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A novel process for the covalent immobilization of laccases on silica gel and its application for the elimination of pharmaceutical micropollutants

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1 **ABSTRACT**

2 In the present work, pharmaceutical micropollutants degradation by laccase immobilized on silica through
3 an innovative process is proposed. The influence of different parameters on the immobilization conditions
4 was evaluated by a 2³ full factorial design, and parameters leading to the highest activity were identified.
5 Under these conditions, laccase activity reached 14 ± 2 U g⁻¹ of silica with a protein immobilization yield of
6 35%. The biocatalyst characterization did not show any change in pH and thermal stabilities but enhanced
7 the long-term storage of laccases. Immobilized *T. versicolor* laccases were then tested to remove four
8 pharmaceutical micropollutants (amoxicillin, ciprofloxacin, carbamazepine and sulfamethoxazole) in the
9 presence of redox mediators (syringaldehyde, p-coumaric acid, and ABTS). High removal yields (50-100%
10 according to the pollutant) were obtained within 4 h of treatment due to the synergistic effect of laccase-
11 mediator biotransformation and adsorption on the support. Overall, the pharmaceuticals' removal
12 efficiency was highly influenced by their physicochemical properties; however, the presence of redox
13 mediators impacted not only the oxidation mechanism but also the interactions between the biocatalyst
14 and micropollutants. Finally, the reusability of the biocatalyst was proved during 7 degradation cycles.

15

16 **Keywords:** Laccase; silica preactivation in gas phase; covalent immobilization; redox mediators;
17 pharmaceutical micropollutants.

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28 1. INTRODUCTION

29 World wide access to pharmaceutical products (PPs) has improved living conditions for the benefit
30 of society. Nonetheless, the broad use of pharmaceuticals also results in their continuous release into the
31 environment as unchanged compounds or metabolites that are inefficiently removed by traditional
32 wastewater treatment plants (WWTPs) (Grandclément et al. 2017). For instance, PPs have been detected in
33 water sources and even in drinking water at concentrations as low as micrograms or nanograms per liter
34 (Ebele et al. 2017). At these low concentrations, pharmaceuticals have the potential to cause short and
35 long-term toxicity, endocrine-disrupting effects in aquatic fauna and human beings. Moreover, the
36 presence of a low quantity of antibiotics in treated wastewaters can also result in antibiotic-resistance in
37 bacteria (Grenni et al. 2018).

38 To address this environmental issue, advanced processes such as extraction, adsorption on
39 activated carbon, ozonation, Fenton oxidation, and electrochemical techniques have been introduced to
40 WWTPs. In order to meet a high quality of the treated water, these tertiary treatments combined with
41 conventional biological treatments allow the oxidation and/or the removal of PPs. However, limitations
42 such as higher cost, formation of hazardous by-products and low efficiency have been reported (Luo et al.
43 2014, Teodosiu et al. 2018).

44 An alternative to chemical and/or physical methods for removing PPs in water is the use of enzyme-
45 catalyzed processes. The bioremediation with biocatalysts such as laccases, lipases or tyrosinases can be
46 considered as environmentally friendly processes because they consume fewer chemicals, water, and
47 energy and produce less waste than other chemical oxidative processes (Naghdi et al. 2018). In particular,
48 laccases receive special attention since they can catalyze the oxidation of phenolic and non-phenolic
49 compounds by only using molecular oxygen that is reduced to water. Hence, since the past two decades,
50 several reports have confirmed the effectiveness of laccases to remove a broad range of pollutants,
51 including pharmaceuticals (Morsi et al. 2020). Nevertheless, the depletion of recalcitrant micropollutants
52 needs the addition of redox mediators. When they react with laccase, these mediators lead to highly
53 reactive and stable radical species, thus reacting with micropollutants (Parra Guardado et al. 2019).

54 Despite all advantages of enzymatic treatment of wastewaters, the use of laccases in environmental
55 applications presents some drawbacks related to their limited stability in solution, difficult recovery, and
56 reusability, resulting in high operational costs. However, these disadvantages can be overcome by
57 immobilization (Sheldon and van Pelt 2013). In a recent review, Sakerian et al. (2020) presented the
58 progress made in the development and implementation of immobilized laccase and horseradish peroxidase
59 for the degradation of hazardous micropollutants in water. Different types of supports have been used,
60 such as porous membranes (De Cazes et al. 2014; Nguyen et al. 2016a) as well as solid particulate supports
61 such as silica, magnetic nanoparticles, chitosan microspheres or Amberlite beads (Fortes et al. 2017; Jiang
62 et al. 2005; Patel et al. 2014; Spinelli et al. 2013). Among the different types of carriers, silica-based
63 materials are highly suitable for enzyme immobilization due to their excellent biocompatibility. Moreover,
64 the hydrophilicity of the surface of silica particles can be modulated to promote enzyme immobilization not
65 only by adsorption but also via covalent bonds (Patel et al. 2014; Spinelli et al. 2013; Zdarta et al. 2018).

66 Immobilization methods and conditions play an important role in the process for which
67 optimization is indispensable to obtain efficient biocatalyst without enzyme leakage through working cycles
68 (Cantone et al. 2013).

69 Covalent immobilization, which creates strong covalent bonds between the enzyme and the
70 activated groups on the support's surface significantly enhances enzyme reusability and reduces enzyme
71 leaching. Among the potential cross-linkers involved in biocatalyst design, glutaraldehyde is probably the
72 most widely used reagent (Shakerian et al. 2020). Cross-linking reactions are generally carried out in the
73 liquid phase, but limitations related to the diffusion phenomenon of glutaraldehyde in the liquid media may
74 lead to incomplete activation of the immobilization support. Moreover, during activation of supports in
75 liquid phase, glutaraldehyde can not only react with reactive groups of the surface but also with other
76 glutaraldehyde molecules, dimerizing or even polymerizing and then grafting groups could not be
77 accessible to aminogroups of enzymes. In addition, when preparing biocatalysts on a large scale, the
78 disposal of solutions containing unreacted glutaraldehyde remains a serious environmental problem.

79 To overcome these drawbacks, a novel process of covalent grafting of laccase onto functionalized silica gel
80 particles has been developed; it involves the activation of the solid support with glutaraldehyde in vapor
81 phase before enzyme grafting. The effects of operating parameters (i.e. concentration and pH of the
82 enzymatic solution, contact time during the immobilization process) were evaluated by full factorial design.
83 The biocatalyst obtained was then characterized in terms of activity, stability at different pH and
84 temperature conditions, and long-term storage and compared to the performance of the free enzyme.
85 Finally, immobilized laccase was tested for the degradation of PPs containing different antibiotics
86 (amoxicillin, ciprofloxacin, sulfamethoxazole) and an anti-epileptic (carbamazepine) without or in the
87 presence of redox-mediators (p-coumaric acid, syringaldehyde, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-
88 sulphonic acid)) and the reusability of the biocatalyst was also evaluated for several degradation cycles.

89

90 **2. MATERIALS AND METHODS**

91 *2.1 Chemicals and enzymes*

92 The pharmaceuticals (amoxicillin (AMX), ciprofloxacin (CIP), carbamazepine (CBZ) and
93 sulfamethoxazole (SMX)), the mediators (p-coumaric acid (PCA), syringaldehyde (SYR), 2,2'-azino-bis-(3-
94 ethylbenzothiazoline-6-sulphonic acid) (ABTS)), the cross-linker agent glutaraldehyde (GLU) (25% (w/v)
95 aqueous solution) and all other chemicals were purchased from Sigma-Aldrich. Stock solutions (100 mg L⁻¹)
96 of AMX and SMX were prepared in citrate-phosphate buffer 0.1 M, pH 7. Given the low water solubility of
97 CIP, the antibiotic was pre-dissolved in hydrochloric acid (0.1 N) and then in citrate-phosphate buffer pH 7.
98 The compounds CBZ, SYR and PCA, were prepared in pure ethanol at a concentration of 1.6 10⁴ mg L⁻¹ for
99 the pharmaceutical and 115 mM for the mediators. Fresh ABTS solution (5 mM) was prepared just before
100 use in citrate-phosphate buffer pH 7.

