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DEGRADATION OF THE COMPOUND 4-NITROPHENOL, BY MEANS OF AN ELECTROCHEMICAL BIOSENSOR OF PURIFIED LACCASE ENZYME "MYCELIOPHTHORA THERMOPHILA"

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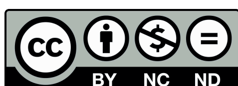

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Abstract: In the last decades, the contamination of the terrestrial ecosystem and aquifers has increased due to the indiscriminate use of a group of highly polluting and difficult to degrade organic compounds, such as phenolic compounds used at industrial level (paints, antibiotics, etc.); these compounds have a molecular structure containing at least one phenol group (aromatic ring linked to a hydroxyl functional group, so they have been classified as primary pollutants to be degraded). In this work we present the results obtained from the purification of the Lacase Enzyme "Myceliophthota Thermophila", by Fast Protein Liquid Chromatography (FPLC) and electrophoresis, used in the design of a biosensor capable of degrading the compound 4-Nitrophenol (4-NF), the electrocatalytic efficiency of this was followed by cyclic voltammetry obtaining a good response for the degradation of 4-NF obtaining as detection limit of 7. 189 ppm, limit of quantification of 25.165 ppm with a Jo exchange current density of $76.2x10^{-3}$ µA/ cm^{-2}, by UV-Vis spectrophotometry a linear trend was obtained with a slope of -0.04 AU/ days and correlation coefficient of 0.9954. By means of this information it was corroborated that the biosensor is a good candidate in the degradation of 4-NF as a function of time, the FT-IR spectroscopic technique shows a structural change of the enzyme Lacase when it is used in the degradation of 4-NF.

Keywords: *Myceliophthota Thermophila*, 4-Nitrophenol, Electrochemical Biosensor.

INTRODUCTION

During the last few decades, freshwater has presented great problems of scarcity, threat and danger; studies carried out by specialists and international organizations on the water balances of the planet report that only 0.007% of freshwater is available for various human uses. It is predicted that by the year 2025 more than two thirds of humanity will suffer from water stress (L'vocich et al., 1995) (Simonovic, 2000). This is due to population growth and the indiscriminate use, waste and pollution of the vital liquid, as part of the anthropogenic activities that humans carry out on a daily basis. It is important to start looking for viable and safe alternatives to recover water contaminated by toxic waste. The few freshwater resources that exist on the planet are located in only six countries: Canada, USA, Brazil, Russia, India and China, since 40% of the world's rivers are concentrated in these countries (Shiklomanov, 2000). Currently, the problem of lack of water is more emphasized, since worldwide there are severe droughts that are beginning to cause tensions and conflicts over water, both in developed and developing countries, affecting economic issues, human health, and environmental impacts; these occupied the first place in the number of disasters that affected 1% or more of the world's population during the years 1963-1992 (Esparza, 2014; Ortega, 2013). That is why, many scientists have been given the task of searching for viable and environmentally friendly alternatives generating new materials, treatments and separation technologies capable of improving the recovery and/or elimination process of phenolic compounds coming from the pharmaceutical industry, resins, plastics, textiles, rubber, explosives, tanneries, electrochemical processes, paints, paper, etc., and their derivatives in polluted waters that contain resistive, corrosive and difficult to degrade toxic wastes; therefore, they are one of the priority pollutants to be reduced in the effluents of polluted waters, since their presence in these is highly harmful to the environment and to human beings (Traiam, 1989).

The toxicity of phenolic compounds depends on their structural and chemical properties, their degree of toxicity depends

on the substituent groups that accept electrons versus electron donors, among the acceptor groups are nitro and chlorine, with 4-nitrophenol being the priority toxic compound among monosubstituted phenolic compounds, along with 4-chlorophenol, which is why the US Environmental Protection Agency (USEPA) (Buikema, et al., 1979; USEPA, 2022), classifies phenol and 4-Nitrophenol within the 21 highly polluting and priority phenolic compounds due to the frequency of appearance and persistence in polluted waters since they affect human health when ingested or in contact with the skin can cause skin changes in a short time, when ingested or inhaled, causes headache, drowsiness, nausea, and cyanosis (blue discoloration of the lips, ears, and fingernails); the Council of the European Union (CCE, 1985) mentions that 4-Nitrophenol is included within the 9 priority phenolic pollutants. These compounds are mutagenic substances in low and high concentrations affect living organisms. The Central Pollution Control Commission (CPCB) and the USEPA consider both phenol, 4-Nitrophenol and 4-Nitrophenol as highly dangerous pollutants, for which reason they have established maximum permissible limits of 1mg/L, 20mg/L and 0.5 mg/L respectively.

