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Paper:

Pérez-Grisales, M., Castrillón-Tobón, M., Copete-Pertuz, L., Plácido, J. & Mora-Martínez, A. (2019).
Biotransformation of the antibiotic agent cephadroxyl and the synthetic dye Reactive Black 5 by *Leptosphaerulina* sp.
immobilised on Luffa (*Luffa cylindrica*) sponge. *Biocatalysis and Agricultural Biotechnology*, 18, 101051
<http://dx.doi.org/10.1016/j.bcab.2019.101051>

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1 **Biotransformation of the antibiotic agent cephadroxyl and the**
2 **synthetic dye Reactive Black 5 by *Leptosphaerulina* sp.**
3 **immobilised on Luffa (*Luffa cylindrica*) sponge**

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11

12 **Abstract**

13 In the present work, immobilisation of *Leptosphaerulina* sp., a Colombian native
14 fungus, improved the biotransformation of pollutants (Remazol black 5 (RB5) dye
15 and cephadroxyl (CPD) antibiotic) in aqueous systems. Four different natural
16 immobilisation matrices (charcoal, luffa sponge, wood chips and cork) were tested

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17 in order to select the most suitable for *Leptosphaerulina* sp. biomass augmentation.
18 Luffa sponge was selected qualitatively as the most appropriate material for the
19 immobilisation of *Leptosphaerulina* sp. CPD and RB5 biotransformation was
20 performed with immobilised and suspended *Leptosphaerulina* sp. cultures on luffa
21 sponge. The luffa sponge-immobilised fungus exhibited a considerable removal of
22 CPD (~100%) and RB5 (91.9%). The luffa sponge-immobilised *Leptosphaerulina* sp.
23 achieved a higher CPD removal than the suspended cultures (~100% vs 94.4%,
24 respectively, on day 15). RB5 experiments revealed a higher removal (91.9% for
25 immobilised fungus vs 87% for suspended fungus, on day 15) and a faster
26 transformation of RB5 in luffa sponge-immobilised cultures than that of free cultures
27 (26.3 decolourisation % per day for immobilised cultures vs 18.2 decolourisation %
28 per day for suspended cultures). Additionally, luffa sponge immobilisation also
29 improved *Leptosphaerulina* sp. production of laccase (Lac) and manganese
30 peroxidase (MnP) (e.g. at day 3, Lac and MnP in immobilised culture were 84% and
31 76%, respectively, higher than suspended culture during CDP removal, and 83%
32 and 5% in bio-treatment of RB5). These results evidenced the potential of Luffa
33 (*Luffa cylindrica*) sponge-immobilised *Leptosphaerulina* sp. as a strategy to enhance
34 the biodegradation process of recalcitrant compounds, to facilitate biomass recycling
35 and to be used in the process scale-up.

36

37 **Keywords:** White-rot fungi; Ligninolytic enzymes; Immobilisation; Antibiotics;
38 Decolourisation.

39 **Capsule:**

40 *Luffa (Luffa cylindrica)* sponge-immobilised *Leptosphaerulina* sp. efficiently removed
41 the antibiotic agent cephadroxyl and the synthetic dye Reactive Black 5.

42

43 **1. INTRODUCTION**

44 Synthetic dyes and antibiotics are chemicals, with inappropriate wastewaters
45 disposal, involved in water sources contamination (Khan et al., 2013). Synthetic dyes
46 are mainly used by the textile industry because their high chemical stability and
47 resistance to oxidising agents and microbial attacks (Bhatia et al., 2017). However,
48 these characteristics complicate their removal from textile industry wastewaters
49 (Wang et al., 2013). Synthetic dyes improper disposal is associated with ecological
50 and health issues. Antibiotics are xenobiotic compounds whose function is to inhibit
51 bacterial growth and are one of the most widely consumed pharmaceuticals in the
52 world (Čvančarová et al., 2015; Lucas et al., 2016). The incorrect disposal of
53 wastewaters with antibiotics alter the natural microbiota of the environment and
54 favour the appearance of increasingly resistant pathogenic bacterial strains (Copete-
55 Pertuz et al., 2018; Xu et al., 2015).

56 Reactive Black 5 (RB5) and the antibiotic cephadroxyl (CPD) are recalcitrant
57 compounds frequently utilised as a relevant model to evaluate novel methods for
58 dyes and antibiotics degradation (Adnan et al., 2014; Enayatizamir et al., 2011;
59 Oliveira et al., 2018; Serna-Galvis et al., 2017). Additionally, these compounds are
60 relevant for Colombia's environment as they have been frequently reported in

61 Colombian water resources (Botero-Coy et al., 2018; Plácido et al., 2016). RB5
62 constitutes 50% of azo dyes employed in the textile industry (Copete-Pertuz et al.,
63 2018; Nabil et al., 2014). RB5 ingestion through contaminated food and water is
64 associated with cancer development and allergic reactions in the respiratory tract
65 (Hussain et al., 2013; Usha et al., 2011). CPD belongs to the group of β -lactam
66 antibiotics and it is associated with toxicity and resistant bacteria proliferation in
67 natural waters (Etebu and Arikekpar, 2016). CPD is frequently distributed and
68 consumed in Colombia; however, its concentration in Colombian wastewater is
69 unknown (Pallares & Martínez, 2012; Serna-Galvis et al., 2017). Other antibiotics
70 such as azithromycin, ciprofloxacin and norfloxacin, can be found in Colombian
71 wastewater at levels above $1 \mu\text{g L}^{-1}$ (Botero-Coy et al., 2018).

72 Antibiotics and dyes removal from wastewaters use conventional methods such as
73 chemical, physical and biological methods; however, they are not able to completely
74 eliminate or remove these compounds (Efligenir et al., 2014; Patel and Bhatt, 2013;
75 Rizzo et al., 2013; Verlicchi et al., 2012). Non-conventional methods such as
76 activated carbon, coagulation, membrane filtration and irradiation are used for dyes
77 and antibiotics removal; although, their use had major disadvantages as they have
78 high costs and utilise toxic oxidising reagents (Adnan et al., 2015; Wang et al., 2013).
79 Traditional activated sludge systems are not recommended for antibiotics removal
80 as they create a favourable environment for the development and propagation of
81 microbial resistance, due to the continuous exposure of bacteria to antibiotics at sub-
82 inhibitory concentrations (Bouki et al., 2013; Rizzo et al., 2013). Therefore, it is
83 necessary to develop novel, economic and environmental friendly methodologies for

84 the removal and degradation of these recalcitrant compounds.

