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RESEARCH ARTICLE

Degradation of diclofenac, trimethoprim, carbamazepine, and sulfamethoxazole by laccase from *Trametes versicolor*: Transformation products and toxicity of treated effluent

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ABSTRACT

The degradation of diclofenac (DCF), trimethoprim (TMP), carbamazepine (CBZ), and sulfamethoxazole (SMX) by laccase from *Trametes versicolor* was investigated. Experiments were conducted using the pharmaceuticals individually, or as a mixture at different initial concentrations (1.25 and 5 mg/L each). The initial enzymatic activity of all the treated samples was around 430–460 $U_{(DMP)}$/L. The removal of the four selected pharmaceuticals tested individually was more effective than when tested in mixtures under the same conditions. For example, 5 mg DCF/L was completely removed to below its detection limit (1 μg/L) within 8 h in the individual experiment vs. after 24 h when dosed as a mixture with the other pharmaceuticals. A similar trend was visible with other three pharmaceuticals, with 95 vs. 39%, 82 vs. 34% and 56 vs. 49% removal after 48 h with 5 mg/L of TMP, CBZ, and SMX tested individually or as mixtures, respectively. In addition, at the lower initial concentration (1.25 mg/L each), the removal efficiency of TMP, CBZ, and SMX in mixtures was lower than that obtained at the higher initial concentrations (5 mg/L each) during both the individual and combined treatments. Four enzymatic transformation products (TPs) were identified during the individual treatments of DCF and CBZ by *T. versicolor*. For TMP and SMX, no major TPs were observed under the experimental conditions used. The toxicity of the solution before and after enzymatic treatment of each pharmaceutical was also assessed and all treated effluent samples were verified to be non-toxic.

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Laccase; *T. versicolor*; pharmaceutical degradation; transformation product; toxicity assessment; wastewater treatment

1. Introduction

Pharmaceutically active compounds (PhACs) such as diclofenac (DCF), trimethoprim (TMP), carbamazepine (CBZ), and sulfamethoxazole (SMX) have been regularly reported in wastewater effluent, ground water, surface water, and even drinking water (Kolpin et al. 2002; Morasch et al. 2010; Santos et al. 2010; Vulliet and Cren-Olivé 2011; Al Aukidy et al. 2012; Ratola et al. 2012; Padhye et al. 2014). Their occurrence has been attributed to their inefficient removal by wastewater treatment plants (WWTPs) (Kümmerer 2009; Miège et al. 2009; Luo et al. 2014). Although found in trace concentrations, the occurrence of PhACs in the environment has attracted much attention recently due to possible undesirable effects including development of bacterial resistance in the environment (Zhang et al. 2009), ecotoxicological impacts (Santos et al. 2010; Hai et al. 2018), and effects on endocrine systems of some aquatic organisms (Hoeger et al. 2005; Fent et al. 2006). Several advanced oxidation processes (e.g. ozonation, UV photolysis, and UV/H_2O_2) have been explored to degrade these PhACs. However, a number of toxic transformation products (TPs) were reported in case of some advanced oxidation processes (Alharbi et al. 2017). Therefore, the removal of PhACs from water without production of toxic TPs during the treatment processes is important to improving water quality (Alharbi et al. 2017; Alharbi and Price 2017).

The degradation of PhACs using enzymes secreted by white rot fungi (WRF) has recently attracted interest as a method to obtain high removal efficiency and non-toxic effluents (Yang et al. 2013). WRF comprises a group of fungi which secret extracellular enzymes

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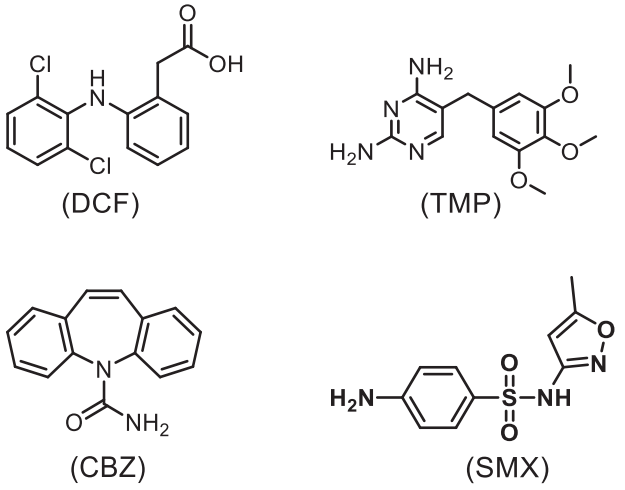

Figure 1. Chemical structures of the four PhACs selected. Diclofenac, trimethoprim, carbamazepine and sulfamethoxazole is denoted by DCF, TMP, CBZ and SMX, respectively.

capable of degrading lignin, a complex natural compound protecting softwood in trees. WRF secret lignin-modifying enzymes, namely peroxidases, for example, lignin peroxidases and manganese-dependent peroxidases and laccase (Reddy 1995). Unlike peroxidases, laccase can catalyse pollutant degradation using oxygen dissolved in the reaction mixture.