Due to the aforementioned, various investigations have been carried out to find new materials, technologies and methods to reduce or degrade the pollution caused by these compounds in order to minimize ecological damage, environmental impact and on humans, methods have been established electrochemical (Zhang et. al., 2007; Esplugas et. al., 2002; Alves et. al., 2005; Lazo et. al., 2007; Lazo 2009; Ahmaruzzaman et. al., 2011) of oxidative, photocatalytic degradation, adsorption with modified clays, carbon monospheres, activated carbon with copper, the first methods have the advantage of obtaining a direct degradation depending on the medium, a disadvantage of the electrochemical method is the energy loss due to the lack of a homogeneous current, and the formation of reactions high schools; photocatalytic degradation generates by-products (CO_2 being one of the causes of global warming); the absorption process being the most used method to control contamination by organic and inorganic compounds; In the literature it is reported that there are various enzymes such as the enzyme Laccase polyphenol oxidase that belongs to the family of blue multicopper oxidases, they are cuproproteins capable of catalyzing the oxidation of various phenolic compounds, aromatic amines and various biological processes, since it uses oxygen molecular as an electron acceptor, reducing itself to two water molecules by means of the transfer of four electrons from the corresponding substrate without forming toxic intermediate compounds, with a monomeric structure with a molecular mass between 36 and 80 KDa, it is active in a pH range of 2.0 to 8.5, is very stable since it has an isoelectric point of 2.6 and 4.5. (Eggert, et al., 2008).

The Laccase enzyme has been shown to degrade recalcitrant compounds, it was found in the sap of the Lac tree (Rhus Vernicifera) (Yoshida, 1883), and in 1896 Bertrand and Laborde demonstrated that it is also found in fungi, being more stable extracellular proteins and Its purification is simpler, its active center is formed by 4 Cu atoms distributed in three redox sites called T1, T2 and T3 to it, the name of multicopper oxidases is attributed to it (Baldrian, 2006), in figure 1, it is shown shows the schematic of the structure of Laccase (Jones et. al., 2015).

Figure 2, shows the scheme of the catalytic cycle of the laccase enzyme, which is carried out through a pong mechanism that begins when the copper that occupies the T1 position

oxidizes the substrate (phenols and anilines) generating a free radical (Petersen et al, 1978), where the extracted electron is transferred to the His-Cys-His tripeptide found in laccase, reaching the T2/T3 site where molecular oxygen is reduced to water (Baldrian, 2006).

The electrochemical study carried out by Lozano et al., showed that the biosensor built with carbon paste and Laccase enzyme presented good efficiency in the degradation of the 4-Nitrophenol compound as a function of time, verifying this by means of a UV-Vis spectroscopic study. this being a viable path to the search for new environmentally friendly materials in the degradation of highly polluting phenolic compounds (Lozano et. al, 2018) continuing with the investigation carried out electrochemically in this work, the purification of the Lacase enzyme is shown, which was used to build a new biosensor following the methodology of Lozano et. col., to identify the structural changes that take place during the degradation of 4-NF, a FT-IR spectrophotometric study was performed.

EXPERIMENTAL METHODOLOGY

LACCASE ENZYME PURIFICATION "MYCELIOPHTHOTA THERMOPHILA"

From the Deni Lite lls powder, produced from a gene of the thermophilic fungus "Myceliophthota Thermophila" (MtL), Novo Nordisk, the laccase enzyme was obtained for its purification by means of Fast Protein Liquid Chromatography (FPLC) Q-Sepharose (Hi-Prep 16/10 QFF, Bio-Rad), flow rate of 5 $mLmin^{-1}$ and pressure of 0.4 MPa; purification by electrophoresis, a 12% acrylamide polymer solution was prepared using a 180 V electrical source for 45 min.

