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Copper-induced Production of Laccases for Lignin Depolymerisation and Micropollutant Degradation by Laccase-mediator Systems

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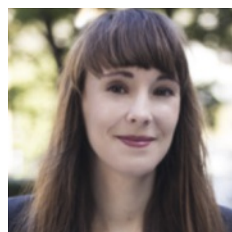
Abstract: Contaminants deriving from human activities represent a constantly growing threat to our environment and have a direct impact on plant and animal health. To alleviate this ecological imbalance, biocatalysis offers a green and sustainable alternative to conventional chemical processes. Due to their broad specificity, laccases are enzymes possessing excellent potential for synthetic biotransformations in various fields as well as for the degradation of organic contaminants. Herein, we produced laccases in submerged cultures of *P. ostreatus* and *T. versicolor* in three different media. The fungi/medium combination leading to the highest enzymatic activity was malt extract (2%) + yeast extract (3%) + glucose (0.8%). Laccase production was further increased by supplementing this medium with different concentrations of Cu^{2+}, which also provided a better understanding of the induction effect. Additionally, we disclose preliminary results on the interaction of laccases with mediators (ABTS and violuric acid - VA) for two main applications: lignin depolymerisation with guaiacylglycerol-β-guaiacyl ether (GBG) as lignin model and micropollutant degradation with Remazol Brilliant Blue (RBB) as enzymatic bioremediation model. Promising results were achieved using VA to increase depolymerization of GBG dimer and to enhance RBB decolorisation.

Keywords: Laccase mediators · Lignin depolymerisation · Micropollutant degradation



Lauriane Pillet obtained her Bachelor in Biochemistry from the University of Geneva before graduating from Ecole Polytechnique Fédérale de Lausanne (EPFL) with a Master in Chemical Engineering and Biotechnology in 2019. After working in the pharmaceutical industry, she started her PhD in the group of Prof. Dr. Francesca Paradisi in Bern, where she focuses on enzyme engineering for the design of efficient, sustainable and intensified continuous catalytic bioprocesses.

Rémy Dufresne studied at HES-SO Valais/Wallis and graduated as Biotechnology Engineer in 2005. He then worked as a research assistant at the Dublin City University before joining a pharmaceutical company where he focused on the production of anti-cancer drugs. He was then hired by HES-SO Valais/Wallis in the frame of a joint project on nanotechnologies with an industrial partner before joining the group of Prof. Dr. Simon Crelier. There, he manages the DSP (Downstream Processing) laboratory and teaches biochemistry, bioanalytics and supervises DSP lab sessions. His research activities include purification and characterisation of biomolecules, microencapsulation as well as anaerobic digestion.

Simon Crelier studied chemical engineering at EPFL, where he also obtained his PhD in the field of enzyme technology. After a postdoctoral fellowship at UC Berkeley in the labs of Prof. Dr. J. M. Prausnitz and Prof. Dr. H. W. Blanch, he worked for 10 years in the food industry. In 2003, he joined the HES-SO Valais/Wallis where he teaches downstream processing and enzyme technology. His research interests include purification of biomolecules, biosorption, microencapsulation and anaerobic digestion

1. Introduction

Laccases (EC 1.10.3.2) are blue multicopper isozymes that are able to oxidise a wide range of phenols or arylamines. The demand for their production has grown in proportion to their steadily increasing use at industrial scale.[1,2] They are highly versatile in nature and are prevalent in plants, bacteria, fungi and in some insects.[1–4] These extracellular enzymes are secreted as a pool of isoforms that can play diverse physiological roles. Plant and bacterial laccases belong to the group of low redox potential laccases, while fungal laccases include both medium and high redox potential enzymes.[4,5] Laccases are usually glycosylated with 15–30% covalently linked carbohydrates, imparting them additional structural and thermal stability. The active site of laccases is composed of four copper atoms subclassified into different types of clusters (T1, T2 and T3) depending on their structural features.[3–8]

These enzymes display a low specificity and nearly hundred different compounds have been identified as laccase substrates. In nature, they are capable of degrading structures as complex and recalcitrant as lignin. This abundant aromatic polymer is currently regarded as a sustainable precursor for the production of new value-added products.[4] However, due to strong bonds within lignin, its degradation and valorisation remain a challenge.[2] In comparison with chemical oxidation methods, enzymatic degradation operates under milder conditions.[2] In some white-rot fungi, laccases are able to oxidise aromatic compounds exceeding their own redox potential with the help of natural chemical mediators that behave like electron shuttles.[2,4] The mediator first gets oxidised by laccase, reduces a co-mediator which can diffuse away and in turn oxidises any substrate that is not able to directly interact with the active site due to steric hindrance.[9] These laccase-mediator systems (LMS) greatly increased the scope of potential laccase applications. Synthetic mediators are toxic and usually inactivate laccases at concentrations above 1 mM. For that reason, mediators such as HBT (1-hydroxybenzotriazole), VA (violuric acid) or NHA (N-hydroxyacetanilidine) are being investigated, the most efficient bearing N-OH groups and having a high unpaired electron density.[10] However, neither the exact electron transfer

pathway nor the detailed dioxygen reduction mechanism is completely understood.[5]

