 1.384 mM.

The natural structure of melanin is rarely reported due to the challenge of obtaining homogeneous and soluble components. Although determining the structure of melanin is beyond the scope of this work, the ^{1}H NMR spectroscopy signature (Fig. 2) of melanin extracted from *A. nidulans* was compared with the signature of commercial L-DOPA melanin and the available data in the literature. In order to achieve adequate solubility for the NMR analyses, the melanin samples were prepared in DMSO-d_6 or D_2O and sonicated. Nevertheless, a precipitate was still present in each case. The commercial L-DOPA melanin led to a broadened ^{1}H NMR signature, characteristic of high molecular

weight polymers (Fig. 2a). The spectra recorded for the extract of *A. nidulans* showed both sharp and broad lines (Fig. 2b and c). This indicates that the samples are mixtures of small molecules (sharp lines) and species of higher molecular weight that may be related to the presence of oligomeric and polymeric melanin structures. It is interesting to note that after two D_2O washes/extractions of the remaining precipitate of the extract, a broader ^1H NMR signature was obtained, closer to the signature of the commercial L-DOPA melanin (Fig. 2d). These results are in agreement with ^1H NMR data previously reported for melanin extracts and commercial melanin.[28–31]

The UV-Vis spectrum showed the typical peak (Fig. S1), ranging from 200 to 300 nm in different concentrations. It is a specific light absorption profile for aromatic compounds and tends to decrease in absorbance with increasing wavelength. Studies that characterized melanin extracted from different fungi showed similar spectrum patterns.[32,33]

Finally the ground state redox potential of the so extracted melanin was characterized and a midpoint potential of 227 mV vs RHE (Reversible Hydrogen Electrode), was found (Fig. S2). This value is very close to the average redox potential of LPMO enzymes reported in literature (aroun 200-220 mV).

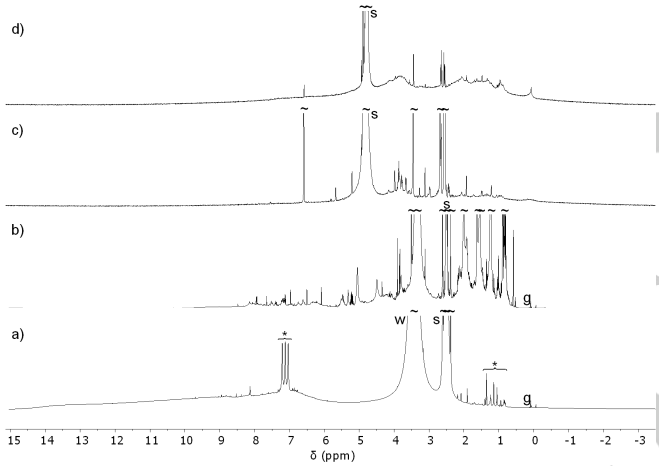


Figure 2. ^1H NMR spectra (298 K, 600 MHz) of (a) commercial L-DOPA melanin in DMSO-d_6, (b) extracted melanin in DMSO-d_6, (c) extracted melanin in D_2O, (d) extracted melanin in D_2O after two D_2O washing with 30 min sonication of the remaining precipitate, *: impurities, w: water, s: solvent, g: grease. A similar vertical scaling was applied for spectra a) and b), as well for spectra c) and d).

Light-induced electron transfer from melanin to LPMO (*An*AA9 and *Tt*AA9)

The ability of melanin to reduce and activate a C4-oxidizing *An*AA9A[15] and C1/C4-oxidizing *Tt*AA9E[34] enzymes during photo-sensitized incubation on phosphoric acid swollen cellulose – PASC, was investigated (Fig. 3). These were benchmarked against incubations containing the standard condition for activation of LPMO, i.e., AscA at 1 mM, dark-yellow chromatograms (Fig. 3a, 3e). The reactions were carried out in three hours, and a concentration of 0.1 mM of melanin was sufficient to trigger the LPMOs activity in the absence of ascorbic acid (AscA) in photosensitized conditions. After quantification of the soluble cello-oligosaccharides produced by the *Tt*AA9E enzyme (Fig. 3b), it was found that incubation with 1 mM of AscA had produced 102 mg/L, instead with 0.1 mM of melanin the same enzyme produced 14 mg/L of the oligosaccharides. Even in dark conditions (black line in Fig. 3a, 3e) a certain level of LPMO activation was detected. Control reactions performed in the absence of the AA9 enzymes under illumination (gray lines in Fig 3a, 3e) or in darkness showed the inability of the melanin itself or in combination with copper, ascorbate or hydrogen peroxide to oxidatively cleave the cellulose (Fig. S3). The high-performance anion-exchange chromatography (HPAEC) analysis relative to the oligosaccharides released from LPMOs confirmed the same pattern for both AscA and melanin Fig. 3b; therefore, the regioselectivity of the enzymes was not affected using a different reducing agent, as it was previously observed[26] for the *Tt*AA9E. Photo-sensitized natural melanin achieved approximately 13% of LPMO activity with only a tenth of the reducing power in molar amount compared to ascorbic acid. These results prompted us to set up a comparative time-course experiment where the *Tt*AA9E was incubated with same molar amount of reductant (0.1 mM of AscA, or 0.1 mM of melanin, or combination of both) and sampled many times during 16 hours incubation (960 minutes). In Fig 3c the chromatograms are reported at the endpoint (16 hours) and can be noted that incubation with melanin outperformed AscA in amount of oligosaccharides released: 17 mg/L compared to 12 mg/L respectively (Fig. 3d), approximately 40% more oligosaccharide. Instead, looking at earlier time points (30', 60' and 180' minutes) an opposite scenario would have been found, having the AscA a faster release of oligosaccharides compared to melanin. Noteworthy is also the incubation with both AscA and melanin together (summing 0.2 mM of reducing power), which saturated the maximum activity of the LPMO earlier then both reductants applied alone, leading to a very fast release of oligosaccharide in the first 60 minutes (80% of the total obtained in 960 minutes). In supplementary figure Fig. S4, are reported the HPAEC chromatograms relative to the oligosaccharides released for all the incubations at each time point.

