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Research Article

Laccase Inhibition by Mercury: Kinetics, Inhibition Mechanism, and Preliminary Application in the Spectrophotometric Quantification of Mercury Ions

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The noncompetitive inhibition of laccase by mercury ions is reported, in particular focusing their effect over the enzyme catalytic activity. The enzymatic kinetics were obtained for different substrates (caffeic acid, gallic acid, and catechol), where caffeic acid displayed the greatest enzymatic activity. The laccase inhibition by mercury ions permitted to establish the inhibition effect through a mixed model (that actually displayed a behavior closer to that of the noncompetitive inhibitors) when evaluated by means of UV-Vis spectrophotometry, using caffeic acid as an electron donor. A mercury concentration of 2 mM led to 35% enzymatic inhibition after only a 2-minute incubation period. This method was used for quantification of mercury ions in aqueous solution, showing a detection limit of 15 ± 1 ppm. Therefore, this work presented a novel perspective for the determination of the toxic Hg(II) ions that can be readily implemented into environmental remediation methods.

1. Introduction

Laccase is a multicopper enzyme commonly found in plants, fungi, and bacteria [1]; this enzyme is related to diverse functions, such as lignin synthesis and plant protection structures, sporulation, lignin degradation, and pathogenesis (fungi), of the mentioned organisms [2, 3]. The laccases have been studied for possible applications in the paper industry, due to their lignin degradation capacity [4], as well as in the treatment of wastewater contaminated by phenolic compounds [5]. This enzyme also has the capacity to oxidize polyphenols, diamines, and other types of compounds [6]. The transformation of the substrate is carried out by means of redox processes of the copper atoms distributed in three catalytic centers: type 1, strong absorption in the visible region $\varepsilon > 3{,}000\ M^{-1}{\cdot}cm^{-1}$ at 600 nm, spectrum of EPR with $A_{||} < 95 \times 10^{-4}\ cm^{-1}$; type 2 or normal center, undetectable UV absorption, line of the EPR having a typical shape corresponding to low molecular weight Cu complexes; and type 3 or coupled binuclear center, strong absorption in the near UV with $\lambda_{max} = 330$ nm, with no EPR signal, which occurs through coupling of the two antiferromagnetic copper ions [7, 8]. These catalytic centers have been characterized by means of electron paramagnetic resonance (EPR) [8, 9].

The measurement of the enzymatic activity and the inhibiting effects should lead to a betterment of the biologic catalyst knowledge and its possible applications in the near future. There are certain organic and inorganic compounds that inhibit the enzymatic activity, such as Mn^{2+}, Hg^{2+}, Co^{2+}, and Cd^{2+}, sulfates, nitrates and chlorides, fatty acids, sulfhydryl groups, quaternary ammonium detergents, and cysteine [10–12].