85 White-rot fungi (WRF) is a novel method for removing antibiotics and dyes from
86 aqueous streams (Adnan et al., 2014; Čvančarová et al., 2014; Daâssi et al., 2013;
87 Prieto et al., 2011). The ascomycete fungus *Leptosphaerulina* sp., isolated from
88 lignocellulosic material in the Valle de Aburrá (Medellin, Colombia) (Chanagá Vera
89 et al., 2012; Plácido et al., 2016), has efficiently degraded different synthetic organic
90 dyes and antibiotics including RB5 and CPD (Chanagá Vera et al., 2012; Copete-
91 Pertuz et al., 2019; Copete-Pertuz et al., 2018; Copete et al., 2015; Plácido et al.,
92 2016). The success obtained in the transformation of these pollutants opened the
93 opportunity for scaling up this wastewater treatment process. Therefore, it is
94 necessary to test strategies to increase enzymes production, biotransformation rates
95 and reduce production costs.

96 Fungal immobilisation is a methodology that facilitates the scale up of wastewater
97 treatments and improves the process efficiencies and removal rate (Couto, 2009; Li
98 et al., 2015). Immobilised fungal cultures had higher efficiencies than those in
99 suspension because immobilisation protects the fungal mycelia from shear damage,
100 decreases the viscosity of the culture broth, and recreates the conditions in which
101 the fungus naturally grows (Barry et al., 2009; Couto, 2009; Daássi et al., 2013; Li et
102 al., 2015). *Funalia trogii* immobilised on calcium alginate microspheres achieved a
103 higher decolourisation percentage (93.8%) of the acid dye black 5 than the fungus
104 in suspension (88%) (Park et al., 2006). Similarly, Calcium-alginate-immobilised
105 *Corioloopsis gallica*, *B. adusta*, *T. versicolour* and *T. trogii* achieved high removal
106 levels (85%, 70.9%, 75.3% and 72.2%, respectively) of the Lanaset gray G dye

107 (Daâssi et al., 2013). The use of polymeric gels for fungal immobilisation has many
108 limitations associated with low mechanical resistance and lack of freedom for
109 biomass proliferation, which is why other materials have been considered to support
110 fungal growth (Couto, 2009).

111 The aim of this research was to select the optimum natural supporting material for
112 the immobilisation of the Colombian native fungus *Leptosphaerulina* sp. and to
113 assess the capability of immobilised *Leptosphaerulina* sp. for the biotransformation
114 of model pollutants (RB5 dye and CPD antibiotic) in aqueous systems. This article
115 sought to study for the first time the effect of *Leptosphaerulina* sp. immobilisation for
116 the transformation of pollutants and enzymes production, the effect of fungal
117 immobilisation for antibiotic transformation and the possible use of Colombia's
118 natural materials as support matrices for fungal immobilisation.

119

120 **2. MATERIALS AND METHODS**

121 **2.1. Chemicals**

122 Reactive Black 5 (RB5) (azoic dye, λ_{\max} = 598 nm) was donated by Fabricato-
123 Tejicondor S.A. Cephadroxyl monohydrate 92.9% (CPD) was obtained from
124 syntofarma. Glucose, yeast extract, ammonium molybdate, monobasic potassium
125 phosphate, zinc sulphate heptahydrate, peptone, tetraborate sodium decahydrate,
126 sodium acetate and malt extract, bought from Carlo Erba. Ammonium L-(+)-tartrate
127 98% and 2,6-dimethoxyphenol 99% (DMP) was obtained from Alfa Aesar.

128 Manganese sulphate heptahydrate, iron sulphate heptahydrate, ammonium
129 sulphate, acetic acid, sodium chloride, formic acid, potassium chloride, tartaric acid,
130 hydrogen peroxide, acetonitrile, methanol and Mueller-Hinton agar were bought from
131 Merck. 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt
132 98% (ABTS) from Sigma-Aldrich.

133 **2.2. Microorganism and culture conditions**

134 *Leptosphaerulina* sp. was obtained from PROBIOM research group's
135 microorganisms collection (CECT 20913) (Copete et al., 2015; Plácido et al., 2016).
136 The fungus was maintained in malt extract agar at 4 °C until use. Mycelium from a
137 10–days old culture was homogenised and employed as inoculum in the removal
138 process (Copete et al., 2015). Experiments were carried out in a culture medium (pH
139 5.6) containing 10 g L⁻¹ glucose, 5 g L⁻¹ peptone, 2 g L⁻¹ ammonium tartrate, 1 g L⁻¹
140 yeast extract, 1 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ KCl and 0.5 g L⁻¹ MgSO₄ · 7H₂O, and 1 mL
141 mineral solution [100 mg L⁻¹ B₄O₇Na₂ · 10H₂O, 70 mg L⁻¹, ZnSO₄ · 7H₂O, 50 mg L⁻¹
142 FeSO₄ · 7H₂O, 10 mg L⁻¹ (NH₄)₆Mo₇O₂₄ · 4H₂O and 10 mg L⁻¹ MnSO₄ · 7H₂O] (Copete-
143 Pertuz, et al., 2018). This work was authorised by the Autoridad Nacional de
144 Licencias Ambientales (ANLA) under the research permit No. 8 de 2010 (Resolución
145 324 de 2014) and the Ministerio de Ambiente y Desarrollo Sostenible of Colombia
146 with the agreement No. 96 of 2014 to genetic resources access.

147

148

149 **2.3. Evaluation of natural support materials for the immobilisation of**
150 ***Leptosphaerulina* sp.**

151 Four different natural materials (charcoal, luffa sponge, wood chips and cork) were
152 obtained from a local store (Medellín, Colombia). They were evaluated in order to
153 select the most appropriate immobilisation material for *Leptosphaerulina* sp. The
154 supporting materials were placed in 250 mL Erlenmeyer flasks with 100 mL of culture
155 medium. The supporting materials initial load (charcoal, 5 g; luffa sponge, 1 g; wood
156 chips, 5 g; cork of 5 cm², 3 g) was selected based on each material characteristics.
157 The luffa sponge and wood chips initial load corresponded to the values reported by
158 El-Sherif et al., (2013) and Mahmoud (2007), respectively. After the support
159 materials were added into the Erlenmeyer flasks, these were inoculated with 5 mL
160 of *Leptosphaerulina* sp. homogenised and incubated at 160 rpm, 28 °C for 15 days
161 (Ehlers and Rose, 2005; Kasinath, 2003). The controls for this experiment were
162 *Leptosphaerulina* sp. cultures without supporting material and the supporting
163 material with culture medium and without fungal strain. All assays were performed
164 in triplicate. The experiment response variable was the amount of biomass retained
165 in each of the supports, which was determined qualitatively by observing the
166 biomass growth in the material during the days 3, 7, 12 and 15. The biomass growth
167 was classified in no growth (–), low growth (+), medium growth (++), and significant
168 growth (+++).

