ADSORPTION AND BIOLOGICAL REMOVAL OF BASIC GREEN 4 DYE USING WHITE-ROT FUNGI IMMOBILIZED ON AGAVE TEQUILANA WEBER WASTE

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ABSTRACT

The fungi *Trametes versicolor* and *Pleurotus ostreatus* were used to remove the triphenylmethane dye Basic Green 4, which is a toxic compound used in dye industries and in aquaculture as a biocide and fungicide. *Agave tequilana* Weber was evaluated as a support and biosorbent because it has high quantities of nutrients and good adsorption capabilities, and its leaves are a by-product of the tequila industry. The kinetics of the uptake of Basic Green 4 by agave fiber showed a relatively fast rate of dye adsorption. At 29 °C, the dye adsorption properties were described by the Freundlich isotherm model, with a K_f value of 0.5683 (mg g^{-1}) ((L g^{-1})$^{1/n}$)$^{-1}$. The fungal culture immobilized on agave fiber and without added glucose was found to provide the best option for pigment removal, obtaining 99.3% discoloration of 200 ppm dye with non-saturated support and 79 U L^{-1} laccase production during 4 days of treatment at 29 °C in Erlenmeyer flasks. Toxicity tests revealed that the immobilization of fungi in agave fiber decreases the toxicity compared with immobilization of fungi in agave leaves. Additionally, when the fiber was previously saturated with the dye and then immobilized, an increase in the effectiveness of the system resulted due to the removal of the dye by biological degradation and by adsorption to the support. In a bubble column reactor, under these conditions, the discoloration was 90% of 50 ppm in 9 days, or two removal cycles, and the toxicity of the degradation products decreased.

KEYWORDS: *Agave tequilana* Weber, Basic Green 4, biodegradation, co-substrate, toxicity.

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1 INTRODUCTION

Industrial dyes can be removed using physical-chemical methods, such as adsorption or chemical degradation, which are expensive, thus limiting their application [1]. Kalyani *et al.* [2] reported that textile dyes and effluents have toxic effects on the germination time and biomass production of many plant species that play an important role in the ecological function, protect the soil from erosion, and provide organic matter to retain soil fertility. The dye Basic Green 4 is used in aquaculture as a medical disinfectant, and in the textile industry, and it is highly toxic to humans and animals [3]. Although physical and chemical treatments to remove pollutants in water have been described [1, 4], the removal of dyes has been difficult and expensive; therefore, researchers have been focused on the biological removal of dyes. Various microorganisms, such as *Pseudomonas pseudomallei* [5], *Citrobacter* sp. [6], *Bacillus subtilis* [7], *Nocardia corallina* [8], *Rhodotorula* sp. [9], *Rhodotorula rubra* [9], *Phanerochaete chrysosporium* [10], and *Cunninghamella elegans* [11], were found to be capable of discoloring triphenylmethane dyes. Maalej-Kammoun *et al.* [3] used enzymes from white rot fungi to remove Basic Green 4 along with laccase from *Trametes versicolor* at an initial concentration of 0.1 U ml^{-1}; these enzymes removed 97% of the Basic Green 4, but it was determined that laccase is not stable. Furthermore, it was found that such microorganisms do not need preconditioning to produce non-specific extracellular enzymes that can degrade xenobiotic compounds, including dyes.

The aim of this research was to evaluate the use of a fungal culture immobilized on *Agave tequilana* Weber for the adsorption and fungal degradation of Basic Green 4.

2 MATERIALS AND METHODS

2.1 Microorganisms and culture media

Trametes versicolor and *Pleurotus ostreatus* fungal strains were obtained from the Applied Biotechnology

Laboratory of Javeriana University, Bogotá, Colombia. The strains were reactivated in wheat bran extract agar at 29 °C for 8 days with a medium containing 175 g L^{-1} wheat bran, 10 g L^{-1} glucose, 5 g L^{-1} peptone, 2 g L^{-1} yeast extract, 0.1 g L^{-1} KH_2PO_4, 0.05 g L^{-1} $MgSO_4*H_2O$, 0.076 g L^{-1} $MnSO_4$, and 0.1 g L^{-1} chloramphenicol to prevent bacterial contamination [12]. The wheat bran was soaked for 1 h, and then squeezed to obtain the liquid extract that was used to mix the medium components; the solid residue was not used. The fungal strains were preserved by colonizing agar discs and storing them in Eppendorf tubes with wheat bran extract agar at 4 °C, as described by Pedroza *et al.* [13].

2.1.1 Fungal biomass production

Fungal biomass was produced under two different conditions, solid culture and liquid culture. Wheat bran extract medium [12] with 18 g L^{-1} agar was used for the solid culture. Each fungus was seeded with a colonized disc that was put into a Petri dish with new agar, and the fungi were incubated for 8 days at 29 °C. For the liquid culture, wheat bran extract medium was used. Ten agar discs, each colonized with the fungus, were added to 100 ml of liquid medium, and the fungi were incubated for 8 days at 29 °C and shaken at 120 rpm.