Industrialisation resulted in a severe contamination of water bodies through a variety of pollutants: pharmaceuticals, plasticizers, herbicides, fertilizers, polycyclic aromatic hydrocarbons (PAHs) or synthetic dyes.[5] Laccases are an efficient and eco-friendly tool to tackle this issue because of their ubiquity.[2,8] In order to meet the ever-increasing demand for laccases in oxidative processes such as lignin degradation and enzymatic bioremediation, low cost and efficient processes are required.[5] While some strategies focus on enzyme immobilisation, site-directed mutagenesis or the development of novel biocatalytic materials,[2] we have focused on the understanding of laccase production in different natural media. In the present study, we produced laccases in submerged cultures, using two well-documented fungi: *Trametes versicolor*[1] and *Pleurotus ostreatus*.[11] We achieved process intensification by first selecting the best of three cultivation media and then by testing the impact of copper supplementation on laccase production, since Cu^{2+} has been shown to both activate the gene responsible for its expression and to enhance its activity.[5,6,12] To keep downstream processing and production costs to a minimum a clarified, dialfiltered and concentrated culture supernatant was used for two applications of laccase-mediator systems (LMS). In a lignin depolymerisation context, we were able to show for the first time that violuric acid (VA) is a suitable mediator for the degradation of lignin phenolics. Using the same LMS, we have successfully degraded Remazol Brilliant Blue (RBB), an anthraquinone dye and documented organic pollutant.

2. Materials and Methods

2.1 *Fungi Cultures*

Pleurotus ostreatus and *Trametes versicolor* strains were a generous gift from Dr. Jonas Margot at EPFL. *P. ostreatus* and *T. versicolor* were grown in three different media at pH 4.5: malt extract (2% w/w), malt extract (2% w/w) + yeast extract (3% w/w) + glucose (0.8% w/w), and UAB defined medium (44.4 mM glucose, 35.84 mM N, 14.79 mM P, 1.99 mM Cl^-, 20.11 mM SO_4^{2-}). A 1x1 cm square from a solid-state fungi culture was inoculated in 400 mL medium. The shake-flask was incubated at 25 °C and 130 rpm for 26 days. Laccase and peroxidase activities were monitored on a daily basis in the culture supernatant. Glucose and maltose concentrations were measured every two days by HPLC on an Aminex HPX 87 H column from BioRad (300 x 7.8 mm) using 5 mM H_2SO_4 in Milli-Q water as an isocratic eluent, a flow rate of 0.6 mL/min, a column temperature of 35 °C, an injection volume of 10 µL and RI (refractive index) detection.

2.2 *Enzyme Assays*

Assays were performed in 96-well plates with a thermoregulated spectrophotometer (Molecular Devices, Spectramax 340). Laccase activity was measured *via* absorbance increase at 420 nm during the oxidation of ABTS (purchased from Roche) into $ABTS^+$. 10 µL of a 10 mM ABTS solution was mixed to 180 µL of 100 mM acetate buffer pH 4.5. 10 µL of laccase culture supernatant was added and absorbance was readily measured at 420 nm and 20 °C for 3 minutes and at 10 seconds intervals.[13]

Total peroxidase activity was determined by following absorbance at 468 nm during the oxidation of DMP (2,6-dimethoxyphenol, purchased from Merck) into coerulignone. The following solutions were mixed: 26 µL of 50 mM KF, 20 µL of 18 mM DMP, 2 µL of 0.1 M H_2O_2, 96 µL of 100 mM sodium tartrate buffer pH 4.5, 4 µL of 5 mM $MnSO_4$. 52 µL of peroxidase-containing sample were placed into a 96-well plate. Finally, 148 µL of the previously described mixture were added. Absorbance was readily measured at 468 nm and 30 °C during 5 minutes and at 10 seconds intervals.[12,13]

Activity was expressed in international units ([U], *i.e.* µmol of substrate converted per minute) per mL of sample ([U/mL]). The molar extinction coefficients ε are 36'000 $M^{-1}\cdot cm^{-1}$ and 53'200 $M^{-1}\cdot cm^{-1}$ for $ABTS^+$ at 420 nm and 20 °C and coerulignone at 468 nm and 30 °C, respectively. An optical path length of 0.5 cm in the wells was determined.

2.3 *Effect of Copper on Laccase Activity*

The effect of Cu^{2+} on laccase activity was tested on a purified *T. versicolor* laccase (purchased from Merck) with concentrations ranging from 0.1 to 1 mg/mL and on laccase culture supernatant. Four copper concentrations were tested by adding $CuSO_4$ to the acetate buffer: 0, 0.25, 0.5 and 1 mM. Laccase activity was then measured as previously described.

2.4 *Trametes versicolor Cultures with Varying Copper Concentrations*

The fungi/culture medium combination leading to the highest laccase activity was supplemented with different concentrations of copper. *T. versicolor* was cultured in malt extract (2 %) + yeast extract (3 %) + glucose (0.8 %) at pH 4.5 during 38 days with 0, 0.25, 5 and 1 mM $CuSO_4$ following the same procedure as previously described. No copper was present in the acetate buffer used for activity measurements.

2.5 *Downstream Processing*

Fungi suspension cultures were filtered, and the clear filtrate concentrated by ultrafiltration. The retentate was then used without further purification for the various applications. This renders the process more ecofriendly, profitable and mimics the natural conditions in which laccases degrade lignin or micropollutants. A depth filter (Seitz K200 from Pall Corporation) was used for medium clarification. Ultrafiltration was performed with a 200 cm^2, 10 kDa nominal NMWC hydrophilic membrane (Sartocon®-Slice 200 Cassettes) mounted on a Sartorius Slice 200 filtration unit (Sartorius Stedim). Starting from a culture supernatant volume of approx. 350 mL, retentate was concentrated to a final volume of 70 mL.