169

170 **2.4. Biotransformation of CPD and RB5 by immobilised *Leptosphaerulina***
171 **sp.**

172 The biotransformation assays utilised 250 mL conical flasks containing 100 mL of
173 liquid medium (pH 5.6) (**Section 2.2**) supplemented with CPD (15256 $\mu\text{g L}^{-1}$) or RB5
174 (200 mg L^{-1}) and a luffa sponge disk (approximately 3 cm thick and 2 g weight).
175 Flasks were inoculated with 5 mL of mycelium previously homogenised (Copete-
176 Pertuz et al., 2018), and later incubated at 28 °C and 160 rpm during 15 days. The
177 cultures were sampled on days 3, 7, 12 and 15. As sampling volume, 4 mL were
178 withdrawn from the flasks at each time point. CPD removal, antimicrobial activity,
179 and RB5 decolourisation percentage were used as response variables. CPD and
180 RB5 removal were followed by high performance liquid chromatography (HPLC) and
181 UV-VIS spectrophotometry, respectively. Additionally, ligninolytic enzymes activities,
182 protein concentration and the retained biomass dry weight were evaluated. All
183 experiments were carried out in triplicate. The *t*-test was utilised to establish the
184 differences between immobilised and suspended cultures with and alpha of 0.05.
185 The *t*-test analyses were performed in the R software version 3.4.3.

186 Six assays were used as controls: 1) *Leptosphaerulina* sp. cultured with luffa sponge
187 and without RB5 or CPD (LC). 2) *Leptosphaerulina* sp. cultured without luffa sponge
188 and without RB5 or CPD (HM). 3) *Leptosphaerulina* sp. cultured with antibiotic and
189 without luffa sponge (AC). 4) *Leptosphaerulina* sp. cultured with dye and without luffa
190 sponge (CC). 5) Culture media with antibiotic and luffa sponge, without
191 *Leptosphaerulina* sp. inoculum (AM). 6) Culture media with dye and luffa sponge,
192 without *Leptosphaerulina* sp. inoculum (CM).

193 **2.5. Determination of ligninolytic enzymatic activities**

194 Lac activity was measured by monitoring the oxidation of ABTS (3 mM) in sodium
195 tartrate buffer (0.1 M, pH 3.0) (Ciullini et al., 2008; Zhou et al., 2014). Similarly, the
196 VP activity was determined from the oxidation of ABTS (3 mM) in buffer solution
197 sodium tartrate (pH 3.0, 0.1 M) and H₂O₂ (0.1 mM) (Camarero et al., 1999; Copete
198 et al., 2015). Lac and VP activities were monitored spectrophotometrically at a
199 wavelength of 420 nm and a molar extinction coefficient of (ϵ_{420} , 36000 M⁻¹ cm⁻¹).
200 MnP activity was measured based on the oxidation of 2,6-dimethoxyphenol (DMP,
201 1 mM), in sodium acetate (pH 4.5, 0.1 M) with H₂O₂ (0.1 mM) and MnSO₄ (1 mM) at
202 469 nm (ϵ_{469} , 27500 M⁻¹ cm⁻¹) (Mizuno et al., 2009). Enzymatic activity was
203 determined in triplicate and expressed in units (U) of enzyme per milligram of protein
204 (U mg⁻¹). U is defined as the amount of enzyme that catalyses 1 μ mol of substrate
205 in 1 min (Ciullini et al., 2008; Enayatzamir, 2009).

206 **2.6. Determination of protein concentration and dry weight**

207 The protein concentration in the fungal extract was determined by the Bradford's
208 method. A calibration curve was constructed from bovine serum albumin solutions
209 (Bradford, 1976). The fungal biomass dry weight was determined by measuring the
210 amount of biomass retained in the support material. The support material was dried
211 before and after *Leptosphaerulina* sp. growth. The support material was dried until
212 constant weight at 60 °C in a convection oven (Precisa®). This procedure was done
213 in triplicate.

214

215 **2.7. Chromatographic analysis of antibiotic**

216 CPD removal was determined by reverse phase high performance liquid
217 chromatography (RP)-HPLC (Thermo Scientific DIONEX UltiMate 3000) equipped
218 with a Diode Array Detector (DAD) and a Thermo scientific Acclaim 120 column (C-
219 18 5 μ m, 4.6x100mm). A homogeneous mixture of acetonitrile (C₂H₃N) and formic
220 acid buffer (CH₂O₂) (10 mM, pH 3), 20/80 (% v/v) was used as mobile phase (Serna-
221 Galvis, et al., 2017). The chromatography utilised 20 μ L of the samples, a mobile
222 phase flow of 1 mL min⁻¹ and a fixed wavelength of 225 nm for the DAD detector.

223 **2.8. Residual antibacterial activity assays**

224 As additional method to determine the degree of antibiotic elimination, the residual
225 antibacterial activity (AA) of CPD and its transformation products were evaluated
226 through the Kirby-Bauer test with the gram-positive bacteria *Bacillus cereus* as the
227 indicator bacterial strain. Petri dishes with Mueller-Hinton Agar were inoculated with
228 15 μ L of the bacterial suspension (optical density of 0.6 at 580 nm). When the agar
229 solidified, 6 mm holes were made in its surface. 30 μ L of sample (antibiotics or
230 transformation products) covered each hole and the petri dishes incubated at 37 °C
231 for 24 h. The AA was determined based on the measurement of the inhibition halos
232 of the samples (Copete-Pertuz et al., 2018; Čvančarová et al., 2015; Čvančarová et
233 al., 2013).

