

Biological Treatment of Pharmaceutical-Based Contaminants with Oxidoreductase Enzymes

Subjects: Engineering, Environmental

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The worldwide access to pharmaceuticals and their continuous release into the environment have raised a serious global concern. Pharmaceuticals remain active even at low concentrations, therefore their occurrence in waterbodies may lead to successive deterioration of water quality with adverse impacts on the ecosystem and human health. To address this challenge, there is an evolving trend toward the search for effective methods to ensure efficient purification of both drinking water and wastewater. Biocatalytic transformation of pharmaceuticals using oxidoreductase enzymes, such as peroxidase and laccase, is a promising environmentally friendly solution for water treatment, where fungal species have been used as preferred producers due to their ligninolytic enzymatic systems.

Keywords: oxidoreductases ; pharmaceuticals ; enzyme immobilization ; biodegradation ; wastewater

1. Occurrence and Toxicity of Pharmaceutical Active Compounds

Hundreds of tons of pharmaceutical compounds are annually dispensed and consumed worldwide [1]. Pharmaceutical active compounds (PhAC) are a group of hazardous contaminants and their fate in water bodies is an increasing environmental concern due to their high consumption, their persistence and activity, and their potential adverse effects on environmental health [2].

PhAC are chemical substances used to diagnose, prevent, treat and change diseases, infections, or discomforts [3]. A wide variety of human and animal medicines such as analgesics, non-steroidal anti-inflammatory drugs (NSAID), antibiotics, lipid regulators, beta-blockers, synthetic hormones and X-rays contrast media has become essential to ensure the health and well-being of the population and, therefore, to increase their life expectancy [4].

The consumption of PhAC is set to increase in future years, due to population aging and improvements in health standards. Accordingly, several pharmaceuticals have been found in wastewater effluents, drinking water and in rivers at low concentrations ranging from ng/L to µg/L [5][6]. As can be seen in **Figure 1**, PhAC can be generated by different sources (e.g., agricultural activities, domestic and hospitals), as well as are designed to be biologically active and may affect non-target organisms. Although pharmaceuticals are usually designed with a single mechanism of action and target, non-target organisms may have receptors and, therefore, unexpected effects may result from unintended exposure [7].

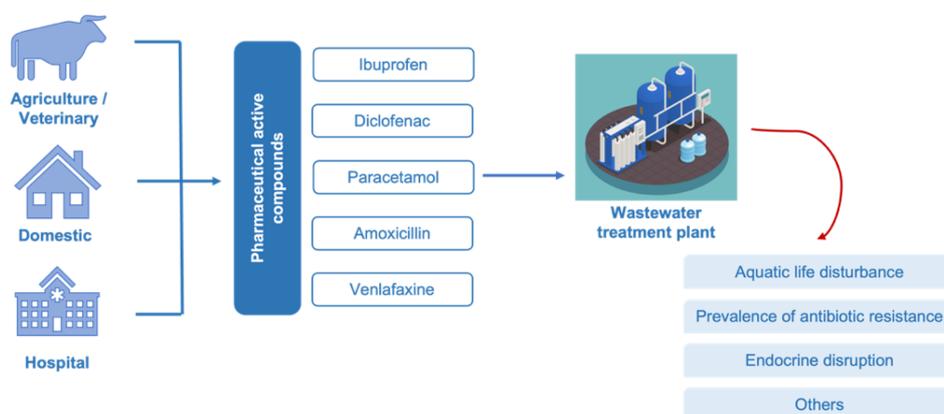


Figure 1. Path of pharmaceuticals active compounds from the use to the disposal in the environment with some of the ecological side effects.

The prevalence of antibiotics in aquatic environments has become an important concern worldwide with the increasing occurrences of antibiotic-resistant bacteria and resistance genes in wastewater and even drinking water, due to their

intensive use for veterinary, agricultural and human medical purposes [8][9]. Another major concern is their ability to interfere with the endocrine system to provoke undesired effects/disruption of homeostasis [5][10]. Continuous exposure to estrogens that are able to mimic and/or antagonize the effects of endogenous hormones produces adverse effects on the reproduction, development, and metabolism of aquatic organisms [11].

Most PhAC are currently not included in routine or regulatory control programs, which makes them candidates for future legislation. In this sense, the Commission Implementing Decision (EU) 2020/1161 has established a watch list of potential water pollutants for Union-wide monitoring in the field of water policy. Thus, azole pharmaceuticals, antibiotics and antidepressants such as clotrimazole, fluconazole, miconazole, amoxicillin, ciprofloxacin, sulfamethoxazole (SMX), trimethoprim and venlafaxine are some of the substances or groups of pharmaceuticals comprised [12].

The environmental concern related to the presence of PhAC in surface and groundwater is related to quantity, as well as their persistence and potential harm to human and aquatic life. The subsistence of trace pharmaceuticals and other contaminants in drinking water is a public health concern, since the potential chronic health effects associated with long term ingestion are scarcely known [13]. Although some compounds do not produce acute toxicity in the aquatic environment at low concentrations, constant release may lead to chronic and undesired synergistic effects with other compounds [14].

2. Enzymatic Biodegradation

Enzymes are catalysts that conduct, within the mild conditions of temperature and pH, chemical reactions at a remarkably high rate, efficiency and specificity [15]. Several enzyme systems have been used for the efficient transformation and degradation of organic pollutants and have been shown to oxidize and degrade the pollutants into smaller intermediates [16].