ELECTROCHEMICAL CHARACTERIZATION

ELECTRODE PREPARATION

A carbon paste was prepared in a 1:1 ratio of 99.99% monocrystalline grade graphite (alpha AESAR) and nujol oil (Fluka), mixing thoroughly until an easy-to-handle paste was obtained. Said paste was placed in a 0.5 cm cavity of an electrode made of Teflon that has a vitreous carbon bar as contact. Later, 12 mg of the laccase enzyme solution was placed on the surface of the carbon paste for its physical adsorption, leaving dry for two hours.

ELECTROLYTE SOLUTION

The electrolytic solution was prepared in a phosphate buffer solution of pH 6.4, so that the enzyme will not be denatured.

ELECTROCHEMICAL SYSTEM

All electrochemical experiments were performed on a BAS 100W Potentiostat, using a typical three-electrode electrochemical cell. A mercurous sulphate reference electrode, a graphite rod counter electrode and Lacassa enzyme modified carbon paste (EPCML) as working electrode.

SPECTROSCOPIC STUDY BY UV-VIS

The spectroscopic studies were carried out using a Perkin-Elemer Lambda 35 UV/ Vis Spectrometer, which has a tungsten and deuterium lamp, the spectra were obtained at 280 and 310 nm/min to determine the region where the Laccase Enzyme absorbs, and 250 at 500 cm^{-1} with a scanning speed of 240 nm/ min

INFRARED SPECTROSCOPY (FT-IR)

The Infrared Spectrophotometer studies were carried out using an S50 FT-IR equipment, in a wavelength interval of 500 to 4000 cm^{-1}.

