Enhancement of ligninolytic enzymes production and decolourising activity in *Leptosphaerulina* sp. by co–cultivation with *Trichoderma viride* and *Aspergillus terreus*

Ledys S. Copete-Pertuz[1], Felipe Alandete-Novoa[1], Jersson Plácido[2], Guillermo A. Correa-Londoño[3], 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 *Centre for Cytochrome P450 Biodiversity, Institute of Life Science, Medical School, Swansea University, Swansea, SA2 8PP, Wales, UK.*

3 *Departamento de Ciencias Agronómicas, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia – Sede Medellín, Calle 59A No 63 – 20, Medellín, Colombia.*

Abstract

This work investigated fungal co–culture as inducer of ligninolytic enzymes and decolourising activity in the Colombian strain *Leptosphaerulina* sp., an ascomycete white-rot fungus isolated from lignocellulosic material. *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus terreus*, *Trichoderma viride*, *Fusarium* sp. and *Penicillium chrysogenum* were tested as *Leptosphaerulina* sp. inducers. The best fungal combinations in terms of enzyme production, fungal growth and decolourising activity were selected from solid media experiments. Response surface methodology (RSM) was utilised to optimise enzyme production and decolourising

*Corresponding author.
Tel.: +57(4)4309339;
E-mail address: almora@unal.edu.co

activity in liquid media. Solid media assays evidenced *T. viride* and *A. terreus* as the best *Leptosphaerulina* sp. inducers. The RSM identified a triple co–culture inoculated with *T. viride* (1000 µL) and *A. terreus* (1000 µL) into a 7–day culture of *Leptosphaerulina* sp. as the best treatment. This triple combination significantly improved ligninolytic enzymes production and Reactive Black 5 dye removal when compared to the *Leptosphaerulina* sp. monoculture and previously used chemical inducers. These results demonstrated the potential of fungal co–culture as an environmentally–friendly method to enhance *Leptosphaerulina* sp. enzymes production and decolourising activity.

Keywords: White-rot fungi; Fungal inducers; Fungal co–culture; Bioremediation; Response surface methodology.

1. Introduction

Laccases (Lacs: *p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2) are phenol oxidases belonging to the group of multicopper oxidase proteins. In nature, Lacs are involved in lignin degradation, morphogenesis, sporulation, pigments production, formation of fruiting bodies and plant pathogenesis (Rivera-Hoyos et al., 2013; Giardina et al., 2015). Lacs are also effective in various biotechnological processes such as biofuel production, textiles finishing (Abd El Monssef et al., 2016; Plácido & Capareda, 2015), and the biodegradation of environmental pollutants (Tortella et al., 2013). The environmental applications include the transformation and degradation of compounds such as synthetic dyes, pharmaceuticals and pesticides (Bagewadi, et al., 2017; Copete-Pertuz et al., 2018; Zeng et al., 2017).

Fungal Lacs are the most studied type of Lacs and produced principally by basidiomycetes (Kuhar et al., 2015). In recent years, the number of reported Lacs from ascomycetes has increased. One of these ascomycetes is *Leptosphaerulina* sp., a native fungus from Colombia. *Leptosphaerulina* sp. produces high amounts of laccase (Lac) and manganese peroxidase (MnP). The ability of these enzymes to fully decolourise several synthetic dyes has been proved by different previous articles (Chanagá Vera et al., 2012; Copete et al., 2015; Plácido et al., 2016).

The use of *Leptosphaerulina* sp. for industrial applications (pollutant removal, delignification or bioethanol production) requires large quantities of enzymes (Liu et al., 2016); making their inducers a necessity to reach the desired enzyme production levels. Copper sulphate and ethanol have favoured the production of Lac in *Leptosphaerulina* sp. (Copete et al., 2015). Other chemical inducers such as ferulic acid, veratryl alcohol and 2,5–xylidine enhanced the Lac activity production (Piscitelli et al., 2011). However, some of them are expensive and in some cases, depending on the concentration used, have a negative impact on the environment (Kuhar & Papinutti, 2014; Pan et al., 2014). Therefore, to improve the production of ligninolytic enzymes by *Leptosphaerulina* sp., it is necessary to find more appropriate inducers, both economically and environmentally.

An alternative enhancing method is microbial co–culture. The combination of microorganisms has favoured Lac production and the degradation of recalcitrant contaminants in soil and water sources (Mikesková et al., 2012; Pan et al., 2014). The three most common co–culture strategies are: the co–culture of two ligninolytic

fungi, the addition of *Gongronella* sp. and the co–culture with members of the Trichoderma genus. First, the co–culture of two ligninolytic fungi, *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* improved Lac production between 1 and 3–times compared with the individual cultures (Chi et al., 2007). Similarly, Qi-He et al. (2011) found that co–cultivation of the white-rot fungi *Phlebia radiata* and *Dichomitus squalens* significantly stimulated the Lac expression after the fourth day of culture. Second, the co–culture with *Gongronella* sp. W5 increased the Lac activity in *Panus rudis* 25–times more than cultures using copper / *o*-toluidine as Lac activity inducers (Pan et al., 2014; Wei et al., 2010). Finally, one of the most studied fungal co–cultures includes the use of Trichoderma genus. *Trichoderma* species such as *T. atroviride, T. harzianum* and *T. longibrachiatum* increased the production of Lacs in mixed cultures (Baldrian, 2004; Flores et al., 2009; Wei et al., 2010; Zhang et al., 2006). Flores et al. (2009) demonstrated that co-cultures of *P. ostreatus* and *Trichoderma* spp. produced 6–times more Lac activity than the respective monocultures. Additionally, Trichoderma species increased Lac activity in *Lentinula edodes* (20–fold*)* and *Coprinus comatus* (2,6–fold) (Ma & Ruan, 2015; Savoie et al., 1998)*.*

Fungal co–culture offers a novel, environmental and economic option for enhancing enzymatic and decolourising activities for other white rot fungi; however, this induction method has not been tested in ascomycetes fungi such as *Leptosphaerulina* sp. Additionally, the effect of Colombian native fungal isolates have not been tested in co-culture and as ligninolytic enzymes inducers. Therefore, the aim of this article was to enhance the production of ligninolytic enzymes and the

biodegradation of the Reactive Black 5 (RB5) dye by using co–cultures of *Leptosphaerulina* sp. with other fungi of biotechnological interest. First, the *Leptosphaerulina* sp. biocompatibility with other fungi in solid media was evaluated on potato dextrose agar (PDA) with ABTS and/or RB5. The most compatible fungi were later evaluated using a response surface methodology (RSM) in a central composite 2^2 + star design to obtain the best co–culture conditions for enhancing *Leptosphaerulina* sp. enzymatic and decolourising activities.

2. MATERIALS AND METHODS

2.1. Chemicals

Reactive Black 5 (RB5) (azoic dye, λ_{max}= 598 nm, from DyStar) was kindly donated by Fabricato–Tejicondor S.A. from Medellín, Colombia. Glucose, yeast extract, peptone, zinc sulphate heptahydrate, monobasic potassium phosphate, tetraborate sodium decahydrate, ammonium molybdate and sodium acetate were obtained from Carlo Erba. 2,6-dimethoxyphenol 99% (DMP) and ammonium L-(+)-tartrate 98% were products of Alfa Aesar. Manganese sulphate heptahydrate, potassium chloride, tartaric acid, acetic acid, iron sulphate heptahydrate and hydrogen peroxide were obtained from Merck. 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt 98% (ABTS) and veratryl alcohol 96% were products of Sigma - Aldrich.

