Decolorization of CI Reactive Blue 222 by immobilized basidiomycetes in response to different carbon and nitrogen inputs

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ABSTRACT - (Decolorization of CI Reactive Blue 222 by immobilized basidiomycetes in response to different carbon and nitrogen inputs). Reactive dyes are found in the final effluents of the textile industry and cannot be removed by conventional treatment processes. The use of basidiomycetes appears to be an effective strategy to degrade dye molecules. In this paper, the parameters that favor decolorization of diazo dye were assessed using basidiomycetes immobilized in *Luffa cylindrica*. Different concentrations of saccharose and urea were assessed, in addition to the introduction of an enriched synthetic effluent. Results showed that the best decolorization occurred at the highest concentration of saccharose and the lowest of urea. It was observed a high biosorptive capacity of the solid support, which decreased when the effluent was enriched with saccharose and urea due to consequent increase in microbial activity. Using the enriched effluent, *Pleurotus ostreatus* decolorized about 70% within 48 hours, and *Trametes villosa* decolorized 58% after 240 hours. *Peniophora cinerea* did not respond to the conditions tested.
Keywords: azo dye, biodegradation, *Pleurotus ostreatus*, textile effluents

RESUMO - (Descoloração do corante reativo azul CI222 por basidiomicetos imobilizados em resposta às diferentes entradas de carbono e nitrogênio). Os corantes reativos são encontrados nos efluentes finais da indústria têxtil. Quando esses efluentes são tratados por processos convencionais, os corantes não são removidos. O tratamento que empregou basidiomicetos mostrou ser capaz de degradar a molécula de corante. Neste trabalho avaliou-se parâmetros que favoreceram a descoloração do corante diazo usando basidiomicetos imobilizados sob *Luffa cylindrica*. Foram avaliadas diferentes concentrações de sacarose e ureia, além da introdução de efluente sintético enriquecido. Os resultados mostraram que a melhor descoloração ocorreu nas maiores concentrações de sacarose e nas menores concentrações de ureia. Observou-se a capacidade de adsorção do suporte sólido, que diminuiu quando o efluente foi enriquecido com sacarose e ureia, devido ao consequente aumento da ação microbiana. *Pleurotus ostreatus* com efluente enriquecido descoloriu cerca de 70%, dentro de 48 horas e *Trametes villosa* descoloriu em torno de 60% após 240 horas. *Peniophora cinerea* não respondeu às condições testadas.
Palavras-chave: biodegradação, corante azo, efluentes têxteis, *Pleurotus ostreatus*

Introduction

There is an extensive variety of synthetic textile dyes that are grouped together according to their similarities in chemical composition and applications, and about ⅔ of them have chromophores of azo groups. The textile reactive ones have significant applications in the market worldwide, due to their broad range of color shades, easy use and low energy consumption in the process. These compounds enter in the textile process through the dying bath, and their efficiency in fiber fixing vary from 60% to 90%. However, the discharge of colorful effluents to the environment is undesirable, not only due to the presence of color itself, but also for potential carcinogenic and/or mutagenic effects, and formation of toxic products. Additionally, azo groups can break down under reductive conditions to release toxic and hydro-soluble amines which, have increased mobility in the systems and makes, this type of effluent problematic (Aguiar *et al.* 2010, Zaharia & Suteu 2012, Kyzas *et al.* 2013).

The inefficiency of physical and chemical processes has led to the association of these treatments with biological ones, which seem to be promising

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strategies for the removal of residual dyes from wastewaters (Senthilkumar *et al.* 2012, Kiran *et al.* 2013, Álvarez *et al.* 2013, Almeida & Corso 2014). Fungi, especially basidiomycetes, have various advantages for their application in this kind of treatment, as well as in biodegradation, due to the action of enzymes of the ligninolytic complex, which allow them to catalyze the degradation of different compounds, including dyes (Bibi and Bhatti 2012, Kalpana *et al.* 2012, Malachova *et al.* 2013, Moreira-Neto *et al.* 2013). Neto (2011), assessed the decolorization of the CI Reactive Blue 19 by basidiomycetes, and found that *Peniophora cinerea*, *Pleurotus ostreatus* and *Trametes villosa*, were are able the dye under conditions of high salinity and high pH. These authors observed the most significant decolorization in the *in vivo* treatment, with a predominance of laccase production (Neto *et al.* 2011, Moreira-Neto *et al.* 2013).

