

Novel Laccase Biosensors from Manihot Esculenta, Crantz -Associated Fungi: A Biotechnological Approach to Dopamine Quantification

Mariana Pontes Vieira

Centro Universitário da FEI

Patrícia Alessandra Bersanetti

Federal University of São Paulo

Vitor Stabile Garcia

University of Coimbra

Gerhard Ett

SENAI/CIMATEC

Andreia de Araújo Morandim-Giannetti (✉ andreia.morandim@gmail.com)

Centro Universitário da FEI

Research Article

Keywords: Laccase. Fungi. Chitosan biosensor. Dopamine. Quantification

Posted Date: February 2nd, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-3911769/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

This study investigates the association of endophytic fungi with the leaves and stems of cassava (*Manihot esculenta* Crantz), leading to the isolation and identification of 23 fungal strains. Of these, 18 fungi were associated with stems and 5 with leaves. Subsequent analysis revealed laccase activity in 7 fungi isolates, predominantly within those derived from stems. Notably, the fungus LV2, identified as a strain of *Xylaria* sp., exhibited the highest laccase activity ($0.03 \text{ U.mg}^{-1}.\text{min}^{-1}$). Optimal LV2 fungus growth and enzyme activity conditions were established at 11 days, pH 6.70, and 29°C ($0.051 \text{ U.mg}^{-1}.\text{min}^{-1}$). Utilizing the LV2 enzymatic extract, we developed a chitosan-based enzyme biosensor with STMP crosslinking for immobilization to detect low dopamine concentrations. The biosensor demonstrated optimal performance at pH 5.1 and 47.8°C , achieving a detection limit of $0.30 \mu\text{mol.L}^{-1}$ for dopamine.

1. Introduction

Polyphenol oxidase enzymes, classified as oxidoreductases, include laccases (benzenediol: oxygen oxidoreductases - EC 1.10.3.2), which are dimeric or tetrameric glycoproteins typically containing four copper atoms per monomer or phenol oxidase. Other examples of this enzyme class are peroxidases, horseradish peroxidase, and bilirubin oxidase [1–4]. These enzymes consist of approximately 500 amino acids and have molecular weights in the 60–85 kDa range, with a carbohydrate content of up to 25% [5–6]. Fungi, mainly white rot fungi, predominantly produce laccases. They can also be derived from plants, insects, archaea, bacteria, and other fungi [5, 7–9]. However, studies on enzyme production from fungi, including basidiomycetes, have been more prevalent due to the significant biodiversity of this microorganism class [9–11].

These enzymes catalyze the oxidation of several compounds, including phenolic compounds, amines, polycyclic aromatic hydrocarbons, methoxy-substituted phenols, and certain inorganic compounds. The reaction proceeds through phenoxyl radicals, reducing O_2 to H_2O via electron transfer [9, 12], hydrogen atom transfer, and ionic oxidation [13–14]. This process occurs at their active sites, which contain four copper atoms categorized into three types: T1 (mononuclear copper), T2 (typical copper), and T3 (coupled binuclear copper). T1 and T2 sites act as the primary electron acceptors, while T3 is reduced by intramolecular transfer of two electrons from T1 and T2 [9, 14].

Due to the diverse oxidation reactions catalyzed by laccases, they find applications in various industrial sectors, including food, cosmetic, textile, and pharmaceutical industries, waste treatments, biofuel production, and biocatalysis in biotechnological processes [5, 9, 15]. They are also utilized in organic synthesis for crosslinking polymeric materials and producing antibiotics, therapeutic drugs, anti-inflammatory drugs, and antioxidants [9, 16]. Moreover, laccases are involved in fiberboard synthesis, pith removal, pulp bleaching, paper production, and the development of biosensors [9, 17–19].

The study of biosensors, analytical devices that integrate biological material with transducers for analytical purposes, has been ongoing since 1970 for quantifying phenolic compounds. Recently, enzymatic biosensors using oxidoreductases, including laccase, have garnered increasing attention [20–22]. According to data extracted from Scopus databases using structured keywords such as "biosensor" and "laccase", 667 works, including articles and reviews, have been published since 1992, with 193 published after 2020. These studies focus on biosensor production for assessing several phenolic compounds. Notably, dopamine, a catecholamine with essential biological functions as a hormone and neurotransmitter linked to the central nervous, endocrine, and cardiovascular systems, serves as a marker for several diseases, such as schizophrenia, dementia, Alzheimer's, Parkinson's, and HIV infection [23–25]. The literature review reveals 58 works published since 1997 concerning laccase-based biosensors for dopamine quantification, obtained through a search using the keywords "biosensor," "laccase," and "dopamine" in Scopus databases (Fig. 1). However, limited literature exists on the use of chitosan matrices for biosensor production, with only one article reporting amperometric biosensors for quantifying dopamine and other phenolic compounds, such as pyrogallol, epicatechin, gallic acid, 1,2-dihydroxybenzoic acid, caffeic acid, chlorogenic acid, rutin, and catechin [26]. This underscores the significance of developing other biocomponents using this highly applicable and cost-effective biopolymer.

Considering the importance of exploring novel sources of laccases and developing new devices capable of quantifying neurotransmitters like dopamine at low concentrations, this study investigates the production of laccases from fungi associated with cassava stems and leaves. The optimal conditions for obtaining laccase from the most promising fungus (LV2) are also examined. Subsequently, the laccase-rich extract from LV2 produces chitosan biosensors, optimizing their operating conditions for quantifying dopamine at low concentrations.

2. Materials and Methods

2.1. Materials

Endophytic fungi were isolated from stems and leaves of cassava (*Manihot Esculenta*, Crantz) and maintained at Centro Universitário FEI. Potato dextrose agar (PDA), chitosan (low molecular weight), syringaldazine, malt extract, glucose, peptone, serum ovalbumin, ammonium sulfate, graphite (100 mesh), Tween 80, trisodium trimetaphosphate (STMP), polyvinylpolypyrrolidone (PVPP), coomassie blue, L-dopamine, gentamicin sulfate, and sodium hydroxide pellets ($\geq 98\%$) were supplied by Sigma–Aldrich (USA). Acetic acid, sodium acetate, copper sulfate, potassium phosphate monobasic, sodium phosphate monobasic, magnesium sulfate heptahydrate, phosphoric acid, sodium hypochlorite, and absolute ethanol were purchased from Neon (Brazil). Nanopure water ($> 18 \text{ M}\Omega$) was prepared using a Millipore purifier (USA).

2.2. Attainment of endophytic fungi

Leaves and stems of cassava were submitted to the sterilization process using sodium hypochlorite (2% w/v), ethanol (70% v/v), and autoclaved water. Afterward, the material was cut aseptically and submitted to growth in a potato dextrose agar (PDA) medium autoclaved. Gentamicin sulfate ($30 \mu\text{g.mL}^{-1}$) was auditioned in this medium. The cultivation occurred at 23°C , in the absence of light, in a Panasonic MIR-254 Refrigerator Incubator (Panasonic Healthcare, Japan), monitoring the development of the fungi until each species reached 1 to 2 cm of diameter (ten days). Subsequently, subculturing was carried out to obtain pure fungi lineages in 10-mL sealed tubes containing a PDA culture medium. The samples were placed in a refrigerator at 4°C [2].

All the isolated fungi (ST1 - *Microdochium lycopodium*, ST2 - *Colletotrichum* sp., ST3 - *Cladosporium* sp., ST4 - *Colletotrichum brevisporum*, ST5 - *Alternaria* sp., ST6 - *Phyllosticta elongate*, ST7 - *Phomopsis* sp., ST8 - *Diaporthe phaseolorum*, ST9 - *Diaporthe endophytica*, ST10 - *Vouchered mycorrhizae*, ST11 - *Phanerochaete australis*, ST12 - *Diaporthe caatingaensis*, ST13 - *Stenocarpella maydis*, ST14 - *Annulohyphoxylon stygium*, ST15 - *Sordariomycetes* sp., ST16 - *Colletotrichum gloeosporioides*, ST17 - *Phanerochaetaceae* sp., ST18 - *Guignardia mangiferae*, LV1 - *Cladosporium xanthochromaticum*, LV2 - *Xylaria* sp., LV3 - *Phaeosphaeria podocarpi*, LV4 - *Peniophora* sp., and LV5 - *Gloeotinia temulenta*) were identified by using automated sequencing by capillary electrophoresis in an ABI 3500 Genetic Analyzer (Applied Biosystems). The alignment of nucleotide sequences produced was compared with reference sequences from GenBank for each fungus.