Laccase secreted by different fungi may have different redox potential (Piscitelli et al. 2011). The degradation of the four selected PhACs, namely, DCF, TMP, CBZ, and SMX by laccase from *Trametes versicolor* has been previously investigated (Marco-Urrea et al. 2009; Tran et al. 2010; Margot et al. 2015; Ji et al. 2016). In addition, the use of redox-mediators as the electron acceptor during enzymatic treatments has been found to improve biodegradation of some PhACs (Touahar et al. 2014; Margot et al. 2015). The effectiveness of DCF degradation by enzymatic treatments has been reported to vary. Laccase from *T. versicolor*, either crude or purified (i.e. commercially available), was found to achieve high DCF removal (>70%) (Tran et al. 2010; Margot et al. 2013; Nguyen et al. 2014a, 2014b); and the use of a mediator was not found to affect DCF removal (Tran et al. 2010). However, laccase from other species was found to be less effective at PhACs degradation compared to *T. versicolor* (Lloret et al. 2010; Nguyen et al. 2014a, 2014b, 2015). CBZ degradation by laccase from *T. versicolor* can vary significantly, depending on the initial concentration, enzymatic activity, and incubation time. For example, about 38% and only 5% of 0.01 and 4.72 mg CBZ/L were removed by laccase secreted by *T. versicolor* after 4 and 96 h of incubation, respectively (Tran et al. 2010; Ji et al. 2016).

In the limited studies on the degradation of the two antibiotics TMP and SMX by enzymatic treatment, TMP was reported to be poorly removed by laccase from *T. versicolor* (Touahar et al. 2014; Arca-Ramos et al. 2016). Contradictory reports on the ability of laccase from *T. versicolor* to degrade SMX can be noted in the literature. For example, 77% of 50 mg SMX/L was removed by laccase from *T. versicolor* after 1 h of incubation (Rahmani et al. 2015), whereas in a separate study, laccase from *T. versicolor* did not degrade SMX (at 20–25 mg/L) within 72 h of incubation (Margot et al. 2015). However, the addition of a redox-mediator was able to achieve a complete elimination within 6 h of incubation. Therefore, it is important that the degradation of DCF, TMP, CBZ, and SMX by laccase from *T. versicolor* and also the identification of any potentially toxic TPs be more thoroughly investigated.

The main aim of this work was to critically assess the ability of laccase from *T. versicolor* to degrade four selected PhACs, namely, DCF, TMP, CBZ, and SMX. This investigation included the degradation of each PhAC individually and in a mixture at different PhAC concentrations. A particular aim was to investigate the formation and fate of TPs during the enzymatic treatment, including confirmation of the molecular structure of the previously identified TPs using their *m/z* ratio and mass fragmentation patterns. In addition, the relative toxicity of the treated mixtures with the original compounds was compared using a typical bacterial toxicity test. The combined investigation of the TPs and the toxicity of the test solution following enzymatic degradation address a significant gap in the literature.

2. Materials and methods

2.1. PhACs and enzyme

Four PhACs including (DCF, TMP, CBZ, and SMX were chosen for this study (Figure 1). Analytical grade PhACs were purchased from Sigma-Aldrich (Castle Hill, Australia). Commercial laccase powder from *T. versicolor* (activity ≥0.5 U/mg) was also purchased from Sigma-Aldrich.

2.2. Enzymatic activity assay

The activity of laccase was measured using a previous method by Paszczynski et al. (1991). Briefly, the oxidation of a substrate of laccase, namely, 2,6-dimethoxy phenol (DMP), at 10 mM was monitored for 2 min at room temperature in 100 mM sodium citrate buffer (pH 4.5) solution. The change in absorbance at 468 nm due to degradation of DMP by laccase was monitored

by a UV-visible spectrophotometer (DR3900, Hach, Loveland, CO). Laccase activity (expressed as $U_{(DMP)}$/L) was calculated using the molar extinction coefficient of 49.6 mM^{-1} cm^{-1}.