2.2 *Agave tequilana* Weber material

Agave tequilana Weber leaves were collected from 8-year-old plants grown in Tequila, Jalisco, Mexico. The leaves were washed, cut into squares (0.5 x 0.5 cm), dried at 60 °C in an oven, and then packed in polyethylene bags; each bag contained the same amount of leaves by weight. The remaining leaves were used for fiber production according to a traditional method, which consists of removing the fleshy part of the leaves using a sharp instrument to expose and remove the fiber then by rinsing and drying in the sun [14]. Both the bags with leaves and those with fiber were sterilized using gamma irradiation (^{60}Co) at a dose of 21 to 38 kGy, which was performed at the Instituto Nacional de Investigaciones Nucleares of Mexico (ININ). A sterilization test on the fiber and leaf samples in nutritive agar and potato dextrose agar was carried out. The chemical characterization of *Agave tequilana* leaves was reported by Guillén-Jiménez *et al.* [15], who evaluated reducing sugars using 3,5-dinitrosalicylic acid [16], total nitrogen [17], total phosphorus [17], and humidity [17].

2.3 Adsorption studies

2.3.1 Adsorption experiments

Adsorption tests were performed in duplicate by mixing 100 ml of an aqueous solution of 200 ppm Basic Green dye with 0.4 g of *Agave tequilana* fiber as a sorbent in a 250-ml Erlenmeyer flask. The flasks were shaken at 120 rpm and 29 °C; 5 ml samples were sampled every 6 h during the first 24 h, and then, every day for 8 days. The residual dye in the supernatant solutions was analyzed spectrophotometrically at 620 nm, and Basic Green dye concentration was calculated using a standard curve.

The adsorption of agave samples with several initial dye concentrations was carried out using a batch method, as described above. In each experiment, 0.4 g of agave fibers with 100 ml of an aqueous solution of the Basic Green 4 dye at concentrations of 200, 180, 170, 160 and 150 ppm were equilibrated for 4 days at 29 °C and 120 rpm. Initial and final dye concentrations were determined as described above.

2.3.2 Equilibrium modelling in a batch system

To examine the adsorption mechanism, pseudo-first-order and pseudo-second-order kinetic models are generally used to test experimental data, assuming that the measured concentrations are equal to the surface concentrations [18].

Pseudo-second order chemical reaction kinetics [19] (Eq. 1) provides the best correlation of the experimental data for the removal of organic pollutants and metals from an aqueous solution by natural adsorbents and activated carbon.

$$\frac{t}{q} = \frac{1}{K_{2,ads} q_e^2} + \frac{1}{q_e} t \quad (1)$$

where, q (mg g^{-1}) is the amount of adsorbed pollutant on the adsorbent at time t, q_e is the dye amount of dye (mg g^{-1}) biosorbed at equilibrium, and $K_{2,ads}$ (g mg^{-1} min^{-1}) is the constant rate of second-order adsorption.

To estimate the adsorption capacities at various initial concentrations of Basic Green 4, the two well-known equilibrium adsorption isotherm models, the Langmuir and Freundlich isotherms, were used.

The Langmuir isotherm model is described in its linear form by Equation 2:

$$\frac{1}{q_e} = \frac{1}{q_m} + \frac{1}{q_m K C_e} \quad (2)$$

where, q_m (mg g^{-1}) and K (l mg^{-1}) are Langmuir constants that indicate the maximum adsorption capability of the fiber [4]. Additionally, for the Langmuir isotherm, the separation factor (R_L) was calculated (Eq. 3):

$$R_L = \frac{1}{1 + K C_o} \quad (3)$$

where, C_o is the initial concentration of the dye [20].

The Freundlich isotherm model in its logarithmic form is given by Equation 4:

$$Log \frac{X}{m} = Log K + \frac{1}{n} Log C_f \quad (4)$$

where, K (mg g^{-1}) and n (dimensionless) are Freundlich constants that indicate the adsorption capability and adsorption intensity, respectively [4].

2.4 Immobilization protocols

The immobilization protocols were carried out using two different support configurations: agave fiber and agave leaf squares (0.5 x 0.5 cm). One gram of either agave fiber or agave squares and 1% (w/v) fungal biomass were added to 50 ml of Radha Culture Medium (RCM), modified to

consist of 2.5 g L^{-1} glucose, 2 g L^{-1} KH_2PO_4, 0.05 g L^{-1} NH_4Cl, 0.5 g L^{-1} $MgSO_4*7H_2O$, 0.1 g L^{-1} $CaCl_2*2H_2O$, 100 µg L^{-1} thiamine-HCl, and a 10 ml solution of trace elements (0.5 g L^{-1} $MnSO_4$, 0.1 g L^{-1} $FeSO_4*7H_2O$, 0.1 g L^{-1} $ZnSO_4*7H_2O$) with a final pH of 4.5 that was prepared separately and then added to the medium [21]. After 1, 4 and 7 days of incubation at 29 ºC, the supports (agave fiber or agave leaves) were removed from the medium and observed by scanning electron microscopy (SEM) to verify the immobilization process [22].

2.5 Preparation of samples for SEM

Samples were prepared according to the procedure proposed by Bozzola and Russell [22]. First, samples were fixed with 2% (w/v) glutaraldehyde for 2 h, and then rinsed three times for 15 min each time with a 0.1 M phosphate buffer. A second step for fixation was carried out with 1% (w/v) osmium tetroxide for 2 h, and the samples were then rinsed three times with a 0.1 M phosphate buffer for 15 min each time, followed by dehydration with ethanol for 10 min at each of the different concentrations (10-100%, v/v). The samples were then dried, mounted on a specimen stub, and finally metal-coated to be examined using a JSM-5800 LV scanning electron microscope (JEOL, Japan).

