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Enzymatic biosensor for the electrochemical detection of 2,4-dinitrotoluene biodegradation derivatives

Marcela C. Rodríguez [a], Mariela R. Monti [b], Carlos E. Argaraña [b], Gustavo A. Rivas [a,*]

[a] *INFIQC, Departamento de Físico Química, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina*
[b] *CIQUIBIC, Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina*

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Abstract

In this work, we demonstrate for the first time that 4-methyl-5-nitrocatechol (4M5NC) and 2,4,5-trihydroxytoluene (2,4,5-THT), two compounds obtained from the 2,4-DNT biodegradation are recognized by polyphenol oxidase as substrates. An amperometric biosensor is described for detecting these compounds and for evaluating the efficiency of the 2,4-DNT conversion into 4M5NC in the presence of bacteria able to produce the 2,4-DNT-biotransformation. The biosensor format involves the immobilization of polyphenol oxidase into a composite matrix made of glassy carbon microspheres and mineral oil. The biosensor demonstrated to be highly sensitive for the quantification of 4M5NC and 2,4,5-THT. The analytical parameters for 4M5NC are the following: sensitivity of $(7.5 \pm 0.1) \times 10^5$ nAM^{-1}, linear range between 1.0×10^{-5} and 8.4×10^{-5} M, and detection limit of 4.7×10^{-6} M. The sensitivity for the determination of 2,4,5-THT is $(6.2 \pm 0.6) \times 10^6$ nAM^{-1}, with a linear range between 1.0×10^{-6} and 5.8×10^{-6} M, and a detection limit of 2.0×10^{-7}. Under the experimental conditions, it was possible to selectively quantify 4M5NC even in the presence of a large excess of 2,4-DNT. The suitability of the biosensor for detecting the efficiency of 2,4-DNT biotransformation into 4M5NC is demonstrated and compared with HPLC-spectrophotometric detection, with very good correlation. This biosensor holds great promise for decentralized environmental testing of 2,4-DNT.

Keywords: Biosensor; Glassy carbon paste; Polyphenol oxidase; 2,4-Dinitrotoluene; 4-Methyl-5-nitrocatechol; 2,4,5-Trihydroxytoluene

1. Introduction

Electrochemical biosensors have demonstrated to be extremely useful in different fields [1]. They present important advantages compared to other biosensors, connected to the sensitivity of electrochemical techniques, the possibility of portability and miniaturization, and the relative low-cost of electrochemical devices [1]. They have been successfully used for the determination of a wide range of analytes like glucose [2], neurotransmitters [3], alcohols [4], nucleic acids [5], and pollutants [6,7] among others.

Carbon materials have received special attention for the preparation of electrochemical biosensors [8]. In fact, glassy carbon, carbon fibers, and graphite in different forms have been used for the preparation of electrochemical biosensors [1]. However, just few examples of the application of glassy carbon composites for developing enzymatic biosensors have been proposed. Wang et al. [9] and Rodríguez and Rivas [10] have described the electrocatalytic properties of a glassy carbon paste electrode towards different analytes as well as the usefulness for developing enzymatic electrodes. Girault et al. [11] have also proposed a composite based on micro-glassy carbon particles and a polystyrene polymer.

The aim of this work is to propose an enzymatic biosensor based on the use of a glassy carbon composite and polyphenol oxidase (PPO) for the quantification of compounds derived from the biodegradation of 2,4-dinitrotoluene (2,4-DNT). These compounds have never been described before as substrates of PPO.

PPO is a cuproprotein widely distributed in nature, responsible for the oxidation of phenol and catechol to the corresponding quinones [12].

* Corresponding author. Tel.: +54 351 4334169/80; fax: +54 351 4334188.
E-mail address: grivas@mail.fcq.unc.edu.ar (G.A. Rivas).

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The nitroaromatic compound 2,4-DNT is a precursor in toluene diisocyanate synthesis used for the production of polyurethanes. This compound is also an important intermediate in the manufacturing of the explosive 2,4,6-trinitrotoluene. Improper disposal of waste streams generated by the industrial activity and military operations have resulted in contamination of the environment with 2,4-DNT. The U.S. Environmental Protection Agency (EPA) includes 2,4-DNT into the priority pollutant list [13,14] because of its toxicity [15–17], carcinogenicity [17,18], and its widespread environmental occurrence. Therefore, manufacturing wastes are specifically regulated by the U.S. EPA.