2.3. Attainment of enzymatic extracts

During the attainment of enzymatic extracts, the isolated mycelia of fungi were frozen with liquid N_2 , ground in the presence of PVPP in a mini processor Cadence Easy Cut Colors MPR514 (USA), and submitted to enzymatic extraction using a sodium acetate buffer (0.2 mol.L^{-1} , pH 4.6). Subsequently, the enzymatic extracts from the fungi were submitted to centrifugation in a Hettich Universal 32R Refrigerated Centrifuge (Germany) for 50 min at 4,000 rpm and 4°C .

All the enzymatic extracts attainment was analyzed by the Bradford method [27] to determine their protein concentration and also by the activity of laccases through the analysis of absorbance at 525 nm after syringaldazine oxidation ($\epsilon = 6.5 \times 10^4 \text{ M}^{-1}.\text{cm}^{-1}$), present in the concentration of 1 g.L^{-1} on a Quimis Spectrophotometer (Brazil). All reactions were processed for 10 min at 23°C . The enzyme activity expressed in Unit (U) was defined as the number of enzymes that catalyze $1 \mu\text{mol}$ of syringaldazine in one minute under the assay's reaction conditions. The fungus with the highest laccase production was submitted to growth in a specific liquid medium to obtain laccase (LML).

2.4. Laccase production in a liquid medium (LML)

During the growth of the fungi, a liquid medium composed of a malt extract solution (1.25% w/v), Tween 80 (0.10% w/v), CuSO_4 (0.005% w/v), KH_2PO_4 (1.0 g.L^{-1}), Na_2HPO_4 (0.26 g.L^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.50 g.L^{-1}), glucose (10.0 g.L^{-1}) and peptone (1% m/v) was prepared. This medium was autoclaved for 15 min at 1.5 atm, followed by the addition of $30 \mu\text{g.mL}^{-1}$ of gentamicin sulfate. All fungi that showed high

laccase activity were grown for 12 days at 25°C, 120 rpm, and evaluated for laccase activity using the syringaldazine method.

After this stage, the influence of growth time, temperature, and pH of the microorganisms that showed higher laccase measurement (LV2) was evaluated in a liquid medium. The activity of laccases was assessed through the analysis by the syringaldazine method after growth at different times, pHs, and temperatures. The culture media were transferred to Falcon tubes and vortexed for two min in the presence of glass beads. The culture broths were centrifuged at 4,000 rpm for 45 min at 4°C, and the supernatants were separated for the laccase activity determination.

The influence of pH, time, and temperature was studied using the Central Composite Rotatable Design (CCRD) with two center points. pH, time, and temperature were treated as input variables, and the laccase activity was treated as an output variable (Table 1).

The data were analyzed with the Statistica 14.0.0.15 software (TIBCO Software Inc, USA), and the results were evaluated using contour surface analysis. The optimal conditions of pH, time, and temperature for the growth of the LV2 fungus to produce laccase were optimized by developing the desirability function. New cultivation was performed under the best condition for fungal growth (time = 11 days, pH = 6.7, and temperature = 29°C), and the enzyme extract attainment was applied to produce chitosan biosensors for dopamine quantification.

Table 1
pH, time, and temperature used to produce laccase.

Coded variable			Uncoded variable		
pH	Time (day)	Temperature (°C)	pH	Time (day)	Temperature (°C)
$-\sqrt{2}$	0	0	2.64	9.0	29.0
-1	-1	-1	4.00	5.0	23.0
-1	-1	+1	4.00	5.0	35.0
-1	+1	-1	4.00	13.0	23.0
-1	+1	+1	4.00	13.0	35.0
0	$-\sqrt{2}$	0	6.00	2.3	29.0
0	0	$-\sqrt{2}$	6.00	9.0	18.9
0	0	0	6.00	9.0	29.0
0	0	0	6.00	9.0	29.0
0	$+\sqrt{2}$	0	6.00	15.7	29.0
0	0	$+\sqrt{2}$	6.00	9.0	39.1
+1	-1	-1	8.00	5.0	23.0
+1	-1	+1	8.00	5.0	35.0
+1	+1	-1	8.00	13.0	23.0
+1	+1	+1	8.00	13.0	35.0
$+\sqrt{2}$	0	0	9.36	9.0	29.0

2.5. Production of enzymatic biosensors

The polymeric matrix was prepared for laccase immobilization. Chitosan was solubilized in a 5% (m/V) acetic acid solution using a 1% concentration. An STMP solution of 2% (m/V) was added to this mixture. The system was under agitation for 30 minutes, dried in an oven at 60°C, and ground in a mortar. After this step, 0.375g of graphite, 0.05g of the polymeric matrix (200 µL of laccase were added - enzymatic

activity = $0.45 \text{ U.mg}^{-1}.\text{min}^{-1}$), and 0.075g of mineral oil were mixed. The system was subjected to homogenization for 5 min and used to produce biosensors.

FTIR analyses were performed in the Fourier Transform Infrared Spectroscopy Nicolet with Attenuated Total Reflectance (FTIR-ATR) model 6700 (USA). The data were obtained using 64 scans per spectrum at a spectral resolution of 4 cm^{-1} in the wavenumber range from 4,000 to 450 cm^{-1} . The X-ray diffractometer (XRD-7000 Model, Shimadzu, Japan) obtained the XRD data. The operating voltage and current were maintained at 40 kV and 35 mA, respectively. The diffraction spectra were collected using the $\theta-2\theta$ method. The samples were scanned in the 2θ 5–40° ranges in steps of 0.015°. The radiation (Cu K α -ray source) wavelength was 1.5406 Å. The crystallinity index was determined using the ratio between the crystalline and total areas.

2.6. Optimization of dopamine quantification

The influence of pH and temperature on dopamine quantification was studied using the Central Composite Rotatable Design (CCRD) with two center points. pH and temperature were treated as input variables, and the maximum anode and cathode currents (I , μA) as the response variable (Table 2). The data were analyzed with the Statistica 14.0.0.15 software (TIBCO Software Inc, USA). The results were evaluated using contour surface analysis and determining the ideal condition to work within the quantifications to determine the lowest concentrations possible. The concentration of the dopamine solution used during the study was $3.3 \mu\text{mol.L}^{-1}$, and it was prepared in phosphate buffer, modifying the pH according to the stipulated statistical planning.

Table 2
pH and temperature used to determine the best condition
for dopamine quantification.

Coded variable		Uncoded variable	
pH	Temperature (°C)	pH	Temperature (°C)
$-\sqrt{2}$	0	5.6	45
-1	-1	6.0	30
-1	+1	6.0	60
0	$-\sqrt{2}$	7.0	23.8
0	0	7.0	45
0	0	7.0	45
0	$+\sqrt{2}$	7.0	66.2
+1	-1	8.0	30
+1	+1	8.0	60
$+\sqrt{2}$	0	8.4	45

All electrochemical experiments by cyclic voltammetry were performed in a conventional three-electrode cell with a working volume of 120 mL using a METROHM AUTOLAB-BP25483 Potentiostat (Brazil). A potential interval between 0.5 and 1 mVs⁻¹ was used during the analysis. Two sweeps per analysis were used at a rate of 0.3 mV.s⁻¹ and a time interval of 24.4ms. The software used to carry out the studies was NOVA 2.0, and Origin 2018 (USA) was used to evaluate the results. The produced biosensor sat as the working electrode, an Ag/AgCl. KCl is a reference electrode, and platinum foil is an auxiliary electrode. After this step, solutions with different dopamine concentrations were analyzed (0.17 to 1.67 $\mu\text{mol.L}^{-1}$), and the minimum possible concentration to be evaluated using the produced biosensor was determined. For this, analyses were carried out by cyclic voltammetry using the same parameters described.

3. Results and Discussion

Pure fungal lineages associated with cassava leaves (LV) and stems (ST) were successfully isolated. Table 3 shows the identification of eighteen fungi from the stems and five fungi from the leaves. Notably, six fungi derived from the stems (ST1 – *Microdochium lycopodium*, ST6 - *Phyllosticta elongata*, ST9 - *Diaporthe endophytica*, ST12 - *Diaporthe caatingaensis*, ST13 - *Stenocarpella maydis*, and ST15 -

Sordariomycetes sp) and one fungus from the leaves (LV2 - *Xylaria* sp.) displayed significant laccase activity.