2.6 Selection of the biocarrier configuration and effect of the addition of co-substrate on dye removal

The selection of the biocarrier configuration and the effect of glucose as a co-substrate on dye removal were performed using a block design with the treatments and controls (presented in Table 1). The tests were performed in triplicate, and a statistical analysis was performed using Tukey's test after verification of the assumption data confidence level of 95% using SAS 9.0 ® program for Windows.

TABLE 1 - Block design for the selection of the biocarrier and the effect of the addition of glucose on the dye removal.

Treatment	Culture
1	*P. ostreatus* + glucose + leaves
2	*P. ostreatus* + glucose + fiber
3	*P. ostreatus* + glucose + free cells
4	*P. ostreatus* + leaves
5	*P. ostreatus* + fiber
6	*P. ostreatus* + free cells
7	*T. versicolor* + *P. ostreatus* + glucose + leaves
8	*T. versicolor* + *P. ostreatus* + glucose + fiber
9	*T. versicolor* + *P. ostreatus* + glucose + free cells
10	*T. versicolor* + *P. ostreatus* + leaves
11	*T. versicolor* + *P. ostreatus* + fiber
12	*T. versicolor* + *P. ostreatus* + free cells
Control 1	Leaves + glucose
Control 2	Leaves
Control 3	Fiber + glucose
Control 4	Fiber
Control 5	Absolute control

For each treatment, 50 ppm aliquots of dye were added to 50 ml of RCM, with or without modification by 2.5 g L^{-1} glucose supplement [21] in a 100-ml Erlenmeyer flask. The solution was inoculated with 1% (w/v) (0.5 g) free fungal biomass or 1 g of support (without previous saturation), with immobilized fungal biomass. Then, percentage of discoloration [23], laccase activity [24], manganese peroxidase (MnP) activity [25], lignin peroxidase activity (LiP) [26], and absorption in the UV-VIS range were measured. As abiotic controls, Erlenmeyer flasks containing 50 ml of RCM medium and 1 g of non-saturated agave fiber or 1 g of non-saturated agave leaves were incubated without microorganisms. Additionally, treatments with only dye medium in the same conditions served as the absolute control. All experiments were performed in triplicate and in the dark to avoid false positives that could result from discoloration associated with light.

2.7 Dye removal using a bubble column reactor

The selected support was saturated with dye and used to immobilize the fungi in solid wheat bran extract agar. For the reactor tests, a glass column (38 cm length, 7.5 cm diameter, total volume 1000 ml) was used. The operational conditions were as follows: 750 ml of 50 ppm Basic Green 4, aeration of 1.091 L min^{-1} at 20 °C, and 7 g of previously saturated and inoculated support. To estimate the reactor's performance with the best configuration of the support, the number of cycles of operation was determined. Samples of 15 ml were sampled for analysis every 24 h for 3 days, for each cycle. At the end of the first cycle, the medium was removed, and the reactor was replenished with fresh medium (50 ppm of dye); the process was repeated for 3 consecutive cycles in duplicate. The measured dependent variables were percentage of discoloration [23], laccase enzyme [24], and UV-VIS spectra. To determine if the performance of the system is diminished with the increasing process time, Tukey's test was performed with a confidence level of 95% using the statistical program SAS 9.0 for Windows. Bioassays were monitored at the beginning and end of each cycle to assess changes in toxicity.

2.8 Toxicity assays pre- and post-treatment

Toxicity assays were performed using two different organisms to compare the dye effects in two different trophic levels.

2.8.1 Bioassays with *Artemia salina*

Artemia salina is a micro crustacean used in toxicity assays. Nauplii of *Artemia* were obtained from hatching cysts, which were hydrated for 24 h in saltwater (20 g L^{-1} NaCl). Approximately 50 mg of *Artemia salina* eggs were placed in an Erlenmeyer flask with 350 ml of saltwater. Cysts were kept in constant agitation with strong aeration at 28 °C, and were exposed to natural or artificial light for 24 h. Acute toxicity assays were performed in plastic containers that contained 10 nauplii with 5 ml of water containing 5 g L^{-1} NaCl. Toxicity tests were performed in triplicate for 24 h. At the end of the test, the number of dead bodies was counted in each container; an organism was considered to be dead if no movement was observed.

Lethal dose values (LD_{50}) (the concentration at which half of the population dies) were calculated using the Finney program (DOS). LD_{50} values less than 1000 ppm are considered to be toxic levels [27, 28].

2.8.2 Bioassays with *Lactuca sativa*

The *Lactuca sativa* variety of Grandes Lagos (edible plant) was also used for analysis to determine the sensitivity to the toxic zinc sulfate ($ZnSO_4$). The following concentrations were evaluated: 10, 20, 40 and 100 mg L^{-1}.The solutions for analysis were made from a stock solution of 200 mg L^{-1}. For wastewater, three dilutions with a factor of 0.5 were used. Reconstituted hard water was used as a negative control.

The assays were performed in Petri dishes that contained filter paper saturated with 3 ml of the substances under evaluation. Twenty-five *Lactuca sativa* seeds of equal size were added to the Petri dishes, which were covered and lined with wet paper towels to avoid moisture loss and placed in the dark for 120 h (5 days) at 22 ±2 °C. Replicates were performed in triplicate for each solution tested; a positive control was used for each concentration, and reconstituted hard water was used as a negative control. At the end of the exposure time, the length of the radical was measured in each seedling using graph paper [29]. The results were analyzed with the statistical program Probit to calculate the concentration that produced 50% inhibition (LD_{50}/ED_{50}).

