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Biocatalysts immobilization onto nanostructured supports with application in the biotransformation of emerging pollutants

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To Laura and Fernando

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Biocatalysts immobilization on nanostructured supports with application in the biotransformation of emerging pollutants

By

Raul Garcia Morales

Abstract

Biotransformation of emerging pollutants in water samples using biocatalysts such as laccases, provides an environmentally attractive alternative to complement conventional wastewater treatments. However, loss and inactivation of enzymes are challenges to solve for their potential use in water treatment applications. The work included in this thesis explores the development of new biocatalyst using a new laccase from *Pycnoporus sanguineus* CS43 strain. First, the biotransformation of contaminants with laccases in free form was carried out to demonstrate their removal potential. Secondly, through enzyme immobilization using nanomaterials as supports to improve the stability and catalytic properties of laccase enzymes.

In order to demonstrate the biotransformation potential of laccases for emerging pollutants, two studies were carried out, in which the effect of enzymes in their free form was analyzed. First, a synthetic matrix was used, followed by using groundwater as a real matrix. The first work evidenced an enhanced biotransformation capability to remove common emerging pollutants such as bisphenol A, 4-nonylphenol, 17-α-ethynylestradiol and triclosan, achieving biotransformation efficiencies of 89–100 % using synthetic samples. In groundwater, biotransformation ranged from 55 to 93 %. The second study also focused in proving the biocatalytic ability of laccases, in this case biotransformation in synthetic samples ranged from 50 – 97 % for diclofenac, β-Naphthol, 2,4 dichlorophenol and 5,7-diiodo-8-hydroxyquinoline, while for groundwater laccases biotransformed more than 53% for all compounds.

Enzyme immobilization onto nanomaterials was achieved using the covalent approach. Process optimization and later studies demonstrated that the proposed procedure using 3-aminopropyltriethoxysilane (APTES) as coupling agent and the cross-linker glutaraldehyde (GLU) as a laccase binder, can enhance inherent properties of laccase enzymes such as high thermostability and pH stability Furthermore, the biocatalyst was assessed in the biotransformation of emerging pollutants such as acetaminophen and diclofenac showing more than 90% biotransformation in synthetic and real samples.

Consequently, due to the promising results obtained in the aforementioned studies, where the enzymatic treatment was evaluated under mild reaction condition, this biocatalytic approach represents a good alternative in the treatment of organic pollutants in water samples.

Contents

Chapter 1

Introduction

Chapter 1

Introduction

Water is an essential natural resource for the survival of all known life forms, and is required for most human activities including agriculture, industry and personal consumption. Therefore, ensuring water quality is a fundamental environmental and public health issue [1]–[3].

The unregulated use of pharmaceuticals, coupled with the overproduction of compounds for human consumption have led to an increase in chemical substances that are released to the environment. Currently, the presence of emerging pollutants in water is receiving increasing attention. Emerging pollutants are substances that are currently not included in routine or regulatory control programs even though have been detected in the environment in range of ng/L to µg/L, which makes them candidates for future legislation due to its adverse effects and/or persistency [4], [5]. This category includes pharmaceuticals, personal hygiene products, surfactants, industrial additives and numerous chemical compounds that are being continuously discharged into the aquatic systems [6]. Some of these compounds have been identified as possible environmental threats to living organisms due to their demonstrated toxic potential and even a considerable amount of emerging pollutants are classified as endocrine disruptors compounds (EDCs) [7]. In addition, some emerging pollutants are not easily degraded, which can lead to their accumulation in different trophic levels [8], [9].

Since the lack of regulation, monitoring and disposal or discharge for emerging pollutants, these have been widely spread to the environment. For this reason recent attention on this area has been paid, especially for wastewater treatment since most conventional wastewater treatment plants (pollutants sink) have not been designed for the elimination of these compounds [6], [10]. This issue poses important challenges in the design of new treatment plants and the development of methods focused on the elimination of such substances [11].

In the last decades there has been a growing interest in treatments focused on the elimination of emerging pollutants through strategies based on biological processes [12]. A promising method is based on the use of oxidoreductase enzymes for the biotransformation of pollutants with similar molecular structure to the enzyme substrates, that include polyphenolic structures and aromatic amines [12], [13]. The use of these enzymes has been described by studies in which their applicability to detoxification and biotransformation of several substances in wastewater effluents of different industries, such as petrochemical, textile, tanneries, and distilleries [14].

Laccases (EC 1.10.3.2) also known as fungal laccases (benzenediol: oxygen reductase) and are the main enzymes type involved in delignification by white rot fungi (*Pycnoporus cinnabarinus*, *Coriolopsis rigida,* among others*)*. These enzymes are extracellular glycoproteins that generally contain four copper atoms in their active site (Figure 1), but other enzymes with two, three, and even six copper atoms have been described [15]. The molecular weight of these enzymes ranges from 50 to 70 kDa, with an isoelectric point between 2.6 and 4.5. They also contain cysteine and histidine residues, which are involved in the binding of copper atoms. Their pH and

optimum temperature range from 2-5, 40-65 ˚C, respectively, and will vary according to the fungal species [15]–[17].

In the case of laccases from *Pycnoporus sanguineus CS43,* which are the enzymes used in this work, it was found that this fungus was able to produce two major isoforms (LacI and LacII). An isoform is an enzyme that differ in amino acid sequence but catalyze the same chemical reaction. Then both isoforms presented a molecular weight of 68 kDa, optimal pH range of 2-4 and optimal temperature range of 40-60 °C. They also presented high pH (5-8) and temperature (25-60 °C) stability, as well as tolerability to organic solvents and some ionic substances [18].

Particularly, the catalytic efficiency of laccases used in this work can be ascribed to dissimilarity in their active site, specifically in the region of substrate binding. A previous study found that different amioacid sequence in the active site of LacI and LacII, exhibited steric hindrance, different polar environment for each and different mode to form hydrogen bonds. Hence, although enzymes are very specific, differences in structure could be the reason why laccases are non-specific, but selective for phenolic and amino compounds [19].

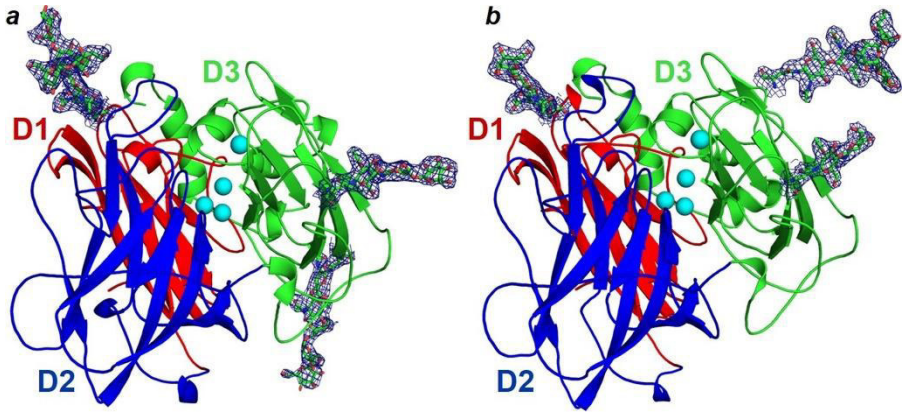

Figure. 1. Ribbon representation of the 3-D structure of laccases from *Pycnoporus sanguineus* CS43: LacI (a) and LacII (b). Domains 1–3 are shown in red, blue and green, respectively. Copper ions are displayed as cyan spheres. The carbohydrate residues in the electron density maps are shown as green sticks. Adapted from [19].

According to laccase substrate reactivity, unlike most oxidoreductase enzymes; laccases use molecular oxygen as an oxidizing agent, and are capable of carrying out the monoelectronic oxidation of diphenols and aromatic amines by removing an electron and a proton from hydroxyl or amino groups, generating phenoxyl or amino radicals, respectively. The oxidation of the latter allows for the generation of the superoxide anion, which along hydrogen peroxide are precursors of the hydroxyl radical. This radical can be responsible for the initial degradation of complex lignocellulose, and also for similar phenolic substrates [20], [21].

As laccases is a nonspecific enzyme, can oxidize different substrates, and their reactivity will vary according to the chemical structure of each substrate. The presence of functional electron-acceptor groups (EAGs) such as amide groups (-$CONR_2$), carboxylic groups (-COOH), halogens (-X), and the nitro group (-NO_2)

promote a deficiency of electrons in the molecule (Figure 2), making them less susceptible to oxidative catabolism. Conversely, electron donor functional groups (EDGs) such as the amine ($-NH_2$), hydroxyl (-OH), alkoxy (-OR), alkyl (-R), and acyl (-COR) groups make molecules more susceptible to electrophilic attack by oxygenases (Figure 3) [22], [23].

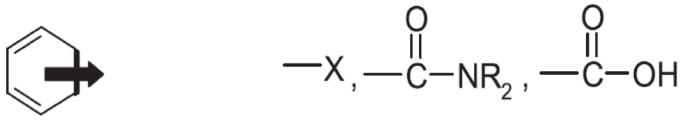

Figure 2. Electron Acceptor Functional Groups

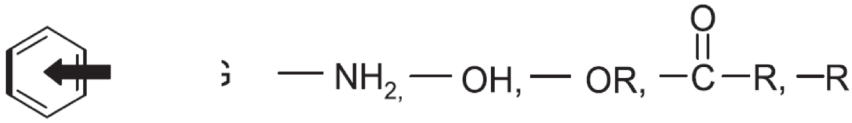

Figure 3. Functional electron donor groups.

For water treatment purposes, enzymes are commonly used in their free form, i.e. dispersed in a liquid medium, which hinders enzyme recovery after treatment. Consequently, free enzymes can only be used once, leading to an increase in both production and purification costs [24]. Furthermore, in free form, enzymes are susceptible to harmful agents the medium and their stability and activity can be affected by these complex matrices, such as the case of highly contaminated wastewater [25] .

An appropriate approach to overcome these drawbacks is enzyme immobilization, which consists in the fixation of an enzyme in an inert and insoluble material through chemical or physical processes, where the biocatalyst is physically located in a well-defined region of space [26], [27]. Some of the characteristics that are improved or modified with immobilization are: (i) wider optimal temperature and pH in comparison with their free form counterpart; (ii) greater stability under pH or temperature changes; (iii) increased activity; (iv) facile enzyme recovery from the reaction medium facilitating its reuse. Additionally, this process prolongs their shelf life compared to enzymes in free form [28].

As shown in Figure 4, the main components in an immobilization system are the enzyme (can be single o multi enzyme system), the support, and the method of union between the enzyme and the support [28]. Nevertheless, simple enzyme immobilization may not achieve the desired enhancement of enzymatic activity or stability, as it may lead to deformation or even denaturalization of enzymatic structures resulting in loss of activity. Hence, the optimal immobilization conditions need to be explored for every specific enzyme, making this technique challenging/ labor intensive [21], [29], [30].

For this particular work, a chemical immobilization approach using covalent binding was selected. This method leads to better outcomes with highly stable preparations and better enzyme retention compared to other physical-based immobilization techniques, like adsorption. Thus, the immobilized enzyme is not detached from the support matrix during the treatment process and can be reused [31], [32].

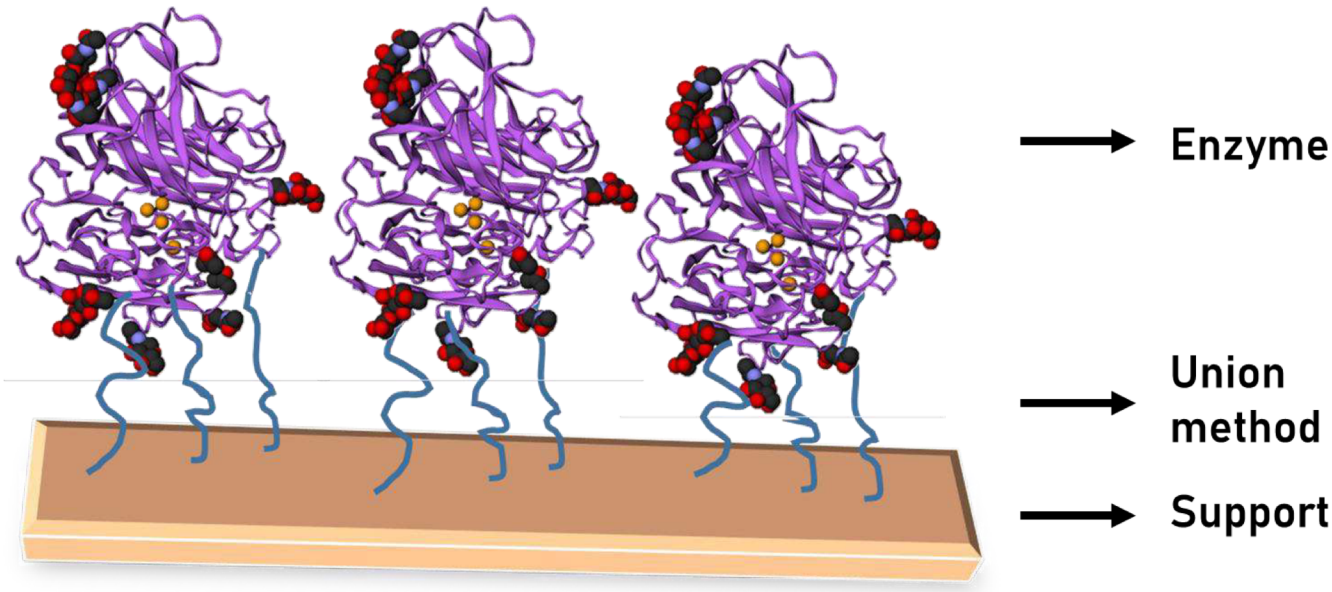

Figure 4. Schematic representation of the components in an enzymatic immobilization system

Another important issue in recent years is the development of supports for enzyme immobilization; among them, nanostructured materials have desirable properties that can improve these processes. One of the main advantages of these materials is their large surface areas which allow greater enzymatic loading and lower resistance to mass transfer for substrates. Besides, they can be synthesized with a high chemical stability and specific functional groups in their surface to facilitate functionalization [30], [33], [34].

In particular for the present work, commercially available titanium (IV) oxide nanoparticulated (titania) has been selected, this material possesses the desired characteristics mentioned above. Among important features of this materials are its specific surface of approx. 50 m^2/g, crystal structure composed of anatase and rutiles phases, and considerable photoactivity. Figure 5 shows the characterization by transmission electron microscopy (TEM) of titanium dioxide nanoparticles, it is possible to observe that material consist in aggregates of some hundred nanometer in size with primary particles of average diameter of 21 nm [35].

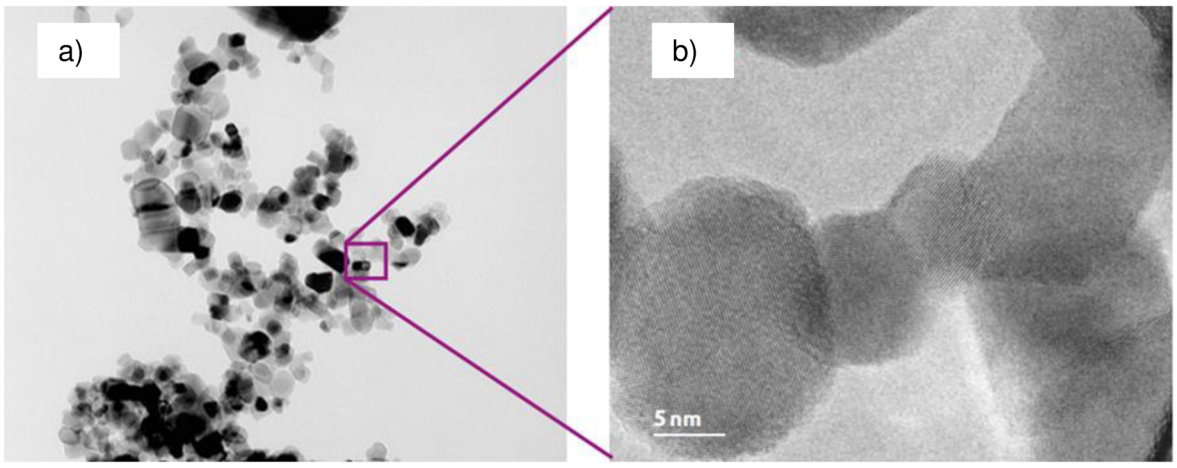

Figure 5. TEM images of titanium dioxide nanoparticles; a) aggregates and agglomerates and b) primary crystals. Figure modified from www.aerosil.com [35].

In view of the aforementioned, the present thesis work focused on studying the effect of a new variety of laccase-type oxidoreductase enzymes obtained from the fungus *Pycnoporus sanguineus* CS43 in free and immobilized form, for the biotransformation of emerging pollutants. The covalent immobilization technique was coupled with nanostructured materials as supports. UV-Visible Spectroscopy and RP-HPLC were used for the qualitative and quantitative monitoring of the biotransformation process.

1.1 Motivation

Despite the fact that water is a renewable resource, its availability has been gradually reduced in an alarming way as demands for its use increase, where the food and industrial sectors stand out [36], [37]. Water is highly vulnerable to deterioration in quality, and the appearance of emerging pollutants lacking of regulation by government or environmental agencies is a cause of great concern. The aforementioned represents serious environmental threats due to the known toxicity and persistence shown by this type of substances [38].

The motivation for this work is the development an environmentally friendly process based on ligninolytic enzymes for the removal of phenolic type emerging pollutants to improve water quality, as well as improving their stability to different agents such as pH, temperature and deactivating substances by means of enzymatic immobilization onto nanomaterials.

1.2 Problem Statement and Context

Currently, traditional wastewater treatment, such as sedimentation, coagulation, and activated sludge; as well as more harsh methods, offer the elimination and inactivation of those pollutants that are high concentrations, but may have high costs that tend to limit the sustainability of the water treatment process, in addition they are not very efficient in eliminating emerging pollutants that are at very low concentrations.

As a result of the aforementioned, there is ongoing concern for the improvement of wastewater treatments. Thus, new treatment methods have emerged facilitating and speeding up the removal and/or degradation of contaminants. Recently, there has been greater interest in the development of treatments involving biological systems, which offer advantages such as (i) compatibility with matrices; (ii) decrease use of toxic supplies; (iii) avoiding the addition of extra compounds in the remediation processes; (iv) generating by-products with low toxicity.

Within the scope of these systems, one that has been highly effective in biotransforming emerging pollutants aqueous media has been the use of enzymes specifically, using ligninolytic enzymes which react over phenolic type compounds reaching almost total degradations.

Among the disadvantages of enzymatic processes for the degradation of compounds is that their application is complicated because they need many factors to remain stable and retain their activity in complex matrices. In addition, in their free form, they can only be used in a single treatment cycle, so they may not be efficient at the time of making a treatment on a larger scale than in the laboratory.

Therefore, there are two main problems from this context: first, emerging pollutants that are not eliminated by conventional water treatment and secondly the need to increase the stability of enzymes used in the removal of emerging pollutants.

1.3 Objectives

General objective

Immobilize oxidoreductase laccase-type enzymes from the *Pycnoporus sanguineus* CS43 fungus onto titanium dioxide nanostructured, for its application in the biotransformation study of emerging pollutants in water samples.

Specific Objectives

- To study the catalytic efficiency of laccase enzymes in their free form in the biotransformation of emerging pollutants.
- Qualitative and quantitative analysis of the biotransformation process of emerging pollutants of interest in synthetic and groundwater samples due to free laccase action by UV-Vis spectroscopy and RP-HPLC.
- Carrying out enzymatic immobilization through the method of covalent bonding.
- Evaluation of the enzymatic immobilization process onto titania nanoparticles, through measurements of pH and optimal temperature and stability of the immobilized biocatalyst.
- Quantitative analysis of the biotransformation process of emerging pollutants of interest in synthetic and groundwater samples due to immobilized laccase action by using UV-Vis spectroscopy and RP-HPLC.

1.4 Hypothesis

The use of a new variety of laccase-type enzymes from the fungus *Pycnoporus sanguineus CS43*, both in its free form and immobilized onto titania nanostructured materials, will generate high yields in the biotransformation of selected emerging pollutants in synthetic and real water samples.

1.5 References

[1] J. E. Leal, A. N. Thompson, and W. A. Brzezinski, “Pharmaceuticals in drinking water: Local analysis of the problem and finding a solution through awareness,” *J. Am. Pharm. Assoc.*, vol. 50, no. 5, pp. 600–603, 2010.

[2] D. J. Lapworth, N. Baran, M. E. Stuart, and R. S. Ward, “Emerging organic contaminants in groundwater: A review of sources, fate and occurrence,” *Environ. Pollut.*, vol. 163, pp. 287–303, 2012.

[3] J. C. G. Sousa, A. R. Ribeiro, M. O. Barbosa, M. F. R. Pereira, and A. M. T. Silva, “A review on environmental monitoring of water organic pollutants identified by EU guidelines,” *Journal of Hazardous Materials*, vol. 344. Elsevier, pp. 146–162, 15-Feb-2018.

[4] B. Petrie, R. Barden, and B. Kasprzyk-Hordern, “A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring,” *Water Res.*, vol. 72, no. 0, pp. 3–27, 2014.

[5] S. Lecomte, D. Habauzit, T. D. Charlier, and F. Pakdel, “Emerging estrogenic pollutants in the aquatic environment and breast cancer,” *Genes*, vol. 8, no. 9. Multidisciplinary Digital Publishing Institute, p. 229, 15-Sep-2017.

[6] J. Rivera-Utrilla, M. Sánchez-Polo, M. Á. Ferro-García, G. Prados-Joya, and R. Ocampo-Pérez, “Pharmaceuticals as emerging contaminants and their removal from water. A review,” *Chemosphere*, vol. 93, no. 7, pp. 1268–1287, 2013.

[7] N. Bolong, A. F. Ismail, M. R. Salim, and T. Matsuura, “A review of the effects of emerging contaminants in wastewater and options for their removal,” *Desalination*, vol. 238, no. 1–3, pp. 229–246, 2009.

[8] J. Liu, G. Lu, Z. Xie, Z. Zhang, S. Li, and Z. Yan, “Occurrence, bioaccumulation and risk assessment of lipophilic pharmaceutically active compounds in the downstream rivers of sewage treatment plants,” *Sci. Total Environ.*, vol. 511, pp. 54–62, 2015.

[9] N. Pi, J. Z. Ng, and B. C. Kelly, “Bioaccumulation of pharmaceutically active compounds and endocrine disrupting chemicals in aquatic macrophytes: Results of hydroponic experiments with Echinodorus horemanii and Eichhornia crassipes,” *Sci. Total Environ.*, vol. 601–602, no. 1, pp. 812–820, 2017.

[10] M. Gros, M. Petrović, A. Ginebreda, and D. Barceló, “Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes,” *Environ. Int.*, vol. 36, no. 1, pp. 15–26, 2010.

[11] S. Rodriguez-Mozaz *et al.*, “Pharmaceuticals and pesticides in reclaimed water: Efficiency assessment of a microfiltration-reverse osmosis (MF-RO) pilot plant,” *J. Hazard. Mater.*, vol. 282, pp. 165–173, Jan. 2015.

[12] M. Asgher, H. N. Bhatti, M. Ashraf, and R. L. Legge, “Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system,” *Biodegradation*, vol. 19, no. 6, pp. 771–783, 2008.

[13] H. Harms, D. Schlosser, and L. Y. Wick, “Untapped potential: exploiting fungi in bioremediation of hazardous chemicals,” *Nat. Rev. Microbiol.*, vol. 9, no. 3, pp. 177–192, Mar. 2011.

