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Enzymatic degradation of tetracycline by *Trametes versicolor* laccase in a fluidized bed reactor

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23 **Keywords** : *Biocatalysts, laccase immobilization, gelatin beads, fluidized bed reactor,*
24 *tetracycline degradation*

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27 **1. Introduction**

28 Over the last few decades, worldwide population growth and ageing have led to increased
29 consumption of pharmaceutically active compounds (PhACs). However, many of these
30 compounds are resistant to conventional treatments used in Wastewater Treatment Plants
31 (WWTPs), and their residues have been detected in surface water and sometimes even in
32 drinking water (Couto et al., 2019; Petrie et al., 2015; Mirsaei et al., 2018). The occurrence
33 and persistence of PhACs in water circuits represents a major global concern due to their
34 negative impact on the environment and health (aquatic toxicity, endocrine disruption,
35 development of antibiotic resistance in some bacteria, etc.) (Grenni et al, 2018; Vieira et al.,
36 2021). Consequently, a number of recent research projects have focused on the development
37 of tertiary treatments to remove the resistant compounds. They include adsorption with
38 activated carbon (Rajapaksha et al., 2019), membrane processes (Khanzada et al., 2020),
39 advanced oxidation processes (Kanakaraju et al., 2018) and hybrid processes (Grandclement
40 et al., 2017).

41 Among these processes, bioremediation involving enzymes appears to be a promising option
42 because enzymatic reactions are carried out under mild conditions (pH, temperature).

43 Particular attention has been focused on oxydo-reductases and especially laccases, which only
44 need the dissolved oxygen in water to degrade a broad range of micro pollutants (Varga et al.,
45 2019; Bilal et al., 2019; Morsi et al., 2020). However, due to the high cost of enzymes and the

46 difficulty of recovering them once they are dissolved in the reaction medium, immobilization
47 of the enzymes onto a support would appear to be a cost-effective solution for improving the
48 biocatalyst stability and allowing their re-used (Basso & Serban, 2019). Furthermore, the use
49 of immobilized catalysts enables continuous operation, which is important for large-scale
50 operations. In a recent review on the potential of free and immobilized oxidative enzymes for
51 wastewater treatment, Zdarta et al (2022) highlighted the key role of the immobilization
52 method and the type of solid support on the activity and stability of the biocatalyst. Indeed, a
53 significant loss of activity during the immobilization step can considerably reduce the
54 economic interest of the process.

55 Among the large family of oxidative enzymes, laccases have been successfully used for
56 antibiotic degradation after being immobilized on various inorganic or organic supports
57 including membranes (de Cazes et al., 2014; Taheran et al.,2017), monoliths (Ahmad et al.,
58 2020) and solid particles (Shi et al.,2014; Wen et al. 2019; Zdarta et al., 2020). Enzymes
59 immobilized on solid particles are currently used in stirred tanks. However, if a continuous
60 process is envisaged, a packed-bed (PBR) or a fluidized-bed reactor (FBR) would be more
61 convenient. In FBRs, the continuous ascendant fluid flow maintains the particles of
62 biocatalyst in suspension, avoiding risks of column clogging and preferential flow paths.
63 Mass transfer in such reactors is also facilitated, but high shear stress can denature the
64 biocatalyst (Dal Magro et al., 2021). Despite this drawback, fluidized-bed reactors are widely
65 used for wastewater treatment with whole cells (Swain et al., 2018) and recently, the use of
66 immobilized laccases in FBRs has been reported for the biotransformation of bisphenol A
67 (Piao et al., 2019) and estrogens (Sun et al., 2021).

68 A previous study (Harguindeguy et al., 2021) reported a promising sustainable process for
69 preparing a gelatin-based support for enzyme immobilization. The gelatin beads, which were
70 obtained by coacervation of gelatin droplets in a polyethylene glycol solution, were cross-

71 linked with glutaraldehyde allowing, on one hand, the improvement of their mechanical and
72 thermal properties and, on the other hand, the covalent bonding of laccases on the surface of
73 the beads. These biocatalytic beads present low density, requiring low fluid velocity to
74 fluidize them. Energy consumption and shear stress are limited, avoiding any risk of laccase
75 denaturation. Such active beads therefore appear to be good candidates for use in FBRs.
76 The present work aims to study the feasibility of micro-pollutant depletion by the laccase of
77 *Trametes versicolor* immobilized onto gelatin beads in an FBR. As explained in the
78 introduction, immobilization generally enhances enzymes stability whereas FBR are very
79 interesting because they operate under continuous conditions and improve the mass transfer of
80 substrates towards active sites. From different types of micro-pollutants (pharmaceuticals,
81 personal care products, pesticides), we selected an antibiotic (tetracycline (TC)) as a model
82 substrate. TC is one of the most widely used veterinary antibiotics in Europe (European
83 Medicine Agency, 2021) and is consequently frequently found in aquatic systems, from
84 municipal wastewaters to seawaters (Abejon et al., 2015). Moreover, the accumulation of TC
85 in wastewater treatment plants is responsible for the proliferation of antibiotic resistant
86 bacteria (ARB), which represents a real health risk for humans and animals (Nguyen et al.,
87 2021). Furthermore, previous studies have demonstrated that TC can be degraded by the
88 laccase of *Trametes versicolor* without requiring a mediator (de Cazes et al., 2014).
89 Firstly, the stability of the immobilized laccases with respect to pH, temperature and during
90 storage was studied in a continuously stirred-tank reactor. Then, the active-beads were used in
91 an FBR and experiments were carried out to evaluate the effect of operating parameters (fluid
92 velocity, oxygen supply by bubbling air) on TC removal. Finally, the acute toxicity of the TC
93 solution before and after enzymatic treatment was evaluated by Microtox® assays.

94

95 **2. Materials and Methods**

96 2.1 Chemicals and enzyme

97 Gelatin porcine skin, (MW \approx 50 – 100 kDa, bloom 300, type A, CAS 9000-70-8),
98 polyethylene glycol (PEG, molecular weight 10000, CAS CAS 25322-68-3), glutaraldehyde
99 aqueous solution grade II (25% in H₂O, CAS : 1111-30-8), laccase (from *Trametes versicolor*,
100 CAS 80498-15-3), and 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt
101 (CAS 30931-67-0), also known as ABTS, were purchased from Sigma-Aldrich. Stock
102 solution of TC (Sigma Aldrich, CAS 60-54-8) was prepared in ultra-pure water (pH 6). Buffer
103 solutions were made from citric acid (CAS 77-92-9), and di-sodium hydrogen phosphate
104 dehydrate (CAS 10028-24-7). All these chemicals were of ACS reagent grade.