101 3-Aminopropyl-functionalized silica gel (particle size 40 - 63 µm) from Sigma-Aldrich was used as
102 support for laccase immobilization. Three different laccase preparations were tested. Laccase powder from
103 *Trametes versicolor* (ref 38429, activity ≥ 0.5 U mg⁻¹ and ref 51639, activity ≥ 10 U mg⁻¹) (TVL) was
104 purchased from Sigma-Aldrich. TVL ref 51639 was used for the determination of immobilization conditions

105 and oxidation reactions with mediators; the rest of the experiments where this enzyme was used were
106 done with TVL ref 38429. Commercial laccase produced by submerged fermentation from *Myceliophthora*
107 *thermophila* (59.5 g L⁻¹ of pure laccase) (MTL) was provided by Novozymes (Denmark). Laccases from
108 *Pycnoporus sanguineus* CS43 (PSL) kindly provided by Dr. Roberto Parra (ITESM, Mexico) were obtained
109 from a tomato medium as described by Ramirez-Cavazos et al. (2014).

110

111 *2.2 Laccase activity assay and protein estimation*

112 The laccase activity was determined by measuring the oxidation of 1 mM ABTS at 420 nm ($\epsilon_{420} =$
113 $36000 \text{ M}^{-1} \text{ cm}^{-1}$) in 0.1 M citrate-phosphate buffer (pH 4) at 25 °C (standard conditions). One enzyme
114 activity unit (U) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min. Immobilized
115 laccase activity was assayed by incubating approximately 8 mg of biocatalyst (dry weight) in a 50 mL
116 solution of ABTS (1 mM) prepared in a citrate-phosphate buffer (0.1 M, pH 4) at 25 °C with continuous
117 stirring. The absorbance at 420 nm was monitored every minute for 10 minutes. Immobilized laccase
118 activity was expressed in U g⁻¹ of solid.

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

124

125 *2.3 Laccase immobilization*

126 Laccases were covalently immobilized on the surface of silica gel particles. Firstly, the silica gel
127 particles were activated with GLU in vapor phase. Two different vessels containing respectively 100 mg of
128 3-Aminopropyl-functionalized silica gel (uniformly dispersed) and 15 mL of 25% (w/v) GLU solution were
129 placed into a closed container (5 L) which was kept at 30°C for different periods (18, 30 and 42 h). Then, the
130 GLU-activated powder was placed in 5 mL of an enzymatic solution containing 107, 203 or 300 U of one of

131 the studied laccases (TVL, MTL, and PSL) in 0.1 M citrate-phosphate buffer pH 4.3, 5.66 or 7. The solution
132 was maintained for 1, 1.5 or 2 h at 25 °C under stirring. Finally, the enzyme-activated solid was washed four
133 times with citrate-phosphate buffer (0.1 M, pH 4) and stored at 4 °C. The whole process is summarized in
134 Figure 1.

135 The supernatant from the enzyme grafting process and the solutions produced from the four
136 washing steps were stored for protein determination. The bound protein expressed as a percentage was
137 calculated as the ratio between the amount of protein immobilized and the protein content in the initial
138 enzymatic solution.

139

140 *2.4 Experimental design and statistical analysis*

141 In this study, 2³ full-factorial experimental design was used to evaluate the influence of three
142 parameters (concentration of enzyme solution (X₁), immobilization time (X₂) and pH of enzyme solution
143 (X₃)) in the activity of immobilized laccase. For each factor, three levels were selected: low level (-1), high
144 level (+1) and basic level (zero) (Table 1). A total of 10 experimental runs, including two replicates at the
145 central point, were carried out in duplicate using MTL as a model enzyme. The obtained responses are
146 shown in Table 2. From experimental data, analysis of variance (ANOVA) was done, and regression
147 coefficients were obtained using Statistica™ software. Curvature check for the response was assessed and,
148 if significant, considered. Moreover, the proportion of variance explained by the model obtained was given
149 by the multiple coefficient of determination R².

150 The regression equation based on the first-order model with three parameters (X₁, X₂ and X₃) and
151 their interactions is presented as follows:

152

$$153 Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (1)$$

154

155 Where Y_i represents the predicted response (the activity of immobilized laccase); X₁, X₂, and X₃ are the
156 coded levels of the factors enzyme concentration, immobilization time and pH of immobilization. The

157 regression coefficients are: b_0 the constant regression coefficient; b_1 , b_2 , b_3 and b_{12} , b_{13} , b_{23} , and b_{123} the
158 regression coefficients for the main and interaction effects, respectively.

159

160 *2.5 Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR)*

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

165

166 *2.6 Characterization of free and immobilized laccase*

167 *Optimum pH and pH stability*

168 The pH of maximum laccase activity (free and immobilized forms) was investigated using 1 mM
169 ABTS in 0.1 M citrate-phosphate buffer (pH 3-7); other reaction conditions were the same as those
170 described in section 2.2. The relative activity was calculated as the ratio between the activity at each pH,
171 and the maximum attained.

172 The effect of pH on the enzyme stability was studied by incubating laccase in 0.1 M citrate-
173 phosphate buffer (pH 4 and 7) at 25 °C for 24 h. Samples were taken and transferred to standard conditions
174 for activity measurement (see part 2.2). The residual activity was calculated in reference to the initial
175 activity value obtained at each pH.

176

177 *Optimum temperature and thermostability*

178 The effect of temperature (15-65°C) on laccase activity was determined by measuring the activity at
179 the corresponding temperature; other reaction conditions were the same as those described in part 2.2.
180 The relative activity was calculated as the ratio between the activity at each temperature, and the
181 maximum attained.

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

187

188 *Storage stability*

189 Storage stability of free and immobilized laccase was investigated at 4°C. For this, both laccase
190 formulations were kept in a citrate-phosphate buffer (0.1 M pH 7). The residual activities were obtained
191 after 1 and 2 months by measuring the remaining activity of each enzyme at standard conditions.

192

193 *2.7 Oxidation of pharmaceuticals by immobilized laccase*

194 Degradation experiments were run in 25 mL glass cells at 25°C under continuous stirring to ensure
195 O₂ saturation of the reaction medium and dark conditions to avoid light oxidation. Reaction media (5 mL)
196 contained a mixture of all pollutants in 0.1 M citrate-phosphate buffer pH 7 and one redox mediator (ABTS,
197 PCA, and SYR) at a concentration of 520 µM. The concentration of each micropollutant in the reaction
198 mixture was fixed at 20 mg L⁻¹ (molar concentration: 55 µM AMX, 79 µM SMX and 60 µM CIP) except for
199 CBZ with only 10 mg L⁻¹ (molar concentration: 85 µM). The main objective of this work is the demonstration
200 that this new process of immobilization in vapor phase, result on enough active solids for the degradation
201 of pharmaceuticals, indeed, these relatively high concentrations of micropollutants were chosen to have
202 the necessary precision HPLC-MS of analysis. Reactions started when 2.5 U (164 ± 18 mg) of immobilized
203 TVL was added to the medium. The degradation process was monitored for 4 h by withdrawing samples at
204 regular times. After sampling, aliquots were centrifuged at 10,000 g to separate the reaction medium from
205 the biocatalyst, and the liquid phase was filtered with CHROMAFIL PTFE-20/30 filters to be immediately
206 analyzed with HPLC-MS.