[14] C. Barrios-Estrada, M. de Jesús Rostro-Alanis, B. D. Muñoz-Gutiérrez, H. M. N. Iqbal, S. Kannan, and R. Parra-Saldívar, “Emergent contaminants: Endocrine disruptors and their laccase-assisted degradation – A review,” *Science of the Total Environment*, vol. 612. Elsevier, pp. 1516–1531, 15-Jan-2018.
[15] J. Yang, W. Li, T. B. Ng, X. Deng, J. Lin, and X. Ye, “Laccases: Production, Expression Regulation, and Applications in Pharmaceutical Biodegradation.,” *Front. Microbiol.*, vol. 8, p. 832, 2017.
[16] J.-A. A. Majeau, S. K.Brar, R. DayalTyagi, S. K. Brar, and R. D. Tyagi, “Laccases for removal of recalcitrant and emerging pollutants,” *Bioresour. Technol.*, vol. 101, no. 7, pp. 2331–2350, Apr. 2010.
[17] C. A. Gasser, E. M. Ammann, P. Shahgaldian, and P. F.-X. X. Corvini, “Laccases to take on the challenge of emerging organic contaminants in wastewater,” *Appl. Microbiol. Biotechnol.*, vol. 98, no. 24, pp. 9931–9952, Dec. 2014.
[18] L. I. Ramírez-Cavazos *et al.*, “Purification and characterization of two thermostable laccases from Pycnoporus sanguineus and potential role in degradation of endocrine disrupting chemicals,” *J. Mol. Catal. B Enzym.*, vol. 108, pp. 32–42, 2014.
[19] M. Orlikowska *et al.*, “Structural studies of two thermostable laccases from the white-rot fungus Pycnoporus sanguineus,” *Int. J. Biol. Macromol.*, vol. 107, pp. 1629–1640, Feb. 2018.
[20] A. Kunamneni, A. Ballesteros, F. J. Plou, and M. Alcalde, “Fungal laccase – a versatile enzyme for applications,” *Biotechnological*, pp. 233–245, 2007.
[21] V. Madhavi and S. S. Lele, “Laccase: properties and applications,” *BioResources*, vol. 4, no. 4, pp. 1694–1717, Aug. 2009.
[22] S. Yanga *et al.*, “Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: A critical review,” *Bioresour. Technol.*, vol. 141, pp. 97–108, Aug. 2013.
[23] L. N. Nguyen *et al.*, “Removal of pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals and pesticides by Trametes versicolor: Role of biosorption and biodegradation,” *Int. Biodeterior. Biodegradation*, vol. 88, pp. 169–175, Mar. 2014.
[24] A. Xenopoulos, “A new, integrated, continuous purification process template for monoclonal antibodies : Process modeling and cost of goods studies,” *J. Biotechnol.*, vol. 213, pp. 42–53, 2015.
[25] J.-A. Majeau, S. K.Brar, and R. DayalTyagi, “Laccases for removal of recalcitrant and emerging pollutants,” *Bioresour. Technol.*, vol. 101, no. 7, pp. 2331–2350, Apr. 2010.
[26] I. Chibata and 千畑一郎., *Immobilized enzymes, research and development*. Kodansha, 1978.
[27] N. R. Mohamad, N. H. C. Marzuki, N. A. Buang, F. Huyop, and R. A. Wahab, “An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes.,” *Biotechnol. Biotechnol. Equip.*, vol. 29, no. 2, pp. 205–220, Mar. 2015.
[28] S. Ba, A. Arsenault, T. Hassani, J. P. Jones, and H. Cabana, “Laccase

immobilization and insolubilization: from fundamentals to applications for the elimination of emerging contaminants in wastewater treatment," *Crit. Rev. Biotechnol.*, vol. 33, no. 4, pp. 404–418, Dec. 2013.

[29] C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan, and R. Fernandez-Lafuente, "Improvement of enzyme activity, stability and selectivity via immobilization techniques," *Enzyme and Microbial Technology*, vol. 40, no. 6. Elsevier, pp. 1451–1463, 02-May-2007.

[30] M. Chen, G. Zeng, P. Xu, C. Lai, and L. Tang, "How Do Enzymes 'Meet' Nanoparticles and Nanomaterials?," *Trends Biochem. Sci.*, vol. 42, no. 11, pp. 914–930, Nov. 2017.

[31] M. Asgher, M. Shahid, S. Kamal, and H. M. Nasir Iqbal, "Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology," *J. Mol. Catal. B Enzym.*, vol. 101, pp. 56–66, Mar. 2014.

[32] M. Bilal *et al.*, "Immobilized ligninolytic enzymes: An innovative and environmental responsive technology to tackle dye-based industrial pollutants – A review," *Sci. Total Environ.*, vol. 576, pp. 646–659, Jan. 2017.

[33] M. Asgher, M. Bilal, H. M. N. Iqbal, Y. Yan, and H. Cheng, "Multi-point enzyme immobilization, surface chemistry, and novel platforms: a paradigm shift in biocatalyst design," *Crit. Rev. Biotechnol.*, vol. 39, no. 2, pp. 202–219, Feb. 2018.

[34] A. Arca-Ramos *et al.*, "Assessing the use of nanoimmobilized laccases to remove micropollutants from wastewater," *Environ. Sci. Pollut. Res.*, vol. 23, no. 4, pp. 3217–3228, 2016.

[35] Evonik, "AEROXIDE®, AERODISP® and AEROPERL® titanium dioxide as photocatalyst - Technical Information 1243." pp. 1–12, 2013.

[36] P. Chowdhary, A. Raj, and R. N. Bharagava, "Environmental pollution and health hazards from distillery wastewater and treatment approaches to combat the environmental threats: A review," *Chemosphere*, vol. 194. Pergamon, pp. 229–246, 01-Mar-2018.

[37] S. N. Zulkifli, H. A. Rahim, and W. J. Lau, "Detection of contaminants in water supply: A review on state-of-the-art monitoring technologies and their applications," *Sensors and Actuators, B: Chemical*, vol. 255. Elsevier, pp. 2657–2689, 01-Feb-2018.

[38] L. Yu and I. Yildiz, "Energy and Water Pollution," in *Comprehensive Energy Systems*, vol. 1–5, Elsevier, 2018, pp. 950–979.

Chapter 2

Biotransformation of Endocrine-Disrupting Compounds in Groundwater: Bisphenol A, Nonylphenol, Ethynylestradiol and Triclosan by a Laccase Cocktail from *Pycnoporus sanguineus* CS43.

This chapter has been published as:

R. Garcia-Morales et. al.

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This chapter correspond to a peer-reviewed article by Garcia-Morales et al., published in the indexed journal Water, Air, and Soil Pollution

2.1 Summary of the chapter

This chapter presents the use of laccase enzymes for biotransformation in synthetic and groundwater samples of endocrine-disruptor compounds (EDCs), such as bisphenol A, 4-nonylphenol, 17-α-ethynylestradiol and triclosan, percentages around 50–100 % were achieved.

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Biotransformation of Endocrine-Disrupting Compounds in Groundwater: Bisphenol A, Nonylphenol, Ethynylestradiol and Triclosan by a Laccase Cocktail from *Pycnoporus sanguineus* CS43

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Abstract The biodegradation of organic compounds present in water at trace concentration has become a critical environmental problem. In particular, enzymatic oxidation by fungal laccases offers a promising alternative for efficient and sustainable removal of organic pollutants in water. In this work, the biocatalytic ability of laccases from the *Pycnoporus sanguineus* CS43 fungus was evaluated. A filtered culture supernatant (laccase cocktail) evidenced an enhanced biotransformation capability to remove common endocrine-disruptor compounds (EDCs), such as bisphenol A, 4-nonylphenol, 17-α-ethynylestradiol and triclosan. A biodegradation of around 89–100 % was achieved for all EDCs using synthetic samples (10 mg L^{-1}) and after the enzymatic treatment with 100 U L^{-1} (50.3 U mg^{-1}). The biodegradation rates obtained were fitted to a first order reaction. Furthermore, enzymatic biocatalytic activity was also evaluated in groundwater samples coming from northwestern Mexico, reaching biotransformation percentages between 55 and 93 % for all tested compounds. As far as we know this is the first study on real groundwater samples in which the enzymatic degradation of target EDCs by a laccase cocktail from any strain of *Pycnoporus sanguineus* was evaluated. In comparison with purified laccases, the use of cocktail offers operational advantages since additional purification steps can be avoided.

Keywords Laccases · *Pycnoporus sanguineus* · Endocrine disrupting compound (EDC) · Groundwater

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1 Introduction

Over the last few decades, the water pollution by micropollutants from anthropogenic sources, such as pharmaceuticals, manufacturing additives or personal-care products, has become one of the most urgent issues to be solved (Lloret et al. 2012). Due to their widespread presence in the environment and their toxic activity even at low concentrations (pgL^{-1} – ngL^{-1}; Debaste et al. 2014), the detection and quantification of endocrine-

disruptor compounds (EDCs) have received increased attention in water quality management and health care, since their presence has been detected in rivers, lakes, groundwater (Sacher et al. 2001; Vega et al. 2007) and other sources of drinkable water (Robert et al. 2011; Stanford and Weinberg 2007).

EDCs are a group of environmental chemicals, from synthetic and natural origin, known for their negative influence on the endocrine system of living organisms (LaFleur and Schug 2011). Several studies have demonstrated that these chemicals mimic hormones or interfere with the action of endogenous hormones (Filby et al. 2007; Cabana et al. 2007a). Removal of EDCs traces in wastewater treatment plants (WWTP) has been a constant challenge due to higher concentrations that can remain in the treated waters. The accumulation of EDCs in the environment has been linked to cancer proliferation, mutations and reproduction disruption in fish, amphibians, birds and mammals, including humans (LaFleur and Schug 2011). Typical EDCs of anthropogenic origin with estrogen-like action include bisphenol A (BPA), 4-nonylphenol (NP) and 17-α-ethynylynyl estradiol (EE2); (Cajthaml et al. 2009), as well as antibacterial triclosan (TCS); (see Fig. 1). Due to EDCs that are slowly biodegraded under aerobic conditions, some of them can persist for more than 40 years as observed in estuary sediments (Miller et al. 2008).

Under such circumstances, the increasing accumulation of EDCs and bactericides in the environment has motivated the investigation of methods with the ability to reduce or inactivate these chemicals. Previous works have been directed on UV exposure as well as advanced oxidation processes such as ozonation (Esplugas et al. 2007). Although these methods produce efficient yields of removal/inactivation, they are expensive and may generate by-products with higher toxicity (Lloret et al. 2012). A promising approach to overcome these limitations is oxidation of EDCs by employing ligninolytic enzymes such as manganese peroxidase and laccases (Suzuki et al. 2003). The use of fungal laccases (blue copper polyphenoloxidase, E.C.1.10.3.2) offers a high potential to degrade and detoxify recalcitrant environmental pollutants (Tsutsumi et al. 2001; Torres et al. 2003).

An exhaustive search concerning laccases from *Pycnoporus sanguineus* was carried out, and to the best of our knowledge, there are no previous studies about the transformation of BPA, NP, EE2 and TCS using this strain in particular. Moreover, it was found that only a few works (Tsutsumi et al. 2001; Saito et al. 2004; Cabana et al. 2007a; Torres-Duarte et al. 2012) have studied the biotransformation of the target EDCs simultaneously with laccases from other commercial strains.

Due to the biotransformation, efficiency of organic compounds by ligninolytic enzymes in water represents an interesting option for environmental and industrial applications; in this work, a filtered culture supernatant containing a cocktail of laccases from *P. sanguineus* CS43 was assayed with synthetic and groundwater samples coming from northwestern Mexico. In order to establish a sustainable methodology for the biodegradation of BPA, NP, EE2 and TCS, a treatment avoiding the use of mediators and under mild conditions was developed.

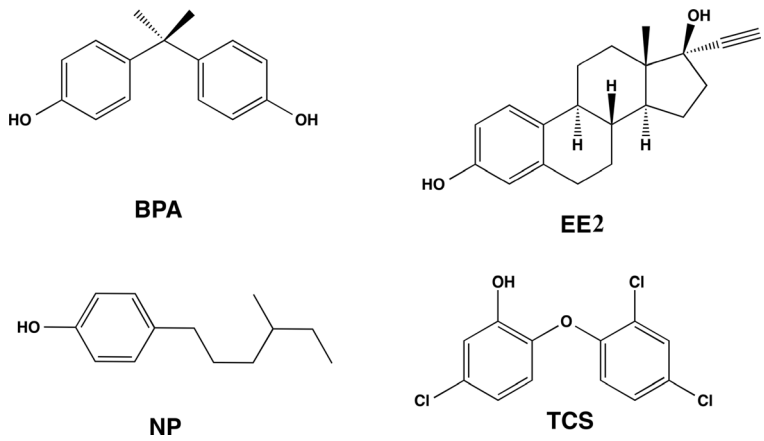

Fig. 1 Schematic structures of target EDCs: bisphenol A (BPA), 17-α-ethynylynyl estradiol (EE2), 4-nonylphenol (NP) and triclosan (TCS)

2 Experimental Section

2.1 Enzyme Laccase and Reagents

Laccases from *P. sanguineus* CS43 were obtained from a tomato medium as described in our previous study (Ramírez-Cavazos et al. 2014a). Mycelia were removed from the culture supernatant by filtration using two tangential flow filters in series, with pore sizes 0.5 and 0.2 μm. After that, the 0.2-μm filtered culture supernatant (laccase cocktail) was ultra-filtered by using a membrane cut-off of 10 kDa. The ultrafiltration process avoids the presence of lower molecular weight solutes present in the culture that can represent an environmental risk. Standards of BPA, NP, EE2 and TCS (high purity grade), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate (ABTS), dibasic sodium phosphate and citric acid salt were obtained from Sigma Aldrich, USA. Methanol, acetonitrile and ethanol (trace analysis quality) were supplied from Fisher Scientific, Tedia and Fermont, respectively.

2.2 Enzyme Characterization

The presence of two abundant laccase isoforms, denominated Lac I and Lac II, in the filtered culture supernatant (laccase cocktail) obtained from a tomato medium is described in our previous work (Ramírez-Cavazos et al. 2014b) To establish the conditions of biodegradation on the target EDCs (i.e. BPA, NP, EE2 and TCS), the purification, characterization and stability information of the two laccase isoforms were applied and used as previously described.

2.3 Enzymatic Activity Assay

Spectrophotometric measurements were performed in a micro-plate reader Omega FLUOstar. The enzyme activity of 20 μL aliquots of appropriately diluted laccase cocktail or purified enzyme was assayed with 5 mM ABTS as the substrate in pH 3 buffer McIlvain (0.2 M sodium phosphate dibasic/citric acid 0.1 M), 25 °C, at 420 nm (ε_{nm}=36,000 M^{-1} cm^{-1}). The enzyme activities were expressed as international units (U), defined as the amount of enzyme necessary to produce 1 μmol of product formed per minute.

2.4 Enzymatic Treatment

Stock solutions of 100 mg L^{-1} were prepared by dissolution of the standards in a mixture of ultrapure water–ethanol 50–50 % (*v*/*v*). Enzymatic reactions were carried out in 10 % (*v*/*v*) McIlvaine buffer (dibasic sodium phosphate 0.2 M/citric acid 0.1 M) pH 5 containing 10 mg L^{-1} of each analyte (using aliquots from stock solutions). Reactions were performed at room temperature and started by adding 100 U L^{-1} (50.3 U mg^{-1}) of laccase. In order to perform a qualitative and quantitative biodegradation of EDCs, UV–vis spectrophotometry and HPLC-UV chromatography techniques were employed. Average values and standard deviations of each reaction were calculated from three independent replicates; blanks and negative controls were prepared and measured at the same time. The catalytic constants of the enzyme were determined varying the concentration of EDCs until catalytic saturation; the transformation rate values were fitted to the Michaelis–Menten equation. In this work, with the only purpose of comparison among the catalytic profile of our cocktail and other reported laccases, a kinetic parameter K_{app} was defined as apparent for $K_{m.}$

2.4.1 UV–Vis Qualitative Analysis

The enzymatic treatment was carried out at room temperature in 5 mL reaction mixture containing 10 mg L^{-1} of each analyte, 10 % (*v*/*v*) buffer McIlvain pH 5 and 100 U L^{-1} (50.3 U mg^{-1}) of laccase; then the solution was vortex-mixed briefly for homogenizing and sheltering from light. To detect changes in the absorbance spectrum in a range of 200–500 nm, aliquots of 3 mL were taken from each treatment and blank of the analyte (without laccase treatments). The aliquots were measured in quartz cells at time 0, 2, 6 and 48 h using a Hach DR 500 spectrophotometer. After monitoring the maximum absorption wavelength, the parameters for the qualitative analysis by HPLC-UV were established.

2.4.2 Quantitative Analysis by HPLC-UV

Determination of EDCs was obtained through a HPLC system coupled to an UV–Vis detector (Agilent Technologies) and a reverse-phase column Agilent Eclipse XDE-C18 150×4.6 mm, 5 μ. A reverse-phase column Agilent Eclipse XDE-C18 150×4.6 mm, 5 μ, was used for the chromatographic measurements. The

final reaction mixture was performed at 25 °C in 1 mL vial containing 10 mg L^{-1} of each analyte, 10 % (*v*/*v*) buffer McIlvain pH 5 and 100 U L^{-1} (50.3 U mg^{-1}) of laccase; then the solution was vortex-mixed briefly for homogenizing and sheltering from light. The enzymatic treatments were measured by triplicate. An injection volume of 20 μL and 1 mL min^{-1} gradient elution by means of (A) acetonitrile (ACN) and (B) 10 mM phosphate buffer (pH 3.5) were applied. The gradient program was set as follows: 0–11 min, 25 % (A); 11–23 min, 95 % (A) and 23–30 min, 25 % (A). BPA, NP, TCS and EE2 were detected at three wavelengths, 206, 290 and 275 nm. The chromatographic analysis was carried out up to 12 h and the extent of the reaction was estimated by the decrease of the corresponding analyte peak analyzed by HPLC-UV chromatography technique and quantified using a calibration curve.

3 Results and Discussion

3.1 Enzyme Characterization

According to Ramírez-Cavazos et al. (2014b), the laccase isoforms produced by *P. sanguineus* in tomato juice medium and present in the crude extract show a relative activity of 65 % at 25 °C, increasing up to 100 % at 40 °C; the optimal pH values were observed in the acidic region. Table 1 summarized the pH range where laccase exhibit relative activity 85 % with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate (ABTS); 2,6-dimethoxyphenol (DMP) and guaiacol (+) as substrates at 25 °C. Based on these results, a temperature of 25 °C and pH 5 were set to perform the degradation profiles of micropollutants since laccases still maintain very high catalytic activity; at the same time, it avoids the use of severe sample pretreatments (e.g. thermal or acidic process) which involves changes in matrix nature (water/groundwater samples).

Table 1 pH range where laccase isoforms exhibit a high relative activity and stability by using different substrates

pH range (relative activity 85 %)			
	ABTS	DMP	Guaiacol
Lac I	2–4	3–5	3.5–5.5
Lac II	2–3	2–4.5	3–5

3.2 Qualitative Analysis by UV–vis Absorption Spectrophotometry

Figure 2 shows the spectroscopic analysis by UV absorption for the selected EDCs before and after enzymatic treatment for their biotransformation. As the figure shows, changes in UV-spectra were apparent for all analytes after 2 h of treatment with laccase cocktail. It is noteworthy that spectra of blank analytes (controls) showed no significant changes after 48 h of monitoring. Bathochromic shifts were observed by the displacement of absorption bands around 275 nm to longer wavelengths (red shifts), and hypochromic shifts were recorded by the reduction in the absorbance intensity around 275 nm for EE2 and total disappearance for BPA. Bands around 275–290 nm are characteristic for phenolic compounds, indicating a clear biotransformation of these EDCs by laccase cocktail. Absorption bands were also observed at 300, 250 and 229 nm for BPA, NP and TCS, respectively, but not for EE2. For this EDC, a spectral saturation was observed nearly to 200 nm (not shown in Fig. 2). An explanation for all these new bands can be the possible formation of by-products, which possess a different spectral behavior. The preliminary UV-absorption study shows the potential role of this cocktail in the biotransformation of EDCs.

3.3 Biodegradation Study of *EDCs* by Laccase Cocktail

A quantitative analysis was performed in order to determine the biotransformation percentage of target EDCs by the laccase cocktail. Figure 3 shows the biodegradation profiles for each analyte due to enzymatic treatment as well as the chromatograms corresponding to the peak of interest at different times of enzymatic treatment.

A decrease in the signal intensity is observed in chromatograms, which indicates the biotransformation of the EDCs. It is noticed that around 5.0–5.5 h of enzymatic oxidation are enough to achieve a biodegradation higher than 89 % for all the analytes. These results are consistent since UV-absorption spectra of EDCs show significant changes after 6 h of treatment (shown in Fig. 2). The slight differences among EDCs in biotransformation percentage can be attributed to their

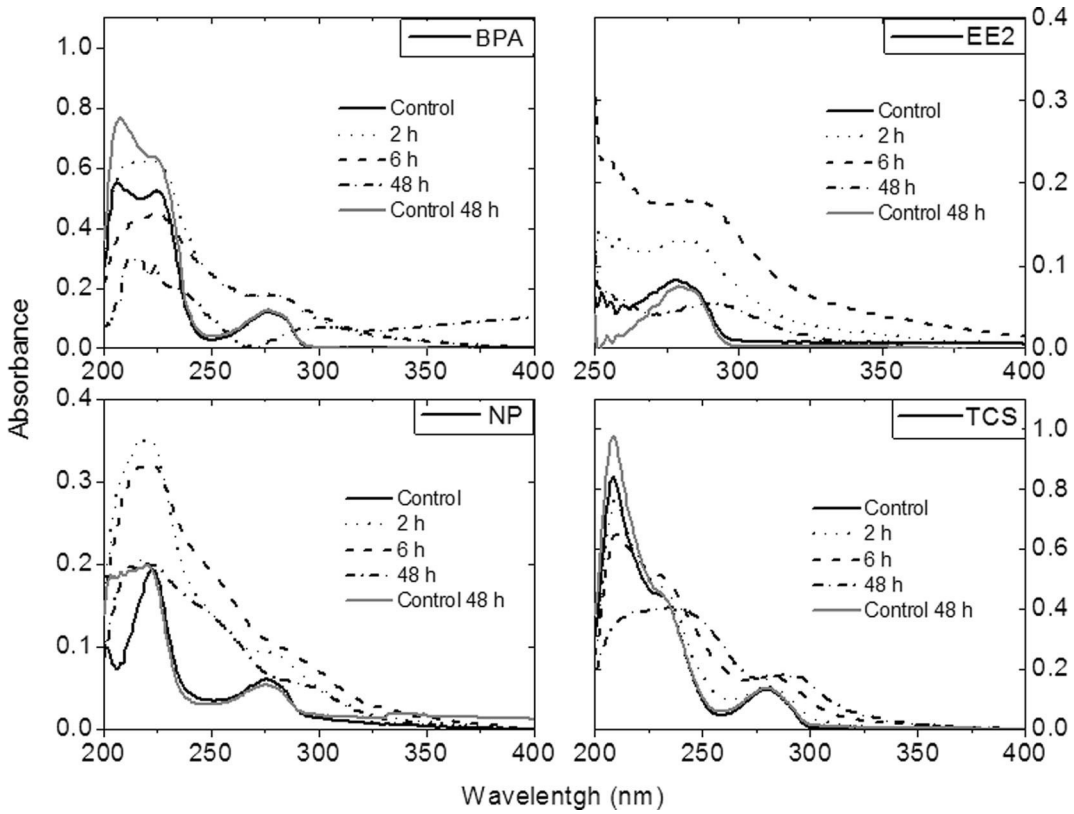

Fig. 2 UV-spectroscopic analysis for enzymatic treatment by using 100 U L^{-1} laccase cocktail at pH 5 and 25 °C

chemical structures (see Fig. 1). Since laccase is an oxidoreductase which couples the one electron oxidation of phenolic substrates, the presence of electron donating functional groups (EDFG) or electron withdrawing functional groups (EWFG) play an important role in reactivity (Yang et al. 2013; Nguyen et al. 2013). According to Yang et al. (2013), EDFG such as hydroxyl (−OH), amines ($-NH_2$), alkoxy (−OR), alkyl (−R) and acyl (−COR) groups induce the electrophilic attack by oxygenase enzymes which generates oxidation of molecules (Tadkaew et al. 2011). Moreover, the presence of EWFG reduces the efficiency of enzymes for attacking the analytes since groups such as amide ($-CONR_2$), halogen (−X) and nitro ($-NO_2$) produce an electron deficiency. The high biotransformation percentage of EE2 (100±0.56 % in 5 h) can be explained by the presence of the hydroxyl group in the aromatic structure, thus making EE2 more susceptible to oxidation by laccase. In the case of NP, the percentage was 93±2.93 % after 6 h of enzymatic treatment. This compound also features hydroxyl and alkyl groups in the aromatic structure which can contribute to its degradation. The next compound with higher biodegradation percentage was TCS (90± 0.94 % in 5.5 h). A particular characteristic of TCS is the presence of both EDFG and EWFG, in spite of the effect of chlorinated groups (EWFG), it seems that the strong electro-donor hydroxyl group makes this molecule appropriate for the laccase oxidation. BPA is another polyphenolic molecule with EDFG, containing two –OH groups and two −R groups; after enzymatic treatment, it presented a 89±1.05 % of biotransformation. As reported previously by Cabana et al. (2007a), TCS were eliminated to a lesser degree, compared with NP. In this work, TCS and BPA were less susceptible to degradation by laccase cocktail than NP and EE2. Studies of enzymatic treatment with commercially available laccase from *Trametes versicolor* report fast enzyme inactivation after reacting conditions with BPA. This is due to the interactions between the radicals from BPA

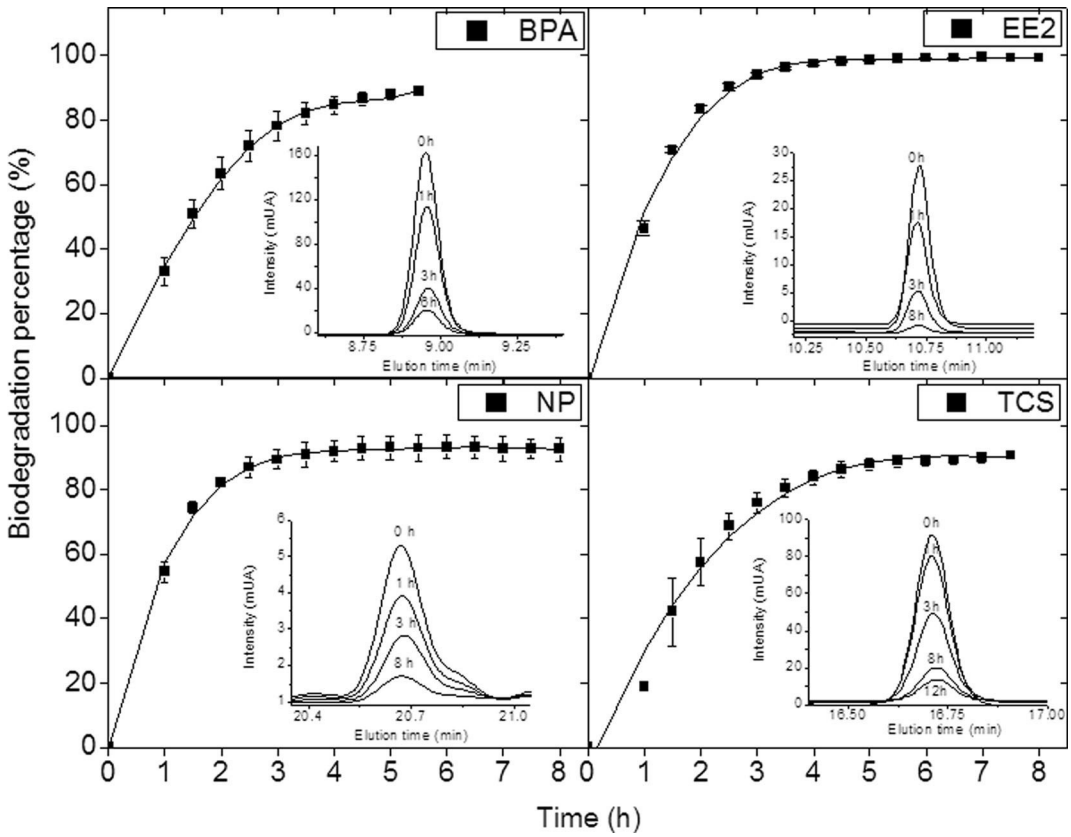

Fig. 3 Biodegradation profile of 10 mgL^{-1} synthetic samples for BPA, EE2, NP and TCS by using 100 U L^{-1} laccase cocktail at pH 5 and 25 °C. Each graph presents the maximum areas of peak taken from the respective chromatograms at different times of enzymatic treatment

and the enzyme (Cabana et al. 2007b). According to Cabana et al. (2007b), the removal efficiency is directly related to the fungal strain, the culture conditions used and the nature of the xenobiotic molecules.