234

235

236

237 **2.9. Evaluation of decolourisation of RB5 in aqueous solution**

238 Decolourisation of RB5 was followed spectrophotometrically (Shimadzu UV-1800
239 spectrophotometer) at 598 nm and was expressed in terms of decolourisation
240 percentage ($D\%$) (**Equation 1**) (Forootanfar et al., 2016; Shedbalkar et al., 2008):

$$241 \quad D\% = \frac{A_0 - A_t}{A_0} \times 100 \quad \text{Equation 1}$$

242 Where A_0 corresponds to the initial absorbance and A_t to the absorbance after the
243 sampling time.

244

245 **3. RESULTS**

246 **3.1. Evaluation of different materials for the immobilisation of** 247 ***Leptosphaerulina* sp.**

248 Four natural materials (charcoal, luffa sponge, wood chips and cork) were evaluated
249 as immobilisation supports for *Leptosphaerulina* sp growth. The immobilisation
250 experiments did not have RB5 or CPD to avoid any external factor influencing the
251 fungal growth on the supporting material. **Table 1** displays the qualitative growth
252 measurements for the different supports. Charcoal was the only material without
253 visible *Leptosphaerulina* sp. growth; this lack of growth corresponded to an inhibition
254 produced by compounds in the charcoal surface or by compounds released from it
255 into the liquid media. Although visible growth was not observed, the fungus was still
256 active; this was identified by the presence of enzymatic activities in the samples

257 (data not shown). On wood chips and cork, *Leptosphaerulina* sp. had medium and
258 low proliferation, respectively. On wood chips, *Leptosphaerulina* sp. grew in specific
259 areas but its distribution did not follow any pattern. On cork, small bodies proliferated
260 on the material's corners and indentations; however, the fungal growth on cork was
261 lower than that of luffa sponge. In luffa sponge, *Leptosphaerulina* sp. exhibited a
262 significant proliferation. In this material, *Leptosphaerulina* sp. biomass distributed
263 principally inside the luffa porous matrix and slightly on the outer surface. As the
264 most significant *Leptosphaerulina* sp. biomass proliferation was observed in the luffa
265 sponge (**Table 1**), this material was selected as the immobilisation matrix for the
266 subsequent biotransformation assays.

267 **3.1. Biotransformation of cephadroxyl using suspended and immobilised** 268 ***Leptosphaerulina* sp.**

269 The CPD removal was compared between the immobilised fungus (AL) and the
270 suspended fungus (AC) during 15 days using as controls, assays with suspended
271 and immobilised fungus without CPD and with luffa and CPD and without fungus.
272 **Figure 1** exhibits the CPD removal percentage and the antibacterial activity
273 percentage (AA %) vs time for the immobilised and suspended *Leptosphaerulina* sp.
274 cultures. The abiotic control achieved a 20% reduction of the CPD initial
275 concentration ($15256 \mu\text{g L}^{-1}$); indicating sorption of CPD into the luffa sponge. In the
276 immobilised and suspended cultures, the CPD concentration decreased
277 considerably (~77.7%) on the third day; after this day, the removal increased at a
278 constant rate (1.8%/day) until day 12. The CPD reduction was superior in the
279 immobilised experiment especially at day 15 when the immobilised culture reached

280 almost 100% removal (**Figure 1A**). In contrast, at day 15, the suspended cultures
281 removed 94.4% of CPD (**Figure 1B**). The immobilised experiment achieved the
282 largest CPD removal % using a mixture of removal mechanisms, including sorption
283 in the luffa sponge, enzymatic degradation in the liquid media and enzymatic
284 degradation of the sorbed antibiotic in the luffa sponge. The *t*-test performed to the
285 results from day 15 evidenced significant differences between the CPD removal
286 percentages of immobilised and suspended cultures (p -value < 0.001). In contrast,
287 the *t*-test performed to the results from day 3, 7 and 12, indicated that the two
288 treatments were not statistically different (p -value > 0.05) (**Supplementary**
289 **material**).

290 Antimicrobial activity (AA) of CPD and its degradation products against *Bacillus*
291 *cereus* was evaluated for the immobilised and suspended cultures (**Figure 1**). In
292 both experiments, the inhibition halo was observed only at day 0 (average diameter
293 of 10.33 ± 0.08 mm), which corresponds to the maximum CPD concentration in the
294 liquid medium ($15496 \mu\text{g L}^{-1}$). The samples from day 3, 7, 12 and 15 did not generate
295 an inhibition halo, indicating that the initial three days of biotransformation with
296 immobilised or suspended *Leptosphaerulina* sp. were enough to significantly reduce
297 CPD concentration and completely reduce the AA.

298 Lac, MnP and VP enzymatic activities were determined for the suspended and
299 immobilised cultures and their results are described in **Figure 2**. In both cultures, the
300 enzymatic activities increased during the first three days followed by a constant
301 enzymatic activity reduction. The immobilised *Leptosphaerulina* sp. experiment
302 achieved the highest expression of the three ligninolytic enzymes measured; Lac

303 (5.56 U mg⁻¹), VP (8.29 U mg⁻¹) and MnP (5.75 U mg⁻¹). During the initial three days,
304 the immobilised culture achieved an enzymatic production 6, 2.5 and 5.5–times
305 higher than the suspended culture's Lac, VP and MnP activities, respectively (**Figure**
306 **2**). The period of maximum enzymatic production correlates with the maximum CPD
307 removal velocity (26%/day). In suspended cultures, VP activity maximum expression
308 was detected at day 7 (3.13 U mg⁻¹); whereas, for Lac (0.92 U mg⁻¹) and MnP (1.03
309 U mg⁻¹) it was detected on the third day. The decreasing trend in the enzymatic
310 activity observed after the third or seventh day, in immobilised and suspended
311 cultures is correlated with the depletion of carbon or nitrogen sources (Copete-
312 Pertuz et al., 2018).

313 The control experiments demonstrated the eliciting effect that the antibiotic produced
314 to the suspended cultures' VP expression. This positive effect was greater during
315 day 7, when the VP activity, in the suspended culture with CPD, was almost three
316 times higher than the VP activity for the suspended control without antibiotic. The
317 Lac activity was similar in both cases; whereas, the MnP production was higher in
318 the control than that of the suspended culture with CPD.

319 Similar to the suspended cultures, the control experiments exhibited the influence of
320 luffa immobilisation in the production of VP and Lac. The immobilised culture control
321 achieved a greater expression of VP and Lac than the suspended culture control. In
322 contrast, MnP activity was higher in the suspended control than that of the
323 immobilised control. The differences in the enzymatic activities between the
324 immobilised *Leptosphaerulina* sp. with CPD and the controls without CPD

325 demonstrated the elicitation of these enzymatic activities by the combination of luffa
326 sponge immobilisation and CPD presence.

327 **Figure 3** describes the dry biomass obtained in the different experiments. These
328 results exhibited a difference between the controls biomass and the immobilised
329 biomass. The biomass immobilised on the luffa sponge control without pollutant
330 doubled the amount of biomass from the immobilised assay with antibiotic. The
331 coexistence of high enzymatic activity and low biomass concentration is associated
332 with the nutritional sources or with CPD inhibition. As CPD enhance the production
333 of enzymes the nutritional sources utilised for producing biomass were reduced,
334 whereas, in absence of CPD, the sources are used for biomass growing instead of
335 been used for enzymatic production. Additionally, if CPD produce growth inhibition,
336 the fungi can produce additional enzymes to attack the inhibitory compound.

