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Coriolopsis trogii MUT3379: A Novel Cell Factory for High-Yield Laccase Production

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Abstract: *Coriolopsis trogii* is a basidiomycete fungus which utilizes a large array of lignin-modifying enzymes to colonize and decompose dead wood. Its extracellular enzymatic arsenal includes laccases, i.e., polyphenol oxidases of relevant interest for different industrial applications thanks to their ability to oxidize a diverse range of natural and synthetic compounds. In this work, the production of laccases in *C. trogii* MUT3379 was explored and improved. From an initial production of ca. 10,000 U L^{-1}, the fermentation process was gradually optimized, reaching a final yield of ca. 200,000 U L^{-1}. An SDS-PAGE analysis of the secretome highlighted the presence of a main protein of ca. 60 kDa showing laccase activity, which was designated as Lac3379-1 once its primary sequence was established by tandem mass spectrometry. The characterization of Lac3379-1 revealed a remarkable enzymatic stability in the presence of surfactants and solvents and a diversified activity on a broad range of substrates, positioning it as an interesting tool for diverse biotechnological applications. The high-yield and robust production process indicates *C. trogii* MUT3379 as a promising cell factory for laccases, offering new perspectives for industrial applications of lignin-modifying enzymes.

Keywords: laccase; *Coriolopsis trogii*; cell factory; white-rot fungi; basidiomycetes; high-yield

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1. Introduction

Greenhouse gases are dangerously increasing as society still relies on fossil fuels for the production and processing of everyday products [1]. The consequent increase in global temperature and climate destabilization not only affect the ecosystems, but also have an impact on human life and on socioeconomic networks, in an inter-dependent and intertwining cycle [2]. One of the main goals of industrial biotechnology is the development of sustainable processes that can reduce our dependence on fossil fuels and artificial chemicals, helping us with climate-resilient development. Enzymes play a pivotal role for biotechnology to help achieve climate neutrality. These proteins catalyze a wide variety of reactions that can be implemented in industrial processes to reduce chemical use and to limit water and energy consumption. Thanks to their striking versatility, enzymes can also favor the usage of renewable feedstocks in place of virgin resources, thus representing one of the pillars of circular bioeconomy. It has been estimated that, compared to classical chemical-grounded processes, enzyme-based manufacturing procedures can save up to 155 kg CO_2 per kg of product generated, thus resulting in an overall potential of enzymes in climate change mitigation from 1 to 2.5 bt of CO_2 emissions reduction per year [2,3].

Filamentous fungi are currently used for the production of ca. 50% of enzymes employed in industrial settings such as cellulases, pectinases, amylases, laccases, and proteases [4]. Indeed, their efficient secretion system makes them excellent workhorses

for the production of different extracellular proteins that can be easily recovered from the fermentation broth and then applied for the manufacturing of food, feed, textiles, detergents, biofuels, paper, etc. [5]. Additionally, filamentous fungi often secrete cocktails of various enzymes which allow the synergistic hydrolysis of complex polymers [6]. Therefore, unrefined sugars and even lignocellulosic waste can be used as carbon and energy sources in fungal fermentations, lowering the overall cost of enzyme production.

Laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductases) are polyphenol oxidases belonging to the multicopper oxidase (MCO) protein superfamily. They are known to be versatile enzymes capable of oxidizing a wide range of phenolic and non-phenolic substrates, releasing water as a by-product [7,8]. In nature, laccases are widely distributed among plants, insects, and both prokaryotic and eukaryotic microorganisms, but some of their most efficient producers are white-rot fungi. This group of basidiomycetes has evolved a complex system of lignin-modifying enzymes (LMEs), that is used for different physiological processes including wood decay, and comprises several isoenzymes of laccases and peroxidases (lignin peroxidases, manganese peroxidases, versatile peroxidases, and dye-decolorizing peroxidases), together with various auxiliary enzymes [9]. Laccases and peroxidases catalyze one-electron oxidations, leading to the formation of highly reactive and non-specific free radicals that are able to attack lignin structure, causing the selective cleavage of linkage bonds [10,11]. This oxidative system is thus able to efficiently break down lignin polymers, making accessible cellulose and hemicellulose as sugar platforms contained in the lignocellulosic biomasses [12].

The industrial interest in laccases relies on the possibility to accommodate different natural and synthetic substrates in their catalytic site [13]. This versatility makes this class of enzymes a promising tool for integrating biotechnological processes in various industries and developing eco-friendly procedures not only in lignocellulosic biorefineries, but also for textiles processing, wastewater treatment and biocatalysis in general [14–16]. Although white-rot fungi are known for their ability to produce large amounts of laccases in nature, the sustainable use of these enzymes is still limited by a lack of efficient processes for their production, resulting in an unaffordable market price. Screenings for the identification of naturally occurring high producer strains, coupled with the implementation of well-designed growth media and robust and cost-effective fermentation processes, is therefore crucial for the industrial application of these enzymes [17].

Coriolopsis trogii is a basidiomycete fungus which is known to utilize a system of lignin-modifying enzymes, including laccases and peroxidases, to colonize and decompose dead wood [18]. Compared to other white-rot fungi as *Phanerochaete crysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor*, whose ligninolytic systems have been extensively investigated, *C. trogii* stands as a less explored lignin-degrading platform. The aim of this work was to explore the production of laccases in *Coriolopsis trogii* MUT3379, a strain isolated from decaying wood collected in "Parco della Mandria" in Turin, Italy, and to improve the application of this white-rot fungus as a cell factory for the production of lignin-modifying enzymes.

2. Materials and Methods

2.1. Strain, Reagents, and Culture Media

The fungal strain used was *Coriolopsis trogii* MUT00003379 (abbreviated as MUT3379) from the collection Mycotheca Universitatis Taurinensis (Turin, Italy). The fungal mycelium was preserved in a solution of nutrient glycerol, obtained by solubilizing 8 g L^{-1} of nutrient broth (DIFCO, Franklin Lakes, NJ, USA) and 200 g L^{-1} of glycerol (CARLO ERBA Reagents, Cornaredo, Italy) in demineralized water, and stored as Working Cell Banks (WCB) at −80 °C. The strain was routinely propagated by growing it on Malt Extract Agar (MEA; 20 g L^{-1} malt extract (Costantino & C, Favria, Italy), 20 g L^{-1} casein peptone (Organotechnie, La Courneuve, France), and 20 g L^{-1} agar agar (HiMedia, Modautal, Germany)), at 25 °C for 7 days.

The liquid fermentation media used in this work were obtained by solubilizing the necessary raw materials in demineralized water. Medium pH was corrected by adding HCl or NaOH solutions, followed by sterilization at 121 °C for 20 min. All of the media used were selected from those described in the BCSMedDat database owned by BioC-CheM Solutions (http://bioc-chemsolutions.com (accessed on 4 July 2024)). All media components and reagents were purchased from Costantino & C (Favria, Italy), Diamalteria Italiana (Milan, Italy), CARLO ERBA Reagents (Cornaredo, Italy), Organotechnie (La Courneuve, France), Roquette (Lestrem, France), or Merck KGaA (Darmstadt, Germany) unless otherwise indicated.