3 RESULTS AND DISCUSSION

3.1 Determination of *Agave tequilana* sorption capability

According to Guillén-Jiménez *et al.* [15], agave leaves contain 4.4 g reducing sugars, 1.42 mg total nitrogen, and 260.27 mg total phosphorus per 100 g in dry matter, indicating that this waste is a rich source of nutrients and can stimulate the biodegradation of pollutants. Additionally, Iñiguez-Covarrubias *et al.* [30] determined that agave leaves have a high α-cellulose content that can enhance the adsorption of the dye to the support surface due to the interaction of the agave's functional groups with the dye. Materials consisting of lignin, cellulose and hemicelluloses with polyphenolic groups play an important role in binding dyes, and do so through different mechanisms [31]. Generally, adsorption on natural materials takes place by complexation, ion exchange, and hydrogen bonding [32].

The adsorption kinetics of basic green 4 are shown in Fig. 1. It can be observed that the adsorption is fast, there is a notable decrease in the dye concentration after 24 h, and equilibrium was reached after 4 days for *Agave tequilana* fiber. The concentration of dye decreased from 25.9 to 2.06 mg, indicating that 23.84 mg of Basic Green 4 was adsorbed by 1 g of agave fiber at 29 ºC. The pseudo-first order and pseudo-second order constants were calculated, and it was determined that the experimental data provided a better fit to the pseudo-second order kinetic model, with the correlation coefficient suggesting a strong correlation (R^2=0.9987), while the correlation coefficient was not as strong (R^2=0.9633) in the pseudo-first order kinetic model. The values obtained for pseudo-second order kinetics were K_2 = 0.206 and q_e=24.33. The pseudo-second order model is based on the sorption capacity onto the solid phase, which predicts the sorption behavior over the whole range studied [33]. Akar *et al.* [34] observed that their experimental results showed good agreement with the pseudo-second order kinetic model, and support the assumption behind the model that the biosorption is due to chemisorption.

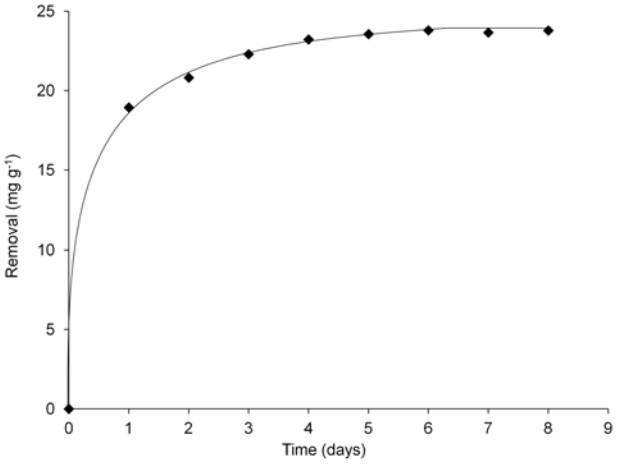

FIGURE 1 - Effect of contact time on Basic Green 4 adsorption to *Agave tequilana* fiber at 25 °C.

The values of the Langmuir and Freundlich constants are shown in Table 2. The coefficient of correlation (R^2= 0.904) indicates that the Freundlich model provides a better fit for the experimental data of Basic Green 4 on agave fiber. Table 2 shows that the value of *n* is greater than one unit (*n*=1.24), demonstrating that Basic Green 4 was favorably adsorbed by the agave fiber; although the Freundlich coefficient of correlation was slightly higher than that of Langmuir, the Langmuir constant values also indicate that agave fiber is a good adsorbent. This was further confirmed with the R_L value, which is between 0 and 1, indicating a favorable adsorption [20].

TABLE 2 - Isotherm constants for Basic Green 4 on *Agave tequilana* at 25 °C.

Isotherm	*Parameters*	
Langmuir		
	q_m (mg g^{-1})	111.1
	K_a (L mg^{-1})	0.0027
	R_L	0.649
	R^2	0.902
Freundlich		
	K_F	0.5683
	n	1.24
	R^2	0.904

The lignin cellulosic by-products have been studied as adsorbents for synthetic dyes, and authors such as Al-

len and Koumanova [35] claimed that activated carbon adsorbs better than these adsorbents; materials, such as wood or peat, are also highly absorbent but cheaper. Several authors have measured the adsorption of different natural materials with the dye Basic Green 4; for example, Guo *et al.* [36] determined that the q_m of rice husk for this dye was 511 mg g^{-1}, and Garg *et al.* [37] determined the q_m for treated sawdust for this dye was 74.5 mg g^{-1}. Khattri and Singh [38] performed an adsorption study with by-products from industrial processes and determined that the q_m for neem sawdust was lower than the value mentioned previously (3.42 mg g^{-1}). According to the results shown in Table 2, the q_m determined using the Langmuir isotherm on the adsorption of fiber with Basic Green 4 is 111.1 mg g^{-1}, indicating that the q_m value for different supports depends on the system applied to adsorption processes.

3.2 Fungal immobilization on Agave biomass

According to the SEM photos, incubation for 4 days is long enough to achieve fungal immobilization in RCM modified with glucose. Previous results have shown that after 4 days of treatment of Black Reactive 5, immobilized *T. versicolor* showed 95% discoloration with 4 days of immobilization and 0.769 g of attached biomass [39]. Herein, it was observed that after 4 days, the microorganisms adhered to the *Agave* biomass (leaves and fiber) (Fig. 2). In some pictures, deterioration of the material could be observed, which could be related to the ligninocellulosic characteristics of the supports, and the erosive and oxidative potential presented by the white rot fungi [23]. It has been reported that lignin degradation occurs via oxidative attack of the enzymes produced by the fungi; then, the simple products from lignin degradation, such as protocatechuic acid or muconic acid, enter the fungal hyphae and are incorporated into intracellular catabolic routes [40].

