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Elimination of Isoxazolyl-Penicillins antibiotics in waters by the ligninolytic native Colombian strain *Leptosphaerulina* sp. considerations on biodegradation process and antimicrobial activity removal

Ledys S. Copete-Pertuz[1], Jersson Plácido[2], Efraím A. Serna-Galvis[3], Ricardo A. Torres-Palma[3], Amanda Mora[1*]

1 *Grupo de Investigación Producción Estructura y Aplicación de Biomoléculas (PROBIOM), Escuela de Química, Facultad de Ciencias, Universidad Nacional de Colombia – Sede Medellín, Calle 59A No 63-20, Medellín, Colombia.*

2 Centre for Cytochrome P450 Biodiversity, Institute of Life Science, Medical School, Swansea University, Swansea, SA2 8PP, Wales, UK.

3 Grupo de Investigación en Remediación Ambiental y Biocatálisis (GIRAB), Instituto de Química, Facultad de Ciencias Exactas y Naturales, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia.

[*] Corresponding author.

Tel.: +57(4)4309339;

E-mail address: almora@unal.edu.co

17 **Abstract**

18 In this work, *Leptosphaerulina* sp. (a Colombian native fungus) significantly removed
19 three Isoxazolyl-Penicillin antibiotics (IP): oxacillin (OXA, 16000 μg L^{-1}), cloxacillin
20 (CLX, 17500 μg L^{-1}) and dicloxacillin (DCX, 19000 μg L^{-1}) from water. The biological
21 treatment was performed at pH 5.6, 28 °C, and 160 rpm for 15 days. The
22 biotransformation proccess and lack of toxicity of the final solutions (antibacterial
23 activity (AA) and cytotoxicity) were tested. The role of enzymes in IP removal was
24 analysed through *in vitro* studies with enzymatic extracts (crude and pre-purified)
25 from *Leptosphaerulina* sp., commercial enzymes and enzymatic inhibitors.
26 Futhermore, the applicabililty of mycoremediation process to a complex matrix
27 (simulated hospital wastewater) was evaluated. IP were considerably abated by the
28 fungus, OXA was the fastest degraded (day 6), followed by CLX (day 7) and DCX
29 (day 8). Antibiotics biodegradation was associated to laccase and versatile
30 peroxidase action. Assays using commercial enzymes (*i.e.* laccase from *Trametes*
31 *versicolor* and horseradish peroxidase) and inhibitors (EDTA, NaCl, sodium acetate,
32 manganese (II) ions) confirmed the significant role of enzymatic transformation.
33 Whereas, biomass sorption was not an important process in the antibiotics
34 elimination. Evaluation of AA against *Staphylococcus aureus* ATCC 6538 revealed
35 that *Leptosphaerulina* sp. also eliminated the AA. In addition, the cytotoxicity assay
36 (MTT) on the HepG2 cell line demonstrated that the IP final solutions were non-toxic.
37 Finally, *Leptosphaerulina* sp. eliminated OXA and its AA from synthetic hospital
38 wastewater at 6 days. All these results evidenced the potential of *Leptosphaerulina*

39 sp. mycoremediation as a novel environmentally friendly process for the removal of
40 IP from aqueous systems.

41

42 **Keywords:** White-rot fungi; Ligninolytic enzymes; Antibiotics degradation;
43 Biotransformation; Wastewater treatment; Hospital wastewaters.

44

45 **1. INTRODUCTION**

46 Antibiotics are therapeutic agents which prevent or inhibit the growth of
47 microorganisms (Gothwal & Shashidhar, 2015; Kümmerer, 2009). These
48 compounds are widely used in human and veterinary medicine (Chen & Zhou, 2014;
49 Du & Liu, 2012; Gothwal & Shashidhar, 2015); therefore, worldwide exists a high
50 demand for antibiotics reaching 100000-200000 tons per year (Becker et al., 2016;
51 Kümmerer, 2003). A significant fraction of antibiotics (50-80%) is excreted through
52 faeces and urine (Ahmed et al., 2015; Santos et al., 2012; Solliec et al., 2016). These
53 compounds are also reaching the environment from soil fertilization procedures with
54 livestock manure and through domestic and hospital wastes (Chen et al., 2014;
55 Gothwal & Shashidhar, 2015; Kang et al., 2016).

56 The presence of antibiotics in the environment has been reported since 1930, but
57 only in the 90s their presence in water bodies became a subject of concern.
58 Antibiotics released into the environment can promote resistant bacteria and their
59 proliferation. Thereby, many antibiotics become ineffective against human and

60 animal pathogens, incrising health risks (Becker et al., 2016; Chen et al., 2014;
61 Homem & Santos, 2011; Özcengiz & Yilmaz, 2017; Sarmah et al., 2006; Xu et al.,
62 2015).

63 Isoxazolyl-Penicillins (IP) are a group of semisynthetic antibiotics with a nucleus of
64 6-aminopenicillanic acid, their structures consist of a β-lactam fused to a thiazolidine
65 ring (Apelblat & Bešter-Rogač, 2015). IP treat infections caused by *Gram*-positive
66 bacteria such as *Staphylococcus aureus*, *Bacillus cereus,* and *Streptococcus*
67 *pneumoniae* (Cha et al., 2006; Hou & Poole, 1971; Sunder et al., 2007; Yamada &
68 Sato, 1962). IP action focuses on the inhibition of cell wall synthesis and murein
69 assembly (Gothwal & Shashidhar, 2015). This pharmaceutical group includes
70 oxacillin (OXA) (3-phenyl-5-methyl-4- isoxazolyl-penicillin), cloxacillin (CLX) (3-(2-
71 chlorophenyl)-5-methyl-4-isoxazolyl-penicillin) and dicloxacillin (DCX) (3-(2,6-
72 dichlorophenyl)-5-methyl-4-isoxazolyl-penicillin) (Apelblat & Bešter-Rogač, 2015).
73 IP's chemical structure makes them resistant to degradation by conventional
74 chemical and biological methods (Fernández-Fernández et al., 2013). These
75 penicillin antibiotics are of special interest because they are largely consumed for
76 the treatment of skin infections and as follow-up therapy after intravenous treatment
77 for osteomyelitis (Giraldo Aguirre et al., 2016). In consequence, IP have been
78 detected in wastewater and recently found in natural waters at concentrations of mg
79 L^{-1} (Serna-Galvis et al., 2016).

80 Conventional wastewater treatment plants (WWTPs) are not designed to remove
81 specific compounds such as pharmaceuticals, personal care products, and

82 agrochemicals products from waters (Rodríguez-Delgado et al., 2016). The leading
83 methods for removing antibiotics from wastewater include physical (activated carbon
84 adsorption, membrane filtration, coagulation and flocculation) (Bolong et al., 2009),
85 advanced oxidation processes (AOPs) (Giraldo-Aguirre et al., 2015; Villegas-
86 Guzman et al., 2015) and biological processes such bioadsorption and activated
87 sludge systems (De Cazes et al., 2014; Nguyen et al., 2014). However, in the AOPs
88 case most of them demand high energy consumption, high operating costs and they
89 may generate toxic by-products (Frade et al., 2014). Whereas, bioadsorption only
90 transfer the pollutants from liquid phase to biomass and activated sludge treatments
91 do not efficiently remove these substances (Badia-Fabregat et al., 2017; Ding et al.,
92 2016; Gothwal & Shashidhar, 2015; Homem & Santos, 2011; Larcher & Yargeau,
93 2011).