2.2. Microorganisms

Leptosphaerulina sp. (CECT 20913), *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus terreus*, *Trichoderma viride*, *Fusarium* sp. and *Penicillium chrysogenum* were obtained from PROBIOM research group's microorganisms collection (Chanagá Vera et al., 2012; Plácido et al., 2016). The fungi were maintained in potato dextrose agar (PDA) at 4 °C until use. 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 with the agreement No. 96 of 2014 to genetic resources access.

2.3. Solid media studies

2.3.1. Co–culture biocompatibility and enzymatic production

Leptosphaerulina sp. was co–cultivated in six combinations (**Table 1**): 1 (*Leptosphaerulina* sp.–*A. niger*), 2 (*Leptosphaerulina* sp.–*A. fumigatus*), 3 (*Leptosphaerulina* sp.–*A. terreus*), 4 (*Leptosphaerulina* sp.–*T. viride*), 5 (*Leptosphaerulina* sp.–*Fusarium* sp.) and 6 (*Leptosphaerulina* sp.–*P. chrysogenum*). Petri dishes with PDA were supplemented with 0.5 mM ABTS, as a ligninolytic enzyme indicator (Plácido et al., 2016). The Petri dish was divided in two halves: on one side, *Leptosphaerulina* sp., and 4 cm in front of it, the other fungal strain. After inoculation, the Petri dishes were incubated at 28 °C for 15 days. The response variables were the growth area and the ligninolytic activity area percentage (LAAP) (green and/or violet halo) (Crowe & Olsson, 2001; Plácido et al., 2016). Fungal and halos growth were followed by photographic records collected every 24 h. The results were reported at days 0, 3, 7, 12 and 15. Green or violet halos

indicated a reaction between the ligninolytic enzymes and the ABTS (Crowe & Olsson, 2001; Hiscox et al., 2010; Plácido et al., 2016). All procedures were performed in triplicate with their monocultures as controls (Wei et al., 2010; Zhang et al., 2006). The images were analysed with the image J software (National Institutes of Health, version 1.51j8, 2017) (Ferreira & Rasband, 2012). The fungal growth was determined measuring the growth area (cm^2) in image J and the LAAP was calculated using **Equation 1**:

$$Ligninolytic\ activity\ area\ \% = \frac{halo\ area\ (green\ or\ violet)}{Petri\ dish\ total\ area} \times 100 \qquad \textbf{Equation 1}$$

Where the Petri dish total area was 68.26 cm^2, and the halo area was determined by the image J software (National Institutes of Health, version 1.51j8, 2017).

2.3.2. Co–culture biocompatibility and decolourising activity

The co–cultures (**Table 1**) and monocultures' decolourising activities were evaluated in Petri dishes with PDA media supplemented with the RB5 dye (50 µM). The fungi were inoculated and incubated similarly as explained in **Section 2.3.1.** The response variable was the percentage change of colour intensity (PCI) (Abd El-Rahim et al., 2003) which was calculated with **Equation 2**.

$$\frac{\Delta CI}{CI_0}\% = \frac{|CI_k - CI_0|}{CI_0} \times 100 \qquad \textbf{Equation 2}$$

Where $\boldsymbol{CI_k}$ is the colour intensity of the medium at day k and $\boldsymbol{CI_0}$ is the medium colour intensity at day 0.

The colour reduction was followed by photographic records collected every 24 h. Based in our previous work, the results were reported on days 0, 3, 7, 12 and 15, as these days are associated with significant moments for enzymes production and/or decolourising activities using *Leptosphaerulina* sp. (Copete et al., 2015; Plácido et al. 2016). All procedures were performed in triplicate and the images were analysed with the image J software (National Institutes of Health, version 1.51j8, 2017) (Ferreira & Rasband, 2012). The two co–cultures with the highest enzymatic activities and/or colour reduction on solid medium were selected for the liquid medium experiment. The area under the curve (AUC) method was used as part of the solid media results' statistical analysis. The AUC was obtained by summing the area of the trapezoids under the curve of LAAP *vs* time and the PCI *vs* time, respectively (Becker et al., 2016). AUCs were analysed using the software R version 3.4.3 employing an one–way analysis of variance (ANOVA) and the Dunnett's test. Statistical significance was defined with an alpha of 0.05.

2.4. Response surface methodology (RSM)

A response surface based on a face–centred central composite 2^2 + star design was applied to determine the best conditions to enhance *Leptosphaerulina* sp. enzymes production and RB5 degradation in co–culture with *T. viride* and *A. terreus* in liquid media. The design evaluated the effect of three factors: *T. viride* inoculum size (X_1), *A. terreus* inoculum size (X_2) and the addition time of *T. viride* and/or *A. terreus* (X_3). The RSM used 20 assays with six central points and 10 degrees of freedom for the experimental error. All the factors had three levels, *T. viride* inoculum size (X_1: 0, 500

and 1000 µL), *A. terreus* inoculum size (X_2: 0, 500 and 1000 µL) and addition time (X_3: 0, 3 and 7 days). The factors and levels combination utilized in the experiment is described in **Table 2**.

The experimental design assays were performed in 250 mL Erlenmeyer flasks with 100 mL of culture medium (pH 5.6) containing 10 g L^{-1} glucose, 2 g L^{-1} ammonium L-(+)-tartrate, 5 g L^{-1} peptone, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} yeast extract, 0.5 g L^{-1} $MgSO_4$. $7H_2O$ and 0.5 g L^{-1} KCl, 1 mL mineral solution [100 mg L^{-1} $B_4O_7Na_2$. $10H_2O$, 70 mg L^{-1}, $ZnSO_4$. $7H_2O$, 50 mg L^{-1} $FeSO_4$. $7H_2O$, 10 mg L^{-1} $MnSO_4$. $7H_2O$ and 10 mg L^{-1} $(NH_4)_6Mo_7O_{24}$. $4H_2O$] (Guillén et al, 1992) and supplemented with RB5 (200 mg L^{-1}). Flasks were inoculated with 5 mL of *Leptosphaerulina* sp. from 10-day-old culture, previously homogenised (Copete et al., 2015). *T. viride* and *A. terreus* were inoculated according to the experimental design described in **Table 2**. After inoculation, the cultures were incubated at 28 °C and 160 rpm for 15 days. Monocultures of the fungi with and without dye were used as controls. Non-inoculated controls (dye without fungus) were also utilised. As sampling volume, 4 mL were withdrawn from the conical flasks at each time point. The response variables (ligninolytic enzyme activities, protein concentration and the decolourising activity of RB5) were measured at days 3, 7, 12 and 15. The results were analysed with the statistical program Statgraphics Centurion XVI®. A second order model was fitted to each response variable mean.