In addition, some studies have reported the presence of carbon and nitrogen sources acting as co-substrates, favoring decolorization and stimulating enzyme production (Levin *et al.* 2010, Andrade *et al.* 2013, Gahlout *et al.* 2013). Hou *et al.* (2004), reported laccase production associated with low nitrogen concentration in culture. Viswanath (2014) considered that the best carbon source for fungi to grow and produce laccase is glucose, whose concentration varies depending on the fungal species used. Mazmanci & Unyayar (2005) observed the predominance of laccase production during CI Reactive Black 5 decolorization by *Furnalia trogii* immobilized in *L. cylindrica*, and concluded that high decolorization rates were associated with enzyme production. Pedrosa-Rodriguez and Rodriguez-Vasquez (2013) studied the treatment of Kraft liquor by *T. versicolor* and observed high decolorization associated with enzyme production, including laccase, when the C/N ratio was high.

Another important factor to consider in biotechnological processes is the use of a support medium, such as *L. cylindrica*, which proved to be efficient for immobilizing biomass of filamentous fungi. This efficiency is due to presence of cellulose, hemicellulose and lignin in its fibers, all compounds that inducers the production of lignocellulolytic enzymes, (Mazmanci and Unyayar 2005, Saratale *et al.* 2011, Saab *et al.* 2013). Mazmanci and Unyayar (2005) observed that *F. trogi*-immobilized with *L. cylindrica* showed an increase in the tolerance to high dye concentrations. In addition, a positive decolorization, and a decrease in organic load of effluents from the paper industry were observed using *T. versicolor* immobilized with polyurethane, in bioreactors (Pedroza-Rodríguez & Rodríguez-Vázquez 2013).

In this context, the current study aimed at assessing the influence of introducing low-cost nutrient concentrations at different times, during the decolorization of Cl Reactive Blue 222 by basidiomycetes immobilized with *L. cylindrica*, as well as optimization the fungal metabolism to override the adsorptive capacity of this support.

Material and methods

Microorganisms -The species *Trametes villosa* (Sw.) Kreisel CCIBt 2628, *Peniophora cinerea* (Pers.) Cooke CCIBt 2541 and *Pleurotus ostreatus* (Jacq.) P. Kumm. CCIBt 2347, obtained from the Collection of Algae, Cyanobacteria and Fungi Cultures of the Institute of Botany (CCIBt) were used in this study. Fungi cultures were maintained in 2% Malt Extract Agar (MEA), at 25 ± 2 ºC.

Dye - The dye CI Reactive Blue 222, (Golden Technology), was measured in an aqueous solution, and prepared at room temperature with deionized water.

Stage 1: Effect of different saccharose and urea concentrations as nutrient sources.

Culture medium; A basic medium (BM) composed of: 0.049 g L^{-1} $CuSO_4$; 0.2 g L^{-1} K_2HPO_4; 0.05 g $L^{-1}MgSO_4$ and 0.016 g L^{-1} $MnSO_4$, was dissolved in distilled water and, different concentrations of saccharose and urea were added as carbon and nitrogen source respectively (table 1).

Culture conditions: fragments of 2 cm^2 of *Luffa cylindrica*, (3 ± 0.25 g) were placed into 250 mL Erlenmeyer flasks, containing, 150 mL water, and sterilized, in the autoclave, 1 atm of pressure, for 40 minutes. Thereafter, all the water was drained, and 150 mL BM was added to the system which was sterilized for 15 minutes. Fragments of *L. cylindrica* remained submerged for 24 hours in the BM, and then the medium was drained completely. Three disks of mycelium grown onto MEA (Ø = 0.5 cm) were inserted into the treated *L. cylindrica* supports. Each cultivation systems was incubated separately for 10 days at 25 ± 0.2 ºC.

