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Enhancing Laccase Production by *Trametes hirsuta* GMA-01 Using Response Surface Methodology and Orange Waste: A Novel Breakthrough in Sugarcane Bagasse Saccharification and Synthetic Dye Decolorization

Guilherme Guimarães Ortolan [1], Alex Graça Contato [2], Guilherme Mauro Aranha [1], Jose Carlos Santos Salgado [1,3], Robson Carlos Alnoch [1,2] and Maria de Lourdes Teixeira de Moraes Polizeli [1,2,*]

[1] Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto 14040-901, São Paulo, Brazil; hortolan.gui@usp.br (G.G.O.); guilhermearanha@usp.br (G.M.A.); robsonalnoch@usp.br (R.C.A.)
[2] Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto 14049-900, São Paulo, Brazil; alexgraca.contato@gmail.com
[3] Department of Chemistry, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto 14040-901, São Paulo, Brazil
* Correspondence: polizeli@ffclrp.usp.br; Tel.: +55-(16)-3315-4680

Abstract: *Trametes hirsuta* GMA-01 was cultivated in a culture medium supplemented with orange waste, starch, wheat bran, yeast extract, and salts. The fungus produced several holoenzymes, but the laccase levels were surprisingly high. Given the highlighted applicability of laccases in various biotechnological areas with minimal environmental impact, we provided a strategy to increase its production using response surface methodology. The immobilization of laccase into ionic supports (CM-cellulose, DEAE-agarose, DEAE-cellulose, DEAE-Sephacel, MANAE-agarose, MANAE-cellulose, and PEI-agarose) was found to be efficient and recuperative, showcasing the technical prowess of research. The crude extract laccase (CE) and CM-cellulose-immobilized crude extract (ICE) showed optimum activity in acidic conditions (pH 3.0) and at 70 °C for the CE and 60 °C for the ICE. The ICE significantly increased thermostability at 60 °C for the crude extract, which retained 21.6% residual activity after 240 min. The CE and ICE were successfully applied to sugarcane bagasse hydrolysis, showing 13.83 ± 0.02 μmol mL^{-1} reducing sugars after 48 h. Furthermore, the CE was tested for dye decolorization, achieving 96.6%, 71.9%, and 70.8% decolorization for bromocresol green, bromophenol blue, and orcein, respectively (0.05% (*w*/*v*) concentration). The properties and versatility of *T. hirsuta* GMA-01 laccase in different biotechnological purposes are interesting and notable, opening several potential applications and providing valuable insights into the future of biotechnological development.

Keywords: laccases; *Trametes hirsuta*; decolorization; immobilization; response surface methodology

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1. Introduction

In recent years, bioprospection research has focused on the search for new molecules, genes, enzymes, processes, organisms, and other products in biodiversity [1,2]. Scientific and economic exploration of these bioresources is essential for countries' development and sovereignty [3,4].

Among filamentous fungi, the mushroom-forming *Trametes* genus is described as a source of enzymes and other metabolites with a wide range of applications, such as anti-cancer molecules, enzymatic cocktails composition, and even biomaterial constitution [5–8]. *Trametes hirsuta* could be used in multiple biotechnological processes and bioremediation efforts, especially regarding the degradation of contaminant dyes [9] or environmental pollutant molecules such as bisphenol-A or phthalic acid esters [10,11].

One of the main interests of bioprospection research is the decomposing of organic matter by fungal enzymes, especially those involved in the degradation of plant cell walls and their constitutive polymers, cellulose, hemicellulose, and lignin [12–14]. Some of the crucial enzymes involved in lignin degradation and depolymerization are laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2), a group of highly efficient, robust, and versatile multi-copper oxidases capable of oxidizing phenolic and non-phenolic compounds assisted or not by mediators [15].

The principal usefulness of this enzyme is the application of molecular oxygen (O_2) as the final electron acceptor for the electrons removed from the substrate, producing water (H_2O) as a byproduct [16]. Many studies were developed exploring the potential application of laccases in the degradation of environmental pollutants, such as bisphenol-A [17–20], pharmaceuticals [21–23], and azo dyes [24–26]. Other laccases applications occur in various industries, especially the paper, textile, food, and pharmaceutical industries [27–29].

Laccases are also utilized in enzymatic cocktails or as part of lignocellulosic biomass pretreatment due to their capability to oxidize lignin, thus modifying its structural properties and allowing for other enzymes to reach the cellulose or hemicellulose fibers [30,31]. Multiple strategies apply biological pretreatment to achieve an eco-friendly process and valorize waste biomass [32–34].

Immobilization techniques are constantly being developed and enhanced to improve these and other biotechnological applications of laccases and other enzymes. One possible immobilization strategy is ionic adsorption, which consists of ionic exchange reactions between functional groups in the backbone of the polymeric column and ionizable amino acid side chains [35].

This study explored a laccase with high catalytic efficiency from *T. hirsuta*. Our group isolated, identified, and characterized the *T. hirsuta* strain investigated in this work [36], which showed multiple interesting characteristics, such as noteworthy antioxidant capacities. The laccase production was optimized using a statistical methodology followed by immobilizing the crude extract in ionic supports. The characterization of free and immobilized laccases was performed regarding multiple physical and chemical parameters, such as optimum temperature and pH, thermostability, and stability to pH. The laccase-crude extract was used in synthetic dye discoloration and sugarcane bagasse saccharification, aiming for their potential applications in bioremediation and the formulation of enzymatic cocktails for lignocellulosic biomass degradation.

