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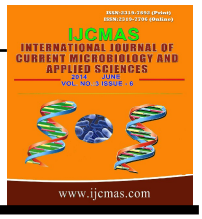

Original Research Article

Discoloration and biodegradation of two dyes by white-rot fungi *Perreniporia tephropora* MUCL 47500 isolated in Gabon

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Keywords

Perreniporia tephropora, enzyme activities, discoloration, biodegradation, dyes

A B S T R A C T

The potential of *Perreniporia tephropora* MUCL 47500 to discolour two textile dyes, Reactive blue 4 and Methyl Orange, was evaluated. Optimal rowth temperature (30°C) was determined. The effect of culture conditions (temperature, pH and concentration) on the discoloration of Reactive blue 4 and Methyl Orange were evaluated during growth of the fungi in culture liquid media. This fungus on culture liquid media containing Reactive blue 4 produces different enzymatic activities like laccase and Mn peroxidase, which have an important role in biodegradation. The biodegradation of dyes was monitored by UV-Vis spectrometer and HPLC analyses. This study demonstrated and reinforced the capacity of tropical basidiomycete *Perreniporia tephropora* to degrade the two dyes

Introduction

The textile industry is one that uses a high volume of synthetic dyes (Zollinger, 1991). Environmental contamination has been pinpointed as one of the greatest problems of modern society, mainly due to population explosion and the increased industrial activities such as synthetic dyes (production of textile, paints, fibres, plastics, leather, paper, mineral oils, waxes, foodstuffs and cosmetics) (Pavko, 2011).

Previous studies reported that dyes contribute to the mutagenic activity of aquatic fauna and flora (Banat et *al.*, 1996; Asgher *et al*., 2006; Chung et *al*., 1992). In addition, this may have a significant impact on human health as well as of other animals (Pierce, 1994). Generally, dyes have complex molecular structures which make them resist elimination from the environment, resulting in direct and indirect exposure of human life and other

organisms to their effects. These dyes are characterized by the presence of groups such as azo- bonds, aromatic rings, sulphonic acid, etc. The treatment of wastewater containing synthetic dyes generally involves chemical and physical methods such as ultrafiltration, ozone oxidation, coagulation, precipitation, adsorption and ionizing radiation (Pavko, 2011). Different studies have presented the many advantages of biological treatment of wastewater with. In fact, microbial processes provide a promising alternative to existing technologies because they are more environmentally friendly and less costly in attenuating the toxicity of dyes (Borchert and Libra, 2001; Lopez *et al*., 2002; Hadibarata and Tachibana, 2009). Several microorganisms, such as fungi, bacteria and yeasts, have ability to discolour and even completely transform many dyes under optimum conditions. The capability of white-rot fungi to removal synthetic dye is due to extracellular enzyme production such as laccases, lignin peroxidases (Lip) and manganese peroxidases (MnP) (Attéké *et al*.,2013; Mounguengui *et al*., 2013; Novotny *et al*., 2004). Several medium factors can influence the ability of fungi in biodegradation, such as temperature, concentration of dye, pH and the chemical structure of dye. In previous study, we have isolated white-rot fungi which have potential to discolour different dyes (Attéké et *al*., 2013). In the present work, we have investigated fungal degradation/discoloration of textile dyes, Reactive blue 4 and Methyl Orange, using *Perreniporia tephropora* and their effect on enzyme production.

Materials and Methods

Organism and culture conditions

A pure culture of fungus *Perreniporia tephropora* MUCL 47500 was obtained from Mycothèque de Louvain-la-Neuve, Belgium. Fungus was conditioned and replicated in tubes containing malt-agar medium at 25°C. These tubes were then preserved and maintained at 4°C before use. The composition of the solid growth medium used in this study is same that is used by Attéké *et al*. 2013.

