Enzymatic degradation of recalcitrant pharmaceutical micropollutants

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Génie des procédés

École doctorale GAIA

Unité de recherche : Institut Européen des Membranes (UMR 5635)

DEGRADATION ENZYMATIQUE DE MICROPOLLUANTS RECALCITRANTS D'ORIGINE PHARMACEUTIQUE

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Le 10 Mai 2019

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Résumé

Ce travail concerne l'étude de la dégradation enzymatique de micropolluants pharmaceutiques récalcitrants présents dans l'eau. Tout d'abord, les efficacités de trois laccases différentes issues respectivement de : *Pycnoporus sanguineus* CS43, *Trametes versicolor* (*Tv*) et *Myceliophtora thermophila* ont été comparées lors d'essais de dépollution de solutions modèles renfermant trois antibiotiques (amoxicilline, ciprofloxacine et sulfaméthoxazole) et un antiépileptique (carbamazépine). Les essais ont été réalisés avec les laccases libres en présence ou non de médiateurs redox. L'impact de plusieurs paramètres opératoires sur les performances des enzymes a également été étudié. Puis, une nouvelle méthode d'immobilisation des laccases impliquant l'activation du support (microparticules à base de silice commerciales) par du glutaraldéhyde en phase vapeur a été mise au point et optimisée en utilisant la méthodologie de plans d'expériences. Après immobilisation, la laccase *Tv* s'est avérée être la plus active. Des essais de dégradation en présence de médiateurs redox ont confirmé l'efficacité de l'enzyme immobilisée et sa possible réutilisation lors de cycles successifs. La toxicité des solutions après traitement a été évaluée par des tests Microtox®. La laccase *Tv* a également été immobilisée sur des nanoparticules non commerciales à base de silice ou d'argile ainsi que sur des composites à base de silice et d'argile. La laccase *Tv* immobilisée sur les supports composites riches en silice a montré une plus grande réactivité et de meilleures performances pour l'élimination des composés cibles.

Mots-clés : Laccase, Enzymes immobilisées, Médiateurs redox, Micropolluants pharmaceutiques, Biodégradation.

Abstract

This work is focused on the study of the enzymatic depletion of recalcitrant pharmaceutical micropollutants in water. The potential degradation of three antibiotics (amoxicillin, ciprofloxacin and sulfamethoxazole) and one anti-epileptic (carbamazepine) was studied with three laccases: *Pycnoporus sanguineus* CS43, *Trametes versicolor* (*Tv*) and *Myceliophtora thermophila*. Free laccase systems were evaluated for pharmaceuticals depletion on model solutions in the presence or absence of redox mediators and the impact of several parameters on the performance of laccases for degradation were studied. The enzymes were then immobilized on different solid supports: commercial silica, laboratory synthetized nano-silica and clay based composite nanomaterials and used for degradation tests. A novel methodology for the covalent binding of laccases onto carriers was developed by using glutaraldehyde in vapour phase and the best immobilization conditions were determined through a 2^3 full factorial design. The immobilized *Tv* shown the highest activity and was tested in presence of redox mediators. Moreover, the reusability was evaluated in several degradation cycles and the toxicity of the solutions after treatment was assessed with the Microtox® test. In comparison to laccase immobilized on commercial silica, the *Tv* supported on laboratory synthetized materials showed higher activity and a better performance for the removal of target compounds.

Keywords: Laccase, Enzyme immobilization, Redox mediators, Pharmaceutical micropollutants, Biodegradation.

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List of abbreviations

ABTS: 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)

AMX: Amoxicillin

AOP: Advanced oxidation process

CAS: Conventional activate sludge

CBZ: Carbamazepine

CIP: Ciprofloxacin

CLEAs: Cross-linking enzyme aggregates

ECs: Emerging contaminants

EC_{50}: Proportion of sample that causes a 50% reduction in bacteria bioluminescence

EDG: Electron donating groups

ET: Electron transfer

EWG: Electron withdrawing groups

GLU: Glutaraldehyde

HAT: Hydrogen atom transfer

HPLC-MS: High performance liquid chromatography mass spectrometry

K_{ow}: Coefficient of partition octanol-water

LiP: Lignin peroxidase

MBR: Membrane bioreactor

MnP: Manganese peroxidase

MTL: *Myceliophthora thermophila* laccase

PCA: p-coumaric acid

PSL: *Pycnoporus sanguineus* laccase

SiNPs: Silica nanoparticles

SMX: Sulfamethoxazole

SYR: Syringaldehyde

TVL: *Trametes versicolor* laccase

WRF: White rot fungi

WWTP: Wastewater treatment plant

Synthèse des travaux en français

I. Contexte

Une eau de bonne qualité est essentielle à la santé humaine et à l'équilibre écologique et à la santé des écosystèmes. Toutefois, ces dernières années, le développement des techniques analytiques a permis la détection de polluants présents à de très faibles concentrations dans les eaux. Ces micropolluants, de par leur accumulation due à leur persistance, présentent une nouvelle menace pour les ressources en eau. Ces composés sont en effet difficilement biodégradables et peuvent avoir des effets potentiellement négatifs sur la santé humaine et l'environnement. Parmi les grandes catégories de contaminants émergents, les composés d'origine pharmaceutiques sont parmi les plus souvent retrouvés dans les eaux en raison leur consommation toujours plus importante en raison de la croissance et du vieillissement de la population. Des quantités importantes de molécules pharmaceutiques non utilisées, ainsi que leurs sous-produits métabolisés, sont continuellement rejetés dans la nature via ou non les effluents des stations d'épuration des eaux usées (STEP). Les effluents des hôpitaux et des industries pharmaceutiques qui présentent des concentrations de l'ordre µg L^{-1} sont évidemment aussi des contributeurs importants à cette pollution.

Ainsi, des résidus de différents types de composés pharmaceutiques (antibiotiques, analgésiques, anti-inflammatoires, antidépresseurs, etc.) ont été détectés dans les eaux usées, les eaux de surface et souterraines, adsorbés sur les sédiments et même dans l'eau potable dans le monde entier (Sui et al., 2015; Tran et al., 2018; Yang et al., 2017). Les concentrations retrouvées dans les plans d'eau varient de quelques ng à plusieurs µg L^{-1}. Dans l'eau des rivières, on trouve couramment des concentrations de l'ordre de la dizaine à la centaine de ng L^{-1}, ces valeurs étant généralement plus faibles que celles observées dans les effluents des stations d'épuration des eaux usées. En outre, les niveaux de contamination des eaux souterraines sont plus faibles que ceux observés dans les eaux de surface ; les concentrations rapportées sont inférieures à 10 ng L^{-1}.

A l'échelle mondiale, la présence de ces micropolluants pharmaceutiques dans l'environnement est préoccupante même si l'ampleur de leurs effets sur la santé des

organismes aquatiques et terrestres est encore largement inconnue. Bien que les niveaux de contamination trouvés soient relativement faibles, ces molécules sont biologiquement actives même à de très faibles concentrations et peuvent ainsi engendrer des effets chroniques suite à une exposition continue et prolongée. Ces effets chroniques comprennent entre autre la perturbation ou l'altération du système endocrinien, l'augmentation des cas de certains types de cancer, le développement d'une résistance aux antibiotiques. Par ailleurs, l'exposition aux produits pharmaceutiques sur les communautés microbiennes naturelles a aussi des effets néfastes qui ont été récemment rapportés (Barra Caracciolo et al., 2015; Gogoi et al., 2018).

La présence de ces molécules pharmaceutiques et de leurs métabolites dans les eaux indique que les traitements classiques des effluents réalisés dans les STEPs sont inefficaces sur ce type de pollution. En effet, ces composés pharmaceutiques de par leur structure complexe et généralement polycyclique sont difficilement biodégradables. Bien que certains d'entre eux puissent être transférés, principalement par adsorption sur les solides en suspension dans les effluents, la grande majorité reste néanmoins présente dans les effluents traités. Il est donc nécessaire de trouver des options de traitement efficaces permettant l'élimination efficace et complète de ces polluants.

Au cours de la dernière décennie, de nombreuses études ont porté sur l'élimination de produits pharmaceutiques dans des eaux et des effluents contaminés via des procédés physico-chimiques, microbiologiques ou d'oxydation avancée. Même si ces études ont donné des résultats prometteurs, ces différents procédés se sont révélés efficaces uniquement pour l'élimination de certains groupes de composés pharmaceutiques. De plus, les coûts d'exploitation élevés, la variabilité des rendements obtenus et la formation dans certains cas de sous-produits toxiques constituent également un obstacle à l'application de ces nouvelles technologies (Luo et al., 2014).

L'utilisation d'enzymes peut être une envisagée comme une alternative intéressante pour la résolution de ce problème. En général, les enzymes sont capables de catalyser des réactions spécifiques, le plus souvent dans des conditions opérationnelles modérées (pH, température, force ionique), ce qui permet de réduire la consommation d'énergie et de rendre le procédé plus écologique. Ces biocatalyseurs peuvent transformer efficacement et rapidement les polluants de l'environnement à des concentrations très diverses ;

contrairement aux microorganismes, leur action n'est pas gênée par la toxicité des molécules traitées. Différents types d'enzymes extraites de bactéries et de champignons ont été utilisées avec succès pour l'élimination de micropolluants toxiques (Demarche et al., 2012). En particulier, les enzymes issues de cultures de champignons de pourriture blanche ou fibreuse (*White rote fungi* WRF) et impliquées dans le processus de dégradation de la lignine (lignine peroxydase (LiP), peroxydase de manganèse (MnP), peroxydase versatile (VP) et laccase) sont également capables d'éliminer une large gamme de xénobiotiques (Cabana et al., 2007; Cruz-Morató et al., 2014).

Parmi la famille des oxydo-reductases, les laccases, grâce à la présence de quatre atomes de cuivre dans leur site actif, sont capables de catalyser l'oxydation de substrats en présence simplement d'oxygène dissous (Fig. 1) (Giardina et al., 2010). Ainsi, les laccases peuvent catalyser l'oxydation d'une large gamme de composés phénoliques et aromatiques, ceci dans une large gamme de températures et de pH. De plus, le fait que cette enzyme ne nécessite que de l'oxygène comme co-substrat représente un avantage supplémentaire pour l'application des laccases par rapport aux peroxydases qui utilisent le peroxyde d'hydrogène comme co-substrat. De nombreuses études ont mis en évidence le potentiel des laccases vis-à-vis de la bioremédiation d'effluents contaminés par différents types de polluants organiques (Jin et al., 2016; Salazar-López et al., 2017; Suda et al., 2012; Tran et al., 2010)

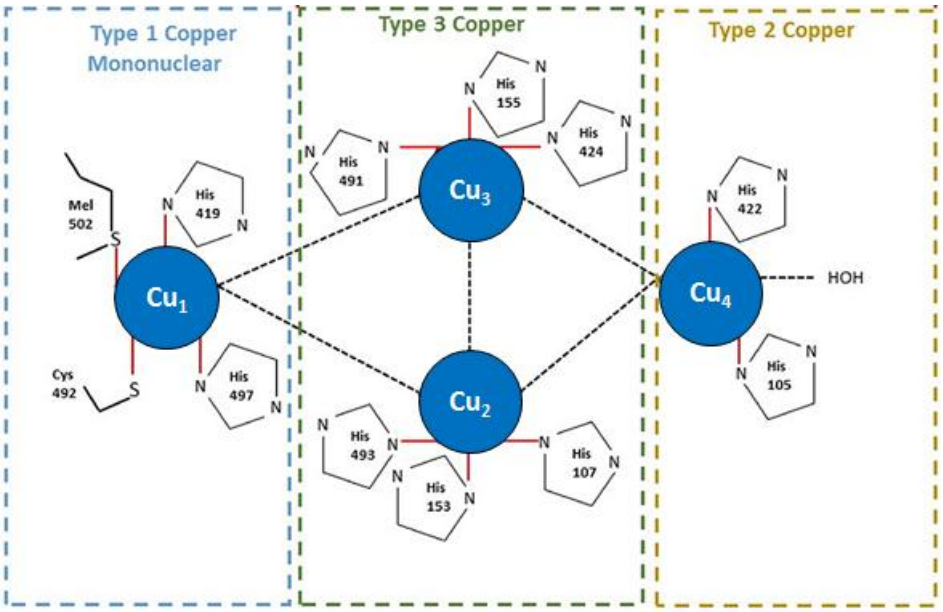

Fig. 1. Représentation schématique du centre active de cuivre dans la laccase (Adapté avec permission de Barrios-Estrada et al., 2018)

Cependant, l'efficacité du traitement dépend des propriétés physico-chimiques des micropolluants et de la souche fongique dont la laccase a été extraite. Par ailleurs, la plupart des polluants pharmaceutiques présents dans l'eau présentent des structures non phénoliques pour lesquels les laccases ne présentent qu'une affinité limitée. Néanmoins, l'utilisation de médiateurs redox qui agissent comme une "navette électronique" entre l'enzyme et le substrat permet d'étendre les capacités d'oxydation des laccases à une gamme plus large de substrats. Ainsi, il est possible d'oxyder des composés chimiques qui en principe ne sont pas des substrats naturels des laccases (Fig. 2) (Cañas and Camarero, 2010).

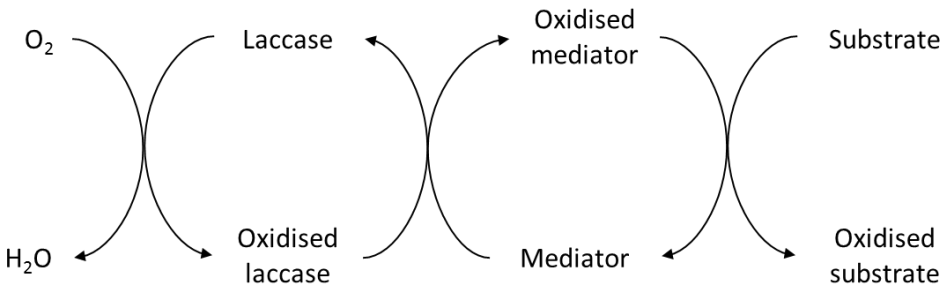

Fig. 2. Schéma du système médiateur laccase.

Généralement, les procédés de traitement enzymatique sont mis en œuvre dans des réacteurs discontinus en présence d'enzymes libres (sous forme de solutions brutes ou purifiées). Toutefois, ce mode de fonctionnement n'est pas adapté aux applications à plus grande échelle, comme dans une STEP, où le procédé doit fonctionner en mode continu. Afin que le biocatalyseur ne soit emporté avec les effluents traités et ne les contamine, il est indispensable de prévoir un moyen de le retenir au sein du réacteur. De plus, l'utilisation de laccases libres présente d'autres inconvénients : leur stabilité en solution est limitée, leur récupération et réutilisation est difficile voire impossible, ce qui entraîne des coûts d'exploitation élevés. Ces inconvénients peuvent être surmontés par l'emploi d'enzymes immobilisées. L'immobilisation des enzymes peut être réalisée sur une grande variété de supports solides à base de matériaux d'origine inorganique, organique et hybride ou composite. Le choix du bon support d'immobilisation est important car ses caractéristiques physico-chimiques influencent fortement les propriétés finales de l'enzyme immobilisée. Ainsi, plusieurs aspects tels que la présence de nombreux groupes fonctionnels et une bonne

résistance chimique et mécanique doivent être pris en compte (Zdarta et al., 2018). D'après la littérature, les supports inorganiques, tels que la silice et les argiles, représentent une excellente option pour l'immobilisation des enzymes en raison de leur grande biocompatibilité, leur grande surface spécifique, la présence de nombreux groupes fonctionnels différents, leur stabilité exceptionnelle, leur faible coût et leur réutilisation potentielle (Zucca and Sanjust, 2014).

Le choix de la méthode d'immobilisation est également extrêmement important ; il dépend des caractéristiques du support choisi, des propriétés de l'enzyme à immobiliser et du procédé auquel le biocatalyseur immobilisé sera appliqué. Parmi les différentes méthodes d'immobilisation disponibles, la liaison covalente semble être la technique la plus appropriée pour les applications dans le domaine de l'eau et des eaux usées puisque cette approche implique l'attachement multipoint d'enzymes au support, ce qui améliore considérablement la stabilité et la réutilisabilité des enzymes et réduit leur lixiviation (Gasser et al., 2014). Des laccases immobilisées par covalence sur différents supports solides ont été appliquées pour l'élimination de polluants pharmaceutiques dans l'eau avec des résultats encourageants (Barrios-Estrada et al., 2018b; De Cazes et al., 2014; Ji et al., 2016; Kumar and Cabana, 2016). Néanmoins, la recherche de nouveaux matériaux et de protocoles d'immobilisation pour le développement de nouveaux biocatalyseurs répondant aux besoins des applications industrielles et environnementales reste d'une importance capitale.

II. Objectifs de recherche

Les principaux objectifs de cette thèse sont d'une part l'étude des potentialités de différentes laccases (2 laccases commerciales et 1 mélange de laccases de *Pycnoporus sanguineus* CS43 préparé à la School of Engineering and Sciences of the Tecnológico de Monterrey au Mexique) vis-à-vis de l'élimination de différents micropolluants pharmaceutiques, à savoir trois antibiotiques (amoxicilline, ciprofloxacine et sulfaméthoxazole) et un anti-épileptique (carbamazépine) en présence ou non de médiateurs redox et d'autre part le développement d'une méthode immobilisation adaptée

à de nouveaux supports, à savoir des nanoparticules à base de silice et d'argile développés dans le cadre de l'ANR POLPHARMA.

Les principales étapes de l'étude seront :

- L'étude de la réactivité des différentes enzymes (sous forme libre) vis-à-vis de différents substrats en réacteur batch
- La mise au point de la méthode d'immobilisation sur des particules de silice commerciales
- Le greffage de l'enzyme la plus réactive sur les nouveaux matériaux et l'étude des potentialités du support actif vis-à-vis de l'élimination des micropolluants.

III. Présentation du manuscrit

Les résultats expérimentaux des travaux réalisés sont rassemblés dans le présent manuscrit qui se compose de six chapitres.

- Le chapitre 1 présente brièvement le contexte et les objectifs de recherche de l'étude.

- Le chapitre 2 présente l'état de l'art sur la problématique des micropolluants présents dans les effluents et l'environnement, leur toxicité et la difficulté de leur élimination. Différentes technologies de traitement conventionnelles et émergentes sont décrites avant de focaliser sur les potentialités du traitement par voie enzymatique. L'objectif est de donner un aperçu des connaissances actuelles relatives à l'élimination de micropolluants par les laccases (libre et immobilisées).

- Le chapitre 3 est la transcription d'un article de recherche publié dans la revue « Process Biochemistry ». Cette partie regroupe les résultats des essais de dégradation par les différentes laccases sous forme libre en présence ou non de médiateurs redox, l'objectif de ces expériences étant de voir si et comment les enzymes dégradent les différents substrats afin de mieux comprendre les

mécanismes impliqués dans la biotransformation des composés pharmaceutiques. Ainsi, les performances des trois enzymes sélectionnées ont été comparées, les effets des conditions opératoires (pH et température) et des médiateurs redox (type) sur l'efficacité d'élimination des micropolluants ciblés ont été étudiés.

- Le chapitre 4 présente la méthode d'immobilisation développée pour permettre un greffage optimum des laccases sur des particules de taille nanométrique. Cette méthode implique l'activation du support (particules de silice commerciale) par du glutaraldéhyde en phase vapeur. L'identification des paramètres influençant le rendement d'immobilisation et l'efficacité du biocatalyseur après immobilisation a été réalisée grâce à un plan d'expériences. Les performances catalytiques de l'enzyme immobilisée ont été comparées à celle de l'enzyme libre avec le substrat modèle (ABTS) et avec le mélange de micropolluants ciblés en présence de médiateurs redox. La réutilisation de l'enzyme immobilisée a été évaluée au cours de plusieurs cycles consécutifs de dégradation.

- Le chapitre 5 présente d'une part l'immobilisation de la laccase commerciale issue de *Trametes versicolor* sur les différents nanomatériaux (nanoparticules de silice, microparticules d'argile et nanocomposites de silice et d'argile) développés dans le cadre du projet ANR POLPHARMA et d'autre part leur utilisation de ces supports activés pour l'élimination des composés pharmaceutiques. L'étude compare dans un premier temps, les potentialités de ces différents supports vis-à-vis de l'immobilisation d'enzyme en termes de rendement d'immobilisation des protéines et d'activité spécifique du solide activé. Puis ces supports actifs ont été utilisés pour le traitement de solutions modèles de micropolluants en présence d'un médiateur redox et les résultats obtenus sont discutés en reliant l'efficacité d'épuration et les propriétés du support d'immobilisation.

- Enfin, le mémoire se termine par le chapitre 6 qui résume les conclusions générales de l'étude et présente les perspectives pour les travaux futurs.

IV. Résultats et Conclusions

Pour répondre à l'objectif de notre étude à savoir : l'évaluation des potentialités des laccases pour la dépollution des eaux renfermant des micropolluants pharmaceutiques, deux approches différentes ont été suivies : le traitement enzymatique au moyen de systèmes de laccases libres et l'utilisation d'enzymes immobilisées sur différents supports solides. Pour les systèmes à laccase libre, le potentiel oxydant d'un extrait brut d'une souche native du champignon de pourriture blanche *Pycnoporus sanguineus* (PSL) a été comparé à ceux d'enzymes commerciales : la laccase de *Myceliophthora thermophila* (MTL) et la laccase de *Trametes versicolor* (TVL) commercialisées respectivement par les sociétés Novozymes et Sigma.

Comme cela a été décrit au chapitre 3, le traitement enzymatique en présence d'enzymes libres seules s'est révélé inefficace pour la biotransformation des composés pharmaceutiques. Seule l'amoxicilline (AMX), un antibiotique, a été partiellement éliminée. Mais les résultats obtenus montrent que l'importance de l'élimination dépend de la laccase utilisée et des conditions de réaction étudiées. En particulier, des conditions légèrement acides (pH 5-6) permettent d'améliorer la dégradation. Les contre-performances observées ont été attribuées aux propriétés physico-chimiques des produits pharmaceutiques testés, les rendant ainsi résistants à l'attaque des laccases. En effet les structures chimiques de ces micropolluants sont assez différentes de celles des substrats habituels des laccases. Afin d'essayer d'élargir le spectre d'action des laccases étudiées, des médiateurs redox ont été ajoutés au milieu réactionnel. Les résultats obtenus ont mis en évidence l'effet positif de l'addition d'un des 3 médiateurs testés à savoir : les médiateurs naturels syringaldéhyde (SYR) et acide p-coumarique (PCA) et du médiateur synthétique 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Cependant, il a été observé que l'amélioration des performances dépend du système laccase-médiateur utilisé. Des pourcentages de dégradation intéressants ont été observés en présence d'ABTS et de SYR. Mais les meilleurs rendements de dégradation des molécules non phénoliques ont été obtenus en présence de SYR ; ces bonnes performances sont à mettre en relation avec la formation de radicaux très réactifs et stables lors de l'oxydation du SYR par les laccases. Par ailleurs, il faut noter que les laccases commerciales ont montré de meilleures performances que l'extrait brut de *P. sanguineus*. Dans l'ensemble, le pouvoir oxydant des laccases a été

augmenté suite à l'ajout de médiateurs, mais aucun des médiateurs testés n'a rendu possible la dégradation de la carbamazépine (CBZ). Quoi qu'il en soit, le potentiel de dégradation des systèmes de laccases libres-médiateurs pour le traitement de certains produits pharmaceutiques récalcitrants a été démontré.

Dans un second temps, une méthode d'immobilisation des laccases sur des supports solides de taille micrométrique (microparticules commerciales de gel de silice fonctionnalisées) a été développée et les résultats obtenus ont été présentés dans le chapitre quatre. Compte tenu de la taille du solide (100 µm), l'activation des billes de silice par le glutaraldéhyde (GLU) a été réalisée en phase vapeur de façon à limiter les risques d'agrégation. A notre connaissance, c'est la première fois que cette technique d'activation est utilisée pour l'immobilisation de laccase en vue de procédés de remédiation. Un plan expérimental complet (2^3) a été utilisé pour étudier l'effet des différents paramètres opératoires (temps d'activation, concentration en enzymes, temps de contact, pH) sur l'efficacité de l'immobilisation. Selon le modèle obtenu, les conditions permettant d'obtenir les billes les plus intéressantes en termes d'activité (14±2 U g^{-1} de billes) et de rendement d'immobilisation des protéines (35%) sont : une durée d'activation de 18 h et un temps de contact de 2 h en présence d'une solution enzymatique renfermant 107 U à pH 7.

Il a ensuite été démontré que l'immobilisation covalente de la laccase sur les billes de silice permet d'améliorer sa stabilité sur une gamme de valeurs de pH (pH de 4 à 7) et de températures (de 25 à 55 °C) mais surtout d'améliorer sa stabilité lors du stockage à long terme par rapport à la forme libre. Néanmoins, après immobilisation, les laccases ne sont toujours pas capables d'oxyder des produits pharmaceutiques ciblés. Cependant, tout comme lors des essais avec l'enzyme libre, l'ajout de médiateurs redox a rendu possible la biotransformation des produits pharmaceutiques. Après 3h de réaction, les pourcentages d'élimination sont compris entre 40-100% selon le substrat et le médiateur considéré. De plus, il a été observé que la disparition des produits pharmaceutiques analysés résultait de l'action combinée de mécanismes de dégradation enzymatique et d'adsorption sur le support, l'importance de l'un ou l'autre de ces mécanismes étant fonction du composé pharmaceutique considéré. En particulier, on a mis en évidence que l'élimination de l'AMX et du SMX est principalement due à la biodégradation, même s'il est possible que l'adsorption de ces composés ait pu jouer un rôle important dans le processus oxydatif en

améliorant le transfert d'électrons entre la laccase et le substrat. Dans le cas de la ciprofloxacine (CIP) et de la CBZ, la disparition de ces composés du milieu réactionnel résulte uniquement de phénomènes d'adsorption. Cela a été attribué à leur affinité pour être adsorbé sur les solides. Dans l'ensemble, les médiateurs redox générant des radicaux phénoxylés (SYR et PCA) semblent plus efficaces pour la biotransformation des produits pharmaceutiques, le SYR étant le plus efficace. Néanmoins, les résultats de tests Microtox®, ont montré que l'utilisation du SYR entraine une augmentation de la toxicité de l'effluent après traitement contrairement à l'ajout d'ABTS, ce dernier médiateur étant en outre le moins toxique des 3 médiateurs testés. Enfin, il a été montré qu'après immobilisation, le biocatalyseur pouvait être réutilisé efficacement pendant sept cycles consécutifs en présence de SYR. Compte tenu de l'intérêt de ce médiateur, des recherches devront être faites pour trouver une solution à l'augmentation de la toxicité engendrée lors de son utilisation.

Le cinquième chapitre de cette thèse a été consacré à l'étude de l'immobilisation de la laccase sur différents supports inorganiques. Des nano-composites de silice (SiNPs), d'argiles (argile Y et SWy-3) et de silice/argile (95/5 et 50/50) présentant des caractéristiques différentes en termes de composition chimique, de taille et de surface spécifique ont été utilisés pour réaliser l'immobilisation covalente de la laccase de *T. versicolor* selon la méthode développée au chapitre 4. Les résultats obtenus ont montré que les cinq supports présentaient des capacités d'immobilisation différentes. La surface spécifique, le type et la composition du matériau semblent donc avoir une influence sur l'immobilisation. Il a été observé que les matériaux à base de silice permettent de greffer de plus grandes quantités de protéines sous forme actives. Ceci est particulièrement évident lorsque l'on compare les performances des nano-composites. Le biocatalyseur est d'autant plus actif que la concentration en silice est plus élevée dans le composite. Ainsi, le nanocomposite 50/50 est le meilleur support en termes d'activité spécifique élevée (185 U mg^{-1}) et de rendement d'immobilisation des protéines (30%), respectivement. Les particules à base de silice seule sont également intéressantes puisqu'elle présente également une activité spécifique élevée (110 U mg^{-1}).

Ces 2 supports ont donc été retenus et les performances des 2 biocatalyseurs obtenus ont été comparées lors d'essais de dégradation du mélange des micropolluants

pharmaceutiques en présence de SYR. Quel que soit le biocatalyseur testé, des rendements de dégradation élevés (50-100%) ont été atteints pour les quatre produits pharmaceutiques étudiés ; ceci en raison de l'action synergique de l'oxydation catalysée par laccase et de la remarquable capacité de sorption des supports. Il est intéressant de noter que même lorsque les deux biocatalyseurs ont montré des efficacités de dépollution similaires, les mécanismes impliqués dans l'élimination des micropolluants du milieu réactionnel sont différents. En raison de la capacité de sorption modérée du support des particules de SiNPs, l'élimination des micropolluants est vraisemblablement à attribuer à la laccase mais il est possible que l'adsorption des substrats sur la silice ait contribué à accélérer et à intensifier la dégradation catalysée par laccase immobilisée. Par contre, alors que le nanocomposite 50/50 présente une capacité de sorption remarquable, cela ne permet pas d'améliorer la biotransformation des micropolluants. En d'autres termes, la seule adsorption des produits pharmaceutiques ne permet pas d'améliorer la dégradation de composés récalcitrants. A notre connaissance, c'est la première fois que des matériaux composites silice/argile sont utilisés comme supports d'immobilisation des laccases pour le traitement des polluants pharmaceutiques et les résultats semblent encourageants. Les résultats présentés ici soulignent le rôle important des propriétés des matériaux supports dans le processus d'immobilisation ainsi que dans la performance du biocatalyseur. En ce sens, nous avons démontré l'avantage d'utiliser des matériaux polyvalents permettant à la fois le greffage de l'enzyme et l'adsorption des composés à éliminer. Leur mise en œuvre au sein des procédés de dépollution permet ainsi, de réaliser en une seule étape, l'élimination d'un mélange de composés dont certains sont non biodégradables mais ont la possibilité de s'adsorber et d'autres qui au contraire ne sont pas capables de s'adsorber mais qui peuvent être biodégradés.

Les travaux réalisés ici démontrent le potentiel d'utilisation des laccases pour le traitement enzymatique des micropolluants pharmaceutiques dans l'eau, mais il est important de considérer certains aspects pour le développement futur de cette recherche. La performance des laccases dans cette thèse a été étudiée principalement à l'aide d'eaux usées synthétiques. Il est important d'évaluer la performance des systèmes libres et des systèmes immobilisés dans des conditions réelles d'eaux usées. En ce sens, les essais avec des systèmes enzymes-médiateurs dans les eaux usées réelles à très faibles concentrations

de micropolluants, comme on les rencontre habituellement dans ces eaux, devraient être fondamentaux pour évaluer l'impact des conditions réelles sur l'efficacité du traitement. Un autre aspect important à considérer est l'extension du traitement, puisque toutes les expériences réalisées dans le cadre de ce travail sont à l'échelle du laboratoire. En ce sens, la conception d'un réacteur (p. ex. bioréacteur enzymatique à membrane, lit garni) serait la meilleure option puisqu'elle permettrait également d'évaluer l'efficacité du traitement en fonctionnement continu.

L'immobilisation de la laccase a permis d'augmenter la stabilité et la réutilisabilité de l'enzyme qui est cruciale pour les applications à grande échelle. Par conséquent, la méthode d'immobilisation proposée devrait être optimisée afin de maximiser les rendements d'immobilisation et la charge d'activité sur les porteurs pour rendre le processus plus rentable. De plus, d'autres essais devraient être effectués avec des SiNP et des nanocomposites 50/50 pour caractériser complètement ces biocatalyseurs et évaluer leur éventuelle réutilisation.

Dans cette étude, il a été démontré que l'ajout de médiateurs a amélioré la dégradation et a élargi le spectre des composés dégradés par la laccase. Cependant, l'ajout continu de ces composés à la réaction entraîne une augmentation des coûts et de la toxicité de l'effluent traité. Il est recommandé d'étudier l'effet du type du médiateur et de sa concentration sur les rendements de dégradation et la toxicité afin de mettre au point un processus de traitement vraiment efficace et efficient. De plus, à la fin de la thèse, une nouvelle approche pour la réutilisation du SYR a été étudiée en immobilisant le médiateur sur des supports solides. Comme les résultats préliminaires semblaient prometteurs, nous croyons qu'il vaut la peine d'étudier en profondeur cette technologie.

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Chapter 1

Introduction

1. Introduction

1.1 Background

Good quality water is essential to sustain human well-being and ecological balance of ecosystems. However, in the recent years the detection of new and emerging pollutants, presenting persistent and accumulative characteristics has highlighted a new threat to water resources due to their potentially negative impacts to human health and the environment. Among the large categories of emerging contaminants, pharmaceutical compounds are among the most commonly present in waters due to their universal use as a consequence of an aging and growing population, and their non-biodegradable and pseudo persistence nature. Significant amounts of un-metabolized and un-used pharmaceuticals as well as their by-products are continuously discharged into water bodies, together with untreated wastewater and effluents from wastewater treatment plants (WWTPs). Thus, residues of different types of pharmaceutical compounds (e.g. antibiotics, analgesics, anti-inflammatories, antidepressants, etc.) have been detected in sewage, surface and groundwater compartments, adsorbed on sediments and even in drinking water all over the world (Sui et al., 2015; Tran et al., 2018; Yang et al., 2017).

The occurrence of pharmaceutical compounds in the environment is of global concern and the extent of their impacts on the health of aquatic and terrestrial organisms is widely unknown. Pharmaceutical pollutants have been found in water bodies at concentrations from ng to several μg L^{-1}, hospital and drug manufacturing effluents are obviously the major contributors with concentrations in the higher μg L^{-1} range. In river water are commonly found in the range of tens to hundreds of ng L^{-1}, which are usually lower than WWTP effluent. In comparison to surface water, lower contamination levels in ground water were reported with less than tens ng L^{-1}. These concentration levels are relatively low but some of these molecules are biologically active even at very low concentrations and can produce chronic effects as the consequence of a continuous and prolonged exposure. Such chronic effects include disruption or alteration of the endocrine system. Moreover, increased incidents of some types of cancer, development of antibiotic resistance and detrimental effects on natural microbial communities have been also

documented as potential effects of pharmaceuticals exposure (Barra Caracciolo et al., 2015; Gogoi et al., 2018).

The constant detection of pharmaceutical products and their metabolites in water bodies indicates that conventional WWTPs are ineffective for the removal of these types of substances. Indeed, the degradation of pharmaceutical compounds implies an important challenge due to their low bioavailability and complex structure. Although some pharmaceutical compounds can be removed from water during conventional wastewaters treatment, mainly by adsorption on suspended solids and/or biodegradation, still a large proportion remains in treated effluents. It is therefore necessary to find effective treatment options allowing the efficient and complete removal of these pollutants. Over the last decade, many studies have investigated the removal of pharmaceuticals from water and wastewater using physicochemical, microbiological or advanced oxidation processes. Even if these studies led to promising results, the treatment processes evaluated showed to be effective only for the removal of certain groups of pharmaceutical compounds. In addition, the high cost of operation, the variable efficiencies obtained and the possible formation of hazardous by-products also poses as an obstacle for the successful application of these technologies (Luo et al., 2014).

A potential alternative to the application of conventional treatment processes is the use of enzymes. Generally, enzymes are able to catalyze specific reactions, mostly under moderate operational conditions (i.e. pH, temperature, ionic strength) which results in lower energy consumption and a more environmentally friendly process. These biocatalysts can effectively transform environmental pollutants over a wide range of concentrations at a rapid rate without the risk of being withdrawn by the effect of these compounds as observed in conventional wastewater treatment plants or bioreactors based on activated sludge processes. Different types of enzymes extracted from organisms such as bacteria and fungi have been described to potentially oxidize hazardous pollutants (Demarche et al., 2012). In this sense, the use of white rot fungi cultures (WRF) and their lignin modifying enzymes (lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccase) have attracted a lot of attention in recent years for their ability to remove a wide range of xenobiotics (Cabana et al., 2007; Cruz-Morató et al., 2014).

Particularly interesting, laccases are oxidative enzymes containing four cupper atoms in the active site which enable them to catalyze single electron oxidation of substrates with the concomitant reduction of molecular oxygen to water (Giardina et al., 2010). In this way, laccases can catalyze the oxidization of a broad range of phenolic and aromatic compounds. The use of laccases is attractive since they can carry out the oxidation of substrates over a wide range of temperatures and pH values. Moreover, the fact that this enzyme only requieres oxygen as co-substrate represent an additional advantage for the application of laccases in comparison to peroxidases. Thus, numerous studies have indicated that laccases present a great potential in the bioremediation of contaminated effluents with different types of organic pollutants (Jin et al., 2016; Salazar-López et al., 2017; Suda et al., 2012; Tran et al., 2010). However, the removal efficiency of these pollutants depends on their physicochemical properties and the fungal strain from which the laccase has been extracted. Most of the pharmaceutical pollutants found in water bodies present non-phenolic structures which are not always suitable for laccase oxidation. The substrate range of laccases can be extended by redox mediators that act as an “electron shuttle” between the enzyme and substrate. In this way, it is possible to oxidize chemical compounds that in principle are not laccase substrates (Cañas and Camarero, 2010).

Generally, enzymatic treatment processes are implemented in batch reactors with free enzymes (either in crude or purified form). However, larger scale applications such as in a WWTP need to be operated in continuous mode. Thus, the retention of the enzyme in the reactor is necessary in order to avoid the laccase being washout with the treated effluent. Furthermore, the use of free laccases present some drawbacks related to their limited stability in solution, difficult recovery and reusability which results in high operational costs. These disadvantages can be overcome by the immobilization of enzymes on different supports. To date, immobilization of enzymes has been carried out on a broad variety of support materials from inorganic, organic and hybrid/composite origin. The proper selection of a support material is of important matter since its physicochemical characteristics strongly influence the final properties of the immobilized enzyme. Thus, several aspects such as the presence of numerous functional groups and good chemical and mechanical resistance need to be considered (Zdarta et al., 2018). In this sense, inorganic support materials, such as silica and clays, represent an excellent option for enzyme immobilization due to their great

biocompatibility, large specific surface area, presence of many different functional groups, exceptional stability, low cost and reusability (Zucca and Sanjust, 2014).

The selection of the immobilization method is also extremely important and depends on the characteristics of the support chosen, the properties of the enzyme to immobilize and the process to which the immobilized biocatalyst will be applied. Among the different immobilization methodologies available, covalent binding seems to be the most suitable technique for water and wastewater applications since this approach involves the multipoint attachment of enzymes to the support which significantly enhance enzyme stability, reusability and reduce enzyme leaching (Gasser et al., 2014). Laccases covalently immobilized on different solid carriers have been applied for the removal of pharmaceutical pollutants in water with encouraging results (Barrios-Estrada et al., 2018b; De Cazes et al., 2014; Ji et al., 2016; Kumar and Cabana, 2016). Therefore, a continuous searching of new materials and immobilization protocols for the development of new biocatalysts meeting the needs of industrial and environmental applications is of important matter.