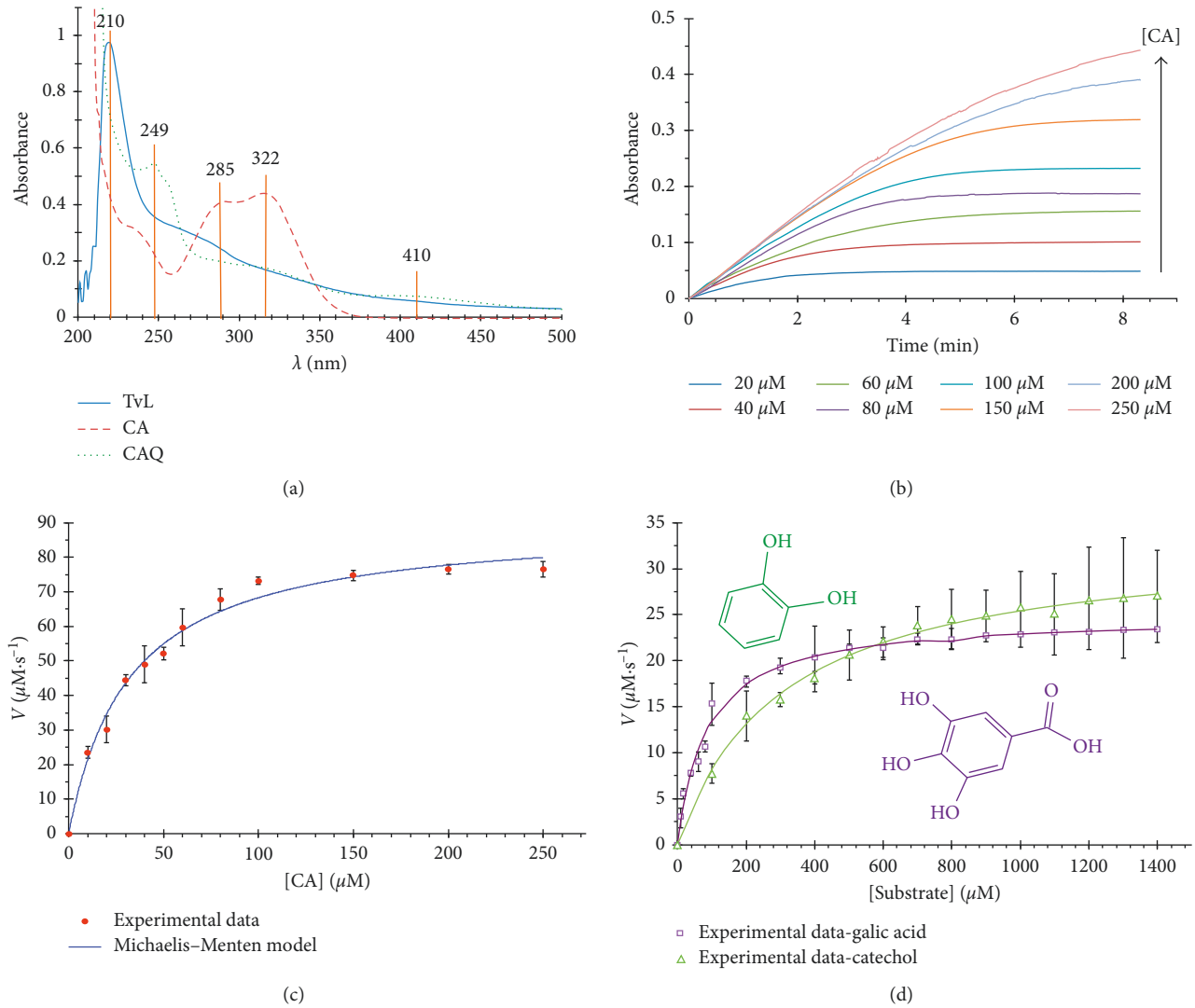

Figure 1: (a) Absorption spectra of the TvL, CA, and CAQ; (b) absorbance as a function of time for the oxidation reaction of different CA concentrations ($\lambda = 410$ nm); (c) spectrophotometric determination of the Michaelis–Menten kinetics for CA oxidation by TvL; (d) spectrophotometric determination of the Michaelis–Menten kinetics for GA and CT oxidation by TvL. All tests were carried out at ambient temperature in acetate buffer (0.1 M, pH 4.5).

The presence of these metal ions in the environment has dangerously damaged it and given rise to various harmful effects to human health, all of them sharing a common anthropogenic origin, since they derive from mining and coal burning industrial activities [13]. Mercury exists in various forms: inorganic (ionic and metallic), to which humans are exposed to as an occupational hazard, and organic (ethylmercury, methylmercury, and phenylmercury, mainly). These forms differ in their degree of toxicity and the effects on the nervous, digestive, and immunologic systems, as well as on lungs, kidneys, skin, and eyes [14–16].

Several mercury(II) ion inhibition studies have been reported using various enzymes, such as laccase (*Daedalea quercina, Leptographium qinlingensis*), where mercury turned out to be the most potent inhibitor, attaining up to 98% inhibition at an ion concentration of 10 mM [10, 11]; cellulose (*Schizophyllum commune*), which exhibits high sensibility toward mercury and modifies the spectrophotometric features of the enzyme [17]; invertase (yeast), which gives a larger inhibition respect to Ag^+ ions and an inhibition from 10^{-7} M [18]; α-amylase (*Paecilomyces variotii*), which displays a relative activity of 77% when adding a 10 mM Hg^{2+} concentration [19]; 5-aminolevulinic dehydratase acid (corn), where the mercury(II) ions modify the affinity toward the substrate and reaction rate, with the results based on the evaluation of K_m and V_{max} [20]; and xylanase (*Trichoderma inhamatum*), which displays an enzymatic activity of 14.4% (2 mM) and 4.0% (10 mM) for xylanase type 1, as well as an activity of 15.6% (2 mM) and 5.9% (10 mM) mercury(II) [21].

