



Degradation of the Textile Dye Reactive Black 5 by Basidiomycetes

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ABSTRACT

Reactive Black 5 (RB5) is one of the synthetic reactive dyes most used in the textile industry, due to its solubility in water and reactive groups which form covalent bonds within the fiber. In the process of dyeing fabrics, however, it is estimated that 12-14% of dyes are released into the effluent. This work evaluated the biodegradation of RB5 dye, adsorbed in polyurethane foam, by basidiomycetes (*Phanerochaete chrysosporium* ATCC 24725, *Pleurotus ostreatus* and *Pleurotus floridae*). Results were evaluated considering the partial- or total medium discoloration, the adsorption capacity of the dye in the polyurethane foam (PUF) and the respirometric measurements. The results showed that *Phanerochaete chrysosporium* was able to partially degrade 50 mg L⁻¹ of RB5 in pH 6.0, when cultivated in Petri dishes. When this microorganism was cultivated in PUF cubes saturated with RB5 solution (50 mg L⁻¹, pH 6.0), CO₂ production reached an accumulated value of 2.16 mg on the fifteenth day, revealing the growth of the microorganism and consequently the contaminant degradation, which was used as the source of nutrients.

Keywords: biodegradation, *phanerochaete chrysosporium*, respirometric method.

Degradação do corante têxtil Preto Reativo 5 por basidiomicetos

RESUMO

O corante Preto Reativo 5 (RB5 - *reactive black 5*) é um dos corantes reativos sintéticos mais utilizados na indústria têxtil, devido à sua solubilidade em água e a grupos reativos que formam ligações covalentes com a fibra. No processo de tingimento de tecidos, no entanto, estima-se que 12-14% dos corantes sejam liberados para o efluente. O objetivo deste trabalho foi avaliar a biodegradação do corante RB5, adsorvido em espuma de poliuretano, por basidiomicetos (*Phanerochaete chrysosporium* ATCC 24725, *Pleurotus ostreatus* e *Pleurotus florida*). Os resultados foram avaliados considerando a descoloração parcial ou total do meio, a capacidade de adsorção do corante na espuma de poliuretano e a medida respirométrica. Os resultados mostraram que *Phanerochaete chrysosporium* foi o mais eficiente, sendo capaz de



degradar parcialmente RB5 na concentração de 50 mg L⁻¹ em pH 6.0, quando cultivado em pacas de Petri. Quando este microrganismo foi cultivado em cubos de espuma de poliuretano saturada com solução de RB5 (50 mg L⁻¹, pH 6.0) a produção de CO₂ atingiu o valor acumulado de 2.16 mg no décimo quinto dia, revelando o crescimento microbiano e a degradação do contaminante, que foi utilizado como fonte de nutrientes.

Palavras-chave: biodegradação, método respirométrico, *Phanerochaete chrysosporium*.

1. INTRODUCTION

The textile industry is one of the most important in the world. The global textile and clothing business in 2017 is estimated to be worth about US \$4.395 trillion. The current global apparel market is estimated at approximately US\$ 1.15 trillion, which makes up nearly 1.8% of the world Gross Domestic Product (GDP) (Textile Mates, 2018). In 2017, China was the top-ranked global textile exporter (37.2 percent of the global market share), followed by the European Union (with 23 percent of the global market share), and India (Statista, 2018).

The technology *Dye Clean* became a reference in the textile chemistry world market, consisting of dyeing cellulosic fibers with reactive dyes, such as Reactive Black 5 (RB5 - CAS Registry Number: 12225-25-1/17095-24-8). Like all reactive dyes, the RB5 is highly soluble in water and difficult to degrade due to its polyaromatic (Fan *et al.*, 2009). Another troubling fact is the production of aromatic amines due to the degradation of this type of dye with azo bonds, which are highly carcinogenic (Vilar *et al.*, 2011), while also being considered highly recalcitrant, toxic and mutagenic substances for various aquatic species (Vedrenne *et al.*, 2012).