Biological treatment for cleaning up polluted environments has became a potential alternative to conventional physical and chemical methods since it presents several advantages, such as low-cost and it provides a definitive solution. 2,4-DNT-degrading bacteria have been isolated from contaminated sites around the world [19–21]. All bacterial strains appear to follow the same degradation pathway (Scheme 1) [19,20]. This catabolic pathway has been extensively investigated at biochemical and genetic levels in the degrading bacteria *Burkholderia* sp. strain DNT and *Burkholderia cepacia* strain R34 [21–28]. The first step in the pathway involves a 2,4-DNT dioxygenase (DntA) that catalyzes the oxidation of 2,4-DNT to 4-methyl-5-nitrocatechol (4M5NC) with concomitant release of nitrite [21,28]. The second nitro group is removed from the aromatic ring by a monooxygenase (DntB) yielding 2-hydroxy-5-methylquinone (2H5MQ) that in turn is reduced to 2,4,5-trihydroxytoluene (2,4,5-THT) by a reductase (DntC) [22,23]. The resulting 2,4,5-THT undergoes a *meta*-cleavage catalyzed by a dioxygenase (DntD) producing 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOHA) [24,25]. A CoA-dependent methylmalonate semialdehyde dehydrogenase (DntE) and a bifunctional isomerase/hydrolase (DntG) catalyze the subsequent reactions to produce pyruvate and propionyl-CoA, which finally enter the central metabolic pathways of the cell [26].

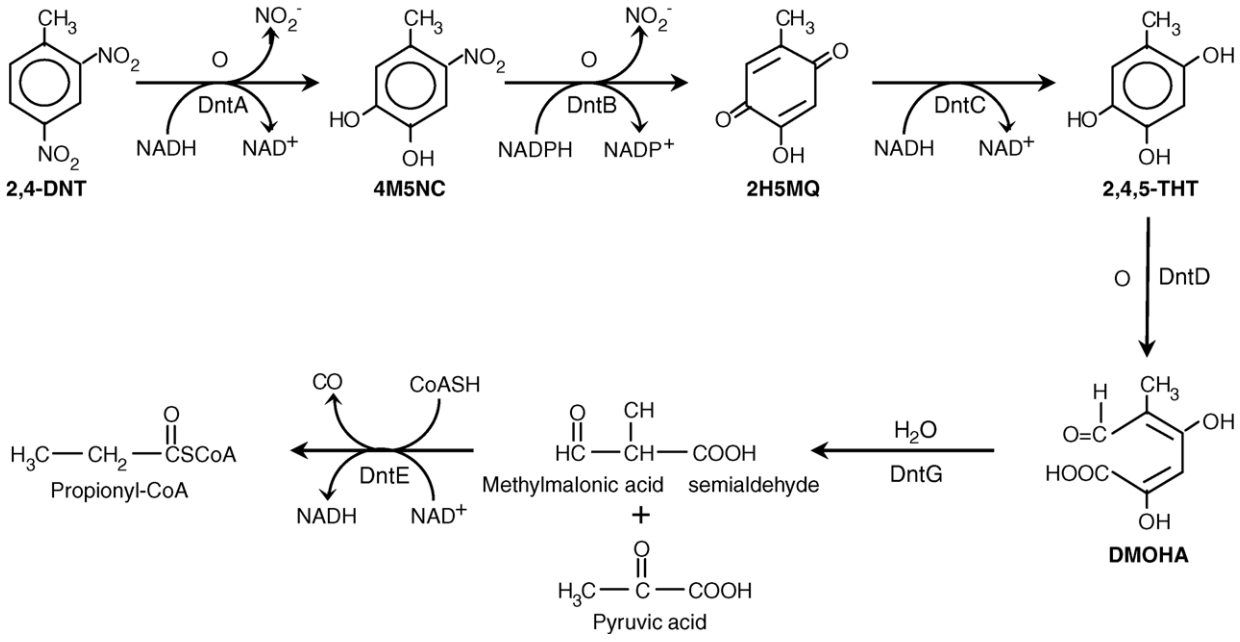
Previous reports have demonstrated the development of bioremediation strategies that exploit the ability of aerobic bacteria to completely degrade 2,4-DNT presents in polluted materials. Fluidized-bed and soil slurry reactors are effective ex situ methods for treatment of wastewaters and polluted soils, respectively [29–32]. To ensure the success of these bioremediation strategies, several factors must be optimized on the engineering design. Particularly, a real time monitoring of the biodegradation process is an important factor in the operation of the bioreactor system [32].