2. Materials and Methods

2.1. Materials

ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), Ponceau S, Victoria Blue B salt, bromocresol green sodium salt, bromophenol blue salt, Methyl Red salt, Trypan blue salt, orcein, Congo red salt, carboxymethylcellulose sodium salt (from wood pulp), locust bean (from *Ceratonia siliqua* seeds), polygalacturonic acid sodium salt (>95%, from citrus fruit), xylan (>90%, from beechwood), *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-xylanopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, DEAE (diethylaminoethyl)-agarose, DEAE-cellulose, and DEAE-Sephacel were purchased from Sigma–Aldrich (Saint Louis, MO, USA). The supports CM (carboxymethyl)-cellulose, DEAE (diethylaminoethyl)-cellulose, MANAE (monoamine-*N*-aminoethyl), and polyethyleneimine (PEI)-agarose were prepared and activated as described elsewhere [37]. Debranched arabinan (>95%, from sugar beet), β-glucan (>95%, from barley), and xyloglucan (>95%, from tamarind seed) were purchased from Megazyme (Wicklow, Ireland). Methylene Blue salt and Gentiant Violet B salt were purchased from Synth (Diadema, SP, Brazil). Orange waste was obtained through local commerce (Ribeirão Preto, SP, Brazil). The exploded sugarcane bagasse (SP80-3280) was granted by the company Pedra Agroindustrial S/A (Serrana, SP, Brazil) and was previously characterized [38]. It was composed of leaves, stems, and straw. All other chemicals were of the highest purity and analytical grade.

2.2. Fungal Growth

T. hirsuta GMA-01 (GenBank MN625447) was maintained in potato dextrose agar (PDA) in Petri dishes. For the initial enzymatic screening, *T. hirsuta* was cultured as described elsewhere [39]. Cultures were performed for a total of 10 days.

To laccase production, three mycelial disks (10 mm) were transferred from the dishes to a 125 mL Erlenmeyer flask with varying amounts of minced (4 mm) sun-dried orange waste, 2 g starch, 1 g wheat bran, 0.2 g yeast extract, plus mineral Vogel mineral medium [40]. Orange waste was obtained through local commerce and consisted of pulp and peels. The initial moisture content of the solid was 80%, and cultures were conducted for 14 days at 28 °C. To obtain the crude enzymatic extracts, 20 mL of distilled water was added to the solid-state cultures, which were macerated, filtrated, and centrifuged (11,950× *g*, 4 °C) for 10 min. The supernatant was collected and used in the assays, as modified from [41].

Laccase production was optimized in solid-state fermentations performed as described above but at various temperatures and quantities of orange waste, according to the experimental design described below.

2.3. Enzymatic Assays

Laccase activity was determined using 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as the substrate. The assay added 100 µL of enzymatic extract to 800 µL of ABTS substrate (0.03% *w*/*v* in sodium acetate buffer, pH 5.0, 50 mmol L^{-1}) (total volume of 900 µL). The ABTS oxidation was monitored by increasing absorbance at 420 nm (ε_{420} = 36 mmol L^{-1} cm^{-1}) for 5 min. Under assay conditions, one unit of activity (U) was defined as the amount of enzyme capable of releasing 1 µmol of product equivalents per min. Laccase activity was expressed in U mL^{-1} [42].

The activities for the holocellulases presented in crude extract were determined with the following natural substrates: carboxymethylcellulose sodium salt (endoglucanase), β-glucan (β-glucanase), beechwood xylan (endo-1,4-β-xylanase), locust bean (mannanase), polygalacturonic acid sodium salt (polygalacturonase), debranched arabinan (endo-1,5-α-arabinanase), and xyloglucan (xyloglucanase). The standard assay was performed with 25 µL of 1% substrate solution (*w*/*v*), 10 µL of 50 mmol L^{-1} sodium acetate buffer, pH 5.0, and 15 µL of enzymatic crude extract. After incubation, 50 µL of the assay samples were added to 50 µL of 3,5-dinitrosalicylic acid (DNS) [43]. The mixture was then boiled at 98 °C in a thermal cycler for 5 min, and the reducing sugars were determined by absorbance at 540 nm in the microplate readers (Spectramax M2, Molecular Devices, San Jose, CA, USA). When adequate, a standard curve of D-glucose or other monosaccharides (xylose, mannose, monogalacturonic acid, and arabinose) was used to estimate the equivalents of reducing sugar concentrations. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzes the release of 1 µmol of reducing sugar per minute under assay conditions.

For determination of activity using the synthetic substrates *p*-nitrophenyl-β-D-glucopyranoside (β-D-glucosidase), *p*-nitrophenyl-β-D-cellobioside (cellobiohydrolase), *p*-nitrophenyl-β-D-galactopyranoside (β-D-galactosidase), and *p*-nitrophenyl-β-D-xylanopyranoside (β-D-xylosidase), a standard assay was carried out by mixing 25 µL of 2 mmol L^{-1} substrate solution (*w*/*v*), 10 µL of 50 mmol L^{-1} sodium acetate buffer, pH 5.0, and 15 µL of crude enzymatic extract. After 20 min, 50 µL of Na_2CO_3 (0.5 mol L^{-1}) was added to stop the enzymatic reaction [12]. The release of *p*-nitrophenolate was measured at 410 nm in a microplate reader using the *p*-nitrophenol as standard (Molecular Devices, Sunnyvale, CA, USA). One unit of enzymatic activity was determined as the amount of enzyme that catalyzes the release of 1 µmol of *p*-nitrophenolate per minute under assay conditions.

2.4. Response Surface Methodology to Optimize Laccase Production

A central composite design (CCD) was used to evaluate the influence of different variables in producing laccases to obtain the best conditions. The design consisted of assays with two independent variables, temperature (°C) and orange waste (g), on 5 levels

(−1.41; −1; 0; +1; +1.41). The effect of the independent variables was evaluated over the response variable of laccase activity (U mL^{-1}). The results were adjusted for a second-order polynomial equation. For the construction of the experimental design, the runs shown in Table 1 were used, consisting of assays after two weeks of cultivation.

Table 1. CCD planning matrix with 3 repetitions on the central point for laccase activity optimization.