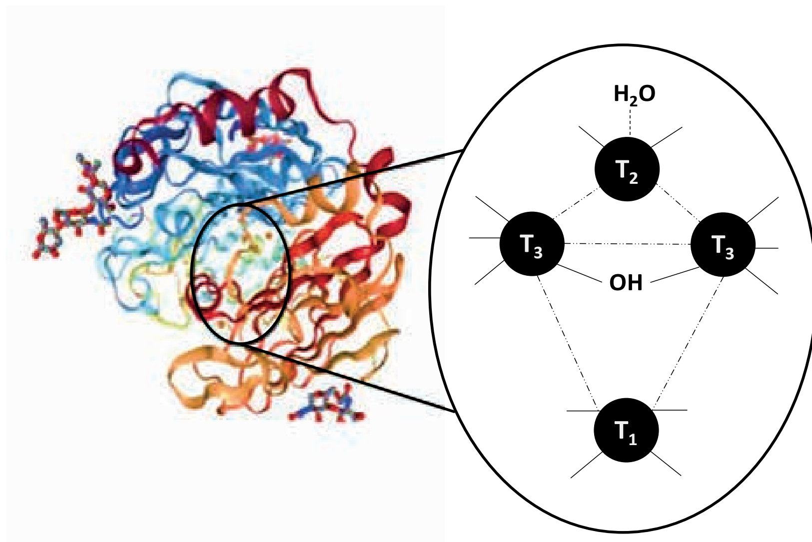

Figure 1. Scheme of the structure of the Laccase Enzyme

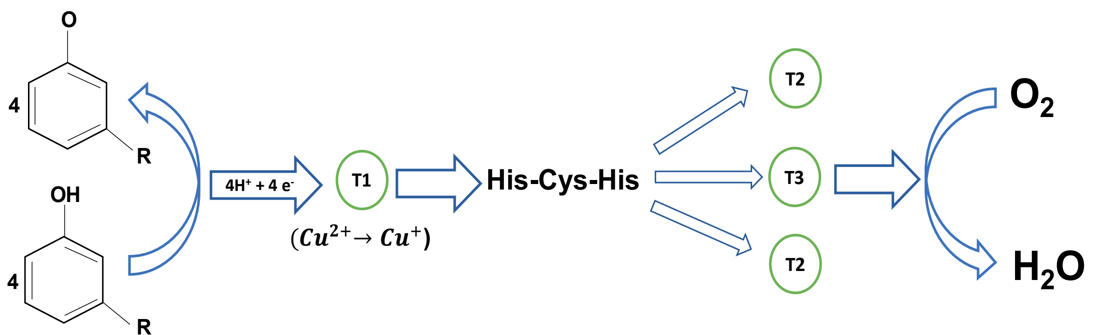

Figure 2. Diagram of the catalytic cycle of the laccase enzyme

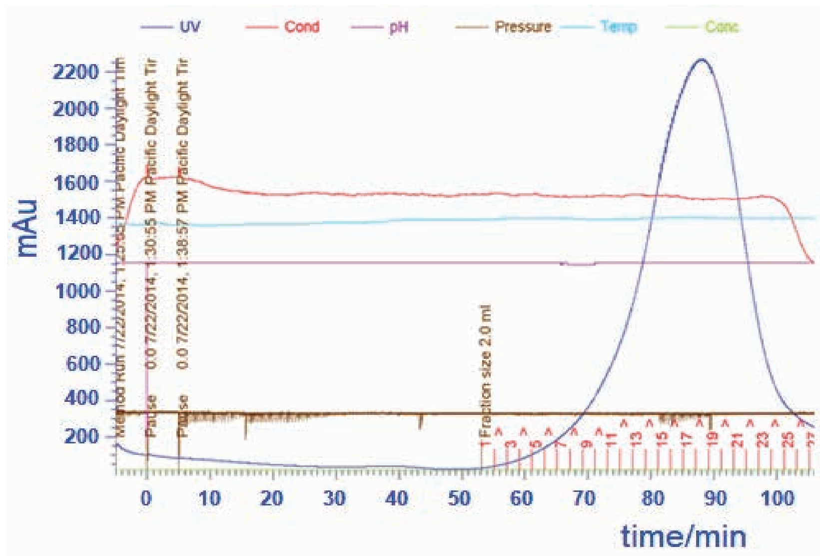

Figure 3. Identification of pure Laccase Enzyme by size exclusion chromatography

RESULTS AND DISCUSSION

PURIFICATION OF THE LACCASE ENZYME

Laccase enzyme was purified by size exclusion chromatography (SEC), using 50 mM TRIS-HCl at pH 6.4, with a HiPrep Q FF 16/10 column at a wavelength of 280 nm, 10 batches of the enzyme were obtained. Purified laccase enzyme with an average yield of 25 mg of protein per 50 grams of commercial Deni Lite powder. Figure 3, shows the results obtained from the chromatographic study where it can be seen that the dialyzed Laccase enzyme is completely free of impurities.

The purification of the laccase enzyme was also verified by electrophoresis, in Figure 4, it is observed that the sample obtained from the Laccase enzyme is completely free of impurities, thus having a totally pure product.

Image 5 shows the 10 mL batch of the already purified Laccase enzyme with a concentration of 61.9 mg/mL, which will be used to perform the electrochemical and spectrophotometric studies for the degradation of 4-Nitrophenol.

A UV-Vis study of the laccase enzyme was performed to verify purification.

UV-VIS OF THE PURIFIED LACCASE ENZYME

In figure 6, the UV-vis spectrum of the laccase enzyme is shown, which presents an absorption at the wavelength of 216 nm.

To the verifying that the laccase enzyme was pure, a study of the enzymatic activity was carried out to identify its maximum potential.

ENZYMATIC ACTIVITY

The study of the enzymatic activity was carried out using an acid substrate [2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonic)] (ABTS) as it is the first effective mediator to obtain laccase, which allows it to have various applications. at the industrial level (Collins et. al., 1998). Figure 7 shows the kinetics of MtL type Michaelis Menten obtained in 10 samples of the laccase enzyme in a concentration range of 0.01 to 2.3 mM, in a time interval of 1 min and waiting intervals between each sample of 5 min, obtaining a reaction rate of 1.8841 mmol/min-1 mL-1 by consuming 0.01408 mmol/min-1L-1 of the substrate per minute. Table 1 shows the values of V_{max} and K_{max}, obtained with the value of the intersection slope.

