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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 *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.

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 $\mu g\ L^{-1}$ 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 catalyze specific reactions under moderate operational conditions preventing undesired side-reactions [8,9].

Particullarly, 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 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

92 one antiepileptic (carbamazepine) in free enzyme systems was evaluated. Firstly
93 specific aspects such as the effect of pH and temperature were assessed for the
94 potential improvement of the treatment. Then the performance of each laccase with
95 selected natural (syringaldehyde and p-coumaric acid) and synthetic (ABTS) redox
96 mediators was compared in terms of degradation efficiency. In this sense, a cyclic
97 voltammetry study was carried out in order to understand the interactions between
98 redox mediators and substrates and the mechanism involved for the oxidation of each
99 compound.
100
101 **2. MATERIALS AND METHODS**
102 *2.1. Chemicals and enzymes*
103 The pharmaceuticals (amoxicillin (AMX), ciprofloxacin (CIP), carbamazepine
104 (CBZ) and sulfamethoxazole (SMX)), mediators (2,2'-azino-bis-(3-ethylbenzothiazoline-
105 6-sulphonic acid)) (ABTS), p-coumaric acid (PCA), syringaldehyde (SYR)) and all other
106 chemicals were purchased from Sigma-Aldrich. The structure of pharmaceuticals and
107 redox mediators is shown in Table. 1. Stock solutions (100 mg L^{-1}) of AMX, CIP and SMX
108 were prepared in citrate-phosphate buffer 0.1 M, pH 7. Considering the lack of
109 solubility in water of CBZ the stock solution of this compound was prepared in pure
110 ethanol at a concentration of 15625 mg L^{-1}. ABTS solution (5 mM) was prepared just
111 before use in buffer citrate-phosphate pH 7. Stock solutions of SYR and PCA (114.9
112 mM) were prepared in pure ethanol.
113 Three different laccase preparations were tested. Laccases from *Pycnoporus*
114 *sanguineus* CS43 (PSL) were obtained from a tomato medium according to Ramirez-

115 Cavazos et al. [28] with some modifications. In short, mycelia were removed from the
116 culture supernatant by filtration using two tangential flow filters in series, with
117 respective pore sizes of 0.5 and 0.2 µm. The 0.2 µm filtrate (laccase cocktail) was then
118 filtered on a 10 kDa ultrafiltration membrane. The resulting enzymatic preparation is a
119 laccase cocktail containing at least two isoenzymes. A complete characterization of
120 such laccase cocktail has been reported by Orlikowska et al. [29]. Laccase powder from
121 *Trametes versicolor* (ref 51639, activity ≥10 U mg^{-1}) (TVL) was purchased from Sigma-
122 Aldrich. Commercial laccase produced by submerged fermentation from
123 *Myceliophthora thermophila* (59.5 g L^{-1} of pure laccase) (MTL) was provided by
124 Novozymes (Denmark).
125
126 *2.2. Laccase activity assay*
127 The laccase activity was determined by measuring the oxidation of 0.5 mM
128 ABTS solution prepared in citrate-phosphate buffer (0.1 M, pH 4). The reaction was
129 monitored at 420 nm (ε_{420} = 36000 M^{-1} cm^{-1}) with a spectrophotometer (Shimadzu UV-
130 2401PC). One enzyme activity unit (U) was defined as the amount of enzymes that
131 oxidized 1 µmol of ABTS per min.
132
133 *2.3. Pharmaceuticals oxidation by laccases*
134 Degradation experiments were run in 100 or 250 ml Erlenmeyer flasks at 25°C
135 under dark conditions to avoid light oxidation and continuous stirring to ensure O_2
136 saturation of the reaction medium. Reaction mixtures (50 or 100 ml) contained one or
137 a mixture of all pollutants in buffer citrate-phosphate (0.1 M) pH 7; each pollutant

138 concentration was fixed at 20 mg L^{-1} (molar concentration: 55 µM AMX, 79 µM SMX
139 and 60 µM CIP) except in the case of CBZ where it was 10 mg L^{-1} (molar concentration:
140 85µM). Some experiments were also carried out at different pH (citrate-phosphate
141 buffer 0.1 M pH in the range of 3-7) and different temperatures (15 – 40 °C).
142 To assess the effectiveness of redox-mediator for enhancing the bio-oxidation
143 of micropollutants, experiments were carried out as described above after adding one
144 of the following mediators (ABTS, PCA and SYR) at a concentration of 520 µM to the
145 reaction medium. Mediator:substrate molar ratios up to 9:1 (depending on the
146 pharmaceutical) were tested in order to ensure the effective removal of the
147 micropollutants due to the mediator action. Reactions were initiated by adding one of
148 the three enzyme preparations at the concentration of 1 200 U L^{-1}. To monitor the
149 degradation process, samples of 1 mL were withdrawn every 24 h when there was no
150 redox-mediator in the reaction medium. When using mediators, samples were taken
151 every 20 minutes during the first three hours and then every hour until 8 h. It is worth
152 noting that the number of samples taken was limited in order to keep the variation of
153 reaction volume below 15%. After sampling, aliquots were inactivated in a water bath
154 at 100 °C for 5 minutes and filtered with CHROMAFIL Xtra H-PTFE-20/30 filters to
155 immediately be analyzed by HPLC-MS. In order to verify the repeatability of the
156 treatments all experiments were carried out several times and at least duplicated. The
157 results reported in all figures correspond to the average of the experiments with their
158 corresponding standard deviations that are given in figures or captions. Controls
159 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 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} 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