94 A novel alternative to physical and chemical treatments are biological processes
95 using white-rot fungi (WRF) and their non-specific and extracellular ligninolytic
96 enzymes. This method, which could be implemented in WWTPs as secondary or
97 tertiary treatments, appears as a viable option for the removal of antibiotics
98 (Čvančarová et al., 2015; De Araujo et al., 2017; Tortella et al., 2013). In addition to
99 high catabolic degradative potential, processes based on WRF are a low cost and
100 an environmentally friendly method (Osma et al., 2010). WRF produce laccase (Lac,
101 E.C 1.10.3.2), manganese peroxidase (MnP, E.C 1.11.1.13), lignin peroxidase (LiP,
102 E.C 1.11.1.14) and versatile peroxidase enzymes (VP, E.C 1.11.1.16). These
103 enzymes have high redox potential, which makes them able to oxidise large number
104 of organic pollutants.

105 Some biotransformation studies with WRF have emphasised on pollutants such as
106 polyaromatic hydrocarbons, synthetic dyes, and pesticides (Migliore et al., 2012;
107 Williams et al., 2007). Fungal strains have shown positive results for antibiotics
108 elimination. For instance, *Mucor ramannianus* and *Gloeophyllum striatum* have
109 efficiently removed enrofloxacin (Parshikov et al., 2000; Wetzstein et al., 1997).
110 Prieto et al. (2011) reported that *T. versicolor* remarkably eliminated ciprofloxacin
111 and norfloxacin. Similarly, Cvancarova et al. (2015) reported the elimination of
112 ciprofloxacin, ofloxacin and norfloxacin by *Irpex lacteus* and *T. versicolor*. *Pleurotus*
113 *ostreatus* degraded oxytetracycline (Migliore et al., 2012), sulfamethoxazole, and
114 trimethoprim (De Araujo et al., 2017). However, under author knowledge, the
115 biotransformation of IP by WRF has not been reported.

116 *Leptosphaerulina* sp., a Colombian ascomycete strain from lignocellulosic material
117 in the Valle de Aburrá (Antioquia, Colombia), has efficiently degraded synthetic dyes
118 (Chanagá Vera et al., 2012; Copete et al., 2015; Plácido et al., 2016). However, the
119 capabilities of this fungus for degrading other recalcitrant compounds are still
120 unknown. Due to the high expression of ligninolytic enzymes (Lac and MnP),
121 *Leptosphaerulina* sp. was considered herein as a potential method to remove
122 antibiotics from aqueous systems (Copete et al., 2015; Plácido et al., 2016). The aim
123 of this work was to evaluate the capability of the Colombian isolate *Leptosphaerulina*
124 sp. and its ligninolytic enzymes for the biotransformation of OXA, CLX and DCX
125 (Isoxazolyl-Penicillins) in aqueous systems. Initially, the participation of enzymatic or
126 sorption processes was determined. During IP bio-treatment, the enzymatic
127 activities (Lac, MnP, LiP and VP), reducing sugars, protein concentration and the

128 antibiotics removal were followed. Assays with enzymatic extracts (crude and pre-
129 purified), commercial enzymes and enzymatic inhibitors were performed. The
130 antimicrobial activity removal against *S. aureus* and cytotoxicity towards HepG2 cell
131 line were also assessed. Finally, the application of the bio-treatment on a syntethic
132 hospital wastewater containing OXA was evaluated.

133

134 2. MATERIALS AND METHODS

135 *2.1. Chemicals*

136 The IP antibiotics were utilised as their corresponding sodium salts: oxacillin (OXA)
137 95% (from Sigma-Aldrich), cloxacillin (CLX) 92.3% (from Syntopharma S.A) and
138 dicloxacillin (DCX) 98.2% (from Research Pharmaceutical) (see chemical structures
139 in **Table 1**). Glucose, peptone, yeast extract, monobasic potassium phosphate, zinc
140 sulphate heptahydrate, tetraborate sodium decahydrate, ammonium molybdate,
141 sodium acetate, malt extract, calcium chloride dehydrate and ammonium chloride
142 were bought from Carlo Erba. Ammonium L-(+)-tartrate 98% and 2,6-
143 dimethoxyphenol 99% (DMP) was obtained from Alfa Aesar. Manganese sulphate
144 heptahydrate, iron sulphate heptahydrate, potassium chloride, ammonium sulphate,
145 sodium chloride, formic acid, tartaric acid, hydrogen peroxide, acetic acid, sodium
146 sulphate, acetonitrile, methanol and Mueller-Hinton agar were bought from Merck.
147 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt 98%
148 (ABTS), veratryl alcohol 96%, 1-hydroxybenzotriazol (HBT), 3-(4,5-Dimethylthiazol-
149 2-yl)-2,5-diphenyltetrazolium bromide > 98% (MTT), dimethyl sulfoxide (DMSO),

150 Dulbecco's modified Eagle's medium (DMEM), ethylenediaminetetraacetic acid
151 (EDTA) and doxorubicin were obtained from Sigma - Aldrich. Fetal bovine serum
152 (FBS) was purchased from Invitrogen. Urea was bought from Panreac.

153

154 **Table 1.** Chemical structures of IP.

IP	Chemical structure
Oxacillin (OXA)	
Cloxacillin (CLX)	
Dicloxacillin (DCX)	

155 ### *2.2. Microorganism and culture conditions*

156 *Leptosphaerulina* sp. was isolated from lignocellulosic material in the Valle de Aburrá
157 (Medellín, Colombia) and conserved in the collection of microorganisms of the
158 research group PROBIOM (CECT 20913) (Chanagá Vera et al., 2012; Copete et al.,
159 2015). The fungus was maintained in malt extract agar at 4 °C until use. Mycelium
160 from 10-days-old culture was homogenized and used as inoculum in the degradation
161 process (Copete et al., 2015). This work was authorised by the Autoridad Nacional
162 de Licencias Ambientales (ANLA) under the research permit No. 8 de 2010
163 (Resolución 324 de 2014) and the Ministerio de Ambiente y Desarrollo Sostenible of
164 Colombia with the agreement No. 96 of 2014 to genetic resources access.

165 ### *2.3. Isoxazolyl-Penicillins biotransformation experiments*

166 Biotransformation assays were carried out in water containing the antibiotics spiked
167 individually (OXA, 16000 μg L^{-1}; CLX, 17500 μg L^{-1}; DCX, 19000 μg L^{-1}), 10 g L^{-1}
168 glucose, 2 g L^{-1} ammonium tartrate, 5 g L^{-1} peptone, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} yeast
169 extract, 0.5 g L^{-1} $MgSO_4$. $7H_2O$, 0.5 g L^{-1} KCl and 1 mL mineral solution [100 mg L^{-1}
170 $B_4O_7Na_2$. $10H_2O$, 70 mg L^{-1}, $ZnSO_4$. $7H_2O$, 50 mg L^{-1} $FeSO_4$. $7H_2O$, 10 mg L^{-1}
171 $MnSO_4$. $7H_2O$ and 10 mg L^{-1} $(NH_4)_6Mo_7O_{24}$. $4H_2O$] (Guillén et al., 1992). The pH of
172 the medium remained at pH 5.6, which in our previous work was found as the optimal
173 pH value for the fungal strain (Copete et al., 2015) and it coincided with the natural
174 pH of IP solutions; additionally, this operational pH was helpful to maintain the
175 antibiotics stability.

