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THE CONTRIBUTION OF H_2O_2 PRODUCED BY *Aspergillus niger* IN VAT BLUE DYE DISCOLORATION: ENHANCEMENT BY A STATISTICAL OPTIMIZATION METHODOLOGY

CONTRIBUCIÓN DEL H_2O_2 PRODUCIDO POR *Aspergillus niger* EN LA DECOLORACIÓN DE AZUL A LA CUBA: MEJORAMIENTO MEDIANTE UNA METODOLOGÍA DE OPTIMIZACIÓN

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Abstract

The dumping of dyes into bodies of water represents an environmental and health problem due to its toxicity. A solution to this problem could be the application of mycorremediation. So, in this work *Aspergillus niger* CDBB-H-175l was investigated in the discoloration (DC) of vat blue dye. A combination of statistical methodologies was applied to optimize DC; initiating with a 2^{4-1} fractional factorial design (FFD), to evaluate; dye concentration, exposure time, pH and agitation. Results showed a minimum and maximum DC of 15% and 43%, respectively. By using a first-order model DC increased further to 63%. Additional experiments enhanced DC to 90% in step 7 of a steepest ascent design, and this was improved to 94% at a higher concentration of 521 mg/L with the application of surface response methodology. Of the 63% total DC determined in the first-order model, 28% was attributed to the glucose oxidase activity (1.98 U/mL) and the H_2O_2 produced by *A. niger*, and 72% due to physisorption to the cell wall. This is the first report where H_2O_2 produced by *Aspergillus niger* is implicated in dye decoloration.

Keywords: *Aspergillus niger*, vat blue, discoloration, hydrogen peroxide, optimization.

Resumen

El vertimiento de colorantes en cuerpos de agua representa un problema ambiental y de salud, debido a su toxicidad. Una solución a dicho problema podría ser la aplicación de la micorremediación. Por lo que en este trabajo se investigó a *Aspergillus niger* CDBB-H-175 para la decoloración (DC) de azul a la cuba. Para ello, se aplicó una combinación de metodologías estadísticas; iniciando con un diseño factorial fraccionado 2^{4-1} (DFF), para evaluar; la concentración del colorante, tiempo de exposición, pH y la agitación. Los resultados mostraron un mínimo y máximo de DC del 15% y 43%, respectivamente. Usando el modelo de primer orden la DC aumentó a 63%. Con la optimización la DC mejoró a 90% en el paso 7 de la metodología de ascenso más pronunciado, un 94% con 521 mg/L de colorante, empleando una metodología de superficie de respuesta. Del 63% DC total, determinado en el modelo de primer orden, el 28% fue atribuido a la actividad de glucosa oxidasa (1.98 U/ml) y al H_2O_2 producidos por *A. niger*, y el 72% fue debido a fisisorción a la pared celular. Este es el primer reporte en el que el H_2O_2 producido por *Aspergillus niger* está involucrado en la decoloración.

Palabras clave: *Aspergillus niger*, azul a la cuba, decoloración, peróxido de hidrógeno, optimización.

1 Introduction

Wastewater treatment is currently a priority for reducing environmental pollution. Interest in wastewater treatment from the textile industry has escalated (Hachem *et al*., 2001; Lopez *et al*., 2002; Constapel *et al*., 2009) because dyes constitute an important class of pollutants due to their toxicities (Pupo *et al*., 2013; Türgay *et al*., 2011; Muz *et al*., 2017). Industrial wastewater becomes colored as an inevitable result of the dyeing processes since approximately 1-20% of the color is discarded (Venkatesha, 2012).

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The complex aromatic structures of synthetic dyes make them quite stable and therefore more recalcitrant toward biodegradation. Dyes are classified as anionic (direct, acidic, and reactive), cationic (basic) or non-ionic (dispersed) (Srinivasan *et al*., 2010). Dyes that have received the most attention, and that are widely used throughout the world, are the azo types (–N=N–, chromophoric functional group) and anthraquinones (Barrios *et al*., 2015).

They are used industrially to dye cotton and cellulose fibers, generating a health risk due to their secondary metabolites, such as aromatic amines, that have been well-documented as potential carcinogenic agents (Pupo *et al*., 2013; Muz *et al*., 2017). It has also been reported that dyes are the cause of allergies, dermatitis, skin irritation and cancer in humans. In general, the textile, foodstuff, dyeing, printing, electroplating, tanning, cosmetics, and paint industries are the main sources of water pollution due to poor waste management and the release of effluents into the environment (Venkatesha, 2012; Gusain *et al*., 2016).

