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

Susana Pérez-Grisales[1†], Marian Castrillón-Tobón[1†], Ledys S. Copete-Pertuz[1*], Jersson Plácido[2], Amanda L. Mora-Martínez[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 Institute of Life Science, Medical School, Swansea University, Swansea, SA2 8PP, Wales, UK.

Abstract

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

*Corresponding authors:

Tel.: +57(4)4309339;

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

† These authors contributed equally to this work

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

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

Capsule:

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

1. INTRODUCTION

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

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

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

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

the removal and degradation of these recalcitrant compounds.

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

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

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

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

2. MATERIALS AND METHODS

2.1. Chemicals

Reactive Black 5 (RB5) (azoic dye, λ_{max}= 598 nm) was donated by Fabricato-Tejicondor S.A. Cephadroxyl monohydrate 92.9% (CPD) was obtained from syntofarma. Glucose, yeast extract, ammonium molybdate, monobasic potassium phosphate, zinc sulphate heptahydrate, peptone, tetraborate sodium decahydrate, sodium acetate and malt extract, 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, ammonium sulphate, acetic acid, sodium chloride, formic acid, potassium chloride, tartaric acid, hydrogen peroxide, acetonitrile, methanol and Mueller-Hinton agar were bought from Merck. 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt 98% (ABTS) from Sigma-Aldrich.

2.2. Microorganism and culture conditions

Leptosphaerulina sp. was obtained from PROBIOM research group's microorganisms collection (CECT 20913) (Copete et al., 2015; Plácido et al., 2016). The fungus was maintained in malt extract agar at 4 °C until use. Mycelium from a 10–days old culture was homogenised and employed as inoculum in the removal process (Copete et al., 2015). Experiments were carried out in a culture medium (pH 5.6) containing 10 g L^{-1} glucose, 5 g L^{-1} peptone, 2 g L^{-1} ammonium tartrate, 1 g L^{-1} yeast extract, 1 g L^{-1} KH_2PO_4, 0.5 g L^{-1} KCl and 0.5 g L^{-1} $MgSO_4$. $7H_2O$, 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} $(NH_4)_6Mo_7O_{24}$. $4H_2O$ and 10 mg L^{-1} $MnSO_4$. $7H_2O$] (Copete-Pertuz, et al., 2018). 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. Evaluation of natural support materials for the immobilisation of Leptosphaerulina sp.

Four different natural materials (charcoal, luffa sponge, wood chips and cork) were obtained from a local store (Medellín, Colombia). They were evaluated in order to select the most appropriate immobilisation material for *Leptosphaerulina* sp. The supporting materials were placed in 250 mL Erlenmeyer flasks with 100 mL of culture medium. The supporting materials initial load (charcoal, 5 g; luffa sponge, 1 g; wood chips, 5 g; cork of 5 cm^2, 3 g) was selected based on each material characteristics. The luffa sponge and wood chips initial load corresponded to the values reported by El-Sherif et al., (2013) and Mahmoud (2007), respectively. After the support materials were added into the Erlenmeyer flasks, these were inoculated with 5 mL of *Leptosphaerulina* sp. homogenised and incubated at 160 rpm, 28 °C for 15 days (Ehlers and Rose, 2005; Kasinath, 2003). The controls for this experiment were *Leptosphaerulina* sp. cultures without supporting material and the supporting material with culture medium and without fungal strain. All assays were performed in triplicate. The experiment response variable was the amount of biomass retained in each of the supports, which was determined qualitatively by observing the biomass growth in the material during the days 3, 7, 12 and 15. The biomass growth was classified in no growth (–), low growth (+), medium growth (++), and significant growth (+++).

2.4. Biotransformation of CPD and RB5 by immobilised Leptosphaerulina sp.

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

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

2.5. Determination of ligninolytic enzymatic activities

Lac activity was measured by monitoring the oxidation of ABTS (3 mM) in sodium tartrate buffer (0.1 M, pH 3.0) (Ciullini et al., 2008; Zhou et al., 2014). Similarly, the VP activity was determined from the oxidation of ABTS (3 mM) in buffer solution sodium tartrate (pH 3.0, 0.1 M) and H_2O_2 (0.1 mM) (Camarero et al., 1999; Copete et al., 2015). Lac and VP activities were monitored spectrophotometrically at a wavelength of 420 nm and a molar extinction coefficient of (ε_{420}, 36000 M^{-1} cm^{-1}). MnP activity was measured based on the oxidation of 2,6-dimethoxyphenol (DMP, 1 mM), in sodium acetate (pH 4.5, 0.1 M) with H_2O_2 (0.1 mM) and $MnSO_4$ (1 mM) at 469 nm (ε_{469}, 27500 M^{-1} cm^{-1}) (Mizuno et al., 2009). Enzymatic activity was determined in triplicate and expressed in units (U) of enzyme per milligram of protein (U mg^{-1}). U is defined as the amount of enzyme that catalyses 1 µmol of substrate in 1 min (Ciullini et al., 2008; Enayatzamir, 2009).