2.5. Enzymatic activities and protein quantification

The MnP activity was obtained spectrophotometrically by measuring the oxidation of DMP (1 mM) at 469 nm (ε_{469}= 27500 M^{-1} cm^{-1}) in sodium acetate buffer (0.1 mM, pH 4.5). Lac and versatile peroxidase (VP) activities were measured by following the enzymatic oxidation of ABTS (3 mM) at 420 nm (ε_{420}= 36000 M^{-1} cm^{-1}) in sodium tartrate buffer (0.1 M, pH 3) with and without H_2O_2 (0.1 mM), respectively. The lignin peroxidase (LiP) activity was measured using the veratryl alcohol oxidation (2 mM) at 310 nm (ε_{310}= 9300 M^{-1} cm^{-1}) in sodium tartrate buffer (0.1 M, pH 3). The protein concentration was measured following the Bradford method (Bradford, 1976). Enzymatic activities were expressed as units (U) per milligram (mg) of protein, where one unit was defined as the amount of enzyme that oxidises one µmol of substrate per minute.

2.6. Decolourisation of RB5

The RB5 decolourisation was followed spectrophotometrically at 598 nm and was expressed in terms of decolourisation percentage (*D%*) (**Equation 3**) (Forootanfar et al., 2016; Shedbalkar et al., 2008):

$$D\% = \frac{A_0 - A_t}{A_0} \times 100 \qquad \textbf{Equation 3}$$

Where A_0 is the initial dye absorbance and A_t is the absorbance at the *t* sampling time.

3. RESULTS AND DISCUSSION

3.1. Solid medium experiments

3.1.1. Growth of fungi on solid medium with ABTS

The biocompatibility of *Leptosphaerulina* sp. with other fungi was determined by the changes in the fungal growth areas during 15 days (**Figure 1**). **Figure 1A** illustrates monocultures growth areas at day 15. At that time, *T. viride* and *Fusarium* sp. monocultures displayed a growth area 20% higher than *Leptosphaerulina* sp. monoculture. *A. niger* monoculture grew similar as *Leptosphaerulina* sp. monoculture; whereas, *A. fumigatus*, *A. terreus*, and *P. chrysogenum* monocultures grew 17, 25 and 40% less than *Leptosphaerulina* sp. monoculture. **Figure 1B** depicts the fungal co–cultures (**Table 1**) growth areas at day 15. In co–culture 1, *A. niger* grew 1.6–times lower than *Leptosphaerulina* sp. In contrast, all the other co–cultures reached greater growth areas than *Leptosphaerulina* sp., *A. fumigatus* 13%, *T. viride* 14.6% and *Fusarium* sp. 19%, *A. terreus* 100% and *P. chrysogenum* 100%. The low growth of *Leptosphaerulina* sp. could be associated with mutual inhibition or hyphal interference. Whereas, *Leptosphaerulina* sp. high growth may be related to fungal cooperation (Boddy & Heilmann-Clausen, 2008; Fukami et al., 2010). *A. terreus* was the only fungus with similar growth in both monoculture and co–culture (growth area= 0.5) (**Figure 1A, 1B**).

3.1.2. Ligninolytic activity on solid medium with ABTS

The Petri dish area with a violet and/or a green colour, produced from the oxidation of ABTS, determined the ligninolytic activity. The areas were measured on days 0, 3, 7, 12 and 15 (**Figure 2**). Most of the fungi (*A. niger, A. fumigatus, A. terreus, Fusarium* sp. and *P. chrysogenum*) did not express ligninolytic activity in

monoculture (**Figures 2A, 2B, 2C, 2E, 2F**), whereas, *T. viride* and *Leptosphaerulina* sp. monocultures showed ligninolytic activity (**Figure 2D, Figure 1**, **2 supplementary material**). In Leptosphaerulina sp. a violet halo was observed after the production of a small green halo (Plácido et al., 2016), whereas T. viride exhibited a green halo. Co–cultures with *Leptosphaerulina* sp. and other fungi without ligninolytic activity in monoculture (co–cultures 1, 2, 3, 5 and 6) reached a ligninolytic activity area percentage (LAAP) up to 70% (**Figures 2A, 2B, 2C, 2E, 2F**). The LAAP difference between *Leptosphaerulina* sp. monoculture and co–culture may be related to the reduction of *Leptosphaerulina* sp growth area. This reduction was associated to the presence of another fungus in the Petri dish. In nature, interactions between soil fungi are mainly combative (Morón-Ríos et al., 2017). At day 15, the LAAP for co–cultures 1, 2, 3 and 5 was 60, 63, 60 and 68% lower than *Leptosphaerulina* sp. monoculture (**Figures 2A, 2B, 2C, 2E**). Co–culture 6 LAAP was different from *Leptosphaerulina* sp. monoculture during the first seven days, with a six–fold increase from day 3 to 7. In contrast, from day 12 to 15, the LAAP was constant (70%) (**Figure 2F**). *Leptosphaerulina* sp. co–culture with *T. viride,* another ligninolytic fungus (co–culture 4), produced a higher LAAP than *Leptosphaerulina* sp. monoculture, this evidenced a possible synergistic effect between both fungal strains. Similarly, Wei et al. (2010) described a synergistic effect in *Panus rudis* and *Gongronella* sp co–culture, this synergy was evidenced by a colour change (reddish-brown) in solid medium with guaiacol, a chemical used for laccase detection.

Figure 3A illustrates the area under the curve (AUC) obtained from the ligninolytic activity of every co–culture and *Leptosphaerulina* sp. monoculture. The ANOVA of the AUCs from the ligninolytic activity tests indicated significant differences (p–value < 0.05) between at least two of the evaluated cultures (**Table 1 supplementary material**). Dunnett's multiple comparisons test for the AUC of the co–cultures and the *Leptosphaerulina* sp. monoculture indicated that ligninolytic activity of co–cultures 1, 5 and 6 did not differ significantly (p–value > 0.05) from the ligninolytic activity of *Leptosphaerulina* sp. monoculture (**Figure 3**, **Table 2 supplementary material**). Co–cultures 2 and 3 had lower ligninolytic activity compared with *Leptosphaerulina* sp. monoculture (p–value < 0.05) (**Figure 3**, **Table 2 supplementary material**). In contrast, co–culture 4 (*Leptosphaerulina* sp.–*T. viride*) exhibited higher ligninolytic activity than the *Leptosphaerulina* sp. monoculture (p–value < 0.05) (**Figure 3**, **Table 2 supplementary material**). Therefore, *T. viride* produced a significant effect on *Leptosphaerulina* sp. ligninolytic activity production.

The co–cultures enzymatic activity increment was associated with interactions such as cooperation and synergism; whereas, the reduction with antagonism and competition. These differences can also be related with mycelial morphology changes, enzymes secretion and metabolites modification as a result of a reciprocal exchange of chemical signals in the culture medium (Pan et al., 2014). Although, in co–culture the growth of *Leptosphaerulina* sp. was limited by the growth of the other fungus, it was compensated by the high production of ligninolytic enzymes by *T. viride* (Lakshmanan & Sadasivan, 2016).