2.6 *Lignin Dimer Depolymerisation*

Guaiacylglycerol-β-guaiacyl (GBG, purchased from TCI) (1.5 mg/mL, 4.68 mM) and the mediator (equimolar concentration, 4.68 mM) were dissolved in 1 mL of 100 mM sodium acetate buffer pH 4.5. Reaction was started by adding 40 µL of laccase solution with an activity of 1.8 U/mL to 960 µL of the GBG/mediator solution. Reaction mixture was incubated at 40 °C and 400 rpm in a thermomixer. Samples were collected at 1, 2, 5, 10, 15, 20, 30 and 60 minutes and after 24 hours. 10 µL of a 187.3 mM sodium azide solution were added in order to terminate the reaction. This assay was performed in the presence and in the absence of ABTS and VA (purchased from Merck) mediators. The samples of reaction mixture were analysed by HPLC-DAD-API-MS (Agilent technologies 1200 infinity) equipped with a 6130B quadrupole LC/MS module on a Nucleoshell C_{18} column (150 x 4.6 mm, particle size 2.7 µm). Injection volume was 1 µL, flow rate was 0.5 mL/min, temperature 30 °C and detection performed *via* DAD at 276 nm. MS data were collected in scan mode (100-1500 m/z range) in both positive and negative polarity with API (atmospheric pressure ionization).

2.7 *Micropollutant Degradation*

RBB (purchased from Merck) decolourisation was followed by monitoring the decrease in absorbance at 592 nm. RBB (0.25, 0.5 and 1 mM) and the mediator (equimolar concentration), were dissolved in 5 mL of 100 mM sodium acetate buffer pH 4.5.

Reaction was started by adding 10 µL of laccase crude extract with an activity of 1 U/mL to 190 µL of the micropollutant/mediator solution. Absorbance was monitored at 592 nm and 20 °C for 5 hours and at 5 minutes intervals. This assay was performed in the presence and in the absence of ABTS and VA mediators and compared with negative controls. RBB decolourisation ratio was calculated according to Eqn. (1):

$$\%\,Decolorisation = \frac{A_{0,RBB} - A_{RBB}(t)}{A_{0,RBB}} \quad (1)$$

3. Results and Discussion

3.1 *Effect of Copper*

The effect of Cu^{2+} on laccase activity was tested first with different $CuSO_4$ concentrations in order to understand whether laccase production was increased upon addition of copper inducer or simply whether activity itself was enhanced (Fig. 1).

As expected, measured activity increased linearly with laccase concentration. Addition of copper to the assay buffer increases laccase activity by an average factor of 1.75, but Cu^{2+} concentration itself seems to have poor influence on the slope, thus indicating a threshold activation type. This effect was also observed on culture medium supernatant and matched the 1.75-fold increase in laccase activity upon addition of copper. These results support the idea that copper globally increases the activity of laccase in a given pool of expressed isoforms *via* a mechanism that remains to be clarified.

3.2 *Submerged Cultures of Pleurotus ostreatus and Trametes versicolor*

P. ostreatus grew in malt extract and in malt extract + yeast extract + glucose. Laccase produced by *P. ostreatus* reaches a maximum of 0.119 U/mL on day 13 in malt extract (Fig. 2A, blue triangles). In this medium, maltose (initially 8 g/L) slowly decreases from day 10 concomitantly with the increase in laccase activity until day 26 to finally reach a concentration of 5 g/L. The low levels of glucose (1.5 mg/mL) contained in malt extract stay constant throughout the experiment (Fig. 2B and 2C, blue triangles). No to poor laccase activity was observed for *P. ostreatus* in malt extract + yeast extract + glucose (Fig. 2A, red triangles). Herein, glucose and maltose (8 g/L) are slowly used by the fungi from day 10 to day 20 (Fig. 2B and 2C, red triangles). *T. versicolor* also grew in malt extract and in malt extract + yeast extract + glucose. No to poor laccase activity was noticed in malt extract (Fig. 2A, blue circles). In this medium, maltose (8 g/L) is completely consumed from day 5 to day 15. Glucose (1.5 mg/mL) seems to stay more or less constant throughout the experiment (Fig. 2B and 2C, blue circles). Maximum activity produced by *T. versicolor* (1.209 U/mL in malt extract + yeast extract + glucose) is reached on day 24 (Fig. 2A, red rounds) and represents 10-fold the maximum produced by *P. ostreatus* (0.119 U/mL in malt extract). Glucose and maltose (8 g/L) are quickly consumed from day 7 to day 9 and from day 5 to day 10, respectively, concomitantly with the increase in laccase activity. None of the fungi grew in the UAB defined medium, possibly because this medium lacks essential amino acids.