The literature survey shows that most enzymatic remediation studies use oxidoreductase enzymes [17][18]. These enzymes are largely produced by the white-rot fungi (WRF), as extracellular enzymes for the degradation of lignin. WRF and their ligninolytic enzymes, namely lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and Lac, have been demonstrated to be capable of transforming a wide range of compounds. This ability is a result of the structural similarities of several micropollutants to lignin, as well as the fact that ligninolytic enzymes are substrate-nonspecific [19]. Other enzymes are, as well, used as biocatalysts for the remediation of toxic compounds, such as tyrosinase, horseradish peroxidase (HRP) and phenoloxidase [17].

Peroxidases (EC 1.11.1.X) are a vast group of heme-containing oxidoreductases, which use hydrogen peroxide as an electron-acceptor to catalyze oxidative reactions [20], whereas Lac (EC 1.10.3.2) are glycosylated multi-copper oxidases that catalyze one-electron oxidation of various substrates associated with the reduction of molecular oxygen to water, via a radical-catalyzed reaction mechanism [21]. In contrast to peroxidases that require the supply of hydrogen peroxide, Lac only requests oxygen as the final electron acceptor for the oxidation reaction to occur, offering an alternative green approach for the biodegradation of several PhAC [22]. Lac is selective towards phenolic compounds, but due to the non-specificity, they are also able to degrade aromatic amines and related substances, thiol groups, diamines, N-heterocycles and phenothiazines [23].

The active sites of Lac enzyme, as represented in **Figure 2**, contain four copper ions: one type 1 (T1) copper ion and a trinuclear copper cluster (TNC) composed of one type 2 (T2) copper ion coupled to binuclear type 3 (T3) copper ions [24]. The T1 copper site is the primary electron-acceptor for electrons offered by the substrate. The electrons are then moved to the TNC through highly conserved His-Cys-His tripeptide. After that, oxygen reacts with the fully reduced enzyme to form a peroxy-intermediate (PI), and then the PI is transformed back into a native intermediate, through a two-electron reduction. Once the fully reduced state is recovered, the final products are released from the TNC site. This results in the production of four radicals by the oxidation of four molecules of substrate, while one molecule of oxygen is reduced to two molecules of water and four protons (H^+) are consumed from the solution [25][26].

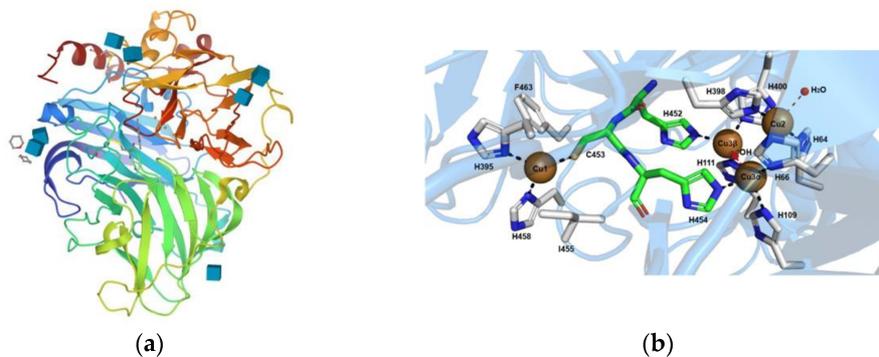


Figure 2. Lac from *Trametes versicolor* (Protein Data Bank code 1KYA) (a). Representation of the different amino acids of the catalytic site that coordinates the T1, T2 and T3 copper sites (b), reprinted from Ref. [27].

The Lac and their active sites with copper ions play a key role in the reduction of oxygen. The redox potential (E°) difference between the T1 copper site and the substrate is one of the major factors that affect the oxidation rate. Accordingly, Lac are classified into three groups: low- (0.4–0.5 V), medium- (0.5–0.6 V) and high- (0.7–0.8 V) redox potential [28][29]. The variety of redox potentials is strictly related to the sources (e.g., bacterial, plant or fungi), due to the difference in the amino acid residues composition around the copper of the first reaction site. Lac from WRF has the highest redox potential, between 0.730 and 0.790 V, with phenylalanine as the non-coordinating axial ligand at the T1 copper site [30]. Among fungal species, Lac have been comprehensively identified from *Ascomycetes* and *Basidiomycetes*. Nonetheless, white-rot basidiomycetes such as *Trametes versicolor*, *Pleurotus ostreatus* or *Cerrena unicolor*, are noted for being efficient lignin degraders and Lac producers [17][31].

Several studies using enzyme extracts have been conducted for biodegradation of PhAC (Table 1). Although high efficiency rates are presented, the enzymatic wastewater treatment process is still relatively expensive, mainly due to the cost of commercial enzymes. One potential solution that may get the process cheaper is the application of crude enzymatic extracts. This might positively influence Lac catalytic activity and stability, as well as avoid the costly process of enzyme purification [20][32]. Furthermore, the crude extract offers efficient oxidation, since the synergistic action of the enzymes and a host of natural mediators and co-factors secreted by the fungi can potentially enhance the general performance [33].

Table 1. Examples of biodegradation of pharmaceutical micropollutants by enzymes.

