Characterization of laccases from *Trametes hirsuta* in the context of bioremediation of wastewater treatment plant effluent

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Abstract

The bioremediation of pharmaceutical compounds contained in wastewater, in an ecological and sustainable way, is possible via the oxidative action of fungal laccases. The discovery of new fungal laccases with unique physico-chemical characteristics pushes researchers to identify suitable laccases for specific applications. The aim of this study is to purify and characterize laccase isoenzymes produced from the Trametes hirsuta IBB450 strain for the bioremediation of pharmaceutical compounds.

Two main laccases mixtures were observed and purified in the extracts and were called Y_n and Y_g. Peptide fingerprinting analysis suggested that Yn was constituted mainly of laccase Q02497 and Y_g of laccase A0A6M5CX58, respectively. Robustness tests, based on tolerance and stability, showed that both laccases were affected in a relatively similar way by salts (KCl, NaCl), organic solvents (ACN, MeOH), denaturing compounds (urea, trypsin, copper) and were virtually unaffected and stable in wastewater. Determination of kinetic constants (Michaelis (K_M), catalytic constant (k_{cat}) and kinetic efficiency ($K=k_{cat}/K_M$)) for the transformation of synthetic hormone 17α-ethynylestradiol and the anti-inflammatory agent diclofenac indicates a lower KM and kcat for laccase Y_n but relative similar K constant compared to Y_g. Synergistic effects were observed for the transformation of diclofenac, unlike 17α-ethynylestradiol. Transformation studies of 17α-ethynylestradiol at different temperatures (4 and 21 °C) indicate a transformation rate reduction of approximately 75-80% at 4 °C against 25% for diclofenac in less than an hour. Finally, the classification of laccases Y_g and Y_n into one of eight groups (group A-H) suggests that laccase Y_g belongs to group A (constitutive laccase) and laccase Y_n belongs to group B (inducible laccase).

Keywords: diclofenac, 17α-ethynylestradiol, kinetic constants, laser diode thermal desorption, laccase A, laccase B

1. Introduction

The increase in the consumption of pharmaceutical products and the growth of the population living in urban centers have led to an increase in the load of wastewater to be treated in municipal wastewater treatment plants (WWTPs) [1–3]. These wastewaters contain various trace organic contaminants, such as pharmaceutical compounds, in concentrations high enough to potentially disrupt aquatic ecosystems. A recent review, presenting results of several hundred publications on the potential toxicity of diclofenac in nature, suggests that there could be harmful effects on the environment. [4]. 17α-ethynylestradiol (EE2), is a recognized as an endocrine disruptor, its concentration in nature has been measured to several tens of ng L^{-1} while negative effects have been observed in some adult male frogs exposed to less than 1 ng L^{-1} [5–7]. The elimination of pharmaceutical compounds by WWTPs remains highly variable. Some of these contaminants such as acetaminophen or ibuprofen are eliminated from wastewater with high efficiency while others such as carbamazepine or temazepam are weakly eliminated [2,3].

To reduce the load of pharmaceutical contaminants in the effluents of WWTPs, the development of new treatment approaches, such as advanced oxidation treatments (AOPs), have been proposed. Although AOPs have proven their effectiveness [8–11], their use generally remains energy-intensive and thus, costly [11,12]. Also, in some cases, AOP may not reduce the toxicity of the treated water [8,13]. Thus, developing green and sustainable AOPs is critical. Biotreatment based on white-rot fungi (WRF), as well as their extracellular enzymes represents an interesting alternative to AOPs [13,14]. For example, high transformation of acetaminophen, ibuprofen, indomethacin, ketoprofen, mefenamic acid, naproxen, etc. in less than 48 h was observed [15–27]. Although the economic benefit of the use of enzyme treatments for WWTPs remains unclear, cost reduction studies show promising results with the reduction of costs such as the price of support materials or the price of enzyme production, and also on the improvement of enzyme activity and stability [13,28,29]. However, the use of WRF in WWTPs poses several difficulties such as their survival under non-sterile conditions and the management of the sludge produced [14]. This is not the case with extracellular enzymes, where different immobilization techniques on solid supports allow their use and recycling [30].

The use of extracellular enzymes for wastewater treatment processes pushes us to consider the choice of the WRF used to produce these enzymes. By considering the application of interest (in this case, the bioremediation of WWTP water), several parameters enter into the equation such as the optimal growth of the fungus resulting in a high yield of laccases (EC 1.10.3.2) and other extracellular enzymes allowing high transformation rate of pharmaceutical compounds in a complex medium. For such application, the *Trametes hirsuta* species of the *Polyporaceae* family looks promising [31]. Indeed, it has been shown to be particularly robust for growth in biosolids, industrial and municipal effluents [32], which is relevant in the context of long-term ecological development. In addition, the supernatant containing the extracellular enzymes (i.e., the crude extract) revealed a strong potential for the degradation of phenolic compounds [33], and their immobilization on a solid support is possible and a topic of current research [29,34,35].

Among the extracellular enzymes excreted by *T. hirsuta*, laccase is the most promising for the development of bioremediation processes for several reasons: i) it does not require a co-substrate (e.g., H_2O_2) to function, since the final electron acceptor is O_2 [36]; ii) it is secreted in large quantities outside the WRF [37]; iii) its redox potential is one of the highest among known fungal laccases (0.780 mV vs. NHE) [38] indicating that it is able to oxidize a wide range of substrates. Indeed, laccase has been used to remove multiple organic contaminants with medium to high transformation rates such as pharmaceuticals (diclofenac, acetaminophen and amoxicillin [13,39,40], hormones (estrone, 17β-estradiol, estriol and EE2 [41]), antiseptics such triclosan

[42] and other trace contaminants such as bisphenol A [43], and iv) no toxicity is observed after treatment of pharmaceutical compounds, unlike the AOPs mentioned above [13].

In the *Polyporaceae* family, to which *T. hirsuta* belongs, genomic studies suggest the presence of several genes coding for the secretion of different laccases.[44]. These laccases, called isoenzymes, have a different amino acid sequence which gives them different physico-chemical properties [45]. The substitution of one or more key amino acids in the active site can strongly modify the interactions between laccase and its substrate [46]. For example, in the case of ABTS, K_M can vary by a factor of a hundred, sometimes between two isoenzymes of the same strain [44,47]. These modifications can also impact the proper oxidation of a substrate and therefore potentially reduce the effectiveness of bioremediation. For example, in the context of the bioremediation of synthetic dyes, the intensity of the UV absorption of Malachite green (at 618 nm) and Remazol Brilliant Blue R (at 600 nm) decreased in both cases by 90% after 24 hours of exposure to the rLACC6 isoenzyme, whereas it only decreased by 5% and 20% respectively after exposure to the rLACC9 isoenzyme for the same duration [48].

While it is highly suspected that a cocktail of isoenzymes should give the best results for the removal of pharmaceutical compounds, it is necessary to first understand the capacity of individual isoenzymes to remove them for future applications in bioremediation. In the context of wastewater bioremediation, it is necessary to consider isoenzymes with optimal physico-chemical properties, and two parameters are crucial: Functionality in a complex matrix and a high potential for transformation of pharmaceutical compounds.

The objective of this study is to characterize the laccase isoenzymes secreted by the strain *T. hirsuta IBB450* for an application to wastewater bioremediation. To do this, the stability and tolerance of the purified isoenzymes were evaluated by exposing them to different denaturants or interfering agents (protease, urea, wastewater, temperature, etc.) and the catalytic performances of the isoenzymes were evaluated using two pharmaceutical compounds of interest, namely diclofenac and 17α-ethynylestradiol.

2. Materials and Methods

2.1. Reagents and chemicals

Water, methanol (MeOH) and formic acid (Optima LC/MS grade) (Fisher Scientific, Ottawa, On, Canada), DCL sodium salt, and EE2 (Sigma Millipore, Oakville, ON, Canada), deuterium-labeled DCL (DCL-d_4) and EE2 (EE2-d4) (CDN-Isotopes, Pointe-Claire, Qc, Canada) were used.

Stock solutions at 1 g L^{-1} of DCL and EE2 in MeOH were stored at –18 °C. Intermediate solutions were prepared from stock solutions in MeOH at a concentration of 20 mg L^{-1}. Stock solutions of internal standards were stored in MeOH at -18 °C at a concentration of 200 µg L^{-1}.