105 2.2 Active bead preparation and characterization

106 2.2.1 Bead preparation:

107 Active beads were prepared according to a previously reported three-step process
108 (Harguindeguy et al, 2021). Firstly, an aqueous gelatin solution (22% w_t, 60°C) was added
109 dropwise with a syringe pump (SP200, flow rate 4 mL min⁻¹) inside an aqueous PEG₁₀₀₀₀
110 solution (40% w/v, 50°C). The PEG solution was then cooled down in an ice bath at 5°C and
111 the formed beads were recovered and washed with distilled water to remove excess PEG. The
112 gelatin beads were then cross-linked and activated by immersion for 1 hour in a 3% v/v
113 glutaraldehyde solution prepared in a citrate phosphate buffer solution (50 mM, pH 7) at 25°C
114 under magnetic stirring (250 rpm). After the activation step, the beads were thoroughly rinsed
115 with the same buffer solution to remove the glutaraldehyde that did not react and were
116 immersed for 2 hours in a solution of *Trametes versicolor* laccase (prepared at 3 U mL⁻¹ in the
117 same citrate phosphate buffer solution) at 25°C under magnetic stirring (250 rpm). At the end
118 of the grafting step, the beads were washed 3 times with the same citrate phosphate buffer
119 solution and stored at 4°C in the buffer until being used. For blank experiments, deactivated

120 beads were obtained by immersion of active beads in boiling water (100°C) for 30 minutes to
121 deactivate the enzymes. The characteristics of the active beads are reported in Table 1.

122 (Table 1)

123

124 2.2.2 Activity assay

125 Enzyme activity measurements are based on the oxidation of 2,2'-Azino-bis (3-
126 ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS). 900 µL of ABTS (1 mM,
127 citrate-phosphate buffer (50 mM, pH 4)) was mixed with 100 µL of laccase solution (10 mg
128 L⁻¹ in the citrate-phosphate buffer) in a spectrophotometric cell and the reaction was
129 monitored with a Shimadzu UV-2401 PC spectrophotometer at 420 nm along with the
130 software UV Probe version 2.21. One unit of enzyme activity (U) was defined as the amount
131 of enzymes that converted 1 µmol of ABTS to its cation radical per minute at pH 4 and 25°C.
132 For immobilized enzymes, 0.7 g of active beads were immersed in 25 mL of ABTS solution
133 (1 mM in citrate-phosphate buffer (50 mM, pH 4, 25°C)), under magnetic stirring (250 rpm),
134 and the absorbance of the solution was monitored every minute for 15 minutes. Both
135 conditions of activity measurement described above are referenced as standard conditions.

136

137 2.2.3 pH and temperature stability

138 pH and thermal stability were determined by incubating free and immobilized enzymes in
139 three citrate phosphate buffer solutions (50 mM, pH 3, 5 or 9) during 24 hours at 25°C or in
140 citrate phosphate buffer solution (50 mM, pH 7) at 60°C during 6 hours. At different times,
141 samples were withdrawn and enzymatic activity was measured with ABTS under standard
142 conditions (1 mM, pH 4, 25°C). Results are expressed as relative activity (i.e. ratio between

143 the activity measured and the initial activity). The experiments were carried out in triplicate
144 and the average value is displayed.

145 2.2.4 Kinetics constants

146 The apparent kinetic parameters of the Michaelis–Menten equation (K_m and V_{max}) of free and
147 immobilized laccase towards TC were determined from initial reaction rate measurements.
148 TC solutions of 2 – 5 – 10 – 15 – 25 ppm were prepared in ultra-pure water (pH 6). The
149 reactions were carried out in batch at 25°C, with an enzyme concentration equivalent to 30 U
150 L^{-1} for both free and immobilized enzymes.

151 2.2.5 Storage stability

152 The storage stability of the free and immobilized enzymes was evaluated, by incubating both
153 laccase forms in a citrate phosphate buffer (50 mM, pH 7) at 4 °C. The residual activity was
154 obtained by measuring the remaining activity every 5 days for 20 days under standard
155 conditions.

156

157 2.3 Tetracycline degradation

158

159 All the TC degradation assays were carried out with a TC solution prepared at 20 ppm in ultra
160 pure water (pH 6). Firstly, TC degradation with both free and immobilized enzymes was
161 studied in a stirred tank reactor (10 mL) at 25°C during 24 hours with an enzyme activity
162 equivalent to 100 U L^{-1} of laccase. Then, degradation assays with active beads were carried
163 out in fluidized-bed reactor operating in recirculation (see Figure 1). A jacketed glass column
164 (height 23 cm ; internal diameter 2 cm) was filled with wet active beads (~ 4 g corresponding
165 to a final enzyme activity of 60 U L^{-1}). The height and porosity of the fixed bed were equal to

166 5 cm and 0.36 respectively. The minimum fluidizing velocity (U_{mf}) was calculated according
167 to eq (1) since the Re value (eq. 2) at U_{mf} was below 20 (Gidaspow, 1994).

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$$169 \quad U_{mf} = \frac{d_p^2}{150} \cdot \frac{\varepsilon_{mf}^3}{(1-\varepsilon_{mf})} \cdot \frac{(\rho_s - \rho_f)}{\mu_f} \cdot g \quad (\text{Eq.1})$$

$$170 \quad Re = \frac{\rho_f U_{mf} d_p}{\mu_f} \quad (\text{Eq.2})$$

171

172 Where d_p is the bead diameter, ε_{mf} is the porosity of the bed at U_{mf} which is assumed to be
173 similar to that of the fixed bed, ρ_s and ρ_f are the density of bead and fluid respectively, μ_f is
174 the fluid viscosity and g is the normal gravity.

175 In the present work, U_{mf} calculated with the mean value of bead diameter (2.0 mm), was 0.21
176 cm min^{-1} (a fluid flow of 40 mL min^{-1}) which corresponded to a Re value of 4.3.

177 TC solution (100 mL) was pumped continuously upwards through the column using a gear
178 pump (micro pump serial number 951990005, Cole Parmer) at different flow rates: 30, 80 and
179 130 mL min^{-1} corresponding to approximately $0.8 U_{mf}$, $2 U_{mf}$ and $3.2 U_{mf}$ respectively. The
180 column and the feed tank temperatures were controlled at 25°C by a water bath with a
181 circulation system. In some experiments, a continuous flow of air (15 mL min^{-1}) was bubbled
182 in the reservoir of feed solution maintaining the reaction medium under oxygen saturation,
183 which was checked by an oxygen sensor (Hamilton oxygen probe).