207 In order to evaluate the reusability of the biocatalyst, experiments were carried out as described
208 above using SYR as the redox mediator and 2.5 U of immobilized TVL. The reaction was followed for 2 h
209 (one cycle). At the end of each cycle, the reaction solution was separated from immobilized TVL by
210 centrifugation, filtered and immediately analyzed with HPLC-MS. 10 mL of citrate-phosphate buffer (0.1 M,
211 pH 7) was added to the TVL-activated solid and after mixing the solid was recovered by centrifugation. This
212 washing procedure was repeated three times, and afterwards, the TVL-activated solid was recovered and
213 used for a new cycle of degradation in the same conditions as described above. The biocatalyst was reused
214 for a total of 9 cycles. Enzymatic degradation was calculated considering the initial and final concentration
215 of micropollutants in the reaction solution for each cycle.

216 All experiments were carried out by duplicate and control without immobilized enzyme nor
217 mediator, control with only 3-Aminopropyl-functionalized silica particles and control with inactive
218 immobilized enzyme and mediator were run in parallel to assess the role of the immobilized enzyme on the
219 removal of the pharmaceuticals except for biocatalyst reusability where only control with inactive
220 immobilized enzyme and mediator was run.

221

222 *2.9 HPLC-MS analysis*

223 Determination of pharmaceuticals concentration was carried out on an HPLC Alliance–Waters
224 e2695 separations module coupled to a MS Micromass Quattro micro API (tri-quadrupole) detector and a
225 Raptor-C18 column (150 x 2.1 mm) with a particle size of 5 µm. The conditions for analysis were: 5 µL of
226 sample injection volume, sample elution at 0.25 mL min⁻¹ with a gradient of (A) 95% water - 5% methanol
227 and (B) 100% methanol. The gradient program was set as follows: 0-3 min, 100% (A); 3-8 min, 100% (B) and
228 8-15 min, 100% (A).

229

230 **3. Results and discussion**

231 *3.1 Immobilization of laccase on silica gel particles and determination of immobilization conditions*

232 Commercial amino-functionalized silica gel microparticles were used for the evaluation of covalent
233 immobilization of laccase. This support was selected since the immobilization of the enzymes on its surface
234 is rapid and easy due to its porous structure (60 Å pore size), large specific surface area (550 m² g⁻¹) and
235 content of amino groups (~1.05 mmol of NH₂ g⁻¹). Indeed, these functional groups allow the establishment
236 of covalent bonds between primary amines on the enzyme and the support thanks to the use of a
237 bifunctional agent like glutaraldehyde (GLU) (Cantone et al. 2013). Moreover, the glutaraldehyde activation
238 of the silica support that exhibited a high density of amino groups (i.e. 1.5 NH₂ per nm²) led to a
239 heterofunctional support. The surface properties of such support offer the possibility to immobilize enzyme
240 through covalent bonds with glutaraldehyde and via ionic exchange or hydrophobic interactions (Barbosa
241 et al. 2013). According to Barbosa et al. (2013), as ionic exchange with amino groups occurs more rapidly
242 than covalent bonds, there is no guarantee that all the enzymes that remained on the support were
243 covalently grafted. Generally, glutaraldehyde activation is carried out in a liquid phase when the solid
244 carrier is submerged in a liquid solution of GLU for a determined time. Nonetheless, while working with
245 small size supports (µm to nm), good dispersion in the GLU solution must be guaranteed to avoid potential
246 agglomeration of the particles and uneven deposition of GLU on the support, which could lead to low
247 immobilization yields. In the present study, it was chosen to perform the covalent immobilization of
248 laccases using GLU in vapor phase (see section 2.3). The silica particles and 25% GLU solution were kept in
249 the same closed vessel at 30°C for different periods. The relatively high volatility of GLU at 30°C ensures its
250 evaporation leading to the deposition of the activating agent on the support as confirmed by the change in
251 color of silica particles (they turn orange). In addition, thanks to the dry environment, the agglomeration of
252 the activated particles has been avoided. GLU vapor cross-linking technique is often used to fabricate
253 biosensors and modify nanofibrous materials (i.e. water insolubility property) (Anh et al. 2002; Destaye et
254 al. 2013). From our knowledge, this is the first investigation where the vapor phase deposition of GLU is
255 used for the immobilization of enzymes onto silica gel particles for bioremediation purposes.

256 Based on preliminary experiments carried out with MTL, three different contact times (18, 30 and
257 42 h) were evaluated. Firstly, it is important to note that, irrespective of the contact time, the silica

258 particles turned from white to dark orange, confirming the formation of aldimines (Schiff linkage (=CHN=)
259 between the free amino groups of silica particle and glutaraldehyde and thus the covalent grafting of
260 laccases (George et al. 1984). Nevertheless, results of activity measurement showed that short activation
261 periods (18 h) yielded a higher specific activity of the immobilized enzyme (Table S1, Supporting
262 Information (SI)). Therefore, GLU activation during 18 h was chosen to carry out all further experiments.

263 To determine the immobilization conditions for the novel process, a design of experiments (DOE)
264 approach was applied to evaluate the parameters influencing and improving MTL immobilization. A 2³ full
265 factorial design was performed where the range of variation of each level was chosen based on theoretical
266 considerations and preliminary experiments. The factors evaluated by the experimental design were the
267 concentration of enzyme solution, time of immobilization and pH of enzyme solution, while the activity
268 expressed by immobilized laccase (U g⁻¹ solid) was considered as the response variable. It is worth noting
269 that GLU time of contact was not considered in the present design since its influence on the response was
270 not significant (data not shown). Thus, a total of 10 experiments fully replicated were performed, and
271 experimental responses are presented in Table 2.

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

274

$$275 Y_i = 3.45 - 0.49X_1 + 0.44X_2 + 0.80X_3 - 0.33X_1X_2 - 0.50X_1X_3 - 0.64X_1X_2X_3 \quad (2)$$

276

277 Table 3 shows the analysis of variance of parameters (ANOVA) and their interactions with statistical
278 significance. The model has a relatively good fit as expressed by the correlation coefficient ($R^2 = 0.908$),
279 meaning that the regression model explains 90% of the total variation of the response, while the residues
280 explain the other 10%. However, the model presented significant curvature ($p = 0.003$) suggesting that the
281 data would be better fitted by another type of design, such as Response Surface Methodology (RSM).
282 Nonetheless, the main objective of this work was to study the influence of the chosen parameters on the
283 response, and for this, the first-order model regression was sufficient, so the model was accepted.

284 The significance of the factors was verified by the p -value statistical parameter. Factors showing a p -
285 value below 0.05, with a confidence level higher than 95% were considered statistically significant for the
286 immobilization process. In this sense, it is important to mention that the final model represented by Eq. 2
287 was run excluding insignificant effects (factors and interactions), as presented in Table 3. The influence of
288 each factor and how they affected the response is expressed in the model's equation and is given by the
289 coefficient of each factor and interaction. The greater the absolute value of the coefficient, the higher its
290 influence on the response. Thus, from Eq. 2 the factors were ranked regarding their significance:

291

292 $X_3 (+) > X_1X_2X_3 (-) > X_1X_3 (-) > X_1 (-) > X_2 (+) > X_1X_2 (-)$

293

294 The most significant factor affecting the activity of immobilized MTL laccase was the pH of the
295 enzyme solution used for immobilization. The model shows that the response was positively affected when
296 the pH increased from 4.33 to 7. The optimal pH of immobilization should be that one promoting the
297 nucleophilic attack of enzyme reactive groups to the GLU activated support (Pezzella et al. 2014).
298 According to Spinelli et al. (2013), neutral or basic conditions promote GLU crosslinking kinetics whereas
299 acidic ones result favorably in the formation of covalent bonds. However, given the low stability of GLU
300 groups at acidic or alkaline pH values, immobilization involving GLU is usually favored at neutral conditions
301 (Barbosa et al. 2013). At these pH values, covalent bonds are established with the terminal amino groups in
302 the protein; but with highly activated glutaraldehyde supports, covalent enzyme-support bonds can be
303 created with other nucleophile groups of the protein leading to a multipoint covalent attachment (Spinelli
304 et al. 2013; Barbosa et al. 2013).