176 The biotransformation process employed liquid cultures in 250 mL conical flasks with
177 100 mL of liquid medium containing antibiotics. Flasks were inoculated with 5 mL of
178 mycelium previously homogenized in a sterilised blender at 8000 rpm for 60 s and
179 later incubated at 28 °C and 160 rpm for 15 days. All assays were performed in
180 triplicate. As sampling volume, 4 mL were withdrawn from the conical flasks at each
181 time point. Enzyme activities, reducing sugars, protein concentration, antibiotics
182 degradation and residual antibacterial activity were monitored during the course of
183 the biotransformation process, at 2, 4, 6, 7, 8 and 15 days. The changes in the
184 antibiotics concentration was followed by high performance liquid chromatography
185 (HPLC) with a diode array detector (DAD).

186 Abiotic (inert *Leptosphaerulina* sp. mycelium with antibiotic) and biotic
187 (*Leptosphaerulina* sp. without antibiotic) controls were prepared as reported by
188 Čvančarová et al. (2015). Non-inoculated controls (antibiotic without fungus) were
189 also performed (Gros et al., 2014). Fungal sorption tests employed *Leptosphaerulina*
190 sp. mycelia cultivated for 8 days. After these days, the fungal biomass was
191 autoclaved (120 °C, 20 min). Then, the inert *Leptosphaerulina* sp. mycelium was
192 combined with each one of the antibiotics (OXA, 16000 μg L^{-1}; CLX, 17500 μg L^{-1};
193 DCX, 19000 μg L^{-1}) in liquid medium (abiotic controls). The abiotic controls were
194 cultivated under the same conditions as before to evaluate the sorption of the
195 antibiotics. The antibiotics were followed by HPLC (Čvančarová et al., 2015).

196

197

198 ### *2.4. OXA biotransformation in a synthetic hospital wastewater (HWW)*

199 A synthetic matrix of hospital wastewater (HWW) was used for evaluating OXA
200 removal (**Table 2**). In this experiment, OXA was chosen for the simulated hospital
201 wastewaters because OXA evidenced the greatest reduction in the previous
202 experiment, therefore, the effects of the HWW on the fungi will be easier detected.
203 The biotransformation process employed 250 mL conical flasks with 100 mL of HWW
204 and OXA (16000 μg L^{-1}). Flasks were inoculated (**section 2.3**) and incubated at 28
205 °C and 160 rpm for 8 days. All assays were performed in triplicate. Antibiotic
206 degradation and residual antibacterial activity were monitored at 2, 4, 6, 7 and 8 days
207 of the biotransformation process.

208

209 **Table 2.** Composition Hospital wastewater (HWW)*.

210
211
212
213
214

Substance	**(g L^{-1})**
$CaCl_2.2H_2O$	0.050
Na_2SO_4	0.100
K_2HPO_4	0.050
KCl	0.100
NH_4Cl	0.050
Urea	1.26
NaCl	2.925

215 *(Antonin et al. (2015); Serna-Galvis et al. (2017))

216

217

218 ***2.5. Enzymatic activities***

219 Lac and VP activities were determined spectrophotometrically (Shimadzu UV-1800)
220 by measuring the oxidation of 3 mM ABTS in 0.1 M sodium tartrate buffer pH 3 (ε_{420},
221 36000 M^{-1} cm^{-1}) in absence and presence of 0.1 mM H_2O_2, respectively. The MnP
222 activity was estimated by measuring the oxidation of 1 mM DMP in 0.1 mM sodium
223 acetate buffer pH 4.5 (ε_{469}, 27500 M^{-1} cm^{-1}). The LiP activity was determined using
224 the 2 mM veratryl alcohol oxidation in 0.1 M sodium tartrate buffer pH 3 (ε_{310}, 9300
225 M^{-1} cm^{-1}). All the enzymatic activities were reported as the amount of enzyme
226 required for oxidizing 1 μmol of substrate in 1 min (U units).

227 ***2.6. Enzymatic inhibition assay***

228 *Leptosphaerulina* sp. was grown with OXA (16000 μg L^{-1}) and inhibitors of Lac
229 (EDTA, 30 mM or NaCl, 300 mM), MnP (sodium acetate, 100 mM) or VP
230 (manganese (II) ions (Mn^{2+}), 0.5 mM). The set of inhibitors and their concentration
231 were selected from the Enzyme Database BRENDA (BRENDA, 2017). These
232 inhibitors were selected because they inhibited ligninolytic enzymes from different
233 types of WRF. Experiments without inhibitors were employed as controls. After 6
234 days, enzymatic activities and AA were determined.

235 ***2.7. Pre-purification of Leptosphaerulina sp. extract with ammonium***
236 ***sulphate ($(NH_4)_2SO_4$)***

237 Crude extracts from *Leptosphaerulina* sp. were freeze-dried or prepurified via
238 ammonium sulphate precipitation. The unfreeze-dried crude extract from

239 *Leptosphaerulina* sp. was saturated sequentially from 50% to 100% with ammonium
240 sulphate, at 4 °C. The proteins were recovered by centrifugation at 13000 rpm for 15
241 min and the pellet was dissolved in 0.1 M sodium acetate buffer (pH 4.5). The sample
242 was dialysed at 4 °C against a 0.1 M sodium acetate buffer (pH 4.5) using a dialysis
243 membrane MWCO 50 KDa (6 Spectra/ Por®). The dialysed product was freeze-dried
244 and used in the *in vitro* degradation of IP.

245 ***2.8. In vitro degradation of IP by ligninolytic enzymes***

246 The IP *in vitro* degradation was conducted in batch reactions using 15 mL vials with
247 3 mL of reaction volume. As controls, the laccase-mediator systems (LMS)
248 experiment was performed with a commercial Lac from *Trametes versicolor* (powder,
249 light brown, ≥0.5 U mg^{-1}). Additionally, horseradish peroxidase (HRP, EC 1.11.1.7,
250 powder, ≥250 U mg^{-1}) was utilized as the peroxidase control. Reactions were
251 initiated by adding either freeze-dried crude extract from *Leptosphaerulina* sp., pre-
252 purified (0.3 mg of protein mL^{-1}) or commercial Lac from *T. versicolor* (2 mg mL^{-1})
253 into a 0.1 M sodium tartrate buffer pH 5.6 containing OXA at 16000 μg L^{-1} and HBT
254 10 mM (as mediator). Peroxidase from horseradish (0.3 mg mL^{-1}) experiment utilised
255 0.1 M sodium acetate buffer (pH 5.6), OXA (16000 μg L^{-1}) and H_2O_2 (0.1 M). The
256 reactions were incubated at 28 °C and 160 rpm for 2 days and the OXA was
257 determined by HPLC.

