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Selection of white-rot fungi to formulate complex and coated pellets for Reactive Orange 165 decolourization

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Abstract Six strains of white-rot fungi isolated from southern Chile were evaluated for their ergosterol/biomass correlation and ligninolytic potential in solid medium to formulate pellets for Reactive Orange 165 (RO165) decolourization. The fungus *Anthracophyllum discolor* was selected to formulate complex pellets (fungal mycelium, sawdust, and activated carbon), coated pellets (complex pellet + alginate) and simple pellets (fungal mycelium). The activity of ligninolytic enzymes (laccase, manganese peroxidase, manganese-independent peroxidase, and lignin peroxidase) was evaluated in both the complex and coated pellets in modified Kirk medium, and the morphology of the pellets was studied using scanning electron microscopy (SEM). Complex pellets of *A. discolor* showed a higher enzymatic production mainly MnP (38 U L^{-1} at day 15) compared to coated and simple pellets. Examinations using SEM showed that both pellets produced a black core that was entrapped by a layer of fungal mycelium. Decolourization of RO165 was demonstrated with all the pellets formulated. However, the highest and fastest decolourization was obtained with complex pellets (100% at day 8). Therefore, complex pellets of *A. discolor* can be used for the biological treatment of wastewater contaminated with RO165.

Keywords: complex pellets, ligninolytic enzymes, RO165 decolourization, white-rot fungi.

INTRODUCTION

The increasing use of synthetic dyes in the textile, paper, cosmetics, leather dyeing, pharmaceutical, and food industries has resulted in serious environmental pollution (Tang et al. 2011). Over 10,000 dyes and 7 x 10^5 ton annual are commercially available worldwide and 5-10% of the dye stuff is lost in the industrial effluents. As a result, they generate a considerable amount of coloured wastewater (Crini, 2006). The economic removal/degradation of polluting dyes is an important current issue, particularly as new regulations regarding industrial effluent discharge are beginning to be enforced (Bibi et al. 2009). The inefficiency of the dyeing process, poor handling of spent effluents and insufficient treatment of wastes from the dyestuff industries has led to dye contamination in the soil and water.

Approximately half of all known dyes are azo dyes, making them the largest group of synthetic colorants used in textile industries. Azo dyes are considered to be toxic to the aquatic biota and are reported to be carcinogenic to humans; some dyes that are non-toxic when they are used but can be transformed into potentially carcinogenic amines if they are released into an aquatic environment (Soares et al. 2002). These compounds can cause problems due to their possible entrance into the food chain of humans and animals, and once they are present in the environment, they begin exerting genotoxic effects in organisms. Therefore, there is a need to remove dyes prior the effluent discharge into water bodies. Azo dyes are in general considered recalcitrant to microbial degradation. However,

microbial degradation of azo dyes has been reported by bacteria (Rajaguru et al. 2000) and white-rot fungi (Martins et al. 2003). The advantage of white-rot fungi (WRF) to degrade azo dyes is related with its oxidative mechanism that avoid the formation of anilines formed by reductive cleavage of azo dyes by bacteria (Chung and Stevens, 1993)

Diverse technologies and reactors have demonstrated the capacity of microorganisms, particularly WRF, to decolorize and remove a wide variety of structurally diverse pollutants including synthetic dyes (Rodriguez-Couto et al. 2003; Rubilar et al. 2012). These fungi possess extracellular ligninolytic enzymes such as laccase and peroxidases and due to the relative lack of specificity have been used to degrade a wide range of organic compounds like dyes (Ramsay et al. 2005; Urra et al. 2006; Eichlerová et al. 2007; Hu et al. 2009; Grassi et al. 2011).

The ability of WRF has opened new prospects for the development of new biological system using microorganisms immobilized in different supports to improve the ligninolytic enzyme production and the capacity to growth and to produce high decolourization. Diverse investigations have shown that different types of organic and inorganic supports allow the fungi pellets formation providing a structural and nutritional function (Walter et al. 2004; Rubilar et al. 2009). In this context, studies developed by Rubilar et al. (2009) reported that pellets of the white-rot fungus *Anthracophyllum discolor* immobilized in a mixture of activated carbon and sawdust demonstrated a high lignin degradation in an airlift reactor (> 72%) and 85% of pentachlorophenol degradation when the pellets were applied in soil. Activated carbon has been used for their effective biomass immobilization and pollutant adsorption (Zhang and Yu, 2000; Ortega-Clemente et al. 2007) and lignocellulosic material have been used as support and carbon source for white-rot fungi (Walter et al. 2004; Lechner and Papinutti, 2006). Therefore, the mixture of support can enhance the enzymatic activity and stability of fungal pellets in a wastewater treatment. However, although the use of support for pellet formation has a key role in degradation process by WRF, no information is available on degradation of highly toxic pollutants such as azo dyes.

Therefore, the aim of this work was to select a strain of WRF that can be utilized to formulate complex and coated fungal pellets to improve decolourization and degradation of Reactive Orange 165 (azo dye).

MATERIALS AND METHODS

Strains and growth media

The WRF *Trametes versicolor* (sp and m-107), *Stereum hirsutum* m-104, *Anthracophyllum discolor*, *Inonotus* sp2 and *Galerina patagonica* sp3 were isolated in different locations in southern Chile (Tortella et al. 2008). These strains were obtained from the Environmental Biotechnology Laboratory at Universidad de La Frontera, Chile. The fungi were transferred from slant cultures to glucose malt extract agar (GMEA) plates (15 g L^{-1} agar, 10 g L^{-1} glucose, 30 g L^{-1} malt extract, pH 5.2) and incubated at 30 ± 2ºC for 7 days.

White-rot fungi selection

The WRF were cultivated in Petri dishes (90 mm) containing GMEA supplemented with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) for determination of laccase, activity observed by the green colorization in the growth medium (Pointing, 1999) and Poly R-478 (100 mg L^{-1}) for determination of peroxidase activity observed through the decolourization of dye from purple to yellow (Pointing, 1999). Each strain was inoculated in the center of each Petri dish with a plug (6 mm) of active mycelium from a 7-day-old culture on GMEA medium and incubated in the dark at 26ºC. The decolourization of Poly R-478 and the oxidation of the medium containing ABTS were measured daily from Petri dishes (mm d^{-1}). The decolourization yield was calculated as the ratio between the diameter of decolourization/oxidation and diameter of fungal growth. To evaluate the ergosterol content similar assay was done in Petri dishes with GMEA medium. Ergosterol content by HPLC and biomass (dry weight) were measured every each 2 days for 10 days at 26ºC. White-rot fungus with the highest ligninolytic potential and ergosterol-biomass correlation was selected for further studies.