Regarding the process of dyeing fabrics, there is an estimate that 12-14% of textile dyes are released into the effluent (Safa *et al.*, 2011). These compounds cause visual pollution, changes in the micro-aquatic biota and photosynthetic processes, and bioaccumulation of toxic substances in aquatic organisms (Kunz *et al.*, 2002). In addition, most of them are classified as toxic, mutagenic and carcinogenic for aquatic organisms and humans (Ahmad *et al.*, 2011; Wang *et al.*, 2014), consisting of a serious ecological problem. The reactive dyes are highly recalcitrant to conventional effluent treatment processes, which, although removing about 80% of the dyes, leave most adsorbed in the sludge (Kunz *et al.*, 2002).

Microbial and enzyme immobilization have been extensively studied in the treatment of dye-based industrial effluents (Bilal *et al.*, 2017), being known as a solid state (SS) cultivation/fermentation process. Polyurethane foam (PUF) is one of the substrates used for fungal SSF, because it increases enzyme production and substrate oxygenation, without causing shear stress to the biomass (Sharari *et al.*, 2013).

White rot fungi (for example, *Phanerochaete chrysosporium*, *Pleurotus* sp, *Trametes* sp, *Ganoderma* sp) are among the most efficient microorganisms in the treatment of xenobiotic molecules, such as synthetic reactive dyes (Wesenberg *et al.*, 2003). Their metabolic capacity to mineralize complex polymers, like lignin, is due to the secretion of non-specific enzymes, which includes Laccases (EC 1.10.3.2), manganese peroxidases (EC 1.11.1.13) and lignin peroxidases (EC 1.11.1.14).

Biodegradation can be assessed by microbial respiration. Respirometric methodologies are capable of evaluating the degradation process of organic waste based on the quantification of CO₂ production or O₂ consumption during metabolic activities. One of the most traditional methods used to assess biodegradation of organic wastes is the Bartha's respirometric assay (Bartha and Pramer, 1965). This is a titrimetric method which measures the CO₂ trapped in a strong alkali after carbon mineralization by the microbial community present in the system (Bartha and Pramer, 1965; ABNT, 1999; Régo *et al.*, 2014). Its use includes the assessment of the complete biodegradation of petroleum wastes, general compounds and organic fertilizers in

soil such as sewage sludge (ABNT, 1999; Régo *et al.*, 2014).

The aim of this study was to use polyurethane foam as matrix support to assess the biodegradation of the RB5 by fungi basidiomycetes *P. ostreatus*, *P. floridae* and *P. chrysosporium* ATCC 24725 in Bartha respirometers.

2. MATERIAL AND METHOD

2.1. Analytical curve

The RB5 dye stock solution was prepared by dissolving dye in Milli-Q water to the concentration of 100 mg L⁻¹. For analytical curve, standard solutions ranging from 1.00 to 30.0 mg L⁻¹ of the dye were used, in parallel with a blank solution of water. The linear analytical calibration of the curve was furnished by a spectrophotometer UV-VIS (Varian Cary 50), in the scan range from 190 to 800 nm. The wavelength of maximum absorption of the dye (598 nm) was used to monitor the concentration of the RB5 during the experiments. The calibration curve of the RB5 described with the equation $y = 0.00262 + 0.02263x$ and the correlation coefficient was 0.99983, indicating a good linearity.

2.2. Preliminary studies

To establish the better condition for RB5 adsorption in polyurethane foam, a full factorial design 2² with central point, as shown in Table 1, was used to check the effect of experimental variables (pH and dye concentration) in the adsorption of the RB5 in PUF. It was therefore determined that the dye concentrations to be used in the experiment were (mg L⁻¹): 12.5, 25 and 50.

Table 1. Full factorial design 2² with central point, used to evaluate the effect of pH and the concentration of Reactive Black 5 dye in its adsorption in polyurethane foam.