258

259

260 ***2.9. Antibiotics chromatographic analysis***

261 In all experiments, the OXA, CLX and DCX removal followed the method described
262 by Serna-Galvis et al. (2016) using reverse phase (RP)-HPLC. The reverse phase
263 (RP)-HPLC (Thermo Scientific DIONEX UltiMate 3000) used a C-18 column (5 μm
264 particle size, 4.6 mm x 250 mm, LiChrosphere® from Merck) and a diode array
265 detector (DAD) set at 225 nm. The mobile phase was acetonitrile (C_2H_3N) / formic
266 acid (CH_2O_2) buffer (10 mM at pH 3), 35/65 (% v/v) for OXA and 58/42 (% v/v) for
267 CLX and DCX, in the isocratic mode. The injection volume was 20 μL. The
268 separation temperature was set at 28 °C and the flow rate at 1 mL min^{-1}. The
269 antibiotic removal percentage was estimated by measuring the changes in the areas
270 of the antibiotics chromatographic peaks at each time against the initial areas (day
271 0). This analysis was complemented with the antimicrobial activity analysis.

272 ***2.10. Residual antibacterial activity assay***

273 The residual antibacterial activity (AA) of the antibiotics and their transformation
274 products from the biotransformation, inhibition, and synthetic wastewater
275 experiments were assessed through the Kirby-Bauer disk diffusion susceptibility test
276 with *Staphylococcus aureus* ATCC 6538 as the indicator microorganism. Petri
277 dishes with Mueller-Hinton agar were inoculated with 15 μL of *S. aureus* suspension
278 (optical density of 0.600 at 580 nm). When the agar solidified, 6 mm holes were
279 made in its surface. Then, 30 μL of sample (antibiotics and/or transformation
280 products) covered each hole and the petri dishes incubated for 24 h at 37 °C. After
281 this, the diameter of the inhibition halo was measured around the holes (Čvančarová

282 et al., 2013; Serna-Galvis et al., 2016). Initially IP concentrations and the appearance
283 of inhibition halos were correlated. In such experiment, it was found that 400 µg L^{-1},
284 438 µg L^{-1} and 475 µg L^{-1} for OXA, CLX and DCX, respectively, generates an
285 inhibition halo of 3 ± 0.07 mm.

286 ***2.11. Reducing sugars and protein quantification***

287 Reducing sugars in liquid medium were quantified through the 3,5-dinitrosalicylic
288 acid (DNS) methodology at 475 nm. Absorbance was transformed into g L^{-1} of
289 glucose by comparison with a glucose standard curve (Ma & Ruan, 2015; Miller,
290 1959). Protein concentration was estimated at 595 nm according to the Bradford
291 protein assay, using bovine serum albumin (BSA) as the protein standard (Bradford,
292 1976).

293 ***2.12. Cytotoxicity assay***

294 The cytotoxicity of the IP degradation products from the biotransformation
295 experiments was determined on the human liver cells-hepatoma (HepG2), using the
296 MTT assay. HepG2 cells were obtained from the American Type Culture Collection
297 (ATCC HB-8065). Cells were seeded into 96-well plates using DMEM with 10% FBS.
298 After 24 h, the fungally-treated samples of OXA, CLX and DCX were added with
299 serial dilutions of 75, 37.5, 18.8, 9.4, 4.7 and 2.3% w/v and incubated for 72 h at 37
300 °C, 5% CO_2. After the initial incubation time, MTT was added and incubated for 3 h
301 at 37 °C. Then, DMSO was added. Finally, the absorbance at 570 nm was measured
302 in a spectrophotometer and the lethal concentration 50 (LC_{50}) was calculated. The

303 assays were performed in two independent experiments and with two replicates by
304 assay. Doxorubicin and untreated cells were utilised as positive and negative
305 cytotoxicity controls, respectively.

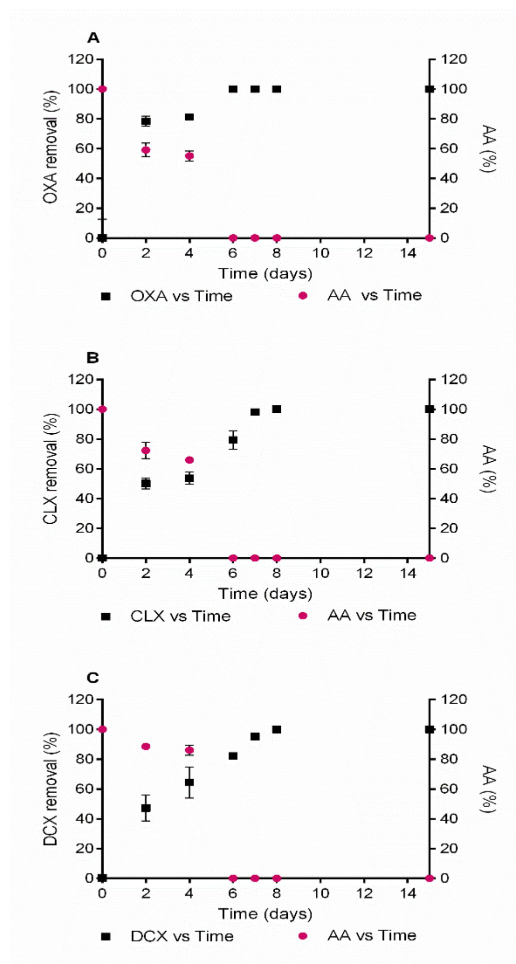
306

307 **3. RESULTS**

308 ***3.1. Removal of IP by Leptosphaerulina sp.***

309 The abatement of OXA, CLX and DCX was determined during 2, 4, 6, 7, 8 and 15
310 days of bio-treatment (**Figure 1**). As seen, *Leptosphaerulina* sp. achieved ~100%
311 removal of the complete set of antibiotics. In general, the fungal biodegradations
312 were rapid (less than 8 days); however, the principal difference among antibiotics
313 was the removal rate. As seen in **Figure 1A**, OXA disappeared on the sixth day and
314 the removal percentage decreased in two principal periods. The first decrease, the
315 most significant one, occurred during the initial two days and achieved ~80% of
316 removal, the second decrease occurred during days 4-6 (~100% removal). AA
317 exhibited the same two reduction periods, a fast decline during the first two days
318 (40%) and the final removal phase within days 4-6. In **Figure 1B**, CLX disappeared
319 on the seventh day. The most significant CLX reduction (50%) occurred on the
320 second day. From day 4 to 6, the removal percentage reached 76%. Similarly, CLX
321 AA was removed 28%, 34% and ~100% during the second, fourth and sixth days,
322 respectively. In **Figure 1C**, DCX vanished on the eighth day and the principal
323 reduction happened during the first two days achieving almost 53% reduction. From
324 day 4 to 6, DCX reduced in 81%. AA was mainly abated between days 4 to 6. In

325 general, on the sixth day, the antibiotics' AA was considerably abated (**Figure 1**). In
326 spite of that, the removal was different in each IP following this order: OXA > CLX >
327 DCX. The OXA removal percentage versus time correlates with the reduction of AA
328 removal percentage versus time (**Figure 1A**). In the case of chlorinated antibiotics
329 (CLX and DCX), when approximately 80% of them was eliminated (at day 6), the AA
330 was null. Similar behaviour was observed when they achieved the greatest removal
331 (7 and 8 day) (**Figures 1B, 1C**).