2.2. Cultivation of *C. trogii* MUT3379 and Optimization of Laccase Production

The cultivation of *C. trogii* MUT3379 was conducted by inoculating a mycelium plug of 1 cm^2 from an MEA plate into a 500 mL baffled flask containing 100 mL of the selected medium. Media evaluated in the primary screening phase for laccase production were BCS006, BCS016, BCS050, BCS052, BCS053, BCS062, BCS071, BCS132, BCS141, BCS148, BCS166, BCS218, BCS255, BCS267, BCS271, BCS284, BCS327, and BCS368, all supplemented with 0.15 mM copper sulphate ($CuSO_4$). Then, for BCS218 (10 g L^{-1} glucose, 10 g L^{-1} malt extract, 4 g L^{-1} yeast extract, 2 g L^{-1} casein peptone, and 2 g L^{-1} $MgCl_2 \cdot 6\ H_2O$), variants of the medium with 1.2, 1.5, or 2× nutrient concentrations and supplemented with 0.15 or 2 mM $CuSO_4$ or copper chloride ($CuCl_2$) were evaluated as well, following the rationale described in the Section 3. In all cases, the liquid cultures were incubated at 25 °C, 150 rpm for a maximum period of 17 days. During cultivation, samples were collected at regular intervals and centrifuged at 3000× *g* for 15 min at 25 °C. Fungal biomass growth was monitored by evaluating the dry weight (g L^{-1}) of the pellet after lyophilization. Culture pH was monitored on the cell-free culture broth with a pH meter. Total sugar consumption was determined by the phenol-sulfuric acid method [19]. Hence, culture supernatants were properly diluted and treated with sulfuric acid 96% (*v*/*v*) and phenol 5% (*w*/*v*). After 15 min of incubation at 55 °C, the concentration of the resulting red-colored product was measured by reading its absorbance at 490 nm. The total sugar amount was then calculated by creating a calibration curve using set concentrations of glucose as the reference standard.

2.3. Laccase Production Monitoring

Laccase production was monitored on all culture supernatants collected by the activity assay on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as substrate [20–22]. Laccase activity was measured spectrophotometrically at 25 °C, using a V530 spectrophotometer (Jasco, Easton, PA, USA) following the oxidation of 0.5 mM ABTS (ε420 nm = 36 mM^{-1} cm^{-1}) at 420 nm for 5 min, in 50 mM sodium acetate, pH 4. Oxygen was incorporated in the reaction mixture by pipetting thoroughly during the addition of the substrate. One unit of activity was defined as the amount of enzyme that oxidized 1 µmol of ABTS per min. The production of laccase activity in culture supernatants was also investigated by polyacrylamide gel electrophoresis (PAGE) performed under non-denaturing conditions (i.e., under NATIVE-PAGE or zymogram analysis). The analysis was carried out on a 3% (*w*/*v*) acrylamide stacking gel, pH 6.8, and a 14% (*w*/*v*) acrylamide running gel at pH 8.9, using a Tris-Glycine buffer 0.5×, pH 6.8, as the running buffer. For detecting laccase activities, gel development was conducted with a solution of 2 mM guaiacol in 50 mM sodium acetate buffer, pH 5, or with 10 mM ABTS in the same buffer. Electrophoresis was also carried out under denaturing conditions (i.e., in sodium dodecyl sulphate SDS-PAGE analysis) on a 14% (*w*/*v*) acrylamide gel and with Coomassie brilliant blue staining to estimate the molecular mass of the produced proteins.

2.4. Laccase Production and Purification

For the purification of the laccase activity, *C. trogii* MUT3379 was first cultivated in BCS218, supplemented with 0.15 mM $CuSO_4$ (BCS218—0.15 mM $CuSO_4$) as a vegetative medium following the inoculum procedure described above. After 144 h of incubation at

25 °C and 150 rpm, 10% (*v*/*v*) of the vegetative culture was transferred to 500 mL baffled flasks containing 100 mL of BCS218 formulated with 1.2× nutrient concentration and supplemented with 2 mM $CuCl_2$ (BCS218 1.2×—2 mM $CuCl_2$). The fermentation was carried out for 15 days under the same incubation conditions, followed by the separation of fungal biomass either via centrifugation at 3000× *g* for 15 min at 25 °C, or via filtration through paper filters to recover the culture supernatants. Total proteins in culture supernatants were precipitated with ammonium sulphate 80% (*w*/*v*). The protein precipitates were then resuspended in 100 mM Tris-HCl pH 8.5 and dialyzed for 72 h against the same buffer for removing unwanted solutes. The laccase was purified from the dialyzed samples using ion-exchange chromatography. The dialyzed protein precipitates were loaded on Amberlite IRA900 (Alfa Aesar, Ward Hill, MA, USA) resin after an equilibration of the column with 100 mM Tris-HCl pH 8.5. Fractions were eluted by a gradient of NaCl from 0 to 1000 mM. Laccase activity was measured in the collected fractions by the activity assay described above, by SDS-PAGE, and by native electrophoresis. Total protein concentration was quantified with the commercial Pierce™ BCA Protein Assay kit (Thermo Scientific™, Waltham, MA, USA).

2.5. Laccase Identification by Tandem Mass Spectrometry

The purified laccase was run on SDS-PAGE gels and visualized with Coomassie brilliant blue in standard conditions. The laccase-protein band was excised as a single polyacrylamide band by the use of a scalpel and processed as follows. The protein was digested with trypsin, the resulting peptides were purified and analyzed by tandem mass spectrometry coupled to high performance liquid chromatography (nanoLC-MS/MS) with a high-resolution Q-Exactive HF instrument (Thermo) coupled with an UltiMate 3000 LC system (Dionex-LC) as previously described [23]. Briefly, the instrument was operated in data-dependent mode. Peptides were desalted on an Acclaim PepMap100 C18 precolumn (5 µm, 100 Å, 300 µm id × 5 mm; Thermo Scientific™, Waltham, MA, USA), and then resolved on a nanoscale Acclaim PepMap 100 C18 column (3 µm, 100 Å, 75 µm id × 50 cm) with a 90 min gradient at a flow rate of 0.2 µL min^{-1}. The gradient was developed from 5% to 25% (*v*/*v*) of (CH_3CN, 0.1% (*v*/*v*) HCOOH) over 75 min, and then from 25% to 40% (*v*/*v*) over 15 min. Peptides were analyzed during scan cycles initiated by a full scan of peptide ions in the ultra-high-field Orbitrap analyzer, followed by high-energy collisional dissociation and MS/MS scans on the 20 most abundant precursor ions (Top20 method). Full-scan mass spectra were acquired from *m*/*z* 350 to 1500 at a resolution of 60,000. For MS/MS fragmentation, ions were selected only with positive charges 2+ and 3+, with a 10 s dynamic-exclusion, and with an AGC target of at least 1×10^5. MS/MS resolution was 15,000. The assembled genome sequence from *Coriolopsis trogii* C001 (GCA_020543525.1) was used as a reference. Initially, all of the possible Stop-to-Stop open reading frames (ORFs) were predicted and transformed into polypeptide sequences with the same strategy as previously described [24]. This proteogenomic database was used to interpret MS/MS spectra using The Mascot Daemon 2.6.1 search engine (Matrix Science, London, UK) with the following parameters: a tolerance of 5 ppm for the parent ions and 0.02 Da for the fragmented ions, Carbamidomethyl (C) as fixed modification, Deamidated (N, Q) and Oxidation (M) as variable modification, and a maximum of 2 trypsin miss-cleavages. Peptides and proteins were identified with an FDR of 0.01 calculated from the relevant decoy database search. A DIAMOND similarity search was then performed to detect the most similar sequences present in the NCBI non-redundant database. Based on the preliminary results, a specific *C. trogii* C001 genome sequence, where the laccase compatible peptides are mapped, was retrieved and analyzed with AUGUSTUS (http://augustus.gobics.de/ (accessed on 4 July 2024)) for the identification and the assembling of exons into the putative full-length polypeptide. The putative protein was then aligned with ClustalW 2.1 to the LC-MS/MS identified peptides in order to identify the most probable deduced sequence for the *C. trogii* MUT3379 laccase. The hypothetical sequence was

then matched against the NCBI non-redundant protein database by the use of BLASTP (https://blast.ncbi.nlm.nih.gov/ (accessed on 4 July 2024)).