3.4 Kinetic Results

Biodegradation rates were fitted to a first order reaction, according with the following equation:

$$In[A] = In[A]_0 - k' t \quad (1)$$

where $[A_0]$ corresponds to the initial concentration of the analytes and $[A]$ is the concentration at a determined time of the reaction; k' is the adjusted rate constant used in the general model and t corresponds to time (in hours). For all the adjustments, an $R^2 > 0.97$ was obtained. As can be seen in Fig. 4, k' constants corresponding to the EDCs were obtained with a similar order of magnitude, in which BPA and TCS presented similar values ($k' = 0.45\ h^{-1}$). According to these results, the reaction order is EE2>NP>TCS=BPA which corresponds with the order of biotransformation percentages for the target analytes.

Some attempts to incorporate this novel technology include the construction of bioreactors (Lloret et al. 2013a, b), immobilization of laccases (Cabana et al. 2009; Torres-Duarte et al. 2012) and the assembly of enzyme electrodes for the biodegradation or monitoring of EDCs (Oguchi 2011). The vast majority of other studies have focused on the effects, quantification and bioremediation of just one EDC at a time. However, complex mixtures of EDCs are suspected of acting together on the endocrine system of organisms (Filby et al. 2007; LaFleur and Schug 2011), which requires a more extensive analysis. Another limitation is the high concentration of required enzyme for removing EDCs, which increases the cost of these processes.

Table 2 summarizes the studies that have been developed using laccases as free enzyme for biotransformation of target EDCs. Some of those works (Cabana et al.

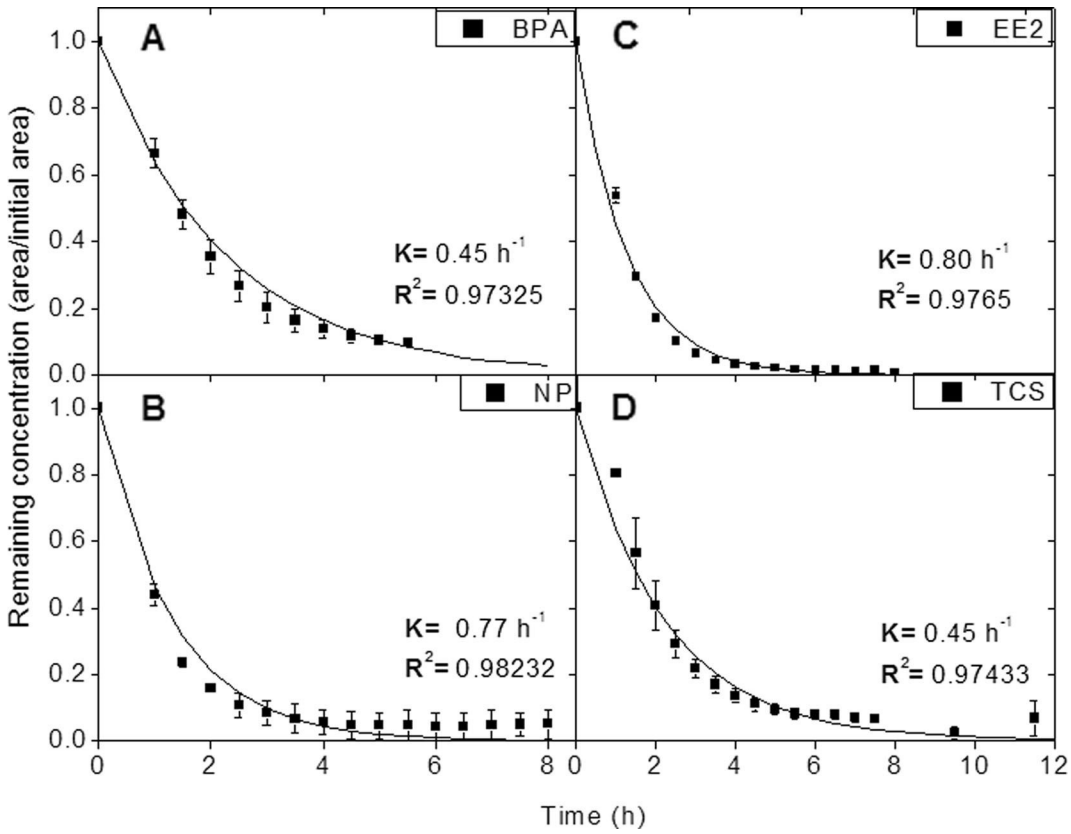

Fig. 4 Biotransformation of EDCs catalyzed by laccase cocktail. Curves were fitted to first order reactions and kinetic rates (*k*) were calculated for the target analytes

2007c) present a comparison in the catalytic efficiency between free and immobilized laccases used for removal of pollutants; others employed crude extracts, purified or recombinant enzymes (Fukuda et al. 2001, 2004; Saito et al. 2003; Cabana et al. 2007a) in biodegradation treatments for synthetic (Kim and Nicell 2006b) or treated waters (Auriol et al. 2008).

The overall effect of the biodegradation method proposed in this study showed comparable removal percentages with respect to previous works using other fungal laccases (see Table 2). In the case of BPA transformation, the calculated K_{app} value shows that there is no strong affinity for this compound in comparison with the K_m value for other EDCs. The removal percentage achieved in this study (89 %) is comparable with the results reported by Torres-Duarte et al. (2012; 85 %) and is even better than previous treatments at 25 °C that reported the use of mediators like 1-hydroxybenzotriazole (56 %), syringaldehyde (60 %) and violuric acid (72 %; Kim and Nicell 2006b). It is important to notice that the use of mediators are very common in enzymatic treatments; however, it adds complexity to the system by augmenting the amount of by-products after the biotransformation of EDCs. Moreover, they are expensive and some has already demonstrated to be toxic, such as PEG (Kim and Nicell 2006c; Murugesan et al. 2010). The best results for BPA degradation (90 %) were obtained only under high temperatures of reaction (Fukuda et al. 2001, 2004; Cabana et al. 2007a) and using elevated laccase concentrations (Saito et al. 2003, 2004). For EE2, the biodegradation percentage obtained in this study was 100 % meanwhile Auriol et al. (2007) achieved a biodegradation of 90 % for (100 ng L^{-1}) after 1 h of treatment with 800 U L^{-1} of laccases from *T. versicolor*, with a Km of 3.9 μM. Then, Auriol et al. (2008) obtained 100 % of removal under the same conditions but using a higher laccase concentration, 20,000 U L^{-1}. Finally, Suzuki et al. (2003) also obtained an EE2 removal of 100 %, but the temperature of reaction was at 30 °C and 1-hydroxybenzotriazole was added as mediator. For NP biotransformation, it can be noted that the highest

Table 2 Catalytic parameters corresponding to the maximal biodegradation of EDCs achieved by different strains of laccases as free enzyme

Laccase strain	Analyte (ppm)	T (°C)	pH	Laccase concentration (U L^{-1})	Catalytic parameters				Mediator/ additive	Removal		Comments	Ref.
					V_{max}/k_T (μM min^{-1})/ (Ms^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})		%	Time (h)		
Trametes versicolor	EE2 (0.1×10^{-3})	25	7	800	$53.4/9.28 \times 10^6$	3.8	–	–	–	90	1	Free laccase with synthetic samples and municipal wastewater after conventional treatment processes in WWTP (treated and filtrated waters)	Auriol et al. (2007)
	EE2 (0.1×10^{-3})	25	7	20,000	–	3.8	0.01	2.23		100	1	Free laccase with synthetic samples and municipal wastewater after conventional treatment processes in WWTP (treated and filtrated waters)	Auriol et al. (2008)
	TCS (5.8)	25	5	3,000	0.92	24	–	–	–	100	4	Free laccase with synthetic samples, oxidation in presence and absence of mediator	Kim and Nicell (2006a)
	TCS (5.8)	25	5	3,000	60	180	–	–	ABTS[a] (0.01 mM)	100	0.5		
	NP (22)	25	5	0.1 mg/mL	–	420	–	–	–	100	1.5	Free laccase with synthetic samples	Catapane et al. (2013)
	NP (220)									60			
	EE2 (3)	30	4.5	600	–	–	–	–	HBT[b] (0.2 mM)	100	8	Free laccase reaction under stirring at 150 rpm	Suzuki et al. (2003)
	NP (50)	30	4.5	100	–	–	–	–	–	60	1	Free laccase reaction under stirring at 150 rpm	Tsutsumi et al. (2001)
	NP (50)			100	–	–	–	–	HBT[b] (0.2 mM)	78	1		
	BPA (50)				–	–	–	–	–	70	1		
	BPA (50)				–	–	–	–	HBT[b] (0.2 mM)	97	1		
	BPA (27.4)	25	5	300	42.7	690	–	–	–	92	2	Free laccase with synthetic samples	Kim and Nicell (2006c)
	BPA (27.0)	25	5	300	42.7	690	–	–	PEG[c] (1.5 μM)	95	2		
	BPA (27.0)	45	5	150	–	–	–	–	–	68	1	Free laccase with synthetic samples	Kim and Nicell (2006b)
	BPA (27.0)	25	5	150	–	–	–	–	–	58	1		
									ABTS[a] (100 μM)	97	1		
									HBT[b] (100 μM)	56	1		
									SA[d] (100 μM)	60	1		
									VLA[e] (100 μM)	72	1		
									TEMPO[f] (100 μM)	52	1		
Coriolopsis gallica UAMH 8260	TCS (25)	25	4.5	0.5–8 U g^{-1}	–	970	1.5	1.5	–	100	n.r.[g]	Free laccase with synthetic samples	Torres-Duarte et al. (2012)
	NP (25)	25	4.5	0.5–8 U g^{-1}	–	420	17.5	42.2	–	100	n.r.[g]		
	BPA (5.0)	25	4.5	0.5–8 U g^{-1}	–	670	13.9	20.7	–	85	18		
Trametes villosa	BPA (500)	60	6	1,500	–	14 100	0.98	–	–	100	1	Recombinant laccase produced in *Aspergillus oryzae*	Fukuda et al. (2001, 2004)
Strain I-4 of the family Chaetomiaceae	BPA (1141)	40	7	50,000	–	10,000	14	1.4	–	99	3	Free laccase with synthetic samples	Saito et al. (2003, 2004)
	NP (1102)	40	7	50,000	–	5,000	1	0.2	–	99	6		
Coriolopsis polyzona	BPA (5)	40	5	10	–	–		–	–	100	4	Crude enzyme preparation	Cabana et al. (2007a)
	NP (5)	50		1						100	8		

Table 2 (continued)

Laccase strain	Analyte (ppm)	T (°C)	pH	Laccase concentration ($U\ L^{-1}$)	Catalytic parameters				Mediator/ additive	Removal		Comments	Ref.
					V_{max}/k_T ($\mu M\ min^{-1}$)/ (Ms^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_{cat}/K_m ($mM^{-1}\ s^{-1}$)		%	Time (h)		
	TCS (5)	50		100						65	8		
Pycnoporus sanguineus	BPA (10.0)	25	5	100	14.98	481.9	–	–	–	89	5.5	Free laccase in synthetic and groundwaters samples	Present study
Sp. CS43	EE2 (10.0)	25	5	100	2.17	32.0[h]	–	–	–	100	5		
Laccase cocktail (*LacI*/*LacII*)	NP (10.0)	25	5	100	23.84	73.6[h]	–	–	–	93	5.5		
	TCS (10.0)	25	5	100	7.76	302.5[h]	–	–	–	90	5.5		

[a] *ABTS* 2,2 0 -azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)

[b] *HBT* 1-hydroxybenzotriazole

[c] *PEG* polyethylene glycol

[d] *SA* syringaldehyde

[e] *VLA* violuric acid

[f] *TEMPO* 2,2,6,6-tetramethoxypiperidine 1-oxyl

[g] *n.r.* not reported

[h] Kapp

affinity for this substrate was obtained with laccase cocktail from *P. sanguineus* CS43, with a K_{app} value of 73.6 μM and obtaining 93 % of removal. A similar study to the present work (i.e. by using free laccase, besides culture supernatant) was developed with laccase obtained from the I-4 strain from the family *Chaetomiaceae* (Saito et al. 2004), in which after 6 h, 100 % of NP (1,102 ppm) was removed but using high amounts of laccase (50,000 U L^{-1}). Lower results were obtained by Tsutsumi et al. (2001), even using HBT as mediator (at 30 °C). In the case of TCS, a removal of 90 % was achieved in this work, only surpassed by works that obtained complete removal employing high amounts of enzyme (Kim and Nicell 2006a) and acidic conditions (Torres-Duarte et al. 2012). Under similar conditions of treatment, this study demonstrated that the overall degradation effect of the enzymatic reaction seemed to be comparable and in some cases, better regarding previous works presented in Table 2. As we reported in a previous study (Ramírez-Cavazos et al. 2014b), the laccase isoforms, present in the crude extract used in this study, are thermostable and highly active up to 70 °C; however, the aim of this work was to develop a sustainable and low-cost water treatment; thus, the reaction was maintained under mild conditions.

4 Application on Groundwater Matrices

Due to the high redox potential of free laccases of a cocktail from *P. sanguineus* CS43 (data not shown), these enzymes can be implemented as an efficient method for the purification of water. In this study, the enzymatic activity was evaluated in real groundwater samples. The samples used in the study were obtained from La Paz Valley, Mexico.

4.1 Experimental Site

La Paz Valley, a desert area located in northwestern Mexico, has a population of 283,000 habitants (Ojeda-Lavin 2012); the water supply for this region is obtained from groundwater resources (87 %). A recent study has revealed a high salinity in groundwaters (287–5352 mg L^{-1}), showing elevated concentrations of chlorides (90–2,960 mg L^{-1}) mainly from NaCl and CaCl; it has a pH range between 6.8 and 8.3. The presence of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, F^- HCO_3^- and SO_4^{2-} and SiO_2 ions suggests water–rock interactions, ion exchange and seawater intrusion, while fluoride concentration is related to hydrothermal fluids; NO_3^- and SO_4^{2-} are related to anthropogenic components. Table 3 shows field parameters and chemical constituents of groundwater samples.

4.2 Biotransformation Behavior of EDCs in Groundwater Samples

Since the majority of studies regarding the biodegradation of EDCs have been carried out by using synthetic (Kim and Nicell 2006b) or treated waters (Auriol et al. 2008), there are scarcely studies that monitor the enzymatic activity in a real matrix with the presence of denaturant substances of laccases (e.g. organic solvents, heavy metals, ions, etc.). In this context, the purpose of this work was also to evaluate the laccase efficiency in a complex matrix for the biotransformation of the BPA, NP, EE2 and TCS. To construct a representative bulk sample, a mixture was assembled with all samples presented in Table 3 by adding equal aliquotes of each one. The reaction mixture was prepared by spiking the bulk groundwater sample with appropriate amounts of each analyte (final concentration of 10 mg L^{-1}) and treated under the conditions described in Section 2.4. Results of biotransformations are displayed in Fig. 5.

As Fig. 5 shows, there is a significant decrease in the biotransformation percentage of TCS (55±2.33 %), which in fact was the analyte with the lowest rate of biodegradation in synthetic samples. For the rest of the analytes, the matrix did not have strong influence in the laccase catalytic ability; BPA 87±1.48 %, EE2 94±0.63 % and NP 81±1.53 %. Although in general a reduction in the removal percentage is observed for all EDCs in comparison with synthetic samples, it is noteworthy that the target compounds are not affected in the same way. This performance can be explained in terms of the variety of components (see Table 3) which are normally found in wastewater effluents and groundwaters by soil filtration. The presence of many ions from soil and antropogenic sources interact with laccases and cause interferences on the biodegradation of analytes. Chloride (Cl^-), halide anions (F^- and Br^-) and hydroxide anion (OH^-) have been reported to bind to the T2 Cu of laccase and interrupt the internal electron transfer between T1 and T2/T3 or to bind near the T1 active site, blocking the access of the substrate to T1 Cu (Margot et al. 2013). Our previous study (Ramírez-Cavazos et al. 2014b) related the presence of some of

Table 3 Field parameters and major ion concentrations in groundwater of selected wells in La Paz

Sample no.	Water use	Land use	Temp (°C)	SEC ($\mu S\ cm^{-1}$)	pH	Ca^{2+} ($mg\ L^{-1}$)	Mg^{2+} ($mg\ L^{-1}$)	Na^{+} ($mg\ L^{-1}$)	K^{+} ($mg\ L^{-1}$)	Cl^{-} ($mg\ L^{-1}$)	HCO_3^{-} ($mg\ L^{-1}$)	SO_4 ($mg\ L^{-1}$)	F^{-} ($mg\ L^{-1}$)	SiO_2 ($mg\ L^{-1}$)
LP-01	Urban	Urban area	28.9	893	6.8	51.0	16.5	67.5	4.07	159	166	37.1	<0.03	19.6
LP-03	Urban	Urban area	29.2	1,537	6.9	103.0	33.7	99.9	5.5	385	185	34.3	<0.05	21.9
LP-07	Urban	Loose topsoil	31.1	2,109	7.1	155.0	60.3	80.9	4.94	521	190	77.1	<0.1	30.7
LP-09	Urban	Dessert land	30.5	865	7.4	52.7	20	81.7	2.71	118	280	47.5	0.08	27.4
LP-13	Urban	Dessert land	31.8	1,155	7.6	43.6	10.1	141	5.5	188	250	53.5	1.47	39
LP-18	Agriculture	Cropland	31.0	5,100	7.0	335.0	186	391	9.12	1,400	578	195	<0.3	37.9
LP-22	Agriculture	Cropland	30.7	7,520	7.0	356.0	131	1,080	10.2	2,260	498	490	<0.3	45
LP-28	Urban-agriculture	Cropland	29.0	6,880	7.0	421.0	186	763	8.94	2,030	634	441	<0.3	47.9
LP-31	Multiple	Cropland	27.1	4,770	7.1	189.0	110	693	3.49	1,140	1,290	228	<0.3	43.5
LP-32	Agriculture	Cropland	29.0	2,589	7.2	237.0	55.9	134	3.59	731	325	50.3	<0.1	33.2
LP-35	Urban	Urban area	31.3	2,751	7.2	255.0	74.5	102	2.97	793	276	64.3	<0.1	29.3
LP-38	Agriculture	Cropland	29.5	799	7.8	44.5	20.7	59.8	2.05	164	186	11.5	0.07	36.2
LP-39	Agriculture	Cropland	29.9	683	7.6	33.3	15.2	58.4	2.25	95.6	218	14	0.06	35.9
LP-40	Urban	Urban area	28.7	8,920	7.2	658.0	344	583	14	2,960	984	243	<0.5	36.2
LP-44	Urban	Urban area	30.2	630	7.2	33.5	15.6	50.1	1.54	89.7	198	14.5	0.2	25.8

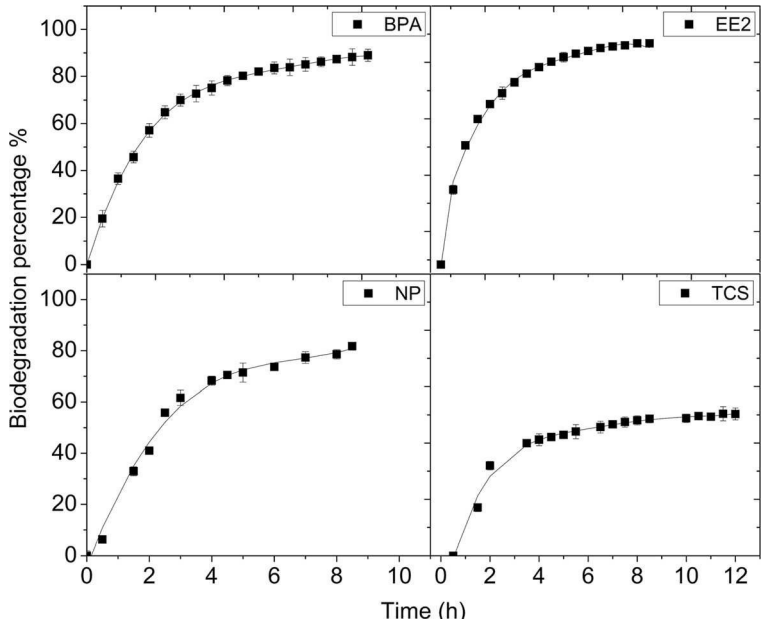

Fig. 5 Biodegradation profiles of BPA, EE2, NP and TCS by using a representative bulk sample from groundwater. A concentration of 100 U L^{-1} laccase cocktail from *P. sanguineus* CS43 and 10 mg L^{-1} EDCs at pH 5 and 25 °C

these components with the decrease in the activity of laccase isoforms in the crude extract used in this work (see Table *4*). The work of Kim and Nicell (2006b) also proved that the presence of these components is related with the decrement of conversion by laccase from *T. versicolor*. Other interferences, such as cyanide (originated by the plastic industry), cause the dissociation at the copper ion from the enzymatic active site, as well as calcium, cobalt and zinc chlorides, which tend to interfere by hydrogen bonding (chaotropic effect; Cabana et al. 2007b).

By taking advantage of the thermostability of *P. sanguines* CS43 laccase (Ramírez-Cavazos et al. 2014b), the degradation rates obtained in groundwater samples would significantly improve by increasing the reaction temperature, above 30 °C in the biodegradation treatment. The implementation of immobilization methods in different materials can be employed to enhance the biodegradation results, as well.