305 In addition, it is well-known that pH also affects the stability of enzymes. In the case of MTL, the
306 enzyme keeps 100% of the initial activity for 24 hours at room temperature under neutral pH (Lloret et al.
307 2010). Moreover, Jiang et al. (2005) also reported pH 7 as the optimal value for the immobilization of a PSL
308 laccase on magnetic chitosan microspheres. These authors reported that this laccase presented low
309 activity and protein loading at more acidic conditions and suggested that the low stability and denaturation
310 of the enzyme as well as the degradation of the support, were at the origin of this behavior.

311 The second and third more significant variables in the model were the interactions among the three
312 studied factors and the interaction of laccase concentration and pH of immobilization, both interactions

313 negatively affecting the response. For example, it was observed that when enzyme concentration and pH
314 were at a low level (107 U and pH 4.33 respectively), the activity of immobilized MTL laccase decreased.
315 The same would be expected if both parameters are present at their high level (330 U and pH 7,
316 respectively, see Table 2). The high and positive coefficient value probably causes such effect for pH. Thus,
317 in order to obtain a more active biocatalyst, enzyme concentration should be set at 107 U and pH at 7.

318 The concentration of enzyme was another parameter that influenced the response negatively. Low
319 amounts of enzymes led to a higher biocatalyst activity with values up to 5.9 and 7.2 U g⁻¹. This behavior
320 was also observed by Fortes et al. (2017) for the immobilization of laccase (Novozym® 51003) on magnetic
321 nanoparticles, and it was attributed to the saturation of the support by the enzyme. An excess of the
322 enzyme on the support may lead to a lack of intermolecular space resulting in mass transfer limitations that
323 affect the dispersion of substrate and products.

324 On the other hand, the immobilization time positively influenced laccase immobilization; the active
325 beads obtained after 2 hours of grafting showed almost twice as much activity (assays 2 and 4 in Table 2).
326 Immobilization time is also a crucial variable for the whole process. Whereas too short contact time can
327 result in a limited amount of laccase molecules reacting with the solid, too long immobilization periods lead
328 to oversaturation of the support, causing the before mentioned mass transfer limitations that result in
329 lower activity (Zheng et al. 2016). In this work, longer immobilization time allowed most of the aldehyde
330 groups to react with the amino-groups in laccase, resulting in a biocatalyst with considerable higher
331 activity.

332 Finally, the last significant parameter was the interaction between laccase concentration and
333 immobilization time with a negative effect. According to the model, a less active biocatalyst would be
334 expected when immobilization time and laccase concentration are either at their low (1 h and 107 U) or
335 high levels (2 h and 300 U).

336 From the obtained model we can conclude that the best conditions that maximize immobilized
337 laccase activity are GLU activation time of 18 h, 107 U of the enzyme, 2 h of grafting and pH 7. Therefore,
338 these conditions were selected as the immobilization parameters for all further experiments.

339 The new immobilization process successfully developed for the immobilization of MTL was then
340 implemented with TVL and PSL. Immobilization protocol was carried out according to the optimal
341 conditions previously established. Nevertheless, Barrios-Estrada et al (2018) have reported that pH 5 was
342 the optimal for TVL and PSL immobilization on ceramic membranes where a cross-linked gelatin was
343 previously deposited. Indeed, some trials of immobilization at pH 5 were carried out with TVL and PSL; but
344 the results shown a similar activity when compared with immobilization at pH 7. Therefore, Table 4 only
345 reports the immobilization yield and the activity of the three immobilized laccases prepared at pH 7.
346 Among the three enzymes, TVL yielded the highest specific activity with 13.6 U g⁻¹ biocatalyst and retained
347 the highest amount of protein (35%). Similar specific activity values (15.8 U g⁻¹ and 17 U g⁻¹ respectively)
348 were obtained for the covalent immobilization of laccase from *C. unicolor* C-139 on silica beads at pH 5
349 (Songulashvili et al. 2012) and *M. thermophila* laccase on Eupergit C carrier at pH 7 (Lloret et al. 2012).

350 In the case of MTL, a large quantity of protein is bound to the support, as suggested by the value of
351 immobilization capacity (38 mg g⁻¹). However, the resulting active beads showed relatively low activity. This
352 is probably due to the age of the commercial preparation of MTL (provided by Novozymes 5 years ago).
353 Since the residual activity was less than 2/3 of the initial activity, a high commercial preparation volume
354 was required to prepare the enzyme solution. Thus, the resulting solution contained large amounts of
355 inactive proteins that competed with the active enzymes to bind to the carrier, leading to a biocatalyst with
356 low activity.

357 Among the different type of active solids, active beads prepared with PSL reported the lowest
358 activity with only 0.76 U g⁻¹. These low activities may have resulted from the presence of impurities in the
359 enzymatic extract. Indeed, Ji et al. (2017) observed a poor performance when they immobilized a crude
360 laccase extract from *P. ostreatus* on titania particles. These authors suggested that the functionalized
361 carrier could have been buried by the non-enzyme components present in the extract, which led to lower
362 enzyme loading and activity. According to their investigation, dilution of the crude extract before
363 immobilization significantly improved laccase loading and apparent activity of the biocatalyst by reducing
364 the competitive effect between the enzyme and the other components to attach to the support. In this

365 study, PSL and MTL extracts were diluted 2 and 9 times, respectively, prior to immobilization; it was
366 probably not enough to make the solid surface more accessible to enzymes which is more evident in the
367 case of PSL.

368 Overall, beads prepared with TVL showed the best results in terms of immobilization yield and
369 activity. Therefore, TVL was chosen to carry out the next stages of the study.

370

371

372 *3.2 Characterization of free and immobilized TVL*

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

381

382 *Fourier Transform Infrared spectroscopy (ATR-FTIR) characterization*

383 The immobilization of TVL on silica gel microparticles was assessed by ATR-FTIR. Figure 2 presents
384 the FTIR spectra of free TVL, untreated 3-Aminopropyl-functionalized silica gel and the silica gel particles
385 after laccase immobilization. Characteristic bands of free laccase were observed at 3300 cm^{-1} (OH and NH
386 vibrations), 2900 cm^{-1} (CH stretch), 1630 cm^{-1} (attributed to a secondary amide structure-CONH), 1350 cm^{-1}
387 (CN stretching of amines) and 1100 cm^{-1} (COC groups) in agreement with the literature (Tavares et al.
388 2015). Pristine silica gel particles presented a strong band centered at 1050 cm^{-1} attributed to the vibration
389 of Si-O-Si and two weak peaks at 1616 cm^{-1} and 1523 cm^{-1} corresponding to Si-OH and primary amine NH
390 groups. After laccase immobilization, the FTIR spectrum still showed the characteristic bands of silica gel

391 particles. However, the specific signals from the enzyme at 1400 cm^{-1} and 1600 cm^{-1} on the active silica
392 spectrum confirm the immobilization of the enzyme on the support. Nonetheless, the decrease in vibration
393 bands attributed to the enzyme suggests that laccase was immobilized in low quantity as confirmed by the
394 immobilization capacity value ($13.5\text{ mg protein g}^{-1}\text{ silica}$) calculated for TVL (Table 4).

395

396

397

398 *Optimum pH and temperature*

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

410 The effect of temperature on TVL activity of the free and immobilized enzyme was investigated in
411 the range $15\text{-}65^\circ\text{C}$ under standard conditions (0.1 M citrate-phosphate buffer pH 4). The optimum
412 temperature for free and immobilized laccase was 55°C (Figure 3b). The Immobilized enzyme showed a
413 broader profile displaying relative activities up to 16% higher than free enzyme for all temperatures tested
414 and retained more than 80% of the activity in the range from $35\text{ to }65^\circ\text{C}$; except at 15°C where free enzyme
415 showed higher relative activity than the immobilized form (58 and 48% respectively).