2.6. Substrate Range

The ability of the purified laccase to oxidize phenolic and nonphenolic compounds was tested using twelve different substrates including guaiacol (ε468 nm = 12 mM^{-1} cm^{-1}), 2,6-dimethylphenol (2,6-DMP, ε468 nm = 49.6 mM^{-1} cm^{-1}), pyrocatechol (ε450 nm = 2.21 mM^{-1} cm^{-1}), pyrogallol (ε450 nm = 4.4 mM^{-1} cm^{-1}), L-3,4-dihydroxyphenylalanine (L-DOPA, ε475 nm = 3.7 mM^{-1} cm^{-1}), potassium hexacyanoferrate(III) ($K_4Fe(CN)_6$, ε405 nm = 0.9 mM^{-1} cm^{-1}), syringaldazine (ε525 nm = 65 mM^{-1} cm^{-1}), syringaldehyde (ε320 nm = 8.5 mM^{-1} cm^{-1}), syringic acid (ε300 nm = 8.5 mM^{-1} cm^{-1}), vanillic acid (ε316 nm = 2.34 mM^{-1} cm^{-1}), and ferulic acid (ε322 nm = 18.6 mM^{-1} cm^{-1}). The assays were performed in triplicate in 50 mM sodium acetate buffer at pH from 3 to 6, or 50 mM Tris-HCl at pH from 6 to 8, and 25 °C.

2.7. Kinetic Properties

Laccase kinetic parameters K_m and k_{cat} were calculated for ABTS, 2,6-DMP, and $K_4Fe(CN)_6$ in 50 mM sodium acetate buffer, pH 4 at 25 °C using increasing concentrations of the substrates (from 0.002 to 2 mM) while maintaining constant the amount of the enzyme. Data were fitted into the classic Michaelis–Menten equation with Kaleidagraph 4.0 (Synergy Software, Reading, PA, USA). The enzyme activity on 0.5 mM ABTS was assayed in the presence of different concentrations of salts (1–1000 mM), surfactants (0.5–5% *v*/*v*), and solvents (5–50% *v*/*v*) in the standard assay conditions above described. Optimal working temperature was found after performing the ABTS oxidation assay at different temperatures (25–80 °C) under standard assay conditions. To assess thermostability, the enzyme was incubated at different increasing temperatures (25–70 °C) for 30, 60, 120, and 240 min. After incubation, the residual activity was assayed on 0.5 mM ABTS under standard assay conditions. Moreover, the stability of the protein was estimated measuring the activity of the purified enzyme under standard assay condition after incubation at different pHs (100 mM sodium citrate pH 3 or 5, 100 mM Tris-HCl pH 7 or 9) at room temperature from 1 to 60 days. All tests were conducted in triplicate.

3. Results

3.1. Fermentation Process Development

To investigate the ability of *Coriolopsis trogii* MUT3379—a white-rot fungus isolated from decaying wood collected in "Parco della Mandria" in Turin and deposited in the Mycotheca Universitatis Taurinensis collection—to produce laccases, a screening of 18 different liquid media, selected among those previously developed for filamentous fungi fermentation by BioC-CheM Solutions, was performed. All of them were supplemented with copper sulphate at 0.15 mM concentration to induce laccase production [25–27]. *C. trogii* MUT3379 grew abundantly in all of the nutritional conditions tested (with the exception of the BCS050 medium), yielding—after 168 h of growth—from 2 g L^{-1} dry weight in BCS071 to ca. 40 g L^{-1} in BCS006, BCS132, and BCS267 (Supplementary Figure S1). An extracellular laccase activity was detectable in all of the screened media (with the exception of BCS368), albeit expressed at different levels and not directly correlated with biomass productivity (Supplementary Figure S1). The highest laccase activity (ca. 10,000 U L^{-1}) was achieved after 168 h of fermentation in BCS218, which was therefore selected for further optimization. The time course of laccase production in BCS218–0.15 mM $CuSO_4$ (Supplementary Figure S2), indicated that the activity started to be detectable after 48–72 h from the inoculum, with an exponential increase from the 168 to the 312 h, reaching a maximum final yield of ca. 60,000 U L^{-1} after 336 h of cultivation. Laccase activity production was inversely proportional to sugar consumption, thus suggesting its association with the late exponential/stationary growth of the fungus (Supplementary Figure S2).

As a first effort to optimize BCS218, the concentration of nutrients in the medium was gradually increased up to two-fold. In parallel, as laccase production is often significantly dependent on copper addition [25–27], $CuSO_4$ supplementation was increased up to 2 mM, and in parallel a different inorganic salt—copper chloride ($CuCl_2$)—was also tested at 0.15 mM and 2 mM concentrations, as a potential inducer. Figure 1 shows that at any concentration of copper salts tested, the increase in nutrient concentration enhanced mycelial growth (Figure 1a,b). Indeed, laccase activity exhibited a nutrient concentration-dependent increase (from ca. 68,000 U L^{-1} at 408 h at 1× nutrient concentration, to ca. 140,000 U L^{-1} at 408 h at 2× concentration; Figure 1c) only in the presence of 0.15 mM copper salt, whereas at 2 mM the results were comparable at any nutrients concentration tested (Figure 1d).

