Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Integrated bioprocess development for laccase production by an endophytic Trametes villosa isolated from cocoa agro-industrial waste

Sophia Alves Luzeiro
Centro Universitário FEI
João Victor Younes Oliveira
Centro Universitário FEI
Gustavo Vieira Olivieri
Centro Universitário FEI
Antonio d'Angelo
University of Naples, University of Naples Federico II
Rosa Turco
University of Naples, University of Naples Federico II
Riccardo Tesser
University of Naples, University of Naples Federico II
Bruna Pratto
Centro Universitário FEI
Andreia de Araújo Morandim-Giannetti
andreia.morandim@gmail.com
Centro Universitário FEI

Research Article

Keywords: Cocoa agro-industrial waste. Endophytic fungi. Laccase. Statistical optimization. Biocatalysis. Dye degradation

Posted Date: August 13th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10607749/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Integrated bioprocess development for laccase production by an endophytic *Trametes villosa* isolated from cocoa agro-industrial waste

Sophia Alves Luzeiro[1], João Victor Younes de Oliveira[1], Gustavo Vieira Olivieri[1], Antonio d'Angelo[2], Rosa Turco[2], Riccardo Tesser[2], Bruna Pratto[1], Andreia de Araújo Morandim-Giannetti[1*]

[1] Department of Chemical Engineering, Centro Universitário FEI, São Bernardo do Campo, São Paulo, Brazil.

[2] University of Naples Department of Chemical Sciences, University of Naples Federico II, Via Cinthia 4, 80126 Naples, Italy

Corresponding Author

*E-mail address: andreia.morandim@gmail.com (A. de A. Morandim-Giannetti)

Phone: +55-011-43532900. Fax: +55-01141095994.

Abstract

Cocoa agro-industrial waste was explored as a source of endophytic fungi for laccase production, resulting in the isolation of 25 microorganisms. Among them, the CC4 (*Trametes villosa*) strain showed the highest production capacity. Statistical optimization demonstrated that laccase production was dependent on cultivation conditions, with maximum specific activity achieved under slightly acidic conditions and moderate temperatures. The production kinetics showed a sigmoidal profile, reaching a maximum specific activity of 171.48 μmol/mg min after five days of cultivation, and was accurately described by the logistic model (R^2 = 0.9871). The adjusted kinetic model successfully represented the laccase-catalyzed oxidation of syringaldazine. The laccase-enriched extract was subsequently immobilized on chitosan to generate a stable biocatalyst for dye degradation, achieving complete decolorization of Remazol Brilliant Blue R and 99.9% decolorization of Bromophenol Blue, while maintaining high catalytic performance during successive reuse cycles. The integration of microbial bioprospecting, process optimization, kinetic modeling, enzyme immobilization, and environmental application demonstrates the potential of cocoa agro-industrial waste as a sustainable source of industrial biocatalysts. This study expands the biotechnological value of cocoa waste and provides an integrated strategy for developing enzymatic processes within the circular bioeconomy and sustainable bioprocess engineering frameworks.

Keywords: Cocoa agro-industrial waste. Endophytic fungi. Laccase. Statistical optimization. Biocatalysis. Dye degradation.

Introduction

Cocoa (*Theobroma cacao* L.) is one of the most economically important tropical crops worldwide, cultivated in Central and South America, West Africa, and Southeast Asia. Global cocoa production has reached approximately 4.7 million tons annually, driven by the growing demand for chocolate and cocoa-derived products [1Alotaibi et al., 2024; Dutra et al., 2023; Tosto et al., 2023]. In Brazil, particularly in the state of Bahia, cocoa production is an important economic activity, generating annual revenues of approximately USD 267 million from the sale of both raw beans and processed products. However, cocoa processing generates substantial agro-industrial waste, as only the beans are used for chocolate production, with the remaining fruit components discarded. It is estimated that approximately 70–80% of the cocoa fruit mass becomes residual biomass during harvesting and processing (3.7 million tons, based only on cocoa pod husk waste) [Barrios-Rodríguez et al., 2022].

From a compositional perspective, this biomass is rich in lignocellulosic and bioactive compounds, typically containing significant amounts of cellulose (19–26%), hemicellulose (8–13%), lignin (14–28%), pectin (6–12%), as well as proteins, minerals, and phenolic compounds [Barrios-Rodríguez et al., 2022; Anoraga et al., 2024; Campos-Vega et al., 2018]. These characteristics highlight cocoa waste as a promising renewable feedstock for biotechnological processes. Consequently, the valorization of cocoa agro-industrial waste has attracted increasing attention as a strategy to reduce environmental burdens while generating high-value bioproducts within the framework of circular bioeconomy and sustainable bioprocess development [Tosto et al., 2023; Afedzi et al., 2023; Alves-Júnior et al., 2021].

Endophytic microorganisms associated with plant tissues have been recognized as promising sources of biotechnologically relevant enzymes. These symbiotic organisms inhabit internal plant tissues without causing apparent harm, contributing to host growth and defense against pathogens [Waqar et al., 2023]. Among them, endophytic fungi have garnered particular

attention for their ability to synthesize bioactive compounds and to produce a broad spectrum of enzymes, including oxidoreductases, lipases, and peptidases. Most endophytic fungi isolated so far belong to the *Ascomycota phylum*, especially the *Sordariomycetes*, *Dothideomycetes*, and *Eurotiomycetes* classes, followed by Basidiomycota (*Agaricomycetes*) and, to a lesser extent, *Mucoromycota* and *Oomycota* [Vidhyashini et al., 2026; Park & Park, 2014; Rajesh & Rai, 2013].

Among fungal oxidoreductases, laccases (EC 1.10.3.2) have emerged as versatile multicopper oxidases capable of oxidizing a wide range of phenolic and non-phenolic substrates while reducing molecular oxygen to water [García-Fuentevilla et al., 2025; Kyomuhimbo et al., 2023; El-Batal et al., 2015]. These enzymes exhibit remarkable catalytic efficiency and thermostability and are widely distributed in fungi, bacteria, plants, and even insects. Their applications span several industrial and environmental processes, including the oxidation of phenolic and aromatic amines, the degradation of dyes, and the removal of heavy metals and cyanide compounds [El-Batal et al., 2015; Le et al., 2025; Sopalun & Iamtham, 2020]. Laccases act as four-electron oxidants, facilitating the breakdown of recalcitrant pollutants into less toxic or more biodegradable forms, thus playing a key role in green biocatalysis [Dutra et al., 2023; Su et al., 2024; Vacalie et al., 2024]. Their immobilized forms, particularly on natural polymers such as chitosan and alginate, provide improved operational stability, reusability, and easier recovery, making them ideal candidates for large-scale wastewater treatment [Kyomuhimbo et al., 2023; Su et al., 2024].

Despite the increasing number of studies on laccase-mediated dye degradation, few have focused on isolating new fungal producers from underexplored substrates such as cocoa residues. A literature survey of the Scopus database revealed that only a limited number of studies have reported the isolation of endophytic fungi from cocoa (*Theobroma cacao*) tissues (11 articles in total). Among these, only one identified laccase-producing strain, namely

Trichothecium sp., was reported [Sartini et al., 2013]. Other studies have explored enzymatic production from *Pestalotiopsis mangiferae* [Lima et al., 2022] and microbial tolerance to heavy metals [Cordoba-Novoa et al., 2023], confirming the potential of cocoa-associated endophytes as sources of industrially valuable enzymes.

The valorization of agro-industrial waste has become an important strategy within circular bioeconomy frameworks, aiming to convert low-value biomass into high-value bioproducts. Cocoa processing generates large amounts of lignocellulosic waste that remain largely underexploited despite their potential as feedstocks for integrated biorefinery systems. In this context, the present study reports, for the first time, the isolation of an endophytic *Trametes villosa* strain from cocoa agro-industrial waste that produces laccase. Beyond identifying a novel fungal source, this work advances the valorization of agricultural residues by proposing an integrated bioprocess framework that combines microbial bioprospecting, statistical optimization of enzyme production, kinetic modeling of laccase biosynthesis, mathematical modeling of the enzyme-catalyzed reaction, enzyme immobilization, and application in dye decolorization. Unlike previous studies that have primarily addressed individual aspects of laccase production or application, the proposed approach establishes a process-oriented link between microbial resource exploration and environmental biotechnology, demonstrating how low-value agro-industrial waste can be transformed into a source of high-value biocatalysts. This integrated strategy contributes to the development of sustainable enzymatic platforms aligned with circular bioeconomy and bioprocess engineering principles.

Materials and Methods

Materials

Endophytic fungi obtained from cocoa waste were isolated and preserved at Centro Universitário FEI. The following reagents were used in the experiments: potato dextrose agar (PDA), syringaldazine(98%), malt extract, glucose (99.5%), peptone, serum ovalbumin (98.0%), ammonium sulfate (99.0%), Tween 80 (≥58.0%), polyvinylpolypyrrolidone (PVPP) (99.0%), Coomassie blue (≥80.0%), gentamicin sulfate (<= 100 %), copper sulfate (98.0%), potassium phosphate monobasic (99.5%), sodium phosphate monobasic (99.0%), magnesium sulfate heptahydrate (98.0%), phosphoric acid (85.0%), sodium hypochlorite (6.0-14.0% active chlorine), and absolute ethanol (99.5%) were obtained from Sigma–Aldrich (USA). Ultrapure water with a resistivity greater than 18 MΩ was produced using a Millipore purification system (USA).