Buffer solution at pH 7 was prepared from monobasic potassium phosphate (ACS grade) (EMD chemicals, Gibbstown, NJ, USA) and dibasic potassium phosphate (ACS grade) from Fisher Scientific. Buffer solution at pH 4 was prepared from glacial acetic acid (ACS grade) (VWR-Anachemia, Mississauga, ON, Canada) and sodium acetate (≥ 99% purity) from Sigma Millipore. Glycerol (GC grade), ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic-acid)] (> 98% purity) and bromophenol blue sodium salt were acquired from Sigma-Millipore. Sodium dodecyl sulfate (SDS), Coomassie brilliant blue R-250, and acrylamide/bis solution used (40% for a ratio of 29:1) (Bio-Rad, Mississauga, On, Canada) were used. Ethanol (95% purity) solution used for the discoloration of the SDS-PAGE gels was purchased from Fisher Scientific. Phosphate Buffered

Saline (PBS) was prepared by adding 8 g of NaCl, 0.2 g of KCl, 1.42 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 to 1 L of Milli-Q water.

2.2. Medium, fungal strain and culture conditions

The fungal strain *T. hirsuta IBB450* was provided from the Culture Collection of the Institute of Biochemistry and Biotechnology (Tbilisi, Georgia). Each culture was carried out under sterile conditions and grew over ten days at 37 °C. The slants were stored at 4 °C on potato starch, glucose, and agar 4%, 20% and 15% (*w/v*) respectively and were renewed every three months. The saline solution used for mycelium storage and washing pellets was 0.87 % *w/v* of NaCl sterile solution. The liquid cultivation medium used for the flask experiment was composed of dextrose, peptone, malt extract, yeast extract and copper sulfate 1%, 0.5%, 0.3%, 0.3% (*w/v*) and 1 mM respectively. All cultures were carried out under pelleted mycelium forms. The method used for pelletization was adapted from Marco-Urrea et al. (2007) [49]. Briefly, four slants (1 cm^2) of *T. hirsuta* from Petri dishes were cultivated in a 500 mL Erlenmeyer flask containing 250 mL of medium solution. After seven days of cultivation (26 °C and at 135 rpm), the solution was filtered, and the fungal biomass was rinsed with sterile saline solution. The fungal biomass was then blended in the saline solution and stored at 4 °C. Two milliliters of the first blended solution was then added in a 1 L Erlenmeyer flask containing 250 mL of cultivation medium. Then, the experience was repeated: after seven days of cultivation at 26 °C and under a 135-rpm orbital agitation, the solution was filtered, and the fungal biomass was rinsed with sterile saline solution. The fungal biomass was then blended in the saline solution and was stored at 4 °C. This solution, hereafter referred as mycelium solution, gives it the most repeatable and reproducible pellet forms.

2.3. Measurement of the enzymatic activity

Laccase activity was measured spectrophotometrically by measuring the oxidation of ABTS at 420 nm. A volume of fifty microliters of enzyme solution was transferred directly into the spectrophotometer cuvette containing 1.950 mL of ABTS solution (0.500 mM in acetate buffer 0.1 mM, pH 4). One unit (U) represents the amount of laccase capable of oxidizing 1 µmol of substrate per minute. Analyses were performed on a UV-Vis spectrophotometer model UV-1800 from Shimadzu. All measurements were made in triplicate.

2.4. Purification and separation of isoenzymes

After 14 days of cultivation, the supernatant was removed from the fungal solution. It was filtered at 1 mm, then centrifuged at ≈ 3000 g for 15 min, finally filtered at 0.22 µm. This crude extract was then concentrated approximately 200 times with Amicon Ultra-15 centrifugal filter units from Sigma Millipore (cut-off 30 and 100 kDa), to obtain only a range of proteins between 30 and 100 kDa into the final extract. Then, 1 mL of the extract was diluted with the PBS solution by a factor of 100 then reconcentrated to 1 mL to remove potential contaminants with Amicon Ultra-15 centrifugal filter units (cut-off 30 kDa).

The apparatus used for separation of the proteins by size exclusion chromatography (SEC) is an AKTA Avant 25 Fast Protein Liquid Chromatography (FPLC) system from GE Healthcare Bio-Sciences (Upsala, Sweden). A first purification was carried out with a Superdex 200 HR 10/30 SEC 24 mL (30 cm × 10 mm, 13 µm

particle size) column also made by GE Healthcare Bio-Sciences. The detection was done by measuring the absorbance at 260 nm and 280 nm. The volume of injection was 250 µL and 60 fractions of 500 µL were collected throughout the separation. Also, a solution containing four SEC standards (2000, 200, 67 and 25 kDa) was separated in a second run to estimate the molecular mass of the proteins presents in each fraction. The enzymatic activity of each fraction was measured by Native-PAGE. The fractions containing the oxidizing activities were mixed and then treated according to the following protocol.

The collected fractions were diluted by a factor of 10 in Q100 buffer (40 mM Tris-Cl pH 8.8, 100 mM NaCl) and purified on a HisTRAP Q FF 1 mL (25 mm × 7 mm, 90 µm particle size) anion exchange chromatography column from GE Healthcare Bio-Sciences. The group of enzymes containing redox activity not retained by the column was collected in the flow-through. The group of enzymes containing oxidation-reduction activity retained by the column is eluted by a NaCl gradient. The gradient of 18 column volumes at 1 mL min^{-1} was from 100 mM to 1000 mM (NaCl in 40 mM Tris-Cl pH 8.8) using the 2-pump system of AKTA Explorer 100 device from GE Healthcare Bio-Sciences. The isoenzymes of interest were subsequently concentrated on an Amicon Ultra-15 centrifugal filter unit (cut-off 10 kDa) and the resulting concentrates were subjected to a change of buffer to PBS. To determine the molecular mass more accurately, a solution containing three SEC standards (66, 43 and 25 kDa) was separated to estimate the molecular mass of laccase isoenzymes purified by a Superdex-75 SEC column.

2.5. Electrophoresis on polyacrylamide gel

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to the Laemmli protocol [50] with a Mini-PROTEAN Tetra Cell device from Bio-Rad. The separation was made with a 9% polyacrylamide gel followed by coloration with Coomassie blue. Then a non-denaturing polyacrylamide gel electrophoresis gel (Native-PAGE) with ABTS staining was performed using the same migrating conditions as for SDS-PAGE without DTT addition and heating. The staining solution used consisted of 1 mM ABTS in acetate buffer at pH 4.

To semi-quantitatively determine the laccase concentration, a known quantity of bovine serum albumin (BSA) was loaded into 5 wells of an SDS-PAGE gel. Then, a known volume of the sample of interest is loaded into 5 more wells. After migration of the proteins through the gel and staining and then discoloration of the gel, the intensity of the bands obtained is measured by the GelAnalyzer software on an unsaturated scan [51]. The intensity of the bands allows to approximate the concentration of the enzyme of interest.

2.6. Identification of laccase isoenzymes by peptide mass fingerprinting

After the migration of ≈ 500 ng of purified isoenzymes through an SDS-PAGE gel (see section 2.5), a slice of gel containing a unique protein band was excised and cut into 1 mm^2 squares and treated by the protocol detailed in the section S-1 (more information in Figure S-1 in the Supporting Information). Briefly, the dehydrated samples thus obtained were enzymatically digested. A first set of samples was digested overnight with stirring at 37 °C with 12.5 ng mL^{-1} of trypsin (MS grade) from Thermo Fisher Scientific (Waltham, MA, USA) in ammonium bicarbonate solution. A second set of samples was obtained by using chymotrypsin (MS grade) from Thermo Fisher Scientific and the same experimental conditions employed with trypsin except that $CaCl_2$ (1 M) must be added during digestion for chymotrypsin. The digested samples were then desalted

through a C18 Zip-Tip column from Thermo Scientific and the solvent was evaporated. The sample was finally reconstituted in a solution of 1% formic acid before being transferred to a glass tube and stored at -20 °C until analysis by liquid chromatography-trapped ion mobility spectrometry-quadrupole-time-of-flight mass spectrometry (LC-TIMS-QTOF).