ELECTROCHEMICAL STUDY

It was carried out using the methodology proposed by Lozano et al. (Lozano et al, 2018), satisfactorily obtaining the same results in the voltammetric analysis, in table 2, the signals obtained are shown.

The electrocatalytic activity of the EPCML biosensor in the degradation of 4-NF was carried out in an electrolytic solution of sulfates at pH 4.6, adding different concentrations of 4-NF, performing a potential sweep between -1440 to 900 mV, in the anodic direction applying a sweep speed of 20 mV/s, 7 readings per day were made at 90 min time intervals, the efficiency was measured for 14 days, obtaining an irreversible process with a response potential variation of DE= 619 mV, the results obtained from the degradation are shown in table 3.

Figure 8 shows the results obtained from the relationship of the anodic peak current vs. the concentration of 4-NF, when using an EPCML electrode, where it is observed that in the initial stage of the enzymatic reaction it is carried out with great effort. speed, this can be related to the number of active sites that the enzyme presents to transform 4-NF. In the second stage, a saturation of these active sites can be observed, which leads to obtaining a linear relationship with a lower slope with respect to the first stage, showing the capacity of the biosensor in the degradation of 4-NF.

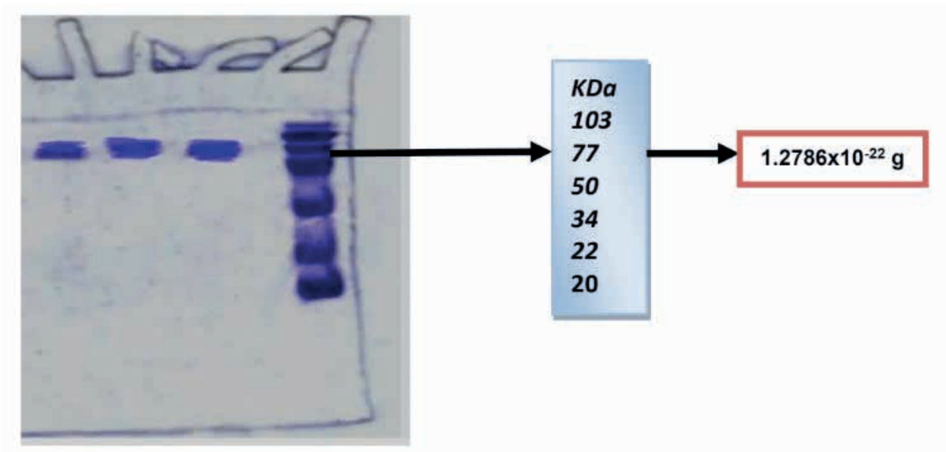

Figure 4. SDS electrophoresis gel obtained by analyzing the sample in the last stage of purification of the laccase enzyme. The high molecular mass marker (P1) was used, the amount of protein supplied in the lanes was 2mg.

Image 5. Lot of 10 mL of the purified Laccase Enzyme

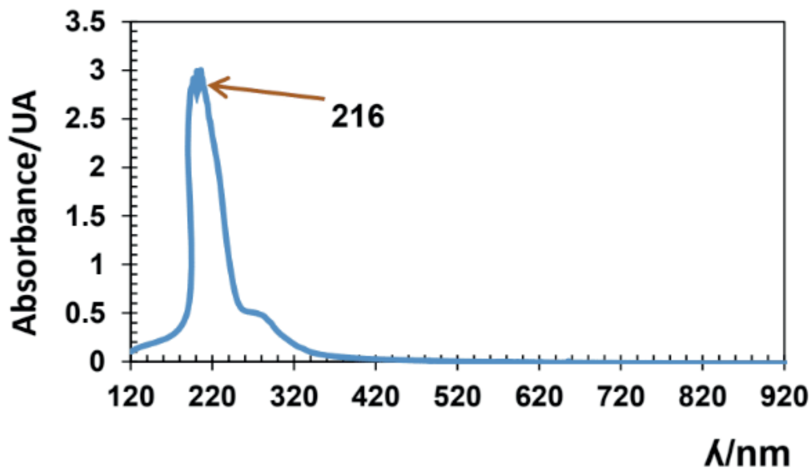

Figure 6. UV-Vis spectrum of the purified Laccase enzyme, scanned at a wavelength of 100 to 1100 nm