Synthetic effluent: the NaCl saline solution (30 g L^{-1}) was sterilized in the autoclave at 121 ºC, 1 atm of pressure, for 20 minutes. After cooled down to 40 ºC, the dye solubilized in 0.5 g L^{-1}, was added, without adjusting the pH.

Treatment system: at day 10 of incubation, 150 mL of the synthetic effluent was added to the cultures. Monitoring was done by taking aliquots of the liquid phase at the initial time and after 24 hours. Flasks without fungi were used as abiotic control (CABio) of the support's biosorption capacity, and flasks with fungi were used to evaluate both the biosorption of the support and the ability of fungal biodegradation (decolorization).

Decolorization: the decolorization ability was assessed spectrophotometrically by the decrease in absorbance at 610 nm, accordingly to Moreira Neto *et al.* (2013).

Data analysis: analyses were made according to a 5 × 5 factorial design, without replicates. Experimental results were assessed with regression analysis and significant differences evaluated with analysis of variance (ANOVA) by using F-distribution (MINITAB, 2016). All percentage data were transformed by using the equation described by Vieira & Hoffman (1989).

Stage 2: optimization of the decolorization treatment system through selected nutritional conditions and the enriched effluent

Preparation of cultures and of the synthetic effluent was carried out as described previously. Low urea concentrations (0% = N1a, 0.0056% = N1b; 0.0112% = N1c, 0.0211% = N1d), were added to the BM medium with a fixing saccharose concentration at 1.2% (C5). Concomitantly, the influence of the enriched synthetic effluent was assessed by adding nutrients (1.2% saccharose and 0.0703% urea - C5N5 - table 1) to the dye solution.

Optimized treatment system: at day 10 of incubation, 150 mL of the enriched synthetic effluent was added to each culture. Monitoring of the complete treatment systems (STc) and of the fungal action (F) isolated was performed by taking aliquots of the liquid phase at the initial time, after 24, 48 and 240 hours after the addition.

Decolorization: the decolorization was assessed as previously described.

Data analysis: analysis of data was performed through ANOVA and, when significant differences ($p \leq 0.05$) were observed, the means were compared by Tukey´s test, using the program MINITAB v. 2016. All data expressed as percentage were transformed according to Vieira & Hoffman (1989).

Results

The results showed that the treatment systems presented from 50% to 63% decolorization depending on the nutritional conditions (table 2), and responded differently to the addition of urea and saccharose depending on the fungi specie. For systems employing *P. ostreatus* ($p = 0.051$) e *P. cinerea* ($p = 0.085$), the best decolorization rates occurred in the absence, or yet in the low nitrogen concentrations, (*i.e.* N1 and N2), but in the case of *T. villosa* ($p = 0.036$) occurred at the biggest carbon concentration, (C5).

Regarding STc (complete treatment systems), which considered the biosorption onto the support medium, and onto the fungal biomass, as well as, the associated biodegradation, *P. ostreatus* showed higher decolorization (about 20%) than the other fungi which decolorized less than 10%. When the fungal action (F), was evaluated separately in relation to nutrients, results were the same as those obtained for the other systems, except in the case of *P. cinerea*, which did not reply to any of them.

The conditions selected in the present study allowed the system overcoming the high biosorption of the support medium, and provided the appropriate conditions for fungal grow, favouring the expression

Table 1. Combination of nutritional concentrations of carbon and nitrogen sources.

Nitrogen concentration (%)	Carbon concentration (%)				
	0,0	0,3	0,6	0,9	1,2
0.0	C1N1	C2N1	C3N1	C4N1	C5N1
0.01125	C1N2	C2N2	C3N2	C4N2	C5N2
0.03094	C1N3	C2N3	C3N3	C4N3	C5N3
0.05063	C1N4	C2N4	C3N4	C4N4	C5N4
0.07031	C1N5	C2N5	C3N5	C4N5	C5N5

of ligninolytic enzymes, which consequently, optimized the decolorization. However, up to 80% of the observed decolorization could be ttributed to the biosorptive capacity of *L. cylindrica.* In this sense, the results indicated that better optimization of the systems was still needed. Other formulations of the culture medium were also evaluated, using low concentrations of urea, according to the requirements of *P. ostreatus* and *P. cinerea*, and maintaining a high saccharose concentration, in order to meet the requirements of *T. villosa*. The systems evaluated at the second stage presented greater decolorization after 24 hours (70 to 78%), than at stage 1. Even when the incubation period was extended up to 240 hours, decolorization only increased in 20% for *P. ostreatus*, 11% for *P. cinerea* and 8% for *T. villosa,* thus demonstrating that during the first 24 hours occurred the highest decolorization.

