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

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Abstract

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

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

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

1. INTRODUCTION

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

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

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

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

Conventional wastewater treatment plants (WWTPs) are not designed to remove 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.

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

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

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

2. MATERIALS AND METHODS

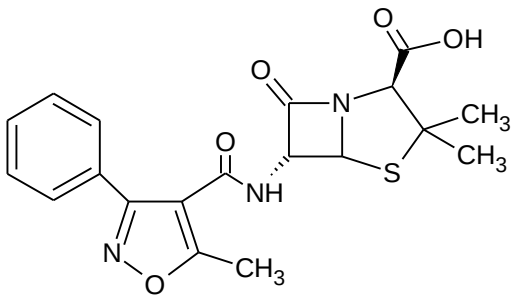
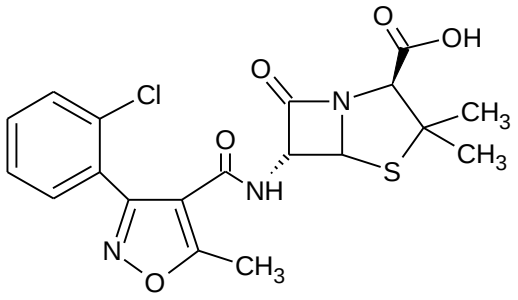
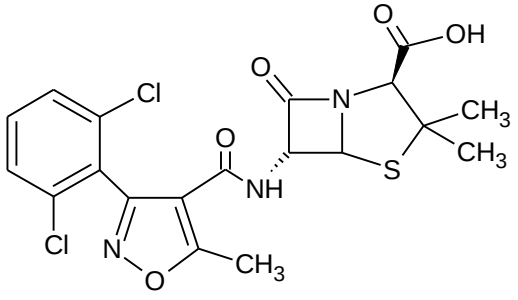
2.1. Chemicals

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

2.2. Microorganism and culture conditions

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

2.3. Isoxazolyl-Penicillins biotransformation experiments

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

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

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

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

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

Table 2. Composition Hospital wastewater (HWW)*.

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

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

2.5. Enzymatic activities

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

2.6. Enzymatic inhibition assay

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

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

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

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

2.8. In vitro degradation of IP by ligninolytic enzymes

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

2.9. Antibiotics chromatographic analysis

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

2.10. Residual antibacterial activity assay

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

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

2.11. Reducing sugars and protein quantification

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

2.12. Cytotoxicity assay

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

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

3. RESULTS

3.1. Removal of IP by Leptosphaerulina sp.

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

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

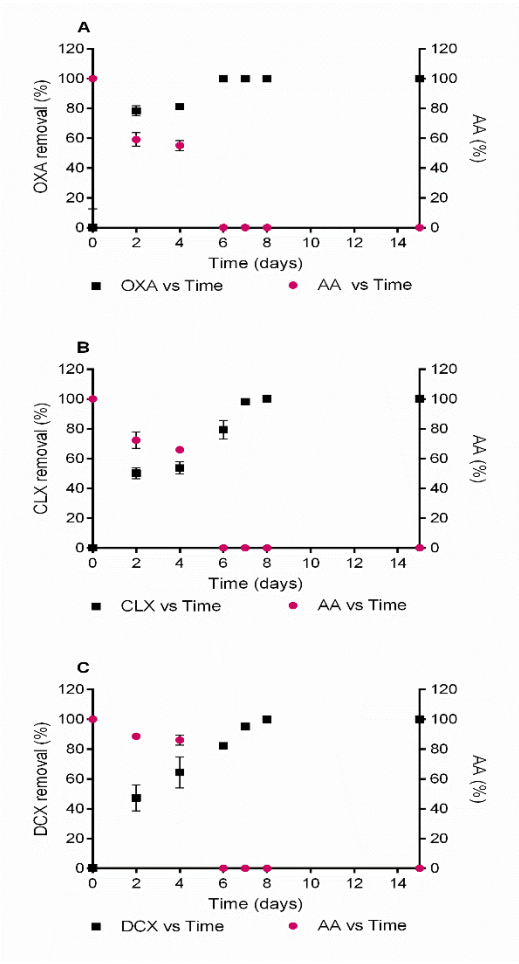


Figure 1. Antibiotic removal % and antibacterial activity (AA) % in the experiments with *Leptosphaerulina* sp. and the antibiotic **A)** OXA, **B)** CLX and **C)** DCX. Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 15 days.