Fungi ST1, ST15, and LV2 exhibited the highest potential for laccase production. These findings align with previous studies by Fillat et al. (2016) and Hassan et al. (2019), which reported the ability of the *Ascomycotas* species, particularly those belonging to the Xylariales, Sordariomycetes, and Dothideomycetes orders, to produce oxidoreductase enzymes like laccases [28–29]. Additionally, Castaño et al. (2015) and Liers et al. (2007) highlighted the laccase expression by *Xylaria* sp., emphasizing the high potential of this species for laccase production [30–31]. Furthermore, Bankole and collaborators (2018) demonstrated laccase production in *Xylaria* polymorpha. These studies provide a rationale for the observed robust laccase activity during the selection of laccase-producing fungi [32].

Table 3
Identification of fungi associated with cassava leaves and stems.

Code	Identified Species	GenBank accession numbers (ITS5 + ITS4)	Phylum	Order	Specific activity (U.mg ⁻¹ .min ⁻¹)
ST1	<i>Microdochium lycopodium</i>	NR_145223	Ascomycota	Xylariales	0.010
ST2	<i>Colletotrichum</i> sp.	MF076603.1	Ascomycota	Glomerellales	0.001
ST3	<i>Cladosporium</i> sp.	MH047202.1	Ascomycota	Capnodiales	0.000
ST4	<i>Colletotrichum brevisporum</i>	KU319458.1	Ascomycota	Glomerellales	0.000
ST5	<i>Alternaria</i> sp.	EF432274.1	Ascomycota	Pleosporales	0.000
ST6	<i>Phyllosticta elongata</i>	EU167584.1	Ascomycota	Botryosphaerales	0.005
ST7	<i>Phomopsis</i> sp.	KM979805.1	Ascomycota	Diaporthales	0.001
ST8	<i>Diaporthe phaseolorum</i>	AY577815.1	Ascomycota	Diaporthales	0.000
ST9	<i>Diaporthe endophytica</i>	AB899789.1	Ascomycota	Diaporthales	0.003
ST10	<i>Vouchered mycorrhizae</i>	AB449173.1	Basidiomycota	Unclassified	0.000
ST11	<i>Phanerochaete australis</i>	o KP135075.1	Basidiomycota	Polyporales	0.000
ST12	<i>Diaporthe caatingaensis</i>	KY085926.1	Ascomycota	Diaporthales	0.003
ST13	<i>Stenocarpella maydis</i>	MF076624.1	Ascomycota	Diaporthales	0.003
ST14	<i>Annulohypoxyton stygium</i>	MG649252.1	Ascomycota	Xylariales	0.000
ST15	<i>Sordariomycetes</i> sp.	MF076626.1	Ascomycota	Sordariomycetes	0.010
ST16	<i>Colletotrichum gloeosporioides</i>	KX197387.1	Ascomycota	Glomerellales	0.001
ST17	<i>Phanerochaetaceae</i> sp.	HM595569.1	Basidiomycota	Polyporales	0.000
ST18	<i>Guignardia mangiferae</i>	EU273524.1	Ascomycota	Botryosphaerales	0.000

Code	Identified Species	GenBank accession numbers (ITS5 + ITS4)	Phylum	Order	Specific activity (U.mg ⁻¹ .min ⁻¹)
LV1	<i>Cladosporium xanthochromaticum</i>	MF473323.1	Ascomycota	Capnodiales	0.000
LV2	<i>Xylaria</i> sp.	KR093861.1	Ascomycota	Xylariales	0.030
LV3	<i>Phaeosphaeria podocarpi</i>	NR_137933.1	Ascomycota	Pleosporales	0.000
LV4	<i>Peniophora</i> sp.	HQ608131.1	Basidiomycota	Russulales	0.000
LV5	<i>Gloeotinia temulenta</i>	DQ235697.1	Ascomycota	Hypocreales	0.000

The functionality and synthesis of laccases are influenced by the distribution of copper atoms within the binding sites and the nutrient concentrations (carbon, nitrogen, inducers, and copper) used during microbial growth and the enzyme expression process [5, 33]. In light of this, the fungi displaying the highest laccase activity (ST1, ST15, and LV2) were cultivated in a nutrient-rich medium designed explicitly for laccase production (LML). The results revealed that the extracts obtained from ST15 - *Sordariomycetes* sp. displayed a laccase activity of 0.040 U.mg⁻¹.min⁻¹, while those from LV2 - *Xylaria* sp. exhibited even higher enzymatic activity at 0.051 U.mg⁻¹.min⁻¹ (Fig. 2). Given the remarkable activity of LV2, it was further subjected to growth in a liquid medium, and specific growth conditions (pH, time, and temperature) were adjusted to enhance laccase production (Table 4).

Table 4
Enzyme activity data as a function of pH, time, and temperature

pH	Time (day)	Temperature	Specific activity (U.mg ⁻¹ .min ⁻¹)
8	5	35	0.000
6	15.7	29	0.019
4	5	35	0.000
6	9	39.1	0.000
6	9	18.9	0.002
8	13	35	0.000
4	13	35	0.000
6	9	29	0.044
6	9	29	0.041
8	13	23	0.034
4	5	23	0.000
4	13	23	0.017
9.36	9	29	0.030
8	5	23	0.000
6	2.3	29	0.000
2.64	9	29	0.000

Through the analysis of the response surfaces (Fig. 3), it was possible to observe that laccase production by the LV2 fungus is significantly influenced by temperature and pH. The results indicate that temperatures above 30°C and below 25°C and pH values below 5.0 and above 8.0 favor enhanced laccase production. These findings align with previous studies by Kittl et al. (2012) and Zhang et al. (2020), who also reported optimal pH conditions of approximately 5.0 and a temperature of 30°C for laccase production and activity in other fungal species [34–35].

The remarkable resemblance between our study's optimal conditions and those reported in the literature further reinforces the validity and reliability of these experimental findings. Additionally, it was possible to find that the growth of the LV2 fungus for periods exceeding 7 days was essential for achieving optimal laccase production. This prolonged cultivation period is likely attributed to the necessary activation of the fungus's metabolic system and the time required for sufficient excretion of the enzyme of interest. The extended growth period contributes to laccase yield and ensures the attainment of enzymatic activity at desirable levels. Overall, this study sheds light on the critical factors influencing laccase production in the

LV2 fungus, offering valuable insights into optimizing growth conditions for enhanced laccase synthesis, which holds great promise for various biotechnological applications.

It was determined that the optimal conditions for laccase production by *Xylaria* sp. (LV2) were a temperature of 29°C, a pH of 6.7, and a cultivation time of 11 days, as evidenced in Fig. 4, confirming the results obtained from response surface analyses. Comparing these data with findings from the literature, a significant enzymatic activity, particularly for LV2, was observed. In studies conducted by Mtibaa et al. (2018), maximum laccase activity of 0.26 U.mg⁻¹ was observed after eight days of growth for the ascomycete *Thielavia* sp. [36]. Chaurasia et al. (2014) reported a maximum laccase activity of 0.36 U.mg⁻¹ after seven days for *Trametes hirsuta* MTCC-1171 [37]. Fillat et al. (2016) observed a maximum laccase activity of 0.25 U.L⁻¹ after 20 days for *Neofusicoccum luteum*, 0.18 U.L⁻¹ after 20 days for *Neofusicoccum australe*, and 0.15 U.L⁻¹ after 30 days for *Hormonema* sp. [28]. Our study's observed optimal conditions for laccase production by *Xylaria* sp. (LV2) provide valuable insights for biotechnological applications, suggesting its potential as an efficient enzyme producer under specific environmental and cultivation conditions. Further growth was performed under this condition after determining the best growth conditions of the LV2 fungus in a liquid medium to produce laccase. The enzyme extract attainment produced a chitosan biosensor for quantifying dopamine in synthetic body fluids.

The extract enriched in laccase was applied to produce the biosensor matrix. Therefore, the polymeric matrix of chitosan cross-linked with STMP was obtained, and later, the enzyme was immobilized. Analyzing the FTIR spectrum (Fig. 5A), the reduction of the band referring to the O-H bond after the synthesis of the matrix and after the immobilization of the enzyme at 3,400 cm⁻¹ is verified. This reduction is due to the presence of interactions between chitosan hydroxyls and both STMP and laccase. It is also possible to verify a significant increase in the band referring to the C-N bond at approximately 1,100 cm⁻¹ due to the presence of the laccase enzyme immobilized in the matrix, as well as at about 990 cm⁻¹ referring to out-of-plane angular deformations of the N-H bonds of the amino acids present in the laccase enzyme. It is also possible to identify a band in the region between 600 and 700 cm⁻¹ referring to the C-S bond as well as a band referring to the presence of the thiocarbonyl group at approximately 1,280 cm⁻¹, which confirms the incorporation of STMP to chitosan due to interactions between the molecules. These data and the enzymatic activity for laccase after obtaining the matrix used to produce the biosensor confirm the effectiveness of enzymatic immobilization.