1.2 Research objectives

The main objectives of this thesis are, on one hand, the study of the potentialities of different laccases (two commercial laccases and a laccase cocktail from *Pycnoporus sanguineus* CS43 prepared at the School of Engineering and Sciences of Tecnologico de Monterrey, Mexico) for the elimination of pharmaceutical micropollutants in the presence or absence of redox mediators; and on the other hand, the development of an immobilization method adapted to new supports, namely nanoparticles based on silica and clay materials developed within the framework of the ANR Polpharma project. The study will be focused on the conversion of antibiotics (amoxicillin, ciprofloxacin and sulfamethoxazole) and anti-epileptic (carbamazepine) compounds as laccase substrates.

The specific objectives that will be accomplished are:

- The study of the reactivity of different enzymes (in free form) towards different substrates in a batch reactor.
- The development of the immobilization method on commercial silica particles.

- The immobilization of the most active enzyme on the new materials and the study of the potentiality of the active supports for the elimination of the pharmaceuticals.

1.3 Thesis outline

This thesis describes the potentiality of three different laccases, in free and immobilized form, for the degradation of recalcitrant pharmaceutical micropollutants. The manuscript consists of six chapters. Chapter 1 introduces the background and research objectives of this study.

Chapter 2 presents the state of the art regarding the occurrence, fate and elimination of pharmaceutical micropollutants in water. Different conventional treatment technologies are described before focusing in enzymatic treatment of these compounds. The objective is to provide an overview of the current knowledge regarding the application of laccases in both free and immobilized forms.

Chapter 3 is composed of a published research article in the Process Biochemistry Journal. This section presents the use of free laccases as well as laccase-mediator systems in batch test conditions. The aim of this work is to investigate if, and how enzymes carry out the degradation process of the different substrates in order to establish the groundwork for the laccase catalyzed transformation of pharmaceutical compounds. Thus, three different enzymes have been evaluated as well as the effects of operational conditions (pH and temperature) and redox mediators (type) on the removal efficiency of target micropollutants.

Chapter 4 presents the immobilization of laccases onto silica gel carriers and the use of the biocatalyst for the degradation of a mixture of pharmaceuticals. A novel protocol for the covalent immobilization of laccases involving the activation of the amino-functionalized support with glutaraldehyde in vapor phase is proposed. In this sense, a statistical experimental design was generated in order to study the parameters affecting the immobilization process. The novel biocatalyst has been completely characterized to properly compare its performance with the free counterpart and applied for the degradation of the target pharmaceuticals in multi-compound solutions in presence of redox mediators. The

reusability of the immobilized enzyme has been evaluated during several consecutive degradation cycles. Additionally, the effectiveness of the enzymatic treatment is also assessed by studying the toxicity of the biotransformation products generated from the enzymatic reactions by Microtox® assays.

Chapter 5 introduces the use of different novel inorganic support nanomaterials for the immobilization of the commercial *Trametes versicolor* laccase and its use for the removal of the target pharmaceutical compounds in batch reactions. This work has been done in the framework of the ANR project POLPHARMA involving the collaboration with different French research laboratories (LCMP-UPMC, BRGM, CEREGE and CIRSEE from SUEZ) which kindly provided the different supports materials to be tested. The study compares the capability of silica nanoparticles, clay microparticles and silica/clay nanocomposites to covalently immobilize laccase based in the protein immobilization yield and the specific activity of each bio-catalytic formulation. The best biocatalysts have been investigated for the simultaneous removal of the target pharmaceuticals with the aid of syringaldehyde as redox mediator. The results obtained are discussed by comparing the different properties of the support materials in order to explain the removal efficiencies observed by each immobilized enzyme.

Finally, the dissertation ends with chapter 6 which summarizes the overall conclusion of the investigation and the perspectives for future works.

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Chapter 2

Literature Review

2. Literature review

2.1. Pharmaceutical compounds as emerging contaminants

In recent decades, studies on the quality assessment of water resources have attracted attention towards a critical problem of contamination due to the occurrence of a variety of newly identified compounds presenting persistent, bioactive and bio-accumulative characteristics (Gogoi et al., 2018; Rodriguez-Delgado and Ornelas-Soto, 2017). This group of substances known as emerging contaminants (ECs) are defined as any synthetic or naturally-occurring chemical or any microorganism that is not routinely monitored or regulated in the environment and has known or suspected potential adverse ecological and human health effects (UNESCO, 2014). Therefore, management of pollution due to ECs presents a new global water quality challenge.

Among ECs, pharmaceuticals are probably one of the most relevant compounds due to their universal use, their physicochemical properties and their capacity to be incompletely removed by conventional wastewater treatment processes (Zenker et al., 2014). They have been detected in several environmental compartments such as surface water, ground water, urban wastewater and even in drinking water (Kanakaraju et al., 2018). Pharmaceuticals represent more than 4000 different molecules with a total consumption of several thousand tons per year for the treatment of human and animal diseases as well as in livestock an aquaculture (de Cazes et al., 2014).

Pharmaceuticals enter the environment through various stages of the product lifecycle from their production and consumption to disposal (Fig. 2.1). In the aquatic environment these compounds are continually introduced via various pathways, such as direct discharges of untreated and treated wastewaters from industries, hospitals, animal husbandry, households and wastewater treatment plants (WWTPs) (Wang and Wang, 2016; Y. Yang et al., 2017). After administration, human pharmaceuticals are excreted as parent forms or as active metabolites and enters the sewage system to reach WWTPs (Klatte et al., 2017). Unfortunately, conventional wastewater treatment processes do not allow the effective elimination of pharmaceuticals from treated water (Bu et al., 2013; Halling-Sorensen et al., 1998). In addition, during treatment pharmaceuticals can be adsorbed on

sewage sludge. Thus, they can reach soils if the sludge is used as fertilizer or if treated water is used for land irrigation (Ebele et al., 2017; Klatte et al., 2017). Similarly, veterinary drugs can be released when animal wastes both solid and liquid are spread on agricultural fields as fertilizers (Ebele et al., 2017). Moreover, pharmaceuticals present in soils can leach from soil into surface and ground waters (Gavrilescu et al., 2015; Heberer, 2002) and/or enter the food chain by crops uptake (Klatte et al., 2017; Rivera-Utrilla et al., 2013).

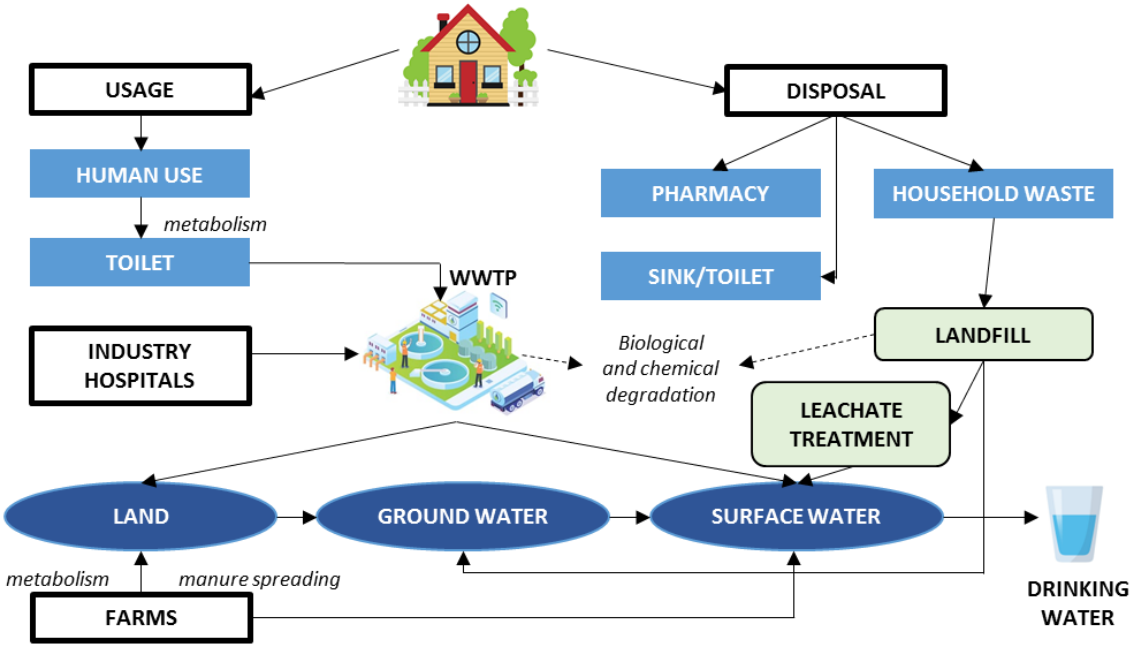


Fig. 2.1. Potential sources and pathways of pharmaceuticals pollution in the environment.

Although pharmaceuticals have been entering the environment for many years, investigation on their occurrence is recent. Since the first detection in the aquatic environment in 1978, the rapid development of high sensitive analytical instruments has allowed to identify more than 275 different pharmaceutical compounds previously undetectable as they usually are present in sewage, surface and groundwater at levels of µg L^{-1} (Zenker et al., 2014). Antibiotics, steroids, antidepressants, beta-blockers, analgesics, anti-inflammatories, lipid-lowering drugs, tranquilizers and stimulants have been detected in surface and ground water bodies in different countries around the world including Germany (Ternes, 1998), Greece (Kosma et al., 2014), Italy (Castiglioni et al., 2004), Spain (Ortiz de

Garcia et al., 2013), South Africa (Archer et al., 2017), United States (Kolpin et al., 2002), China (Bu et al., 2013) Canada and Brazil (Ternes et al., 1999).

The occurrence of pharmaceutical compounds in the environment - due to their non-biodegradable and pseudo persistence nature - is of global concern and the extent of their impacts on aquatic and terrestrial organisms is widely unknown. Short term studies suggest that acute toxic effects of pharmaceutical pollutants are insignificant, but their continuous release and exposure may produce chronic effects (Barra Caracciolo et al., 2015). Potential concerns from prolonged exposure include disruption or alteration of the endocrine system by mimicking, blocking or hampering hormonal functions. Increased incidents of some types of cancer, development of antibiotic resistance and detrimental effects on natural microbial communities have been also documented as potential effects of pharmaceuticals exposure (Barra Caracciolo et al., 2015; Gogoi et al., 2018).

As a result of the insufficient knowledge in terms of toxicity, impacts and behaviours of pharmaceuticals, only few are regularly monitored in the environment and many remain unregulated. At present, different organizations have established directives and legal frameworks to protect and improve the quality of water resources. The United States Environmental Protection Agency (USEPA) has included three pharmaceuticals (erythromycin, 17α-ethinylestradiol (EE2) and nitroglycerin) in their recent contaminant candidate list (CCL-3) together with eight synthetic hormones (Kanakaraju et al., 2018). The European Union released a set of directives including the directive 2000/60/EC which established a strategy to define high risk substances to be prioritized. Then, a set of 33 priority substances and their respective environmental quality standards were ratified by the directive 2008/105/EC. In 2013, the directive 2013/39/EU recommended attention for the monitoring and treatment options for a group of 45 priority substances including two pharmaceuticals (diclofenac and EE2) and a natural hormone estradiol (E2). Finally, in 2015 the watch list of substances for European Union wide monitoring was amended to include three macrolide antibiotics and the natural hormone estrone (E1) (Barbosa et al., 2016).

2.1.1. Antibiotics

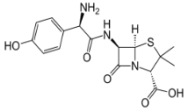
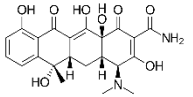
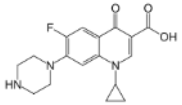
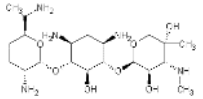
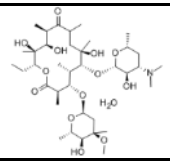
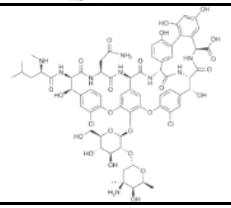
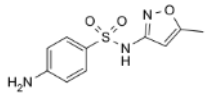
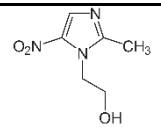
Antibiotics are organic molecules that inhibit the growth of bacteria, most of them have microbial origin but they are also semi or totally synthetic (Michael et al., 2013). Currently, about 250 different antibiotics are available for use in veterinary and human medicine which are classified in different classes according to their chemical structure or mechanism of action (Table 2.1) (Barra Caracciolo et al., 2015; Kümmerer, 2009).The impact of antibiotics in the environment is highly dependent on their consumption habits and their strong intrinsic persistence. For example, antibiotics such as β-lactams are the most used for human therapy (Li, 2014), but sulfonamides, fluoroquinolones, macrolides and trimethoprim are the most frequently detected in both influent and effluent samples worldwide (Tran et al., 2018). Therefore, a representative antibiotic belonging to the β-lactam (amoxicillin), sulfonamide (sulfamethoxazole) and fluoroquinolone (ciprofloxacin) groups were chosen as model substrates to be eliminated in the present research. The structures of these selected antibiotic compounds are presented in Table 2.1.

Amoxicillin (AMX), is a penicillin-type antibiotic which is distinguished by the presence of an extra amino group on its side chain. These antibiotics are of the most important groups in medicinal and veterinary therapy with a consumption reaching 50-70% of the total amount of antibiotic applied in human medicine. AMX and its presence in the environment, have been widely investigated despite its extensive hydrolysis which leads to instability and its rapid degradation to various degradation products (Gozlan et al., 2013).

Sulfamethoxazole (SMX) is an antibiotic which belongs to the family of sulfonamides; these compounds limit bacterial growth by preventing the synthesis of folic acid which is essential for the synthesis of bacterial DNA (Barra Caracciolo et al., 2015). SMX is considered as an emerging contaminant of concern due to its potential adverse effects on ecosystems and human health (Avisar et al., 2009).

Ciprofloxacin (CIP) belongs to a class of synthetic antibiotic agents with a broad range of activity against gram-negative and gram-positive organisms. CIP can inhibit essential enzymes involved for DNA production and it is used for the treatment of severe infections (Le-Minh et al., 2010).

Table 2.1. Classes, consumption and main uses of antibiotics (Adapted from Grenni et al., 2018; with permission).

Class	Antimicrobial effect	Main use	Consumption EU (tons year^{-1})	Example	Chemical structure
β-lactams	Synthesis inhibition of cell wall	Human, veterinary	1779.8-2110.9	**Amoxicillin**	
Tetracyclines	Inhibition of protein synthesis	Human, veterinary	5.9-2942.6	Tetracycline	
Fluoroquinolones	Synthesis inhibition of nucleic acids	Human	186.1-231.5	**Ciprofloxacin**	
Aminoglycosides	Inhibition of protein synthesis	Human, veterinary, plants	4.7-290.8	Gentamicinc	
Macrolides	Inhibition of protein synthesis	Human, veterinary, food additive	252.3-638	Erythromycin A	
Glycopeptides	Synthesis inhibition of cell wall	Human, veterinary	n.r.	Vancomycin	
Sulfonamides	Synthesis inhibition of nucleic acids	Human, veterinary	121.5-826.3	**Sulfamethoxazole**	
Nitroimidazoles	Inhibition of protein synthesis	Human	n.r.	Metronidazole	

n.r.: Not reported. Data of consumption in 1997 from Cars et al. (2001).

The occurrence of these three antibiotics in different environmental matrices has been investigated and some examples are summarized in Table 2.2. In general antibiotics are detected in water bodies at concentrations from ng to several μg L^{-1}, hospital and drug manufacturing effluents are the major contributors with concentrations in the higher μg L^{-1} range (Kümmerer, 2009). For example, CIP has been typically quantified in hospital effluents with concentrations up to 101 μg L^{-1} (Lindberg et al., 2014). Antibiotics in river water are commonly found in the range of tens to hundreds of ng L^{-1}, which are usually lower than WWTP effluent. In comparison to surface water, lower contamination levels in ground water were reported with less than tens ng L^{-1}. Anyway, high concentration of SMX has been quantified in ground water (Table 2.2). Probably due to its weak sorption to soil, reaching ground water by leaching or running off (Carvalho and Santos, 2016). Nonetheless, WWTPs are believed to be one of the main hotspots for the releasing of antibiotic pollutants into the environment as they usually are the final destination of urban and, in some cases, hospital effluents as well as due to their limited efficiency for the removal of these compounds.

The relatively high concentrations of SMX and especially of CIP in WWTP effluents could be related to their high consumption and resistance to biological degradation. However, CIP can conjugate with metal cations present in wastewater to form complex compounds becoming more abundant in sewage sludge (Tiwari et al., 2017). In contrast, despite AMX extensive consumption its occurrence has not been covered frequently. Probably the fast hydrolysis and rapid degradation of the antibiotic in addition to analytical limitations contribute to low detection in aquatic systems as evidenced in the lack of reports for its presence in ground water.

Table 2.2. Occurrence and concentration of antibiotics found in different water bodies.

Compound	**Range of concentration** (ng L^{-1})		
	Surface water	WWTP effluent	Ground water
AMX	BLQ-662	BLQ-190	-
SMX	BLQ-4330	BLQ-1800	BLQ-458
CIP	80	BLQ-5692	0.28-40
Reference	Gibs et al. (2013) Kasprzyk-Hordern et al. (2008) Phuong Hoa et al. (2011)	Guerra et al. (2014) Minh et al. (2009) Rosal et al. (2010) Tran et al. (2016)	López-Serna et al. (2013) Van Stempvoort et al. (2013)

BLQ: below limit of quantification

The continuous input and partial elimination of antibiotics has raised concern about the ecological risk of these compounds in the environment. Antibiotics and their degradation products have the potential to disrupt vital ecosystem processes due to their anti-microbial characteristics being bacteria, fungi and microalgae the organisms more vulnerable. Hence, chronic toxicity and the prevalence of resistance to antibiotics in bacterial species have been associated to the presence of antibiotics in the environment (Michael et al., 2013). Toxic effects of CIP in aquatic organisms was studied by Ebert et al. (2011) in photo autotrophic *Anabaena flos-aquae* (cyanobacteria), *Desmodesmus subspicatus* (green algae) and *Lemna minor* (macrophyte) finding that the antibiotic was more toxic to the cyanobacteria and the macrophyte at the predicted environmental concentration. In other study, cyanobacteria were more vulnerable than green algae to SMX when photosynthetic efficiency was evaluated (van der Grinten et al., 2010). Gonzalez-Pleiter et al. (2013) evaluated the toxicity of different antibiotics both in single and mixture solutions on cyanobacteria and green algae finding that the toxic effects of antibiotics increased when they were in combination with other antibiotics. Exposure to antibiotics may have adverse effects on reproductive systems of different organisms like the freshwater flea *Daphnia magna* and the crustacean *Artemia salina*. In fact, Kim et al. (2007) studied the effect of sulfonamides and trimethoprim on the acute toxicity by employing *D. magna*, *V. fisheri* and the Japanese medaka fish where the freshwater flea resulted as the most susceptible organism in terms of effect/lethal concentrations.

The potential development of antibiotic resistance in natural bacterial strains is of especial concern. Extensive use of both human and animal antibiotics has led to the emergence and spread of antibiotic resistance bacteria resulting in the reduced therapeutic potential of these compounds against human and animal pathogens. The consequences are particularly worrying for bacteria in the aquatic environment since they are continuously exposed to antibiotic residues. For example, Zhang et al. (2009) observed higher prevalence of antibiotic resistance in *Acinetobacter* spp. in downstream samples from a WWTP in comparison to upstream samples, especially for trimethoprim. Additionally, the presence of antibiotics in WWTP can influence the microbial activities and in consequence the performance of the treatment. Gonzalez-Martinez et al. (2014) reported that CIP induced deterioration of the nitrification process even at relatively low concentrations.

2.1.2. Antiepileptic

Carbamazepine (CBZ) is probably the most common anti-seizure drug used worldwide for the control of grand mal and psychomotor epilepsy. Additionally, it is also prescribed for the treatment of bipolar depression and trigeminal neuralgia (Clara et al., 2004). Even when only 2 to 3% of the drug is excreted as the parent compound, CBZ has been frequently detected in natural waters, ground water, soil and sediments in ground water recharging areas (Clara et al., 2004; Sui et al., 2015; Tran et al., 2018). Due to its poor removal in conventional WWTPs and persistence in the environment CBZ was suggested as a marker for anthropogenic contamination (Barra Caracciolo et al., 2015). For this reason, CBZ was also chosen to be part of the pharmaceuticals studied in the present investigation.

Concentration of CBZ detected in influents and effluents of WWTPs around the world varied significantly reaching few µg L^{-1} (Table 2.3). As mentioned before, conventional WWTPs are ineffective for CBZ removal as it presents low biotransformation and limited adsorption to sludge (Naghdi et al., 2018a). Thus, CBZ has been detected at concentrations up to 1075 ng L^{-1} in surface water samples in Berlin (Heberer et al., 2002). In ground water CBZ was detected in 42% of samples collected in 23 European countries with a maximum concentration of 390 ng L^{-1} (Loos et al., 2010). Moreover, ground water studies suggested that CBZ could survive intact after 8 to 10 years of residence in the subsurface (Sui et al., 2015). It was not attenuated during bank filtration which may be one of the reasons for their prevalence in the groundwater (Clara et al., 2004). In fact, soil studies found CBZ tends to accumulate and to be recalcitrant to microbial degradation (Barra Caracciolo et al., 2015).

The potential effects of CBZ have been documented in various recent studies. CBZ was found to reduce bacterial biomass of riverine biofilm (Lawrence et al., 2005), to be toxic to *Hydra attenuata* organism (Quinn et al., 2008) and to decrease the health of *Mytilus galloprovincialis* mussel (Martin-Diaz et al., 2009). Jos et al. (2003) carried out an ecotoxicological evaluation of CBZ using different model systems including *daphnia magna*, *vibrio ficheri*, *chlorella bulgaris*, the plant *allium cepa* and the Vero monkey kidney cells. Although results demonstrated that CBZ did not produced acute toxicity in aquatic biota assayed, chronic and synergistic effects with other chemicals should not be excluded.

Table 2.3. Concentration range of CBZ in effluents from WWTPs.

Country	Effluent concentration (ng L^{-1})	Reference
Spain	69-173	Rosal et al. (2010)
South Africa	279.5	Archer et al. (2017)
Austria	940-1510	Clara et al. (2004)
Greece	28-416.8	Kosma et al. (2014)
Germany	6300	Ternes (1998)
Germany	1630	Heberer et al. (2002)
France	157.3-293.4	Togola and Budzinski. (2008)

2.1.3. Physicochemical properties of the selected pharmaceuticals

Table 2.4 shows the main physicochemical properties of the selected antibiotic and antiepileptic compounds. Some parameters such as water solubility or the coefficient of partition octanol-water (Log K_{ow}) are important values to observe in order to estimate the fate of the pollutants in the environment and during water treatment. In general, compounds presenting lower water solubility and high Log K_{ow} values are expected to show high sorption potential. In this sense, from the selected pharmaceuticals only CBZ could be expected for potential sorption. The acidity constant (pKa) is also an important parameter since the neutral ionic forms of the target pollutants usually behave differently.

Table 2.4. Physicochemical properties of selected pharmaceutical compounds.

Compound	Molecular weight (g mol^{-1})[a]	Solubility in water (mg L^{-1})	pKa[b]	Log K_{ow}[a]
AMX	365.4	3400[e]	2.4 7.4 9.6	0.87
SMX	253.3	610[c]	1.83 5.57	0.89
CIP	331.3	~0*	5.9-6.1 8.7-8.9	0.28
CBZ	236.3	78[d]	13.9	2.45

*soluble in diluted aqueous acid solution.

a: Wang and Wang, (2016); b: Ternes and Joss, (2006); c: Suárez et al., (2008); d: Celiz et al., (2009); e: DRUGBANK (https://www.drugbank.ca/).

2.2. Treatment technologies for pharmaceuticals removal

As mentioned above, most conventional water and WWTPs were not designed for the treatment of wastewater containing pharmaceutical and other emerging contaminants. Typically, WWTPs comprise a primary system of physicochemical treatment and a secondary system that consists of a biological reactor formed by activated sludge. Although some micropollutants can be removed from water during conventional treatment, still a variable proportion remain in the effluent to be discharged to the environment. It is therefore imperative to find effective treatment options not only from a practical point of view, but also from an economical aspect. Over the past few decades, a variety of different physical, chemical and biological technologies have been evaluated for the removal or degradation of pharmaceutical compounds in water. In this section, an overview of the most relevant treatment technologies available for each category is presented. The advantages and drawbacks of the different processes are resumed in Table 2.5.

2.2.1 Physical treatment processes

2.2.1.1 Adsorption

Adsorption is one of the most prevalent physical processes for the removal of contaminants in water and one of the main processes for pharmaceuticals treatment (Wang and Wang, 2016). It is a simple process that enables to transfer molecules in liquid phase to a solid surface thus allowing the removal of pollutants instead of producing more dangerous by-products (Homem and Santos, 2011; Rivera-Utrilla et al., 2013). Overall, the effectiveness of adsorption process depends on both the nature of the pollutant and the type of adsorbent material. Thereby, in order to enhance the removal of pharmaceutical micropollutants different adsorbents have been investigated and developed.

Activated carbon is probably the most traditional adsorbent used in water treatment. It can be used in two different presentations: powdered activated carbon (PCA) and granular activated carbon (GAC). PAC tends to be more efficient due to its higher specific surface and is usually added in contact basins, while GAC is packed in a “column” (Snyder et al., 2007). The performance of activated carbon adsorption mainly depends on the properties of the activated carbon sorbent (i.e. specific surface area, porosity) and on the hydrophobicity and charge of the target compounds (Michael et al., 2013). Moreover, reaction conditions such

as, temperature, pH or contact time can also significantly influence the process. Unfortunately, activated carbon lacks selectivity and when used in complex water matrices competition for adsorption sites between the target pharmaceuticals and other compounds in water (i.e. organic matter) may occur leading to efficiency loss (Mailler et al., 2015).

Table 2.5. Advantages and drawbacks of different technologies in the removal of pharmaceutical micropollutants in wastewater (Adapted from Ahmed et al., 2017; with permission).

Treatment process	Advantages	Drawbacks
Adsorption	Removal of a wide variety of micropollutants	Difficult regeneration of adsorbent materials
		High costs related to the consumption and disposal of used adsorbents
Membrane filtration	Removal of a number of micropollutants including some pharmaceuticals	High costs of operation
		Membrane fouling
		Disposal issue of concentrated sludge
		Low removal of hydrophobic compounds
Coagulation-Flocculation	High removal of turbidity and colour	Ineffective removal of pharmaceutical compounds
	Increased sedimentation rate	Large generation of sludge
		Introduction of coagulant slats in the aqueous phase
Advanced oxidation processes (AOP)	High removal efficiency	High energy consumption
	Selective oxidation favouring disinfection properties	Formation of toxic by-products
		Interference of free radical scavengers
	Short degradation rate	
	Allows degradation and mineralization of pollutants	
Activated sludge	Low capital and operational costs	Low removal efficiency
		Generation of large amounts of sludge
Membrane bioreactor	Effective removal of hydrophobic compounds	High energy consumption
		Membrane fouling
	High quality effluent	Inefficient removal of hydrophilic and persistent compounds
	Small foot print	
		Post-treatment may be required

Although activated carbon is the most used adsorbent, new materials like graphene and carbon nanotubes have also been tested as a possible option for the adsorption of pharmaceutical micropollutants. Graphene presents higher specific surface area than activated carbon and has been proved to remove pharmaceutical compounds (Wang and Wang, 2016). Similarly, the excellent properties of carbon nanotubes led to several studies to investigate their potential for the adsorption of pharmaceuticals like carbamazepine (Ji et al., 2016b) and sulfamethoxazole (Ji et al., 2016a). As in the case of activated carbon, the adsorption capacity of these two materials also depends on the properties on the pollutants. Although these materials are considered as a promising technique for the removal of pharmaceutical compounds, research has to be done in order to investigate the feasibility of the technology at large scale application in continuous processes as well as with real wastewater (Wang and Wang, 2016). Table 2.6 summarizes the removal efficiency of selected pharmaceutical micropollutants via adsorption on different materials.

Table 2.6. Removal of selected pharmaceuticals through adsorption by different materials.

Compound	Type of adsorbent, dose adsorbent	Type of water	Influent concentration (µg L^{-1})	Removal (%)	Reference
AMX	GAC, 30 g L^{-1}	Real wastewater	317 000	95	Putra et al. (2009)
SMX	PAC, 1-20 mg L^{-1}	River water	na	20	Westerhoff et al. (2005)
	PAC, 5 mg L^{-1}	Surface water	0.1	35	Snyder et al. (2007)
	PAC, 50 mg L^{-1}	Real wastewater	0.6	60	Altmann et al. (2014)
	Graphene, 100 mg L^{-1}	Synthetic water	100	34	Yang and Tang (2016)
CIP	PAC, 8-43 mg L^{-1}	Hospital wastewater	15.7	>99	Michael et al. (2013)
CBZ	PAC, 5 mg L^{-1}	Surface water	0.1	70	Snyder et al. (2007)
	GAC, 10 mg L^{-1}	Synthetic water	0.5	100	Yu et al. (2008)
	Graphene	Synthetic water	10 000	97	Rizzo et al. (2015)

na : not available. GAC: granular activated carbon. PAC: powdered activated carbon

2.2.1.2 Membrane filtration

The use of membrane separation processes for water treatment has increased in the last decades. Among the different type of membranes available in the market, ultrafiltration (UF, pore size 10 nm), nanofiltration (NF, pore size 1 nm) and reverse osmosis (RO, pore size

<1 nm) membranes have been proved to effectively remove small size pollutants such as pharmaceutical compounds (Ganiyu et al., 2015). Removal of pharmaceuticals micropollutants in membrane separation can occur through various mechanisms (adsorption, steady state rejection, electrostatic effects) which are dependent on the physicochemical properties of the compound, the feeding solution and the membrane characteristics (Michael et al., 2013).

Overall, studies showed excellent removal yields for all the target pollutants (Košutić et al., 2007; Radjenović et al., 2008; Zhang et al., 2006). Several antibiotics including β-lactams, quinolones, tetracyclines and sulfonamides were highly removed (<90%) after combined treatment with micro filtration (MF) and RO (Watkinson et al., 2007). Kimura et al. (2004) tested two different RO membranes for the treatment of endocrine disrupting compounds and pharmaceutical active compounds obtaining high retention of SMX (70-82%) and CBZ (85-91%). Similarly, Morse and Jackson (2004) reported complete removal for AMX from wastewater by RO.

Despite the good removal reported for the target pharmaceuticals is important to remark that as in adsorption, this treatment process produces a new effluent (i.e. the retentate) where the contaminants are concentrated. Moreover the sensitivity of the membranes to temperature and the presence of organic compounds and dissolved salts in the feeding medium can cause membrane deterioration and fouling which usually decrease the effectivity of the process (Homem and Santos, 2011).

2.2.1.3 Coagulation and flocculation

Coagulation and flocculation represent an important part of conventional water and wastewater treatment plants and are typically applied before sedimentation and filtration in a process better known as clarification. Coagulation involves the addition of chemicals, usually aluminium and iron salts, which react with suspended and colloidal particles to form micro-particles by reducing their repulsive charges (Matilainen et al., 2010). Then, during the flocculation these micro-particles collide and form larger aggregates or flocs that are easily removed by sedimentation (Matilainen et al., 2010; Verma et al., 2012).

The combined process of coagulation-flocculation is usually used in water treatment to decrease turbidity and colour as well as for the removal of pathogens. However, this technology has been found inefficient for the removal of pharmaceutical pollutants. Poor removal could be related to the use of conventional coagulants which were not specifically designed to remove emerging organic micropollutants (Alexander et al., 2012). Nonetheless, partial removal of antibiotics in the range from 20 to 90% depending on the nature of the compound has been achieved using different coagulants (Choi et al., 2008; Stackelberg et al., 2007). For example, Stackelberg et al. (2007) reported that the addition of ferric chloride during the clarification process led to an average reduction of 15% in the concentration of 32 organic compounds, including nine pharmaceuticals. According to these authors, the moderate SMX removal observed could be attributed to hydrolysis reaction with the coagulant and the very low elimination of CBZ resulted of its sorption on suspended particle matter.

2.2.2 Advanced oxidation processes

Advanced oxidation processes (AOPs) represent a very effective alternative for the treatment of recalcitrant pharmaceutical micropollutants that are not removed by conventional water and wastewater treatment technologies. AOPs are oxidative methods based on the generation of radicals ($OH^{\bullet}$, $O_2^{\bullet-}$, $HO_2^{\bullet}$), mainly hydroxyl radicals ($OH^{\bullet}$) (Kanakaraju et al., 2018). These radicals are extremely reactive species that can successfully attack a broad range of organic and inorganic molecules leading to the oxidation or even mineralization of the pollutants (Ahmed et al., 2017; Rivera-Utrilla et al., 2013). Hydroxyl radicals can be generated from oxidizing agents such as ozone (O_3) or hydrogen peroxide (H_2O_2), often combined with metallic or semiconductor catalysts or UV radiation (Homem and Santos, 2011; Rivera-Utrilla et al., 2013). AOPs commonly applied for pharmaceutical water/wastewater treatment could be classified into three categories: photochemical processes, non-photochemical processes and hybrid or combined processes. Examples of the application of some of these processes for the treatment of the target pharmaceuticals are presented hereafter (Fig. 2.2).

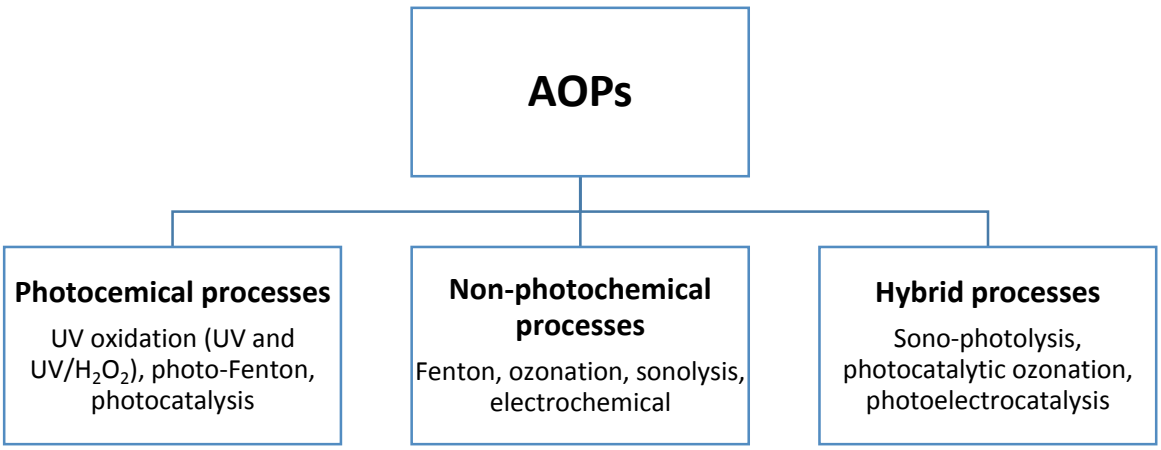


Fig. 2.2. AOPs technologies for removal of pharmaceutical micropollutants in wastewater.

Ozonation and combinations of O_3, with H_2O_2 (O_3/H_2O_2), and O_3 with UV (O_3/UV) have been applied to pharmaceuticals in water as single oxidation method, peroxidation or disinfection step before other treatments. For example, a study carried out by Alharbi et al. (2016) suggested that different doses of ozone could be required for the effective oxidation of four different pharmaceuticals (diclofenac, CBZ, SMX and trimethoprim). The treatment of pharmaceuticals including different classes of antibiotics (β-lactam, sulphonamides) with ozonation led to high removal yields (>50%); however, low mineralization of the pollutants was observed probably due to the presence of persistent by-products formed during the process (Akmehmet Balcıoğlu and Ötker, 2003; Almomani et al., 2013; Andreozzi et al., 2005). Ozonation process could be improved by the addition of H_2O_2 or UV irradiation as demonstrated by Drewes et al. (2008) who used a lab-scale and full-scale O_3/H_2O_2 systems for the removal of several pharmaceuticals including SMX obtaining more than 90% degradation after 2 min contact time.

The application of UV radiation during disinfection step in water treatment processes can lead also to the degradation of pharmaceutical micropollutants. In this method removal efficiencies will depend on the chemical structure and the UV absorption sensitivity of the pharmaceuticals (Kanakaraju et al., 2018). For example, Drewes et al. (2008) reported poor removal for SMX (25-50%) during UV disinfection step. The poor removal was attributed to the characteristics of the water matrix containing dissolved organic carbon that competed for the low UV radiation during the treatment. To overcome this issue, the combination of UV with H_2O_2 (UV/H_2O_2) usually leads to better removals for pharmaceuticals with poor UV

absorption. Enhancement of AMX degradation was reported when UV/H_2O_2 (10 mM H_2O_2) was applied in comparison to only UV treatment (Jung et al., 2012). Vogna et al. (2004) found that UV/H_2O_2 irradiation allowed complete transformation of CBZ after 4 min treatment although low removal (35%) of total organic carbon (TOC) was observed. Borikar et al. (2015) performed pilot-scale experiments in a water treatment plant and compared the efficiency of ozone/H_2O_2 and UV/H_2O_2 processes for the degradation pharmaceuticals obtaining high removal yields for both technologies (92-97%). Among the tested compounds, CBZ, fluoxetine, naproxen and gemfibrozil showed the highest removal with almost complete degradation.

Regarding to Fenton and photo-Fenton oxidation, both processes have been found to be effective for the degradation of pharmaceuticals (Ahmed and Chiron, 2014; Arslan-Alaton and Dogruel, 2004; Ay and Kargi, 2010). Oxidation of AMX by photo-Fenton process was not influenced by the source of irradiation as demonstrated by Trovó et al. (2008) achieving 85 and 89% removal respectively, under black and solar irradiation. Another investigation used doses higher that 5 mg L^{-1} of ferrate (Fe(VI)) for the partial elimination of SMX and CIP (85%) (Lee et al., 2009). Complete removal of CBZ (50 μM) in WWTP effluent was reported when solar photo-Fenton was applied during 30 min (Ahmed and Chiron, 2014). Although high removal yields that can be obtained by the application of Fenton processes, formation of toxic by-products can result from the treatment which could represent a serious drawback for the use of this technology at full scale (Wang and Wang, 2016).

Among the less known AOPs, sonolysis treatment was applied for CIP degradation that was better removed when low frequency was applied (544 kHz) (De Bel et al., 2011). Electrochemical oxidation using TiO_2 anode reported 80% oxidation of pharmaceutical micropollutants including SMX and CBZ. Nonetheless, when ozonation or UV/H_2O_2 systems were applied to these pharmaceuticals, complete removal was observed in shorter contact times in comparison to TiO_2 treatment (Andreozzi et al., 2004).