Compound	Enzyme	Source	PhAC (mg/L)	Reaction Conditions	Enzyme Load (U/L)	Efficiency (%)	Ref.
	LiP	<i>Phanerochaete chrysosporium</i>	5	pH 4, 24 mg/L H ₂ O ₂ , 25 °C, 2 h.	180	100	[34]
Diclofenac	Lac	<i>Trametes versicolor</i>	1	pH 6.5, 25 °C, 5 h.	500	>90	[35]
	Lac	<i>Pycnoporus sanguineus</i>	100	pH 5, 25 °C, 8 h.	100	50	[36]
5,7-Diiodo-8-hydroxyquinoline	Lac	<i>Pycnoporus sanguineus</i>	100	pH 5, 25 °C, 3.5 h.	100	78	[36]
Carbamazepine	Lac	<i>Trametes versicolor</i>	1	pH 6, 35 °C, 24 h.	60	30	[37]
Salicylic acid	Lac	<i>Trametes pubescens</i>	0.001	pH 6.9, 25 °C, 24 h.	100	>90	[38]
17- α -ethynyl estradiol	Lac	<i>Trametes pubescens</i>	0.001	pH 6.9, 25 °C, 24 h.	100	>90	[38]
Sulfamethoxazole	Lac	<i>Phanerochaete chrysosporium</i>	10	pH 4.5, 30 °C, 48 h.	6076	50	[39]
	Lac	<i>Pycnoporus sanguineus</i>	10	30 °C, 72 h.	170	29	[40]
17- β -estradiol	Lac	<i>Trametes hirsuta</i>	5	pH 5, 25 °C, 120 min.	5000	99	[31]
	Lac	<i>Trametes pubescens</i>	0.001	pH 6.9, 25 °C, 24 h.	100	>90	[38]

Compound	Enzyme	Source	PhAC (mg/L)	Reaction Conditions	Enzyme Load (U/L)	Efficiency (%)	Ref.
Tetracycline ¹ Oxytetracycline ²	MnP	<i>Phanerochaete chrysosporium</i>	50	pH 4.8, 0.1 mM Mn ²⁺ , 0.1 mM H ₂ O ₂ , 37 °C, 4 h.	40	73 ¹ 84 ²	[41]
Acetaminophen	Lac	<i>Bjerkandera adusta</i> TBB-03	20	pH 5–7, 25 °C, 2 h.	270	100	[42]
	HRP	<i>Horseradish</i>	6	pH 7.4, 400 μM H ₂ O ₂ , 25 °C, 4 h.	12800	100	[2]
Triclosan	Lac	<i>Trametes versicolor</i>	3	pH 6, 25 °C, 4 h.	2000	52	[43]
Doxorubicin	Lac	<i>Trametes Versicolor</i>	0.25	pH 7, 30 °C, 2 h.	900	100	[44]
Imipramine	Lac	<i>Paraconiothyrium variabile</i>	0.12	pH 5, 37 °C, 6 h.	1600	98	[45]

A huge amount of renewable biomass is generated annually due to agricultural, food and industrial activities. Agricultural and food wastes are often disposed of indiscriminately or burnt off, thereby constituting environmental hazards and contributing to global warming through the generation of greenhouse gases. To achieve environmental sustainability, value-addition to wastes and promotion of advances in the circular economy, agro-food wastes are now being investigated for valorization through bio-enzymatic approach [46]. For fungal cultures, a wide variety of lignocellulosic biomass has been used as an alternative substrate in submerged fermentation, solid-state fermentation or semisolid-state fermentation. Peanut shell, wheat straw, wheat bran, rice straw, rice bran, agave bagasse, sugarcane vinasse, corn bran, fruit peel, tea, sunflower seeds and apple pomace are some of the studied lignocellulosic wastes for Lac production for water remediation [47][48][49][50][51][52][53][54][55][56].

Lonappan et al. [35] present a novel insight on residue valorization and found that apple pomace and pulp and paper solid waste were capable of inducing Lac production by *T. versicolor*. The resulting crude enzyme was effective in the degradation of diclofenac sodium (DCF) at an environmentally relevant concentration (0.5 mg/L).

Kang et al. [42] stimulated the production of *Bjerkandera adusta* TBB-03 Lac using lignocellulosic substrates as the sole enzyme inducer (e.g., ash wood chips). The formed Lac consistently showed a high capability to degrade acetaminophen (APAP) under various conditions. The authors also defended a 22% of improvement in SMX removal in the presence of APAP, that could act as mediator for oxidation. Enzyme suspension with lower purification levels may contain phenolic compounds that act as natural mediators and consequently increase removal efficiency. For instance, Lac from a mushroom substrate, based on sawdust and wheat bran, colonized by *P. ostreatus*, demonstrates significant removal of DCF (90%), bicalutamide (43%) lamotrigine (73%) and metformin (49%), when compared with the control with an uncolonized mushroom substrate [57].

Most Lac used to degrade pharmaceuticals to date are mainly sourced from fungi. However, heterologous expression of Lac genes has attracted increasing attention due to the requirement to find more specific enzymes tailored to the complexity of wastewater matrices [58]. Protein engineering may provide higher enzyme yields and may allow the production of Lac with improved properties such as operational stability, pH stability, solvent stability and thermostability [59][60]. For example, a novel Lac derived from *Bacillus tequilensis* SN4 was demonstrated to be thermo-alkali-stable [60]. Lac Lcc9 from *Coprinopsis cinerea* expressed in *Pichia pastoris* also presented improved activity and stability at neutral and alkaline pH conditions [58]. Moreover, heterologous expressed bacterial Lac in *Escherichia coli* successfully degraded sulfanilamide antibiotics [61].