2.3. Enzymatic experimental procedure

Stock solutions containing 5 g/L of DCF, TMP, CBZ, and SMX were separately prepared in pure methanol, and then stored in amber bottles at −18 °C prior to use. The degradation of 5 mg/L of DCF, TMP, CBZ, and SMX, by laccase from *T. versicolor* was first investigated for the PhACs individually. Following this, the degradation of each PhAC in a mixture with the others was investigated at different initial concentrations of the individual PhACs (5 and 1.25 mg/L). These PhACs have been detected in WWTPs at concentrations ranging from 0.001 to 0.1 mg/L (Luo et al. 2014). In this study, a relatively high concentration (i.e. 1.25 and 5 mg/L) of each PhAC was selected in order to investigate the formation of TPs. To generate TPs at detectable level, it is a common practice to work with higher initial concentrations of contaminants than environmentally relevant (Hata et al. 2010; Jelic et al. 2012; Ji et al. 2016).

To investigate degradation efficiency, commercially available laccase from *T. versicolor* (0.08 g) was added to ultrapure Milli-Q water (50 mL) in aluminum foil covered 250 mL beakers to achieve an initial enzymatic activity of *ca*. 430–460 $U_{(DMP)}$/L. When the targeted enzymatic activity was achieved, the selected PhAC concentrations were added to the enzyme solution in individual beakers. The beakers were incubated on a rotary shaker for 48 h at 80 rpm and 25 °C. The pH of the reaction media was between 6.8 and 6.9 at the start of each experiment. The experiments were carried out in duplicate. Sub-samples were collected after 1, 2, 4, 8, 12, 24, and 48 h of incubation by taking 0.5 mL and adding 0.5 mL of methanol to deactivate the laccase to arrest further degradation. The treated samples were then diluted to fit in the concentration range of the calibration curve prior to analysis by LC-MS to calculate the removal efficiency over time.

For the detection of TPs, 5 mg/L of DCF, TMP, CBZ, and SMX was added to the laccase solution (activity of 430–460 $U_{(DMP)}$/L) in individual beakers, and incubated as described above for 48 h. At the conclusion of incubation, the treated effluent was collected and solid phase extraction (SPE) was used to extract the compounds prior to analysis by LC-MS (Alharbi et al. 2016). Pre-concentration with SPE cartridges increased the sensitivity of the LC-MS analysis to identify the TPs. The Sep-Pak SPE cartridges purchased from Waters (Rydalmere, NSW, Australia) possessed the following properties: C18, 6 cc; 55–105 μm particle size; 500 mg sorbent per cartridge. Before sample loading at a flow rate of 1–5 mL/min, the cartridges were preconditioned with (i) 7 mL dichloromethane and methanol mixture (1:1 *v/v*), (ii) 7 mL of methanol, and (iii) 7 mL of Milli-Q water. The TPs extracted were then eluted from the cartridges using methanol (3 mL) at a flow rate of 1–5 mL/min. This corresponded to a concentration factor of approximately 17.

2.4. Analytical methods

The analysis of the parent PhACs and any TPs were performed by a liquid chromatography-electrospray ionisation-mass spectrometer (LC-ESI-MS) (Shimadzu single quadrupole LC-MS 2020). A series of standard solutions for each parent compound was prepared for calibration purposes at 1, 10, 50, 100, and 200 μg/L. The sample injection volume was 20 μL. The separation of the compounds was carried out by a Kinetex® Pentafluorophenyl 100 A column (100 mm × 3 mm, 2.6 μm) purchased from Phenomenex (Lane Cove, NSW, Australia). The column temperature was maintained at 31 °C. The mobile phase comprised of 0.1% formic acid in Milli-Q water (A) and acetonitrile (B). It was passed at a flow rate of 500 μL/min in the following gradient elution (%B): 0.01 min (5%), 5 min (10%), 20 min (45%), 23 min (90%), 28.1 min (90%), 29 min (10%), 33 min (10%), and 35.01 min (controller stop). Both positive and negative ion modes were used to collect mass spectrometric data from *m/z* 150 to 700. In addition, selected scan mode was used to look for the particular *m/z* of the previously reported TPs of DCF and CBZ after enzymatic treatment. All of the detector parameters were held constant during analysis. These include interface temperature (350 °C), desolvation line (DL) temperature (250 °C), heat block temperature (200 °C), dry gas flow (3 L/min), and nebulizing gas flow (1.5 L/min).

2.5. Toxicity assessment

At the end of the incubation period, the treated and untreated solutions were collected and compounds were extracted using SPE cartridges (Section 2.3) and eluted in 2 mL of methanol. This corresponded to a SPE concentration factor of 25. Samples were stored in 2 mL amber vials and kept in a −18 °C freezer prior to toxicity analysis.