Isolation of Endophytic Fungi

Cocoa waste (fruit peels, stems, and leaves) was initially washed with distilled water and fragmented into small pieces (approximately 0.5 cm) prior to surface sterilization. The fragments were then sequentially immersed in sodium hypochlorite solution (2% w/v), ethanol (70% v/v), and sterile distilled water. After sterilization, the plant fragments were aseptically sectioned and inoculated onto previously autoclaved potato dextrose agar (PDA). Gentamicin sulfate (30 µg/mL) was added to the medium to inhibit bacterial growth. The cultures were incubated at 24 °C in a Panasonic MIR-254 incubator (Panasonic Healthcare, Japan), and fungal growth was monitored until colonies reached a diameter of 1–2 cm (approximately 10 days). Subsequently, each isolate was subcultured to obtain pure fungal strains, which were preserved in sealed 10 mL tubes containing PDA medium and stored at 4 °C.

The fungus with the most significant potential for laccase production was submitted to BPI Biotechnology (Botucatu-SP) and identified by automated capillary electrophoresis sequencing. Total DNA from the sample was extracted, and the ITS rDNA region was amplified using ITS1 and ITS4 primers for identification. PCR products were evaluated on 2% agarose gels stained with Gel Red and visualized under UV light. After this step, the PCRs were purified using Agencourt AMPure XP magnetic beads, and the purified products were quantified by fluorescence using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Sanger sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit, followed by ethanol/EDTA/Sodium Acetate precipitation. Sequencing was performed using an ABI3730xl DNA Analyzer (Applied Biosystems) capillary electrophoresis instrument. After sequencing, the electropherograms were analyzed using Chromas Lite and Geneious 4.8.3 software to obtain the consensus sequence. This sequence was then used to search for similar sequences deposited in NCBI.

Enzymatic Extract Preparation and Evaluation of Laccase Production

The fungal mycelia obtained from pure cultures were rapidly frozen in liquid nitrogen and subsequently macerated with polyvinylpolypyrrolidone (PVPP). The resulting powder was suspended in sodium phosphate buffer (0.2 mol/L, pH 6.0) for enzyme extraction. The crude extracts were then centrifuged using a Hettich Universal 32R refrigerated centrifuge (Germany) at 4,000 rpm for 50 minutes at 4 °C to remove cellular debris. Protein quantification of the supernatants was performed using the Bradford method [Bradford, 1976]. Laccase activity was determined by monitoring the oxidation of syringaldazine (1 g/L) at 525 nm using a UV/VIS spectrophotometer (Kasuaki, Japan). The molar extinction coefficient used for calculations was $\varepsilon = 6.5 \times 10^4$ M cm. All assays were carried out at 23 °C for 10 minutes. The fungal isolate

exhibiting the highest laccase activity was subsequently cultivated in a liquid medium to produce laccase for further experiments. All characterizations were performed in duplicate.

Optimization of Laccase Production

A liquid culture medium was prepared to promote the growth of the fungus that showed the greatest potential for laccase production (CC4). For this purpose, malt extract (1.25% w/v), Tween 80 (0.10% w/v), $CuSO_4$ (0.005% w/v), KH_2PO_4 (1.0 g/L), Na_2HPO_4 (0.26 g/L), $MgSO_4 \cdot 7H_2O$ (0.50 g/L), glucose (10.0 g/L), and peptone (1% w/v) were used. The medium was sterilized by autoclaving for 15 minutes at 1.5 atm and, after cooling, was supplemented with gentamicin sulfate (30 µg/mL) to prevent bacterial contamination. After preparing the medium, the CC4 fungus was fermented in it. The effects of temperature and pH on enzyme production were investigated using liquid cultures grown under different conditions: pH values from 3.3 to 8.8 and temperatures from 20.5 to 37.5 °C. Each fermentation process was carried out for 1 week at 150 rpm. After cultivation, samples were transferred to Falcon tubes containing glass beads and vortexed for two minutes to ensure homogenization. The broths were then centrifuged at 6,000 rpm for 30 minutes at 4 °C, and the resulting supernatants were used to determine laccase activity. The growths were carried out in duplicate.

The influence of the tested parameters was evaluated using a Central Composite Rotatable Design (CCRD) with two central replicates. Temperature and pH were considered independent variables, and specific laccase activity was the response variable. Statistical analyses and contour surface evaluations were performed with Statistica software version 14.0.0.15 (TIBCO Software Inc., USA). Optimal conditions for CC4 growth to increase laccase production were identified using the desirability function. A new culture was maintained under optimal conditions for fungal growth (pH = 5.4 and temperature = 28ºC), and the resulting enzymatic extract was used to study the kinetics of laccase production by the fungus.

Kinetic study – Laccase production

The kinetic study of laccase production by the CC4 fungus was conducted using the Logistic model (Equation 1) [Berkson, 1944] and the Gompertz model (Equation 2) [Gompertz, 1825]. Production was monitored for 9 days, with the pH and temperature used in this stage previously determined during the optimization stage (pH = 5.4 and temperature = 28 °C). All experiments were performed in duplicate, and the parameters were estimated by customizing MATLAB R2024b code using the "lsqnonlin" function and the Levenberg-Marquardt minimization algorithm [Marquardt, 1963; Levenberg,1944].

$$C = \frac{a}{1+b\ exp\ (-kt)} \qquad (1)$$

where C is the specific laccase activity, t is the time, a is the parameter associated with the maximum value (production capacity or upper limit), b is the parameter that affects the initial shape of the curve, and k is the parameter associated with the growth rate.

$$C = a\ exp\ [-b\ exp\ (-kt)\ ] \qquad (2)$$

where C is the specific laccase activity, t is the time, a is the parameter named scale constant, and b and k are parameters associated with the growth rate.

A kinetic study was also conducted on the oxidation of syringaldazine catalyzed by the laccase-enriched extract obtained from the CC4 fungus after 6 days of growth (the ideal period determined by the kinetic study), at 28ºC and pH 5.4. The initial concentrations of syringaldazine (substrate) ranged from 5.5 to 416.25 μmol/L, and the enzyme had an initial activity of 171.475 μmol/mg min. The product concentration (related to catalytic activity) was quantified spectrophotometrically, with aliquots collected every 10 minutes over 300 minutes.

The Michaelis-Menten model [Michaelis & Menten, 1913] was used during this stage. The most diffuse form is reported in equation 3, where v_0 represents the reaction rate, V_{max} is the maximum reaction velocity, K_M is the Michaelis-Menten constant, and $[S]$ is substrate concentration.

$$v_0 = \frac{V_{max}[S]}{K_M + [S]} \quad (3)$$

Instead of applying the equation, this work used a general system of ordinary differential equations (ODEs) based on single-phase isothermal reactor mass balances. Considering the general reaction scheme of an enzyme-catalyzed reaction described by equation 4. The reaction rates for all the reactions are expressed by equations 5-7. The mass balance was described by equations 8-11.

$$E + S \; k_1, \; k_{-1} \leftrightarrow ES \; \; k_2 \rightarrow P + E \quad (4)$$

$$r_1 = k_1[S] \quad (5)$$

$$r_{-1} = k_{-1}[ES] \quad (6)$$

$$r_2 = k_2[ES] \quad (7)$$

$$\frac{d[S]}{dt} = -r_1 + r_{-1} \quad (8)$$

$$\frac{d[E]}{dt} = -r_1 + r_{-1} + r_2 \quad (9)$$

$$\frac{d[ES]}{dt} = +r_1 - r_{-1} - r_2 \quad (10)$$

$$\frac{d[P]}{dt} = r_2 Y \quad (11)$$

The yield (Y) was estimated for each experiment according to equation 12, considering the final product concentration, $[P]_{end}$, and the initial substrate concentration, $[S]_0$.

$$Y = \frac{[P]_{end}}{[S]_0} \quad (12)$$

The reported expressions have been implemented in MATLAB R2024b and solved with the numerical solver "ode15s". Moreover, the model was fitted to the experimental data using the "lsqnonlin" function and the Levenberg-Marquardt minimization algorithm. Finally, the estimated kinetic constants can be used to calculate the K_M and V_{max} parameters according to equations 13 and 14.

$$K_M = \frac{k_{-1} + k_2}{k_1} \quad (13)$$

$$V_{max} = k_2[E_0] \quad (14)$$

Laccase immobilization and application in dye decolorization

After optimizing laccase production conditions using the fungus CC4 (*Trametes villosa*), the resulting laccase-enriched enzyme extract was used both in its free form and immobilized in chitosan to conduct initial tests for treating dye-containing effluents, with the aim of evaluating the extract's efficiency in this process. For this, initially, laccase was immobilized on chitosan activated with glutaraldehyde. To do this, 0.5 g of chitosan was added to 20 mL of a 6 mmol/L glutaraldehyde solution, and the mixture was stirred continuously at 25 °C for 3 h to activate the support by forming reactive aldehyde groups. After the activation step, the chitosan was thoroughly washed with distilled water to remove residual glutaraldehyde. Next, 20 mL of laccase extract from the CC4 fungus was added to the activated support, and immobilization was carried out for 24 h at 5 °C (pH 5.4), thereby forming covalent bonds between the enzyme and the activated chitosan matrix.

After immobilization, the biocatalyst was used to treat effluents containing Remazol Brilliant Blue R or Bromophenol Blue dyes, prepared separately at an initial concentration of 500 mg/L. The reactions were carried out at 35 °C and pH 6.0, with the addition of 100 µL of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) as a redox mediator. All experiments were performed under orbital shaking at 200 rpm for 1 h.