This work presents the results concerning the spectrophotometric study of laccase from *Trametes versicolor* with three different substrates (caffeic acid, gallic acid, and catechol), evaluating their activity and inhibition degrees in the presence of mercury(II), and observing that millimolar mercury concentrations can reduce the laccase reaction extent. The assessment of the enzymatic activity and the

effects of the inhibitors will allow a better knowledge of the biologic catalyst and its possible future applications, like the indirect quantification of the inhibitor itself.

2. Materials and Methods

Laccase from *Trametes versicolor* (EC 1.10.3.2), with an activity of 13.6 U/mg, was obtained from Sigma; sodium acetate trihydrate was from JT Baker; acetic acid glacial was from Laboratorios Lutz, México, 60.05%; caffeic acid, gallic acid, and catechol were from Fluka; ethanol was from Sigma-Aldrich (HPLC degree); $Hg(NO_3)_2$ was from Sigma-Aldrich; and deionized water was from a MilliQ Millipore equipment.

2.1. Kinetic Analyses and Enzymatic Inhibition. The kinetic UV-Vis tests were carried out in a Perkin Elmer Spectrometer Lambda 20. The absorbance of the caffeic acid oxidation was monitored at 410 nm using different substrate concentrations (10–250 μM); the catechol oxidation absorbance was monitored at 390 nm with different substrate concentrations (100–1400 μM); the gallic acid oxidation absorbance was monitored at 385 nm with different substrate concentrations (100–1400 μM). The Hg(II) concentrations for laccase inhibition were 0.05, 0.1, 0.5, 1, 2, 3, and 4 mM. The incubation times for inhibition were 2, 5 and 10 contact minutes. The laccase concentration was 10 $\mu g \cdot mL^{-1}$ for all cases. All tests were carried out at ambient temperature in 1 cm optical path quartz cells in an acetate buffer (0.1 M pH 4.5). The experimental conditions were chosen based on studies reported by our working group [22, 23].

3. Results and Discussion

The laccase is considered a nonspecific enzyme, capable of oxidizing a wide variety of phenolic compounds, which is why this work used substrates like the caffeic acid, gallic acid, and catechol for the kinetic studies.

The system was characterized through UV-Vis spectroscopy from 800 to 200 nm; the results are shown in Figure 1(a): it can be observed that *Trametes versicolor* laccase (TvL) displayed two maximum absorption bands at 210 and 275 nm, whereas the caffeic acid (CA) shows three maximum absorption bands at 241, 285, and 322 nm. The (CA) oxidation product (caffeoquinone, labeled as CAQ) presents three absorption maxima at 250, 322, and 410 nm. The signal of the CAQ at 410 nm allows quantifying the enzymatic reaction product to enable the kinetic studies without interferences. The remaining substrates were also characterized as well as the oxidation products: the catechol (CT) exhibited two absorption bands with maxima at 227 and 270 nm, while the *o*-quinone (Q) presents a maximum absorption at 390 nm. The gallic acid (GA) showed only one maximum absorption band at 260 nm, and its oxidation product showed absorption maxima at 256 and 395 nm.

Figure 1(b) shows the absorbance at 410 nm as a function of time for different CA concentrations. It can be observed that, for each CA concentration, the absorbance increases linearly until attaining reaction equilibrium, where the absorbance remains constant; the time to reach equilibrium