3.2. Biodegradation and sorption processes in the removal of IP

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

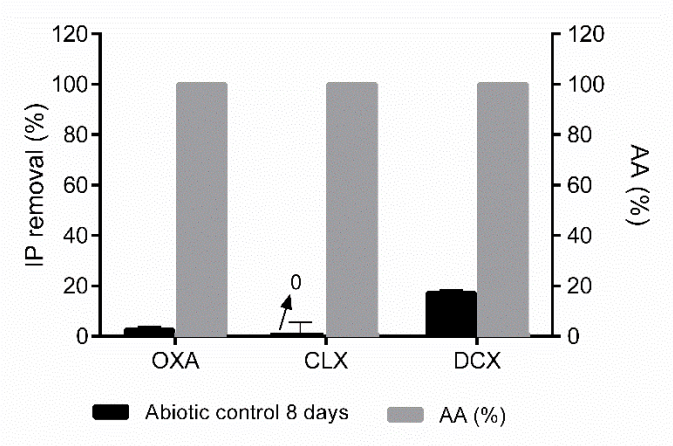


Figure 2. OXA [16000 µg L^{-1}], CLX [17500 µg L^{-1}] and DCX [19000 µg L^{-1}] treated by *Leptosphaerulina* sp. inert from 8 days of growth. Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 8 days.

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.