2.2.3 Biological treatment processes

2.2.3.1 Conventional activated sludge

Conventional activated sludge (CAS) is probably the most common biological treatment used in urban WWTPs. It is intended for the removal of organic matter based on

biological processes (i.e. aerobic, anaerobic systems) (Tran et al., 2018). CAS uses dissolved oxygen to promote the growth of microorganisms that can degrade pollutants at specific operational conditions (Ahmed et al., 2017; Michael et al., 2013). However, this treatment is usually not efficient enough for the removal of persistent micropollutants due to their recalcitrant nature and low quantity.

Elimination and transformation of ECs during CAS is the result of two major processes: sorption and biodegradation (Grandclément et al., 2017). Metabolism and co-metabolism are the mechanisms through which biodegradation can take place, co-metabolism being the most likely to occur since many ECs are toxic or resistant to microbial activity (Tran et al., 2018). Moreover, micropollutants are present in wastewater at too low concentrations to be used as direct growth substrate. Thus, biodegradation of pharmaceuticals in CAS depends on the composition of the microbial community, in this case microorganisms involved in co-metabolic degradation, as well as on the presence of primary growth substrates (organic carbon, ammonium, etc.) (Tran et al., 2013). For example, ammonia-oxidizing bacteria involved in nitrification process could enhance the removal of pharmaceuticals and pesticide micropollutants by means of co-metabolic process as demonstrated by Margot et al. (2015b).

As mentioned before, sorption onto activated sludge can be a significant removal pathway for some compounds. The tendency of micropollutants to accumulate in sludge is related to their physicochemical properties and can be evaluated using either sludge sorption constant (K_d) or the octanol-water partition coefficient (K_{ow}). In general, the higher K_d (>3.2) or K_{ow} values (>4.0) the higher sorption of the compounds to sludge (Joss et al., 2006; Rogers, 1996). However, this prediction may not be accurate for polar or charged compounds such as fluoroquinolone antibiotics for which electrostatic interactions with sludge could result as the main reason for their removal during CAS (Golet et al., 2003).

The combined effect of biodegradation and adsorption CAS systems can result in a wide range of removal efficiencies for ECs as shown in Fig. 2.3. Pharmaceuticals including antibiotics (AMX, CIP, SMX), anti-inflammatory drugs (ibuprofen, naproxen, salicylic acid), hormones (estrone, estriol) showed high removal (>80%) during biological treatment. In contrast, compounds like CBZ seemed to be resistant to biological treatment with average removal below 40%. It is worth noting that negative removals for some micropollutants after

CAS have been observed as result of different processes occurring during treatment. The enzymatic cleavage of metabolites and conjugates which transforms them back into the parent compounds and/or the release of compounds initially trapped in faecal particles during the decomposition of the latter lead to an increase in the concentration in effluents after treatment (Göbel et al., 2007; Grandclément et al., 2017; Verlicchi et al., 2012). Moreover, operational parameters such as sludge retention time (SRT), hydraulic retention time (HRT) and reactor design affect the performance of CAS leading to important variations in removal yields between WWTPs (Grandclément et al., 2017). For example, short SRT and low biomass concentration could lead to partial degradation of micropollutants (Tiwari et al., 2017). Nonetheless, these problems can be overcome by the use of other biological treatment technologies such as membrane bioreactors.

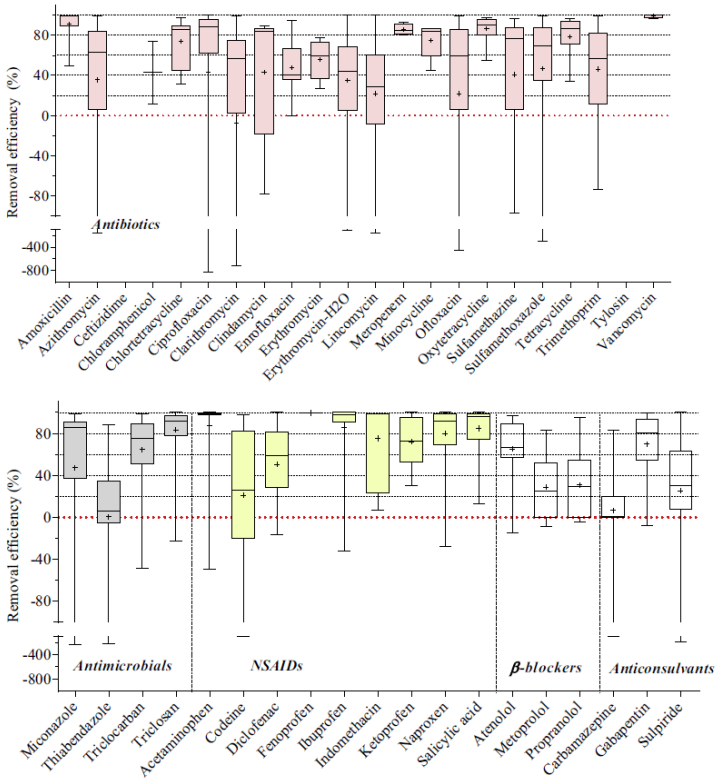


Fig. 2.3. Box–and-whisker plots showing variation in removal efficiencies of various pharmaceuticals in biological wastewater treatment processes. (Tran et al., 2018; with permission).

2.2.3.2 Membrane bioreactor

Membrane bioreactor (MBR) technologies couple activated sludge treatment with a membrane separation process in order to retain the sludge in the system, thus promoting the microbial degradation of organic matter and, in some cases, micropollutants (pharmaceuticals). Typically, in a MBR the membrane module (hollow fibers or flat membranes) is submerged inside the reactor tank conforming a single unit (de Cazes et al., 2014). MBRs are seen as more efficient systems for the removal of micropollutants as they present some advantages in comparison to CAS such as low space requirements, increased SRT in smaller reaction volumes and the almost inexistent presence of suspended solids in the permeate. Nonetheless, in order to achieve such a good performance continuous aeration in the lower part of the membrane is generally necessary in order to ensure oxygen saturation as well as to create turbulence to reduce as possible the membrane clogging and fouling which are the biggest disadvantages of this technology (de Cazes et al., 2014). In addition, MBR operation can be also affected by the sludge age and concentration, composition of wastewater, conductivity, operating temperature and pH (Ahmed et al., 2017).

Regarding to the effectiveness of MBRs for the removal of pharmaceutical micropollutants mixed performance was observed, varying from almost complete removal for some pharmaceuticals to almost negligible degradation for other compounds. Snyder et al. (2007) used a MBR for the treatment of primary effluent of a WWTP finding it effective for the removal of several pharmaceutical micropollutants including caffeine, acetaminophen and SMX. Similarly, Clara et al. (2005) tested a MBR at pilot scale for the removal of pharmaceuticals, musk fragrances and endocrine disrupting chemicals (EDCs) at different SRT and compared the result to the performance of CAS. Compounds like CBZ were not removed by CAS or MBR treatments, while ibuprofen or benzafibrate were highly eliminated (90%). Interestingly, Clara found no difference in treatment efficiencies between MBR and CAS. In the same line, a lab scale MBR was applied for the removal of polar pollutants from a municipal wastewater facility. MBR was found to enhance by 20-50% the degradation of pharmaceuticals showing medium removal (15-80%) in CAS treatment. Nonetheless, in the case of well degradable or recalcitrant pollutants removals were not improved by the MBR process (Weiss and Reemtsma, 2008). Actually, this observation was

reported in different studies where recalcitrant compounds such as CBZ were not eliminated at all after MBR treatment (Bernhard et al., 2006; Radjenović et al., 2009). Table 2.7 present a comparison between the removal efficiency and removal mechanism of target pharmaceuticals in MBR and CAS processes.

Table 2.7. Comparison of removal efficiency of target pharmaceuticals in CAS and MBR processes. (Adapted from Tiwari et al., 2017; with permission).

Pharmaceutical	Removal MBR (%)[a,c]	Removal CAS (%)[a,b]	Biodegradation (%)[c]	Sorption (%)[b,c]
AMX	>75	>75	>80	-
SMX	66	51.9	50-90	1
CIP	89	-	0	98
CBZ	28	<25	<40	<5

a: (Le et al., 2018); b: (Behera et al., 2011); c: (Kim et al., 2014)

2.2.3.3 Enzymatic treatment

Enzymes are versatile bio-catalysts that in recent years have attracted attention as an interesting option for the degradation of persistent pollutants. Generally, enzymatic processes are easy to control and can be operated over a broad range of conditions with low energy consumption (Torres et al., 2003). Enzymes catalyse specific reactions and mostly act under mild conditions (pH, temperature, solvents and ionic strength) which results in low environmental impact (Demarche et al., 2012). Moreover, the high selectivity and specificity of enzymes prevent undesired side reactions, thus making the treatment more cost effective (Demarche et al., 2012). Enzymatic treatment involves chemical processes based on the action of biological catalysis which render several advantages in comparison to conventional physico-chemical and biological processes. These biocatalysts can effectively treat environmental pollutants over a wide range of concentrations at a rapid rate with a lower risk of being inhibited by the effect of these compounds, although such inhibition is more likely to occur at high pollutant concentrations (Karam and Nicell, 1997). Thus, enzymes represent a promising tool for the selective removal of pharmaceutical pollutants from waste streams.

Various enzymes have been described to potentially remove hazardous compounds from the environment. According to Demarche et al. (2012), enzymes like hydrolases (EC3:

lipases, esterases, cellulases), oxidoreductases (EC1: peroxidases, phenoloxidases) and lyases (EC4: decarboxylases, dehydratases) could be used for the biotransformation of pollutants. For example, hydrolases can be used as a pre-treatment for the degradation of biopolymers, whilst oxidoreductases could be applied to attack specific pollutants of different nature (Demarche et al., 2012; Nyanhongo et al., 2007). Oxidoreductases are extracellular enzymes that are generally produced by the white-rot fungi (WRF) for the degradation of lignin, the structural polymer found in wood. This consortium of oxidoreductase enzymes, also known as lignin modifying enzymes (LME), includes lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccase (Mester and Tien, 2000; Naghdi et al., 2018b). These enzymes act by generating highly reactive and non-specific radicals that randomly attack lignin molecules. In this way, LME can be used for the treatment not only of single molecules but also of mixtures, which perhaps is their greatest advantage (Mester and Tien, 2000). Therefore, different studies have reported the use of LME for the biotransformation of various ECs including pharmaceutical compounds such as carbamazepine, diclofenac, naproxen, sulfamethoxazole and tetracyclines (Auriol et al., 2008; Eibes et al., 2011; Melo and Dezotti, 2013; Torres et al., 2003; Wen et al., 2009; Zhang and Geißen, 2010). Further discussion on the properties of oxidative enzymes, with special focus on the application of laccases, is presented in the next section.

2.3 Oxidative enzymes

The oxidative enzymes secreted by WRF can be classified into two main groups: peroxidases and phenol oxidases.

2.3.1. Peroxidases

Peroxidases are heme-containing enzymes which require the presence of peroxides such as hydrogen peroxide (H_2O_2) to catalyse a variety of reactions (Conesa et al., 2002). The catalytic cycle of peroxidases is a three-step reaction (Fig. 2.4). The native enzyme (Fe^{3+}) is oxidized by hydrogen peroxide. This produces water and the oxidized form of peroxidase, called Compound I (Fe^{4+} $-R^{+\bullet}$). Then, Compound I oxidizes one molecule of substrate, resulting in a substrate radical and Compound II (Fe^{4+}). Compound II oxidizes a second

substrate molecule, leading to a second substrate radical and the native peroxidase (reduced state Fe^{3+}) (Conesa et al., 2002; Nakayama and Amachi, 1999; Torres et al., 2003).

Peroxidases are differentiated according to their substrate specificity. Manganese peroxidase (MnP) uses Mn(II) as reducing substrate, whereas lignin peroxidase (LiP) oxidizes non-phenolic and phenolic compounds. Anyhow, the broad substrate observed for these enzymes is the result of their structural properties and high redox potential (1 V) (Demarche et al., 2012; Mester and Tien, 2000).

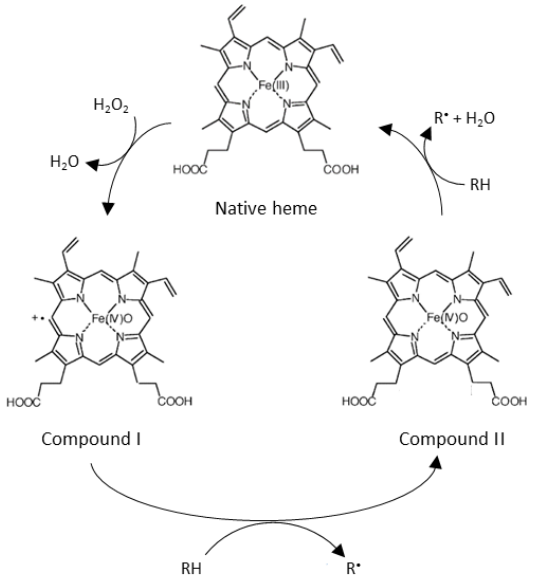


Fig. 2.4. Catalytic cycle of peroxidases (Adapted from Torres et al., 2003; with permission).

2.3.1.1 Lignin peroxidase (LiP; EC 1.11.1.14)

Lignin peroxidase was the first LME discovered in the fungus *Phanerochaete chrysosporium* (Glenn et al., 1983). LiP is a monomeric glycol protein with molecular mass around 40 kDa with a heme group in its active site (Hammel and Cullen, 2008). Thus, this enzyme follows the classical catalytic mechanism of peroxidases which requires peroxide as electron acceptor for the oxidation of phenolic and aromatic non-phenolic compounds with similar structure to lignin. Enzymatic reactions catalysed by LiP include benzylic alcohol oxidations, side-chain cleavages, ring-opening reactions, demethoxylations and oxidative dechlorinations. In this way LiP is able to oxidize substrates with high redox potential such as polycyclic aromatic compounds, polychlorinated byphenyls and dyes (Wesenberg et al., 2003).

2.3.1.2 Manganese peroxidase (MnP; EC 1.11.1.13)

Manganese peroxidase enzyme is also a heme glycoprotein that can catalyse the oxidation and depolymerisation of natural substrates like lignin as well as recalcitrant xenobiotics in the presence of peroxide (Wong, 2009). However, MnP is unique among peroxidases because it prefers Mn^{2+} as its reducing substrate. Following the classical peroxidase catalytic mechanism (Fig. 2.5), Mn^{2+} is oxidized by both MnP I and MnP II compounds, resulting in the formation of Mn^{3+}. Then, Mn^{3+} which is subsequently stabilized by chelation with organic acids (i.e. oxalate) produced by the fungi itself diffuses from the enzyme to oxidize substrates (Conesa et al., 2002). Although compound MnP I is also able to oxidize phenolic compounds (e.g. guaiacol) its efficiency is lower in comparison to MnP II compound (Mester and Tien, 2000).

$$MnP+H_2O_2 \rightarrow MnP\ I+ H_2O$$
$$MnP\ I+Mn^{2+} \rightarrow MnP\ II+ Mn^{3+}$$
$$MnP\ II+Mn^{2+} \rightarrow MnP+ Mn^{3+}+H_2O$$
$$Mn^{3+}+RH \rightarrow Mn^{2+}+R + H^{+}$$

Fig. 2.5. Catalytic cycle of manganese peroxidase

2.3.1.3 Versatile peroxidase (VP; EC 1.11.1.16)

Versatile peroxidase has been considered as a hybrid between MnP and LiP since it combines the substrate specificity of both peroxidases (Wesenberg et al., 2003). VP is able not only to oxidize Mn^{2+} but also phenolic and non-phenolic aromatic compounds that are typical substrates for LiP in the absence of manganese (Wong, 2009).

2.3.2 Phenol oxidases

Phenol oxidases are another group of oxidative enzymes that catalyse the oxidation of phenolic compounds by only using molecular oxygen. They are subdivided into two subgroups: tyrosinases and laccases.

2.3.2.1 Tyrosinase (Tyr; EC 1.10.3.1)

Tyrosinase is a copper-containing protein known as monophenol oxidase or catecholase. Although it shares most of the substrates with laccases, the ability to oxidize tyrosine is exclusive to this enzyme (Demarche et al., 2012). Typically, tyrosinases catalyse two consecutive reactions: (i) the hydroxylation of monophenols with molecular oxygen to form *o*-diphenols; and (ii) the dehydrogenation of *o*-diphenols with oxygen to form *o*-quinones. Quinones are mostly unstable and undergo non-enzymatic polymerization to yield water insoluble substances that can be easily removed by simple filtration (Karam and Nicell, 1997).

2.3.2.2 Laccase (EC 1.10.3.2)

Laccases represent the largest subgroup of blue multicopper oxidases (Giardina et al., 2010) firstly detected in the Japanese tree *Rhus vernicifera* (Kim et al., 2010). Since then, they have been found widely distributed in higher plants, bacteria, insects and fungi (Brijwani et al., 2010; Giardina et al., 2010).

Laccases are characterized for being dimeric or tetrameric glycoproteins that usually contain four copper atoms organized into the active site (Durán et al., 2002; Riva, 2006). Using these redox active copper atoms laccases catalyse the oxidation of a wide variety of substrates, such as phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, aromatic diamines, hydroxyindiols, benzenethiols, inorganic/organic metal compounds and many others, by only using molecular oxygen that is reduced to water (Gasser et al., 2014; Giardina et al., 2010; Kunamneni et al., 2007). Structurally talking, the active center of laccases contains four atoms of copper which are classified into three groups according to their different spectroscopy characteristics (Fig. 2.6): Type 1 (T1) site copper atom absorbs intensely at 600 nm and is responsible for the blue colour, Type 2 (T2) normal copper invisible in the UV-Vis absorption spectrum and the Type 3 (T3) coupled binuclear copper center which absorbs markedly at 330 nm (Claus, 2004; Santhanam et al., 2011). The T1 copper center acts as the primary electron acceptor site where the enzyme catalyses the oxidation of substrates. The T2 and T3 copper centers form a trinuclear copper cluster site where molecular oxygen is reduced to water by accepting the electrons transferred from the T1 site (Polak and Jarosz-Wilkolazka, 2012; Torres et al., 2003).

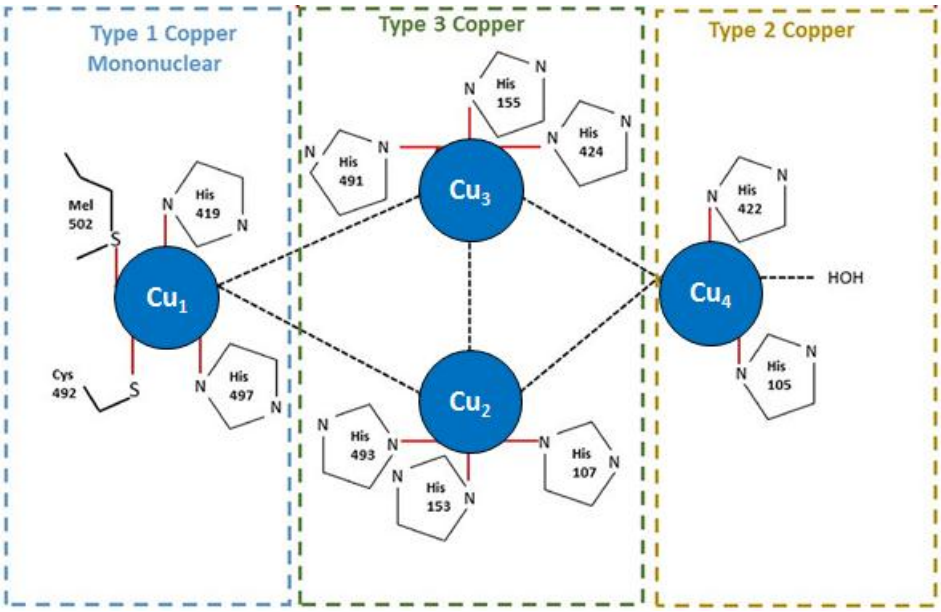


Fig. 2.6. Schematic representation of the copper center in laccase (Adapted from Barrios-Estrada et al., 2018; with permission).

The mechanism of catalysis followed by laccase involves mainly three steps: (i) reduction of the T1 copper site by the substrate, (ii) electron transfer from the T1 site to the T2/T3 copper cluster, and (iii) the reduction of molecular oxygen by the T2/T3 copper sites. This mechanism involves a four-electron reduction of O_2 to water as shown in Fig. 2.7. In the resting state the enzyme is completely oxidized; then, a total reduction of the copper centers (Cu^{2+} to Cu^{+}) by the substrate occurs in T1 site. Electrons are extracted individually from the reducing substrate and transferred to the T2/T3 cluster site resulting in the conversion of the resting form of the enzyme to a fully reduced state. Then, reduction of dioxygen takes place at the T2/T3 cluster site in two steps via the formation of peroxide intermediates and their subsequent reduction to water. In the last step, all four copper centers are oxidized, and O^{2-} is released as a second water molecule. (Wong, 2009). The net result is the oxidation of four molecules of substrate to produce four radicals while reducing one molecule of oxygen to two molecules of water. The radicals formed can then undergo non-enzymatic reactions including hydration, hydrogen abstraction or polymerization for synthesizing new molecules with valuable functions (Claus, 2004; Kudanga et al., 2011; Wesenberg et al., 2003). Laccase attack can also result in the decarboxylation of phenol and methoxyphenolic acids or the demethylation of methoxyl groups. Dehalogenation of

substituents located in the ortho or phara position may also take place in the case of substituted compounds (Majeau et al., 2010).

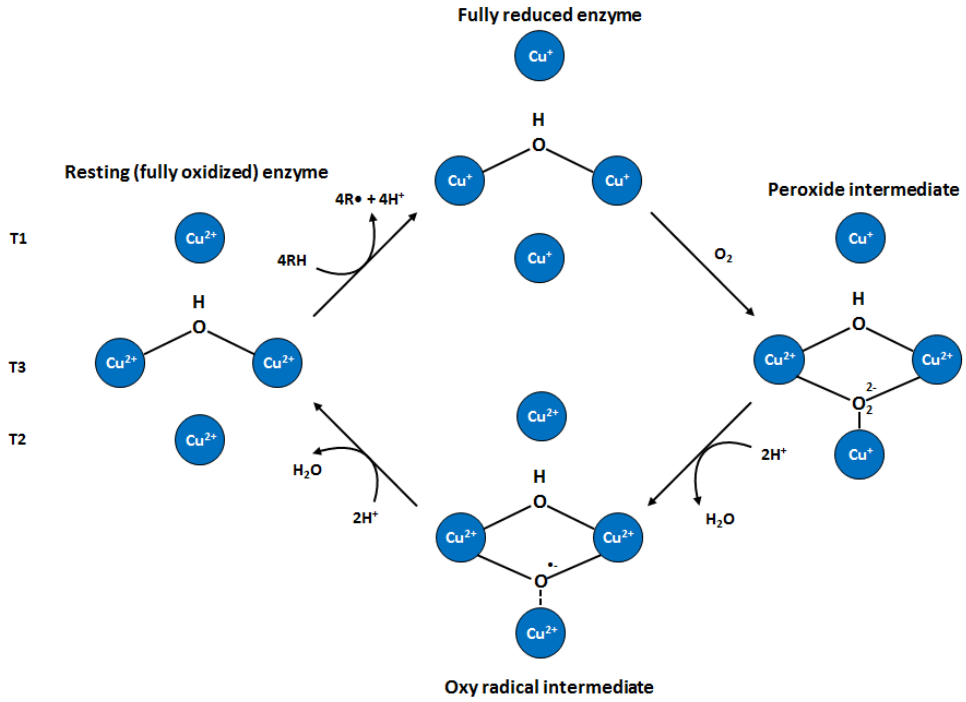


Fig. 2.7. Catalytic cycle of laccase (Adapted from Wong, 2009; with permission).

The effectiveness of laccase for the oxidation of substrates depends on the difference of redox potentials between the T1 copper and the substrate. The pH of the medium also plays a significant role in the enzymatic catalysis reaction because it does not only affects the laccase activity but also the redox potential of the substrate (Polak and Jarosz-Wilkolazka, 2012). Thus, laccases are unable to directly oxidize substrates presenting significantly higher redox potentials (>1.3 V). Additionally, steric hindrance issues could also impede catalytic oxidation of certain substrates (Kudanga et al., 2011). However, oxidation of recalcitrant substrates by laccase can be made possible by the addition of mediators: a group of low-molecular weight organic compounds that after oxidation by laccase, form highly active radicals capable of oxidizing substrates that laccase alone cannot oxidize (Brijwani et al., 2010; Kunamneni et al., 2007; Majeau et al., 2010). The factors affecting laccase oxidation of substrates will be further discussed in the next section.

Overall, the wide substrate range observed in laccases as well as their simple requirements for catalysis make these enzymes suitable and attractive for bioremediation

applications. Therefore, in this investigation laccases were selected to carry out the biotransformation of pharmaceutical micropollutants in synthetic water.

2.4 Application of laccase for pharmaceuticals removal

In this section, the use of laccases for pharmaceutical micropollutants removal is presented. The bibliographic review will focus on the application of laccases for the biotransformation of pharmaceutical compounds from two perspectives: (i) using free laccase in form of crude culture extract or pure enzyme, and (ii) using immobilized laccase.

2.4.1 Removal by free laccase

2.4.1.1 Crude enzyme

The term crude enzyme refers to the direct use of enzymes harvested from microbial cultures. The use of extracellular extracts for the degradation of pharmaceutical compounds instead of living cells may be advantageous in terms of a more controllable and targeted treatment, reaching high reaction kinetics and avoid the addition of nutrients or waiting for the growth of cells (Naghdi et al., 2018b; Stadlmair et al., 2018). Therefore, several research groups have devoted efforts to assess the role of extracellular enzymes in the biotransformation of pharmaceutical compounds. Table 2.8 shows some examples of recent studies reporting the degradation of pharmaceuticals by crude laccase. Guo et al. (2014) used crude enzyme of *Phanerochaete chrysosporium* at different concentrations for the degradation of SMX. Degradation yields up to 50% were obtained when laccase was added at a concentration of 6076 U L^{-1}; however, it was observed that SMX degradation was not directly dependent on laccase activity and similar removal could be achieved at lower laccase concentration. Similarly, the potential of laccase from a *Pycnoporus sanguineus* culture extract was evaluated for the degradation of the antibiotics SMX, CIP, and norfloxacin (NOR) in single and mixture solutions. *In vitro* assays showed CIP and NOR were resistant to laccase degradation, whereas SMX concentration decreased by 29% after 72 h of treatment both in single and mixture reactions (Gao et al., 2018). Low removal of antibiotics was also reported by Suda et al. (2012) who tested a cell free extract from *Trametes versicolor* for the oxidation of four tetracyclines (tetracycline, chlortetracycline, doxycycline and oxytetracycline) obtaining removals in the range from 14-48% within 4 h.

Tran et al. (2010) used a crude enzyme solution with an initial laccase activity of 1500 U L^{-1} for the degradation of ten pharmaceutical compounds at environmentally relevant concentrations (10 µg L^{-1}). Complete removal of diclofenac, naproxen and indomethacin was observed within 12 h of incubation, while partial degradation of the rest of the compounds was observed. In a study carried out by Cabana et al. (2009) the degradation of triclosan (TCS) and other two endocrine disrupting chemicals by and enzymatic preparation from *Coriolopsis polyzona* was assessed. Authors observed the degradation efficiency of the micropollutants significantly varied depending on the compound. Moreover, reaction conditions (pH and temperature) had an important influence in the removal process so that 65% of TCS (5 mg L^{-1}) disappeared after 4 h treatment when the reaction was carried out at pH 5 and 50°C. In the same line, operational conditions were optimized in the attempt to maximize the oxidation of the anti-epileptic CBZ by *Trametes versicolor* crude laccase. The effect of temperature, pH and enzyme concentration were studied by a response surface methodology finding that performing the biotransformation at 35°C and pH 6 with 60 U L^{-1} of enzyme led to 30% removal of the recalcitrant compound (Naghdi et al., 2018a).

These results suggest that the performance of laccase to catalyse the degradation of pharmaceuticals depend on different factors such as substrate type, reaction conditions and enzymes properties. As observed in Table 2.8, the treatment of the same compound by enzymes from different sources can lead to different removal efficiencies. For example, complete degradation of diclofenac was observed with laccase from *Trametes versicolor*, whilst only 50% was attained when *Pycnoporus sanguineus* laccase was employed. However, it is important to consider that in order to properly compare the performance of different enzymes for the degradation of pharmaceutical(s) the operational conditions must be also comparable which is not often reported in literature. Anyhow, using crude enzyme solution brings some advantages. The utilization of crude enzyme without further enzyme purification could reduce the cost of the treatment process. Moreover, the possible presence of natural compounds in the crude solution produced during fungi growth may act as mediators favouring the degradation of some pharmaceuticals (Tran et al., 2010).

Table 2.8. Biotransformation of pharmaceutical micropollutants by crude laccase.

Pharmaceutical	Laccase source	Concentration (mg L^{-1})	Reaction conditions	Efficiency (%)	Reference
Carbamazepine	*Trametes versicolor*	1	60 U mL^{-1} of laccase, 10 mL reaction, pH 6, 150 rpm, 24 h	30	(Naghdi et al., 2018a)
Chlortetracycline	*Trametes versicolor*	5	10 nkat mL^{-1}, 0.5 mL reaction, pH 4.5, 30°C, 150 rpm, 4 h	48	(Suda et al., 2012)
Chloramphenicol	*Trametes hirsuta*	0.5	100 U of laccase, pH 5, 7 days	100	(Navada and Kulal, 2019)
Diclofenac	*Trametes versicolor*	0.5	500 U L^{-1} of laccase, 100 mL reaction, pH 4.5, 50°C, 150 rpm, 5h	99	(Lonappan et al., 2017)
	Pycnoporus sanguineus	10	100 U L^{-1} of laccase, pH 5, 25°C, 8 h	50	(Rodríguez-Delgado et al., 2016)
	Trametes versicolor	0.01	1500 U L^{-1} of laccase, 10 mL reaction, pH 4.5, 30°C, 125 rpm, 12 h	100	(Tran et al., 2010)
Ibuprofen	*Trametes versicolor*	0.01	1500 U L^{-1} of laccase, 10 mL reaction, pH 4.5, 30°C, 125 rpm, 12 h	15	(Tran et al., 2010)
Naproxen	*Trametes versicolor*	0.01	1500 U L^{-1} of laccase, 10 mL reaction, pH 4.5, 30°C, 125 rpm, 12 h	100	(Tran et al., 2010)
Sulfamethoxazole	*Pycnoporus sanguineus*	10	170 U L^{-1} of laccase, 25 mL reaction, 72 h	29	(Gao et al., 2018)
Sulfamethoxazole	*Phanerochaete chrysosporium*	10	96-6076 U L^{-1} of laccase, 5 mL reaction, pH 4.5, 30°C, 48 h	≤ 50	(Guo et al., 2014)
Tetracycline	*Trametes versicolor*	5	10 nkat mL^{-1} of laccase, 0.5 mL reaction, pH 4.5, 30°C, 150 rpm, 4h	16	(Suda et al., 2012)
Triclosan	*Coriolopsis polyzona*	5	100 U L^{-1} of laccase, pH 5, 50°C, 8h	65	(Cabana et al., 2007)

2.4.1.2 Purified enzyme

Purified laccases obtained from different strains of WRF have been used for the removal of pharmaceutical from water as shown in Table 2.9. Tran et al. (2010) used a commercial purified laccase from *Trametes versicolor* for the degradation of ten pharmaceutical micropollutants in spiked buffered water at pH 4.5. Similarly, to the results obtained with crude enzyme, purified laccase allowed fast and complete transformation of diclofenac, naproxen and indomethacin. High removal (>90%) of acetaminophen, imipramine, ketoconazole and TCS was achieved when purified laccase from *Pleurotus ostreatus*, *Paraconiothyrium variable*, *Trametes versicolor* and *Pycnoporus sanguines* were used (Ramírez-Cavazos et al., 2014; Stadlmair et al., 2017; Tahmasbi et al., 2016; Yousefi-Ahmadipour et al., 2016). In contrast, Murugesan et al. (2010) reported that only 57% of TCS was transformed by laccase from *Ganoderma lucidum*. A commercial laccase purified from *Myceliophthora thermophila* has been reported to remove 65% and 40% of diclofenac (5 mg L^{-1}) at pH 4 and 5, respectively, but no significant removal of naproxen was observed (Lloret et al., 2010).

The effect of treatment conditions on the oxidation of diclofenac, mefenamic acid and TCS by commercial *Trametes versicolor* laccase was investigated by Margot et al. (2013). Experiments were carried out in spiked solutions at different pH values, enzyme concentrations, reaction times and temperatures. Results showed that the optimal conditions depended on the compound and that even if all the parameters considered were significant, pH had the greatest influence on the process performance. Thus, pH values between 4.5 to 6.5; temperatures above 25°C and 730 U L^{-1} of laccase led to removal yields higher than 90%. Interestingly, authors observed that the presence of other pharmaceuticals in the reaction mixture influenced the degradation. For example, the removal of diclofenac increased from 25 to 95% when mefenamic acid was also in the reaction medium. Low removal of different antibiotics was observed with the use of purified laccase. De Cazes et al. (2014) reported about 30% elimination of tetracycline treated with commercial *Trametes versicolor* laccase after 24 h reaction. In another work by Prieto et al. (2011), the same commercial purified laccase hardly reached 16% degradation of CIP (5 mg L^{-1}). Table 2.9 shows that not only antibiotics showed recalcitrance to laccase degradation since CBZ was also poorly degraded (<38%) irrespective to the type of laccase used (Nguyen et al., 2014b;

Tran et al., 2010). The inability of laccase to degrade compounds like CBZ has been related to the presence of functional groups (amide) in its structure that impede laccase attack (Yang et al., 2013).

Overall, the results from the available studies demonstrate that laccases, either in crude or purified forms, can degrade a wide variety of pharmaceutical compounds at a broad range of operational conditions. However, most of the investigations cited here were performed a laboratory scale, in synthetic water and low reactional volumes which makes difficult to transpose these results to real wastewater applications. Moreover, some other considerations need to be taken into account for the potential application of these biocatalyst at higher scale such as the non-reusable nature of the enzyme, the possible inactivation due to environmental conditions or their reduced capacity to degrade compounds with considerable higher redox potential (Barrios-Estrada et al., 2018; Gasser et al., 2014). To overcome these limitations new treatment systems including the addition of redox mediators and the immobilization of the enzyme have been developed. In the next sections these options will be explored in more detail.

Table 2.9. Biotransformation of pharmaceutical micropollutants by purified laccase.

Pharmaceutical	Laccase source	Concentration (mg L^{-1})	Reaction conditions	Efficiency (%)	Reference
Acetaminophen	*Pleurotus ostreatus*	0.001	1.5 µM of laccase, 1.5 mL reaction, pH 7.4, 25°C, 20 min	100	(Stadlmair et al., 2017)
Carbamazepine	*Asperguillus oryzae*	5	90 U of laccase, 200 mL reaction	10	(Nguyen et al., 2014b)
	Trametes versicolor	0.01	6000 U L^{-1} of laccase, 10 mL reaction, pH 4.5, 30°C, 125 rpm, 3 h	38	(Tran et al., 2010)
Ciprofloxacin	*Trametes versicolor*	10	16.7 nKat mL^{-1} of laccase, 30°C, 150 rpm, 20 h	16	(Prieto et al., 2011)
Diclofenac	*Asperguillus oryzae*	5	90 µM min^{-1} of laccase, 200 mL reaction	21	(Nguyen et al., 2014b)
	Myceliophthora thermophila	5	2000 U L^{-1} of laccase, 20 mL reaction, pH 4, 25°C, stirring, 8h	65	(Lloret et al., 2010)
	Pleurotus ostreatus	0001	1.5 µM of laccase, 1.5 mL reaction, pH 7.4, 25°C, 20 min	40	(Stadlmair et al., 2017)
	Trametes versicolor	0.01	6000 U L^{-1} of laccase, 10 mL reaction, pH 4.5, 30°C, 125 rpm, 3 h	100	(Tran et al., 2010)
Ibuprofen	*Trametes versicolor*	0.01	6000 U L^{-1} of laccase, 10 mL reaction, pH 4.5, 30°C, 125 rpm, 3 h	38	(Tran et al., 2010)
Imipramine	*Paraconiothyrium variable*	0.12	1.63 U of laccase, 5 mL reaction, pH 4.9, 37°C, stirring, 5.7 h	99	(Tahmasbi et al., 2016)
Ketoconazole	*T versicolor*	300	1 U mL^{-1} of laccase, pH 4.5, 45°C, 6h	98	(Yousefi-Ahmadipour et al., 2016)
Sulfamethoxazole	*Asperguillus oryzae*	5	90 µM min^{-1} of laccase, 200 mL reaction	9	(Nguyen et al., 2014b)
Tetracycline	*Trametes versicolor*	20	0,01 g L^{-1} of laccase, 100 mL reaction, pH6, 25°C, 24 h	30	(De Cazes et al., 2014)
Triclosan	*G lucidum*	0.05	5 U of laccase, 1 mL reaction, pH 4, 30°C, 24 h	57	(Murugesan et al., 2010)

2.4.1.3 Laccase mediator system

The effectiveness of laccase to catalyse the oxidation of pharmaceutical compounds is highly dependent on the redox potential of the enzyme. Moreover, the functional groups within the micropollutant structure also have an effect on the extent of their degradation by laccase. The presence of electron-donating groups (EDG) (e.g. amine (-NH_2), hydroxyl (-OH), alkoxyl (-RO), alkyl (-R), acyl (-COR)) or electron withdrawing groups (EWG) (e.g. amide (-CONR2), carboxylic (-COOH), halogen (-X) and nitro (-NO_2)) could facilitate or oppose resistance to laccase attack (Yang et al., 2013). Thus, poor degradation of non-phenolic pharmaceutical micropollutants has been generally attributed to the presence of EWG in the molecular structure and to higher redox potential compared to laccase.

Degradation efficiency of pharmaceutical micropollutants can be enhanced by using redox mediators, in the so-called laccase-mediator system, that act as electron shuttles between the target compounds and the enzyme. In a laccase mediator system, the enzymatic reaction becomes a two-step process (Fig. 2.8). First, the redox mediator reacts with the enzyme and generates strong oxidizing intermediates that then diffuses away from the enzymatic pocket and, by means of non-enzymatic mechanisms, enable the oxidation of compounds that in principle are not laccase substrates (Cañas and Camarero, 2010).