3.1.3. Decolourising activity of Leptosphaerulina sp. in co–culture

The images' colour intensity was measured over a 15-day period to determine the decolourising activity. The results for days 0, 3, 7, 12 and 15 were used to calculate the percentage change of colour intensity (PCI) (**Equation 2**). *A. niger*, *A. fumigatus*, *T. viride*, *Fusarium* sp. and *P. chrysogenum* monocultures did not show significant decolourising activity (**Figures 4A, 4B, 4D, 4E, 4F**). Whereas, *Leptosphaerulina* sp. and *A. terreus* monocultures had decolourising activity reaching a maximum PCI of 100% and 95.46% at day 15, respectively (**Figure 4C, Figure 3**, **4 supplementary material**). All co–cultures exhibited decolourisation and it increased through time. At day 15, co-cultures 1, 2, and 6 had lower decolourising activity than *Leptosphaerulina* sp. monoculture (100%) (**Figures 4A, 4B and 4F**). Co–cultures 3, 4 and 5 showed similar or slightly higher decolourising activity than *Leptosphaerulina* sp. monoculture (**Figure 4C, 4D and 4E**).

Similar to **Section 3.1.2.**, the RB5 removal in solid media (**Figure 3B**) was analysed with the AUC methodology. The AUC analysis' ANOVA for the RB5 removal indicated significant differences (p–value < 0.05) between at least two treatments (**Table 3 supplementary material**). Therefore, Dunnett's multiple comparison tests were performed. The RB5 removal between co–culture 5 and *Leptosphaerulina* sp. monoculture did not exhibit significant differences (**Figure 3B**, **Table 4 supplementary material**). The RB5 removal by co–culture 1, 2, 3, 4, and 6 were statistically different (p–value < 0.05) than the *Leptosphaerulina* sp. monoculture (**Figure 3B**, **Table 4 supplementary material**). *Leptosphaerulina* sp.–*A. terreus*

and *Leptosphaerulina* sp.–*T. viride* co–cultures achieved greater decolourisation than the *Leptosphaerulina* sp. monoculture. *Leptosphaerulina* sp.–*A. terreus* co–culture showed the highest decolourising activity indicating *A. terreus* as a candidate to facilitate the degradation of RB5 by *Leptosphaerulina* sp. (**Figure 4C**).

In this study, *A. terreus* monoculture did not exhibit ligninolytic activities, but it displayed a high decolourising activity. *A. terreus* strain sorbed the RB5 dye (**Figure 5 supplementary material**) from the medium, which explains the high decolourising activity by *Leptosphaerulina* sp.–*A. terreus* co–culture. These findings concur with previous studies where members of the genus *Aspergillus* have sorbed textile dyes (Assadi & Jahangiri, 2001; Sumathi & Manju, 2000). Similarly, *Fusarium* sp. monoculture did not express ligninolytic activities but exhibited a modest RB5 decolourisation. However, such sorption did not generate a significant synergistic effect in co–cultured with *Leptosphaerulina* sp. *T. viride* monoculture did not show decolourising activity despite having ligninolytic activity, this lack of decolourising activities in ligninolytic enzymes produced by *T. viride* have been reported in previous researches (Murugesan et al., 2007). This may explain why the co–culture of *Leptosphaerulina* sp. and *T. viride* did not exhibit better decolourisation than *Leptosphaerulina* sp. monoculture. Although, Saeed et al. (2009) reported methylene blue removal through sorption by *T. viride;* this behaviour was not observed with the *T. viride* strain used in this article. Additionally, *P. chrysogenum* strain did not exhibit ligninolytic or RB5 decolourising activities and did not have a synergistic relationship in co–culture with *Leptosphaerulina* sp. This behaviour indicated a lack of stress in *P. chrysogenum* strain produced by *Leptosphaerulina*

sp. or its compounds. In contrast to the results obtained in this work, Nayanashree et al. (2015) and Vaidyanathan et al. (2011) reported that *P. chrysogenum* had ligninolytic and/or dye decolourisation activity.

Based on the statistical analyses, *T. viride*, *A. terreus* were selected as the most suitable fungal inducers for the enzymatic and decolourising activities of *Leptosphaerulina* sp. in solid medium; therefore, these two fungal strains were used in the liquid co–culture studies.

3.2. Response surface methodology (RSM)

Table 3 describes the Lac, VP and MnP activities and decolourisation percentage (*D%*) at days 3, 7, 12 and 15 for the treatments evaluated in the RSM (**Table 2**). Assay 4 (*T. viride* 1000 µL, *A. terreus* 1000 µL, added at day 7) expressed the highest Lac activity (2.06 U mg^{-1}) at the twelfth day. This activity was 8–times higher that *Leptosphaerulina* sp. monoculture. Similarly, assay 4 reached the highest VP activity at days 12 (7.32 U mg^{-1}) and 15 (3.60 U mg^{-1}). At day 12, the highest VP activity was almost 36–times higher than *Leptosphaerulina* sp. monoculture. The time of addition was an important factor, when *T. viride* and/or *A. terreus* were added to the culture at day zero the enzymatic activity was low; in contrast, additions at days 3 and 7 resulted in high expression of VP. Similar to the other enzymatic activities, assay 4 achieved the largest MnP production at day 12 and 15. MnP

346 activity (1.75 U mg^{-1}) was 88–times higher than *Leptosphaerulina* sp. monoculture
347 (**Table 3**). LiP activity was not detected in the experiments.

348 **Table 3** also summarises the RB5 *D%* achieved by *Leptosphaerulina* sp. in co–
349 culture with *T. viride* and *A. terreus.* A gradual increase in RB5 *D%* was noticed with
350 increase in bio-treatment time. As expected assay 4 had the highest *D%* of all the
351 treatments. At day 12, assay 4 reached 92%, which was 16% better than the
352 *Leptosphaerulina* sp. monoculture (76%).

353 In general, the RSM results demonstrated that on the twelfth day *Leptosphaerulina*
354 sp.' ligninolytic and decolourising activities were enhanced by co–culture with *T.*
355 *viride* (1000 µL) and *A. terreus* (1000 µL) added at day 7 (assay 4). The RSM results
356 shown that addition time and inoculum size were significant factors in the response
357 variables increment. The kinetics studies correlated the increment in the production
358 of Lac, VP and MnP with RB5 decolourisation (**Table 3**). The enhancement of the
359 ligninolytic enzymes production in *Leptosphaerulina* sp. via co–culture with *T. viride*
360 and *A. terreus* (Lac, VP and MnP) was a determining factor in the increment in RB5
361 decolourisation. Similar synergic effect has been reported by Lade et al. (2012), the
362 authors reported an improvement in the removal of azo dye Rubine GFL by co–
363 cultures of *Aspergillus ochraceus* and *Pseudomonas* sp. SUK1. Response surfaces
364 for Lac, VP, MnP and *D%* at day 12 were illustrated in **Figure 5**.