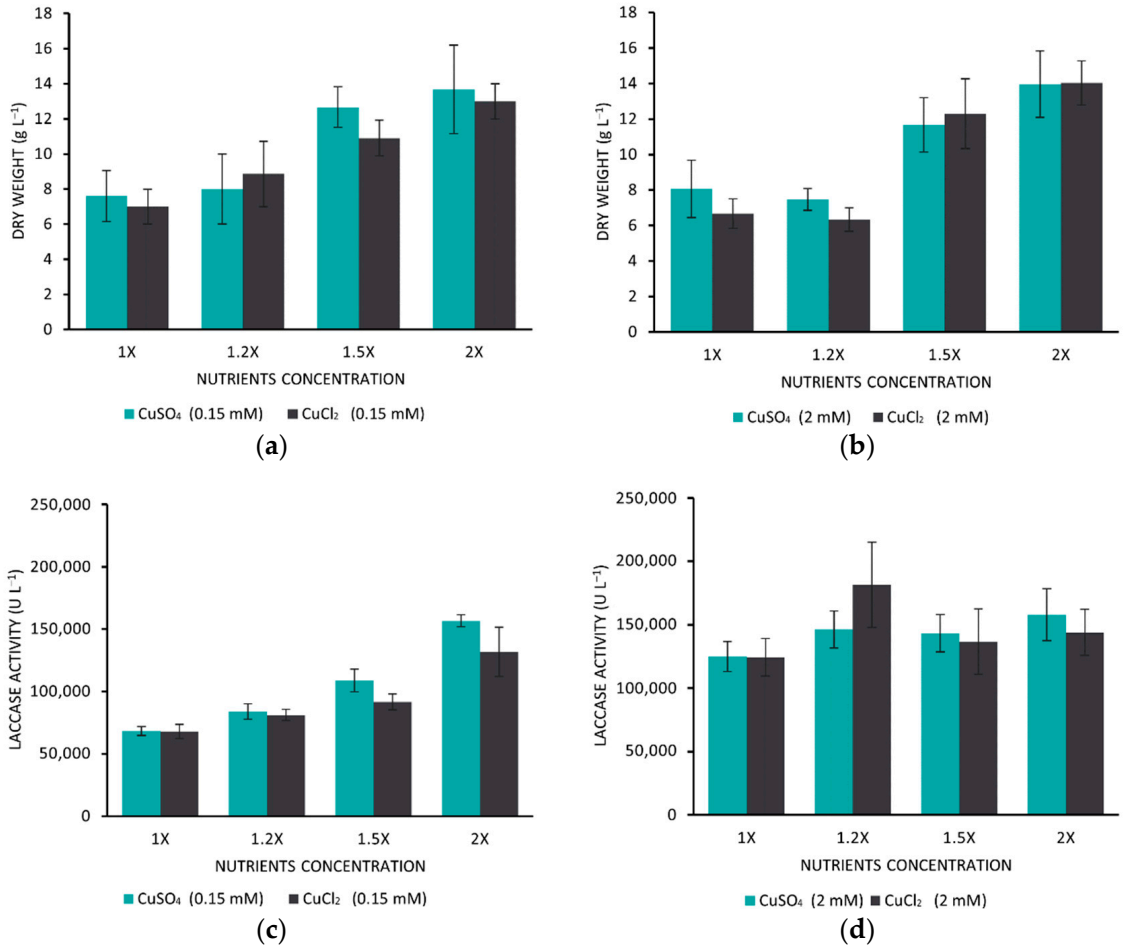


Figure 1. Dry weight (g L^{-1}) (panels (**a**,**b**)) and laccase activity (U L^{-1}) (panels (**c**,**d**)) in relation to the nutrient concentration (1×, 1.2×, 1.5×, and 2×) measured after 408 h of cultivation in BCS218 medium containing 0.15 mM (**a**,**c**) or 2 mM (**b**,**d**) $CuSO_4$ or $CuCl_2$. Data are the average ± standard deviation of three independent fermentations.

This different trend was confirmed following the time course of laccase production (Supplementary Figure S3). At 0.15 mM copper salt, laccase activity increased, reaching its maximum after ca. 300 h of fermentation, being thereafter almost stable until the end of the process (Supplementary Figure S3a,b). In the presence of 2 mM $CuSO_4$ or $CuCl_2$, laccase production continued to increase, reaching its highest value after 408 h from the inoculum (Supplementary Figure S3c,d). These results also indicate that the effects of copper supplementation on fungal growth and laccase production were independent of the nature of the salt used ($CuSO_4$ or $CuCl_2$). Overall, we selected the following combination to proceed with laccase activity production optimization: 1.2× nutrient concentration

and $CuCl_2$ at 2 mM. The highest laccase activity achieved in this condition at 408 h was 181,000 ± 34,000 U L^{-1} (Figure 1d).

The second improvement introduced in *C. trogii* MUT3379 cultivation, was the development of a vegetative step before the laccase production phase. Vegetative cultures are often used in fermentations to prepare fresh biomass for seeding the production medium and are crucial especially in the view of a future scaling-up of the production processes [28]. To this purpose, the best media supporting the fungal growth (i.e., BCS132, BCS052, BCS267, and BCS006, Supplementary Figure S1), all supplemented with 0.15 mM $CuSO_4$, were tested as vegetative media in parallel with BCS218–0.15 mM $CuSO_4$. These cultures were used (after 144 h of cultivation) to inoculate at 10% (*v*/*v*) the selected production medium BCS218 1.2×—2 mM $CuCl_2$. The overall best result (207,000 ± 11,000 U L^{-1}), in terms of laccase activity in the production medium, was obtained using the very same BCS218—0.15 mM $CuSO_4$ as vegetative medium (Figure 2). With all of the other media used as vegetative cultures, laccase production was lower, ranging from 48,000 ± 6000 U L^{-1} with BCS006—0.15 mM $CuSO_4$ as vegetative medium, to 103,000 ± 1000 U L^{-1} with BCS132—0.15 mM $CuSO_4$ (Figure 2). Varying the length of the fungal growth in BCS218—0.15 mM $CuSO_4$ as a vegetative culture did not further improve laccase production: in fact, a comparable laccase activity in BCS218 1.2×—2 mM $CuCl_2$ (ca. 200,000 U L^{-1}) was achieved with durations of the vegetative phase between 120 and 168 h, while adopting shorter or longer vegetative phases led to a reduction of the enzyme activity in the production medium.

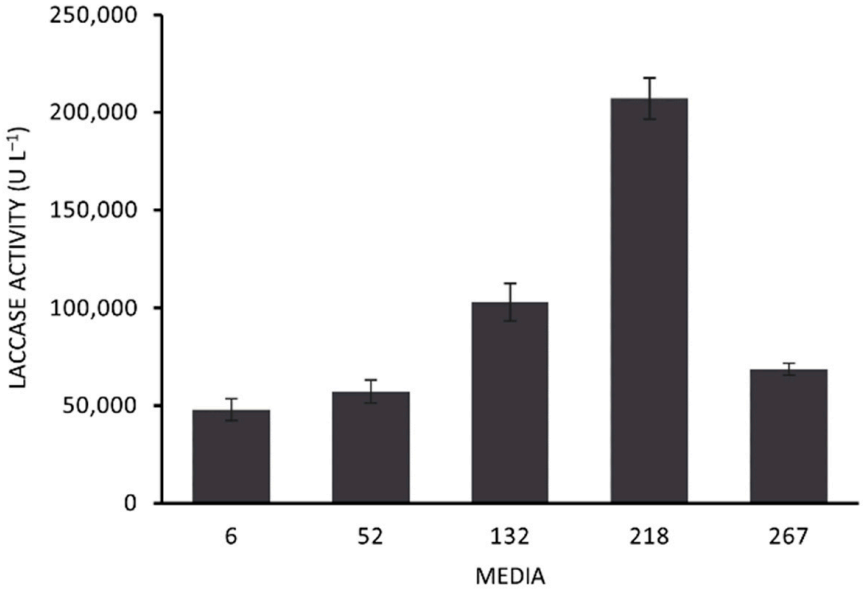


Figure 2. Laccase activity (U L^{-1}) in BCS218 1.2×—2 mM $CuCl_2$ medium inoculated with vegetative cultures obtained by growing *C. trogii* MUT3379 for 144 h in the different media indicated in the graph. Media are here indicated for simplicity only with the last numbers of their identifying code in the BCSMedDat database. Data are the average ± standard deviation of three independent fermentations and are relative to laccase activities detected after 360 h of cultivation in BCS218 1.2×—2 mM $CuCl_2$ production medium.

Finally, Figure 3 shows the time course of *C. trogii* growth and laccase production in the optimized conditions so far investigated. The biomass accumulated exponentially from 72 h of incubation to 144 h, and during this period a consumption of 9 g L^{-1} of total sugars was observed. Laccase started to be produced from 72 h of incubation but the more pronounced increase in production was observed after 240 h. This coincided with the complete depletion of total sugars, indicating that laccases are probably over-produced in response to carbon source starvation [29].

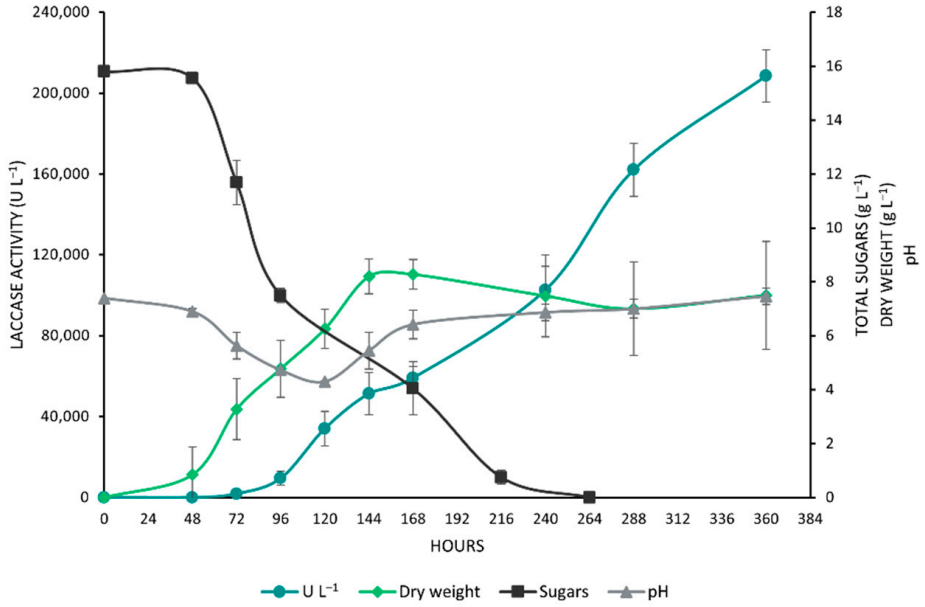


Figure 3. Time courses of laccase activity (U L^{-1}), dry weight (g L^{-1}), sugar consumption (g L^{-1}), and pH in BCS218 1.2×—2 mM $CuCl_2$, inoculated after the introduction of the vegetative step in BCS218—0.15 mM $CuSO_4$. Data are the average ± standard deviation of three independent fermentations.

