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

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ABSTRACT

Bio-catalytic processes have recently attracted attention as an interesting option for the degradation of persistent pollutants as they are capable to catalyze 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 *Myceliophthora 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.
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 peroxydases) since
they catalyze specific reactions under moderate operational conditions preventing undesired side-reactions [8,9].

Particularly, Laccases (1.10.3.2) are able to catalyze single electron oxidation of phenolic moieties via phenoxyl 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 catalyzed reactions is an important matter. Recently, the evaluation of critical aspects such as type and dose of mediator and their influence on 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 mixtures 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.

2. MATERIALS AND METHODS

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 (SRY)) and all other chemicals were purchased from Sigma-Aldrich. The structure of pharmaceuticals and redox mediators is shown in Table. 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 15625 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.

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).

### 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 (\(\varepsilon_{420} = 36000 \text{ M}^{-1} \text{ cm}^{-1}\)) 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.

### 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: 85 µM). 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 analyzed 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.

2.4. HPLC-MS quantitative analysis

Micropollutants concentration during enzymatic treatment was analyzed using a HPLC Alliance – Waters e2695 separations module equipped with a C18 Raptor column (150 x 2.1 mm, 5µm) coupled to a MS Micromass Quattro 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 with the following gradient program: 0-3 min, 100% (A), 3-8 min, 100% (B) and 8-15 min, 100% (A).

2.5. Redox potential determinations

Cyclic voltammetry experiments were carried out in sodium phosphate buffer 0.1 M, pH 7, using a VERSASTAT3 voltammetry analyzer (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. RESULTS AND DISCUSSION

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-catalyzed 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. 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. 1).

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 (-NH₂), alkoxy (-RO), alkyl (-R) and acyl (-COR). On the other hand, EWG such as carboxylic (-COOH), amide (-CONR₂), halogen (-X) and nitro (-NO₂) 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 previously reported by Becker et al. [31] who observed that even if the 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 the work of 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 depend 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 BPA, which is a phenolic compound and then after enzyme oxidation would form phenoxy 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, CMZ, 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 BFA) 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.

3.2. *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. 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 varied depending on the type of laccase considered. According to Fig. 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.

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. 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 favored 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 behavior was also reported by Margot et al. [38] for the removal of diclofenac 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 center 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 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 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.

3.3.1 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. 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. 3a and 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 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).

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 a phenoxy 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 on 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 phenoxy 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%).

3.3.2. Effect of ABTS addition

The effects of ABTS addition on the transformation of pharmaceuticals are presented in Fig. 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 AMX was observed after 2 h of reaction with TVL whereas about 90% of removal was achieved after 3 h with MTL (Fig. 4a). Moreover, it is worth noting that during the first 90 minutes of reaction, ABTS did not favor 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. 4b).

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\(^{+}\)) and dication (ABTS\(^{2+}\)) 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 behavior 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 favor the transformation of pharmaceuticals.

3.3.3. Effect of syringaldehyde addition

The addition of SYR to the pharmaceuticals mixture resulted in high enzymatic degradation (50-100%) (Fig. 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. 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. 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 89-95% 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.

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 catalyzed 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 phenoxy 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 phenoxy 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 phenoxy 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 in this work, can result in the acceleration of the loss of laccase activity by the attack of the radicals formed during mediator oxidation can attack 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 7h 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 [55, 56] has already noticed his 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 24h 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 hours.

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. [57] 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 2).

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 favored 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.

Acknowledgements
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Biotransformation of Carbamazepine by Laccase-Mediator System: Kinetics, By-

Figure captions

**Fig. 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.

**Fig. 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 (◼).

**Fig. 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.

**Fig. 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.

**Fig. 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.
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<td><strong>Carbamazepine (CBZ)</strong></td>
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<td><strong>Syringaldehyde (SYR)</strong></td>
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*Table 1*

Chemical structure of pharmaceuticals and mediators used in this work.
Table 2
Reactivity of selected laccases towards mediators

<table>
<thead>
<tr>
<th>PHARMACEUTICAL</th>
<th>M. thermophila</th>
<th>T. versicolor</th>
<th>P. sanguineus</th>
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<tr>
<td>AMX</td>
<td>SYR (+)</td>
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<td>SMX</td>
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<td>SYR (+)</td>
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<td>SYR (+++)</td>
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</tr>
<tr>
<td>CBZ</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

(+++) very reactive; (+++) reactive; (+) less reactive.
Fig. 1
Fig. 2

(a) Plot showing the relationship between pH and the fraction of C/Co (%).

(b) Bar graph showing the fraction of C/Co (%) at different temperatures (°C).

Temperature (°C)
Fig. 3
Fig. 4

(a)

(b)

(c)
Fig. 5

(a) 

(b) 

(c)