2.1. Laccase-Mediator Catalyzed System

Lac possess a relatively low redox potential (≤ 0.8 V) compared to peroxidase (>1 V). The redox potential difference between the substrate and the enzyme T1 copper affects the oxidative capacity of Lac. For example, Lac efficiently promotes single-electron oxidation of phenols. Accordingly, non-phenolic compounds, with redox potential above 1.3 V, are not directly prone to oxidation by Lac [62]. This lack of affinity of Lac is generally influenced by the distribution of functional groups in the chemical structure of the substrates. Compounds with electron withdrawing groups (EWG) such as carboxylic (-COOH), amide (-CONR₂), halogen (-X) and nitro (-NO₂) have lower enzyme affinity due to the less susceptible to oxidative catabolism [63].

The degradation efficiency of pollutants using Lac can be enhanced by the addition of redox mediators that are easily oxidized by the enzyme to free radicals. The presence of these small molecular weight compounds allows Lac to overcome a kinetic barrier and increase the spectrum of pollutants potentially degraded, as the mediator species have higher redox potentials. These compounds act as an “electron shuttle”, enabling the oxidation of complex substrates by highly reactive radicals that result from mediator oxidation by Lac. These radicals may return to their parent compound through reduction during the oxidation of the target pollutant [33][64].

Table 2 presents a list of redox mediators and their related information. The hydrogen atom transfer (HAT), electron transfer (ET) and ionic mechanisms are the primary mechanisms for mediator oxidation of a compound. The oxidation mechanism of the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2, 2, 6, 6-tetramethyl-1-piperidinyloxy (TEMPO) radicals follows ET and ionic mechanisms, respectively. As well as *p*-coumaric acid, hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI) and syringaldehyde (SA) follow HAT oxidation mechanism [65].

Table 2. Characteristics of redox mediators used in treatment of micropollutants by Lac-mediator systems.

Oxidation Mechanism	Redox Mediator	Origin	Type of Mediator	Free Radical Generated
Electron transfer	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt	Synthetic	ABTS	ABTS ^{•+} ABTS ²⁺
	Hydroxybenzotriazole	Synthetic	N-OH	=N-O [•] Aminoxyl
	N-hydroxyphthalimide	Synthetic	N-OH	=N-O [•] Aminoxyl
	Violic acid	Natural	N-OH	=N-O [•] Aminoxyl
Hydrogen atom transfer	Vanillin	Natural	C ₆ H ₄ (OH) (OCH ₃)	C ₆ H ₅ O [•] Phenoxy
	Syringaldehyde	Natural	C ₆ H ₄ (OH) (OCH ₃)	C ₆ H ₅ O [•] Phenoxy
	Acetosyringone	Natural	C ₆ H ₄ (OH) (OCH ₃)	C ₆ H ₅ O [•] Phenoxy
	<i>p</i> -coumaric acid	Natural	C ₆ H ₄ (OH) (OCH ₃)	C ₆ H ₅ O [•] Phenoxy
Ionic oxidation	2,2,6,6-tetramethylpiperidinyloxy	Synthetic	N-O	N=O [•] Oxo-ammonium

The mediator type and concentration have a major impact on the practicality and feasibility of using Lac in PhAC degradation. Some literature studies that used mediators coupled with enzymes for pharmaceutical transformation are presented in **Table 3**. Naproxen (NPX) is an example of a recalcitrant drug able to resist enzymatic oxidation due to the presence of the EWG carboxyl and the absence of any strong electron-donating groups (EDG). Lac mediated with HBT achieved the greatest removal of NPX (80%) at the highest mediator concentration of 1 mM, while the performance of violuric acid (VLA), another N-OH type mediator, was weaker (~60%). At an inferior dose (0.5 mM), HBT and ABTS mediators still presented very efficient removal rates of NPX [66].

Table 3. Examples of biodegradation of pharmaceutical micropollutants by Lac coupled with reaction mediators.

Compound	Lac Source	Mediator	Reaction Conditions	Enzyme Load (U/L)	Efficiency (%)	Ref.
Diclofenac	<i>Trametes versicolor</i>	1 mM of HBT ¹ and SA ²	0.1 mg/L PhAC. 25 °C, 24 h.	1440	>95 ¹ 80 ²	[33]
Carbamazepine	<i>Trametes versicolor</i>	0.018 mM of ABTS	1 mg/L PhAC. pH 6, 35 °C, 24 h.	60	95	[37]
Sulfamethoxazole	<i>Trametes versicolor</i>	0.5 mM ABTS, SA and AS	20-25 mg/L PhAC. pH 6-7, 25 °C, 2-6 h.	560	100	[64]
Tetracycline Oxytetracycline	<i>Pycnoporus</i> sp. <i>SYBC-L10</i>	1 mM of ABTS	50 mg/L PhAC. pH 6, 5 min, 0 °C.	10,000	100	[22]