3.2. Laccase Purification

The supernatant of *C. trogii* MUT3379 grown in the optimized conditions described above was first subjected to ammonium sulphate precipitation. When the precipitate was analyzed by SDS-PAGE, a prominent band at ca. 60 kDa appeared, together with a few other minor bands at higher or lower molecular masses (Figure 4a). The purity of the enzyme preparation was further improved by ion-exchange chromatography using an IRA-900 ion-exchange resin. Indeed, an SDS-PAGE analysis of the eluted fraction revealed a single band corresponding to the ca. 60 kDa secreted protein already identified in the ammonium sulphate precipitate (Figure 4b).

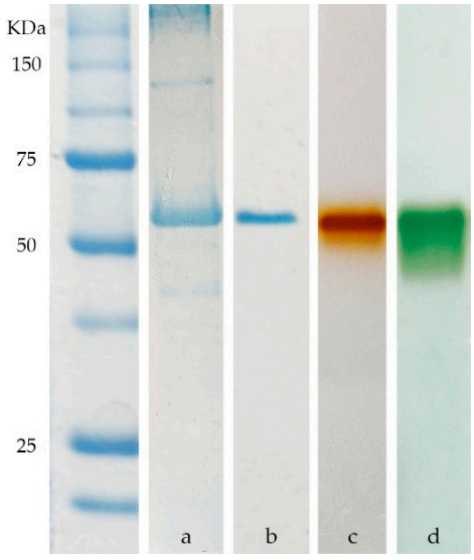


Figure 4. SDS-PAGE analysis of ammonium sulphate precipitate (**a**), and of purified laccase (**b**). Zymograms of purified laccase developed with guaiacol (**c**) and ABTS (**d**). In the first lane, molecular markers (Bio-Rad Laboratories, Hercules, CA, USA) were loaded.

The laccase activity of the protein was revealed by NATIVE page analysis with both guaiacol and ABTS (Figure 4c,d). The process allowed the recovery of ca. 360 mg of pure laccase per litre of culture, with a final yield of 79% and a purification fold of 2.2 (Table 1). At this point, we named this laccase Lac3379-1, as the first laccase identified from *C. trogii* MUT3379.

Table 1. Purification of laccase activity from *C. trogii* MUT3379 fermentation broth.

Purification Step	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U mg^{-1})	Yield (%)	Purification Fold
Culture filtrate	60	13,700	61	226	10	1
$(NH_4)_2SO_4$ precipitation	6	11,200	37	303.4	82	1.3
Ion-exchange chromatography	6	10,900	22	494.3	79	2.2

3.3. Identification of Lac3379-1 by Proteomic Analysis

For the identification of Lac3379-1, the purified protein was subjected to proteomics analysis. The high-resolution tandem mass spectra corresponding to the peptides obtained by Lac3379-1 tripsin digestion were matched with all of the hypothetical ORFs annotated in the genome of *C. trogii* C001 (GCA_020543525.1), which was the only one publicly available from this fungal species. Among positive matches, two peptides, ORF-1_360 (FQLNVIDNMTNHTMLK) and ORF-1_376 (GFAGGINSAILR), mapped onto the genome within contiguous loci, thus suggesting that they could be part of a potential gene encoding for a laccase. The genome sequence spanning around the loci encoding these two peptides was retrieved from the *C. trogii* C001 genome sequence (Supplementary Figure S4). This DNA sequence was first analyzed with AUGUSTUS (http://augustus.gobics.de; (accessed on 4 July 2024)) for identifying and assembling the exons into a single mature polypeptide, and then the further matching of the identified ORFs was performed with the additional peptides sorted from the LC-MS analysis according to the method described in [24]. A total of six peptides were mapped on this hypothetical Lac3379-1 ORF (Figure 5). The identified ORF encoded for a protein with a calculated size of 57.9 kDa. This molecular mass is in agreement with the ca. 60 kDa signal from the SDS-PAGE analysis (Figure 4b). A BLASTP similarity search of the Lac3379-1 hypothetical protein sequence was performed against the NCBI non-redundant protein database and displayed a best match (Identities: 476/535 (89%), Positives: 500/535 (93%), Gaps: 17/535 (3%)) with the Laccase 5 from *Trametes* sp. C30 (Sequence ID: ACO53431.1) (Supplementary Figure S5).

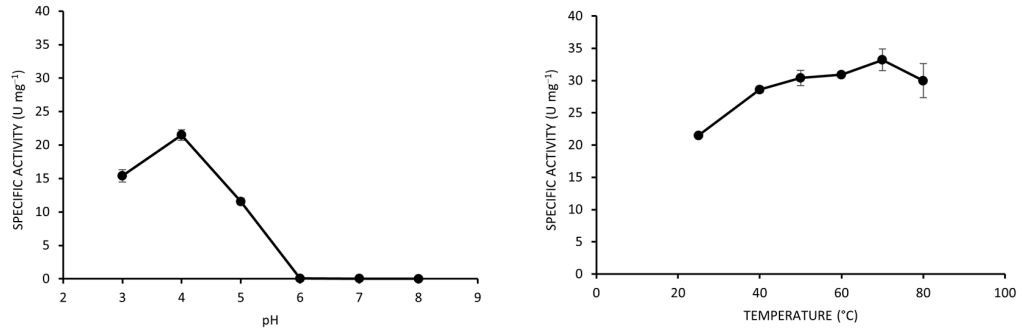
3.4. Lac3379-1 Characterization

The optimal pH and temperature ranges for Lac3379-1 activity were first assessed on ABTS as substrate (Figure 6). Lac3379-1 was found to be more active in acidic buffers with an optimal pH of 4 (22 $\pm$ 1 U mg^{-1}). The activity on ABTS rapidly declined when the pH increased to 6 and no enzymatic activity was observed at pH 7 or 8. The optimal temperatures were in the range from 40 to 80 °C with a maximum of activity at 70 °C.