184 (Fig 1)

185 Blank experiments were carried out without any enzyme or with deactivated beads to ensure
186 that TC self-degradation or adsorption were negligible. To determine the operational stability
187 of the immobilized enzymes, 5 cycles of TC degradation were carried out in the FBR with the
188 same active beads by replacing the TC solution by a fresh one every 24 hours. Between each
189 cycle, the beads were thoroughly rinsed with 50 mL of citrate phosphate buffer (50 mM, pH
190 7) as well as the reactor. The initial activity was considered as the reference value of 100%.

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2.4 Analysis

2.4.1 TC analysis

TC concentration was measured by high-performance liquid chromatography coupled to triple-quadrupole mass spectrometry (HPLC–MS). Samples were injected through a Machery-Nagel C18 column (50 mm x 2 mm) with a Waters e2695 Separations Module, and the 410 m/z fragment was detected with a Micromass Quattro micro API device. Elution was done with an isocratic mobile phase (HPLC grade water, 99% formic acid, and acetonitrile (74.9:0.1:25)) at a flow rate of 2 mL min⁻¹. The amount of TC was quantified with calibration standards prepared by dilution of the TC stock solution in ultra-pure water.

2.4.2 Toxicity assessments

Acute toxicity of the TC solution before and after enzymatic treatment was evaluated by the Microtox® bacteria toxicity test based on the general principles described by Bulich (1979) and Johnson (2005). The toxicity of samples was determined by measuring the effect on the luminescence of marine bacteria *Vibrio fischeri* NRRL B-11177 using a Microtox® Model 500 Analyzer with MicrotoxOmni software (Modern Water Inc.; United Kingdom). The toxicity measurements were carried out for TC solution (1 ppm) before and after 24 hours of laccase treatment (with both free and immobilized laccase). The pH of all the samples was adjusted between 6.5 and 7.5 and the toxicity was evaluated after 5 and 15 minutes of contact with the bacteria. Sample toxicity was expressed in percentage of inhibition effect I (%) compared to a control (salt solution) and calculated by equation 3, where E₀ is the bioluminescence intensity of the control, and E is the bioluminescence intensity of the sample.

$$I \% = \frac{E_0 - E}{E_0} * 100 \quad (\text{Eq.3})$$

Each sample was analyzed in triplicate and the average value is reported in the results section.

216

217 **3. Results**

218 In the figures of this section, each point presented is a mean value of at least three
219 experimental results, the bars of error were calculated by the standard deviation of the
220 measurements.

221 3.1 Stability of immobilized enzymes

222 The stability of free and immobilized enzymes was studied for 3 different pHs (pH 3, 5 and 9)
223 and the results are presented in Figure 2-A. According to this figure, grafting seems to have
224 only a slight effect on laccase stability at pH 5 and 9. Both enzyme forms showed a similar
225 decrease of activity in the range of 20-30% after 24 hours of incubation at pH 5 or 9. On the
226 contrary, when the enzymes were incubated at pH 3, the positive effect of grafting is clearly
227 observed. After 24 hours of incubation, the residual activity of the free enzyme was equal to
228 $30 \pm 3\%$ while the immobilized laccase still presented a remaining activity of $52 \pm 3\%$. The
229 protecting effect of grafting towards deactivation induced by acidic conditions has already
230 been reported in the literature (Wang et al., 2008, Asgher et al., 2017, Guardado et al. 2021).
231 This could be related to the stiffening of the enzyme structure, making it less sensitive to
232 denaturing conditions, especially when multi-covalent bonds are involved, but the hydrophilic
233 properties of the gelatin support could also contribute to maintaining a favorable environment
234 around the enzymes (Rodriguez et al., 2021).

235 Enzymatic stability can also be affected by temperature increase; the denaturation effect
236 depends on the enzyme strain and can be mitigated with grafting. Figure 2-B displays the
237 activity evolution of free and immobilized enzymes incubated at 60°C during six hours. A
238 large decrease in enzyme stability is noticed, especially for free enzymes. In this case, relative
239 activity remains at just $40 \pm 2\%$ after 1 hour of incubation at 60°C; this value drops to $12 \pm$
240 1% after 6 hours incubation. On the contrary, the immobilization step seems to improve

241 thermostability of enzymes, as relative activity remains at $38 \pm 1\%$ after 6 hours at 60°C . As
242 previously reported for acidic pH conditions, grafting appears to prevent structural
243 transformation of enzymes when confronted with relatively high temperatures (Rodriguez et
244 al., 2021).

245 (Fig 2)

246 The storage stability of free and immobilized enzymes was studied in citrate phosphate buffer
247 (50mM, pH7) at 4°C during 20 days. During that time (results not shown), free laccase
248 activity decreased slightly but continuously to $90 \pm 5\%$ of the initial value at the end of the
249 experiment. In the case of immobilized enzymes, an initial decrease in activity was observed
250 within the first days of storage, probably due to the leakage of enzymes that were not
251 covalently bound to the gelatin beads. Non-covalent interactions (Van der Waals or ionic
252 interactions) between laccase and the gelatin chains can also occur during the grafting step.
253 Due to the weakness of these bonds, biocatalysts that have not been covalently grafted can be
254 released into the storage solution. However, the final concentration of enzymes in the storage
255 solution was probably too low and did not allow enzyme activity to be measured. The
256 decrease of bead activity could also result in enzyme distortion promoted by further
257 interactions between laccases and the support as has been already reported (Rodriguez et al.,
258 2021). Nevertheless, after 10 days and until the end of the experiment, the relative activity
259 remained constant and equal to $85 \pm 4\%$ of the initial value. Although no stability increase
260 was observed compared to free enzymes, the covalent grafting method used in this work leads
261 to stable laccase derivatives.