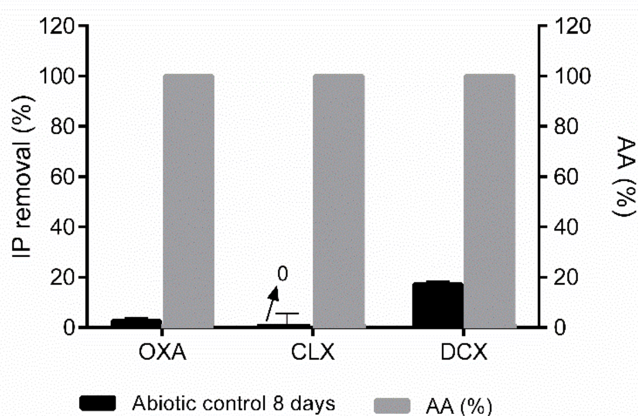


332

333 **Figure 1.** Antibiotic removal % and antibacterial activity (AA) % in the experiments with
334 *Leptosphaerulina* sp. and the antibiotic **A)** OXA, **B)** CLX and **C)** DCX. Experimental
335 conditions: 28 °C, 160 rpm, pH = 5.6, 15 days.

336 ***3.2. Biodegradation and sorption processes in the removal of IP***

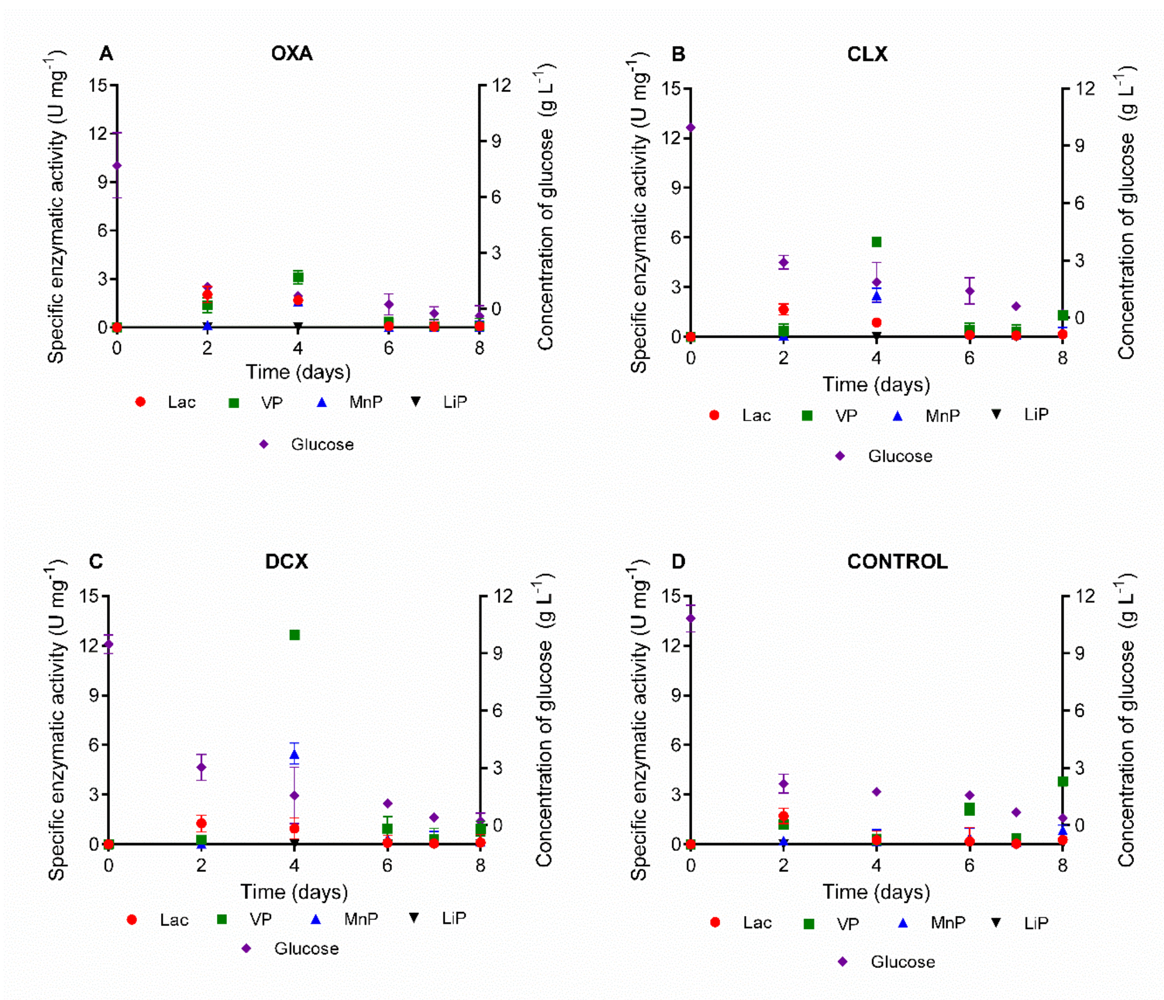
337 To determine the IP sorption by *Leptosphaerulina* sp., biotic, abiotic, and non-
338 inoculated control experiments were performed. **Figure 2** displays the IP sorption by
339 the inert *Leptosphaerulina* sp. mycelium. For all antibiotics, the biomass did not sorb
340 high amounts of antibiotics. CLX was not removed by sorption, while OXA and DCX
341 had less than 3% and 18% of sorption, respectively. The results indicated that IP
342 removal by *Leptosphaerulina* sp. was achieved with low sorption of the antibiotics
343 into the mycelium (abiotic controls). On the other hand, the control experiments
344 demonstrated the preservation of the antimicrobial activity (0% removed, **Figure 2**).
345 Additionally, the antibiotics concentration in the non-inoculated controls did not
346 change during the experiment. Therefore, the antibiotics disappearance can be
347 attributed to biotic factors. To understand the IP degradation mechanism, enzymatic
348 production analyses were assessed.

349 

350 **Figure 2.** OXA [16000 µg L^{-1}], CLX [17500 µg L^{-1}] and DCX [19000 µg L^{-1}] treated by
351 *Leptosphaerulina* sp. inert from 8 days of growth. Experimental conditions: 28 °C, 160 rpm,
352 pH = 5.6, 8 days.

Figure 3 depicts the production profiles of Lac, MnP, LiP and VP by *Leptosphaerulina* sp. during OXA, CLX and DCX removal. The predominant expression of Lac on the second day correlates with the most significant antibiotic reduction (**Figure 1**). In fact, Lac production of 2.05, 1.66 and 1.26 U mg^{-1} (**Figures 3A, 3B, 3C**) coincided with the reduction of 78%, 50% and 47% of OXA, CLX and DCX, respectively (**Figures 1A, 1B, 1C**). Simultaneously, the cultures exhibited a remarkable decrease in glucose concentration evidencing considerable microbial activity.

For all antibiotics, at day 4, VP achieved the highest enzymatic activity (3.11 U mg^{-1} (OXA), 5.74 U mg^{-1} (CLX) and 12.65 U mg^{-1} (DCX)) in the liquid medium, followed by Lac and MnP activities. LiP activity was not detected in any of the experiments (**Figures 3A, 3B, 3C**). As seen in **Figure 3,** the VP production was different in each antibiotic and their activities ranked from top to bottom, DCX > CLX > OXA. Similar behaviour was observed for the MnP, its highest expression was detected in DCX (5.49 U mg^{-1}) followed by CLX (2.51 U mg^{-1}) and OXA (1.62 U mg^{-1}) (**Figures 3A, 3B, 3C**). At day 2, in the biotic control (*Leptosphaerulina* sp. without antibiotic) Lac had significant expression (1.72 U mg^{-1}) and VP reached a similar value (1.19 U mg^{-1}) when compared with the maximum activities obtained (**Figure 3D**). On the fourth day, the control's VP activity was 0.29 U mg^{-1}. In contrast, at day fourth in the OXA, CLX and DCX removal experiments VP activity increased 11, 20 and 44 times, respectively. These results evidenced the participation of ligninolytic enzymes from *Leptosphaerulina* sp. in IP disappearance.