Moreover, given the broad spectrum of light absorbance properties of melanin, an attempt to activate the pigment using UV light, and thus the LPMO reaction, was performed. After 30 minutes of UV irradiation, the melanin precipitated, compromising the feasibility of the assay, and probably unfolding the enzyme too.

Furthermore, other LPMOs available in our lab have been tested to prove the non-specificity of the photosensitization driven by melanin. In Fig. S5 chromatograms of oligosaccharides are shown from the melanin-mediated photosensitization of LPMOs from *Thermoascus aurantiacus* (*Ta*AA9A) and *Myceliophthora thermophila* (*Mt*AA9I), proving the non-specificity of the melanin-mediated photosensitization.

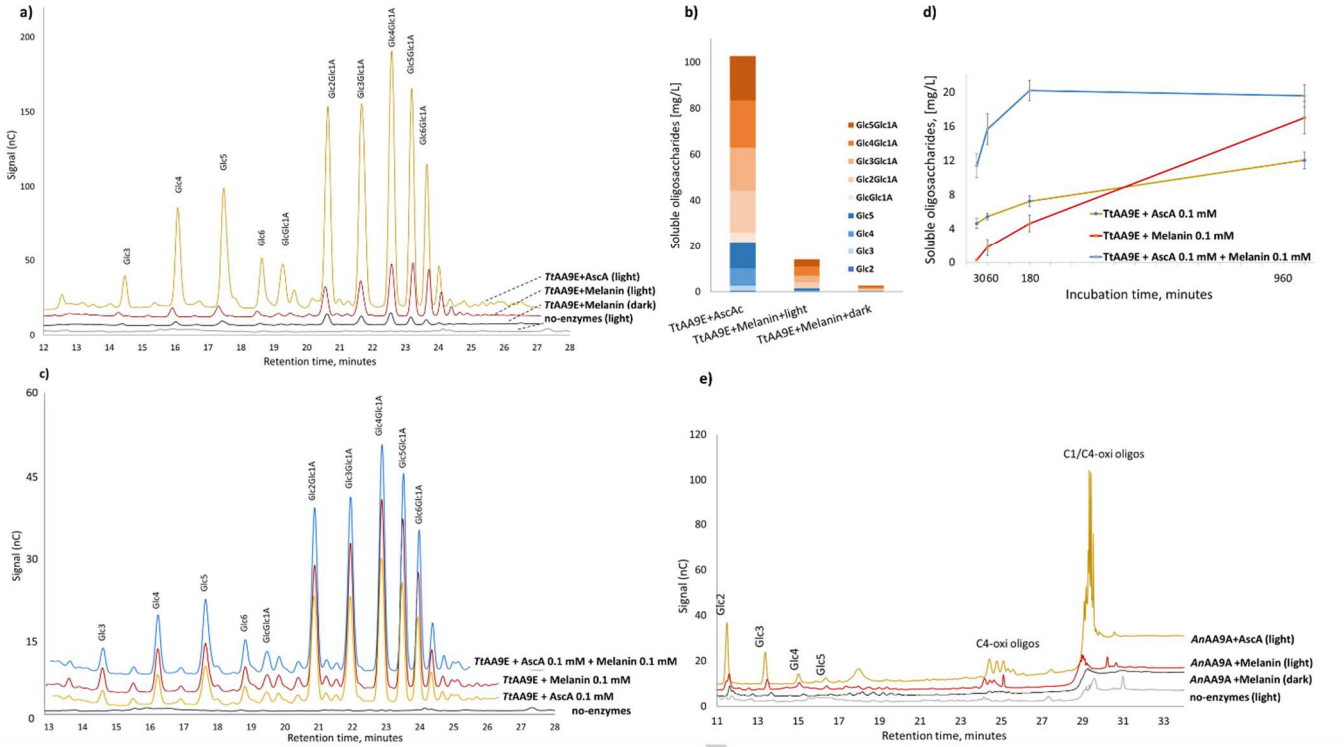


Figure 3. LPMOs incubations with melanin in different settings. a) HPAEC-PAD chromatographic profile of soluble cellulose oligosaccharides by 1µM of *Tt*AA9E with 1 mM AscA (dark-yellow); 0.1 mM photoinduced melanin (red); 0.1 mM melanin in darkness (black). Incubation time 3 hours. b) Quantification of the oligosaccharides from a) using purified standard for cellobiose (Glc2), cellotriose (Glc3), cellotetraose (Glc4) and cellopentaose (Glc5). The oxidized version were obtained by end-point incubation with CDH enzymes so generating cellobionic acid (GlcGlc1A), cellotrionic acid (Glc2Glc1A), cellotetraonic acid (Glc3Glc1A), cellopentaonic acid (Glc4Glc1A). c) HPAEC-PAD chromatographic profile of oligosaccharides produced by *Tt*AA9E with 0.1 mM AscA (dark-yellow); 0.1 mM melanin (red); 0.1 mM melani + 0.1 mM of AscA (blue); no-enzymes control containing cellulose, melanin and AscA (black); 16 hours incubation. d) Quantification of the oligosaccharides from c) plus other time points of same conditions (30', 60', 180' minutes). e) HPAEC-PAD chromatographic profile of cellulose solubilized oligosaccharides by 1µM of *An*AA9A with 1 mM AscA (dark-yellow); 0.1 mM photoinduced melanin (red); 0.1 mM melanin in darkness (black); Grey line shows the negative control: cellulose with AscA and Melanin irradiated by light. Incubation time 3 hours. General conditions for all incubations (if not differently indicated): 0.5% (w/v) PASC, 50 mM sodium acetate buffer pH 6.0; 50 °C with shaking at 1000 rpm; irradiation with white light (210 µmol photons/m^2/s) was provided by LED diodes or kept in the dark. All experiments were done in triplicates.