Compound	Lac Source	Mediator	Reaction Conditions	Enzyme Load (U/L)	Efficiency (%)	Ref.
Chloramphenicol	<i>Trametes hirsuta</i>	0.5 mM of SA, vanillin, ABTS and α -naphthol	10 mg/L PhAC. pH 5, 25 °C, 48 h.	50,220	100	[32]
Ketoconazole	<i>Trametes versicolor</i>	1 mM of HBT	300 mg/L PhAC. pH 4.5, 45 °C, 6 h.	1000	98	[67]
Naproxen	<i>Pleurotus ostreatus</i>	1 mM of ABTS	0.5 mg/L PhAC. 25 °C, 8 h.	0.26	80	[66]
Olsalazine	<i>Aspergillus aculeatus</i>	2 mM of ABTS ¹ , <i>p</i> -Coumaric acid ² and HBT ³	100 mg/L PhAC. pH 5, 29 °C, 48 h.	-	99 ¹ 98 ² 94 ³	[68]
Atenolol	<i>Trametes versicolor</i>	0.5 mM of TEMPO	2.7 mg/L PhAC. pH 7, 25 °C, 4 h.	5000	80	[69]

Low molecular weight mediators allow complex compounds to access the active site of the enzyme. The oxidation of HBT by Lac generates small aminoxyl radicals that can remove the H atom from the O-H bond of phenolic substrates and consequently can create the phenoxyl radicals. The aforementioned mechanism may be responsible for the significant improvement in the degradation of salicylic acid [70].

In contrast to the low efficiency of the Lac degradation process without mediators, Lac coupled with ABTS revealed complete degradation of tetracycline (TC) and oxytetracycline (OTC) after only 4 min and 5 min, respectively [22]. Naghdi and co-authors [37] observed that 95% removal of carbamazepine (CBZ) was possible due to the combination of ABTS with crude Lac from *T. versicolor*. Furthermore, they reported that the enzyme concentration had a quadratic effect on biotransformation since an optimum level of Lac activity led to a rapid generation of ABTS radicals and caused an efficient transformation of CBZ, but further addition of enzyme to the solution increased the collisions and interactions and could block enzymes active sites. In the case of Lac-catalyzed degradation of the antibiotic chloramphenicol (CAP), Navada and co-authors [32] concluded that natural mediators (SA and vanillin) exhibited lower K_m values than the reactions mediated by the synthetic mediators (ABTS and α -naphthol), showing that the natural mediators have a higher affinity to Lac than CAP and, therefore, increased the rate of oxidation reactions.

Ideally, during the oxidation of the pollutant, only oxygen is consumed in the catalytic cycle. However, the consumption of the mediator during the reaction is also possible. In this case, "Lac enhancer" is a more accurate term. Margot and co-authors [64] investigated and showed the potential of the Lac-mediator system for the degradation of the antibiotic SMX with ABTS, SA and acetosyringone (AS). From their results, neither ABTS, SA nor AS acted as catalysts, since these three mediators were consumed during the reaction, with a mediator/pollutant molar ratio between 1.1 and 16. The authors proposed an alternative model of oxidation in which Lac oxidize mediators to reactive radicals, that can transform into more stable products and react with each other or with pollutants.

Bankole and co-authors [68] demonstrated the effectiveness of ABTS, HBT and *p*-coumaric acid as redox mediators in the degradation of olsalazine by Lac from *Aspergillus aculeatus*. Furthermore, they observed that the increase in degradation efficiency was proportional to an increase in the concentration of mediators till a threshold value is reached, and at this point, no significant enhanced degradation of the pharmaceuticals occurs. This result is in line with the general trend observed in the literature that such threshold concentrations depend on the source of Lac, the target compound and the mediator used [33].

On the other hand, Lac catalyzed reaction with TEMPO, which produces the oxoammonium cation, was able to greatly promote atenolol (ATL) transformation in an aqueous solution, with complete removal after 12 h [69]. However, Wang et al. [69] also found that Lac would be deactivated more rapidly in the reactions with higher mediator concentration as a result of distortion or blockage of enzyme active sites by radicals. Other findings show that free radicals produced by the oxidation of a mediator can destabilize the enzyme by reacting with the aromatic amino residues on its outer surfaces [66].

Catalytic degradation using ligninolytic enzymes such as Lac with redox mediators may represent an alternative clean strategy for PhAC removal from the water matrix. However, the viability of this solution in real treatment systems is limited due to the necessity for high concentrations of mediator and the formation of several remediation products in concentrations eventually higher than the original pollutant [64].

2.2. Transformation Mechanisms and Toxicity Evaluation

Enzymes transform complex compounds into simpler substances and it is unknown whether pharmaceuticals are metabolized by remedying enzymes to less or more toxic products. There is also a lack of knowledge on the structure of the metabolites resulting from pharmaceuticals degradation process. Therefore, some authors have studied the transformation pathways, as well as metabolites toxicity or estrogenic activity.

Considering molecular weight and chemical structure, Kózka et al. [74] observed three main types of transformations of a series of antidepressants and immunosuppressants carried out by fungal ligninolytic enzymes. The first one is chemical oxidation and occurred for clomipramine, mianserin, sertraline, fluoxetine and citalopram. The second transformation is straight demethylation or demethylation coupled with other reactions such as oxidation or deamination, and was observed for clomipramine, mianserin, sertraline and venlafaxine transformation. The third type of transformation is the oxidative cleavage of the molecule into two parts of comparable size and was observed for fluoxetine and paroxetine.