In the following sections, we demonstrate the affinity of PPO for two important compounds originated from the biological degradation of 2,4-DNT and propose the use of the glassy carbon paste electrode modified with PPO as an important tool to evaluate the efficiency of the biodegradation process.

2. Experimental

2.1. Reagents

Catechol and mushroom polyphenol oxidase (or Tyrosinase E.C. 1.14.18.1, 1030 U/mg) were purchased from SIGMA. 2,4-dinitrotoluene (2,4-DNT) (99% of purity) was obtained from Fluka. 4-Methyl-5-nitrocatechol (4M5NC) and 2,4,5-trihydroxytoluene (2,4,5-THT) were kindly provided by Jim C. Spain, Tyndall Air Force Base, FL. 2-Hydroxy-5-methylquinone (2H5MQ) was obtained by spontaneous oxi-



Scheme 1. Pathway for 2,4-DNT degradation in *B. cepacia* strain R34. The enzymes are: DntA, 2,4-dinitrotoluene (2,4-DNT) dioxygenase; DntB, 4-methyl-5-nitrocatechol (4M5NC) monooxygenase; DntC, 2-hydroxy-5-methylquinone (2H5MQ) reductase; DntD, 2,4,5-trihydroxytoluene (2,4,5-THT) oxygenase; DntG, 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOHA) isomerase/4-hydroxy-2-keto-5-methyl-6-oxo-3-hexenoate hydrolase; DntE, methylmalonate semialdehyde dehydrogenase. NO_2^-, nitrite; CoASH, coenzyme A.

dation of 2,4,5-THT in KH_2PO4 buffer (20 mM, pH 6.7) as previously described [22]. 2,4-Dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOHA) was produced from 2,4,5-THT using the DntD oxygenase from *E. coli* JMP109 pJS76 [27]. Glassy carbon, carbon microparticles (carbon spherical powder 0.4–12 μm, type 2) were purchased from Alfa AEsar. Other chemicals were reagent grade and used without further purification.

Ultrapure water ($\rho = 18\ M\Omega$) from a Millipore-MilliQ system was used for preparing all the solutions. A 0.050 M phosphate buffer solution pH 7.40 was employed as supporting electrolyte in the amperometric determinations.

2.2. Subcloning and expression of dntA genes in Ralstonia eutropha JMP289

2.2.1. Bacterial strains and media

E. coli JM109 strains carrying pJS37 or pJS76 plasmids [27] were supplied by Jim C. Spain, Tyndall Air Force Base, FL. *R. eutropha* JMP289 [33] was provided by Dr. Bernardo González, Pontificia Universidad Católica de Chile, Santiago de Chile. *E. coli* and *R. eutropha* strains were cultured in Luria Bertani (LB) medium [34] with agitation (225 rpm) at 37 and 28°C, respectively.

2.2.2. Polimerase chain reaction (PCR) amplification and cloning of dntA genes

The *dntA* (*dntAaAbAcAd*) genes that encode the 2,4-DNT dioxygenase from *Burkholderia* sp. strain DNT [28] were obtained from pJS37 plasmid by PCR amplification of a 5.2-kb fragment. Primers used were: *dntA*c-for 5′-CATGACCTCACCCTCACC-3′ and *dntA*c-rev 5′-CTCACAGGAAGATCATCAGG-3′. PCR was performed in a 50-μL reaction mixture by using standard cycling conditions: 95 °C for 5 min and 35 cycles consisting of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 5 min, followed by 10 min at 72 °C. The *dntA* amplified fragment was cloned into pGEM-T Easy vector (Promega, Madison, USA) to obtain the pMRM8 plasmid.

2.2.3. dntA delivery plasmid construction and conjugation assay

The 5.2 kb fragment presents in pMRM8 plasmid was subcloned into the unique *Not*I site of pUT mini-Tn5 Hg^r [35] obtaining the pUT-MRM4 delivery vector that allowed the insertion of the *dntA* genes into *R. eutropha* JMP289 chromosome.