375

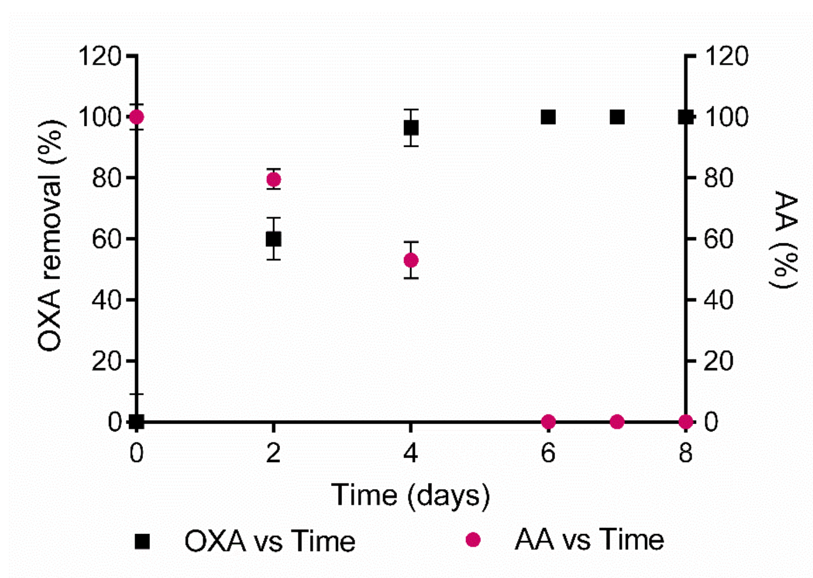
376 **Figure 3.** Specific enzymatic activities and glucose concentration for IP removal experiment
377 **A**) OXA, **B**) CLX and **C**) DCX. **D**) biotic control (*Leptosphaerulina* sp. without antibiotic).
378 Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 8 days.

379

380 ### *3.3. OXA biotransformation in a synthetic hospital wastewater (HWW)*

381 To evaluate the effect of a complex matrix, a hospital wastewater effluent (HWW)
382 was simulated (**Table 2**) and used as liquid medium (**section 2.3**) for the
383 biotransformation process. The experiment tested the removal of OXA and AA

384 (**Figure 4**) by *Leptosphaerulina* sp. In HWW, OXA was reduced 60% during the two
385 initial days of treatment with *Leptosphaerulina* sp. This reduction is lower than the
386 obtained in the liquid medium experiment (80%, **Figure 1A**). This revealed an initial
387 effect of HWW on the fungus activity. On the fourth day, *Leptosphaerulina* sp.
388 achieved a greater removal of OXA (96%) and AA (47%). Similar to the liquid
389 medium results, on the sixth day, antibiotic and AA were not detected in HWW by
390 the quantification methods used. In this synthetic water, *Leptosphaerulina* sp.
391 produced MnP, Lac and VP. At day 2, MnP was the largest activity detected (1.5 U
392 mg^{-1}); whereas, at the same time Lac and VP were 3-time lower than MnP (~0.5 U
393 mg^{-1}). The largest expression of Lac (1.37 U mg^{-1}) and VP (1.24 U mg^{-1}) activities
394 were observed on the sixth and seventh day, respectively.

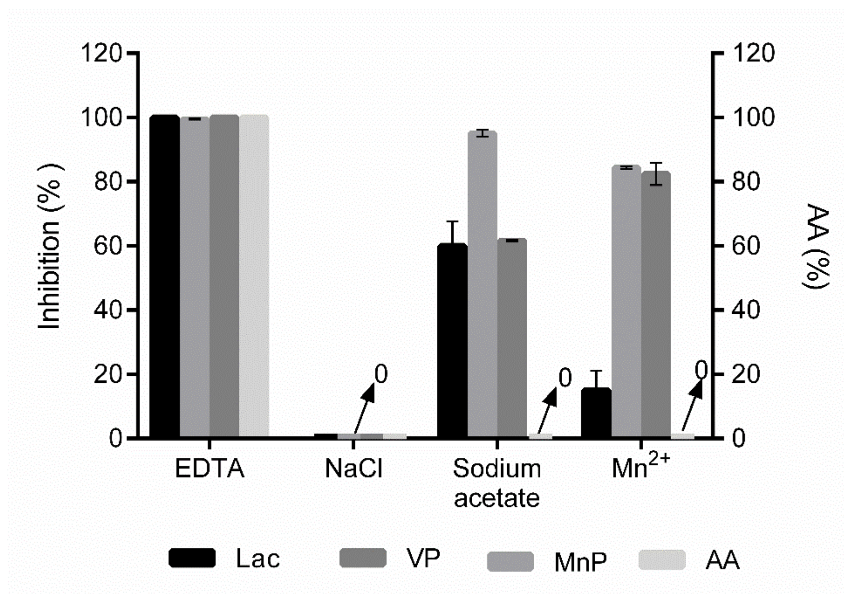


395

396 **Figure 4.** OXA removal % and antibacterial activity (AA) % in the experiments with
397 *Leptosphaerulina* sp. in HWW. Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 8 days.

398 ***3.4. Enzymatic inhibitors studies***

399 Enzymatic inhibition studies confirmed the participation of Lac, MnP and VP on IP
400 removal. **Figure 5** illustrates the inhibitory effect of EDTA, NaCl, sodium acetate and
401 Mn^{2+} on the enzymatic activity of Lac, MnP and VP from *Leptosphaerulina* sp. EDTA
402 inhibited all enzymes, sodium acetate inhibited 60%, 95% and 62% of Lac, MnP and
403 VP, respectively. Whereas, Mn^{2+} inhibited 15%, 84% and 82% of Lac, MnP and VP,
404 respectively. In contrast, NaCl did not inhibit the enzymatic expression, this
405 behaviour disagrees with the ones reported for other microorganisms in the Enzyme
406 Database BRENDA (BRENDA, 2017).



407

408 **Figure 5.** Influence of the EDTA (30 mM), NaCl (300 mM), sodium acetate (100 mM) and
409 Mn^{2+} (100 mM) on Lac, MnP, VP and AA during the removal of OXA by *Leptosphaerulina*
410 sp. Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 6 days.