183 The potential of each one of three laccases to transform pharmaceutical active
184 compounds was initially evaluated without mediators. Firstly, each pharmaceutical was
185 treated individually with 60 U of each laccase. After 72 h of enzymatic oxidation no
186 sign of laccase-catalyzed degradation for SMX, CIP and CBZ was found by any of the
187 enzymes tested (results not shown). In contrast the antibiotic AMX was effectively
188 transformed (Fig. 1). From the enzymes tested, PSL showed the highest affinity for
189 AMX reaching 72% removal. Commercial laccases TVL and MTL presented similar
190 performance with 58% degradation at the end of the reaction. In the case of PSL the
191 first signs of oxidation were visible after 24 h of reaction.
192 No conversion was detected in controls without enzyme, except for AMX where
193 a decrease in concentration was observed. Belonging to the β-lactam penicillins, AMX
194 is an antibiotic known to be unstable in aqueous solutions due to its fast hydrolysis and
195 degradation to various sub-products [30]. Nonetheless, the self-degradation of AMX
196 was much lower in comparison to reactions where enzymes were added, evidencing
197 the effective catalytic action of laccases on the antibiotic removal (Fig. 1).
198 Affinity of laccase towards substrates is influenced mainly by the redox
199 potential of the specific laccase and the presence of strong electron donating
200 functional groups (EDG) or electron withdrawing functional groups (EWG) [23].
201 Functional EDG prone to be attacked by laccase include hydroxyl (-OH), amines ($-NH_2$),
202 alkoxy (-RO), alkyl (-R) and acyl (-COR). On the other hand, EWG such as carboxylic (-
203 COOH), amide ($-CONR_2$), halogen (-X) and nitro ($-NO_2$) may prevent electron
204 abstraction from occurring by forming a steric shield [9]. Hence, removal efficiencies
205 obtained in this study can be attributed to the chemical structure of the

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

215 The antibiotic SMX contains an amine (EDG) and a sulfonamide (EWG) group in
216 its structure. The influence of both groups on the degradation has not been clearly
217 established. In some studies effective removal of the pharmaceutical was reported
218 [32], whereas in others the sulfone group has been related with its recalcitrance to
219 degradation [33]. Guo et al. [32] reported that white-roth fungus *P. chrysosporium*
220 could oxidize SMX. In contrast, Margot et al. [25] observed SMX was recalcitrant to
221 purified TVL oxidation in absence of redox mediators. Interestingly, higher removal of
222 the antibiotic was obtained by fungal cultures in comparison to purified laccase as
223 demonstrated by Gao et al. [34] who observed High oxidation yields of SMX by using *P.*
224 *chrysosporium* and *P. sanguineus* fungal cultures; but visible lower catalytic effects
225 were obtained when the corresponding purified enzyme extracts were used. Possibly,
226 the effect of other enzymes or compounds present in fungal cultures enhanced SMX
227 removal. In addition, low oxidation of SMX would be also attributed to the redox
228 potential of selected laccases. The redox potential of sulfonamides is estimated to be

229 in the range of 0.858-1.158 V which is considerable high in comparison to redox
230 potential of most laccases (0.5-0.8 V). Indeed, these compounds are recalcitrant to the
231 oxidation by laccase [35]. In the case of CIP and CBZ both pharmaceuticals possess
232 strong EWG. The amide group in CBZ and the presence of the carboxyl and halogen
233 group in CIP make them highly resistant to laccase oxidation [33,36]. The lack of
234 oxidation observed in this study agrees with the work of Ji et al. [37] who reported less
235 than 5% removal of CBZ by free laccase treatment.

236 Considering the limited oxidative performance of laccases towards
237 pharmaceuticals in single substrate assays, simultaneous conversion of AMX, SMX, CIP
238 and CBZ in mixtures, was carried out to study potential substrate
239 mediation/competition effect in the reaction. Investigation on the effect of substrate
240 mixtures on enzyme specificity is of high interest to assess the applicability of enzymes
241 as a treatment option since treated wastewaters generally contain a complex mixture
242 of pollutants. In these experiments the concentration of SMX, CIP and CBZ remain
243 unchanged in the reaction medium, confirming their recalcitrance to enzymatic
244 oxidation (data not shown). Laccase affinity towards these substrates was not
245 influenced by the presence of others micropollutants in the reaction. Nonetheless,
246 their presence results also on a very slight decrease of the AMX concentration.
247 However, this decrease is difficult to be considered as result of the enzymatic
248 degradation because of the self-oxidation of this substrate is of the same order of
249 magnitude.