The oxidizing capability of the laccase was then compared on a further eleven different substrates including guaiacol, 2,6-dimethylphenol (2,6-DMP), and syringaldazine (Supplementary Figure S6). Although Lac3379-1 oxidized all of the substrates, the maximal specific activities were recorded for potassium hexacyanoferrate ($K_4Fe(CN)_6$, 22 $\pm$ 2.6 U mg^{-1}), followed by 2,6-DMP (7 $\pm$ 0.6 U mg^{-1}) and syringaldazine (6.6 $\pm$ 0.6 U mg^{-1}). In general, the activity of the enzyme was confirmed as strongly dependent on acidic pH, with pH 4 to 5 being the optimal condition. A slight activity at an alkaline pH was observed only in the case of pyrogallol and L-DOPA as substrates, but we cannot exclude that it may be due to an autoxidation phenomenon of these molecules in alkaline buffers, as previously observed [30,31].

```
Lac_3379-1      MARFQSLLTFITLSLVASVYAAIGPVADLTISNGAVSPDGFSRQAILVNDVFPSPLITGN
ORF-1_360a      -----------------------------------------QAILVNDVFPSPLITGN
                                                         ******************

Lac_3379-1      KASCFLHRRSPHMLTCLQGDRFQLNVIDNMTNHTMLKSTSIHWHGFFQHGTNWADGPAFV
ORF-1_360       --------------------FQLNVIDNMTNHTMLK-----------------------
                                    ****************

ORF-1_360a      K-------------------------------------------------------------
                *

Lac_3379-1      NQCPISTGHAFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPIVVYDPQDPHKSLYDVDDE
ORF-1_360       -------------------------------------------------------------
Lac_3379-1      STVITLSDWYHVAAKLGARFPPGSDSTLINGLGRSITTLNADLAVVSVTQGKRYRFRLVS
ORF-1_360       -------------------------------------------------------------

Lac_3379-1      LACDPNWAFSIDNHNMTIIEADSVSTKPHTVDSITIFTAQRYSFVLIADQPVDNYWIRAN
ORF-1_360       -------------------------------------------------------------
Lac_3379-1      PNVGNRGFAGGINSAILRYDGAPPVEPTTVQPPSVIPMVEADLTTFDSRPAPGRPVPGGV
ORF-1_376.      ------GFAGGINSAILR-----------------------------------------
                      ************

Lac_3379-1      DLALNLLFSFNGSNFFINGASFVPPTVPVLLQILSGAQAAQDLLPSGSVFTLPPNADIEI
ORF-1_360       -------------------------------------------------------------
Lac_3379-1      SMPAGAAGAPHPFHLHGHSFAVVRSAGQTGFNFENPVFRDVVSTGTPAANDNVTIRFRTD
ORF-3_317b      ------------------------SAGQTGFNFENPVFR---------------------
                                        ***************

Lac_3379-1      NPGPWFLHCHIDFHLDAGFAVVLAEDTPDVKAVNPVPKAWEDLCPTFNALAPGDT
ORF-3_317       -------------------------------AVNPVPK-----------------
                                              *******

ORF-3_317a      ------------------------------------AWEDLCPTFNALAPGDT
                                                    ******************
```

Figure 5. Hypothetical sequence of Lac 3379-1. The LC-MS peptides matching with the laccase deduced sequence are indicated. Alignment of the LC-MS peptides was performed with ClustalW and peptides names are reported in the sequence alignment. * indicates positions which have a fully conserved residue.



(**a**) (**b**)

Figure 6. Effect of different pHs (**a**) and temperatures (**b**) on Lac3379-1 activity on ABTS as substrate. In panel a, the activity was measured at 25 °C, while in panel b the activity was measured at pH 4. Data are expressed as activity units per mg of protein and are the average ± standard deviation of three independent measurements.

Kinetic parameters K_m and k_{cat} of Lac3379-1 were calculated using ABTS, $K_4Fe(CN)_6$, or 2,6-DMP as substrates. A typical Michaelis–Menten kinetic was observed in all of the cases. The enzyme efficiency was also evaluated by calculating the k_{cat}/K_m ratio. Results are summarized in Table 2. The lowest Km value (2.64 μM) and, consistently, the highest turnover rate (154.1 s^{-1}) and catalytic efficiency (58.4 s^{-1} μM^{-1}), were found for ABTS. Catalytic efficiency for $K_4Fe(CN)_6$ and 2,6-DMP were ca. 2000 and 225 times lower than that for ABTS, respectively.

Table 2. Summary of kinetic parameters calculated for ABTS, 2,6-DMP, and $K_4Fe(CN)_6$. Data are the average ± standard deviation of three independent measurements.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
ABTS	2.6 ± 0.6	154.1 ± 0.8	58.4 ± 13
2,6-DMP	105.5 ± 8	27.5 ± 0.6	0.26 ± 0.02
$K_4Fe(CN)_6$	76.9 ± 14	2.2 ± 0.07	0.029 ± 0.004

The activity of Lac3379-1 was then assayed in the presence of different salts (NaCl, KCl, $CaCl_2$), surfactants (Tween 20, Tween 80, Triton X-100, IGEPAL CA630, sodium dodecyl sulfate (SDS), and Nonidet P40), and solvents (ethanol, methanol, acetonitrile, and dimethylsulfoxide), performing the measurements in standard assay conditions, i.e., on 0.5 mM ABTS at 25 °C in 50 mM sodium acetate pH 4 (Figure 7). The oxidizing activity of Lac3379-1 gradually declined as the salt concentration increased (Figure 7a), independently of the type of salt added. Although ca. 100% of the activity measured in control conditions (i.e., without salts) was maintained at 1 mM NaCl, KCl, or $CaCl_2$, it then declined ca. 40% at 10 mM, and ca. 80–90% at 100 mM, being almost negligible at 1 M.

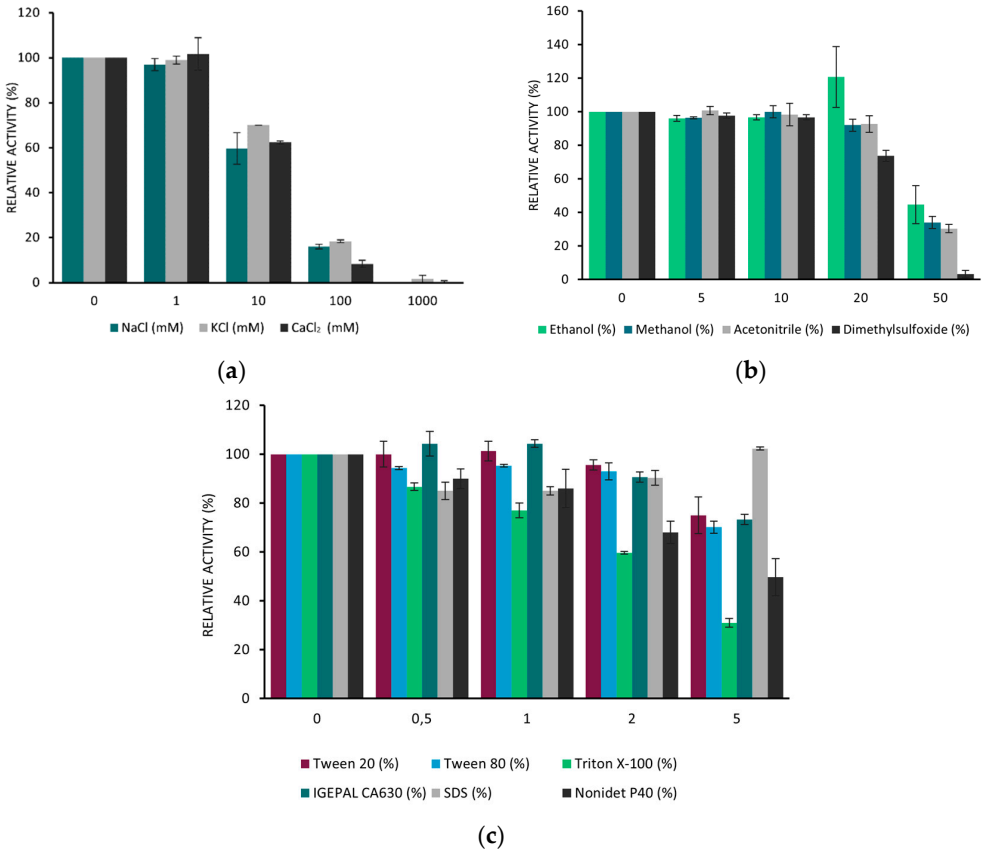
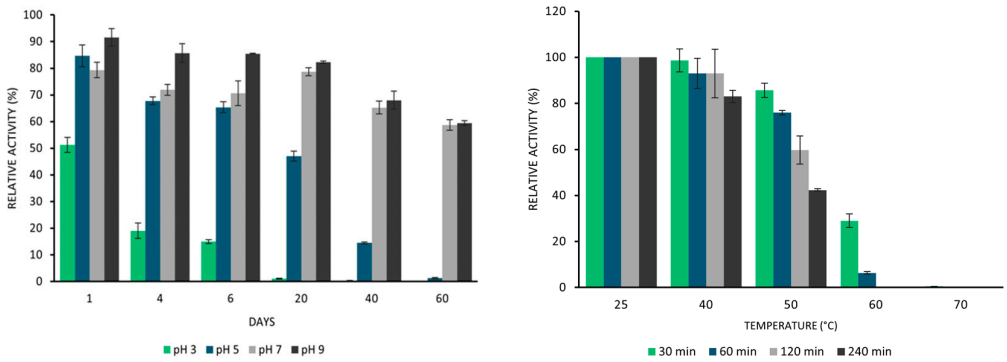


Figure 7. Relative laccase activity in the presence of salts (**a**), solvents (**b**), and surfactants (**c**). 100% corresponds to the activity recorded in standard assay conditions (equal to 22 ± 1 U mg^{-1}). Data are the average ± standard deviation of three independent measurements.