Table 4 Effect of inhibitors on purified laccase activities

	IC_{50} (mM)		Complete inhibition (mM)	
Substrates	Lac I	Lac II	SD Lac I	SD Lac II
NaF	0.08	0.02	16	8
NaCl	65	14	2,000[a]	2,000[a]
NaN_3	6.20E-06	6.90E-07	16	16
Na_2SO_4	800[b]	800[b]	800[a]	800[a]

Laccase activity was measured using ABTS as the substrate at pH 3 (modified from Ramírez-Cavazos et al. 2014b)

[a] Values refer to the respective highest concentration tested where a complete inhibition was not observed

[b] Values refer to the respective highest concentration tested where 50 % inhibition was not observed

5 Conclusions

Biotransformation of BPA, EE2, NP and TCS using a laccase cocktail from *P. sanguineus* CS43 was studied and compared in both synthetic and real groundwater samples. A removal higher than 89 % was achieved for all selected analytes in synthetic samples, which were achieved using free laccase enzyme and avoiding the use of mediators. In terms of the biotransformation on real groundwater samples, a decrease in degradation percentages caused by the interaction of the ions present in the complex matrix were clearly observed. This study reveals the high biocatalytic efficiency of this cocktail composed by LacI and LacII for the removal of common EDCs with low amounts of laccase activity (100 U L^{-1}) and treatment time in comparison with other studies. Consequently, laccase cocktail from *P. sanguineus* strain CS43 represents a promising alternative for biotransformation systems with operational advantages such as less purification steps. To enhance the biotransformation process, further work will be focused on testing this enzyme under conditions above 25 °C. Immobilization

methods as well as scaling-up bioreactors using these biomaterials can represent an opportunity to study and to design novel biodegradation technologies.

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Compliance with Ethical Standards All co-authors have approved and agree with the contents of the manuscript; there is no conflict of interest or financial interest to report. This research does not involve any kind of study in humans or animals. We certify that the submission is original work and is not under consideration by another journal.

References

Auriol, M., Filali-Meknassi, Y., Tyagi, R. D., & Adams, C. D. (2007). Laccase-catalyzed conversion of natural and synthetic hormones from a municipal wastewater. *Water Research, 41*, 3281–8. doi:10.1016/j.watres.2007.05.008.

Auriol, M., Filali-Meknassi, Y., Adams, C. D., et al. (2008). Removal of estrogenic activity of natural and synthetic hormones from a municipal wastewater: efficiency of horseradish peroxidase and laccase from Trametes versicolor. *Chemosphere, 70*, 445–52. doi:10.1016/j.chemosphere.2007.06.064.

Cabana, H., Jiwan, J.-L. H., Rozenberg, R., et al. (2007a). Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus Coriolopsis polyzona. *Chemosphere, 67*, 770–8. doi:10.1016/j.chemosphere.2006.10.037.

Cabana, H., Jones, J. P., & Agathos, S. N. (2007b). Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: a review. *Engineering in Life Science, 7*, 429–456.

Cabana, H., Jones, J. P., & Agathos, S. N. (2007c). Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. *Journal of Biotechnology, 132*, 23–31. doi:10.1016/j.jbiotec.2007.07.948.

Cabana, H., Jones, J. P., & Agathos, S. N. (2009). Utilization of cross-linked laccase aggregates in a perfusion basket reactor for the continuous elimination of endocrine-disrupting chemicals. *Biotechnology and Bioengineering, 102*, 1582–92. doi:10.1002/bit.22198.

Cajthaml, T., Kresinová, Z., Svobodová, K., & Möder, M. (2009). Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi. *Chemosphere, 75*, 745–50. doi:10.1016/j.chemosphere.2009.01.034.

Catapane, M., Nicolucci, C., Menale, C., et al. (2013). Enzymatic removal of estrogenic activity of nonylphenol and octylphenol aqueous solutions by immobilized laccase from Trametes versicolor. *Journal of Hazardous Materials, 248–249*, 337–46. doi:10.1016/j.jhazmat.2013.01.031.

Debaste, F., Songulashvili, G., & Penninckx, M. J. (2014). The potential of Cerrena unicolor laccase immobilized on mesoporous silica beads for removal of organic micropollutants in wastewaters. *Desalination and Water Treatment, 52*, 2344–2347.

Esplugas, S., Bila, D. M., Krause, L. G. T., & Dezotti, M. (2007). Ozonation and advanced oxidation technologies to remove endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) in water effluents. *Journal of Hazardous Materials, 149*, 631–42. doi:10.1016/j.jhazmat.2007.07.073.

Filby, T. A., Neuparth, K., Thorpe, R., et al. (2007). Health Impacts of estrogens in the environment, considering complex mixture effects. *Environmental Health Perspectives, 115*, 1704–1710.

Fukuda, T., Uchida, H., Takashima, Y., et al. (2001). Degradation of bisphenol A by purified laccase from Trametes villosa. *Biochemical and Biophysical Research Communications, 284*, 704–6. doi:10.1006/bbrc.2001.5021.

Fukuda, T., Uchida, H., Suzuki, M., et al. (2004). Transformation products of bisphenol A by a recombinant Trametes villosa laccase and their estrogenic activity. *Journal of Chemical Technology and Biotechnology, 79*, 1212–1218. doi:10.1002/jctb.1115.

Kim, Y., & Nicell, J. A. (2006a). Laccase-catalysed oxidation of aqueous triclosan. *Journal of Chemical Technology and Biotechnology, 81*, 1344–1352. doi:10.1002/jctb.1507.

Kim, Y. J., & Nicell, J. A. (2006b). Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A. *Bioresource Technology, 97*, 1431–1442.

Kim, Y. J., & Nicell, J. A. (2006c). Laccase-catalyzed oxidation of bisphenol A with the aid of additives. *Process Biochemistry, 41*, 1029–1037. doi:10.1016/j.procbio.2005.11.012.

LaFleur, A. D., & Schug, K. A. (2011). A review of separation methods for the determination of estrogens and plastics-derived estrogen mimics from aqueous systems. *Analytica Chimica Acta, 696*, 6–26.

Lloret, L., Hollmann, F., Eibes, G., et al. (2012). Immobilisation of laccase on Eupergit supports and its application for the removal of endocrine disrupting chemicals in a packed-bed reactor. *Biodegradation, 23*, 373–86.

Lloret, L., Eibes, G., Feijoo, G., et al. (2013a). Application of response surface methodology to study the removal of estrogens in a laccase-mediated continuous membrane reactor. *Biocatal Biotransformation, 31*, 197–207. doi:10.3109/10242422.2013.815745.

Lloret, L., Eibes, G., Moreira, M. T., et al. (2013b). Improving the catalytic performance of laccase using a novel continuous-flow microreactor. *Chemical Engineering Journal, 223*, 497–506. doi:10.1016/j.cej.2013.03.018.

Margot, J., Bennati-Granier, C., Maillard, J., et al. (2013). Bacterial versus fungal laccase: potential for micropollutant degradation. *AMB Express, 3*, 63. doi:10.1186/2191-0855-3-63.

Miller, T. R., Heidler, J., Chillrud, S. N., et al. (2008). Fate of triclosan and evidence for reductive dechlorination of triclocarban in estuarine sediments. *Environmental Science and Technology, 42*, 4570–4576. doi:10.1021/es702882g.

Murugesan, K., Chang, Y.-Y., Kim, Y.-M., et al. (2010). Enhanced transformation of triclosan by laccase in the presence of redox mediators. *Water Research, 44*, 298–308. doi:10.1016/j.watres.2009.09.058.

Nguyen, L. N., Hai, F. I., Yang, S., et al. (2013). Removal of trace organic contaminants by an MBR comprising a mixed culture of bacteria and white-rot fungi. *Bioresource Technology, 148*, 234–41. doi:10.1016/j.biortech.2013.08.142.

Oguchi S (2011) Detection and decomposition of bisphenol-a. US Pat. App. 12/999,469 1.

Ojeda-Lavin A (2012) Proyecciones de la población 2010–2050. In: Mex. Gov. Off. Data Base, CONAPO 2012. http://www.conapo.gob.mx/es/CONAPO/Documento_Metodologico_Proyecciones_2010_2050. Accessed 16 Jan 2015.

Ramírez-Cavazos, L. I., Junghanns, C., Nair, R., et al. (2014a). Enhanced production of thermostable laccases from a native strain of Pycnoporus sanguineus using central composite design. *Journal of Zhejiang University. Science. B, 15*, 343–52.

Ramírez-Cavazos, L. I., Junghanns, C., Ornelas-Soto, N., et al. (2014b). Purification and Characterization of two thermostable laccases from Pycnoporus sanguineus and potential role in degradation of endocrine disrupting chemicals. *Journal of Molecular Catalysis B: Enzymatic, 104*, 32–42.

Robert, K, L ERI, Marion, J, et al. (2011). Assessment of micropollutants from municipal wastewater—combination of exposure and ecotoxicological effect data for Switzerland. In: García-Einschlag FS (ed) Waste Water-Eval. Manag. pp 31–54.

Sacher, F., Lange, F. T., Brauch, H.-J., & Blankenhorn, I. (2001). Pharmaceuticals in groundwaters analytical methods and results of a monitoring program in. *Journal of Chromatography A, 938*, 199–210.

Saito, T., Hong, P., & Kato, K. (2003). Purification and characterization of an extracellular laccase of a fungus (family Chaetomiaceae) isolated from soil. *Enzyme and Microbial Technology, 33*, 520–526. doi:10.1016/S0141-0229(03)00158-3.

Saito, T., Kato, K., & Yokogawa, Y. (2004). Detoxification of bisphenol A and nonylphenol by purified extracellular laccase from a fungus isolated from soil. *Journal of Bioscience and Bioengineering, 98*, 64–6. doi:10.1016/S1389-1723(04)70243-1.

Stanford, B. D., & Weinberg, S. D. (2007). Isotope dilution for quantitation of steroid estrogens and nonylphenols by gas chromatography with tandem mass spectrometry in septic, soil, and groundwater matrices. *Journal of Chromatography A, 1176*, 26–36.

Suzuki, K., Hirai, H., Murata, H., & Nishida, T. (2003). Removal of estrogenic activities of 17beta-estradiol and ethinylestradiol by ligninolytic enzymes from white rot fungi. *Water Research, 37*, 1972–5. doi:10.1016/S0043-1354(02)00533-X.

Tadkaew, N., Hai, F. I., McDonald, J. A., et al. (2011). Removal of trace organics by MBR treatment: the role of molecular properties. *Water Research, 45*, 2439–51. doi:10.1016/j.watres.2011.01.023.

Torres, E., Bustos-Jaimes, I., & Le Borgne, S. (2003). Potential use of oxidative enzymes for the detoxification of organic pollutants. *Applied Catalysis B: Environmental, 46*, 1–15. doi:10.1016/S0926-3373(03)00228-5.

Torres-Duarte, C., Viana, M. T., & Vazquez-Duhalt, R. (2012). Laccase-mediated transformations of endocrine disrupting chemicals abolish binding affinities to estrogen receptors and their estrogenic activity in zebrafish. *Applied Biochemistry and Biotechnology, 168*, 864–76. doi:10.1007/s12010-012-9825-2.

Tsutsumi, Y., Haneda, T., & Nishida, T. (2001). Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. *Chemosphere, 42*, 271–276.

Vega, D., Agüí, L., & González-Cortés, A. (2007). Electrochemical detection of phenolic estrogenic compounds at carbon nanotube-modified electrodes. *Talanta, 71*, 1031–8. doi:10.1016/j.talanta.2006.05.071.

Yang, S., Hai, F. I., Nghiem, L. D., et al. (2013). Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. *Bioresource Technology, 141*, 97–108. doi:10.1016/j.biortech.2013.01.173.

Chapter 3

Biotransformation kinetics of pharmaceutical and industrial micropollutants in groundwaters by a laccase cocktail from *Pycnoporus sanguineus* CS43 fungi.

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3.1 Summary of the chapter

The biocatalytic activity of laccases in their free form was analyzed using a range of emerging pollutants in synthetic and groundwater samples. Removal ranged between 50 – 97 % for diclofenac, β-Naphthol, 2,4 dichlorophenol and 5,7-diiodo-8-hydroxyquinoline.

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Biotransformation kinetics of pharmaceutical and industrial micropollutants in groundwaters by a laccase cocktail from *Pycnoporus sanguineus* CS43 fungi

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ABSTRACT

In this work, the biocatalytic ability of laccases from filtered culture supernatant of *Pycnoporus sanguineus* was evaluated without mediators and under mild reaction conditions. This 100 U L^{-1} laccase cocktail removed 50% Diclofenac, 97% β-Naphthol and 71% 2,4 Dichlorophenol within 8 h of reaction and 78% for 5,7-Diiodo-8-hydroxyquinoline within 3.5 h; at initial concentrations of 10 mg L^{-1} and at 25 °C. Furthermore, this enzyme cocktail also removed in excess of 53% all tested compounds in a real groundwater sample from northwestern Mexico. In comparison with purified laccases, the use of cocktail offers operational advantages since additional purification steps can be avoided.

1. Introduction

The contamination of surface waters, groundwaters and drinking water supplies with persistent, bioactive and bioaccumulative substances is a critical environmental issue due to their potential health and ecological effects (Petrović et al., 2003). Agrochemicals, pharmaceuticals, personal care products, household chemicals and raw materials for industry are used intensively in daily life, therefore they are also introduced into the environment by direct disposal, surface water run-off, leakage from landfills and effluents from wastewater treatment plants (WWTPs) (Caliman and Gavrilescu, 2009). The elimination of these emerging pollutants from discharges is desirable because of potential damages to environmental organisms even at low concentrations (Bolong et al., 2009; Gavrilescu et al., 2015).

Most frequently types of micropollutants detected in water supplies are industrial pollutants (Deblonde et al., 2011) and pharmaceutical compounds such as antibiotics, analgesics and anti-inflammatory drugs (Tijani et al., 2014). Since WWTPs are not well-designed to remove harmful pollutants present at trace concentrations (Caliman and Gavrilescu, 2009), the present research is aimed to propose, design and study a laccase-based bioconversion method for diclofenac sodium (DFC), 5,7-Diiodo-8-hydroxyquinoline (DHQ), β -Naphthol (β-NP) and 2,4-dichlorophenol (2,4-DCP). DCF, a non-steroidal anti-inflammatory drug prescribed as antipyretic analgesic (Zhang et al., 2008b) is classified as a harmful environmental pollutant because of its toxicity and biomagnification in the food chain (Naidoo and Swan, 2009). Due to its extensive use, DCF has been found in several water supplies, even in groundwater at concentrations between 3.6 and 580 ng L^{-1} (Einsiedl et al., 2010; Stuart et al., 2012). DHQ (or iodoquinol) is an antibiotic prescribed in the treatment of amebiasis and vaginal infections by trichomonas (Nagata et al., 2012); DHQ constitutes a harmful pollutant due to its mutagenic and persistence properties (He et al., 2005; Howard and Muir, 2011) associated with neuropathies (Baumgartner et al., 1979). β-NP is a hazardous industrial pollutant that is also widely detected in the environment; it is employed in the production of dyes and pharmaceutical compounds and is found in the shale oil as well (Roch

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and Alexander, 1995). β-NP has been proved to be refractory and dangerous to wildlife and human beings (Croera et al., 2008). Last but not least, 2,4-dichlorophenol (2,4-DCP), which is the precursor of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), is one of the most abundant chlorophenols found in water supplies (House et al., 1997). 2,4-DCP has been detected in groundwater at concentrations ranging from 52 to 3300 ng L^{-1} (Padkel et al., 1992; Rudel et al., 1998; Fava et al., 2005). This compound shows very high toxicity and low biodegradability, and some studies (Tessier et al., 2000) demonstrated potential food safety issues due to its capability to bioaccumulate in various organisms through the food chain. Under these circumstances, 2,4-DCP was included on the priority pollutant list by the US Environmental Protection Agency (EPA) and the European Union (Callahan et al., 1979).

A combination of UV and advanced oxidation processes such as ozonation (Esplugas et al., 2007) have been shown to produce efficient removal/inactivation of micropollutants. However, these processes are expensive (Lloret et al., 2012) and may generate by-products with higher toxicity. Sein et al. (2008) reported the presence of two toxic products as result of the oxidation of diclofenac by ozone, diclofenac- 2,5-iminoquinone and 5-hydroxydiclofenac. A promising approach to overcome such limitations is the application of enzymes from ligninolytic fungi, such as laccase, lignin and manganese peroxidase to eliminate a wide variety of aromatic compounds under mild conditions (Cajthaml et al., 2009). In particular, the use of laccase from *Pycnoporus sanguineus* has been extensively reviewed and to the best of our knowledge there are no studies on the biotransformation of DFC, β-NP and 2,4-DCP in water using this strain in particular or the bioconversion of DHQ. Moreover, assays of enzyme catalyzed oxidation of organic pollutants in groundwater have not been reported previously. In order to establish a methodology for the biotransformation of these pollutants, in this work, a filtered culture supernatant, which contains a cocktail of laccases from *P. sanguineus* CS43, was assayed in synthetic and groundwater samples from northwestern Mexico. The results obtained using mild conditions of reaction and avoiding mediators show that ligninolytic enzymes can biotransform DFC, DHQ, β-NP and 2,4-DCP, and thus this methodology can represent an interesting option for environmental and industrial applications.

2. Materials and methods

2.1. Enzyme laccase and reagents

Laccases from *P. sanguineus* CS43 were obtained from a tomato medium as described in our previous study (Ramírez-Cavazos et al., 2014a). In short, mycelia were removed from the culture supernatant by filtration using two tangential flow filters in series, with pore sizes 0.5 and 0.2 μm. After that, 0.2 μm filtered culture supernatant (laccase cocktail) was ultra-filtered using a membrane cut-off of 10 kDa. Standards of DFC, DHQ, β-NP and 2,4-DCP (high purity grade), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate (ABTS), dibasic sodium phosphate and citric acid salt were obtained from Sigma Aldrich, USA. Methanol, acetonitrile and ethanol (trace analysis quality) were supplied from Fisher Scientific, Tedia and Fermont, respectively.

2.2. Enzyme characterization

The presence of two abundant laccase isoforms, denominated Lac I and Lac II, in the filtered culture supernatant (laccase cocktail) obtained from a tomato medium is described in our previous work (Ramírez-Cavazos et al., 2014a). Tests to detect other ligninolytic enzymes in the culture supernatant (e.g. lignin peroxidase and manganese peroxidase) were carried out as described below.

Total peroxidase activity was first determined by using an indirect method reported by Eggert et al. (1996) adding to the laccase activity assay (section 2.3) a volume of H_2O_2 to 100 mM final concentration and recording the difference in absorbance caused by the possible presence of peroxidases. On the other hand, lignin peroxidase (LiP), manganese peroxidase (MnP) and manganese-independent peroxidase (MiP) activities were also measured using specific standard methods as following: (1) LiP activity was determined by the oxidation of veratryl alcohol as described by Arora and Gill (2001). The reaction mixture contained 1 ml sodium tartrate buffer 125 mM pH 3.0, 500 μL veratryl alcohol 10 mM; 500 μL H_2O_2 of 2 mM and 500 μL of enzyme extract. The reaction was started by adding hydrogen peroxide and the production of veratraldehyde was determined at 310 nm ($\varepsilon_{310} = 9300\ M^{-1}\ cm^{-1}$). (2) MnP activity was determined by the method of Wariishi et al. (1992). The assay mixture contained 0.5 mM $MnSO_4$ in 50 mM sodium malonate (pH 4.5), and the reaction was started by the addition of 10 μL H_2O_2 to give a final concentration of 0.4 mM. The formation of Mn (III)-malonate complexes was followed at 270 nm ($\varepsilon_{270} = 11\ 590\ M^{-1}\ cm^{-1}$). (3) Manganese-independent peroxidase activity (MiP) was calculated from the peroxidase activity of MnP assay detected in the absence of Mn^{2+} ions (Vyas et al., 1994).

2.3. Enzymatic activity assay for laccase

Spectrophotometric measurements were performed in a microplate reader Omega FLUOstar. The enzyme activity of 20 μL aliquots of appropriately diluted laccase cocktail was assayed with 5 mM ABTS as substrate in buffer McIlvain (0.2 M sodium phosphate dibasic/citric acid 0.1 M), pH 3, 25 °C at 420 nm (ε nm = 36 000 $M^{-1}cm^{-1}$). The enzyme activities were expressed as international units (U).

2.4. Enzymatic treatment

For DCF and 2,4-DCP stock solutions of 100 mg L^{-1} were prepared by dissolution of standards in ultrapure water; in the case of DHQ and β-Naftol a mixture of methanol-water 50-50% (v/v) was employed as solvent. Enzymatic reactions were carried out in 10% (v/v) McIlvaine buffer (dibasic sodium phosphate 0.2 M/citric acid 0.1 M) pH 5 containing 10 mg L^{-1} of each analyte (using aliquots from stock solutions). Reactions were performed at room temperature and started by adding 100 U L^{-1} of laccase. The solution was vortex-mixed briefly for homogenizing and protected from light. Quantitative analysis of micropollutants transformation was performed by HPLC-UV chromatography. All treatments and blank samples were prepared and measured in triplicate. The catalytic parameters of the enzyme were determined by varying the analyte concentrations until catalytic saturation. The transformation rate values were fitted to the Michaelis–Menten equation. The half substrate concentration of saturation which is considered as an apparent Km (Km_{app}) and the V_{max} values were calculated for each analyte for comparison with published data for purified laccases.

2.4.1. HPLC quantitative analysis

The quantitative analysis of DCF, DHQ, β-NP and 2,4-DCP was performed on a HPLC 1200 system (Series Rapid Resolution LC System Agilent Technologies, Santa Clara, USA) coupled to a UV–Vis detector. A reverse-phase column Agilent Eclipse XDE-C18 of 150 × 4.6 mm and particle size of 5 μm was used for the chromatographic measurements. 20 μL of sample were injected and eluted at 1 mL min^{-1} with a gradient of (A) acetonitrile (ACN) and (B) 10 mM phosphate buffer (pH 3.5). The gradient program was set as follows: 0–11 min, 25% (A), 11–23 min, 95% (A) and 23–30 min, 25% (A). DHQ, DCF, 2,4-DCP and β-NP were detected at 3

wavelengths: 206 nm, 290 nm and 275 nm. Average values and standard deviations for all reactions were calculated from three independent replicates; blanks and negative controls were prepared and measured at the same time.

2.4.2. Application on groundwater matrices: sample collection and analysis

Fifteen samples were collected from agricultural and domestic water production wells, from the La Paz valley, a desert area located in northwestern Mexico. Sampling and analyses procedures for major and minor chemical components are identical to those used in Mahlknecht et al. (2004). Briefly, temperature, pH, electrical conductivity and dissolved oxygen were measured in the field with a multiparameter probe (WTW Multi 350i). The pH probe was calibrated before each measurement. Alkalinity was determined on untreated water samples by volumetric titration (0.02 N H_2SO_4) to pH 4.3. In each sampling site, polyethylene bottles (pre-rinsed with tri-distillate water) were filled with filtered (0.45 μm) groundwater samples. Then, samples for anion and cation measurements were stored, in pre-rinsed high density polyethylene bottles (Nalgene) at 4 °C. The cations and anions were determined by Activation Laboratories Ltd. in Ancaster, Ontario, with inductive-coupled plasma mass spectrometry (ICP-MS) and ion chromatography, respectively.

Samples from the 15 different groundwater were pooled to construct a representative bulk sample with the following physicochemical characteristics: (conductivity of 3146 μScm^{-1}, temperature of 30 °C, pH 7.2, 197.8 mgL^{-1} of Ca^{2+}, 85.3 mgL^{-1} of Mg^{2+}, 292.3 mgL^{-1} of Na^{+}, 5.29 mgL^{-1} of K^{+}, 868.9 mgL^{-1} of Cl^{-}, 417.2 mgL^{-1} of HCO_3^{-}, 133.4 mgL^{-1} of SO_4^{2-} and 0.264 mgL^{-1} of F^{-}). The reaction mixture was prepared by spiking the bulk groundwater sample with appropriate amounts of each analyte (final concentration of 10 mg L^{-1}) and treated under the conditions described above.

3. Results and discussion

3.1. Enzyme characterization

Preliminary assays did not detect other ligninolytic enzymes (e.g. lignin peroxidase and manganese peroxidase) in the culture supernatant. Peroxidase activity, as determined by ABTS oxidation in the presence of H_2O_2, was not detectable, there was no detection of LiP activity (H_2O_2-dependent veratryl alcohol oxidation) as well. Moreover, the peroxidase activity could not be ascribed to an MnP-type enzyme either since it was not dependent on the presence of free Mn(II). According to Ramírez-Cavazos et al. (2014b) the laccase isoforms produced by *P. sanguineus* in tomato juice medium show a relative activity of 65% at 25 °C, increasing up to 100% at 40 °C. Reaction was determined to be optimal between pH 3 to 5 using DMP, guaiacol and ABTS as substrates (Ramírez-Cavazos et al., 2014b). Therefore, laccase assays in this work were performed at 25 °C and pH 5.