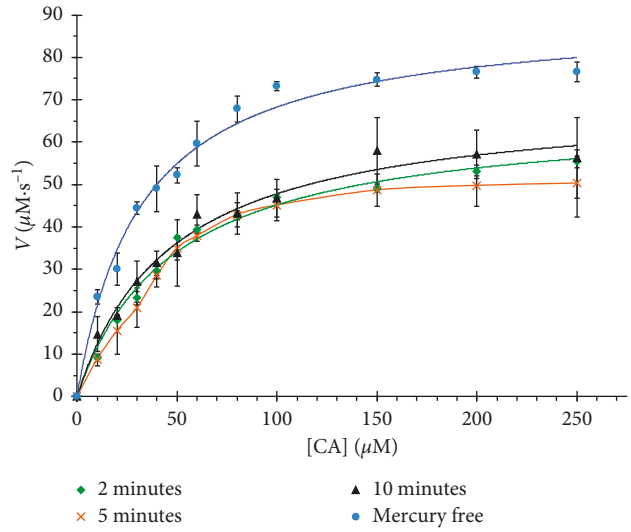

Figure 2: Spectrophotometric determination of the Michaelis–Menten kinetics for the CA oxidation by TvL in 1 mM mercury ion concentrations at different incubation times. The kinetics were determined in acetate buffer (0.1 M, pH 4.5), monitoring the CA enzymatic oxidation at 410 nm.

Table 1: Kinetic constants for different inhibition times of TvL by mercury(II), monitoring the CAQ absorbance at 410 nm in acetate buffer ($n = 3$).

Inhibition time (minutes)	K_m (μM)	V_{max} ($\mu M \cdot s^{-1}$)	K_{cat} (μMmL·μg^{-1})	k_{cat}/K_m ($mL \cdot \mu g^{-1}$)
0	43 ± 4	90 ± 3	9.0 ± 0.3	0.28 ± 0.03
2	55 ± 5	53 ± 2	5.3 ± 0.2	0.10 ± 0.01
5	58 ± 7	51 ± 3	5.1 ± 0.3	0.09 ± 0.04
10	57 ± 5	56 ± 3	5.6 ± 0.3	0.10 ± 0.03

increases with the substrate concentration, although it can be stated that at 8 minutes, all concentrations reached equilibrium. Furthermore, it is also observed that the slope of the linear segment increases with substrate concentration up to 100 μM, where the said slope ceases to increase, indicating enzymatic saturation. The slope of the linear segments in these kinetic plots represents the absorbance change as a function of time; hence, in accordance with the Lambert–Beer law [24], the slope represents also a concentration change of the reaction product as a function of time, in other words, the enzymatic reaction rate.

Figure 1(c) shows the results of fitting the Michaelis–Menten kinetic model into the initial enzymatic reaction rate as a function of the CA concentration [25–29], giving a $K_m = 43 \pm 4\,\mu$M and a $V_{max} = 90 \pm 3\,\mu M \cdot s^{-1}$. Figure 1(d) shows the initial enzymatic reaction rate as a function of the GA and CT concentration also with the Michaelis–Menten fitting; the kinetic constants obtained for these substrates were 85 ± 1 μM and 307 ± 18 μM, respectively, whereas the maximal rates were 24 ± 7 and 33 ± 6 $\mu M \cdot s^{-1}$. These results indicate that the TvL exhibits a greater affinity toward CA since the K_m for this substrate is the smallest. Notwithstanding the saturation for the GA and CT happened at higher substrate concentrations, for both cases, the initial