The LC-TIMS-QTOF system from Bruker Daltonics (Bremen, Germany) was composed of a nanoElute liquid chromatograph coupled to a TimsTOF Pro ion mobility mass spectrometer with a Captive Spray nanoelectrospray ion source, also manufactured by Bruker Daltonics. Digested peptides from ≈ 250 ng of each sample were injected into an Acclaim PepMap100 C_{18} trap column (5 mm × 0.3 mm, 5 µm particle size) from Thermo Fisher Scientific with a constant flow of 4 µL min^{-1} then eluted into a PepSep C_{18} column (25 cm × 75 µm, 1.9 µm particle size) from Bruker Daltonics. The mobile phase was composed of 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Peptides were eluted over a 120 min gradient from initial conditions (95% A, 5% B) to final conditions (63% A, 37% B) at a flow rate of 500 nL min^{-1}. Data was acquired using data-dependent auto-MS/MS with a *m/z* 100-1700 range, with parallel accumulation serial fragmentation (PASEF) acquisition enabled. The number of PASEF scans was set to 10 (1.27 s duty cycle) and a dynamic exclusion of 0.4 min *m/z*-dependent isolation window and a collision energy of 42 eV. The target intensity was set to 20000, with an intensity threshold of 2500.

Raw files were analyzed using MaxQuant software version 1.617.0 and the Uniprot *T. hirsuta* database (Taxon ID: 5327, version: 04/10/2018, 63 entries). The parameters used for the MaxQuant analysis (with the TIMS-DDA type in the group-specific parameters) were as follows: 2 cleavage errors were allowed; the fixed modification was carbamidomethylation on cysteine. Variable changes included in the analysis were oxidation of methionine, acetylation of N-terminal proteins, and carbamylation of proteins (K, N-terminal). A mass tolerance of 10 ppm was used for precursor ions and a tolerance of 20 ppm was used for fragment ions. More details are given in section S-1. The scores returned by the identification algorithm are based on probability of matching the mass spectrum to the database sequences of interest [52].

2.7. Identification and properties of the laccase isoenzymes

The percentage of similarity between two amino acid sequences was carried out with the SIM-Alignment Tool for protein sequences software (Expasy (https://web.expasy.org/sim/)). The theoretical mass and the isoelectric point were generated by Expasy Compute pI/Mw tool (https://web.expasy.org/compute_pi/). The N-glycosylation sites were predicted by NetNGlyc 1.0 (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0).

The optimal pH of the purified isoenzymes was measured between pH 2 and 8. The initial activities at pH 4 were ≈ 500 U L^{-1}. To measure the optimal pH, a citrate buffer (pH 2-3.5), an acetate buffer (pH 4-5.5) and a phosphate buffer (pH 6-8) at 0.1 M were employed. The buffer solution employed for the measurement of activity with ABTS was adjusted according to the pH studied.

The Michaelis-Menten kinetic constants were determined according to Lineweaver and Burk methodology [53] using ABTS as a substrate. K_M (mol L^{-1}) is the ratio of the slope of the line to the y-intercept. The maximum speed V_{max} (mol L^{-1} min^{-1}) is the ratio of the constant K_M to the slope of the line. The catalytic constant k_{cat} is the ratio between V_{max} and the laccase concentration. The kinetic efficiency constant K (=k_{cat}/K_M ; L s^{-1} mol^{-1}) is the ratio of the constant k_{cat} to the constant K_M. The initial enzymatic concentration

was ≈ 250 U L^{-1}. A total of 9 solutions of ABTS at different concentrations were prepared. The most concentrated solution was at $4.5'10^{-4}$ M and the dilutions were made in series from a factor of 2 up to a factor of 256. The solvent was an acetate buffer at pH 4 (acetate buffer) at 0.01 M.

2.8. Characterization and comparison of the laccase isoenzymes for application to bioremediation

2.8.1. Quantification of trace organic contaminants by laser diode thermal desorption-triple quadrupole mass spectrometry

Quantitative analysis of DCL and EE2 before and after treatment with purified laccases was carried out by a laser diode thermal desorption-triple quadrupole mass spectrometry (LDTD-QqQ-MS) system composed of an LDTD source made by Phytronix (Quebec, QC, Canada) and a TQ-S micro triple quadrupole Xevo made by Waters (Milford, MA, USA). Before introducing the samples into the microwell plate, all the wells were treated with a volume of 4 µL of a coating solution (268 µM EDTA, 1.4% NH_4OH, MeOH-Water 3:1 *v/v*) then dried in an oven at 60 °C for 10 min. A coating has been added to improve the signal of the analytes [54]. To improve the repeatability of the analyses, 30 µL internal standards intermediate solution was mixed with 30 µL of each sample. Then 4 µL of this mix was introduced into the wells of the LDTD plate and dried in an oven at 60 °C for 15 min. The desorption of the compounds took place with a laser power pattern going from 0% to 45% of its maximum power in 3 s and remaining at 45% for 2 s. The carrier gas flow rate was fixed at 3 L min^{-1}. The current applied to the corona needle was fixed at 3 µA. The temperature of the source was set to 150 °C and the flow rate of the carrier cone was fixed at 10 L h^{-1}. The analysis was carried out in positive mode. The complete analysis time per sample was 13.2 sec. The instrument was set in MRM mode and two fragment ions were measured: one for quantification and the other for identification (more information in Table S-1 in the Supporting Information).

2.8.2. Tolerance and stability tests

The tolerance tests of purified laccases were carried out using organic solvents (MeOH and ACN), and salts (NaCl and KCl) as interfering agents. The tests with the solvents and the salts were carried out according to the same protocol: the sample was spiked with one of the salts (NaCl or KCl) at 50 mM or with one of the solvents (MeOH ACN) at 20 % *v/v*. At the same time, laccase (≈ 500 U L^{-1}) was mixed with the interfering agent for 5 minutes before laccase activity being measured. An effluent from a regional sewage treatment plant in the province of Québec, Canada also served as an interfering agent. The sample was prepared by mixing the ABTS solution concentrated 20 times and the effluent according to a 5:95 volumetric ratio. The pH of the effluent was adjusted to pH 7 by addition of HCl at 10 N, then to pH 4. The final activity was ≈ 500 U L^{-1}, and the activity was measured at these two pH values. On the other hand, laccase activity was measured at pH 7 (phosphate buffer at 0.1 M), and another sample was prepared at pH 4 (section 2.3.).

The activity of the enzymes of interest (≈ 500 U L^{-1}) was measured after exposure to different denaturing compounds: 100 mM urea solution at pH 7 for 48 h, 100 mM copper sulfate solution at pH 4 for 48 h, 10 mg L^{-1} trypsin solution at pH 7 for 48 h and sewage treatment plant effluent at pH 7 for 48 h The pH was stabilized at 7 with phosphate buffer (0.1 M) or at pH 4 with acetate buffer (0.1 M). At the same time, the same manipulation was carried out without denaturing. Sample containing denatured laccase was used to correct

for potential fluctuations. The tubes were placed in the dark on an orbital shaker and stirred at 130 rpm. In parallel, the activity of the enzymes was measured after being heated at 60.2 °C. After 0, 5, 10, 15, 25, 25 min, a sample of 50 µL was analyzed immediately. The pH was stabilized at 7 with phosphate buffer (0.01 M).

2.8.3. Transformation of pharmaceuticals

A kinetic study was carried out over 6 h for EE2 and 48 h for DCL transformation, at pH 7 (5 mM of potassium phosphate), with ≈ 150 and 750 U L^{-1} of laccase for the elimination of EE2 and DCL respectively. Briefly, a final concentration of the enzymes of interest was added to a 50, 100, 150, 250 and 500 µg L^{-1} solution of pharmaceutical compounds. The reaction medium was placed on an orbital shaker and stirred in the dark at 130 rpm. The samples were withdrawn after contact times of 0 h, 1 h and 3 h for EE2 and 0 h, 24 h and 48 h for DCL. The samples taken were placed into the wells of the LDTD plate and analyzed immediately (see section 2.8.1). Finally, kinetics constants were obtained using the Lineweaver and Burk method (see section 2.7). In parallel, in order to evaluate the synergistic effects between the two isoenzymes, a 50:50 w/w mixture was prepared with the same conditions mentioned above for EE2, DCL and ABTS.