365 The Lac production model's ANOVA evidenced that the most important variables
366 were the linear effect of *T. viride* inoculum size*,* the linear and quadratic effect of
367 addition time, and the interaction between *T. viride* inoculum size and addition time

(**Table 5 supplementary material**). The VP production model's ANOVA identified as significant the linear effect of *T. viride* inoculum size, the linear effect of *A. terreus* inoculum size, the linear and quadratic effect of addition time and the interaction between *T. viride* inoculum size and addition time (**Table 6 supplementary material**). The MnP production model's ANOVA identified as significant variables the linear effects of *T. viride* and *A. terreus* inoculum sizes (**Table 7 supplementary material**). The *D%* model's ANOVA determined as significant variables the linear and quadratic effect of *A. terreus* inoculum size, and the interaction between *T. viride* and *A. terreus* inoculum sizes (**Table 8 supplementary material**). The R^2 of the models indicated that they could explain 75.5%, 89%, 72%, and 76% of the variation of the Lac, VP, MnP and *D%*, respectively. The regression equations that fitted to the models for Lac, VP, MnP and *D%* at day 12 are shown below (**Equations 5, 6, 7, 8**):

$$Lac = 0.646774 + 0.000425072X_1 + 0.000600754X_2 - 0.307581X_3 + 4.25071 \times 10^{-7}X_1X_2 + 0.000129406X_1X_3 + 0.000110956X_2X_3 - 6.92675 \times 10^{-7}X_1^2 - 8{,}78224 \times 10^{-7}X_2^2 + 0.0349727X_3^2 \quad \textbf{Equation 5}$$

$$VP = 0.96518 + 0.000258485X_1 - 0.000520679X_2 - 0.759508X_3 + 0.00000178952X_1X_2 + 0.00071413X_1X_3 + 0.000242988\,X_2X_3 - 0.0000015854\,X_1^2 - 1.00251 \times 10^{-8}X_2^2 + 0.0872629X_3^2 \quad \textbf{Equation 6}$$

$$\log(MnP) = -3.19889 - 0.000247804X_1 + 0.0015558X_2 - 0.46444X_3 + 0.00000215874X_1X_2 + 0.000304643X_1X_3 + 0.0000600017X_2X_3 - 7.26598X_1^2 - 0.00000130196X_2^2 + 0.0551799X_3^2 \quad \textbf{Equation 7}$$

$$D\% = 76.6866 - 0.0338426X_1 + 0.0347534X_2 - 3.70321X_3 + 0.0000077794\,X_1^2 + 0.0000296666X_1X_2 + 0.00102074X_1X_3 - 0.000039327X_2^2 + 0.00174X_2X_3 + 0.379897X_3^2 \quad \textbf{Equation 8}$$

A. terreus monoculture (control) removed 27, 49, 62 and 68% of the dye at days 3, 7, 12 and 15, respectively. However, enzymatic activities were not detected. *T. viride* monoculture (control) exhibited the lowest removal with values between 31% and 36%. In contrast, *Leptosphaerulina* sp. monoculture removed 54, 72, 79 and 81% of RB5 at days 3, 7, 12 and 15, respectively. In fact, the co–culture treatment was significant better than *Leptosphaerulina* sp., *T. viride* and *A. terreus* monocultures. *T. viride* and *Leptosphaerulina* sp. monocultures exhibited a decrease in the enzymatic activities when the culture media was supplemented with RB5.

Our results described for the first time the application of fungal co–culture using three strains (*Leptosphaerulina* sp., *T. viride* and *A. terreus*) for enhancing ligninolytic enzymes production and RB5 removal in liquid medium. The triple fungal combination achieved better *D%*, Lac and MnP activities (8, 88 and 1.2–times, respectively) than the co–cultures of *Pleurotus florida* and *Rhizoctonia solani* reported by Kumari & Naraian (2016). The highest RB5 *D%* was reached by co–culturing the three fungi, but the combination of *A. terreus* and *Leptosphaerulina* sp. also reached high decolourisation. This suggested that *A. terreus* did not contribute

409 on the enzyme production, but it made a significant contribution on the RB5 removal,
410 this decolourising activity was also observed in the solid media experiments (**Figure**
411 **5 supplementary material**).

412 The Lac activity decreased when 1000 µL of *T. viride* and/or *A. terreus* were added
413 at day 0 (assay 7, 11 and 15). In this case, the fungal inducers inhibited
414 *Leptosphaerulina* sp. growth and its enzymatic production. These fungi grew faster
415 than *Leptosphaerulina* sp. and took the nutrients from the culture medium (*e.g.* assay
416 7 and 15). In contrast, the addition of these fungi at day 7 remarkably increased the
417 ligninolytic enzymes production (assay 4, at day 12). Regardless of the inoculum
418 size, at day 7 the response variables increased as *Leptosphaerulina* sp. has grown
419 considerably in the culture medium. The presence of significant biomass from the
420 induced fungus had an advantage when fungal inducers with fast growth are used
421 in mixed culture (Baldrian, 2004).

422 Fungal co–culture showed an evident increment in RB5 decolourising activity due to
423 the increment in the production of laccase and peroxidases. The increment of
424 enzymatic production in co–cultured microorganisms is a result of cross-species
425 and/or cross-genera interactions (Hamza et al.,2018). Fungal co–cultures adapt
426 more efficiently to complex and variable environmental conditions than monocultures
427 since co-cultures produce a greater enzymatic diversity, can utilise intermediate
428 metabolites for further mineralisation and facilitate the transformation of pollutants
429 into non-toxic compounds (Hamza et al., 2018). In the future, fungal co-cultures can
430 be identified for specific types of fungi and for specific enzymatic activities, therefore,

431 the inoculum for an specific biotechnological process will be a mixed fungal culture
432 instead of a monoculture. A service for the identification of these novel inoculums
433 and selling them to the enzymes industry can be a future route to commercialising
434 this research.

435 *Leptosphaerulina* sp. cultured with copper sulphate (500 µM) and ethanol (9 g L^{-1})
436 as inducers produced 3–times more Lac than *Leptosphaerulina* sp. without inducer
437 (Copete et al., 2015). Whereas, in the present study, the use of *T. viride* and *A.*
438 *terreus* as fungal inducers increased 8–times the Lac activity produced by
439 *Leptosphaerulina* sp. Therefore, the co–culture of *Leptosphaerulina* sp. with *T. viride*
440 and *A. terreus* is a superior method to enhance ligninolytic activities production than
441 chemical inducers previously tested. The positive effects of fungal inducers include
442 the increment of pollutants removal, costs reduction by the absence of additional
443 chemicals and the lack of toxic intermediary compounds. These results pave the way
444 for future applications of fungal co–culture in textile wastewater treatments, enzyme
445 production and the transformation of other pollutants. Future works will focus on
446 understanding the synergic effect of fungal co–culture in the production of isozymes,
447 the treatment of novel pollutants and the metabolic interactions among fungi.

448

449 **4. CONCLUSIONS**

450 Co–culture was proved as an effective method for enhancing *Leptosphaerulina* sp.
451 enzymatic and decolourising activities. Solid culture experiments demonstrated *T.*

viride and *A. terreus* as the most compatible strains with *Leptosphaerulina* sp. and RSM experiments revealed the importance of fungal inducer, inoculum size, and addition time. The best *Leptosphaerulina* sp. co–culture combination was *T. viride* (1000 µL) and *A. terreus* (1000 µL) added at day 7. This combination increased enzymes production (Lac 8–times, VP 36–times, MnP 88–times) and RB5 removal (1.2–times) *vs* monoculture. The use of fungal co–culture as inducers obtained superior results than previously used chemical inducers. These results revealed the potential of co–cultivation as an alternative for enzymatic induction and pollutants bioremediation.