To evaluate the stability and reuse of the immobilized enzyme, three consecutive treatment cycles were performed. At the end of each cycle, the immobilized material was recovered, washed with phosphate buffer (pH 6.0) to remove residual substrates and reaction products, and reused in the subsequent cycle. Dye degradation was monitored by UV-Vis spectroscopy, measuring the decrease in absorbance at 590 nm, the characteristic wavelength used to track decolorization of dye solutions. Degradation efficiency was calculated based on the percentage reduction in absorbance relative to the initial dye concentration.

Results and Discussion

Fungi Isolation and Laccase Production

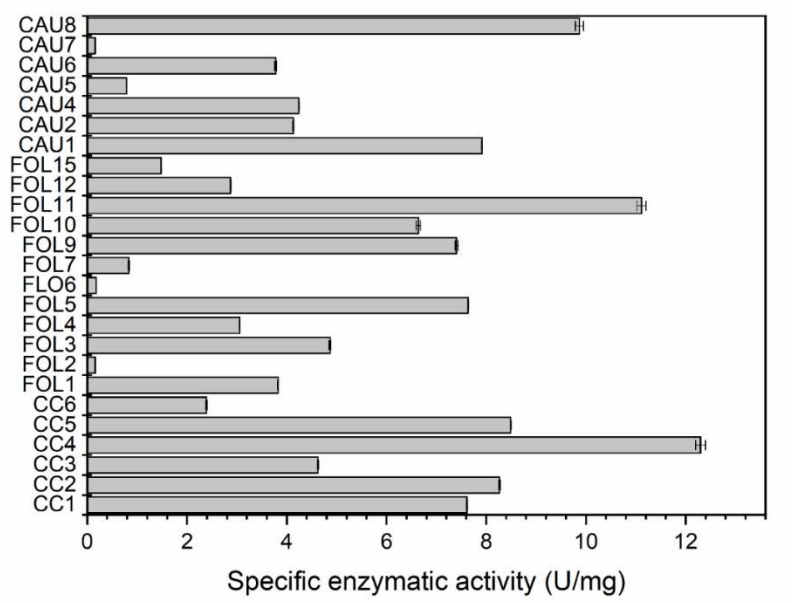
The isolation of endophytic fungi from cocoa agro-industrial waste yielded 25 microorganisms from the fruit peels, stems, and leaves of *Theobroma cacao* L., with 12 strains (48%) from leaves, 7 (28%) from stems, and 6 (24%) from fruit peels. The predominance of microorganisms from leaf tissues suggests that leaves constitute an ecological niche particularly favorable to colonization by endophytic fungi. From a physiological perspective, leaves are metabolically active organs characterized by intense carbon flux from photosynthesis. A significant fraction of the newly fixed carbon is transported through the mesophyll and vascular tissues as soluble sugars, organic acids, and other low-molecular-weight compounds, creating nutrient-rich microhabitats that can support endophytic microbial communities [Emoh et al., 2025; Osogo et al., 2025; Hardoim et al., 2015; 32. Arnold & Lutzoni, 2007]. In addition to primary metabolites, leaves also accumulate a wide range of phenolic compounds and secondary metabolites that can act as selective factors, shaping endophytic diversity [Thakur & Kharwal, 2025; Revathy & Kesavan, 2024].

As aerial organs directly exposed to the atmosphere, leaves continuously interact with microorganisms originating from the phyllosphere, airborne spores, and surrounding plant

surfaces. This constant microbial influx facilitates colonization events and promotes the establishment of several endophytic fungi in internal tissues [Thakur & Kharwal, 2025]. Furthermore, microstructural features of leaves, including stomatal openings, cuticular microfractures, and mesophyll intercellular spaces, provide potential entry points and protected niches for microbial persistence. These structural and metabolic features together increase the likelihood of endophyte recruitment and maintenance in leaf tissues compared with plant organs with higher lignin content [Aryakia, 2025].

Similar distribution patterns have been widely reported in perennial crops and woody plants, in which leaf tissues often harbor richer, more diverse endophytic fungal communities than structurally rigid organs [Arnold & Lutzoni, 2007]. The relatively high number of microorganisms obtained from cocoa waste reinforces the hypothesis that agro-industrial by-products constitute an underexploited reservoir of microbial diversity. In the context of cocoa waste, which is often considered low-value biomass, however, current results demonstrate that these materials can harbor a wide range of endophytic fungi with relevant potential for biotechnology.

In this context, preliminary screening of the isolated fungi revealed substantial variability in laccase production capacity among strains. Although several fungi exhibit enzymatic activity, the CC4 strain showed the highest specific laccase activity among those evaluated (Fig. 1). This result corroborates previous data on endophytic fungal communities, in which enzyme production is strongly species-dependent and influenced by intrinsic genetic and physiological factors.



306

307 **Fig. 1.** Laccase production by fungi isolated from cocoa waste.

308

309 Molecular identification of the CC4 fungus revealed 100% sequence identity with
310 *Trametes villosa*, a basidiomycete species widely recognized for its high laccase production
311 capacity (Fig. 2). Although *T. villosa* has been extensively studied as a source of laccase, its
312 isolation as an endophyte from agro-industrial cocoa residues has not been previously reported.
313 This finding is particularly relevant since most studies describe *Trametes* species as wood-
314 decomposing fungi associated with forest ecosystems. The present result, therefore, expands
315 the known spectrum of *T. villosa* sources and highlights cocoa residues as a new and sustainable
316 source of laccase-producing fungi.

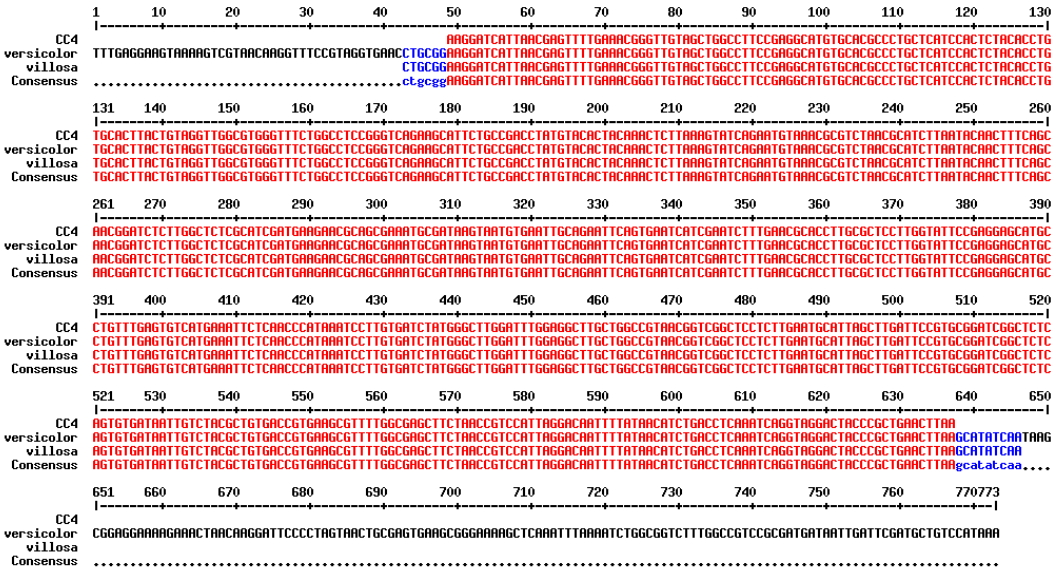


Fig. 2. Molecular identification of fungal isolate CC4 as *Trametes villosa*.

Optimization of Culture Conditions for Laccase Production

The growth of the microorganism CC4 (*Trametes villosa*) was initially investigated under different pH and temperature conditions to determine the optimal parameters for laccase production (Table 1). The pH and temperature of the fermentation process were systematically varied, and their effects on enzyme biosynthesis were evaluated. The response variable was the specific laccase activity, calculated as the ratio between enzymatic activity, determined by the syringaldazine oxidation assay, and protein concentration.

The experimental data were analyzed using Statistica 14.0.0.15, enabling evaluation of each variable's influence on the process (Fig. 3, Table 2) and estimation of their corresponding effects. As shown in Fig. 3A, both pH and temperature exerted a strong influence on laccase production by the endophytic fungus CC4. The investigated temperature range (18.9–39.1 °C) and pH interval (2.64–9.36) were selected based on conditions commonly reported for fungal growth and laccase biosynthesis in filamentous fungi, as well as on preliminary cultivation assays designed to encompass both suboptimal and potentially inhibitory conditions.

The results indicate that moderate temperatures, ranging from approximately 25 to 29 °C, combined with mildly acidic conditions, favored higher specific laccase activity (Fig. 3B). In contrast, elevated temperatures and alkaline conditions significantly reduced enzyme production. This behavior is consistent with the physiological characteristics of white-rot fungi and with the biochemical properties of fungal laccases, whose biosynthesis and catalytic performance are highly dependent on environmental conditions. The influence of pH extends beyond fungal growth and directly affects several physiological processes associated with laccase production. Slightly acidic conditions are frequently associated with increased secretion of extracellular oxidoreductases, whereas alkaline environments may impair enzyme synthesis, secretion, and maturation. Furthermore, extracellular pH influences nutrient availability, metal ion solubility, and protein folding processes, all of which may affect enzyme biosynthesis and catalytic performance [Kiaei et al., 2025].