The removal of EE2 and DCL was followed as a function of time at 4 °C and 21 °C at 400 µg L^{-1} for 6 h and 98.5 h respectively. The initial laccase activities were of 100 U L^{-1} and 1000 U L^{-1} for the elimination of EE2 and DCL, respectively. For EE2, an aliquot of 30 µL was taken at the following times: 0, 1, 3 and 6 h. For DCL, an aliquot was taken for analysis at time 0, 19, 26, 38, 50, 62.5, 73 and 98.5 h. These aliquots were then processed following the protocol described in Section 2.8.1 The activity was monitored during the experiments and a blank control was used. To ensure that the change in volume and temperature was not significant during the sampling times, the initial solution volume was set to 10 mL. Therefore, total sampled volume was < 2% of the initial volume. Regarding EE2, the experiment was repeated with 3 different concentrations: 100 µg L^{-1}, 200 µg L^{-1}, and 600 µg L^{-1}. The K_M and k_{cat} could be briefly determined to identify the limiting parameter. In the case of DCL, the experiment was repeated twice at 4000 µg L^{-1}.

2.8.4. Statistical analysis

Statistically significant differences for robustness tests were determined by Student's t test at the α=0.05 level. For the determination of the enzyme kinetic constants by the linearization method of Lineweaver and Bulk, errors were expressed as a function of the confidence interval. The confidence interval was expressed as a function of the standard error of the slope or of the ordinate using Student's t value at α=0.05 for *n*-2 degrees of freedom. Calculations were carried out in Microsoft Excel (version 365).

3. Results

3.1. Purification of isoenzymes

Fourteen days after culturing the fungus, the enzymatic activity of the crude extract was around 5 kU L^{-1} and approximately 800 kU L^{-1} after concentration by centrifugation and filtration. Separation of the concentrated extract by SDS-PAGE (Figure S-2) indicated the presence of largely predominant proteins at ≈ 65 kDa. The presence of proteins ≈ 10-15 kDa was also detected in lower amounts. In parallel, proteins at ≈ 50, 38, 34 and 28 kDa were also present in trace amounts. Finally, no protein with a mass greater than 100 kDa was observed.

Separation of the same concentrated crude extract by Native-PAGE using ABTS as staining dye, a technique capable of separating and detecting enzymes with oxidizing activity, indicated the presence of two colored bands (Figure S-3). The upper band containing the oxidizing enzymes was called "Y_n" and the lower band "Y_g". This notation will be kept throughout the publication for simplicity. According to our results from GelAnalyzer, the proportion of Y_n to Y_g was ≈ 1 to 2.

To purify the oxidizing enzymes, the crude extract was separated by FPLC-SEC (Figure 1). Three peaks were observed: The first peak at an elution volume of ≈ 8 mL was the dead volume (V_0), the second peak at an elution volume of ≈ 14 mL was composed of proteins of molecular mass around 60 kDa (V_{e1}) and the third peak at an elution volume of ≈ 19 mL was composed of proteins of around 10 kDa (V_{e2}). V_{e1} is the only peak containing proteins with oxidative activity (Figure 1, b).

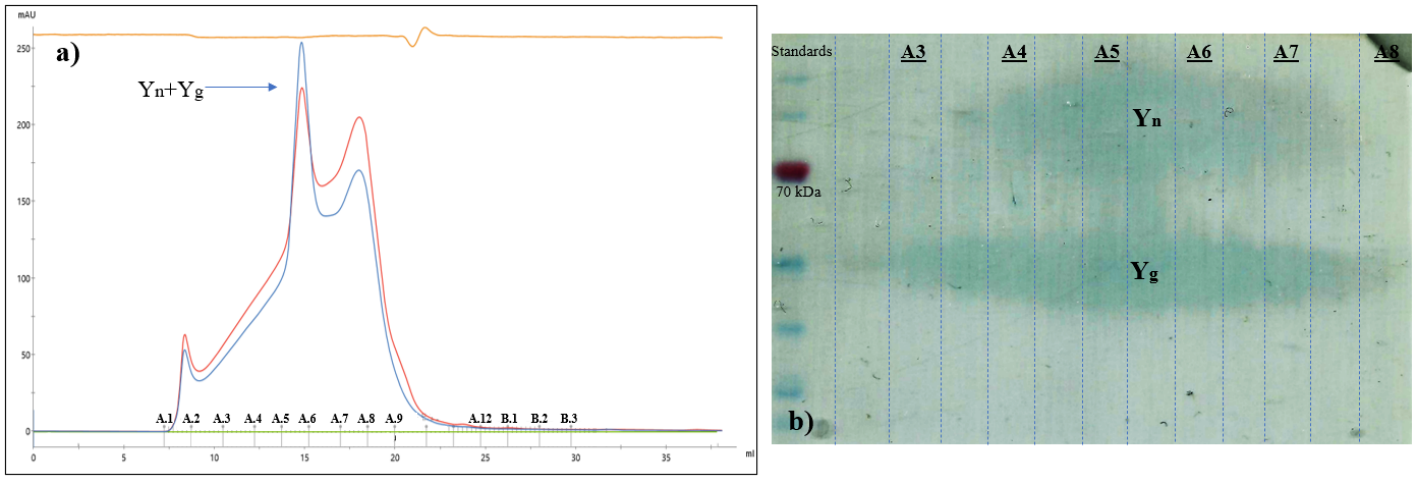

Figure 1. a) Chromatogram obtained by FPLC-SEC showing the proteins contained in the crude extract. b) Native-PAGE of the collected fractions. Orange line: Conductivity; Blue line: 280 nm; red line: 260 nm.

These two bands containing Y_n and Y_g (V_{e1}, Figure 1, b) were cut and separated again in a second SDS-PAGE gel (Figure S-3). These results demonstrated that the proteins ≈ 65 kDa had ABTS-oxidizing activity and that they had a similar apparent mass but different charges. Then, the collected fractions containing the enzymatic activity after FPLC-SEC purification were separated by FPLC-anion exchange chromatography (Figure 2). These results revealed the presence of two peaks: the first with V_{e3} ≈ 32 mL and the second with V_{e4} ≈ 37 mL (Figure 2, a). All fractions were collected, including the two peaks and the flow-through, and were migrated through a Native-PAGE gel (Figure 2, b and c). V_{e3} consists of Y_g, but Y_n was observed for V_{e4}. Y_n was not retained by the anionic resin and was present in the flow-through (Figure 2, b). This method allowed to efficiently separate and purify the two groups of oxidizing enzymes present in the crude extract. The enzymes thus purified were then submitted to peptide mass fingerprinting by LC-TIMS-QTOF for identification.