2.6. Determination of protein concentration and dry weight

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

2.7. Chromatographic analysis of antibiotic

CPD removal was determined by reverse phase high performance liquid chromatography (RP)-HPLC (Thermo Scientific DIONEX UltiMate 3000) equipped with a Diode Array Detector (DAD) and a Thermo scientific Acclaim 120 column (C-18 5µm, 4.6x100mm). A homogeneous mixture of acetonitrile (C_2H_3N) and formic acid buffer (CH_2O_2) (10 mM, pH 3), 20/80 (% v/v) was used as mobile phase (Serna-Galvis, et al., 2017). The chromatography utilised 20 µL of the samples, a mobile phase flow of 1 mL min^{-1} and a fixed wavelength of 225 nm for the DAD detector.

2.8. Residual antibacterial activity assays

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

2.9. Evaluation of decolourisation of RB5 in aqueous solution

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

$$D\% = \frac{A_0 - A_t}{A_0} \times 100 \qquad \textbf{Equation 1}$$

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

3. RESULTS

3.1. Evaluation of different materials for the immobilisation of Leptosphaerulina sp.

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

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

3.1. Biotransformation of cephadroxyl using suspended and immobilised Leptosphaerulina sp.

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

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

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

Lac, MnP and VP enzymatic activities were determined for the suspended and immobilised cultures and their results are described in **Figure 2**. In both cultures, the enzymatic activities increased during the first three days followed by a constant enzymatic activity reduction. The immobilised *Leptosphaerulina* sp. experiment 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

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

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

3.2. RB5 Decolourisation

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

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

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

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

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

4. DISCUSSION

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

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

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

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

The different results obtained by the combination of immobilisation and pollutant can be associated with different *Leptosphaerulina* sp. morphologies. Fungal morphology can be influenced by different environmental factors such as reactor geometry, agitation speed, airflow and culture media which can generate variability in the enzymatic expression (Krull et al., 2013; Naghdi et al., 2018). RB5 removal by immobilised *Leptosphaerulina* sp. was associated with dye sorption by the solid 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.

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

5. CONCLUSIONS

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

6. ACKNOWLEDGEMENTS

The authors would like to thank the research system of the Universidad Nacional de Colombia for the financial support provide by the grant No. 35945 “Biotransformación de antibióticos β-lactámicos por el aislado fúngico nativo *Leptosphaerulina* sp. y sus enzimas ligninolíticas”. L.S. Copete-Pertuz would like to thank the Grupo de 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

512 **7. REFERENCES**

513 Adnan, L. A., Rahim, A., & Yusoff, M. (2014). Biodegradation of Bis-Azo Dye Reactive Black 5 by
514 White-Rot Fungus Trametes gibbosa sp . WRF 3 and Its Metabolite Characterization.
515 https://doi.org/10.1007/s11270-014-2119-2

516 Adnan, L. A., Sathishkumar, P., Mohd Yusoff, A. R., & Hadibarata, T. (2015). Metabolites
517 characterisation of laccase mediated Reactive Black 5 biodegradation by fast growing
518 ascomycete fungus Trichoderma atroviride F03. *International Biodeterioration and*
519 *Biodegradation*, *104*, 274–282. https://doi.org/10.1016/j.ibiod.2015.05.019

520 Barry, D. J., Chan, C., & Williams, G. A. (2009). Morphological quantification of filamentous fungal
521 development using membrane immobilization and automatic image analysis. *J Ind Microbiol*
522 *Biotechnol*, 787–800. https://doi.org/10.1007/s10295-009-0552-9

523 Bertrand, B., Martínez-Morales, F., & Trejo-Hernández, M. R. (2017). Upgrading Laccase Production
524 and Biochemical Properties: Strategies and Challenges. *Biotechnology Progress*, *33*(4), 1015–
525 1034. https://doi.org/10.1002/btpr.2482

526 Bhatia, D., Sharma, N. R., Singh, J., & Kanwar, R. S. (2017). Biological methods for textile dye
527 removal from wastewater: A Review. *Critical Reviews in Environmental Science and*
528 *Technology*, *3389*(November), 0–0. https://doi.org/10.1080/10643389.2017.1393263

529 Botero-Coy, A. M., Martínez-Pachón, D., Boix, C., Rincón, R. J., Castillo, N., Arias-Marín, L. P., …
530 Hernández, F. (2018). ‘An investigation into the occurrence and removal of pharmaceuticals in
531 Colombian wastewater.’ *Science of the Total Environment*, *642*, 842–853.
532 https://doi.org/10.1016/j.scitotenv.2018.06.088