The pH was adjusted to 5.5 with hydrochloric acid (0. 5 N) before autoclaving at 121°C for 15 min. The culture medium was transferred into 250-mL Erlenmeyer flasks and each reagent (enzymatic revelation) or dye, depending on its final concentration, was added. The mixture was shaken, then 70 ml of liquid culture media were transferred in flasks of 100 mL. The liquid culture media were constituted the same way as the solid culture media, except for the absence of agar. Each test was replicated four times.

Dyes and reagents

The two dyes Reactive blue 4 (RB4) and Methyl Orange (MO) were obtained from Sigma-Aldrich (Sigma-Aldrich Chimie sarl, Saint Quentin Fallavier, France); malt extract and agar were obtained from Acros Organics (Fisher Scientific SAS, Strasbourg, France); ABTS (acid, 2, 2-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid), TFA (trifluoroacetic acid) and ethyl acetate were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Methanol for HPLC was obtained from Carlo Erba Réactifs (Carlo Erba Reactifs-SDS, Val de Reuil, France). Glucose was obtained from Prolabo (VWR International S.A.S, Strasbourg, France).

All minerals to culture media were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

Growth and optimization of physico-chemical parameters for discoloration

The optimum growth temperature was evaluated by measuring growth diameters at different temperatures (15, 22, 30, 35, 40 and 45°C) on solid culture media. For dye discoloration on liquid culture medium, each Erlenmeyer flask (100 mL) contained liquid culture medium (70 mL) and the dye (0.3 g/L). One disc of agar of 1 cm diameter containing the fungus was added. The culture media were incubated for 15 days (30°C and humidity 75%) under static conditions. Erlenmeyer flasks controls were also prepared (dye alone or fungus alone in culture medium). Every 2, 4, 6, 8, 13 and 15 days, Erlenmeyer flasks were removed to evaluate the activities of laccase and Mn peroxidase, as well as the rate of discoloration of the culture media containing RB4 (0.3 g/L). An aliquot (3 mL) of each culture media was withdrawn at the different time intervals. Each aliquot was centrifuged at 40000 rpm for 15 minutes using an Eppendorf 5702 centrifuge (Eppendorf France S.A.S, Le Pecq, France) to isolate the fungal mass. The supernatant phase was used to determine discoloration by measuring the absorbance at the maximum absorption wavelength (λ_{max}) of the respective dyes using a UV-Vis (Varian) Cary Scan spectrophotometer (Varian, Inc., Palo Alto, USA). All tests were conducted four times and the results presented are averages with the corresponding standard deviations. The percentage discoloration was calculated according to the formula:

$$\text{Discoloration (\%)} = \frac{(\text{initial absorbance} - \text{observed absorbance})}{\text{initial absorbance}} \times 100$$

Enzymatic activities

Ligninolytic enzymatic activities on liquid medium were measured in various supernatants containing RB4 or not. The method used was described by Atteke *et al.,* 2013. The activities of manganese peroxidase and laccase were measured using a UV-Vis (Varian) Cary Scan Spectrophotometer. Laccase activity was determined in a reaction mixture containing ABTS by measuring the optical density at 420 nm. Manganese peroxidase activity was determined to the method using phenol red at 610 nm in the presence of Mn^{2+}. All enzymatic reactions were carried out at 30ºC. All enzyme assays were conducted four times and the average rates were calculated to represent the enzyme activity. One unit of enzyme activity U was defined as the amount that catalysed the production of one micromole of substrate per minute per millilitre of reaction mixture.

Biodegradation analysis

After complete decolorization, one part of supernatant (20 mL) was centrifuged at 40000 rpm for 15 min with an Eppendorf 5702 centrifuge. An equal volume of ethyl acetate was used to extract metabolites from clear supernatant 4 times. Organic phases were grouped in different Eppendorf tubes for evaporation under vacuum using Univapo 150 H centrifuge evaporator (UniEquip GmbH, Martinsried/Munich, Germany). The same procedure was used for control culture media (fungus alone in medium). The HPLC analysis has been described by Mounguengui *et al*. (2007) using the Empower software. During UV–vis spectral analysis, changes in absorption spectra in discoloured medium (200–800 nm) were recorded in comparison with the results from the control runs. HPLC analysis was carried on Supelco Discovery C18 (250 mm × 4.6 mm i.d.) column