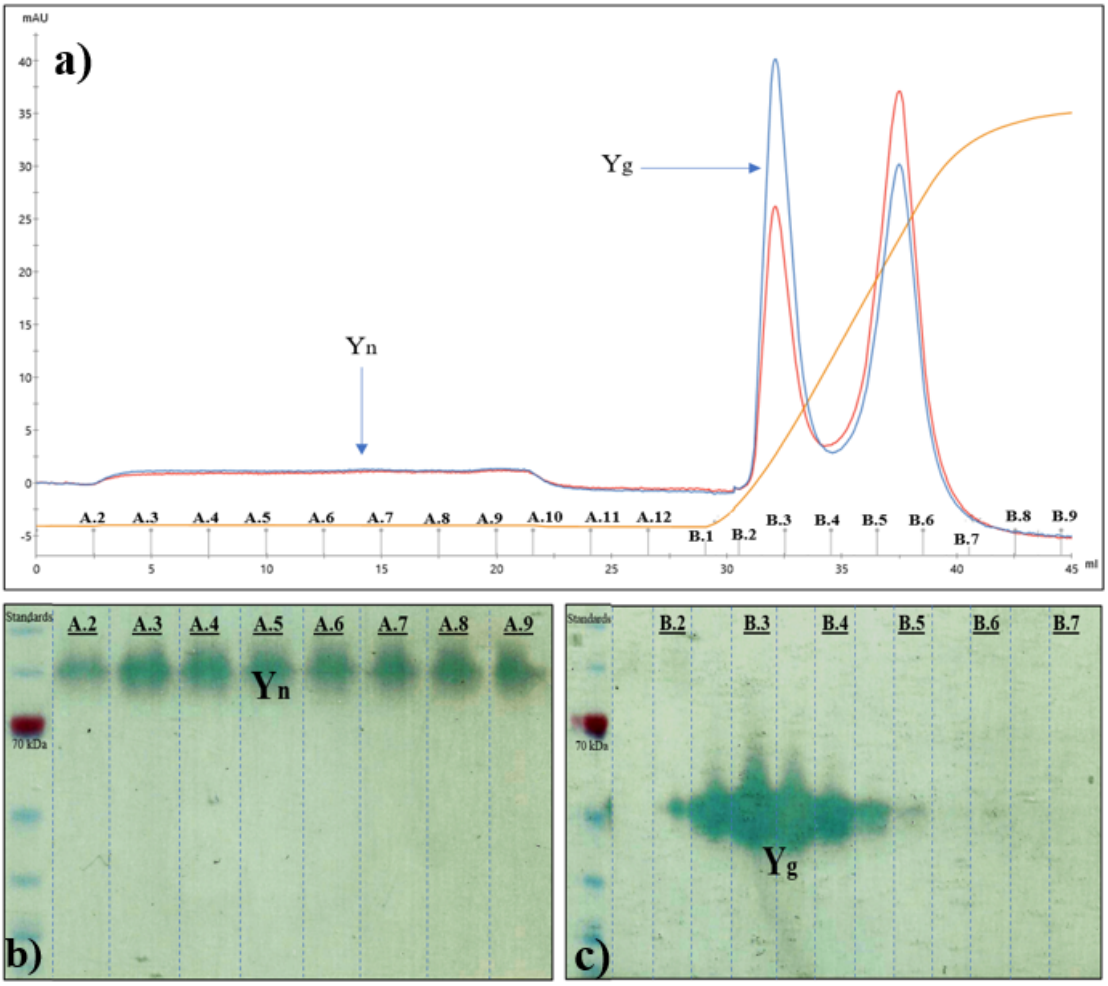

Figure 2. a) Chromatogram obtained by FPLC-anion exchange chromatography showing the proteins contained in the previously purified extract by FPLC-SEC (Figure 1). b & c) Native-PAGE of the collected FPLC fractions. Orange line: Conductivity; Blue line: 280 nm; red line: 260 nm.

3.2. Tentative identification of laccase isoenzymes

Based on the peptide mass fingerprinting using trypsin, two peptides of interest from isoenzyme Y_n were identified (Table 1). One of the peptides was unique and corresponds to the A0A6M5CX58 laccase, while the use of the leading razor protein, i.e., the protein with the best score, was necessary to match the second peptide to this same laccase.

Three peptides of interest resulting of the Y_g isoenzyme were identified. Two of these peptides are not unique but, the use of the leading razor protein allows it to be affiliated with laccase Q02497. The third peptide was unique to laccase A0A0A7M1X9. No other laccases were detected, so it would seem that ≈ 90% of the observed signal intensity is due to laccase Q02497 and ≈ 10% is due to laccase A0A0A7M1X9. All identified and named isoenzymes of laccase were from the *T. hirsuta* strain (TRAHI in Uniprot).

Table 1. Main results from peptide mass fingerprinting analysis of Y_n and Y_g using trypsin for digestion.

Protein Name	Unique peptides Y_n + leading razor protein	Unique peptides Y_g + leading razor protein	Score	Intensity in Y_n fraction	Intensity in Y_g fraction
Laccase F (A0A0A7M1X9)	0	1+0	114	0	4.6×10^5
Laccase (Q02497)	0	0+2	191	0	5.5×10^6
Laccase 1 (A0A6M5CX58)	1+1	0	124	1.6×10^7	8.3×10^4

Digestion with chymotrypsin produced many more peptides of interest (Table 2). In total, 45 peptides of interest resulting from the digestion isoenzyme Y_n by chymotrypsin were identified and 40 of them corresponded to laccase A0A6M5CX58 with a signal intensity around ten times greater than the peptides for other laccases. Several different laccases could constitute the rest of the mixture, with relatively similar intensities between them (e.g., peptides corresponding to laccase A0A2Z2CBW5, A0A0A7M289, A0A0A7685, A0A0A7421), but the sum of their intensities does not exceed 10% of the intensities of the peptides corresponding to laccase A0A6M5CX58.

For isoenzyme Y_g, 42 peptides of interest were identified and 26 of them corresponded to laccase Q02497 with an intensity approximately ten times greater than the peptides specific to laccase F (Table 2, Y_g). The presence of unique A0A6M5CX58 laccase peptides in the Y_g extract at high concentration would indicate possible contamination of the extract (2.2×10^8 *versus* 9.9×10^7, table 2). However, this contamination is less visible with trypsin digestion (1.6×10^7 *versus* 8.3×10^4, table 1). The purification of the sample used for digestion with chymotrypsin was carried out after the digestion with trypsin and came from a different batch. This batch was not used in any other experiment.

Table 2. Main results from peptide mass fingerprinting analysis from the digestion of Y_n and Y_g by chymotrypsin.

Protein Name	Unique peptides Y_n	Unique peptides Y_g	Score	Intensity in Y_n fraction	Intensity in Y_g fraction
Laccase F (A0A0A7M1X9)	0	6	67	0	4.5×10^7
Laccase (Q02497)	0	2	323	6.6×10^5	2.7×10^8
Laccase 1 (A0A6M5CX58)	19	7	323	2.2×10^8	9.9×10^7

Peptide mass fingerprinting analysis of Y_n and Y_g by trypsin and chymotrypsin are complementary and allowed to conclude that: i) Y_n is composed of 96% of laccase A0A6M5CX58 and 4% of a mixture of other laccases and ii) Y_g is composed of 89% of laccase Q02497 and of 7% laccase F (Figure S-4). For sake of clarity, we will continue to call laccase Y_n and laccase Y_g these two mixtures.

3.3. Characterization of the laccase isoenzymes for application to bioremediation

3.3.1. Tolerance and stability tests

Results of tolerance tests are shown in Figure 3.A. A decrease in activity of ≈ 50% for both laccases was observed when exposed to organic solvents (MeOH or ACN) at 25% *v/v* concentration. On the other hand, the activity drops to ≈ 40% for both laccases in the presence of 50 mM NaCl or KCl. However, laccase Y_g appears to be more tolerant than laccase Y_n towards these salts. The enzymatic activity of both laccases after acidification of the wastewater effluent to pH 4 is not significantly different from the reference activity conditions (deionized water at pH 4).

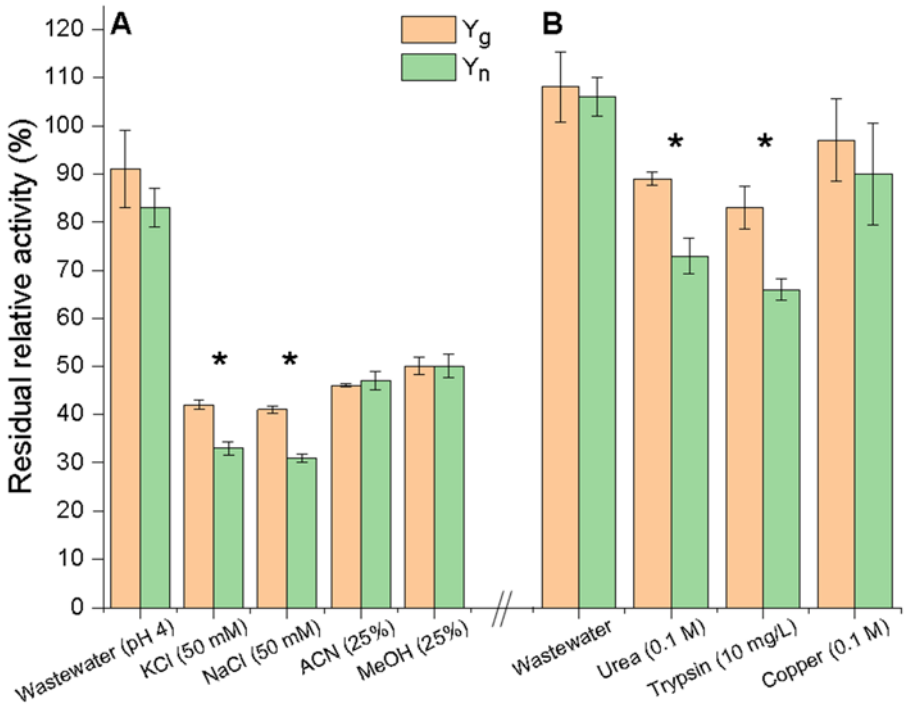

Figure 3. A. Mean residual relative activity (%) of laccase Y_n and Y_g in the presence of diverse interferences. B. Mean residual relative activity (%) of laccase Y_g and Y_n after 48 h of exposure to various denaturants. Error bars indicate the standard deviations (n=3). Asterisks (*) indicate that the observed difference is statistically significant ($p < 0.05$).