Many different low molecular weight molecules have been used as mediators. The artificial mediators include compounds such as 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), violuric acid (VA), N-hydroxyphtalamide (HPI), 2,2',6,6'-Tetramethylpiperidine-N-oxyl (TEMPO) (Kunamneni et al., 2008). Moreover in recent years, the use of natural compounds generated during the degradation of lignin by WRF have been also investigated for their potential application in bioremediation processes (Camarero and Ibarra, 2005; Cañas and Camarero, 2010).

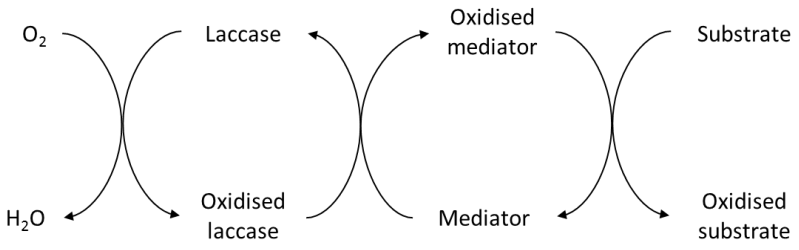


Fig. 2.8. Schematic diagram of laccase mediator system.

Mediators can be classified according to the oxidation mechanism they follow to promote the oxidation of substrates: hydrogen atom transfer, electron transfer and ionic oxidation. Mediators following the hydrogen atom transfer (HAT) mechanism include compounds that have N-hydroxyl functional groups in their chemical structure (e.g. 1-hydroxylbenzotriazole, violuric acid). For HAT mechanism, the mediator undergoes the abstraction of a hydrogen atom to form nitroxyl radicals (N-O$^{\bullet}$) that will then react with target substrates (Kurniawati and Nicell, 2007). These types of mediators may be suitable for mediating the transformation of compounds with weak C-H bonds (Cantarella et al. 2003). The electron transfer (ET) route is followed by laccase-ABTS system. ABTS is an azino mediator that is oxidized by laccase to produce ABTS cation radicals (ABTS$^{\bullet+}$ or ABTS^{2+}) that will then serve as a shuttle for electron transfer between the target substrate and the enzyme (Kunamneni et al., 2007). ABTS has been proposed as mediator for the transformation of compounds with relatively low oxidation potential (Catarella et al., 2003). Finally, TEMPO follows ionic mechanism to favour the oxidation of primary and secondary alcohols (Fabrini et al., 2002). It is oxidized by laccase forming an oxoammonium ion (N=O$^{\bullet}$) which subsequently oxidizes the substrate and is reduced to N-hydroxy-TEMPO. This species may undergo acid-induced disproportionation or be re-oxidized by laccase, resulting in the regeneration of the oxoammonium ion (Kurniawati and Nicell, 2007). Table 2.10 summarizes the characteristics of the most studied mediators.

Several studies have demonstrated improvement in the removal of pharmaceutical micropollutants by the addition of mediators. Prieto et al. (2011) reported that the addition of ABTS increased the oxidation yields of CIP from 16 to 98% and of NOR from zero to 34% after 30 h treatment. Lloret et al. (2010) compared the removal of naproxen and diclofenac by *Myceliophthora thermophila* laccase and laccase-HBT system at pH 4 and 5. The authors showed that laccase-HBT was able to enhance the removal of diclofenac by 30% and 25% at pH 4 and 5, respectively; whereas naproxen removal was 60% and 40% respectively. In another study Hata et al. (2010) observed that the aminoxyl radicals generated in the laccase-HBT system could reduce the concentration of CBZ by 60% when a repeated dose of the mediator was added to the reaction medium.

Laccase-mediator systems performance has been found to be dependent on the mediator concentration. The removal of CBZ was enhanced (from 47% to 95%) when the

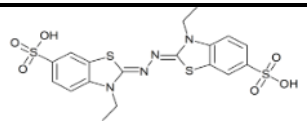
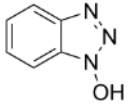
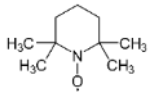
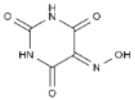
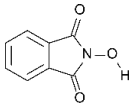
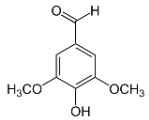
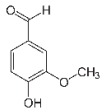
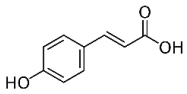
concentration of ABTS increased from 6 to 14 µM in combination with *Trametes versicolor* laccase (Naghdi et al., 2018a). Blanquez et al. (2016) studied the removal of CIP and NOR by testing different natural mediators including SYR, acetosyringone and methyl syringate (500 µM) at alkaline conditions. Results showed that acetosyringone was the best mediator achieving almost complete removal (>90%) for the two antibiotics in 24 h. However, when these authors increased by five-fold the concentration of mediators higher removal of the antibiotics was observed, leading to six and four-fold increased degradation of CIP and NOR respectively for acetosyringone and 17 and 20-fold enhanced removal in the case of methyl syringate (Blanquez et al., 2016). In another study, Murugesan et al. (2010) reported that using a mediator to pollutant ratio of 5:1, at a mediator concentration of 1mM, led to considerably better removal of TCS (up to 85%) using SYR or HBT compared to using laccase treatment alone. Margot et al., (2015a) investigated the removal of SMX with *Trametes versicolor* laccase mediated by ABTS, SYR and acetosyringone finding that mediators were consumed at a mediator-pollutant molar ratio of 1.1-16. However, increasing the mediator concentration beyond a limit may not affect the removal of the micropollutants as suggested by Ashe et al. (2016) who reported that naproxen was efficiently eliminated by *Pleurotus ostreatus* laccase in the presence of VA or HBT, but increasing concentration of VA from 0.5 to 1 mM caused no improvement in the removal of the drug.

The capacity of mediators to improve the degradation of pharmaceuticals is compound specific. For example, Ding et al. (2016) compared the performance of SYR and HBT (0.5 mM) for the removal of mixtures of sulfonamide, tetracycline and quinolone antibiotics. SYR showed better removal than HBT for sulfonamides and tetracyclines. Weng et al. (2012) investigated the effect of six mediators on degradation of sulfadimethoxine and sulfamanomethoxine by laccase. These authors reported that ABTS and VA provided the fastest transformation, while relatively slower transformation was observed with SYR and 4-hydroxybenzyl alcohol and at last no significant impact of 4-hydroxyacetophenone and 4-cyanopheno was evidenced (Weng et al., 2012). A screening of seven mediators for the degradation of trace organic pollutants showed that ABTS and VA were better at the transformation of phenolic compounds such as oxybenzone and pentachlorophenol, whilst non-phenolic compounds like naproxen were better removed by VAN and HBT (Ashe et al., 2016).

An issue regarding the use of mediators is the possibility that radicals generated from the reaction could inactivate the laccase. The activity of laccase was reduced by 90% after 8 h of contact with HBT mediator (Hata et al., 2010). Similarly, Ashe et al. (2016) reported a loss of activity of laccase after the addition of mediators. For example, when TEMPO, HPI, SA or VAN were added the loss of laccase activity was negligible (<10%) but also low pollutant removal was observed. In contrast, a significant loss in laccase activity was observed after the addition of VA, HBT or ABTS but the highest removals were obtained with these mediators (Ashe et al., 2016). Moreover, the extent of inactivation of the enzyme depends on the concentration of the mediator. Nguyen et al. (2014a) observed that laccase lost 14-39% of its activity when HBT dose was in the range from 0.1-1 mM.

The use of laccase-mediator systems for the treatment of pharmaceutical micropollutants shows promising results. However, the application of laccase-mediator systems in continuous operation is limited due to the necessity of constant addition of mediator which at the end increases the cost of the treatment and makes the process more difficult to operate (Nguyen et al., 2014b). Moreover, in some cases the use of mediator may increase the toxicity of the treated effluent as shown for ABTS and SYR (Kim and Nicell, 2006; Nguyen et al., 2014b). Thereby, the laccase-mediator treatment results ineffective or even adverse by creating another environmental problem. In order to develop an effective removal process based on the use of laccase and redox mediator, aspects such as the selection of the appropriate mediator and its optimal concentration and the monitoring of toxicity after treatment are critical and need to be investigated in detail.

Table 2.10. Characteristics of redox mediators used in laccase-mediator systems for the degradation of pharmaceutical micropollutants.

Compound	Type of mediator	Free radicals generated	Oxidation mechanism	Chemical structure	Substrates	References
ABTS[A]	Synthetic	$ABTS^{+\bullet}$ $ABTS^{2+}$	ET		AMX[I], CBZ[J], CIP[K], NOR[L], SPD[M], STZ[N]	Naghdi et al. (2018a); Parra Guardado et al. (2019); Prieto et al. (2011); Rodríguez-Rodríguez et al. (2012)
HBT[B]	Synthetic	Aminoxyl	HAT		CBZ, NPX[O], TCS[P], DCF[Q]	Ashe et al. (2016); Hata et al. (2010); Lloret et al. (2010); Murugesan et al. (2010)
TEMPO[C]	Synthetic	Oxoammonium (N=O•)	Ionic		NPX	Ashe et al.(2016)
VA[D]	Synthetic	Aminoxyl	HAT		SPD, STZ, NPX	Ashe et al. (2016); Rodríguez-Rodríguez et al. (2012)
HPI[E]	Synthetic	Aminoxyl	HAT		NPX	Ashe et al. (2016)
SYR[F]	Natural	Phenoxyl	HAT		SMX[R], TCS, CIP, DCF	Lloret et al. (2010; Murugesan et al. (2010); Parra Guardado et al. (2019)
VAN[G]	Natural	Phenoxyl	HAT		NPX	Ashe et al. (2016)
PCA[H]	Natural	Phenoxyl	HAT		AMX, SMX, CIP	Parra Guardado et al. (2019)

A: 2,2-azino-nis (3-ethylbenzothiazoline-6-sulfnoic acid); B: 1-hydroxibenzotriazole; C: 2,2,6,6-tetramethylpiperidinyloxyl; D: Violuric acid; E: N-hydroxyphthalimide; F: Syringaldehyde; G: Vanillin; H: *p*-coumaric acid; I: Amoxicillin; J: Carbamazepine; K: Ciprofloxacin; L: Norfloxacin; M: Sulfapyridine; N: Sulfathiazole; O: Naproxen; P: Triclosan; Q: Diclofenac; R: Sulfamethoxazole.

2.4.2 Removal by immobilized laccase

Although batch experiment setups are convenient for assessing the feasibility of using laccases for the removal of micropollutants, larger scale applications such as in a WWTP need to be operated in continuous mode. This necessitates that enzymes are retained in the reactor since continuous addition of enzymes, as mentioned above, would lead to increased treatment costs (Gasser et al., 2014). Moreover, large scale applications of enzymes often get hampered by their lack of long-term operational stability, susceptibility to inactivation and their difficult recovery or reusability. Enzyme immobilization is a potential solution for these problems since it allows continuous operation and reuse of the biocatalyst.

Enzyme immobilization is a process in which an enzyme is located or confined into a matrix or support, resulting in insoluble forms that retain catalytic activity (Datta et al., 2013). There are several reasons for using enzymes in immobilized form; immobilization enhances enzyme stability, protects enzyme from denaturation and facilitates handling and recovery as well as their reuse over several reaction cycles maintaining good catalytic efficiency (Cabana et al., 2009; Ji et al., 2017; Patel et al., 2014; Sheldon, 2007; Zheng et al., 2016). According to several published reviews, methods for immobilization can be classified in different ways (An et al., 2015; Fernández-Fernández et al., 2013; Mohamad et al., 2015; Sheldon and van Pelt, 2013). However, there are typically three different types of immobilization approaches: (i) binding to a carrier, (ii) cross-linking, and (iii) entrapment/encapsulation (Fig. 2.9).

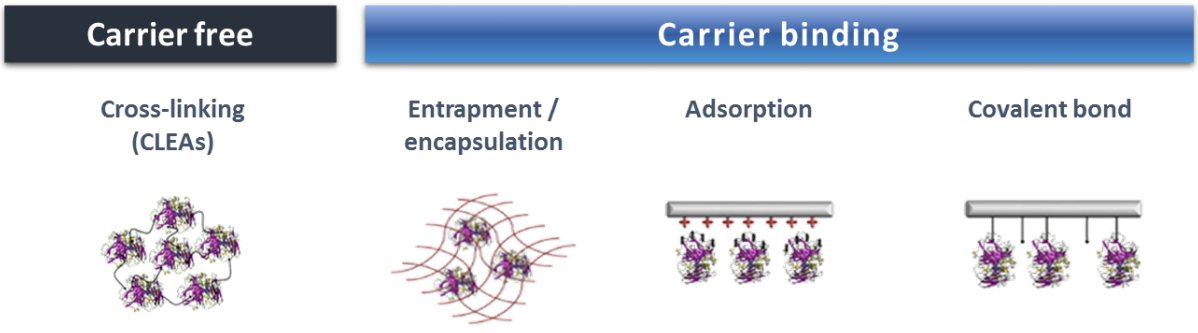


Figure 2.9. Immobilization of enzymes.

The immobilization of enzymes to supports can be by adsorption (hydrophobic or Van der Waals interactions), ionic forces or covalent bonds, and can be performed on different types of material carriers. These materials are generally divided into organic (e.g. chitosan, alginate, polyamide), inorganic (silica, alumina, clay, active carbon) and hybrid/composite materials (silica-magnetite, chitosan-silica) (Cantone et al., 2013; Datta et al., 2013; Zdarta et al., 2018a).

The immobilization via adsorption involves the enzyme being physically adsorbed or attached onto the support material. Adsorption is based on the creation of non-specific interactions mainly via hydrogen bonds, ionic or hydrophobic interactions. Thus, conformational changes in the enzyme structure are limited which results in the retention of high catalytic activity (Hernandez and Fernandez-Lafuente, 2011). Immobilization by covalent binding is based on the reaction of functional groups of the support material with functional groups of the enzyme (mainly $–NH_2$, -SH, and –OH) resulting in the formation of strong chemical bonds which significantly increases laccase stability and reduces its leakage (Datta et al., 2013). Encapsulation refers to the confinement of the enzyme within semipermeable spheres made of polymers (Rochefort et al., 2008); whilst, entrapment is based on the retention of the enzyme within a polymeric grid or fibers which assures the integrity of the enzyme structure (Mohamad et al., 2015). The cross-linking involves the generation of intramolecular links between the molecules of the enzyme by means of bifunctional compounds such as GLU or carbodiimides. In this way, enzymes in the form of cross linking enzyme crystals (CLECs) or cross linking enzyme aggregates (CLEAs) form stable structures without the use of a solid supports (Cao et al., 2003; Sheldon and van Pelt, 2013).

Table 2.11 presents the main advantages and disadvantages of the different immobilization techniques. Overall, immobilization methods involving the formation of chemical bonds provide stable attachment and reduce enzyme inactivation rates; however, the activity of the biocatalyst could be reduced due to the modification of the native enzyme structure induced by the covalent bonds (Sheldon, 2007). In contrast, adsorption and entrapment methods typically have fewer effects on the structure of the enzyme but provide less stability during the reaction (Durán et al., 2002). It is clear the immobilization process significantly influences the properties of the immobilized enzyme as the

immobilization method determines the process specification of an enzyme such as cost, catalytic activity, effectiveness and deactivation kinetics (Naghdi et al., 2018b).

Table 2.11. Advantages and drawbacks of immobilization techniques.

Immobilization technique	Strength of interactions	Advantages	Drawbacks
Adsorption	Weak	No enzyme modification Simple and inexpensive Reusability of support	Lower efficacy level Desorption of enzymes
Covalent bond	Strong	Strong and stable interactions Multipoint attachment Relatively simple method Variety of supports available	Competitive inhibition issues Enzyme chemical modification Loss of functional conformation of enzyme
Encapsulation	Weak	No enzyme modification Protection of enzyme Cost effective method	Less concentration of enzymes Generation of unwanted products
Entrapment	Weak/strong	No enzyme modification Fast method Low cost	Enzyme leaking Pore diffusion limitations Low level of industrial application
Cross-linking	Strong	No support needed High strength of interactions	Polyfunctional reagents are required Denaturation or structural modification by cross-linker

The selection of the immobilization method should be done not only taking into account the characteristics of each technique but also the type of biocatalyst to immobilize and the bio-catalytic process in which immobilized enzyme will be applied. In this sense, covalent binding seems to be the most suitable option for laccase immobilization for water and wastewater treatment applications since this approach typically yields more stable biocatalyst compared to other immobilization methods. Stability is a key factor if the immobilized enzymes will be used in tertiary wastewater treatment processes in which the enzymes are exposed to harsh environmental conditions (i.e. high pH, turbulent flows, denaturing agents) (Gasser et al., 2014). In comparison to the other immobilization techniques (adsorption, encapsulation) where the binding forces are too weak to prevent enzyme leakage, covalent immobilization results in a more stable attachment of the laccase

on the support (Sheldon, 2007). Thus, covalent binding of laccases on solid materials for pollutants removal has been intensively investigated (Cabana et al., 2009; Jahangiri et al., 2014; Liu et al., 2012; Lloret et al., 2012; Mohammadi et al., 2018; Pezzella et al., 2014; Xu et al., 2013).

According to Zdarta et al. (2018) the immobilization methodology has a strong influence in the selection of the carrier. However, the selected support not only serves as a surface where the enzyme can be attached, but its physical and chemical properties also have an influence in the properties of the immobilized enzyme (Cao et al., 2003). Materials with numerous functional groups facilitate covalent immobilization and multipoint attachment of the laccase molecules. In this way the stability of the biocatalyst is improved, however it might also lead to diffusional limitations in transport of substrates (Zdarta et al., 2018a). Inorganic materials such as mesoporous silica and hollow carbon spheres (Fortes et al., 2017; Shao et al., 2019), organic carriers of natural origin like chitosan or coconut fibers (Cristóvão et al., 2012; Zheng et al., 2016) and even hybrid materials such as magnetic chitosan/clay beads (Aydemir and Güler, 2015) can be used for covalent immobilization. Organic and inorganic supports are an interesting option from the point of view of economy and stability, whilst hybrid and composites materials are attractive because they can be specially design for specific applications resulting in an improvement of the whole process.

Inorganic materials are especially suitable for covalent immobilization of laccases for water treatment applications due to all the properties they display. These materials show exceptional mechanical and chemical stability properties which ensure the integrity of the biocatalyst during the process (Zucca and Sanjust, 2014). Inorganic carriers also have many different functional groups (i.e hydroxyl, carboxyl, carbonyl) that facilitates enzyme attachment and support functionalization with modifying agents like GLU or 3-aminpropyltriethoxyxilane (APTES) which is convenient for covalent immobilization (Zucca and Sanjust, 2014). Another advantage is the large specific surface area (up to 1500 m^2 g^{-1}) inorganic carriers offer allowing high retention of enzyme (Magner, 2013; Thielemann et al., 2011). Moreover, these materials are relatively easy and cheap to obtain due to their abundant presence in nature and their simple synthesis procedure. Thus, inorganic supports can be obtained in different shapes and sizes and even form part of hybrid or composite materials. The versatility showed by inorganic materials is important since activity of the

covalent bonded laccase usually depends on the size, specific area and shape of carrier material (dos Santos et al., 2015; Mohamad et al., 2015).

In this sense, the use of inorganic (nano)particles are growing interest since they provide a large surface area for enzyme binding leading to higher laccase loading at the same time that minimizes diffusional limitations (Ansari and Husain, 2012; Zdarta et al., 2018b). For instance, *Trametes versicolor* laccase was covalently immobilized onto TiO_2 nanoparticles and the resulting biocatalyst was used for the removal of the recalcitrant CBZ (Ji et al., 2016b). In comparison to free laccase the active TiO_2 nanoparticles showed improved stability allowing degrading 60% of the pharmaceutical in combination with PCA as redox mediator. These authors observed a minimal loss of enzymatic activity at the end of 96 h of reaction which was attributed to the improved conformational strength of the laccase on the support and the decrease of mass transfer resistance. In another study by Ji et al. (2016), the potentiality of cross-linked carbon nanotubes-based bio-catalytic membranes was investigated for the degradation of a mixture of five micropollutants including CBZ, diclofenac and ibuprofen in an enzymatic membrane reactor (EMR). The covalently immobilized membrane with laccase from *Trametes versicolor* yielded to high laccase loading and specific activity values (140 μg cm^{-2} and 0.40 U cm^{-2}), and showed enhanced stability for long term storage and under hydraulic shear stress. The bio-catalytic membrane was able to degrade micropollutants at a high extent (46-90%) while keeping 84% of its original activity after 48 h of reaction which indicates the good stability of the immobilized enzyme.

As shown in Table 2.12 broad range of support materials and immobilization techniques have been used for the preparation of biocatalyst for the degradation of pharmaceutical compounds in both synthetic and real wastewaters. For example, Kumar and Cabana (2016) reported the immobilization of laccase as CLEAS on magnetic nanoparticles (MAC-CLEAs) for the simultaneous treatment of 13 pharmaceuticals. The MAC-CLEAs showed enhanced storage stability over more than one year at 4˚C and the capacity to be reused even after 150 batch cycles with ABTS as substrate. Moreover, the biocatalyst was extremely efficient for the degradation of the mixture of pharmaceuticals achieving high removal yields (40-99%) for 10 of the 13 compounds including almost complete degradation

of mefenamic acid, acetaminophen, diclofenac atenolol and epoxy-CBZ within 12 h of reaction.

Immobilization of laccases onto different supports not only improves enzyme stability for the degradation of the pharmaceuticals but also, in some cases, the carrier plays a complementary role in the removal process as demonstrated in several studies (Cabana et al., 2009; Dai et al., 2016; Naghdi et al., 2017; Nguyen et al., 2016; Shao et al., 2019). Shao et al. (2019) immobilized laccase onto hollow carbon nanospheres by both physical adsorption and covalent binding. The adsorbed biocatalyst showed the best results in terms of high enzyme loading (835 mg g^{-1}) and excellent thermal, pH and long storage stability and reusability. The biocatalysts were then used for the removal of tetracycline and CIP hydrochloride obtaining high removal yields (up to 99%) for the two compounds which was attributed to the synergistic action of adsorption on the support and enzymatic degradation since the biocatalyst support showed high sorption capacity towards the antibiotics (55% for tetracycline and 77% for CIP).

Similarly, Nguyen et al. (2016) observed that removal of bisphenol A, diclofenac, CBZ and SMX by laccase adsorbed on granular activated carbon in a packed-bed column was via the simultaneous adsorption and degradation processes, especially for the removal of the recalcitrant CBZ and diclofenac compounds. Authors suggested that the co-adsorption of laccase and micropollutants onto the carrier facilitated enzymatic degradation due to the enhancement of electro transfer between the laccase and substrate molecules after adsorption on the support surface. In this context, results interesting to explore the possibility of using support materials offering properties for combining enzyme immobilization and adsorption of both pollutants and reaction by-products. Such systems could lead to efficient processes were the enhanced bioconversion of pollutants due to the immobilization of enzyme would be reinforced by adsorption in order to obtain cleaner effluents in one step treatment. Therefore, future research should focus on the development of new support/sorbent materials as well as innovative immobilization techniques leading to multifunctional biocatalyst showing high catalytic properties for the removal of toxic environmental pollutants.

Table 2.12. Removal of pharmaceutical compounds by immobilized laccase.

Pharmaceutical	Enzyme source	Immobilization technique	Support material	Reaction conditions	Removal (%)	Reference
Acetaminophen	*Trametes versicolor*	Cross-linking	Magnetic CLEAs	50 µg L^{-1} pollutant, 1000 U L^{-1} of laccase, 10 mL reaction, pH 7, 30°C, 6 h in batch reactions with wastewater	27	(Arca-Ramos et al., 2016)
	Trametes versicolor	Cross-linking	MAC-CLEAs	100 µg L^{-1} pollutant, 1000 U L^{-1} of laccase, 10 mL reaction, pH 7, 20˚C, 12 h in batch reactions	97	(Kumar and Cabana, 2016)
Amoxicillin	*Trametes versicolor*	Covalent immobilization	TiO_2 multichannel membrane	10 µg L^{-1} pollutant, 1mM SYR, 5 L reaction, pH 6, 25°C, 24 h in EMR	95	(Becker et al., 2016)
Carbamazepine	*Trametes versicolor*	Covalent immobilization	TiO_2 nanoparticles	20 µM pollutant, 1mM PCA, 0,5 U ml^{-1} of laccase, pH 7 in reactor	60	(Ji et al., 2016b)016
	Trametes versicolor	Adsorption	Cross-linked carbon nanotube membranes	20 µM pollutant, 0.45 U cm^{-2} of laccase, pH 5.5, 48 h in membrane cell	48	(Ji et al., 2016a)
	Trametes versicolor	Adsorption	Biochar	20 ng L^{-1} pollutant, 20 mL reaction, 25°C, 24 h in batch reaction with wastewater	66	(Naghdi et al., 2017)
Clofibric acid	*Trametes versicolor*	Adsorption	Cross-linked carbon nanotube membranes	20 µM pollutant, 0.45 U cm^{-2} of laccase, pH 5.5, 48 h in membrane cell	45	(Ji et al., 2016a)
Ciprofloxacin	*Trametes versicolor*	Covalent immobilization	TiO_2 multichannel membrane	11 µg L^{-1} pollutant, 1mM SYR, 5 L reaction, pH 6, 25°C, 24 h in EMR	93	(Becker et al., 2016)
Diclofenac	*Asperguillus oryzae*	Adsorption	Activated carbon	2.5 mg L^{-1} pollutant, 100 mL reaction, 25°C, 2 h in batch reactions	90	(Nguyen et al., 2016)
	Pleurotus florida	Covalent immobilization	Poly(lactic-co-glycolic acid) nanofiber	50 mg L^{-1} pollutant, 4 U of laccase, 25 mM SYR, 1 mL volume, pH 4, 30°C, 5h	100	(Sathishkumar et al., 2012)

Table 2.12. Removal of pharmaceutical compounds by immobilized laccase (continuation).

Pharmaceutical	Enzyme source	Immobilization technique	Support material	Reaction conditions	Removal (%)	Reference
Diclofenac	*Trametes versicolor*	Cross-linking	MAC-CLEAs	100 µg L^{-1} pollutant, 1000 U L^{-1} of laccase, 10 mL reaction, pH 7, 20˚C, 12 h in batch reactions	95	(Kumar and Cabana, 2016)
Ibuprofen	*Trametes versicolor*	Adsorption	Cross-linked carbon nanotube membranes	20 µM pollutant, 0.45 U cm^{-2} laccase, pH 5.5, 48 h in membrane cell	60	(Ji et al., 2016a)
Mefenamic acid	*Trametes versicolor*	Cross-linking	MAC-CLEAs	100 µg L^{-1} pollutant, 1000 U L^{-1} of laccase, 10 mL reaction, pH 7, 20˚C, 12 h in batch reactions	99	(Kumar and Cabana, 2016)
Sulfamethoxazole	*Asperguillus oryzae*	Adsorption	Carbon activated	2.5 mg L^{-1} pollutant, 100 mL volume, 25°C, 2 h in batch reactions	92	(Nguyen et al., 2016)
	Trametes versicolor	Covalent immobilization	CPC silica beads	50 mg L^{-1} pollutant, 1 U mL^{-1} of laccase, 1mM HBT, 5 mL reaction, pH 5, 40°C, 1 h in batch reactions	76	(Rahmani et al., 2015)
	Cerrena unicolor	Cross-linking	Magnetic CLEAs	100 mg L^{-1} pollutant, 20 U ml^{-1} of laccase, 5 µM ABTS, pH 7, 25°C, 48 h in batch reactions	29	(J. Yang et al., 2017)
Sulfathiazole	*Trametes versicolor*	Covalent immobilization	CPC silica beads	50 mg L^{-1}pollutant, 1 U ml^{-1} of laccase, 1mM HBT, 5 mL reaction, pH 5, 40°C, 1 h in batch reactions	85	(Rahmani et al., 2015)
Tetracycline	*Trametes versicolor*	Covalent immobilization	Mono-channel ceramic membrane	20 mg L^{-1} pollutant, 0,01 g L^{-1} of laccase, pH6, 25°C, 24 h in batch reactions	56	(De Cazes et al., 2014)
Tetracycline	*Cerrena unicolor*	Cross-linking	Magnetic CLEAs	100 mg L^{-1} pollutant, 20 U mL^{-1} of laccase, pH 7, 25°C, 48 h in batch reactions	65	(J. Yang et al., 2017)
Triclosan	*Trametes versicolor*	Encapsulation	Carbon nanotube modified electrospun fibrous membranes	10 mg L^{-1} pollutant, 2 mg of laccase, 30 mL reaction, pH 7, 25°C, 5 h in batch reactions	100	(Dai et al., 2016)

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Chapter 3

Effect of redox mediators in pharmaceuticals degradation by laccase: A comparative study

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3. Effect of redox mediators in pharmaceuticals degradation by laccase: A comparative study

Abstract

Bio-catalytic processes have recently attracted attention as an interesting option for the degradation of persistent pollutants as they are capable to catalyse specific reactions at mild conditions and low environmental impact. In this work the potential to transform pharmaceutical micropollutants of a novel laccase from *Pycnoporus sanguineus* CS43 was compared to the commercial laccases *Trametes versicolor* and *Myceliophtora thermophila*. In the absence of redox mediators micropollutants were resistant to degradation, except for the antibiotic amoxicillin that was transformed by all laccases. The influence of natural and synthetic redox mediators (syringaldehyde, p-coumaric acid and ABTS) on the laccase oxidation system was investigated. Results showed the degradation of a complex mixture of pharmaceuticals is both compound and redox mediator dependent. Syringaldehyde resulted as the best redox mediator allowing the highest degradation yields of the antibiotics amoxicillin (80%), sulfamethoxazole (100%) and ciprofloxacin (40%) within 3 h treatment. Overall, commercial laccases showed better catalytic performance in comparison to *P. sanguineus* CS43 laccase especially in the presence of redox mediators. The successful transformation of pharmaceuticals by the combined action of different laccases and redox mediators demonstrate the potential of these systems for the removal of complex pollutant matrices.

Keywords: Laccase, Pharmaceutical micropollutants, Redox mediators, Bio degradation

3.1. Introduction

Pharmaceuticals are synthetic or natural chemicals found in prescription medicines, therapeutic and veterinary drugs with a worldwide annual consumption estimated to 100 000 tons per year [1]. The broad use of pharmaceuticals leads to its continuous release into the environment as intact substances or metabolites that are ineffectively removed by conventional wastewater treatment plants [2,3]. Hence, pharmaceuticals are detected in water bodies at concentrations from µg L^{-1} to ng L^{-1} representing a threat to aquatic ecosystems and human health [4,5].

The degradation of pharmaceutical compounds implies an important challenge due to their low bioavailability and complex structure. To date, several processes for the removal of these pollutants have been studied, including conventional techniques (activated sludge, filtration, coagulation, flocculation, sedimentation) advanced oxidation processes (ozonation, UV irradiation, Fenton oxidation), adsorption and membrane processes [3,6]. Recently, the use of "hybrid processes", which are a combination of two or more treatment processes, was reviewed since it seems to be an effective way to remove micropollutants [7]. Nonetheless, the high cost of operation, the inconstant efficiencies obtained and the possible formation of hazardous by-products are major drawbacks to their successful application of these technologies [3].

A promising alternative treatment is the use of ligninolytic enzymes obtained from white rot fungi cultures (laccases, peroxidases, manganese peroxidases) since they catalyse specific reactions under moderate operational conditions preventing undesired side-reactions [8,9].

Particullarly, Laccases (1.10.3.2) are able to catalyse single electron oxidation of phenolic moieties via phenoxy radicals with the reduction of oxygen to water. Furthermore, laccases catalyze the oxidization of phenolic compounds, amines, methoxy-substituted phenols and some inorganic compounds [10]. Numerous studies have indicated that laccases present a great potential in the bioremediation of contaminated effluents with dyes [11], phenolic compounds [12], pesticides [13], pharmaceuticals and personal care products [14–18]. The oxidation effectiveness of substrates by laccase depends on factors such as the difference of redox potential between laccase T1 copper and substrate, pH of the medium

and chemical structure of substrates [19]. However, non-phenolic compounds like pharmaceuticals are not always suitable for laccase oxidation since their redox potential is usually higher than the redox potential of most laccases produced by fungi [20]. Moreover, the presence of certain functional groups in the structure of the substrate can greatly influence its transformation. Functional groups like hydroxyl and amines, better known as electron donating groups (EDG) make substrates more susceptible to laccase attack. Whereas electron withdrawing functional groups including amides, nitro and carboxylic groups will prevent laccase oxidation resulting in a more difficult process [9].

Laccase catalysis can be extended by the inclusion of redox mediators that act as electron shuttle between the enzyme and substrate. This mechanism involves the oxidation of the mediator by laccase, which results in the production of highly reactive and stable radical species that diffuse away from enzyme's active site to react with other substrates [21]. Thereby, it is possible to oxidize chemical compounds that in principle are not substrates of laccase. Mediators differ from each other in terms of optimal reaction conditions, substrate specificity and the mechanism followed to oxidize substrates (electron transfer, hydrogen atom transfer and ionic oxidation) [21,22]. Hence, understanding the role of mediators in laccase catalysed reactions is an important matter. Recently, the evaluation of critical aspects such as type and dose of mediator and their influence of laccase stability and effluent toxicity for the removal of trace organic compounds was reported [23]. However, this study is only focused on the performance comparison of a laccase with different mediator combinations, similarly to most of the previous investigations evaluating laccase-mediator systems for the removal of environmental pollutants [24,25]. Only few investigations have studied and compared the effect of different type of oxidative enzymes on the treatment of organic pollutants alone or in combination with several redox mediator compounds [26,27]. Therefore, research on the performance of different laccase-mediator combinations under identical operational conditions for the removal of pharmaceutical pollutants is still limited. A study with this focus would allow properly comparing and understanding the synergy between different laccase formulations and mediators and their effect in the oxidation of pharmaceutical compounds.

In the present work the potential of three different laccases (*Myceliophthora thermophila*, *Trametes versicolor* and *Pycnoporus sanguineus* CS43) for the degradation of

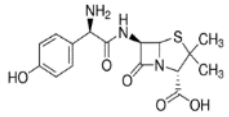
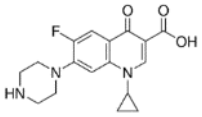
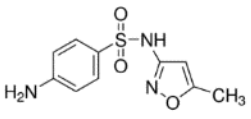
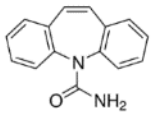
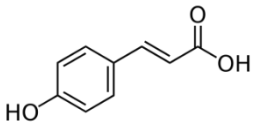
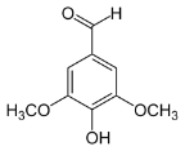
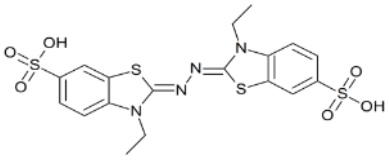
three antibiotics (amoxicillin, ciprofloxacin and sulfamethoxazole) and one antiepileptic (carbamazepine) in free enzyme systems was evaluated. Firstly, specific aspects such as the effect of pH and temperature were assessed for the potential improvement of the treatment. Then, the performance of each laccase with selected natural (syringaldehyde and p-coumaric acid) and synthetic (ABTS) redox mediators was compared in terms of degradation efficiency. In this sense, a cyclic voltammetry study was carried out in order to understand the interactions between redox mediators and substrates and the mechanism involved for the oxidation of each compound.

3.2. Materials and methods

3.2.1. Chemicals and enzymes

The pharmaceuticals (amoxicillin (AMX), ciprofloxacin (CIP), carbamazepine (CBZ) and sulfamethoxazole (SMX)), mediators (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) (ABTS), p-coumaric acid (PCA), syringaldehyde (SYR)) and all other chemicals were purchased from Sigma-Aldrich. The structure of pharmaceuticals and redox mediators is shown in Table. 3.1. Stock solutions (100 mg L^{-1}) of AMX, CIP and SMX were prepared in citrate-phosphate buffer 0.1 M, pH 7. Considering the lack of solubility in water of CBZ the stock solution of this compound was prepared in pure ethanol at a concentration of 15,625 mg L^{-1}. ABTS solution (5 mM) was prepared just before use in buffer citrate-phosphate pH 7. Stock solutions of SYR and PCA (114.9 mM) were prepared in pure ethanol.

Table 3.1. Chemical structure of pharmaceuticals and mediators used in this work.

ANTIBIOTICS			ANTIEPILEPTIC
Amoxicillin **(AMX)**	Ciprofloxacin **(CIP)**	Sulfamethoxazole **(SMX)**	Carbamazepine **(CBZ)**
			
MEDIATORS			
p-coumaric acid **(PCA)**	Syringaldehyde **(SYR)**	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (**ABTS**)	
			

Three different laccase preparations were tested. Laccases from *Pycnoporus sanguineus* CS43 (PSL) were obtained from a tomato medium according to Ramirez-Cavazos et al. [28] with some modifications. In short, mycelia were removed from the culture supernatant by filtration using two tangential flow filters in series, with respective pore sizes of 0.5 and 0.2 µm. The 0.2 µm filtrate (laccase cocktail) was then filtered on a 10 kDa ultrafiltration membrane. The resulting enzymatic preparation is a laccase cocktail containing at least two isoenzymes. A complete characterization of such laccase cocktail has been reported by Orlikowska et al. [29]. Laccase powder from *Trametes versicolor* (ref 51639, activity ≥10 U mg^{-1}) (TVL) was purchased from Sigma-Aldrich. Commercial laccase produced by submerged fermentation from *Myceliophthora thermophila* (59.5 g L^{-1} of pure laccase) (MTL) was provided by Novozymes (Denmark).

3.2.2. Laccase activity assay

The laccase activity was determined by measuring the oxidation of 0.5 mM ABTS solution prepared in citrate-phosphate buffer (0.1 M, pH 4). The reaction was monitored at 420 nm (ε_{420} = 36000 M^{-1} cm^{-1}) with a spectrophotometer (Shimadzu UV-2401PC). One enzyme activity unit (U) was defined as the amount of enzymes that oxidized 1 µmol of ABTS per min.

3.2.3. Pharmaceuticals oxidation by laccases

Degradation experiments were run in 100 or 250 ml Erlenmeyer flasks at 25°C under dark conditions to avoid light oxidation and continuous stirring to ensure O_2 saturation of the reaction medium. Reaction mixtures (50 or 100 ml) contained one or a mixture of all pollutants in buffer citrate-phosphate (0.1 M) pH 7; each pollutant concentration was fixed at 20 mg L^{-1} (molar concentration: 55 µM AMX, 79 µM SMX and 60 µM CIP) except in the case of CBZ where it was 10 mg L^{-1} (molar concentration: 85µM). Some experiments were also carried out at different pH (citrate-phosphate buffer 0.1 M pH in the range of 3-7) and different temperatures (15 – 40 °C).