250 The simultaneous presence of micropollutants in a reaction media can affect
251 the removal efficiency in the system. The results presented until now in literature are

252 sometimes contradictory and depend on the mixture of pharmaceuticals studied. For
253 example, Ji et al. [14] who studied the free enzymatic removal of five representative
254 pharmaceuticals (bisphenol-A (BPA) , diclofenac (DCF), clofibric acid, ibuprofen (IBP)
255 and CBZ) observed that degradation yield in mixtures of three molecules was doubled
256 respect to samples with only one pharmaceutical. The increase in degradation was
257 attributed to the presence of BPA, which is a phenolic compound and then after
258 enzyme oxidation would form phenoxyl radicals that can act as redox mediator
259 between laccase and other micropollutants more recalcitrant to enzymatic
260 degradation. On the contrary, Margot et al. [38] observed that removal yield was four
261 times lower in the presence of mefenamic acid and diclofenac compared to single
262 compound solution. Authors suggest that a competitive effect between substrates
263 takes place thus reducing removal yields. Nguyen et al. [33] studied the simultaneous
264 removal of , SMX, CMZ, DCF and BPA with a commercial laccase. No effect of the
265 mixture of micropollutants on the removal was observed. These authors attributed the
266 resistance (SMX and CBZ) or the vulnerability (DCF and BFA) to laccase oxidation to the
267 chemical structure of each compound. Seems like the cocktail effect of micropollutants
268 mixtures in laccase catalytic system depends on the presence of more reactive
269 compounds that could promote the removal of recalcitrant ones [39]. Indeed, the
270 presence of different compounds in the reaction media is strongly dependent on the
271 mixture studied. In the present work the degradation of AMX observed could have an
272 influence of the degradation of other micropollutants in the mixture.
273 Furthermore, effective removal of pharmaceutical mixtures directly depends on
274 the type of enzyme and compound as demonstrated by Stadlmair et al. [27]. These

275 authors tested different types of oxidative enzymes for the multiple conversions of
276 acetaminophen, DCF and sotalol and they observed that inhibition or enhancement
277 effect in case of horseradish peroxidase did not occur with laccase from *Pleurotus*
278 *ostreatus*. The poor degradation performance obtained in this investigation could be
279 also related to the operational conditions which were not optimized. Indeed, enzyme
280 reactivity generally depends on conditions such as pH and temperature. The effect of
281 these parameters was studied only for AMX since it was the most sensitive compound
282 to enzymatic degradation.
283
284 *3.2. Effect of pH and temperature in the oxidation of AMX by laccases*
285 Removal of AMX by enzymatic treatment was investigated at different pH.
286 Laccase produced by *P. sanguineus, M. thermophila* and *T. versicolor* were used for this
287 purpose. In order to evaluate AMX self-degradation, similar experiments were carried
288 out by replacing the enzymatic solution by the same amount of corresponding buffer.
289 Results obtained are reported in Fig. 2a.
290 As observed in the control reactions, AMX in aqueous solution was less stable
291 at acidic conditions in comparison to neutral pH values. After 48 h at pH 3 around 80%
292 of the compound was disappeared. At higher pH values, self-degradation decreased
293 and stabilized at around 30% (pH 5-7). It is important to note that after enzyme
294 addition, no AMX removal was observed at pH 3 by any of the laccases used.
295 Moreover, AMX transformation yield significantly variated depending on the type of
296 laccase considered. According to Fig. 2a the addition of MTL leads to a slight decrease
297 of AMX concentration in comparison to the control as long as pH is less or equal to 5.

298 However, at pH values higher than 5, no considerable transformation was observed
299 regarding to the control curve.
300 The TVL presented a completely different reactivity towards AMX in function of
301 pH. No oxidation occurred when pH was in the range from 3 to 5. Nonetheless, when
302 pH was higher than 5, AMX concentration significantly decreased in comparison to
303 control reactions (residual concentrations at pH 6 was 52% versus 73% and 34% versus
304 69% at pH 7). The best performance of TVL was thus achieved between pH6 and 7. PSL
305 was definitely the best enzyme for the AMX degradation. In comparison to controls
306 this enzyme allowed to improve AMX degradation from pH 4 to 7. The best
307 performance was observed between pH 5 and 6. In such conditions AMX was almost
308 completely removed (residual concentration less than 1% and 4% at pH 5 and 6
309 respectively).
310 Optimal pH depends on the laccase properties as well as on the substrate
311 properties, since pH can alter the charge of the compound and the configuration shape
312 of the enzyme which is fundamental for the access or the binding of substrates to the
313 active site [38]. These results are in good agreement with the conversions reported by
314 Lloret et al [40]. and Margot et al. [38], who reported an optimal pH around 5.5 for the
315 degradation of several micropollutants. Even though in some cases oxidation can be
316 observed at low pH values, acidic conditions may lead to complete enzyme
317 deactivation. According to the results presented in this work pH 6 would represent the
318 balance between laccase activity and stability to maximize AMX oxidation capacity;
319 therefore, pH 6 was chosen to evaluate the influence of temperature in the catalytic
320 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