533 Bouki, C., Venieri, D., & Diamadopoulos, E. (2013). Detection and fate of antibiotic resistant bacteria
534 in wastewater treatment plants: A review. *Ecotoxicology and Environmental Safety*, *91*, 1–9.
535 https://doi.org/10.1016/j.ecoenv.2013.01.016

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Camarero, S., Ibarra, D., Martınez, M. J., & Martınez, A. T. (2005). Lignin-Derived Compounds as Efficient Laccase Mediators for decolorization of recalcitrant dye.pdf. *Appl. Environ. Microbiol.*, *71*(4), 1775–1784. https://doi.org/10.1128/AEM.71.4.1775–1784.2005

Camarero, S., Martínez, M., & Martínez, A. T. (2012). Understanding lignin biodegradation for the improved utilization of plant biomass in modern biorefineries. *Biofuels, Bioproducts and Biorefining*, *6*(3), 246–256. https://doi.org/10.1002/bbb

Camarero, S., Sarkar, S., Ruiz-Dueñas, F. J., Martínez, M. J., & Martínez, Á. T. (1999). Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. *Journal of Biological Chemistry*, *274*(15), 10324–10330. https://doi.org/10.1074/jbc.274.15.10324

Chanagá Vera, X., Plácido Escobar, J., Marín Montoya, M., & Yepes Pérez, M. D. S. (2012). Hongos Nativos con Potencial Degradador de Tintes Industriales en el Valle de Aburrá , Colombia. *Revista Facultad Nacional Agronomía de Medellín*, *65*(2), 6811–6821.

Ciullini, I., Tilli, S., Scozzafava, A., & Briganti, F. (2008). Fungal laccase, cellobiose dehydrogenase, and chemical mediators: Combined actions for the decolorization of different classes of textile dyes. *Bioresource Technology*, *99*(15), 7003–7010. https://doi.org/10.1016/j.biortech.2008.01.019

Copete-Pertuz, L. S., Alandete-Novoa, F., Plácido, J., Correa-Londoño, G. A., & Mora-Martínez, A. L. (2019). Enhancement of ligninolytic enzymes production and decolourising activity in Leptosphaerulina sp. by co–cultivation with Trichoderma viride and Aspergillus terreus. *Science of The Total Environment*, *646*, 1536–1545. https://doi.org/10.1016/j.scitotenv.2018.07.387

Copete-Pertuz, L. S., Pérez-Grisales, M. S., Castrillón-Tobón, M., Guillermo, A., Londoño, C., García, G. T., & Martínez, A. L. M. (2018). Decolorization of Reactive Black 5 Dye by Heterogeneous Photocatalysis with TiO2 /UV. *Revista Colombiana de Química*, *47*(63), 36–44. https://doi.org/doi.org/10.15446/rev.colomb.quim.v47n2.67922

Copete-Pertuz, L. S., Plácido, J., Serna-Galvis, E. A., Torres-Palma, R. A., & Mora, A. (2018). Elimination of Isoxazolyl-Penicillins antibiotics in waters by the ligninolytic native Colombian strain Leptosphaerulina sp. considerations on biodegradation process and antimicrobial activity removal. *Science of the Total Environment*, *630*, 1195–1204. https://doi.org/10.1016/j.scitotenv.2018.02.244

Copete, L. S., Chanagá, X., Barriuso, J., López-Lucendo, M. F., Martínez, M. J., & Camarero, S. (2015). Identification and characterization of laccase-type multicopper oxidases involved in dye-decolorization by the fungus Leptosphaerulina sp. *BMC Biotechnology*, *15*(74), 1–13. https://doi.org/10.1186/s12896-015-0192-2

Couto, S. R. (2009). Dye removal by immobilised fungi. *Biotechnology Advances*, *27*(3), 227–235. https://doi.org/10.1016/j.biotechadv.2008.12.001

Čvančarová, M., Moeder, M., Filipová, A., & Cajthaml, T. (2015). Biotransformation of fluoroquinolone antibiotics by ligninolytic fungi - Metabolites, enzymes and residual antibacterial activity. *Chemosphere*, *136*, 311–320. https://doi.org/10.1016/j.chemosphere.2014.12.012

Čvančarová, M., Moeder, M., Filipova, A., Reemtsma, T., & Cajthaml, T. (2013). Biotransformation of the antibiotic agent flumequine by ligninolytic fungi and residual antibacterial activity of the

transformation mixtures. *Environmental Science and Technology*, *47*(24), 14128–14136. https://doi.org/10.1021/es403470s

Daássi, D., Mechichi, T., Nasri, M., & Rodriguez-Couto, S. (2013). Decolorization of the metal textile dye Lanaset Grey G by immobilized white-rot fungi. *Journal of Environmental Management*, *129*, 324–332. https://doi.org/10.1016/j.jenvman.2013.07.026