To assess the effectiveness of redox-mediator for enhancing the bio-oxidation of micropollutants, experiments were carried out as described above after adding one of the following mediators (ABTS, PCA and SYR) at a concentration of 520 µM to the reaction medium. Mediator:substrate molar ratios up to 9:1 (depending on the pharmaceutical) were tested in order to ensure the effective removal of the micropollutants due to the mediator action. Reactions were initiated by adding one of the three enzyme preparations at the concentration of 1 200 U L^{-1}. To monitor the degradation process, samples of 1 ml were withdrawn every 24 h when there was no redox-mediator in the reaction medium. When using mediators, samples were taken every 20 minutes during the first three hours and then every hour until 8 h. It is worth noting that the number of samples taken was limited in order to keep the variation of reaction volume below 15%. After sampling, aliquots were inactivated in a water bath at 100 °C for 5 minutes and filtered with CHROMAFIL Xtra H-PTFE-20/30 filters to immediately be analysed by HPLC-MS. In order to verify the repeatability of the treatments all experiments were carried out several times and at least duplicated. The results reported in all figures correspond to the average of the experiments with their corresponding standard deviations that are given in figures or captions. Controls without enzyme neither mediator as well as controls with mediator but no enzyme were run in parallel to assess the role of enzymes on the removal of the pharmaceuticals.

3.2.4. HPLC-MS quantitative analysis

Micropollutants concentration during enzymatic treatment was analysed using a HPLC Alliance – Waters e2695 separations module equipped with a C18 Raptor column (150 x 2.1 mm, 5 µm) coupled to a MS Micromass Quatro micro API (tri-quadripole) detector. 5 µl of sample were injected and two eluents, namely eluent A (95% water – 5% methanol) and eluent B (100% methanol), were passed through the column at a flow rate of 0.25 ml min^{-1} in the following gradient program: 0-3 min, 100% (A), 3-8 min, 100% (B) and 8-15 min, 100% (A).

3.2.5. Redox potential determinations

Cyclic voltammetry experiments were carried out in sodium phosphate buffer 0.1 M, pH 7, using a VERSASTAT3 voltammetry analyser (Princeton Applied Research). A cell of 250 ml was used in all voltammetry studies. The electrochemical cell consisted of an Ag/AgCl reference electrode, a platinum wire counter electrode and a graphite working electrode. Scan rates of 0.5 V s^{-1} were applied whereas the concentration of substrates and mediators were in the range of 0.2 – 3 mM according to molar concentrations used in degradation reactions.

3.3. Results and discussion

3.3.1. Oxidation of pharmaceuticals by laccases

The potential of each one of three laccases to transform pharmaceutical active compounds was initially evaluated without mediators. Firstly, each pharmaceutical was treated individually with 60 U of each laccase. After 72 h of enzymatic oxidation no sign of laccase-catalysed degradation for SMX, CIP and CBZ was found by any of the enzymes tested (results not shown). In contrast the antibiotic AMX was effectively transformed (Fig. 3.1). From the enzymes tested, PSL showed the highest affinity for AMX reaching 72% removal. Commercial laccases TVL and MTL presented similar performance with 58% degradation at

the end of the reaction. In the case of PSL the first signs of oxidation were visible after 24 h of reaction.

No conversion was detected in controls without enzyme, except for AMX where a decrease in concentration was observed. Belonging to the β-lactam penicillins, AMX is an antibiotic known to be unstable in aqueous solutions due to its fast hydrolysis and degradation to various sub-products [30]. Nonetheless, the self-degradation of AMX was much lower in comparison to reactions where enzymes were added, evidencing the effective catalytic action of laccases on the antibiotic removal (Fig. 3.1).

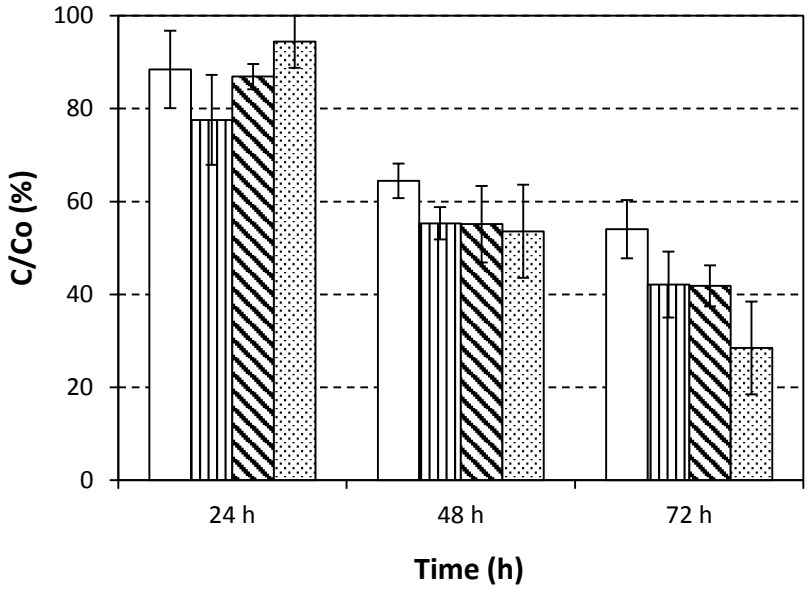


Fig. 3.1. Oxidation of AMX in single substrate by laccases from *M. thermophila* (▥), *P. sanguineus* (▨) and *T. versicolor* (▧), control without enzyme (□) at 25 °C and pH 7.

Affinity of laccase towards substrates is influenced mainly by the redox potential of the specific laccase and the presence of strong electron donating functional groups (EDG) or electron withdrawing functional groups (EWG) [23]. Functional EDG prone to be attacked by laccase include hydroxyl (-OH), amines ($-NH_2$), alkoxy (-RO), alkyl (-R) and acyl (-COR). On the other hand, EWG such as carboxylic (-COOH), amide ($-CONR_2$), halogen (-X) and nitro ($-NO_2$) may prevent electron abstraction from occurring by forming a steric shield [9]. Hence, removal efficiencies obtained in this study can be attributed to the chemical structure of the pharmaceuticals. Effective oxidation of AMX can be explained by the presence of a group

hydroxyl attached to an aromatic ring that enables the catalytic action of laccases. The slight difference in removal efficiency among laccases may be due to the redox potential of the enzyme that varies depending on the fungal source. Indeed, the AMX degradation results discussed above are in good agreement with the removal of AMX by TVL was previously reported by Becker et al. [31] who observed that even if self-degradation of AMX in blanks runs was obvious, the observed complete oxidation of this antibiotic in an enzymatic membrane reactor after 24 h of treatment was the result of the enzymatic oxidation.

The antibiotic SMX contains an amine (EDG) and a sulfonamide (EWG) group in its structure. The influence of both groups on the degradation has not been clearly established. In some studies effective removal of the pharmaceutical was reported [32], whereas in others the sulfone group has been related with its recalcitrance to degradation [33]. Guo et al. [32] reported that white-rot fungus *P. chrysosporium* could oxidize SMX. In contrast, Margot et al. [25] observed SMX was recalcitrant to purified TVL oxidation in absence of redox mediators. Interestingly, higher removal of the antibiotic was obtained by fungal cultures in comparison to purified laccase as demonstrated by Gao et al. [34] who observed high oxidation yields of SMX by using *P. chrysosporium* and *P. sanguineus* fungal cultures; but visible lower catalytic effects were obtained when the corresponding purified enzyme extracts were used. Possibly, the effect of other enzymes or compounds present in fungal cultures enhanced SMX removal. In addition, low oxidation of SMX would be also attributed to the redox potential of selected laccases. The redox potential of sulfonamides is estimated to be in the range of 0.858-1.158 V which is considerable high in comparison to redox potential of most laccases (0.5-0.8 V). Indeed, these compounds are recalcitrant to the oxidation by laccase [35]. In the case of CIP and CBZ both pharmaceuticals possess strong EWG. The amide group in CBZ and the presence of the carboxyl and halogen group in CIP make them highly resistant to laccase oxidation [33,36]. The lack of oxidation observed in this study agrees with literature. Ji et al. [37] who reported less than 5% removal of CBZ by free laccase treatment.

Considering the limited oxidative performance of laccases towards pharmaceuticals in single substrate assays, simultaneous conversion of AMX, SMX, CIP and CBZ in mixtures, was carried out to study potential substrate mediation/competition effect in the reaction. Investigation on the effect of substrate mixtures on enzyme specificity is of high interest to

assess the applicability of enzymes as a treatment option since treated wastewaters generally contain a complex mixture of pollutants. In these experiments the concentration of SMX, CIP and CBZ remain unchanged in the reaction medium, confirming their recalcitrance to enzymatic oxidation (data not shown). Laccase affinity towards these substrates was not influenced by the presence of others micropollutants in the reaction. Nonetheless, their presence results also on a very slight decrease of the AMX concentration. However, this decrease is difficult to be considered as result of the enzymatic degradation because of the self-oxidation of this substrate is of the same order of magnitude.

The simultaneous presence of micropollutants in a reaction media can affect the removal efficiency in the system. The results presented until now in literature are sometimes contradictory and depends on the mixture of pharmaceuticals studied. For example, Ji et al. [14] who studied the free enzymatic removal of five representative pharmaceuticals bisphenol-A (BPA), diclofenac (DCF), clofibric acid, ibuprofen (IBP) and CBZ) observed that degradation yield in mixtures of three molecules was doubled respect to samples with only one pharmaceutical. The increase in degradation was attributed to the presence of bisphenol-A, a phenolic compound and then after enzyme oxidation would form phenoxyl radicals that can act as redox mediator between laccase and other micropollutants more recalcitrant to enzymatic degradation. On the contrary, Margot et al. [38] observed that removal yield was four times lower in the presence of mefenamic acid and diclofenac compared to single compound solution. Authors suggest that a competitive effect between substrates takes place thus reducing removal yields. Nguyen et al. [33] studied the simultaneous removal of SMX, CBZ, DCF and BPA with a commercial laccase. No effect of the mixture of micropollutants on the removal was observed. These authors attributed the resistance (SMX and CBZ) or the vulnerability (DCF and BPA) to laccase oxidation to the chemical structure of each compound. Seems like the cocktail effect of micropollutants mixtures in laccase catalytic system depends on the presence of more reactive compounds that could promote the removal of recalcitrant ones [39]. Indeed, the presence of different compounds in the reaction media is strongly dependent on the mixture studied. In the present work the degradation of AMX observed could have an influence of the degradation of other micropollutants in the mixture.

Furthermore, effective removal of pharmaceutical mixtures directly depends on the type of enzyme and compound as demonstrated by Stadlmair et al. [27]. These authors tested different types of oxidative enzymes for the multiple conversions of acetaminophen, DCF and sotalol and they observed that inhibition or enhancement effect in case of horseradish peroxidase did not occur with laccase from *Pleurotus ostreatus*. The poor degradation performance obtained in this investigation could be also related to the operational conditions which were not optimized. Indeed, enzyme reactivity generally depends on conditions such as pH and temperature. The effect of these parameters was studied only for AMX since it was the most sensitive compound to enzymatic degradation.

3.3.2. Effect of pH and temperature in the oxidation of AMX by laccases

Removal of AMX by enzymatic treatment was investigated at different pH. Laccase produced by *P. sanguineus, M. thermophila* and *T. versicolor* were used for this purpose. In order to evaluate AMX self-degradation, similar experiments were carried out by replacing the enzymatic solution by the same amount of corresponding buffer. Results obtained are reported in Fig. 3.2a.

As observed in the control reactions, AMX in aqueous solution was less stable at acidic conditions in comparison to neutral pH values. After 48 h at pH 3 around 80% of the compound was disappeared. At higher pH values, self-degradation decreased and stabilized at around 30% (pH 5-7). It is important to note that after enzyme addition, no AMX removal was observed at pH 3 by any of the laccases used. Moreover, AMX transformation yield significantly variated depending on the type of laccase considered. According to Fig. 3.2a the addition of MTL leads to a slight decrease of AMX concentration in comparison to the control as long as pH is less or equal to 5. However, at pH values higher than 5, no considerable transformation was observed regarding to the control curve.

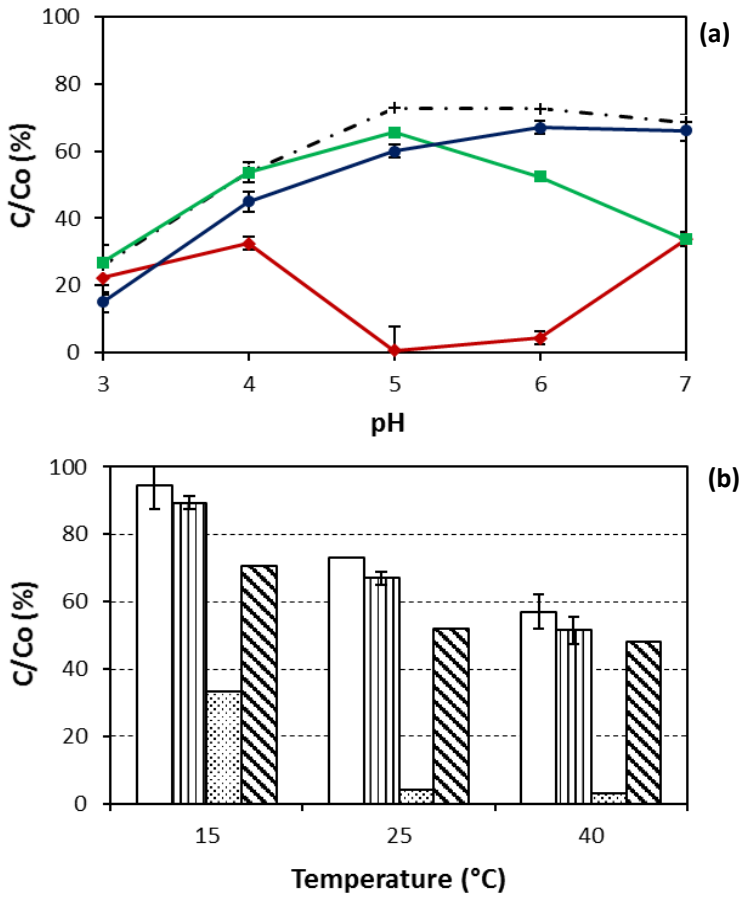


Fig. 3.2. (a) Oxidation of AMX at different pH by laccases from *M. thermophila* (●), *P. sanguineus* (◆) and *T. versicolor* (■), control without enzyme (+) after 48 h at 25 °C. (b) Effect of temperature on the oxidation of AMX at pH 6 after 48 h by laccases from *M. thermophila* (▥), *P. sanguineus* (▨) and *T. versicolor* (▧), control without enzyme (□).

The TVL presented a completely different reactivity towards AMX in function of pH. No oxidation occurred when pH was in the range from 3 to 5. Nonetheless, when pH was higher than 5, AMX concentration significantly decreased in comparison to control reactions (residual concentrations at pH 6 was 52% versus 73% and 34% versus 69% at pH 7). The best performance of TVL was thus achieved between pH6 and 7. PSL was definitely the best enzyme for the AMX degradation. In comparison to controls this enzyme allowed to improve AMX degradation from pH 4 to 7. The best performance was observed between pH 5 and 6. In such conditions AMX was almost completely removed (residual concentration less than 1% and 4% at pH 5 and 6 respectively).

Optimal pH depends on the laccase properties as well as on the substrate properties, since pH can alter the charge of the compound and the configuration shape of the enzyme

which is fundamental for the access or the binding of substrates to the active site [38]. These results are in good agreement with the conversions reported by Lloret et al. [40] and Margot et al. [38], who reported an optimal pH around 5.5 for the degradation of several micropollutants. Even though in some cases oxidation can be observed at low pH values, acidic conditions may lead to complete enzyme deactivation. According to the results presented in this work pH 6 would represent the balance between laccase activity and stability to maximize AMX oxidation capacity; therefore, pH 6 was chosen to evaluate the influence of temperature in the catalytic system.

The effect of temperature on the removal of AMX was evaluated at 15, 25 and 40°C. From Fig. 3.2b it can be noticed that the increase of reaction temperature leads to an increase of AMX self-degradation from 6% at 15 °C to nearly 40% at 40°C. This is not surprising, since most of chemical reactions are favoured by temperature. AMX contains an EDG prone to laccase catalytic activity; hence laccase properties will be determinant to find the best reactivity conditions. Regardless of the tested temperature, MTL did not improve considerably the removal of AMX. These results were expected since this enzyme presented little activity at pH 6. After the addition of TVL, residual content of AMX decreased as temperature increased. The highest AMX removal (around 50%) was obtained at 40°C. However, if self-degradation is taken into account, no significant bio-oxidation increase was observed when passing from 25°C to 40°C. The best oxidation performance was obtained with PSL reaching removal values up to 80% and 90% after only 24 h at 25 and 40°C respectively. Afterwards reaction slowed down –probably due to the decrease of substrate concentration- even so the final AMX concentrations were less than 4 and 3% respectively. It is also important to remark that PSL showed good oxidation performance even at 15 °C. After 24 h 65% of AMX was successfully transformed.

Similar behaviour was also reported by Margot et al. [38] for the removal of DCF and mefenamic acid by *T. versicolor* laccase, removal increased from 10 to 25 °C but then no additional removal was observed at higher temperatures. Authors suggested that optimal temperature depends on the substrate properties since laccase denaturation is less likely to occur in the temperature range tested. The three laccases tested in this work are produced by different strains of white rot fungi; therefore, they are expected to display different catalytic properties. Moreover, commercial TVL and MTL are purified or partially purified

enzymes contrary to the laccase cocktail from PSL. The better performance of PSL could be attributed to the presence of at least two laccase isoforms in the culture supernatant as well as some impurities. All these components in PSL supernatant could have protected the active centre from pH and temperature variations making the laccase cocktail the same or more stable than the purified isoforms as suggested by Ramirez-Cavazos et al. [41].

3.3.3. Oxidation of pharmaceuticals by laccases in the presence of redox mediators

In this section the mediated action of two natural mediators: p-coumaric acid and syringaldehyde was compared with the influence of the synthetic mediator ABTS for the oxidation of the pharmaceutical active compounds in mixtures. As mentioned in section 3.1, factors such as molecular structure of the micropollutant, redox potential of the enzyme, type and concentration of mediator can strongly influence the degradation process. In this sense we chose to work with mediator compounds that follow different oxidation mechanisms: hydrogen atom transfer (SYR and PCA) and electron transfer (ABTS). Mediator concentration was fixed at 520 µM to provide a high mediator:substrate molar ratio to allow the effective removal of the studied pharmaceuticals by laccase-mediator systems as demonstrated by Murugesan et al. [42]. Finally, all degradation reactions as well as control reactions were followed for at least 3 h.

3.3.3.1. Effect of p-coumaric acid addition

Degradation yields obtained after PCA addition variate in function of the substrate-enzyme couple as shown in Fig. 3.3. It is important to note that no effect of PCA addition on the transformation of pharmaceuticals was observed in control reactions, suggesting that degradation was carried out effectively by the laccase-mediator system. The same observation can be done for all the couples enzyme-mediator studied.

In the case of TVL, addition of PCA leads to a slow but continuous oxidation of SMX and CIP (Fig. 3.3a and 3.3c). Respectively 31% and 40% of these compounds were removed after 2 h of reaction; afterwards their concentrations remained nearly constant. However, TVL was still unable to oxidize AMX contrary to PSL which allowed removing 40% of AMX in less than 40 minutes of reaction. Again, the increase of reaction time did not lead to an

enhancement of AMX removal; the residual concentration after 2 h stayed constant at 60% of the initial concentration. Regarding to CBZ, no sign of degradation was observed after PCA addition. In fact, it was not transformed by any couple laccase-mediator studied confirming the recalcitrance of this antiepileptic to biodegradation (data not shown).

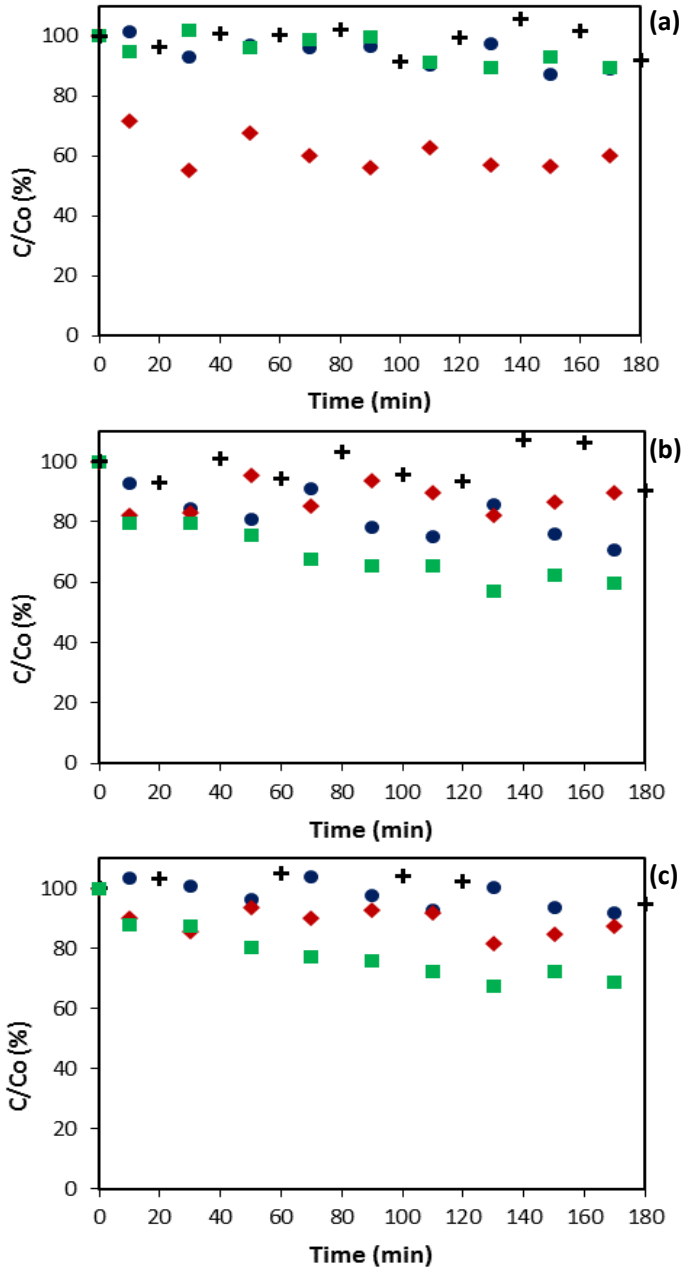


Fig. 3.3 Residual concentration of (a) AMX, (b) SMX and (c) CIP in mixture after 170 minutes treatment with laccase-PCA mediator system: *M. thermophila* (●), *P. sanguineus* (◆) and *T. versicolor* (■). Control without enzyme (+) at 25 °C and pH 7. Standard deviation less than 10% from duplicate experiments.

The mechanism of reaction followed by PCA for substrate oxidation is based on hydrogen atom transfer (HAT) in which mediator remove a hydrogen atom to create phenoxyl radical. Nevertheless, the effectiveness of this oxidation process is also related to the redox potential of PCA which altogether with the affinity of the enzyme for the mediator and the reactivity/stability of radicals formed will control the yield of laccase-mediator reactions [40,43]. Following this mechanism PCA has been reported as an excellent laccase mediator, especially for the removal of recalcitrant pollutants such as polycyclic aromatic hydrocarbons (PAH) [43,44]. In this way, PCA was reported to enhance the removal of the recalcitrant CBZ in hybrid membrane reactors, both in single [37] and mixture micropollutant solutions [39]. The improved degradation of CBZ in the mentioned works could be attributed to the improved stability of immobilized enzyme used in the bioreactors compared with free laccase. Although in the present investigation PCA did not enhance CBZ oxidation, its effect in the removal of other persistent pollutants was validated.

In this study from all the enzymes, TVL showed higher affinity to PCA since better degradation yields were observed specially towards recalcitrant pharmaceuticals such as CIP. Although redox potential of PCA was high enough to start oxidizing the pharmaceuticals, most likely phenoxyl radicals formed were not stable to continue with the process. For instance, visible effects were observed at the end of three hours with relatively low oxidation ratios (10-40%).

3.3.3.2. Effect of ABTS addition

The effects of ABTS addition on the transformation of pharmaceuticals are presented in Fig. 3.4. The presence of ABTS in the reaction medium allowed the oxidation of AMX and SMX by TVL and MTL. Degradation percentage observed for AMX by TVL was higher than the obtained with MTL; a complete removal of AMX was observed after 2 h of reaction with TVL whereas about 90% of removal was achieved after 3 h with MTL (Fig. 3.4a). Moreover, it is worth noting that during the first 90 minutes of reaction, ABTS did not favour the oxidation of SMX by any of both laccases. Visible effects were only observed after 2 h with removal efficiencies up to 30 and 50% by MTL and TVL respectively (Fig. 3.4b).

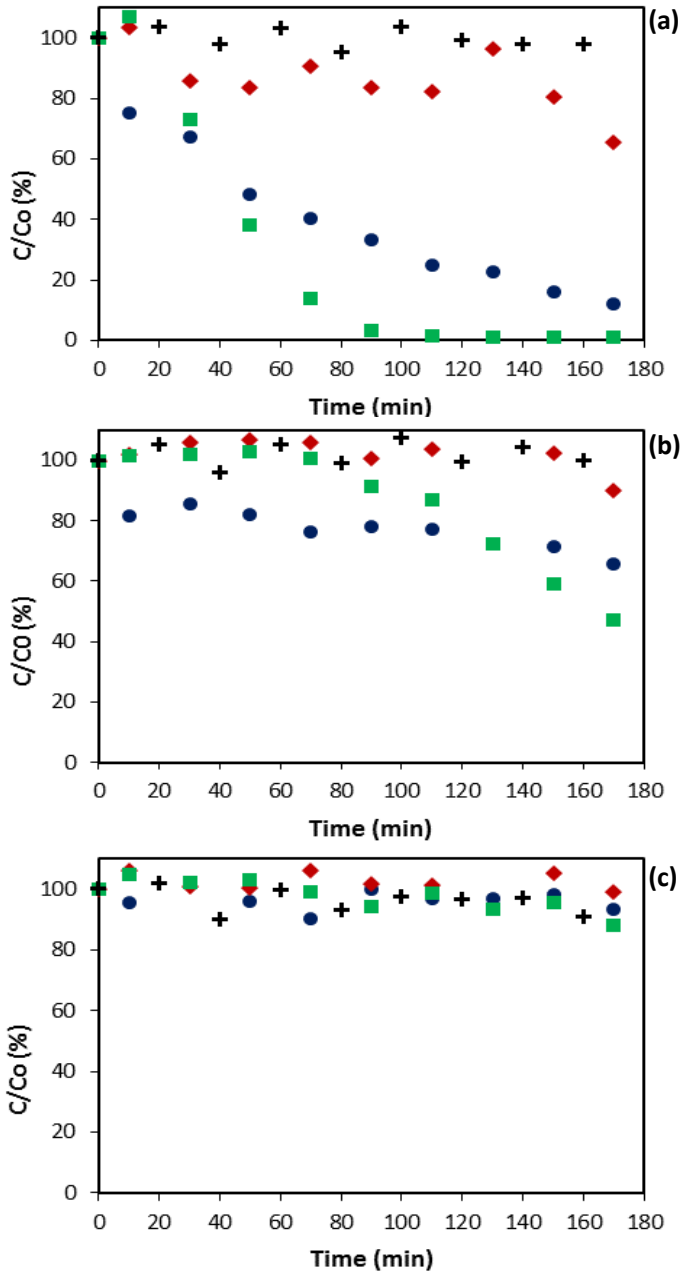


Fig. 3.4. Time course degradation of AMX (a), SMX (b) and CIP (c) by laccases in presence of ABTS as mediator at pH 7 and 25 °C. *M. thermophila* (●), *P. sanguineus* (◆) and *T. versicolor* (■). Control without enzyme (+). Standard deviation less than 10% from duplicate experiments.

Few studies have reported the degradation of SMX by laccase-mediator systems [25,34,45]. Margot et al. [25] obtained similar removal yields to those obtained in this work (30%) with *T. versicolor* laccase in presence of ABTS in pure compound solutions but at longer reaction times (10 h). ABTS is an azino mediator that undergoes oxidation by means of electron transfer mechanism and has been proposed for the transformation of

compounds with relatively weak C-H bonds [46]. Nonetheless, in general, the reactivity of mediators towards substrates in laccase-mediator systems varies depending on the functional groups in the substrate [21]. SMX presents a phenylamine in its structure whereas AMX shows a phenol group. Since both groups are susceptible for laccase degradation, phenol may be an easier substrate to be transformed due to the presence of electron-donating substituents at the benzene ring which decreases the electrochemical potential [47].

Last assumption was confirmed by means of the electrochemical analysis carried out with substrates and mediators. When AMX or SMX were added to a solution of ABTS an increase in the oxidation current for both cation ($ABTS^{\bullet+}$) and dication ($ABTS^{\bullet 2+}$) forms was observed. ABTS dication current increased from 11.5 to 23 mA in presence of AMX and from 11.5 to 13.5 mA for SMX. This phenomenon was previously reported by Bourbonnais et al. [48] for the oxidation of veratryl alcohol with ABTS. According to these authors, during cyclic voltammetry determinations the mediator is oxidized at the electrode and then radicals formed diffuse in the solution to oxidize the substrate. In the case of ABTS the reaction relies on the two electrons oxidation of substrate that regenerates the cation radical at the electrode, resulting in a current increase compared to the oxidation of ABTS alone. The fact that the increase of oxidation current for AMX is significantly higher than for SMX suggest that radicals formed during AMX oxidation are more stable and regenerates faster at the electrode making easier AMX transformation in comparison to SMX.

Independently of the enzyme tested, the presence of ABTS did not allow CIP oxidation. These results contrast with those reported by Prieto et al. [49] where almost complete degradation of CIP (i.e. 97%) by *T. versicolor*-ABTS system was achieved but in their case the reaction was carried out for 30 h. In addition to the difference of reaction times, the absence of CIP biodegradation could be related to the difference of fungal species used to produce the laccases. It may also result from a competition with AMX and SMX oxidation. ABTS cation radicals reacted first with AMX and SMX during 3 h, by that time radicals may not be stable or available anymore to continue with the oxidation which would limit the reaction [24]. Further research would be necessary to determine the optimal reaction parameters as well as the factors limiting the reaction (i.e. availability of ABTS and laccase stability).

On the contrary to the results obtained for TVL and MTL, ABTS did not significantly react with PSL since lower degradation percentages were obtained with this couple. A different behaviour was reported by Gao et al. [34] who tested a laccase extract from *P. sanguineus* for the degradation of antibiotics, among them CIP and SMX, in single and mixture solution. They carried out reactions at 30 °C in the presence of ABTS (1 mM) obtaining high transformation yields (85-100%) but 72 h were necessary to achieve these removal yields. The high transformation yields obtained by Gao could be influenced by the operational conditions used in their experiments, higher temperatures and mediator concentration as well as longer reaction times may favour the transformation of pharmaceuticals.

3.3.3.3. Effect of syringaldehyde addition

The addition of SYR to the pharmaceuticals mixture resulted on a high enzymatic degradation (50-100%) (Fig. 3.5). Among all pharmaceuticals studied, SMX was removed with the highest efficiency by all laccases. MTL showed the best performance with almost 100% degradation within the first 10 minutes of reaction (Fig. 3.5b). TVL and PSL also completely transformed SMX after 1.5 and 2.5 h. These results are better to those reported by Shi et al. [50] who observed almost a complete degradation of SMX after 30 min with a laccase from *Echinodontium taxodii* but in the presence of higher SYR concentration (1 mM).

Similarly, AMX was well removed by the couple MTL-SYR attaining 80% removal within the first 30 minutes. After this time the reaction stabilized and no significant increase in degradation was observed before the end of the experiment. In the case of TVL and PSL moderate transformation yields of AMX were obtained; 50% of removal was achieved at the end of 3 h (Fig. 3.5a). Increasing the reaction time up to 8 h showed no significant enhancement in degradation; after 4h AMX removal leveled off around to 70%. Degradation of AMX in presence of different concentrations of SYR (0.1 and 1 mM) was described by Becker et al. [31]. These authors reported removal yields from 89 to 95% depending on the mediator concentration. Interestingly, authors observed that the use of SYR slightly reduced removal yield of AMX in comparison with the treatment with only laccase. Such negative effect was not observed in this study, on the contrary, enhanced oxidation of AMX was observed for all laccases tested.

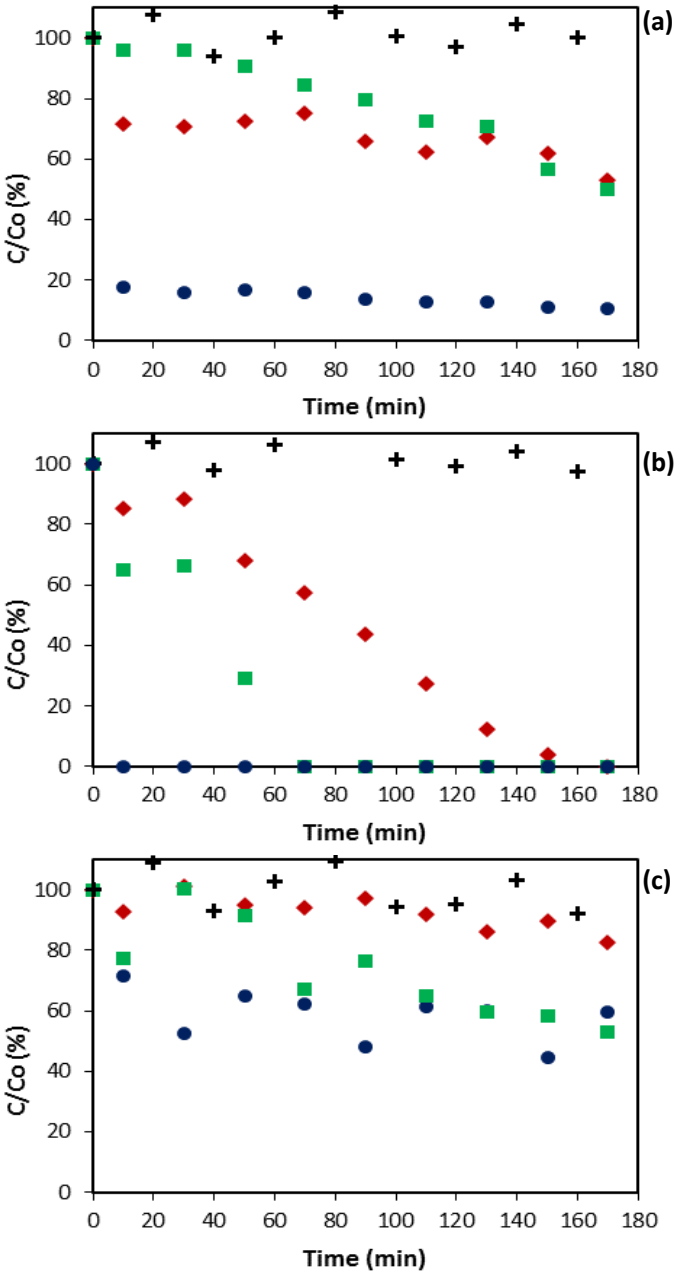


Fig. 3.5. Oxidation of AMX (a), SMX (b) and CIP (c) by laccases in presence of SYR as mediator at pH 7 and 25 °C. *M. thermophila* (●), *P. sanguineus* (◆) and *T. versicolor* (■). Control without enzyme (+). Standard deviation less than 10% from duplicate experiments.

SYR was able to oxidize CIP altogether with MTL and TVL attaining 40% removal after 3h of reaction and up to 60% at the end of 8 h. To our knowledge, results obtained in this study represent the highest CIP degradation yield reported for a laccase catalysed oxidation by free enzymes using SYR as mediator. Laccase-SYR system was previously tested for the degradation of CIP with very low removal yields [36,51]. For example, Ding et al. [36]

observed negligible removal of CIP by free laccase. Moreover, the degradation was not improved by the presence of SYR even at concentrations as high as 2 mM.

Mediator SYR belongs to the group of substituted phenols compounds that have been proved as suitable laccase mediators [26,52]. The mechanism of reaction followed by SYR for the oxidation of non-phenolic substrates is the generation of phenoxyl radicals by HAT [53]. Structurally, SYR presents a phenol group which is easily oxidized by laccase and two methoxy substituents in ortho position that increase the stability of phenoxyl radicals. Therefore, the efficiency of SYR as mediator in terms of speed and conversion yield is related to the high concentration and stability of the phenoxyl radicals formed during oxidation reactions [47]. This actually explains the high and fast conversion yields obtained in this study.

Overall, from all mediators studied the best results were obtained with SYR. The presence of SYR accelerates degradation of the pharmaceuticals within the first 3 h of reaction, after this time no further transformation was observed. Various hypotheses could explain the threshold observed: loss of mediators and/or laccase activity, enhancement of the concentration of oxidation products, etc. all of these possibilities could have a negative influence on the degradation rate. For example, some authors have demonstrated that an excess of mediators, as is the case of this work, can result on the acceleration of the loss of laccase activity by the attack of the radicals formed during mediator oxidation to enzyme's catalytic sites [24,37,54]. However, in this work samples taken from the supernatant still showed enzymatic activity for the three laccases after 7 h of reaction (results not presented). Indeed, it is also possible a competition for the oxidative process between the pharmaceuticals and oxidation products formed. De Cazes [16,55] has already noticed this effect for the degradation of tetracycline and erythromycin with immobilized enzymes. The authors observed a decrease of the degradation yield with time up to stabilization of antibiotics concentration after 24 h of reaction. Then, substrates solutions were replaced by fresh ones and the initial degradation activity was reached again. They were able to repeat the same cycles during more than 200 h.

As mentioned at the beginning of this section, CBZ was not transformed by any laccase-mediator system assayed. Poor removal of CBZ was previously reported even in the presence of redox mediators like SYR or ABTS [37,54]. As previously discussed, the low

removal amounts can be attributed to the presence of strong EWG in its structure making the pharmaceutical not suitable for biodegradation. Moreover, recent evidence suggests that reaction conditions could play a determinant role in CBZ degradation. Naghdi et al. [56] observed that addition of ABTS increased degradation of CBZ from 30% to 82% in 24 h by a laccase extract from *T. versicolor*, but when reaction conditions were optimized to 35 °C, pH 6, with 60 U L^{-1} of enzyme concentration and 18 µM of mediator up to 95% removal was obtained. Further investigation should be done in order to find the conditions allowing CBZ degradation in the matrix.

Generally, the addition of redox mediators resulted in the increase of pharmaceuticals degradation. However, the degradation yield depends on the mediator type and laccase used. The best combination of mediator/laccase leading to the highest removal of AMX was TVL in combination with ABTS. In the case of SMX the antibiotic was better removed by MTL and SYR. Similarly, CIP highest removal was obtained by both MTL and TVL in presence of SYR.