Run	Temperature °C (x_1)	Orange Waste g (x_2)	Experimental Activity (U mL^{-1})	Predicted Activity (U mL^{-1})	Residue
1	−1 (24)	−1 (3.0)	3.575	3.655	−0.08042
2	1 (32)	−1 (3.0)	3.656	5.284	−1.62817
3	−1 (24)	1 (4.0)	4.692	5.062	−0.37032
4	1 (32)	1 (4.0)	7.444	9.362	−1.91808
5	−1.41 (22)	0 (3.5)	1.083	1.175	−0.09247
6	1.41 (34)	0 (3.5)	7.458	5.355	2.10293
7	0 (28)	−1.41 (2.8)	7.347	6.547	0.79962
8	0 (28)	1.41 (4.2)	11.625	10.414	1.21083
9	0 (28)	0 (3.5)	11.208	11.223	−0.01497
10	0 (28)	0 (3.5)	11.222	11.223	−0.000
11	0 (28)	0 (3.5)	11.215	11.223	−0.00797

A residual value was calculated for each of the experimental runs. It measures the difference between the predicted and the experimental activities. Therefore, high residue, in absolute values, indicates a non-predictive model.

The α axial points were chosen following the relation $\alpha = k^{1/2}$, where k represents the number of evaluated factors (independent variables). For k = 2, $\alpha = \pm 1.41$ [44]. The estimated response Y to the variables, where Y is the laccase activity in U mL^{-1}, can be approximated by the quadratic polynomial equation (Equation (1)):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon \quad (1)$$

where β_0 is the intercept, β_1, and β_2 are the first-order model coefficients, β_{12} is the ratio between products, β_{11} and β_{22} are the second-order model coefficients, x_1 is the temperature variable, x_2 is the orange waste mass variable, and ε is the term representing the residual error of the model. A Student's *t*-test was performed to investigate the statistical significance of the regression coefficients. The regression coefficients used to express the fit quality of the mathematical models obtained and their statistical significance levels were determined through the *F* test (Fisher's test). The statistical software Statistica v.10.0 (StatSoft, Tulsa, OK, USA) was used to analyze all the experimental data using the residual error.

2.5. Immobilization on Ionic Adsorption Resins

A standard immobilization assay was carried out with the supports DEAE-cellulose, MANAE-agarose, PEI-agarose, DEAE-Sephacel, CM-cellulose, MANAE-cellulose, and DEAE-agarose according to [45]. The supports were washed with 10 mmol L^{-1} Tris -HCl (pH 7.0) buffer solution. Then, the crude enzymatic extract was added to the support in a 1:10 ratio and agitated by stirring rolls at 4 °C. Laccase assays were performed after 30-, 60-, and 120-min immobilization to evaluate the best support for adsorption. Immobilization yield (Y) (Equation (2)), immobilization efficiency (E_f) (Equation (3), and recovered activity (R_A) (Equation (4)) were calculated.

$$Y(\%) = \frac{U_A - U_R}{U_A} * 100 \quad (2)$$

$$E_f(\%) = \frac{U_H}{U_A - U_R} * 100 \quad (3)$$

$$R_A(\%) = \frac{U_H}{U_A} * 100 \quad (4)$$

where Y is the immobilization yield in %, U_A is the total number of units (U) offered for immobilization, U_R is the residual number of units (U) in solution after the immobilization procedure, U_H is the total number of immobilized units (U), E_f is the immobilization efficiency in %, and R_A is the recovered activity in % [46,47].

2.6. Reuse of Immobilized Enzymes

The reusability of the immobilized crude extract in the best-evaluated support was investigated. After the immobilization procedure described above, the immobilized crude extract was incubated in Tris/HCl buffer (10 mmol L^{-1}, pH 7.0) and at its optimal temperature (60 °C). Activity assays were performed sequentially after a 15 min incubation using the ABTS protocol described above. The results were expressed as relative activity compared to the original activity obtained before the first wash. The washes were carried out with the immobilization buffer (Tris/HCl 10 mmol L^{-1}, pH 7.0) followed by centrifugation (3500× g, 4 °C, 3 min). The supernatant was withdrawn, and the pellets were suspended in the immobilization buffer [18].

2.7. Biochemical Characterization of Free and Immobilized Enzymes

The optimum temperatures of free (CE) and immobilized laccases (ICE) were determined using ABTS (0.03% w/v in 50 mmol L^{-1} sodium acetate buffer, pH 5.0) as the substrate at temperatures ranging from 30 to 80 °C. The thermostability of laccase was investigated by measuring residual activity at the previously determined optimum temperature (70 °C for the CE and 60 °C for the ICE) after incubation of the free and immobilized enzymes in the absence of a substrate after different time intervals.

Similarly, optimum pH values were investigated for the free and immobilized laccases using ABTS (0.03% w/v) as the substrate and the McIlvaine buffer [48], with pH values from 2.0 to 7.0. Assays were performed at the previously determined optimum temperature (70 °C for the CE and 60 °C for the ICE), and the results were expressed as U mL^{-1}. The stability of the free and immobilized laccases to pH was evaluated by measuring the post-incubation residual activity, in the absence of a substrate, of the enzymes in their optimum pH (3.0) conditions. These assays were incubated in ice baths to reduce thermal inactivation as much as possible. The sequential enzymatic assays were performed at different periods with the ABTS assay.

2.8. Saccharification of Sugarcane Bagasse

The saccharification experiment was conducted with a final volume of 1 mL and 3% (w/v) of steam-exploded sugarcane bagasse. In each experiment, 0.1 mL of sodium acetate buffer (final concentration of 50 mmol L^{-1}, pH 5.0) was added to 0.9 mL of enzymatic extract and incubated at 50 °C [38]. A negative control was made by incubating the sugarcane bagasse with 1 mL of sodium acetate buffer (50 mmol L^{-1}, pH 5.0). After 24 and 48 h of hydrolysis, aliquots were removed and centrifuged (10 min, 10,000× g, 4 °C). The colorimetric technique determined the reducing sugars released [43]. Results were expressed as µmol of sugar mL^{-1}.