Vat dyes represent approximately 15% of the total consumption of the textile industry, and they are mainly used in the dyeing of cotton fibers, silks, and celluloses. Anthraquinones are characterized by their condensed molecular structure that is highly and stronger resistant to biodegradation (Pupo *et al*., 2013). They are insoluble in water, but dissolve under alkaline conditions (Sirianuntapiboon *et al*., 2006). Among the dyes currently available, vat blue, also known as indanthrene blue, was the first of the indanthrene dyes prepared by Bonn in 1901. This dye produces coloration that is very stable upon washing and exposure to sunlight, but it does not resist chlorination. Despite its excellent qualities, practically no new anthraquinone-based dyes have been developed during the past 20 years (Chang *et al*., 2009). The high resistance of this dye to microbial treatments and its high stability make it necessary to develop adequate treatment methods to remove it from wastewater without producing other contaminants that may cause environmental damage. During the past 20 years, microorganisms have been extensively used to eliminate dyes from industrial wastewater based on degradation or adsorption processes, and a variety of dyes can be adsorbed by living or inactive microorganisms (Sirianuntapiboon *et al*., 2006). This process is known as biosorption. Variations among the chemical structures of the dyes give rise to various interactions between the dyes and the biosorbents. Fu and Virarahvan (2001a and 2002b) reported the removal of acid blue 29 (anionic), basic blue 9 (cationic), Congo red (anionic) and dispersed red 1 (non-ionic) in liquid media through biosorption by the inactive biomass *Aspergillus niger*. These authors demonstrated that *A. niger* was able to remove the dyes from aqueous solutions, and they suggested that the carboxyl, amino, phosphate, and lipid groups of the *A. niger* cellular biomass play important roles in the biosorption of the dyes (Colak *et al*., 2009). These results showed that the use of biomaterials as sorbents for the treatment of textile wastewater is an alternative to conventional methods of treatment. The ability to remove the dyes and the growing conditions and the physiological age of the fungus that may favor the biosorption process or discoloration (DC) were not documented. However, varying such conditions could affect improvements in the efficiency of the biosorption process.

Therefore, in the present study, the effects of the culture conditions of *A. niger* were investigated to improve the biosorption process of vat blue by an active biomass. For this purpose, a series of experimental statistical designs were used for optimizing the abiotic conditions that generate the best efficiencies of DC in a liquid medium.

2 Materials and method

2.1 Microorganism

The strain *A. niger* CDBB-H-175 was obtained from the National Collection of Microbial Strains and Cell Cultures of the Centro de Investigacion y de Estudios Avanzados del Instituto Politécnico Nacional. This strain was grown on potato dextrose agar (PDA) at 28 °C for 5 days. After the colony growth phase, 6 mm diameter agar disks were added to each 500-mL Erlenmeyer flask with 170 mL of Wunder medium (Wunder *et al*., 1994). The flasks were incubated at 28 °C, 125 rpm for 72 h, and then the biomass was filtered with a 1-mm pore diameter metal filter and the humidity was determined to inoculate 1.5 g of dry weight of biomass per liter.

2.2 Preparation of the dye solution and determination of dye discoloration

The vat blue dye was provided by Químicos y Colorantes SA. de CV.

Table 1. Characteristics of vat blue.

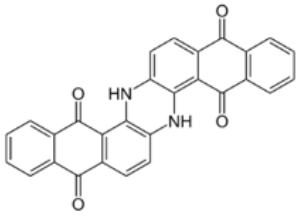
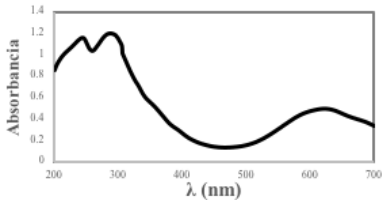
Name: Vat blue Chemical formula: $C_{28}H_{14}N_2O_4$ Water solubility: <0.1 g/100 mL a 21°C	Molecular weight: 442.43 g/mol
Molecular structure	Adsorption spectrum
	

Table 2. Adsorption conditions to be selected and the values established for the biosorption of vat blue on the active mycelium of *A. niger*.

	Levels	
Variable	-1	1
Dye concentration (mg/L)	50	100
pH	3	5
Agitation (rpm)	60	180
Contact time (min)	30	90

The chemical structure, the UV/Vis adsorption spectrum and other specific characteristics of the dye are shown in Table 1. Vat blue is used to dye cotton and rayon fabrics (chemicals and dyes).

The vat blue concentration was determined using a Shimadzu UV-1800 UV spectrophotometer at a wavelength of 630 nm. The percentage of decolorization of vat blue was defined as the difference in the concentration of the dye before and after adsorption and was obtained using equation (1):

$$Discoloration(\%) = \frac{(C_i - C_f)}{C_i} \times 100 \qquad (1)$$

where C_i and C_f are the initial and final concentrations of the dye (mg/L), respectively.