From the three redox mediators SYR showed as the most effective mediator allowing the degradation of AMX, SMX and CIP in short periods of time. The effectiveness of SYR to mediate the oxidation of pharmaceuticals was followed by ABTS and PCA which presented the slowest reaction yields. MTL and TVL were the most reactive enzymes showing high affinity to SYR (Table 3.2).

Table 3.2. Reactivity of selected laccases towards mediators.

Pharmaceutical	Laccase		
	M. thermophila	*T. versicolor*	*P. sanguineus*
AMX	SYR (++)	ABTS (+++)	SYR (+)
SMX	SYR (+++)	SYR (++)	SYR (+)
CIP	SYR (+++)	SYR (+++)	-
CBZ	-	-	-

(+++) very reactive; (++) reactive; (+) less reactive.

3.4. Conclusions

Results of this work show that without mediators, laccases only oxidized amoxicillin. Operational conditions had an important influence in the removal yield of the antibiotic that was favoured at acidic conditions (pH 5-6).

In presence of redox mediators, pharmaceuticals degradation was both mediator and laccase dependent. Syringaldehyde and ABTS showed as the best redox mediators allowing the highest transformation yields (50-100%) in less than 3 h. However, there is not a broad spectrum mediator since carbamazepine was not transformed by any laccase-mediator system. From our knowledge this is the most detailed study presenting the enzymatic degradation of amoxicillin in laccase-mediator systems that was better degraded by laccase in presence of ABTS but closely followed by syringaldehyde. Results suggest that amoxicillin oxidation occurs by the ET mechanism of reaction but analysis of the reaction products will be needed to confirm this assumption. The best degradation yields of ciprofloxacin and sulfamethoxazole were reached by using a commercial laccase and syringaldehyde as mediator. These results show that pharmaceuticals containing phenolic groups are removed depending on the redox potential of the mediator and the stability of the radicals formed. Overall, commercial laccases presented better performance for the degradation of micropollutants in laccase-mediator systems. Nonetheless, the data obtained by the comparison of performance among the three different laccase formulations in the model study under the same controlled conditions offer valuable information of the potential use of this biocatalyst for the treatment of complex pollutant matrices where different oxidative characteristics are often needed.

This study demonstrates the potential utility of laccase-mediator systems for treatment of complex micropollutant mixtures. Notwithstanding the progress realized, many drawbacks like stabilization or decay of the enzymatic activity are still present. Further studies to improve these enzymatic processes are being carried out, they include the study of their ecotoxicity. Moreover, the study of the immobilization of different laccase-mediator systems in order to reuse the enzymes is also under way.

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Chapter 4

Novel method for the covalent immobilization of laccases on silica gel and its application for the elimination of pharmaceutical micropollutants

4. Novel method for the covalent immobilization of laccases on silica gel and its application for the elimination of pharmaceutical micropollutants

Abstract

In the present work a novel methodology for the covalent immobilization of laccase onto silica gel carriers by using glutaraldehyde in vapour phase is proposed. The influence of different parameters on the immobilization conditions were evaluated by a 2^3 full factorial design. Through regression analysis the process was well fitted by a linear polynomial equation (R^2=0.90899; Adj-R^2=0.8559) under which laccase activity reached 14 ± 2 U g^{-1} of beads with a protein immobilization yield of 35%. The biocatalyst was characterized and then tested in the removal of pharmaceutical micropollutants in the presence of redox mediators. High removal yields (40-100%) were obtained within 3 h of treatment as the result of the synergistic effect of laccase-mediator biotransformation and adsorption on the support. Moreover, the reusability of the biocatalyst was proved during 7 degradation cycles. Although the addition of redox mediators allowed high removal yields, these compounds led to the increase in the acute toxicity of the effluent as suggested by the Microtox® assay, which variated on the type of mediator. Notwithstanding the toxicity issue, the biocatalyst proposed in this investigation could be a promising alternative for the application of enzymatic treatment in bioremediation applications.

Keywords: Laccase, covalent immobilization, redox mediators, pharmaceutical micropollutants, Toxicity test

4.1. Introduction

World wide access to pharmaceutical products (PPs) has improved living conditions in benefit of the society. Nonetheless, the broad use of pharmaceuticals also results in their continuous release into the environment as unchanged compounds or metabolites that are inefficiently removed by traditional wastewater treatment plants (WWTPs) (Grandclément et al., 2017). For instance, PPs have been detected in water sources and even in drinking water at concentrations as low as micrograms or nanograms per liter (Heberer, 2002). At these low concentrations pharmaceuticals have the potential to cause short and long term toxicity, endocrine disrupting effects in aquatic fauna and humans. Moreover, the presence of low quantity of antibiotics in treated wastewaters can also result on antibiotic-resistance in bacteria (Luo et al., 2014; Voulvoulis et al., 2015).

To address this environmental issue advanced processes such as extraction, adsorption on activated carbon, ozonation, Fenton oxidation and electrochemical techniques have been introduced to WWTPs. In order to meet high quality of the treated water these tertiary treatments combined to conventional biological treatments allow the oxidation and/or the removal of PPs but limitations such as higher cost, formation of hazardous by-products and low efficiency have been reported (Luo et al., 2014).

An alternative to chemical and/or physical methods for the removal of PPs in water is the use of enzyme catalysed processes. The bioremediation with biocatalyst such as laccases, lipases or tyrosinases can be considered as environmentally friendly processes because they consume less chemicals, water, energy and produce less waste in comparison to other chemical oxidative processes (Zdarta et al., 2018). In particular laccases receive special attention since they are able to catalyse the oxidation of phenolic and non-phenolic compounds by only using molecular oxygen that is reduced to water. Hence, since the past two decades several reports have confirmed the effectiveness of laccases for the removal of a broad range of pollutants including pharmaceuticals (Barrios-Estrada et al., 2018; De Cazes et al., 2014; Ding et al., 2016).

Despite all advantages of enzymatic treatment of wastewaters, the use of laccases in environmental applications present some drawbacks related to their limited stability in solution, difficult recovery and reusability which results in high operational costs. However,

these disadvantages can be overcome by immobilization of enzymes on different supports such as silica, magnetic nanoparticles, chitosan microspheres or Amberlite beads (Fortes et al., 2017; Jiang et al., 2005; Patel et al., 2014; Spinelli et al., 2013). Among the different types of carriers, silica-based materials are highly suitable for enzyme immobilization due to their great biocompatibility. Moreover, the hydrophilicity of the surface of silica particles can be modulated in order to promote enzyme immobilization not only by adsorption but also via covalent bonds (Patel et al., 2014; Zdarta et al., 2018). Covalent immobilization is a rapid method based in the creation of strong covalent bonds between the enzyme and the activated groups on the surface of the support (Fernández-Fernández et al., 2013). The result is a multipoint attachment of the enzyme to the support that significantly enhances enzyme reusability and reduces enzyme leaching.

However, in order to develop a successful immobilization method, the properties of the enzyme and the type of support have to be evaluated since parameters such as immobilization yield and efficiency are highly influenced by the carrier material (Sheldon and van Pelt, 2013). Moreover, immobilization conditions play an important role in the process for which optimization is indispensable. Thereby, a continuous searching of new materials and immobilization protocols is necessary in order to provide a wide spectrum of biocatalyst that can be effectively applied in industrial processes.

In the present study, a novel method for the covalent immobilization of laccases and the potential application of the biocatalyst for the degradation of PPs in water is proposed. Laccase immobilization was carried out on commercial functionalized silica gel particles that were previously activated with glutaraldehyde in vapour phase. The effects of concentration and pH of the enzymatic solution and the contact time during the immobilization process were evaluated by a full factorial design. The biocatalyst obtained was then characterized in terms of activity, stability at different pH and temperature conditions and long-term storage and compared to the performance of free enzyme. Finally, immobilized laccase was tested for the degradation of PPs containing different antibiotics (amoxicillin, ciprofloxacin, sulfamethoxazole) and an anti-epileptic (carbamazepine) and the reusability of the biocatalyst was also evaluated during several degradation cycles.

4.2. Materials and methods

4.2.1 Chemicals and enzymes

The pharmaceuticals (amoxicillin (AMX), ciprofloxacin (CIP), carbamazepine (CBZ) and sulfamethoxazole (SMX)), the mediators (p-coumaric acid (PCA), syringaldehyde (SYR), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)), the cross-linker agent glutaraldehyde (GLU) (25% aqueous solution) and all other chemicals were purchased from Sigma-Aldrich. Stock solutions (100 mg L^{-1}) of AMX and SMX were prepared in citrate-phosphate buffer 0.1 M, pH 7. Given the low water solubility of CIP, the antibiotic was pre-dissolved in hydrochloric acid (0.1 N) and then in buffer citrate-phosphate pH 7. The compounds CBZ, SYR and PCA were prepared in pure ethanol at a concentration of 15625 mgL^{-1} for the pharmaceutical and 114.9 mM for the mediators. Fresh ABTS solution (5 mM) was prepared just before use in buffer citrate-phosphate pH 7.

3-Aminopropyl-functionalized silica gel (particle size 40 - 63 µm) from Sigma-Aldrich was used as support for laccase immobilization. Three different laccase preparations were tested. Laccase powder from *Trametes versicolor* (ref 38429, activity ≥0.5 U mg^{-1} and ref 51639, activity ≥10 U mg^{-1}) (TVL) was purchased from Sigma-Aldrich. TVL ref 51639 was used for the determination of immobilization conditions and oxidation reactions with mediators; the rest of the experiments where this enzyme was used were done with TVL ref 38429. Commercial laccase produced by submerged fermentation from *Myceliophthora thermophila* (59.5 g L^{-1} of pure laccase) (MTL) was provided by Novozymes (Denmark). Laccases from *Pycnoporus sanguineus* CS43 (PSL) were obtained from a tomato medium as described by Ramirez-Cavazos et al. with some modifications. In short, mycelia were removed from the culture supernatant by filtration using two tangential flow filters in series, with respective pore sizes of 0.5 and 0.2 µm. Afterwards, the 0.2 µm filtrate (laccase cocktail) was concentrated on a 10 kDa ultrafiltration membrane (Ramírez-Cavazos et al., 2014).

4.2.2 Laccase activity essay and protein estimation

The laccase activity was determined by measuring the oxidation of 1 mM ABTS at 420 nm (Ɛ420 = 36000 M^{-1} cm^{-1}) in 0.1 M citrate-phosphate buffer (pH 4) at 25 °C. One enzyme

activity unit (U) was defined as the amount of enzyme that oxidized 1 µmol of ABTS per min. Immobilized laccase activity was assayed by incubating approximately 8 mg of biocatalyst (dry weight) in 50 ml solution of ABTS (1 mM) prepared in citrate-phosphate buffer (0.1 M, pH 4) at 25 °C with continuous stirring. The absorbance at 420 nm was monitored every minute during a period of 10 minutes. Immobilized laccase activity was expressed in U g^{-1} solid.

Protein concentrations were estimated by the method of Lowry using bovine serum albumin as a standard (Lowry et al., 1951). The quantity of protein immobilized on the support was calculated by the difference between the amount of protein initially added and the recovered in the supernatant and washing solutions. All spectrophotometric measurements were carried out on a Shimadzu UV-2401PC spectrophotometer.

4.2.3 Laccase immobilization

Laccases were covalently immobilized on the surface of silica gel particles. Firstly, the silica gel particles were activated with GLU in vapour phase. 100 mg of 3-Aminopropyl-functionalized silica gel were uniformly dispersed on the bottom of an aluminium plate (35 mL) and placed inside a glass vessel (100 mL) containing approximately 15 mL of 25% GLU solution into a closed vessel (5 L) which was kept at 30°C for different periods of time (18, 30 and 42 h). Then, the GLU-activated powder was placed in 5 mL of enzymatic solution containing 107, 203 or 300 U of one of studied laccases (TVL, MTL and PSL) in 0.1 M buffer citrate-phosphate pH 4.3, 5.66 or 7. The solution was maintained for 1, 1.5 or 2 h at 25 °C under stirring so that the free GLU –CHO groups in the silica could react with amino groups of enzymes. Finally, the enzyme-activated solid was washed with buffer citrate-phosphate (0.1 M, pH 4) and stored at 4 °C. The supernatant from enzyme grafting process and the solutions produced from the four washing steps were stored for protein determination.

Bound protein expressed as percentage was calculated as the ratio between the amount of protein immobilized and the protein content in the initial enzymatic solution.

4.2.4 Experimental design and statistical analysis

In this study, 2^3 full-factorial experimental design was used to evaluate the influence of three parameters (concentration of enzyme solution (X_1), immobilization time (X_2) and pH of enzyme solution (X_3)) in the activity of immobilized laccase. For each factor three levels were selected: low level (-1), high level (+1) and basic level (zero) (Table 4.1). A total of 10 experimental runs, including two replicates at the central point were carried out in duplicate using MTL as a model enzyme. The obtained responses are shown in Table 4.2. From experimental data, analysis of variance (ANOVA) was done and regression coefficients were obtained using Statistica™ software. Curvature check for the response was assessed and if significant considered. Moreover, the proportion of variance explained by the model obtained was given by the multiple coefficient of determination R^2.

The regression equation based on the first-order model with three parameters (X_1, X_2 and X_3) and their interactions is presented as follows:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (1)$$

Where Y_i represents the predicted response (activity of immobilized laccase); X_1, X_2 and X_3 are the coded levels of the factors enzyme concentration, immobilization time and pH of immobilization. The regression coefficients are: b_0 the constant regression coefficient; b_1, b_2, b_3 and b_{12}, b_{13}, b_{23} and b_{123} the regression coefficients for the main and interaction effects respectively.

Table 4.1. Factor levels used according to the 2^3 factorial design.

Variable	Level		
	-1	**0**	**1**
X_1: Concentration of enzyme solution (U)	107	203	300
X_2: Immobilization time (h)	1	1.5	2
X_3: pH of enzyme solution	4.33	5.66	7

4.2.5 Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR)

The presence of functional groups on the enzyme and support before and after immobilization was evaluated by FTIR spectroscopy. Analysis was done using a Nicolet "Nexus" spectrometer (ThermoFisher) equipped with a diamond ATR "Golden Gate" plate. The scan range was 400-4000 cm^{-1} using 128 scans per spectrum with a resolution of 4 cm^{-1}.

4.2.6 Characterization of free and immobilized laccase

Optimum pH and pH stability

The pH of maximum laccase activity (free and immobilized) was investigated using 1 mM ABTS in 0.1 M citrate-phosphate buffer (pH 3-7). The relative activity was calculated as the ratio between the activity at each pH and the maximum attained.

The effect of pH on the enzyme stability was studied by incubating laccase in 0.1 M citrate-phosphate buffer (pH 4 and 7) at 25 °C during 24 h. Samples were taken and transferred to standard conditions for activity measurement. The residual activity was calculated in reference to the initial activity value obtained at each pH.

Optimum temperature and thermostability

The effect of temperature (15-65°C) on laccase activity was determined by measuring the activity at the corresponding temperature under the standard conditions described for both free and immobilized enzymes. The relative activity was calculated as the ratio between the activity at each temperature and the maximum attained.

Studies of thermal stability of immobilized laccase were carried out by measuring the residual activity of the laccase exposed to different temperatures (25, 45 and 55°C) in 0.1 M citrate-phosphate buffer (pH 7) for different incubation periods and compared with the results obtained with free laccase. Samples were transferred to standard reaction conditions to determine the laccase activity with ABTS as previously described.

Storage stability

Storage stability of free and immobilized laccase was investigated at 4°C. For this, both laccase formulations were kept in plastic tubes containing buffer (0.1 M) citrate-

phosphate pH 7. The residual activities were obtained after 1 and 2 months by measuring the remaining activity of each enzyme at standard conditions.

4.2.7 Oxidation of pharmaceuticals by immobilized laccase

Degradation experiments were run in 25 mL glass cells at 25°C under continuous stirring to ensure O_2 saturation of the reaction medium and dark conditions to avoid light oxidation. Reaction media (5 mL) contained a mixture of all pollutants in buffer 0.1 M citrate-phosphate pH 7 and one redox mediator (ABTS, PCA and SYR) at a concentration of 520 µM. Micropollutants concentration was fixed at 20 mg L^{-1} except for CBZ where it was only 10 mg L^{-1}. Reactions started when 2.5 U (164 ± 18 mg) of immobilized TVL were added to the medium. The degradation process was monitored during 4 h by withdrawing samples at regular times. After sampling, aliquots were centrifuged at high speed for five minutes to separate the reaction medium from the biocatalyst; then, the medium was filtered with CHROMAFIL Xtra H-PTFE-20/30 filters to immediately be analysed by HPLC-MS.

In order to evaluate the reusability of the biocatalyst, experiments were carried out as described above using SYR as redox mediator and 2.5 U of immobilized TVL. The reaction was followed for 2 h (one cycle). At the end of each cycle the reaction solution was separated from immobilized enzyme by centrifugation, filtered and immediately analysed by HPLC-MS. Then, immobilized enzyme was washed three times with buffer citrate-phosphate (0.1 M, pH 7) and afterwards used for a new cycle of degradation in the same conditions as described above. The biocatalyst was reused for a total of 9 cycles. Enzymatic degradation was calculated considering the initial and final concentration of micropollutants in the reaction solution for each cycle.

All experiments were carried out by duplicate and control without immobilized enzyme neither mediator, control with only 3-Aminopropyl-functionalized silica particles and control with inactive immobilized enzyme and mediator were run in parallel to assess the role of immobilized enzyme on the removal of the pharmaceuticals except for biocatalyst reusability where only control with inactive immobilized enzyme and mediator was run.

4.2.8 Microtox test

Acute toxicity of the mixture of PPs before and after enzymatic treatment was evaluated by the Microtox® bacteria toxicity test based on the general principles described by ISO (2007) (Karczmarczyk et al., 2014). The toxicity of samples was determined by measuring the effect on the luminescence of marine bacteria. Bacterial luminescence was measured using a Microtox® Model 500 Analyzer (Modern Water Inc.; United Kingdom). The bacteria used in this method was the strain *Vibrio fischeri* NRRL B-11177. This bacterium emited luminescence during growth relating to extracellular respiration which was linked itself to cell activity. As the activity of the cell could be reduced by the presence of toxic elements, bioluminescence is therefore a very good indicator of state of the bacterium and thus of the global toxicity of the sample. This device allowed for the acute toxicity test with the help of the software MicrotoxOmni®.

The test performed in this investigation is called 81.9% basic test were a serie of dilutions starting from 81.9% of the initial sample concentration are carried out by adding 22% NaCl solution to allow *Vibrio fischeri* normal activity and therefore luminescence emission. Before measuring the bacteria luminescence, pH of the samples were adjusted between 6.5 and 7.5 with sodium hydroxide or sulfuric acid, followed by a 0.2 µm-filtration with syringe filters in order to eliminate any precipitate or solid matter in the solution. The results were expressed as $EC_{50,15}$ which corresponds to the concentration of sample (% v/v) that causes a 50% reduction in light emission of the luminescent bacteria after 15 min of contact. The EC_{50} values reported in this study are expressed as percentage (% v/v) of the initial sample and were estimated according to the Basic Test protocol of the software.

4.2.9 HPLC-MS analysis

Determination of pharmaceuticals concentration was carried out on a HPLC Alliance – Waters e2695 separations module coupled to a MS Micromass Quatro micro API (tri-quadripole) detector and a Raptor-C18 column (150 x 2.1 mm) with particle size of 5 µm. The conditions for analysis were: 5 µL of sample injection volume, sample elution at 0.25 mL min^{-1} with a gradient of (A) 95% water - 5% methanol and (B) 100% methanol. The gradient program was set as follows: 0-3 min, 100% (A); 3-8 min, 100% (B) and 8-15 min, 100% (A).

4.3. Results and discussion

4.3.1 Immobilization of laccase on silica gel particles and determination of immobilization conditions

Commercial amino-functionalized silica gel micro particles were used for the evaluation of covalent immobilization of laccase. This support was selected once the immobilization of enzyme on its surface is rapid and easy due to its porous structure (60 Å pore size), large specific surface area (550 m^2 g^{-1}) and content of amino groups (~1.05 mmol of NH_2 g^{-1}).

For this type of immobilization, glutaraldehyde - a bifunctional agent - is added to form covalent bonds between primary amines on the enzyme and an amino functionalized support (Cantone et al., 2013). Generally, this step is carried out in liquid phase when the solid carrier is submerged in a liquid solution of GLU for a determined period of time. Nonetheless, while working with small size supports (micro to nanoparticles) a good dispersion in the GLU solution has to be guaranteed in order to avoid potential agglomeration of the particles and an uneven deposition of GLU on the support that could lead to low immobilization yields. In the present study a novel method for the covalent immobilization of laccases on commercial amino functionalized silica gel micro particles is proposed.

The new immobilization protocol consists in crosslinking laccase on the support previously activated by using GLU in vapour phase (Fig. 4.1). The relatively high volatility of GLU at room temperature allows its evaporation, resulting in the deposition of the activating agent on the support in a dry environment. The GLU vapour phase activation step was performed by keeping in the same closed vessel silica particles and 25% GLU solution for different periods of time. Temperature was set at 30°C to ensure GLU evaporation. After the designated time, silica gel particles which were originally white became orange making evident the reaction of GLU on the solid (Fig. 4.2). The coloration on silica particles was uniform and no agglomeration was observed.

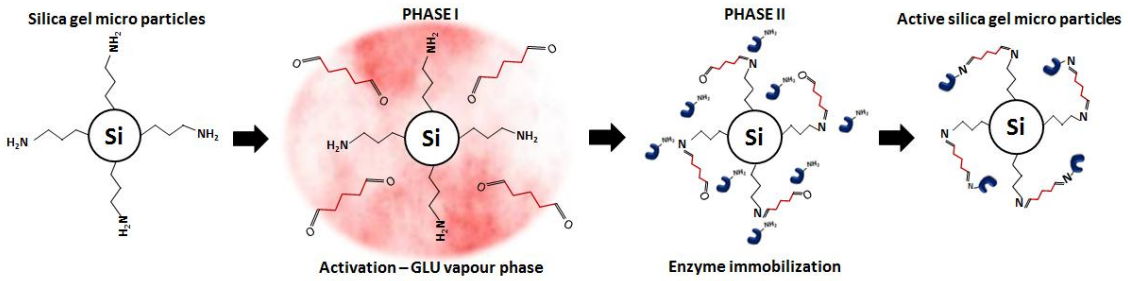

Fig. 4.1. Covalent immobilization of laccase con silica gel via activation with glutaraldehyde in vapour phase.

Based on preliminary experiments, three different contact times (18, 30 and 42 h) were evaluated. Results showed that short activation periods (18 h) yielded to higher specific activity of immobilized enzyme (data not shown). Therefore, GLU activation during 18 h was chosen to carry out all further experiments. GLU vapour cross-linking technique is often used for the fabrication of biosensors and for the modification of nanofibrous materials (i.e. water insolubility property) (Anh et al., 2002; Destaye et al., 2013). From our knowledge this is the first investigation where vapour phase deposition of GLU is used for the immobilization of enzymes onto silica gel particles for bioremediation purposes.

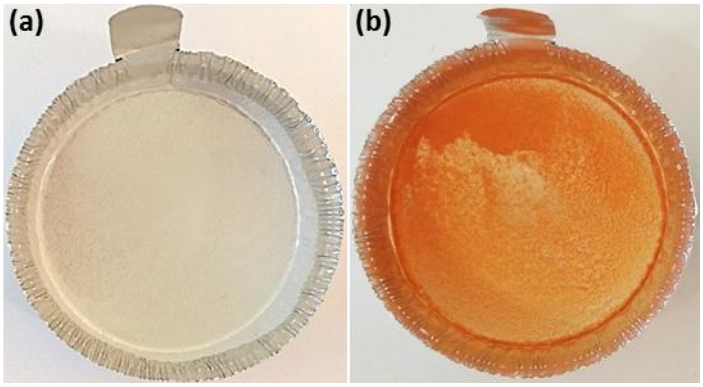

Fig. 4.2. Silica gel microparticles (a) before and (b) after activation with GLU in vapour phase.

In order to determine the immobilization conditions for the novel method, a design of experiments (DOE) approach was applied to evaluate the parameters influencing and improving laccase immobilization. A 2^3 full factorial design was performed where the range of variation of each level was chosen based on theoretical considerations as well as on preliminary experiments. The factors evaluated by the design were concentration of enzyme

solution, time of immobilization and pH of enzyme solution for which the activity expressed by immobilized laccase (U g^{-1} solid) was considered as the response variable. It is worth noting that GLU time of contact was not considered in the present design since its influence on the response was found to not be significant (data not shown). Thus, a total of 10 experiments fully replicated were performed and experimental responses are presented in Table 4.2.

Table 4.2. Values for the response (activity of biocatalyst) according to the 2^3 factorial design

Assay	Factors			Activity of immobilized laccase (U g^{-1} solid)	
	X_1	X_2	X_3	Observed value	Predicted value
1	-1	-1	-1	2.77	2.52
2	-1	-1	1	3.90	3.83
3	-1	1	-1	2.92	2.77
4	-1	1	1	7.26	6.64
5	1	-1	-1	1.73	1.90
6	1	-1	1	2.80	3.79
7	1	1	-1	3.25	3.41
8	1	1	1	2.83	2.73
9	0	0	0	2.50	2.37
10	0	0	0	2.23	2.37
11	-1	-1	-1	2.22	2.52
12	-1	-1	1	3.81	3.83
13	-1	1	-1	2.66	2.77
14	-1	1	1	5.97	6.64
15	1	-1	-1	2.02	1.90
16	1	-1	1	4.83	3.79
17	1	1	-1	3.63	3.41
18	1	1	1	2.57	2.73
19	0	0	0	2.50	2.37
20	0	0	0	2.23	2.37

Using the experimental data and by applying multiple regression analysis a first order model predicting the response was obtained:

$$Y_i = 3.45 - 0.49X_1 + 0.44X_2 + 0.80X_3 - 0.33X_1X_2 - 0.50X_1X_3 - 0.64X_1X_2X_3 \quad (2)$$

The statistical significance of the model was evaluated by the analysis of variance (ANOVA) as shown in Table 4.3.

The model has a good fit as expressed by the correlation coefficient (R^2= 0.908) meaning that the regression model explains 90% of the total variation of the response, whilst the other 10% is explained by the residues. The model presented significant curvature (p = 0.003) suggesting that the data would be better fitted by other type of design such as Response Surface Methodology (RSM). Nonetheless, the main objective of this work was to study the influence of the chosen parameters on the response and for this the first order model regression was enough, so the model was accepted. Moreover, the residuals plots and normal data distribution obtained from the raw residual analysis also validated the model.

Table 4.3. Analysis of variance (ANOVA) for the first order model for activity of immobilized laccase.

Factor	SS	df	MS	*F*	*p*
Curvature	3.76	1	3.76	13.59	0.0031
X_1	3.86	1	3.86	13.97	0.0028
X_2	3.08	1	3.08	11.12	0.0059
X_3	10.17	1	10.17	36.79	0.0001
X_1:X_2	1.69	1	1.69	6.13	0.0292
X_1:X_3	3.97	1	3.97	14.36	0.0026
X_1:X_2:X_3	6.61	1	6.61	23.89	0.0004
Error	3.32	12	0.28		
Total SS	36.46	19			

SS=sum of squares; df=degrees of freedom; MS=mean square; F=F distribution value; X_1=concentration enzyme solution; X_2=immobilization time; X_3=pH enzyme solution.
R^2=0.90899; Adj-R^2=0.8559

The significance of the factors was verified by p-value statistical parameter. Factors showing a p-value below 0.05, with a confidence level higher than 95% were considered as statistically significant for the immobilization process. In this sense, it is important to mention that the final model represented by Eq. 2 was run excluding insignificant effects (factors and interactions) as observed in Table 4.3. The influence of each factor and the way they affected the response is expressed in the model's equation and is given by the

coefficient of each factor and interaction. Thus, from Eq. 2 the factors were ranked regarding to their significance:

$$X_3\ (+) > X_1X_2X_3\ (-) > X_1X_3\ (-) > X_1\ (-) > X_2\ (+) > X_1X_2\ (-)$$

where the sign in parentheses describes if the effect in the response is either positive or negative.

The most significant factor affecting the response was pH of enzyme solution. The model shows that response was positively affected when pH passed from the low (pH 4.33) to the high level (pH 7). Given the low stability of GLU groups at acidic or alkaline pH values, immobilization using GLU activated supports is usually favoured at neutral conditions (Barbosa et al., 2013). At these pH values the most reactive amino group in the protein tends to be terminal amino group which are more likely to react with the polymer (cyclic hemiacetal oligomer) form of GLU to form stable structures (Barbosa et al., 2013; Spinelli et al., 2013). Moreover, GLU crosslinking kinetics at neutral or basic conditions are faster in comparison to acidic conditions resulting favourable for the formation of covalent bonds. (Spinelli et al., 2013).

The pH of immobilization also affects laccase stability. It is known that MTL can retain 100% of its initial activity at pH 7 and room temperature for at least 24 h (Lloret et al., 2010). It is possible that the high stability of laccase at these conditions (room temperature and neutral pH) favoured immobilization in comparison to pH 4. Jiang et al. (2005) also reported pH 7 as the optimal value for the immobilization of *P. sanguineus* laccase on magnetic chitosan microspheres. Authors suggested the low stability and denaturation of the enzyme as well as the degradation of the support as the cause for the low laccase activity and protein loading at more acidic conditions.

The second and third more significant variables in the model were the interactions among the three studied factors and the interaction of laccase concentration and pH of immobilization, both interactions negatively affecting the response. For example, it was observed that when enzyme concentration and pH were in the low level (107 U and pH 4.33) the response decreased. Same would be expected if both parameters are present at their high level (Table 4.2). Such effect is probably caused by the high and positive coefficient

value for pH. Thus, in order to achieve a higher response, enzyme concentration should be at the -1 level (107 U) and pH at the +1 level (pH 7).

The following parameter affecting the response was the concentration of enzymatic solution which influenced the response in a negative way. Low amounts of enzyme led to higher activity of the biocatalyst with values up to 5.9 and 7.2 U g^{-1}. This behaviour was also observed by Fortes et al. (2017) for the immobilization of laccase on magnetic nanoparticles and it was attributed to the saturation of the support by the enzyme. An excess of enzyme on the support may lead to a lack of intermolecular space resulting in the occurrence of mass transfer limitations that affects the dispersion of substrate and products (Fortes et al., 2017).

On the other hand, the immobilization time had a positive influence on laccase immobilization; the active beads obtained after 2 hours of grafting showed almost twice as much activity (experiments 2 and 4). Immobilization time is also crucial variable for the whole process. Whereas too short contact time can result in a limited amount of laccase molecules reacting with the solid, too long immobilization periods lead to oversaturation of the support causing the mass transfer limitations before mentioned that reflects in lower activity (Zheng et al., 2016). In this work longer immobilization time allowed to most of the aldehyde groups to be blocked by amino-groups in laccase resulting in a biocatalyst with considerable higher activity.

Finally, the last significant parameter was the interaction between laccase concentration and time of immobilization with a negative effect. According to the model, low activity would be expected when both time of immobilization and laccase concentration are either at their low or high levels. From the obtained model we can conclude that the best conditions to maximize the response are when laccase concentration is at the low level and when time and pH of immobilization are fixed at their highest level. Thereby, GLU activation time of 18 h, 107 U of enzyme, 2 h of grafting and pH 7 were selected as the immobilization parameters for all further experiments.

The successful development of an immobilization method depends mainly on the type of support and enzyme chosen since they directly influence performance parameters such as immobilization yield. In this sense, TVL and PSL were tested with the aim of evaluating the performance of this novel immobilization method with different laccases.

Immobilization protocol was carried out according to the optimal conditions previously established. Nevertheless, immobilization trials were also carried out at pH 5 since this pH was previously reported as the optimum pH for the immobilization of TVL and PSL on ceramic membranes (Barrios-Estrada et al., 2018).

The highest activity was observed when immobilization was carried out at pH 7 for both PSL and TVL (Table 4.4). Nonetheless it is worth noting that no significant difference in the measured activity was observed between pH 5 and 7. Among the three enzymes TVL yielded the highest specific activity with 13 and 13.6 U g^{-1} biocatalyst at pH 5 and pH 7 respectively and retained the highest amount of protein (35%). Similar specific activity values were obtained for the covalent immobilization of *C. unicolor* C-139 on silica beds at pH 5 (Songulashvili et al., 2012) and *M. thermophila* on Eupergit C carrier at pH 7 (Lloret et al., 2012).

Table 4.4. Immobilization yield and activity in the immobilization of laccases on 3-Aminopropyl-functionalized silica micro particles at pH 7.

Enzyme	Activity (U g^{-1} biocatalyst)	Immobilization capacity (mg g^{-1} biocatalyst)	Bound protein (%)
MTL	4.5 ± 2.1	37.9 ± 15.4	21.6 ± 10.3
PSL	0.76	N.D	N.D
TVL	13.6 ± 2.3	13.5 ± 6.2	35.4 ± 4.7

N.D: Not determined due to the low activity measured

In the case of MTL, a large quantity of protein bounded to the support as suggested by the value of immobilization capacity (38 mg g^{-1}) (Table 4.4), however, the resulting active beads showed a quite low activity. This is probably due to the age of the commercial preparation of MTL which was provided by Novozymes 5 years ago. Since the residual activity was less than 2/3 of the initial activity, a high volume of commercial preparation was required to prepare the enzyme solution. The resulting solution therefore contained large amounts of inactive proteins that competed with the active enzymes to bind to the carrier, leading to a biocatalyst with low activity.

Among the different types of active solids, active beads prepared with PSL reported the lowest activity with only 0.41 U g^{-1} and 0.76 U g^{-1} at pH 5 and 7 respectively. These low

activities may be resulted from the presence of impurities in the enzymatic extract. Indeed, Ji et al. observed a poor performance when they immobilized a crude laccase extract from *P. ostreatus* on titania particles. These authors suggested that the functionalized carrier could have been buried by the non-enzyme components present in the extract. Thus, the surface area available on the support and the cross linking interaction between the enzyme and support were reduced which led to lower enzyme loading and activity (Ji et al., 2017). According to their investigation, dilution of the crude extract before immobilization significantly improved laccase loading and apparent activity of the biocatalyst by reducing the competitive effect between the enzyme and the other components to attach on the support. In this study, PSL and MTL extracts were diluted 2 and 9 times respectively prior to immobilization; it was probably not enough to make the surface of the solid more accessible to enzymes which is more evident in the case of PSL.

Overall beads prepared with TVL showed the best results in terms of immobilization yield and activity. Therefore, TVL was chosen to carry out the next stages: characterization of immobilized enzyme and degradation of pharmaceutical micropollutants.

4.3.2 Characterization of free and immobilized TVL

Characterization of the resulting biocatalyst is important due to the shift in optimum operating conditions that can occur after laccase immobilization. This change is basically due to the interactions between the support and enzyme that usually change the conformation of laccase. Successful immobilization can lead to an enzyme showing increased stability towards harsh environmental conditions in comparison to the soluble form (Mohamad et al., 2015; Rodrigues et al., 2013). In this section the activity and stability of free and immobilized laccase were compared at different values of pH, temperature and for long term storage. Moreover, Fourier Transform Infrared spectroscopy (ATR-FTIR) characterization was done in order to confirm the presence of laccase on the surface of the carrier due to the covalent binding.

Optimum pH and temperature

Optimal pH of activity for free and immobilized TVL was investigated in the pH range from 3 to 7 in 0.1 M citrate-phosphate buffer at room temperature. From Fig. 4.3a can be observed that pH did not significantly influence the activity of immobilized enzyme in comparison to free enzyme. Actually both immobilized and free laccase showed a similar activity versus pH profile, except at pH 6 where relative activity of immobilized laccase was on average 15% lower than free enzyme. Moreover, both free and immobilized systems exhibited the maximum activity at pH 3. According to Jiang et al. (2005) immobilization can create a microenvironment surrounding the enzyme where an unequal partitioning of H^+ and OH^- concentrations caused by electrostatic interactions with the support can cause a change in optimal pH. The fact that a shift in optimal pH did not occur in this study suggests that electrostatic interactions were not involved during immobilization and the process was rather carried out through covalent interaction as observed in other investigations (Jiang et al., 2005; Misra et al., 2014).

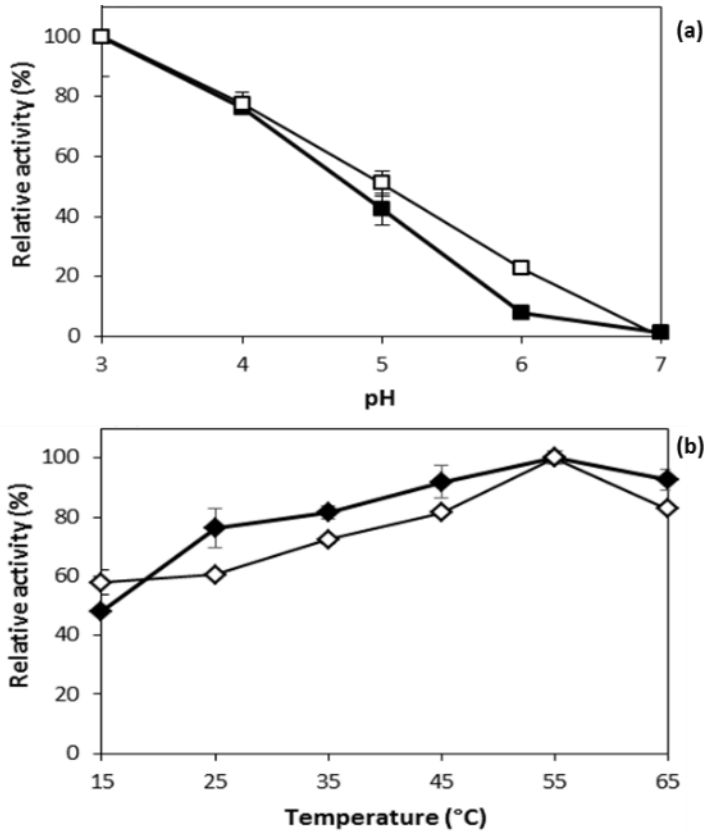

Fig. 4.3. Effect of (a) pH and (b) temperature on free and immobilized TVL. pH (3-7) and temperature (15-65°C) were tested with 1 mM ABTS in 0.1 M citrate-phosphate buffer. Free and immobilized enzymes are represented by open and closed symbols respectively.