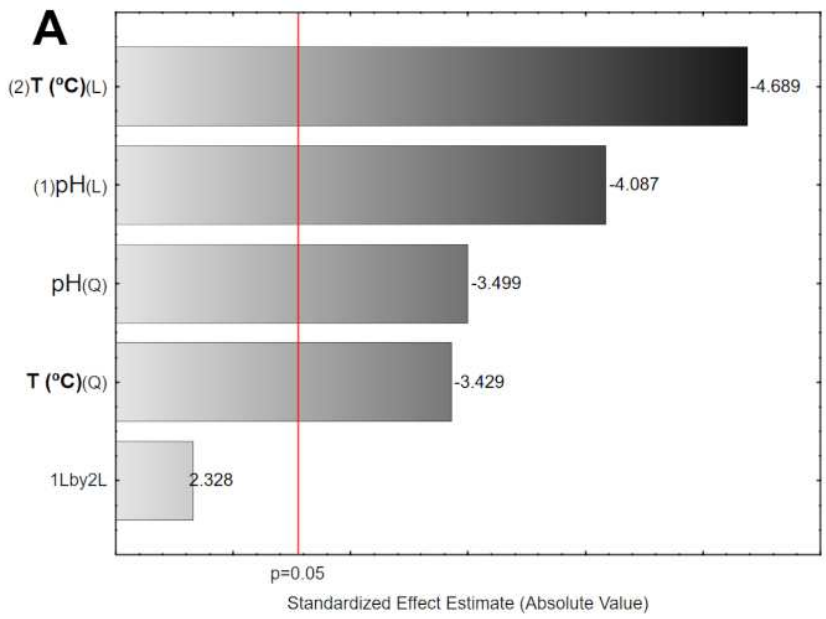
Copper availability also plays an important role in laccase production. Since copper sulfate was included in the culture medium, its presence likely contributed to enhanced enzyme biosynthesis. Copper acts both as an essential cofactor and as a physiological inducer of laccase production in many basidiomycetes. During enzyme maturation, copper atoms are incorporated into the highly conserved catalytic centers of multicopper oxidases, ensuring the formation of the active enzyme and promoting efficient electron transfer during catalysis. The structural stability of laccase is likewise dependent on cultivation conditions [Kiaei et al., 2025; Jeyabalan et al., 2025]. Fungal laccases contain four copper atoms organized into one T1, one T2, and a binuclear T3 catalytic center responsible for electron transfer and oxygen reduction. Deviations from optimal pH and temperature conditions may alter protein conformation and the coordination environment of these metal centers, thereby reducing catalytic efficiency and accelerating enzyme deactivation. Therefore, the enhanced enzyme production observed under mildly acidic conditions and moderate temperatures likely reflects both favorable fungal

359 metabolism and preservation of the structural integrity of the multicopper oxidase system. An
360 interaction effect between pH and temperature was also observed, with laccase production
361 reaching its maximum when both variables were simultaneously adjusted to values near the
362 optimal region. Deviations from these conditions led to a marked decline in specific activity,
363 highlighting the importance of balancing cultivation parameters to maximize enzyme
364 production and stability [Kiaei et al., 2025; Jeyabalan et al., 2025; Zhang et al., 2026].
365

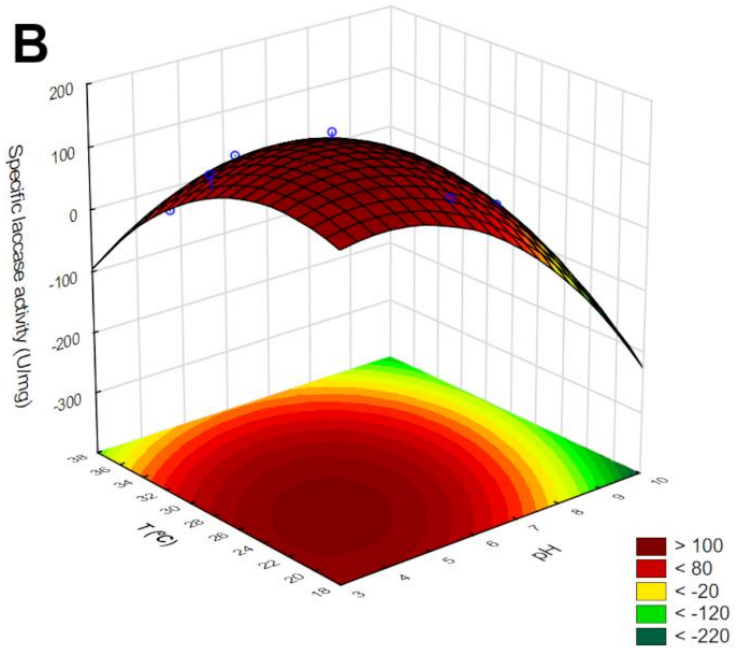
366 **Table 1** Enzyme activity data for laccase and protein concentration.

pH	T (ºC)	Laccase activity (µmol/mL min)	Protein concentration (mg/mL)	Specific Laccase Activity (µmol/mg min)
3.3	29	79.323 ± 0.218	0.618 ± 0.021	128.523 ± 3.916
4.0	35	3.024 ± 0.225	0.597 ± 0.033	5.062 ± 0.101
8.0	35	3.342 ± 0.225	0.610 ± 0.037	5.478 ± 0.039
6.0	29	84.662 ± 0.196	0.590 ± 0.027	143.651 ± 6.874
4.0	23	78.831 ± 0.435	0.705 ± 0.182	115.608 ± 0.293
8.0	23	20.610 ± 0.225	1.007 ± 0.036	20.089 ± 0.217
6.0	37	37.138 ± 0.218	1.002 ± 0.021	37.093 ± 0.977
8.8	29	6.815 ± 0.196	0.761 ± 0.328	9.812 ± 3.973
6.0	20	71.738 ± 0.413	0.637 ± 0.013	112.648 ± 2.900
6.0	29	72.692 ± 0.413	0.651± 0.023	111.719 ± 3.248

367



368



369

370 **Fig. 3.** (A) Influence of variables on laccase production by the fungus CC4 and (B) Laccase

371 production by the fungus CC4 as a function of culture conditions.

372

373

374

375

376

Table 2 Estimation of effects on specific enzymatic activity.

Factor	Effect estimates of temperature and pH on specific enzymatic activity (µmol/mg min); R-sqr=.9364; MS Residual=473.9296									
	Effect	Std.Err.	t(4)	p	-95.% (Cnf.Limt)	+95.% (Cnf.Limt)	Coeff.	Std.Err. (Coeff.)	-95.% (Cnf.Limt)	+95.% (Cnf.Limt)
Mean/Interc.	128.07	15.33	8.35	0.00	85.50	170.63	128.07	15.33	85.50	170.63
(1)pH(L)	-63.85	15.62	-4.09	0.02	-107.22	-20.48	-31.92	7.81	-53.61	-10.24
pH(Q)	-74.15	21.20	-3.50	0.02	-133.00	-15.31	-37.08	10.60	-66.50	-7.65
(2)**T (ºC)**(L)	-72.72	15.51	-4.69	0.01	-115.77	-29.66	-36.36	7.75	-57.89	-14.83
T (ºC)(Q)	-68.82	20.07	-3.43	0.03	-124.54	-13.09	-34.41	10.04	-62.27	-6.55
1L by 2L	50.69	21.77	2.33	0.08	-9.75	111.13	25.34	10.88	-4.88	55.57

The fitted quadratic model adequately represented the experimental data, yielding a coefficient of determination (R^2) of 0.9364. To further evaluate the model's statistical significance and the contribution of each factor, an analysis of variance (ANOVA) was performed (Table 3). Both the linear and quadratic effects of pH and temperature were statistically significant ($p < 0.05$), confirming that these variables strongly influenced laccase production. Temperature contributed the most to the model, as indicated by the highest F-value for its linear effect ($F = 21.99$, $p = 0.0094$), followed by the linear effect of pH ($F = 16.71$, $p = 0.0150$). Although the interaction between pH and temperature was not statistically significant at the 95% confidence level ($p = 0.0804$), its inclusion improved the model's predictive performance. Furthermore, the adjusted coefficient of determination ($R^2 = 0.8568$) demonstrated the robustness of the fitted model for predicting optimal cultivation conditions for laccase production.

Table 3 Analysis of variance (ANOVA) of the quadratic model fitted to specific laccase activity

Factor	Var.:Specific laccase activity (µmol/mg min); R-sqr=.93637; Adj:.85684, 2 factors, 1 Blocks, 10 Runs; MS Residual=473.9296				
	SS	df	MS	F	p
(1)pH(L)	7917.93	1	7917.93	16.70698	0.015007
pH(Q)	5800.86	1	5800.86	12.23992	0.024929
(2)**T (ºC)**(L)	10421.26	1	10421.26	21.98904	0.009383
T (ºC)(Q)	5571.69	1	5571.69	11.75637	0.026566
1L by 2L	2569.29	1	2569.29	5.42125	0.080397
Error	1895.72	4	473.93		
Total SS	29794.26	9			

The desirability analysis identified pH 5.4 and 28 °C as the optimal cultivation conditions for maximizing specific laccase activity (Fig. 4). These findings confirm the strong dependence of laccase biosynthesis on cultivation conditions and demonstrate that optimal production results from the combined effects of fungal physiology, copper-mediated regulation, and preservation of enzyme structural stability. Similar cultivation conditions have been reported for laccase production by other white-rot fungi, including the findings of Kiaei et al. (2025), reinforcing the importance of moderately acidic environments and intermediate temperatures for maximizing fungal laccase production.