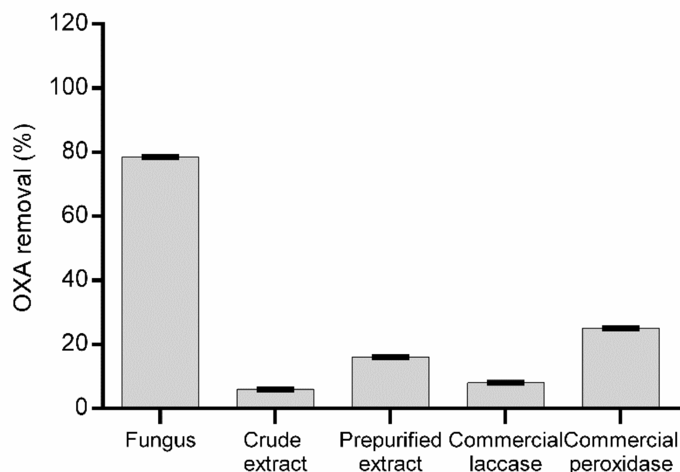
411

412 The AA was evaluated at the end of the process. EDTA produced complete
413 enzymatic inhibition which caused a lack of AA removal; in contrast, the assays with
414 partial enzymatic inhibition achieved significant AA removal. These results confirmed
415 the enzymatic nature of the antibiotics biotransformation by *Leptosphaerulina* sp.
416 Additionally, they evidenced that low enzymatic activities can lead to AA removal.

417 ***3.5. Enzymatic in vitro studies***

418 To study the individual degrading ability of the enzymatic machinery produced by the
419 fungus, *in vitro* essays with crude and pre-purified extracts were considered. In
420 addition, commercial enzymes were evaluated and compared with enzymatic
421 extracts from *Leptosphaerulina* sp. In these experiments, OXA was employed
422 because it was the antibiotic most rapidly degraded by *Leptosphaerulina* sp. **Figure**
423 **6** illustrates the percentage of IP removal after two 2 days of treatment. The crude
424 extract (which contains Lac: 1.12 U mg^{-1}; VP: 1.77 U mg^{-1} and MnP: 0.28 U mg^{-1})
425 removed 6% of OXA. Whereas, the pre-purified extract (Lac: 1.16 U mg^{-1}; VP: 1.12
426 U mg^{-1} and MnP: 0.20 U mg^{-1}) eliminated 16% of OXA. The tests carried out with the
427 commercial Lac achieved an 8% reduction of OXA initial concentration. The
428 comparison between the fungus' enzymatic extracts and the commercial enzymes
429 evidenced the pre-purified extract as the most suitable option for *in vitro* removal.
430 On the other hand, the experiment with the commercial peroxidase (which eliminates
431 25% of OXA, **Figure 6**) correlated with the results from the in vivo experiments with
432 *Leptosphaerulina* sp. (**Figure 3A**), where a high VP activity was associated with OXA
433 biotransformation. Under *in vitro* conditions, commercial Lac alone did not degrade

434 considerably the antibiotic; while commercial peroxidase achieved a significant
435 reduction in the OXA removal percentage.



436

437 **Figure 6**. OXA removal by *Leptosphaerulina* sp. and its enzymes, crude, pre-purified
438 extracts of *Leptosphaerulina* sp., laccase from *Trametes versicolor* with HBT, and
439 peroxidase from horseradish. After 2 days of bio-treatment.

440

441 ***3.6. Cytotoxicity studies***

442 The toxicity of OXA, CLX and DCX solutions after 8 days of fungal treatment was
443 assessed on the HepG2 cell line. The test established a LC_{50} > 75 % w/v (**Table 3**),
444 which refers to the concentration of IP or the transformation products (TPs) that
445 cause the death of half HepG2 cells. The treatment with *Leptosphaerulina* sp. did
446 not change the LC_{50} in relation to the initial solutions of IP. Additionally, LC_{50} of IP
447 and their degradation products were 17.65 times higher than the positive control
448 (doxorubicin, **Table 3**).

449 **Table 3**. LC_{50} for solutions of IP before and after 8 days of bio-treatment with

450 *Leptosphaerulina* sp. evaluated on HepG2 cells.

Sample	LC_{50} HEPG2 (% w/v)
OXA before bio-treatment	>75
OXA after bio-treatment	>75
CLX before bio-treatment	>75
CLX after bio-treatment	>75
DCX before bio-treatment	>75
DCX after bio-treatment	>75
Control (doxorubicin)	4.25

451

452 **4. DISCUSSION**

453 To our knowledge, this is the first study reporting the biotransformation of IP by
454 *Leptosphaerulina* sp. and its ligninolytic enzymes. *Leptosphaerulina* sp. removed
455 high concentrations of IP (40 µM: 16.0 mg L^{-1} OXA: 17.5 mg L^{-1} CLX: 19.0 mg L^{-1}
456 DCX) in eight or less days. This biotransformation time is shorter than reported in
457 homologous previous works with β-lactam antibiotics and fungi. Lucas et al. (2016)
458 reported 96% β-lactam antibiotics (initial concentration 10 µg L^{-1}) elimination by *T.*
459 *versicolor* ATCC 42530 on 15 days. In that case, the authors worked at
460 concentrations 1000 times lower than the reported in the present article. Therefore,
461 under the experimental conditions of this article, *Leptosphaerulina* sp. was able to
462 eliminate large concentrations of antibiotics and in shorter time (less than 8 days)

463 than other fungi previously reported. *T. versicolor* required at least 14 days to
464 degrade fluoroquinolones such as ciprofloxacin, norfloxacin and ofloxacin
465 (Čvančarová et al., 2015). *P. chrysosporium*, *Bjerkandera* sp. R1 and *B. adusta*
466 completely abated sulfamethoxazole within 14 days (Cruz-Morató et al., 2013).
467 Sulfanilamide and sulfapyridine were transformated by *T. versicolor* in a 10% and
468 95.6%, respectively, after 15 days (Schwarz et al., 2010).

469 Gros et al. (2014) reported that in HWW, *T. versicolor* degraded 98.5% of the
470 fluoroquinolone antibiotic ofloxacin (10 mg L^{-1}) by the eighth day. The partial or
471 complete elimination of antibiotics on HWW depends on the fungi and the antibiotic
472 evaluated. In this complex matrix, the process difficulty increases because the
473 inoculated fungi will compete with others microorganisms growing in the wastewater
474 for nutrients and space (Badia-Fabregat et al., 2017). Other factors that can affect
475 the efficiency of the HWW fungal treatment are the chemical composition of the
476 wastewater, the pH, the configuration of the reactor and the addition of nutrients
477 (Anastasi et al., 2010).

478 From **Figure 1,** it is noticeable that CLX and DCX required more days than OXA to
479 be biodegraded by *Leptosphaerulina* sp. These differences in the IP removal time
480 can be attributed to the presence or absence of chlorine in their aromatic moiety
481 (**Table 1**). The biodegradation of chlorinated antibiotics (CLX and DCX) was slower
482 than the non-chlorinated one (OXA). The slower transformation of compounds with
483 halogen groups correlates with an electron deficiency produced by the halogen
484 groups in the aromatic moiety (Rodríguez-Delgado et al., 2016). Additionally,

485 electron-withdrawing substituents such as chloro, fluoro and nitro can inhibit the
486 oxidation of organic pollutants by fungal laccases (Abadulla et al., 2000). Therefore,
487 the biodegradation by *Leptosphaerulina* sp. depends on the chemical structure of
488 the IP and the antibiotic recalcitrance is a function of the increment of chlorines in
489 the molecule (Çabuk et al., 2012).

490 The bioprocess with *Leptosphaerulina* sp. also completely abated the AA. This is a
491 remarkable result because antimicrobial activity elimination should be guaranteed in
492 antibiotic-wastes treatment. The lack of AA produces antibiotics biologically inactive
493 limiting the proliferation of antibiotic-resistant bacteria. The absence of AA at the end
494 of the biotransformation process suggested that the transformation products also
495 lack of AA.