Pellets formulation

For the preparation of the inoculum, 100 mL of modified Kirk medium containing 10 g L^{-1} glucose, 2 g L^{-1} peptone, 2 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 0.1 g L^{-1} $CaCl_2$, 2 mg L^{-1} thiamine, and mineral salts (10 mL L^{-1}) were prepared in Erlenmeyer flasks (500 mL), and the pH was adjusted to 4.5 with 20 mM acetate buffer. After autoclaving, a sterile thiamine stock solution was added to a final concentration of 2 mg L^{-1}. The flasks were inoculated with three glucose malt agar plugs (5 mm diameter) of active mycelium from the fungus *A. discolor* selected in the previous screening assay and incubated at 30ºC in the dark. After 7 days, the fungal mycelium was aseptically homogenized in a sterile blender for 1 min and stored at 4ºC for further pellet formation.

Three types of pellets were prepared: complex (mycelium immobilized on sawdust and powdered activated carbon) and coated (complex pellet coated with calcium alginate) and simple (only mycelium) used as control. All pellets were formulated in Erlenmeyer flasks containing 100 mL of modified Kirk medium. Simple pellets (mycelium-only controls) were prepared by adding 2 mL of fungal inoculum (approximately 30 mg), complex pellets were prepared by mixing 15/15/30 mg of sawdust/powdered activated carbon/mycelium (Ortega-Clemente et al. 2007) and coated pellets were formulated for entrapped of complex pellets with Ca-alginate polymer. Coated pellets were performed using a Na-alginate solution (2%) that was dispensed into a $CaCl_2$ solution (3% w/w) by shaking. After 30 min, the beads were collected from the solution, washed with distilled water and allowed to harden for 30 min in a solution of $CaCl_2$ (3% w/w). Finally, the beads were washed with 0.7% NaCl under sterile conditions. All the solutions used in these experiments were autoclaved previously. All the experiments were performed in triplicate and incubated in a rotary shaker at 100 rpm at 26ºC for 15 days. Microphotographs of pellets were taken using a scanning electron microscope (SEM) to determine the surface structure of fungal pellets. Additionally, a sample of pellets was taken and biomass was determined by dry weight after drying at 60ºC to constant weight.

Ligninolytic enzyme activity by pellets of white-rot fungus

Pellets of *A. discolor* (simple, complex and coated) were evaluated for laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) production. Pellets prepared previously (3.0 g wet pellets) and 100 mL of modified Kirk medium were added into a 250 mL Erlenmeyer flask. Cultures were incubated in a rotary shaker at 100 rpm at 26ºC, for 15 days. Laccase, MnP and LiP activities were monitored every 2 days in the liquid culture. All assays were run in triplicate.

Decolourization of RO165 by pellets of a white-rot fungus

Complex, coated and simple pellets of *A. discolor* were evaluated for RO165 decolourization. Approximately 3.0 g wet pellets and 100 mL of modified Kirk medium with 100 mg L^{-1} Reactive Orange 165 (RO165) were added into a 250 mL flask. The flasks were incubated at 26ºC by shaking (100 rpm) for 11 days. Samples were collected periodically and centrifuged at 5,000 rpm for 15 min at 4ºC. Spectrophotometric method was used to monitor the optical density at 493 nm by Spectronic Genesys 2PC UV-Visible spectrophotometer. The residual RO165 in the culture medium was quantified by standard curve (R^2 = 0.998) and the percentage of decolourization of dye was calculated with the following formula:

$$\% \text{ dye removal} = \frac{A\text{-}B}{B}$$

Where A is the absorbance value after 1 hr of incubation and B is the absorbance value at time *t.*

Analyses

Ergosterol in solid medium. A method described by Marin et al. (2006) was used. The calculated recovery of ergosterol was approximately 80% for the concentrations found in this study. The fungal mycelium from Petri dishes was extracted with 40 mL of 10% KOH in methanol by magnetic stirring for 30 min. A 10 mL aliquot was transferred to a screw-cap tube and placed in a hot water bath (60ºC) for 20 min. The tubes were transferred to room temperature and allowed to cool. Three millilitres of water

and 2 mL hexane were added to the tubes, which were then vortexed for 2 min. After separation of the upper hexane layer, the sample was transferred to a 10 mL vial. The hexane extraction was repeated twice using 2 mL each time. The extracts were combined and evaporated to dryness under a stream of nitrogen. The dry extracts were dissolved in 2 mL of methanol, filtered through 0.45 µm cellulose acetate membrane and finally transferred to a 5 mL vial. The high-performance liquid chromatography equipment consisted of a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with a 20-µL loop and a Merck Hitachi L-7455 diode array detector. The detector was set at 282 nm, and the column was a RP-18 (Superspher RP-C18, 5 µm, 4.6 x 150 mm). The mobile phase was methanol, with a flow rate of 1 mL min^{-1}. Ergosterol standard for the calibration line (R^2 = 0.99) was purchased from Sigma (St Louis, MO). Finally, the biomass dry weight was determined after drying at 60ºC to constant weight.

Scanning electron microscopy (SEM). Photomicrographs of complex pellets were taken using a scanning electron microscope (SEM) in Centre of Biological Engineering, Universidade do Minho. To prepare samples for testing, the core of each sample was cut into pieces of the same length using a flame-sterilized knife. Fixation of the sample was performed with glutaraldehyde (2.5%) at 4ºC for 1.5 hrs, cacodylate salt (0.1 M, pH 7.0) for 30 min, osmium tetroxide (1%), and dehydration with acetone, followed by drying and metallization with gold.

Ligninolytic enzyme activities in liquid medium. Ligninolytic enzymes were analyzed in the culture medium after centrifugation at 5,000 *g* for 10 min. The MnP activity was monitored by the peroxide-dependent degradation of 2,6-dimethoxyphenol (DMP) (De Jong et al. 1994). The reaction mixture contained 50 mM sodium malonate (pH 4.5) 1 mM DMP, 1 mM $MnSO_4$, and up to 600 µL supernatant in a volume of 1 mL. The reaction was initiated by the addition of 0.4 mM H_2O_2. Laccase was assayed by the peroxide-independent degradation of 2,6- dimethoxyphenol (De Jong et al. 1994). For both enzymatic reactions, the products were measured at 468 nm in a spectrophotometer (Spectronic Genesys 2PC). MiP activity was determined in a reaction mixture containing 200 µL of 250 mM sodium malonate (pH 4.5), 50 µL of 20 mM 2.6-DMP, 100 µL of 20 mM EDTA, and 550 µL of supernatant. LiP activity was determined with veratrylic alcohol as a substrate (Tien and Kirk, 1988). One unit of enzymatic activity was defined as the quantity of enzyme needed to produce 1 µmol of oxidized product and was expressed in U L^{-1}. The daily values of different enzyme activities (Lac, MnP, MiP and LiP) were used to determine the accumulated enzyme activity.

RESULTS AND DISCUSSION

White-rot fungi selection

Fungi isolated from various geographic sites in southern Chile were evaluated to select an efficient strain with high ligninolytic activity and high ergosterol/biomass correlation in solid medium. The strains evaluated were selected using the standard decolourization method with Poly R-478 (Rigas and Dritsa, 2006) that is associated with the ability to produce peroxidases (MnP and LiP) and, ABTS oxidation that is associated with the ability to produce laccase (Pointing, 1999). We analyzed the relationship between fungal growth and the ability to produce ligninolytic enzymes (Figure 1) and we calculated the decolourization (Poly R-478) and colourization (ABTS) yields.