The pUT-MRM4 delivery vector was introduced for propagation into *E. coli* SY327 (λ*pir*) [36], and then transferred to *E. coli* S17-1 (λ*pir*) [35] that was finally used as donor strain. The *R. eutropha* JMP289 transconjugants were obtained by biparental conjugation using the filter mating method [35] and were selected on LB agar plates containing 20 μg/mL rifampin and 0.05 mM merbromide. A transconjugant that showed a positive PCR amplification of *dntA* genes using its genomic DNA and transformed 2,4-DNT to 4M5NC in microplate assays [27], was selected to perform the 2,4-DNT biotransformation assay.

2.2.4. 2,4-DNT biotransformation assay

R. eutropha transconjugant cells from stationary phase LB culture were harvested by centrifugation, washed twice, and suspended (absorbance$_{600}$ = 0.50) in KH_2PO_4 buffer (50 mM, pH 7.5) supplemented with 85 μM 2,4-DNT. After discrete incubation times, cells were removed from medium by centrifugation at 10,000 g for 10 min and the supernatant fluid was filtered through a 0.22 μm membrane (Millipore). Finally, the supernatant fluid was stored at −70 °C until analysis. Controls without cells or 2,4-DNT were performed.

2.3. Apparatus

The electrochemical measurements were carried out with an EPSILON potentiostat (BAS). The electrodes were inserted into the cell (BAS, Model MF-1084) through its Teflon cover. A platinum wire and Ag/AgCl, 3 M NaCl (BAS, Model RE-5B) were used as counter and reference electrodes, respectively. All potentials are referred to the latter. A BAS Cell-Stand C3 gave the convective transport during the amperometric determinations.

The glassy carbon paste electrode (GCPE) (90.0 and 10.0%, w/w, glassy carbon microparticles and mineral oil, respectively) was prepared by mixing in an agata mortar the glassy carbon microparticles (90.0%, w/w) with mineral oil (Aldrich) (10.0%, w/w). The graphite paste electrode (gCPE) was prepared in a similar way by using graphite powder (Fisher # 38). The composites electrodes containing PPO were prepared in the following way: the desired amount of enzyme (4.0%, w/w) was mixed with mineral oil in an agata mortar for 3 min followed by the incorporation of glassy carbon powder and mixing for additional 15 min. A portion of the resulting paste was packed firmly into the cavity (3.0 mm diameter) of a Teflon tube. The electric contact was established through a stainless steel screw. A new surface was obtained by smoothing the electrode onto a weighing paper.

Reversed-phase high-performance liquid chromatography (HPLC) was used to quantify 2,4-DNT and its degradation product 4M5NC as follows. The supernatant fluid was injected in an Allsphere ODS-1 column (5 μm, 250 by 4.6 mm; Alltech) with 13.5 mM trifluoroacetic acid–acetonitrile (50:50) as mobile phase at a flow rate of 1.0 mL/min. Both compounds were detected by UV absorbance at 254 nm with a Spectra-Physics Variable Wavelength Detector (Westshore Technologies).

Spectrophotometric determinations of 2,4,5-THT were performed with a Shimadzu UV-1601 spectrophotometer.

2.4. Procedure

All the experiments were conducted at room temperature. The amperometric experiments were carried out by applying the desired potential and allowing the transient current to decay prior to the addition of the analyte and the subsequent current monitoring.

3. Results and discussion

It was demonstrated that GCPE represents a good alternative for the detection of different compounds due to the inherent

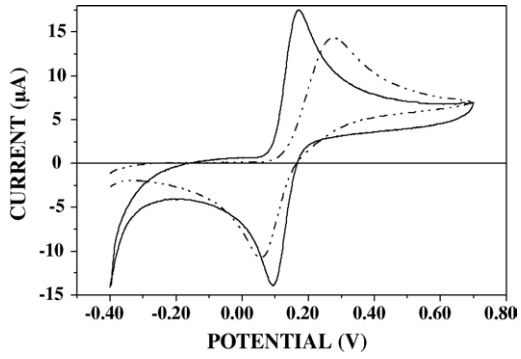


Fig. 1. Cyclic voltammograms for 5.0×10^{-4} M catechol at bare graphite paste electrode (gCPE) (dotted line) and at bare glassy carbon paste electrode (GCPE) (solid line). Supporting electrolyte: 0.050 M phosphate buffer solution, pH 7.40. $v = 0.100$ V s^{-1}. GCPE and gCPE: 90.0% (w/w) carbon powder and 10.0% (w/w) mineral oil.