Solvent tolerance was observed at concentrations up to 20% (*v*/*v*), at which more than 70% of the enzyme activity measured in the control condition (i.e., without solvents) was maintained. Remarkably, Lac3379-1 maintained 45 ± 11% of its relative activity in the presence of 50% (*v*/*v*) ethanol, and 34 ± 3.6% and 30 ± 2.5% at 50% (*v*/*v*) in the presence of methanol and acetonitrile, respectively (Figure 7b).

Among the surfactants, Tween 20, Tween 80, Igepal CA630, and SDS in concentrations from 0.5 to 2% (*v*/*v*) did not affect the enzyme activity, which was comparable to the one measured in the absence of surfactants. Even at 5% (*v*/*v*), the exposure to these molecules did not dramatically reduce the Lac3379-1 activity. In particular, the enzyme activity seemed not affected at the highest concentration tested of SDS (Figure 7, panel c), showing a remarkable tolerance to this surfactant. Lac3379-1 was less tolerant to Triton X-100, since its residual activity was inversely proportional to the surfactant concentration, although approximately 30% activity was still maintained at the concentration of 5% (*v*/*v*).

The long-term stability of Lac3379-1 was then evaluated by incubating the enzyme at room temperature at pH 3, 5, 7, and 9 for up to 60 days. As visible in Figure 8, panel a, the protein maintained its oxidizing capacities for more than a month when diluted in neutral or alkaline buffers. In these conditions the enzyme conserved 60% of its initial activity even after 60 days of incubation. In acidic solutions, the laccase activity declined more rapidly. Indeed, at pH 3, it was reduced to 15% after 6 days of incubation, being almost null after 20 days. At pH 5, ca. 50% of the initial activity remained detectable after 20 days, being then reduced to ca. 15% after 40 days of incubation.



(**a**) (**b**)

Figure 8. Relative laccase activity measured after incubation at different pHs (**a**) and temperatures (**b**). 100% corresponds to the activity recorded in standard assay conditions (equal to ca. 22 ± 1 U mg^{-1}). Data are the average ± standard deviation of three independent measurements.

Thermostability was evaluated after incubation for 30, 60, 120, and 240 min at increasing temperatures, from 25 to 70 °C. The protein maintained its enzymatic activity up to 50 °C. When incubated at 60 °C for 30 and 60 min, the protein conserved the 29% and the 6% of its initial activity, respectively, while it was completely inactivated after incubation at 70 °C (Figure 8b).

4. Discussion

White-rot fungi such as *Phanerochaete crysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor* have been extensively studied for the degradation and transformation of the highly recalcitrant phenolic polymer constituting lignin. Interestingly, fungal laccases and peroxidases possess oxidative properties able to act on different substrates making them suitable for many different industrial applications that go beyond the sole treatment of lignocellulosic materials [32]. However, the lack of robust production processes limits the application of white-rot fungi as cell factories for the production of these enzymes at low cost. Consequently, our attention focused on the less exploited basidiomycete *Coriolopsis trogii*, which was previously reported to colonize and decompose dead wood and that

could represent a potential promising candidate to produce novel laccases [18]. Our work was driven by the will to develop, from the beginning, a robust production process which could then meet the biotechnological needs, overcoming the current bottlenecks in the use of these biocatalysts.

To this purpose, 18 different production media were evaluated for cultivating *C. trogii* MUT3379. It was found that extracellular laccase activity production in this fungus was not always proportional to fungal growth, and it was associated with the stationary phase, confirming what has been previously reported in the literature [29]. Indeed, the production of lignin-modifying enzymes in white-rot fungi is often associated with the stationary phase of growth and it is subjected to catabolite repression, depending on a combination of different factors undergoing an as of yet poorly elucidated complex regulation system [29]. As reported for other fungal strains, *C. trogii* laccase production was highly dependent on the addition of copper salts. Indeed, copper salts are one of the most commonly used laccase inducers [33–35] as copper is both needed for the correct synthesis of the protein catalytic site and it serves as an activator of the ACE transcriptional factors, which switch on the expression of laccase encoding genes [16,36]. Notably, at the lowest copper concentration that we tested (0.15 mM), laccase production by *C. trogii* increased proportionally to nutrient concentrations. Instead, when we added 2 mM copper, the effect of nutrient concentrations became less relevant, allowing their reduction in the cultivation medium. The use of less concentrated media might represent an advantage during fungal fermentations, with a positive impact on scaling up by reducing broth viscosity and foaming, consequently favoring aeration and mixing, and facilitating recovery protocols [37]. An additional advantage of the optimized medium BCS218 1.2×—2 mM $CuCl_2$ that we finally used for laccase production, was that *C. trogii* MUT3379 grew in it as small defined pellets, thus facilitating the mycelium removal via filtration. The laccase activity was then recovered from the cultivation broth and the lack of complex soluble and insoluble nutrients in BCS218 medium permitted a clear permeate to be obtained from which the enzyme was easily purified. Finally, the overall production process and its scalability were improved by the introduction of a vegetative cultivation step, which led to the remarkable laccase activity of ca. 200,000 U L^{-1} after 360 h from the inoculum, also reducing the time needed to achieve the maximum productivity. In these conditions, the SDS-PAGE gel of the fungal secretome showed the net predominance of a ca. 60 kDa band corresponding to the laccase named Lac3379-1 after its identification by proteomic analysis (calculated molecular mass of 57.9 kDa). Lac3379-1 purification, based on protein precipitation followed by chromatographic separation, led to a final remarkable yield of 79%.

Thanks to the improvement of the upstream titer and the efficiency of the downstream process, the laccase production level obtained in this work highly exceeds that previously reported in the literature for other *C. trogii* strains. Laccase production in *C. trogii* Han751 was investigated by An et al. [38], who achieved a final production of ca. 8600 U L^{-1}, while Bao et al. [39] cloned and successfully expressed *C. trogii* Mafic-2001 laccase in *Pichia pastoris* with a production titer of 720 U L^{-1}. Different *Coriolopsis* spp. were also investigated for laccase production, with *Coriolopsis gallica* being the most frequently studied [40–42]. *C. gallica* 1184 was reported by Songulashvili et al. to produce laccase activity at a comparable level with our strain, in a 50 L bioreactor [41]. However, in this case, the production medium was definitively richer than the BCS218 medium, containing for instance 50 g L^{-1} of glucose and 17 g L^{-1} of peptone, which negatively impacted both the cost and the scalability of the upstream and downstream processes. In fact, the purification yield of *C. gallica* 1184 laccase stood at 16.2% only, pointing out a significant loss of laccase activity during the protein recovery [41].