In our study, the six evaluated strains produced Poly R-478 decolourization at different level, and four strains presented ABTS colorization (Figure 1). With respect to the decolourization of Poly R-478, the strains that showed the highest decolourization were *A. discolor, T. versicolor* sp and *Inonotus* sp with 13.6, 11.0 and 10.0 mm d^{-1}, respectively. However, the major difference observed between these strains was the yield. The yield is the ratio between the decolourization of Poly R-478 or oxidation of ABTS and radial fungal growth, indicating that when yield is near 1.0 the fungal growth is associated with high secretion of enzymes. Therefore, the results demonstrated that *A. discolor* was the strain that showed the highest ability to produce peroxidase (MnP) with a yield value of 1 (Figure 1).

In relation to the ABTS oxidation, the strains that showed highest ability to produce laccase were *T. versicolor* sp, *T. versicolor* m-107 and *S. hirsutum* m-104 with 7.0, 5.0 and 5.0 mm d^{-1}, respectively. The yield for these strains was between 0.7 and 0.88, indicating that the ABTS oxidation is less associated with fungal growth. The strain *A. discolor* and *G. patagonica* showed no visible ABTS oxidation. Different results were reported by Tortella et al. (2008) for the same strains but using different culture composition. This difference could be due to that enzyme production in solid medium

depends on different factors such as nutrient limitation, substrate composition and nitrogen source (Leonowicz and Grzywnowicz, 1981; Machado et al. 2005; Kachlishvili et al. 2006).

Similar studies have been reported for white-rot fungi selection (De Koker et al. 2000; Martins et al. 2003; Mendonça et al. 2008). Mendonça et al. (2008) evaluated comparatively *Ganoderma australe* and *Ceriporiopsis subvermispora* in the biodegradation of ABTS and Poly R-478 in liquid medium, and in the pre-treatment of *Eucalyptus globulus* wood chips. Laccase was detected in liquid and wood cultures with *G. australe* and *C. subvermispora* produce Lac and MnP when grown in liquid medium and only MnP was detected during wood decay. ABTS was totally depleted by all strains after 8 days of incubation while Poly R-478 was degraded up to 40% with *G. australe* strain and up to 62% by *C. subvermispora* after 22 days of incubation (Mendonça et al. 2008).

Another parameter used to select fungal strains was ergosterol/biomass correlation (Figure 2). This indicator is appropriate to select a strain with a high capacity for fungal biomass production in order to obtain a strain for the production of pellets that can be used in dye decolourization. Ergosterol is a sterol found in cell membranes of fungi and microalgae with the advantage of indicating only viable biomass, since it is quickly degraded after the cell's death (Gutarowska and Zakowska, 2009). Ergosterol content has been used to estimate fungal biomass in various environments because there is a strong correlation between ergosterol content and fungal dry mass (Newell et al. 1988; Bermingham et al. 1995; Montgomery et al. 2000; Barajas-Aceves et al. 2002). However, the amount of ergosterol in fungal tissue is not constant and depends on several factors, such as age of the culture, the developmental stage and the growth conditions (type of growth media, pH and temperature) (Suberkropp et al. 1993).

In our study total mycelia ergosterol ranged from 48 to 104 mg g^{-1}, and total dry biomass ranged from 223 to 526 mg in the six strains evaluated (Figure 2). The ergosterol content was closely correlated with dry biomass production during the growth of each of the fungal species and r^2 was > 0.92 in all fungal strains (Table 1). Similar results were obtained by Montgomery et al. (2000) in pure cultures of six fungal species originating from soil and plant materials.

Table 1. Linear regression for ergosterol content versus dry biomass and r^2 values of pure cultures of six fungal strains.

Fungal strains	Lineal regression	r^2
Stereum hirsutum sp1	y = 118.52x - 2.6267	0.9395
Trametes versicolor m-107	y = 173.56x + 0.5565	0.9968
Trametes versicolor sp	y = 135.94x - 1.3185	0.9656
Galerina patagonica sp3	y = 194.5x - 0.9711	0.9228
Inonotus sp2	y = 293.35x - 1.9521	0.9615
Anthracophyllum discolor	y = 228.5x + 0.1994	0.9872

Therefore, the results obtained in solid medium demonstrated that *A. discolor* has the metabolic and growth capacity to formulate pellets containing activated carbon and sawdust that are coated with alginate. This agrees with the results of Tortella et al. (2008), who described the strain *A. discolor* as very promising for its use in future studies of the degradation of pollutants and indicates its potential for use in biotechnological applications.

Pellets morphology evaluation

Complex and coated pellets of *A. discolor* were evaluated after 7 days of incubation at 26ºC in Kirk medium to determine the size and shape of fungal pellets (Figure 3). The pellets were approximately 4 and 5 mm in diameter for the complex and coated pellets, respectively. Complex pellets showed a stable sphere of irregular surface composed of hyphal agglomeration formed by the effect of agitation during cultivation (Figure 3a) whereas coated pellets were sphere of homogeneous surface due to calcium alginate coating (Figure 3b). The biomass of complex and coated pellets was determined to examine the capacity of coated pellets to grow inside alginate beads after 10 days of incubation. After

7 days of incubation, complex pellets produced 20% more biomass than coated pellets, and at the end of the incubation, the difference decreased by 15% (data non-shown).

SEM analysis was developed in outside and inside of both pellets (complex and coated) in order to analyze the surface of pellets and presence of activated carbon and sawdust as supports. Results of SEM analysis showed that both pellets contained a black core of activated carbon and sawdust that was entrapped inside pellets. Fungal hyphae were not detected in the core of pellets due to the low transference of oxygen (Figure 4a). Fungal mycelia on the surface of complex pellets showed hyphae agglomeration (Figure 4b) whereas the SEM of surface of coated pellets shows a smooth surface without mycelia, demonstrating the efficiency of the encapsulation process (Figure 4c). Calcium alginate completely surrounded the pellets, however, fungal mycelia under of surface of coated pellets was observed (Figure 4d). Eichlerová et al. (2007) presented SEM micrographs after 14 days of cultivation of *Dichomitus squalens* and showed substantial morphological changes in mycelia growing in a media containing Orange G. The hyphae deformations were more intensively manifested in solid media than in liquid culture.

The use of activated carbon has been investigated as an adsorbent of several organic compounds (Lin et al. 2005). It also acts to stimulate secondary metabolism and produces more ligninolytic enzymes due to the physical properties of activated carbon and nutrient extraction from sawdust (Zhan and Yu, 2000). The presence of sawdust provides different compounds such as hemicelluloses, lignin and cellulose. Therefore, it is not surprising that immobilized fungi can produce extracellular secondary metabolites.