Analyzes via DRX (Fig. 5B) corroborate the data obtained via FTIR, verifying that crystallinity increased after matrix synthesis and reduced after laccase enzyme immobilization. This fact can be explained by introducing a salt (trisodium trimetaphosphate-STMP) into the matrix, which is responsible for forming cross-links between the chitosan chains. However, it is possible to note that the crystallinity index of the matrix decreased with the incorporation of the enzyme laccase due to the breaking of the chemical bonds between the chitosan chains and the modification of the functional groups of the material. These modifications may result in a breakage or modification in the crystalline structure of chitosan, leading to

a decrease in the crystallinity index (%). Lacase can also interact with the chitosan matrix through non-covalent bonds or Van der Waals forces. These interactions can disturb the organization of chitosan chains, making the matrix less crystalline and more amorphous. A crystallinity percentage of 25.41% was verified for chitosan, 45.46% for chitosan after modification with STMP (CH-STMP), and 37.06% after laccase immobilization (CH-STMP-laccase).

The matrix obtained was used in the biosensor production, and during the optimization stage of the analysis conditions, its sensitivity was evaluated as a function of pH and temperature changes. Analyzing the results of cyclic voltammograms (Fig. 6A), it is verified that both variables interfere with increasing the response signal referring to the cathodic reaction (I , μA); however, the temperature interferes more significantly in the anodic response (I , μA). It is also possible to confirm that the analysis at pHs from 5.0 to 6.0 showed the most incredible sensitivity. The best results were achieved at temperatures between 40 and 50°C (Fig. 6B). It is also important to emphasize that the temperature favors the increase in the intensity of the response signal referring to the anodic reaction (oxidation). However, a reduction in the signal intensity related to the cathodic reaction can also be verified, making it difficult to control the enzymatic catalysis, leading to errors in the quantifications.

During the analysis of the voltammograms, it is possible to verify a positive redox potential with a value of approximately 0.2 V due to the movement of two electrons after the oxidation of dopamine. A reduction peak of approximately -0.25 V was also verified during cathodic scanning but with lesser intensity, which suggests that the reaction has low reversibility. Data analysis shows that, with increasing pH, there is a shift in the signal referring to the cathode peak (reduction reaction) to more negative potentials due to the increased difficulty of proton transfer at basic pH during dopamine analysis, which indicates that the dopamine oxidation reaction is pH-dependent. The separation between the anodic and cathode peak potentials was approximately 0.45 V, and the ratio of the anodic to cathode peak currents was about 0.53 A, which corroborates the low reversibility of the observed reaction. It is also possible to follow that with increasing temperature, there is a reduction in the peak of the cathodic response, indicating that, with increasing temperature, the occurrence of the reverse reaction becomes more difficult.

In this context, the best condition for dopamine quantification with the produced biosensor was also established (pH = 5.06 and temperature = 47.8°C) (Fig. 7). This condition allowed the quantification of dopamine in concentrations up to $0.30 \mu\text{mol.L}^{-1}$ (Fig. 8). Lower concentrations did not show satisfactory results. Li et al. (2021) verified the best biosensor responses at pH 7.3 and a temperature of 44.3°C [38]. Molinnus et al. (2018) also showed greater sensitivity of laccase biosensors when using a pH equal to 7.4 [39]. These works corroborate the obtained results and confirm the biosensor's efficiency for quantifying dopamine at low concentrations.

4. Conclusion

Twenty-three fungi from cassava leaves and stems were analyzed in this study to verify laccase activity. *Xylaria* sp. (LV2) showed the most significant potential to produce this enzyme. Therefore, a study was conducted to determine the best growth conditions, with an ideal growth time of 11 days, a pH equal to 6.7, and a temperature of 29°C.

It was also possible to verify the efficiency of applying the enzymatic extract obtained in the production of biosensors using the new CH-STMP immobilization matrix, optimizing its performance conditions for quantifying dopamine in concentrations of up to 0.30 $\mu\text{mol.L}^{-1}$ (pH = 5.06 and T = 47.8).

Declarations

Credit authorship contribution statement

MPV, VSG, and AAMG collected and analyzed data. PAB, GE, and AAMG carried out the manuscript writing. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest/competing interests that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analyzed during this study are included in this published article.

Acknowledgments

This work was supported by grants #2015/19273-2 and #2022/09183-0 from the São Paulo Research Foundation (FAPESP).

References

1. Zhang W, Zhang Z, Ji L, Lu Z, Liu R, Nian B, Hu Y (2023) Laccase immobilized on nanocomposites for wastewater pollutants degradation: current status and future prospects. *Bioprocess Biosyst Eng* 46(11):1513–1531.
2. Hoyos LV, Chaves A, Grandezz D, Medina A, Correa J, Ramirez-Castrillon M, Valencia D, Caicedo-Ortega NH (2023) Systematic screening strategy for fungal laccase activity of endophytes from *Otoba gracilipes* with bioremediation potential. *Fungal Biol* 127(9):1298–1311.
3. Wang S, Huang W, Feng Z, Tian X, Wang D, Rao L, Tan M, Roongsawang N, Song H, Wenxia Jiang Bai W (2023) Laccase-mediated formation of hydrogels based on silk-elastin-like protein polymers with ultra-high molecular weight. *Int J Biol Macromol* 231:123239.

4. Zhou M, Fakayode OA, Ren M, Li H, Liang J, Yagoub AEA, Fan Z, Zhou C (2023) Laccase-catalyzed lignin depolymerization in deep eutectic solvents: challenges and prospects. *Bioresour Bioprocess* 10(1):1–22.
5. Kyomuhimbo HD, Brink HG (2023) Applications and immobilization strategies of the copper-coated laccase enzyme; a review. *Heliyon* 9(2):e13156.
6. Wang YF, Xu H, Feng L, Shen XF, Wang C, Huo XK, Tian X-G, Ning J, Zhang B-J, Sun C-P, Deng S (2019) Oxidative coupling of coumarins catalyzed by laccase. *Int J Biol Macromol* 135:1028–1033.
7. Yang X, Gu C, Lin Y (2020) A novel fungal laccase from *Sordaria macrospora* k-hell: expression, characterization, and application for lignin degradation. *Bioprocess Biosyst Eng* 43:1133–1139.
8. Zhang LB, Deng ZQ, Qiu TT, Zhu F, Ye XY (2023) Characterization of a laccase isolated from *Trametes hirsuta* and its application in the oligomerization of phenolic compounds. *Fungal Biol* 127(1–2):872–880.
9. Hahn V (2023) Potential of the enzyme laccase for synthesizing and derivating antimicrobial compounds. *World J Microbiol Biotechnol* 39(4): 1–27.
10. Eren B, Zenger O, Başığmez HİO, Peşint GB (2023) Enhanced laccase separation from fermentation medium using cryogel columns. *J Biotechnol* 364:58–65.
11. Reis AC, Kolvenbach BA, Nunes OC, Corvini PF (2020) Biodegradation of antibiotics: the new resistance determinants—part II. *N Biotechnol* 54:13–27.
12. Yue SJ, Song C, Li S, Huang P, Guo SQ, Hu HB, Wang W, Zhang XH (2019) Synthesis of cinnabaric acid by metabolically engineered *Pseudomonas chlororaphis* GP72. *Biotechnol Bioeng* 116(11):3072–3083.
13. Morandim-Giannetti AA, Carvalho TS, Andrade Neto JC, Bersanetti, PA (2023) DSC evaluation of cross-link development induced by laccases in corneas. *J Therm Anal Calorim* 148(1):63–68.
14. Zhu P, Wang Y, Li G, Liu K, Liu Y, He J, Lei J (2019) Preparation and application of a chemically modified laccase and copper phosphate hybrid flower-like biocatalyst. *Biochem Eng J* 144:235–243.
15. Tarasov A, Stozhko N, Bukharinova M, Khamzina E (2023) Biosensors based on phenol oxidases (laccase, tyrosinase, and their mixture) for estimating the total phenolic index in food-related samples. *Life* 13(2):291.
16. Bersanetti PA, Escobar VH, Nogueira RF, Ortega, FS, Schor P, Morandim-Giannetti AA (2019) Enzymatically obtaining hydrogels of PVA crosslinked with ferulic acid in the presence of laccase for biomedical applications. *Eur Polym J* 112:610–618.
17. Singh G, Kumar S, Afreen S, Bhalla A, Khurana J, Chandel S, Aggarwal A, Arya SK (2023) Laccase mediated delignification of wasted and non-food agricultural biomass: Recent developments and challenges. *Int J Biol Macromol* 235:123840.
18. Negi BB, Das C (2023) Mycoremediation of wastewater, challenges, and current status: A review. *Bioresour Technol Rep* 22:101409.