416

417 *pH stability and thermostability*

418 Industrial applications often require enzymes showing high stability at extreme operational
419 conditions (e.g. pH, temperature, presence of organic solvents). Therefore, pH and thermal stability of
420 immobilized TVL were evaluated at pH 4 and 7 and 25°C for 24 h and compared to that of the free enzyme.
421 Results reported in Figure 4a confirms that laccases are very stable at pH 7; deactivation profiles obtained
422 at pH 7 were nearly similar for both biocatalyst forms (0% and less than 10% of deactivation in 24 h for
423 immobilized and free enzyme respectively). Moreover, it can be noted that immobilization enhances
424 laccase stability in the case of short storage duration (i.e. up to 6 h) at pH 4. After 6 h of incubation residual
425 activity of the free enzyme was equal to 64%, while immobilized enzymes retained 87% of the initial
426 activity. Similar enhancement on stability at acidic pH was observed when laccase was immobilized on
427 granular activated carbon (Nguyen et al. 2016b) and fumed silica microparticles (Gamallo et al. 2018). The
428 authors stated that improved stability could be related to the rigidification of the enzyme after
429 immobilization, which makes it less susceptible to denaturation.

430 Nevertheless, at the end of 24 h, the residual activity of free and immobilized laccase was practically
431 the same (56 and 61% respectively). However, the decrease in activity may be due to the release of non-
432 covalently immobilized enzymes. Indeed, as suggested by Barbosa et al. (2013), the activated silicate
433 particles can be considered as hetero-functional support where laccases can also be immobilized through
434 non-covalent interactions (i.e. ionic or hydrophobic interactions) which can be affected by acidic conditions.
435 Thermostability was evaluated when both free and immobilized (Figure 4b) laccases were incubated in
436 citrate-phosphate buffer (pH 7) at selected temperatures in the range from 25 to 55 °C. It was observed
437 that the activity of immobilized laccase at 45°C and 55°C dropped more rapidly than that of the free
438 enzyme within the first 4 h (Figure 4b). However, for longer incubation times at 55°C, the residual activity of
439 immobilized enzymes remained constant (around 25%), while those of free enzymes continued decreasing
440 to reach 6% after 24 h of incubation. Incubation at 45°C resulted in a similar loss of activity for both free
441 and immobilized laccases with residual activity values of 45% and 52%, respectively, after 24 h. At 25°C, low
442 thermal inactivation was observed with less than 10% loss on activity for both free and immobilized
443 laccases.

444 Overall, results showed that the immobilization of TVL on silica particles did not improve the
445 enzyme's thermostability. A similar observation was reported by Fortes et al. (2017) for the thermal
446 stability of *Aspergillus oryzae* laccase immobilized on magnetic nanoparticles at 60°C. These authors
447 observed a higher kinetic constant of enzyme deactivation for the immobilized enzyme than for the free
448 form. Even if immobilization can be considered as a powerful tool to improve enzyme stability, it is evident
449 that the preparation protocol played an important role in the poor stability increase observed in this study.
450 For instance, Sanchez et al. (2016) demonstrated that two different immobilized preparations of bovine
451 trypsin treated at similar inactivation conditions suffered very different conformational changes and,
452 therefore, different residual activity. In fact, catalytic capacity is closely related to the 3D structure of the
453 enzyme which can be drastically impacted by high temperatures (Tavares et al. 2015). The fast thermal
454 inactivation of immobilized TVL compared to the free TVL could be attributed to faster or more drastic
455 changes in the enzyme structure, but it can also be due to enzyme desorption (Tavares et al. 2015; Galvão
456 et al. 2018). A new set of experiments would be necessary to confirm the phenomenon behind the low
457 thermostability observed in this investigation.

458

459 *Storage stability*

460 To assess storage stability of both free and immobilized enzymes, a solution of free enzymes (152 U
461 mL⁻¹) and active beads (100 mg mL⁻¹) were kept at 4°C in citrate-phosphate buffer (0.1 M, pH 7), and
462 residual activity was measured after one and two months. Results showed that immobilized laccase
463 retained 72% and 47% of its initial activity after one and two months of storage, respectively. In contrast,
464 free laccase's residual activity, which was around 44% at the end of the first month, could no longer be
465 measured after two months of storage. These results confirm that covalent attachments improve enzyme
466 stability by reducing the occurrence of changes in the active conformation of the immobilized enzymes.

467

468 *3.3 Oxidation of pharmaceuticals by immobilized laccase*

469 At first, reactions were carried out in a batch reactor by adding only the immobilized biocatalyst to
470 the micropollutant solution. After 48h, no significant bio-oxidation was observed for any pharmaceutical
471 confirming the recalcitrance of these micropollutants to laccase degradation, as already reported with free
472 enzymes by Parra Guardado et al. (2019). These authors attributed the low removal to the complex
473 chemical structure of the compounds.

474 In view of improving the removal of the selected pharmaceuticals, redox mediators were added to
475 the reaction media. Redox mediators are compounds that laccase can easily oxidize resulting in the
476 production of radical species with higher oxidative capacity than the laccase itself. In this work, the
477 compounds SYR, PCA, and ABTS were chosen since they have already demonstrated to enhance the
478 oxidation of AMX, SMX, and CIP in free enzyme systems (Parra Guardado et al. 2019).

479 Time course degradation for the pharmaceuticals studied by immobilized laccase-mediator systems
480 and the effect of the adsorption-based removal by the inactivated laccase immobilized on the same
481 support is shown in Figure 5. First of all, we can notice that for AMX (Figure 5a) and SMX (Figure 5b), the
482 decrease in the concentration observed by the enzymatic reaction is higher than the adsorption
483 phenomenon. Regarding CIP, the decrease observed is similar except when ABTS is used as a mediator; in
484 this case, the adsorption observed is relatively lower. Finally, for CBZ, the decrease of the concentration is
485 similar for adsorption or bio-reaction experiments.

486 If only adsorption results are considered (open symbols), we can observe an increase in the
487 adsorption phenomenon during the first 30 minutes, then the concentration stabilizes. Moreover,
488 adsorption depends on the mediator used; 40% of AMX, SMX, and CIP are adsorbed in the presence of PCA
489 and SYR, whereas when ABTS is used as a mediator, the adsorption phenomenon decreases (< 20% for AMX
490 and CIP, 30% for SMX). CBZ is the highest adsorbed molecule, around 50%, regardless of the mediator used.
491 It is important to notice that the adsorption of the antibiotics by the pristine silica support was less than
492 10% for AMX, SMX and CIP and only 18% of CBZ after 4 h (Fig. S1, SI).

493 The removal of organic pollutants via adsorption by the support has been extensively described. For
494 example, Cabana et al. (2009) reported adsorption values from 40 to 60% for nonylphenol, bisphenol A and

495 triclosan by inactivated laccase covalently immobilized on Celite R-633 supports. CIP hydrochloride
496 adsorption reached 77% when mesoporous carbon nanospheres were used (Shao et al. 2019). Inactive
497 immobilized laccase on Magnetic Cu^{2+} -chelated silica particles removed 38% of pentachlorophenol by
498 adsorption (Wang et al. 2012). As discussed above, the highest adsorption observed was for CBZ, which has
499 the highest octanol/water partition coefficient ($\text{Log } K_{ow}=2.45$) and the lowest water solubility (78 mg L^{-1})
500 (Celiz et al. 2009). Regarding the antibiotics, lower adsorption for AMX and SMX would be expected as
501 judged from their corresponding partition coefficients and water solubility values: 0.87 and 3400 mg L^{-1} for
502 AMX and 0.89 and 610 mg L^{-1} for SMX (Wishart et al. 2006). In the case of CIP, a $\text{Log } K_{ow}$ value of 0.28 but an
503 extremely low solubility (less than 1 mg L^{-1}) in water explains its high adsorption (Wishart et al. (2006).