VARIABLE	Level (-1)	Level (0)	Level (+1)
pH	6	7	8
Dye concentration (mg L ⁻¹)	12.5	25	50
Experiment	Dye concentration		pH
1	-	-	-
2	+	-	-
3	-	+	+
4	+	+	+
5	0	0	0
6	0	0	0
7	0	0	0

The pH was adjusted using NaOH 0.1 and 0.01 mol L⁻¹, HCl 0.1 and 0.01 mol L⁻¹ solutions. The polyurethane foam was ceded by the Tecnotrater Group (Federal University of Paraná State - Brazil), having the following composition: polyethylene glycerol, 4.4- diphenylmethane diisocyanate, glycerol, water and Montmorillonite 3%.

To achieve the maximum saturation of the dye in the matrix, adsorption studies were carried out introducing 100 mg of PUF cubes (1 cm x 1 cm x 1 cm) into 50 mL of RB5 dye solution, being shaken (120 rpm) at room temperature (25 °C), with 3 mL samples being collected at 15 and 30 minutes. However, as the experiment progressed, magnetic agitators were also used. Samples of the supernatant (3 mL) were collected at 10-minute intervals for 2 hours, in order to verify the saturation of the adsorbent.

The amount of dye taken up and the percentage of the dye removed by the PUF were calculated by applying Equations 1 and 2, respectively.

$$q = \frac{C_o - C_f}{m} \cdot V \quad (1)$$

$$\% \text{ Removal} = 100 \cdot \frac{C_o - C_f}{C_o} \quad (2)$$

In which “q” is the amount of dye adsorbed by the adsorbent (mg g^{-1}), “Co” is the initial dye concentration placed in contact with the adsorbent (mg L^{-1}), “Cf” is the dye concentration (mg L^{-1}) after the batch adsorption procedure, “m” is the mass of adsorbent (g) and “V” is the volume of dye solution (L).

2.3. Microorganisms

P. ostreatus, *P. floridae* and *P. chrysosporium* ATCC 24725 were cultivated on sterile Potato Dextrose Agar (PDA), in Petri dishes, at $26^\circ\text{C} \pm 2^\circ\text{C}$ for seven days, being kept at $+4^\circ\text{C}$ for up to 30 days.

Inoculum was prepared by transferring an aliquot of the mycelium to a 250 mL Erlenmeyer flask containing 50 mL of sterile PDA, and incubating it for seven days at $26^\circ\text{C} \pm 2^\circ\text{C}$. The cell suspension was collected at the end of cultivation by adding 50 mL of sterile peptone water solution (1 g L^{-1}), 0.2% of Tween 80, 3 g of glass beads (3 mm diameter) and a magnetic bar. The suspension was subjected to agitation for 15 minutes, with sufficient rotation to promote cell dispersion. Cells were quantified using a Neubauer chamber and also by counting using Spread Plate technique, on PDA (Spier *et al.*, 2006).

2.4. Biodegradation of RB5 dye

In order to optimize RB5 biodegradation by each fungus, a full factorial design 2^2 with central point was performed in Petri dishes, as shown in Table 1 (the same conditions evaluated for adsorption of RB5 in PUF).

Biodegradation experiments were performed in 9 mm Petri dishes, containing 30 mL of the RB5 dye added with agar-agar (1.5%), and adjusted pH. The inoculation occurred in wells located in the central part of each plate, with the addition of 100 μL of the cellular suspension. The plates were then incubated at $26^\circ\text{C} \pm 2^\circ\text{C}$. Radial growth was measured when one of the fungi reached the edge of the plate, in quadruple, using a digital caliper. The growth speed was determined by the division of the growth average observed by the number of days the experiment occurred (mm day^{-1}) (Miyashira *et al.*, 2010)

After measuring the growth rate, the Petri dishes were reincubated at $26^\circ\text{C} \pm 2^\circ\text{C}$ for five days, to visually evaluate the total or partial discoloration of the media.

2.5. Respirometric Method

The respirometric assays were conducted considering the methodology described first by Bartha (1965), detailed at ABNT - NBR Method 14283/1999 (ABNT, 1999).

