



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

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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 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-treatment of RB5). These results evidenced the potential of Luffa sponge-immobilised *Leptosphaerulina* sp. as a strategy to enhance the biodegradation of recalcitrant compounds, to facilitate biomass recycling and to be used in the process scale-up.

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., 2018b; 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

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(Copete-Pertuz et al., 2018a; 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 and Martínez, 2012; Serna-Galvis et al., 2017). Other antibiotics such as azithromycin, ciprofloxacin and norfloxacin, can be found in Colombian wastewater at levels above $1 \mu\text{g L}^{-1}$ (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; Daássi 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., 2018a,b, 2019; 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, bio-transformation rates and reduce production costs.

Fungal immobilisation is a methodology that facilitates the scaleup 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 *Corioloopsis 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 (Daássi 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, $\lambda_{\text{max}} = 598 \text{ nm}$) was donated by Fabricato-Tejicondor S.A. Cephadroxyol 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} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 mL mineral solution [100 mg L^{-1} $\text{B}_4\text{O}_7\text{Na}_2 \cdot 10\text{H}_2\text{O}$, 70 mg L^{-1} , $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg L^{-1} $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg L^{-1} $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ and 10 mg L^{-1} $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$] (Copete-Pertuz et al., 2018b). 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 (Medellin, 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 et al., 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 $\mu\text{g L}^{-1}$) 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., 2018b), 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 an 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 $\text{M}^{-1} \text{cm}^{-1}$). 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 $\text{M}^{-1} \text{cm}^{-1}$) (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 et al., 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 5 μm , 4.6 \times 100 mm). A homogeneous mixture of acetonitrile ($\text{C}_2\text{H}_3\text{N}$) 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., 2018b; Čvančarová et al., 2013, 2015).

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):

$$D\% = \frac{A_0 - A_t}{A_0} \times 100 \quad (1)$$

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;

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 ^a
Charcoal	-
Luffa sponge	+++
Wood chips	+
Cork	+

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

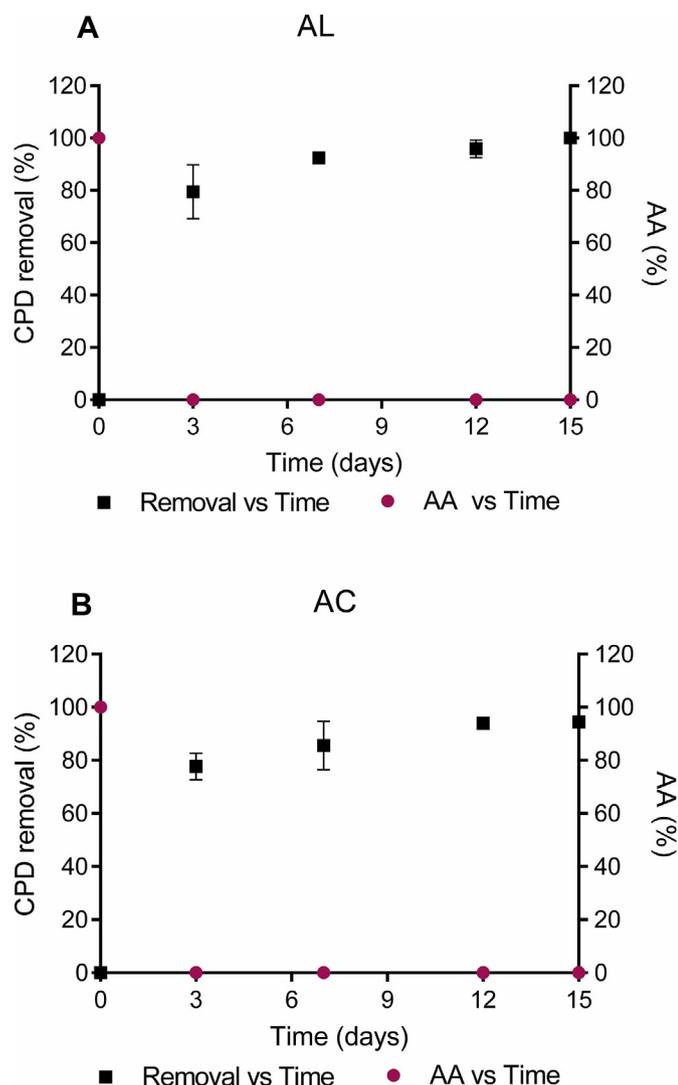


Fig. 1. CPD removal % and AA % over time. A) AL *Leptosphaerulina sp.* immobilised, B) AC *Leptosphaerulina sp.* in suspension. Experimental conditions: 28 °C, 160 rpm, pH = 5.6, 15 days. Data points are means and standard deviations (n = 3). There were no significant differences between AL and AC results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

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.