19. Bai Y, Jing Z, Ma R, Wan X, Liu J, Huang W (2023). A critical review of enzymes immobilized on chitosan composites: characterization and applications. *Bioprocess Biosyst Eng* 46(11):1539–1567.
20. Silva LI, Ferreira FD, Freitas AC, Rocha-Santos TA, Duarte AC (2009) Optical fiber biosensor coupled to chromatographic separation for screening of dopamine, norepinephrine and epinephrine in human urine and plasma. *Talanta* 80(2):853–857.
21. Leite OD (2003) Synergic effect studies of the bi-enzymatic system laccase–peroxidase in a voltammetric biosensor for catecholamines. *Talanta* 59(5): 889–896.
22. Jiaojiao X, Pengyun W, Bin Z, Onyinye AI (2021). Enhancing electrochemical sensing for catechol by biomimetic oxidase covalently functionalized graphene oxide. *Bioprocess Biosyst Eng* 44:343–353.
23. Ohshiro K, Sasaki Y, Minami T (2023) An extended-gate-type organic transistor-based enzymatic sensor for dopamine detection in human urine. *Talanta* 7:100190.
24. Wang J, Huang R, Qi W, Su R, He Z (2022) Construction of biomimetic nanozyme with high laccase- and catecholase-like activity for oxidation and detection of phenolic compounds. *J Hazard Mater* 429:128404.
25. Shervedani RK, Amini A (2012) Direct electrochemistry of dopamine on gold–*Agaricus bisporus* laccase enzyme electrode: Characterization and quantitative detection. *Bioelectrochemistry* 84:25–31.
26. Vlamidis Y, Gualandi I, Tonelli D (2017) Amperometric biosensors based on reduced GO and MWCNTs composite for polyphenols detection in fruit juices. *J Electroanal Chem* 799:285–292.
27. Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
28. Fillat Ú, Martín-Sampedro R, Macaya-Sanz D, Martín JA, Ibarra D, Martínez MJ, Eugenio ME (2016) Screening of eucalyptus wood endophytes for laccase activity. *rocess Biochem* 51(5):589–598.
29. Hassan N, Rafiq M, Rehman M, Sajjad W, Hasan F, Abdullah S (2019) Fungi in acidic fire: A potential source of industrially important enzymes. *Fungal Biol Rev* 33(1):58–71.
30. Castaño JD, Cruz C, Torres E (2015) Optimization of the production, purification and characterization of a laccase from the native fungus *Xylaria* sp. *Biocatal Agric Biotechnol* 4(4):710–716.
31. Liers C, Ullrich R, Pecyna M, Schlosser D, Hofrichter M (2007) Production, purification and partial enzymatic and molecular characterization of a laccase from the wood-rotting ascomycete *Xylaria polymorpha*. *Enzyme Microb Technol* 41(6–7):785–793.
32. Bankole PO, Adekunle AA, Govindwar SP (2018) Biodegradation of a monochlorotriazine dye, cibacron brilliant red 3B-A in solid state fermentation by wood-rot fungal consortium, *Daldinia concentrica* and *Xylaria polymorpha*: Co-biomass decolorization of cibacron brilliant red 3B-A dye. *Int J Biol Macromol* s 120:19–27.
33. Unuofin JO, Okoh AI, Nwodo UU (2019) Utilization of agroindustrial wastes for the production of laccase by *Achromobacter xylosoxidans* HWN16 and *Bordetella bronchiseptica* HSO16. *J Environ Manage* 231: 222–231.

34. Kittl R, Mueangtoom K, Gonaus C, Khazaneh ST, Sygmund C, Haltrich D, Ludwig R (2012) A chloride tolerant laccase from the plant pathogen ascomycete *Botrytis aclada* expressed at high levels in *Pichia pastoris*. J Biotechnol 157(2):304–314.
35. Zhang C, You S, Liu Y, Wang C, Yan Q, Qi W, Su R, He Z (2020) Construction of luffa sponge-based magnetic carbon nanocarriers for laccase immobilization and its application in the removal of bisphenol A. Bioresour Technol 305:123085.
36. Mtibaa R, Barriuso J, Eugenio L, Aranda E, Belbahri L, Nasri M, Martínez MJ, Mechichi T (2018) Purification and characterization of a fungal laccase from the ascomycete *Thielavia* sp. and its role in the decolorization of a recalcitrant dye. Int J Biol Macromol 120:1744–1751.
37. Chaurasia PK, Yadav RS, Yadava S (2014) Purification and characterization of yellow laccase from *Trametes hirsuta* MTCC-1171 and its application in synthesis of aromatic aldehydes. Process Biochem 49(10):1647–1655.
38. Li J, Yu J, Sun Z, Liu H, Wang X (2021) Innovative Integration of Phase-Change Microcapsules with Metal–Organic Frameworks into an Intelligent Biosensing System for Enhancing Dopamine Detection. ACS Appl. Mater. Interfaces 13(35):41753–41772.
39. Molinnus D, Hardt G, Siegert P, Willenberg HS, Poghosian A, Keusgen M, Schöning MJ (2018) Detection of adrenaline in blood plasma as biomarker for adrenal venous sampling. Electroanalysis 30(5):937–942.

Figures

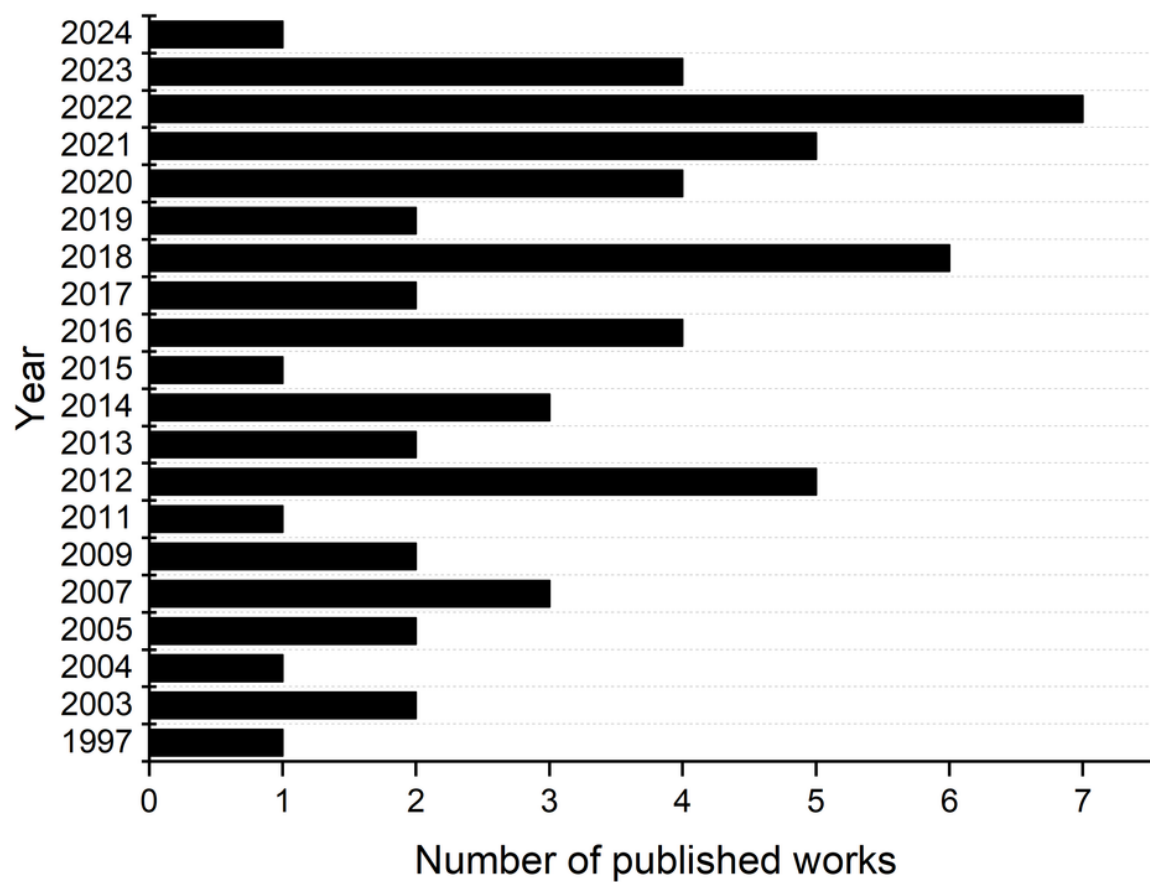


Figure 1

Relevant studies on enzymatic biosensors for dopamine quantification have been published since 1997.