262 3.2 TC degradation

263 Previous studies have demonstrated that TC is degraded by *Trametes versicolor* laccase (De
264 Cazes). TC was therefore chosen as a PhACs model compound to evaluate the performance of

265 enzymatic beads in a stirred tank reactor (STR) and an FBR. Firstly, apparent kinetics
266 constants of laccase towards TC were determined with free and immobilized enzymes;
267 experiments were carried out with the same enzyme activity (30 U L^{-1}). The V_{\max} values
268 (0.001 and $0.002 \text{ } \mu\text{mol.L}^{-1}.\text{min}^{-1}$ for free and immobilized enzymes respectively) towards TC
269 were very low compared to those estimated at the same enzyme concentration with ABTS
270 (0.029 and $0.033 \text{ } \mu\text{mol L}^{-1} \text{ min}^{-1}$ for free and immobilized enzymes respectively)
271 (Harguindeguy et al, 2021). Since ABTS is known as a specific substrate for laccases, it is not
272 surprising that this enzyme shows a higher reactivity for this substrate. In addition, as the
273 V_{\max} values of both enzyme forms were quite similar, it is not possible to conclude whether or
274 not immobilization improves the reactivity of the enzymes towards TC. Nevertheless, it
275 appears that immobilized laccase has a slightly higher affinity towards TC than free laccase;
276 the K_m value is lower for the immobilized form ($62.8 \text{ } \mu\text{mol L}^{-1}$) than for the free form (88.2
277 $\text{ } \mu\text{mol L}^{-1}$). However, it should be noted that this positive effect on laccase affinity was lesser
278 than that observed by De Cazes et al. These authors reported that the K_m of laccase covalently
279 grafted to a gelatin layer deposited on a ceramic membrane was 5 times lower than that of free
280 laccase (De Cazes et al., 2014).

281 The first experiments of TC gradations of were carried out with a 20 ppm TC solution in a
282 stirred tank reactor with both free and immobilized laccases at the same concentration (100
283 U.L^{-1}); the results obtained are presented in Figure 3. After a similar decrease of TC
284 concentration for both enzyme forms within the first two hours of reaction, the degradation
285 rate slowed down more drastically with free enzymes to reach a final TC removal of $50 \pm 2\%$
286 after 24 hours. When active beads were used, the residual TC concentration was $30 \pm 1\%$. As
287 experiments carried out with inactivated beads resulted on a TC degradation less than 4 %, it
288 can be concluded that TC removal is mainly the result of laccase activity.

289 The immobilization clearly improves the stability of enzymes in operational conditions and
290 seems to preserve the biocatalyst from shear stress denaturation. De Cazes et al. have
291 observed similar results with the same laccase of *Trametes versicolor* covalently grafted to a
292 ceramic membrane previously coated with a gelatin layer. They reported that TC degradation
293 increased from $30 \pm 7\%$ up to $56 \pm 8\%$ when the laccases were immobilized (De Cazes et al.,
294 2014). This improvement of enzyme stability can be related to a favorable environment
295 created by gelatin. More generally, grafting to a biopolymer helps enhance the stability of
296 proteins without altering the flexibility necessary for their catalytic activity (Ma et al., 2018;
297 Asgher et al., 2017).

298 The TC degradation tests in the FBR were carried out at 25°C with a 20 ppm TC solution and
299 recirculating flow of $80 \text{ mL}\cdot\text{min}^{-1}$ (i.e. $2 U_{mf}$). It should be noted that the reaction volume and
300 enzyme concentration were different from those used for experiments done in the stirred-tank
301 reactor (100 mL instead of 10 mL and 60 U L^{-1} instead of 100 U L^{-1} respectively). The results
302 obtained are reported in Table 2. As already reported for stirred tank reactor, no adsorption or
303 TC auto degradation occurred in the FBR. Blank experiments showed that $98 \pm 2\%$ of the TC
304 remained in the reactor after 24 hours whereas this percentage decreased to $56 \pm 4\%$ when
305 active beads were used. This later value is lower than that observed in the stirred-tank reactor
306 ($35 \pm 1\%$). However, when reaction volume and enzyme concentration are taken into account,
307 the average degradation rate (estimated over 24 hours) is almost 5 times higher in the FBR
308 ($1.59 \text{ mg h}^{-1} U_{enzymes}^{-1}$) than in the stirred tank reactor ($0.35 \text{ mg h}^{-1} U_{enzymes}^{-1}$). The larger
309 amount of TC in the FBR probably reduced the substrate limiting issues and thus explained
310 the higher degradation rate observed. In addition, even if we are still in laminar conditions
311 (flow velocity = 0.42 cm s^{-1} , $Re = 8.5$), the hydrodynamic conditions and beads movement
312 observed could also play a role by reducing the boundary layers and thus facilitating the
313 contacts and exchanges between the substrate and the enzymes.

314 (Fig 3)

315 (Table 2)

316 3.3 Effect of the recirculation flow rate and air bubbling

317 To improve the efficiency of TC degradation, the effect of the circulation flow rate and air
318 bubbling were also investigated. Experiments were carried out at 3 different recirculation
319 rates (30 mL min⁻¹, 80 mL min⁻¹, and 130 mL min⁻¹, corresponding respectively to the
320 following linear velocities: 0.16, 0.42 and 0.69 cm.s⁻¹ and Re values equal to 3.2, 8.5 and
321 13.8), without or with air bubbling (15 mL min⁻¹). An oxygen sensor was used to verify that
322 the air bubbling maintained the oxygen saturation of the reaction medium in the feed tank.
323 The results are reported in figure 4.

324 When no air was added (open symbols), the percentage of degradation increased with the
325 velocity of the fluid. At a velocity of 0.16 cm s⁻¹, which is lower than the minimum fluidizing
326 velocity (0.21 cm s⁻¹, corresponding to 40 mL min⁻¹), the degradation rate of TC is very low
327 and only 7% of TC is degraded in 24 hours. On the contrary, 50% and 70% of degradation
328 were achieved in the reactor when the circulation flow were fixed to 2 and 3.2 U_{mf}
329 respectively. Increasing the flow rate allows particles to be separated from each other. At flow
330 rates of 80 and 130 mL min⁻¹, 95% and 100% respectively of the beads were in fluidized
331 conditions. The circulation of the fluid around the particles was thus facilitated leading to
332 better exchange between the catalyst and the substrate. On the contrary, in fixed-bed reactors,
333 since all particles are in contact with each other, the access to all active sites is probably
334 limited; it is also possible that the fluid does not flow homogeneously through the bed
335 creating inhomogeneous reaction zones inside the reactor. Furthermore, even though the
336 increased flow rate reduces the residence time in the reactor, it enables renewal of the
337 substrate close to the active sites of the enzymes that favors degradation. Similar results were

338 reported by Piao et al. (2019) who observed an increase in bisphenol degradation from 50% to
339 80% in 6 hours by increasing the flow rate from 1.2 mL min⁻¹ to 7.2 mL min⁻¹ in an FBR
340 using porous silica as a laccase immobilization support.