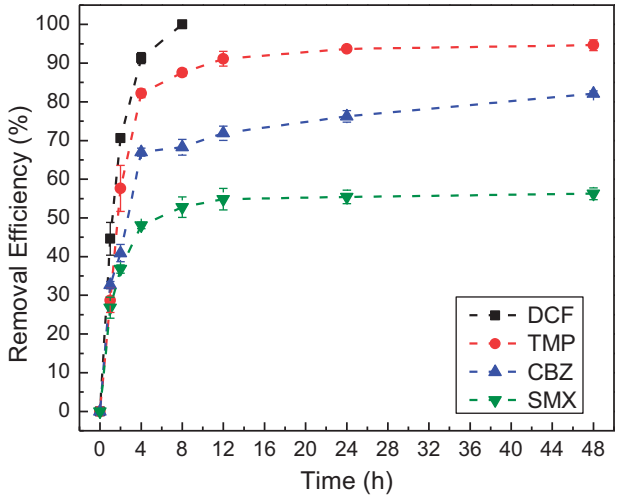

Figure 2. The individual degradation of 5 mg/L of each pharmaceutical (DCF, TMP, CBZ, and SMX) by laccase from *T. versicolor*.

The bacterial luminescence toxicity screen (BLT-Screen) developed by van de Merwe and Leusch (2015) was used to assess the toxicity of samples before and after the treatment. An aliquot of naturally bioluminescent bacteria, *Photobacterium leiognathi*, was incubated with a serial dilution of the sample extracts in a phosphate buffered saline solution. After 30 min, luminescence was measured on a Fluostar Omega plate reader (BMG Labtech, Ortenberg, Germany). The luminescence inhibition was calculated relative to a negative control. The concentration of the sample required to inhibit bacterial luminescence by 20% was denoted IC20. It was computed for each sample by linear regression of the response between 0 and 40% inhibition. Results are presented as a relative toxicity unit (rTU), which is the reciprocal of the IC20.

3. Results and discussion

3.1. Laccase-catalysed degradation of PhACs

Laccase-catalysed degradation of a compound comprises the transition of an electron from the compound to laccase in presence of a co-factor (i.e. dissolved oxygen) (Yang et al. 2013; Asif et al. 2017; 2018). However, the extent of degradation by laccase is significantly affected by the molecular properties of the target compounds such as the presence of aromatic or heterocyclic rings, electron donating groups (EDGs), coupled bonds, and/or readily oxidized substitutions (Tran et al. 2010). In addition, laccase can efficiently degrade phenolic PhACs, while the degradation of non-phenolic PhACs is only possible if the redox-potential of laccase is high enough or PhACs contain one or more EDGs (Lloret et al. 2010; Nguyen et al. 2014b, 2015). Enzyme-catalysed reactions generally follow the Michaelis-Menten kinetic model, thus, it is also possible that degradation of PhACs by laccase may have attained an equilibrium after certain extent of removal (Berg, 2002). All the PhACs selected for this study were non-phenolic and their removal by laccase from *T. versicolor* during individual and combined treatments are discussed in the following sections.

3.1.1. Degradation of individual PhACs

The individual degradation of 5 mg/L of each PhAC by laccase from *T. versicolor* was evaluated over an incubation period of 48 h. The individual degradation of the four selected PhACs by laccase appears to be influenced by their molecular properties. Experiments showed different reactivity of the four selected PhACs toward the applied enzymatic treatment (Figure 2). The initial degradation rates (10^{-4} mgL^{-1} s^{-1}) of the four PhACs were calculated during the first 4 h of the incubation time. The maximal degradation rates of the four PhACs (10^{-4} mgL^{-1} s^{-1}) were 5.0 ± 0.2 (DCF), 3.9 ± 0.3 (TMP), 2.8 ± 0.1 (CBZ), and 2.55 ± 0.07 (SMX).

Five mg DCF/L was degraded to below the experimental detection limit, that is, 1 μg/L within 8 h of incubation, in agreement with a previous study (Margot et al. 2013). The chemical structure of DCF includes an aromatic amine (an EDG) which may enhance its oxidation by laccase (Nguyen et al. 2014b). For CBZ and TMP, fast degradation rates were initially observed (0–4 h of incubation time) during individual treatments of 5 mg/L of each PhAC. Then, both PhACs were gradually degraded from 4 to 48 h of incubation time to achieve total elimination of 95 and 82% for TMP and CBZ, respectively. This finding confirms the capacity of laccase from *T. versicolor* to efficiently degrade CBZ without using a redox mediator (Tran et al. 2010). This result somewhat contradicts previous literature findings on the capacity of laccase from *T. versicolor* to degrade CBZ. While previous studies have indicated that the presence of the electron withdrawing functional group 'amide' could generate an electron deficiency and consequently may reduce the susceptibility of CBZ to enzymatic oxidation (Nguyen et al. 2014b), our results indicate that susceptibilities do exist, and provides new insight into CBZ degradation by laccase from *T. versicolor*.