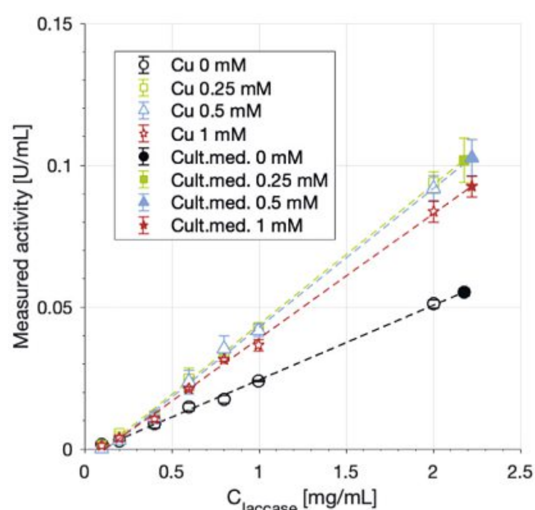


Fig. 1. Effect of copper addition ($CuSO_4 \cdot 5\ H_2O$) in the activity measurement buffer on laccase activity assay. Linear fits were performed on commercial laccases from *T. versicolor* with known concentration (open markers). Culture medium supernatant samples are indicated by filled markers. Laccase concentration for the culture medium samples being unknown, it was estimated from the 0 mM linear fit.

Laccases are produced as a pool of isozymes encoded by a complex set of genes whose regulation is still poorly understood, and their expression in fungi depends on environmental conditions such as temperature, pH, inductors, culture conditions and medium composition.[14–18] Several authors noticed an increase in laccase activity concomitantly to a rapid increase of glucose uptake and showed that laccase production was related to stress response elements in the promoters of genes encoding for laccases.[16,18] Our observations are in accordance with these results. It is generally accepted that nitrogen depletion causes laccase production and that high carbon to nitrogen ratios are required for laccase production.[14,15] This could explain why no laccase activity was produced in malt extract, due to its low nitrogen content.[16] However, results obtained with *P. ostreatus* are somewhat unusual, high laccase activity levels having been obtained by several authors in solid-state and submersed cultures.[17–21] Park *et al*.[22] found that apple pomace, a medium rich in carbohydrates, dietary fibers, minerals and phenolic compounds, enhanced *P. ostreatus* laccase activity. Impact of copper supplementation on laccase expression was studied as well by Diaz *et al*.[23] and Zhu *et al*.[24] The reasons for low laccase activity observed in *P. ostreatus* could include differences in the structure, stability, activity or expression levels of the different isoforms as well as the absence of nutrients or laccase inducers in the culture medium. Our observations are in accordance with other authors who observed similar variations of the inducer effect depending on the strain, the culture medium and the growing conditions.[25,26] Our results however do not fully clarify the laccase expression mechanism and a more systematic investigation would be needed. While many studies focused on improving laccase production by changing culture conditions or by adding inducers, reasons for enhancement of laccase production are scarce.[27]

3.3 *Trametes versicolor Cultures with Varying Copper Concentrations*

Inducers such as copper ions, nitrates or aromatic compounds were previously shown to significantly increase laccase mRNA.[14,15,19,20] However, results from the literature show no consensus regarding the effect of copper on laccase production and activity. In order to both shed light on this aspect and to intensify our laccase production process, we cultivated *T. versicolor* in malt extract + yeast extract + glucose supplemented with four different concentrations of copper (Fig. 3). The same laccase activity profile as previously was obtained in the four cultures. During the first fungi metabolism (iodophase), activity in the 0 and 0.25 mM are similar (0.40–0.50 U/mL on day 14), whereas it is slightly increased for the 0.5 mM (0.73 U/mL on day 14) and greatly increased (1.70 U/mL on day 14) for the 1