electroactivity of CG microparticles [9,10]. Fig. 1 shows the voltammetric behavior of 5.0×10^{-4} M catechol at bare graphite paste (dotted line) and at bare glassy carbon paste (solid line) electrodes. An important decrease in the peak potential separation (0.280 and 0.164 V for gCPE and GCPE, respectively) as well as a decrease in the peak currents ratio (1.8 and 1.2 for gCPE and GCPE, respectively) is obtained at the GCPE, demonstrating the improved electroactivity of the composite based on the glassy carbon microparticles.

The first step in the biodegradation of 2,4-DNT is its conversion into 4M5NC by the action of DntA (Scheme 1, step 1). Based on the structure of this compound, we evaluated the affinity of PPO towards 4M5NC. As it is widely known [12], PPO generates quinones from phenols and catechols that can be electrochemically detected at an adequate potential, usually below 0.00 V. Fig. 2A shows the amperometric recording obtained at −0.100 V at GCPE modified with PPO after successive additions of 1.0×10^{-5} M 4M5NC. The reduction current obtained after each addition of the catechol derivative indicates that 4M5NC is recognized by PPO, making possible its detection from the reduction of the quinone enzymatically generated. A fast response is obtained in the presence of this compound and the resulting calibration plot shows a sensitivity of $(7.5 \pm 0.1) \times 10^5$ nAM^{-1} with a linear range between 1.0×10^{-5} and 8.4×10^{-5} M, and a detection limit of 4.7×10^{-6} M. Similar experiments performed with a GCPE without PPO did not show any response (not shown). Therefore, we are demonstrating for the first time that 4M5NC is substrate of PPO and that it is possible to evaluate its concentration by electrochemical techniques.

A control experiment was performed to check the response of the bioelectrode in the presence of 2,4-DNT. Fig. 2B shows the amperometric recording at −0.100 V for successive additions of 1.0×10^{-5} M 2,4-DNT. Due to the absence of hydroxyl groups in the molecule, PPO cannot recognize this compound as substrate and there is no conversion of 2,4-DNT into the corresponding quinone. Therefore, no reduction currents were observed even for high-concentrations of this compound, indicating that under these conditions there are no quinones enzymatically generated to be reduced at the working potential.

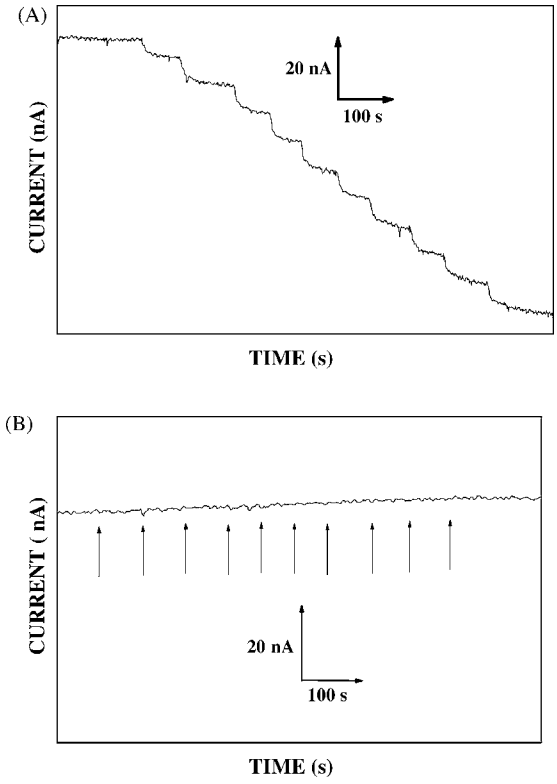


Fig. 2. Current–time profiles for successive additions of 1.0×10^{-5} M 4M5NC (A) and 2,4-DNT (B). Working potential: −0.100 V. PPO-GCPE: 4.0% (w/w) PPO, 8.0% (w/w) mineral oil, and 88.0% (w/w) GC powder. Supporting electrolyte: 0.050 M phosphate buffer solution, pH 7.40.