The species evaluated responded differently to nitrogen concentrations. In the case of *T. villosa* a greater response was observed at the lowest concentrations tested, (N1bC5) and *P. ostreatus* responded positively ($p < 0.05$) to the highest nitrogen concentration evaluated at stage 2 (N1dC5), while *P. cinerea* did not respond to the conditions tested. However, when the fungal system was compared with the abiotic control, the fungal system provided less decolorization than at stage 1 (2% for *P. ostreatus* and *P cinerea*, and 7% for *T. villosa*). These results showed that the new nutrient formulation favoured the biosorption onto the solid support and the fungal biomass.

At stage 1, the high concentrations of both saccharose and urea, favoured the increase in fungal biomass. Thus maximum concentrations of both nutrients used in stage 1 (N5C5) were added to the

Table 2. Decolorization of CI Reactive Blue 222 through treatment systems *in vivo* employing *Pleurotus ostreatus*, *Peniophora cinerea* and *Trametes villosa*, individually immobilized in *Luffa cylindrica.*

Treatment	Decolorization on the systems of in vivo treatment [%]					
	Pleurotus ostreatus		Peniophora cinerea		Trametes villosa	
	STc	F	STc	F	STc	F
C1N1	47,98	18,03	58,13	4,16	44,56	0
C1N2	38,54	8,6	44,8	0	50,35	5,1
C1N3	46,17	16,23	41,28	0	44,62	0
C1N4	27,39	0	55,24	1,26	45,16	0
C1N5	45,69	15,74	41,96	0	48,84	3,59
C2N1	28,2	0	47,25	0	49,45	4,19
C2N2	43,47	13,53	46,51	0	0	4,75
C2N3	48,9	18,95	13,67	0	46,44	1,19
C2N4	8,06	0	44,85	0	44,55	0
C2N5	34,77	4,82	52,91	0	42,88	0
C3N1	28,51	0	51,21	0	45,45	0,2
C3N2	46,8	16,86	49,62	0	50,14	4,89
C3N3	41,18	11,24	55,81	1,84	50,27	5,01
C3N4	43,5	13,56	48	0	45,57	0,32
C3N5	33,73	3,78	39,75	0	46,96	1,71
C4N1	45,87	15,92	63,67	9,7	49,79	4,54
C4N2	54,49	24,55	50,4	0	53,14	7,89
C4N3	41,18	7,57	38,55	0	42,72	0
C4N4	43	13,06	44,25	0	48,18	2,92
C4N5	41,66	11,71	25,74	0	46,28	1,03
C5N1	17,77	0	59,56	5,59	54	8,74
C5N2	47,99	18,04	55,56	1,58	50,74	5,48
C5N3	37,51	22,46	49,36	0	50,99	5,74
C5N4	17,34	0	54,16	0,19	47,41	2,16
C5N5	14,64	0	52,38	0	54,18	8,92

synthetic effluent in an attempt to favour fungal metabolism. All evaluated treatments showed a reduction in the dye adsorption by the support medium, regardless of the nitrogen concentration in the culture. The systems with *P. ostreatus* and *T. villosa* responded positively ($p < 0.05$) to the decolorization, according to the formulation of the synthetic effluent and the contact time (figure 1). *P. ostreatus* (figure 1a)