3.3 Selection of the configuration of the biocarrier and effect of the addition of glucose as a co-substrate on dye removal

There is currently no published data on Basic Green 4 removal by adsorption or biological degradation using leaves or fibers of *Agave tequilana* Weber. This study is important because the results may justify the use of a resource waste as sorbent and fungal support in dye removal processes.

According to the statistical analysis results, there are significant differences between treatments ($p<0.0001$), showing that, under the experimental conditions, the highest discolorations were obtained using *T. versicolor* and *P. ostreatus* immobilized in agave fiber without glucose (T11) and with glucose (T8), with discoloration values of 99.3% and 99.2%, respectively (Table 3); however, there were no differences between T11 and T8 ($p>0.05$), suggesting that both could be used and that the fungal culture obtains simple carbon sources from the fiber of agave. While the dye biotransformation is carried out, the fungi can produce enzymes such as cellulases or hemicellulases that hydrolyze glycosidic bonds to free monomers and disaccharides, such as cellobiose and glucose [41]. Laccase can be induced by the lignin present in the vegetal residue [39]. Iñiguez-Covarrubias *et al.* [30] claimed that the concentration of α-cellulose (64.8%) in agave is higher than in other plant fibers, while the lignin (15.9%) content of agave leaves is lower than that of other woods, such as *Pinus ocarpa* and *Eucalyptus*, which have 27.4% and 18.2% lignin, respectively. Additionally, the amount of total reducing sugars in the leaves of agave is 4.4% (w/w), which could maintain the fungal growth [15].

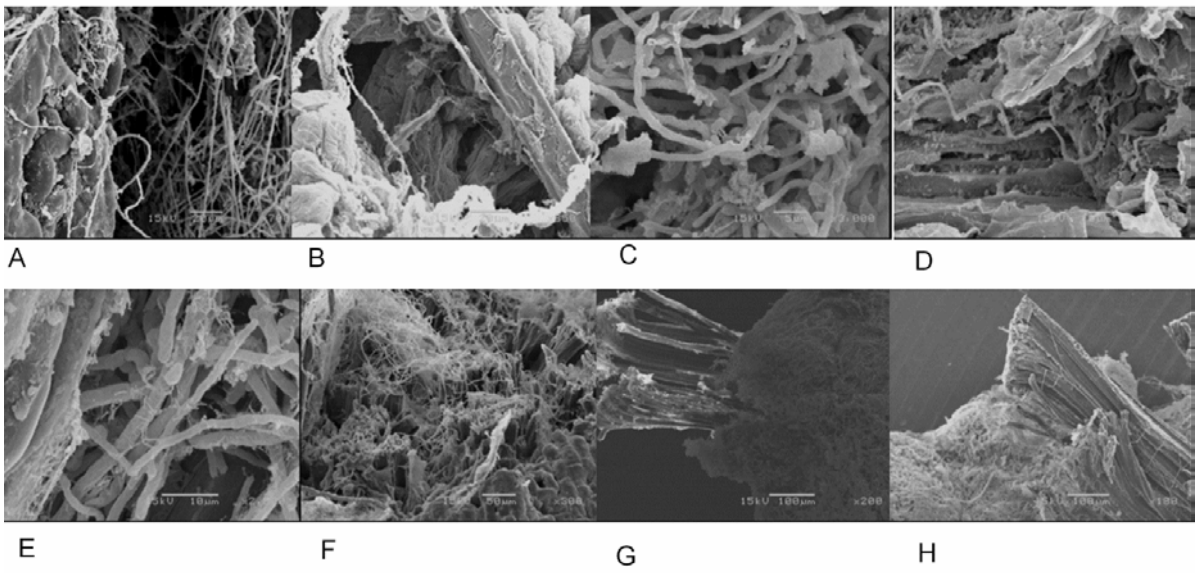

FIGURE 2 - SEM of immobilized supports (*T. versicolor* leaves, 4 days (A); *T. versicolor* leaves, 7 days (B); *T. versicolor* fiber, 4 days (C); *T. versicolor* fiber, 7 days (D); *P. ostreatus* stalk, 4 days (E); *P. ostreatus* stalk, 7 days (F); *P. ostreatus* fiber, 4 days (G); *P. ostreatus* fiber, 7 days (H)).

TABLE 3 - Block design results of the percentage of discoloration and laccase (U L^{-1}) production (4 days of treatment, 120 rpm, 29 °C, 50 ppm Basic Green 4).