The effect of temperature on TVL activity of free and immobilized enzyme was investigated in the range 15-65°C under standard conditions (0.1 M citrate-phosphate buffer pH 4). The optimum temperature for free and immobilized laccase was 55°C (Fig. 4.3b). Immobilized enzyme showed a broader profile displaying relative activities up to 16% higher in comparison to free enzyme for all temperatures tested and retained more than 80% of the activity in the range from 35 to 65°C; except at 15°C where free enzyme showed higher relative activity in comparison to the immobilized form (58 and 48% respectively).

pH stability and thermostability

Thermostability was evaluated when both free and immobilized laccases were incubated in buffer citrate-phosphate (pH 7) at selected temperatures in the range from 25 to 55 °C. It was observed that the activity of immobilized laccase at 45°C and 55°C dropped more rapidly than that of free enzyme within the first 4 h (Fig. 4.4b). However, for longer incubation times at 55°C, the residual activity of immobilized enzymes remained constant (around 25%) while those of free enzymes continued decreasing to reach 6% after 24 hours of incubation. Incubation at 45°C led to similar activity loss for both free and immobilized laccases with residual activity values of 45% and 52% respectively at the end of 24 h. At 25°C low thermal inactivation was observed with less than 10% loss on activity for both free and immobilized laccases.

Overall, immobilization increased TVL resistance to temperature especially in the case of long-term exposure. Similar observation was reported by Fortes et al. (2017) for the thermal stability of *Asperguillus oryzae* laccase immobilized on magnetic nanoparticles at 60°C. These authors observed a higher kinetic constant of enzyme deactivation (*k*) for free enzyme than for immobilized form. Nonetheless, at the long term, immobilization enhanced laccase thermal stability allowing to keep the same residual activity for almost 5 h, whilst those of free enzyme continued decreasing with incubation time. Lettera et al. (2015) also observed increased stability when immobilized recombinant laccase POXA 1b from *P. ostreatus* on Epoxy activated poly(methacrylate) beads in the range from 25 to 65°C with a two-fold increase in enzyme half-life ($t_{1/2}$) values. It seems clear that supports offer a stabilizing environment to enzyme making it more resistant to extreme conditions.

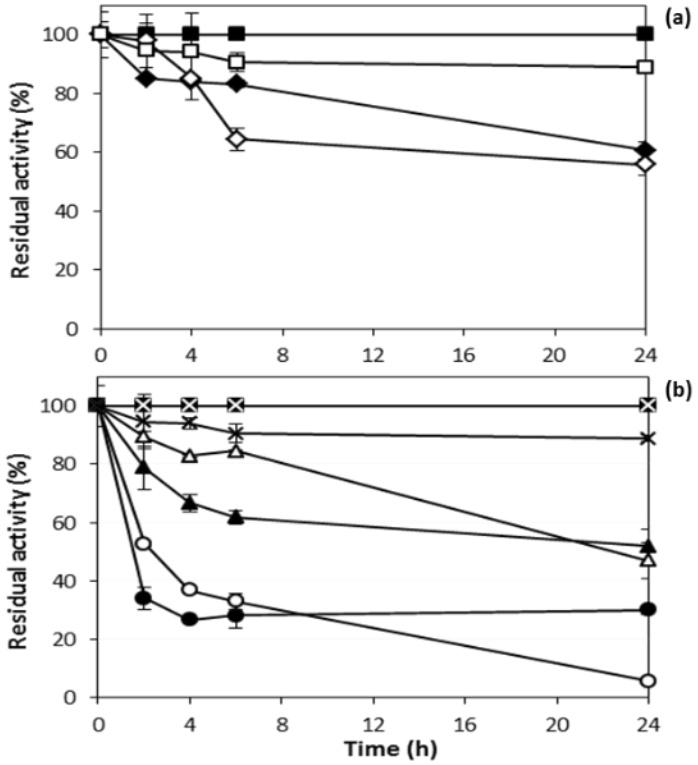

Fig. 4.4 pH (a) and thermal stability (b) of free and immobilized TVL on silica particles. pH values: 4 (◇) and 7 (□). Temperature values: 25°C (×), 45°C (△) and 55°C (○). The open and closed symbols represent free enzyme and immobilized enzyme respectively.

Storage stability

In general, stability of enzymes in solution is affected during storage, resulting in a gradual reduction of their activity. To assess the storage stability of both free and immobilized enzymes, a solution of free enzymes (152 U mL^{-1}) and active beads (100 mg mL^{-1}) were kept at 4°C in buffer citrate-phosphate (0.1 M, pH 7) and residual activity was measured after one and two months. Results showed that immobilized laccase retained 72% and 47% of its initial activity after one and two months of storage respectively. In contrast, residual activity of free laccase almost reached 44% at the end of the first month. After two months of storage, it was not possible to measure the residual activity as fungi flocs were observed in the media. The lost in activity observed in the immobilized enzyme could be attributed to the natural loss of laccase activity during time. Nonetheless, these results show that storage stability was improved by the immobilization process in comparison to free enzyme. It is well known that the multipoint fixation of enzyme to supports via covalent bond increases stability by reducing the occurrence of changes in the active conformation of the immobilized enzyme (Sari et al., 2006).

Fourier Transform Infrared spectroscopy (ATR-FTIR) characterization

The immobilization of TVL on silica gel micro particles was assessed by ATR-FTIR. Fig. 4.5 presents the FTIR spectra of free TVL, untreated 3-Aminopropyl-functionalized silica gel and the silica gel particles after laccase immobilization. Characteristic bands of free laccase were observed at 3300 cm^{-1} (OH and NH vibrations), 2900 cm^{-1} (CH stretch), 1630 cm^{-1} (attributed to a secondary amide structure-CONH), 1350 cm^{-1} (CN stretching of amines) and 1100 cm^{-1} (COC groups) in agreement with literature (Tavares et al., 2015). Pristine silica gel particles presented a strong band centered at 1050 cm^{-1} attributed to the vibration of Si-O-Si and two weak peaks at 1616 cm^{-1} and 1523 cm^{-1} corresponding to Si-OH and primary amine NH groups. After laccase immobilization, FTIR spectrum showed the characteristic bands of silica gel particles. Moreover, the presence of the specific signals from the enzyme at 1400 cm^{-1} and 1600 cm^{-1} on the active silica spectrum confirms the immobilization of the enzyme on the support. Nonetheless, the decrease in vibration bands attributed to the enzyme suggests that laccase was immobilized in low quantity as confirmed by the immobilization capacity value (13.5 mg protein g^{-1} silica) calculated for TVL (Table 4.4).

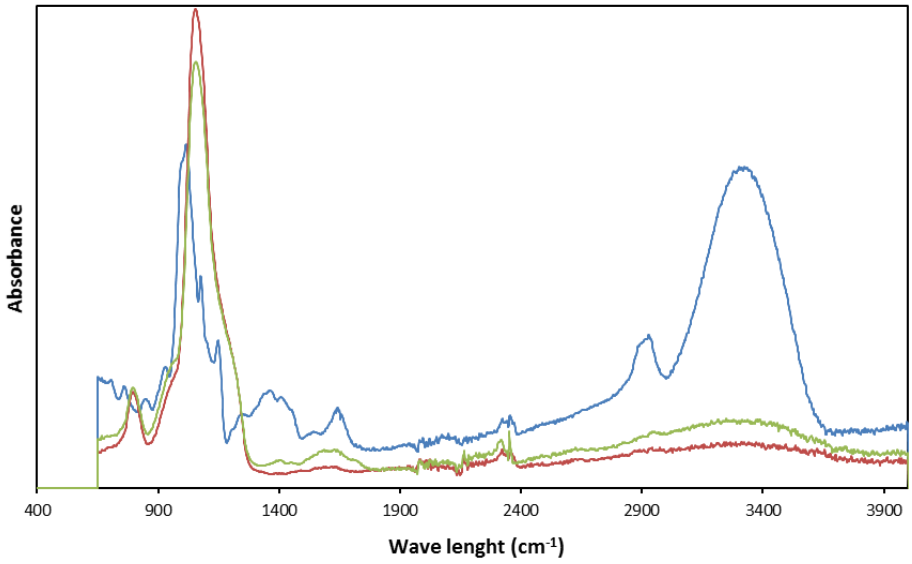

Fig. 4.5. FITR-ATR spectra of laccase (blue line), untreated 3-aminopropyl functionalized silica gel (red line) and TVL immobilized on silica gel particles (green line).

4.3.3 Oxidation of pharmaceuticals by immobilized laccase

The capacity of immobilized TVL for the oxidation of a mixture of four pharmaceutical micropollutants was evaluated in batch reactions. As a first attempt micropollutants were treated directly with the immobilized biocatalyst and the oxidation efficiency was assessed.

After 48 h no significant oxidation was observed for any pharmaceutical (data not shown). This observation confirmed the recalcitrance of these micropollutants to laccase degradation which was already reported by Parra Guardado et al. (2019). In this previous work, the potential of free laccases (MTL, PSL and TVL) for the biotransformation of the four pharmaceuticals into both single-component and mixture solutions was investigated; in all cases studied, bio-oxidation was negligible. These authors attributed the poor removal to the complex chemical structure of the compounds which make them not easy to be oxidized by laccase.

Taking into account the inability of immobilized enzyme to transform the selected pharmaceuticals, redox mediators were added to the reaction and their role in the oxidation process was evaluated. Redox mediators are compounds that laccase can easily oxidize resulting in the production of radical species with higher oxidative capacity than the laccase itself. In this work the compounds SYR, PCA and ABTS were chosen since they have already demonstrated to enhance the oxidation of AMX, SMX and CIP in free enzyme systems (Parra Guardado et al., 2019). Mediator concentration was fixed at 520 μM and reactions were followed for 4 h. Time course degradation of AMX, CIP, SMX and CBZ by immobilized laccase-mediator systems as well as the adsorption-based removal by the support is shown in Fig. 4.6.

Firstly, the adsorption capacity of the support was studied. Tests were carried out with both untreated particles and inactivated immobilized particles. No significant adsorption of the antibiotics by the pristine support was observed (less than 10%); only 18% of CBZ was adsorbed after 4 h (data not shown). On the other hand, tests with inactivated biocatalyst reported adsorptions that varied from 10% to 40% for AMX, 30% to 40% for SMX, 13% to 40% for CIP and 40% to 52% for CBZ (Fig. 4.6). Higher adsorption observed with this control in comparison to untreated particles could be attributed to interaction between the denatured enzymes, which “coated” the silica carrier, and the micro pollutants; thus, increasing its sorption capacity.

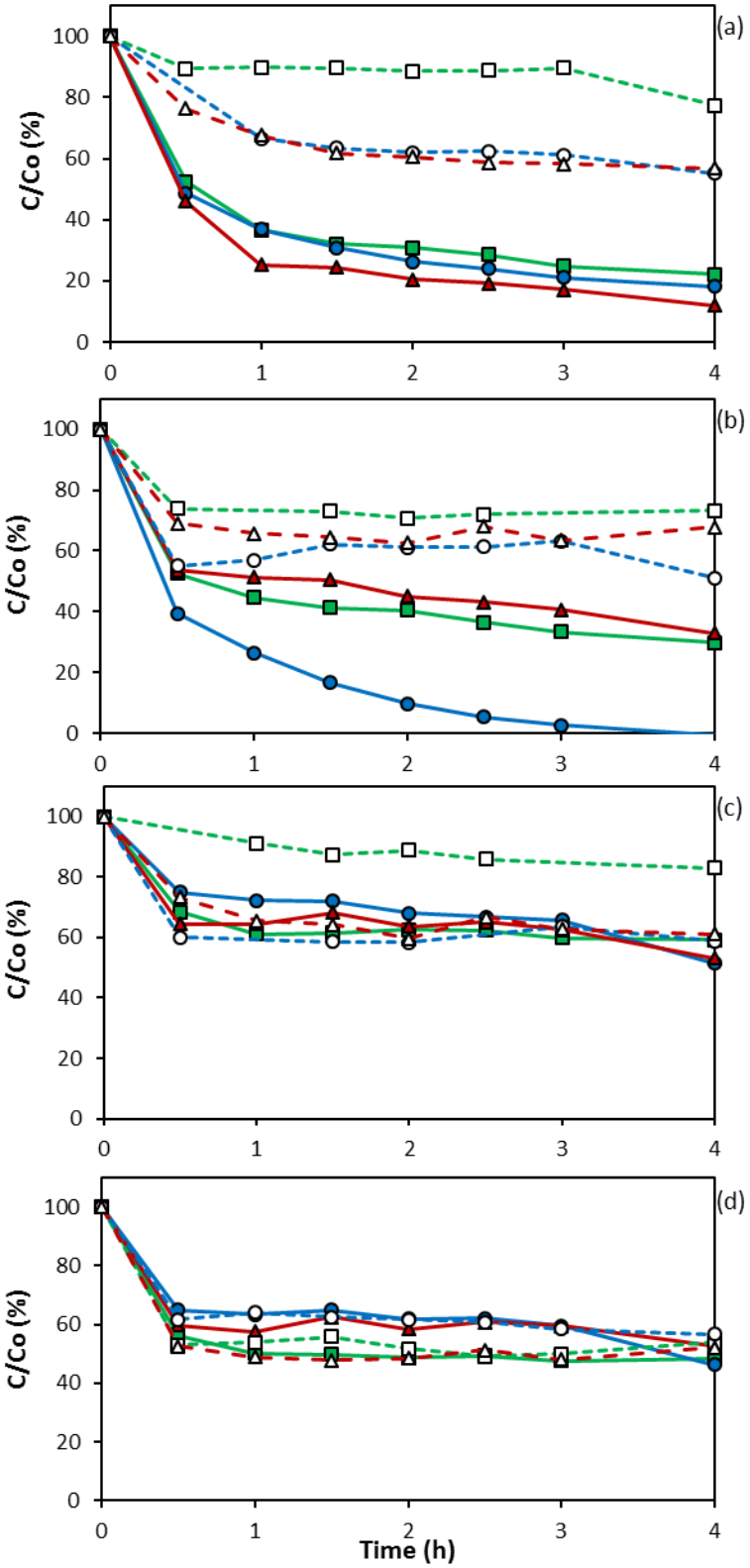

Fig. 4.6. Residual concentration of (a) AMX, (b) SMX, (c) CIP and (d) CBZ in mixture after 4 h treatment with immobilized TVL on silica particles and different redox mediators: ABTS (■), SYR (●) and PCA (▲). The open symbols represent the removal by adsorption on the support with inactivated laccase. Measurements were carried out by duplicate, with standard deviations less than 15%.

Removal of organic pollutants via adsorption by the support has been extensively described. For example, Cabana et al. (2009) reported adsorption values from 40 to 60% for nonylphenol, bisphenol A and triclosan by inactivated laccase covalently immobilized on Celite R-633 supports. Ciprofloxacin hydrochloride adsorption reached 77% when mesoporous carbon nanospheres were used (Shao et al., 2019). Inactive immobilized Magnetic Cu^{2+} -chelated silica particles removed 38% of pentachlorophenol by adsorption (Wang et al., 2012).

As observed in Fig. 4.6 the extent of removal due to adsorption varied depending on the pharmaceutical and it could be associated to each compound's hydrophobicity. The highest adsorption observed was for CBZ which has the highest octanol/water partition coefficient (Log K_{ow}=2.45) and the lowest water solubility (78 mg L^{-1}) (Celiz et al., 2009; Pyka et al., 2006). Regarding to the antibiotics low sorption for AMX and SMX would be expected as judged from their corresponding partition coefficients and water solubility values: 0.87 and 3400 mg L^{-1} for AMX and 0.89 and 610 mg L^{-1} for SMX (Suárez et al., 2008; Wang and Wang, 2016). In the case of CIP a Log K_{ow} value of 0.28 but an extremely low solubility in water explains its high adsorption (Al-Omar, 2005; Takacs-Novak et al., 1992).

Overall, the proportion of micropollutants removed by adsorption was practically the same for the four pharmaceuticals (30-50%). However, in presence of ABTS the adsorption of CIP and AMX was remarkably different in comparison to SYR/PCA control reactions (Fig. 4.6c). If attention is paid to Fig. 4.6, ABTS seemed to reduce the proportion of micropollutants adsorbed on the support in comparison to the other mediators. Interestingly, this effect was mainly observed on antibiotic compounds. In a previous work, Parra Guardado et al. (2019) observed that the addition of ABTS, SYR or PCA in the absence of laccase had no effect in the transformation of target pharmaceuticals. Thus, results obtained in this investigation could have resulted from the interaction between inactivated enzyme and ABTS. Nevertheless, no explanation about the nature of the possible interactions occurring in the system can be provided; even when experiments were replicated the tendency of ABTS reducing the adsorption of pharmaceutical on the solid was corroborated. A deeper insight in the effect of mediator-protein-pollutant interactions would be needed in order to understand the adsorption mechanism of pharmaceuticals on the support, which is out of the scope of this investigation.

Although part of the observed elimination was caused by adsorption on the solid carrier, the action of immobilized enzyme-mediator system in the removal of the target pharmaceuticals was evident, especially for SMX and AMX. In presence of SYR complete transformation of the antibiotic SMX was achieved within 4 h of treatment (Fig. 4.6b). The use of ABTS and PCA also allowed removing 53% and 54% of SMX respectively in 30 minutes but afterwards the concentration remained practically constant until the end of the experiment. Regarding to AMX, the three mediators led to similar results with transformation yields in the range of 78-89% as observed in Fig. 4.6a. However, PCA was the mediator allowing the highest oxidation with only 11% of AMX remaining after 4 h. As with SMX, AMX oxidation occurred mainly during the first 60 minutes of reaction, after which the residual concentration remained almost constant regardless to the mediator used. According to Fig. 4.6c and 4.6d removal of CIP and CBZ occurred within the first 30 minutes of treatment. CBZ removal was entirely by adsorption as confirmed by control reactions with inactivated biocatalyst. Regarding to CIP, no effect of SYR and PCA mediators in the antibiotic's removal was observed which was also attributed to adsorption on the support. However, in presence of ABTS as shown by the control, CIP transformation was caused by enzymatic biodegradation.

Overall, results indicate that removal of these micropollutants by immobilized laccase-mediator system is both compound and mediator dependent. Marked difference in the removal of SMX, AMX and in some point of CIP and CBZ was observed. The main reason could be the difference in laccase-mediator oxidative capacity and the affinity of the pharmaceuticals for adsorption. Based on the global removal efficiency of the system, micropollutants clearly differentiate in two groups: the highly degradable pharmaceuticals and the recalcitrant pharmaceuticals. The latter group is formed by CBZ and CIP, compounds presenting high hydrophobicity and complex chemical structure that are very resistant to laccase oxidation even in presence of redox mediators (Hata et al., 2010). Belonging to the highly degradable group, AMX and SMX are antibiotics that possess functional groups prone to be attacked by laccase (hydroxyl and amine groups respectively). The highest oxidation rates of AMX and SMX were achieved when SYR or PCA were present in the reaction medium. Interestingly both mediators follow the hydrogen atom transfer mechanism of reaction in which after oxidation, phenoxyl radicals are produced to act as electron shuttle

between the laccase and substrate (Cañas and Camarero, 2010). Indeed, phenoxyl radicals formed were highly reactive and stable to carry out the simultaneous transformation of AMX and SMX.

Nonetheless, it is important to note that the high removal obtained for AMX, for SMX specially and in some extent for CIP could not be totally attributed to laccase-catalysed oxidation but to a combined contribution of adsorption and degradation. As shown in Fig. 4.6 adsorption of pharmaceuticals reached equilibrium 30 minutes after the start of the reaction, then laccase oxidation effects ongoing to be more evident. In other words, adsorption of micropollutant on immobilized enzyme could have help and in some extent enhance laccase degradation. Nguyen et al. observed similar behaviour for the removal of the recalcitrant CBZ and SMX with immobilized laccase on granular activated carbon (GAC) in a packed bed reactor. These authors suggested that enhancement in laccase degradation for both compounds could be possibly due to the improved electron transfer between laccase and substrate after adsorption on the support surface (Nguyen et al., 2016a). Such effect is especially evident for SMX when SYR acted as mediator leading to the complete transformation of the antibiotic in less than 4 h.

Results obtained in this investigation are consistent with previous studies reporting the elimination of pharmaceutical micropollutants with immobilized laccase. For example, Becker et al. (2016) evaluated the simultaneous degradation of 38 antibiotics at environmentally relevant concentrations (10 µg L^{-1}) in an enzymatic membrane reactor (EMR). After 24 h, 32 out of the 38 antibiotics were removed in a high extent (>50%) including 94% for AMX, 97% for SMX and 93% for CIP when SYR was present at a high concentration (1 mM). Cross-linked carbon nanotubes bio-catalytic membranes containing enzyme physically adsorbed were used for the treatment of a mixture of bisphenol-A, carbamazepine, diclofenac, clofibric acid and ibuprofen in an EMR obtaining removal yields from 40 to 90% depending on the compound (Ji et al., 2016a). Yang et al. used immobilized laccase in magnetic cross-linked enzyme aggregates combined with ABTS for the removal of different types of antibiotics including sulfamethoxazole (100 mg L^{-1}) in batch reactions obtaining 30% degradation after 48 h (Yang et al., 2017). Similarly, De Cazes et al. (2014) reported that free and immobilized laccase degraded 56% and 30% respectively of tetracycline after 24 h.

In a previous work, the high affinity of free laccase-SYR system for AMX and SMX was also observed, although degradation yields obtained depended on the laccase used (MTL, TVL or PSL) (Parra Guardado et al., 2019). Free TVL (1.2 U mL^{-1}) in presence of SYR reached 50% removal for AMX in 3 h and complete elimination of SMX in 1.5 h. In the present investigation, considerably lower amounts of immobilized enzyme (0.5 U mL^{-1}) achieved comparable or even higher removal yields for both micropollutants within the same period of time. These results suggest that the novel immobilization method proposed in this work positively influenced the stability of TVL which coupled with the adsorption capacity of the biocatalyst enhanced the removal of pharmaceutical pollutants. Therefore, the high removal yields obtained in this model study by the combined action of adsorption-degradation altogether with the enhanced stability of the enzyme highlights the potential of using this biocatalyst for the treatment of effluents containing similar type of recalcitrant compounds.

4.3.4 Reusability of immobilized laccase

The biocatalyst was reused in a total of 9 cycles (2 h each cycle) for the removal of selected micropollutants using SYR as redox mediator. At the end of each degradation cycle the biocatalyst was washed with buffer solution (see materials and methods, section 4.2.7) and added to a fresh solution containing the mixture of pharmaceuticals and mediator.

As shown in Fig. 4.7 the percentage of removal varied depending on the micropollutant. The extent of degradation of AMX over the first four cycles was relatively constant (~60%). After, removals gradually decreased to reach 43% at the end of the ninth cycle (Fig. 4.7a). Moreover, it is important to note that levels of combined removal (degradation/adsorption) remained practically constant until the seventh cycle; afterwards no difference between degradation and adsorption was observed. In contrast, enzymatic oxidation of SMX occurred only during the first two cycles with high oxidation yields: 82% and 57% (Fig. 4.7b). Subsequent removal was entirely due to adsorption on the support (~40%). As expected CIP and CBZ removal was entirely by adsorption with average elimination of 30% and 50% for each compound (data not shown).

The loss of efficiency observed for the degradation of AMX and SMX by immobilized enzyme has been extensively reported in different studies and is usually attributed to the

high molar mass products formed during the reaction that could inhibit or bury the active center of laccase leading to mass transfer resistance (Ji et al., 2016b; Xu et al., 2013). The possible inhibition of immobilized laccase could be explained by the loss in its capability to simultaneously transform both antibiotics. Whereas SMX was highly removed only in the first two cycles, AMX was continuously transformed during 7 cycles. There is the possibility that interactions between the active biocatalyst and the substrates/by-products formed during the first cycles caused conformational changes in the enzyme that narrowed its capacity to oxidize both compounds. Moreover, the preference for AMX transformation over SMX during the following cycles could be linked to its chemical structure and the presence of functional groups which make AMX more prone to laccase oxidation in comparison to SMX (Yang et al., 2013).

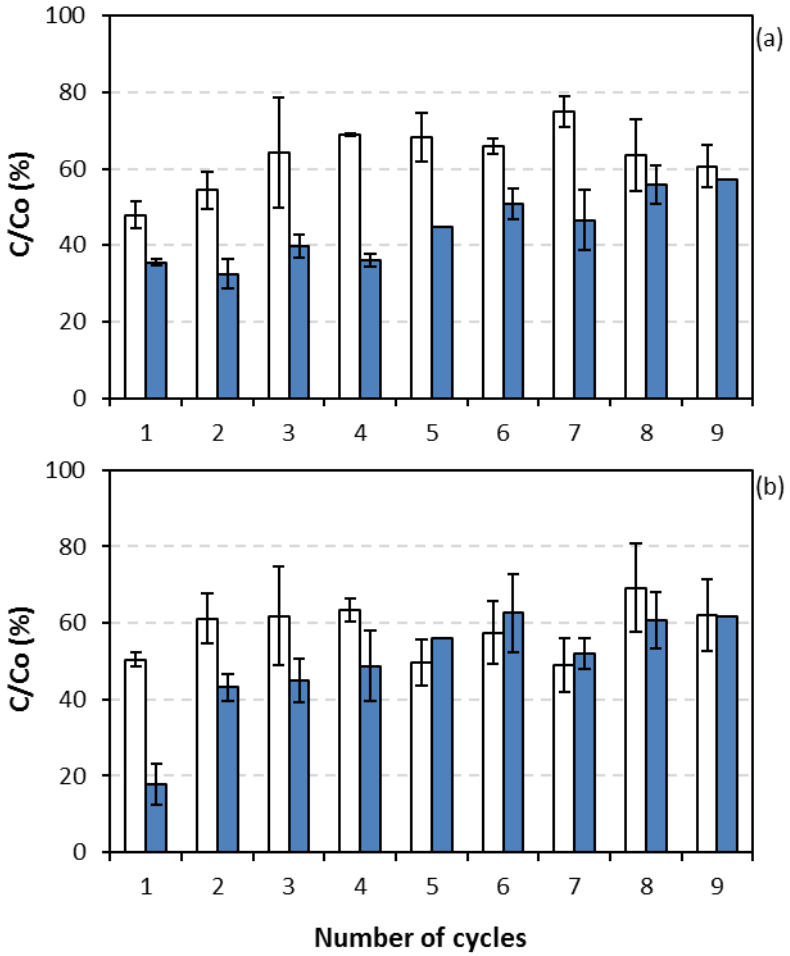

Fig. 4.7. Removal of (a) AMX and (b) SMX in a mixture by immobilized laccase and SYR as redox mediator in consecutive cycles at 25°C and pH 7. Adsorption of each micropollutant on the support with inactivated laccase is represented by white bars.

4.3.5 Toxicity assessment

The acute toxicity of the effluent after enzymatic treatment with the different mediators was evaluated by the Microtox® assay. This test is used to investigate the general toxicity of samples by monitoring the change in light emission of the bioluminescent bacteria *Vibrio fischeri* before and after sample addition. The obtained data was used to calculate the EC_{50}, which is the percentage of sample dilution (v/v) that causes a 50% reduction in bacteria bioluminescence. Fig. 4.8 shows the results obtained for the untreated solutions at t=0 h (mix of pharmaceuticals + mediator) and samples after 4 h treatment with immobilized laccase -mediator systems (6 U, 0.52 mM respectively). According to Niemirycz et al. (2007), results from the EC_{50} values (15 minutes) can be classified as follow: Non-toxic samples: EC_{50}>100%; low –toxic samples: 10<EC_{50}≤100%; toxic samples 1%≤EC_{50}≤10%; and, highly-toxic samples EC_{50}<1%.

Results suggest that ABTS was the mediator showing the lowest toxic effect. Before treatment, the control samples containing only the mixture of the pharmaceuticals and ABTS showed an EC_{50} value of 103%, meaning that this solution was not toxic for the microorganisms. The analysis of the reaction by-products after 4 h of treatment with immobilized laccase and ABTS, showed a slightly increase in the toxicity with an EC_{50} value of 95% which can be considered as a low-toxic sample. These results contrast to those reported by Kim and Nicell (2006) for the degradation of triclosan using ABTS as redox mediator. This authors observed that the addition of ABTS (20 μM) to the reaction increased the toxicity of the effluent by 120 times in contrast with the untreated sample. This was attributed to the generation of ABTS reactive radicals which may have attacked and damaged the bacteria. In this study, even with a considerably higher concentration of ABTS (520 μM) the increase in the toxicity was practically negligible. Probably, the radicals formed during the reaction after interacting with the mixture of pharmaceuticals were not reactive enough to cause damage to the bacteria.

Regarding to PCA, no change in the toxicity of the samples was observed when comparing before (55%) and after laccase treatment (54%). However, it is important to note that, according to the untreated samples, the addition of PCA resulted more toxic to the bacteria than ABTS. Fillat et al. (2010) reported that the toxicity of laccase-PCA treated flax pulp effluent was below the emission limit stablished by the Spanish legislation; however, no

further explanation was given. A lack of information in literature about the toxicity assessment of PCA restricts giving any explanation about the behaviour of PCA.

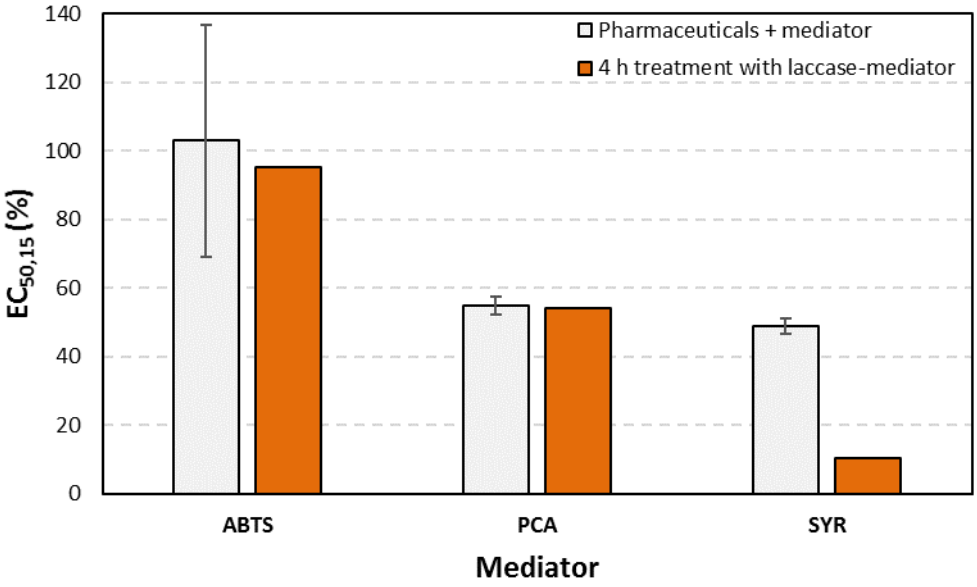

Fig. 4.8. Comparison of toxicity after the enzymatic treatment of pharmaceuticals in the presence of different redox mediators.

The toxicity of the test media treated with laccase-SYR led to the most toxic effect on *V. fischeri* after 15 minutes of contact (EC_{50}=11%). Even the untreated solution containing SYR reported the lowest EC_{50} (49%) from the three mediators evaluated. The higher toxicity of SYR has been previously reported in different investigations (Becker et al., 2016; Fillat et al., 2010; Nguyen et al., 2016b, 2014). For example, Fillat reported that the toxicity of laccase-SYR effluent was 10 times higher than that of laccase-HBT effluent from a flax pulp treatment process. The increase in toxicity of effluents treated with SYR has been related to the generation of intermediate species and/or the degradation products that are more toxic than the parent compounds (Fillat et al., 2010). According to Becker et al. (2016) oxidation of aromatic compounds such as quinolones and tetracyclines can lead to more toxic by-products as is the case in this work. Moreover, the higher toxicity observed by SYR could be also related to its initial concentration in the reaction. SYR doses in the range of 10-50 µM significantly decreased the toxicity of the effluent from an enzymatic membrane reactor (EMR) containing several trace organic compounds in comparison to a 100 µM dose (Nguyen

et al., 2016b). In another work, Becker et al. (2016) reported that even at t=0 h the luminescence of bacteria was inhibited when SYR was added at a high concentration (1000 µM). These authors attributed the toxicity of SYR in the luminescent bacteria to the antimicrobial activity of the mediator. Thus, the low EC_{50} values observed in the untreated samples for SYR and possibly for PCA could be also related to the antimicrobial effect of these compounds. Further research on this matter as well as on the optimal dose for SYR leading to lower toxicity would be necessary for the successful use of this mediator, however this is beyond the scope of this study.

4.4. Conclusions

In this study, *Trametes versicolor* laccase was covalently immobilized onto commercial silica gel particles through a novel immobilization method involving the activation of the support with glutaraldehyde in vapour phase. This is the first work reporting the use of this activation technique for the immobilization of laccase on solid supports for bioremediation applications. An experimental design was proposed in order to identify the most important factors involved in the immobilization process. According to the model generated the most important factor affecting immobilization was the pH. The immobilization conditions found to maximize the activity showed by the biocatalyst were 18 h of GLU activation time, enzyme concentration of 107 U, 2 h of contact time and pH 7. Overall, in comparison to free enzyme, immobilization improved enzyme stability over a range of pH and temperature conditions as well as for long storage.

The potentiality of using the biocatalyst for the removal of pharmaceuticals was evaluated in osmosed water containing a mixture of four representative pharmaceutical micropollutants. High removal yields (40-100%) were achieved when redox mediators were present in the reaction medium. It was observed that redox mediators generating phenoxyl radicals were the most effective for the biotransformation of the pharmaceuticals, with SYR showing the best performance. However, the use of SYR considerably increased the toxicity of the effluent, whilst ABTS led to the lowest toxic treatment. The higher toxicity of SYR could be related to the high dose of the mediator used in the treatment that led to the generation of intermediate species and formation of more toxic by-products. Compared to

free laccase, immobilized enzyme removed the micropollutants more effectively due to the combined action of adsorption on the support and laccase catalysed oxidation. In addition, reusability of the biocatalyst was proved for the continuous removal of the pharmaceuticals during seven cycles. The results presented in this study show that the novel immobilization method proposed and the resulting biocatalyst are a promising tool to increase the application of enzymes in bioremediation processes.

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Chapter 5

Versatile inorganic supports for laccase immobilization and enhanced removal of pharmaceutical micropollutants in water

5. Versatile inorganic supports for laccase immobilization and enhanced removal of pharmaceutical micropollutants in water

Abstract

In this work, several biocatalysts were developed based on the immobilization of *Trametes versicolor* laccase on different clay, silica and silica/clay nanocomposite supports. The highest yields of enzyme immobilization were obtained when laccase was covalently bonded to silica and silica/clay nanocomposites with specific activity values of 109 and 185 U g^{-1} respectively. These biocatalysts were applied for the biotransformation of a mixture of the pharmaceuticals amoxicillin (AMX), ciprofloxacin (CIP), sulfamethoxazole (SMX) and carbamazepine (CBZ) in batch treatments and in the presence of syringaldehyde as redox mediator. Results showed all these pharmaceuticals were eliminated in a high extent (45-100%) at a contact time of less than 180 minutes due to the complementary action of laccase degradation and adsorption on the two supports. In comparison to laccase immobilized onto silica nanoparticles (SiNPs), the immobilized nanocomposite showed higher oxidative capacity and selective sorption properties towards the target pharmaceutical compounds, thus demonstrating the advantages of using these type of supports for bio-catalytic and environmental applications.

Keywords: Laccase, covalent immobilization, pharmaceutical micropollutants, biodegradation, water pollution

5.1. Introduction

The use of enzymes for bio-catalytic conversion of pollutants in bioremediation processes is an environmental friendly technology that has gained a lot of attention in recent years. Enzymes display excellent properties (high activity, selectivity and specificity) which allow them to catalyse complex reactions under mild experimental conditions (Misra et al., 2014). Particularly, laccases are high potential oxidative enzymes showing large substrate specificity towards aromatic compounds, amines, methoxy-substituted phenols and some inorganic compounds (Gasser et al., 2014). According to several reports, laccases are a promising candidate for the remediation and treatment of contaminated water and soil with recalcitrant pollutants such as dyes (Pezzella et al., 2014), phenolic compounds (Gianfreda and Bollag, 1994), endocrine disrupting contaminants (EDC) (Debaste et al., 2014), pharmaceutical products (Kumar and Cabana, 2016) and pesticides (Jin et al., 2016).

The use of enzymes in their free form presents some major disadvantages that limit their application in large scale processes. The relatively low stability of laccases and their susceptibility to inactivation due to unfavourable environmental conditions (i.e. inhibitory agents, extreme values of pH and temperature) are perhaps the main drawbacks (Gasser et al., 2014). Moreover, free enzymes are difficult to separate from the medium which limits their reusability and increases operational costs. In order to overcome such limitations, immobilization of enzymes on different kinds of support materials has been investigated as an effective way to improve laccases properties (Sheldon and van Pelt, 2013). There are plenty reports highlighting the benefits of laccases immobilization including longer storage, stability, reusability and increased tolerance to extreme conditions (Cabana et al., 2009; Ji et al., 2017; Patel et al., 2014; Zheng et al., 2016). Nonetheless, in order to achieve such benefits a proper selection of the immobilization method and support material is determinant since they greatly influence the final properties of the biocatalyst (Zdarta et al., 2018a).

Conceptually, physical and chemical interactions are the two basic methods through which enzyme immobilization can be accomplished. Physical methods include the entrapment of enzyme within a tridimensional matrix, encapsulation in polymers or adsorption of the enzyme to the support by electrostatic interactions, whereas chemical techniques by means of covalent bonding assures the irreversible binding of the enzyme to

the solid matrix (Sheldon, 2007). Covalent immobilization is an interesting and widely used technique to insolubilize enzymes due to its simplicity and specificity. Immobilization by covalent binding is based on the reaction of functional groups of the support material with functional groups of the enzyme (mainly reactive amino acid residues such as $–NH_2$, -SH and –OH) resulting in the formation of strong chemical bonds which significantly increases laccase stability and reduces its leakage (Datta et al., 2013).

Immobilization of enzymes has been carried out on a broad variety of support materials from organic and inorganic origin. The most relevant developments in support materials for immobilization from a historical perspective were recently reviewed by Zdarta et al. (2018a). According to these authors, three factors are determinant for the selection of a support for immobilization of enzymes: (i) the presence of numerous functional groups, (ii) good sorption properties, and (iii) good thermal, chemical and mechanical resistance as well as operational stability. In this sense, inorganic supports such as silica and montmorillonites represent an excellent option for laccase immobilization. These supports show large specific surface area and many different functional groups (i.e hydroxyl, carboxyl, carbonyl) that facilitates enzyme attachment and support functionalization by using anchorage agents like glutaraldehyde (GLU) or 3-aminpropyltriethoxyxilane (APTES) which are both convenient for covalent immobilization (Zucca and Sanjust, 2014). Moreover, these inorganic materials are relatively cheap and abundant in nature which facilitates their use and their application as enzymatic supports.