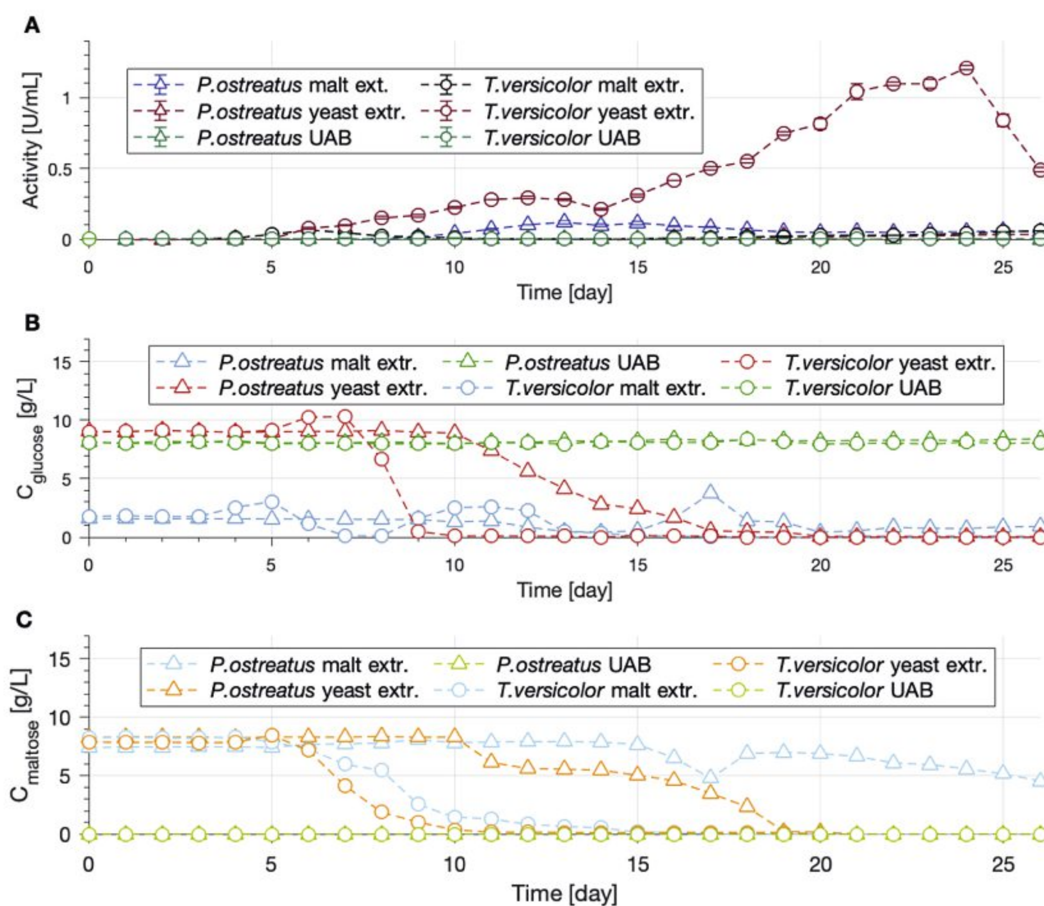


Fig. 2. Evolution of A) laccase activity, B) glucose concentration and C) maltose concentration versus time during submerged cultures of *P. ostreatus* and *T. versicolor* in three different media: malt extract (2%), malt extract (2%) + yeast extract (3%) + glucose (0.8 %) and UAB defined medium.

mM trial. During the second fungi metabolism, laccase activity reaches maxima of 1.21, 1.37, 1.58 and 3.32 U/mL for 0, 0.25, 0.5 and 1 mM Cu^{2+}, respectively (Fig. 3A). Copper supplementation increases laccase activity by factors 1.1, 1.3 and 2.7, respectively. Herein copper supplementation boosts laccase activity by a factor that increases with copper concentration, in contrast with the results presented in section 3.1.

It is worth noting that the culture medium was diluted 20 times in the copper-free acetate buffer used for activity measurements. These elements indicate that more laccases were likely produced by the fungi. While other authors measured increased quantities of laccase mRNA[19,20] or increased laccase activities upon copper supplementation,[14,19,28] no study on the combined effect of copper on laccase activity itself and on its increased expression was published to our knowledge. Determining the threshold for the copper concentration required to increase laccase activity would conclusively shed light on this aspect and help determining the optimal copper concentration for laccase production.[27–29] Low peroxidase activity was also produced in the culture medium (Fig. 3B) with a maximum activity of 0.2 U/mL. Copper supplementation seems to have no influence on peroxidase activity or activation, which is expected, peroxidases being copper-independent enzymes.

3.4 *Phenolic Lignin Model Dimer Depolymerisation with Laccase-mediator Systems*

While laccases possess the ability to react with the phenol hydroxyl groups of lignin (10–30%), non-phenolic subunits (70–90%) are more recalcitrant and require LMS or lignin peroxidase.[30] LMS were found to act on the non-phenolic part of lignin,[31,32] but little is known about their effect on the phenolic part. It therefore seemed relevant to us to study the latter, especially because polymerisation can occur, which would go against our goal.[34] Laccase-catalysed reactions of GBG, a lignin model dimer compound mimicking phenolic subunits of lignin, have been previously investigated in the presence of HBT and ABTS mediators. Hilgers *et al*. found that the HBT effect was insignificant because of its slow oxidation kinetics compared to GBG oxidation, resulting in extensive polymerisation of GBG as observed for laccase without mediator.[30] Munk *et al*. also observed this effect.[33] These results are contradictory with other studies that showed a much higher lignin degradation efficiency with laccase/HBT than with laccase alone.[35] In contrast, Hilgers *et al*. found that GBG was oxidised by both laccase and by readily formed $ABTS^{+}$, resulting in a complex comprising ABTS and GBG.[30] It therefore seemed relevant to first study the interactions of laccases and ABTS with GBG as a point of comparison. Violuric acid (VA) has been described as particularly efficient to oxidise the non-phenolic part of lignin,[32] but no study on its effect on the phenolic part has been reported to date. We therefore tested this promising mediator on GBG with a commercial, purified laccase (Merck) and with crude extract laccase in order to understand the effect of peroxidase (Fig. 4). The different products were identified using accurate mass determination. Scheme 1 shows the structure of GBG and of the two mediators used in this study.

When laccases alone are used, GBG ($C_{17}H_{20}O_6$) peak area decreases as a function of time while a peak corresponding to a GBG dimer ($C_{34}H_{38}O_{12}$) increases. GBG dimer then decreases concomitantly with the formation of GBG trimer ($C_{51}H_{56}O_{18}$), thus indicating a polymerisation formed by coupling of phenoxy radicals *via* C–C bond formation (Fig. 4A). Tetramers or pentamers might even be formed in later reaction steps and could not be seen, polymer being usually not detectable by HPLC. This confirms the results obtained by Hilgers *et al*.[30] who also noticed an extensive GBG polymerisation upon addition of laccase. Laccase alone therefore creates radicals that can further react and result in polymerisation. The same reaction profile as with the purified laccase is observed with culture medium supernatant on GBG. However, the GBG