(Sigma-Aldrich Chimie sarl, Saint Quentin Fallavier, France) at 30°C with a mobile phase moving at the rate of 1.5 mL min^{-1}. The separation of compounds was performed using distilled water containing 0.05% TFA and methanol containing 0.05% TFA with a gradual program. 52% methanol was used for 5 minutes and increased to 70% for 10 minutes. It was then increased to 95% for 25 minutes and then decreased to 52% for 2 minutes.

Results and Discussion

Optimum growth temperature of *Perreniporia tephropora* on solid medium

The results of these trials are represented on table 1. Fungal colonies grew differently with temperature. For temperatures between 30°C and 40°C, the growth rates were between 12.08 mm day^{-1} and 12.92 mm day^{-1}. At temperatures lower or higher than this temperature range, the growth rates were lower than 10. In addition, between 30 and 40°C (figure 1), mycelium was compact and covered the entire Petri dish at six days. Our study shows that the optimum growth temperature was 30°C and at lower temperatures, growth slowed. This optimum temperature is similar to that found for *Ganoderma lucidum* (Jayasinghe *et al*., 2008) and for other fungi (Urek and Pazarlioglu, 2007; Duarte *et al*., 2012).

Optimal discoloration parameters

For the maximization of dye discoloration (RB4 and MO) by *Perreniporia tephropora*, different experiments were conducted to find the optimum conditions of pH, temperature and dye concentration in liquid medium. The effect of initial pH (3.0-7.0) was investigated and it was found that the highest percent discoloration rates occurred at pH 4.5 to 5.5 (Figure 1). The maximum discoloration was found at pH 5.5: 94.3 ± 0.7 % for RB4 and 90± 1.2 % for MO compared to neutral pH 7: 50± 0.2 % for RB4 and 41.5± 1.2 % for MO. pH has a considerable effect on synthetic dye discoloration and the optimal pH for discoloration is often between 4.5 to 11 for most of the dyes (Rohilla *et al*., 2012; Chen *et al*., 1999). Acidic pH is often observed during fungal growth and dye discoloration.

The study temperature ranged from 15-45°C. Maximum discolorations were observed at 30°C: 95.18 ± 0.65% for RB4 and 92.03±0.21 for MO compared to 22°C: 60.40 ± 0.44% for RB4 and 51± 0.37% MO and 35°C: 88.61 ± 0.22% for RB4 and 81± 0.7% for MO as presented in figure 2. The discoloration activity was significantly suppressed at 15°C and 45°C. This might be due to the loss of cell viability or deactivation of the enzymes responsible for discoloration. In addition, the discoloration of dyes may be through fungal reaction which relies on optimal temperature and pH (Rohilla *et al*., 2012; Attéke *et al*., 2013).

The biodegradation abilities of fungus can be enhanced by gradually exposing them to higher concentrations of synthetic dyes. Adaptation of fungi toward toxic or recalcitrant compounds is found to be very useful in improving the rate of discoloration process.

In the present study, the effect of varying concentration of dyes (0.05 to 1.5 g/L) on discoloration was also investigated. The discoloration efficiency of the fungus was found to be higher (> 80%) with

concentrations lower than 0.5 g/L under static conditions at 30°C (Figure 3). In addition, growth of the fungus was strongly inhibited at higher dye concentrations (1.5 g/L). This high concentration of dye may be toxic to metabolic activity. Similar findings have been observed (Atteke *et al.,* 2013; Gopinath *et al.,* 2009). Generally, difficulties of dye degradation by fungus may be due to higher molecular mass, structural complexity and the presence of inhibitory groups such as sulphides, chlorides and aromatics in the dyes (Hu *et al*., 2001).