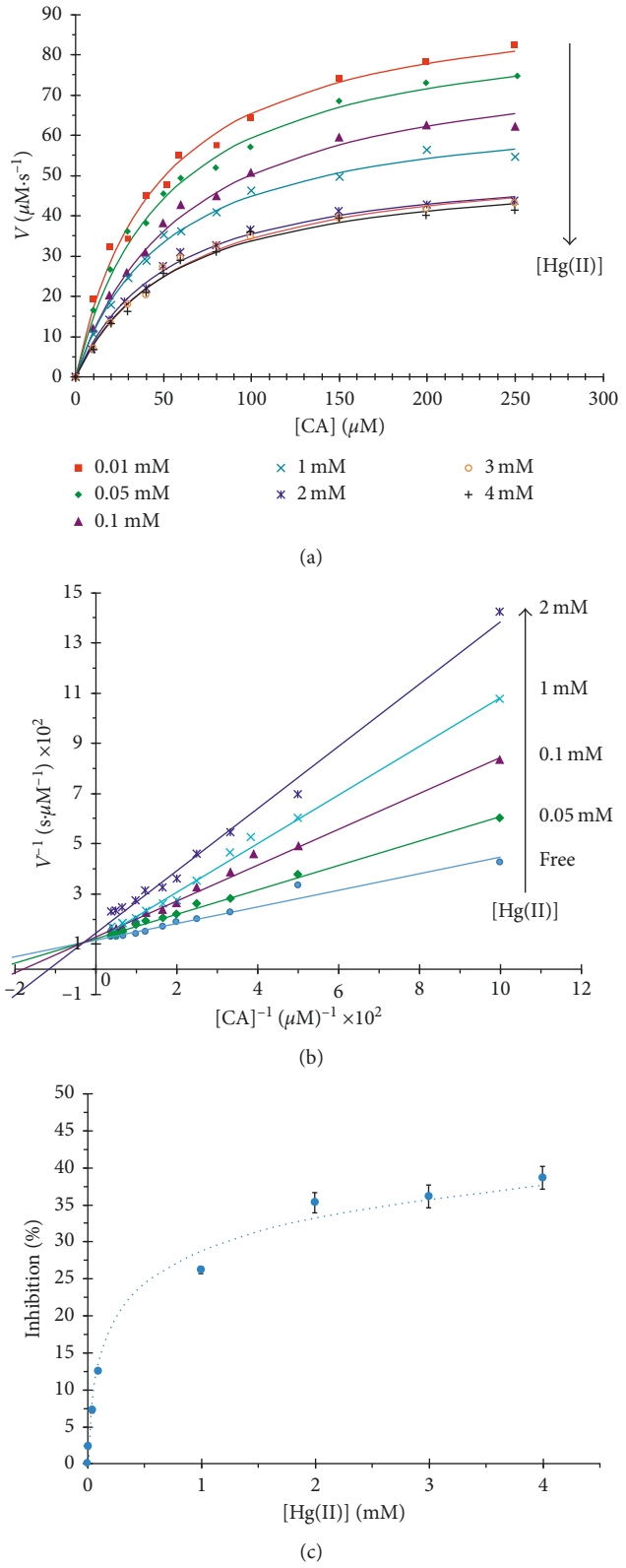

Figure 3: (a) Enzymatic reaction rate as a function of CA concentration for different mercury(II) concentrations; (b) double-reciprocal plot (1/V versus 1/[CA]) for the system TvL-CA inhibited for different Hg (II) concentrations; (c) inhibition percent plot of the system TvL-CA at 200 μM of CA and 2-minute incubation.

Table 2: Kinetic parameters for the system TvL-CA as a function of the mercury(II) concentration ($n = 3$).

[Hg(II)] (mM)	K_m (μM)	V_{max} (μM·s^{-1})	K_{cat} (μMmL·μg^{-1})	k_{cat}/K_m (mL·μg^{-1})
0	43 ± 4	90 ± 3	9.0 ± 0.3	0.28 ± 0.03
0.01	46 ± 3	95 ± 3	9.5 ± 0.2	0.20 ± 0.01
0.05	49 ± 3	88 ± 2	8.8 ± 0.3	0.20 ± 0.02
0.1	57 ± 4	79 ± 2	7.9 ± 0.2	0.13 ± 0.01
1	53 ± 4	54 ± 2	5.4 ± 0.2	0.11 ± 0.01
2	55 ± 5	53 ± 2	5.3 ± 0.2	0.10 ± 0.01
3	56 ± 6	52 ± 5	5.2 ± 0.5	0.09 ± 0.01
4	54 ± 5	53 ± 3	5.3 ± 0.3	0.10 ± 0.01

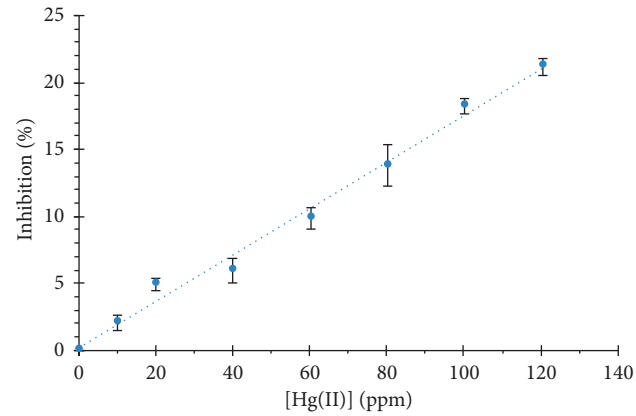

Figure 4: Calibration plot for the initial oxidation rate of 200 μM CA per 10 TvL·μg·mL^{-1} as a function of mercury concentration. The kinetics were obtained in acetate buffer (0.1 M, pH 4.5), monitoring the enzymatic oxidation of CA at 410 nm with 2-minute incubation.