Dubey, M. K., Zehra, A., Aamir, M., Meena, M., Ahirwal, L., Singh, S., … Bajpai, V. K. (2017). Improvement strategies, cost effective production, and potential applications of fungal glucose oxidase (GOD): Current updates. *Frontiers in Microbiology*, *8*(JUN), 1–22. https://doi.org/10.3389/fmicb.2017.01032

Efligenir, A., Déon, S., Fievet, P., Druart, C., Morin-Crini, N., & Crini, G. (2014). Decontamination of polluted discharge waters from surface treatment industries by pressure-driven membranes: Removal performances and environmental impact. *Chemical Engineering Journal*, *258*, 309–319. https://doi.org/10.1016/j.cej.2014.07.080

Ehlers, G. A., & Rose, P. D. (2005). Immobilized white-rot fungal biodegradation of phenol and chlorinated phenol in trickling packed-bed reactors by employing sequencing batch operation. *Bioresource Technology*, *96*(11), 1264–1275. https://doi.org/10.1016/j.biortech.2004.10.015

El-Sherif, M. F., Youssef, A. S., Hassan, M. A., Hassan, H. M. G., & El-Aassar, S. A. (2013). Immobilization and Solid-State Fermentation Methods for Chitinase Production from. *Life Science Journal*, *10*(4), 3036–3043.

Enayatizamir, N., Tabandeh, F., Rodríguez-Couto, S., Yakhchali, B., Alikhani, H. A., & Mohammadi, L. (2011). Biodegradation pathway and detoxification of the diazo dye Reactive Black 5 by Phanerochaete chrysosporium. *Bioresource Technology*, *102*(22), 10359–10362. https://doi.org/10.1016/j.biortech.2011.08.130

Enayatzamir, K., Alikhani, H. A., & Rodríguez Couto, S. (2009). Simultaneous production of laccase and decolouration of the diazo dye Reactive Black 5 in a fixed-bed bioreactor. *Journal of Hazardous Materials*, *164*(1), 296–300. https://doi.org/10.1016/j.jhazmat.2008.08.032

Etebu, E., & Arikekpar, I. (2016). Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *Ijambr*, *4*, 90–101.

Fernández, J. A., Henao, L. M., Pedroza-Rodríguez, A. M., & Quevedo-Hidalgo, B. (2009). Inmovilización de hongos ligninolíticos para la remoción del colorante negro reactivo 5. *Revista Colombiana de Biotecnología*, *11*(1), 59–72.

Forootanfar, H., Rezaei, S., Zeinvand-Lorestani, H., Tahmasbi, H., Mogharabi, M., Ameri, A., & Faramarzi, M. A. (2016). Studies on the laccase-mediated decolorization, kinetic, and microtoxicity of some synthetic azo dyes. *Journal of Environmental Health Science and Engineering*, *14*(1), 7. https://doi.org/10.1186/s40201-016-0248-9

García-Santamarina, S., & Thiele, D. J. (2015). Copper at the fungal pathogen-host axis. *Journal of Biological Chemistry*, *290*(31), 18945–18953. https://doi.org/10.1074/jbc.R115.649129

Hussain, S., Maqbool, Z., Ali, S., Yasmeen, T., Imran, M., Mahmood, F., & Abbas, F. (2013). Biodecolorization of reactive black-5 by a metal and salt tolerant bacterial strain Pseudomonas sp. RA20 isolated from Paharang drain effluents in Pakistan. *Ecotoxicology and Environmental Safety*, *98*, 331–338. https://doi.org/10.1016/j.ecoenv.2013.09.018

Iqbal, M., & Edyvean, R. G. J. (2005). Loofa sponge immobilized fungal biosorbent: A robust system

for cadmium and other dissolved metal removal from aqueous solution. *Chemosphere*, *61*(4), 510–518. https://doi.org/10.1016/j.chemosphere.2005.02.060

Jeon, J. R., Baldrian, P., Murugesan, K., & Chang, Y. S. (2012). Laccase-catalysed oxidations of naturally occurring phenols: From in vivo biosynthetic pathways to green synthetic applications. *Microbial Biotechnology*, *5*(3), 318–332. https://doi.org/10.1111/j.1751-7915.2011.00273.x

José Pallares, C., & Martínez, E. (2012). Implementación de un programa de uso regulado de antibióticos en 2 unidades de cuidado intensivo medico-quirúrgico en un hospital universitario de tercer nivel en Colombia. *Infectio*, *16*(4), 192–198. https://doi.org/10.1016/S0123-9392(12)70013-9

Kasinath, A., Novotný, Č., Svobodová, K., Patel, K. C., & Šašek, V. (2003). Decolorization of synthetic dyes by Irpex lacteus in liquid cultures and packed-bed bioreactor. *Enzyme and Microbial Technology*, *32*(1), 167–173. https://doi.org/10.1016/S0141-0229(02)00279-X

