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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

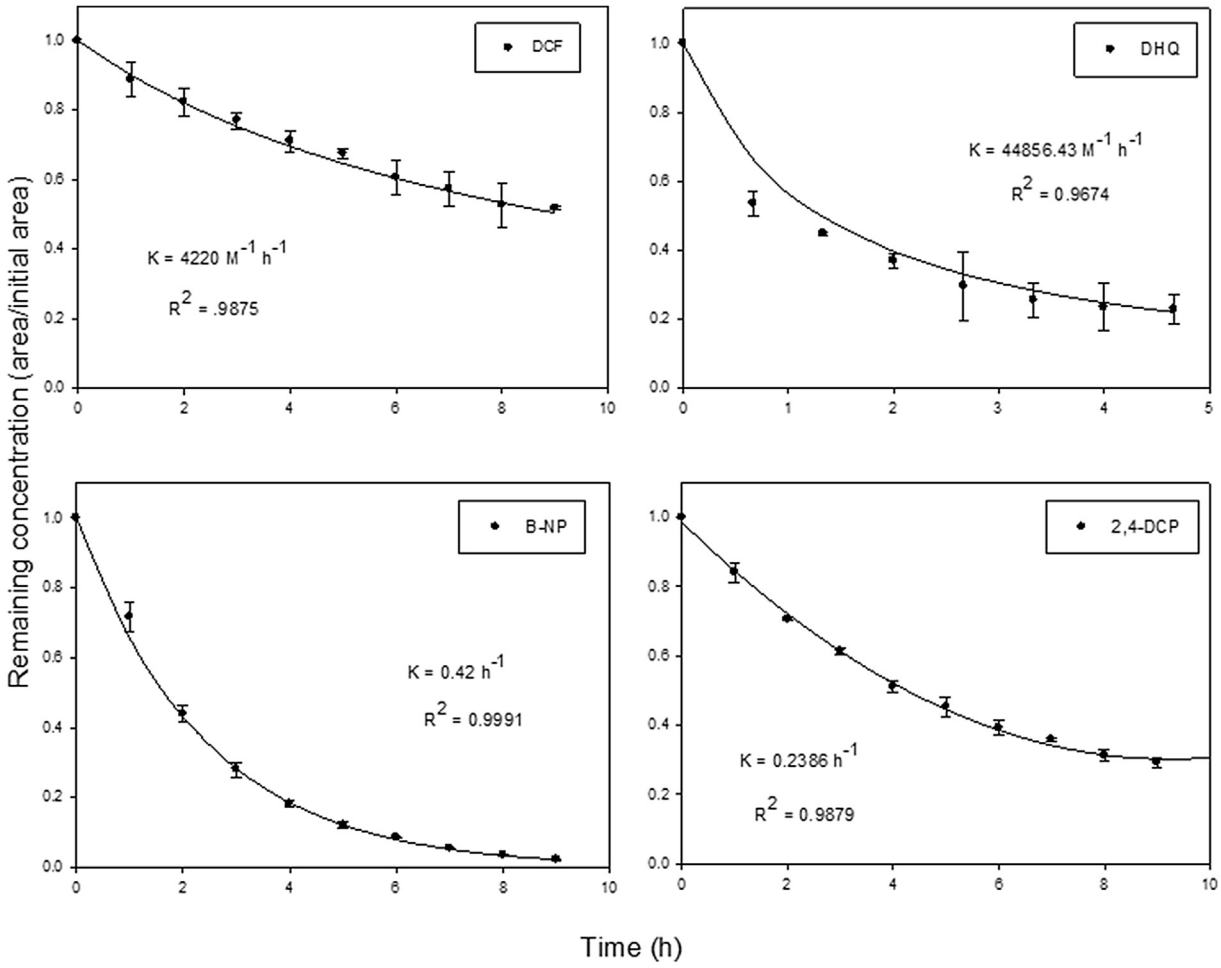

Fig. 1. Biotransformation profiles of 10 mg L^{-1} synthetic samples of DCF, DHQ, β-NP and 2,4-DCP by using 100 U L^{-1} laccase cocktail at 25 °C and pH 5. The curves were fitted to first (β-NP and 2,4-DCP) or second (DCF and DHQ) order of reaction.

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]; 1 mM	4		99	0.5	$k = 2.618$[a]		
					7		98	24	$k = 0.182$[a]		
				SA[c]; 1 mM	4		99	2	$k = 1.028$[a]		
					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]; 1 mM			96				
	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]; 1 mM			27				
	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]; 1 mM			98				
	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]; 0.5 mM			65	8			
				SA[c], 1 mM			100	1			
				VA/V[e], 1 mM			100	24			
				CA[f], 1 mM			92	24			
				FA[g], 1 mM			83	24			
	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).

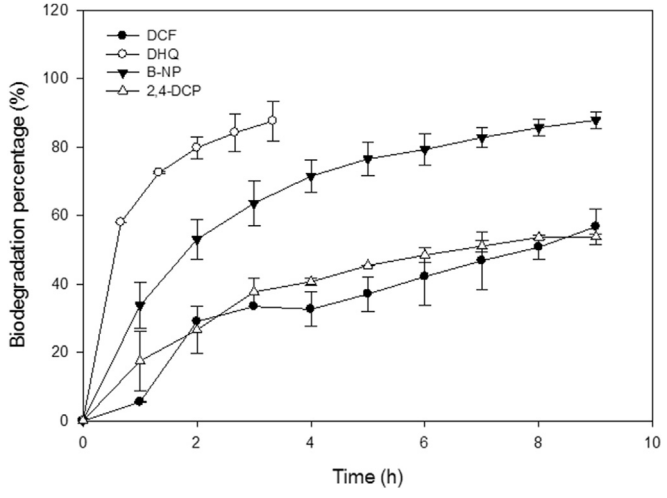

Fig. 2. Biotransformation profile of DCF, DHQ, β-NP and 2,4-DCP on a representative bulk sample from groundwater. A concentration of 100 UL^{-1} laccase cocktail (from *P. sanguineus* CS43) and 10 mg L^{-1} of each micropollutant at 25 °C and pH 5 were employed.

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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