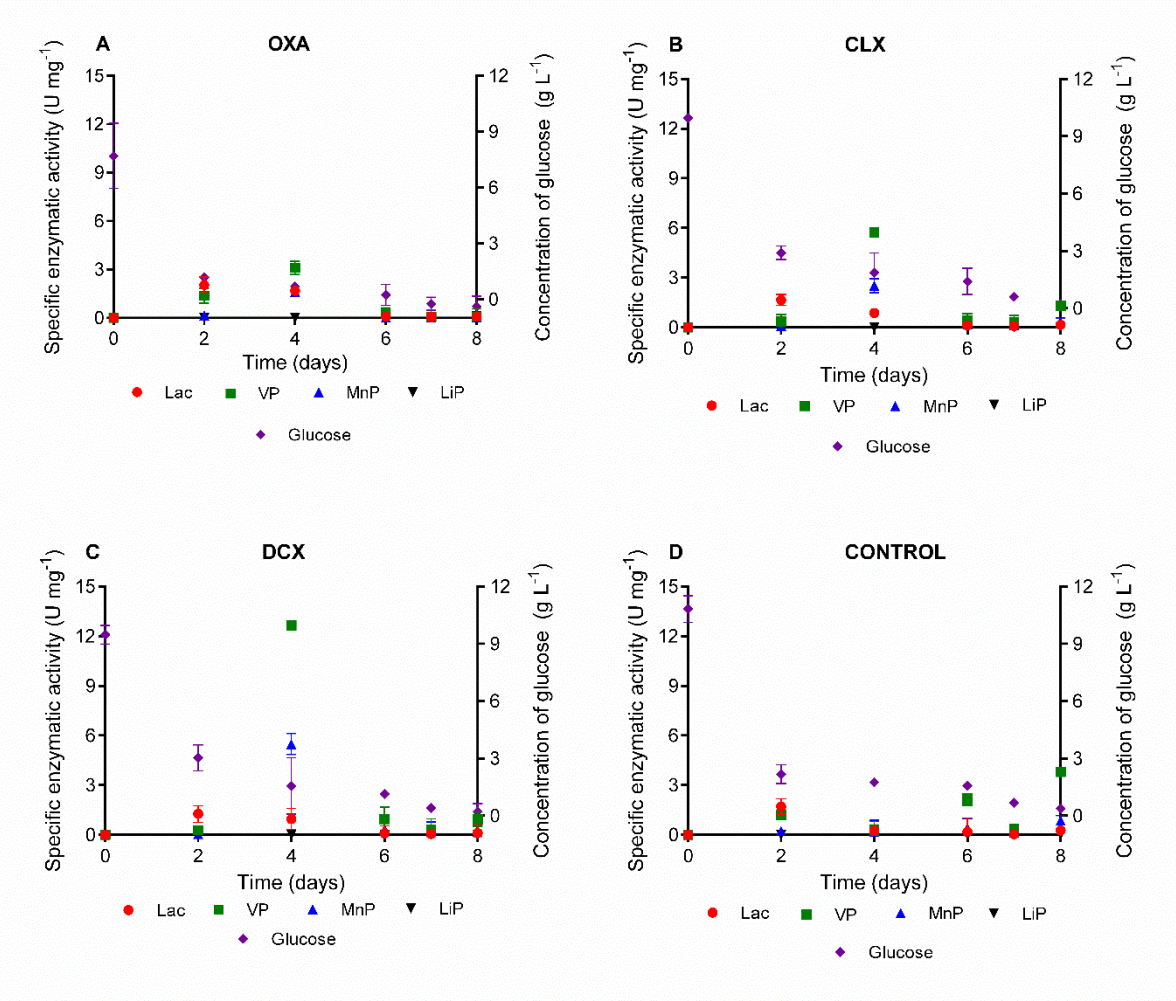


Figure 3. Specific enzymatic activities and glucose concentration for IP removal experiment **A**) OXA, **B**) CLX and **C**) DCX. **D**) biotic control (*Leptosphaerulina* sp. without antibiotic). Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 8 days.

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

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

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

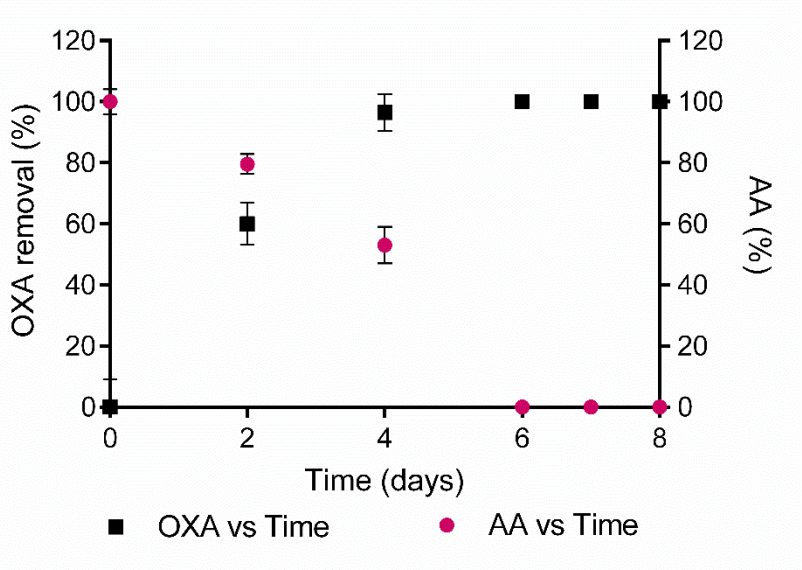


Figure 4. OXA removal % and antibacterial activity (AA) % in the experiments with *Leptosphaerulina* sp. in HWW. Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 8 days.