344 are expected to display different catalytic properties. Moreover, commercial TVL and
345 MTL are purified or partially purified enzymes contrary to the laccase cocktail from
346 PSL. The better performance of PSL could be attributed to the presence of at least two
347 laccase isoforms in the culture supernatant as well as some impurities. All these
348 components in PSL supernatant could have protected the active center from pH and
349 temperature variations making the laccase cocktail the same or more stable than the
350 purified isoforms as suggested by Ramirez-Cavazos et al. [41].
351
352 *3.3. Oxidation of pharmaceuticals by laccases in the presence of redox mediators*
353 In this section the mediated action of two natural mediators: p-coumaric acid
354 and syringaldehyde was compared with the influence of the synthetic mediator ABTS
355 for the oxidation of the pharmaceutical active compounds in mixtures. As mentioned
356 in section 1 factors such as molecular structure of the micropollutant, redox potential
357 of the enzyme, type and concentration of mediator can strongly influence the
358 degradation process. In this sense we chose to work with mediator compounds that
359 follow different oxidation mechanisms: hydrogen atom transfer (SYR and PCA) and
360 electron transfer (ABTS). Mediator concentration was fixed at 520 µM to provide a
361 high mediator:substrate molar ratio to allow the effective removal of the studied
362 pharmaceuticals by laccase-mediator systems as demonstrated by Murugesan et al.
363 [42]. Finally, all degradation reactions as well as control reactions were followed for at
364 least 3 h.
365
366 *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 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).

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 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

390 such as polycyclic aromatic hydrocarbons (PAH) [43,44]. In this way, PCA was reported
391 to enhance the removal of the recalcitrant CBZ in hybrid membrane reactors, both in
392 single [37] and mixture micropollutant solutions [39]. The improved degradation of
393 CBZ in the mentioned works could be attributed to the improved stability of
394 immobilized enzyme used in the bioreactors compared with free laccase. Although in
395 the present investigation PCA did not enhance CBZ oxidation, its effect on the removal
396 of other persistent pollutants was validated.

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

403

404 *3.3.2. Effect of ABTS addition*

405 The effects of ABTS addition on the transformation of pharmaceuticals are
406 presented in Fig. 4. The presence of ABTS in the reaction medium allowed the
407 oxidation of AMX and SMX by TVL and MTL. Degradation percentage observed for AMX
408 by TVL was higher than the obtained with MTL; a complete removal AMX was
409 observed after 2 h of reaction with TVL whereas about 90% of removal was achieved
410 after 3 h with MTL (Fig. 4a). Moreover, it is worth noting that during the first 90
411 minutes of reaction, ABTS did not favor the oxidation of SMX by any of both laccases.

412 Visible effects were only observed after 2 h with removal efficiencies up to 30 and 50%
413 by MTL and TVL respectively (Fig. 4b).
414 Few studies have reported the degradation of SMX by laccase-mediator
415 systems [25,34,45]. Margot et al. [25] obtained similar removal yields to those
416 obtained in this work (30%) with *T. versicolor* laccase in presence of ABTS in pure
417 compound solutions but at longer reaction times (10 h). ABTS is an azino mediator that
418 undergoes oxidation by means of electron transfer mechanism and has been proposed
419 for the transformation of compounds with relatively weak C-H bonds [46].
420 Nonetheless, in general, the reactivity of mediators towards substrates in laccase-
421 mediator systems varies depending on the functional groups in the substrate [21]. SMX
422 presents a phenylamine in its structure whereas AMX shows a phenol group. Since
423 both groups are susceptible for laccase degradation, phenol may be an easier
424 substrate to be transformed due to the presence of electron-donating substituents at
425 the benzene ring which decreases the electrochemical potential [47].
426 Last assumption was confirmed by means of the electrochemical analysis
427 carried out with substrates and mediators. When AMX or SMX were added to a
428 solution of ABTS an increase in the oxidation current for both cation ($ABTS^{\bullet+}$) and
429 dication ($ABTS^{\bullet 2+}$) forms was observed. ABTS dication current increased from 11.5 to
430 23 mA in presence of AMX and from 11.5 to 13.5 mA for SMX. This phenomenon was
431 previously reported by Bourbonnais et al. [48] for the oxidation of veratryl alcohol with
432 ABTS. According to these authors, during cyclic voltammetry determinations the
433 mediator is oxidized at the electrode and then radicals formed diffuse in the solution
434 to oxidize the substrate. In the case of ABTS the reaction relies on the two electrons

435 oxidation of substrate that regenerates the cation radical at the electrode, resulting in
436 a current increase compared to the oxidation of ABTS alone. The fact that the increase
437 of oxidation current for AMX is significantly higher than for SMX suggest that radicals
438 formed during AMX oxidation are more stable and regenerates faster at the electrode
439 making easier AMX transformation in comparison to SMX.