Discoloration tests on various dyes

The discoloration different dyes (RB4 and MO) using *Perreniporia tephropora* fungus was conducted on liquid culture media. The dyes evaluated in this study contain different chemical groups that are degradable by white rot fungi. The degradation efficiency of the test fungi as determined based on measured of absorbance supernatants in liquid media are shown in figure 4.

These results show the discoloration capacity of *Perreniporia tephropora* fungus cultured in the media containing 0.3 g/L dye (MO or RB4). After 15 days, the discoloration rates were to 70.61± 3.82% and 85.75± 2.22% respectively to MO and RB4 (Figure 4). Some studies have shown that different fungi such as *Pycnoporus sanguineus* (Attéké *et al*., 2013), *Ganoderma* species (Levin et al., 2004) and *Hexagonia apiaria* (Mounguengui *et al*., 2013) discolour RB4, Orange G and Congo Red efficiently in solid and liquid media. The fungus *Perreniporia tephropora* is very poorly known in biodegradation and bioremediation, but has shown good results in this study (discoloration rate higher to 60% for 15 days with a dye concentration of 0.3 g/L) compared to other white-rot fungi described in the literature (Bibi and Bhatti, 2012; Rigas and Drista, 2006). It should be noted that after 4 days of incubation, the media containing MO and RB4 gradually become less coloured. The drastic colour change was observed in the eye, especially with RB4 between 6 and 8 days. These colours changes resulted in changes of UV-VIS absorption values during this period and these absorption values may have been due to a reaction of the dyes with enzymes secreted by the fungal mycelia (Attéké *et al*., 2013). There was no abiotic loss of RB4 and MO within 15 days of incubation, indicating that discoloration of dyes by *Perreniporia tephropora* was due to biological mechanisms rather than adsorption (data not shown). Therefore, this fungus could be economically interesting in future biodegradation applications. Enzyme activities (laccase and manganese peroxidase) were determined based respectively on an ABTS assay (Lac) and phenol red assay (MnP) (Figure 5) on liquid media containing 0.3 g/L RB4 or no (control).

No lignin peroxidase activity was observed in the media. Low laccase (Lac) and manganese peroxidase (MnP) activities were observed in the control media (without dye). These two activities are known to be involved in dye degradation by fungi (Moreira-Neto *et al*., 2013; Arantes *et al*., 2007). The largest amounts of laccase were secreted after 2 days: 13.63 U/mL and 6 days: 19.87 U/mL on media containing RB4. Under the same conditions, *Pycnoporus sanguineus* has produced below 6 U/mL of laccase activity and below 2 U/mL of MnP activity (Attéké *et al.,* 2013).

Table.1 Radial growth rates of fungus on solid culture medium at temperatures from 15°C to 45°C

temperature (ºC)	15	22	25	30	35	40	45
growth rate (mm day^{-1})	3.89	7.35	9.63	12.92	11.61	12.08	4.57

Figure.1 Discoloration of two synthetic dyes by *Perreniporia tephropora* at different pH on liquid culture medium during one week. Each test is replicated four times

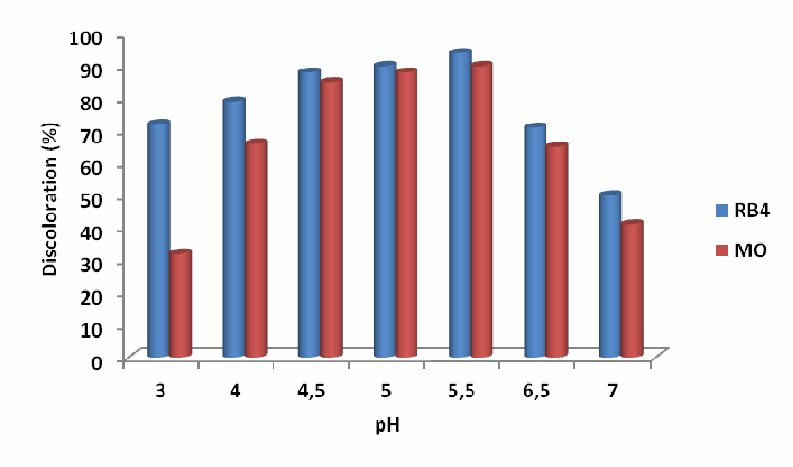


Figure.2 Discoloration of two synthetic dyes by *Perreniporia tephropora* at different temperatures on liquid culture medium during one week. Each test is replicated four times.