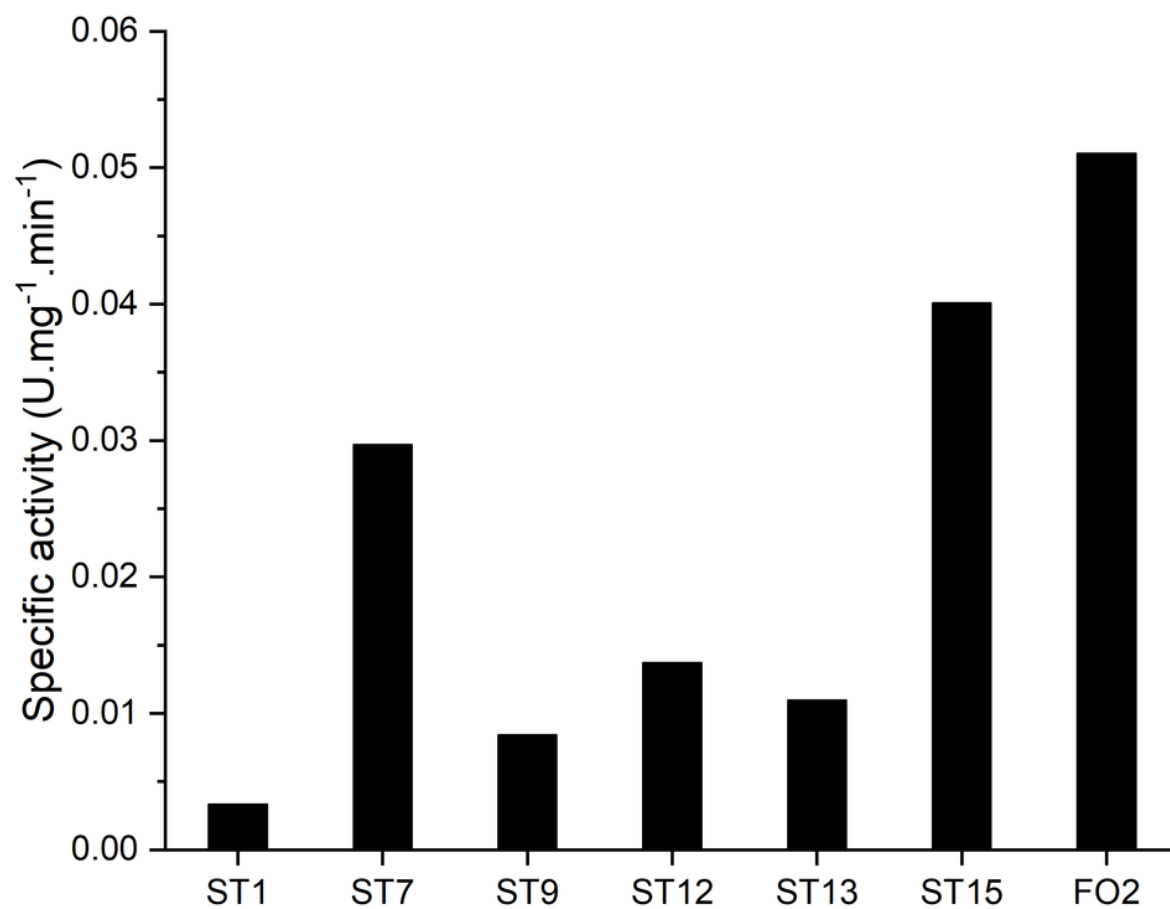


Figure 2

Specific activity for enzymatic extracts submitted to growth in an LML

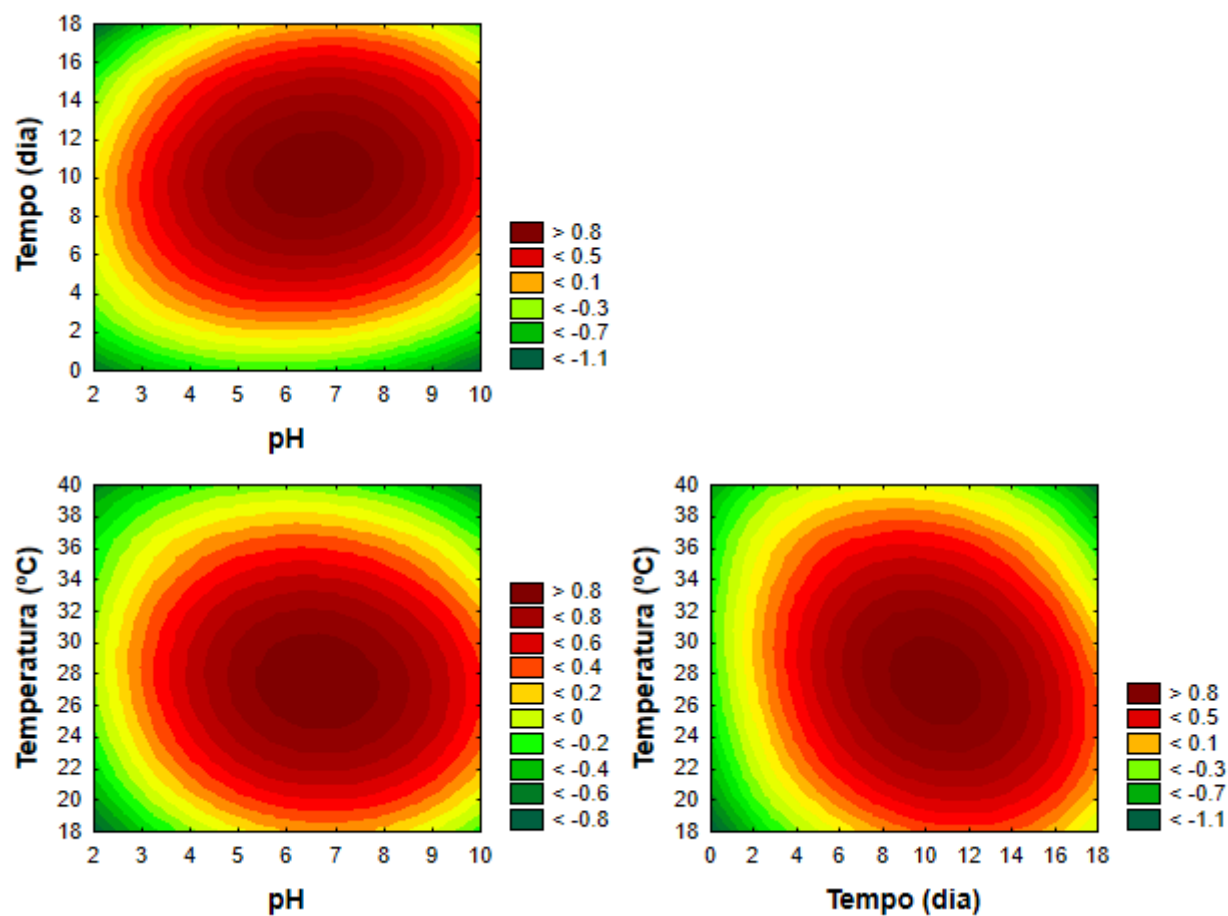


Figure 3

Influence of time, temperature, and pH on laccase production

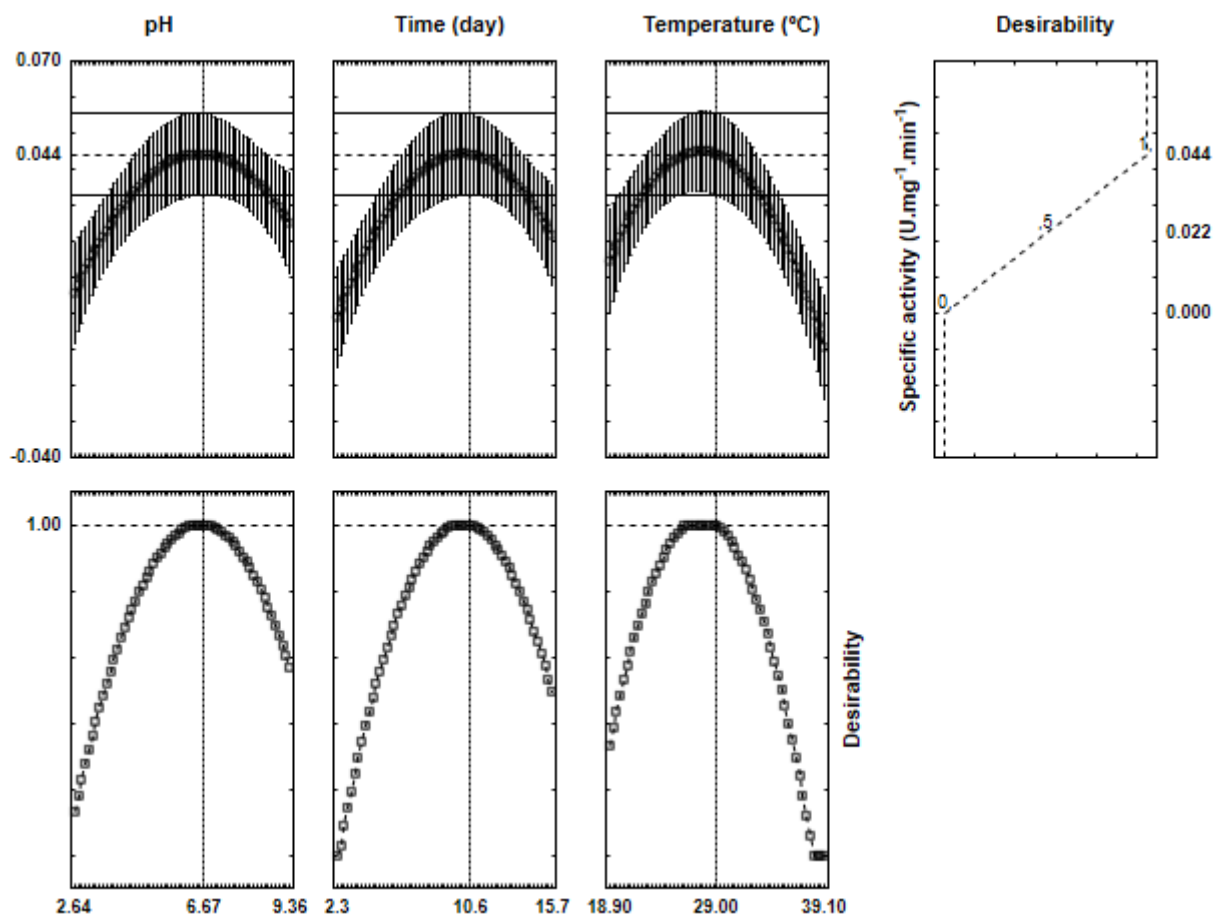


Figure 4

Optimal growth conditions of the LV2 fungus for laccase production