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

451 On the contrary to the results obtained for TVL and MTL; ABTS did not
452 significantly react with PSL since lower degradation percentages were obtained with
453 this couple. A different behavior was reported by Gao et al. [34] who tested a laccase
454 extract from *P. sanguineus* for the degradation of antibiotics, among them CIP and
455 SMX, in single and mixture solution. They carried out reactions at 30 °C in the presence
456 of ABTS (1 mM) obtaining high transformation yields (85-100%) but 72 h were
457 necessary to achieve these removal yields. The high transformation yields obtained by

458 Gao could be influenced by the operational conditions used in their experiments,
459 higher temperatures and mediator concentration as well as longer reaction times may
460 favor the transformation of pharmaceuticals.
461
462 *3.3.3. Effect of syringaldehyde addition*
463 The addition of SYR to the pharmaceuticals mixture resulted on a high
464 enzymatic degradation (50-100%) (Fig. 5). Among all pharmaceuticals studied, SMX
465 was removed with the highest efficiency by all laccases. MTL showed the best
466 performance with almost 100% degradation within the first 10 minutes of reaction
467 (Fig. 5b). TVL and PSL also completely transformed SMX after 1.5 and 2.5 h. These
468 results are better to those reported by Shi et al. [50] who observed almost a complete
469 degradation of SMX after 30 min with a laccase from *Echinodontium taxodii* but in the
470 presence of higher SYR concentration (1 mM).
471 Similarly, AMX was well removed by the couple MTL-SYR attaining 80% removal
472 within the first 30 minutes. After this time the reaction stabilized and no significant
473 increase in degradation was observed before the end of the experiment. In the case of
474 TVL and PSL moderate transformation yields of AMX were obtained; 50% of removal
475 was achieved at the end of 3 h (Fig. 5a). Increasing the reaction time up to 8 h showed
476 no significant enhancement in degradation; after 4h AMX removal leveled off around
477 to 70%. Degradation of AMX in presence of different concentrations of SYR (0.1 and 1
478 mM) was described by Becker et al. [31]. These authors reported removal yields from
479 89-95% depending on the mediator concentration. Interestingly, authors observed that
480 the use of SYR slightly reduced removal yield of AMX in comparison with the treatment

481 with only laccase. Such negative effect was not observed in this study, on the contrary,
482 enhanced oxidation of AMX was observed for all laccases tested.
483 SYR was able to oxidize CIP altogether with MTL and TVL attaining 40% removal
484 after 3h of reaction and up to 60% at the end of 8 h. To our knowledge, results
485 obtained in this study represent the highest CIP degradation yield reported for a
486 laccase catalyzed oxidation by free enzymes using SYR as mediator. Laccase-SYR
487 system was previously tested for the degradation of CIP with very low removal yields
488 [36,51]. For example, Ding et al. [36] observed negligible removal of CIP by free
489 laccase. Moreover, the degradation was not improved by the presence of SYR even at
490 concentrations as high as 2 mM.
491 Mediator SYR belongs to the group of substituted phenols compounds that
492 have been proved as suitable laccase mediators [26,52]. The mechanism of reaction
493 followed by SYR for the oxidation of non-phenolic substrates is the generation of
494 phenoxyl radicals by HAT [53]. Structurally, SYR presents a phenol group which is easily
495 oxidized by laccase and two methoxy substituents in ortho position that increase the
496 stability of phenoxyl radicals. Therefore, the efficiency of SYR as mediator in terms of
497 speed and conversion yield is related to the high concentration and stability of the
498 phenoxyl radicals formed during oxidation reactions [47]. This actually explains the
499 high and fast conversion yields obtained in this study.
500 Overall, from all mediators studied the best results were obtained with SYR.
501 The presence of SYR accelerates degradation of the pharmaceuticals within the first 3
502 h of reaction, after this time no further transformation was observed. Various
503 hypotheses could explain the threshold observed: loss of mediators and/or laccase