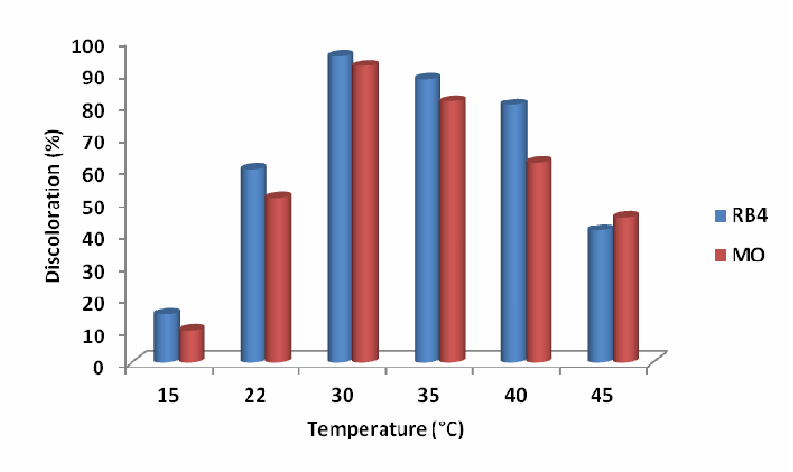


Figure.3 Discoloration of two synthetic dyes by *Perreniporia tephropora* at different concentrations on liquid culture medium during one week. Each test is replicated four times

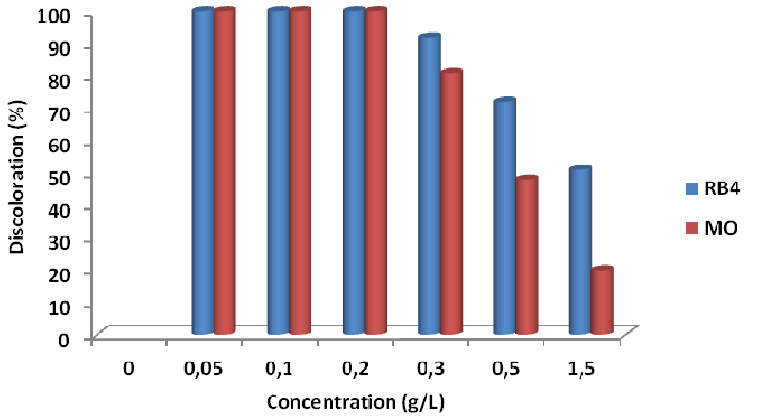


Figure.4 Discoloration of two synthetic dyes (MO 0.3 g/L and RB4 0.3 g/L) by *Perreniporia tephropora* in liquid culture medium during 15 days at 30°C. Each test is replicated four times.