Hydrogen peroxide

Evidence that natural photosensitizer molecules like lignin or quinones can generate H_2O_2 alongside promoting long-range light-induced electron transfer (LIET) advocates for its careful detection and/or quantification. [16,34] Figures 4 and 5 present data on the quantification of H_2O_2 evolved by photosensitized melanin (derived from *A. nidulans*) and detected using the horseradish peroxidase (HRP) method based on the Amplex™ Red reagent. Due to the nature of the assay, exposure of the samples to light was done before mixing with the detection solution to avoid artificial resorufin formation by exposing the reagent to light.

The time course experiment (Fig. 4, 5 and supplementary material S6) shows that in dark conditions, melanin did not accumulate a detectable amount of H_2O_2 (detection limit <0.3 µM). One cannot exclude that if a minor amount had been produced, it could have been decomposed by the melanin itself, due to its reactive oxygen species (ROS) scavenger ability.[36] Therefore an exogenous amount of hydrogen peroxide (15 µM) was added to the melanin in darkness, yet it was not degraded for the entire length of the measurements (960 minutes, Fig. S6). This result also confirms the role of melanin as a compatible reducing agent (electron donor) for LPMO since cellulose oxidation was obtained when melanin and the enzyme were incubated in dark conditions (Fig. 3), although the oligosaccharides produced by both *Tt*AA9E and *An*AA9A were in a basal-minimal amount. Yet, when melanin was exposed to white light, a constant accumulation of H_2O_2 was detected over time in a linear trend: reaching a concentration of ~14 µM of H_2O_2, corresponding to 2,9 µM/h production rate for the first 300 minutes (Fig. 4). The latest detection points at 960 min showed around 23 µM of H_2O_2, corresponding to an hourly production of 1.1 µM/h, assuming that it was produced continuously. The same experiment was conducted in the presence of AscA, a reducing molecule known to interact with photoexcited melanin, working as ultimate sacrificial molecule so donating electron eventually leading to higher production of H_2O_2, as also earlier demonstrated by Wielgus and Sarna for melanin derived from bovine eyes [35], and for other natural photosensitizers i.e. phycobilisomes, chlorophyllin [19,22] and catecholamines from insects [36]. Indeed, when melanin was exposed to light and in the presence of 0.5 mM AscA, the hourly production of H_2O_2 was 10,5 µM/h for the first 180 minutes. Interestingly the final time point at 960 min showed a lower residual amount of H_2O_2 (about 45 µM), determining a decrease of the hourly production to 2,75 µM/h (Fig. 4). This suggests an intrinsic ROS scavenger activity of melanin and ascorbate after a first prooxidant burst induced by light. Overall, the amount of hydrogen peroxide produced by melanin in this setting is compatible with previous experiments where it was added exogenously to keep constant activation of LPMO by Bissaro and co-workers.[22]

Moreover, in Fig. 5 the LPMO enzymes and the cellulose substrate were added to the photoactivated melanin setup resulting in the absence of hydrogen peroxide formation, indicating the ability of the enzymes to efficiently utilize H_2O_2 as a co-substrate for the lytic oxidation of cellulose. At each tested sampling point, the amount of H_2O_2 was kept below the detection limit, indicating that LPMO enzymes had enough activity and dosage to consume it.

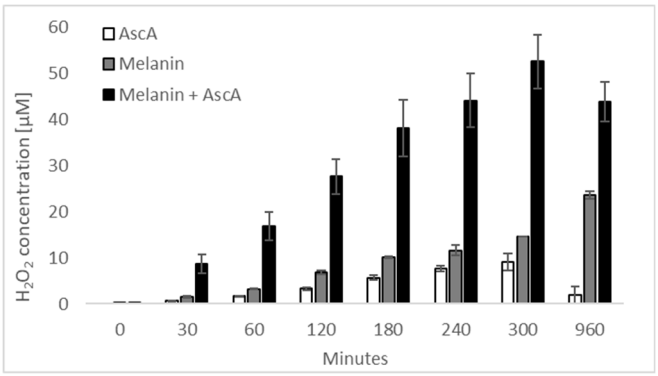


Figure 4. Photocatalytic melanin-related H_2O_2 production over time. All samples were exposed to light. Black bars: melanin 0.1 mmol exposed to white light. Grey bars: melanin 0.1 mM and AscA 0.5 mM exposed to white light. White bars: AscA 0.5 mM exposed to white light (no melanin control). Error bars indicate the standard deviation of 3 different replicas.

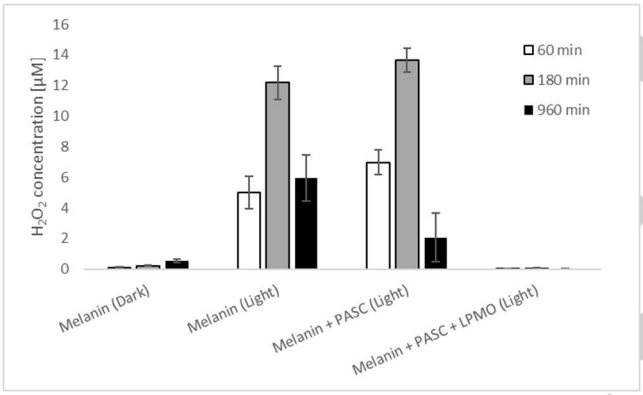


Figure 5. Photocatalytic melanin-related H_2O_2 production with LPMO at selected time points. Melanin is presented in dark and light conditions, all the other conditions are instead performed only in light. Black bars: 960 minutes reaction time. Grey bars: 180 minutes. White bars: 60 minutes. Error bars indicate the standard deviation of 3 different replicas.