Each Bartha respirometer (ABNT, 1999) received 100 mg of PUF, which were saturated with dye RB5 (50 mg L^{-1}) (sole carbon and nitrogen source to the microorganisms), and the pH was adjusted to 6.0. This substrate was inoculated with 1 mL of the *P. chrysosporium* cell suspension. As the inoculum solution had 1.8×10^6 colony forming unity (CFU) mL^{-1} ; this led to an initial cell concentration of 1.8×10^4 CFU g^{-1} PUF.

The experiment took place for 15 days at room temperature (25°C). The CO_2 generated during microbial respiration was measured every 24 hours, by removing KOH solution (0.2 N) of the respirometric flask and replacing it daily. The residual KOH was titrated with standard HCl (0.1 N) after addition of 1 mL of a solution of barium chloride to precipitate carbonate ions. Thus, the total amount of CO_2 produced as a function of incubation time could be calculated and plotted.

The values of CO_2 were obtained according to Equation 3 (Régo et al., 2014):

$$\text{CO}_2 \text{ (mg)} = (A - B) \times 50 \times f_{\text{HCl}} \times 0.044 \quad (3)$$

Where “A” is the volume of HCl (0.1 N) used to titrate the blank (mL), “B” is the volume of HCl (0.1 N) used to titrate the sample (mL), and “ f_{HCl} ” is the factor for HCl (0.1 N). The values 50 and 0.044 were used to transform the CO_2 from micromoles to milligrams (Régo *et al.*, 2014).

3. RESULTS AND DISCUSSION

3.1. Preliminary adsorption study

Figure 1 illustrates the decay of sample absorbance during the factorial design study of the adsorption of the RB5 dye in PUF.

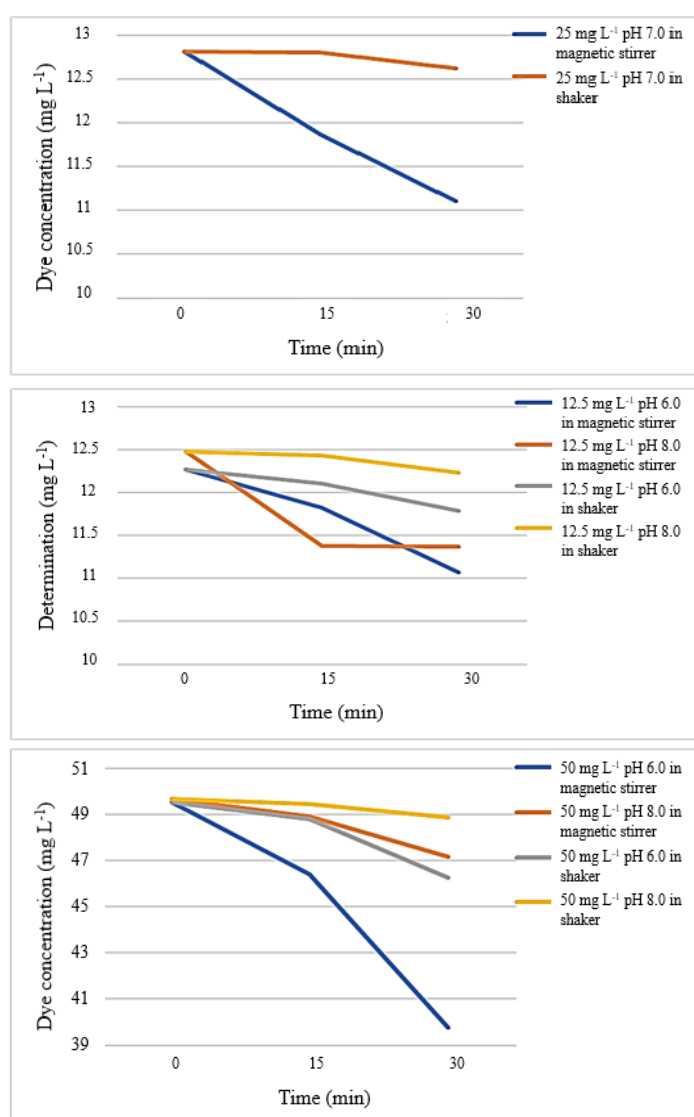


Figure 1. Absorbance variation of the samples during the factorial design study, checking the effect of pH and dye concentration in the adsorption of Reactive Black 5 in polyurethane foam.