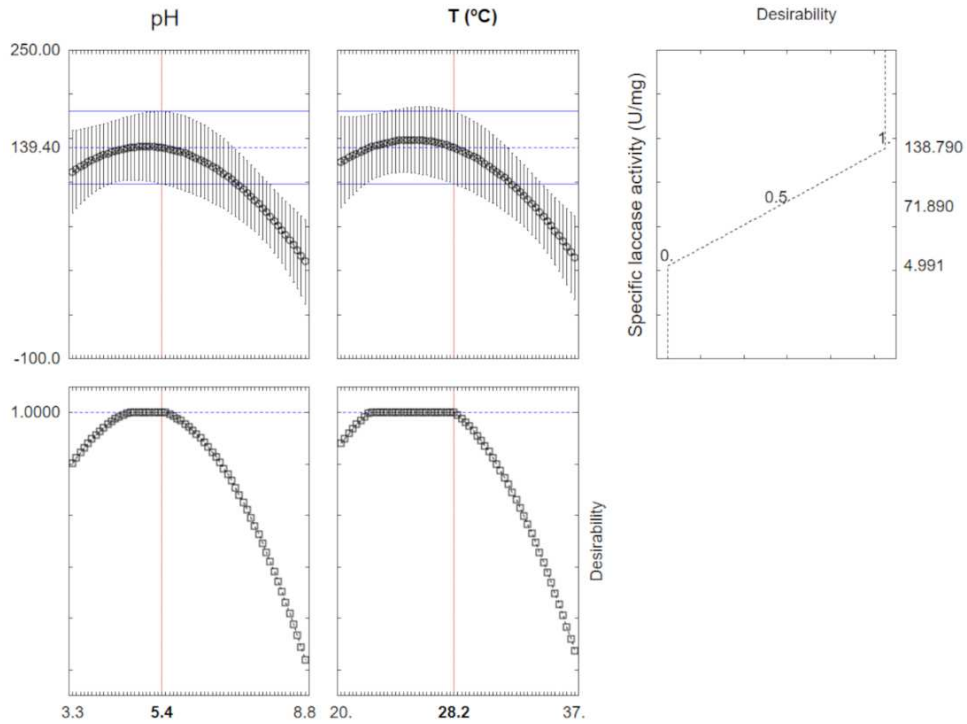


Fig. 4. Response surface and desirability analysis for laccase production by *Trametes villosa* as a function of pH and temperature.

Kinetic analysis and modeling of laccase production

The experimental data describing laccase production during fungal cultivation are presented in Table 4. A progressive increase in laccase activity, protein concentration, and

409 specific laccase activity was observed during the first six days of cultivation, reaching a
410 maximum specific activity of 171.48 μmol/mg min on day 5. This behavior reflects the active
411 phase of fungal growth and enzyme biosynthesis, during which cellular metabolism is directed
412 toward the synthesis and secretion of extracellular enzymes. This production profile indicates
413 that laccase biosynthesis is predominantly associated with the fungus's active growth phase,
414 suggesting a close relationship between fungal physiological development and enzyme
415 production. After this period, a pronounced decline in laccase activity was observed, indicating
416 reduced enzyme productivity and stability during the later stages of cultivation.

417 The decrease in laccase activity is likely associated with physiological and biochemical
418 changes occurring during the stationary phase of fungal growth. These factors may include
419 nutrient depletion, accumulation of metabolic by-products, extracellular proteolytic activity,
420 and progressive enzyme deactivation. In addition, prolonged cultivation can compromise the
421 stability of multicopper oxidases such as laccase, reducing their catalytic performance over
422 time. These observations highlight the importance of identifying an optimal harvesting time to
423 maximize enzyme recovery and overall process productivity.

424 To quantitatively describe the production profile, Logistic and Gompertz models were
425 implemented in MATLAB to estimate the kinetic parameters (Table 5) and generate the
426 corresponding fitted curves (Fig. 5). Both models adequately represented the experimental data,
427 yielding high coefficients of determination ($R^2 > 0.98$). The Logistic model provided a slightly
428 superior fit ($R^2 = 0.9871$) compared with the Gompertz model ($R^2 = 0.9839$), indicating a more
429 accurate representation of the observed production dynamics.

430 The temporal evolution of laccase production exhibited a characteristic sigmoidal
431 profile, as is commonly observed in microbial growth-associated processes. This pattern
432 comprises an initial adaptation phase, followed by a rapid production stage, and a subsequent
433 stabilization period as the system approaches its maximum production capacity. The superior

434 performance of the Logistic model suggests that laccase biosynthesis in Trametes villosa is
435 closely associated with the physiological development of the fungal culture, enabling the model
436 to accurately describe the progression of enzyme production throughout cultivation.

437 The graphical comparison between the Logistic and Gompertz models (Fig. 5) further
438 supports the suitability of the Logistic equation for representing laccase biosynthesis by *T.*
439 *villosa*. The closer agreement between the Logistic model and the experimental data,
440 particularly during the early production stages, indicates that enzyme production exhibits
441 growth-associated behavior, with biosynthesis increasing rapidly as fungal biomass develops
442 and gradually approaches a maximum production capacity. Although both models successfully
443 captured the overall production trend, the Logistic model more accurately reproduced the
444 experimental sigmoidal profile and therefore represents a more reliable predictive tool for
445 process optimization and production scheduling.

446

447 **Table 4** Specific laccase activity data as a function of fungal cultivation time

Time (Day)	Laccase activity (μmol/mL min)	Protein concentration (mg/mL)	Specific Laccase Activity (μmol /mg min)
0	0.055 ± 0.000	0.017 ± 0.003	3.226 ± 0.570
1	0.083 ± 0.039	0.026 ± 0.000	3.176 ± 1.497
2	3.635 ± 0.245	0.534 ± 0.008	6.803 ± 0.360
3	23.971 ± 0.122	0.452 ± 0.008	52.998 ± 0.632
4	66.489 ± 5.992	0.583 ± 0.008	113.959 ± 8.769
5	87.757 ± 3.329	0.512 ± 0.015	171.475 ± 11.657
6	92.132 ± 1.292	0.600 ± 0.008	153.681 ± 0.180
7	46.800 ± 1.175	0.529 ± 0.000	88.527 ± 2.222
8	29.631 ± 1.175	0.527 ± 0.011	56.257 ± 1.045

448

449 Beyond its descriptive capabilities, kinetic modeling provides a useful framework for
450 predicting the optimal cultivation window to maximize laccase productivity. The estimated
451 parameters can be used to identify the optimal harvest time, minimize productivity losses from

prolonged cultivation, and inform future scale-up strategies for industrial laccase production. The identification of the optimal production window is particularly important for industrial applications, since harvesting the culture after the production peak may result in significant losses of enzymatic activity and overall process productivity. Similar sigmoidal production profiles and optimal production periods have been reported for fungal laccases produced by white-rot fungi, including the findings of Kiaei et al. (2025) [Kiaei et al., 2025], reinforcing the importance of kinetic modeling as a valuable tool for improving bioprocess efficiency, enzyme recovery, and industrial process design.

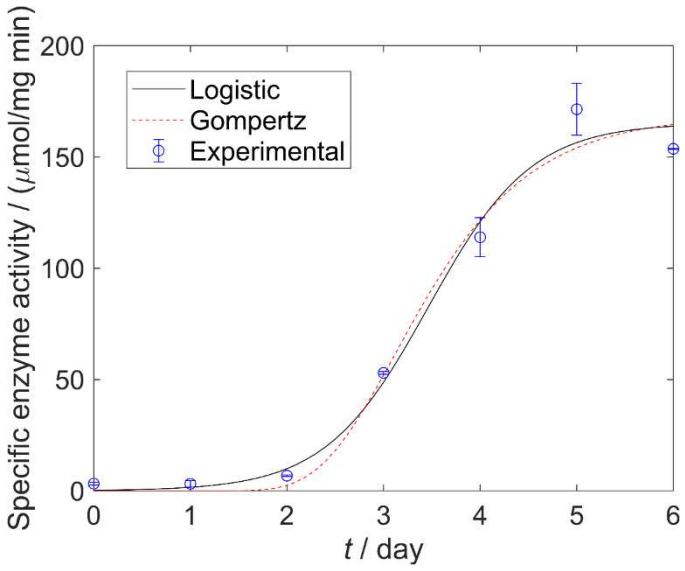


Fig. 5. Laccase production profile.

Table 5 Parameters of the Logistic and the Gompertz models.

Parameter	Logistic Model	Gompertz Model
a / (μmol/mg min)	165.3	168.9
b	664.5	54.46
k / day^{-1}	1.876	1.278
R^2	0.9871	0.9839

The kinetic model describing the oxidation of syringaldazine catalyzed by the laccase-enriched extract from *Trametes villosa* (CC4) was used to evaluate the catalytic behavior of the

enzymatic system across a range of substrate concentrations. The experimental and obtained product concentration profiles are presented in Figure 6. As expected, product formation increased with increasing initial substrate concentration, demonstrating the enzyme's ability to efficiently catalyze substrate oxidation over a wide concentration range. The proposed model successfully reproduced the experimental reaction profiles and accurately described product formation over time. Under all evaluated conditions, the model captured both the initial phase of the reaction and the progression toward the experimentally observed final plateau. Although complete substrate conversion was not achieved, experimentally determined yields ranged from 82% to 91%, indicating that most of the substrate was converted to product under the evaluated conditions.