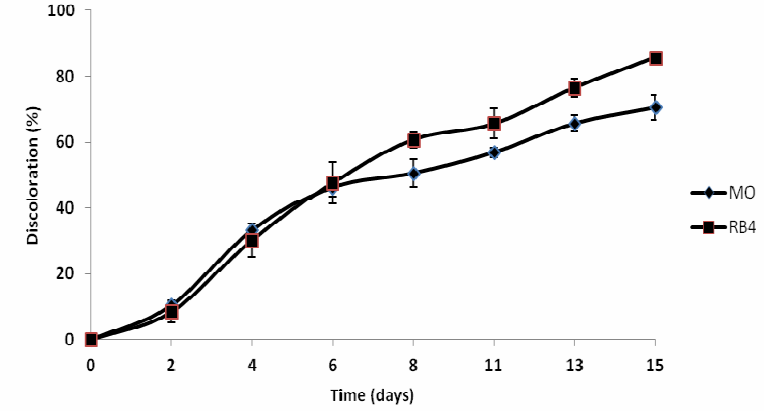
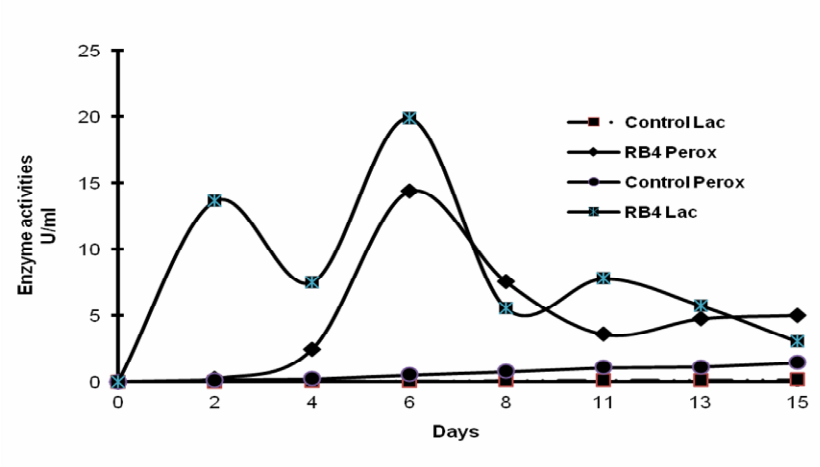


Figure.5 Enzyme activities (laccase (Lac) and manganese peroxidase (MnP)) during discoloration by *Perreniporia tephropora* on liquid culture medium containing RB4 0.3 g/L. Each test is replicated four times.