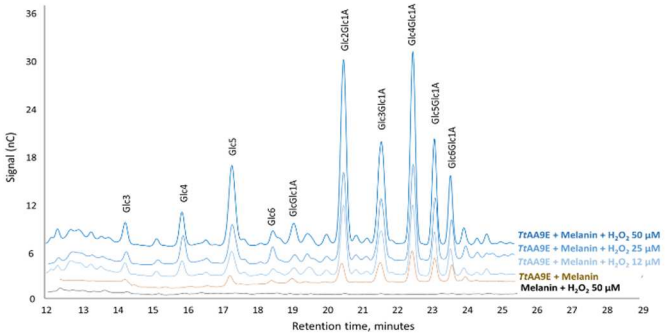


Figure 6. HPAEC-PAD chromatographic profile of soluble cellulose oligosaccharides by 1 µM of *Tt*AA9E incubated with 0.1 mM (dark-yellow); to this setting an increasing amount of H_2O_2 was added: 12 µM light-blue; 25 µM medium-blue; 50 µM dark-blue. Black line represent the negative control lacking the enzyme. General conditions for all incubations: 0.5% (w/v) PASC, 50 mM sodium acetate buffer pH 6.0; 180 minutes at 50 °C with shaking at 1000 rpm in darkness.

Finally to prove that the melanin has sufficient reducing ability to activate the Cu[II] to Cu[I] an experiment in darkness was performed where *Tt*AA9E was incubated with melanin and exogenous amount of H_2O_2 in a range of 12 to 50 (µM), Fig. 6. The results show increasing solubilization of cellulose at increasing dosages of hydrogen peroxide confirming the ability of melanin to serve as reducing agent for LPMO also in darkness.

Hydrogen peroxide intracycle

Light-driven enzymatic processes for AA9 have been described previously using thylakoids isolated from photosynthetic microorganisms, chlorophyllin, lignin[19], quinones from insect's exoskeleton[36] hematite[21], and a vanadium-doped titanium dioxide (V-TiO_2) system[37]. Bissaro and co-workers[22] have also shown that light could tune *in situ* generation of H_2O_2, which would fuel the oxidation of polysaccharides. Essentially, natural phenols can donate and accept electrons with appropriate mediators through a mechanism that varies the oxidation and reduction state of phenols.

The electron transfer properties of melanin have been known for a long time.[38] Exposure to ionizing radiation and other radiation sources (such as visible light) has boosted this property, as demonstrated in the double reaction system of NADH oxidation and ferrocyanide reduction, where melanin acts as an electron transfer agent.[39] Figure 7 depicts a possible mechanism of how melanin can be involved in a light-driven enzymatic system. In the photoreduction process and oxygenated situations, melanin can reduce O_2 to H_2O_2 as shown by our results and in agreement with literature[40] and the latter is used as co-substrate by the AA9 LPMOs after a successful reduction of Cu(II) to Cu(I). In this regard, we speculate on the possible direct electron transfer from melanin to the AA9 LPMO (as proven by the successful cellulose oxidation observed in dark conditions for *Tt*LPMO9E [Fig. 3A] also in presence of H_2O_2 [Fig. 6]), perhaps accelerated by photoexcitation. Whether a more efficient electron transfer would be possible due to light absorbance is demonstrated only at the level of H_2O_2 evolution (Fig. 4). The addition of a second reducing species, such as ascorbate, drove melanin to an even higher rate (and amount) of H_2O_2 production, preventing the expected natural melanin auto-oxidation after many cycles of electron donation, as it has already been described that melanin auto-oxidation competes with donor oxidation.[41] Although delayed, the autooxidation of melanin will occur and can lead to further polymerization and precipitation; moreover, the production of H_2O_2 also accelerates photo-bleaching.[42] Finally, the bleaching of melanin is also accelerated by binding to Cu[II], suggesting a possible involvement of Fenton-like processes.[43] This reaction would produce ROS such as hydroxyl and hydroperoxyl radicals and superoxide anions, yet in our control experiments with Cu[II], melanin and H_2O_2 these had not caused oxidation of cellulose without the LPMO enzymes.

10.1002/cctc.202300602

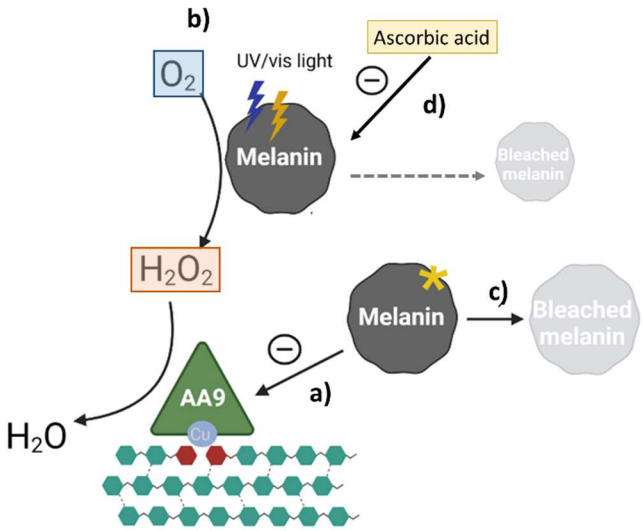


Figure 7. Possible mode of photosensitization from melanin to LPMO-AA9 based on the presented results and literature. (a) Melanin can reduce LPMO enzymes via donation of electron due to a favorable reduction potential, also in darkness. b) Upon UV or visible light irradiation melanin can also reduce O_2 to H_2O_2, a co-substrate of LPMO. c) Both options lead to the autooxidation of melanin (causing bleaching or precipitation). d) By the use of ascorbic acid as sacrificial molecule or reducing agent, an electron can be transferred to the melanin filling the hole vacated by the previous transfer of the photoexcited electron. This also prevent the oxidation of melanin that remain reduced for longer period at the expenses of the ascorbate. Eventually after having spent all the molecules of ascorbic acid, the melanin will start oxidizing.