2.9. Synthetic Dye Decolorization

For the assays, three concentrations (0.005%, 0.01%, 0.05% w/v) were used for each dye (Ponceau S, Victoria Blue B, bromocresol green, bromophenol blue, Methyl Red, Methylene Blue, and Gentiant Violet B). An assay mixture containing 2 mL of dye solution prepared with sodium acetate buffer (0.05 mol L^{-1}, pH 5.0) and 2 mL of crude extract diluted to include a final activity of 3 U was incubated in the dark at 28 °C [49]. All three concentrations of dyes were tested. After 24 h, the decrease in absorbance was measured at the maximum absorbance wavelength of each dye in a UV-VIS Shimadzu Spectrophotometer

(Kyoto, Japan). A boiled laccase (98 °C, 10 min) solution was utilized as a blank for each dye concentration. The decolorization was calculated in percentage (Equation (5)).

$$Decolorization(\%) = \frac{A_F - A_0}{A_0} \quad (5)$$

where A_0 is the dye's initial absorbance at the maximum absorbing wavelength, and A_F is the final absorbance at the maximum absorbing wavelength.

2.10. Statistical Analysis

All analyses were performed in triplicate when applicable. The data were expressed as a means ± standard deviation. Differences between means at the 5% ($p < 0.05$) level were considered significant. For the CCD, one-way ANOVA was performed with the coefficients obtained at a 5% significance level. For the hydrolysis, results for 24 h were compared with those for 48 h using two-way ANOVA followed by the Bonferroni post hoc test. Statistical significance was determined by $p < 0.05$.

3. Results and Discussion

3.1. Enzymatic Screening

The results of enzymatic assays for holocellulases and laccase are shown in Figure 1. Enzymes assayed with natural substrates presented lower activity if compared to the activities on synthetic substrates. The enzymatic levels detected in the crude extract were endoglucanase (0.026 ± 0.004 U mL^{-1}), mannanase (0.122 ± 0.009 U mL^{-1}), β-glucanase (0.235 ± 0.022 U mL^{-1}), endo-1,4-β-xylanase (0.201 ± 0.016 U mL^{-1}), endo-1,5-α-arabinanase (0.171 ± 0.019 U mL^{-1}), and xyloglucanase (0.032 ± 0.013 U mL^{-1}). One exception in this scenario happened with polygalacturonase, which had an activity of 0.410 ± 0.001 U mL^{-1}. On the other hand, most enzymes assayed with synthetic substrates presented higher activities. β-D-galactosidase and β-D-glucosidase presented 0.431 ± 0.006 and 0.556 ± 0.022 U mL^{-1} of activity, respectively. Other enzymes, like cellobiohydrolase (0.072 ± 0.016 U mL^{-1}) and β-D-xylosidase (0.058 ± 0.006 U mL^{-1}), had lower activities. These values are compatible with other studies that found low cellulase activity and hemicellulase production by white-rot fungi [50,51].

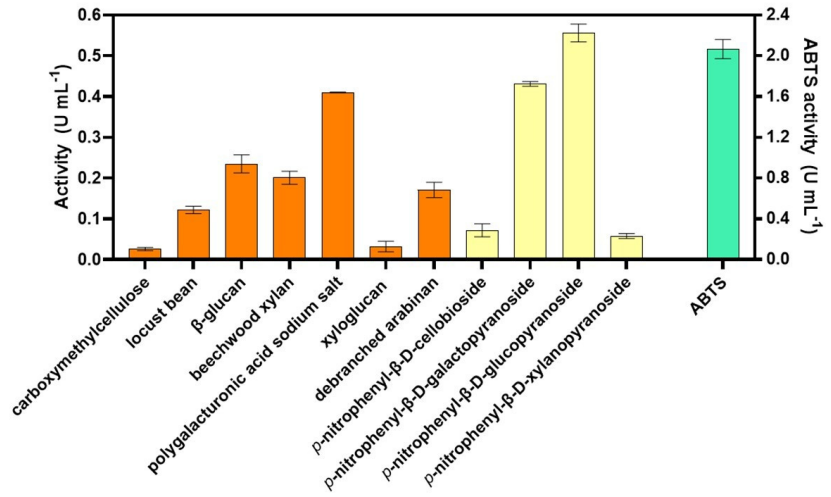

Figure 1. Enzyme activities (U mL^{-1}) for the tested substrates. Orange bars indicate activities assayed with natural substrates. Yellow bars indicate activities assayed with synthetic substrates. The green bar indicates activity assayed with ABTS (data plotted on the right Y-axis). Error bars indicate mean ± standard deviation.

As expected for a white-rot, basidiomycete fungus, laccase, assayed with a synthetic substrate (ABTS), was the enzyme with higher activity in the crude extract, with an activity

of 2.066 ± 0.094 U mL^{-1}. High laccase production by white-rot fungi has already been reported in the literature, and thus, these fungi are often investigated as efficient laccase producers [52–54]. Therefore, we chose to continue our investigation with this enzyme.

3.2. Optimization of Culture Conditions for Laccase Production

First, a complete CCD, with four axial points and three central points, was proposed to optimize the *T. hirsuta* culture conditions regarding the amount of orange waste (g) and temperature (°C). Both variables were level-coded as −1.41, −1, 0, +1, and +1.41. Then, 11 independent cultures were performed in different conditions. The temperature (°C), orange waste (g), and laccase activity (dependent variable, U mL^{-1}) were used in the CCD.

The scientific literature presents orange waste media as an inducer of laccase production by filamentous fungi [41,49]. A Pareto diagram of effects and a contour surface graph were plotted. The contour plot demonstrated the highest enzymatic activity at the 0-level for temperature (28 °C) and 1.41-level for orange waste (4.2 g) (Figure 2a), and the quadratic and linear temperature effects were statistically significant (Figure 2b). The culture in the optimized orange waste medium resulted in 11.625 U mL^{-1} of laccase activity. This represents an increase of 5.6-fold when compared to the previously obtained laccase activity (2.066 U mL^{-1}).