peak area decreases more slowly than with purified laccase, and the amount of GBG dimer also decreases at a slower rate. This indicates that the low levels of peroxidases present in the culture medium do not influence lignin model dimer depolymerisation. Culture medium supernatant + ABTS and VA (Fig. 4C and B) both show a decrease in GBG and mediator peak area. If polymerisation starts occuring as seen by the presence of GBG dimer with both mediators, no GBG trimer was detected, thereby indicating that ABTS and VA are efficient to prevent GBG further polymerisation. Instead, radical cations readily formed by laccase result in C_α oxidation of GBG and mediator coupling to GBG, suggesting that once ABTS or VA is grafted on phenolic lignin subunit, further polymerisation is blocked. Our results are in accordance with Munk *et al*. who suggested a grafting of mediators to lignin to explain their EPR results[33] and with Hilgers *et al*. who observed stable GBG-ABTS adducts.[30] Herein, a product corresponding to the mass detected by Hilgers *et al*.[30] *via* HPLC-DAD-API-MS was detected (Scheme 2A). A product corresponding to the mass of the oxidised form of GBG coupled to VA with a similar structure than the one described by Hilgers*et al*.[30] for HBT was also identified (Scheme 2B). The exact structure of this molecule remains to be determined.

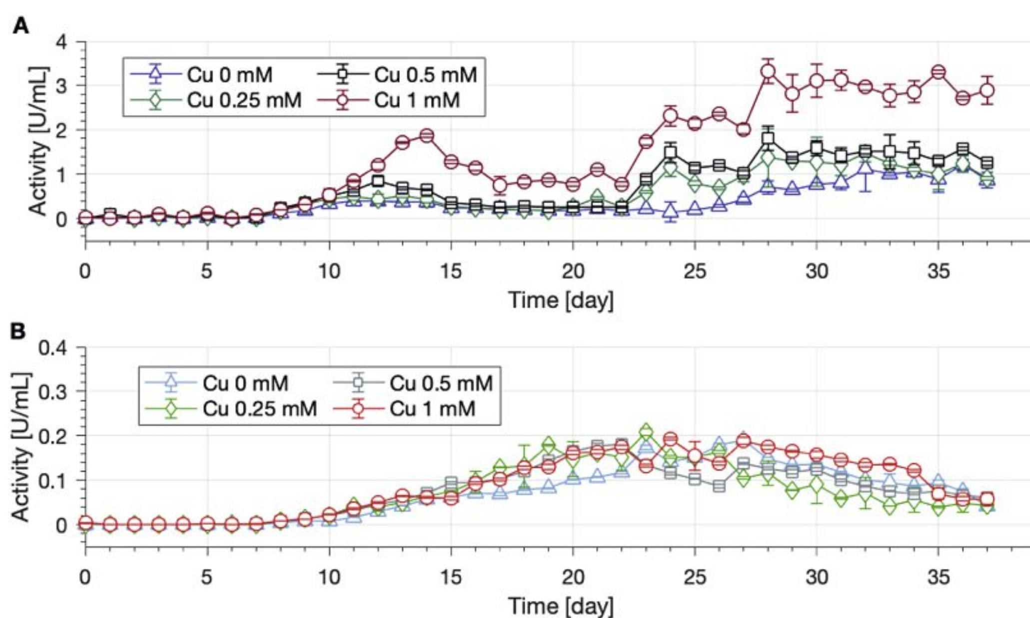


Fig. 3. A) laccase activity and B) peroxidase activity versus time for *T. versicolor* cultures in malt extract (2%) + yeast extract (3%) + glucose (0.8 %) supplemented with four different concentrations of Cu^{2+}.

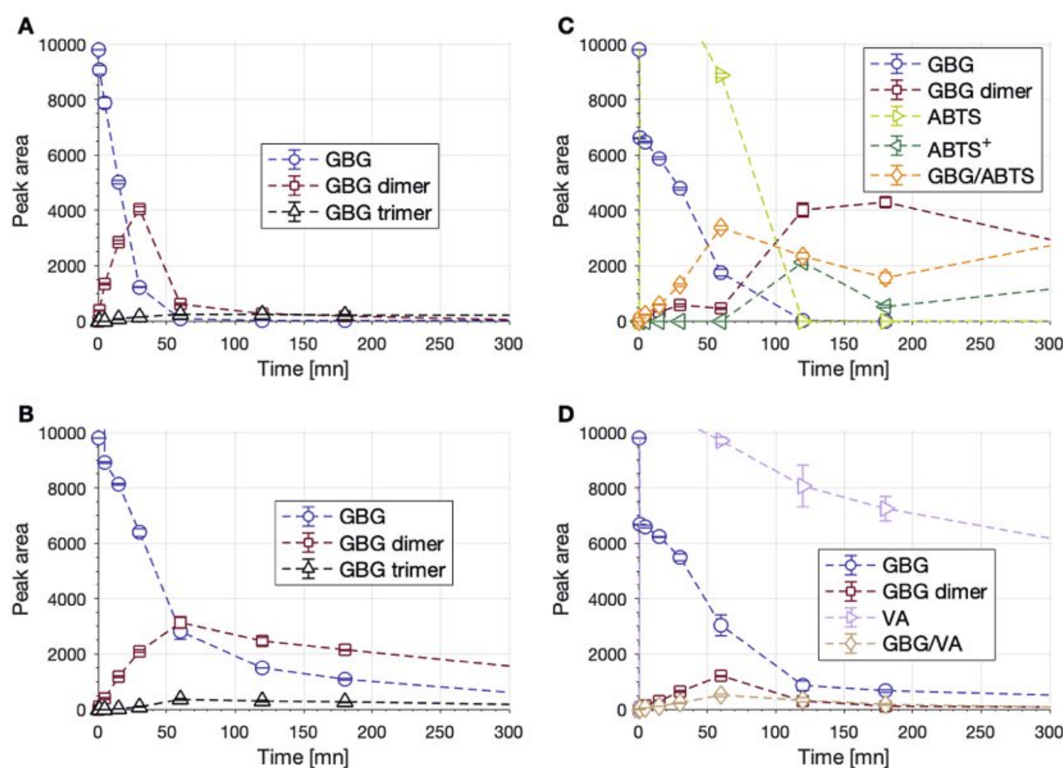
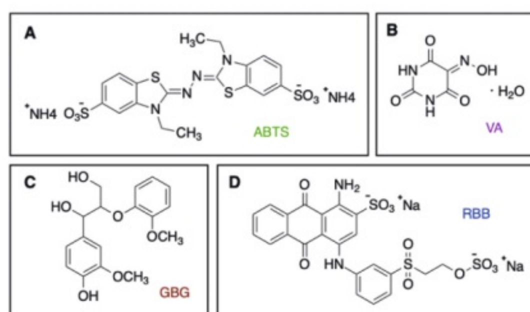
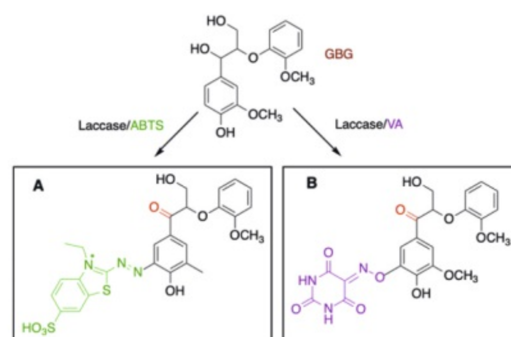