Khan, R., Bhawana, P., & Fulekar, M. H. (2013). Microbial decolorization and degradation of synthetic dyes: A review. *Reviews in Environmental Science and Biotechnology*, *12*(1), 75–97. https://doi.org/10.1007/s11157-012-9287-6

Krull, R., Wucherpfennig, T., Eslahpazir, M., Walisko, R., Melzer, G., Hempel, D. C., … Wittmann, C. (2013). Characterization and control of fungal morphology for improved production performance in biotechnology. *Journal of Biotechnology*, *163*(2), 112–123. https://doi.org/10.1016/j.jbiotec.2012.06.024

Li, X., Xu, J., Toledo, R. A. De, & Shim, H. (2015). Bioresource Technology Enhanced removal of naproxen and carbamazepine from wastewater using a novel countercurrent seepage bioreactor immobilized with Phanerochaete chrysosporium under non-sterile conditions. *BIORESOURCE TECHNOLOGY*, *197*, 465–474. https://doi.org/10.1016/j.biortech.2015.08.118

Lucas, D., Badia-Fabregat, M., Vicent, T., Caminal, G., Rodríguez-Mozaz, S., Balcázar, J. L., & Barceló, D. (2016). Fungal treatment for the removal of antibiotics and antibiotic resistance genes in veterinary hospital wastewater. *Chemosphere*, *152*, 301–308. https://doi.org/10.1016/j.chemosphere.2016.02.113

Mahmoud, D. (2007). Immobilization of invertase by a new economical method using wood sawdust waste. *Australian Journal of Applied Science*, *1*(4), 364–372.

Masran, R., Zanirun, Z., Bahrin, E. K., Ibrahim, M. F., Lai Yee, P., & Abd-Aziz, S. (2016). Harnessing the potential of ligninolytic enzymes for lignocellulosic biomass pretreatment. *Applied Microbiology and Biotechnology*, *100*(12), 5231–5246. https://doi.org/10.1007/s00253-016-7545-1

Máximo, C., Amorim, M. T. P., & Costa-Ferreira, M. (2003). Biotransformation of industrial reactive azo dyes by Geotrichum sp. CCMI 1019. *Enzyme and Microbial Technology*, *32*(1), 145–151. https://doi.org/10.1016/S0141-0229(02)00281-8

Mitkowski, N. A., & Browning, M. (2004). Leptosphaerulina australis associated with intensively managed stands of Poa annua and Agrostis palustris. *Canadian Journal of Plant Pathology*, *26*(2), 193–198. https://doi.org/10.1080/07060660409507131

Mizuno, H., Hirai, H., Kawai, S., & Nishida, T. (2009). Removal of estrogenic activity of iso-butylparaben and n-butylparaben by laccase in the presence of 1-hydroxybenzotriazole. *Biodegradation*, *20*(4), 533–539. https://doi.org/10.1007/s10532-008-9242-y

Nabil, G. M., El-Mallah, N. M., & Mahmoud, M. E. (2014). Enhanced decolorization of reactive black 5 dye by active carbon sorbent-immobilized-cationic surfactant (AC-CS). *Journal of Industrial and Engineering Chemistry*, *20*(3), 994–1002. https://doi.org/10.1016/j.jiec.2013.06.034

Naghdi, M., Taheran, M., Brar, S. K., Kermanshahi-pour, A., Verma, M., & Surampalli, R. Y. (2018). Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes. *Environmental Pollution*, *234*, 190–213. https://doi.org/10.1016/j.envpol.2017.11.060

Oliveira, L. M. F., Nascimento, M. A., & Lopes, R. P. (2018). Removal of Beta-Lactams Antibiotics through Zero-Valent Copper Nanoparticles, *29*(8), 1630–1637.

Park, C., Lee, B., Han, E. J., Lee, J., & Kim, S. (2006). Decolorization of acid black 52 by fungal immobilization. *Enzyme and Microbial Technology*, *39*(3), 371–374. https://doi.org/10.1016/j.enzmictec.2005.11.045

Patel, V. R., & Bhatt, N. S. (2013). Involvement of ligninolytic enzymes of Myceliophthora vellerea HQ871747 in decolorization and complete mineralization of Reactive Blue 220. *Chemical Engineering Journal Journal*, *233*, 98–108. https://doi.org/10.1016/j.cej.2013.07.110

Plácido, J., Chanagá, X., Ortiz-Monsalve, S., Yepes, M., & Mora, A. (2016). Degradation and detoxification of synthetic dyes and textile industry effluents by newly isolated Leptosphaerulina sp. from Colombia. *Bioresources and Bioprocessing*, *3*(6), 1–14. https://doi.org/10.1186/s40643-016-0084-x

Prieto, A., Möder, M., Rodil, R., Adrian, L., & Marco-Urrea, E. (2011). Degradation of the antibiotics norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products. *Bioresource Technology*, *102*(23), 10987–10995. https://doi.org/10.1016/j.biortech.2011.08.055

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., … Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the Total Environment*, *447*, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