Results of the stability tests are shown in Figure 3.B. The activity of neither laccase decreases with the presence of copper sulfate. However, the presence of urea or trypsin decreases the activity of the two laccases, but laccase Y_n was significantly more affected than laccase Y_g. No significant decrease in laccase activity was measured after exposure to wastewater effluent (filtered or not, data not shown). Finally, we observed greater stability of Y_n laccase compared to Y_g laccase at acidic pHs (data not shown).

3.3.2. Removal kinetics of EE2 and DCL by laccase Y_g and Y_n

The elimination kinetics of the two compounds were relatively similar for the two isoenzymes, which is reflected by a relatively similar K constant: $(4.5 \pm 0.3)\times10^{1}$ L s^{-1} mol^{-1} and $(4\pm1)\times10^{1}$ L s^{-1} mol^{-1} for DCL and $(2.1\pm0.4)\times10^{3}$ L s^{-1} mol^{-1} and $(1.5\pm0.2)\times10^{3}$ L s^{-1} mol^{-1} for EE2 for laccase Y_g and laccase Y_n respectively (Table 3). However, the K_M is globally higher for laccase Y_g than for laccase Y_n with a result of 9.8±0.7 and 4±1 µM for DCL and 5.6±0.9 and 3.7±0.5 µM for EE2, respectively (Table 3). Conversely, k_{cat} is overall lower for the laccase Y_g compared to laccase Y_n with a result of $(2.7\pm0.2)\times10^{-2}$ min^{-1} and $(9\pm3)\times10^{-3}$ min^{-1} for

DCL and $(7\pm1)\times10^{-1}$ min^{-1} and $(3.4\pm0.4)\times10^{-1}$ min^{-1} for EE2 (Table 3). Therefore, the constant K, being the ratio between the constant k_{cat} and K_M, was relatively similar in both laccases, as mentioned earlier. For the case of EE2 and ABTS, no case of synergy effect was observed contrary to the case of DCL. The impact of the synergy is positive and increases performance by approximately 50% (Table 3).

Table 3. Kinetic constants (K_M, k_{cat}, K) obtained by the two isoenzymes individually and mixed (1:1, *w/w*) taking ABTS, DCL and EE2 as substrate at pH 7. Error indicates the 95% confidence interval.

Kinetic constant	Isoenzyme	ABTS	DCL	EE2
K_M (µM)	Y_g	30.2±0.4	9.8±0.7	5.6±0.9
	$Y_g + Y_n$	32.0±0.6	1.2±0.2	4±1
	Y_n	33.7±0.7	4±1	3.7±0.5
k_{cat} (min^{-1})	Y_g	$(2.36\pm0.02)\times10^{4}$	$(2.7\pm0.2)\times10^{-2}$	$(7\pm1)\times10^{-1}$
	$Y_g + Y_n$	$(1.75\pm0.03)\times10^{4}$	$(4.3\pm0.8)\times10^{-3}$	$(5\pm2)\times10^{-1}$
	Y_n	$(1.04\pm0.04)\times10^{4}$	$(9\pm3)\times10^{-3}$	$(3.4\pm0.4)\times10^{-1}$
K ($L\ s^{-1}\ mol^{-1}$)	Y_g	$(1.30\pm0.02)\times10^{7}$	$(4.5\pm0.3)\times10^{1}$	$(2.1\pm0.4)\times10^{3}$
	$Y_g + Y_n$	$(9.1\pm0.2)\times10^{6}$	$(6\pm1)\times10^{1}$	$(1.9\pm0.9)\times10^{3}$
	Y_n	$(5.1\pm0.1)\times10^{6}$	$(4\pm1)\times10^{1}$	$(1.5\pm0.2)\times10^{3}$

3.3.3. Removal of DCL and EE2 at different temperatures by laccase Y_g

For EE2, after the first hour, approximately 80% and 30% of removal was observed at 21 and 4 °C, respectively. After 6 h, removal was complete at both temperatures. Thus, the ratio of removal (21 *vs* 4 °C) was approximately 3.2 after a contact time of 1 h. Changing the initial concentration of EE2 (100, 200, and 600 µg L^{-1}) gives us ratios of 3.3, 3.0, and 3.1 respectively (Figure 4 and Figure S-5) with an overall similar elimination pattern, regardless of the concentration. This demonstrates that the elimination kinetics at 4 °C are reduced by about 67 to 70% compared to 21 °C, which can be explained by a reduction of a factor 4 in the k_{cat} (the K_M was relatively similar, data not shown).

In the case of DCL, after 20 h, about 40 and 20% removal was observed at 21 and 4 °C, respectively. Then the difference in removal kinetics observed between the two temperatures are much less clear, with a decrease of about 20 and 25% from the 20th hour to the 40th h. Finally, between the 40th and the 96th hours, the elimination kinetics of DCL at 4 and 21 °C were close, with a ratio of around 1.33 (Figure 4). The same trend was observed at 4000 µg L^{-1}. Finally, the time required to eliminate 80% of the residual DCL is little impacted by its initial concentration, respectively 400 or 4000 µg L^{-1} (approximately 90 h) (Figure S-6).Thus, enzyme activity remained constant throughout the experiment (Figure 4).

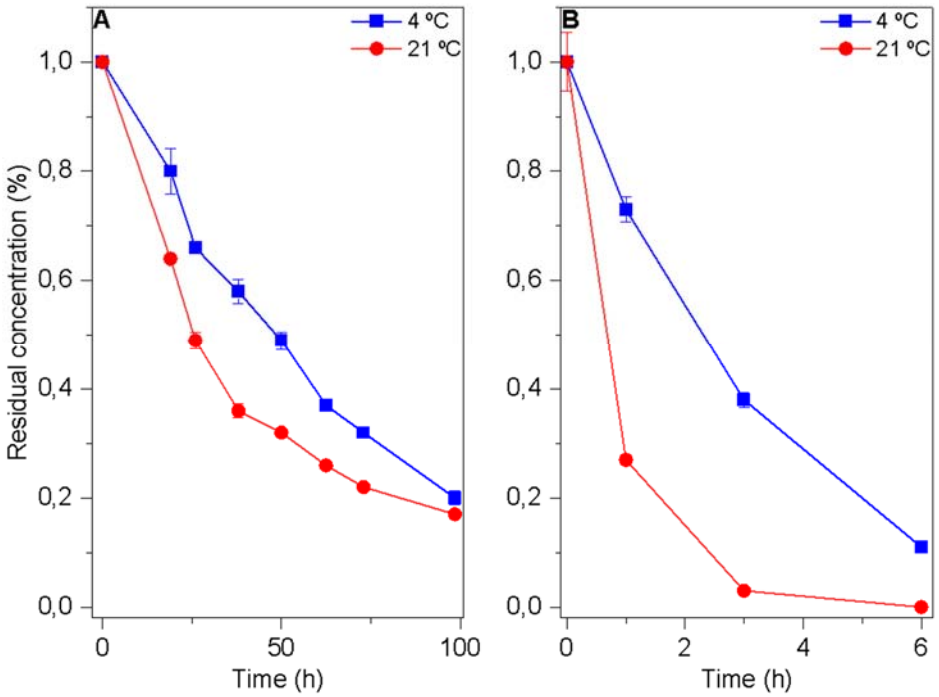

Figure 4. Relative elimination of DCL (A) and EE2 (B) at 400 µg L^{-1} (%) as a function of time (h) at 4 and 21 °C and pH 7, by laccase Y_g.