337 **3.2. RB5 Decolourisation**

338 Similar to the procedure executed in the CPD biotransformation, the RB5 dye
339 decolourisation was evaluated with immobilised (CL) and suspended cultures (CC)
340 during 15 days, the experiment included the measurement of decolourisation
341 percentage ($D\%$) and ligninolytic activities. **Figure 4** presents the $D\%$ of RB5 in both
342 the *Leptosphaerulina* sp. suspended and immobilised cultures. During the first 3
343 days, the immobilised culture had a higher decolourisation rate (26.3 $D\%/day$) than
344 the suspended culture (18.2 $D\%/day$). At the third day, the immobilised culture's $D\%$
345 was 86.8%; whereas, for the suspended culture was 59.2% (p -value < 0.01). For the
346 duration of the experiment, the immobilisation experiment's $D\%$ was higher than that

347 of the suspended culture. From **Figure 4**, the control without the fungus and without
348 luffa did not produced RB5 removal. Likewise, the control assay using the support
349 matrix without the fungus demonstrated that sorption on the luffa sponge has a small
350 participation (9%) in RB5 decolourisation. Therefore, the RB5 removal exhibited by
351 the immobilised and suspended cultures is due to biological and enzymatic
352 mechanisms and not to external factors such as light or sorption in the Erlenmeyer
353 flask or the immobilisation matrix. At days 3, 7, and 12, the immobilised and
354 suspended cultures were statistically different (p -value < 0.05). In contrast, at day
355 15, the immobilised experiment's $D\%$ (91.9%) was numerically higher than that of
356 the suspended culture (87%), but their difference was not statistically significant (p -
357 value > 0.05) (**Supplementary material**). The greatest difference between
358 immobilised and suspended culture was the decolourisation rate; the immobilised
359 experiment reached the maximum $D\%$ at the 7 day, half of the time required by the
360 suspended culture. This rise in the decolourisation rate was an evidence of the
361 positive effect of luffa sponge immobilisation on *Leptosphaerulina* sp. decolourising
362 activity.

363 As well as for CPD, Lac, MnP and VP enzymatic activities were determined for all of
364 the previously mentioned assays. **Figure 5** describes the three enzymatic profiles
365 for the immobilised and suspended cultures and the control assays. On the third day,
366 the luffa immobilised-fungus expressed the highest Lac activity (0.48 U mg^{-1}) (**Figure**
367 **5A**); whereas, the suspension culture reached its maximum activity (0.22 U mg^{-1}) on
368 the seventh day. The VP activity profile for the immobilised and suspended
369 experiments reached their maximum values on the seventh day with 0.76 U mg^{-1}

370 and 1.68 U mg⁻¹, respectively. For the MnP activity, the suspended culture exhibited
371 (1.02 U mg⁻¹) a slightly greater maximum activity than the immobilised fungus (0.89
372 U mg⁻¹); in both cases, the maximum activity was reached on the seventh day. The
373 *Leptosphaerulina* sp. cultured without luffa sponge and without pollutant achieved
374 the highest MnP activity.

375 Finally, the dry biomass from the immobilised and suspended culture was measured
376 at the end of the process. **Figure 3** shows the average biomass dry weight obtained
377 for *Leptosphaerulina* sp. cultured on luffa sponge with (CL) and without (LC) dye. A
378 considerable lower amount of biomass grew in the luffa sponge in the experiments
379 with dye compared with the experiment without dye. RB5 generated a reduction in
380 *Leptosphaerulina* sp. growth; however, it was lower than the growth reduction
381 generated by CPD.

382

383 **4. DISCUSSION**

384 This article is the first report showing fungal immobilisation for antibiotics removal
385 and the first report about the effect of immobilisation in the biotransformation of RB5
386 by *Leptosphaerulina* sp. Additionally, this article is one of the first reports of
387 Colombia's natural materials used as support matrix for fungal immobilisation. The
388 natural matrices experiment demonstrated that the immobilisation of
389 *Leptosphaerulina* sp. required lignocellulosic materials with high porosity and large
390 porous size. This affirmation was supported by the absence of growth in charcoal
391 (non-lignocellulosic material) and the reduced growth in cork and wood chips.

392 Although charcoal, cork and wood chips have high porosity, the porous size is
393 smaller making them unsuitable to support fungal growth. Luffa sponge has a fibrous
394 network with high porosity, significant surface area and larger pore size making this
395 material ideal for fungal biomass immobilisation (Sriharsha et al., 2017). The
396 significant biomass in the luffa sponge suggests that this material could emulate the
397 conditions in which *Leptosphaerulina* sp. grows naturally. Luffa sponge has similar
398 composition (cellulose 50-60%, hemicellulose 25-28% and lignin 10-12%) (Saeed
399 and Iqbal, 2013) as other lignocellulosic materials (turf grasses) utilised as substrate
400 by other *Leptosphaerulina* genus members (Mitkowski and Browning, 2004).
401 Previous studies have evaluated luffa sponge as a support for the immobilisation of
402 filamentous fungi such as *P. chrysosporium*, *Trichoderma viride* and *Funalia trogii*
403 **(Table 2)**. The biocompatibility and biomass distribution between *Leptosphaerulina*
404 sp. and luffa sponge is similar to *P. chrysosporium* immobilisation in luffa sponge
405 (Iqbal and Edyvean, 2005). In that study, *P. chrysosporium* hyphae significantly grew
406 in the internal cavities of the fibrous network.

407 *Leptosphaerulina* sp. immobilised in luffa sponge increased the CPD removal % and
408 the *D*% compared with the suspended culture. However, CPD and RB5 removal
409 increases were different. On one hand, the immobilised CPD removal increased on
410 the final measurement point compared with the suspended culture, this improvement
411 was correlated with a significant production of ligninolytic enzymes and a
412 considerable reduction in the biomass proliferation on the luffa sponge. As no other
413 authors have evaluated fungal immobilisation for antibiotics removal, the comparison
414 with other research works included other pharmaceutical compounds **(Table 2)**. The

415 CPD removal % obtained by the luffa immobilised culture reached higher removals
416 than other immobilised fungi treating other pharmaceutical compounds. *P.*
417 *chrysosporium* BKM-F-1767 immobilised in wood sawdust obtained 80% removal
418 and ~100% removal of carbamezepine and naproxen, respectively (Li et al., 2015).
419 On the other hand, RB5 removal (92%) improved during the initial days (3 to 7)
420 instead of the final measurement point (15 day). This decolourisation rate
421 improvement achieved a 50% reduction in the decolourisation time compared with
422 the suspension culture. The RB5 removal obtained by *Leptosphaerulina* sp.
423 immobilised in luffa sponge (92%) is higher than that of *Trametes pubescens*
424 immobilised in stainless steel sponges (74%) and lower than that of *Trametes*
425 *versicolor* immobilised in luffa sponges (98%). In both cases, the RB5 concentrations
426 were lower (30 and 150 mg L⁻¹) than the employed in this study (200 mg L⁻¹). The
427 positive effect of fungal immobilisation has also been demonstrated for removing
428 other dyes such as brilliant green, reactive blue 98, evans blue, and acid blue 74
429 (**Table 2**).