The best adsorption result was found using the magnetic stirrer, at pH 6.0. This variation was possible because the magnetic stirrer provided better agitation than the shaker, creating a whirlwind able to plunge and completely soak all the PUF cubes in the RB5 solution, improving the retention of textile dye. In relation to the pH, it should be emphasized that pH changes the surface load of the adsorbent and the degree of ionization of different pollutants. This variable can change the adsorption process by decoupling functional groups present in the active sites of the adsorbent (Mall et al., 2014).

3.2. Adsorption study of the RB5 due on the support.

The adsorption experiment was conducted with RB5 dye at 50 mg L^{-1} in pH 6.0 and pH 7.0. The maximum adsorption of RB5 dye in PUF occurred after 110 minutes of stirring in both pH, as illustrated in Figure 2. The increase in the concentration of RB5 observed at 120 minutes indicates the desorption/adsorption equilibrium of the PUF was achieved, ending the experiment.

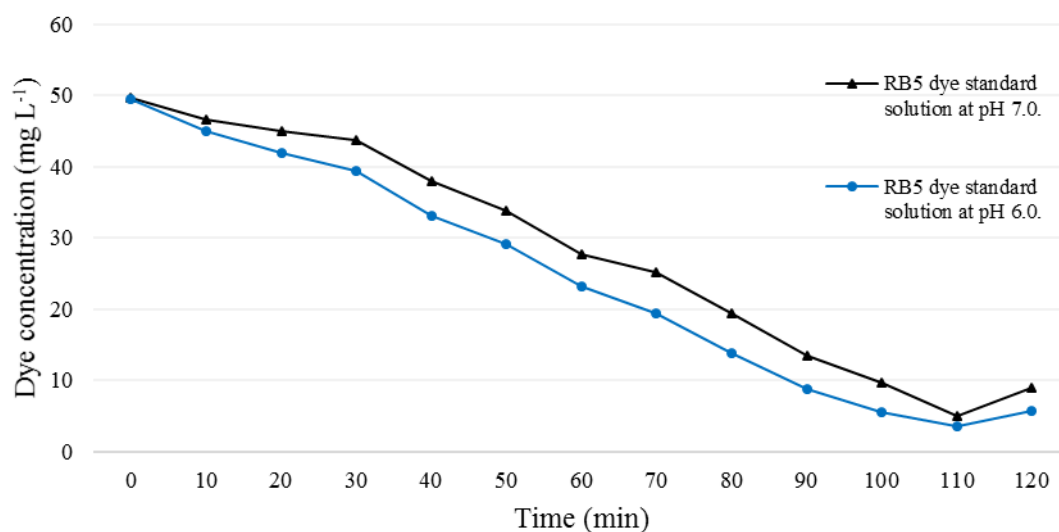


Figure 2. Adsorption efficiency of RB5 50 mg L^{-1} in polyurethane foam, at pH 6.0 and pH 7.0.

It was noted that the acidification of solution (pH 7.0 to pH 6.0) increases the adsorption of RB5 at PUF. This occurs precisely because of the dissociation of the ions present in the molecules of the textile dye and the ionization of the amino groups present in the PUF composition. However, this alternative wasn't addressed in this work, because after the adsorption of the dye in the PUF, this matrix was used as support and the textile dye as a nutrient source to the fungal culture, restricting the study to this range of work. Table 2 shows the percentage and quantification of RB5 dye (50 mg L^{-1}) adsorbed by PUF.

Table 2. Results of the adsorption experiments mentioning the percentage and the quantity of Reactive Black 5 dye (50 mg L^{-1}) adsorbed by polyurethane foam.