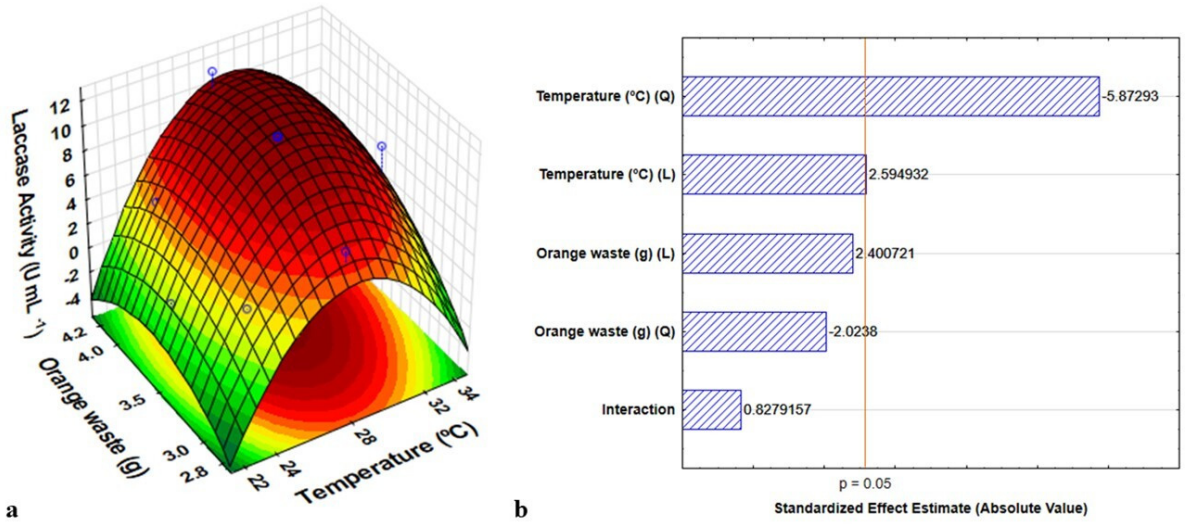

Figure 2. Contour plot (**a**) and Pareto chart of effects (**b**) regarding the influence of carbon source (g) and temperature (°C) in the dependent variable laccase activity (U mL^{-1}).

The statistically significant coefficients ($p < 0.05$; $R^2 = 0.91$) were verified by one-way ANOVA (Table 2) and were further incorporated in the polynomial equation obtained for *T. hirsuta* (Equation (6)).

$$EnzymaticActivity\left(UmL^{-1}\right) = 11.223 - 4.002\left(temperature^2\right) + 1.482\ (temperature) \quad (6)$$

Table 2. ANOVA test for the model obtained after culture optimization.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F_{calc}	F_{tab}
Model	717.212	10	71.712	15.56	3.35
Residual	36.871	8	4.609		
Lack of fit	34.147	6	5.691	4.18	19.33
Error	2.723	2	1.361		
Total	754.082	10			

$R^2 = 0.91$; $p < 0.05$.

The response surface methodology allowed for the evaluation of various temperatures and orange waste values. Temperature seems to be a very important factor. Runs 5, 6, 9,

10, and 11 all had 3.5 g of orange waste at different temperatures. The enzymatic activity detected for the run performed at 28 °C (run 10) is 10.36-fold higher than the one detected for the run performed at 22 °C (run 5). On the other hand, more significant temperatures had an antagonistic effect on the laccase activity. The run performed at 34 °C (run 6) presented an enzymatic activity 1.5-fold lower than the one detected in run 10, at 28 °C. Furthermore, carbon sources also seem to be an influential factor. The most considerable laccase activity was detected for run 8, performed at 28 °C and 4.2 g of orange waste.

3.3. Ionic Immobilization

Ionic adsorption is an immobilization strategy subject to the action of carrier enzymes. It presents an easy operation and a more diversified possible support option [55,56]. To determine the best support for ionic immobilization among DEAE-agarose, DEAE-cellulose, DEAE-Sephacel, CM-cellulose, MANAE-agarose, MANAE-cellulose, and PEI-agarose, multiple parameters regarding laccase activity were measured after immobilization periods for each tested support (Table 3).

Table 3. Immobilization parameters were calculated for each support and time.

Ionic Supports	Time (min)								
	30			60			120		
	Iy	***Ef***	***Ra***	***Iy***	***Ef***	***Ra***	***Iy***	***Ef***	***Ra***
DEAE-cellulose	9.0 ± 0.7	12.9 ± 0.4	6.5 ± 0.3	7.9 ± 0.6	10.2 ± 0.1	5.6 ± 0.4	9.1 ± 0.7	9.9 ± 0.6	6.5 ± 0.2
MANAE-agarose	20.6 ± 0.2	32.5 ± 1.1	18.0 ± 0.7	20.9 ± 0.3	29.8 ± 0.2	18.1 ± 0.3	21.0 ± 0.1	27.3 ± 0.2	17.9 ± 0.1
PEI-agarose	6.2 ± 0.3	19.3 ± 0.1	5.8 ± 0.3	4.9 ± 0.5	19.6 ± 0.2	4.5 ± 0.3	4.2 ± 0.7	19.0 ± 0.2	3.8 ± 0.3
DEAE-Sephacel	12.0 ± 0.4	23.2 ± 0.7	7.6 ± 0.3	9.4 ± 0.1	20.8 ± 0.5	6.6 ± 0.4	10.0 ± 0.3	17.6 ± 0.1	7.1 ± 0.4
CM-cellulose	**93.1 ± 3.4**	**62.7 ± 0.8**	**91.9 ± 1.1**	**65.7 ± 1.3**	**61.9 ± 0.6**	**62.5 ± 0.8**	**58.4 ± 0.2**	**58.7 ± 0.5**	**56.0 ± 0.9**
MANAE-cellulose	13.7 ± 0.4	15.8 ± 0.2	6.3 ± 0.4	9.7 ± 0.1	14.3 ± 0.1	4.1 ± 0.1	10.4 ± 0.1	14.5 ± 0.7	4.3 ± 0.4
DEAE-agarose	19.5 ± 0.1	**55.3 ± 0.4**	15.5 ± 0.1	20.1 ± 0.6	53.1 ± 0.7	15.5 ± 0.6	14.7 ± 2.1	**52.8 ± 1.9**	10.4 ± 0.8

Iy: Immobilization yield (%); ***Ef***: immobilization efficiency (%); ***Ra***: recovered activity (%).