3.2. Biotransformation of DCF, DHQ, β-NP and 2,4-DCP by laccase cocktail

The laccase cocktail degraded approximately 50% of DCF within 8 h, 78% of DHQ was biotransformed in just 3.5 h. In the case of β-NP and 2,4-DCP, the bioconversion of approximately 97% and 71%, was achieved in 8 h, respectively (Fig. 1).

Since laccase is an oxidoreductase which couples the electron oxidation of phenolic substrates, the presence of electron donating functional groups (EDFG) or electron withdrawing functional groups (EWFG) plays an important role in its reactivity (Nguyen et al., 2013; Yang et al., 2013a). According to Yang et al. (2013a), EDFG such as hydroxyl (−OH), amines (−NH2), alkoxy (−OR), alkyl (−R) and acyl (−COR) groups are susceptible for electrophilic attack by oxygenase enzymes. Moreover, the presence of EWFG reduces the affinity of enzymes for biotransformation on these compounds since groups such as amide ($-CONR_2$), halogen (−X) and nitro ($-NO_2$) produce an electron deficiency (Tadkaew et al., 2011). The slight differences of the biotransformation percentage between the compounds can be attributed to their chemical structures. Thus, the bioconversion percentage of β-NP (97%) can be explained by the presence of the hydroxyl group attached to the aromatic ring that facilitates electrophilic attack by laccase. The next compounds with higher biotransformation were DHQ and 2,4-DCP (78% and 71%, respectively); a particular characteristic of DHQ and 2,4-DCP is the presence of both EDFG and EWFG in their structure. In spite of the effect of iodine (in DHQ) and chlorine (in 2,4-DCP) halogens (EWFG), the strong electro-donor hydroxyl group apparently made these molecules appropriate for the oxidation by laccase. In the case of DCF, the biotransformation percentage was 50% after 8 h of enzymatic treatment. DCF contains a carboxylic acid group, which is a strong EWG; however, this compound contains an aromatic amine, an EDG-type functional group, which may decrease the affinity of the enzyme. A similar result was reported by Almansa et al. (2004) who investigated biotransformation of different dyes by laccase. Carboxyl groups were identified to be responsible for the increased recalcitrance of the dyes, while hydroxy-substituted dyes were most susceptible to enzyme action.

3.3. Reaction kinetics

The biotransformation rates of β-NP and 2,4-DCP were fitted to a first order reaction, according to the following equation:

$$ln[A] = ln[A]_0 - k't \quad (1)$$

where $[A_0]$ corresponds to the initial concentration of the analytes (mg L^{-1}) and [A] is the concentration (mg L^{-1}) at a particular reaction time; k' (h^{-1}) is the adjusted rate constant used in the general model and t corresponds to time (in hours). In case of DHQ and DCF the bioconversion rates were adjusted to a second order reaction, according to the equation:

$$\frac{1}{[A]} = \frac{1}{[A]_0} + k't \quad (2)$$

where $[A_0]$ corresponds to the initial concentration of the analytes (mg L^{-1}) and [A] is the concentration (mg L^{-1}) at a particular reaction time; k' (M^{-1} h^{-1}) is the adjusted rate constant used in the general model and t corresponds to time (in hours). For all the adjustments a $R^2 > 0.96$ was obtained (shown in Fig. 1). The first order biotransformation analysis showed that k' for β-NP (0.42 h^{-1}), is higher than that for 2,4-DCP (0.23862 h^{-1}). In the case of DHQ, the velocity of rate degradation was very high ($k'_{DHQ} = 44856.43$ M^{-1} h^{-1}) in contrast with the result observed for DCF ($k'_{DCF} = 4220$ M^{-1} h^{-1}). In terms of affinity enzyme-substrate (Km^{app} value) the analyte order would be DQH> 2,4-DCP > DCF> β-NP (Table 1).

Table 1 summarizes the studies that have been performed using free laccases for the enzymatic biotransformation of the target micropollutants. In this work, a bioconversion of 50% was obtained for DCF, which is better than the percentages obtained by Yang et al. (2013b), who reported 27% removal using mediators. Meanwhile, Margot et al. (2013b) reported 31% of degradation at pH 5.5 and 25 °C. Almost complete bioconversion (>95%) of DCF was achieved using mediators such as 1-Hydroxybenzotriazole (Nguyen et al., 2013), syringaldehyde, violuric acid (VA) (Lloret et al., 2010, 2013) or with elevated amounts of enzyme (2000 U L^{-1}) (Marco-Urrea

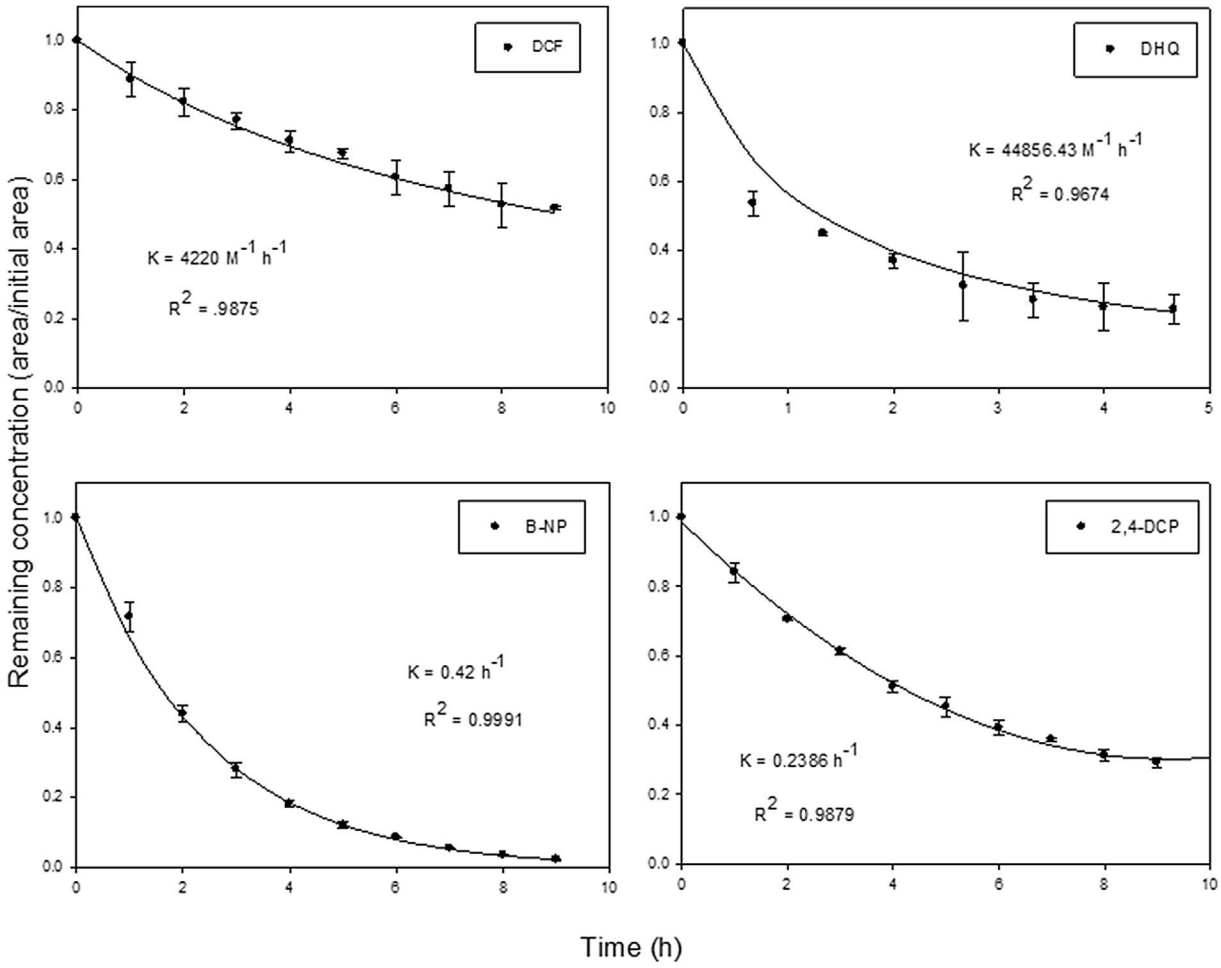

Fig. 1. Biotransformation profiles of 10 mg L^{-1} synthetic samples of DCF, DHQ, β-NP and 2,4-DCP by using 100 U L^{-1} laccase cocktail at 25 °C and pH 5. The curves were fitted to first (β-NP and 2,4-DCP) or second (DCF and DHQ) order of reaction.

et al., 2010; Lloret et al., 2010, 2013). Complete elimination of DCF was obtained at the expense of extremely long treatment times of 1 day (Nguyen et al., 2014), 3 days (Badia-Fabregat et al., 2014) and even 7 days (Margot et al., 2013a) by using enzymatic reactions. Studies that report the degradation of DHQ by laccase have not been conducted up to date. In this study, 78% of biotransformation was achieved for DHQ. On the other hand, only two studies have reported the degradation of β-NP with immobilized commercial laccase. Krastanov (2000) studied the degradation of β-NP using free laccase from *Pyricularia oryzae*, observing no degradation of the compound; a complete removal (100 mg L^{-1} of analyte) was achieved only after a two-step treatment with the co-immobilization of laccase and tyrosinase in a fixed-bed tubular bioreactor (see Table 1). Lante et al. (2000) reported the immobilization of a commercial laccase from *P. oryzae* in a spiral-wound asymmetric polyethersulphone membrane reactor, obtaining 18% of β-NP degradation at initial concentrations of 100 mg L^{-1}, pH 6.6 and 35 °C (data not shown in Table 1), a lower efficiency compared to the 97% of biotransformation obtained for β-NP in this study. The results showed in the aforementioned studies prove the promising application of the *P. sanguineus* laccase (employed in this work) for biotransformation treatment of β-NP in water, showing high rates of degradation even as free enzyme. A bioconversion of 71% was achieved for 2,4 DCP in this work. Higher removals were obtained in previous studies since they achieved almost complete removal, but by using free laccase at acidic conditions (Jia et al., 2012), temperatures around 30 and 50 °C (Zhang et al., 2008a; Gaitan et al., 2011; Qin et al., 2012; Xu et al., 2013) or employing high amounts of enzyme (Zhang et al., 2008a).

Under similar conditions of treatment, this study demonstrated that the overall degradation efficiency was comparable and in some cases, better compared to previous reports presented in Table 1. As we reported in a previous study (Ramírez-Cavazos et al., 2014b) the laccase isoforms, present in the crude extract used in this study, are thermostable and highly active up to 70 °C; however the aim of this work was to develop an efficient water treatment, avoiding the use of mediators and under mild conditions.

It is important to notice that the use of mediators is very common in laccase treatments, however it adds complexity to the system by augmenting the amount of by-products after the biotransformation of micropollutants (Murugesan et al., 2010); moreover, additives used as stabilizing agents, such as PEG, are expensive and some are toxic (Kim and Nicell, 2006a).

4. Biotransformation behavior of DCF, DHQ, β-NP and 2,4 DCP in groundwater samples

Biotransformation of DCF and DHQ in groundwater samples were similar to those observed in synthetic buffer solution whereas degradation of β-NP and 2,4-DCP decreased from 97% to 86% and from 71% to 53%, respectively (Fig. 2). Enaud et al. (2011) reported that chloride inhibits the oxidation of ABTS, but not that of

Table 1
Kinetic and catalytic coefficients corresponding to the maximal biodegradation of target micropollutants achieved by different of laccases as free enzyme.

Compound	Laccase sources	Laccase concentration (U L^{-1})	Analyte (mg L^{-1})	Mediator	pH	T (°C)	Removal %	Time (h)	Kinetic (k) and catalytic (V_{max}, Km^{app}) coefficients	Comments	Ref.
DCF	*Trametes versicolor*	210	20.0	–	5	25	96	288	–	Wastewater effluents samples	(Margot et al., 2013a)
					6		100	144			
					7		100	192			
	Streptomyces cyaneus	220		–	5		80	288			
					6		80	288			
					7		60	288			
	Trametes versicolor	2000	5.0	–	4	25	99	4	$k = 0.643$[a]	Continuous magnetic stirring; synthetic samples	(Lloret et al., 2013)
					7		27	24	$k = 0.004$[a]		
				HBT[b];	4		99	0.5	$k = 2.618$[a]		
				1 mM	7		98	24	$k = 0.182$[a]		
				SA[c];	4		99	2	$k = 1.028$[a]		
				1 mM	7		50	24	$k = 0.014$[a]		
	Trametes versicolor	25	1.0	–	4.5	25	100	72	–	Biotransformation study with carbon isotope	(Badia-Fabregat et al., 2014)
	Trametes versicolor	35	0.1	–	4.5	28	70	24	–	Removal of trace organic compounds by crude enzyme extract	(Nguyen et al., 2013)
				HBT[b];			96				
				1 mM							
	Trametes versicolor	40	1.45	–	4.5	25	No removal[d]	22	–	Removal of trace organic compounds rotary shaker at 120 rpm	(Yang et al., 2013b)
				HBT[b];			27				
				1 mM							
	Trametes versicolor	73	20	–	5.5	23	31	2.48	–	response surface method for the determination of optimal conditions	(Margot et al., 2013b)
	Trametes versicolor	35.0	0.1	–	4.5	25	73	24	–	Removal of trace organic compounds by crude enzyme extract at 70 rpm	(Nguyen et al., 2014)
				HBT[b];			98				
				1 mM							
	Trametes versicolor	2000	40	–	4.5	25	75	4.5	–	shaking conditions at 135 rpm	(Marco-Urrea et al., 2010)
							95				
	Myceliophthora thermophila	2000	5	–	5	22	40	8	–	Free laccase treatment of synthetic sample with natural/synthetic mediators	(Lloret et al., 2010)
				HBT[b];			65	8			
				0.5 mM							
				SA[c],			100	1			
				1 mM							
				VA/V[e],			100	24			
				1 mM							
				CA[f],			92	24			
				1 mM							
				FA[g],			83	24			
				1 mM							
	Pycnoporus sanguineus Sp. CS43 Laccase cocktail (LacI/LacII)	100	10	–	5	25	50	8	$k = 0.014$[a] $V_{max} = 5.7245$[h] $Km^{app} = 1076.76$[i]	Synthetic and groundwaters sampleswith Free laccase	Present study
DHQ	*Pycnoporus sanguineus Sp. CS43 Laccase cocktail (LacI/LacII)*	100	10	-	5	25	78	3.5	$k = 0.113$[a] $V_{max} = 11.41$[h] $Km^{app} = 61.31$[i]	Synthetic and groundwaters with Free laccase	Present Study
β-NP	*Pyricularia oryzae*	20 000	100	–	6.5	25	No removal[j]	0.25	–	Free laccase with synthetic samples	(Krastanov 2000)
	Pycnoporus sanguineus Sp. CS43 Laccase cocktail (LacI/LacII)	100	10	-	5	25	97	8	$k = 0.420$[k] $V_{max} = 44.96$[h] $Km^{app} = 1221.07$[i]	Synthetic and groundwaters with Free laccase	Present Study
2,4-DCP	*Trametes versicolor*	854 mg L^{-1}	50	–	6	50	83	6	$k = 1.590$[k]	Free laccase with synthetic samples	(Xu et al., 2013)
	Trametes versicolor	0.2 mg L^{-1}	815	–	4.5	25	94	2	$k = 2.280$[k]	Free Laccase with synthetic samples	(Jia et al., 2012)
	Trametes pubescens	10	15	–	6	40	99	8	$k = 1.147$[k]	Free laccase in presence of a mixture of dichlorophenols, degradation in shake flasks at 200 rpm.	(Gaitan et al., 2011)
	Amillariella mellea	100	75	–	6.5	40	97	10	–	Free laccase with synthetic samples	(Qin et al., 2012)
	Coriolus versicolor	297000	10	–	5.5	30	94	10	$k = 4.80 \times 10^{7}$[k]	Free laccase with synthetic samples	(Zhang et al., 2008a)
	Pycnoporus sanguineus Sp. CS43	100	10		5	25	71	8	$k = 0.2386$[k] $V_{max} = 2.21$[h] $Km^{app} = 224.44$[i]	Synthetic and groundwaters with free laccase	Present Study

Table 1 (*continued*)

Compound	Laccase sources	Laccase concentration (U L^{-1})	Analyte (mg L^{-1})	Mediator	pH	T (°C)	Removal %	Time (h)	Kinetic (*k*) and catalytic (V_{max}, Km^{app}) coefficients	Comments	Ref.
	Laccase cocktail (LacI/LacII)										

[a] Second order reaction, (L/(mg h)).
[b] HBT; 1-hydroxybenzotriazole.
[c] SA; syringaldehyde (SA).
[d] No removal was observed using extracellular extract of *T. versicolor*; 27% of removal was reported in presence of mediator HBT and degradation >99% was achieved using *T. versicolor* whole-cell.
[e] VA/V; Violuric acid/Vanilin.
[f] CA; p-coumaric acid.
[g] FA; ferulic acid.
[h] V_{max} = μM/min.
[i] Km^{app} = the half substrate concentration of saturation, considered as an apparent Km (units, μM).
[j] No removal was observed using free laccase from *P. oryzae* and a complete degradation was reported after a two-step treatment with co-immobilized laccase and tyrosinase in a fixed-bed tubular bioreactor.
[k] First order reaction, (hr^{-1}).

anthraquinonic compounds. Also, depending on which ions are present, different effects in the catalytic performance of the enzyme are observed (Zilly et al., 2011), which may be provoked by conformational changes in the active site that determine how deeply substrates penetrate into the pocket (Hakulinen et al., 2008). For example, chloride (Cl^-), halide anions (F^-, Br^-) and hydroxide anion (OH^-), have been reported to bind to the T2 Cu of laccase and interrupt the internal electron transfer between T1 and T2/T3, or to bind near the T1 active site, thus blocking the access of the substrate to T1 Cu (Margot et al., 2013a). In our previous study (Ramírez-Cavazos et al., 2014b), the presence of some of these components was related with the decrease in the activity of laccase isoforms in the crude extract used in this work. Kim and Nicell (2006b) reported that Bisphenol A biotransformation, using laccase from *Trametes versicolor*, was also negatively impacted by the presence of nitrite, thiosulfate, Cu(II), Fe(III), cyanide, fluoride and chloride (Kim and Nicell, 2006b). Other interferences, such as cyanide and calcium cause dissociation of the copper ion in the enzymatic active site, while cobalt and zinc chlorides tend to interfere by hydrogen bonding (chaotropic effect) (Cabana et al., 2007).

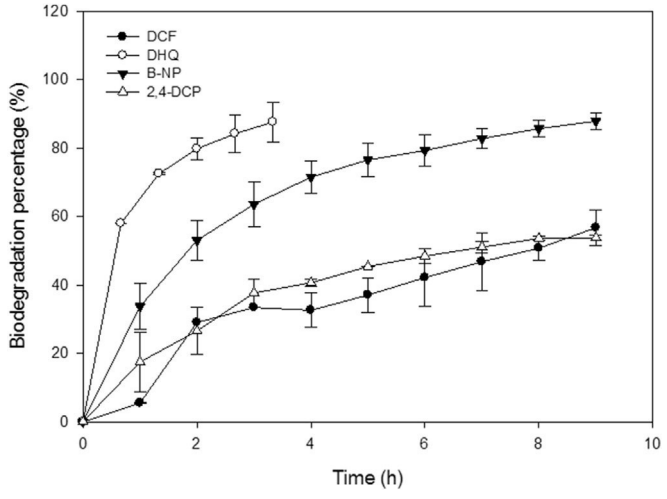

Fig. 2. Biotransformation profile of DCF, DHQ, β-NP and 2,4-DCP on a representative bulk sample from groundwater. A concentration of 100 UL^{-1} laccase cocktail (from *P. sanguineus* CS43) and 10 mg L^{-1} of each micropollutant at 25 °C and pH 5 were employed.

5. Conclusions

The biotransformation of the micropollutants DCF, DHQ, β-NP and 2,4 DCP using a laccase cocktail from *P. sanguineus* CS43 was studied in both synthetic buffers and real groundwater samples. The degradation percentages obtained in synthetic samples for DCF (50%), DHQ (78%), β-NP (97%) and 2,4 DCP (71%) were achieved using free laccase enzyme and avoiding the use of mediators. Degradation of DCF and DHQ was not affected by groundwater constituents which decreased the biotransformation efficiency of the enzyme for β-NP and 2,4 DCP. This study reveals a high biocatalytic efficiency of the cocktail composed by LacI and LacII for the removal of micropollutants due to the low amounts of laccase employed (100 UL^{-1}). Consequently, laccase from *P. sanguineus* strain CS43 represents a promising alternative for biotransformation of trace pollutants in complex matrices.

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References

Almansa, E., Kandelbauer, A., Pereira, L., Cavaco-Paulo, A., Guebitz, G.M., 2004. Influence of structure on dye degradation with laccase mediator systems. Biocatal. Biotransfor. 22, 315–324. http://dx.doi.org/10.1080/10242420400024508.
Arora, D.S., Gill, P.K., 2001. Comparison of two assay procedures for lignin peroxidase. Enzym Microb. Technol. 28, 602–605. http://dx.doi.org/10.1016/S0141-0229(01)00302-7.
Badia-Fabregat, M., Rosell, M., Caminal, G., Vicent, T., Marco-Urrea, E., 2014. Use of stable isotope probing to assess the fate of emerging contaminants degraded by white-rot fungus. Chemosphere 103, 336–342. http://dx.doi.org/10.1016/j.chemosphere.2013.12.029.
Baumgartner, G., Gawel, M.J., Kaeser, H.E., Pallis, C.A., Rose, F.C., Schaumburg, H.H., Thomas, P.K., Wadia, N.H., 1979. Neurotoxicity of halogenated hydroxyquinolines: clinical analysis of cases reported outside Japan. J. Neurol. Neurosurg. Psychiatry 42, 1073–1083. http://dx.doi.org/10.1136/jnnp.42.12.1073.
Bolong, N., Ismail, A.F., Salim, M.R., Matsuura, T., 2009. A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination 239, 229–246. http://dx.doi.org/10.1016/j.desal.2008.03.020.
Cabana, H., Jones, J.P., Agathos, S.N., 2007. Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: a review.