Similar to other fungal laccases, Lac3379-1 was found active on a broad range of molecules, mostly in an acidic environment. It presented the highest activity on ABTS, potassium hexacyanoferrate, and 2,6-DMP. The calculated kinetic parameters suggested a very high affinity for ABTS with a catalytic efficiency of 58.4 ± 13 s^{-1} μM^{-1}, almost

reaching the so-called 'catalytic perfection' [43]. Although fungal laccases often show a high affinity for ABTS, the Lac3379-1 Km value was lower in comparison to the previously characterized laccases from *C. trogii* Mafic-2001 (with a Km value of 90 µM) and from *Coriolopsis rigida* (two laccases with Km values of 11 and 12 µM, respectively) [39,44]. This Lac3379-1 high affinity for ABTS suggests its possible use in industrial settings, where ABTS is often employed as a laccase redox mediator to enhance the oxidative power of the enzyme against more recalcitrant substrates [45]. For instance, the *C. gallica* laccase-mediator system was previously proposed as an effective solution to treat and reduce the phytotoxicity of poly-azo dye [46]. Laccase-mediator systems are also often employed for removing pharmaceutical compounds from wastewaters [47]; for example, Wang et al. described tetracycline removal from seawater [48]. The laccase/mediator system was also used to enhance the laccase performance in lignin valorization [49,50]. In a study of Rico et al., laccase-mediated lignin degradation was optimized using methyl syringate as a mediator, resulting in a 30% increase in lignin removal from eucalypt wood. This improvement correlated with a better saccharification of the biomass to be used as feedstock for biofuel production [51].

In terms of salt tolerance and pattern of activity at different temperatures and pHs, Lac3379-1 resembled the features of other fungal laccases [7] such those from *C. trogii* Mafic-2001 [39] and *C. gallica* 1184 [41]. Conversely, regarding stability at different temperatures and pHs, Lac3379-1 was notably distinct. For instance, the stability of *C. trogii* Mafic-2001 laccase significantly declined after incubation at 50 °C for 60 min, whereas Lac3379-1 retained approximately 40% of its relative activity even after 240 min of incubation at the same temperature [39]. Additionally, Lac3379-1 demonstrated considerable stability when incubated at pH seven and nine, unlike other laccases such as those obtained from *C. gallica* TCK, and its mutant counterpart T906, whose stability quickly declined when incubated at pH levels above four [42].

Indeed, Lac3379-1 was found particularly tolerant to surfactants, in that up to 2% (*v*/*v*) did not significantly alter its oxidative potential. This represents an important feature for the industrial application of this laccase. Surfactants are widely used as additives in textile industries, not only as detergents during textile washing processes but also as wetting agents for unprocessed textile fibers. The resulting wastewaters are often rich in surfactants that could limit the efficiency of the laccase-mediated removal of toxic dyes [52,53]. Surfactants were also used to increase the availability of polycyclic aromatic hydrocarbons (PAHs) in contaminated soils [54,55], where laccases are employed for biodegrading these molecules [56,57].

Another peculiar characteristic of Lac3379-1 was its tolerance to different solvents up to concentrations of 20% (*v*/*v*). Considering the widespread use of organic solvents for solubilizing phenolic substrates, susceptibility to solvents may represent a relevant limiting factor for laccase application in biocatalysis [45]. Interestingly, Lac3379-1 was found particularly tolerant to DMSO, more than the previously reported laccase from *Trametes trogii* LK13 [58]. Lac3379-1 retained 74.0 ± 3.2% of its enzymatic activity even in the presence of 20% (*v*/*v*) DMSO, while *T. trogii* laccase maintained only 58.7 ± 3.9% of its relative activity in the presence of 5% (*v*/*v*) DMSO [58]. Notably, the solvent tolerance profile of Lac3379-1 was similar to the one of the yellow laccase produced by *C. gallica* NCULAC F1 [59].

To conclude, *C. trogii* MUT3379 was found to be an excellent cell factory for the production of a novel laccase, the first identified as produced by this strain. Optimizing its fermentation process led to a 20-fold improvement in the laccase production titer, reaching a remarkable level of ca. 200,000 U L^{-1}, which is, to the best of our knowledge, the highest one ever achieved by *C. trogii* strains. Moreover, by adopting the medium BCS218 and its variants, a simplified purification process, consisting of two steps only, was implemented. To further explore the *C. trogii* MUT3379 ligninolytic system while improving the sustainability of the enzyme production, fermentations on different residual biomasses, including pruning and agricultural waste, will be further evaluated. In parallel, innovative

strain improvement strategies will be investigated to further enhance the laccase production titer, making the industrial exploitation of this enzyme even more appealing. Indeed, the biochemical characterization of purified Lac3379-1 revealed promising enzyme features suitable for various industrial applications. Confirming this, *BioC-CheM Solutions* has recently applied Lac3379-1 for the bio-bleaching of cotton fibers and for the bioremediation of textile wastewater. Additional potential applications of Lac3379-1 worthy of exploration will be delignification and whitening in pulp and paper industries, as well as biocatalysis in polymer synthesis and functionalization.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10070376/s1, Figure S1: (a) Dry weight (g L^{-1}) and (b) extracellular laccase activity (U L^{-1}) obtained cultivating *Coriolopsis trogii* MUT3379 in 18 differently composed media at 25 °C and 150 rpm for 168 h. All cultivation media tested are hereby indicated for simplicity only with the last numbers of their identifying code in the *BCSMedDat* database (owned by *BioC-CheM Solutions)* and were all supplemented with 0.15 mM $CuSO_4$; Figure S2: Time courses of extracellular laccase activity (U L^{-1}), pH, and sugar consumption (g L^{-1}) kinetics in BCS218—0.15 mM $CuSO_4$. Data are the average ± standard deviation of three independent fermentations; Figure S3: Time courses of extracellular laccase activity (U L^{-1}) obtained in the different BCS218 formulations (medium as-it-is-1×, or with increased nutrient concentrations of 1.2, 1.5 or 2×) supplemented with 0.15 mM $CuSO_4$ (a), 0.15 mM $CuCl_2$ (b), 2 mM $CuSO_4$ (c), or 2 mM $CuCl_2$ (d); Figure S4: Genome sequence from *C. trogii* C001 of the region around the LC-MS identified peptides; Figure S5: BLASTP alignment of the hypothetical Lac 3379-1 sequence against the non-redundant protein database retrieved as the best match; Figure S6: Lac3379-1 activity at 25 °C and at different pHs on $K_4Fe(CN)_6$ (a), 2,6-DMP (b), syringaldazine (c), guaiacol (d), pyrogallol (e), pyrocatechol (f), L-DOPA (g), syringaldehyde (h), syringic acid (i), vanillic acid (l), and ferulic acid (m). Data are expressed as activity units per mg of purified Lac3379-1 and are the average ± standard deviation of three independent measurements.

Author Contributions: Conceptualization, L.M. and F.B. (Fabrizio Beltrametti); Data curation, F.B. (Fabrizio Beltrametti); Formal analysis, F.B. (Fabrizio Beltrametti); Funding acquisition, F.M. and F.B. (Fabrizio Beltrametti); Investigation, L.M. and M.B.; Methodology, M.B., F.B. (Francesca Berini), and F.B. (Fabrizio Beltrametti); Project administration, F.B. (Fabrizio Beltrametti); Software, J.A.; Supervision, F.B. (Francesca Berini) and F.B. (Fabrizio Beltrametti); Validation, F.B. (Francesca Berini), F.M. and F.B. (Fabrizio Beltrametti); Writing—original draft, L.M.; Writing—review and editing, F.B. (Francesca Berini) and F.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Authors Luca Mellere and Fabrizio Beltrametti were employed by the company BioC-CheM Solutions S.r.l. Author Martina Bellasio was employed by the company Actygea S.r.l. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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