The best immobilization parameters were obtained from CM-cellulose after 30 min. The immobilization yield was 93.1%, the immobilization efficiency was 62.7%, and the recovered activity was 91.9%. Other works have reported efficient laccase immobilization in MANAE-agarose [17]. However, the recovered activities were lower than the one obtained for CM-cellulose. Therefore, this ionic resin was selected for the following experiments with an immobilization time of 30 min. CM-cellulose is covered in carboxyl groups (COO^-), which can bind to positively charged amino acid residues belonging to the protein's external surface, arginine, and/or lysine. Other supports like DEAE-agarose have positively charged amine groups ($-NH^+$) [57], while PEI-agarose also contains amine groups but with a more complex polymeric structure [58]. These supports interact with negatively charged residues on the laccase, such as aspartate and glutamate. However, the interaction strength may vary depending on the charge densities and the polymer chain flexibilities [59]. For example, they might form weaker or less stable interactions with the enzyme than CM-cellulose, leading to lower immobilization efficiency and recovered activity.

Ionic bonding between an enzyme and a support is a simple, effective, and process-favorable immobilization process because of its lower costs, easy enzyme desorption when needed, and vast possibilities of available ionic supports [60].

A high recovered activity is perhaps the most critical parameter because it evaluates how much of the original offered enzymatic activity is still available after the immobilization procedure. It is also important to note that other molecules and/or proteins are subsequently immobilized into the supports of the crude extract utilized for immobilization.

The immobilized crude extract also presented high reusability, a crucial parameter for the practical and/or commercial application of laccases [61]. After 20 cycles of washing and measurement, the immobilized *T. hirsuta* crude extract retained 68.0% of residual laccase activity (Figure 3), characterizing the crude extract/CM-cellulose pair as an essential and relevant biocatalyst.

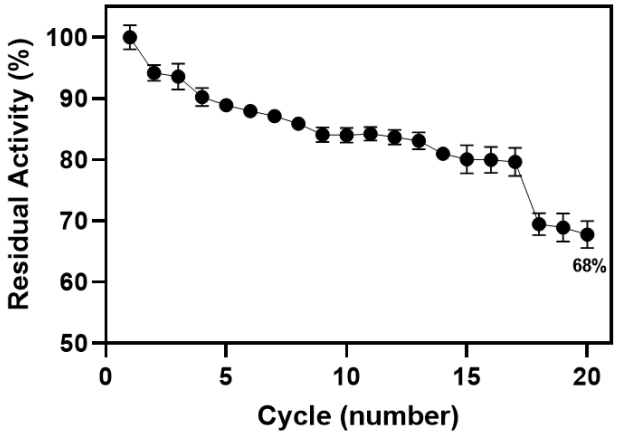

Figure 3. Reusability of the CMC-immobilized crude enzymatic extract after 20 cycles.

This is especially interesting because the interaction between laccase and CM-cellulose is a type of ionic adsorption and, therefore, weaker than a covalent bond. The immobilized crude extract might be used for up to 15 reaction cycles while retaining more than 80% of its original activity. When the activity is lost, high salt concentrations might be used to wash the immobilization derivative so that the support might be renewed and ready for a new immobilization procedure.

3.4. Influence of Temperature on Laccase Activity

The optimum temperature was 70 °C for the CE and 60 °C for ICE despite also showing high laccase activity at 70 °C and 80 °C (Figure 4a). The shift in optimum temperature is probably due to the co-immobilization of components other than the laccase itself and/or structural modification in the tertiary protein structure of the enzyme [62,63].

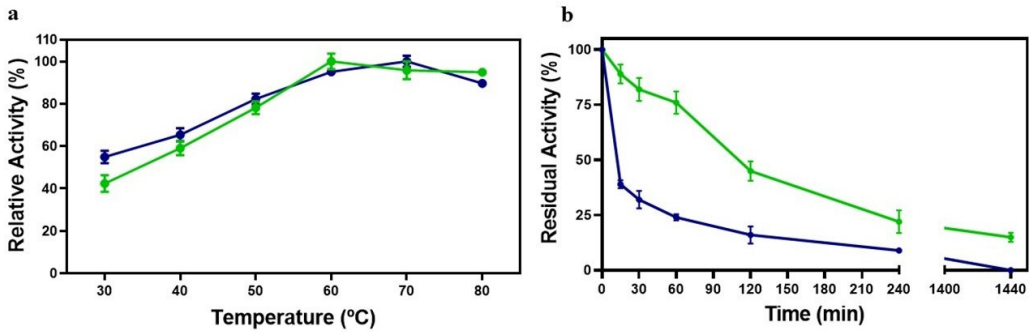

Figure 4. Optimum temperature (**a**) and thermostability (**b**) for the *T. hirsuta* enzymatic extracts containing laccase. (●) Crude extract laccase; (●) immobilized crude extract laccase. Thermostability was evaluated at the optimum temperature: 70 °C for the crude extract (CE) and 60 °C for immobilized crude extract (ICE).

The enzymatic thermostability aimed at industrial applications is a crucial parameter to be studied because the immobilization process generally increases the enzymatic stability. The enzyme thermostability was measured at their optimum temperatures: 70 °C for the CE and 60 °C for the ICE. The ICE presented the highest thermostability, retaining 45% of activity after 120 min incubation, while the CE retained 16% of activity after the same time. After 240 min, the CE lost 91% of its activity, while the ICE lost 78.4% (Figure 4b). This happened due to the thermal inactivation of the free enzyme molecules [64]. Immobilization on CM-cellulose successfully managed to provide higher thermostability for *T. hirsuta* laccase.