One the disadvantage of complex pellets is that the biomass due to hyphae agglomerations surrounding the pellets could cause a breakdown of complex pellet when are used for prolonged incubation time (Rubilar et al. 2009). These morphological changes of complex pellets could be diminished by alginates coating due to that this polymer present various advantages such as biodegradability, hydrophilicity, low density and mechanical stability, all of which make them highly suitable for producing alginate beads. The properties of the beads can be affected by the immobilization process, by the effects of alginate viscosity, by the length of time in the $CaCl_2$ bath, and by the $CaCl_2$ concentration (Yakup et al. 2004). Some reports have demonstrated that re-coating of pellets reduces the viability of pellets entrapped in Ca-alginate beads (Ramsay et al. 2005).

Ligninolytic enzyme activities of *A. discolor* pellets

Ligninolytic activity and accumulated ligninolytic activity during the incubation of complex, coated and simple pellets of *A. discolor* in liquid medium for 15 days are shown in Figure 5a, 5b and 5c. The main enzyme produced by the three types of pellets was MnP followed by MiP. Lacasse and LiP were produced in a lesser extent. Accumulated Lac activity was 18.5 U L^{-1}, 32.1 U L^{-1} and 0.4 U L^{-1} in the complex, coated and simple pellets, respectively. Accumulated MnP activity was 147.9 U L^{-1}, 98.2 U L^{-1} and 93.1 U L^{-1} for the complex, coated and simple pellets, respectively.Accumulated MiP peroxidase activity was 69 U L^{-1}, 34.2 U L^{-1} and 27.9 U L^{-1} for the complex, coated and simple pellets, respectively. Finally, the accumulated LiP activity was 4.3 U L^{-1}, 11.4 U L^{-1} and 18.6 U L^{-1} for the complex, coated and simple pellets, respectively. Based on these results, we observed that the accumulated activity of Lac and MiP increased significantly due to the introduction of sawdust and activated carbon during pellet formulation (complex and coated) in comparison with simple pellets; on the other hand, accumulated MnP activity was the highest in complex pellets (Figure 5a). The maximum peak of enzyme activity for the complex pellet was observed on day 15 (38 U L^{-1} for MnP). On the other hand, for the coated and simple pellets, the maximum peaks for MnP were at day 13.

Similar results were obtained by Rubilar et al. (2009). The authors demonstrated that complex pellets of *A. discolor* had a high stability with a high MnP production in comparison with simple pellets, during lignin degradation in an airlift reactor. Ortega-Clemente et al. (2007) demonstrated that pellets of *T. versicolor* formulated with sawdust and activated carbon (triple pellets) showed MnP and Lac average values 30% higher than in double pellets (only sawdust). Our results suggest that increasing MnP activity with complex pellet could be associated with highest dyes degradation.

Decolourization of RO 165 by pellets of *A. discolor*

RO165 decolourization by complex, coated and simple pellets of *A. discolor* was evaluated during 11 days of incubation and the results are shown in Figure 6. We can observe that the dye RO165 was degraded by the three types of pellets; however complex pellets showed a faster decolourization compared to coated and simple pellets. At day 4 complex pellets achieved 87% of dye decolourization while simple and coated pellets obtained 64 and 34%, respectively. At day 8 complex pellets achieved 100% of decolourization while simple and coated pellets achieved 100% of decolourization after 11 days of incubation. During the first 6 days, the RO165 decolourization by coated pellets was slower compared to complex and simple pellets. It is due to that in coated pellets the penetration of fungal mycelium in layer of alginate is slower.

The highest and fastest RO165 decolourization by complex pellets could be related to MnP production (Figure 5). In this context, studied developed by Urra et al. (2006) showed that the decolourization of 100 mg L^{-1} of RO165 by *P. chrysosporium* was 91% after 15 days, and they suggested that RO165 was degraded by the oxidative action of MnP present in the medium concordant with our result. Baldrian and Šnajdr (2006) evaluated the decolourization of azo dyes by different white-rot fungi, demonstrating that the fastest degradation of Poly B-411 was performed by the strains with high levels of Lac and MnP.

In relation to complex pellets Rubilar et al. (2009) reported that complex pellets of *A. discolor* containing sawdust and activated carbon exhibited higher MnP activity level than simple pellets; therefore, this led to a higher level of pollutant degradation. This effect can be explained by the presence of a microenvironment inside fungal pellets that changes in the presence of supports, thus improving the enzymatic activity and hence the decolourization (Zhang and Yu, 2000).

The absorbance spectrum of RO165 during the incubation with complex pellets changed drastically, showing a peak at 493 nm at 0 day and no peak at 11 days. This reduction corresponded to a shift in the coloration of the medium from orange to yellow, demonstrating the dye degradation (Figure 7). Similar behaviour was observed with simple and coated pellets of *A. discolor* (data non-shown). At the end of decolourization, all the pellets were transferred to flasks containing ethanol for 24 hrs in order to identify the dye adsorbed in the mycelium. In our study, no dye was detected adsorbed onto mycelium.

CONCLUDING REMARKS

Some general, promising conclusions may be derived for the results here obtained:

- Chilean white-rot fungus *A. discolor* showed high level of peroxidases and high biomass/ergosterol correlation demonstrating a great potential for pellets formulation to dyes decolourization.

- Complex pellets of *A. discolor* showed a higher enzymatic production mainly MnP compared to coated and simple pellets.

- Decolourization of RO165 was demonstrated with all the pellets formulated. However, the highest and fastest decolourization was obtained with complex pellets.

The highest and faster RO165 decolourization by complex pellets was correlated with enzymatic activity of MnP and the change in the absorbance spectrum demonstrated the degradation of the dye.

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REFERENCES

BALDRIAN, P. and ŠNAJDR, J. (2006). Production of ligninolytic enzymes by litter-decomposing fungi and their ability to decolorize synthetic dyes. *Enzyme and Microbial Technology*, vol. 39, no. 5, p. 1023-1029. **[CrossRef]**

BARAJAS-ACEVES, M.; HASSAN, M.; TINOCO, R. and VAZQUEZ-DUHALT, R. (2002). Effect of pollutants on the ergosterol content as indicator of fungal biomass. *Journal of Microbiological Methods*, vol. 50, no. 3, p. 227-236. **[CrossRef]**

BERMINGHAM, S.; MALTBY, L. and COOKE, R.C. (1995). A critical assessment of the validity of ergosterol as an indicator of fungal biomass. *Mycological Research,* vol. 99, no. 4, p. 479-484. **[CrossRef]**

BIBI, I.; BHATTI, H.N. and ASGHER, M. (2009). Decolourisation of direct dyes with manganese peroxidase from white rot basidiomycete *Ganoderma lucidum*-IBL-5. *The Canadian Journal of Chemical Engineering*, vol. 87, no. 3, p. 435-440. **[CrossRef]**

CHUNG, K.-T. and STEVENS JR., B. (1993). Degradation of azo dyes by environmental microorganisms and helminths. *Environmental Toxicology and Chemistry,* vol. 12, no. 11, p. 2121-2132. **[CrossRef]**

CRINI, G. (2006). Non-conventional low-cost adsorbents for dye removal: A review. *Bioresource Technology*, vol. 97, no. 9, p. 1061-1085. **[CrossRef]**

DE JONG, E.; FIELD, J.A. and DE BONT, J. (1994). Aryl alcohols in the physiology of ligninolytic fungi. *FEMS Microbiology Reviews*, vol. 13, no. 2-3, p. 153-187. **[CrossRef]**

DE KOKER, T.H.; ZHAO, J.; ALLSOP, S.F. and JANSE, B.J.H. (2000). Isolation and enzymatic characterization of South African white-rot fungi. *Mycological Research*, vol. 104, no. 7, p. 820-824.