341 Figure 5 shows that at low recirculation rates (30 and 80 mL min⁻¹) the injection of air (closed
342 symbols) considerably improves the degradation rate of TC. Indeed, when the velocity of the
343 solution is lower than minimum fluidizing velocity (0.8 U_{mf}), adding air to the system
344 increased the degradation of TC from 7% to 40% in 24 hours. This could be due to the
345 presence of small air bubbles observed around the active beads. These small air bubbles
346 transported from the feed tank by the flow caused expansion of the bed and increased
347 agitation around the beads, leading to better exchanges between the catalyst and the substrate.
348 Moreover, the addition of air also allowed a high concentration of oxygen to be maintained
349 near the active sites. The same effect was observed in the FBR for a flow rate of 80 mL.min⁻¹
350 (i.e 2 U_{mf}) where the TC degradation was enhanced from 45% to 70%. Nevertheless, the
351 opposite behavior was obtained at the highest recirculation flow applied (squares : 130
352 mL.min⁻¹, 3.2 U_{mf}). In this case, the air bubbling resulted in a decrease of the percentage of
353 TC degradation at 24 hours from 70% (without air bubbling) to 50% (with air bubbling). This
354 decrease probably results from the presence of numerous and large air bubbles dragged by the
355 higher flow into the FBR. Indeed, the presence of these air bubbles may mean that some
356 active sites were not continuously in contact with the solution, leading to a decrease of the
357 reaction rate. It is also possible that the high recirculation flow combined with the presence of
358 gas bubbles, induced unfavorable shear stresses on the enzymes, leading to their denaturation
359 and thus to a decrease in the efficiency of the degradation process.

360 (Fig 4)

361 3.4 Operational stability of the biocatalyst

362 The operational stability of the immobilized laccase inside the FBR was evaluated in cyclic
363 experiments carried out during 5 degradation cycles of 24 hours. The experiments were
364 carried out at a laccase concentration of 100 U L^{-1} and at recirculation rate of 100 mL min^{-1}
365 without air addition. At the end of each cycle, the reaction medium was removed and the
366 active beads were rinsed with a citrate-phosphate buffer (50 mM, pH7). The reactor was then
367 fed with a fresh TC solution. The evolution of TC concentration during these 5 cycles is
368 reported in the figure 5. It should be noted that no loss of activity was observed during the
369 three first cycles since the evolution of TC concentration was quite similar and a TC
370 degradation of 57% was achieved after 24 hours for these three cycles. Afterwards, a slight
371 decrease in the degradation efficiency was observed during the 4th and 5th cycles and the TC
372 degradation was only 47% at the end of the cycles. Nevertheless, these experiments
373 demonstrate the excellent stability of the immobilized laccases which were still active and
374 able to degrade more than 45% of the TC of a 20 ppm TC solution after 120 h of use.

375 (Fig 5)

376 3.5 Toxicity assays

377 Degradation does not always mean that the toxicity of the effluent is reduced as has already
378 been reported in the literature (Beker et al., 2016). Therefore Microtox assays were performed
379 on TC samples before and after enzymatic treatment with both free and immobilized laccase.
380 As the concentrations used in this project are quite far from those found in real effluents, the
381 degradation tests were carried out in batch with a 1 ppm TC solution and 60 U L^{-1} of free or
382 immobilized enzymes. The results obtained are presented in figure 7. The initial TC solution
383 showed inhibition effects of 29% and 42% after 5 and 15 minutes of contact respectively.
384 After 24 hours of enzyme treatment, these values almost doubled when free enzymes were
385 used (57 and 73% respectively) whereas they decreased to 19 and 25 %, respectively, when

386 the enzymes were immobilized on the gelatin beads. These results are surprising and seem to
387 indicate that the degradation products obtained with free enzymes could be different and more
388 toxic towards *Vibrio fischeri* than those generated by immobilized enzymes. A difference
389 between the oxidation mechanisms of free and immobilized laccases is possible, resulting in
390 various degradation products with different toxicity levels. However, the difference in toxicity
391 may also be the result of the presence of additives in the commercial enzyme solution. Indeed,
392 Microtox assays performed on a solution of free laccase prepared at 60 U mL⁻¹ showed
393 inhibition effects of 39% and 50% after 5 and 15 minutes of contact respectively. On the
394 contrary, no inhibition was observed when Microtox assays were performed on water placed
395 in contact with active beads for 24 hours. The rinsing of the active beads carried out after the
396 grafting step probably allowed the removal of the toxic species present in the commercial
397 enzyme solution.

398 Microtox results show that the treatment of TC solution with immobilized laccases enable a
399 decrease in the toxicity of TC towards a specific bacterium (*Vibrio fischeri*). Nevertheless,
400 other bioassays with other microorganisms must be performed to conclude on the safety of
401 TC degradation products.

402 (Fig 6)

403 **4. Conclusion**

404 Laccase of *Trametes versicolor* was covalently immobilized onto gelatin beads to be used in
405 an FBR for degradation of TC. The results obtained showed that TC depletion depends on the
406 operating conditions. High feed flow rates and air bubbling in reaction mixture can prevent
407 mass transfer limitation and/or reaction limitation due to lack of oxygen. A good operational
408 stability was observed for 5 successive degradation cycles of 24 hours. Thus, this grafted
409 biocatalyst combined with an FBR seems to be a promising alternative to assure a high
410 degradation rate as well as possible recyclability of the catalyst. Furthermore, the

411 biodegradability of gelatin beads is a real advantage for the development of a sustainable
412 depolluting process. However, the active beads preparation needs further improvement in
413 order to reduce bead sizes and increase the immobilization yield (only 30% in the present
414 study). Modeling and simulation studies will be also necessary to scale-up the process for
415 waste-water treatment and to conclude on its economic viability.

416

417 **CRedit authorship contribution statement**

418 Marine Harguindeguy: investigation, formal analysis, writing of the original draft; Céline
419 Pochat-Bohatier: conceptualization, supervision, formal analysis, writing - review & editing;
420 José Sanchez-Marcano : conceptualization, formal analysis, writing - review & editing; Marie-
421 Pierre Belleville : conceptualization, supervision, formal analysis, writing - review & editing

422

423 **Conflicts of interest**

424 There are no conflicts to declare.

425

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Table 1: Active bead characteristics.

Diameter (mm)	Density (kg m ⁻³)	Specific activity (U g ⁻¹)
2 ± 0.2	1112 ± 13	0.1±0.01

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579 **Table 2:** Degradation of a TC solution (20 ppm) after 24 hours of reaction in two types of
580 reactors: stirred tank reactor 10 mL in volume and 100 U L⁻¹ of enzymatic activity and FBR
581 100 mL and 60 U L⁻¹ respectively (temperature 25°C ; flow rate in FBR: 80 mL min⁻¹).