504 activity, enhancement of the concentration of oxidation products etc. All of these
505 possibilities could have a negative influence on the degradation rate. For example,
506 some authors have demonstrated that an excess of mediators, as is the case in this
507 work, can result on the acceleration of the loss of laccase activity by the attack of the
508 radicals formed during mediator oxidation can attack enzyme's catalytic sites
509 [24,37,54]. However, in this work samples taken from the supernatant still showed
510 enzymatic activity for the three laccases after 7h of reaction (results not presented).
511 Indeed, it is also possible a competition for the oxidative process between the
512 pharmaceuticals and oxidation products formed. De Cazes [55, 56] has already noticed
513 his effect for the degradation of tetracycline and erythromycin with immobilized
514 enzymes. The authors observed a decrease of the degradation yield with time up to
515 stabilization of antibiotics concentration after 24h of reaction. Then substrates
516 solutions were replaced by fresh ones and the initial degradation activity was reached
517 again. They were able to repeat the same cycles during more than 200 hours.
518 As mentioned at the beginning of this section, CBZ was not transformed by any
519 laccase-mediator system assayed. Poor removal of CBZ was previously reported even
520 in the presence of redox mediators like SYR or ABTS [37,54]. As previously discussed,
521 the low removal amounts can be attributed to the presence of strong EWG in its
522 structure making the pharmaceutical not suitable for biodegradation. Moreover,
523 recent evidence suggests that reaction conditions could play a determinant role in CBZ
524 degradation. Naghdi et al. [57] observed that addition of ABTS increased degradation
525 of CBZ from 30% to 82% in 24 h by a laccase extract from *T. versicolor*, but when
526 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

550 transformed by any laccase-mediator system. From our knowledge this is the most
551 detailed study presenting the enzymatic degradation of amoxicillin in laccase-mediator
552 systems that was better degraded by laccase in presence of ABTS but closely followed
553 by syringaldehyde. Results suggest that amoxicillin oxidation occurs by the ET
554 mechanism of reaction but analysis of the reaction products will be needed to confirm
555 this assumption. The best degradation yields of ciprofloxacin and sulfamethoxazole
556 were reached by using a commercial laccase and syringaldehyde as mediator. These
557 results show that pharmaceuticals containing phenolic groups are removed depending
558 on the redox potential of the mediator and the stability of the radicals formed. Overall,
559 commercial laccases presented better performance for the degradation of
560 micropollutants in laccase-mediator systems. Nonetheless, the data obtained by the
561 comparison of performance among the three different laccase formulations in the
562 model study under the same controlled conditions offer valuable information of the
563 potential use of this biocatalyst for the treatment of complex pollutant matrices where
564 different oxidative characteristics are often needed.
565 This study demonstrates the potential utility of laccase-mediator systems for
566 treatment of complex micropollutant mixtures. Notwithstanding the progress realized,
567 many drawbacks like stabilization or decay of the enzymatic activity are still present.
568 Further studies to improve these enzymatic processes are being carried out, they
569 include the study of their ecotoxicity. Moreover, the study of the immobilization of
570 different laccase-mediator systems in order to reuse the enzymes is also under way.
571

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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.

Table 1

Chemical structure of pharmaceuticals and mediators used in this work

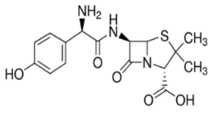
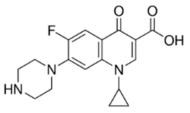
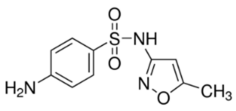
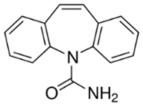
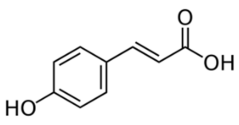
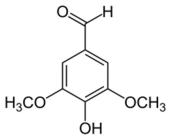
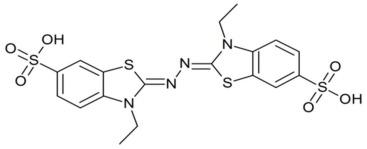
ANTIBIOTICS			ANTIEPILEPTIC
Amoxicillin **(AMX)**	Ciprofloxacin **(CIP)**	Sulfamethoxazole **(SMX)**	Carbamazepine **(CBZ)**
			
MEDIATORS			
p-coumaric acid **(PCA)**	Syringaldehyde **(SYR)**	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (**ABTS**)	
			

Table 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.