Table 3: Percent recoveries for the mercury determination in synthetic samples ($n = 3$).

$[Hg(II)]_0$ (ppm)	Inhibition (%)	$[Hg(II)]_{rec.}$ (ppm)	Recovery %
30	5.4 ± 0.3	30 ± 2	100 ± 3.3
110	18.5 ± 0.4	106 ± 6	96.4 ± 5.4
200	29.1 ± 1.4	160 ± 14	80.0 ± 7.0

rates are small as compared with those of the CA, which exhibits high rates at small concentrations. Therefore, the CA was chosen as the substrate for all subsequent studies.

3.1. Inhibition Time. In order to enable an adequate proposal for the same incubation time for TvL inhibition by Hg (II) ions, a study was carried out at 2-, 5-, and 10-minute incubation with constant concentrations of 1 mM mercury. The results are shown in Figure 2, where it can be graphically observed that the kinetic behaviors did not display a significant change for the studied inhibition times. It can also be observed that the 1 mM mercury concentration was capable of inhibiting by about 25% of the catalytic activity of

Table 4: Indirect analytical methods for quantification of Hg(II) found in the literature that are based on the principle of enzymatic inhibition.

	Detection method	LOD (ppm)	Linear interval (ppm)	pH	Incubation time (min)	Comment	Ref.
Invertase biosensor	Amperometric	0.001	0.01–0.04	0.1 M NaOH	10	Biosensor coupled to a batch injection analysis	[32]
Invertase immobilized	Thermometric	0.005	0.005–0.08	7.0	2–6	Coupling to FIA	[33]
GOx biosensor	Amperometric	0.05	2–32	7.0	—	Nafion- and MnO_2-modified CPE	[34]
Invertase-mutarotase-GOx biosensor	Conductimetric	0.005	—	6.5	10–20	—	[35]
GOx free	Colorimetric	0.015	0.005–1	5.0	2	—	[36]
Alcohol ox. and horseradish peroxidase free	Fluorescence	2.5×10^{-6}	5.0×10^{-6}–0.05	7.5	20	—	[37]
Sucrose biosensor	Amperometric	—	0.002–0.2	6.0	20	—	[38]
GOx biosensor	Amperometric	—	—	7.0	30	—	[39]
GOx and laccase biosensor	Amperometric	0.03	—	5.0	—	—	[40]
GOx	Amperometric	0.5	0.4–36	7.0	—	—	[41]
Laccase free	Colorimetric	15	10–120	4.5	2	—	This work

the TvL. The Michaelis–Menten (K_m) constants for the different inhibition times are shown in Table 1 together with their maximal rates (V_{max}), the catalytic constants (K_{cat}), and the catalytic efficiencies (k_{cat}/K_m). All these values show that there is no meaningful difference among the different inhibition times since all the constant values are statistically equal. Therefore, 2-minute incubation will be the contact time for the forthcoming sections.

3.2. Effect of the Inhibitor Concentration. Figure 3(a) shows the effect of the mercury ion concentrations on the TvL catalytic efficiency as a function of varying concentrations of the metal ion (0–4 mM). The enzyme contact time with the inhibitor was 2 minutes for all concentrations with $10\,\mu g \cdot mL^{-1}$·TvL, for each experiment. It can be clearly noted that the mercury concentration increments resulted in V_{max} decrements (Figure 3(a) and Table 2). Further, the Michaelis–Menten constant increased lightly, which indicates a mixed inhibition mechanism [30, 31]. In order to further clarify this inhibition mechanism, the Lineweaver–Burk double-reciprocal plots were evaluated for different inhibitor concentrations (Figure 3(b)).