504 As already stated, in the presence of ABTS, the adsorption of CIP and AMX was remarkably different
505 compared to SYR or PCA control reactions. If attention is paid to Figure 5, ABTS seemed to reduce the
506 proportion of micropollutants adsorbed on the support in comparison to the other mediators. These results
507 might be explained by assuming interactions or adsorption of ABTS on inactivated enzymes leading to a
508 competition for adsorption sites between antibiotics and mediators.

509 Although part of the observed elimination was caused by adsorption on the solid carrier, the action
510 of the immobilized enzyme-mediator system in removing the target pharmaceuticals was evident for SMX
511 and AMX. In the presence of SYR, complete transformation of the antibiotic SMX was achieved within 4 h of
512 treatment (Figure 5b). The use of ABTS and PCA also allowed removing 53% and 54% of SMX, respectively,
513 in 30 minutes. Regarding AMX, the three mediators led to similar results with transformation yields in the
514 range of 78-89%, as observed in Figure 5a. According to Figure 5c, removal of CIP occurred within the first
515 30 minutes of treatment, and no effect of SYR and PCA mediators in the antibiotic's removal was observed;
516 the decrease of CIP concentration was attributed to adsorption on the support. However, in ABTS presence,
517 CIP removal was due to enzymatic degradation as shown by the control. CBZ removal was entirely by
518 adsorption, as confirmed by control reactions with inactivated biocatalyst.

519 Overall, results indicate that removal of these micropollutants by immobilized laccase-mediator
520 systems is both compound and mediator dependent. A marked difference in the removal of SMX, AMX and

521 at some point of CIP and CBZ was observed. The main reason could be the difference in laccase-mediator
522 oxidative capacity and the tendency of the pharmaceutical molecules to be adsorbed. Based on the global
523 removal efficiency of the system, micropollutants clearly differentiate into two groups: the biodegradable
524 pharmaceuticals and the recalcitrant ones. The latter group is formed by CBZ and CIP, compounds
525 presenting high hydrophobicity and complex chemical structure that are very resistant to laccase oxidation
526 even in the presence of redox mediators (Parra Guardado et al. 2019; Hata et al. 2010). Belonging to the
527 biodegradable group, AMX and SMX are antibiotics that possess functional groups prone to be attacked by
528 laccase (OH and NH₂, respectively). The highest oxidation rates of AMX and SMX were achieved when SYR
529 or PCA was present in the reaction medium. Interestingly both mediators follow the hydrogen atom
530 transfer mechanism of reaction in which after oxidation, phenoxy radicals are produced to act as an
531 electron shuttle between the laccase and substrate (Cañas and Camarero 2010). Indeed, phenoxy radicals
532 formed were highly reactive and stable to carry out the simultaneous transformation of AMX and SMX.

533 Nonetheless, it is important to note that the high removal obtained for AMX, for SMX especially and
534 to some extent for CIP, could not be totally attributed to laccase-catalyzed oxidation but a combined
535 contribution of adsorption and degradation. As shown in Figure 5, the adsorption of pharmaceuticals
536 reached equilibrium 30 minutes after the start of the reaction, then laccase oxidation effects ongoing to be
537 more evident. In other words, adsorption of micropollutant on immobilized enzyme could have helped and,
538 to some extent, enhanced laccase degradation. Nguyen et al. (2016b) observed similar behavior for the
539 removal of the recalcitrant CBZ and SMX with immobilized laccase on granular activated carbon in a packed
540 bed reactor. These authors suggested that enhancement in laccase degradation for both compounds could
541 possibly be due to the improved electron transfer between laccase and substrate after adsorption on the
542 support surface. Such an effect is especially evident for SMX when SYR was the mediator leading to the
543 complete transformation of the antibiotic in less than 4 h.

544 The high affinity of the free laccase-SYR system for AMX and SMX was also observed in a previous
545 work (Parra Guardado et al. 2019). Free TVL (1.2 U mL⁻¹) in the presence of SYR reached 50% removal for
546 AMX in 3 h and complete elimination for SMX in 1.5 h. In the present investigation, considerably lower

547 amounts of immobilized enzyme (0.5 U mL^{-1}) achieved comparable or even higher removal yields for both
548 micropollutants within the same reaction time. These results suggest that the novel immobilization process
549 proposed in this work positively influenced the stability of TVL, which, coupled with the adsorption capacity
550 of the biocatalyst, enhanced the removal of pharmaceutical pollutants.

551

552 *3.4 Reusability of immobilized laccase*

553 The biocatalyst was reused in a total of 9 cycles (2 h each cycle) for the removal of selected
554 micropollutants using SYR as a redox mediator. At the end of each degradation cycle, the biocatalyst was
555 washed with buffer solution (see section 2.7) and added to a fresh solution containing the mixture of
556 pharmaceuticals and mediator.

557 As shown in Figure 6, the percentage of removal is micropollutant dependant. Even if the
558 dispersion of results seems quite large (for instance, see the bars of errors), some trends can be identified
559 regarding the contribution of the enzymatic reaction to the depletion. Indeed, if the enzymatic degradation
560 contribution is estimated as the difference between the total removal (reaction+adsorption, blue bars) and
561 the adsorption (white bars), for AMX, the enzymatic biodegradation is more important during the first 7
562 cycles (between 12 to 28%), and then it decreases for the last two cycles (8 and 3% respectively). In
563 contrast, for SMX the evolution of this difference is much clear, decreasing continuously for the first 8
564 cycles (from 32% to 8%), while only the adsorption phenomenon was observed for the last cycle. As
565 expected, CIP and CBZ removal were entirely by adsorption with an average elimination of 30% and 50% for
566 each compound (Fig. S2, S1). Nevertheless, explanations are not simple because we have to consider that
567 these experiments were done in mixtures. There is a competition for adsorption sites in such complex
568 systems, and the reactivity of the immobilized laccase towards each one of the molecules studied is also
569 different.

570 The loss of efficiency observed for the degradation of micropollutants by immobilized enzymes has
571 been extensively reported in different studies, and many explanations could be given: from loss of
572 enzymatic activity to inhibition of the active center of laccase by the accumulation of high molar mass

573 products formed during the reaction leading to mass transfer resistance (De Cazes et al. 2014; Ji et al.
574 2016). Indeed, the possible inhibition of immobilized laccase could be explained by the loss in its capability
575 to transform both antibiotics simultaneously. Whereas SMX was highly removed only in the first two cycles,
576 AMX was continuously transformed for 7 cycles. There is the possibility that interactions between the
577 active biocatalyst and the substrates/by-products formed during the first cycles caused conformational
578 changes in the enzyme that narrowed its capacity to oxidize both compounds. Moreover, the preference
579 for AMX transformation over SMX during the following cycles could be linked to its chemical structure and
580 the presence of functional groups that make AMX more prone to laccase oxidation in comparison to SMX
581 (Yang et al. 2013).

582

583 **4. CONCLUSIONS**

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

593 The potentiality of using the biocatalyst for the removal of pharmaceuticals was evaluated in
594 osmosed water containing a mixture of four representative pharmaceutical micropollutants. High removal
595 yields (40-100%) were achieved when redox mediators were present in the reaction medium. It was
596 observed that redox mediators generating phenoxy radicals were the most effective for the
597 biotransformation of the pharmaceuticals, with SYR showing the best performance. Compared to free
598 laccase, immobilized enzyme removed the micropollutants more effectively due to the combined action of

599 adsorption on the support and laccase catalyzed oxidation. Furthermore, the biocatalyst reusability was
600 proved for the continuous removal of the pharmaceuticals during seven cycles. The results presented in
601 this study show that the novel immobilization process proposed, and the resulting biocatalyst is a promising
602 tool to increase the application of enzymes in bioremediation processes. However, as the use of redox
603 mediators may increase the toxicity of the effluent as well as the cost of the water treatment, further works
604 are needed to find solutions allowing retention and reuse of the mediators.