Eng. Life Sci. 7, 429–456. http://dx.doi.org/10.1002/elsc.200700017.
Cajthaml, T., Kresinová, Z., Svobodová, K., Möder, M., 2009. Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi. Chemosphere 75, 745–750. http://dx.doi.org/10.1016/j.chemosphere.2009.01.034.
Caliman, F.A., Gavrilescu, M., 2009. Pharmaceuticals, personal care products and endocrine disrupting agents in the environment – a review. Clean. Soil, Air, Water 37, 277–303. http://dx.doi.org/10.1002/clen.200900038.
Callahan, M.A., Slimak, M.W., Gabel, N.W., May, I., Fowler, C., Freed, J.R., Jennings, P., Durfee, R., Whitmore, F.C., Maestri, B., Mabey, W., Holt, B.R., Gould, C., 1979. Water-related Environment Fate of 129 Priority Pollutants Vol. II: Halogenated Aliphatic Hydrocarbons Halogenated Ethers Monocyclic Aromatics Phthalate Esters Polycyclic Aromatic Hydrocarbons Nitrosamines Miscellanelus Compounds. US Environmental Protection Agency, pp. 412–432.
Croera, C., Ferrario, D., Gribaldo, L., 2008. In vitro toxicity of naphthalene, 1-naphthol, 2-naphthol and 1,4-naphthoquinone on human CFU-GM from female and male cord blood donors. Toxicol. Vitr. 22, 1555–1561. http://dx.doi.org/10.1016/j.tiv.2008.06.004.
Deblonde, T., Cossu-Leguille, C., Hartemann, P., 2011. Emerging pollutants in wastewater: a review of the literature. Int. J. Hyg. Environ. Health 214, 442–448. http://dx.doi.org/10.1016/j.ijheh.2011.08.002.
Eggert, C., Temp, U., Eriksson, K.L., 1996. The ligninolytic system of the white rot fungus pycnoporus cinnabarinus: purification and characterization of the laccase. Appl. Environ. Microbiol. 62, 1151–1158.
Einsiedl, F., Radke, M., Maloszewski, P., 2010. Occurrence and transport of pharmaceuticals in a karst groundwater system affected by domestic wastewater treatment plants. J. Contam. Hydrol. 117, 26–36. http://dx.doi.org/10.1016/j.jconhyd.2010.05.008.
Enaud, E., Trovaslet, M., Naveau, F., Decristoforo, A., Bizet, S., Vanhulle, S., Jolivalt, C., 2011. Laccase chloride inhibition reduction by an anthraquinonic substrate. Enzyme Microb. Technol. 49, 517–525. http://dx.doi.org/10.1016/j.enzmictec.2011.07.007.
Esplugas, S., Bila, D.M., Krause, L.G., Dezotti, M., 2007. Ozonation and advanced oxidation technologies to remove endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) in water effluents. J. Hazard Mater. 149, 631–642. http://dx.doi.org/10.1016/j.jhazmat.2007.07.073.
Fava, L., Orrù, M.A., Crobe, A., Barra Caracciolo, A., Bottoni, P., Funari, E., 2005. Pesticide metabolites as contaminants of groundwater resources: assessment of the leaching potential of endosulfan sulfate, 2,6-dichlorobenzoic acid, 3,4-dichloroaniline, 2,4-dichlorophenol and 4-chloro-2-methylphenol. Microchem J. 79, 207–211. http://dx.doi.org/10.1016/j.microc.2004.10.009.
Gaitan, I.J., Medina, S.C., González, J.C., Rodríguez, A., Espejo, A.J., Osma, J.F., Sarria, V., Alméciga-Díaz, C.J., Sánchez, O.F., 2011. Evaluation of toxicity and degradation of a chlorophenol mixture by the laccase produced by trametes pubescens. Bioresour. Technol. 102, 3632–3635. http://dx.doi.org/10.1016/j.biortech.2010.11.040.
Gavrilescu, M., Demnerová, K., Aamand, J., Agathos, S., Fava, F., 2015. Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. N. Biotechnol. 32, 147–156. http://dx.doi.org/10.1016/j.nbt.2014.01.001.
Hakulinen, N., Andberg, M., Kallio, J., Koivula, A., Kruus, K., Rouvinen, J., 2008. A near atomic resolution structure of a Melanocarpus albomyces laccase. J. Struct. Biol. 162, 29–39. http://dx.doi.org/10.1016/j.jsb.2007.12.003.
He, L., Jurs, P.C., Kreatsoulas, C., Custer, L.L., Durham, S.K., Pearl, G.M., 2005. Probabilistic neural network multiple classifier system for predicting the genotoxicity of quinolone and quinoline derivatives. Chem. Res. Toxicol. 18, 428–440. http://dx.doi.org/10.1021/tx049742m.
House, W.A., Leach, D., Long, J.L.A., Cranwell, P., Smith, C., Bharwaj, L., Meharg, A., Ryland, G., Orr, D.O., Wright, J., 1997. Micro-organic compounds in the Humber rivers. Sci. Total Environ. 194–195, 357–371. http://dx.doi.org/10.1016/S0048-9697(96)05375-2.
Howard, P.H., Muir, D.C.G., 2011. Identifying new persistent and bioaccumulative organics among chemicals in commerce II. Pharm. Environ. Sci. Technol. 45, 6938–6946. http://dx.doi.org/10.1021/es201196x.
Jia, J., Zhang, S., Wang, P., Wang, H., 2012. Degradation of high concentration 2,4-dichlorophenol by simultaneous photocatalytic-enzymatic process using TiO2/UV and laccase. J. Hazard Mater 205–206, 150–155. http://dx.doi.org/10.1016/j.jhazmat.2011.12.052.
Kim, Y.J., Nicell, J.A., 2006a. Laccase-catalyzed oxidation of bisphenol A with the aid of additives. Process Biochem. 41, 1029–1037. http://dx.doi.org/10.1016/j.procbio.2005.11.012.
Kim, Y.J., Nicell, J.A., 2006b. Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A. Bioresour. Technol. 97, 1431–1442. http://dx.doi.org/10.1016/j.biortech.2005.06.017.
Krastanov, A., 2000. Removal of phenols from mixtures by co-immobilized laccase/tyrosinase and polyclar adsorption. J. Ind. Microbiol. Biotechnol. 24, 383–388.
Lante, A., Crapisi, A., Krastanov, A., Spettoli, P., 2000. Biodegradation of phenols by laccase immobilised in a membrane reactor. Process Biochem. 36, 51–58. http://dx.doi.org/10.1016/S0032-9592(00)00180-1.
Lloret, L., Eibes, G., Lú-Chau, T.A., Moreira, M.T., Feijoo, G., Lema, J.M., 2010. Laccase-catalyzed degradation of anti-inflammatories and estrogens. Biochem. Eng. J. 51, 124–131. http://dx.doi.org/10.1016/j.bej.2010.06.005.
Lloret, L., Eibes, G., Moreira, M.T., Feijoo, G., Lema, J.M., 2013. On the use of a high-redox potential laccase as an alternative for the transformation of non-steroidal anti-inflammatory drugs (NSAIDs). J. Mol. Catal. B Enzym 97, 233–242. http://dx.doi.org/10.1016/j.molcatb.2013.08.021.
Lloret, L., Hollmann, F., Eibes, G., Feijoo, G., Moreira, M.T., Lema, J.M., 2012. Immobilisation of laccase on Eupergit supports and its application for the removal of endocrine disrupting chemicals in a packed-bed reactor. Biodegradation 23, 373–386. http://dx.doi.org/10.1007/s10532-011-9516-7.
Mahlknecht, J., Schneider, J.F., Broder, J.M., Navarro de León, I., Bernasconi, S.M., 2004. Groundwater recharge in a sedimentary basin in semi-arid Mexico. Hydrogeol. J. 12, 511–530. http://dx.doi.org/10.1007/s10040-004-0332-6.
Marco-Urrea, E., Pérez-Trujillo, M., Cruz-Morató, C., Caminal, G., Vicent, T., 2010. Degradation of the drug sodium diclofenac by trametes versicolor pellets and identification of some intermediates by NMR. J. Hazard Mater. 176, 836–842. http://dx.doi.org/10.1016/j.jhazmat.2009.11.112.
Margot, J., Bennati-Granier, C., Maillard, J., Blánquez, P., Barry, D.A., Holliger, C., 2013a. Bacterial versus fungal laccase: potential for micropollutant degradation. Amb. Express 3, 63. http://dx.doi.org/10.1186/2191-0855-3-63.
Margot, J., Maillard, J., Rossi, L., Barry, D.A., Holliger, C., 2013b. Influence of treatment conditions on the oxidation of micropollutants by Trametes versicolor laccase. N. Biotechnol. 30, 803–813. http://dx.doi.org/10.1016/j.nbt.2013.06.004.
Murugesan, K., Chang, Y.-Y., Kim, Y.-M., Jeon, J., Kim, E., Chang, Y., 2010. Enhanced transformation of triclosan by laccase in the presence of redox mediators. Water Res. 44, 298–308. http://dx.doi.org/10.1016/j.watres.2009.09.058.
Nagata, N., Marriott, D., Harkness, J., Ellis, J.T., Stark, D., 2012. Current treatment options for Dientamoeba fragilis infections. Int. J. Parasitol. Drugs drug Resist 2, 204–215. http://dx.doi.org/10.1016/j.ijpddr.2012.08.002.
Naidoo, V., Swan, G.E., 2009. Diclofenac toxicity in Gyps vulture is associated with decreased uric acid excretion and not renal portal vasoconstriction. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149, 269–274. http://dx.doi.org/10.1016/j.cbpc.2008.07.014.
Nguyen, L.N., Hai, F.I., Yang, S., Kang, J., Leusch, F.D.L., Roddick, F., Price, W.E., Nghiem, L.D., 2013. Removal of trace organic contaminants by an MBR comprising a mixed culture of bacteria and white-rot fungi. Bioresour. Technol. 148, 234–241. http://dx.doi.org/10.1016/j.biortech.2013.08.142.
Nguyen, L.N., Hai, F.I., Yang, S., Kang, J., Leusch, F.D.L., Roddick, F., Price, W.E., Nghiem, L.D., 2014. Removal of pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals and pesticides by Trametes versicolor: role of biosorption and biodegradation. Int. Biodeterior. Biodegrad. 88, 169–175. http://dx.doi.org/10.1016/j.ibiod.2013.12.017.
Padkel, H., Couture, G., Roy, C., Masson, A., Locat, J., Gélinas, P., Lesage, S., 1992. Groundwater Contamination and Analysis at Hazardous Waste Sites. In: Chapter 13- Developing Methods for the Analysis of Toxic Chemicals in Soil and Groundwater: the Case of Vile Mercier, Quebec, Canada, vol. 4. CRC press, p. 381.
Petrović, M., Gonzalez, S., Barceló, D., 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. Trac. Trends Anal. Chem. 22, 685–696. http://dx.doi.org/10.1016/S0165-9936(03)01105-1.
Qin, R.-B., Zhu, X.-F., Wu, K., Zhang, J.-J., Zhao, H.-K., 2012. Condition optimization for degradation of chlorophenols using laccase from Amillariella mellea. Huan Jing Ke Xue 33, 2470–2474.
Ramírez-Cavazos, L.I., Junghanns, C., Nair, R., Cárdenas-Chávez, D., Hernández-Luna, C., Agathos, S., Parra, R., 2014a. Enhanced production of thermostable laccases from a native strain of Pycnoporus sanguineus using central composite design. J. Zhejiang Univ. Sci. B 15, 343–352.
Ramírez-Cavazos, L.I., Junghanns, C., Ornelas-Soto, N., Cárdenas-Chávez, D., Hernández-Luna, C., Demarche, P., Enaud, E., García-Morales, R., Agathos, S.N., Parra-Saldivar, R., 2014b. Purification and characterization of two thermostable laccases from Pycnoporus sanguineus and potential role in degradation of Endocrine Disrupting Chemicals. J. Mol. Catal. B Enzym 108, 32–42. http://dx.doi.org/10.1016/j.molcatb.2014.06.006.
Roch, F., Alexander, M., 1995. Biodegradation of hydrophobic compounds in the presence of surfactants. Environ. Toxicol. Chem. 14, 1151–1158. http://dx.doi.org/10.1002/etc.5620140705.
Rudel, R.A., Melly, S.J., Geno, P.W., Sun, G., Brody, J.G., 1998. Identification of alkylphenols and other estrogenic phenolic compounds in wastewater, Septage, and groundwater on Cape Cod, Massachusetts. Environ. Sci. Technol. 32, 861–869. http://dx.doi.org/10.1021/es970723r.
Sein, M., Zedda, M., Tuerk, J., Schmidt, T.C., Golloch, A., Von Sonntag, C., 2008. Oxidation of diclofenac with ozone in aqueous solution. Environ. Sci. Technol. 42, 6656–6662. http://dx.doi.org/10.1021/es8008612.
Stuart, M., Lapworth, D., Crane, E., Hart, A., 2012. Review of risk from potential emerging contaminants in UK groundwater. Sci. Total Environ. 416, 1–21. http://dx.doi.org/10.1016/j.scitotenv.2011.11.072.
Tadkaew, N., Hai, F.I., McDonald, J.A., Khan, S.J., Nghiem, L.D., 2011. Removal of trace organics by MBR treatment: the role of molecular properties. Water Res. 45, 2439–2451. http://dx.doi.org/10.1016/j.watres.2011.01.023.
Tessier, L., Boisvert, J.L., Vought, L.B., Lacoursie, J.O., 2000. Effects of 2,4-dichlorophenol on the net-spinning behavior of hydropsyche slossonae larvae (Trichoptera; Hydropsychidae), an early warning signal of chronic toxicity. Ecotoxicol. Environ. Saf. 46, 207–217. http://dx.doi.org/10.1006/eesa.1999.1897.
Tijani, J.O., Fatoba, O.O., Madzivire, G., Petrik, L.F., 2014. A review of combined advanced oxidation technologies for the removal of organic pollutants from water. Water, Air, Soil Pollut. 225, 1–30. http://dx.doi.org/10.1007/s11270-014-2102-y.
Vyas, B.R.M., Bakowski, S., Šašek, V., Matucha, M., 1994. Degradation of anthracene by selected white-rot fungi. FEMS Microbiol. Ecol. 14, 65–70. http://dx.doi.org/10.1111/j.1574-6941.1994.tb00091.x.
Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese (II) Oxidation by manganese

Peroxidase from the Basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of chelators. J. Biol. Chem. 267, 23688–23695.

Xu, R., Zhou, Q., Li, F., Zhang, B., 2013. Laccase immobilization on chitosan/poly(vinyl alcohol) composite nanofibrous membranes for 2,4-dichlorophenol removal. Chem. Eng. J. 222, 321–329. http://dx.doi.org/10.1016/j.cej.2013.02.074.

Yang, S., Hai, F.I., Nghiem, L.D., Price, W.E., Roddick, F., Moreira, M.T., Magram, S.F., 2013a. Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. Bioresour. Technol. 141, 97–108. http://dx.doi.org/10.1016/j.biortech.2013.01.173.

Yang, S., Hai, F.I., Nghiem, L.D., Roddick, F., Price, W.E., 2013b. Removal of trace organic contaminants by nitrifying activated sludge and whole-cell and crude enzyme extract of Trametes versicolor. Water Sci. Technol. 67, 1216–1223. http://dx.doi.org/10.2166/wst.2013.684.

Zhang, J., Liu, X., Xu, Z., Chen, H., Yang, Y., 2008a. Degradation of chlorophenols catalyzed by laccase. Int. Biodeterior. Biodegrad. 61, 351–356. http://dx.doi.org/10.1016/j.ibiod.2007.06.015.

Zhang, Y., Geißen, S.U., Gal, C., 2008b. Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. Chemosphere 73, 1151–1161. http://dx.doi.org/10.1016/j.chemosphere.2008.07.086.

Zilly, A., da Silva Coelho-Moreira, J., Bracht, A., Marques de Souza, C.G., Carvajal, A.E., Koehnlein, E.A., Peralta, R.M., 2011. Influence of NaCl and Na_2SO_4 on the kinetics and dye decolorization ability of crude laccase from Ganoderma lucidum. Int. Biodeterior. Biodegrad. 65, 340–344. http://dx.doi.org/10.1016/j.ibiod.2010.12.007.

Chapter 4

Biotransformation of emerging pollutants in groundwater by laccase from *P. sanguineus* CS43 immobilized onto titania nanoparticles

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4.1 Summary of the chapter

Laccase immobilization was achieved by means of a covalent approach where 3-aminopropyltriethoxysilane (APTES) was used as coupling agent and the cross-linker glutaraldehyde (GLU) functioned as laccase binder. Laccases were successfully immobilized onto titania nanoparticles, enhancing thermal and pH stability. Furthermore, immobilized laccase was assessed in the biotransformation of emerging pollutants like acetaminophen and diclofenac showing more than 90% removal in synthetic and real samples.

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Biotransformation of emerging pollutants in groundwater by laccase from *P. sanguineus* CS43 immobilized onto titania nanoparticles

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ABSTRACT

Biotransformation of organic compounds by using biocatalysts such as laccases provides an environmentally attractive alternative to supplement conventional wastewater treatment. However, loss and inactivation of enzymes are challenges to solve for their potential use in water treatment applications. Titania presents a high chemical stability and easy functionalization, which are important characteristics in a support used for immobilization processes. Therefore, in this study, the immobilization of laccase enzymes produced by *Pycnoporus sanguineus* CS43 onto titania nanoparticles (~21 nm) was optimized. Surface modification of the support was carried out by using different weight% (wt%) of 3-aminopropyltriethoxysilane (APTES) as a coupling agent and the cross-linker glutaraldehyde (GLU) as a laccase binder. Free and immobilized enzymatic activity were measured based on 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays and were compared regarding apparent activity and activity recovery. In addition, the effect of ionic strength on the immobilization process was studied, finding that low ionic strength (25 mM phosphate buffer) promote a high activity recovery, greater than 90% and even higher than previous reports. Immobilized laccases showed high thermostability at 50 and 60 °C (half-lives of 45.7 and 3.7 h, respectively), and high stability at low pH values of 2 and 3 (half-lives of 31.8 and 107.1 h respectively). Furthermore, the biocatalyst was assessed in the biotransformation of emerging pollutants such as acetaminophen and diclofenac by using 100 UL^{-1} of immobilized laccase at pH 4; the maximum biotransformation percentage of DCF was 68% after 8 h, and more than 90% biotransformation of ACE was reached after 2 h of treatment.

1. Introduction

The increasing consumption of chemical products is an urgent global issue since many of these enter the environment directly or indirectly, causing pollution of the water bodies [1]. Among these products are the pharmaceutical active compounds (PhACs) or pharmacologically active metabolites [2], which are acquired without medical prescriptions and with annual production volumes typically in the kiloton to megaton range [3–5].

Currently, for example, more than eighty PhACs have been detected in aquatic media such as wastewater, surface water, groundwater and even tap water, which some of them are well-known to be persistent compounds [2,6]. According with Rivera-Utrilla et al. [7], most of these PhACs are considered as *emerging pollutants*, since they are currently not included in routine or regulatory control programs even though have been detected in the environment, which makes them candidates for future legislation due to its adverse effects and/or persistency [7,8].

It has been shown that PhACs have potential ecological and human health risks and are significantly toxic to aquatic species, even at trace levels ranging from ngL^{-1} to μgL^{-1} [9], In a recent report, the Word Health Organization (WHO) acknowledged that wastewater-impacted surface water and groundwater can reach PhACs concentrations of 0.1 μgL^{-1} [10]. Some negative impacts of PhACs include feminization of male organisms, growth inhibition, and carcinogenicity, which are further enhanced through bioaccumulation. In particular, acetaminophen and diclofenac are a matter of environmental concern due to their incomplete removal in wastewater treatment plants and their potential endocrine disruption, kidney and reproduction toxicity. In

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addition, it has been demonstrated that they can cause neurological effects in some aquatics organisms [11–15].

Although conventional wastewater treatments are effective in eliminating most of the organic compounds, many drugs and by-products are released into the environment [16]. Enzymatic biotransformation of organic pollutants has progressively attracted increasing interest in the development of alternative treatments due to its outstanding advantages, including high activity on a broad range of substrates as well as less toxic biotransformation by-products [17,18]. Laccases (E.C.1.10.3.2) are enzymes that belong to the family of multicopper oxidases, that catalyze the oxidation of various aromatic substrates monoelectronically to their corresponding radicals in the presence of molecular oxygen as an electron acceptor and its own reduction to water [18].

Enzymes in their free form are very difficult to recover from an aqueous sample [19]; therefore, they can only be used once for water treatment applications. This leads to increased costs since more enzyme needs to be produced and purified [20]. In addition, the stability and activity of free enzymes are affected in complex matrices, e.g., in the presence of highly polluted wastewater [21]. A suitable approach to overcome these drawbacks is enzymatic immobilization, that, in general, increases the stability and prolongs the shelf-life of enzymes compared to their free use [22].

The use of nanostructured materials for enzymatic immobilization has gained a lot of ground as they possess properties that can improve the immobilization process [23]. Titanium (IV) oxide nanoparticulate (titania) is a good candidate as a support in the enzymatic immobilization processes, since it provides large surface areas, allowing a higher enzyme loading and reduced mass transfer resistance for substrates. In addition, titania has high chemical stability and can be easily functionalized. Moreover, titania offers covalent binding that provides a strong bond between the enzyme and its support matrix via chemical reaction, by using bifunctional crosslinking reagents. This method leads to better outcomes with highly stable preparations compared to other immobilization techniques based on adsorption interactions [24]. Consequently, the immobilized enzyme is not detached from the support matrix during the treatment process and can be reused [25].

In this study, the immobilization of purified laccase enzymes from fungus *Pycnoporus sanguineus* CS43 was performed by using supports based on nanostructured titania. Characterization of the immobilized biocatalyst was also carried out. In addition, the biotransformation of acetaminophen and diclofenac was evaluated using both free and immobilized enzyme under mild acidic reaction conditions and avoiding mediators. Finally, biotransformation of DCF and ACE was carried out on spiked groundwater samples, showing a great potential for the removal of the target pollutants.

2. Experimental

2.1. Materials

Laccases (*P. sanguineus CS43*) were obtained from a tomato medium as described in our previous work [26]. Briefly, mycelia were recovered from the tomato medium supernatant after 10 days of culture by filtration (0.2 µm pore size). Then, it was concentrated by ultrafiltration with a tangential-flow filter (Membrane cut-off of 10 kDa, Sartorius Sartojet). The ultra-filtered sample was purified with a DEAE-cellulose ion exchange column eluted with 20 to 300 mM phosphate buffer pH 6.0 at a flow rate of 2 mL/min. Finally, laccase fractions were collected and concentrated.

High purity grade standards of acetaminophen (ACE) and diclofenac (DFC); 2,20 −azino-bis (3-ethylbenzthiazoline-6-sulfonate (ABTS); dibasic sodium phosphate and citric acid salt were obtained from Sigma Aldrich, USA. Acetonitrile and anhydrous ethanol (HPLC grade) were supplied from Tedia. Titanium oxide nanoparticles (TiO_2) (Degussa, P25) were supplied by Sigma-Aldrich; 3-Aminopropyl triethoxysilane (APTES) from Sigma and glutaraldehyde (GLU) from Merck Millipore.

2.2. TiO_2 modification and laccase immobilization

The immobilization method was modified from Hou et al. [27]. For TiO_2 functionalization, 400 mg of TiO_2 were first dispersed in 15 mL of anhydrous ethanol and disaggregated with an ultrasonic bath sonicator for 5 min followed by probe sonication during 15 min. The corresponding amount of APTES for each wt% (2, 3, 5 and 7%) was diluted in anhydrous ethanol, and this solution was added dropwise to the TiO_2–ethanol suspension. Then, more anhydrous ethanol was added until a concentration of 6 mg mL^{-1} of TiO_2 was reached. The mixture was placed under reflux for 24 h at 65 °C. Afterwards, nanoparticles were separated by filtration and washed with anhydrous ethanol. Finally, the TiO_2-nanoparticles functionalized with APTES (TiO_2-APTES) were dried under vacuum at room temperature. For GLU functionalization, TiO_2-APTES nanoparticles were ground into a fine powder and 50 mg were dispersed in a solution of 4% (v:v) of GLU previously dissolved in a 200 mM phosphate buffer solution (pH 7), and left to react for 12 h. Unreacted GLU was removed by centrifugation and re-dispersed in phosphate buffer; this washing step was repeated 3 times. For enzyme immobilization, TiO_2-APTES nanoparticles functionalized with GLU were suspended in 5 mL of laccase solution (1500 UL^{-1}) for 72 h at 20 °C (TiO_2-Lac). Afterwards, the unreacted laccase was removed by centrifugation and re-dispersed in phosphate buffer solution, repeating this washing step 3 times.

2.3. Assessment of laccase activity

Laccase activity was determined by monitoring the oxidation rate of ABTS to $ABTS^+$ at 420 nm in a microplate reader Omega FLUOstar Spectrophotometer at 25 °C. For determination of free enzyme activity, 20 µL of a laccase solution and 20 µL of aqueous ABTS (5 mM) were added to 160 µL of pH 3 phosphate–citrate buffer solution. 20 µL of pH 3 phosphate–citrate buffer solution were used instead of the free laccase as a blank. For immobilized laccase activity, 50 mg of TiO_2-Lac were dispersed in 45 mL of pH 3 phosphate–citrate buffer solution, and 5 mL of 5 mM ABTS solution were added to initiate the reaction. Before each UV measurement, the solution was rapidly filtered through a PTFE 0.2 µm syringe filter to remove TiO_2-Lac. In both cases, one unit (U) of laccase activity was defined as the amount of laccase forming 1 µmol of $ABTS^+$ per minute.

To evaluate the performance of the immobilization process, activity recovery was defined as the experimental activity of the immobilized laccase divided by the laccase activity theoretically immobilized onto the support. The theoretically immobilized activity was calculated as the difference between the initial laccase activity, before immobilization process, and the residual activity after the process.

2.4. Stability of the immobilized enzyme

Stability of immobilized laccase under different pH was examined by using phosphate–citrate buffer solution with pH ranging from 2.0 to 8.0, at 25 °C. The residual activity of TiO_2-Lac nanoparticles was measured daily for up to 15 days, and results were compared with the initial activity. Initial characterization of laccases isolated from *P. sanguineus* CS43 showed a wide range thermostability in free form (25–75 °C) [26]. Therefore, a similar range of stability 25 to 80 °C was tested in order to compare free and immobilized enzymes, and to determine if this properties were retained or improved by the immobilization process. A 25 mM phosphate buffer (pH 7) was used as incubator solution.

The residual activity of the TiO_2-Lac nanoparticles was measured until their inactivation. For all stability experiments, three replicates were made. The pH (acidic region) and thermal inactivation *versus* time was modelled by using the 3-parameter model of Aymard and Belarbi [28].

$$((A)t/(A)0) = C^{*}\exp(-a^{*}t) + (1 - C)^{*}exp(-b^{*}t) \quad \text{Eq. (1)}$$

The ratio (A)t/(A)0 represents the enzyme activity remaining after time t. Exponents *a* and *b* are complex expressions of rate constants. The constant C indicates the weight between the two exponential parts of the equation. The values of the parameters for this model were obtained by curve fitting in the plot of the residual enzyme activity *versus* time. GraphPad Prism 6.0 software was used for these calculations. Finally, half-life values were calculated according to Eq. (1) at (A)t/(A)0 = 0.5.