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Supplementary data

E-supplementary data of this work can be found in online version of the paper.

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639

List of Tables and Figures

662 **Table 1.** Fungal co–cultures.

Co–culture	Fungi
1	*Leptosphaerulina* sp.–*A. niger*
2	*Leptosphaerulina* sp.–*A. fumigatus*
3	*Leptosphaerulina* sp.–*A. terreus*
4	*Leptosphaerulina* sp.–*T. viride*
5	*Leptosphaerulina* sp.–*Fusarium* sp.
6	*Leptosphaerulina* sp.–*P. chrysogenum*

663

664 **Table 2.** Experimental design to evaluate RB5 removal and enzyme activities by

665 *Leptosphaerulina* sp. in co–culture with *T. viride* and *A. terreus*.

Assay	*T. viride* (µL)	*A. terreus* (µL)	Time of addition (day)
1	500	500	3
2	0	1000	7
3	500	500	7
4	1000	1000	7
5	500	500	3
6	500	500	3
7	1000	1000	0
8	0	0	0
9	500	500	0
10	1000	500	3
11	0	1000	0
12	500	500	3
13	500	500	3
14	500	0	3
15	1000	0	0
16	0	500	3
17	500	500	3
18	1000	0	7
19	500	1000	3
20	0	0	7

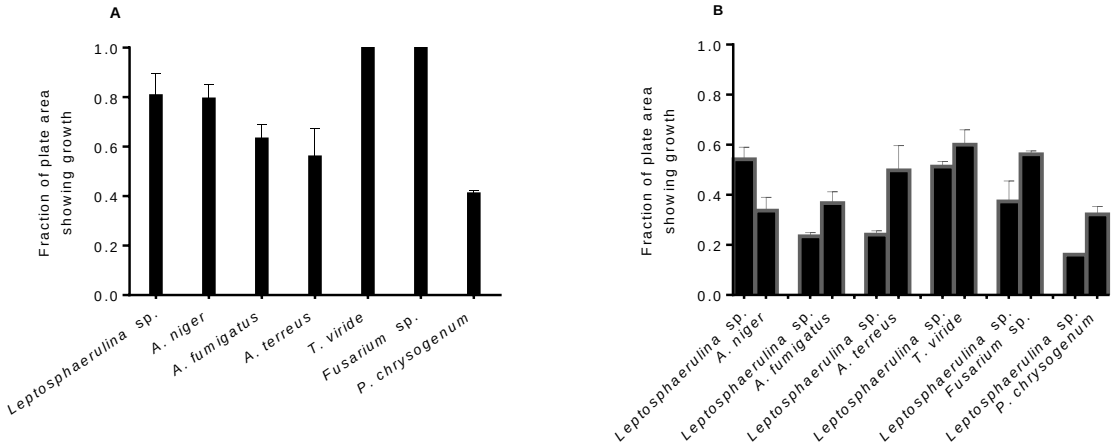
666

667 **Table 3.** Lac, VP, MnP activities and *D%* during RB5 removal by *Leptosphaerulina* sp. in co–culture v

668 *terreus.*

Assay	Lac (U mg^{-1})				VP (U mg^{-1})				MnP (U mg^{-1})				*D%*		
	Day				Day				Day				Day		
	3	7	12	15	3	7	12	15	3	7	12	15	3	7	12
1	1.58	1.15	0.77	1.75	4.91	5.36	1.10	1.01	0.28	0.05	0.13	0.10	67.92	71.49	76.9
2	1.73	1.40	0.74	1.67	5.22	3.03	0.87	0.72	0.08	0.06	0.07	0.04	74.36	76.50	78.6
3	1.82	1.07	0.90	0.41	5.20	3.48	2.39	0.77	0.36	0.07	0.12	0.09	64.67	67.11	68.2
4	1.78	1.13	2.06	0.88	5.21	4.65	7.32	3.60	0.29	0.15	1.75	0.72	55.97	72.99	91.7
5	1.50	1.21	0.61	1.68	4.79	5.15	1.12	1.18	0.03	0.04	0.05	0.21	71.72	72.83	75.6
6	1.47	1.23	0.59	1.42	4.84	5.55	1.25	1.48	0.04	0.17	0.04	0.13	66.20	78.27	78.2
7	0.00	0.67	0.53	0.90	0.00	0.98	0.88	0.62	0.00	0.02	0.24	0.04	44.12	67.63	71.7
8	1.57	1.30	0.34	0.08	5.00	3.58	0.20	0.19	0.03	0.01	0.03	0.04	51.34	57.66	75.6
9	0.70	1.39	1.16	1.16	0.81	1.55	1.30	0.10	0.06	0.02	0.08	0.01	56.63	75.84	76.8
10	0.83	0.49	0.34	0.49	1.50	0.89	0.10	0.11	0.03	0.04	0.08	0.10	24.64	49.76	71.5
11	0.17	0.41	0.40	1.08	0.12	0.36	0.42	1.01	0.00	0.02	0.08	0.03	73.01	75.90	76.3
12	1.41	1.24	0.59	1.52	4.93	6.36	1.02	1.10	0.32	0.18	0.10	0.14	50.40	57.78	71.7
13	1.48	1.20	0.74	1.54	5.00	5.65	0.06	0.19	0.02	0.04	0.09	0.08	49.09	78.77	79.2
14	0.98	0.44	0.55	0.88	3.85	0.51	0.40	0.11	0.02	0.03	0.06	0.06	33.94	35.56	50.9
15	0.00	0.06	0.36	0.00	0.00	0.01	0.01	0.06	0.00	0.01	0.01	0.03	27.73	38.28	51.1
16	1.22	0.26	0.48	0.35	4.32	2.33	0.38	0.52	0.01	0.00	0.02	0.01	65.41	65.81	67.9
17	1.62	1.22	0.63	1.79	4.59	5.11	1.17	0.92	0.00	0.04	0.03	0.07	60.62	78.04	78.2
18	1.08	0.63	0.84	0.36	3.83	3.58	3.66	1.22	0.02	0.03	0.06	0.03	45.66	48.86	49.3
19	1.26	0.43	0.18	0.20	4.10	2.77	0.86	0.89	0.13	0.00	0.02	0.02	52.46	65.03	65.0
20	1.63	1.45	0.26	0.09	4.35	3.77	0.13	0.33	0.00	0.02	0.02	0.03	47.88	62.50	75.6