For SMX, only 56% of the initial concentration was removed within 48 h. It could be concluded that DCF was highly susceptible to the oxidation by laccase from *T. versicolor*, whereas SMX was the most resistant

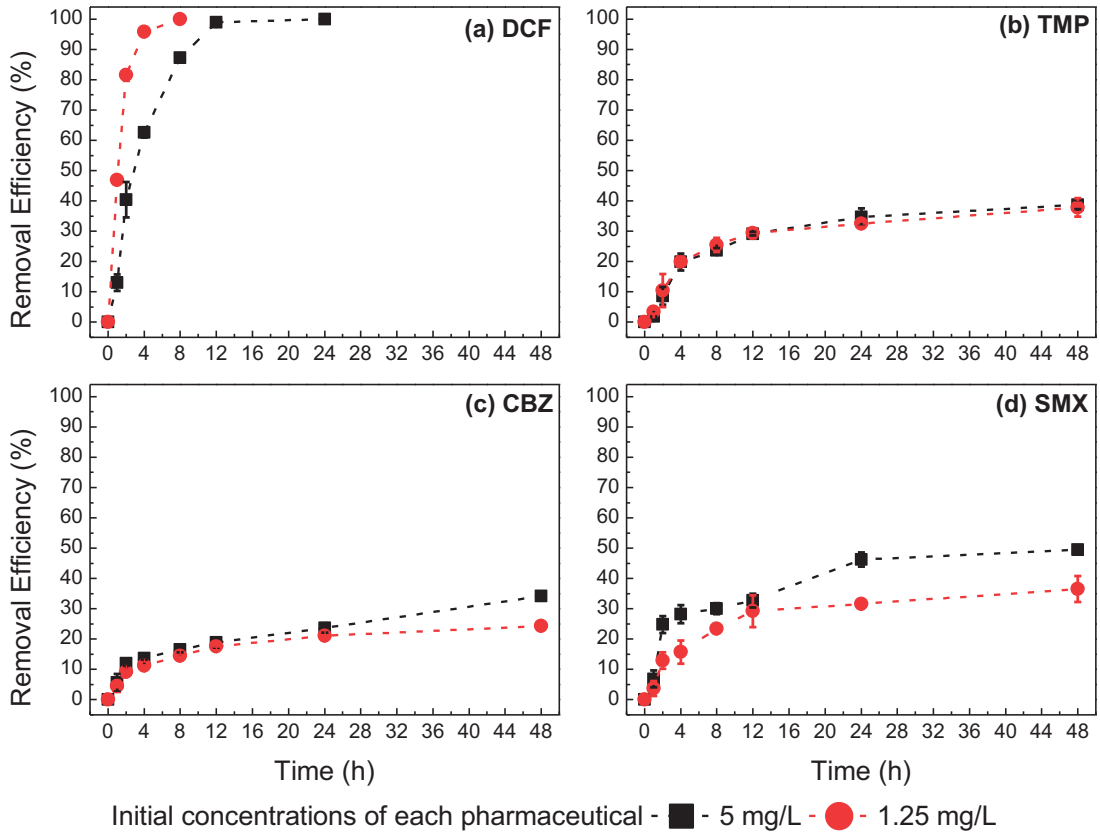

Figure 3. The effect of initial concentrations during degradation of (a) DCF, (b) TMP (c) CBZ, and (d) SMX in mixtures by laccase from *T. versicolor*.

PhAC during individual treatments under the applied conditions.

3.1.2. Degradation of PhACs in mixtures

The effect of different initial concentrations (i.e. 1.25 and 5 mg/L) of the four selected PhACs in mixtures during treatments by laccase from *T. versicolor* was also investigated (Figure 3). The degradation of 5 mg/L of each PhAC in mixture showed initial rates (10^{-4} mgL^{-1} s^{-1}) of 2.23 ± 0.05 (DCF), 0.7 ± 0.1 (TMP), 0.46 ± 0.05 (CBZ), and 1.7 ± 0.2 (SMX). In addition, at initial PhAC concentrations of 1.25 mg/L each, the initial degradation rates (10^{-4} mgL^{-1} s^{-1}) in mixture were 1.41 ± 0.03 (DCF), 0.17 ± 0.01 (TMP), 0.15 ± 0.02 (CBZ), and 0.22 ± 0.04 (SMX). Independent of the initial PhAC concentrations, the results confirmed that the removal efficiency during the mixture treatments was not as effective as the individual treatments (Figures 2 and 3). The degradation of the four selected PhACs during combined treatments are likely affected by the molecular properties of each PhAC, the total PhAC concentrations, and the competition between the four PhACs to be oxidized by laccase from *T. versicolor*.