	Standard solution of RB 5 dye 50 mg L^{-1} without pH adjustment (pH 7.0)	Standard solution of RB 5 dye 50 mg L^{-1} at pH 6.0
Concentration in the solution after adsorption (mg L^{-1})	5.2	3.8
% of mass RB5 in the solution	10.4	7.6
Adsorbed concentration (mg L^{-1})	44.7	46.2
% of RB5 adsorbed	89.5	92.4
q (mg g^{-1})	22.35	23.1

The percentage of RB5 dye adsorption by polyurethane foam (92.4%) is close to the values mentioned by Mori and Cassella (2009), which observed 93% adsorption of violet crystal dye by PUF after 4 hours of stirring.

Góes *et al.* (2016) studied the adsorption of red dye samples by four modified polyurethane foams. PUF without cellulose adsorbed 83.8% of the dye, PUF with cellulose 75.7%, PUF with modified cellulose (1:1) 90.9%, and PUF with modified cellulose (3:1) 84.3%.

The difference in the percentage of mass adsorbed in other works, when compared to the values obtained, may be due to factors such as the presence of different ionic groups in foams, the dimensions, densities and quantities of each piece used (which directly interferes with the surface area), the presence, quantity and arrangement of the ionizable groups in each foam, favoring the adsorption of several dye molecules or rendering unusable some reactive sites due to possible steric impediments, the dye-loading used, as well as the agitation conditions (temperature, concentration, pH and agitation speed) employed in each of the adsorption tests.

In the present work, the use of PUF with nitrogenous groups was favorable for the adsorption of the RB5 textile dye, which in aqueous solution has four negatively charged sulfate groups.

3.3. Evaluation of radial growth and discoloration

Cell suspensions were counted in Neubauer chamber and PDA plates, showing similar values (Table 3), indicating that fungi had viable cells in the inoculation.

Table 3. Number of fungi cells in the microbial suspensions used as inoculums.

Fungi	Neubauer Chamber Count (cel mL ⁻¹)	Count on PDA Plates (CFU mL ⁻¹)
<i>Pleurotus floridae</i>	9 x 10 ⁵	1.1 x 10 ⁵
<i>Pleurotus ostreatus</i>	3 x 10 ⁵	1.5 x 10 ⁵
<i>Phanerochaete chrysosporium</i>	1.8 x 10 ⁸	1.2 x 10 ⁸

PDA: potato dextrose agar; CFU colony forming unity.

P. chrysosporium presented higher radial growth speed (RGS) than *Pleurotus* in all conditions studied, certainly due to the higher number of cells inoculated in the experiment (Table 3). While *Pleurotus* inoculum had 1.1 - 1.5 x 10⁵ colony forming unity (CFU) (considering that 100 µL were inoculated in the wells, at the center of the Petri dishes), *P. chrysosporium* inoculum had 1.2 x 10⁸ CFU.

It's well known that the performance of a biological process is significantly influenced by the size and viability of the inoculum (Pirt, 1976). In this study RGS was probably influenced by factors such as change in nutrients, presence of inhibitors, spore germination, and concentration of the inoculum. Increasing inoculum size leads to an increase of the dividing (active) cell fraction, influencing growth rate (dX/dt) which is defined as: dX/dt = µX (where µ is the specific growth rate, and X is the inoculum size).

Although this is a difference in inoculation, Ottoni *et al.* (2013) also had better discoloration results of RB5 dye with *P. chrysosporium* (and *Trametes versicolor*) than with *P. ostreatus*. They also observed that in Petri dishes RB5 was decolorized only at a concentration equal or below 100 mg L⁻¹, being the best results obtained at 50 mg L⁻¹.

Comparing the genus *P.*, it was observed that in most of the conditions *P. ostreatus* showed higher RGS than *P. floridae*. To *P. floridae* the higher RGS occurred at pH 6.0, at the dye's lowest concentration (7.36 mm day⁻¹), while for *P. ostreatus* it occurred at 25 mg L⁻¹, at pH 7 (6.97 mm day⁻¹). Muthukumaran *et al.* (2017) in their study showed that the *P. ostreatus* was able to remove 64% of the RB5 dye (Figure 3).