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Reactor type		TC residual after	Mean degradation rate (mg h ⁻¹ .Uenzymes ⁻¹)
		24 hours (%)	
Stirred tank	Inactivated beads	96 ± 3	-
	Active beads	35 ± 1	0.35
Fluidized bed	Inactivated beads	98 ± 2	-
	Active beads	56 ± 4	1.59

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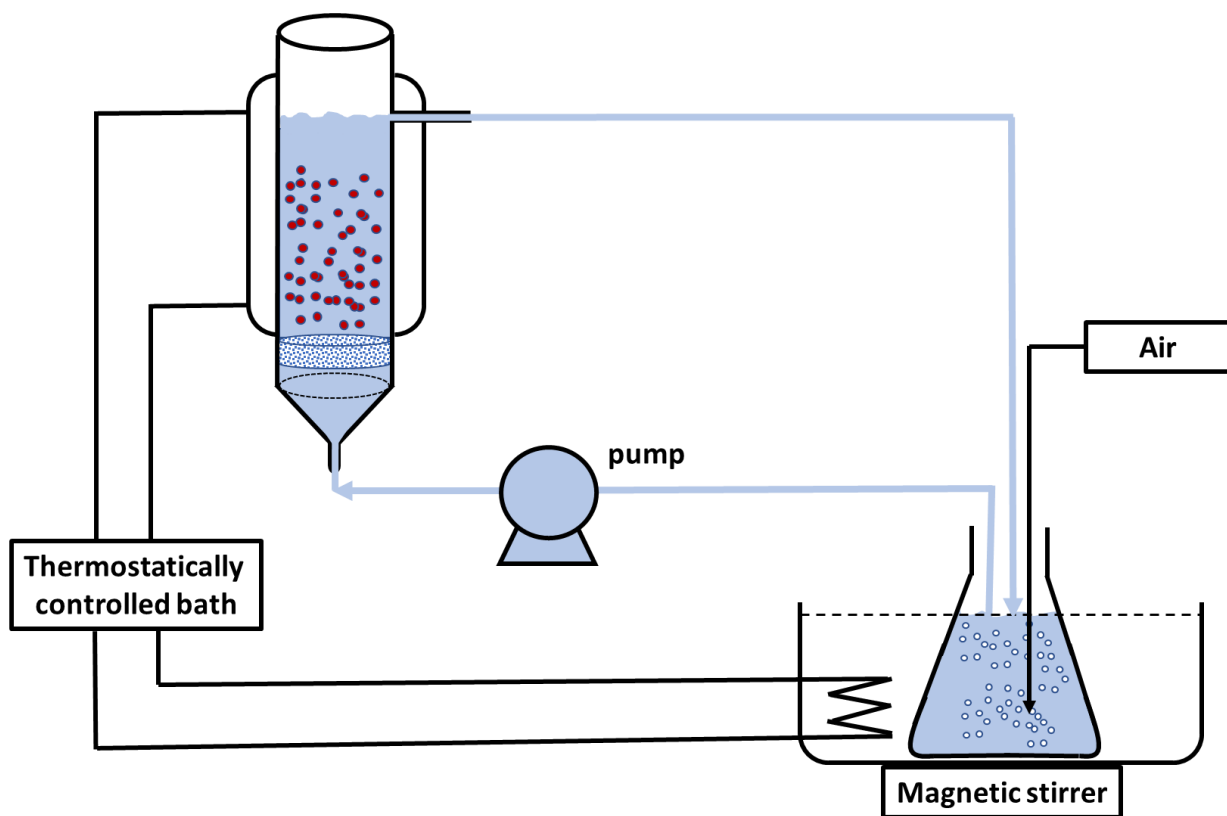
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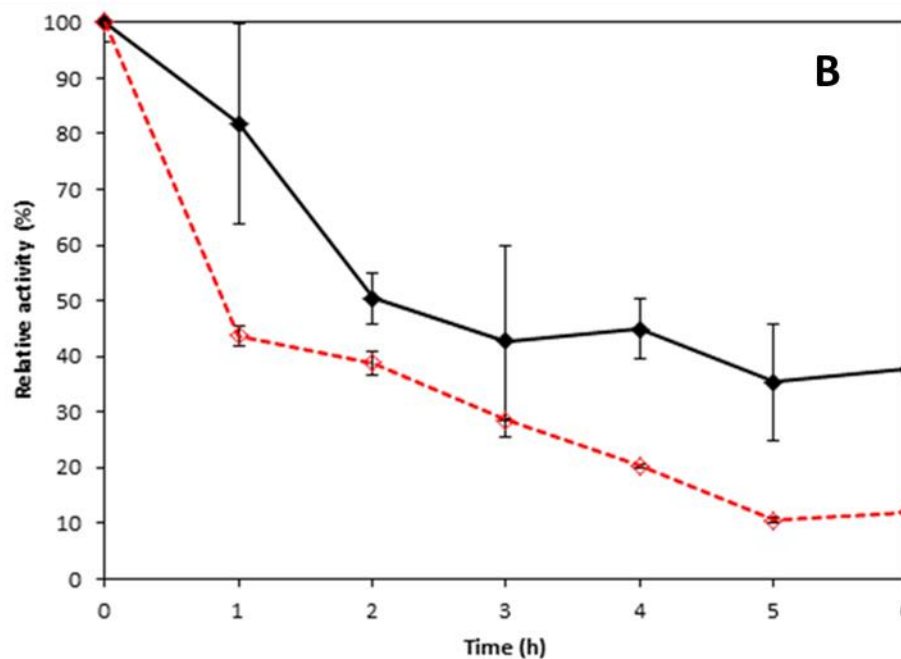
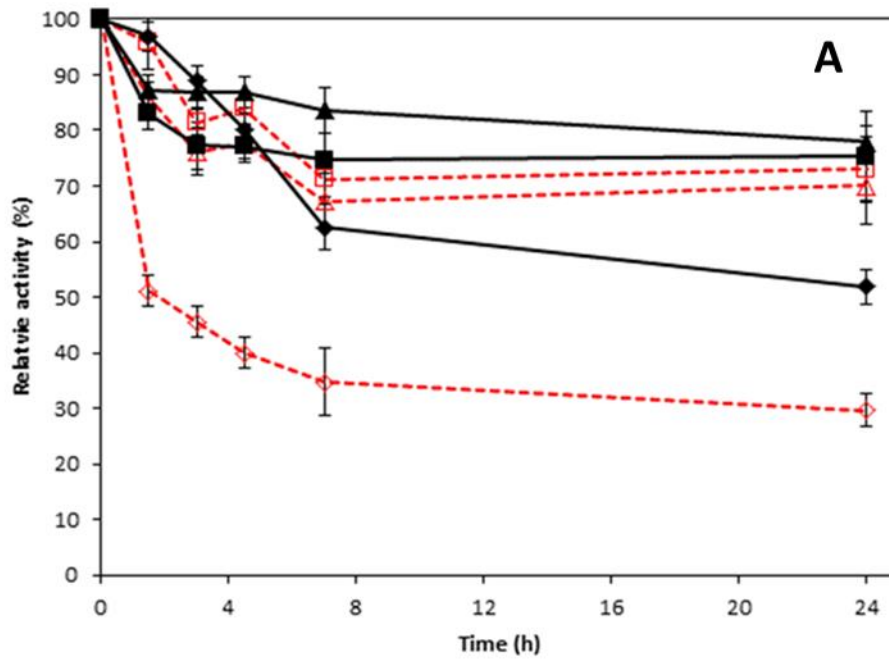
592 **Figure 1:** Enzymatic fluidized-bed reactor (FBR).