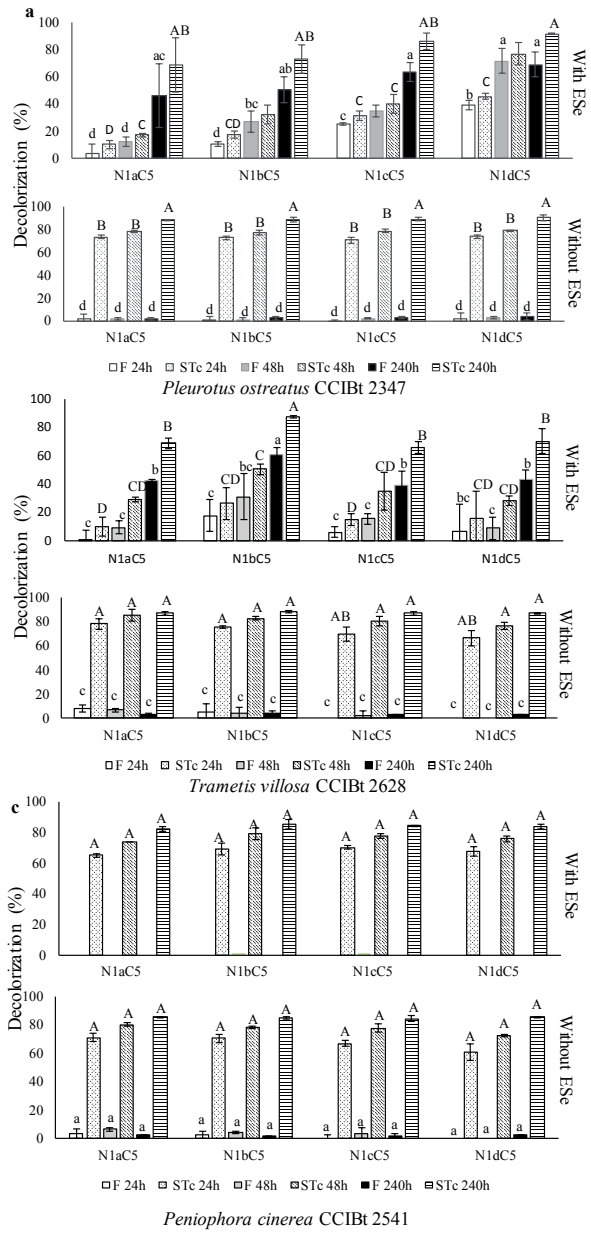

Figure 1. Comparison ofthe effect of nutrients in the CI Reactive Blue 222 *in vivo* decolorization employing *Pleurotus ostreatus* CCIBt 2347 (a), *Trametes villosa* CCIBt 2628 (b) and *Peniophora cinerea* CCIBt 2541 (c), with and without nutritional additive in (ESe), up to 240 hours of incubation, in the systems of complete treatment (STc) and treatment without the abiotic effect of the control (F).

and *T. villosa* (figure 1b) became more efficient under this new culture conditions due to an increased availability of free dye in the liquid phase, and also to the nutrients added to the effluent that might have favoured the ligninolytic metabolism.

P. ostreatus showed the highest decolorization rate (76.39%), in 48 hours when the abiotic control was discounted (71.30%), or when used in the non-enriched effluent, after 240 hours (89.76%), although these differences were not significant ($p \geq 0,05$). This fact indicates that the degradative metabolism of this fungus was able to match the adsorptive capacity of the immobilization support (figure 1a), and showed a positive effect on the decolorization with the increase in nitrogen concentration (figure 1a, $p = 0.00$). In contrast, *T. villosa* presented greater decolorization, (61.12%), at the lowest nitrogen concentration (N1bC5), associated with a longer contact time. Decolorization by *T. villosa*-STc was 87.58 %, similar to those registered when non-enriched effluent was used (88.05%). *P. cinerea* showed 85% of decolorization, in the presence of the non-enriched effluent, but in a longer contact time (240 hours). This fungus either exhibited the best decolorization responses after 48 hours, in the absence of nitrogen (6.17%) or at the lowest concentration evaluated (4.16%). The decolorization observed in the treatment with the enriched effluent was equal to the decolorization in the abiotic control. Therefore, the introduction of nitrogen into the culture, or into the effluent, did not stimulated the development of the degradation metabolism of this fungus. In this case, observed that the main action for decolorization was biosorption, presenting from 70 to 85% of decolorization, in the period considered while the highest percentage of decolorization occurred in the first 24 hours, and approximately 10% in a longer contact time (figure 1c).