Conclusion

The discovery of melanin as photo-excitable natural pigment confirms the intriguing synergism between the biotic photodegradation of plant cell walls through biocatalytic strategies. Furthermore, we provide evidence on the ability of melanin to efficiently sustain the catalytic cycle of the bioresources-degrading LPMO enzymes at dosages 10-fold lower than the normal state of the art for similar applications based on ascorbate. Moreover, the biochemical implications are various: melanin could activate several AA9 LPMO enzymes not only the one produced by the same organism (*A. nidulans*) from which it was extracted. Therefore, proving non-specificity towards the enzymatic partner. This, coupled with the abundance and ease of extraction for melanin compared to lignin heteropolymer represents an interesting bio-derived, photoactivable solution for *in-situ* hydrogen peroxide generation i.e. for application with redox enzymes. Nowadays the bioeconomy sector based on lignocellulose is looking for alternatives for greener productions, and redox enzymes depended on hydrogen peroxide are considered the most attractive for the production of biochemicals i.e. UPO, peroxidases, lignin-peroxygenases to name few.

The ability to perform double electron donation (reduction of Cu[II] to Cu[I]) for the activation of the LPMO, plus the supply of electrons for the photo-reduction of oxygen into hydrogen peroxide, makes fungal melanin an ideal platform to be further exploited for the sustainable biocatalysis. An interesting approach would be to test the redox complexity of melanin from different fungi to screen its potential uses as an inexpensive sacrificial molecule for the nascent photo-biocatalysis field.

Experimental Section

Fungal melanin extraction and quantification

For extracting fungal L-DOPA melanin, *Aspergillus nidulans* A773 (genotype: pyrG89;wA3;pyroA4) obtained from the Fungal Genetics Stock Center (FGSC, St Louis, MO) was plated into Potato-Dextrose-Agar (PDA) and incubated at 25 °C for five days with a photoperiod of 12 h light/12 h darkness. 11-mm discs were made in the solid medium with a cork borer for the inoculum. One disc was added to 150 mL of an *A. nidulans* liquid minimal medium described by Segato et al.[44] but containing 0.5 % (w/v) of glucose as a carbon source. The fungus was incubated for 20 days at 28 °C at 200 rpm with a photoperiod of 12 h light/12 h darkness. The liquid medium was filtrated on Whatman paper n°1, 0.02 % (w/v) of sodium azide was added to the supernatant and stored at 4 °C for later use. The mycelium was discarded. The pigment was extracted as described by Gonçalves *et al.*[33] Briefly, the supernatant samples were acidified to pH 1.5 with 6 mol/L HCl and incubated overnight at room temperature for the melanin precipitation. After this, the precipitate was centrifuged at 4500 g for 15 minutes, and the supernatant was discarded. Next, the pellet was washed with 40 mL deionized H_2O, centrifuged at 4500 g for 15 minutes, and the supernatant was discarded. This step was repeated two more times to deacidify the melanin. The residual pellet was resuspended in 40 mL of citrate-phosphate buffer pH 6.0. L-DOPA melanin (Sigma ref. M8631) was used as a standard for quantification. Solubilization of all melanin samples and the synthetic one was adapted from the protocol described by Fernandes et al.[45] The melanin sample was dissolved in a solution of 1 mol/L NaOH (w/v) with 10% DMSO (v/v) for 1 h at 80 °C and then centrifuged at 3000 g for five minutes. The supernatant was used for quantification. The synthetic melanin used for the standard curve varied its concentration from 0.0005 to 0.1 mg/mL The samples were read on 405 nm in the UV-Vis spectrophotometer Lambda 25 (PerkinElmer®). The supernatant melanin was also submitted to a scan in the range of 200-800 nm. For this, the extracted melanin was diluted 25-fold, 50-fold, and 70-fold in citrate-phosphate buffer pH 6.0.

Nuclear Magnetic Resonance spectroscopy

^{1}H NMR analyses of the commercial L-DOPA (Sigma ref. M8631) and the melanin extracted from *A. nidulans* were performed at 25 °C using a JEOL JNM-ECZ600R/S3 spectrometer operating at 14.1 T (600.17 MHz for ^{1}H) equipped with a double resonance ROYAL™ probe. Samples were in 600 µL D_2O or DMSO-d_6 (7.3 mg of commercial L-DOPA and 31.5 mg of extracted melanin) and sonicated for 2 h to improve the solubility of the melanin samples. In each case, a solid deposit remained visible at the bottom of the tube. The spectra were recorded using a spectral width of 20.0 ppm centered at 5.0 ppm, 4 s relaxation delay, 2.6 µs high-power RF pulse width (flip angle of 30°), 2.2 s acquisition time, and 512, 1024 or 4000 scans. Processing was performed using MestReNova 14.3.0-30573. It comprised zero-filling (total of 256k points), cosine square multiplication of the free induction decay and Fourier transform, phase correction, baseline correction, and chemical shift referencing of the spectrum. The ^{1}H spectra were referenced using the residual signal of the solvent (HDO: 4.79 ppm; DMSO-d_5: 2.50 ppm).

Cyclic Voltammetry

CV for melanin, was performed at room temperature using PGSTAT30 Potentiostat (Autolab) at ambient temperature with a normal three-electrode configuration consisting of an edge plane pyrolytic graphite (EPPG) as a working electrode (ProSense, 3.0-mm diameter), an Ag/AgCl electrode (Metrohm) as a reference electrode, and a platinum-wire auxiliary electrode. The electrode was soaked in 1 mM melanin suspension in 50 mM sodium acetate buffer pH 6.0 and kept under dark condition. Prior the CV analysis, the sample was purged for 10 minutes with N2 for oxygen depletion and kept under N2 overpressure all along the measurement. The CV is recorded using a scan rate of 100 mV. The midpoint ground state redox potential is converted to mV vs RHE (reversible hydrogen electrode) using the following equation:

$$E(RHE) = E(Ag/AgCl) + 0.199 + 0.059 * pH$$

AA9 LPMO assay

The enzymes used in this work, *An*LPMO9A from *A. nidulans*, *Tt*LPMO9E from *Thielavia terrestris*, *Mt*AA9I from *M. thermophila,* and *Ta*AA9A from *T. auranticus,* were obtained as described by Kadowaki et al.[46] and Zarattini et al.[47] For the assay the generic reaction mixture contained 1 µM of LPMO, 0.5 % (w/v) PASC, and 50 mM of sodium acetate buffer pH 6.0. As a reducing agent, 1 mM ascorbic acid (AscA) or 0.1 mmol/L melanin was used. Reactions were incubated at 50 °C with shaking at 1000 rpm with visible light (210 µmol photons/m^2/s) or darkness. Different incubations time were applied as specified in each figure. Also some samples contained 0.1 mM of AscA also specified were meaningful. At the end of incubation, the samples were centrifuged at 15.000 *g* for 3 min. The supernatant was filtrated through an Amicon Ultra 0.5 mL filter with a cut-off of 3 kDa to retain the melanin. Native and oxidized oligosaccharides were analyzed by high-performance anion-exchange chromatography HPAEC on a Dionex ICS-6000 system equipped with pulsed-amperometric detection (PAD) and a CarboPac PA1 analytical column (2 x 250 mm), with the elution performed as described by Kadowaki et al.[46] The detection and quantification of native oligosaccharides from DP2 to DP5 was done by using pure standards from Megazymes Ltd, while the relative C1-oxidized version were obtained by end point incubation with cellobiose

dehydrogenase enzyme *Mt*CDH until disappearing of the native oligosaccharide was reached accordingly to previous literature protocol.[48]
Careful consideration should be given to the melanin injection into the HPAEC, particularly if in the presence of soluble melanin. This is able to pass the usual filters used for sample preparation and eventually when injected will permanently coat the PA1 column resin causing possibly a shift in the retention times of the oligosaccharides. It is recommended to first condition the column with blank samples containing melanin to achieve consistent retention shift through the entire experiment.

H_2O_2 production assay
The rate of H_2O_2 production was assessed using MyQubit Amplex™ Red Peroxide Assay (Thermo Fisher Scientific) following the manufacturer's protocol. The working solution contained 100 µmol/L Amplex™ Red reagent, 0.2 µmol/mL of horseradish peroxidase (HRP), and 1X PBS buffer pH 7.4 in a final volume of 240 µL. The fluorescent emission from the resorufin was measured after 5 min of incubation. For each condition, three independent replicates were done. The experiments were set as follows: 0.1 mM melanin, and where indicated 0.5 mM AscA, or 1 µM of *Tt*LPMO9E, or 1% w/w PASC. Visible light was provided at 210 µmol photons/m^2/s in continuous, dark conditions were achieved by working in a dark room.[49]

Keywords: AA9 LPMO • photobiocatalysis • L-DOPA melanin • Bioresources • Biorefinery