Treatment	Culture	% Discoloration	Laccase (U L^{-1})
1	*P. ostreatus* + glucose + leaves	95.5 ± 2.9	18.5 ± 4.75
2	*P. ostreatus* + glucose + fiber	97.8 ± 2.7	14.9 ± 1.56
3	*P. ostreatus* + glucose + free cells	86.2 ± 1.67	0.6 ± 1.11
4	*P. ostreatus* + leaves	94.4 ± 2.6	22.3 ± 5.03
5	*P. ostreatus* + fiber	99.2 ± 0.07	1.6 ± 1.37
6	*P. ostreatus* + free cells	92.7± 2.87	3.2 ± 1.94
7	*T. versicolor* + *P. ostreatus* + glucose + leaves	98.4 ± 0.81	39.9 ± 1.34
8	*T. versicolor* + *P. ostreatus* + glucose + fiber	99.3 ± 0.22	162 ± 1.21
9	*T. versicolor* + *P. ostreatus* + glucose + free cells	87.7 ± 1.17	1.3 ± 0.33
10	*T. versicolor* + *P. ostreatus* + leaves	99 ± 0.12	26.4 ± 1.24
11	*T. versicolor* + *P. ostreatus* + fiber	99.3 ± 0.11	79.1 ± 1.05
12	*T. versicolor* + *P. ostreatus* + free cells	86.9 ± 0.89	0.5 ± 0.17
Control 1	Leaves + glucose	80.8 ± 1.55	0 ± 0
Control 2	Leaves	84.2 ± 3.18	0 ± 0
Control 3	Fiber + glucose	86.2 ± 2.75	0 ± 0
Control 4	Fiber	88.8 ± 2.68	0 ± 0
Control 5	Absolute control	11.2 ± 3.24	0 ± 0

Treatments with *P. ostreatus* (T1-T6) alone resulted in discolorations of approximately 86% and 99%, meaning that fungal immobilization, glucose supplementation and the adsorption capability of the support are the factors that influence dye removal. Dye discoloration with the supports without immobilized microorganisms shows the ability of the agave to be a biosorbent for the system proposed.

According to the statistical analysis for laccase production, there are highly significant differences between treatments ($p<0.0001$), showing that, under the evaluated experimental conditions, the highest expressions of laccase were obtained using a mixed fungal culture containing *T. versicolor* with *P. ostreatus* immobilized in agave fiber with glucose supplement (T8), and the same culture and support without glucose (T11), which yielded values of 162 U L^{-1} and 79 U L^{-1}, respectively. The difference between T8 and T11 showed that the presence of co-substrate possibly favors the enzymatic activity because a simple carbon source generates the primary metabolism activation of the fungus and, therefore, the enzyme production for degradation of the compounds in the medium [42]. Additionally, the glucose assimilation generates a decrease in pH, favoring the optimal conditions of enzyme activity [43]. Additionally, acids such as oxalic acid are generated that help to chelate Mn^{+3}, and act as oxidants in distant areas of influence of the hyphae [44].

Treatments with only *P. ostreatus* (T1-T6) produced laccase enzyme levels between 0.65 and 22.28 U L^{-1}. This concentration was the highest in the treatment with the immobilized fungus, showing that the agave leaves and fiber acted as an inductor of enzymatic activity. This may be due to agave being a lignified material with a greater abundance of aromatic compounds. Moreover, in all treatments, it was observed that the presence of *Agave tequilana* Weber in fiber and leaves generates an increase in the discoloration of the medium, thus providing additional benefits from the treatment. Additionally, the statistical analysis shows that the addition of glucose did not significantly affect the amount of discoloration.

On the other hand, the MnP and LiP production did not present significant effects on the treatment ($p = 0.4794$, $p = 0.2794$), as the presence of these enzymes could not be observed in any of the treatments (Fig. 3). Thus, these enzymes are not related to the discoloration, and the behavior of the microorganisms is different for each treatment. Heinfling *et al.* [45] suggested that commercial dyes cannot be decolorized by MnP and LiP, which are produced by some white-rot fungal strains; additionally, these authors determined that the enzymes can be inhibited by these dyes.

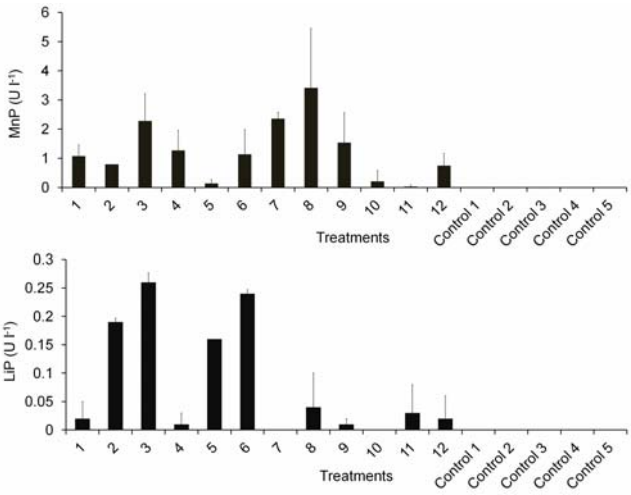

FIGURE 3 - Manganese peroxidase and lignin peroxidase activities in the block design treatments (Conditions: 4 days of treatment, 120 rpm, 29 °C, 50 ppm Basic Green 4).

Different spectral curves of the degradation samples of different treatments were collected via spectrophotometry. The results showed the disappearance of the peak corresponding to the initial dye, and the appearance of new peaks likely corresponding to products obtained from the dye discoloration and support degradation (Fig. 4). In the dye degradation, the intermediary leucomalachite green may be formed, which is colorless and absorbs in the UV region (260 nm). In treatment 11, which corresponds to *T. versi-*

color and *P. ostreatus* immobilized on fiber, a very high peak at 300 nm was observed, which may correspond to degradation intermediaries of the lignin present in the support, such as phenylpropyl alcohol, that are part of the lignin molecule. The appearance of the peaks in the treatment spectrum shows that fungi use the support as a carbon source, and degrade compounds of the support to generate the additional intermediates observed in the peaks.