3.5. Influence of pH on Laccase Activity

Another crucial parameter for selecting and applying an enzyme in any bioprocess is the pH of activity. Fungal laccases generally have an optimal activity pH in acid values and lose much of their oxidative potential in pH values greater than 5.8 [65]. Different assays were performed at the previously determined optimum temperature (70 °C for the CE and 60 °C for the ICE) and various pH values for free and immobilized laccases. The optimum pH obtained for the CE and ICE was 3.0 (Figure 5). The values agree with other *T. hirsuta* laccases in the literature [53] (Figure 5a).

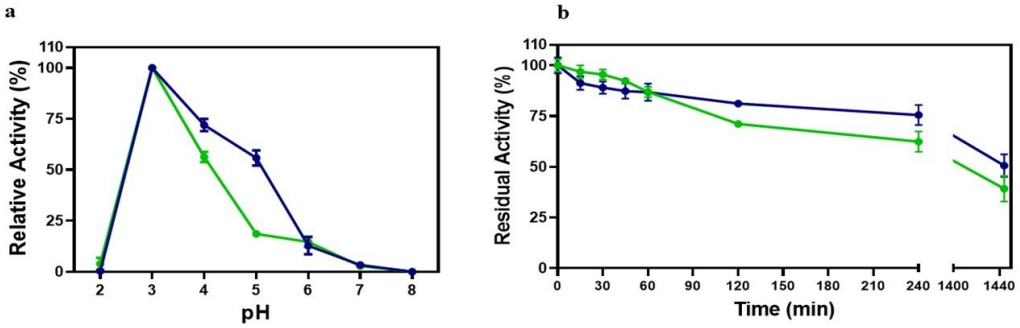

Figure 5. Optimum pH (**a**) and pH stability (**b**) for the *T. hirsuta* enzymatic extracts containing laccase. The stability tests were performed at 4 °C. (●) Crude extract laccase; (●) immobilized crude extract laccase.

Considering short incubation times (up to 45 min of the assay), the CE retained 87.3% of its original laccase activity, while the ICE retained 92.3%. After 60 min of incubation at the optimum pH (pH 3.0), both the CE and ICE demonstrated high levels of laccase activity. The crude extract retained a significant portion of its original activity, indicating good stability in its free form for short-term applications. The ICE also maintained the activity. This result suggests that the enzyme is currently well preserved in both forms. By 120 and 240 min, a decline in activity was observed in ICE. In contrast, the CE showed increased stability, retaining a higher percentage of its activity. At 1440 min, the CE retained 50% of its original activity, while the ICE retained 40% (Figure 5b). Therefore, the immobilization process in CM-cellulose did not show significant differences concerning the influence of the laccase pH of *T. hirsuta*.

3.6. Sugarcane Bagasse Hydrolysis

The CE was used to hydrolyze sugarcane bagasse for 24 and 48 h. After 24 h and 48 h, 7.06 ± 0.05 μmol mL^{-1} and 13.83 ± 0.02 μmol mL^{-1} of reducing sugars were released, respectively (Figure 6). A similar result was observed with immobilized laccase. This indicates that the immobilization process of *T. hirsuta* laccase for application in sugarcane bagasse hydrolysis is unnecessary due to the similar results obtained from applying crude extract directly. However, the immobilization of laccase is more stable and could offer a positive outcome in saccharification with more extensive time. Further studies would be interesting for better biochemical knowledge of the protein structure and the modeling of laccases since several were identified in the genome of fungi from the *Trametes* genus. In-depth biochemical studies, including techniques such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and molecular dynamics simulations, could reveal fundamental details about the structural conformation of laccase, the binding sites of substrates and cofactors, and the electron transfer pathways [66]. Not all are studied in this context. Additionally, it is possible to suggest that the complementation of crude extracts with enzymes or enzymatic extracts from other non-basidiomycete fungi would be an exciting strategy, and the use of enzymatic extracts from *T. hirsuta* could also be an attractive strategy to improve lignocellulosic biomass hydrolysis, revealing its potential for application in the biofuels industry, especially in the production of bioethanol

from the breakdown of plant biomass into fermentable sugars, since multiple cellulases, hemicellulases, and ligninases acting together might reach better results [67–69].

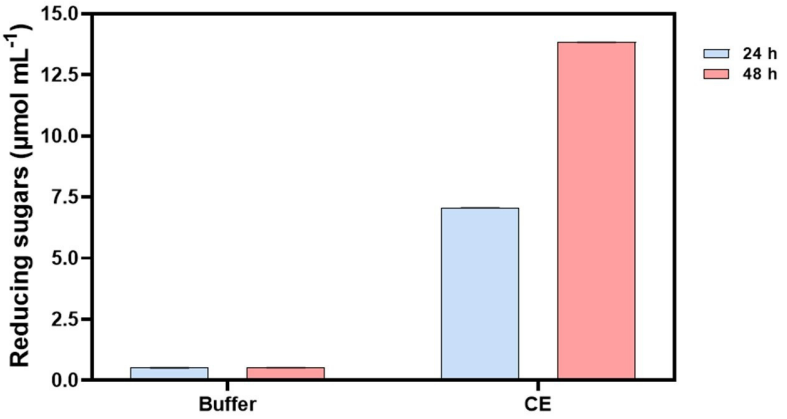

Figure 6. Sugarcane bagasse hydrolysis after 24 and 48 h with *T. hirsuta* crude extract. **Buffer.** Negative control; **CE.** *T. hirsuta* crude extract.

3.7. Decolorization of Various Synthetic Dyes

Different dyes for laboratory and industrial uses were selected for the trials. The diluted crude extract of *T. hirsuta* containing 3 U of laccase was incubated for 24 h at 28 °C with various dyes (0.005–0.05%) in sodium acetate buffer (0.05 mol L^{-1}, pH 5.0). A temperature of 28 °C was selected for the decolorization assay to mimic the temperature found in sewage, where the industrial effluents are deposited [70]. By conducting the assay at 28 °C, we aimed to replicate the real-world conditions under which the laccases from white-rot fungi would operate in a wastewater treatment context [71,72].