[1] R. J. B. Cordero, A. Casadevall, *Fungal Biol Rev* **2017**, *31*, 99–112.
[2] C. Coleine, J. E. Stajich, L. Selbmann, *Trends Ecol Evol* **2022**, DOI 10.1016/J.TREE.2022.02.002.
[3] H. C. Eisenman, A. Casadevall, *Appl Microbiol Biotechnol* **2012**, *93*, 931–940.
[4] K. D. Hyde, J. Xu, S. Rapior, R. Jeewon, S. Lumyong, A. T. Grace Niego, P. D. Abeywickrama, J. V S Aluthmuhandiram, R. S. Brahamanage, S. Brooks, A. Chaiyasen, K. W. Thilini Chethana, P. Chomnunti, C. Chepkirui, B. Chuankid, N. I. de Silva, M. Doilom, C. Faulds, E. Gentekaki, V. Gopalan, P. Kakumyan, D. Harishchandra, H. Hemachandran, S. Hongsanan, A. Karunarathna, S. C. Karunarathna, S. Khan, J. Kumla, R. S. Jayawardena, J.-K. Liu, N. Liu, T. Luangharn, A. G. Patrick Macabeo, D. S. Marasinghe, D. Meeks, P. E. Mortimer, P. Mueller, S. Nadir, K. N. Nataraja, S. Nontachaiyapoom, W. Penkhrue, C. Phukhamsakda, U. Shaanker Ramanan, A. R. Rathnayaka, R. B. Sadaba, B. Sandargo, B. C. Samarakoon, D. S. Tennakoon, R. Siva, T. Suwunwong, B. Thongbai, N. Thongklang, D. Wei, S. Nuwanthika Wijesinghe, J. Winiski, J. Yan, E. Yasanthika, M. Stadler, *Fungal Divers* **2019**, *97*, 1–136.
[5] M. Lübeck, P. S. Lübeck, **2022**, DOI 10.3390/microorganisms10040753.
[6] G. Espina, J. Atalah, J. M. Blamey, *Front Bioeng Biotechnol* **2021**, *9*, 654.
[7] S. Hüttner, A. Johansson, P. Gonçalves Teixeira, P. Achterberg, R. B. Nair, *Fungal Biol Biotechnol* **2020**, *7*, 1–17.
[8] Z. Zhao, E. Kim, C. Y. Chen, J. R. Rzasa, Q. Zhang, J. Li, Y. Tao, W. E. Bentley, J. P. Lumb, B. Kohler, G. F. Payne, *Mater Chem Front* **2022**, DOI 10.1039/d2qm00106c.
[9] J. D. Nosanchuk, R. E. Stark, A. Casadevall, *Front Microbiol* **2015**, *6*, 1463.
[10] X. Wang, L. Kinziabulatova, M. Bortoli, A. Manickoth, M. A. Barilla, H. Huang, L. Blancafort, B. Kohler, J. P. Lumb, *Nat Chem* **2023**, DOI 10.1038/s41557-023-01175-4.
[11] J. Dighton, T. Tugay, N. Zhdanova, *FEMS Microbiol Lett* **2008**, *281*, 109–120.
[12] A. Casadevall, R. J. B. Cordero, R. Bryan, J. Nosanchuk, E. Dadachova, *Microbiol Spectr* **2017**, 509–514.
[13] L. K. Caesar, N. L. Kelleher, N. P. Keller, *Fungal Genetics and Biology* **2020**, *144*, DOI 10.1016/J.FGB.2020.103477.
[14] M. Szilágyi, F. Anton, I. Pócsi, T. Emri, *J Basic Microbiol* **2018**, *58*, 440–447.
[15] G. Jagadeeswaran, L. Gainey, A. J. Mort, *AMB Express* **2018**, *8*, DOI 10.1186/s13568-018-0701-5.
[16] C. R. F. Terrasan, M. V. Rubio, J. A. Gerhardt, J. P. F. Cairo, F. J. Contesini, M. P. Zubieta, F. L. de Figueiredo, F. L. Valadares, T. L. R. Corrêa, M. T. Murakami, T. T. Franco, G. J. Davies, P. H. Walton, A. Damasio, *Microbiol Spectr* **2022**, DOI 10.1128/SPECTRUM.02125-21.
[17] B. Bissaro, V. G. H. Eijsink, *Essays Biochem* **2023**, 20220250.
[18] C. E. Meyer, C. Schoenenberger, R. P. Wehr, D. Wu,and C. G. Paliva, *Macromol Biosci* **2021**, 21, 2100249
[19] D. Cannella, K. B. Möllers, N. U. Frigaard, P. E. Jensen, M. J. Bjerrum, K. S. Johansen, C. Felby, *Nat Commun* **2016**, *7*, DOI 10.1038/ncomms11134.
[20] J. Kim, T. V. T. Nguyen, Y. H. Kim, F. Hollmann, C. B. Park, *Nature Synthesis* **2022**, *1:3*, *1*, 217–226.
[21] D. Wang, M. R. Kao, J. Li, P. Sun, Q. Meng, A. Vyas, P. H. Liang, Y. S. Wang, Y. S. Y. Hsieh, *J Agric Food Chem* **2022**, *70*, 9941–9947.
[22] B. Bissaro, E. Kommedal, Å. K. Røhr, V. G. H. Eijsink, *Nat Commun* **2020**, *11*, DOI 10.1038/s41467-020-14744-9.
[23] B. M. Blossom, D. A. Russo, R. K. Singh, B. Van Oort, M. B. Keller, T. I. Simonsen, A. Perzon, L. F. Gamon, M. J. Davies, D. Cannella, R. Croce, P. E. Jensen, M. J. Bjerrum, C. Felby, *ACS Sustain Chem Eng* **2020**, *8*, 9301–9310.
[24] A. G. v. Sepulchro, V. V. O. A. Pellegrini, L. D. Dias, M. M. A. S. Kadowaki, D. Cannella, I. Polikarpov, *Biofuel Research Journal* **2021**, *8*, 1454–1464.
[25] P. M. R. Higasi, J. A. Velasco, V. O. A. Pellegrini, E. A. de Araújo, B. A. França, M. B. Keller, C. A. Labate, B. M. Blossom, F. Segato, I. Polikarpov, *Carbohydr Polym* **2021**, *260*, DOI 10.1016/j.carbpol.2021.117814.
[26] J. Velasco, A. G. V. Sepulchro, P. M. R. Higasi, V. O. A. Pellegrini, D. Cannella, L. C. De Oliveira, I. Polikarpov, F. Segato, *ACS Sustain Chem Eng* **2022**, *10*, 16969–16984.
[27] E. G. Kommedal, C. F. Angeltveit, L. J. Klau, I. Ayuso-Fernández, B. Arstad, S. G. Antonsen, Y. Stenstrøm, D. Ekeberg, F. Gírio, F. Carvalheiro, S. J. Horn, F. L. Aachmann, V. G. H. Eijsink, *Nat Commun* **2023**, *14*, 1063.
[28] Z. Ye, Y. Lu, S. Zong, L. Yang, F. Shaikh, J. Li, M. Ye, *Process Biochemistry* **2019**, *76*, 203–212.
[29] M. Ye, G. yi Guo, Y. Lu, S. Song, H. yan Wang, L. Yang, *Int J Biol Macromol* **2014**, *63*, 170–176.
[30] S. Zong, L. Li, J. Li, F. Shaikh, L. Yang, M. Ye, *Appl Biochem Biotechnol* **2017**, *182*, 669–686.
[31] Q. Liu, J. Xiao, B. Liu, Y. Zhuang, L. Sun, *International Journal of Molecular Sciences 2018, Vol. 19, Page 3736* **2018**, *19*, 3736.
[32] C. Pacelli, A. Cassaro, A. Maturilli, A. M. Timperio, F. Gevi, B. Cavalazzi, M. Stefan, D. Ghica, S. Onofri, *Appl Microbiol Biotechnol* **2020**, DOI 10.1007/s00253-020-10666-0.
[33] R. C. R. Gonçalves, H. C. F. Lisboa, S. R. Pombeiro-Sponchiado, *World J Microbiol Biotechnol* **2012**, *28*, 1467–1474.
[34] B. Westereng, D. Cannella, J. Wittrup Agger, H. Jørgensen, M. Larsen Andersen, V. G. H. Eijsink, C. Felby, *Scientific Reports 2015 5:1* **2015**, *5*, 1–9.
[35] A. R. Wielgus, T. Sarna, *Photochemistry and Photobiology*, **2008**, 84: 683–691683
[36] E. G. Kommedal, F. Sæther, T. Hahn, V. G. H. Eijsink, *Proceedings of the National Academy of Sciences* **2022**, *119*, e2204510119.
[37] B. Bissaro, Z. Forsberg, Y. Ni, F. Hollmann, G. Vaaje-Kolstad, V. G. H. Eijsink, *Green Chemistry* **2016**, *18*, 5357–5366.
[38] E. V. Gan, H. F. Haberman, I. A. Menon, *Arch Biochem Biophys* **1976**, *173*, 666–672.
[39] E. Dadachova, R. A. Bryan, X. Huang, T. Moadel, A. D. Schweitzer, P. Aisen, J. D. Nosanchuk, A. Casadevall, *PLoS One* **2007**, *2*, DOI 10.1371/journal.pone.0000457.
[40] M. Tada, M. Kohno, Y. Niwano, *J Clin Biochem Nutr* **2010**, *46*, 224–228.
[41] L. Migliaccio, M. Gryszel, V. Derek, A. Pezzella, E. D. Głowacki, *Mater Horiz* **2018**, *5*, 984–990.
[42] J. P. L. Franco Cairo, F. Mandelli, R. Tramontina, D. Cannella, A. Paradisi, L. Ciano, M. R. Ferreira, M. V. Liberato, L. B. Brenelli, T. A. Gonçalves, G. N. Rodrigues, T. M. Alvarez, L. S. Mofatto, M. F. Carazzolle, J. G. C. Pradella, A. F. Paes Leme, A. M. Costa-Leonardo, M