Fig. 4. Evolution of peak area at 276 nm versus time for the reactions of phenolic lignin model dimer compound (GBG) with laccase-mediator systems (LMS). A) laccase crude extract + GBG B) purified laccase + GBG + ABTS C) laccase crude extract + GBG D) laccase crude extract + GBG + VA.



Scheme 1. Molecular structure of A) ABTS and B) VA, the two mediators used in this study, and of C) GBG and D) RBB, the phenolic lignin model dimer and the micropollutant used for applications, respectively.



Scheme 2. Molecular structure of A) ABTS/GBG complex also found by Hilgers *et al.*[30] B) Potential VA/GBG complex found in this study (to be confirmed).

This is in agreement with the results of Longe *et al.*,[32] who noticed a 49% depolymerisation rate versus 43% for VA and ABTS, respectively. Albeit GBG/VA (Scheme 2B) has a similar structure as GBG/HBT,[30] the redox potential of VA is much higher than the one of HBT, thereby explaining its higher efficiency in the prevention of polymerisation. This is confirmed by Li *et al*.[36] who found that violuric acid allowed *T. versicolor* laccase to oxidise non-phenolic lignin model compounds much faster than HBT. Bourbonnais *et al*. showed that oxidation of HBT by laccases was 85 times slower than oxidation of ABTS.[37] Picart *et al*. even showed the efficiency of VA in a multi-step biocatalytic depolymerisation of lignin.[37] Our model system would need further investigation in order to unveil mechanisms governing the effect of violuric acid on lignin depolymerisation. This reaction could be repeated with other synthetic lignin models and analysed by EPR spectroscopy, FT-IR, pyrolysis-GC-MS, quantitative ^{31}P, 2D HSQC NMR or size exclusion chromatography (SEC) as performed by several authors.[38–40]

3.5 *Dye Degradation*

Laccases have also shown a great potential to replace conventional expensive chemical methods in the degradation of a large variety of organic pollutants such as endocrine disruptors, pharmaceuticals or dyes.[41,42] LMS have been used in several studies for the degradation of micropollutants with HBT,[43,45,48] ABTS,[43,45,48] acetosyringone,[44] syringaldehyde and many other mediators.[45] Natural mediators such as vanillin and syringaldehyde[45–48] have been found to be particularly efficient for the degradation of pesticides[45] and chloramphenicol.[46] As an organic contaminant model, we have tested the ability of LMS to degrade Remazol brilliant blue (RBB), a water-soluble anthraquinone dye used in textile industries which can damage both aquatic and vegetative life if contaminated water is used for irrigation. The same LMS as for lignin depolymerisation were tested on RBB degradation in order to understand if ABTS or VA could also help in bioremediation processes with a different type of compound. As for the GBG depolymerisation, RBB degradation was tested with culture medium supernatant laccase alone and in the presence of ABTS or VA mediators (Fig. 5). A maximum decolourisation percentage of 50% was obtained with laccase alone, while the addition of ABTS allowed a decolourisation percentage of only 38%. As seen in Scheme 2, ABTS and RBB have similar structures, and the addition of ABTS to the reaction might have reduced RBB degradation for the benefit of ABTS. This could also be due to the spectrophotometric method used. Pype *et al* reported a significant difference (up to 10%) appearing between the level of RBB degradation and the level of obtained decolourisation thanks to a mechanistic study on the degradation of RBB by laccases.[49] Interestingly, VA enabled a much higher decolourisation degree of 70%. VA therefore participates in the oxidation of RBB and helps its degradation *via* either a special degradation mechanism or a faster kinetics. Our results are in accordance with Ashe *et al.*, who studied the effect of seven mediators on trace organic contaminants and found that non phenolic compounds were best removed using HBT or VA.[48] Violuric acid therefore seems to be a very promising mediator for dye degradation. Ashe *et al.* found that VA efficiently degraded selected compounds without causing significant toxicity in the effluent.[48]