Kasonga et al. [72] proposed the metabolic pathways for ibuprofen (IBP) and CBZ based on detected intermediates by a fungal consortium of Lac, LiP and MnP. The IBP transformation pathway appeared to result from hydroxylation with addition of hydroxyl group to 1,2-dihydroxy-IBP or carboxylation reaction leading to the substitution of the methyl group by carboxylic group to form IBP carboxylic acid. Furthermore, the CBZ metabolic pathway was presented in four routes. The first route proposed was oxidation or hydrolysis to iminostilbene. The second route consisted of oxidation reactions of the carbons on the aromatic benzene group to CBZ-2,3-quinone. The third route combined hydroxylation, hydrolysis and then oxidation to iminoquinone. The fourth and principal metabolic route started with oxidation or epoxidation and the end products were acridone, 10,11-dihydro-10-hydroxy-CBZ and 9-hydroxymethyl-10-carbamoyl acridan. Likewise, according to Naghdi et al. [37], 10,11-dihydro-10,11-dihydroxy-CBZ and 10,11-dihydro-10,11-epoxy-CBZ are considered to be the primary metabolites from CBZ oxidation by Lac-ABTS. Moreover, toxicity tests revealed that these products had no estrogenic effect.

In another study related to the degradation of DCF by Lac, the authors identified 3'-hydroxydiclofenac, 4'-hydroxydiclofenac, and 5-hydroxydiclofenac as the major transformation products [35].

Yang et al. [73] demonstrated that immobilized *C. unicolor* Lac was effective in detoxification of TC antibiotics and identified three transformation products with LC-TOF-MS. According to the authors, TC is first oxidized by Lac to the corresponding ketone and then the amino group is bi-demethylated to form the second transformation product. The final product results from oxidation, followed by water elimination and dehydrogenation.

Lac oxidation of SMX in presence of ABTS possibly results in two products from pharmaceutical degradation and a third one from ABTS oxidation by Lac, designated 3-ethyl-6-sulfonate benzothiazolinone imine [64]. In another study, Tian et al. [22] demonstrated that TC was first oxidized by Lac-ABTS to OTC as the major transformation product and proposed a degradation pathway including deamination, demethylation and dehydration.

The assessment of the overall toxicity of the treated effluents is essential to provide a more complete outlook of the environmental relevance of the enzymatic treatments in real practical applications. Several metabolites result from enzymatic water treatment and additional toxicological information by means of bioassays is imperative. These studies are able to describe the ecotoxicity and the estrogenic activity of the resulting metabolites.

Presently, there are examples of pharmaceutical enzymatic degradation that produce fewer toxic compounds and lack of estrogenicity effect. A micro-toxicity study with *Pseudokirchneriella subcapitata*, *Candida albicans*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae* revealed that Lac-HBT treated ketoconazole and its isolated metabolites, such as 1-(4-{4-[2-(2,4-dichloro-phenyl)-2-imidazol-1-ylmethyl-[1,3]dioxolan-4-ylmethoxy]-phenyl}-4-oxy-piperazin-1-yl)-ethanone, suffered a decrease in the toxicity levels. The presence of the oxygen atom in the structure of metabolites reduces their lipophilicity and decreases their toxicity [67]. Furthermore, Lac-SA mediated system led to the transformation of CAP in chloramphenicol aldehyde and had less toxicity for microbial growth than mediators vanillin, ABTS and α -naphthol [32].

Spina et al. [74] showed that crude Lac from *Trametes pubescens* MUT 2400 was very active against all target micropollutants as ketoprofen, present in real municipal wastewater. Estrogenic analysis and toxicological tests with *Raphidocelis subcapitata* and *Lepidium sativum* showed a clear ecotoxicity reduction of treated wastewater. Similarly, Sun et al. [31] reported that a concentrated Lac isolated from *Trametes hirsuta* was capable of effectively metabolize 17 β -estradiol (E2) more than 99% and potentially lead to a reduction of the estrogenic activity of E2. Through the combination

of 13C-isotope labelling with high-resolution mass spectrometry, the dimers, trimers and tetramers were recognized as the primary by-products of E2 metabolism.

Contrarily, Becker et al. [75] verified that Lac mediated with SA effectively removes (>50%) a broad range of antibiotics after 24 h. However, this enhanced degradation induces unspecific toxicity. Furthermore, Feng et al. [69] verified that the transformation of ATL via Lac/TEMPO-catalyzed reaction greatly reduced the mortalities of zebrafish (*Danio rerio*) eggs, but the degradation products and the residual TEMPO still possess toxicity (approximately 40%). This transformation mainly involved hydroxylation, carbonylation, C–O bond cleavage and coupling reactions.

2.3. Immobilized Biocatalytic System

The practical application of freely suspended enzymes exhibits high activity in the biotransformation processes. Nevertheless, the free form is limited by the low stability and high cost of production for large scale implementation due to the impossibility of recovery [76][77]. To overcome such limitations, several studies have already demonstrated that enzyme activity and stability can be improved by immobilization on solid supports [17].