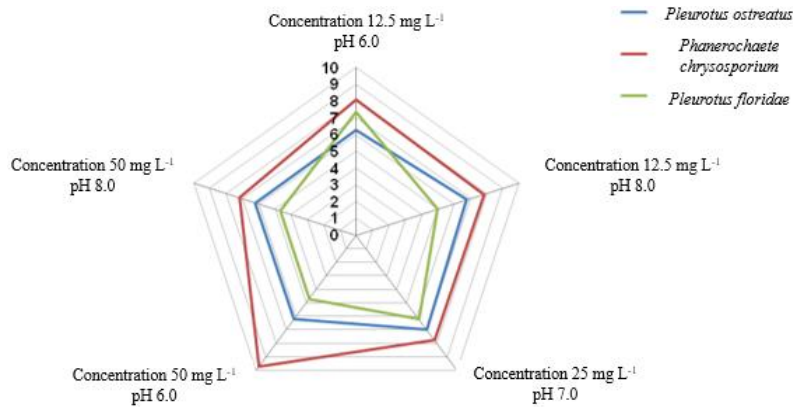


Figure 3. Radial growth speed (mm day^{-1}) of *P. ostreatus*, *P. floridae* and *P. chrysosporium* cultivated on Reactive Black 5 dye solution solidified with agar-agar (1.5 g L^{-1}) in Petri dishes.

Although it was visually observed that the color of the dye (chromophore group) in the cultivation media was not totally degraded, *P. chrysosporium* left them with a rose hue color, indicating their partial biodegradation. This color changing was also observed by Bonugli-Santos *et al.* (2013) using the fungus *Peniophora sp.*

Considering the partial degradation observed, RGS values obtained in *P. chrysosporium* cultivation were used to verify the existence of secondary interactions between the variables (dye concentration and pH). According to the response surface chart (Figure 4), *P. chrysosporium* biodegrades RB5 more efficiently when cultivated in higher dye concentration (50 mg L^{-1}) and lower pH (6.0), as shown by a red color in surface chart. Thus, the biodegradation efficiency increases from green to red color, which used lower pH and higher concentrations.

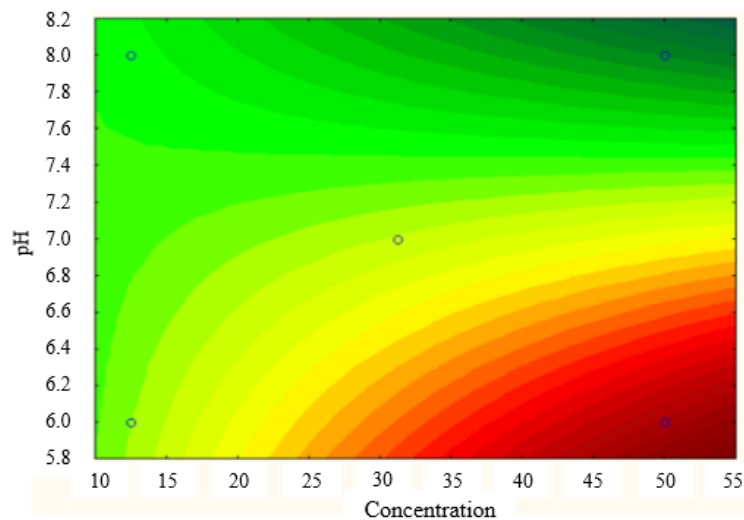


Figure 4. The response surfaces chart about *P. chrysosporium* ATCC 24725 growth when cultivated in Reactive Black 5 dye solutions in different pHs, in Petri dishes.

Radha *et al.* (2005) observed that the growth of *P. chrysosporium* and the corresponding discoloration process were essentially controlled by the pH of the medium, depending on the nature of the substrate used. Their studies with different dyes showed better biodegradation results at lower pH (4.0 – 6.0). Permpornsakul *et al.* (2016) also concluded that a higher discoloration of RB5 by *P. chrysosporium* is obtained at pH 6.0 than at pH 7.0 or 8.0, corroborating with this study.