DCF was highly degradable by laccase from *T. versicolor* during both the individual and combined treatments. This observation is consistent with that in a previous study which reported complete removal of DCF by laccase (Tran et al. 2010). For TMP, CBZ, and SMX, better removal efficiency was achieved during treatment of the mixture at a higher initial concentration (5 mg/L each). Our results are consistent with those in a previous study where the efficiency of removal of several non-phenolic PhACs by laccase-enzymatic membrane reactor increased at a higher initial concentration (Nguyen et al. 2016).

3.1.3. Effect of incubation time on degradation

In this study, degradation of PhACs by laccase was evaluated over an incubation period of 48 h, and samples were collected after regular intervals to investigate the significance of incubation time. It is clear from Figure 2 that individual PhAC degradation by laccase was fast initially, and the majority of degradation occurred within first 8 h. For DCF, a complete removal was achieved within the first 8 h of incubation time when DCF was tested individually as well as in a mixture of PhACs at an initial concentration of 1.25 mg/L (Figures 2 and 3(a)). On the other hand, the degradation of CBZ, SMX, and TMP in mixtures was observed to be rapid during the first 12 h of incubation time

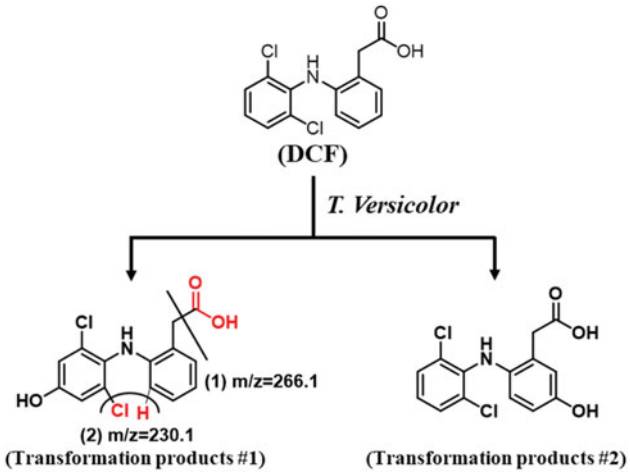

Figure 4. The two isomers of DCF TPs identified in this study. Fragmentation of TP #1 is shown in red.

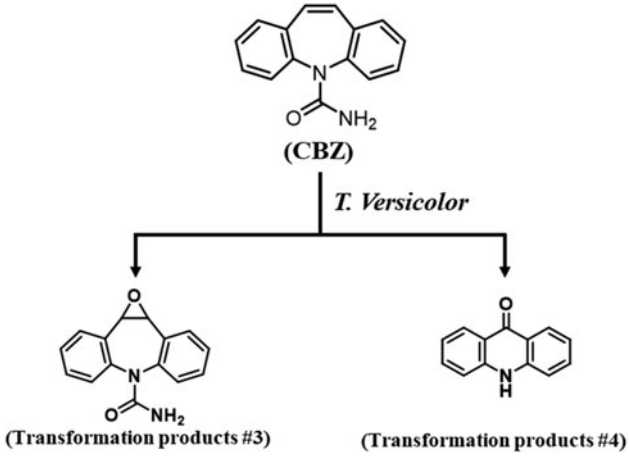

Figure 5. CBZ TPs identified in the current study.

(Figures 3(b–d)). Then, their degradation by laccase slowed down from 13 to 24 h of incubation, and almost stopped for the remaining duration. This cease of degradation is probably due to the inactivation of laccase and/or kinetic limitations (Khlifi et al. 2010; Ashe et al. 2016). Therefore, incubation time is an important design criterion for determining the size of the bioreactor during the development of a treatment process. It should be carefully selected as overestimation could result in increasing the capital cost of the treatment process.

3.2. Formation of TPs

The formation of TPs during the degradation of DCF, TMP, SMX, and CBZ by laccase from *T. versicolor* was investigated using LC-MS in both negative and positive scan mode (range, *m/z* 150 to 700). In addition, a selected ion mass mode was also used to detect the previously reported TPs during enzymatic treatments using their particular *m/z*. A total of four enzymatic TPs (two for DCF and two for CBZ) were detected at low abundance during the degradation by laccase. On the other hand, there were no TPs observed during degradation of TMP and SMX in the MS spectrum in either positive or negative scan mode.