496 *Leptosphaerulina* sp. enzymes profile varied depending on the antibiotic. OXA had
497 VP and Lac as the principal activities, whereas, in CLX and DCX, VP represented
498 the main activity. The expression of VP by *Leptosphaerulina* sp. during CLX or DCX
499 biotransformation is higher than the reported by *Bjerkandera adusta* in the
500 degradation of non-chlorinated pharmaceuticals such as carbamazepine, ketoprofen
501 and trimethoprim (Touahar et al., 2014). Additionally, VP activity from
502 *Leptosphaerulina* sp. increased when the amount of chlorine atoms in antibiotics
503 augmented (3.11 U mg^{-1} for OXA, 5.74 U mg^{-1} for CLX, 12.65 U mg^{-1} for DCX),
504 suggesting that VP has a significant role in the transformation of these molecules.
505 Other authors also proved the VP ability to eliminate chlorinated compounds and its
506 participation in the transformation of halogenated phenols (Longoria et al., 2008).

507 VP from *B. adusta* strain UAMH 8258 produced the oxidative dehalogenation of
508 pesticide molecules such as dichlorophen. Similarly, VP from *Pleurotus eryngii* was
509 able to degrade 2,4-dichlorophenol (Davila-Vazquez et al., 2005; Pozdnyakova et
510 al., 2013).

511 Regarding the comparison between the enzymes from *Leptosphaerulina* sp. (crude
512 and pre-purified extracts) and commercial Lac for OXA degradation (**Figure 6**). The
513 pre-purified extract (which contains Lac, VP and MnP) removed 2 times the removal
514 percentage of OXA in comparison with commercial Lac. This confirmed that in
515 addition to Lac, the other enzymes secreted by *Leptosphaerulina* sp. are involved in
516 OXA removal. The low OXA removal percentage observed in the crude extract may
517 be associated to the non-presence of all the degrading machinery (*i.e.,* biomass and
518 intracellular enzymes) involved in the bio-treatment with *Leptosphaerulina* sp.
519 (Cajthaml, 2015; Čvančarová et al., 2015). In contrast, commercial peroxidase
520 exhibited the largest OXA elimination during enzymatic *in vitro* studies. This
521 established the participation of peroxidases on IP elimination. As previously reported
522 by Copete et al. (2015), *Leptosphaerulina* sp. can also secret different types of
523 oxidases such as glucose-methanol-choline (GMC) oxido-reductase family, NADH
524 oxidases, dye-decolorizing peroxidases, catalases and copper-containing oxidases,
525 which may participate in the biotransformation process.

526 Enzymatic activities of Lac, VP and MnP were completely inhibited by EDTA (30
527 mM) (**Figure 5**). The inhibitory effect of EDTA has been reported in other fungi
528 (Asgher et al., 2013; Forootanfar et al., 2011). Surprisingly, the use of NaCl in

529 *Leptosphaerulina* sp. increased the production of enzymes, which diverge from the
530 reported by Nagai et al. (2002), who found that NaCl 300 mM had a strong inhibitory
531 effect (90%) on Lac from *Agaricus blazei*. The inhibitory effect of Mn^{2+} has been
532 observed in VP, Lac and MnP from other fungi (Baldrian, 2004; Martinez et al., 1996;
533 Mester & Field, 1998). The inhibition assays confirmed the preponderant action of
534 enzymes on IP degradation. Surprisingly, although inhibitors such as Mn^{2+} or acetate
535 reduced the enzymatic activities in *Leptosphaerulina* sp. the remaining activities
536 were sufficient to eliminate AA. This remarks the strong transforming action of the
537 fungal enzymes on the considered antibiotics.

538 The enzymatic process that lead to the transformations of IP could be associated to
539 a cleavage of their β-lactam ring structure by ligninolytic enzymes and electron
540 abstraction from the aromatic ring (Marx et al., 2015). Antibiotics such as IP contain
541 a reactive and unstable cyclic amide (β-lactam), which is susceptible to chemical
542 and enzymatic transformation (Deshpande et al., 2004). In fact, the loss of
543 antimicrobial activity (**Figure 1**) can be associated with modifications by
544 *Leptosphaerulina* sp. enzymes on the β-lactam moiety; furthermore, the generation
545 of free radicals activators (Martínez et al., 2005; Rivera-Hoyos et al., 2013) allows
546 ligninolytic enzymes to be active on a high diversity of organic substrates including
547 antibiotics. As reported by Hofrichter (2002) certain non-phenolic aromatic
548 substances (as IP) could be modified for one-electron abstraction from the aromatic
549 ring.

550 Finally, the toxicity analysis indicated that the resultant solutions from IP bio-
551 treatment were non-toxic (**Table 3**). Moreover, the combination of these results with
552 the pollutants degradation and elimination of antimicrobial activity highlight the future
553 use of *Leptosphaerulina* sp. as an effective alternative to remediate water polluted
554 with IP antibiotics.

555

556 **5. CONCLUSIONS**

557 The Colombian isolate *Leptosphaerulina* sp. and its ligninolytic enzymes were able
558 to biotransform Isoxazolyl-Penicillins (OXA, CLX and DCX) in aqueous matrices.
559 *Leptosphaerulina* sp. achieved ~100% removal of antibiotics and antimicrobial
560 activity in all the IP within 8 days or less (OXA day 6, CLX day 7 and DCX day 8).
561 Additionally, the biotransformation products were non-toxic and without antibiotic
562 activity. Under the experimental conditions of this study, IP removal was associated
563 with the production of Lac and VP in all antibiotics and MnP was significant for the
564 high removal percentages of CLX and DCX. *In vitro* studies confirmed the enzymatic
565 nature of the biotransformation of IP by *Leptosphaerulina* sp. Additionally,
566 *Leptosphaerulina* sp. demonstrated its ability to significantly remove OXA and AA
567 using synthetic hospital wastewaters conditions. These results highlight the
568 opportunity to develop a biotechnological process based in *Leptosphaerulina* sp. for
569 the treatment of wastewaters polluted with antibiotics.

570

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587

588 **Nomenclature**

589 AA Antibacterial activity

590 ABTS 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium
591 salt

592 AOPs Advanced oxidation processes

593 BSA Bovine serum albumin

594 CLX Cloxacillin

595 DAD Diode array detector

596 DCX Dicloxacillin

597 DMEM Dulbecco's modified Eagle's medium

598 DMP 2,6-dimethoxyphenol

599 DMSO Dimethyl sulfoxide

600 DNS 3,5-dinitrosalicylic acid

601 EDTA Ethylenediaminetetraacetic acid

602 FBS Fetal bovine serum

603 HBT 1-hydroxybenzotriazol

604 HPLC High performance liquid chromatography

605 HRP Horseradish peroxidase

606 HWW Hospital wastewater

607 IP Isoxazolyl-Penicillin

608 MnP Manganese peroxidase

609 MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

610 NaCl Sodium chloride

611 Lac Laccase

612 LC_{50} Lethal concentration 50

613 LiP Lignin peroxidase

614 LMS Laccase-mediator systems

615 OXA Oxacillin

616 TPs Transformation products

617 U Units

618 VP Versatile peroxidase

619 WRF White-rot fungi

620 WWTPs Wastewater treatment plants

621 ʎ Wavelength

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