669



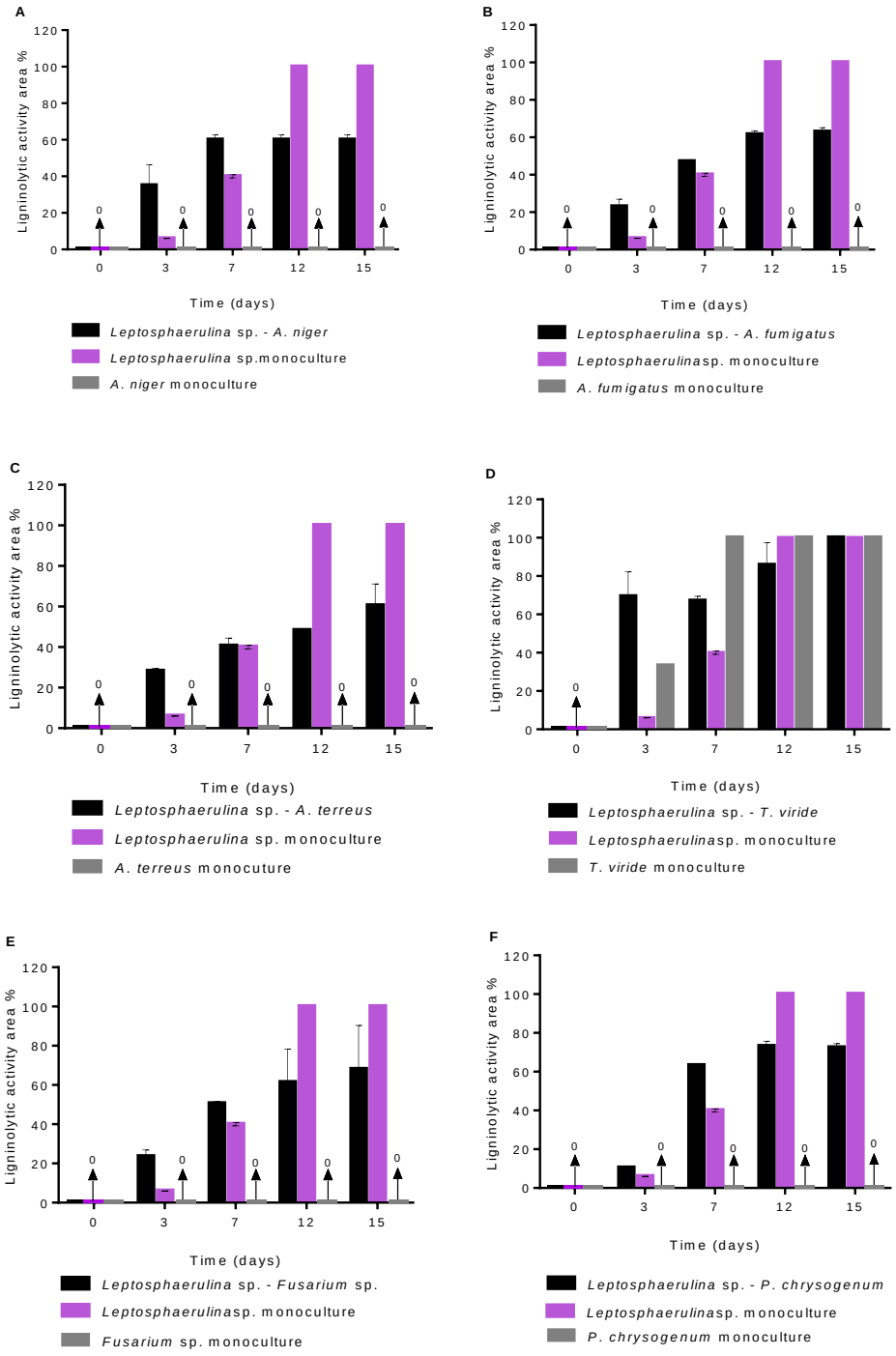
670

671 **Figure 1.** Growth areas of *Leptosphaerulina* sp., *A. niger*, *A. fumigatus*, *A. terreus*,

672 *T. viride*, *Fusarium* sp. and *P. chrysogenum* presented as a fraction of the total area

673 of the plate. **A)** monocultures at day 15, **B)** co–cultures at day 15. Cultures on PDA

674 with ABTS, 28 °C.



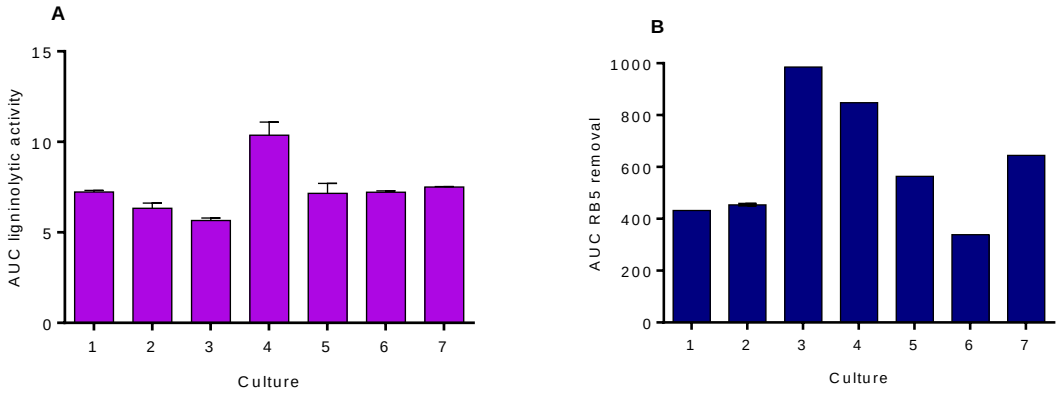
675

676 **Figure 2.** Ligninolytic activity of *Leptosphaerulina* sp. paired against **A)** *A. niger,* **B)**

677 *A. fumigatus,* **C)** *A. terreus*, **D)** *T. viride*, **E)** *Fusarium* sp., and **F)** *P. chrysogenum*

678 presented as percentages of the total area of the plate. Cultures on PDA with ABTS,

679 at 28 °C.