605

606

607 **Declarations**

608

609 **Ethics approval and consent to participate**

610 Not applicable

611

612 **Consent for publication**

613 Not applicable

614

615 **Availability of data and materials**

616 The datasets used and/or analyzed during the current study are available from the corresponding
617 author on reasonable request, additional information and results are given in supplementary
618 material.

619

620 **Competing interests**

621 The authors declare that they have no competing interests

622

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627

628 **Authors' contributions**

629 ALPG carried out the experimental work of enzymatic degradation of antibiotics with laccases and
630 mediators, SDB worked on the design and analysis of the experimental modelling, MPB designed
631 degradation experiments and contributes to the scientific discussion, JSM supervised all of the
632 work, contributed with the discussions and wrote the whole manuscript. All of the authors revised
633 the manuscript.

634

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Table 1. Factor levels used according to the 2³ factorial design.

Variable	Level		
	-1	0	1
X ₁ : Concentration of enzyme solution (U)	107	203	300
X ₂ : Immobilization time (h)	1	1.5	2
X ₃ : pH of enzyme solution	4.33	5.66	7

Table 2. Values for the response (activity of the biocatalyst) according to the 2³ factorial design

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

X1=concentration enzyme solution; X2=immobilization time; X3=pH enzyme solution.

Table 3. Analysis of variance (ANOVA) for the first-order model for the activity of immobilized laccase.

Factor	SS	df	MS	F	p
Curvature	3.76	1	3.76	13.59	0.0031
X ₁	3.86	1	3.86	13.97	0.0028
X ₂	3.08	1	3.08	11.12	0.0059
X ₃	10.17	1	10.17	36.79	0.0001
X ₁ :X ₂	1.69	1	1.69	6.13	0.0292
X ₁ :X ₃	3.97	1	3.97	14.36	0.0026
X ₁ :X ₂ :X ₃	6.61	1	6.61	23.89	0.0004
Error	3.32	12	0.28		
Total SS	36.46	19			

SS=sum of squares; df=degrees of freedom; MS=mean square; F=F distribution value; X₁=concentration enzyme solution; X₂=immobilization time; X₃=pH enzyme solution.

R²=0.90899; Adj-R²=0.8559

Table 4. Immobilization yield and activity in the immobilization of laccases on 3-Aminopropyl-functionalized silica microparticles at pH 7.

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

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

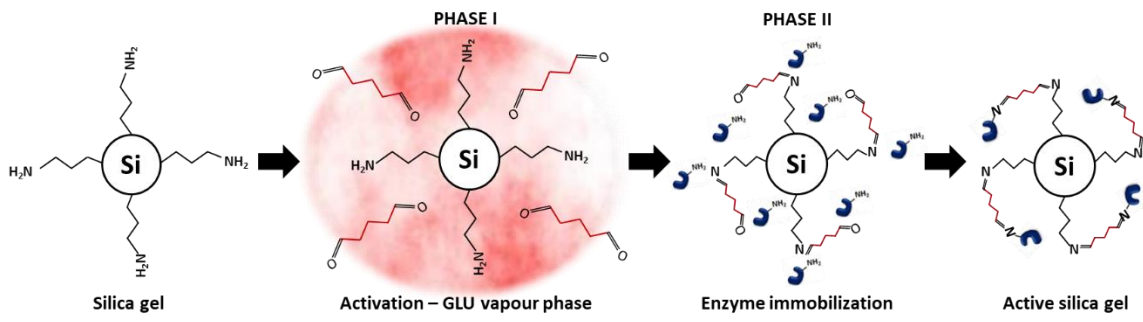


Fig. 1. Covalent immobilization of laccase on silica gel via activation with glutaraldehyde (GLU) in vapor phase.

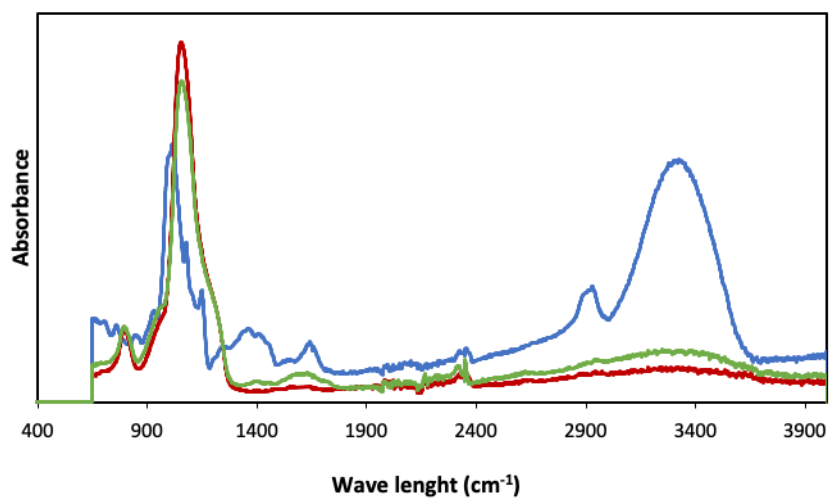


Fig. 2. FITR-ATR spectra of laccase (blue line), untreated 3-aminopropyl functionalized silica gel (red line) and TVL immobilized on silica gel particles (green line).

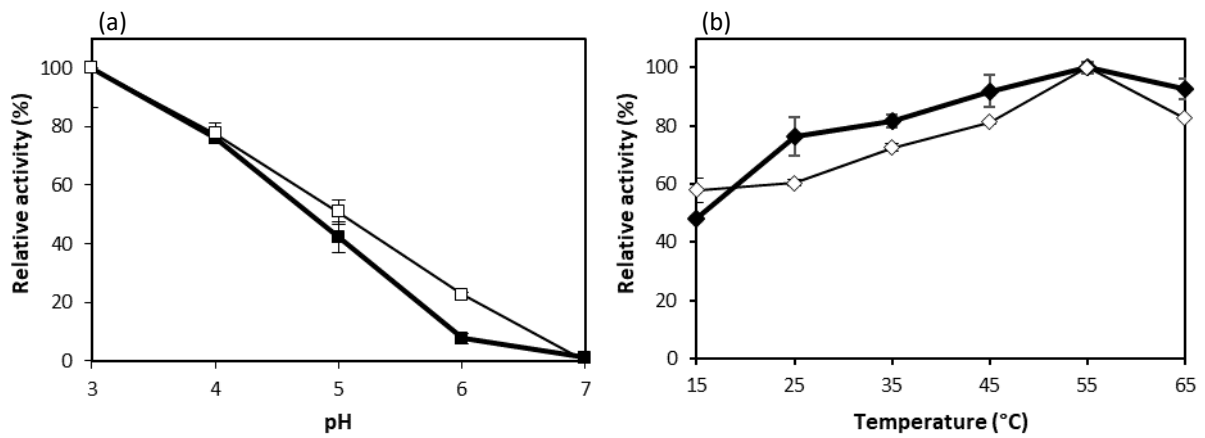


Fig. 3. Effect of (a) pH and (b) temperature on free and immobilized TVL. pH (3-7) and temperature (15-65°C) were tested with 1 mM ABTS in 0.1 M citrate-phosphate buffer. Free and immobilized enzymes are represented by open and closed symbols respectively.