Saeed, A., & Iqbal, M. (2013). Loofa (Luffa cylindrica) sponge: Review of development of the biomatrix as a tool for biotechnological applications. *Biotechnology Progress*, *29*(3), 573–600. https://doi.org/10.1002/btpr.1702

Serna-Galvis, E. A., Ferraro, F., Silva-Agredo, J., & Torres-Palma, R. A. (2017). Degradation of highly consumed fluoroquinolones, penicillins and cephalosporins in distilled water and simulated hospital wastewater by UV254 and UV254/persulfate processes. *Water Research*, *122*, 128–138. https://doi.org/10.1016/j.watres.2017.05.065

Shedbalkar, U., Dhanve, R., & Jadhav, J. (2008). Biodegradation of triphenylmethane dye cotton blue by Penicillium ochrochloron MTCC 517. *J Hazard Mater*, *157*(2–3), 472–479. https://doi.org/10.1016/j.jhazmat.2008.01.023

Sriharsha, D. V., Kumar R., L., & Savitha, J. (2017). Immobilized fungi on Luffa cylindrica: An effective biosorbent for the removal of lead. *Journal of the Taiwan Institute of Chemical Engineers*, *80*, 589–595. https://doi.org/10.1016/j.jtice.2017.08.032

Usha, S. M., Sasirekha, B., Bela, R. B., Devi, S., Kamalini, C., Manasa, G. A., & Neha, P. M. (2011). Optimization of Reactive Black 5 dye and Reactive Red 120 dye degradation. *Journal of Chemical and Pharmaceutical Research*, *3*(6), 1089–1096. https://doi.org/0975-7384

706 Verlicchi, P., Al Aukidy, M., & Zambello, E. (2012). Occurrence of pharmaceutical compounds in
707 urban wastewater: Removal, mass load and environmental risk after a secondary treatment-A
708 review. *Science of the Total Environment*, *429*, 123–155.
709 https://doi.org/10.1016/j.scitotenv.2012.04.028

710 Wang, Z. W., Liang, J. S., & Liang, Y. (2013). Decolorization of Reactive Black 5 by a newly isolated
711 bacterium Bacillus sp. YZU1. *International Biodeterioration and Biodegradation*, *76*, 41–48.
712 https://doi.org/10.1016/j.ibiod.2012.06.023

713 Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., … Meng, W. (2015). Occurrence of antibiotics and
714 antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river.
715 *Chemosphere*, *119*, 1379–1385. https://doi.org/10.1016/j.chemosphere.2014.02.040

716 Yildirim, S. C., & Yesilada, O. (2015). A comparative study on decolorization of reactive azo and
717 indigoid dyes by free/immobilized pellets of Trametes versicolor and Funalia trogii. *Journal of*
718 *Enviromental Biology*, *36*(November), 1393–1400.

719 Zabłocka-godlewska, E., Przystas, W., & Grabinska-Sota, E. (2017). Efficiency of decolorization of
720 different dyes using fungal biomass immobilized on different solid supports Grabi nska-Sota.
721 *Brazilian Journal of Microbiology*, *9*, 285–295. https://doi.org/10.1016/j.bjm.2017.06.010

722 Zhou, P., Fu, C., Fu, S., & Zhan, H. (2014). Purification and characterization of white laccase from
723 the white-rot fungus Panus conchatus. *BioResources*, *9*(2), 1964–1976.
724 https://doi.org/10.15376/biores.9.2.1964-1976

725 Zucca, P., Cocco, G., Sollai, F., & Sanjust, E. (2016). Fungal laccases as tools for biodegradation of
726 industrial dyes. *Biocatalysis*, *1*(1), 82–108. https://doi.org/10.1515/boca-2015-0007

727

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	**Growth[1]**
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.
Leptosphaerulina sp.	Luffa sponge	CPD	15256 µg L^{-1}	~100%	This work
Leptosphaerulina sp.	Luffa sponge	RB5	200 mg L^{-1}	91.9%	This work
Trametes versicolor	Luffa sponge	RB5	150 mg L^{-1}	98%	(Fernández et al., 2009)
P. chrysosporium BKM-F-1767	Wood dust	Naproxen	1000 µg/leach	~100%	(Li et al., 2015)
		Carbamezepine	1000 µg/leach	80%	
Polyporus picipes (RWP17), *Gleophylum odoratum (DCa)*	Polypropylene Washer	Evans Blue	100 mg L^{-1}	~100%	(Zabłocka-godlewska et al., 2017)
		Brilliant green	100 mg L^{-1}	85%	
Trametes pubescens	Stainless steel sponges	RB5	30 mg L^{-1}	74%	(Enayatzamir et al., 2009)
Trametes versicolor	Free pellets	Acid blue 74	50 mg L^{-1}	96.8%	(Yildirim & Yesilada, 2015)
		Reactive Blue 198	50 mg L^{-1}	91.3%	
Funalia trogii	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)