For DCF, two isomer TPs with molecular ions of *m/z* 310 in a negative scan mode were detected (TP #1 and #2; Figure 4). These TPs were eluted through the LC-MS column at 18.5 and 17.8 min, respectively, before DCF elution at 21.5 min. Based on their polarities, TP #1 was formed by hydroxylation of the benzene ring that contains the two chlorine atoms, and had the highest abundance. The fragment ions obtained by MS of DCF TP #1 confirmed the previously identified structure in the literature: it has a molecular weight of 311 g/mol, with molecular formula $C_{14}H_{11}Cl_2NO_3$. Collision-induced dissociation of the [M–H]- molecular ion of TP #1 produced ions at *m/z* of 266.1 and 230.1. Mass losses from the [M–H+] ion were then calculated to be 44 (CO_2) and 80 (CO_2 and HCl) g/mol, respectively (Figure 4). These TPs were also identified during the oxidation of DCF by ozone, but at a higher abundance (Alharbi et al. 2016). They were formed mainly via hydroxylation at the para position of the amine group of the benzene rings, including 4′-OH diclofenac and 5-OH diclofenac (Marco-Urrea et al. 2010). Thus, their molecular structures differ by one extra oxygen atom with respect to DCF, without any changes in the benzene ring equivalence and double bond. One of them was produced at a relatively high abundance compared to the other. However, once the DCF was completely removed, both TPs were no longer detected, which indicates that they were also highly degradable by laccase.

For CBZ, two TPs (TP #3 and #4; Figure 5) were detected at low abundance during degradation of CBZ by laccase. These TPs eluted at 14.6 and 15.4 min on the reverse-phase column and had molecular ions at *m/z* 253 and 196 in positive scan mode, respectively. Therefore, their molecular weights were determined to be 252 and 195 g/mol, with molecular formula of ($C_{15}H_{12}N_2O_2$) and ($C_{13}H_9NO$), respectively. The formation of both TPs during degradation of CBZ by laccase has been documented previously (Hata et al. 2010; Jelic et al. 2012; Ji et al. 2016). TP #3 was identified as 11-epoxy-carbamazepine and consists of one extra oxygen atom with respect to molecular formula of CBZ. TP #4 was identified as (9(10H)-acridone). Two other TPs with molecular weights of 253 and 180 g/mol, were reported during degradation of CBZ in another study (Jelic et al. 2012). However, none of

Table 1. Comparison of degradation of 5 mg/L of each PhAC by different oxidation processes.

Treatment process	CBZ	DCF	SMX	TMP	Reference
Ozonation					
Dose (mg/L)	1.6	2.3	4.5	2.8	Alharbi et al. (2016)
Removal (%)	100	100	100	100	
#TPs	7	11	13	15	
Solution toxicity	Toxic	Toxic	Non-toxic	Non-toxic	
UV photolysis					
Exposure time (min)	60	8	8	90	Alharbi et al. (2017)
Removal (%)	25	100	100	71	
#TPs	13	15	12	5	
Solution toxicity	Toxic	Toxic	Non-toxic	Non-toxic	
UV/H_2O_2					
Exposure time (min)	60	20	NA	90	
H_2O_2 dose (g/L)	0.12	0.12	NA	0.12	
Removal (%)	99	100	NA	97	
#TPs	12	NA	NA	8	
Solution toxicity	Non-toxic	Non-toxic	NA	Non-toxic	
Purified laccase from *T. versicolor*					
Incubation time (h)	48	8	48	48	This study
Enzymatic activity (U/L)	430–460	430–460	430–460	430–460	
Removal (%)	82	100	56	95	
#TPs	2	2	0	0	
Solution toxicity	Non-toxic	Non-toxic	Non-toxic	Non-toxic	

NA: data not available; TPs: transformation products.

these products was detected in the current study using both negative and positive selected ion mode, which indicated that they were not formed under the applied experimental conditions. TP #3 has been previously identified during the degradation of CBZ by UV with H_2O_2, whereas TP #4 was detected during both direct UV photolysis and also UV with only a small amount of H_2O_2 (Alharbi et al. 2017).

In conclusion, the laccase-mediated degradation of DCF, TMP, CBZ, and SMX by laccase did not produce any major TPs under the experimental conditions used and LC-MS parameters employed. In addition, the detection of minor TPs was challenging due to the formation of many minor peaks in the LC chromatogram from the enzyme matrix. Most of these peaks were also observed after the treatment of each PhAC by laccase and had *m/z* ranging from 150 to 700 in either negative or positive MS scan mode. Consequently, the detection of new minor PhAC TPs could not be readily achieved. For DCF and CBZ, the detection of minor TPs was achieved by using their previously reported *m/z* in the literature. On the other hand, there was insufficient data in literature regarding the production of TPs during degradation of SMX and TMP by *T. versicolor*. For SMX, all the previous studies have treated SMX in the presence of other PhACs and/or mediators in order to improve SMX degradation (Shi et al. 2014; Margot et al. 2015). Therefore, the reported TPs were not fully transformed from SMX alone as there were other chemical compounds involved in the applied treatment. Additionally, in previous studies, the chemical structures of some identified SMX TPs consisted of the chemical structures of the mediators used. For TMP, there has not been any study to date on the formation of TPs by laccase treatment.