430 The different results obtained by the combination of immobilisation and pollutant can
431 be associated with different *Leptosphaerulina* sp. morphologies. Fungal morphology
432 can be influenced by different environmental factors such as reactor geometry,
433 agitation speed, airflow and culture media which can generate variability in the
434 enzymatic expression (Krull et al., 2013; Naghdi et al., 2018). RB5 removal by
435 immobilised *Leptosphaerulina* sp. was associated with dye sorption by the solid
436 matrix (9%), sorption by the fungal biomass and enzyme production by the fungus.

437 This combined mechanism explains the faster degradation observed on the first days
438 of the immobilised *Leptosphaerulina* sp. assay.

439

440 The production of ligninolytic enzymes in cultures with antibiotics have been
441 dependent on the physicochemical characteristics of the antibiotic (Naghdi et al.,
442 2018). In this case, CPD phenolic structure can act as an inducer for the enzymatic
443 activities or act as laccase mediator (Camarero et al., 2012; Jeon et al., 2012). In
444 contrast, the immobilised culture with RB5 did not produce high enzymatic activities,
445 however, this culture was able to significantly reduce the decolourisation time and
446 achieve higher decolourisation than the suspended culture. The high decolourising
447 activity associated with low enzymatic activities can be explained by the production
448 of iso-enzymes, which can have more affinity for RB5 (Copete et al., 2015).
449 Lignocellulosic materials as a support matrix can trig Iso-enzymes expression as this
450 type of material stimulates the production of ligninolytic enzymes and favours the
451 attachment of fungal biomass by simulating fungal growth in nature (Jeon et al.,
452 2012; Masran et al., 2016). Additionally, as the biomass was in contact with the
453 supporting matrix, cell wall associated oxidases could participate in the degradation
454 of the pollutants, these enzymes can explain the high decolourising activity with low
455 suspended ligninolytic activities (García-Santamarina and Thiele, 2015; Zucca et al.,
456 2016). The enzymes involved in the biotransformation process of the antibiotic are
457 not limited to those evaluated in this research, although, they are the most commonly
458 reported. The production and participation of other enzymes depends on the nature
459 and structure of the pollutants in the culture media.

460 As the CPD removal by immobilised and suspended cultures had similar values
461 during the majority of the process and the only statistical difference between them
462 was obtained during the final day. The selection of immobilised over suspended
463 cultures for CPD removal should be focused not only in the removal percentage but
464 also in the other advantages demonstrated by the immobilised *Leptosphaerulina* sp.
465 CPD treatment with immobilised cultures were able to produce higher amounts of all
466 the enzymatic activities. Additionally, this hyper production can include the
467 production of other iso-enzymes or other enzymes. Technically, immobilisation
468 allows biomass re-usage and facilitates the *in-situ* operation as it can be easily
469 transported. As the processing time for removing RB5 was significantly reduced by
470 immobilised *Leptosphaerulina* sp. this process is a better option for textile
471 wastewater treatment and future process scale-up. Fungal immobilisation can
472 improve the enzymes quality, activity, or type (Bertrand et al., 2017; Dubey et al.,
473 2017). These differences can generate benefits such as greater affinities for
474 substrates, higher redox potentials and greater stability (Bertrand et al., 2017; Dubey
475 et al., 2017). *Leptosphaerulina* sp. immobilisation in luffa sponge is an alternative to
476 scale-up and reutilise *Leptosphaerulina* sp. biomass for different type of pollutants.
477 Future research will be focused on the evaluation of immobilised biomass recycling,
478 the simultaneous removal of pollutants and reactor configuration selection for
479 scaling-up the *Leptosphaerulina* sp. pollutants biotransformation process.

480

481 5. CONCLUSIONS

482 Luffa sponge was selected as the optimum supporting material for the immobilisation
483 of the Colombian native fungus *Leptosphaerulina* sp. Suspended and immobilised
484 *Leptosphaerulina* sp. cultures achieved significant removal of RB5 and CPD (>90%).
485 However, *Leptosphaerulina* sp. immobilised on Luffa sponge exhibited better
486 characteristics for removing CPD and RB5 from aqueous systems than suspended
487 cultures. RB5 removal time was reduced more than 50% by immobilised
488 *Leptosphaerulina* sp. Immobilised cultures for CPD biotransformation significantly
489 improved the production of ligninolytic enzymes, in contrast, immobilised cultures for
490 RB5 removal did not achieve high enzymes production. The removal of both
491 pollutants included a combined removal mechanism comprising sorption on the
492 immobilising matrix, biomass sorption and enzymatic degradation. These results
493 demonstrated the potential of *Leptosphaerulina* sp. immobilisation as a viable
494 strategy for enhancing pollutants removal and facilitating the industrial application of
495 *Leptosphaerulina* sp. bio-transformations.

496

497 6. ACKNOWLEDGEMENTS

498 The authors would like to thank the research system of the Universidad Nacional de
499 Colombia for the financial support provide by the grant No. 35945 “Biotransformación
500 de antibióticos β -lactámicos por el aislado fúngico nativo *Leptosphaerulina* sp. y sus
501 enzimas ligninolíticas”. L.S. Copete-Pertuz would like to thank the Grupo de
502 Investigación en Remediación Ambiental y Biocatálisis (GIRAB) at Universidad de

503 Antioquia (Medellin, Colombia) for suppling the cephadroxyl and the HPLC and, the
504 Chocó state Government and the Departamento Administrativo de Ciencia,
505 Tecnología e Innovación, Colombia (COLCIENCIAS) for the financial support via the
506 granting of “Formación de capital humano para el departamento del Chocó -
507 Doctorado Nacional” (Convocatoria No 694 de 2014) for her PhD studies. Dr Jersson
508 Plácido would like to thank the support provided by the European Regional
509 Development Fund / Welsh Government funded BEACON+ research program
510 (Swansea University).