2.5. Determination of kinetic parameters

Michaelis–Menten kinetic parameters Km of the biocatalytic nanoparticles were determined by measuring the laccase activity with ABTS as a substrate under different concentrations, ranging from 5 to 250 mM. The values of the kinetic parameters were obtained by non-linear curve fitting in the plot of reaction rate *versus* substrate concentration, following the next equation:

$$V = (\text{Vmax}^{*}[S]/(\text{Km} + [S]) \quad \text{Eq. (2)}$$

Where V is the reaction rate, [S] is the substrate concentration and Vmax represent the maximum rate achieved by the bio-catalytic system. The Km Michaelis constant is the substrate concentration at which the reaction rate is half of Vmax and is often used as the indication of the enzyme-substrate affinity.

2.6. Enzymatic treatment of pharmaceuticals

Stock solutions (500 mgL^{-1}) were prepared by dissolution of the standards in ultrapure water. For free enzymatic treatment, reactions were adjusted to 1 mL adding 100 µL of phosphate/citric acid buffer pH 4, and the corresponding volume of stock solution to reach 10 mgL^{-1} of the target analyte and ultrapure water. All experiments were performed at room temperature and started by adding 100 UL^{-1} (50.3 U mg^{-1}) of free laccase.

For TiO_2-Lac nanoparticles, 50 mg of nanoparticles were suspended in 10 mL solution, containing 10 mgL^{-1} of the target pollutant. Then, the mixture was placed into beaker, protected from light to avoid TiO_2 photo-degradation, and then stirred at 500 rpm. The pH of the solution was adjusted using 10% (v/v) phosphate/citric acid buffer pH 4.

HPLC-UV (Agilent Technologies 1200 series) chromatography was employed in order to quantitatively record the biotransformation of the target pollutants. The chromatographic method was based on a reverse-phase column (Agilent Eclipse XDB-C18, 5 µ, 150 × 4.6 mm) using an injection volume of 20 µL and a flux of 1 mL/min, with gradient elution by means of (A) acetonitrile and (B) 25 mM phosphate buffer (pH 3). The gradient program was set as follows: 0 min 10% (A); 9 min, 90% (A) and 12 min, 10% (A). ACE and DCF were detected at three wavelengths, 210, 230 and 280 nm. Chromatographic analysis was carried out for up to 8 h and the level of the reaction was estimated by the decrease of the corresponding analyte peak and quantified using a calibration curve. Average values and standard deviations of each reaction were calculated from three independent replicates; blanks and negative controls were prepared and measured at the same time.

3. Results

3.1. Optimization of covalent immobilization onto TiO_2

3.1.1. The effect of ionic strength

Purified laccases from *Pycnoporus sanguineus* CS43 were used in all experiments. As previously reported, Lac I and Lac II are the most prevalent enzymes, and preliminary assays have not detected other ligninolytic enzymes (e.g., lignin peroxidase or manganese peroxidase) in the culture supernatant [26,29]. Laccase enzymes were immobilized onto titanium dioxide nanoparticles using a modified covalent method from that described by Hou et al., [30]. Basically, in this immobilization process, during incubation at pH 7, titania nanoparticles and enzymes obtain opposite charges which increases their mutual attraction. Furthermore, the covalent bonding formed among the enzyme and the functionalized support allows a higher laccase loading [31].

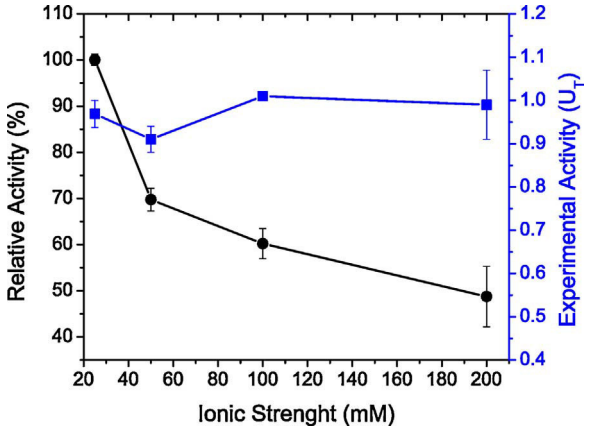

Fig. 1. Effect of ionic strength on the immobilization process of laccase enzymes.

Since incubation plays an important role, the effect of ionic strength (provided by the buffer solution) in the immobilization process was studied, as it is well known that the presence of ions in the medium can inhibit free enzymes. In order to test the effect of buffer concentration during incubation, TiO_2 was first functionalized with 2 wt% APTES and subsequently with 4% GLU. Then, laccase enzymes and the functionalized support were incubated in various media at different ionic strength in a range of 25–200 mM (pH 7, phosphate buffer).

As can be seen in Fig. 1, there was an increasing trend in recovered activity as ionic strength decreases. At 200 mM activity recovery was 50%, whereas for 25 mM it was close to 100%. At the same time, it was observed that experimental activity was maintained over all tested media at about 1U_T. These results are related to the fact that chemical reactions involving enzymes must be carried out under mild conditions (neutral pH, room temperature, and low ionic strength) in order to minimize the unavoidable conformational changes [32,33]. Moreover, a low ionic strength during incubation facilitates a first ion adsorption and a second covalent binding between the aldehyde groups of the support and the primary amino groups of the enzyme. Based on these results, an ionic strength of 25 mM (phosphate buffer) was used for further experiments.

3.1.2. Effect of APTES (wt%) on the immobilization process

For TiO_2 functionalization, silane coupling agents are frequently used to attach molecules with a second terminal functionality for further modification; a typical self-assembled monolayer agent is 3-aminopropyl(triethoxy)silane (APTES) wich has been reported as grafting in several semiconductors including titania nanoparticles [34].

In this work, the functionalization of TiO_2 with different amounts of APTES (2–7 wt%) was evaluated. All materials with different grade of functionalization were subsequently modified with 4% GLU and incubated with 14 U_T of initial laccase activity in order to analyze the effect of APTES concentration on the immobilization process. As shown in Fig. 2, the increase in APTES wt% allows a considerable retention of experimental immobilized activity that could be associated with the enzyme amount that has been attached to the support. As can be observed, at 2 wt% the experimental activity was about 1 U_T, and a very pronounced increment was observed when 5 wt% is tested, giving a result of 14 U_T. Similar values of experimental activity were obtained with 7 wt%. According to this tendency, this behavior is due to the increment of anchoring sites produced by APTES functionalization of TiO_2. It was also observed that in all cases activity recoveries were close to 100%. Activity preservation also seems to be given by the low ionic

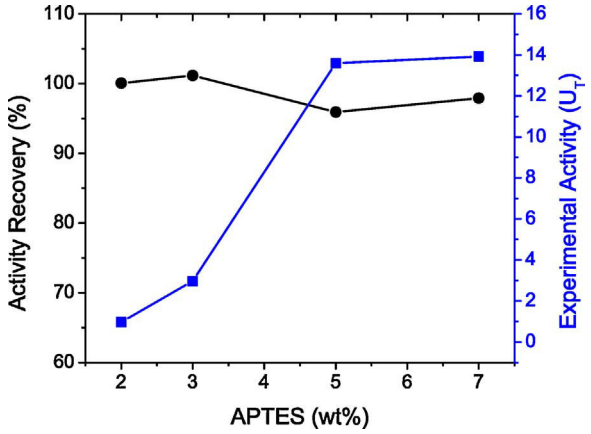

Fig. 2. Effect of APTES amount (wt%) on the immobilization process of laccase enzymes.

strength used in the incubation process. These results are higher than those obtained by other authors, e.g., as reported by Hou et al. (2014) with an optimal result of 79% for activity recovery [27]. In another work, the use of Celite R-633 for immobilization of laccase from *Coriolopsis polyzona* was investigated by using APTES-GLU functionalization, obtaining a 55% of recovery activity [35]. The recoveries observed in the aforementioned reports help highlight the effectiveness of the method proposed in this work for immobilizing laccase enzymes onto TiO_2 nanoparticles.

3.2. Characterization of immobilized enzyme (TiO_2-Lac)

3.2.1. Effect of pH and temperature on TiO_2-Lac activity

As can be seen in Fig. 3a, the optimal pH values for TiO_2-Lac were observed in the same acidic region from 2 to 4, but it was also observed an increase in relative activity with more than 90% in contrast to 65% for free laccases at pH 4. Beyond pH 5, there is a similar behavior between TiO_2-Lac and the free laccases. Regarding the effect of temperature, for immobilized laccases more than 85% of relative activity was obtained between 25 and 60 °C (Fig. 3b); this range was wider in comparison with free laccases (40 to 60 °C). For both free laccases and TiO_2-Lac, there is a pronounced deactivation effect above 60 °C, losing around 15–30% in relative activity.

3.2.2. pH and temperature stability of TiO_2-Lac

Immobilized laccases were incubated under different pH values ranging from 2 to 8, during 360 h. As shown in Fig. 4, under acidic conditions (pH 2 −4) there is still some activity remaining (between 20 and 50%,) whereas, for slight acid to basic conditions, the activity was well preserved (65–100%). Results of thermal stability (Fig. 5) showed that laccases immobilized onto titania nanoparticles were stable at 40 °C, having a residual activity of 90% after 72 h incubation. For 50 °C,

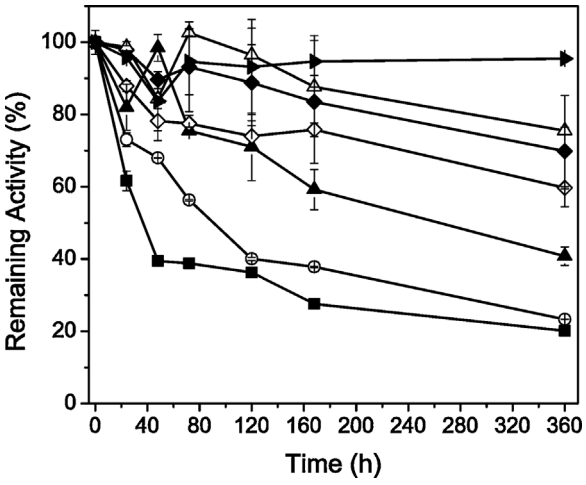

Fig. 4. Stability studies of immobilized laccases at different pH values: pH 2 (■), pH 3 (○), pH 4 (▲), pH 5(◊), pH 6 (♦), pH 7 (△), pH 8(►). Data points represent the means of three replicates ± SD.

residual activity fell below 60%; whereas for temperatures beyond 60 °C, the activity was drastically reduced.

The values of half-life for immobilized and free enzyme at different conditions (pH and temperature) are summarized in Table 1. Data of half-lives at different pH values showed that TiO_2-Lac presented a substantial improvement over those of free laccases. The values of half-lives at different temperatures suggest that immobilization provide a better stability, since a remarkable improvement is observed at temperatures above 60 °C in contrast to free laccases. Improved stability of TiO_2-Lac may be due to the strong chemical bonding between laccase and the support, that offers structural rigidity and reducing the manifestation of drastic conformational changes of the enzyme under extreme conditions, thus leading to a better stability [30,36,37].

3.2.3. Kinetics parameters of TiO_2-Lac and free laccases

Table 2 shows the kinetic constants for TiO_2-Lac and the free laccases. Since in the present study a conjugate of purified enzymes (Lac I and lac II) was used, the kinetic constants are shown as apparent constants. As can be seen in Table 2, in the case of free laccases, substrate affinity (Km_{app}) was 23.55 ± 3.65 μM, and for TiO_2-Lac 21.24 ± 1.92 μM. These values fall into the range of those reported for laccases from other *Pycnoporus* families [26]. Although the immobilization process did not generate any significant change over the Km_{app} values, it is important to take into account that there was a good preservation of the affinity between immobilized enzyme and substrate.

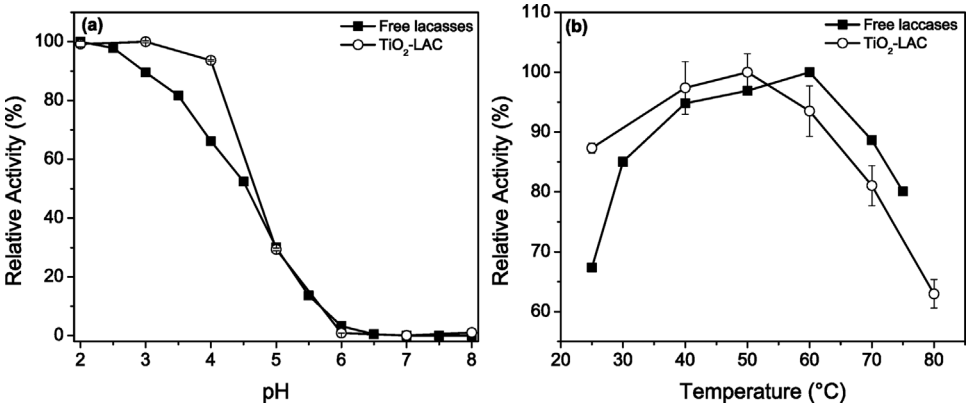

Fig. 3. (a) Effect of pH at 25 °C and (b) Temperature on both free and immobilized laccase activities.

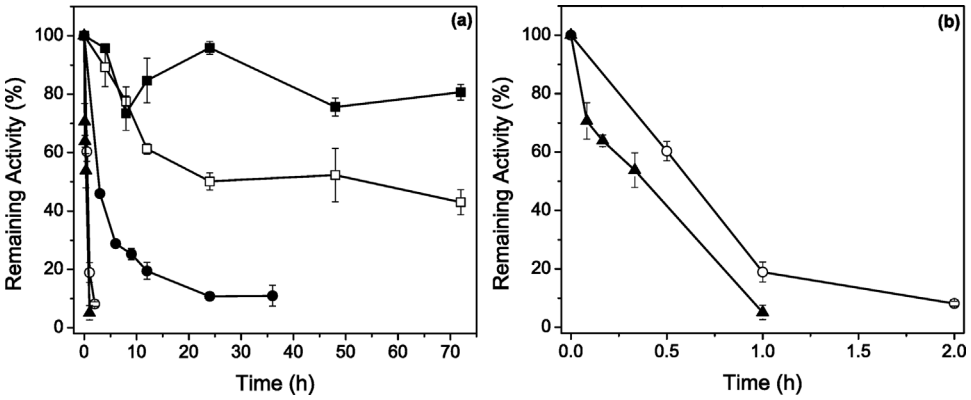

Fig. 5. Thermal stability at pH 7 for: (a) immobilized laccases at different temperature values; 40 °C (■), 50 °C (□), 60 °C (●); (b) amplification of stabilities at 70 °C (○) and 80 °C (▲). Data points represent the means of three replicates ± SD.

Table 1
Half-lives of TiO_2-Lac and free laccases at different pH values (at room temperature), and different temperatures (at pH 7). Calculated half-lives by the biexponential Eq. (1).

Parameter	TiO_2-Lac	R^2	Free enzyme	R^2
pH Value				
2	31.85	0.95	5.17	0.99
3	107.10	0.97	31.60	0.99
4	261.70	0.86	81.75	0.98
5	> 360	0.92	75.29	0.99
6	> 360	–	290.45	0.98
7	> 360	–	> 480	–
8	> 360	–	> 480	–
Temp. (°C)				
40	> 72	–	> 1012	–
50	45.70	0.81	73.64	0.98
60	3.69	0.92	3.25	0.97
70	0.55	0.97	0.18	0.99
80	0.30	0.95	0.02	0.99

Table 2
Kinetic constants for TiO_2-Lac and free laccases, and their optimal pH and temperature values.

KM_{app}(μM)		Optimal pH		Optimal Temperature (°C)	
Free	TiO_2-Lac	Free	TiO_2-Lac	Free	TiO_2-Lac
23.55 ± 3.65	21.24 ± 1.92	2–3	2–4	40–60	25–60

3.3. Application on the biotransformation of pollutants

3.3.1. Biotransformation of ACE and DCF by free and immobilized laccases

ACE and DCF are pharmaceutical compounds that have shown toxic impact on aquatic ecosystems, specially related to endocrine disruption and reproductive disorders, and their bioaccumulation can also create toxic effects in humans [38]. These compounds as well as their by-products have been detected in both surface water and groundwater [39–41].

In this work, the biotransformation of ACE and DCF was first studied individually, i.e., each compound was treated separately, and second, by a simultaneous system in which a mixture of both compounds was analyzed. Treatments were applied on a synthetic matrix composed of ultrapure water (UPW) and 100 UL^{-1} of free or immobilized laccases at pH 4. As can be seen in Fig. 6a, for the individual treatment and free enzyme, ACE undergoes a rapid biotransformation above 90% within the first 2 h, whereas, for DCF removal above 50% is obtained after 8 h of treatment. Following with the individual treatment, the use of TiO_2-Lac for biotransformation of ACE and DCF (Fig. 6a) shows that the removal of ACE was close to 90% with an increase in the treatment time (4 h). In the case of DCF, removal above 50% was obtained in less time using TiO_2-Lac (4 h) than free laccase (8 h). As mentioned previously, in this work simultaneous treatment (ACE-DCF) was also carried out (Fig. 6b) since both ACE and DCF are commonly found together in aquatic systems, which shows a variety of effects on the performance of pollutant removal [42,43]. In the case of the biotransformation of ACE-DCF by using free laccase, the removal of ACE was not certainly affected by the addition of DCF into the reaction mixture, but for DCF, its biotransformation was reduced with a removal around 30% after 8 h of treatment. The use of TiO_2-Lac for the biotransformation of ACE-DCF seems to have a similar behavior in the case of ACE, although slightly affected, showing almost 90% removal after 4 h. Whereas, DCF showed an improvement in its biotransformation with a 68% after 8 h. It is important to take into account that pollutant adsorptions on TiO_2 is considered insignificant (data not shown) as mentioned in previous works [44,45].

Laccase reactivity based on substrate functional groups has been described by the presence of electron donating groups (EDG) such as hydroxyl (-OH), amines (-NH_2), alkoxy (-OR), alkyl (-R) and acyl (-COR) groups that are susceptible for electrophilic attack by oxygenase enzymes, conversely, electron withdrawing groups (EWG) reduce enzyme affinity since groups such as amide (-$CONR_2$), halogen (-X) and nitro (-NO_2) produce an electron deficiency [46,47]. Consequently, the high biotransformation percentage for ACE observed in the different treatments carried out in this work can be explained by the presence of the hydroxyl and amine group attached to the aromatic ring; the presence of these functional groups facilitate electrophilic attack by laccase (Fig. 7a). The low biotransformation percentage for DCF (20–68%), can be associated to the fact that although DCF molecule contains an amine group (Fig. 7b), two atoms of chloride are attached to the aromatic ring which are strong EWG, producing an electron deficiency and decreasing the affinity of the enzyme [46]. Moreover, the improvement shown on DCF biotransformation with immobilized laccases can be attributed to the fact that, at pH 4, TiO_2-Lac show higher stability than free laccases (half-lives of 261.70 h and 81.75 h respectively, see Section 3.2.3), which means that more enzymatic activity is preserved and thus leading to a higher biotransformation capability. In addition, the immobilization process favors the interaction between enzyme and DCF, allowing a better diffusion of the substrate [48,49].

3.3.2. Biotransformation of ACE and DCF by free and immobilized laccases in groundwater

Groundwater samples used in this work present a chemical composition mainly influenced by calcite and dolomite. Their concentration of major ions, Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, NO_3^- and HCO_3^-, is also related with water-rock interaction. The natural pH in this samples was between 6.77 and 7.88, indicating neutral to slightly alkaline water conditions in the area.

Treatments were carried out by using a matrix composed of groundwater (GW) and 100 UL^{-1} of free/TiO_2-Lac for both individual and simultaneous system at pH 4. Neither ACE nor DCF were detected by previous analyses of the groundwater samples used in this study.

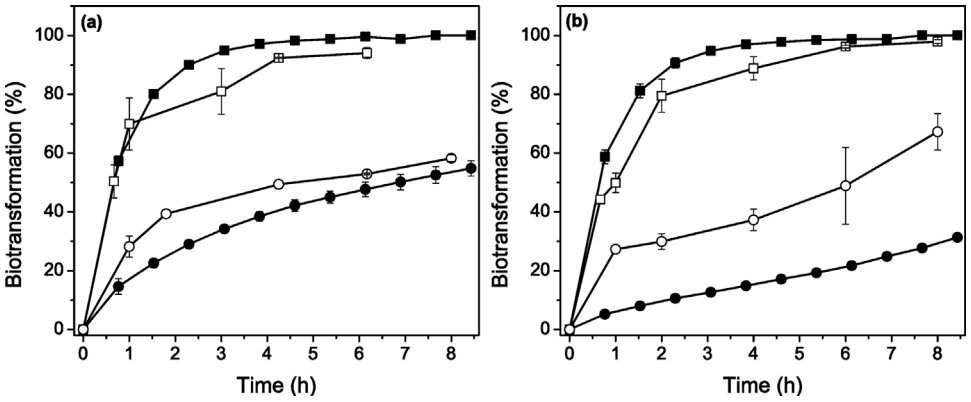

Fig. 6. Biotransformation behavior in UPW for ACE and DCF treated (a) individually and (b) simultaneously. ACE (■) and DCF (●) for free laccases, and ACE (□) and DCF (○) for TiO_2-Lac.

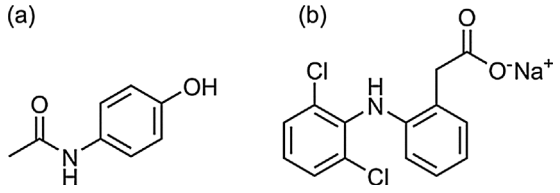

Fig. 7. Schematic structures of target pollutants: a) acetaminophen (ACE) and b) sodium diclofenac (DCF).

However, maximum concentrations of this compounds reported in the literature range between 0.025 and 2.05 mgL^{-1} [39]. Consequently groundwater samples were spiked to treatments at a concentration of 10 mgL^{-1} each. In the case of individual treatment, the biotransformation of ACE and DCF by using free laccases can be seen in Fig. 8a. For ACE, a rapid removal of 97% within the first 4 h was reached, which shows similar behavior to biotransformation observed when using synthetic matrix. In contrast, DCF removal was 34% for groundwater matrix after 8 h of treatment. The use of TiO_2-Lac is also shown in Fig. 8a. In this case, biotransformation was affected for both ACE and DCF with a removal of 84% (4 h) and 33% (8 h) respectively. It is known that the presence of ions in the medium can inhibit free enzymes by obstruction of the active site, for example, halide anions (F, Cl and Br) and hydroxide anion (OH), have been reported to bind to the T2 Cu of laccase and interrupt the internal electron transfer between T1 and T2/T3, or to bind near the T1 active site, thus blocking the access of the substrate to T1 Cu [50], other ions such as nitrite, thiosulfate, Cu (II), Fe (III), and cyanide have an effect as they reduce the catalytic power of enzymes [51]. Also, the presence of metal ions can affect the catalytic activity of laccases; calcium has been shown to cause dissociation of the copper ion in the enzymatic active site, while cobalt and zinc chlorides tend to interfere by hydrogen bonding [52].

The effect on biotransformation for the simultaneous system (ACE-DCF) by using free laccase was also evaluated (Fig. 8b). As all similar cases, the removal of ACE was not affected, obtaining 96% after 4 h of reaction; but in the case of DCF poor results were obtained, with only 20% of biotransformation after 8 h of treatment. Although the use of TiO_2-Lac for biotransformation of the simultaneous system ACE-DCF seems to have a slight lower performance for ACE, showing 89% removal after 4 h, nonetheless, the biotransformation of DCF was significantly improved with a 58% removal after 8 h.

In previous works, the biotransformation of ACE has been reported using different enzymatic treatments, e.g., the use of laccase from *Trametes versicolor* in synthetic samples leads to more than 90% biotransformation after 4 h [53]; Ba et al. [54], tested the biotransformation of ACE in municipal wastewaters (MWW) and hospital wastewater (HWW), resulting in 80–100% in 8 h of treatment [54]. The use of immobilized laccase has also been studied by using alginate, showing a maximum removal of 94% in 4 h [55]. Cross-linked enzyme aggregates (CLEAs) of laccases has also been reported for ACE biotransformation, resulting in more than 90% at 8 h when is used in synthetic samples, whereas in MWW and HWW leads to a complete biotransformation in 24 h [56]. Hachi et al. [57] used immobilized laccase from *Trametes hirsuta* to transform ACE in spiked individual and binary solutions, where the immobilized laccase was able to remove 70% in the binary system while only 25% in the individual system was obtained after 48 h [57].