Oliveira-Neto, A. Damasio, G. J. Davies, C. Felby, P. H. Walton and F. M. Squina, *Green Chem.,* **2022**, 24, 4845

[43] W. Korytowski, T. Sarna, *Journal of Biological Chemistry* **1990**, *265*, 12410–12416.

[44] F. Segato, A. R. L. Damásio, T. A. Gonçalves, R. C. de Lucas, F. M. Squina, S. R. Decker, R. A. Prade, *Enzyme Microb Technol* **2012**, *51*, 100–106.

[45] B. Fernandes, T. Matamá, D. Guimarães, A. Gomes, A. Cavaco-Paulo, *Pigment Cell Melanoma Res* **2016**, *29*, 707–712.

[46] M. A. S. Kadowaki, S. Magri, M. O. de Godoy, A. V. Monclaro, M. Zarattini, D. Cannella, *Enzyme Microb Technol* **2021**, *143*, 109704.

[47] M. Zarattini, M. Corso, M. A. Kadowaki, A. Monclaro, S. Magri, I. Milanese, S. Jolivet, M. O. de Godoy, C. Hermans, M. Fagard, D. Cannella, *Communications Biology 2021 4:1* **2021**, *4*, 1–13.

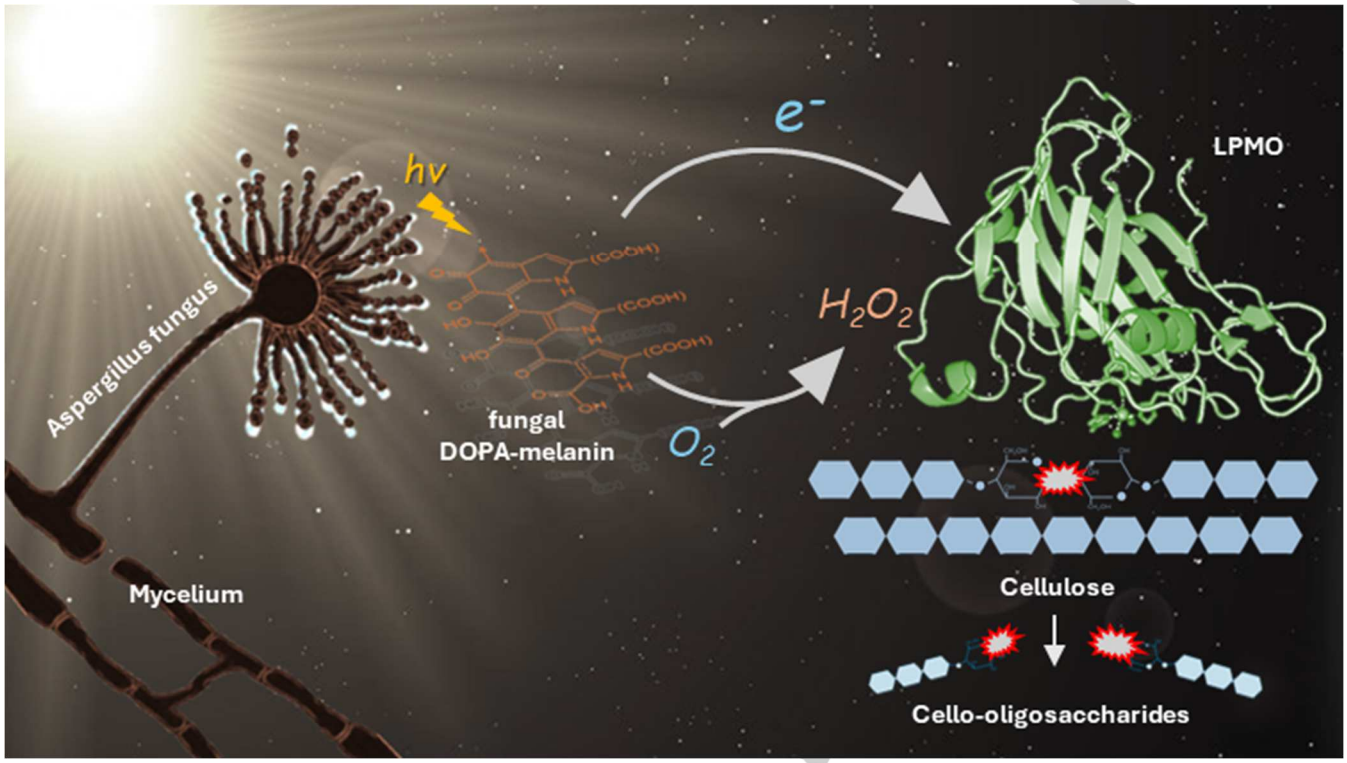
[48] D. Cannella, N. Weiss, C. W. C, Hsieh, S. Magri, M. Zarattini, J. Kuska, N. Karuna, L. G. Thygesen, I. Polikarpov, C. Felby, T. Jeoh and H. Jorgensen, *Cellulose* **2023**, 30, 6259–6272

[49] B. Zhao, F. A. Summers, R. P. Mason, *Free Radic Biol Med* **2012**, *53*, 1080–1087

WILEY-VCH

RESEARCH ARTICLE

TOC



Photosensitation of fungal melanin provides photo-excited electrons and H_2O_2-cosubstrate to fungal AA9-LPMO enzymes. An example of natural photobiocatalytic system with component sorted all from the same organism.