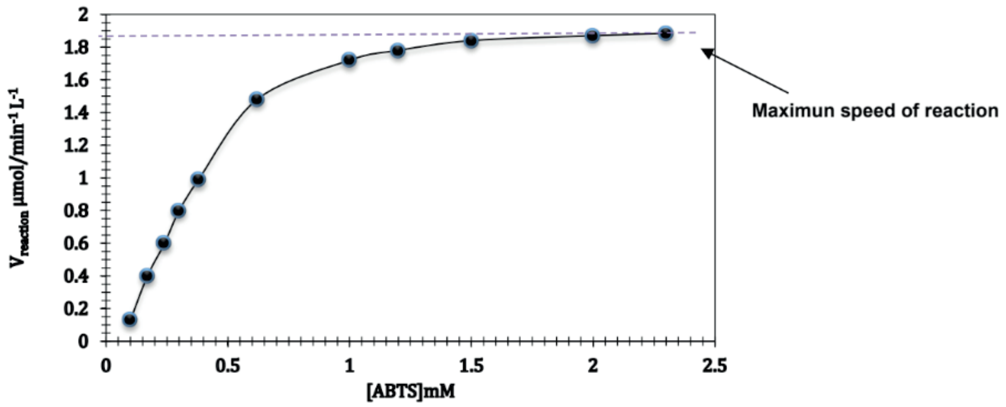

Figure 7. Kinetics of MtL type Michaelis Menten at 25°C using ABTS as substrate in 50 mM phosphate buffer, pH 6.4, at different substrate concentrations from 0.01 to 2.3 Mm.

	VALUE	STANDARD ERROR
$V_{máx/mmol/mi^{-1}L^{-1}}$	1.8845	0.03115
$K_{máx/mM}$	0.4587	0.05672

Table 1, values obtained from Vmax and Kmax in the kinetics of MtL

Electrode	Epa/mV	Epc/mV
EPC	-----	------
4-NF/PC	-913	-665
EPC/Laccase enzyme	-483 y -273	-273
4-NF/EPCML	-484 y -274 Two potential shoulders at 42 and 590	-273

Table 2, values obtained from Epa and Epc by VC in an electrolytic medium of phosphates pH 6.4

[4-NT]/mM	I_{pa}/µA
2.00x10^{-1}	45.2
4.00x10^{-2}	40.0
8.00x1^{0-}3	37.0
1.60x10^{-3}	34.2
3.20x10^{-4}	26.8
6.40x10^{-5}	26.5
1.28x10^{-5}	25.6

Table 3, values obtained from i_{pa} at different concentrations of 4-NT

Table 3 shows the results obtained from the electrochemical degradation of the 4-Nitrophenol (4-NF) compound at a scanning speed of 20 mV/s, on the electrochemical biosensor at low concentrations.

Figure 9 shows the UV-Vis spectrum of the degradation study of the 4-NF compound when using the same EPCML, it can be clearly seen that there is a decrease in absorbance at a wavelength of 320 nm as a function of the time. The data present a linear relationship between the absorbance as a function of time (No. of days) for the degradation of the 4-NF compound.

Through the UV-Vis spectrophotometric study, the 320 nm signal is obtained where the degradation of 4-NF as a function of time is clearly observed. Figure 10 shows the graph obtained from the absorbance (AU) vs. time (days), in the degradation of 4-NF at a wavelength of 320 nm.

As can be seen, there is a linear degradation as a function of time with a slope of -0.04 AU/days and a correlation coefficient of 0.9954, which corroborates that the laccase enzyme sensor is capable of degrading the 4-NF compound as a function of time. showing good electrocatalytic efficiency both electrochemically and spectroscopically. A FT-IR study was carried out to verify if there are structural changes of the Laccase Enzyme before and after having carried out the degradation of 4-NF on the EPCML. Figure 11 shows the spectra obtained.