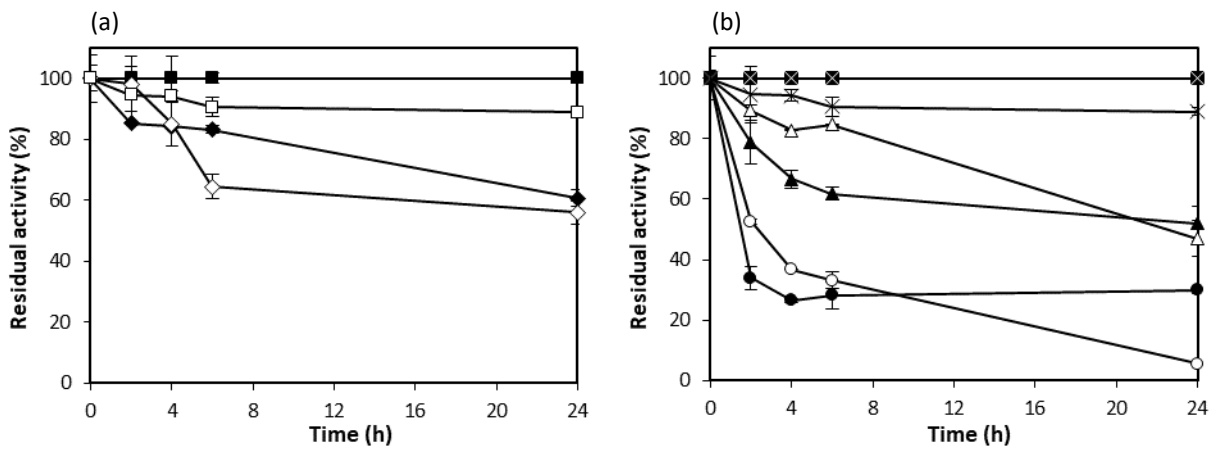


Fig. 4. (a) pH and (b) thermal stability of free and immobilized TVL on silica particles. pH values: 4 (◆) and 7 (□). Temperature values: 25°C (×), 45°C (▲) and 55°C (●). The open and closed symbols represent free enzyme and immobilized enzyme respectively.

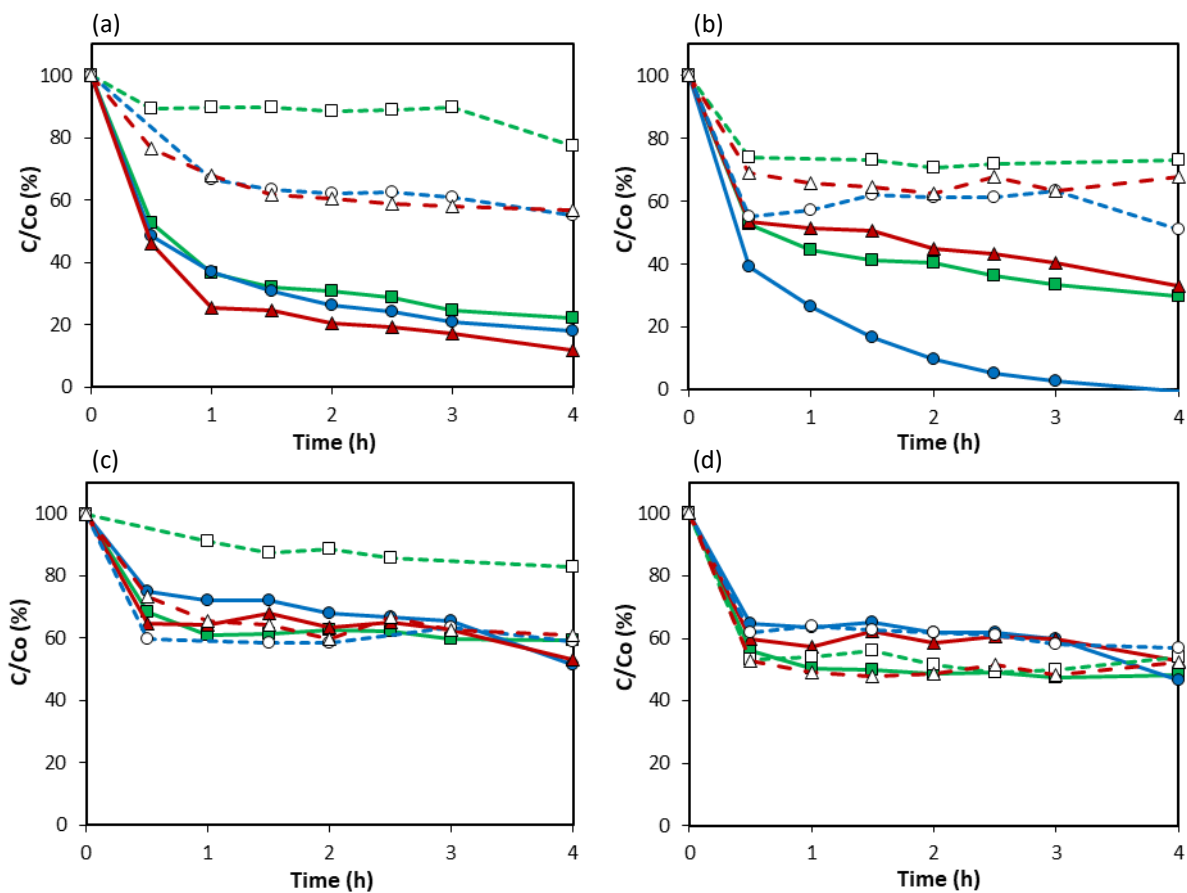


Fig. 5. Residual concentration of (a) AMX, (b) SMX, (c) CIP and (d) CBZ in mixture after 4 h treatment with immobilized TVL on silica particles and different redox mediators: ABTS (■), SYR (●) and PCA (▲). The open symbols represent the removal by adsorption on the support with inactivated laccase. Measurements were carried out by duplicate, with standard deviations less than 15%.

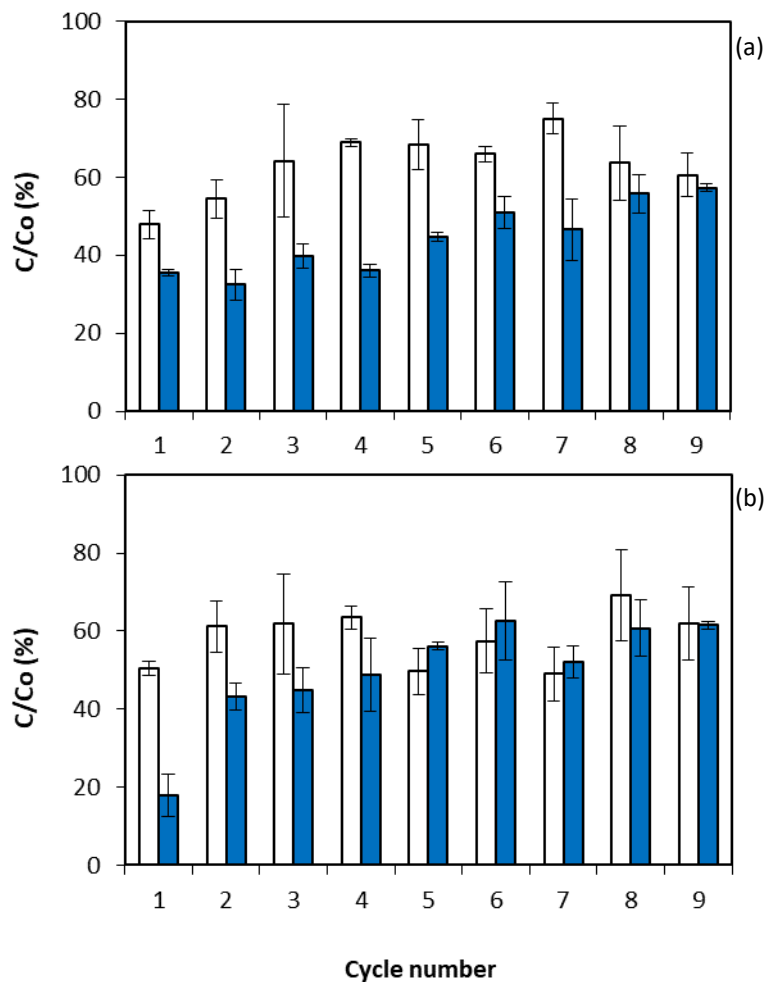


Fig. 6. Removal of (a) AMX and (b) SMX in a mixture by immobilized laccase and SYR as redox mediator in consecutive cycles at 25°C and pH 7 (blue bars). Adsorption of each micropollutant on the support with inactivated laccase is represented by white bars.