511

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TABLE AND FIGURE CAPTIONS

Table 1. Growth of fungi on materials used for biomass immobilisation after fifteen days of the experiment. The experiment was carried out in the absence of antibiotic or dye.

Table 2. Comparative table of immobilised microorganisms used for removing pollutants from aqueous systems.

Figure 1. CPD removal % and AA % over time. **A)** AL *Leptosphaerulina* sp. immobilised, **B)** AC *Leptosphaerulina* sp. in suspension. Experimental conditions: 28 °C, 160 rpm, pH= 5.6, 15 days. Data points are means and standard deviations (n= 3). There were no significant differences between AL and AC results at any moment (p-value > 0.05) except at day 15 (p-value < 0.001).

Figure 2. Evaluation of the ligninolytic enzymatic activities. AL to the immobilisation experiments with CPD. AC are the fungus cultures in suspension with CPD. HM is the cultivation of the fungus in suspension free of CPD antibiotic. LC corresponds to the immobilisation experiments free of CPD. **A)** Specific activity Lac, **B)** Specific activity VP, **C)** Specific activity MnP. Experimental conditions: 28 °C, 160 rpm, pH= 5.6, 15 days.

Figure 3. Quantification of dry weight for immobilisation experiments. AL corresponds to immobilisation assays with antibiotic. CL are the dye-containing immobilisation experiments. LC corresponds to the antibiotic-free dye-free

immobilisation experiment. Experimental conditions: 28 °C, 160 rpm, pH= 5.6, 15 days.

Figure 4. Decolourisation percentage of RB5. CC corresponds to fungus cultures in suspension. CL are the immobilisation assays. Control 1: culture medium with RB5 dye [200 mg L⁻¹], without fungus and without luffa sponge. Control 2: culture medium with RB5 dye [200 mg L⁻¹], with luffa sponge and without fungus. Experimental conditions: 28 °C, 160 rpm, pH= 5.6, 15 days. Data points are means and standard deviations (n= 3). There is a significant difference between CL and CC results at every moment (p-value < 0.01) except at day 15 (p-value > 0.05).

Figure 5. Evaluation of the ligninolytic enzymatic activities. CL correspond to the immobilisation experiment with dye. CC is the fungus culture in suspension with dye. HM is the dye-free suspended fungus culture. LC corresponds to the dye-free immobilisation experiments. **A)** Specific activity Lac for dye containing cultures, **B)** Specific activity VP for dye containing cultures, **C)** Specific activity MnP for dye containing cultures. Experimental conditions: 28 °C, 160 rpm, pH= 5.6, 15 days.

Table 1. Growth of fungi on materials used for biomass immobilisation after fifteen days of the experiment. The experiment was carried out in the absence of antibiotic or dye.

Support material	Growth¹
Charcoal	-
Luffa sponge	+++
Wood chips	+
Cork	+

¹ No growth (-), low growth (+), medium growth (++), and significant growth (+++). Control: *Leptosphaerulina* sp. in suspension had significant (+++) growth.

Table 2. Comparative table of immobilised microorganisms used for removing pollutants from aqueous systems.

Microorganism	Support material	Pollutant	Initial concentration	Removal percentage	Ref.
<i>Leptosphaerulina</i> sp.	Luffa sponge	CPD	15256 µg L ⁻¹	~100%	This work
<i>Leptosphaerulina</i> sp.	Luffa sponge	RB5	200 mg L ⁻¹	91.9%	This work
<i>Trametes versicolor</i>	Luffa sponge	RB5	150 mg L ⁻¹	98%	(Fernández et al., 2009)
<i>P. chrysosporium</i> BKM-F-1767	Wood dust	Naproxen	1000 µg/leach	~100%	(Li et al., 2015)
		Carbamezepine	1000 µg/leach	80%	
<i>Polyporus picipes</i> (RWP17), <i>Gleophyllum odoratum</i> (DCa)	Polypropylene Washer	Evans Blue	100 mg L ⁻¹	~100%	(Zabłocka-godlewska et al., 2017)
		Brilliant green	100 mg L ⁻¹	85%	
<i>Trametes pubescens</i>	Stainless steel sponges	RB5	30 mg L ⁻¹	74%	(Enayatzamir et al., 2009)
<i>Trametes versicolor</i>	Free pellets	Acid blue 74	50 mg L ⁻¹	96.8%	(Yildirim & Yesilada, 2015)
		Reactive Blue 198	50 mg L ⁻¹	91.3%	
<i>Funalia trogii</i>	Free pellets	Acid blue 74	50 mg L ⁻¹	96.1%	(Yildirim & Yesilada, 2015)
		Reactive Blue 198	50 mg L ⁻¹	87.8%	

Figure 1. CPD removal % and AA % over time. **A)** AL *Leptosphaerulina* sp. immobilised, **B)** AC *Leptosphaerulina* sp. in suspension. Experimental conditions: 28 °C, 160 rpm, pH= 5.6, 15 days. Data points are means and standard deviations (n= 3). There were no significant differences between AL and AC results at any moment (p-value > 0.05) except at day 15 (p-value < 0.001)