3.2. Biotransformation of cephadroxyll 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. Fig. 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 ($15256 \mu\text{g L}^{-1}$); 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 (Fig. 1A). In contrast, at day 15, the suspended cultures removed 94.4% of CPD (Fig. 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 (Fig. 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 ($15496 \mu\text{g L}^{-1}$). 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 Fig. 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 (5.56 U mg^{-1}), VP (8.29 U mg^{-1}) and MnP (5.75 U mg^{-1}). During the initial three days, the immobilised culture achieved an enzymatic production 6, 2.5 and 5.5-times higher than the suspended culture's Lac, VP and MnP activities, respectively (Fig. 2). The period of maximum enzymatic production correlates with the maximum CPD removal velocity (26%/day). In suspended cultures, VP activity maximum expression was detected at day 7 (3.13 U mg^{-1}); whereas, for Lac (0.92 U mg^{-1}) and MnP (1.03 U mg^{-1}) it was detected on the third day. The decreasing trend in the enzymatic activity observed after the third or seventh day, in immobilised and suspended cultures is correlated with the depletion of carbon or nitrogen sources (Copete-Pertuz et al., 2018a,b).

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

Similar to the suspended cultures, the control experiments exhibited the influence of luffa immobilisation in the production of VP and Lac. The immobilised culture control achieved a greater expression of VP and Lac than the suspended culture control. In contrast, MnP activity was higher in the suspended control than that of the immobilised control. The differences in the enzymatic activities between the 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.

Fig. 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

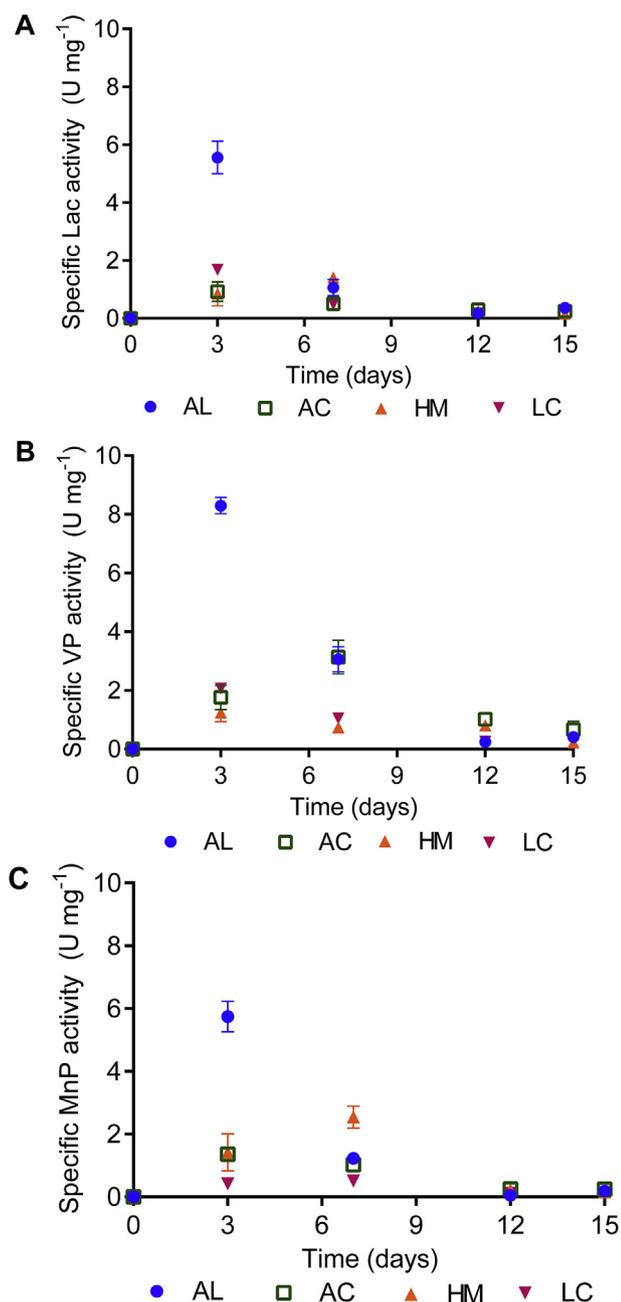


Fig. 2. Evaluation of the ligninolytic enzymatic activities. AL to the immobilisation experiments with CPD. AC are the fungus cultures in suspension with CPD. HM is the cultivation of the fungus in suspension free of CPD antibiotic. LC corresponds to the immobilisation experiments free of CPD. A) Specific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

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.

3.3. 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. Fig. 4 presents the *D*% of RB5 in both the *Leptosphaerulina* sp. suspended and immobilised cultures. During the first 3 days, the

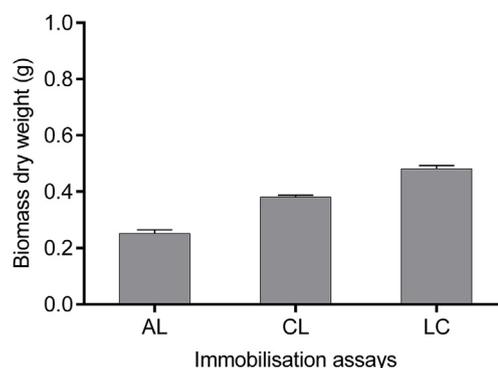


Fig. 3. Quantification of dry weight for immobilisation experiments. AL corresponds to immobilisation assays with antibiotic. CL are the dye-containing immobilisation experiments. LC corresponds to the antibiotic-free dye-free immobilisation experiment. Experimental conditions: 28 °C, 160 rpm, pH = 5.6,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