Table 4 shows the characteristic peaks for the EPCML without being used in the degradation of 4-NF and the EPCML/4-NF, the signals of the 4-NF compound are observed due to the interaction that exists with the laccase enzyme, using adsorption changing its chemical structure temporarily since the main characteristic of enzymes is to return to their natural state at the end of the reaction after a period of time, which makes it efficient to continue using it as a biosensor.

CONCLUSIONS

The results show that the laccase enzyme was purified with the applied methodology, which was confirmed by activity tests and gel electrophoresis, obtaining a maximum speed reached by the enzyme of 1.8845 μmol/min-1 mL^{-1}.

The electrochemical methodology provides evidence that it is possible to degrade a recalcitrant compound such as 4-NF using a laccase enzyme-modified carbon paste electrode (EPCML), a biosensor on which 4-NF reacts electrochemically with a transfer rate charge J_o = $7.62x10^{-2}$ mA/cm^{-2} relatively large.

Statistical parameters for 4-NF degradation were also obtained with a detection limit of 7.189 ppm and quantification of 25.165 ppm, so it can be said that this biosensor is capable of efficiently degrading 4-NF.

The UV-Vis spectroscopic study allowed us to verify that the electrode modified with laccase enzyme (EPCML) is a good candidate for the degradation of 4-NF as a function of time, showing a decrease in absorbance. The FT-IR study gives indications that the degradation of the 4-NF compound is taking place in the hydroxyl groups (OH-), or by the nitro group (NO_2), of the laccase enzyme.

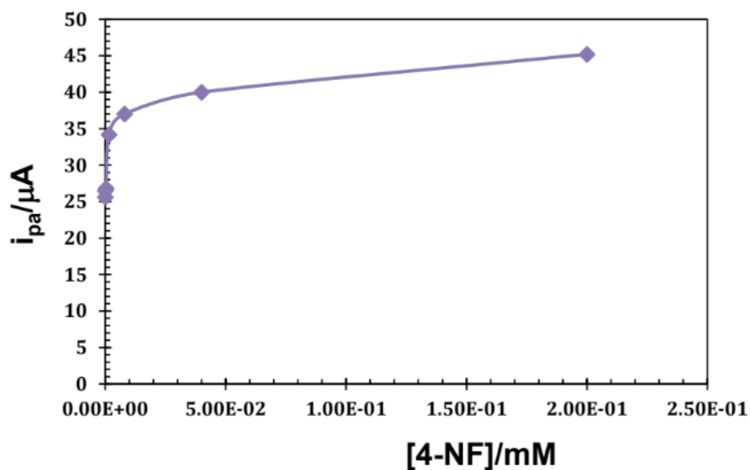

Figure 8. I_{pa} ratio as a function of the concentration of 4-Nitrophenol/EPCML

Electrode	Correlation coefficient (R)	Detection limits/ppm	Quantitation limits/ppm	J_o/µAcm^{-2}
EPCML	0.9879	7.189	25.165	$76.2x10^{-3}$

Table 3. Statistical analysis of the relationship of ipa vs. 4-Nitrophenol concentration

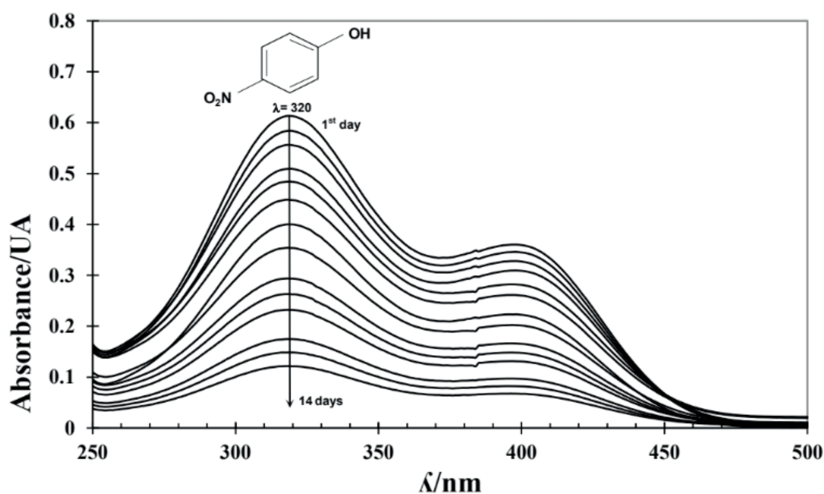

Figure 9. UV-Vis spectra recorded from samples collected from the electrochemical degradation of 4-NF as a function of time. The spectra were obtained by applying a wavelength range from 250 to 500 nm.