3.4. Enzymatic inhibitors studies

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

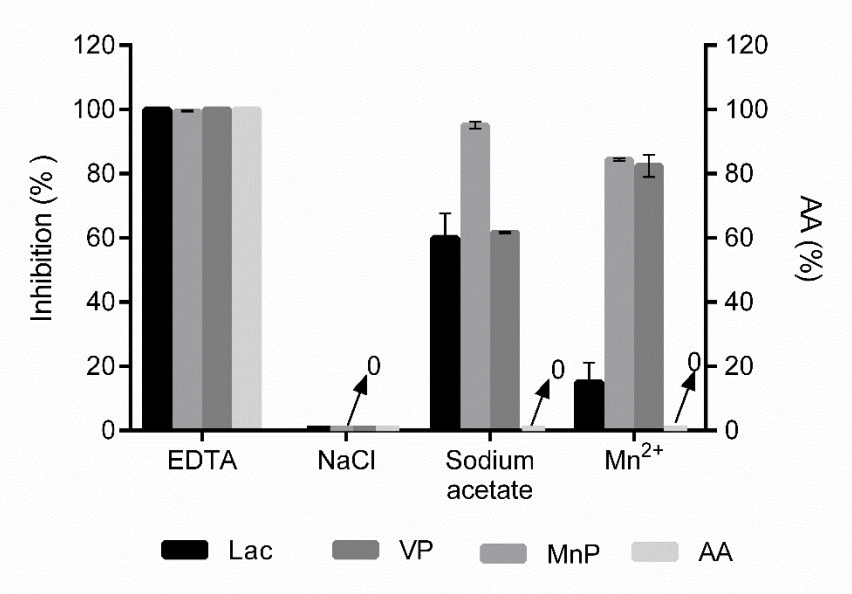


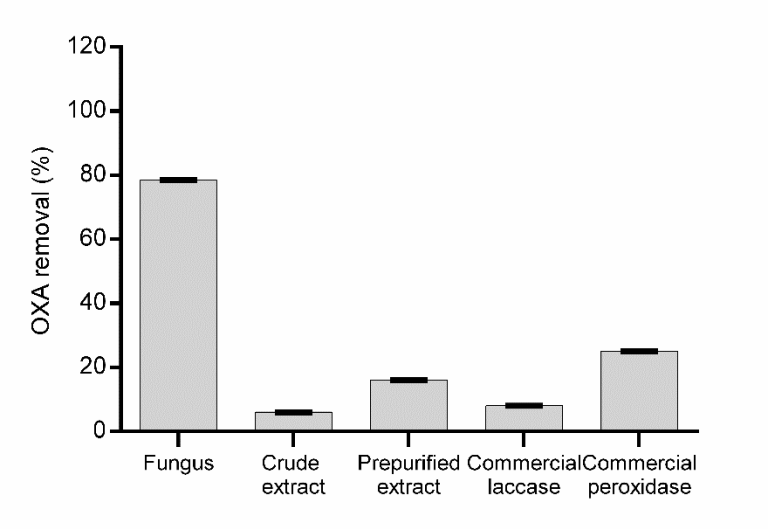
Figure 5. Influence of the EDTA (30 mM), NaCl (300 mM), sodium acetate (100 mM) and Mn^{2+} (100 mM) on Lac, MnP, VP and AA during the removal of OXA by *Leptosphaerulina* sp. Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 6 days.

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

3.5. Enzymatic in vitro studies

To study the individual degrading ability of the enzymatic machinery produced by the fungus, *in vitro* essays with crude and pre-purified extracts were considered. In addition, commercial enzymes were evaluated and compared with enzymatic extracts from *Leptosphaerulina* sp. In these experiments, OXA was employed because it was the antibiotic most rapidly degraded by *Leptosphaerulina* sp. **Figure 6** illustrates the percentage of IP removal after two 2 days of treatment. The crude extract (which contains Lac: 1.12 U mg^{-1}; VP: 1.77 U mg^{-1} and MnP: 0.28 U mg^{-1}) removed 6% of OXA. Whereas, the pre-purified extract (Lac: 1.16 U mg^{-1}; VP: 1.12 U mg^{-1} and MnP: 0.20 U mg^{-1}) eliminated 16% of OXA. The tests carried out with the commercial Lac achieved an 8% reduction of OXA initial concentration. The comparison between the fungus' enzymatic extracts and the commercial enzymes evidenced the pre-purified extract as the most suitable option for *in vitro* removal. On the other hand, the experiment with the commercial peroxidase (which eliminates 25% of OXA, **Figure 6**) correlated with the results from the in vivo experiments with *Leptosphaerulina* sp. (**Figure 3A**), where a high VP activity was associated with OXA 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**).

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

4. DISCUSSION

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

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

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

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

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

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

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

5. CONCLUSIONS

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

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Nomenclature

AA	Antibacterial activity
ABTS	2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium 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

MnP	Manganese peroxidase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
Lac	Laccase
LC_{50}	Lethal concentration 50
LiP	Lignin peroxidase
LMS	Laccase-mediator systems
OXA	Oxacillin
TPs	Transformation products
U	Units
VP	Versatile peroxidase
WRF	White-rot fungi
WWTPs	Wastewater treatment plants
ʎ	Wavelength

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