EICHLEROVÁ, I.; HOMOLKA, L.; BENADA, O.; KOFROŇOVÁ, O.; HUBÁLEK, T. and NERUD, F. (2007). Decolorization of Orange G and Remazol Brilliant Blue R by the white rot fungus *Dichomitus squalens*: Toxicological evaluation and morphological study. *Chemosphere*, vol. 69, no. 5, p. 795-802. **[CrossRef]**

GRASSI, E.; SCODELER, P.; FILIEL, N.; CARBALLO, R. and LEVIN, L. (2011). Potential of *Trametes trogii* culture fluids and its purified laccase for the decolorization of different types of recalcitrant dyes without the addition of redox mediators. *International Biodeterioration & Biodegradation*, vol. 65, no. 4, p. 635-643. **[CrossRef]**

GUTAROWSKA, B. and ZAKOWSKA, Z. (2009). Mathematical models of mycelium growth and ergosterol synthesis in stationary mould culture. *Letters in Applied Microbiology*, vol. 48, no. 5, p. 605-610. **[CrossRef]**

HU, M.; CHAO, Y.; ZHANG, G.; XUE, Z. and QIAN, S. (2009). Laccase-mediator system in the decolorization of different types of recalcitrant dyes. *Journal of Industrial Microbiology and Biotechnology*, vol. 36, no. 1, p. 45-51. **[CrossRef]**

KACHLISHVILI, E.; PENNINCKX, M.; TSIKLAURI, N. and ELISASHVILI, V. (2006). Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *World Journal of Microbiology and Biotechnology*, vol. 22, no. 4, p. 391-397. **[CrossRef]**

LECHNER, B.E. and PAPINUTTI, L. (2006). Production of lignocellulosic enzymes during growth and fruiting of the edible fungus *Lentinus tigrinus* on wheat straw. *Process Biochemistry*, vol. 41, no. 3, p. 594-598. **[CrossRef]**

LEONOWICZ, A. and GRZYWNOWICZ, K. (1981). Quantitative estimation of laccase forms in some white rot-fungi using syringaldazine as a substrate. *Enzyme and Microbial Technology*, vol. 3, no. 1, p. 55-58. **[CrossRef]**

LIN, Y.-B.; FUGETSU, B.; TERUI, N. and TANAKA, S. (2005). Removal of organic compounds by alginate gel beads with entrapped activated carbon. *Journal of Hazardous Materials*, vol. 120, no. 1-3, p. 237-241. **[CrossRef]**

MACHADO, K.C.; MATHEUS, D.C.R. and BONONI, V.L.R. (2005). Ligninolytic enzymes production and Remazol Brilliant Blue R decolorization by tropical Brazilian basidiomycetes fungi. *Brazilian Journal of Microbiology*, vol. 36, no. 3, p. 246-252. **[CrossRef]**

MARIN, S.; MORALES, H.; RAMOS, A. and SANCHIS, V. (2006). Evaluation of growth quantification methods for modelling the growth of *Penicillium expansum* in an apple-based medium. *Journal of the Science of Food and Agriculture*, vol. 86, no. 10, p. 1468-1474. **[CrossRef]**

MARTINS, M.A.M.; LIMA, N.; SILVESTRE, A.J.D. and QUEIROZ, M.J. (2003). Comparative studies of fungal degradation of single or mixed bioaccessible reactive azo dyes. *Chemosphere*, vol. 52, no. 6, p. 967-973. **[CrossRef]**

MENDONÇA, R.T.; JARA, J.F.; GONZÁLEZ, V.; ELISSETCHE, J.P. and FREER, J. (2008). Evaluation of the white-rot fungi *Ganoderma australe* and *Ceriporiopsis subvermispora* in biotechnological applications. *Journal of Industrial Microbiology & Biotechnology*, vol. 35, no. 11, p. 1323-1330. **[CrossRef]**

MONTGOMERY, H.J.; MONREAL, C.M.; YOUNG, J.C. and SEIFERT, K.A. (2000). Determination of soil fungal biomass from soil ergosterol analyses. *Soil Biology and Biochemistry*, vol. 32, no. 8-9, p. 1207-1217. **[CrossRef]**

NEWELL, S.Y.; ARSUFFI, T.L. and FALLON, R.D. (1988). Fundamental procedures for determining ergosterol content of decaying plant material by liquid chromatography. *Applied and Environmental Microbiology*, vol. 54, no. 7, p. 1876-1879.

ORTEGA-CLEMENTE, A.; PONCE-NOYOLA, M.T.; MONTES-HOECASITAS, M.C.; VICENT, M.T.; BARRERA-CORTÉS, J. and POGGI-VARALDO, H.M. (2007). Semi-continuous treatment of recalcitrant anaerobic effluent from pulp and paper industry using hybrid pellets of *Trametes versicolor*. *Water Science & Technology*, vol. 55, no. 6, p. 125-133. **[CrossRef]**

POINTING, S.B. (1999). Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. *Fungal Diversity*, vol. 2, p. 17-33.

RAJAGURU, P.; KALAISELVI, K.; PALANIVEL, M. and SUBBURAM, V. (2000). Biodegradation of azo dyes in a sequential anaerobic aerobic system. *Applied Microbiology and Biotechnology*, vol. 54, no. 2, p. 268-273. **[CrossRef]**

RAMSAY, J.A.; MOK, W.H.W.; LUU, Y.S. and SAVAGE, M. (2005). Decoloration of textile dyes by alginate-immobilized *Trametes versicolor*. *Chemosphere*, vol. 61, no. 7, p. 956-964. **[CrossRef]**

RIGAS, F. and DRITSA, V. (2006). Decolourisation of a polymeric dye by selected fungal strains in liquid cultures. *Enzyme and Microbial Technology*, vol. 39, no. 1, p. 120-124. **[CrossRef]**

RODRIGUEZ-COUTO, S.; MOLDES, D.; LIÉBANAS, A. and SANROMÁN, A. (2003). Investigation of several bioreactor configurations for laccase production by *Trametes versicolor* operating in solid-state conditions. *Biochemical Engineering Journal*, vol. 15, no. 1, p. 21-26. **[CrossRef]**