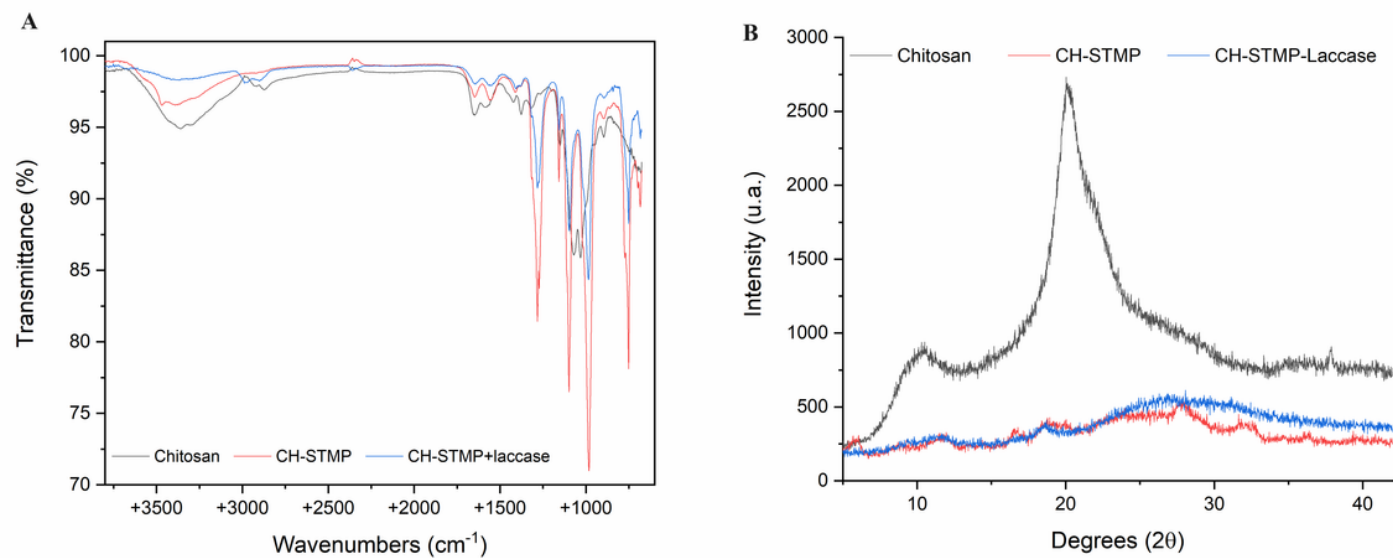


Figure 5

FTIR spectrum (A) and DRX graphics (B) of chitosan, CH-STMP matrix, and matrix after laccase immobilization

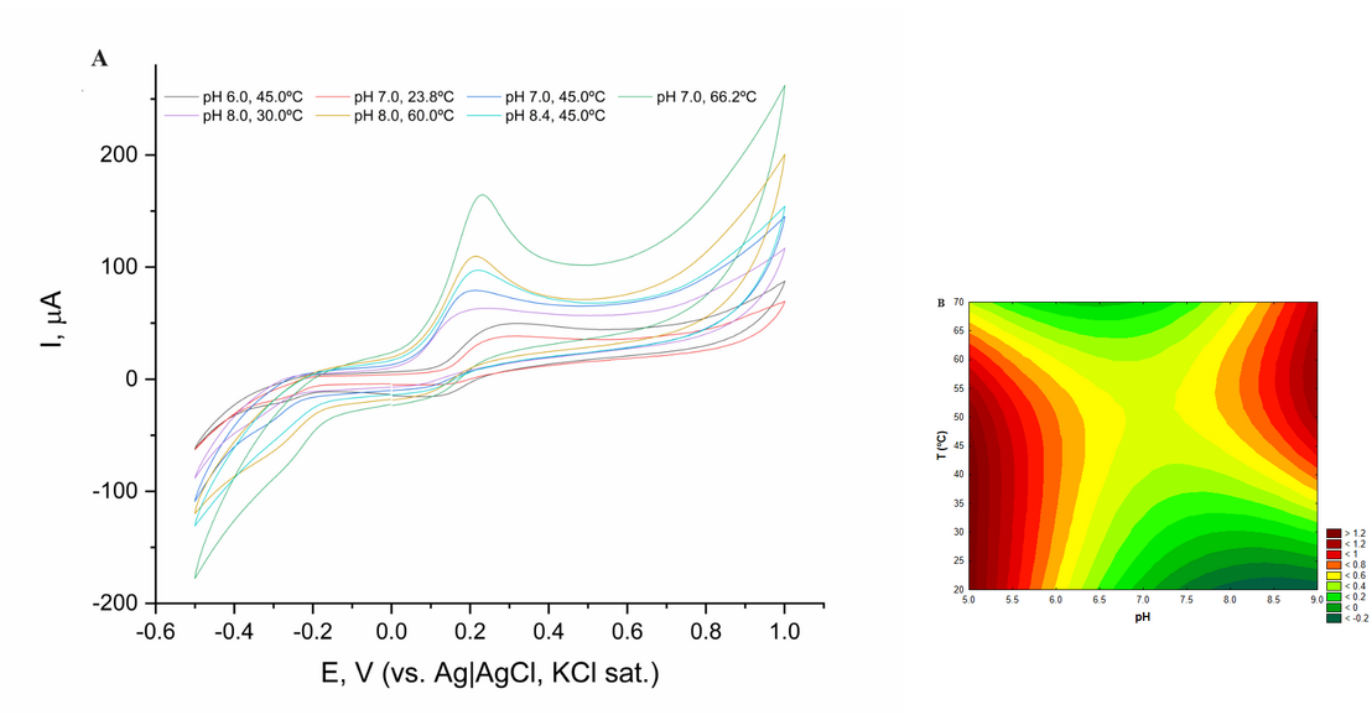


Figure 6

Evaluation of ideal working conditions for dopamine quantification via cyclic voltammetry: (A) Graphics obtained (B) Contour surface for dopamine quantification