In the highest concentration tested (0.05% w/v), bromocresol green (96.6%), bromophenol blue (71.9%), and orcein (70.8%) presented greater decolorization. For the intermediate concentration (0.01% w/v), the dyes that suffered the greatest decolorization were bromocresol green (89.7%), Trypan blue (83.4%), Congo red (73.2), and Victoria Blue B (69.3%). Finally, at the lowest concentration (0.005% w/v), the laccase of *T. hirsuta* caused more decolorization in the dyes bromocresol green (84.6%), Trypan blue (78.5%), and Congo red (72.3%). Other dyes also showed good decolorization, such as Gentian violet at 0.005% (72.1%). Bromocresol green is a dye of the triphenylmethanes group and is used in the textile industry and laboratory tests as a pH indicator. The decolorization obtained in this study for this dye was superior to others in the literature [73,74]. The other dyes selected are potential contaminants since they are commonly used in research laboratories and industries. All decolorizations obtained for all dyes at all concentrations are summarized in Table 4.

Table 4. Decolorization (%) achieved after incubation with 3 U of *T. hirsuta* CE.

Dye	λ_{max} (nm)	0.05% (w/v)	0.01% (w/v)	0.005% (w/v)
Bromophenol blue	610	**71.9 ± 2.4**	65.7 ± 3.4	33.9 ± 7.1
Methylene Blue	665	2.9 ± 1.0	6.3 ± 4.8	52.4 ± 9.8
Trypan blue	580	35.9 ± 8.6	**83.4 ± 0.3**	**78.5 ± 5.2**
Orcein	579	**70.8 ± 2.1**	36.9 ± 9.2	49.0 ± 2.8
Ponceau S	515	0	46.6 ± 0.2	48.2 ± 1.9
Bromocresol green	640	**96.6 ± 2.7**	**89.7 ± 1.2**	**84.6 ± 1.8**
Methyl Red	520	43.6 ± 0.8	34.6 ± 2.0	2.0 ± 0.9
Congo red	498	14.8 ± 5.9	**73.2 ± 0.6**	**72.3 ± 2.1**
Victoria Blue B	615	0	**69.3 ± 0.7**	62.2 ± 3.9
Gentian violet	587	0	59 ± 2.1	**72.1 ± 3.2**

Assays were performed at pH 5.0 and 28 °C. λ_{max}: wavelength corresponding to maximum absorption.

The efficacy of laccase in degrading these dyes can be attributed to its ability to interact with the dye's aromatic rings and substituents, which affect the electron density and stability of the aromatic system. Dyes with electron-donating groups (such as hydroxyl or methoxy groups) generally have higher electron density on the aromatic rings, making them more susceptible to laccase-catalyzed oxidation [75]. This likely explains why bromocresol green, which has phenolic and sulfonic groups [76], showed the highest level of decolorization.

Bromocresol green and bromophenol blue are triphenylmethane dyes, where the aromatic rings are stabilized by electron-withdrawing bromine atoms and electron-donating hydroxyl groups. Laccase attacks these aromatic structures, initiating radical formation and subsequent breakdown of the dye's chromophore, leading to decolorization [77].

The enzyme also showed a high decolorization percentage for orcein, which contains anthraquinone chromophores. The quinone structure in anthraquinone dyes is particularly susceptible to oxidation by laccase because the enzyme can effectively oxidize the hydroquinone form to quinone, disrupting the conjugated system that imparts color to the dye [78].

Azo dyes like Congo red and Trypan blue, characterized by one or more azo bonds (-N=N-) linking aromatic rings, showed moderate decolorization. The azo bond is relatively stable; however, it can be cleaved by laccase, especially in the presence of mediators that facilitate electron transfer [79].

Gentian violet showed lower decolorization rates at higher concentrations (59% at 0.01% and 72.1% at 0.005%). This dye has a triphenylmethane structure which is more oxidation-resistant due to the steric hindrance provided by its three aromatic rings. However, at lower concentrations, the enzyme can effectively access and oxidize the dye molecules [80].

Therefore, dyes with structures that facilitate electron transfer or stabilize radical intermediates are more readily decolorized by laccase. Conversely, more complex or sterically hindered dye structures may require additional mediators or specific conditions to achieve higher decolorization rates [81].

The decolorization results for synthetic dyes can be worse at lower concentrations due to several factors, as seen by Contato et al. [49]. Laccases and other enzymes rely on the presence of substrate molecules (in this case, dye molecules) to catalyze reactions. At lower dye concentrations, the number of available substrate molecules decreases, which can reduce the frequency of enzyme-substrate interactions, leading to slower or less efficient decolorization. Additionally, some enzymes may have a lower binding affinity for the substrate at low concentrations. This can reduce the rate at which the enzyme binds to and processes the dye molecules, further decreasing the overall decolorization effectiveness.

4. Conclusions

This study demonstrated the promising potential of the laccase produced by the fungus *T. hirsuta* GMA-01 for various biotechnological applications. The enzyme proved highly effective in decolorizing synthetic dyes, such as bromocresol green, bromophenol blue, and orcein, highlighting its ability to degrade environmental pollutants, which is valuable for bioremediation processes. Additionally, the laccase showed good performance in the hydrolysis of sugarcane bagasse, indicating its potential use in biofuel production, especially within a circular economy and waste valorization context. Furthermore, the immobilization of laccase on ionic supports, such as CM-cellulose, significantly increased its thermal stability and reusability, proving to be a viable approach for industrial applications requiring reusable enzymes. The production of laccase using orange waste as a carbon source further emphasizes the sustainable and cost-effective nature of the process, aligning with green biotechnology practices.

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and J.C.S.S.; writing—original draft preparation, G.G.O.; writing—review and editing, A.G.C., R.C.A., J.C.S.S. and M.d.L.T.d.M.P.; supervision, A.G.C. and M.d.L.T.d.M.P.; project administration, M.d.L.T.d.M.P.; funding acquisition, M.d.L.T.d.M.P. All authors have read and agreed to the published version of the manuscript.

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