4. Conclusion

In the present work, we have first studied the influence of culture medium on fungal growth and laccase production of two well-known basidiomycetes. We observed unusual results with *P. ostreatus* producing low laccase activity in malt extract while *T. versicolor* showed high laccase activity in malt extract + yeast extract + glucose. Further experiments are required in order to shed light on this phenomenon. Understanding laccase expression mechanism could enhance the development of natural, safe and efficient industrial oxidative processes. We have also shown that addition of copper was efficient to enhance both laccase activity compared to copper-free reaction mixtures and its expression. *T. versicolor* in malt extract + yeast extract + glucose supplemented with 1 mM Cu^{2+} gave the most conclusive results. Additionally, we have shown that violuric acid (VA) was an efficient mediator to favour phenolic lignin model degradation over polymerization and proposed a structure for the identified GBG/VA complex. Finally, we showed that VA efficiently promoted decolourisation of Remazol brilliant blue (RBB), an anthraquinone dye toxic to aqueous environments. The ever-increasing demand of laccases for processes such as delignification, industrial oxidative processes and bioremediation require the production of large quantities of enzymes at low cost. In order to tackle these challenges, we propose several ways to expand our work. First, the impact of further increase in copper concentration in combination with other phenolic inducers should be investigated. Combined phenolic and copper inducers were indeed found to be particularly effective.[22,24,28,29] Then, the effect of VA should be further investigated in parallel with the use of vanillin as an efficient natural mediator for the degradation of lignin and pesticides.[45,48,50] Supplementing such a natural mediator to our culture medium supernatant would reduce process toxicity. Improvement of a process should take into account the cost, the environmental impact, be scalable and economically viable. One of the main limitations to the large-scale application of LMS is their high price, their inactivation effect on laccases, and the potentially toxic derivatives they could generate. In order to overcome these limitations, we propose to co-immobilise laccases and their efficient mediators via various immobilization techniques. This could improve stability, reusability, but also resistance to industrial conditions such as high temperatures, mechanical stress, organic solvents

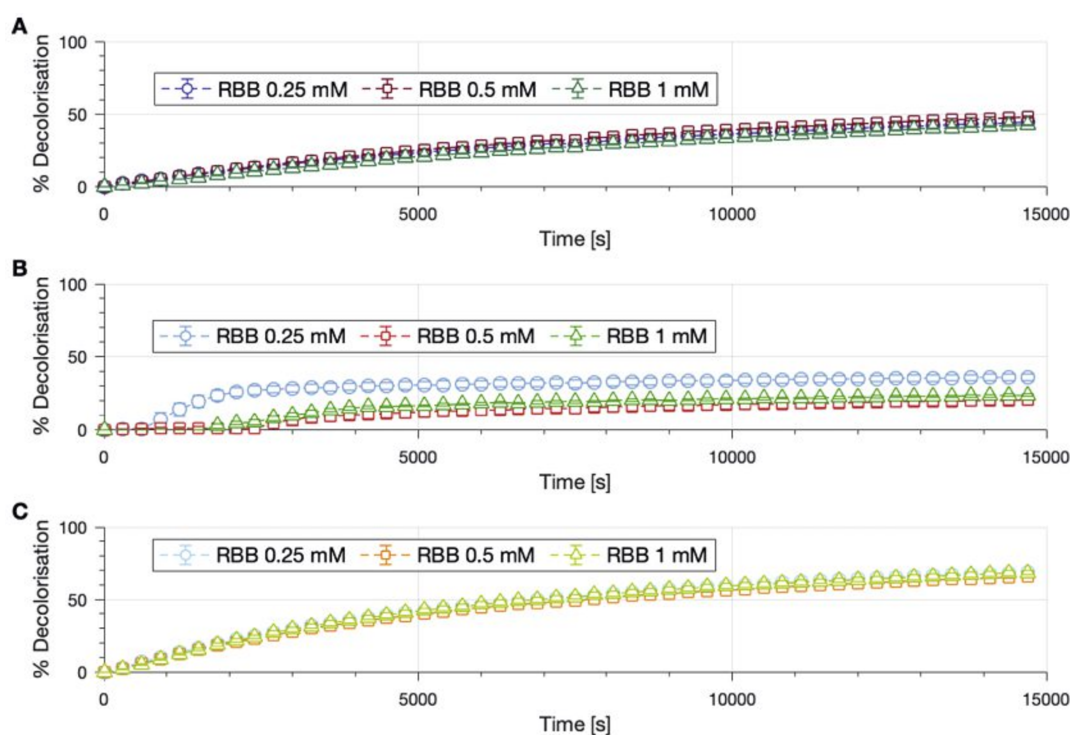


Fig. 5. Evolution of decolourisation % of RBB versus time for A) laccase crude extract B) laccase crude extract and ABTS and C) laccase crude extract and VA at RBB concentrations of 0.25, 0.5 and 1 mM.

and high temperatures or harsh pH values. Immobilisation could also minimise the problem of laccases deactivation observed with N-OH type of mediators. There is indeed an urgent need for sustainable enzymatic processes that would both respect the environment and reach commercial success.

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