3.3. Solution toxicity assessment

Advanced oxidation processes, namely, UV photolysis, UV/H_2O_2, and ozonation may transform the PhACs to a number of potentially toxic and/or resistant TPs (Alharbi et al. 2016, 2017). To check if this was similarly the case with enzymatic degradation, the toxicity of the four PhACs (DCF, TMP, CBZ, and SMX), their laccase-treated solution mixtures, and the pure laccase solution were evaluated. All the tested samples were found to be non-toxic in the BLT-Screen (all $<$2.5 rTU) at the PhAC concentrations tested. To the extent of our knowledge, this is the first report on the toxicity of TMP to bacteria before and after laccase treatment.

The data from this study indicate that the transformation of the selected PhACs by laccase from *T. versicolor* did not lead to the formation of any significantly toxic TPs. A few previous studies have also reported that laccase-mediated degradation of DCF, CBZ, and SMX did not give rise to toxicity in the treated effluent (Marco-Urrea et al. 2010; Margot et al. 2015; Ji et al. 2016). However, by simultaneously monitoring TPs and effluent toxicity, in this study, we are able to infer further that the TPs detected were at very low abundance (Section 3.2) which may not have the ability to affect the toxicity level.

It is interesting to compare the enzymatic degradation of the four selected PhACs with that by advanced oxidation processes (i.e. ozonation, UV photolysis, and UV/H_2O_2 treatment) in terms of

degradation of parent PhAC, formation of TPs, and toxicity assessment of treated solutions (Table 1). These experiments were carried out under the same experimental conditions (i.e. initial PhAC concentrations, individual PhAC treatment, and solution media) and using the same toxicity assessment assay (Alharbi et al. 2016, 2017). Complete elimination of DCF, TMP, CBZ, and SMX was achieved after the reaction with 2.3, 2.8, 1.6, and 4.6 mg/L of ozone, respectively (Alharbi et al. 2016). In addition, UV photolysis was able to completely remove DCF and SMX within a short time of UV exposure (8 min) (Alharbi et al. 2017). On the other hand, TMP and CBZ were reported to be resistant to direct UV photolysis, with approximately 71 and 25% removal after 90 and 60 min of UV exposure, respectively. However, the combination of UV with 0.12 mg H_2O_2/L prior UV exposure significantly improved the removal of TMP and CBZ up to 97 and 99%, respectively. Therefore, laccase from *T. versicolor* was able to achieve higher removals for TMP (95%) and CBZ (82%) than that achieved by direct UV photolysis under the same initial PhAC concentrations.

A wide range of resistant TPs was reported during degradation of the four PhAC by ozone, UV photolysis, and UV/H_2O_2 treatment. In addition, UV photolysis caused an increase in toxicity when applied to DCF, CBZ, and SMX (Alharbi et al. 2017). Similarly, ozonation of CBZ and DCF resulted in an increase in the toxicity of the treated solutions. This was ascribed to the formation of several major TPs that are more toxic than the original PhACs. Therefore, non-toxic effluent and more efficient degradation of TMP and CBZ by laccase than by direct UV photolysis indicate the relative benefit of enzymatic degradation.

4. Conclusion

This study provides novel insights on the enzymatic degradation of four PhACs, namely DCF, TMP, CBZ, and SMX, which are commonly detected in wastewater and wastewater-impacted water bodies. The results of this study revealed that laccase can effectively degrade DCF (100%), TMP (95%), and CBZ (85%), while SMX degradation was moderate (56%). Notably, with the exception of DCF, degradation of the PhACs reduced significantly when the performance of laccase was assessed for the treatment of synthetic wastewater containing the mixture of PhACs. The rate of PhAC degradation was rapid when tested individually as compared to that in mixtures regardless of the initial PhAC concentrations. A total of four TPs were detected at low abundance during the degradation of DCF and CBZ by laccase from *T. versicolor*. For SMX and TMP, TPs were not detected under the applied treatment conditions and LC-MS parameters. All effluent samples treated by laccase from *T. versicolor* were non-toxic based on the bacterial luminescence toxicity assay. The advanced oxidation processes (AOPs) such as ozonation and UV-H_2O_2 may often be more effective than laccase-based treatment systems. However, the treated effluent produced by AOPs can be more toxic than the parent PhACs. Enzyme-based treatments, therefore, offer great potential as an alternative approach to the treatment of pollutants in wastewater effluent. Future studies to verify these important findings in continuous-flow enzymatic bioreactors operated under non-sterile conditions are recommended.

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Disclosure statement

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