It is very important to perform determinations of 4M5NC in the presence of its precursor 2,4-DNT, to analyze the percentage of conversion of this pollutant during the biotransformation process (proposed in the present work). Amperometric determinations of 4M5NC in the presence of elevated concentrations of 2,4-DNT did not show any change, demonstrating that it is possible to measure 4M5NC in the presence of the nitroaromatic compound (not shown).

Another key aspect is the detection of 4M5NC in the medium where the 2,4-DNT biotransformation is occurring. Hence, it is necessary to evaluate the response of the bioelectrode in the presence of the components of the biotransformation medium. Fig. 3A displays the results obtained by exposing the biosensor to 10 additions of 2,4-DNT dissolved in the biotransformation medium without the addition of the corresponding bacteria carrying the DntA oxygenase that converts it into 4M5NC. No response was obtained under these conditions, demonstrating that if the enzymatic biotransformation of 2,4-DNT does not occur, effectively there are no substrates for PPO. Similar amperometric experiments obtained after addition of the medium containing 2,4-DNT and *R. eutropha* JMP289 genetically modified with *dntA* genes are shown in Fig. 3B. Since these genes codified the oxygenase that catalyzes the transformation of 2,4-DNT into 4M5NC, the expected reduction current was obtained, indicating that the biotransformation took place and that the proposed biosensor is able to perform determinations of this process.

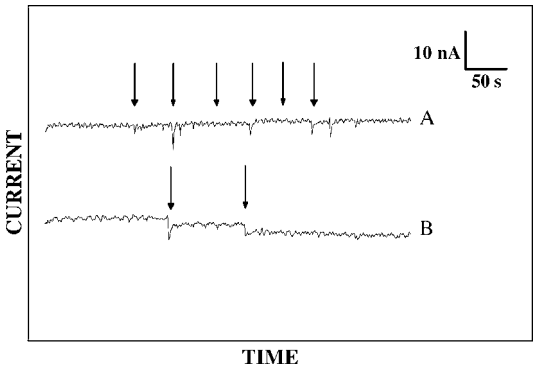


Fig. 3. Amperometric recordings obtained at −0.100 V using a GCPE-PPO for successive additions of: (A) biotransformation medium containing 2,4-DNT in the absence of *R. eutropha* JMP289 genetically modified with *dntA* genes and (B) biotransformation medium containing *R. eutropha* JMP289 genetically modified with *dntA* genes and 2,4-DNT after 1 h of incubation. Other conditions as in Fig. 2.

Fig. 4 shows a comparison between the amperometric and HPLC-spectrophotometric response of 4M5NC generated after different incubation times (first step of Scheme 1). A very good correlation was obtained between these two techniques, demonstrating that GCPE-PPO represents a interesting alternative for performing field determinations due to the lower cost, the shorter time for performing the analysis and comparable sensitivities.

The conversion of 4M5NC into 2,4,5-trihydroxytoluene (2,4,5-THT) is also interesting to follow the 2,4-DNT biodegradation process. The inconvenience is that at neutral pH there is equilibrium between 2,4,5-THT and 2-hydroxy-5-methylquinone that makes impossible the amperometric determination of 2,4,5-THT in 0.050 M phosphate buffer pH 7.40. It is known that 2,4,5-THT is highly stable in acidic solution. For that reason, taking in consideration that PPO possesses a pH activity range from pH 4.5 to 8.5, we checked by spectrophotometry the stability of 2,4,5-THT (previously prepared in 1.00 M HCl to avoid chemical transformations) in 0.100 M acetate buffer solution pH 4.80. During the time of the assay, the compound remained stable, allowing, in this way, the amperometric determination of 2,4,5-THT in this medium.