The convergence of the data series on the y-axis of the Lineweaver–Burk plots is typical of a competitive inhibition mechanism, in which the value of K_m increases although V_{max} does not, whereas the data convergence on the x-axis indicates a noncompetitive mechanism, which in this case K_m does not change, but V_{max} diminishes. The analyses of the Lineweaver–Burk double-reciprocal plots indicate that the mercury inhibition mechanism by TvL is carried out through a mix of the previous models (mixture of the competitive and noncompetitive). However, the nonlinear regressions (Figure 3(a) and Table 2) seem to suggest that the inhibition model is closer to the noncompetitive model. It can also be observed from Figure 3(c) that a mercury concentration of 0.05 mM inhibited the enzymatic response to 7% and the 0.1 mM concentration did so to 12%, whereas the 1 mM concentration inhibited to 25%. Lastly, for 2, 3, and 4 mM metal concentrations, an inhibition of approximately 35% was attained without significant change.

Table 2 shows the enzymatic activity parameters for TvL after being inhibited with different mercury ion concentrations, from which it can be said that, at 2, 3, and 4 mM inhibitor concentrations, effectively the response remains constant. Also, the Michaelis–Menten constant shows a small increase with the mercury concentration, and from 2 mM mercury, it is statistically identical. As expected, the maximum rate value decreases with increasing inhibitor concentration, which proves also that the enzyme is being inhibited. It can also be noted that the catalytic constant (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) diminish with increasing mercury concentration until it remained constant at higher inhibitor concentrations. All these results reveal the possibility to use the TvL inhibition for the mercury(II) quantification in aqueous solution.

3.3. Mercury(II) Determination in Synthetic Samples. Several calibration plots were built in order to determine mercury(II) in synthetic samples as a function of the metal ion concentration, keeping the TvL concentration constant at $10\,\mu g \cdot mL^{-1}$ and 2 minutes in contact with the inhibitor. For this test, synthetic mercury solutions containing 30, 110, and 200 ppm were prepared by dissolving mercury nitrate in deionized water. Figure 4 shows the calibration plot; the method rendered a detection limit of 15 ± 1 ppm and a linear range of 10 to 120 ppm. The detection limit (DL) was calculated using the equation $DL = 3s_y/m_{cal}$, where s_y represents the typical error and m_{cal} represents the slope of the calibration plot. A summary of the results of the mercury(II) determination in synthetic samples is shown in Table 3. For mercury concentrations of 30, 110, and 200 ppm, the percent recovery were as follows: 100, 96.4, and 80.0%, respectively, with errors smaller than 7%.

3.4. Comparison with the Literature. Table 4 shows a comparison between the methods proposed in the literature and that described in the present work for determining mercury(II) in aqueous solution, all of them based on the same enzymatic inhibition principle. It is worthwhile to state that there are only a few methods based on this principle of using free enzymes (not immobilized).

Conversely, there are numerous methods reported using enzymes immobilized on a polymer network to modify Pt, Au, glassy carbon, carbon paste, and printed electrodes, among others. It is also important to point out that a large number of works have used enzymes such as glucose oxidase (GOx), invertase, urease, and others, to be inhibited by mercury, although laccase has been much less studied. The method reported here is comparable with some other methods reported insofar as their detection limit, even though this is not the case for the majority of them, because they report detection limits inferior to those in this work. However, the method reported here presents other advantages because the enzyme is free in solution, apart from the fact that the amount of laccase needed for each analysis is very small ($10\,\mu g{\cdot}mL^{-1}$) as well as the buffer solution (2 mL), and lastly, the analysis cost is indeed small, because they are coupled to a quick and simple procedure.

4. Conclusions

The laccase enzymatic inhibition through mercury(II) ions was reported, establishing a mixed inhibition model (preferable to a noncompetitive inhibition model) by means of UV-Vis spectrophotometry. The TvL enzyme presents higher activity when using caffeic acid as a substrate, in comparison with gallic acid and catechol, fitting in the Michaelis–Menten with constants of 43 ± 4, 85 ± 1, and $307 \pm 18\,\mu M$, respectively. Small mercury(II) ion concentrations did inhibit the TvL enzymatic activity in only 2 contact minutes, without meaningful difference at greater times. Then, the mercury laccase inhibition was used for quantification of the metal ion. The method reported here exhibited a detection limit of 15 ± 1 ppm with a linear dynamic interval of 10–120 ppm mercury ($R^2 = 0.9893$). Further, the method is fast and simply implemented into other procedures of environmental remediation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

There are no conflicts to declare.

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