3.4. Biodegradation Assay of Dye Reactive Black 5 Adsorbed on Polyurethane Foam

After fifteen days of *P. chrysosporium* cultivation in PUF cubes, chromophore group of RB5 dye appears to have been completely degraded, as visually observed. This degradation may have occurred by lignin or manganese peroxidase activity, the production of which was reported to be greatly improved when *P. chrysosporium* was cultivated immobilized in PUF, then comparing with the production in conventional stationary liquid culture (Bilal *et al.*, 2017). Figure 5 shows the concentration of accumulated CO₂ produced during this experiment.

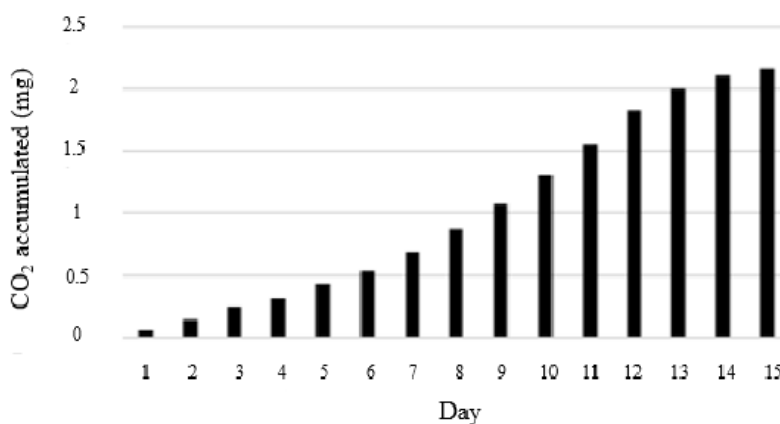


Figure 5. CO₂ accumulated (mg), produced during the biodegradation of the dye Reactive Black 5 (50 mg L⁻¹, pH 6.0) by *P. chrysosporium* cultivated in polyurethane foam cubes.

Respiration measurement directly determines the microbial activity by measuring carbon dioxide produced during microbial respiration, and, indirectly, determines the biodegradation of organic contaminants. Values shown in Figure 5 indicate that during all experiments (15 days) *P. chrysosporium* was degrading RB5. As a diazo dye, RB5 has the presence of a nitrogen double bond in the chromophore group, and respiration values combined with visual discoloration observed seem to indicate that microorganism was degrading RB5, as a carbon and/or nitrogen source.

Enayatizamir *et al.* (2011) investigated RB5 degradation by *P. chrysosporium* immobilized on cubes of nylon sponge. The SS cultivation led to the best results with a discoloration percentage of 90.3% in 72 h for an initial RB5 concentration of 100 mg L⁻¹. Their results showed that the discoloration ability of *P. chrysosporium* was greatly influenced by the support used, being the ability of *P. chrysosporium* to degrade RB5 mainly due to the secretion of the extracellular enzyme MnP.

4. CONCLUSION

It can be observed that the basidiomycete *P. chrysosporium* was the most effective for RB5 dye discoloration, besides having been the fungus studied with better radial growth speed, reaching the average value of 9.712 mm day⁻¹ in Petri dish. The adsorption test has proved that polyurethane foam has great capacity in the retention of the RB5 dye, with efficiency in adsorption of 92.4%, equivalent to 46.2 mg of dye of a solution with initial concentration at 50 mg L⁻¹ and pH 6.0. It was possible to ascertain that the maximum concentration condition (50 mg L⁻¹) of the dye solution RB5 and minimum pH (pH 6.0) was favorable for the development of this research, since the optimal pH value for maximum adsorption also favored the development of the *P. chrysosporium*.

With the use of the respirometric technique, it was possible to observe the increase in the amount of CO₂ produced during the inoculation, reaching the accumulated value on the last day

of monitoring of 2.16 mg of CO₂. It is concluded that the *P. chrysosporium* can efficiently degrade the RB5 dye adsorbed in polyurethane foam, using it as the only source of nutrients.

5. ACKNOWLEDGMENTS

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