All the treatments showed a decrease in absorbance at 610 nm. However, the absorption spectra of the liquid phase that contained the enriched effluent and used *P. ostreatus* and *T. villosa*, exhibited a hypsochromic shift of the maximum absorption wavelength from 610 nm to 526 nm (figure 2), which indicated transformation of the dye molecule. In the case of *P. cinerea* systems, it was observed the disappearance of all absorption peaks of the absorption spectrum, which might be related to a possible biosorptive action.

Discussions

Previous studies have also shown that each fungal strain responds differently to the nutritional

concentrations, Akdogan *et al.* (2014), evaluated the anthraquinone dye CI Reactive Blue 19 (0.05 g L^{-1}) treated by *Coprinus plicatilis*, and observed 80% decolorization in cultures that had received 5 g L^{-1} of urea, after 5 days of incubation. Koyani *et al.* (2013) also observed a high percentage of decolorization of the dye CI Reactive Yellow 145 (0.01 g L^{-1}), treated by *Phanerochaete chrysosporium* in the presence of 0.01 g L^{-1} of urea. On the other hand *P. chrysosporium* exhibited high decolorization (97%) of diazo dye (0.05 g L^{-1}) in the presence of the saccharose and absence of urea Andrade *et al.* (2013).

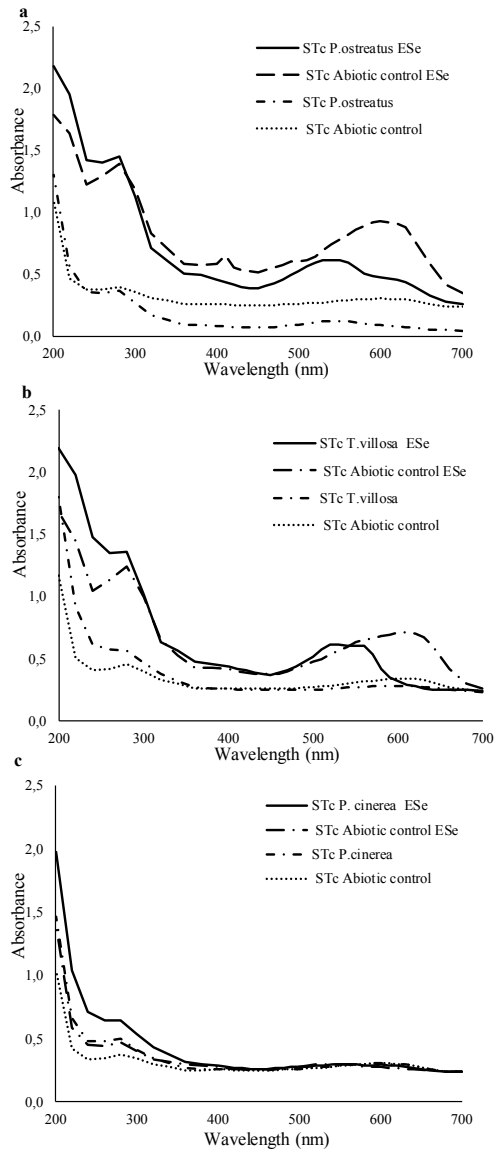

Figure 2: Spectrum of absorption of effluents containing CI Reactive Blue 222, in treatment systems of improved nutritional condition, employing Pleurotus ostreatus CCIBt2347 (a), Trametes villosa CCIBt2628 (b) and Peniophora cinerea CCIBt2541, after 240 hours of incubation, comparing to complete treatment systems (STc) and treatment systems without the abiotic effect of the control (F).

The results these studies indicated that 80% decolorization was associated with the high biosorptive capacity of *L. cylindrica*, as previously documented (Altınışık *et al.* 2010, Cherifi *et al.* 2013, Saab *et al.* 2013, Yu *et al.* 2013). However, Enayatizamir *et al.* (2011) also suggested a positive influence of the ligninolytic support which defines the type and proportion of the enzymes produced, favoring the expression of the enzymes that are necessary for the decolorization process.