During the last decades, scientific attention has focused on the development of new type of supports for enzyme immobilization such as nanoparticles, hybrid and composite materials (Zdarta et al., 2018b). Composite materials represent a very interesting option for enzyme immobilization since they combine properties of two different precursors which maximize their advantages (Grigoras, 2017). In this way it is possible to obtain “tailor made” carriers for a given enzyme and process to be applied, thus facilitating the control of immobilization process (Zdarta et al., 2018a). As result, immobilized enzymes on this type of supports are considerably stabilized which favours the retention of high catalytic properties, enhances enzyme reusability and protects it from conformational changes (Yang et al., 2012). Composites can be obtained by the conjugation of organic-organic, inorganic-inorganic and inorganic-organic precursors. Therefore, different type of compounds

including silica and clays have been combined for the formation of composites and used for catalytic and environmental applications (Addy et al., 2012; Li et al., 2009). However, to the best of our knowledge, there is no previous investigation reporting the use of silica/clay composite as support for laccase immobilization.

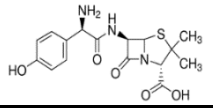
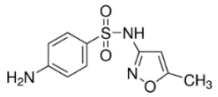
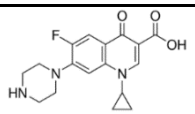
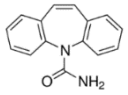
As the research of innovative materials and immobilization methodologies capable to maximize enzyme properties is essential to the development of enzymatic processes at industrial scale, the aim of this work was to perform the efficient immobilization of laccase on different type of silica, clay and nanocomposite supports. For this purpose, covalent immobilization was carried out according to a novel immobilization protocol proposed by Parra et al. (in preparation) which involved the use of GLU in vapour phase as the cross-linking agent. The biocatalysts showing the highest activity were then evaluated in terms of the biotransformation potential of a mixture of pharmaceutical compounds in spiked osmosed water.

5.2. Materials and methods

5.2.1 Chemicals and enzymes

The target pharmaceuticals (amoxicillin (AMX), sulfamethoxazole (SMX), ciprofloxacin (CIP) and carbamazepine (CBZ)), the bifunctional agent for enzyme immobilization (glutaraldehyde (25%) (GLU)), the redox mediator (syringaldehyde (SYR)), the substrate (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)) and all other chemical products were purchased from Sigma-Aldrich. Stock solutions of AMX and SMX were prepared in citrate-phosphate buffer (0.1 M, pH 7); whereas CIP was pre-dissolved in a diluted solution of hydrochloric acid (0.1 N) and then the stock solution was finally prepared in the same buffer pH 7. SYR and CBZ stock solution were prepared in pure ethanol. The structure and physicochemical properties of target pharmaceuticals are shown in Table 5.1. Laccase powder from *Trametes versicolor* (activity ≥1 U mg^{-1} and ≥10 U mg^{-1}) (TVL) was provided from Sigma-Aldrich.

Table 5.1. Structure and physicochemical properties of selected pharmaceutical micropollutants

Compound	Molecular weight ($g\ mol^{-1}$)	Solubility in water ($mg\ L^{-1}$)	Log K_{ow}	Chemical structure
AMX	365.4	3400	0.87	
SMX	253.3	610	0.89	
CIP	331.3	~0	0.28	
CBZ	236.3	78	2.45	

5.2.2 Support materials for immobilization

Six different materials were used as support for immobilization: silica nanoparticles (SiNPs and Si-30R50), clay particles (Y and SWy-3) and nanocomposites (50/50 and 95/5). These materials were developed in the frame of the ANR project POLPHARMA (ANR-15-CE04-0007) by different French research laboratories (LCMP-UPMC, BRGM, CEREGE); their characteristics are summarized in Table 5.2.

Table 5.2. Characteristics of the different supports

Type of material	Supplier	BET specific surface area (m^2g^{-1})	Size	Material kind
Silica nanoparticles				
SiNPs	LCMCP-UPMC	229	37 nm	Silica nanoparticles
Si-30R50[1]	BRGM	45	84 nm	Klebosol 30R50
Clay particles				
Y	BRGM	99	1.3 µm	Montmorillonite Nador, Morocco (98%)
SWy-3		44	1.5 µm	Montmorillonite Wyoming (97%)
Nanocomposites				
50/50	CEREGE/BRGM	73	51 µm	50% Y / 50% Si-30R50
95/5		67	92 µm	95% Y / 5% Si-30R50

1: (Bizi, 2012)

5.2.3 Determination of laccase activity and protein assay

Laccase activity was determined by measuring the oxidation of 1 mM ABTS to its cation radical ($ABTS^{+}$) at a wavelength of 420 nm in buffer citrate-phosphate (pH 4) and 25 °C. One unit (U) of enzyme activity was defied as the amount of enzyme that oxidized 1 µmol of ABTS per min. Activity of immobilized laccase was assayed by incubating the biocatalyst in 50 mL solution of ABTS (1 mM) prepared in citrate-phosphate buffer (pH 4) at 25 °C with continuous stirring. The absorbance at 420 nm was monitored every minute for a period of 10 minutes. Immobilized laccase activity was expressed in Ug^{-1} solid.

Protein assays were performed on supernatants and washing buffers according to the method of Lowry using bovine serum albumin as a standard (Lowry et al., 1951). All spectrophotometric measurements were carried out with a Shimadzu UV-2401PC spectrophotometer.

5.2.4 Laccase immobilization

Laccase was immobilized onto the different supports by the method described by Parra et al. (in preparation). First, activation of the support with GLU in vapour phase was carried out by dispersing 100 mg of each carrier on the bottom of an aluminium plate (35 mL). The plate was placed inside a glass vessel (100 mL) containing approximately 15 mL of 25% (v/v) GLU solution. The vessel was closed and kept at 30°C during 18 h. Enzyme immobilization was performed by incubating the GLU-modified particles in 5 mL solution containing 107 U of laccase in buffer citrate-phosphate (0.1 M) pH 7 at room temperature under continuous stirring for 2 h. The resulting biocatalyst was then washed four times with buffer citrate-phosphate pH 4 and stored at 4 °C.

The enzymatic activity of all biocatalysts as well as the protein content of both supernatants and washing solutions were measured to determine the enzyme load and bound protein yield.

5.2.5 Biotransformation of pharmaceuticals by immobilized laccase onto SiNPs and nanocomposite 50/50

The oxidation of a mixture of four pharmaceuticals by immobilized laccase was investigated in 5.5 mL reaction medium containing AMX, SMX, CIP (20 mg L^{-1}) and CBZ (10 mg L^{-1}) dissolved in citrate-phosphate buffer (0.1 M, pH7) and SYR as redox mediator (520 µM) in 25 mL glass vials at 25°C under continuous stirring to ensure O_2 saturation of the reaction medium and dark conditions to avoid light oxidation. Reactions started when 7 U of immobilized laccase onto SiNPs or nanocomposite 50/50 were added to the reaction medium. Samples were withdrawn at specific time intervals for 3 h to monitor the removal process. After centrifugation at high speed for five minutes, sample supernatants were decanted, filtered with CHROMAFIL Xtra H-PTFE-20/30 filters and immediately analysed by HPLC-MS.

In parallel, experiments with raw SiNPs and nanocomposite 50/50 with and without mediator, as well as controls with immobilized laccase previously inactivated were also carried out to assess the role of adsorption on the removal of the pharmaceuticals. All experiments were carried out by duplicate and results are reported as means with corresponding standard deviations.

5.2.6 HPLC-MS analysis

The concentration of pharmaceutical micropollutants was determined by high-performance liquid chromatography (HPLC) on an Alliance – Waters e2695 separations module HPLC coupled to a MS Micromass Quatro API (triquadropole) detector and a 150 x 2.1 mm Raptor C-18 column (5 µm). A 5 µL sample volume was injected into the column. Elution was performed at a flow rate of 0.25 mL min^{-1} with a gradient of (A) 95% water - 5% methanol and (B) 100% methanol. The gradient program was set as follows: 0-3 min, 100% (A); 3-8 min, 100% (B) and 8-15 min, 100% (A).

5.3. Results and discussion

5.3.1 Immobilization of laccase onto different types of supports

Inorganic materials are widely used as supports for immobilization of enzymes for environmental applications due to their exceptional properties (Zdarta et al., 2018a). In this investigation, five novel supports belonging to different classes of inorganic compounds (silica, clay and silica/clay nanocomposite) were used for the covalent immobilization of laccase (Table 5.2). Immobilization was performed according to a novel protocol proposed by Parra et al. (in preparation) which involved the activation of each support previously functionalized with APTES with GLU in vapour phase. This step allowed easier handling of micro/nanoparticles as it is performed in dry conditions, avoiding - agglomeration of the particles that may occur when activation is performed in a liquid solution. After immobilization, measurements of enzymatic activity of the different biocatalysts as well as protein determination of the supernatants were carried out in order to determine enzyme load and protein immobilization yield. Table 5.3 shows the results obtained for all supports. A comparative analysis of the different parameters in Table 5.3 will allow identifying the best biocatalysts to be further used.

The various supports showed different laccase immobilization capability. Clay carriers allowed moderate enzyme loading and bound protein values. Both clay materials presented similar protein immobilization yields (32-38%) but Y-clay displayed higher specific activity with 57.4 U g^{-1} of support. According to Shen et al. (2002), interactions between clays and enzymes during immobilization can be influenced by the material properties such as clay type, surface area and cation exchange capacity. The higher specific activity obtained for Y clay could be related to its specific surface area (99 m^2 g^{-1}) which is considerable larger than those of SWy-3 clay (44 m^2 g^{-1}). For example, Ahn et al. (2002) compared montmorillonite and kaolinite as supports for laccase immobilization. These authors reported that montmorillonite capacity for enzyme immobilization was 12 times higher in comparison to kaolinite, which was attributed to its higher surface area. The specific area is an important parameter since it determines the enzyme loading capacity of a carrier. Typically, the best support would be that one showing the highest loading capacity, because in this way it is possible to immobilize larger amounts of enzyme with a lower volume of support (dos Santos et al., 2015). However, a higher loading capacity may not always ensure the resulting

biocatalyst will also show high enzyme activity as observed with SWy-3 clay. The Swy-3 clay allowed immobilizing 38% of protein, whereas Y-clay with a twice larger specific surface area attained similar protein immobilization yield (32%). There is the possibility that the higher immobilization capacity of SWy-3 led to an oversaturation of the support with laccase which reflected in low activity. According to literature, this could be due to the steric hindrance of laccase on the surface of clay particles. Steric hindrance occurs when an excessive concentration of enzyme is loaded on the support which leads to undesired protein interactions and flexibility inhibition of enzyme conformation (Fortes et al., 2017). Similar observation was also reported on previous studies dealing with the immobilization of laccases and lipases on magnetic nano and microparticles (Fortes et al., 2017; Guo et al., 2003; Jiang et al., 2009).

Table 5.3. Activity recovery, bound protein and enzyme loading obtained for laccase immobilization on different supports

Type of material	Bound protein (%)	Activity (U g^{-1} biocatalyst)
Silica particles		
SiNPs	48 ± 8	109 ± 10
Clay particles		
Y	33 ± 5	57 ± 7
SWy-3	38 ± 6	40 ± 5
Nanocomposite		
50/50	30 ± 10	185 ± 2
95/5	29 ± 7	24 ± 1

Covalent attachment of TVL on SiNPs allowed high retention of protein which also showed high laccase activity. Actually, this nano-biocatalyst yielded to the greatest protein immobilization yield obtained in the study with an average of 48.2%, as well as the second highest enzyme loading with 108.7 U g^{-1}. The overall good performance of SiNPs for laccase immobilization may be also attributed to the material properties. Moreira et al. (2017) observed that immobilization of TVL onto different types of silica nanoparticles previously modified with APTES and GLU led to high activity yields and improved enzyme stability. It was suggested that immobilization of enzymes onto silica supports could lead to high affinity

for model substrates such as ABTS which directly reflected in high activity yields. Probably, such affinity is the result of an effective enzyme immobilization and reduced diffusional limitations provided by the high surface area and porous structure of silica nanocarriers (Zdarta et al., 2018b).

The use of composite materials as supports for enzyme immobilization represent a very interesting option since they combine the characteristics of different precursors which results in materials showing enhanced properties (Grigoras, 2017). Table 5.2 shows the characteristics of each nanocomposite. The two nanocomposites 50/50 and 95/5 present different particle size, 51 and 92 μm respectively; however, in terms of specific surface area both values are similar. Results show both nanocomposites attained the same protein immobilization yield (∼30%) which is not surprising given that the nanocomposites presented almost the same specific surface area. However, these immobilization yields were the lowest from the five materials evaluated. The quantity of protein attached to the nanocomposites was 18% lower in comparison to SiNPs but nearly the same (3% lower) than the observed with Y-clay beads (one of their precursors). The latter observation is interesting since the specific area of both nanocomposites was reduced by 30% average in comparison to Y-clay (Table 5.2). Even when the specific surface area of nanocomposites was reduced, comparable capacity to Y-clay in terms of enzyme loading was observed.

On the other hand, contrasting results were observed when the specific activity was evaluated. The nanocomposite 50/50 showed the maximum enzyme load in the study with 185.2 U g^{-1}, whereas nanocomposite 95/5 reported the lowest activity with barely 23.7 U g^{-1}. The different ratio of silica/clay of each nanocomposite could be the cause for the different activity showed by each material. Nanocomposite 50/50 presented equal concentration of both Y-clay and Si 30R50, whilst composite 95/5 structure was 95% Y-clay and 5% Si 30R50. Probably, the highest concentration of Si 30R50 in composite 50/50 provided a more suitable environment for TVL which reflected in a considerable higher activity. Therefore, the outstanding results obtained with the nanocomposite 50/50 could be the result of Y-clay allowing the attachment of relatively high quantities of laccase and the highest concentration of silica in the support providing a stable microenvironment for the enzyme.

Considering the best results of bounded protein and enzyme load the SiNPs and nanocomposite 50/50 were selected for the following experiments of biotransformation of pharmaceutical micropollutants.

5.3.2 Biotransformation of pharmaceuticals by immobilized laccase on SiNPs and nanocomposite 50/50 particles

The capacity of immobilized TVL onto SiNPs and nanocomposite 50/50 to transform pharmaceutical micropollutants was assessed in batch reactions. The reaction medium was composed by a mixture of the antibiotics AMX, CIP, and SMX (20 mg L^{-1}); the antiepileptic CBZ (10 mg L^{-1}) and SYR (520 µM) as redox mediator. Enzymatic transformation of each micropollutant for both biocatalysts tested is presented in Fig. 5.1, along with the adsorption-based removals observed in the control experiments.

Results show both biocatalysts yielded to high removal efficiencies ranging from 45 to 100% and 49 to 100% with SiNPs and nanocomposite 50/50 respectively. Nonetheless, the elimination extent depended on the pharmaceutical and biocatalyst used, since removals observed were the outcome of a combined process of adsorption and enzymatic degradation. As shown in Fig. 5.1, the two materials showed different adsorption capacities as demonstrated by control experiments prepared with previously inactivated laccase. The nanocomposite 50/50 exhibited outstanding affinity for CIP that was completely eliminated via adsorption on this support, whereas CBZ was moderately adsorbed (up to 49%) and AMX and SMX removal was negligible (<10%). On the other hand, SiNPs controls showed similar capacity of adsorption for all the compounds with slightly high values in the range from 44 to 65%. Moreover, it is important to mention that neither the modification of the carriers nor the presence of SYR modified the sorption capacity of both supports as observed in their corresponding controls carried out with the pristine materials.

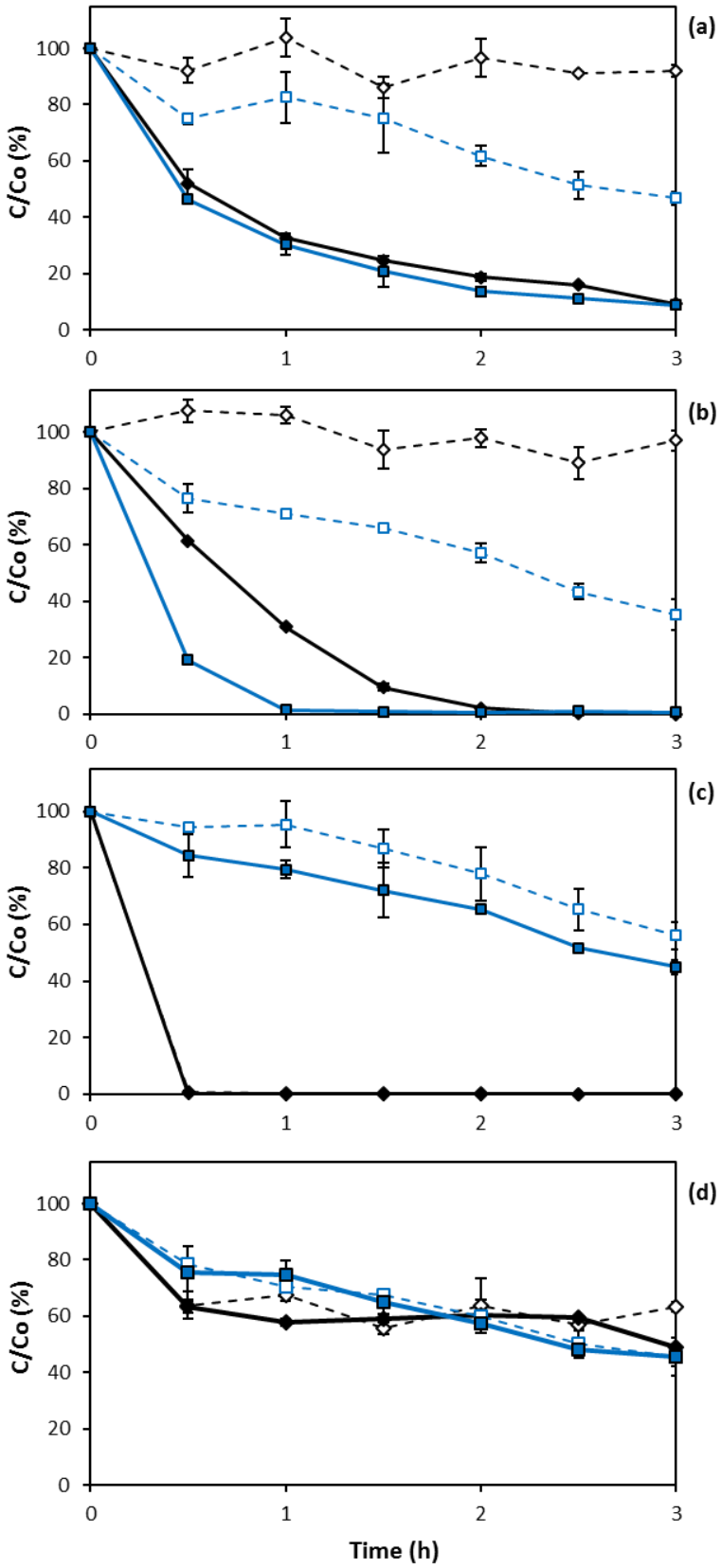


Fig. 5.1. Removal of (a) AMX, (b) SMX, (c) CIP and (d) CBZ in mixture with TVL immobilized onto SiNPs particles (■), and onto nanocomposite 50/50 (◆) over 3 h at pH 7 and with SYR as redox mediator. The open symbols represent the removal by adsorption on the support with inactivated laccase (standard deviation is indicated as error bars).

The different capability of the two carriers to adsorb the micropollutants can be related to each material properties. For example, the presence of various functional groups, good sorption properties and high stability of silica carriers make them an excellent option for the immobilization of oxidative enzymes as well as for the selective sorption of pollutants such as dyes and phenolic compounds (Champagne and Ramsay, 2007; Kim et al., 2011). In a previous work Parra et al. (in preparation) observed comparable sorption removals (30-50%) for a mixture of pharmaceutical micropollutants by inactivated TVL immobilized on commercial silica gel particles. In contrast, the high and selective adsorption of the micropollutants showed by nanocomposite 50/50 probably was the result of the combined characteristics of its inorganic precursors, silica and clay, which are characterized for presenting high surface area and sorption capacity. It is known that montmorillonite clay materials can provide extra adsorption due to their two dimensional layered structure (An et al., 2015).

However, adsorption observed for the pharmaceuticals in this study cannot be only associated to the properties of the solid supports but also to the physicochemical characteristics of each micropollutant. In general, hazardous compounds can be attached to the surface of sorbent materials by means of different mechanisms such as surface adsorption, ion exchange, complexation, chelation or micro-precipitation (Crini, 2006; Michael et al., 2013). Nevertheless, it has been recognized that the efficiency and selectivity of adsorption for pharmaceutical compounds are mostly due to hydrophobic or ion exchange interactions. The affinity of compounds to adsorb and accumulate to solids due to hydrophobic interactions can be evaluated by the octanol-water partition coefficient (K_{ow}). Typically, neutral charged compounds with a log K_{ow}<2.5 are considered to show low sorption potential (Michael et al., 2013). Nonetheless, it is important to know that this assumption cannot be applied to polar or charged molecules since their prediction often leads to underestimations. In the present investigation even when the studied pharmaceuticals showed log K_{ow} values below 2.5 (Table 5.1), the compounds were indeed adsorbed onto the supports. The relatively low Log K_{ow} values of AMX and SMX (0.89 and 0.87 respectively) and the hydrophilic nature of both antibiotics could explain their low adsorption observed on nanocomposite 50/50. The high adsorption of CIP can be attributed to its particular sorption mechanism, which appears to occur by a combination of

hydrophobic forces and electrostatic interactions, being the latter one the most predominant mechanism (Golet et al., 2003). Regarding to CBZ, the removal yields observed by both supports can be related to its Log K_{ow} value of 2.45 which is in the limit between low and moderate adsorption potential as well to its low solubility in water (78 mg L^{-1}). In addition, it is worth noting that elimination of CIP and CBZ was entirely by adsorption as shown in Fig. 5.1c and 5.1d. Fast and complete removal of CIP was obtained by composite 50/50 in only 30 minutes. Whilst in presence of SiNPs residual concentration of CIP decreased constantly to reach 56% at the end of 3 h. According to Fig. 5.1d CBZ removal occurred mainly during the first 30 minutes of treatment with average elimination of 50% for both SiNPs and nanocomposite immobilized biocatalysts.

Even if part of the elimination was associated with the adsorption of the micropollutants to the supports, enzymatic degradation was observed as the major contribution in the removal process for the antibiotics AMX and SMX. The biotransformation of AMX by both immobilized biocatalysts followed almost identical profiles, reaching 91% removal at the end of 3 h (Fig. 5.1a). In the case of SMX, a rapid and complete degradation was observed with laccase immobilized onto SiNPs after 1 h of reaction (see Fig. 5.1b). Similarly, laccase immobilized on composite 50/50 allowed 100% removal of SMX but 2 h were necessary to achieve such transformation. These results confirmed that the solid support played a relevant role in the degradation of these compounds.

According to the results obtained, faster removal of the antibiotics was obtained when TVL was immobilized onto SiNPs, as this carrier was capable of moderately adsorbing AMX and SMX. This observation suggests that adsorption of micropollutants onto active SiNPs may have facilitated the degradation of pharmaceuticals by laccase. Similar effect was reported by various research groups for the removal of dyes and pharmaceutical micropollutants when enzyme was immobilized onto different supports such as granular activated carbon (GAC), alumina and chitosan-Fe membranes (Nguyen et al., 2016; Wen et al., 2015; Zille et al., 2003). According to these authors, the interaction between pollutants and the active sites of immobilized laccase was improved after adsorption resulting in an enhanced removal performance. In this way, the combined action of adsorption and biodegradation allowed to TVL immobilized on SiNPs to achieve high and fast removal yields for both antibiotics which was especially evident for SMX.

Although no adsorption of AMX and SMX was observed on nanocomposite 50/50, however, this activated solid yielded to the same high removal percentages obtained by SiNPs. Nonetheless, longer contact times were necessary to achieve such comparable results. The use of composite/hybrid materials for the covalent immobilization of enzymes have been demonstrated to significantly improve enzyme stability (Aydemir and Güler, 2015; Sathishkumar et al., 2012). Aydemir and Güler (2015) observed that covalent immobilization of TVL on magnetic chitosan-clay composite beads considerably enhanced enzyme stability allowing complete removal of phenol under the optimum conditions. The longer time needed for nanocomposite 50/50 catalysed reactions could be related to diffusional limitations in the transport of substrate created by the multipoint attachment of laccase on the nanocarrier. Nonetheless, even considering these diffusional constraints, immobilized TVL onto composite 50/50 was able to achieve high removal yields.

The high degradation yields observed for AMX and SMX can be related to the presence of functional groups in their structure susceptible for laccase oxidation. The presence of hydroxyl and amino groups directly attached to a benzene ring, in AMX and SMX respectively, make them putative laccase substrates. However, the presence of SYR in the reaction media acting as electron shuttle between immobilized TVL and substrates could be considered as the determinant factor that favoured the transformation of AMX and SMX as reported by (Parra Guardado et al., 2019). In contrast, CIP and CBZ showed recalcitrance to laccase biotransformation. Neither the presence of SYR nor the immobilization of laccase allowed the enzymatic transformation of the pharmaceuticals. Resistance of CBZ and CIP is associated to the presence of strong electron withdrawing functional groups (amide and halogen, respectively) which generates electron deficiency making the compounds less prone to laccase action (Yang et al., 2013).

5.4. Conclusions

In recent years, the simultaneous use of a material for enzyme immobilization and for adsorption has become subject of study to many research groups with the objective to develop advanced technologies able to achieve high removal efficiency of environmental pollutants. Different materials of organic, inorganic and hybrid/composite origin have been

tested for their potential to simultaneously immobilize enzyme and adsorb pharmaceutical micropollutants and results seem encouraging (Cabana et al., 2009; García-Delgado et al., 2018; Ji et al., 2016; Naghdi et al., 2017; Nguyen et al., 2016; Shao et al., 2019). In this regard, results described in the present work highlight the important role of the carrier material properties in the immobilization process of laccase as well as in the performance of the immobilized biocatalyst for the removal of environmental micropollutants.

The five materials evaluated in the present study showed different capability to immobilize laccase. Results revealed that the immobilization process was influenced by both the specific surface area of each carrier and the type of material and its composition. It was found that silica materials had a positive effect in the immobilization of laccase as observed with the composite silica/clay nanoparticles where a higher concentration of silica yielded to the most active biocatalyst. Therefore, composite 50/50 and SiNPs resulted as the best supports in terms of high specific activity (185 U mg^{-1}) and protein immobilization yield (48%), respectively.

The two biocatalysts were then used for the removal of a mixture of pharmaceutical micropollutants in the presence of SYR as redox mediator. Overall, both SiNPs and nanocomposite 50/50 biocatalysts achieved high removal yields for all the pharmaceuticals as the result of increased laccase stability after immobilization and the remarkable sorption capacity of the carriers. High removal of the antibiotics AMX (91%) and SMX (100%) was mainly due to the oxidative action of immobilized laccase, whilst the elimination of CIP (56-100%) and CBZ (50%) was due to adsorption on the carriers. Although both biocatalysts showed similar performance for the removal of the target micropollutants, it was observed that they followed different pathways. High and fast degradation efficiency of micropollutants by immobilized laccase on SiNPs was possibly due to the moderate sorption capacity showed by the support, allowing a better interaction between laccase and pharmaceuticals after the compounds were adsorbed on the silica surface. In contrast, the high removals obtained by the active nanocomposite were the result of an increased stability of the enzyme and a remarkable selective sorption capacity only towards CIP and CBZ. From the best of our knowledge this is the first report of the use of silica/clay nanocomposites as laccase carriers for the removal of environmental micropollutants. The conception of treatment systems using materials showing properties for immobilization of

enzymes and adsorption could be a promising option for the effective biotransformation of toxic mixtures of pollutants at mild conditions to obtain cleaner effluents in one step treatment. In this sense, the biocatalysts herein proposed represent a valuable addition to the current knowledge for the future development of this type of processes in different configurations of enzymatic bioreactors for example.

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Chapter 6

Conclusions and Perspectives

6. Conclusions and perspectives

The objective of this thesis was to evaluate the removal of four model pharmaceutical micropollutants in water (amoxicillin (AMX), ciprofloxacin (CIP), sulfamethoxazole (SMX) and the antiepileptic carbamazepine (CBZ)) by different laccase-based treatments. For this purpose, two different approaches were followed: the enzymatic treatment by means of free laccase systems and the use of immobilized enzymes onto different solid supports. For the free laccase systems, the oxidative potential of a crude extract from a native strain from *Pycnoporus sanguineus* (PSL) white rot fungus was compared to that of the commercially available *Myceliophthora thermophila* (MTL) and *Trametes versicolor* (TVL) purified enzymes. As described in chapter 3, enzymatic treatment resulted ineffective for the biotransformation of the pharmaceutical compounds. Only the antibiotic AMX was partially eliminated, but the extent depended on the laccase used and the reactions conditions assayed which was observed to increase at slightly acidic conditions (pH 5-6). The poor removals observed were attributed to the physicochemical properties of the pharmaceuticals which make them resistant to laccase attack. In an attempt to expand the substrate spectrum of laccases, redox mediators were added to the reaction. Results demonstrated the positive effect of the addition of the natural mediators syringaldehyde (SYR), p-coumaric acid (PCA) and the synthetic compound 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS). However, the performance of the laccase-mediator systems was observed to be mediator dependent. ABTS and SYR yielded to the highest removal efficiencies. In particular, ABTS was observed to enhance the degradation of phenolic compounds. However, SYR was the best redox mediator allowing the highest degradation yields, especially for non-phenolic molecules, as result of the highly reactive and stable radicals formed after its oxidation by laccase. In this sense, commercial laccases showed better performance than the crude extract from *P. sanguineus*. Overall, laccase oxidative potential was increased by the addition of mediators, however it was not possible to find a broad spectrum mediator since CBZ was not transformed by any laccase-mediator system tested. Anyhow, the potential of free laccase-mediator systems for the treatment of recalcitrant pharmaceuticals was demonstrated.

In the fourth chapter, we have presented the work carried out for the immobilization of the laccases. A new methodology was developed for the covalent binding of laccases onto commercial amino-functionalized silica gel micro-particles. The novel method involved the activation of the silica beads with glutaraldehyde (GLU) in vapour phase in order to facilitate the handling of the small particles during this step. This is the first time this activation technique is used for the immobilization of laccase on solid supports for bioremediation applications. In order to stablish the best immobilization conditions, a 2^3 full experimental design was generated to study the effect of different parameters in the process. According to the resulting model, the conditions leading to the highest laccase activity (14±2 U g^{-1} of beads) and protein immobilization yield (35%) were: 18 h of GLU activation time, 107 U of enzyme, 2 h of contact time and pH 7. The covalent immobilization of laccase onto the silica beads allowed to enhance its stability over a range of pH and temperature values as well as to improve its long term storage in comparison to the free form. Even if the stability of laccase was increased after immobilization, the resulting biocatalyst was still unable to catalyze the oxidation of the target pharmaceuticals. However, similarly to free enzyme, the addition of redox mediators significantly increased the transformation of the pharmaceuticals reaching high removal yields (40-100%) within 3 h of reaction. It was observed that removal of pharmaceuticals occurred by the combined action of degradation and adsorption on the support, and the occurrence of one or the other mechanism was compound dependent. AMX and SMX elimination was due to biodegradation, although it was also observed that adsorption may have played an important role in the process by enhancing the electron transfer between laccase and substrates. In the case of CIP and CBZ, the removal was completely by adsorption which was attributed to their high affinity to adsorb on solids. Overall, redox mediators generating phenoxyl radicals were the most effective for the biotransformation of the pharmaceuticals, with SYR showing the best performance. Nonetheless, the use of SYR considerably increased the toxicity of the effluent as suggested by the Microtox® assay; whilst ABTS led to the lowest toxic treatment. Finally, the reusability of the biocatalyst showed that immobilize enzyme could be effectively reused for the degradation of pharmaceuticals for seven consecutive cycles with SYR as mediator. In this sense, a balance between effective removal of the micropollutants and less toxic effluents needs to be found for the successful implementation of the treatment.

The fifth chapter of this thesis was devoted to study the immobilization of laccase onto different inorganic support materials. Silica (SiNPs), clays (Y-clay and SWy-3) and silica/clay (95/5 and 50/50) nanocomposites showing different characteristics in terms of chemical composition, size and specific surface area were used to carry out the covalent immobilization of TVL using the new method developed in chapter 3. Results showed the five supports presented different capability to immobilize laccase and it was mainly influenced by the specific surface area of each carrier as well as by the type and composition of the material. It was observed that silica materials had a positive impact in the immobilization of laccase by allowing binding large quantities of highly active protein. This was especially evident with the nanocomposites, where a higher concentration of silica in the composite (5% vs 50%) yielded to the most active biocatalyst. Thus, nanocomposite 50/50 and SiNPs resulted as the best supports in terms of high specific activity (185 U mg^{-1}) and protein immobilization yield (48%), respectively. The performance of both biocatalysts was compared for the degradation of pharmaceutical micropollutants with SYR as mediator in batch reactions. Both biocatalysts reached high removal yields (50-100%) for the four pharmaceuticals studied due to the synergistic action of laccase catalyzed oxidation and remarkable sorption capacity of the carriers. Interestingly, even when the two biocatalysts showed similar removal yields the mechanism they follow to achieve such results was certainly different. Laccase immobilized onto SiNPs showed high and fast degradation efficiency due to the moderate sorption capacity of the support for the pharmaceuticals, which possibly enhanced the interaction between laccase and pharmaceuticals once the compounds were adsorbed on the support surface. In contrast, even when nanocomposite 50/50 showed remarkable sorption capacity, it was not observed to increase the biotransformation of the micropollutants. In other words, the degradation and adsorption of the pharmaceuticals were carried out by independent processes. In this work, this is the first time that silica/clay composite materials are used as laccase carriers for the treatment of pharmaceutical pollutants and results seem encouraging. The results presented here, highlight the important role of the carrier material properties in the immobilization process as well as in the performance of the resulting biocatalyst for the removal of environmental pollutants. In this sense, we have demonstrated the benefit of using versatile materials showing properties to both immobilize enzymes and adsorb hazardous compounds which could potentially lead to greener and simpler “one step” treatment processes.

The work done here demonstrate the potential of using laccases for the enzymatic treatment of pharmaceutical micropollutants in water, however is important to consider some aspects for the future develop of this research. The performance of laccases in this thesis was investigated mainly using synthetic wastewater. It is important to evaluate the performance of both free and immobilized systems in real wastewater conditions. In this sense, tests with enzymes-mediator systems in real wastewater at the very low concentration of micropollutants, as they are usually encountered in such waters, should be fundamental to assess the impact of real conditions on the effectiveness of the treatment. Another important aspect to consider would be scaling up the treatment, since all the experiments carried out in this work were at laboratory scale. In this sense, the design of a reactor (e.g. enzymatic membrane bioreactor, packed bed) would be the best option as it would also allow to evaluate the effectiveness of the treatment in continuous operation.

Immobilization of laccase allowed to increase the stability and reusability of the enzyme which is crucial for large scale applications. Therefore, the immobilization method proposed should be optimized in order to maximize the immobilization yields and the activity load on the carriers to make the process more cost effective. Additionally, further tests should be done with SiNPs and nanocomposite 50/50 to fully characterize these biocatalysts and evaluate their possible reusability.

In this study, the addition of redox mediators has been demonstrated to improve the degradation and also to expand the spectrum of compounds degraded by laccase. However, the continuous addition of these compounds to the reaction leads to increased costs and higher toxicity of the treated effluent. It is recommended to study the effect of mediator type and concentration on both degradation yields and acute toxicity in order to truly develop an efficient and effective treatment process. Moreover, at the end of the thesis a new approach for the reusability of SYR was investigated by immobilizing the mediator onto solid supports. Since the preliminary results seemed promising, we think it is worth to study in deep this technology.

Résumé

Ce travail concerne l'étude de la dégradation enzymatique de micropolluants pharmaceutiques récalcitrants présents dans l'eau. Tout d'abord, les efficacités de trois laccases différentes issues respectivement de : *Pycnoporus sanguineus* CS43, *Trametes versicolor* (*Tv*) et *Myceliophtora thermophila* ont été comparés lors d'essais de dépollution de solutions modèles renfermant trois antibiotiques (amoxicilline, ciprofloxacine et sulfaméthoxazole) et un antiépileptique (carbamazépine). Les essais ont été réalisés avec les laccases libres en présence ou non de médiateurs redox. L'impact de plusieurs paramètres opératoires sur les performances des enzymes a également été étudié. Puis, une nouvelle méthode d'immobilisation des laccases impliquant l'activation du support (microparticules à base de silice commerciales) par du glutaraldéhyde en phase vapeur a été mise au point et optimisée en utilisant la méthodologie de plans d'expériences. Après immobilisation, la laccase *Tv* s'est avérée être la plus active. Des essais de dégradation en présence de médiateurs redox ont confirmé l'efficacité de l'enzyme immobilisée et sa possible réutilisation lors de cycles successifs. La toxicité des solutions après traitement a été évaluée par des tests Microtox®. La laccase *Tv* a également été immobilisée sur des nanoparticules non commerciales à base de silice ou d'argile ainsi que sur des composites à base de silice et d'argile. La laccase *Tv* immobilisée sur les supports composites riches en silice a montré une plus grande réactivité et de meilleures performances pour l'élimination des composés cibles.

Mots-clés : Laccase, Enzymes immobilisées, Médiateurs redox, Micropolluants pharmaceutiques, Biodégradation.

Abstract

This work is focused on the study of the enzymatic depletion of recalcitrant pharmaceutical micropollutants in water. The potential degradation of three antibiotics (amoxicillin, ciprofloxacin and sulfamethoxazole) and one anti-epileptic (carbamazepine) was studied with three laccases: *Pycnoporus sanguineus* CS43, *Trametes versicolor* (*Tv*) and *Myceliophtora thermophila*. Free laccase systems were evaluated for pharmaceuticals depletion on model solutions in the presence or absence of redox mediators and the impact of several parameters on the performance of laccases for degradation were studied. The enzymes were then immobilized on different solid supports: commercial silica, laboratory synthetized nano-silica and clay based composite nanomaterials and used for degradation tests. A novel methodology for the covalent binding of laccases onto carriers was developed by using glutaraldehyde in vapour phase and the best immobilization conditions were determined through a 2^3 full factorial design. The immobilized *Tv* shown the highest activity and was tested in presence of redox mediators. Moreover, the reusability was evaluated in several degradation cycles and the toxicity of the solutions after treatment was assessed with the Microtox® test. In comparison to laccase immobilized on commercial silica, the *Tv* supported on laboratory synthetized materials showed higher activity and a better performance for the removal of target compounds.

Keywords: Laccase, Enzyme immobilization, Redox mediators, Pharmaceutical micropollutants, Biodegradation.