The stabilization of the peptide structure of the biocatalyst, creating interactions between the enzyme and an immobilization matrix, leads to enzyme stability and resistance improvement towards extreme operational conditions, including strong pH, high temperature or the presence of organic solvents [78]. Furthermore, immobilization allows the easy recovery of the enzyme and offers high reusability in several catalytic cycles without significant loss of its unique properties, which reduces operating costs [79].

The immobilization strategies are divided into methods based on physical or chemical interactions between enzymes and supports [80]. Physical immobilization involves the creation of non-specific interactions via hydrogen bonds, ionic and hydrophobic interactions. The physical methods include entrapment, encapsulation and adsorption, and there is no requirement for the functionalization of the support [17]. On the other hand, chemical immobilization includes enzyme attachment to the matrix by covalent binding or cross-linking [81]. An example of a covalent binding agent is glutaraldehyde that is capable to react with the amine groups at the surface of both enzymes and support through the formation of Schiff's bases and Michael's adducts [82]. Moreover, enzymes can be cross-linked to each other or create a cross-linked enzyme aggregate (CLEA) [83].

Physical adsorption is simpler and leads to higher final enzyme activity. However, desorption or leakage of the immobilized enzyme is common with cycles of use due to the relatively weak binding forces. Oppositely, chemical immobilization leads to partial deformations in the enzyme molecular shape but offers a robust attachment of enzymes to the support [78][84]. The characteristics of the support are essential to define the success of the final biocatalyst, therefore the ideal support for usage of industrial applications should be inert, rigid, inexpensive, eco-friendly and present thermal and mechanical resistances [85]. The support matrices can be classified according to their chemical composition as inorganic materials, organic materials, hybrids and composite materials. A large diversity of support has been developed due to the search for better stability and scale-up performance. Several recent methods of enzyme immobilization are summarized in **Table 4**.

Table 4. Examples of the removal of pharmaceutical compounds by immobilized enzyme.

Support Material	Enzyme	Immobilization Method	PhAC	Reaction Conditions	Efficiency (%)	Ref.
Poly(l-lactic acid)-co-poly(ε-caprolactone) nanofibers	Lac from <i>Trametes versicolor</i>	Encapsulation	Naproxen	1 mg/L PhAC. pH 5, 25 °C, 24 h, 100 rpm.	90	[77]

Support Material	Enzyme	Immobilization Method	PhAC	Reaction Conditions	Efficiency (%)	Ref.
Poly(l-lactic acid)-co-poly(ϵ -caprolactone) nanofibers	Lac from <i>Trametes versicolor</i>	Encapsulation		1 mg/L PhAC. pH 3, 25 °C, 24 h, 100 rpm.	90	[77]
Polyvinylidene fluoride membrane with multi-walled carbon nanotubes	Lac from <i>Trametes hirsuta</i>	Covalent bonding		5 mg/L PhAC. pH 5, 25 °C, 4 h.	95	[78]
Titania nanoparticles	Lac from <i>Pycnoporus sanguineus CS43</i>	Covalent bonding		10 mg/L PhAC. pH 4, 25 °C, 4 h.	50	[86]
Micro-biochar from pine wood (PW) and pig manure (PM)	Lac from <i>Trametes versicolor</i>	Covalent bonding	Diclofenac	0.5 mg/L PhAC. pH 6.5, 25 °C, 5 h (PW) or 2 h (PM).	99	[82]
Chitosan macro-beads	Lac from <i>Trametes versicolor</i>	Covalent bonding		50 mg/L PhAC. pH 3, 25 °C, 4 h, 1:1 M ratio for ABTS:drug.	90	[30]
CLEA	Lac from <i>Trametes versicolor</i>	Cross-linking		0.001 mg/L PhAC. pH 5, 24 h, 22 °C.	90	[83]
Polyacrylonitrile–biochar composite nanofibrous membrane	Lac from <i>Trametes versicolor</i>	Covalent bonding		0.2 mg/L PhAC, pH 4, 8 h, 35 °C.	73	[87]
Polyacrylonitrile–biochar composite nanofibrous membrane	Lac from <i>Trametes versicolor</i>	Covalent bonding	Chlortetracycline	0.2 mg/L PhAC, pH 4, 8 h, 35 °C.	63	[87]
Polyvinylidene fluoride membrane with multi-walled carbon nanotubes	Lac from <i>Trametes hirsuta</i>	Covalent bonding		5 mg/L PhAC. pH 5, 25 °C, 48 h.	27	[78]
Magnetite nanoparticles	HRP LiP	Adsorption		0.35 mg/L PhAC. pH 3, 55 °C, 3 days.	100	[88]
Polyimide aerogels	Lac from <i>Trametes versicolor</i>	Covalent bonding	Carbamazepine	0.02 mg/L PhAC. pH 3, 25 °C, 24 h, 200 rpm.	74	[89]
Pinewood nanobiochar	Lac from <i>Trametes versicolor</i>	Adsorption		0.02 mg/L PhAC. pH 3.5, 25 °C, 24 h, 200 rpm.	80	[76]
Polyamide/polyethylenimine nanofibers	Lac from <i>Trametes versicolor</i>	Covalent bonding	Triclosan	10 mg/L PhAC. pH 7, 25 °C, 20 h, 80 rpm.	74	[90]
Titania nanoparticles	Lac from <i>Pycnoporus sanguineus CS43</i>	Covalent bonding	Acetaminophen	10 mg/L PhAC. pH 4, 25 °C, 4 h.	90	[86]