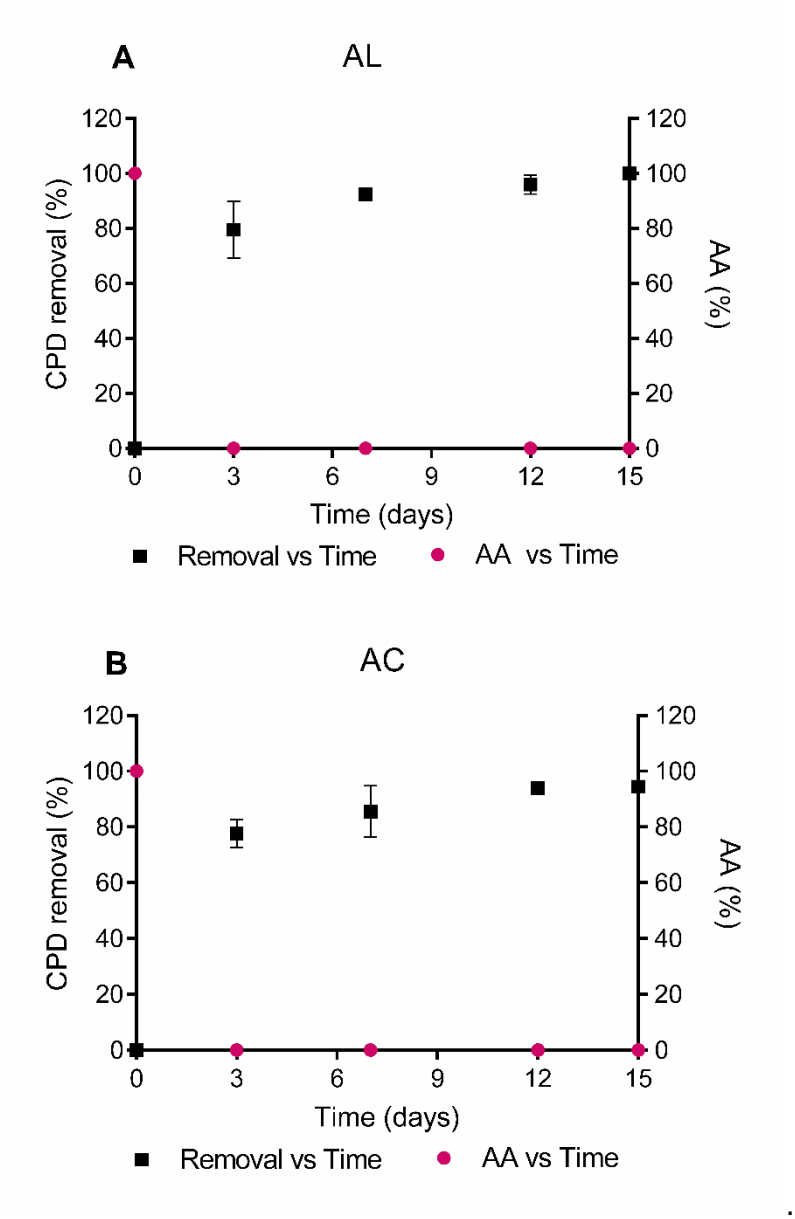

Figure 2. Evaluation of the ligninolytic enzymatic activities. AL to the immobilisation experiments with CPD. AC are the fungus cultures in suspension with CPD. HM is the cultivation of the fungus in suspension free of CPD antibiotic. LC corresponds to the immobilisation experiments free of CPD. **A)**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