Fig. 1

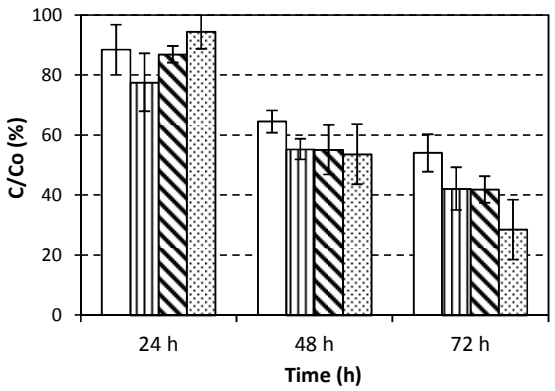


Fig. 2

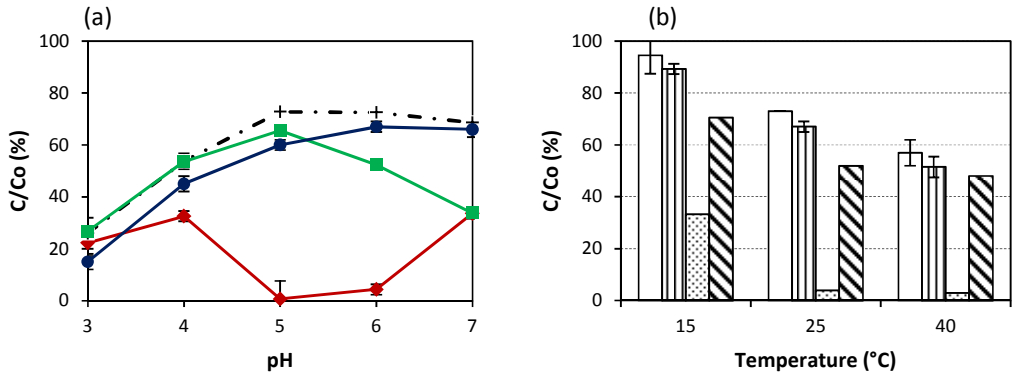

(a)
100
80
60
40
20
0
C/Co (%)
3
4
5
6
7
pH
(b)
100
80
60
40
20
0
C/Co (%)
15
25
40
Temperature (°C)