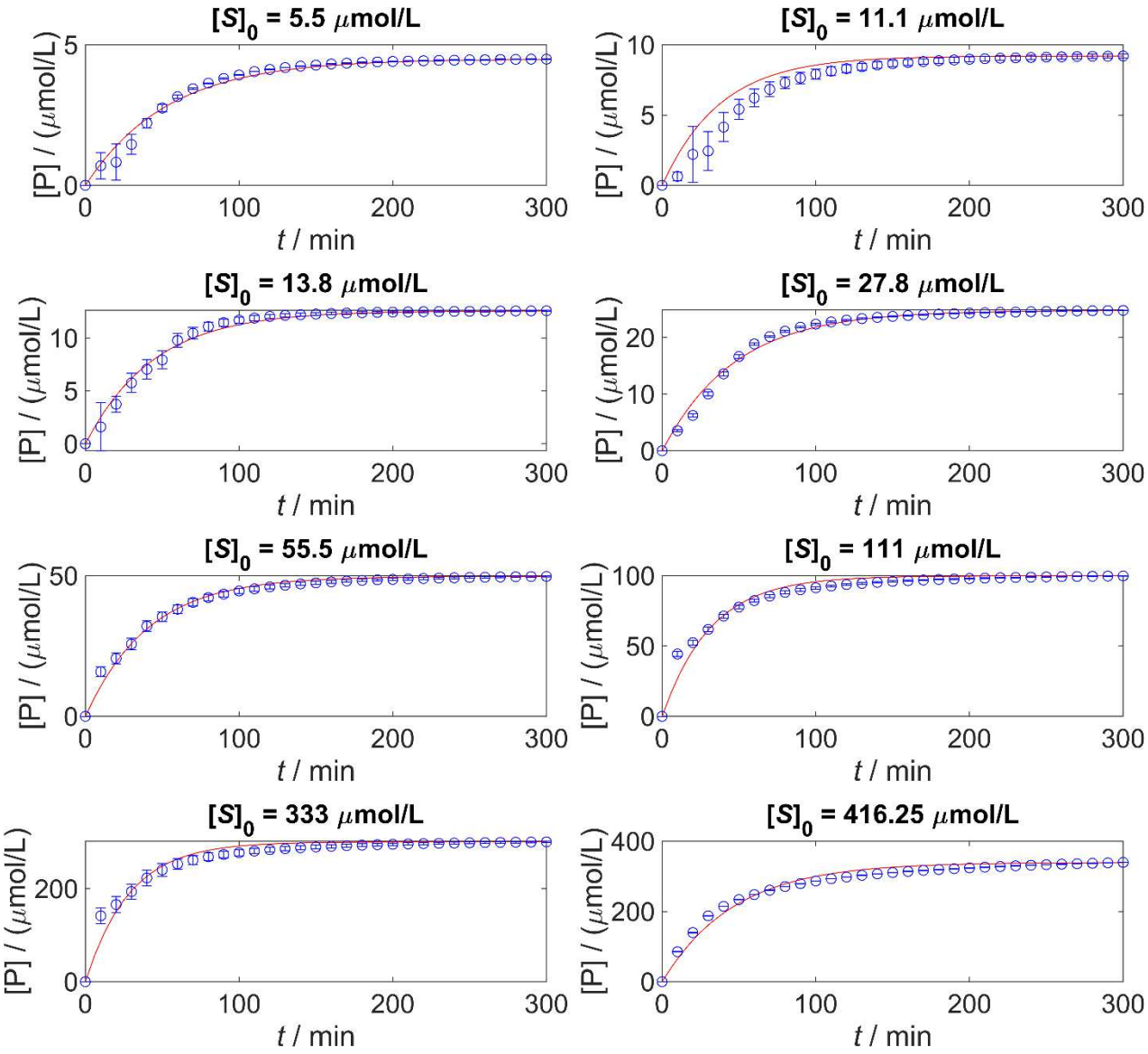


Fig. 6. Product profiles for different initial substrate concentrations: (○), experimental; (—), model.

The estimated kinetic constants and their respective errors are presented in Table 6, while the correlation matrix of the parameters is shown in Table 7. As expected for nonlinear kinetic systems, relatively high correlations were observed between some estimated parameters. This behavior is commonly observed in enzymatic kinetic models based on coupled differential equations, in which multiple combinations of parameters can yield similar reaction profiles. Despite these correlations, the estimated parameters remained physically meaningful and enabled satisfactory reproduction of the experimental data.

Unlike conventional Michaelis-Menten analyses based solely on initial reaction rates, the present approach employs a system of ordinary differential equations to describe the temporal evolution of substrate consumption, enzyme-substrate complex formation, product generation, and enzyme regeneration throughout the reaction period. Consequently, the model provides a more comprehensive representation of the catalytic process and offers greater predictive capacity for process design and optimization. Using the estimated kinetic constants, the maximum reaction rate (V_{max}) and the Michaelis-Menten constant (K_M) were calculated to be 284.65 μmol/L min and 67.38 μmol/L, respectively. The K_M value indicates a moderate affinity of the enzyme for syringaldazine, consistent with the behavior commonly reported for fungal laccases acting on phenolic substrates. At the same time, the relatively high V_{max} value indicates that the laccase-enriched extract has substantial catalytic capacity once the enzyme-substrate complex forms, reflecting the high oxidative potential characteristic of fungal multicopper oxidases.

The catalytic performance observed in this study is directly related to the structural characteristics of laccases. These enzymes contain four copper atoms distributed among the catalytic centers T1, T2, and T3, responsible for electron transfer and the reduction of molecular oxygen to water [Kiaei et al., 2025; Jeyabalan et al., 2025; Zhang et al., 2026]. The experimentally observed efficient oxidation of syringaldazine demonstrates the preservation of these catalytic centers in the laccase-enriched extract produced by *T. villosa*. It confirms the functionality of the multicopper oxidase system under the reaction conditions evaluated.

The inability to achieve complete substrate conversion suggests the presence of factors that limit the reaction's progression under the conditions studied. Such factors may include partial enzyme deactivation during prolonged reaction times, thermodynamic constraints, product accumulation effects, or other physicochemical phenomena not explicitly incorporated into the current model. However, incorporating experimentally determined yields enabled the

model to accurately reproduce the observed reaction plateaus without additional inhibitory terms.

Table 6 Estimated kinetic constant values

	Value	Error
k_1 / min^{-1}	0.13	± 0.02
k_{-1} / min^{-1}	7.10	± 0.51
k_2 / min^{-1}	1.66	± 0.22

Table 7 Correlation matrix of estimated parameters

	k_1	k_{-1}	k_2
k_1	1	0.97	-0.99
k_{-1}	0.97	1	0.96
k_2	-0.99	-0.96	1

Evaluation of the efficiency of laccase in the dye decolorization

To evaluate the practical applicability of the laccase-enriched extract produced by CC4, isolated from cocoa agro-industrial waste, decolorization assays were performed using synthetic effluents containing Remazol Brilliant Blue R (RBBR) and Bromophenol Blue (BPB). The enzymatic extract was evaluated both in its free form and after immobilization on glutaraldehyde-activated chitosan. The results obtained are presented in Table 8.

The free enzymatic extract achieved 82.66% decolorization of RBBR in the first treatment cycle, demonstrating that laccase produced by CC4 efficiently oxidizes anthraquinone-based dyes. In contrast, only 0.15% decolorization was observed for BPB under the same conditions. This difference highlights the influence of dye structure on laccase-mediated oxidation, as substrate accessibility, redox potential, molecular complexity, and steric effects directly affect catalytic efficiency. The distinct behavior observed for the two dyes

suggests that RBBR is more susceptible to direct enzymatic oxidation, whereas BPB requires more favorable catalytic conditions to achieve efficient degradation.

Table 8 Efficiency of applying laccase-enriched extract in the degradation of dyes.

Remazol Brilliant Blue R degradation			
	1st Cycle (%)	**2nd Cycle (%)**	**3rd Cycle (%)**
Free enzyme	82.66 ± 0.40	-	-
Immobilized enzyme	100.00 ± 0.00	95.91 ± 0.03	85.18 ± 0.09
Bromophenol Blue degradation			
	1st Cycle (%)	**2nd Cycle (%)**	**3rd Cycle (%)**
Free enzyme	0.15 ± 0.02	-	-
Immobilized enzyme	99.90 ± 0.39	98.96 ± 0.00	98.79 ± 0.88

A substantial improvement in decolorization efficiency was observed after enzyme immobilization. Complete decolorization of RBBR (100.00%) and nearly complete decolorization of BPB (99.90%) were achieved during the first treatment cycle. The enhanced catalytic performance of the immobilized system can be attributed to improved enzyme stability, preservation of the active conformation, and increased resistance to operational stresses. Immobilization frequently reduces conformational flexibility and protects enzymes against deactivation, thereby extending their catalytic lifetime and improving process efficiency.

The use of chitosan as an immobilization support offers several advantages, including biocompatibility, biodegradability, low cost, and the presence of amino groups suitable for chemical activation. The treatment of chitosan with glutaraldehyde generates reactive aldehyde groups that promote covalent attachment between the support and enzyme molecules. This immobilization strategy minimizes enzyme leaching, enhances operational stability, and facilitates catalyst recovery and reuse. Furthermore, covalent immobilization helps preserve the

structural integrity of the multicopper oxidase system, which is essential for maintaining catalytic activity during repeated operational cycles.