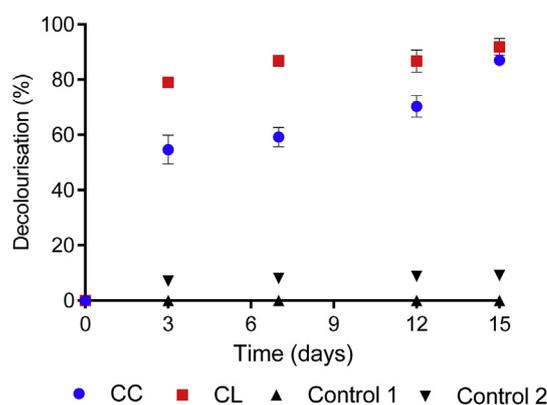


Fig. 4. Decolourisation percentage of RB5. CC corresponds to fungus cultures in suspension. CL are the immobilisation assays. Control 1: culture medium with RB5 dye [200 mg L⁻¹], without fungus and without luffa sponge. Control 2: culture medium with RB5 dye [200 mg L⁻¹], with luffa sponge and without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

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 Fig. 4, the control without the fungus and without luffa did not produce RB5 removal. Likewise, the control assay using the support matrix without the fungus demonstrated that sorption on the luffa sponge has 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.

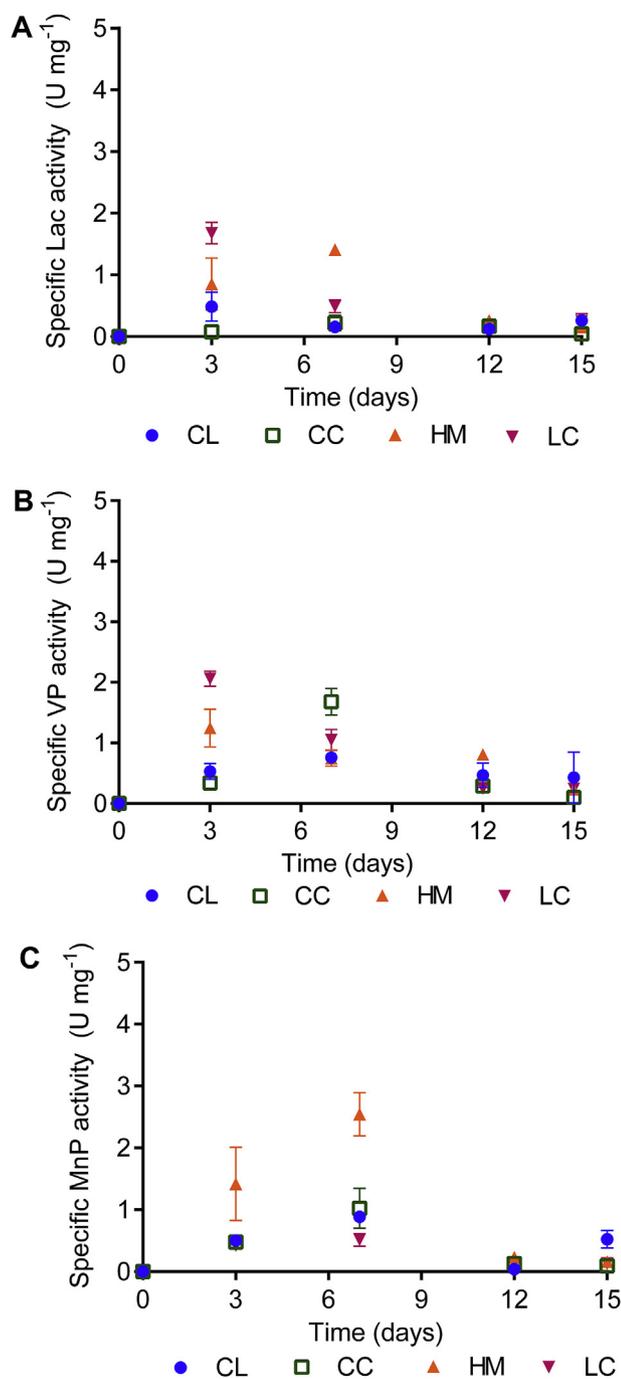


Fig. 5. Evaluation of the ligninolytic enzymatic activities. CL correspond to the immobilisation experiment with dye. CC is the fungus culture in suspension with dye. HM is the dye-free suspended fungus culture. LC corresponds to the dye-free immobilisation experiments. A) Specific activity Lac for dye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

As well as for CPD, Lac, MnP and VP enzymatic activities were determined for all of the previously mentioned assays. Fig. 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}) (Fig. 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. Fig. 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 carbamazepine and naproxen, respectively (Li et al., 2015). On the other hand, RB5 removal (92%) improved during the initial days (3–7) 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 *T. 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

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

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

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

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2019.101051>.

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