RUBILAR, O.; ELGUETA, S.; TORTELLA, G.; GIANFREDA, L. and DIEZ, M.C. (2009). Pelletization of *Anthracophyllum discolor* for water and soil treatment contaminated with organic pollutants. *Journal of Soil Science and Plant Nutrition*, vol. 9, no. 3, p. 161-175. **[CrossRef]**

RUBILAR, O.; TORTELLA, G.; CUEVAS, R.; CEA, M.; RODRÍGUEZ-COUTO, S. and DIEZ, M.C. (2012). Adsorptive removal of pentachlorophenol by *Anthracophyllum discolor* in a fixed-bed column reactor. *Water, Air & Soil Pollution*, vol. 223, no. 5, p. 2463-2472. **[CrossRef]**

SOARES, G.M.B.; AMORIM, M.T.P.; HRDINA, R. and COSTA-FERREIRA, M. (2002). Studies on the biotransformation of novel disazo dyes by laccase. *Process Biochemistry*, vol. 37, no. 6, p. 581-587. **[CrossRef]**

SUBERKROPP, K.; GESSNER, M.O. and CHAUVET, E. (1993). Comparison of ATP and ergosterol as indicators of fungal biomass associated with decomposing leaves in streams. *Applied and Environmental Microbiology*, vol. 59, no. 10, p. 3367-3372.

TANG, W.; JIA, R. and ZHANG, D. (2011). Decolorization and degradation of synthetic dyes by *Schizophyllum sp.* F17 in a novel system. *Desalination*, vol. 265, no. 1-3, p. 22-27. **[CrossRef]**

TIEN, M. and KIRK, T.K. (1988). Lignin peroxidase of *Phanerochaete chrysosprium*. *Methods in Enzymology*, vol. 161, p. 238-249. **[CrossRef]**

TORTELLA, G.; RUBILAR, O.; GIANFREDA, L.; VALENZUELA, E. and DIEZ, M. (2008). Enzymatic characterization of Chilean native wood-rotting fungi for potential use in the bioremediation of polluted environments with chlorophenols. *World Journal of Microbiology and Biotechnology*, vol. 24, no. 12, p. 2805-2818. **[CrossRef]**

URRA, J.; SEPÚLVEDA, L.; CONTRERAS, E. and PALMA, C. (2006). Screening of static culture and comparison of batch and continuous culture for the textile dye biological decolorization by *Phanerochaete chrysosporium. Brazilian Journal of Chemical Engineering*, vol. 23, no. 3, p. 281-290. **[CrossRef]**

WALTER, M.; BOUL, L.; CHONG, R. and FORD, C. (2004). Growth support selection and biodegradation of PCP by New Zealand white-rot fungi. *Journal Environmental Management*, vol. 71, no. 4, p. 361-369. **[CrossRef]**

YAKUP ARICA, M.; BAYRAMOĞLU, G.; YILMAZ, M.; BEKTAŞ, S. and GENÇ, Ö. (2004). Biosorption of Hg^{2+}, Cd^{2+}, and Zn^{2+} by Ca-alginate and immobilized wood-rotting fungus *Funalia trogii*. *Journal of Hazardous Materials*, vol. 109, no. 1-3, p. 191-199. **[CrossRef]**

ZHANG, F. and YU, J. (2000). Decolourisation of Acid Violet 7 with complex pellets of white rot fungus and activated carbon. *Bioprocess and Biosystems Engineering*, vol. 23, no. 3, p. 295-301. **[CrossRef]**

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Figures

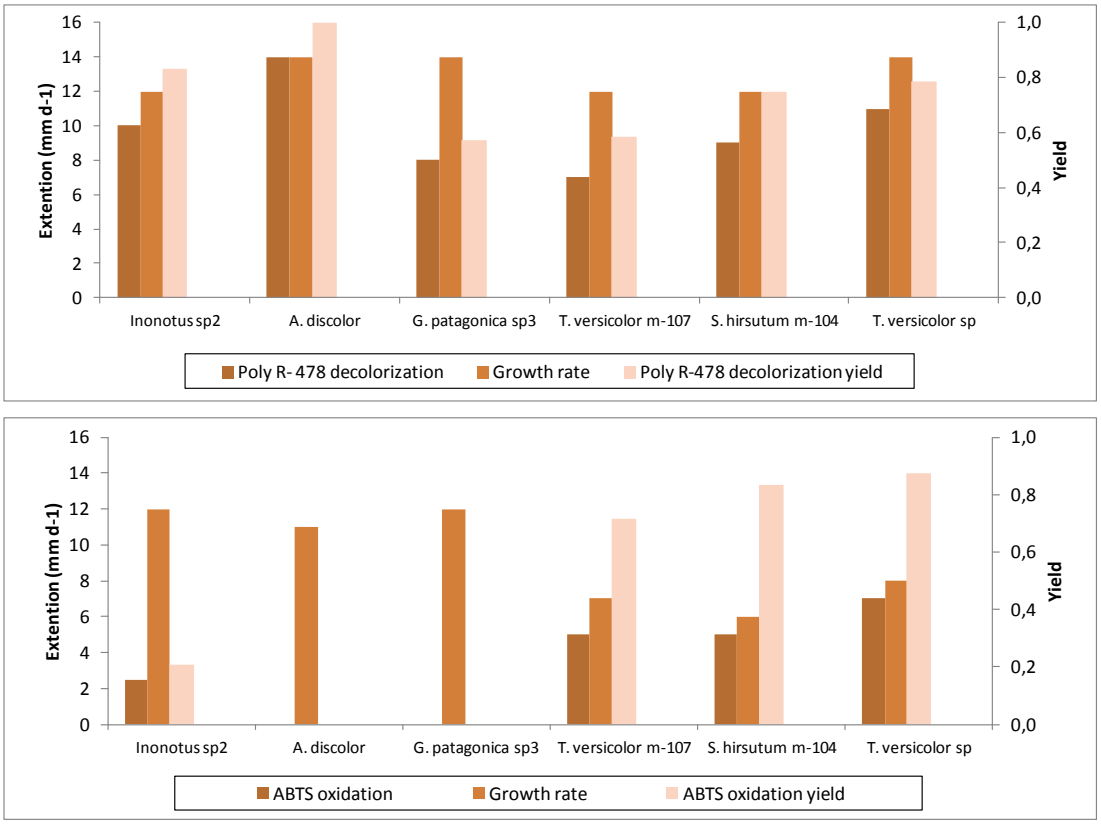

Fig. 1 Decolourization of Poly R-478, growth rate and yield (a), oxidation of ABTS, growth rate and yield (b) of white-rot fungi in GMEA medium after 10 days of incubation at 26ºC.