By incorporating saccharose and urea to the synthetic effluent, the activity of basidiomycetes became more efficient, leading to the transformation of the dye molecule. These nutrients ensure favorable nutritional conditions for ligninolytic metabolism, in addition to decreasing the adsorptive capacity of *L. cylindrica*. Consequently, large quantities of free dye become available in the liquid phase to the *in vivo* systems. Saccharose promoted growth and activated fungal metabolism, by creating high acidity conditions in the culture medium, which favoured dye degradation (Garg & Tripathi 2017). According to Asgher (2013), the enriched effluent, is able to modify the chemical and physical properties of the biossorbent due to the activation or deactivation of the support's surface functional groups, and thus the biosorption is decreased.

A hypsochromic shift of the maximum absorption wavelength (λ_{max}) from 610 nm to 526 nm, was observed in the absorption specters of the liquid phase of the systems using *T. villosa* and *P. ostreatus* after receiving the enriched effluent. Similarly, Mazmanci & Unyayar (2005) studied the methylene blue treatment employing *Trametes versicolor*, and observed a hypsochromic displacement from 597 nm to 562 and 523 nm. According to Kaushik & Malik (2009) displacement of λ_{max} may arise from the cleavage of the dye chromophore center and, depending on the cleavage, the removal of λ_{max} can be either partial or complete. On the other hand, according to Zaharia and Suteu (2012), color of synthetic dyes is due to the presence of chromogene-chromophores structures, which are the electron acceptors, and coloring capacity of dyes is due to the auxochrome groups, the donors of electrons, and, as such, able to be ionized. Thus, considering such reactive molecules, the dyes oxidation caused by the ligninolytic enzymes of the fungus may have changed the conformation of the dye molecule resulting in a change of color in the more visible region.

According to Ciullini *et al.* (2008) laccases are specific to the dye substrate and to certain chemical structures, mainly due to differences in the distribution of electrons, load density and spatial structure of the molecule, which in turn influences decolorization rates. These authors evidenced the fungal capacity of degrading antraquinones by using a simple mix of two laccase isoenzymes in the absence of a redox mediator, under optimum enzyme concentrations, and conditions of temperature and pH. Moreira Neto *et al.* (2013), studied the decolorization of the anthraquinone dyes Cibacron Brilliant Blue H-GR by same fungal species and observed the presence of laccase activity in all cultures. In the case of CI Reactive Blue 222, the diazo dye used in the present study, Zollinger (2003).observed partial degradation, which might indicate that oxidation occurred in the weaker position of the connections. Ballaminut (2017) also evaluated decolorization of this dye employing the same fungi in similar culture and observed laccase production, suggesting a positive correlation between the presence of this enzyme and the discoloration registered during the process. Laccases are able to modify the azo structures, destruct their chromophore groups, and simultaneously generate phenoxyl radicals. Firstly, an electron is abstracted from the ring generating a phenoxyl radical, and the abstraction of a second electron generates an aromatic cation, which can be stabilized by the donors groups that are present in the ring. Laccases are not able oxidize the analogous compounds replaced in the molecule, at the meta position, in isolation, once the strength of the enzyme activation at that position is low. The removal of electrons replaced by halogen groups, or by one nitro group, in the aromatic rings, prevents the formation of cationic radical and, consequently, can inhibit or hinder degradation of this kind of dye (Ciullini *et al.* 2008).

These results demonstrated the ability of the fungi analyzed to transform the dye molecule when conditions are favorable for fungal metabolism, in this case, through the addition of nutrients to the synthetic effluent that minimized both the physiological stress of fungi and dye biosorption to the support. The efficiency of the proposed treatment can be still optimized by adding mediators to the synthetic effluent, in order to extend the catalytic action of the enzyme and act in different patterns of replacements (Amansa *et al.* 2004).

The results of this study also evidenced that, under particular conditions, the fungal activity can be equal or even superseed the biosorption activity of *L. cylindrica*. Therefore, the study presented a promising biotechnological proposal for the treatment of textile effluents in which a biological system is combined with biosorption and biodegradation, with minimum formation of residues once the solid substrate is biodegradable.

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