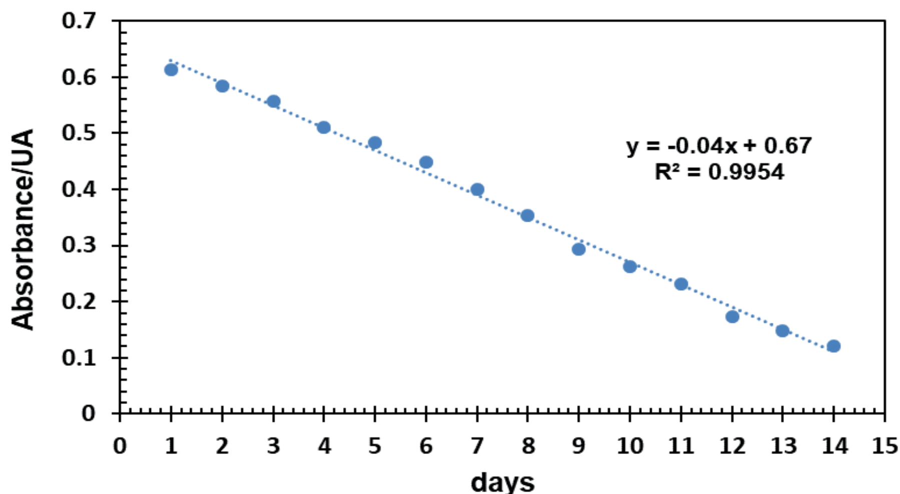

Figure 10. Relation obtained from absorbance vs. days of the degradation process of the 4-NF compound.

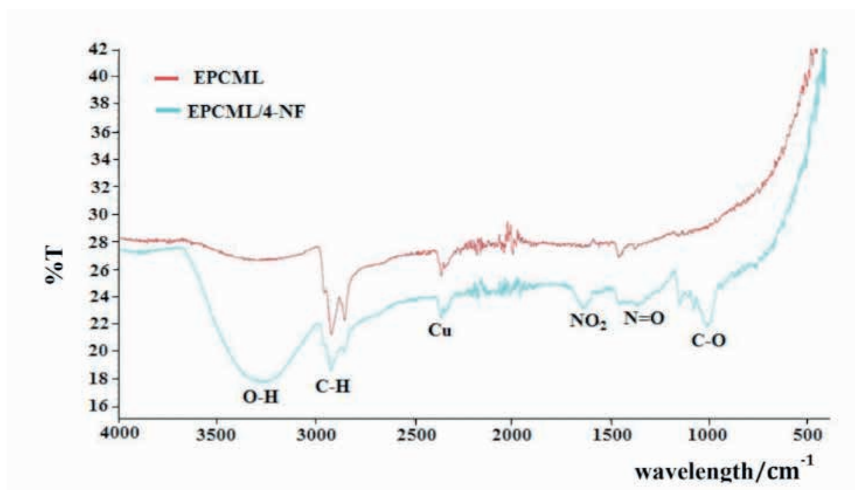

Figure 11. FT-IR spectrum of a sample of the EPCML (red line) and EPCML used in the degradation of the 4-NF compound (blue line).

ELECTRODE	WAVELENGTH/cm^{-1}	FUNTIONAL GROUP
EPCML	1350	N=O
	2380	Cation Cu^{2+}
	2920	Group asymmetric stretch vibration C-H
	3300	stretching O-H
EPCML/4-NF	1020	C-O
	1350	N=O
	1650	Symmetric stretch vibration NO_2
	2380	Cation Cu^{2+}
	2920	Group asymmetric stretch vibration C-H
	3300	stretching O-H

Tabla 4, Main characteristic peaks obtained by FTIR of the EPC and EPCM used in the degradation of 4-NF

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