Biotransformation of DCF by using free laccases has been widely reported at different conditions, giving as result low percent removal among 27% to almost complete but noticing the use of mediators such as 1-Hydroxybenzotriazole, syringaldehyde, violuric acid or with elevated amounts of enzyme at the expense of extremely long treatment times (1-7 days) [58–61]. Nonetheless, few works use immobilized laccases for DCF biotransformation, e.g., laccases from *Thielavia sp.* onto biotitania particles were able to catalyze the 54% removal of DFC after 4 h of incubation, this result was achieved using an activity loading of 2300 UL^{-1} [62]. The use of laccases from *Trametes versicolor* and *Myceliophthora thermophila* immobilized and co-immobilized onto fumed

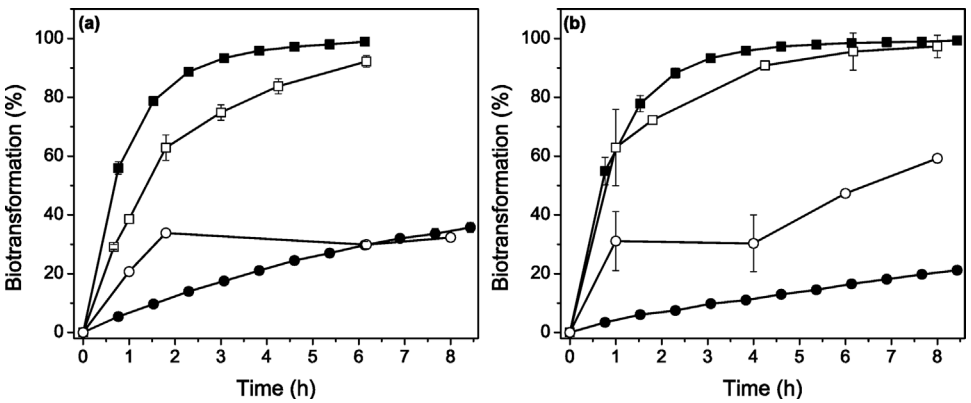

Fig. 8. Biotransformation behavior in GW for ACE and DCF treated (a) individually and (b) simultaneously. ACE (■) and DCF (●) for free laccases, and ACE (□) and DCF (○) for TiO_2-Lac.

silica nanoparticles leads to a non-observed biotransformation for any enzymatic system, showing that this compound is highly recalcitrant toward laccase oxidation under a complex matrix [23].

Based on previous studies, results reported in this work allow to argue that the catalytic power of laccase enzymes from *Pycnopus sanguineus* CS43 on the pharmaceutical biotransformation are comparable and, in some cases, even better. Moreover, treatments were carried out under mild reaction conditions, and avoiding the use of mediators as well as large amounts of enzyme or increasing temperature. Although it is known that enzymatic deactivation may be observed due to the large number of compounds present in complex matrices, in this work the catalytic power of the immobilized enzymes was not reduced in the treatment of groundwater samples. To the best of our knowledge (1) no previous work has addressed the removal of acetaminophen and diclofenac (*emerging pollutants*) simultaneously in groundwater samples via a biocatalyst immobilized onto titania nanoparticles; (2) the present study reports the immobilization of laccase enzymes produced by a native *P. sanguineus* CS43 strain, for which the immobilization process onto titania nanoparticles has not been previously explored. This work addresses for the first time the effect of the ionic strength in the immobilization process of laccase enzymes onto TiO_2, in order to obtain a high activity recovery. Results showed recoveries close to 100%, which are the highest reported so far.

4. Conclusions

In this work, laccases were successfully immobilized onto TiO_2 nanoparticles by using a covalent immobilization approach. The results revealed that the immobilization procedure leads to a complete activity recovery for different amounts of APTES (2–7 wt%). It was also observed that immobilization process improved the pH and thermal stability of laccases. Kinetic parameters for free and immobilized laccases show similar substrate affinities. Biotransformation of ACE and DCF by using free and immobilized laccases from *P. sanguineus* CS43 was carried out in both synthetic and groundwater samples. Consequently, this biocatalytic approach represents a good alternative in the treatment of organic pollutants in complex matrices.

Acknowledgments

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References

[1] A.C. Johnson, R.L. Donnachie, J.P. Sumpter, M.D. Jürgens, C. Moeckel, M.G. Pereira, An alternative approach to risk rank chemicals on the threat they pose to the aquatic environment, Sci. Total Environ. 599–600 (2017) 1372–1381, http://dx.doi.org/10.1016/j.scitotenv.2017.05.039.

[2] N. Pi, J.Z. Ng, B.C. Kelly, Bioaccumulation of pharmaceutically active compounds and endocrine disrupting chemicals in aquatic macrophytes: results of hydroponic experiments with Echinodorus horemanii and Eichhornia crassipes, Sci. Total Environ. 601-602 (2017) 812–820, http://dx.doi.org/10.1016/j.scitotenv.2017.05.137.

[3] E.C. 2011, Proposal for a Directive of the European Parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as Regards Priority Substances in the Field of Water Policy, 2015.

[4] USEPA, Contaminants of Emerging Concern, (2015).

[5] J.E. Leal, A.N. Thompson, W.A. Brzezinski, Pharmaceuticals in drinking water: local analysis of the problem and finding a solution through awareness, J. Am. Pharm. Assoc. 50 (2010) 600–603, http://dx.doi.org/10.1331/JAPhA.2010.09186.

[6] J. Wang, B. He, D. Yan, X. Hu, Implementing ecopharmacovigilance (EPV) from a pharmacy perspective: a focus on non-steroidal anti-inflammatory drugs, Sci. Total Environ. 603–604 (2017) 772–784, http://dx.doi.org/10.1016/j.scitotenv.2017.02.209.

[7] J. Rivera-Utrilla, M. Sánchez-Polo, M.Á. Ferro-García, G. Prados-Joya, R. Ocampo-Pérez, Pharmaceuticals as emerging contaminants and their removal from water. A review, Chemosphere 93 (2013) 1268–1287, http://dx.doi.org/10.1016/j.chemosphere.2013.07.059.

[8] W. Brack, V. Dulio, J. Slobodnik, The NORMAN Network and its activities on emerging environmental substances with a focus on effect-directed analysis of complex environmental contamination, Environ. Sci. Eur. 24 (2012) 29, http://dx.doi.org/10.1186/2190-4715-24-29.

[9] A. Butkovskyi, L.H. Leal, G. Zeeman, H.H.M. Rijnaarts, Micropollutants in source separated wastewater streams and recovered resources of source separated sanitation, Environ. Res. 156 (2017) 434–442, http://dx.doi.org/10.1016/j.envres.2017.03.044.

[10] WHO, Pharmaceuticals in Drinking-water, (2015).

[11] L. Xia, L. Zheng, J.L. Zhou, Effects of ibuprofen, diclofenac and paracetamol on hatch and motor behavior in developing zebrafish (Danio rerio), Chemosphere 182 (2017) 416–425, http://dx.doi.org/10.1016/j.chemosphere.2017.05.054.

[12] M. Kummerová, Z. Stepán, B. Petr, T. Jan, Possible ecological risk of two pharmaceuticals diclofenac and paracetamol demonstrated on a model plant Lemna minor, J. Hazard. Mater. 302 (2016) 351–361, http://dx.doi.org/10.1016/J.JHAZMAT.2015.09.057.

[13] M.M.I. Fekete-Kertesz, Z. Kunglne-Nagy, Ecological impact of micropollutants on aquatic life determined by an innovative sublethal endpoint daphnia magna heartbeat rate,» carpathian, J. Earth Environ. Sci. 11 (2016) 345–354.

[14] R. Nava-Alvarez, a C. Razo-Estrada, S. Garcia-Medina, L.M. Gomez-Olivan, M. Galar-Martinez, Oxidative stress induced by mixture of diclofenac and acetaminophen on common carp (Cyprinus carpio), Water Air Soil Pollut. 225 (2014) 1873–1881, http://dx.doi.org/10.1007/s11270-014-1873-5.

[15] M.V. Lukáš, Čapka, H. Zlámalová Gargošová Using UV–vis spectrophotometry for determining ecotoxicity of selected non-steroidal anti-inflammatory drugs, Fresenius Environ. Bull. 24 (2015) 4758–4762.

[16] B. Quinn, F. Gagné, C. Blaise, Evaluation of the acute, chronic and teratogenic effects of a mixture of eleven pharmaceuticals on the cnidarian, Hydra attenuata, Sci. Total Environ. 407 (2009) 1072–1079, http://dx.doi.org/10.1016/j.scitotenv.2008.10.022.

[17] C.A. Gasser, E.M. Ammann, P. Shahgaldian, P.F.-X.X. Corvini, Laccases to take on the challenge of emerging organic contaminants in wastewater, Appl. Microbiol. Biotechnol. 98 (2014) 9931–9952, http://dx.doi.org/10.1007/s00253-014-6177-6.

[18] J. Yang, W. Li, T.B. Ng, X. Deng, J. Lin, X. Ye, Laccases: production, expression regulation, and applications in pharmaceutical biodegradation, Front. Microbiol. 8 (2017) 832, http://dx.doi.org/10.3389/fmicb.2017.00832.

[19] C. Ji, J. Hou, V. Chen, Cross-linked carbon nanotubes-based biocatalytic membranes for micro-pollutants degradation: performance, stability, and regeneration, J. Membr. Sci. 520 (2016) 869–880, http://dx.doi.org/10.1016/j.memsci.2016.08.056.

[20] A. Xenopoulos, A new, integrated, continuous purification process template for monoclonal antibodies: process modeling and cost of goods studies, J. Biotechnol. 213 (2015) 42–53, http://dx.doi.org/10.1016/j.jbiotec.2015.04.020.

[21] J.-A. Majeau, S. K.Brar, R. DayalTyagi, Laccases for removal of recalcitrant and emerging pollutants, Bioresour. Technol. 101 (2010) 2331–2350, http://dx.doi.org/10.1016/J.BIORTECH.2009.10.087.

[22] S. Ba, A. Arsenault, T. Hassani, J.P. Jones, H. Cabana, Laccase immobilization and insolubilization: from fundamentals to applications for the elimination of emerging contaminants in wastewater treatment, Crit. Rev. Biotechnol. 33 (2013) 404–418, http://dx.doi.org/10.3109/07388551.2012.725390.

[23] A. Arca-Ramos, E.M. Ammann, C.A. Gasser, P. Nastold, G. Eibes, G. Feijoo, J.M. Lema, M.T. Moreira, P.F.X. Corvini, Assessing the use of nanoimmobilized laccases to remove micropollutants from wastewater, Environ. Sci. Pollut. Res. 23 (2016) 3217–3228, http://dx.doi.org/10.1007/s11356-015-5564-6.

[24] M. Asgher, M. Shahid, S. Kamal, H.M. Nasir Iqbal, Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology, J. Mol. Catal. B Enzym. 101 (2014) 56–66, http://dx.doi.org/10.1016/J.MOLCATB.2013.12.016.

[25] M. Bilal, M. Asgher, R. Parra-Saldivar, H. Hu, W. Wang, X. Zhang, H.M.N. Iqbal, Immobilized ligninolytic enzymes: an innovative and environmental responsive technology to tackle dye-based industrial pollutants − A review, Sci. Total Environ. 576 (2017) 646–659, http://dx.doi.org/10.1016/J.SCITOTENV.2016.10.137.

[26] L.I. Ramírez-Cavazos, C. Junghanns, N. Ornelas-Soto, D.L. Cárdenas-Chávez, C. Hernández-Luna, P. Demarche, E. Enaud, R. García-Morales, S.N. Agathos, R. Parra, Purification and characterization of two thermostable laccases from *Pycnoporus sanguineus* and potential role in degradation of endocrine disrupting chemicals, J. Mol. Catal. B Enzym. 108 (2014) 32–42, http://dx.doi.org/10.1016/j.molcatb.2014.06.006.

[27] J. Hou, G. Dong, Y. Ye, V. Chen, Laccase immobilization on titania nanoparticles and titania-functionalized membranes, J. Memb. Sci. 452 (2014) 229–240, http://dx.doi.org/10.1016/j.memsci.2013.10.019.

[28] Christian Aymard, A. Belarbi, Kinetics of thermal deactivation of enzymes: a simple three parameters phenomenological model can describe the decay of enzyme activity, irrespectively of the mechanism, Enzyme Microb. Technol. 27 (2000) 612–618, http://dx.doi.org/10.1016/S0141-0229(00)00258-1.

[29] M. Rodríguez-Delgado, C. Orona-Navar, R. García-Morales, C. Hernandez-Luna, R. Parra, J. Mahlknecht, N. Ornelas-Soto, Biotransformation kinetics of pharmaceutical and industrial micropollutants in groundwaters by a laccase cocktail from *Pycnoporus sanguineus* CS43 fungi, J. Mol. Catal. B Enzym. 101 (2017) 2331–2350, http://dx.doi.org/10.1007/s11270-015-2514-3.

[30] J. Hou, G. Dong, Y. Ye, V. Chen, Enzymatic degradation of bisphenol-A with immobilized laccase on TiO2 sol-gel coated PVDF membrane, J. Memb. Sci. 469 (2014) 19–30, http://dx.doi.org/10.1016/j.memsci.2014.06.027.

[31] J. Hou, G. Dong, B. Luu, R.G. Sengpiel, Y. Ye, M. Wessling, V. Chen, Hybrid membrane with TiO2 based bio-catalytic nanoparticle suspension system for the

degradation of bisphenol-A, Bioresour. Technol. 169 (2014) 475–483, http://dx.doi.org/10.1016/j.biortech.2014.07.031.
[32] C. N.Britos, J. A.Trelles, Development of strong enzymatic biocatalysts for dye decolorization, Biocatal. Agric. Biotechnol. 7 (2016) 228–233, http://dx.doi.org/10.1016/J.BCAB.2016.06.009.
[33] A. Tello, R. Cao, M.J. Marchant, H. Gomez, Conformational changes of enzymes and aptamers immobilized on electrodes, Bioconjug. Chem. 27 (2016) 2581–2591, http://dx.doi.org/10.1021/acs.bioconjchem.6b00553.
[34] Y.-Y. Song, H. Hildebrand, P. Schmuki, Optimized monolayer grafting of 3-aminopropyltriethoxysilane onto amorphous, anatase and rutile TiO2, Surf. Sci. 604 (2010) 346–353, http://dx.doi.org/10.1016/J.SUSC.2009.11.027.
[35] H. Cabana, C. Alexandre, S. N.Agathos, J. Peter Jones, Immobilization of laccase from the white rot fungus Coriolopsis polyzona and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals, Bioresour. Technol. 100 (2009) 3447–3458, http://dx.doi.org/10.1016/J.BIORTECH.2009.02.052.
[36] J. N.Talbert, J. M.Goddard, enzymes on material surfaces, Colloids Surf. B Biointerfaces 93 (2012) 8–19, http://dx.doi.org/10.1016/J.COLSURFB.2012.01.003.
[37] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, J.M. Guisán, R. Fernández-Lafuente, Preparation of a very stable immobilized biocatalyst of glucose oxidase from Aspergillus niger, J. Biotechnol. 121 (2006) 284–289, http://dx.doi.org/10.1016/J.JBIOTEC.2005.07.014.
[38] D. Montes-Grajales, M. Fennix-Agudelo, W. Miranda-Castro, Occurrence of personal care products as emerging chemicals of concern in water resources: a review, Sci. Total Environ. 595 (2017) 601–614, http://dx.doi.org/10.1016/J.SCITOTENV.2017.03.286.
[39] T. aus der Beek, F.A. Weber, A. Bergmann, S. Hickmann, I. Ebert, A. Hein, A. Küster, Pharmaceuticals in the environment-global occurrences and perspectives, Environ. Toxicol. Chem. 35 (2016) 823–835, http://dx.doi.org/10.1002/etc.3339.
[40] Q. Sui, X. Cao, S. Lu, W. Zhao, Z. Qiu, G. Yu, Occurrence, sources and fate of pharmaceuticals and personal care products in the groundwater: a review, Emerg. Contam. 1 (2015) 14–24, http://dx.doi.org/10.1016/j.emcon.2015.07.001.
[41] A.J. Ebele, M. Abou-Elwafa Abdallah, S. Harrad, Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment, Emerg. Contam. 3 (2017) 1–16, http://dx.doi.org/10.1016/j.emcon.2016.12.004.
[42] M.Petrović J.Radjenović, D.Barceló F.Ventura, Rejection of pharmaceuticals in nanofiltration and reverse osmosis membrane drinking water treatment, Water Res. 42 (2008) 3601–3610, http://dx.doi.org/10.1016/J.WATRES.2008.05.020.
[43] T. Thiebault, M. Boussafir, C. Le Milbeau, Occurrence and removal efficiency of pharmaceuticals in an urban wastewater treatment plant: mass balance, fate and consumption assessment, J. Environ. Chem. Eng. 5 (2017) 2894–2902, http://dx.doi.org/10.1016/J.JECE.2017.05.039.
[44] N. Miranda-García, M. Ignacio Maldonado, J.M. Coronado, S. Malato, Degradation study of 15 emerging contaminants at low concentration by immobilized TiO2 in a pilot plant, Catal. Today. 151 (2010) 107–113, http://dx.doi.org/10.1016/J.CATTOD.2010.02.044.
[45] J. Radjenović, Carla Sirtori, Mira Petrović, D. Barceló, S. Malato, Solar photocatalytic degradation of persistent pharmaceuticals at pilot-scale: kinetics and characterization of major intermediate products, Appl. Catal. B Environ. 89 (2009) 255–264, http://dx.doi.org/10.1016/J.APCATB.2009.02.013.
[46] S. Yanga, Faisal I. Hai, Long D. Nghiem, W.E. Price, F. Roddick, M. T.Moreira, S. F.Magram, Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review, Bioresour. Technol. 141 (2013) 97–108, http://dx.doi.org/10.1016/J.BIORTECH.2013.01.173.
[47] L.N. Nguyen, Faisal I. Hai, Shufan Yang, J. Kang, F.D.L. Leusch, F. Roddick, W.E. Price, L.D. Nghiem, Removal of pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals and pesticides by Trametes versicolor: role of biosorption and biodegradation, Int. Biodeterior. Biodegrad. 88 (2014) 169–175, http://dx.doi.org/10.1016/J.IBIOD.2013.12.017.
[48] R. Xu, R. Tang, Q. Zhou, F. Li, B. Zhang, Enhancement of catalytic activity of immobilized laccase for diclofenac biodegradation by carbon nanotubes, Chem. Eng. J. 262 (2015) 88–95, http://dx.doi.org/10.1016/j.cej.2014.09.072.
[49] L.N. Nguyen, F.I. Hai, A. Dosseto, C. Richardson, W.E. Price, L.D. Nghiem, Continuous adsorption and biotransformation of micropollutants by granular activated carbon-bound laccase in a packed-bed enzyme reactor, Bioresour. Technol. 210 (2016) 108–116, http://dx.doi.org/10.1016/j.biortech.2016.01.014.
[50] J. Margot, C. Bennati-Granier, J. Maillard, P. Blánquez, D.A. Barry, C. Holliger, Bacterial versus fungal laccase: potential for micropollutant degradation, AMB Express. 3 (2013) 63, http://dx.doi.org/10.1186/2191-0855-3-63.
[51] Y.-J. Kim, J.A. Nicell, Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A, Bioresour. Technol. 97 (2006) 1431–1442, http://dx.doi.org/10.1016/j.biortech.2005.06.017.
[52] H. Cabana, J.P. Jones, S.N. Agathos, Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: a review, Eng. Life Sci. 7 (2007) 429–456, http://dx.doi.org/10.1002/elsc.200700017.
[53] S. Ba, J.P. Jones, H. Cabana, Hybrid bioreactor (HBR) of hollow fiber microfilter membrane and cross-linked laccase aggregates eliminate aromatic pharmaceuticals in wastewaters, J. Hazard. Mater. 280 (2014) 662–670, http://dx.doi.org/10.1016/j.jhazmat.2014.08.062.
[54] S. Ba, L. Haroune, C. Cruz-Morató, C. Jacquet, I.E. Touahar, J.P. Bellenger, C.Y. Legault, J.P. Jones, H. Cabana, Synthesis and characterization of combined cross-linked laccase and tyrosinase aggregates transforming acetaminophen as a model phenolic compound in wastewaters, Sci. Total Environ. 487 (2014) 748–755, http://dx.doi.org/10.1016/j.scitotenv.2013.10.004.
[55] K. Ratanapongleka, S. Punbut, Removal of acetaminophen in water by laccase immobilized in barium alginate, Environ. Technol. 0 (2017) 1–10, http://dx.doi.org/10.1080/09593330.2017.1301563.
[56] I.E. Touahar, L. Haroune, S. Ba, J.P. Bellenger, H. Cabana, Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals, Sci. Total Environ. 481 (2014) 90–99, http://dx.doi.org/10.1016/j.scitotenv.2014.01.132.
[57] M. Hachi, A. Chergui, A.R. Yeddou, A. Selatnia, H. Cabana, Removal of acetaminophen and carbamazepine in single and binary systems with immobilized laccase from Trametes hirsuta, Biocatal. Biotransform. 35 (2017) 51–62, http://dx.doi.org/10.1080/10242422.2017.1280032.
[58] J. Margot, Julien Maillard, Luca Rossi, D.A. Barry, C. Holliger, Influence of treatment conditions on the oxidation of micropollutants by Trametes versicolor laccase, N. Biotechnol. 30 (2013) 803–813, http://dx.doi.org/10.1016/J.NBT.2013.06.004.
[59] S. Yang, F.I. Hai, L.D. Nghiem, F. Roddick, W.E. Price, Removal of trace organic contaminants by nitrifying activated sludge and whole-cell and crude enzyme extract of Trametes versicolor, Water Sci. Technol. 67 (2013) Accessed 14, September 2017 http://wst.iwaponline.com/content/67/6/1216.
[60] E. Marco-Urrea, Miriam Pérez-Trujillo, Carles Cruz-Morató, G. Caminal, T. Vicenta, Degradation of the drug sodium diclofenac by Trametes versicolor pellets and identification of some intermediates by NMR, J. Hazard. Mater. 176 (2010) 836–842, http://dx.doi.org/10.1016/J.JHAZMAT.2009.11.112.
[61] M. Badia-Fabregat, Mònica Rosell, Glòria Caminal, T. Vicent, E. Marco-Urrea, Use of stable isotope probing to assess the fate of emerging contaminants degraded by white-rot fungus, Chemosphere 103 (2014) 336–342, http://dx.doi.org/10.1016/J.CHEMOSPHERE.2013.12.029.
[62] I.I. Ardao, D. Magnin, S.N. Agathos, Bioinspired production of magnetic laccase-biotitania particles for the removal of endocrine disrupting chemicals, Biotechnol. Bioeng. 112 (2015) 1986–1996, http://dx.doi.org/10.1002/bit.25612.

Chapter 5

Conclusions

5.1 General conclusions

This work demonstrated the efficient biotransformation of several emerging pollutants (ACE, BPA, EE2, NP, TCS, DCF, DHQ, β-NP and 2,4 DCP) by free and immobilized onto TiO_2 laccases from *Pycnoporus sanguineus CS43*. Results showed that biotransformation efficiencies in synthetic (MQ water-buffer) and a complex matrix (groundwater) ranged from 50 to 100 %; these results were obtained under mild conditions, i.e., room temperature (25 C), pH = 5 and 100 U/L of enzymatic activity, in addition avoiding the use of mediators.

In terms of the biotransformation on groundwater samples, a low decrease in biotransformation percentages was observed, and was attributed to the interaction between the enzyme and ions present in the complex matrix.

Enzymatic immobilization onto a nanostructured support was successfully achieved. It was observed that immobilization of laccase enzymes onto TiO_2 nanoparticles by using a covalent immobilization approach leads to a complete activity recovery, and improved pH and thermal stability of laccases.

5.2 Contributions

- Laccase enzymes from *Pycnoporus sanguineus* CS43 strain were evaluated in the biotransformation of several emerging pollutants such as ACE, BPA, EE2, NP, TCS, DCF, DHQ, β-NP and 2,4 DCP.
- Laccase efficiency was assessed in the biotransformation of emerging pollutants on a complex matrix, specifically groundwater samples. To the best of our knowledge there was no research focused on the biotransformation of emerging pollutants in this matrix.
- Laccases immobilization was optimized in terms of the following parameters; ionic strength of buffer for immobilization, variations on APTES concentrations as coupling agent, and variations on GLU concentrations as binding agent. This was the first work to thoroughly study the effect of ionic strength on the immobilization process.
- Laccases from *Pycnoporus sanguineus* CS43 were successfully immobilized onto TiO_2 nanomaterials leading a complete activity recovery, and improvement of pH and thermal stability of laccases. After an intensive literature examination, it was found that the present study showed the highest activity recovery values using titania.

5.3 Future work

Since water treatment by enzymatic processes is a relatively recent issue, improvement and applicability of these treatments is still under development.
Future work for this study will be focused on
(i) testing laccase enzymes under real conditions using more complex water samples,
(ii) analyzing more immobilization methods as well to figure out which performs better,
(iii) characterization of resulting biomaterials, and
(iii) scaling-up the enzymatic process in bioreactors .
This work in turn can represent an opportunity to study and to design novel biotransformation technologies in water treatment.

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