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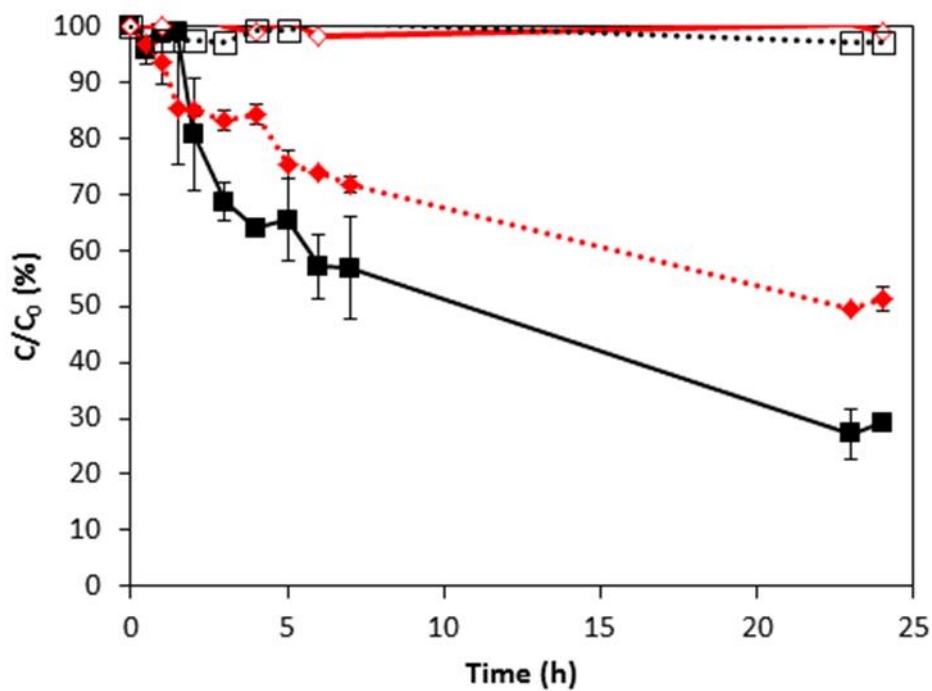
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598 **Figure 2:** Stability of free and immobilized enzymes (A): pH stability of free enzymes (red
 599 dotted line, open symbols) and immobilized enzymes (black continuous line, closed symbols)
 600 during 24 hours at pH 3 (diamonds), pH 5 (circles), and pH 9 (triangles), incubation at 25 °C
 601 in a citrate-phosphate buffer (50 mM); (B) temperature stability of free enzymes (dotted line)
 602 and immobilized enzymes (continuous line) incubated at 60 °C in a citrate phosphate solution
 603 buffer (50 mM, pH 7).

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607 **Figure 3:** TC (20 ppm) degradation with free (red diamonds – dotted line) or immobilized
608 (black squares – continuous line) enzymes at 100 U.L^{-1} , at 25°C for 24 hours. Blanks are
609 represented in open red diamonds (free enzymes) or open black squares (immobilized
610 enzymes).

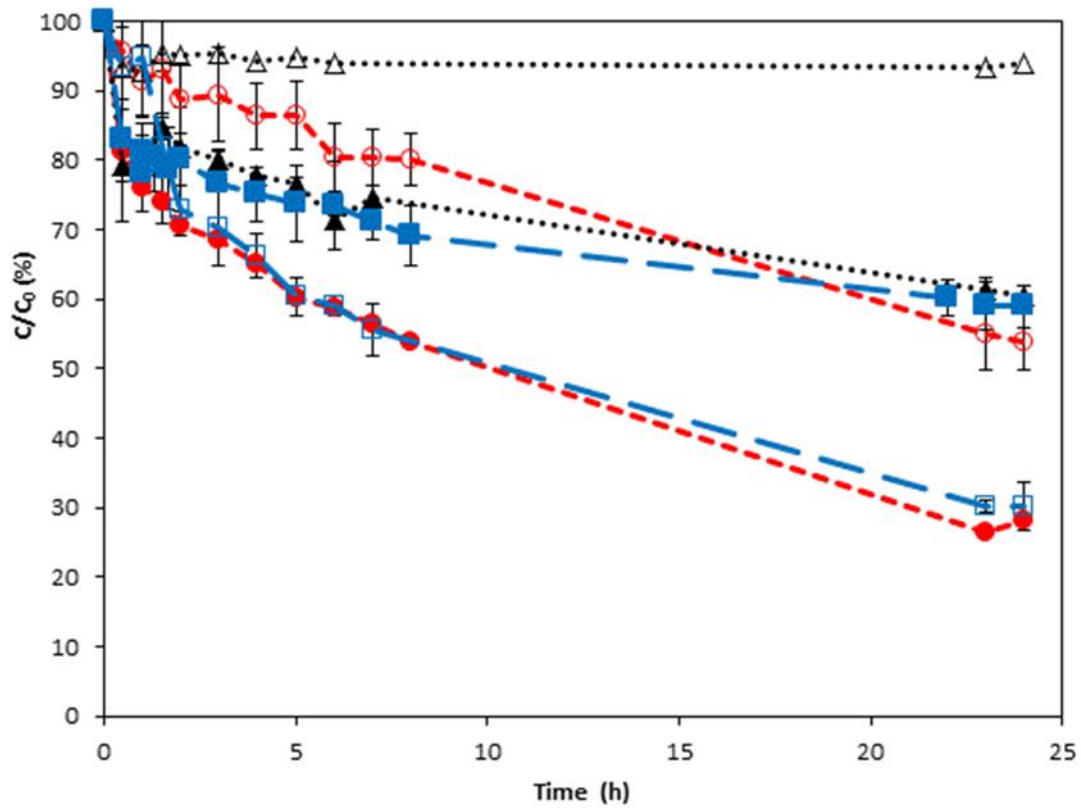
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617 **Figure 4:** Effect of circulation flow rate and oxygen bubbling on TC degradation. (black
618 triangle curve : 30 mL min⁻¹; red circle curve : 80 mL min⁻¹; blue square curve : 130 mL min⁻¹,
619 open symbols : without air bubbling, closed symbols : with air bubbling).