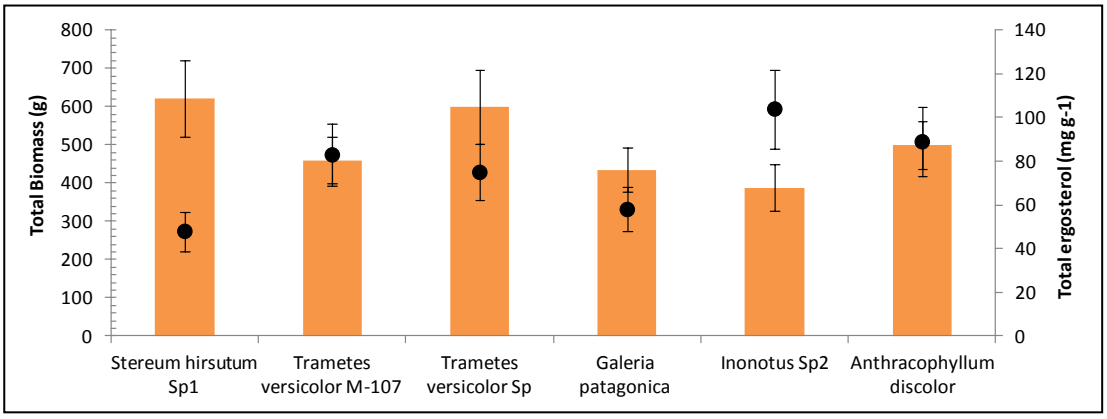

Fig. 2 Total amount of biomass (bars) and total amount of ergosterol of white-rot fungi in GMEA medium after 10 days of incubation at 26ºC.

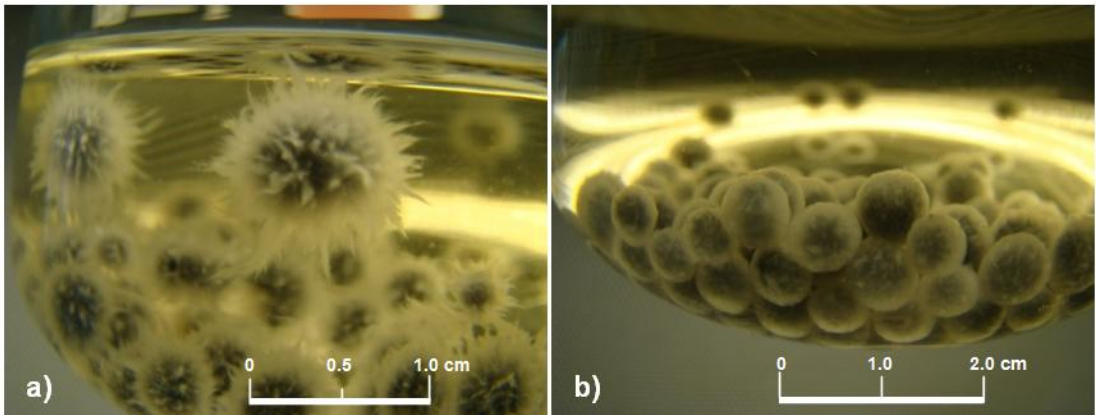

Fig. 3 Complex (a) and coated pellets (b) of *A. discolor* formed after 7 days of incubation at 26ºC.

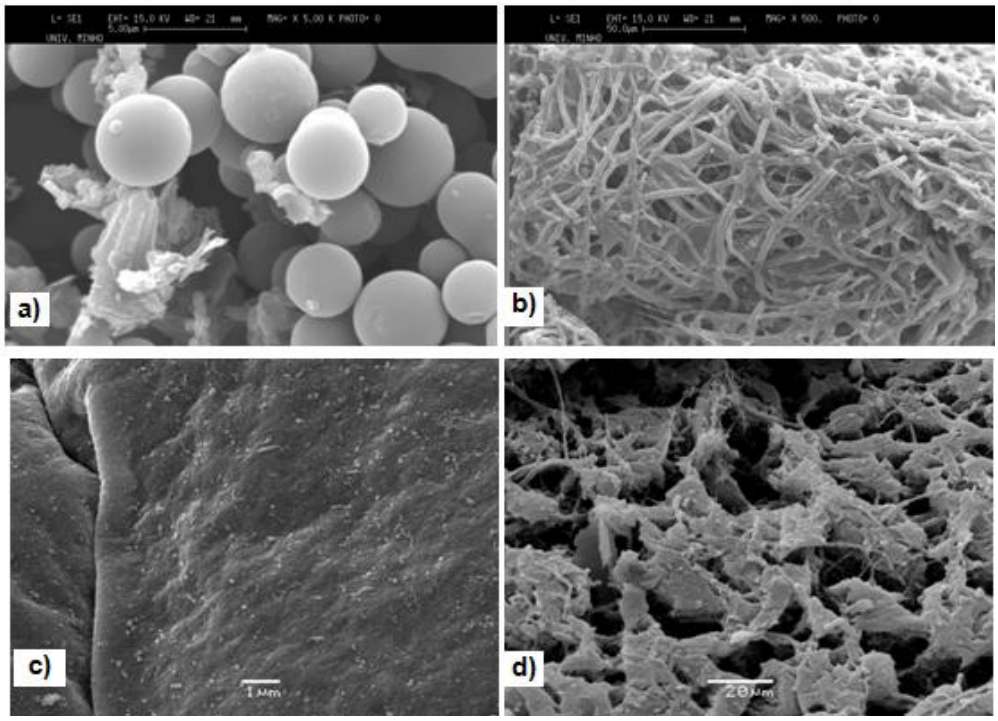

Fig. 4 Scanning electron microscopy of complex pellets of *A. discolor*, after 7 days of incubation. (a) Black core inside both pellets (5 µm); (b) fungal mycelia on the surface of complex pellets (50 µm); (c) surface of coated pellets (1 µm); (d) fungal mycelia under the surface of coated pellets (20 µm).