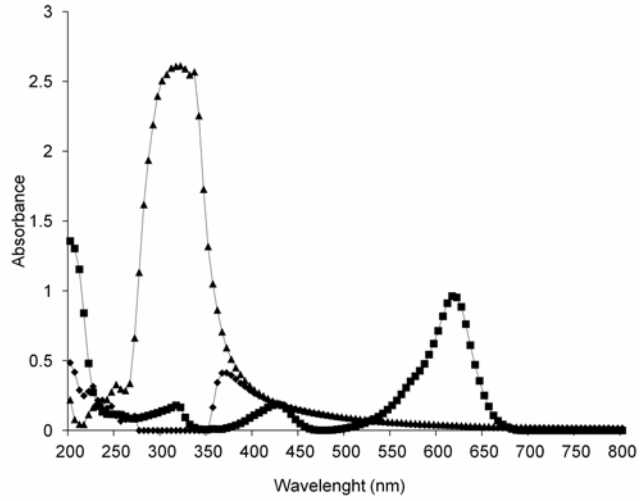

FIGURE 4 - UV/VIS scans of the optimal treatments obtained in the selection of the configuration of the biocarrier, and the effect of the addition of glucose as co-substrate on the dye removal: Initial sample (■), Treatment 8 (♦), Treatment 11(▲).

Additionally, bioassays with *Artemia salina* and *Lactuca sativa* were performed to determine the LD_{50} values after the treatment (Table 4); the test was performed only for the treatment with the highest removal (T11) and controls of supports without microorganisms. In the *Artemia salina* samples with glucose addition and fungi immobilized in leaves and fiber, lower LD_{50} values were obtained when the biomass was immobilized than with free biomass, showing that the organism could be useful to some agave components.

In the controls, it was shown that leaves inhibit the organisms and may have some components that influence the LD_{50} value; on the other hand, the fiber absorbs the dye strongly with the unsaturated support and may decrease the initial toxicity to an extent sufficient for detection.

For *Lactuca sativa* and *Artemia salina* assays, it was observed that fiber diminishes the toxicity more than the leaves do. The values obtained from the leaves show that increases in toxicity are even higher with an initial dye concentration of 250 ppm, perhaps due to some leaves' agave components.

On the other hand, the data obtained from the treatment with fiber and glucose showed no toxicity on *Lactuca,* possibly due to the presence of a co-substrate that may favor the dye transformation and eliminate the toxic intermediaries of the dye. When the fiber was used without glucose, the LD_{50} obtained for *Lactuca* was 57.7 ppm, which could be due to the presence of some degradation intermediates from the dye degradation or lignin fractions of the support. With these toxicity values, it was observed that *Artemia salina* shows higher sensitivity than *Lactuca sativa*, which may be due to the mechanism of action of pollutants on organisms. Few reports have been published that evaluated the toxicity of dyes; Sánchez-Fortún and Barahona [46] have reported the effect of an aquatic pollutant, an organophosphorous compound that is a cholinesterase inhibitor, inhibiting acetylcholinesterase and resulting in excessive accumulation of acetylcholine and overstimulation of central and peripheral cholinergic receptors of *Artemia salina*.

3.4 Dye removal using a bubble column reactor

According to previous results, 3-day cycles of treatment were established, and the support was saturated because high, non-adsorbed concentrations of the dye could be toxic for fungal biomass. This can be inferred from the results of previous tests, where it was found that using a fiber without saturation results in a decrease of laccase production and less color removal (Fig. 5). The support saturated with 23.84 mg of dye per g of fiber (according to saturation assays), and immobilized in solid medium, was put into the reactor, and RCM with 50 ppm was added. Dye removal rates were higher than 90% in the 3rd cycle, and because a saturated support was used, the removal was due to fungi. The comparison of mean values between cycles for the treatments with previously saturated fiber show significant differences between treatments ($p = 0.0036$), suggesting that the system is stable for discoloration until cycle 3, or for 9 days (216 h) under the studied experimental conditions. Significant differences in laccase activity were also shown ($p = 0.0023$), and the highest amount of laccase activity was observed in cycle 1 (67 U L^{-1}). In cycles 2 and 3, a lower enzymatic activity was measured (Fig. 5), showing that laccase is likely acting

TABLE 4 - LD_{50} evaluated in the block design for *Lactuca sativa* (5 days, 22 °C) and *Artemia salina* (24 h, 28 °C).

Treatments	Analysis	LD_{50} (ppm)
Initial concentration of dye (50 ppm)	Acute toxicity with *Artemia salina*	11.897
Leaves without microorganisms(Control 2)	Acute toxicity with *Artemia salina*	2.901
Fiber without microorganisms (Control 4)	Acute toxicity with *Artemia salina*	76.664
Fungi immobilized on fiber (Treatment 11)	Acute toxicity with *Artemia salina*	0.124
Initial concentration of dye (50 ppm)	Acute toxicity with *Lactuca sativa*	85.11
Leaves without microorganisms (Control 2)	Acute toxicity with *Lactuca sativa*	28.3
Fiber without microorganisms (Control 4)	Acute toxicity with *Lactuca sativa*	87.7
Fungi immobilized on fiber (Treatment 11)	Acute toxicity with *Lactuca sativa*	57.7