680

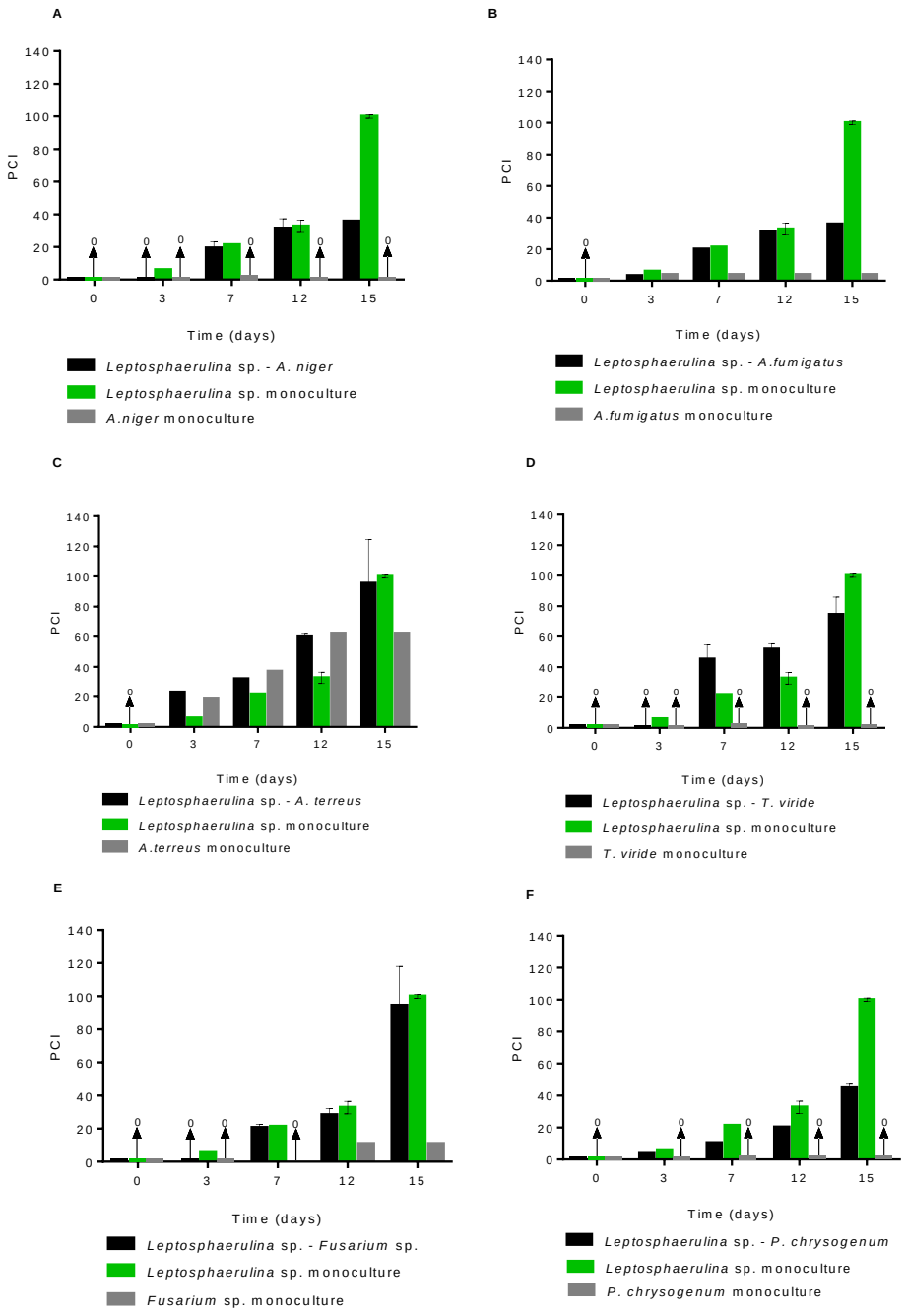
681 **Figure 3.** Area under the curve (AUC), after 15 days of **A)** ligninolytic activity **B)** RB5

682 removal. Culture: 1 (*Leptosphaerulina* sp.–*A. niger*), 2 (*Leptosphaerulina* sp.–*A.*

683 *fumigatus*), 3 (*Leptosphaerulina* sp.–*A. terreus*), 4 (*Leptosphaerulina* sp.–*T. viride*),

684 5 (*Leptosphaerulina* sp.–*Fusarium* sp.), 6 (*Leptosphaerulina* sp.–*P. chrysogenum*);

685 7 (*Leptosphaerulina* sp. monoculture).



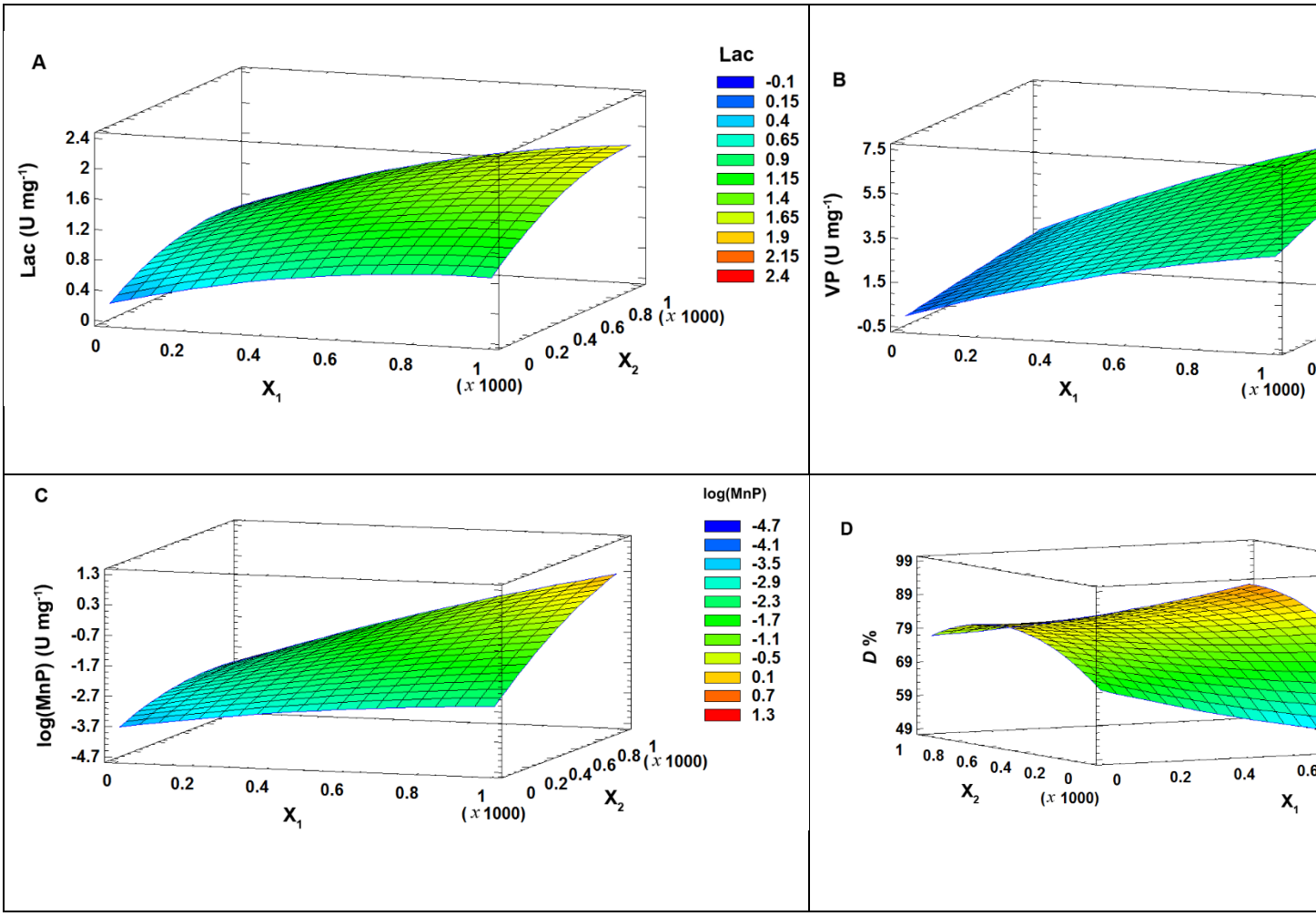
686

687 **Figure 4.** Decolourising activity of *Leptosphaerulina* sp. paired against **A)** *A. niger,*

688 **B)** *A. fumigatus,* **C)** *A. terreus*, **D)** *T. viride*, **E)** *Fusarium* sp., **F)** *P. chrysogenum.*

689 Cultures on PDA with RB5. 28 °C, after 15 days. PCI: percentage change of the

690 colour intensity.



691 **Figure 5.** Response surface for enzyme activities (U mg^{-1}) and RB5 removal (%) by *Leptosphaerulina s*

692 *T. viride* and *A. terreus* **A)** Lac, **B)** VP, **C)** MnP, **D)** *D%* of RB5. Experimental conditions: 28 °C, 160 rp