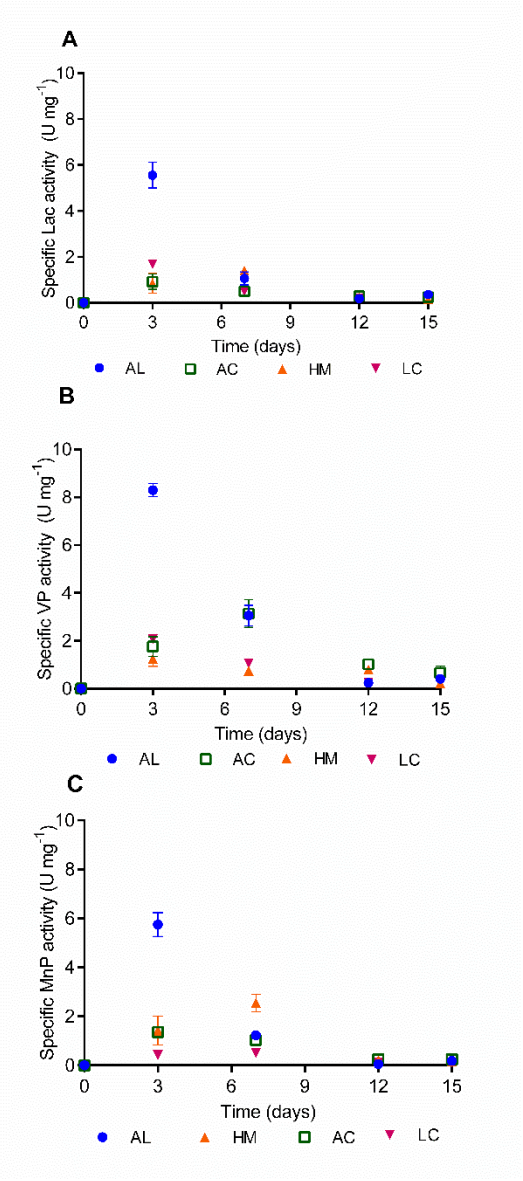

Figure 3. Quantification of dry weight for immobilisation experiments. AL corresponds to immobilisation assays with antibiotic. CL are the dye-containing immobilisation experiments. LC corresponds to the antibiotic-free dye-free immobilisation experiment. Experimental conditions: 28 °C, 160 rpm, pH= 5.6, 15 days.

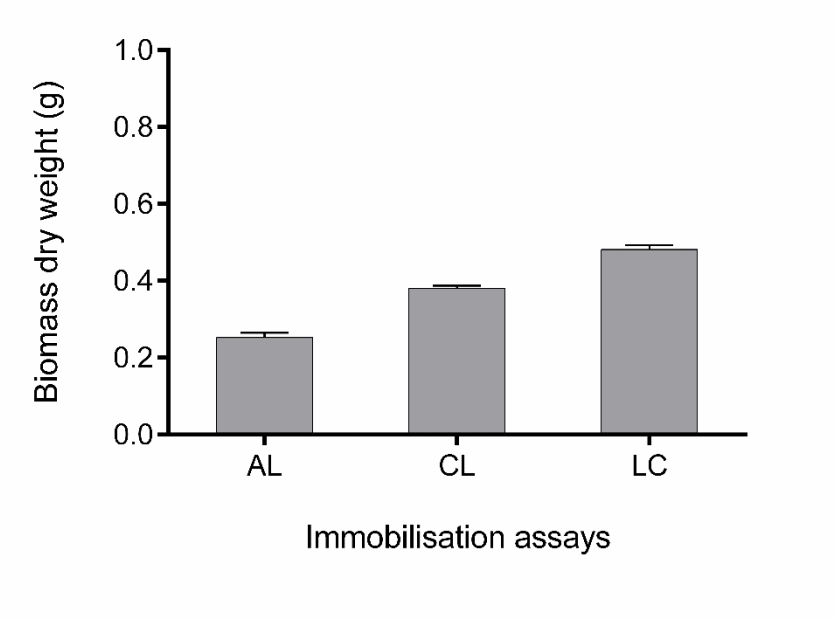

Figure 4. Decolourisation percentage of RB5. CC corresponds to fungus cultures in suspension. CL are the immobilisation assays. Control 1: culture medium with RB5 dye [200 mg L^{-1}], without fungus and without luffa sponge. Control 2: culture medium with RB5 dye [200 mg L^{-1}], with luffa sponge and 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).

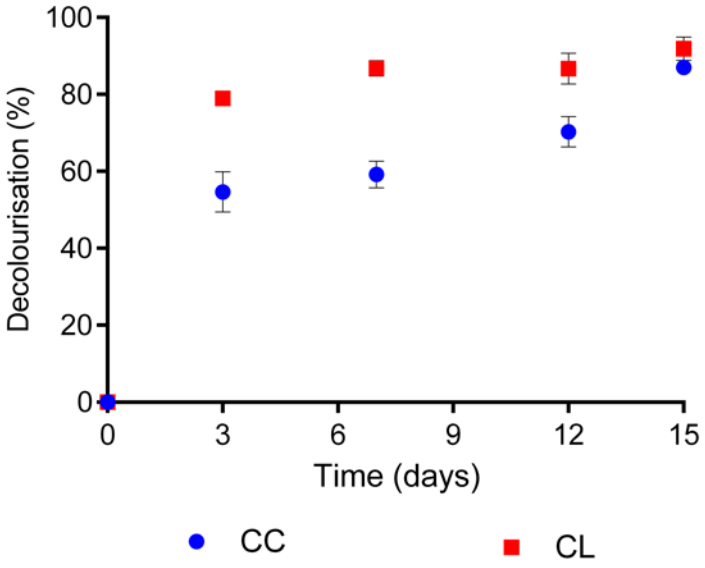

Figure 5. Evaluation of the ligninolytic enzymatic activities. CL correspond to the immobilisation experiment with dye. CC is the fungus culture in suspension with dye. HM is the dye-free suspended fungus culture. LC corresponds to the dye-free immobilisation experiments. **A)** Specific activity Lac 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.