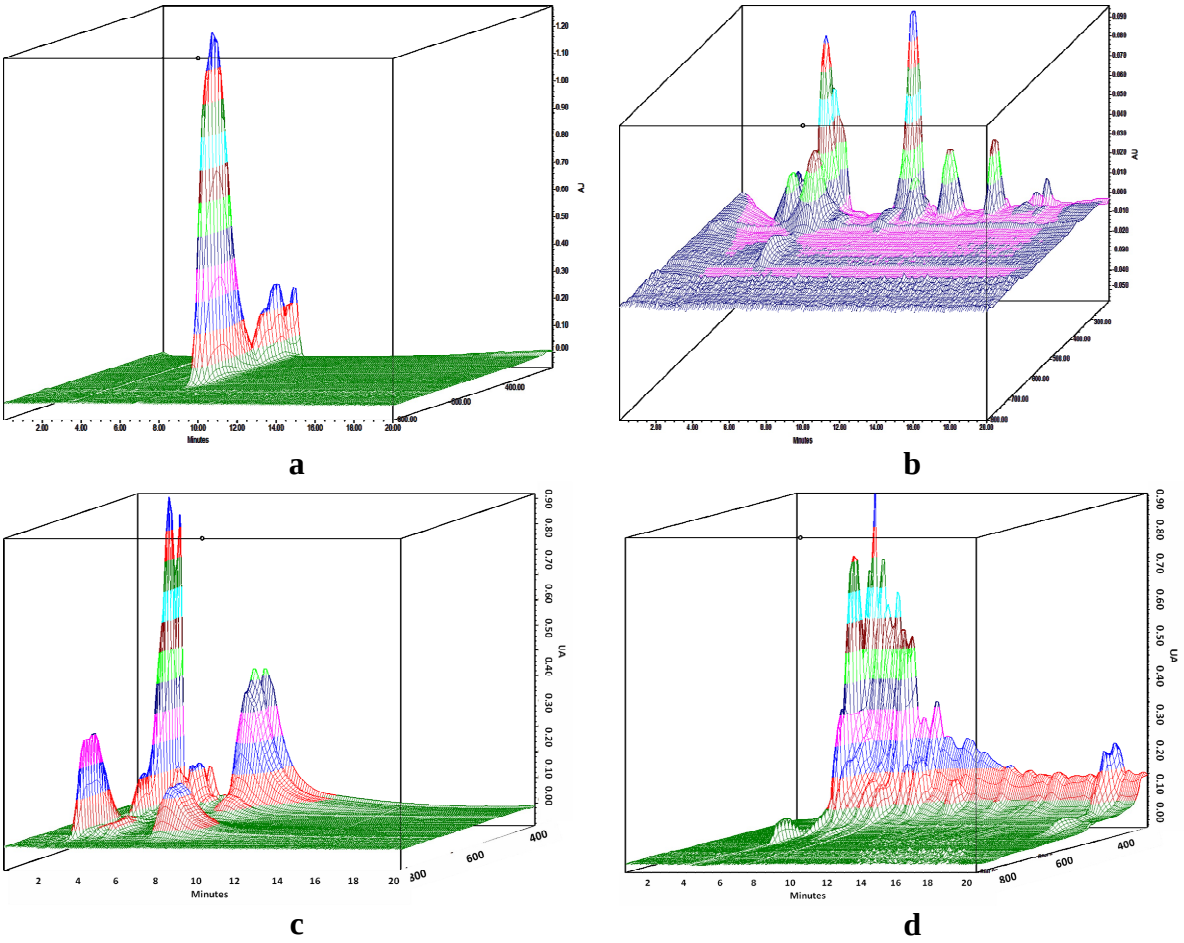


Figure.6 3-D chromatograms of MO control (a), degradation products of MO (b), RB4 control (c) and degradation products of RB4 (d)

The laccase activity is predominant during dyes degradation by different fungi compared to the MnP activity (Valderrama et al., 2003). In our study we have the same trend during this period (15 days). The secretion of Lac and MnP by *Perreniporia tephropora* was greatly enhanced when cultured in media containing RB4. Production of ligninolytic enzymes is affected by many factors, including the medium composition, carbon to nitrogen ratio, pH, temperature and aeration rate (Attéké *et al*., 2013; Mounguengui *et al*., 2013; Bibi and Bhatti, 2012; Rigas and Drista, 2006; Valderrama *et al*., 2003; Gopinath *et al.*, 2009). Moreover, many aromatic compounds with specific concentrations have been widely used to stimulate the production of ligninolytic enzymes (Arora and Gill, 2001). Therefore, many studies have been conducted in an attempt to improve the production of ligninolytic enzymes by white rot fungi (Arora and Gill, 2001; Kaluskar *et al*., 1999). These results suggest that adding of RB4 (0.3 g/L) to a medium can greatly enhance ligninolytic enzyme production and that fungi have the capacity to degrade this dye efficiently.

HPLC analysis showed the formation of new peaks (new compounds) (6c and 6b) with respect the controls (6a and 6b). The HPLC analysis of the control at the beginning of incubation (MO and RB4) showed a major peak at 5.12 min for MO control and at 1.8, 2.5 and 5.3 min retention time for RB4 (Fig. 6a and 6b) with absorbance areas between 280 to 590 nm for MO and 300 to 700 nm for RB4.

The biodegradation of the parent compounds after discoloration showed new peaks between 2 and 16 min retention times for RB4 degradation products and between 2 and 13 min for major peaks of MO degradation products (Fig. 6b and 6d). From HPLC analysis, it is clear that peaks of controls were transformed into different new peaks with altered retention times and absorbances (Olukanni *et al*., 2010; Harshad *et al*., 2012).

The results of this study suggest a great potential of *Perreniporia tephropora* to be used to discolour and degrade two synthetic dyes. The textile dyes (Reactive Blue 4 and Methyl Orange) are well degraded (above 60% for both) under different optimal conditions. Physiochemical parameters (pH 5.5, temperature 30°C, dye concentration 0.3 g/L) had a significant effect on dye discoloration. This fungus produces different enzymes such as Lac and MnP with the dyes in liquid media. For future studies, an identification of degradation products and toxicity of these compounds will be necessary.

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