4. Discussion

4.1. Purification of isoenzymes

Although molecular mass, robustness, and removal performance of laccases Y_n and Y_g are very close, we observe a totally different behavior of their interaction with ions. In fact, ≈ 275 mM of NaCl is sufficient to desorb the Y_g laccase from the anionic column, whereas less than 100 mM would be needed for the Y_n laccase (Figure 2). Chloride ions are often used as competing ions in the literature, with a gradient that can vary from 0 to 1000 mM and a pH between 4.8 and 8.0 [55–58]. Moreover, unlike the laccase Y_g, we observe a very weak migration of the laccase Y_n through the Native-PAGE gel (migrating from the anode towards the cathode, negatively charged, Figure S-2). These two observations could be related, partly explained by a higher pI at the surface of the laccase Y_n.

A strong disparity of the results in connection with molecular mass of the purified proteins was observed according to the method used. For example, the molecular mass of laccase Y_g was 56, 66, or 51 kDa for the theoretical mass, SDS-PAGE and FPLC-SEC, respectively (Figure S-7). The theoretical mass could underestimate the right molecular mass because it does not take into account glycosylation which generally represents 10 to 30% of the mass of the enzyme [59]. As for SDS-PAGE, previous studies indicate that in 40% of cases, the method overestimates or underestimates the exact mass [60,61]. Finally, in the case of size exclusion-FPLC, the particular three-dimensional structure of the laccase (up to 30% glycosylation) [62] could be different from that of the standards, thus giving distorted results. Although the mass of fungal laccase is often between 60 and 70 kDa [63,64], rare exceptions have measured a mass of about 45 kDa [65,66].

4.2. Tentative identification of laccase isoenzymes

The identification of laccases in both mixtures was based on a limited set of entries (63 entries for proteins including 17 for laccase from *T. hirsuta*) in UniProt, therefore only proteins listed in the database can be accurately identified. Few peptides were formed with trypsin digestion, which can be explained by the low abundance of arginine or lysine in laccases (≈ 20-30 for over 500 amino acids) and their distribution through the peptide sequence, either too close or too far apart, thus giving peptides either too small or too large. However, since two complementary digestion methods (trypsin and chymotrypsin) were used and high scores between 67 and 323 were obtained, the results are reliable and the identification has a high confidence, indeed, a score above 40 could be considered high confidence [52] .

Identifying a protein by determining its amino acid sequence can be complex and expensive. Peptide mass fingerprinting is a simpler and less expensive alternative based on enzymatic digestion of proteins to obtain characteristic peptides and database searching. However, it is difficult to evaluate the completeness of the database which limits this technique to study unknown laccases. Therefore, an alternative approach based on the classification of laccases into groups according to physico-chemical parameters will be discussed in section 4.3.5.

4.3. *Characterization and comparison of the laccase isoenzymes for application to bioremediation*

4.3.1. Tolerance and stability tests

Laccase activity is often measured under optimal conditions, far from the actual conditions of use. Activity tests performed in this study were measured a few minutes after the addition of different interfering agents, and for the case of NaCl or KCl, the loss of activity is essentially reversible. Interferences are known to bind with the T2/T3 copper center and prevent electron transfer from the T1 site, more particularly small anions such as chloride or hydroxide [67]. Laccase isoenzyme Y_g seems to be less disturbed than Y_n by chloride ions. The same observation was made with hydroxide anions with a relative activity decrease from 100% for both isoenzymes to 22 and 4% for laccases Y_g and Y_n, respectively, at pH 6 (Figure S-8). Our results agree with the literature [48] which indicates a significant decrease of laccase activity (-10%) from a chloride concentration of 5 mM. Regarding organic solvents, our results are also consistent with the literature [48]. Indeed up to 10% in organic solvent (methanol, acetonitrile) the loss of activity is negligible. In parallel, results show that isoenzymes Y_n and Y_g are weakly affected by wastewater effluent. This result is especially interesting since it demonstrates that the complex composition of the effluent does not appear to significantly disturb the activity of both laccases and therefore the possibility of using laccases in this type of matrix.

The two isoenzymes appear to have close stability (Figure 3.B). The low loss of activity observed with the tested denaturants (100 mM urea, 100 mM copper, 10 mg L^{-1} trypsin, wastewater effluent) indicates a high potential for use, even in a complex matrix. Moreover, the literature indicates that up to 10 mM, most metals do not have a significant negative impact (or few) on laccase activity (e.g., magnesium, copper, zinc, calcium) [48,58,65,66,68,69]. The stability seems slightly greater for the laccase Y_g (*versus* trypsin and urea used as denaturants) except at acid pH where the laccase Y_n has much better stability (data not shown). Equivalent studies have been carried out and strong disparities have been observed between other isoenzymes. For example, laccase α and β retain more than 90% of their initial activities, against 55 and 15% for laccase γ and δ respectively in 25 h tests at pH 4. Generally, laccase is more stable at neutral pH for two reasons: i) high

acidity modifies the ionization of the side chains and disrupts the normal functioning of laccase; and ii) hydroxide anions can interact with the copper core of laccase [70].

Overall, the robustness (stability and tolerance) of the two isoenzymes is relatively close, which could be explained by a similar percentage of glycosylation (based on laccase Q02497 and A0A6M5CX58, results not shown). Indeed, several studies suggest that the good stability of a laccase is directly linked to its percentage of glycosylation, playing the role of a protective shell. Finally, although free laccases lose their activity after one week (in wastewater effluent samples, results not shown) [71,72], it is known that their immobilization on a solid support increases their stability. Indeed, it has been reported that immobilized laccases can conserve 50% of their initial activity after 15 days [72]. In parallel, the present study demonstrates that wastewater effluent does not decrease the activity of laccase which is an essential property and almost never determined for the application of laccases to the bioremediation of sewage.

4.3.2. Removal kinetics of EE2 and DCL by laccase Y_g and Y_n

Since the formation of new substrates for laccase from DCL or EE2 will bias the constants obtained (K_M, k_{cat} and K) like hydroxy-DCL or hydroxy-EE2 [73,74]. We recommend adding the term "apparent" to be more accurate. Very few studies in the literature were available to compare the results concerning pharmaceutical compounds. For example, a K_M of 3.78 µM for EE2 using laccase from *T. versicolor* has been determined in a previous study [41], compared to 4±1 µM in this study. This is explained by equivalent oxidation-reduction power of laccases from *T. versicolor* and *T. hirsuta* [63]. Our results indicate a relatively equivalent K for laccases Y_g and Y_n, thanks to the compensation of K_M and k_{cat}. Similar results have been identified in the literature: laccase α, β and γ have a very close K despite a very strong difference in the K_M, ranging from 22 to 359 µM and in the k_{cat}, ranging from 55 to 647 s^{-1} for the transformation of the ABTS [75,76].

To our knowledge, kinetic studies involving synergistic effects of enzymes on pharmaceutical compounds are rare in the literature. We can quote for example the decolorization of indigo carmine was 15, 10 and 5% by laccase I, II, III respectively and 58% when the three of them were combined [77]. However, a definitive explanation for this phenomenon remains unknown. It is possible that the synergistic effect observed could be explained by a greater affinity of one isoenzyme to the initial compounds and of the other isoenzyme to the transformed compounds. This is possible because several publications have indicated a clear difference in the elimination of certain organic compounds by depending on the isoenzymes [48,75]. However, additional experiments, out of the scope of the present study, are necessary to validate this hypothesis, in particular by using a wide range of pharmaceutical compounds.