Fig.3

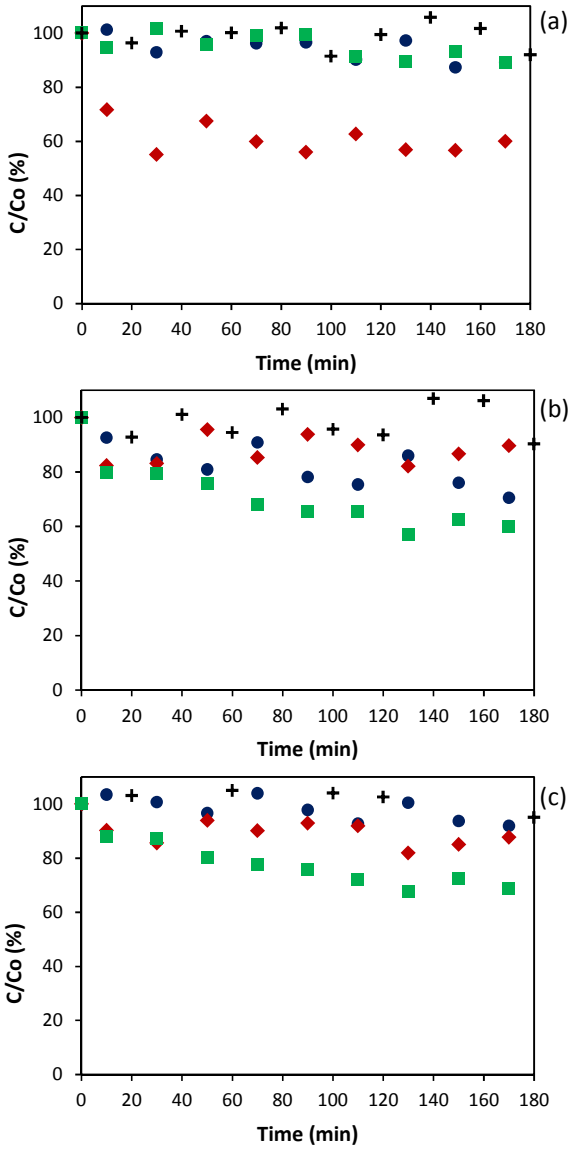


Fig. 4

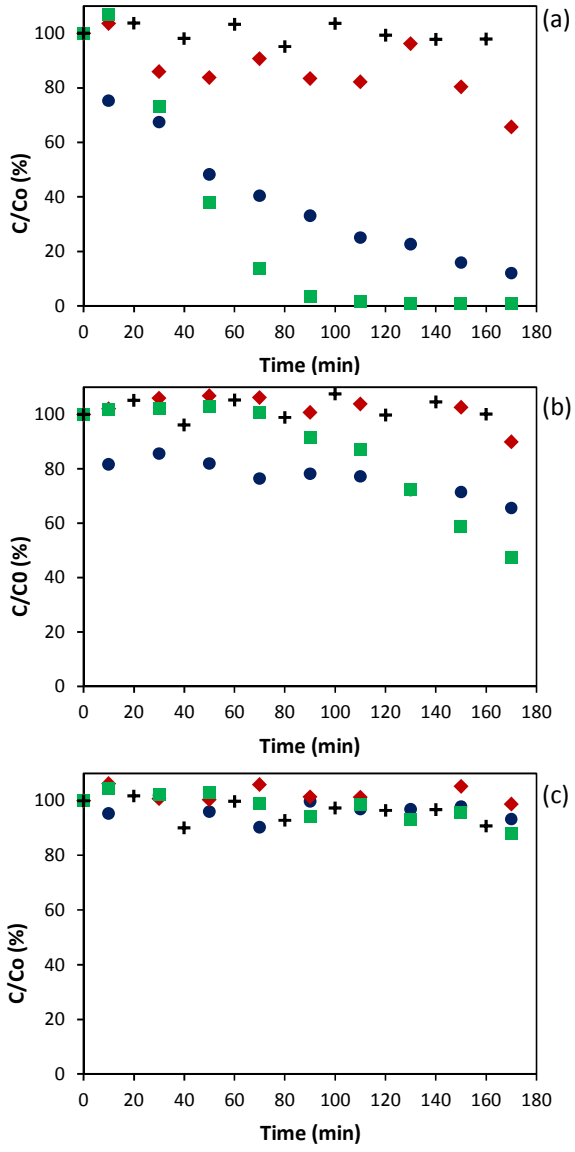
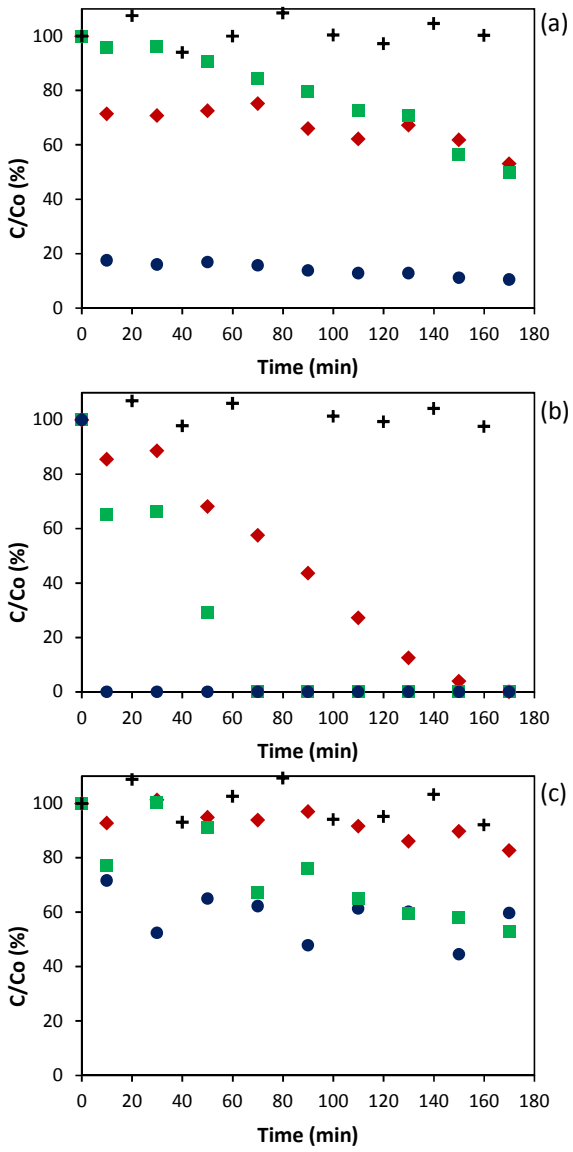
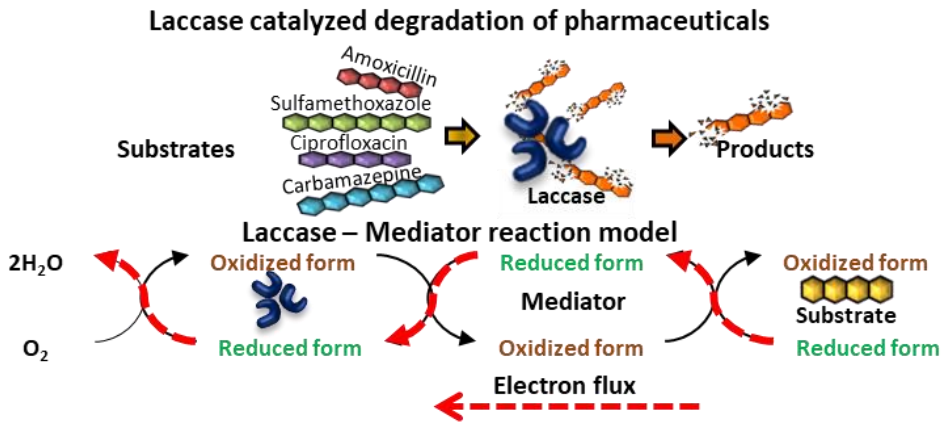


Fig.5




Laccase catalyzed degradation of pharmaceuticals
Substrates
Amoxicillin
Sulfamethoxazole
Ciprofloxacin
Carbamazepine
Laccase
Products
Laccase – Mediator reaction model
2H2O
O2
Oxidized form
Reduced form
Reduced form
Mediator
Oxidized form
Oxidized form
Substrate
Reduced form
Electron flux