Support Material	Enzyme	Immobilization Method	PhAC	Reaction Conditions	Efficiency (%)	Ref.
Commercial silica gel particles	Lac from <i>Trametes versicolor</i>	Covalent bonding	Sulfamethoxazole	20 mg/L PhAC. pH 7, 25 °C, 0.5 h. 520 µM of ABTS.	53	[91]
Commercial silica gel particles	Lac from <i>Trametes versicolor</i>	Covalent bonding	Amoxicillin	20 mg/L PhAC. pH 7, 25 °C, 4 h. 520 µM of ABTS.	80	[91]
M-CLEA	Lac from <i>Cerrena unicolor</i>	Cross-linking		100 mg/L PhAC. pH 6, 25 °C, 48 h.	100	[73]
Mesostructured cellular foam	Lac from <i>Trametes versicolor</i>	Adsorption	Tetracycline	1 mg/L PhAC. pH 5, 25 °C, 1 h.	100	[92]
Bentonite-derived mesoporous materials	Lac from <i>Trametes versicolor</i>	Adsorption		10 mg/L PhAC. 30 °C, 3 h.	60	[84]

A wide range of materials are used for enzyme immobilization. Zarta and co-authors [77] studied Lac immobilization by adsorption and encapsulation using poly(l-lactic acid)-co-poly(ε-caprolactone) (PLCL) electrospun nanofibers. After 24 h, encapsulated Lac biodegraded over 90% of NPX and DFC, contrasting with an adsorbed enzyme which presented lower removal efficiency, 60% for NPX and 80% for DFC. This is mainly justified by the deactivation and elution of enzymes from the support. Dong and co-authors [93] described a mediating system in which Lac was adsorbed over π-π interactions, onto pristine few-layer graphene (FLG) surface. The composite effectively transformed beta-blocker labetalol for more than 10 cycles, as the FLG increases the exposure extent of the catalytic center with the entrapment of the catalytic activity. Graphene oxide and alginate matrix

Immobilization of enzymes provides protection against denaturation and conformational changes. For example, Sharifi-Bonab and co-authors [95] immobilized Lac on graphene oxide (GO) nanosheets, followed by entrapment in alginate biopolymer. The immobilized Lac retained more than 70% of its initial activity after 10 days, as compared to the Lac that became inactive. Furthermore, according to Zarta and co-authors [92], Lac immobilized in mesostructured cellular foam (MCF) silica retained higher activity (80%) over a wider range of temperature and pH. The enzyme immobilized onto MCF + Cu preserved almost 90% of its initial activity after 10 cycles since the MCF has a protective effect towards Lac molecules immobilized onto the surface and into its pores. Similarly, Lac can enter into the narrow mesopores of meso-MIL-53(Al) by undergoing conformational changes and becoming immobilized in the mesopore-MIL-53(Al), thus the entrapment force is significantly higher compared to conventional physical adsorption [97]. In another example, Nguyen and co-authors [80] immobilized Lac on acid-washed granular activated carbon (GAC) via physical adsorption and observed that GAC-bound Lac maintained full activity for up to 8 cycles of continuous application.

Covalent binding of enzymes on solid materials for pollutant removal has been intensively investigated and yields efficient results. Masjoudi and co-authors [78] described a covalent immobilization of Lac on polyvinylidene fluoride (PVDF) membrane modified with multi-walled carbon nanotubes (MWCNT) and observed successful removal of DCF (95% in 4 h) in a mini-membrane reactor. Likewise, Maryšková and co-authors [90] immobilized the Lac onto polyamide/polyethylenimine (PA/PEI) nanofibers, via covalent attachment, with the Lac retaining more than 52% of initial activity after 30 days and successfully degrading triclosan (~70%) and 17α-ethynylestradiol (~50%) in real wastewater effluent. In another work, Lac immobilization on titania nanoparticles (TiO₂), with the functionalizing agent 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde cross-linker, showed remarkable stability at 50 °C and 60 °C and low pH values of 2 and 3 [86].

Compared with literature data, Apriceno and co-authors [30] reported that a quite low value of enzymatic activity (0.02 U of enzyme) was required for 90% of degradation of DCF (50 mg/mL) in 3 h, when the Lac was covalently immobilized on chitosan beads and coupled with ABTS. Yaohua and co-authors [93] also reported an efficient degradation of indole, even after 5 and 10 cycles, using a co-immobilization method, which consisted on the encapsulation of ABTS molecules into the dual-functionalized cellulose beads, followed by covalent binding of Lac.

Magnetic cross-linked enzyme aggregates (M-CLEA) are another example of the enzyme immobilization method, in which amino-functionalized magnetic nanoparticles are used. Yang and co-authors reported that Lac immobilized as M-CLEA eliminated over 80 µg/mL of TC in 12 h [73]. Similarly, CLEA and M-CLEA Lac showed high DCF removal capacity (~80%) at 1 and 5 µg/L pollutant concentrations [83].

The use of immobilized biocatalysts for PhAC transformation leads to sustainable industrial process performance. Despite the promising results, there are still issues to be further investigated such as the production costs of the immobilized enzymes, the possibility of scaling biocatalytic systems, as well as storage stability and the treatment potential of numerous PhAC in real wastewater effluents using enzyme immobilized systems.

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