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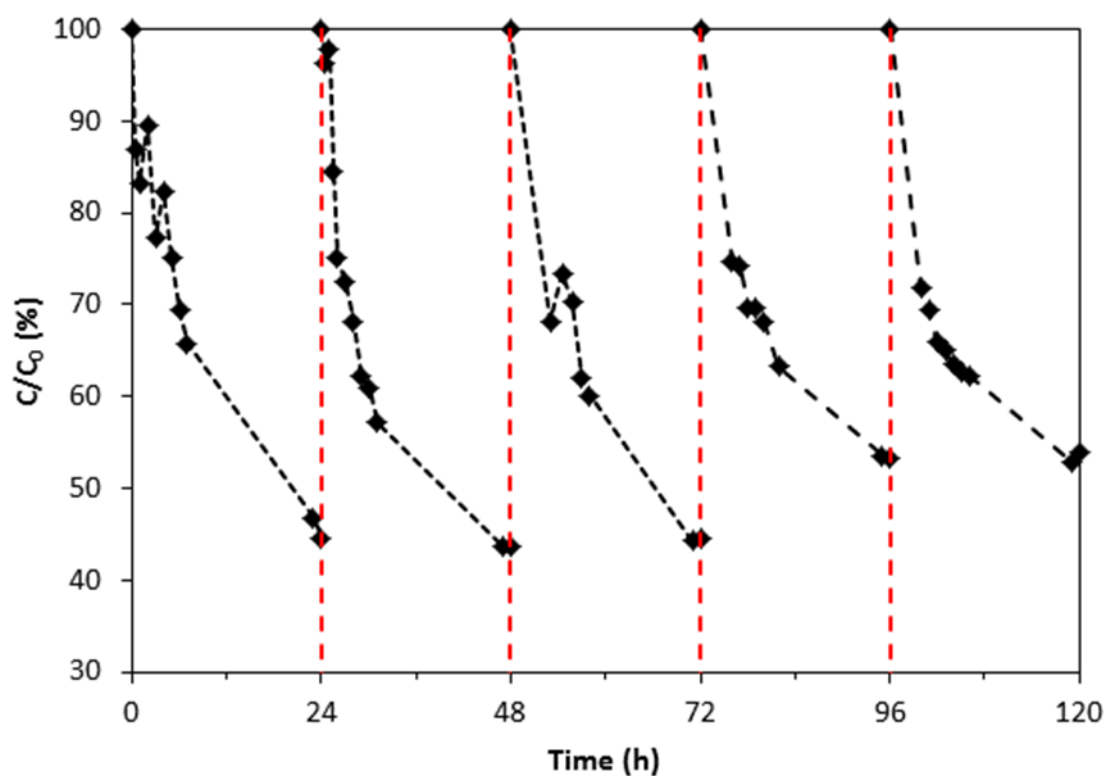
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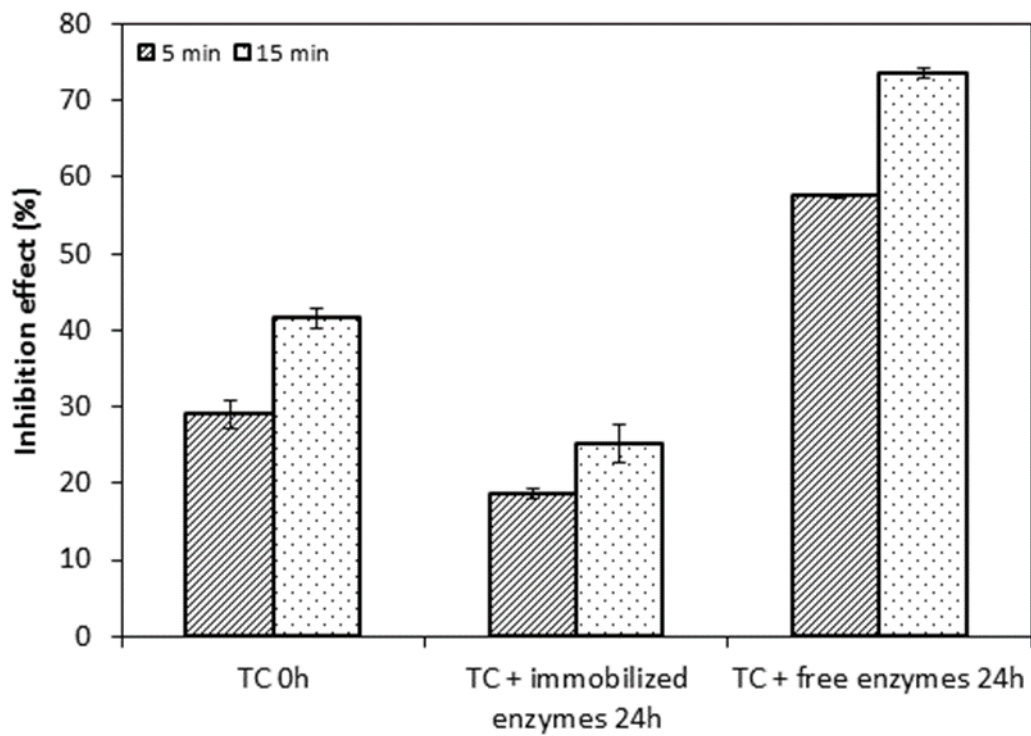
629 **Figure 5:** Operational stability of immobilized laccase inside the FBR (100mL of 20 ppm TC
630 solution prepared in ultra-pure water pH 6, enzymes concentration of 100 U L⁻¹, recirculation
631 flow rate of 100 mL min⁻¹ at 25°C). After each 24 hours period (dotted red line) the reactor
632 was emptied, washed and refilled with a TC fresh solution and a new experiment started. A
633 total of 5 cycles were carried out (total of 120 h).

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640 **Figure 6:** Inhibition effects of TC solution before and after laccase treatment (with free and
641 immobilized enzymes) after 5 minutes (stripes) or 15 minutes (dots) of contact with bacteria
642 *Vibrio fischeri*.

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