The high stability of the immobilized biocatalyst was confirmed through successive reuse experiments. For RBBR, decolorization efficiencies remained at 95.91% and 85.18% during the second and third cycles, respectively. Similarly, BPB decolorization remained above 98% throughout all reuse cycles. These results demonstrate remarkable operational stability and indicate that only limited losses of catalytic activity occurred during repeated applications. The gradual decrease observed after successive cycles is expected in immobilized enzyme systems and may be associated with progressive enzyme deactivation, partial obstruction of catalytic sites by reaction products, or minor catalyst losses during washing and recovery procedures.

From a mechanistic perspective, laccase catalyzes the oxidation of aromatic compounds via one-electron transfer, generating highly reactive radical intermediates. These radicals subsequently undergo fragmentation, rearrangement, polymerization, or cleavage reactions, ultimately leading to dye decolorization. The addition of ABTS as a redox mediator further enhances the enzyme's catalytic performance by facilitating electron transfer between the laccase active site and dye molecules that may not be readily oxidized directly. This mediator-assisted mechanism likely contributed to the high decolorization efficiencies observed for both dyes.

The performance achieved in the present study compares favorably with previously reported laccase-based dye degradation systems (Table 9). Several studies have reported decolorization efficiencies ranging from approximately 70% to 96%, depending on the enzyme source, immobilization strategy, and operational conditions. In contrast, the immobilized laccase produced by T. *villosa* achieved complete decolorization of RBBR and near complete decolorization of BPB while maintaining high catalytic activity across repeated reuse cycles. Moreover, unlike many previously reported systems that rely on purified enzymes, engineered

microorganisms, or complex synthetic supports, the present work employed a laccase-enriched extract from an endophytic fungus isolated from cocoa agro-industrial waste, which was immobilized on a natural, biodegradable support. This combination reinforces the sustainability and economic attractiveness of the proposed biocatalytic platform.

Table 9 Comparison of laccase systems applied to dye degradation.

Catalyst Type	Enzyme Source	Carrier Material	Target Dye	Optimal pH	Optimal Temperature (°C)	Degradation Efficiency (%)	Reusability/Operational Stability	Source
Immobilized	-	Magnetic Metal Organic Frameworks (MMOFs)	Methylene Blue, Crystal Violet	4.5	70	91% to 93% (within 20 min in batch mode)	Retains 89% activity after 10 cycles	Ladole et al., 2020
Immobilized	Not in source	Fe3O4@C-laccase	Malachite Green (MG), Brilliant Green (BG), Crystal Violet (CV)	4.6	55	96.4% to 99.9%	Retained 94.0% to 99.3% efficiency after 10 cycles	Wei et al., 2024
Immobilized	Aspergillus oryzae	Mechanically ground silk fibroin nanofibers (SFNF)	Trypan Blue	5.0	60	76%	-	Miyawaki & Sakai, 2025
Free	Malva parviflora	None	Congo Red	5.0	37	~97.5%	-	Aziz et al., 2023
Immobilized (Purified)	Malva parviflora	Sephadex G-150	Congo Red	5.0	37	69%	-	Aziz et al., 2023
Free	Ganoderma lucidum	Not in source	Orange G, Acid Orange 7, Remazol Brilliant Blue R, Bromophenol Blue, Methyl Green, Indigo Carmine	5.0	30	37.23% to 99.94%	Maintained between 45.06% and 82.04% activity after 7 cycles, depending on the dye	Deng et al., 2024
Free	Peniophora sp. CBMAI 1063 (heterologous expression in Pichia pastoris)	Not in source	Indigo Carmine	3.0	60	93% (within 2 h with syringaldehyde)	-	Otero et al., 2025
Free	Geobacillus stearothermophilus strain MB600	Not in source	Remazol Brilliant Blue R	4.0	75	93% (within 4 h)	-	Bibi et al., 2025
Free	Arthrographis kalrae	None	Remazol Brilliant Blue R (RBBR)	7.0	35	97.18%	-	Yadav et al., 2021
Immobilized	Not in source	Sodium alginate-dopamine/polyethylene glycol (SA-PDA/PEG) composite hydrogels	Reactive Blue 19 (RB-19)	3.0	55	88.38%	Retained 90.23% activity after 6 cycles	Su et al., 2024
Free	Not in source	None	Reactive Blue 19 (RB-19)	3.0	55	12.26%	-	Su et al., 2024
Immobilized		Chitosan	Bromophenol Blue degradation	6	35	99.90%	Retained 98.90% activity after 3 cycles	This work
Immobilized		Chitosan	Remazol Brilliant Blue R	6	35	100.00%	Retained 85.18% activity after 3 cycles	This work

These findings demonstrate that endophytic fungi associated with cocoa agro-industrial waste represent promising sources of oxidative enzymes for environmental biotechnology

584 applications. More broadly, the integration of microbial bioprospecting, enzyme production,
585 immobilization, and wastewater treatment exemplifies a circular bioeconomy approach in
586 which low-value agricultural residues are converted into high-value biotechnological resources.
587 The high catalytic efficiency, operational stability, and reusability of the developed biocatalyst
588 highlight its potential for future applications in sustainable wastewater treatment processes and
589 industrial biocatalysis.
590

591 **Conclusion**

592 This study demonstrated the potential of cocoa agro-industrial waste as a source of
593 biotechnologically valuable endophytic fungi by isolating and characterizing a laccase-
594 producing *Trametes villosa* (CC4). Statistical optimization revealed that laccase production
595 was strongly influenced by cultivation conditions, with maximum specific activity achieved
596 under mildly acidic conditions (pH 5.4) and moderate temperatures (28 °C). Kinetic evaluation
597 showed a characteristic sigmoidal production profile, with maximum specific activity reaching
598 171.48 U/mg after five days of cultivation. Among the evaluated growth models, the Logistic
599 equation provided the best representation of enzyme production dynamics, supporting its
600 application as a predictive tool for process optimization and scale-up.

601 The kinetic model developed for syringaldazine oxidation successfully described the
602 catalytic behavior of the laccase-enriched extract over a broad range of substrate concentrations.
603 The estimated kinetic parameters confirmed the high catalytic potential of the enzyme system
604 and demonstrated the suitability of the proposed modeling approach for representing laccase-
605 catalyzed reactions.

606 Immobilization of the laccase-enriched extract on glutaraldehyde-activated chitosan
607 yielded a stable, reusable biocatalyst that achieved complete decolorization of Remazol
608 Brilliant Blue R and 99.9% decolorization of Bromophenol Blue. The immobilized system

maintained high catalytic performance during successive reuse cycles, demonstrating its potential for practical wastewater treatment applications. Overall, this work presents an integrated bioprocess framework encompassing microbial bioprospecting, process optimization, kinetic modeling, enzyme immobilization, and environmental application. The results reinforce the value of cocoa agro-industrial waste as a reservoir of microbial biodiversity and support its conversion into high-value biotechnological resources within circular bioeconomy and sustainable bioprocess engineering strategies.

CRediT authorship contribution statement

SAL, JVYO, GVO, AA, and AAMG performed material preparation, data collection, and analysis. AAMG, GVO, AA, RT, RT, and BP wrote the first draft of the manuscript, and all authors provided comments on subsequent versions. AAMG, RT, and RT have read and approved the final manuscript.

Acknowledgments

The authors acknowledge Centro Universitário FEI for providing the infrastructure and technical support required for this study. The authors also gratefully acknowledge the financial support provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), under the research projects 2023/17175-0 and 2024/14025-0, as well as by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and collaboration during the BEPE research period (FAPESP 2025/06516-6).

Funding

This work was supported by the São Paulo Research Foundation (FAPESP), Brazil, under research project grant nº 2025/06516-6, 2023/17175-0 and 2024/14025-0.

Declarations

Declaration of Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Declaration of generative AI and AI-assisted technologies in the writing process: During the preparation of this work, the author(s) used Grammarly on a limited basis to improve the manuscript's readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the publication.

Ethical approval: This study did not involve human or animal subjects, ethical approval was not required.

Data Availability

No data was used for the research described in the article.

References

Afedzi, A. E. K., et al. (2023). Valorization of Ghanaian cocoa processing residues as extractives for value-added functional food and animal feed additives – A review. Biocatalysis and Agricultural Biotechnology, 50:102835.

Alotaibi, R. F., et al. (2024). Chromatographic and spectroscopic methods for the detection of cocoa butter in cocoa and its derivatives: A review. Heliyon, 10:e31467.

Alves-Júnior, M., et al. (2021). Functional and morphological analysis of isolates of phylloplane and rhizoplane endophytic bacteria interacting in different cocoa production systems in the Amazon. Current Research in Microbial Sciences, 2:100039.