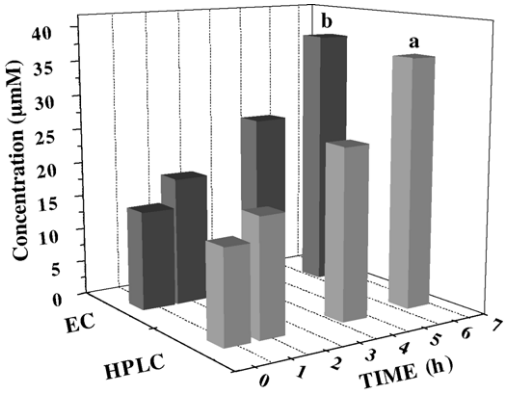
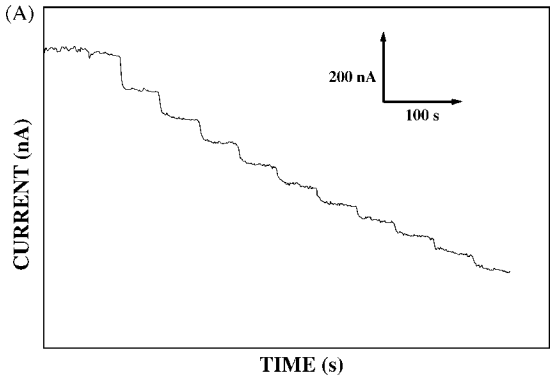


Fig. 4. Incubation time effect on the 4M5NC production. Comparison between HPLC with optical detection (a) and amperometry using GCPE-PPO (b) for the monitoring of the 2,4-DNT biotransformation. (a) Wavelength: 254 nm; (b) working potential: −0.100 V. Other conditions as in Fig. 2.



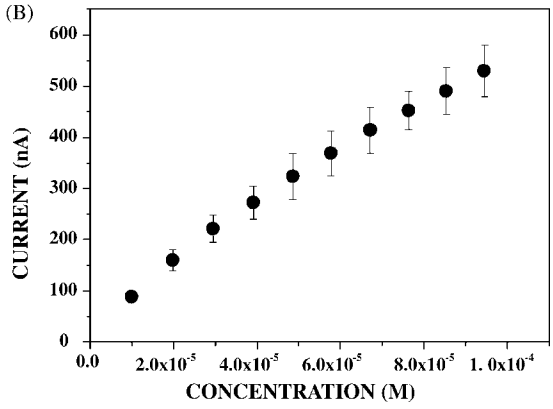


Fig. 5. (A) Current–time profiles for successive additions of 1.0×10^{-5} M 2,4,5-THT using a PPO-GCPE. (B) Calibration plot obtained from (A). Working potential: −0.100 V. Supporting electrolyte: 0.100 M acetate buffer solution, pH 4.80.

Fig. 5 shows amperometric recordings obtained at GCPE-PPO for successive additions of 1.0×10^{-5} M 2,4,5-THT to a 0.100 M acetate buffer solution pH 4.80 using the GCPE-PPO. A very fast response is obtained after the addition of 2,4,5-THT with a sensitivity of $(6.2 \pm 0.6) \times 10^6$ nAM^{-1}, a linear range between 1.0×10^{-6} and 5.8×10^{-6} M and a detection limit of 2.0×10^{-7} M. We are also reporting for the first time the affinity of PPO toward 2,4,5-THT. Therefore, to perform the determinations using the biotransformation medium as a substrate, once the reaction was stopped, the supernatant was treated with 1 M HCl in order to avoid chemical transformations of the trihydroxytoluene, and then proceed regularly.

Finally, the catabolic step catalyzed by the oxygenase DntD involves the transformation of 2,4,5-THT into the aliphatic 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOHA). Amperometric determinations were performed after successive additions of DMOHA and no reduction currents were detected even for high amounts of this compound (not shown) since DMOHA contains hydroxyl groups but it is not an aromatic compound. These results demonstrate that DMOHA is not substrate of PPO under these conditions.

4. Conclusions

We have demonstrated for the first time that the 2,4-DNT biodegradation intermediates 4M5NC and 2,4,5-THT are sub-

strates of PPO. We have also proposed a new alternative to follow the biodegradation process of 2,4-DNT by the action of 2,4-DNT-biodegrading bacteria in a fast, sensitive and simple way by using the GCPE modified with PPO. The different affinity of PPO towards 2,4-DNT and 4M5NC, have made possible to follow the kinetics of the 2,4-DNT biotransformation through the formation of 4M5NC. The suitability of the present scheme to follow this biotransformation, in combination with the widely known advantages of electrochemical devices, opens the doors to exciting applications in the development of sensors capable of operation in a field environment.

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