4.3.3. Removal of DCL and EE2 at different temperatures by laccase Y_g

The rapid transformation and the kinetic constants obtained for EE2 are consistent with previous studies [41,78]. EE2, like many estrogens, is known to be easily transformed by laccase, in particular thanks to the presence of a phenol group within the structure but also a lower oxidation-reduction potential than that of the laccase of the *T. hirsuta* strain (0.40-0.60 V vs. Ag/AgCl for EE2) [79–82]. The removal of DCL by laccases is known [13,74]. In this study, its rate of transformation is almost 100 times lower than that of EE2, which could be explained by the absence of a phenol group and a higher oxidation potential (0.65 V vs. Ag/AgCl for DCL) [83,84]

The drop in temperature had a negative impact on the kinetics of transformation of both pharmaceutical compounds by laccase. However, contrary to the transformation of EE2, the decrease in temperature had a lower impact on DCL transformation rate, especially at 4000 μg L^{-1} (Figure S-6). One of the hypotheses that can explain this observation is that the temperature drop (ΔT = 18 °C) was not sufficient for the Gibbs energy to become unfavorable. Unlike the case of EE2, if the main mechanism of transformation of DCL is by the formation of a free radical, this mechanism could be less affected by low temperatures and could thus explain the small difference in kinetics between the two temperatures. In parallel, the transformation kinetics was also close at 400 or 4000 μg L^{-1} (80% transformation after 90 hours for both, Figure 5 and S-6), which is nearer to order 1 reaction (usually as a radical reaction) than enzymatic reaction (as a Michaelis-Menten reaction). To our knowledge, this study was the first to compare the transformation kinetics of pharmaceutical compounds by purified laccase as a function of temperature at room temperature *versus* low temperature.

4.3.4. Classification of laccases

One of the main limitations in laccase research nowadays is the difficulty of standardizing the results obtained from the characterization of laccases by the different research groups. Thus, it is difficult or impossible to compare results reliably. Another important limitation is the excessive number of existing laccases. Only for fungi of the genus *Trametes*, more than a hundred laccases have been listed and new strains are regularly discovered, it will be difficult to characterize all the isoenzyme for a given application. Another major issue was the difficulty in clearly identifying a laccase without having to determine the entire peptide sequence. Proteomic studies could be an alternative, but it is not possible to know the completeness of the database. Finally, identifying all the peptide sequences of all the isoenzymes seems necessary.

The classification of laccases in groups would allow to solve the three points previously: i) as the constitutive laccase seems to be secreted by all fungi of the class *Polyporaceae* and they share similar physicochemical properties [85,86], it would be possible to use it as a reference isoenzyme for the characterization of inducible isoenzymes and therefore to work semi-quantitatively, ii) to avoid the characterization of all the isoenzymes of each strain for a given application, it would be possible to characterize a single isoenzyme from each group, which would be representative of their set, iii) the exact identification of an isoenzyme by genomic tools is not obligatory to classify it in a group, which does not depend solely on its physico-chemical characteristics. Thus, this will ultimately lead to more concise and effective results for knowledge sharing and will improve the identification of the best enzymes for specific applications, such as the bioremediation of wastewater.

A very promising classification based on the homology of laccase-secreting genes from the fungi of *Polyporaceae* family based on eight groups named A-H was developed by Savinosa et al. [44,45,62,85,87]. In parallel, the research group Savinosa et al., by reviewing the characterization studies available in the literature, observed similar physicochemical properties between the different laccases, because two laccases from the same group will share similar characteristics [44,45].

Unfortunately, the tools allowing the classification of isoenzymes based on physicochemical properties are still underdeveloped. This is why, from the results of this study, and based on the studies already published [44], a set of simple tools for classifying laccases are proposed herein. To classify the two purified isoenzymes in one of the eight groups proposed by Savinosa et al., and based on their results, we identified four discriminating parameters: molecular mass, the half-life time at 60 °C, the K_M using ABTS as substrate and the pI. Table 3 describes the eight isoenzymes (A-H) from *T. hirsuta 072* (which can be considered as

references) [44,85,87] according to the four parameters chosen. For more details concerning the choice of parameters please consult section S-9 in the Supporting Information.

Table 3. Reference isoenzymes from *T. hirsuta 072* according to four key parameters [44,85,87].

	molecular mass	pI	$t_{1/2}$ 60 °C	K_M (ABTS)
Laccase A (*group A*)	≈ 66 kDa	< 5	> 10 min	< 80 µM
Laccase B (*group B*)	≈ 66 kDa	> 6	≈ few min	< 80 µM
Laccase C (*group C*)	> 71 kDa	< 5	≈ few min	> 200 µM
Laccase D (*group D*)	> 71 kDa	> 6	> 10 min	> 200 µM
Laccase F (*group F*)	interm	> 6	interm	interm
Laccase G (*group G*)	> 71kDa	< 5	nd	> 200 µM
Laccase H (*group H*)	interm	< 5	> 10 min	> 200 µM

Note: Interm intermediate value. ND = no defined.

The Y_g and Y_n laccases have similar characteristics with the group A and group B laccases respectively (refer to Table 3). It is manifested by: i) a relatively low molecular mass for both (≈ 66 kDa, determined experimentally by SDS-PAGE), ii) a relatively low isoelectric point for Y_g (< 5, theoretical pI of laccase Q02497) and high for Y_n (> 6, theoretical pI of laccase A0A6M5CX58), iii) Y_g has a relatively long half-life at 60 °C (≈ 30 min, determined experimentally, data not shown) unlike Y_n (< 3 min), iv) Y_g and Y_n have a low K_M (< 80 µM, determined experimentally).Finally, the percentage of similarity of the peptide sequence could be an additional parameter for the classification of an isoenzyme. In our case, the percentage of similarity between laccase Y_g and laccase A is the highest with 88% (using laccase Q02497). A similar result is obtained between laccase Y_n and laccase B with 84% (using laccase A0A6M5CX58). In the future, more tools could be standardized such as SDS-PAGE for the determination of molecular mass and thus obtain comparable results. In parallel, the commercial laccase, such as *T. versicolor* one, could be used as a standard, because it remains commonly used and remains easily accessible.

The similar results of the stability tests between laccase Y_g and Y_n are consistent at similar percentages of glycosylation (probably ≈ 10%), as it is the case for laccases of groups A and B. The difference in the isoelectric point could explain the greater impact of chloride ions on laccase Y_n. The laccase Y_n (group B) has an overall lower K_M and k_{cat} than the laccase Q02497 (group A) [62]. Group B laccases are known to have a low K_M. However, the lack of information on k_{cat} in the literature gives the impression that group B laccases are more efficient. While the results also indicate lower k_{cat}, which does not necessarily make it more efficient, with a similar K constant [62,85]. Finally, the group A-B laccases could be complementary. Indeed, group A laccase, with a high k_{cat}, would quickly and efficiently transform substrates at high concentration, then after acidification of the medium, group B laccase would attack more meticulously at low concentration substrates, thanks to a lower K_M and a high stability at low pH.

5. Conclusion

The objective of this study was to characterize and compare two laccase isoenzymes from the *T. hirsuta* strain for the bioremediation of WWTPs effluent. Laccase Y_g (composed mainly of laccase Q02497 or a closely related laccase) and laccase Y_n (composed mainly of laccase A0A6M5CX58 or a closely related laccase) are the two major isoenzymes of the crude extract, based on peptide mass fingerprinting analysis. Two key parameters for the intended application were evaluated for both isoenzymes: robustness (tolerance and stability) in a complex medium and performance for the transformation of pharmaceutical compounds. Regarding robustness, the two isoenzymes do not seem to be interfered by a WWTPs effluent and preserve

all of their respective activity. The same observation was made concerning stability; the two isoenzymes preserved all of their activity after exposure to a WWTPs effluent for 48 h (filtered or not). The performance tests indicate that laccase Y_g has a higher k_{cat} but also a lower K_M than laccase Y_n for EE2 and DCL thus making the conclusion of this point more nuanced with a relatively similar K constant. Synergistic effects were also observed for DCL. Thus, the Y_n and Y_g isoenzymes could be complementary. The use of laccase for bioremediation at low temperature is possible, and in the case of DCL, the decrease in the rate of transformation remains relatively low (≈ 25%). Therefore, bioremediation of effluents remains possible in cold climates.
Finally, this study proposed a set of tools allowing the classification of isoenzymes based on a few experiments that could improve the efficiency and the comparability of laccase properties between research groups. The results would indicate that laccase Y_g would be from group A while laccase Y_n from group B.

6. Credit authorship contribution statement

Younes EL YAGOUBI: conceptualization, data processing, formal analysis, investigation, methodology, writing – review and editing, writing – original draft. **Bruno LEMIEUX**: formal analysis, investigation, methodology, data curation, software. **Pedro SEGURA**: conceptualization, supervision, resource, investigation, validation, writing – review and editing, writing – original draft. **Hubert CABANA**: supervision, conceptualization, project administration, funding acquisition, resources, writing – review and editing, writing – original draft.

7. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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