658 Anoraga, S. B., Shamsudin, R., Hamzah, M. H., Sharif, S., & Saputro, A. D. (2024). Cocoa
659 by-products: A comprehensive review on potential uses, waste management, and emerging
660 green technologies for cocoa pod husk utilization. Heliyon, 10:e35537.
661 Arnold, A. E., & Lutzoni, F. (2007). Diversity and host range of foliar fungal endophytes: Are
662 tropical leaves biodiversity hotspots? Ecology, 88:541–549.
663 Aryakia, E. (2025). Cryobanking of rare and endangered plant species: Key propagules and
664 genetic stability assessments. Journal of Nature Conservation, 84:126844.
665 Aziz, G. M., Hussein, S. I., M-Ridha, M. J., Mohammed, S. J., Abed, K. M., Muhamad, M. H.,
666 & Hasan, H. A. (2023). Activity of laccase enzyme extracted from Malva parviflora and
667 its potential for degradation of reactive dyes in aqueous solution. Biocatalysis and
668 Agricultural Biotechnology, 50:102671.
669 Barrios-Rodríguez, Y. F., Salas-Calderón, K. T., Orozco-Blanco, D. A., Gentile, P., & Girón
670 Hernández, J. (2022). Cocoa pod husk: A high-pectin source with applications in the food
671 and biomedical fields. ChemBioEng Reviews, 9:462–474.
672 Berkson, J. (1944). Application of the logistic function to bio-assay. Journal of the American
673 Statistical Association, 39:357–365.
674 Bibi, M., Yasmin, A., Murtza, I., Saeed, A., Jamil, M. A., Syed, S. S., & Hai, A. (2025).
675 Degradation of anthraquinone dye Remazol Brilliant Blue R through Geobacillus laccase:
676 Simulation of molecular docking and process optimization by artificial neural network.
677 Results in Engineering, 23:106908.
678 Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram
679 quantities of protein utilizing the principle of protein-dye binding. Analytical
680 Biochemistry, 72:248–254.

681 Campos-Vega, R., Nieto-Figueroa, K. H., & Oomah, B. D. (2018). Cocoa (Theobroma cacao
682 L.) pod husk: Renewable source of bioactive compounds. Trends in Food Science &
683 Technology, 81:172–184.
684 Cordoba-Novoa, H. A., Cáceres-Zambrano, J., & Torres-Rojas, E. (2023). Isolation of native
685 cadmium-tolerant bacteria and fungi from cacao (Theobroma cacao L.) cultivated soils in
686 central Colombia. Heliyon, 9:e22489.
687 Deng, W., et al. (2024). Efficient degradation and detoxification of structurally different dyes
688 and mixed dyes by LAC-4 laccase purified from white-rot fungi Ganoderma lucidum.
689 Ecotoxicology and Environmental Safety, 279:116450.
690 Dutra, J. C. F., et al. (2023). Anaerobic digestion using cocoa residues as substrate: Systematic
691 review and meta-analysis. Energy for Sustainable Development, 72:265–277.
692 El-Batal, A. I., Elkenawy, N. M., Yassin, A. S., & Amin, M. A. (2015). Laccase production by
693 Pleurotus ostreatus and its application in synthesis of gold nanoparticles. Biotechnology
694 Reports, 5:31–39.
695 Emoh, P. C. D., Betote Diboue, P. H., Foko Dadji, G. A., Assam Assam, J. P., Biabi Abite, M.
696 F. A., Tchamgoue Ngalani, E., & Nyegue, M. A. (2025). Morphological and molecular
697 characterization of endophytic fungi isolated from Alstonia boonei De Wild. Scientific
698 African, 27:e02550.
699 García-Fuentevilla, L., Eugenio, M. E., Martín-Sampedro, R., & Ibarra, D. (2025).
700 Applicability of a laccase from the eucalypt wood endophytic fungus Hormonema sp.
701 CECT-13092 for advanced bioethanol production. New Biotechnology, 87:60–71.
702 Gompertz, B. (1825). On the nature of the function expressive of the law of human mortality,
703 and on a new mode of determining the value of life contingencies. Philosophical
704 Transactions of the Royal Society of London, 115:513–585.

Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., & Sessitsch, A. (2015). The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiology and Molecular Biology Reviews, 79:293–320.

Jeyabalan, J., Veluchamy, A., & Narayanasamy, S. (2025). Production optimization, characterization, and application of a novel thermo- and pH-stable laccase from Bacillus drentensis 2E for bioremediation of industrial dyes. International Journal of Biological Macromolecules, 308:142557.

Kiaei, F., Shahverdi, A. R., Delnavazi, M. R., Faramarzi, M. A., & Mojtabavi, S. (2025). Enhanced laccase production by Paramyrothecium roridum under solid-state fermentation: Application in neutral-pH delignification and valorization of acorn shells. International Journal of Biological Macromolecules, 314:146535.

Kyomuhimbo, H. D., & Brink, H. G. (2023). Applications and immobilization strategies of the copper-centered laccase enzyme: A review. Heliyon, 9:e13156.

Ladole, M. R., Pokale, P. B., Patil, S. S., Belokar, P. G., & Pandit, A. B. (2020). Laccase immobilized peroxidase mimicking magnetic metal organic frameworks for industrial dye degradation. Bioresource Technology, 317:124035.

Le, X. A., Dang, T. V., & Kim, M. I. (2025). Peroxidase-laccase dual enzymatic activities of bovine serum albumin-copper hybrid nanoflowers for detecting ascorbic acid and degrading phenolic dyes. International Journal of Biological Macromolecules, 146912.

Levenberg, K. (1944). A method for the solution of certain nonlinear problems in least squares. Quarterly of Applied Mathematics, 2:164–168.

Lima, C. S., et al. (2022). Pestalotiopsis mangiferae isolated from cocoa leaves and concomitant tannase and gallic acid production. Fungal Biology, 126:471–479.

729 Marquardt, D. W. (1963). An algorithm for least-squares estimation of nonlinear parameters.
730 SIAM Journal on Applied Mathematics, 11:431–441.
731 Michaelis, L., & Menten, M. (1913). Die Kinetik der Invertinwirkung. Biochemische
732 Zeitschrift, 49:333–369.
733 Miyawaki, A., & Sakai, S. (2025). Degradation of azo dyes using laccase immobilized on
734 mechanically ground silk fibroin nanofibers. Bioresource Technology, 399:133251.
735 Osogo, A. K., Muyekho, F., Were, H., & Okoth, P. (2025). Deciphering common bean
736 (Phaseolus vulgaris L.) microbiome assemblages reveal mechanistic insights into host-
737 pathogen-microbiome interactions. Genomics, 117:111064.
738 Otero, I. V. R., Haslbeck, M., Santello, L. C., Ferreira, H., Sieber, V., & Sette, L. D. (2025).
739 Heterologous laccase from the marine environment: Purification, characterization, and
740 degradation of synthetic dyes. Biocatalysis and Agricultural Biotechnology, 63:103485.
741 Park, N., & Park, S. S. (2014). Purification and characterization of a novel laccase from
742 Fomitopsis pinicola mycelia. International Journal of Biological Macromolecules, 70:583–
743 589.
744 Rajesh, P. S., & Rai, V. R. (2013). Hydrolytic enzymes and quorum sensing inhibitors from
745 endophytic fungi of Ventilago madraspatana Gaertn. Biocatalysis and Agricultural
746 Biotechnology, 2:120–124.
747 Revathy, M. A. S., & Kesavan, D. (2024). Endophytic fungi of spurred mangrove, Ceriops
748 tagal, and its bioactivity potential: Predominance of Aspergillus species and its ecological
749 significance. Microbe, 4:100144.
750 Sartini, et al. (2013). Production, partial purification and biochemical characterization of
751 polyphenol oxidase from Trichothecium sp. (endophytic fungus of cocoa pod husk). Asian
752 Journal of Microbiology, Biotechnology and Environmental Sciences, 15:621–626.

753 Sopalun, K., & Iamtham, S. (2020). Isolation and screening of extracellular enzymatic activity
754 of endophytic fungi isolated from Thai orchids. South African Journal of Botany, 134:273–
755 279.
756 Su, X., Yang, X., Long, H., Li, Y., Sun, W., Mo, T., & Su, J. (2024). Construction of
757 immobilized laccase hydrogels via sodium alginate-dopamine/polyethylene glycol and its
758 efficient degradation of dyeing wastewater. International Journal of Biological
759 Macromolecules, 279:134929.
760 Thakur, B. K., & Kharwal, N. (2025). The soil-plant-microbe nexus: Mechanisms, interactions,
761 and implications for sustainable agriculture. Pedosphere, in press.
762 Tosto, A., et al. (2023). Simulating cocoa production: A review of modeling approaches and
763 gaps. Agricultural Systems, 206:103614.
764 Vacalie, E., Preda, D., Oancea, P., Leonties, A. R., Aricov, L., & Raducan, A. (2024). Effective
765 degradation of azo dyes by ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))
766 mediated laccase: Kinetic studies. Process Biochemistry, 145:311–319.
767 Vidhyashini, S. V., Sharmila, R., Hussain, S., Petkoska, A. T., & Jeelani, P. G. (2026).
768 Comparative bioactive compound profiling of endophytic fungi and their respective host
769 plants for sustainable drug discovery. Medical Microecology, 100172.
770 Waqar, S., Bhat, A. A., & Khan, A. A. (2023). Endophytic fungi: Unraveling plant–endophyte
771 interaction and the multifaceted role of fungal endophytes in stress amelioration. Plant
772 Physiology and Biochemistry, 206:108174.
773 Wei, Y., Xie, W., Wang, X., Chong, Q., Li, S., & Chen, Z. (2024). Photothermal degradation
774 of triphenylmethane dye wastewater by Fe_3O_4@C-laccase. International Journal of
775 Biological Macromolecules, 282:137053.

776 Yadav, A., Yadav, P., Singh, A. K., Sonawane, V. C., Bharagava, R. N., & Raj, A. (2021).
777 Decolourisation of textile dye by laccase: Process evaluation and assessment of its
778 degradation bioproducts. Bioresource Technology, 340:125591.
779 Zhang, C., Wang, C., Zhang, X., Yao, C., Zhu, L., Chai, X., ... & Qi, W. (2026). Recent
780 advances in laccase: From molecular characteristics to advanced applications in
781 biocatalysis, immobilization, and nanozymes. Journal of Environmental Chemical
782 Engineering, 121035.