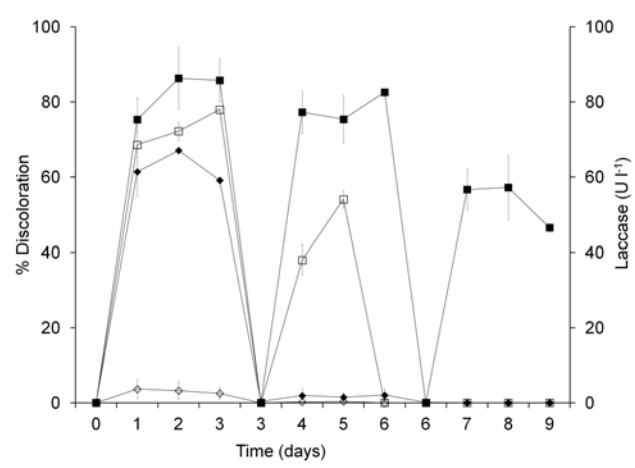

FIGURE 5 - Operative cycles: Percent discoloration with treatments with saturated fiber (■), Laccase (U L^{-1}) with treatments with saturated fiber (♦), Percent discoloration with treatments with non-saturated fiber (□), Laccase (U L^{-1}) with treatments with non-saturated fiber (◊).

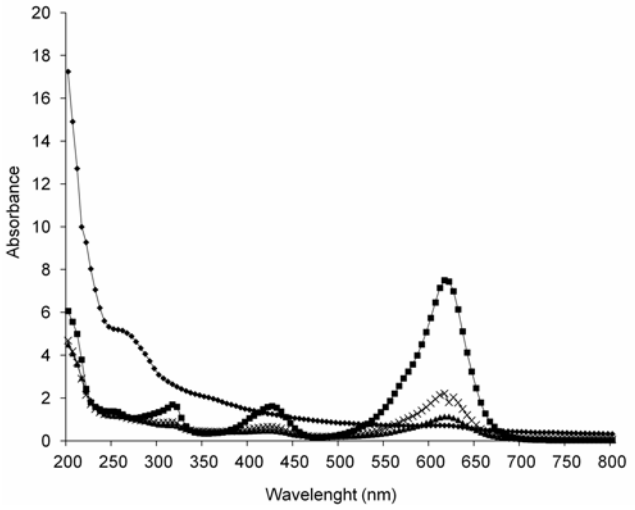

FIGURE 6 - UV/VIS scans of cycles for saturated fiber and 50 ppm of dye: Initial sample (■), cycle 1 (♦), cycle 2 (▲), and cycle 3 (x).

on products of dye degradation, or molecules that support degradation. Other enzymes, such as tyrosinases or other peroxidases, that degrade the dye, may be produced and continue to act on dye degradation [47]. In the UV/VIS scan (Fig. 6), peaks in the visible and ultraviolet region are present from cycle 1, whereas in cycles 2 and 3, a gradual decrease of the initial peak of the dye was observed. This trend can be attributed to the action of non-quantified enzymes that may diminish the intensity in UV peaks observed at approximately 280 nm. The correlation of variables between cycles was positive and significant for cycle 1 ($p = 0.009$), showing that the laccase is related to discoloration, thus affecting the dye structure, breaking the azo group, and facilitating the partial mineralization of the dye. Maalej-Kammoun *et al.* [3] reported that with high concentrations of laccase, degradation is more effective and fast; on the other hand, they reported that high concentrations of Basic Green 4 could inhibit enzyme production. When the fiber is saturated with the dye prior to fungal immobilization, a gradual adaptation process of the fungi to the dye occurs, allowing the fungi to resist stress and to maintain active metabolism when in contact with the dye in the liquid medium and, in turn, producing laccase. Additionally, the fungi immobilized in a natural support, such as agave fiber, promote the ligninolytic growth conditions of the fungal environment. This explains why the fungus produces high quantities of enzyme that are optimal for the support of degradation and for its use as a carbon source. On the other hand, it is possible that different immobilization processes adhere different amounts of biomass and affect the biocarrier efficiency.

According to results reported by Chhabra *et al.* [48], peaks in the UV/VIS scan are generated due to laccase's activity as a degrading agent of Basic Green 4.They claim that the peaks correspond to N-methylated peaks, di-demethylated forms and dibenzyl methane, and they also found that benzaldehyde was one of the final degradation compounds.

From the toxicity assays, it was observed that prior saturation of the fiber not only improved the amount of discoloration and the enzymatic production but also significantly decreased the toxicity, resulting in a LD_{50} >1000 ppm for *Artemia salina* in the first cycle; however, this characteristic is only observed in cycle 1. For cycle 2, the LD_{50} is 0.7176 ppm. The toxicity of the samples decreased, possibly due to the presence of laccase which eliminates toxic compounds, thus producing simpler compounds and partially mineralizing the compound. Additionally, when the support is saturated, the initial concentrations of the dye in liquid medium could be lower, facilitating the reactor operation and increasing the process efficiency.

4 CONCLUSIONS

Agave fiber is a good adsorbent for Basic Green 4; a pseudo-second order model adequately described the kinetic process of biosorption. The adsorption isotherm follows a Freundlich model, and the results show that the adsorption capacity of agave fiber is similar to that of other lignocellulosic materials. The adsorption of the dye by agave fiber and the biological treatment of the dye using two immobilized white-rot fungi notably diminished the dye's toxicity. The immobilization of fungi on agave fiber is a cheap and effective option for Basic Green 4 dye biodegradation.

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