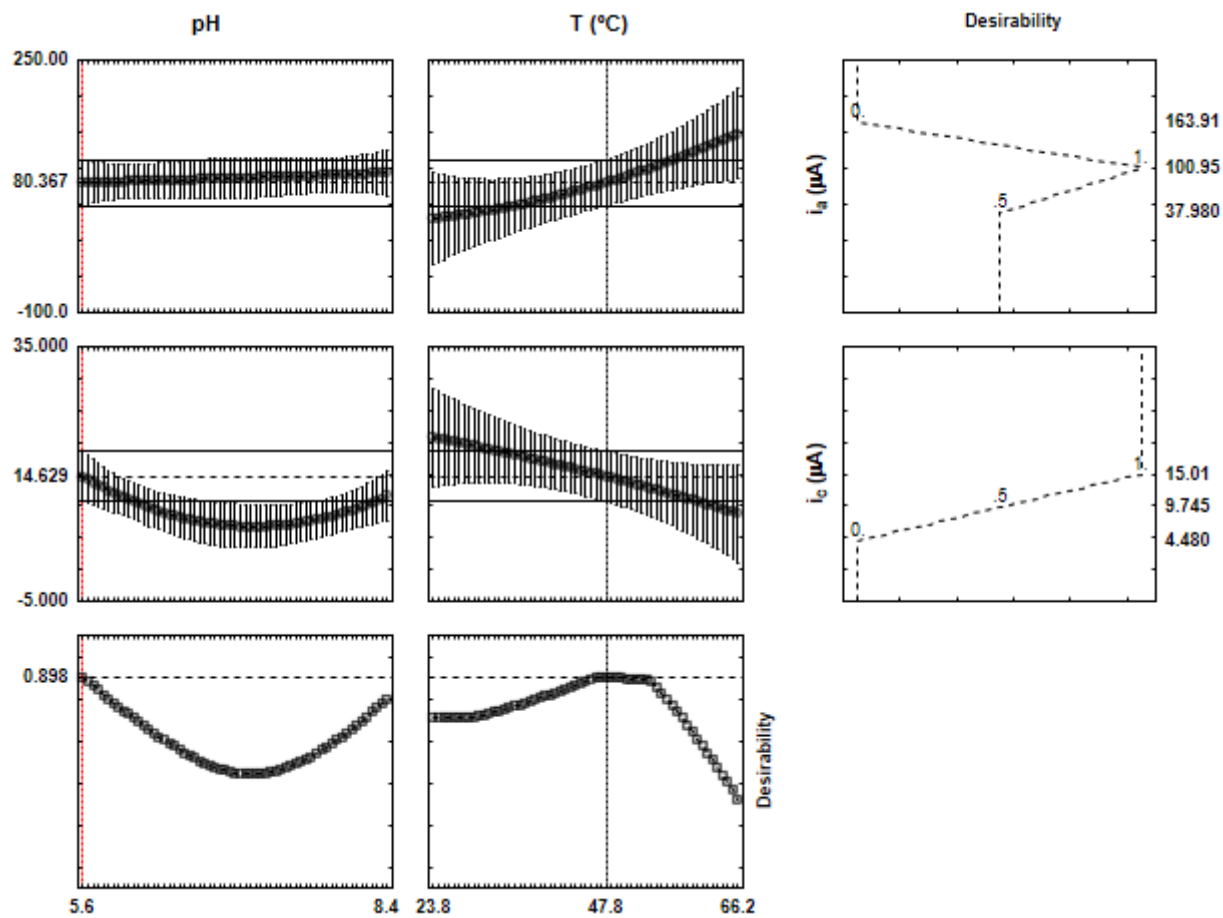


Figure 7

Optimization of dopamine analysis conditions by cyclic voltammetry

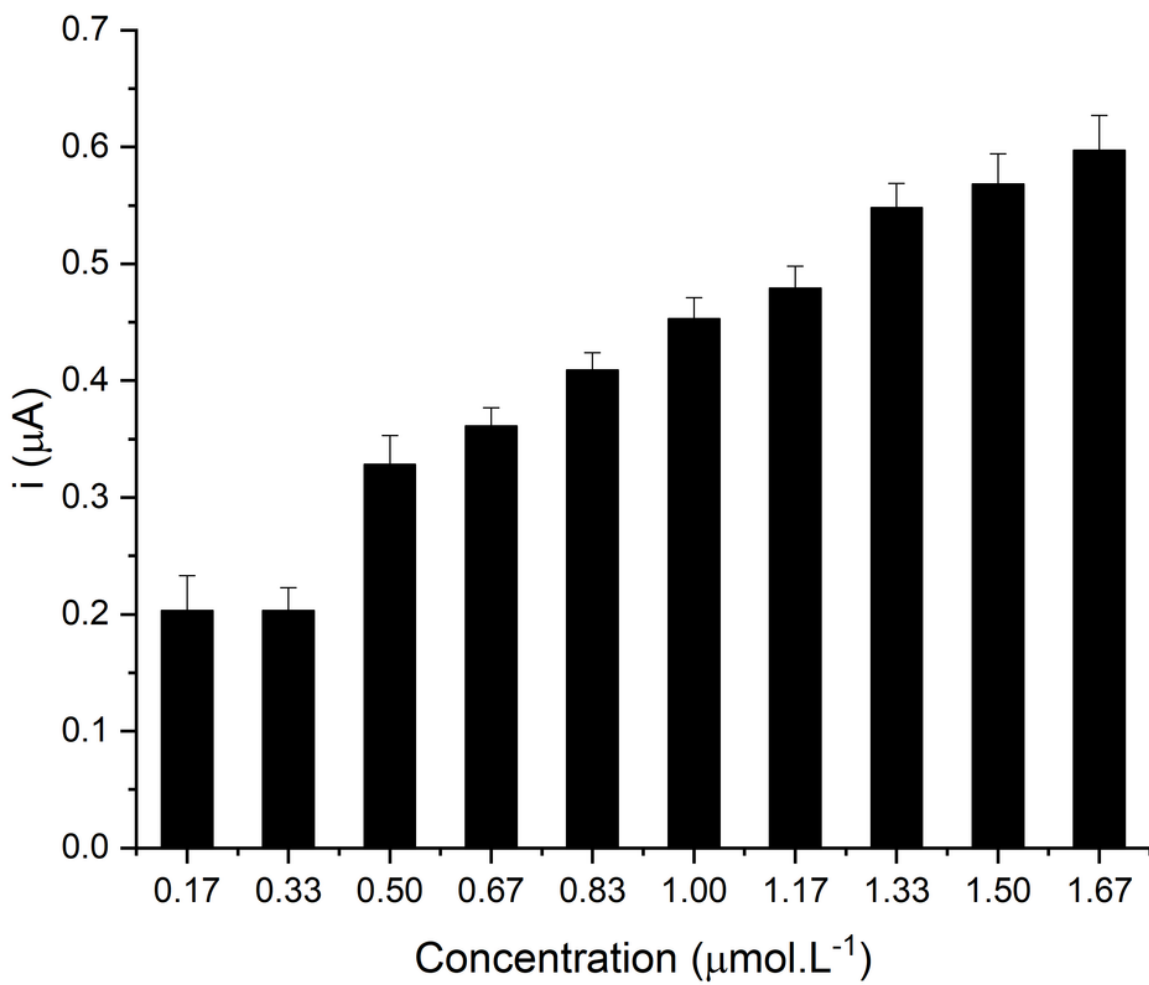


Figure 8

Anode current as a function of dopamine concentration