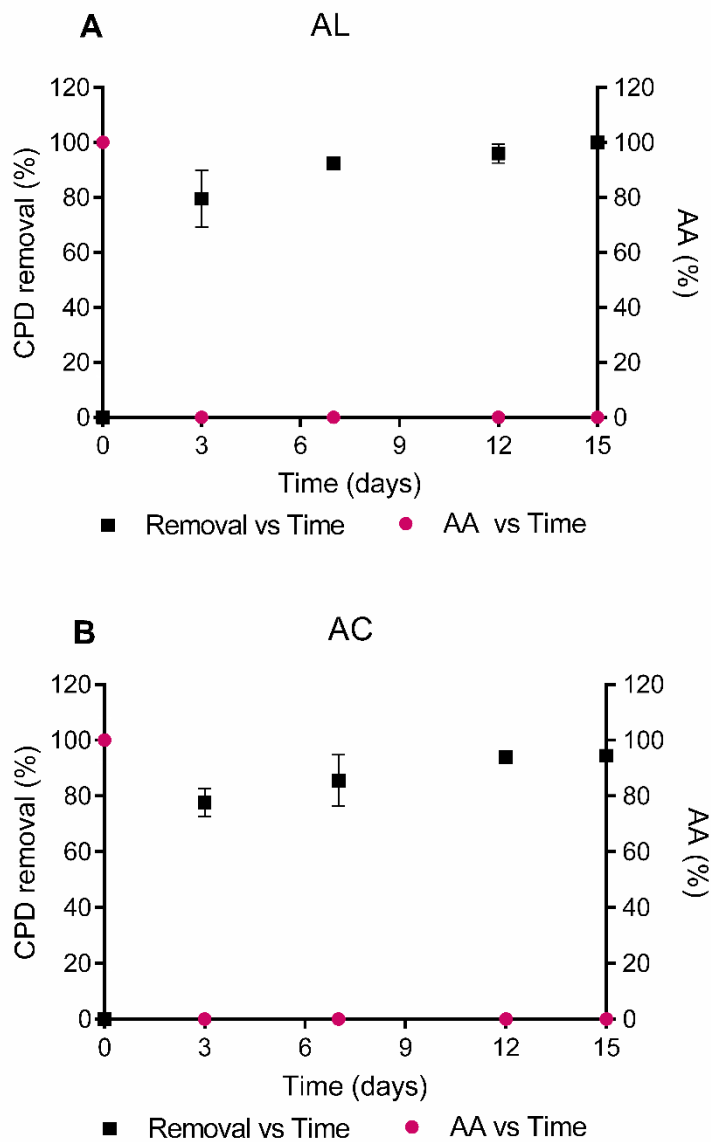


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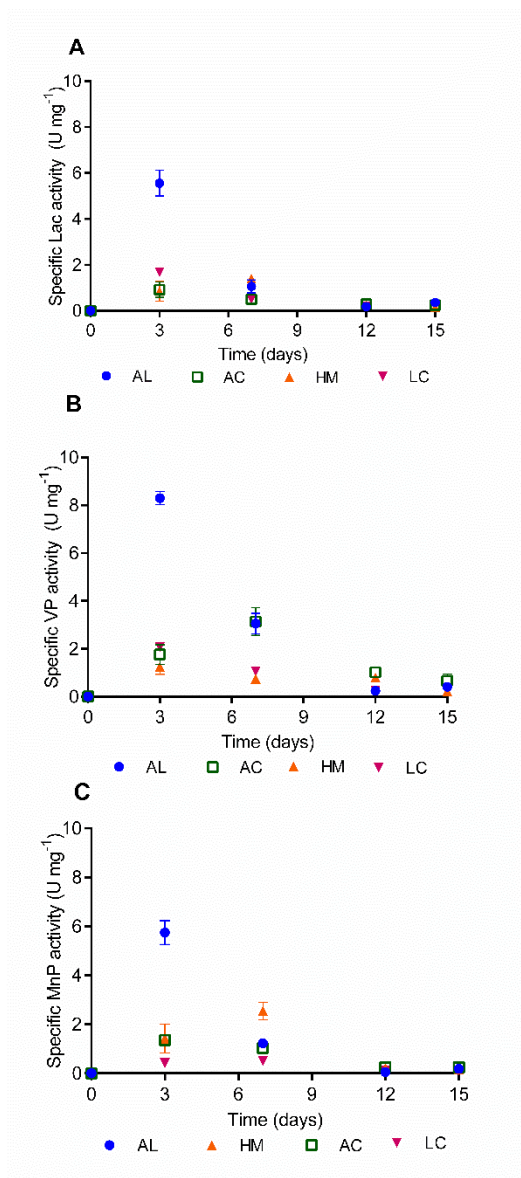


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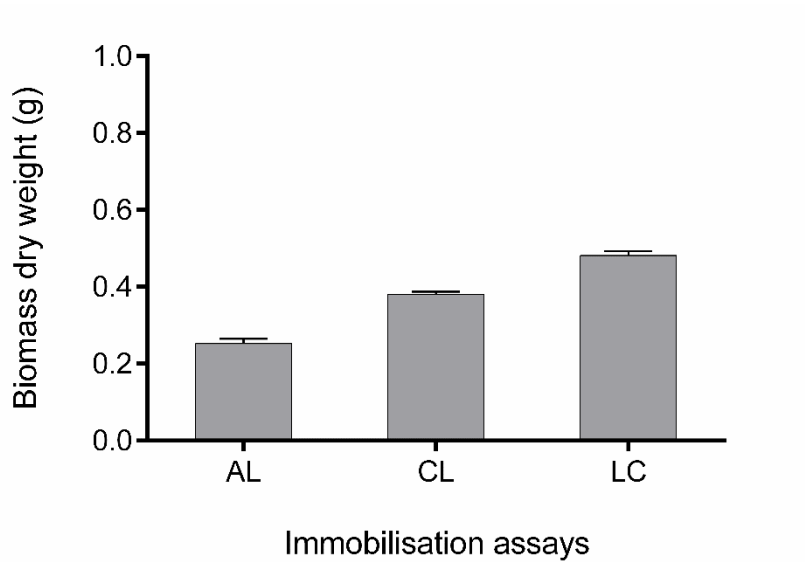


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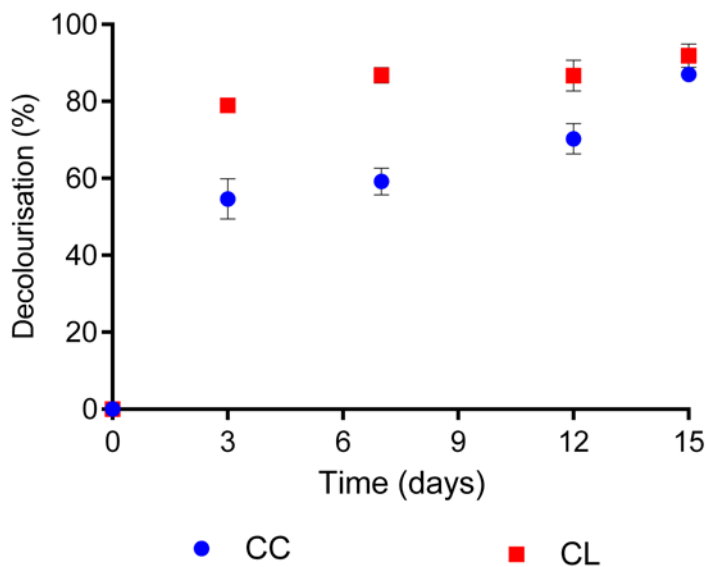


Figure 5. Evaluation of the ligninolytic enzymatic activities. CL correspond to the immobilisation experiment with dye. CC is the fungus culture in suspension with dye. HM is the dye-free suspended fungus culture. LC corresponds to the dye-free immobilisation experiments. **A)** Specific activity Lac for dye containing cultures, **B)** Specific activity VP for dye containing cultures, **C)** Specific activity MnP for dye containing cultures. Experimental conditions: 28 °C, 160 rpm, pH= 5.6, 15 days.