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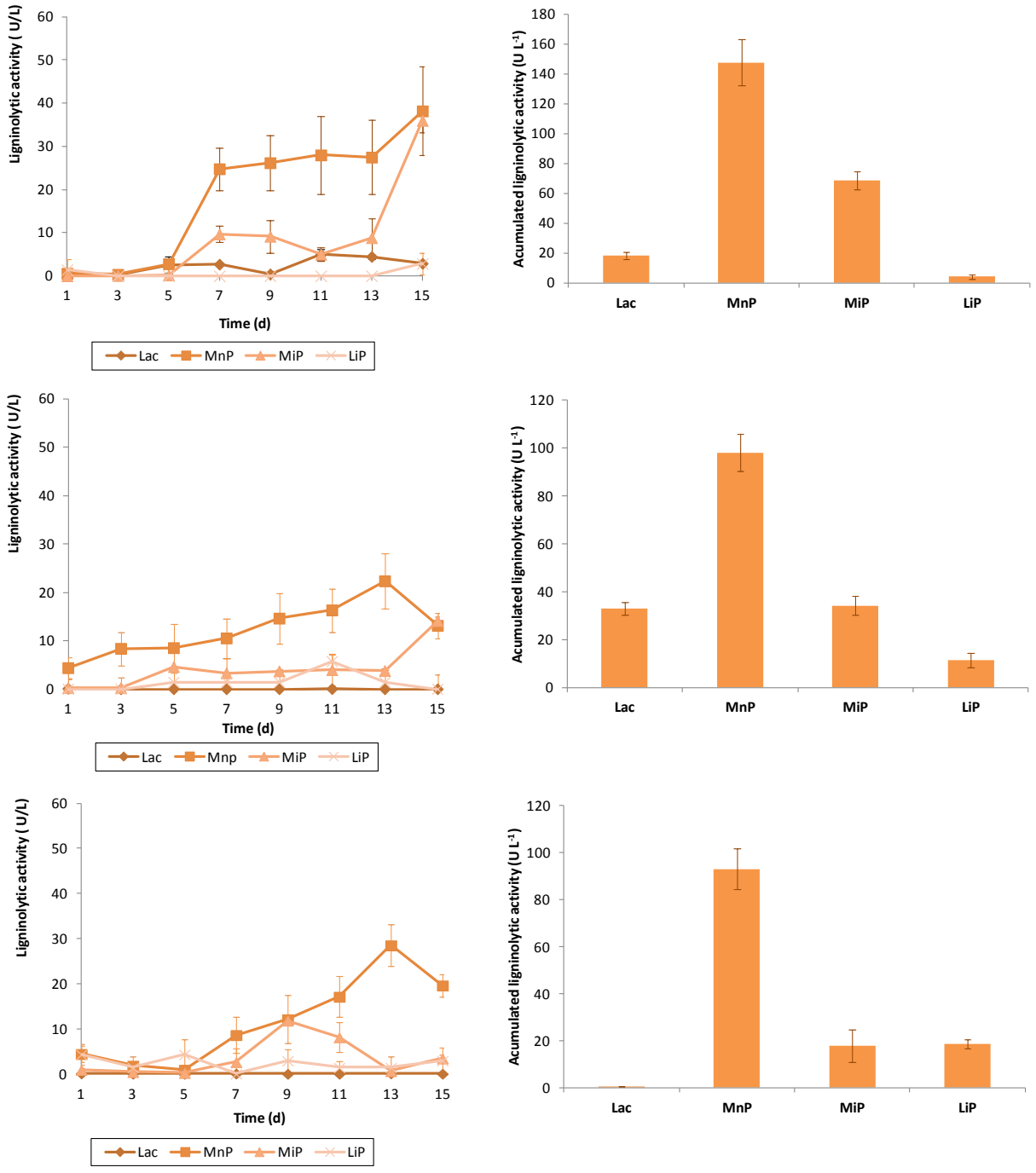

Fig. 5 Ligninolytic enzyme activities (left) and accumulated ligninolytic activity (right) of *A. discolor* pellets in liquid medium after 15 days of incubation at 26ºC. (a) Complex pellets; (b) Coated pellets; (c) Simple pellets.

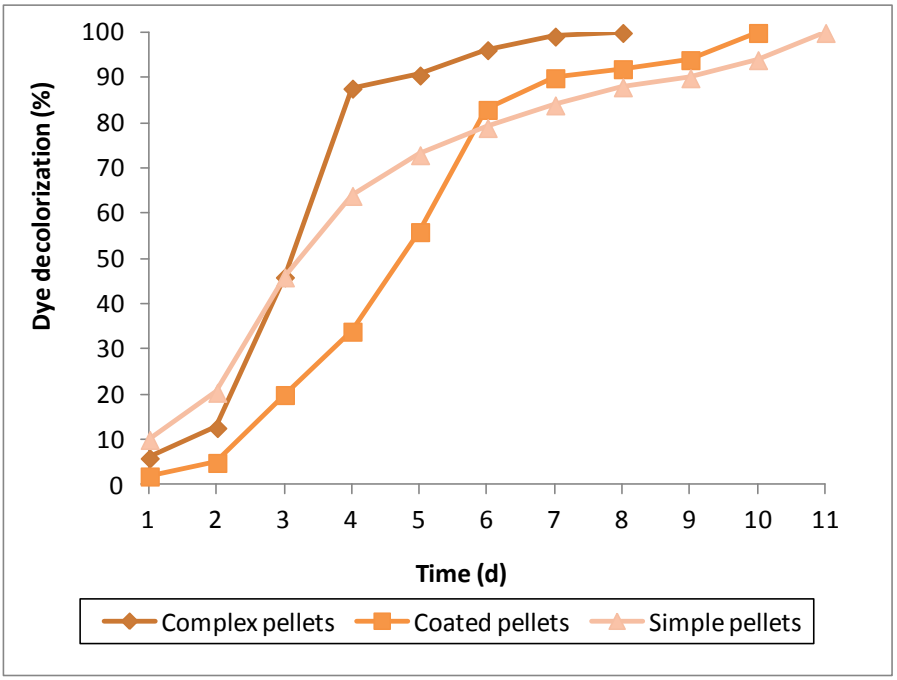

Fig. 6 Decolourization of RO165 by complex, coated and simple pellets of *A. discolor* after 11 days at 26ºC.

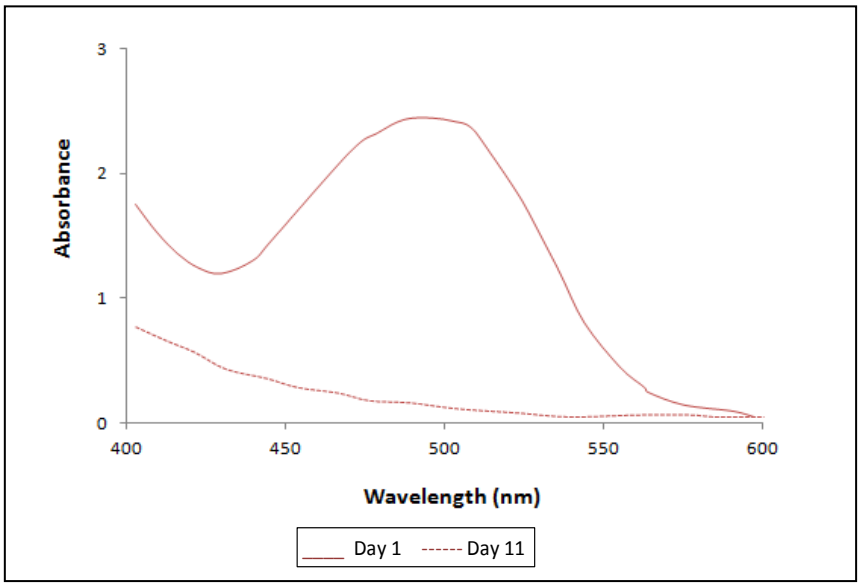

Fig. 7 Absorbance spectrum of RO165 at time 0 and after 11 days of incubation with complex pellets of *A. discolor* at 26ºC.

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