The amount of dye adsorbed by the biomass of *A. niger* at equilibrium was calculated using equation (2):

$$q_e = \frac{(C_i - C_f)}{W} V \qquad (2)$$

where q_e is the amount of dye adsorbed per gram of biosorbent at equilibrium (mg/g), C_i and C_f are the initial and final concentration of the dye (mg/L), respectively, V(L) is the volume of the solution containing the colorant, and W(g) is the weight of the biosorbent used.

2.3 Improvement of the vat blue decolorization capacity of A. niger

2.3.1 Selection of fungi growing conditions

The selection of variables that affect vat blue biosorption by an *A. niger* active fungal biomass was performed by using a 2^{4-1} fractional factorial experimental design (FFD). The variables were selected based on the report by Mustafa *et al*., 2017. Table 2 shows the values proposed for each evaluated variable (independent variables): dye concentration, pH, agitation, and exposure time. The response variable was the percentage of discoloration (DC), the percentage of adsorption (Qe), to a 3 days old active fungal biomass.

The relationship between the culture conditions and discoloration was determined using a generalized linear model (GLM) method, which is a flexible generalization of an ordinary linear regression, with the aim of describing its effect on the biosorption of the dye on the fungal biomass. In the same way, a multiple linear regression method (REG) was applied to determine the culture conditions that affect the discoloration.

Improvement of the ability of the fungus to discolor the dye through the application of a succession of linear models (method of steepest ascent).

Experimental conditions were established to obtain the maximum increase in discoloration, for which a first-order model was obtained by applying a 2^2 factorial experimental design with central points. Once it was known what relationship the selected experimental conditions affected, a new design could

be applied to improve biosorption of the dye. For this, the steepest ascent method was applied, which is a procedure that moves sequentially over the path of steepest ascent. The first-order adjusted model that defines the increase in discoloration is given by $\hat{Y} = \beta_0 + \sum_(i = 1)^{\kappa} \beta_i x_i$ and the first-order response surface, i.e. the contours of $\hat{Y}$, is a series of parallel lines. The direction of steepest ascent is the one where $\hat{Y}$ increases most rapidly. It is usually taken as the path of steepest ascent to the line that passes through the center of the region of interest and that is normal to the adjusted surface. Therefore, increases over the trajectory are proportional to the regression coefficients β_i (Montgomery, 2014).

Determination of the maximum discoloration by a surface response methodology

Once the stationary point of the discoloration of the medium was determined, optimum culture conditions were determined for *A. niger* by using a central composite design (CCD) of a response surface methodology. This consists of a factorial 2^k (+1 and -1) with n_f runs, 2k axial runs or star (±1.414), calculated from equation (3), where α is the axial distance of the point and n is the number of independent variables ($n = 2$), and n_c central runs (0); this methodology is efficient in adjusting a second-order model.

$$\alpha = (2^n)^{1/4} \tag{3}$$

2.4 Statistical analysis

Each experiment was carried out in triplicate. The experimental data were subjected to an analysis of variance (ANOVA). A comparison of means was used via the method of least significant difference (LSD) with an α of 0.05, in which different letters have different statistical significances and the same letters have the same statistical significance. Results are reported as the mean ± the standard deviation. The CCD was estimated using the Design-Expert statistical package version 10, using the natural values.

2.5 Glucose oxidase activity (GOX)

The GOX activity was evaluated by measuring the production of hydrogen from glucose added as a substrate in the assay. For this, a modality coupled with horseradish peroxidase was used, with 2,2'-azino -bis(3-ethylbenzothiazolin-6-sulphonic acid) (ABTS) as the substrate. The absorbance readings were performed at 420 nm and each unit of activity was defined as 1 μmol of hydrogen peroxide generated per minute under standard conditions in the assay.

3 Results and discussion

3.1 Variable selection

The parameters of the culture (dye concentration, pH, agitation, and exposure time) were considered as independent variables of the decolorization process (response variable), which occurred primarily through the biosorption of blue dye to the cells of the biomass of *A. niger*. The selection of the most significant variables for the discoloration was achieved by an FFD with an experimental matrix of eight runs, shown in Table 3, which includes the coded values, predicted values and the capacity of adsorption (Qe). Each experiment was carried out in triplicate.

Table 3. Matrix of the 2^{4-1} fractional factorial design and experimental results.

Run No.	Dye (mg/L)	pH	Agitation (rpm)	Time (min)	Experimental DC (%)	Predicted DC (%)	Qe (mg/g)
1	50 (-1)	3 (-1)	60 (-1)	30 (-1)	15.65d ± 3.75	14.65	4.05f
2	100 (1)	3 (-1)	60 (-1)	90 (1)	36.97b ± 4.1	36.95	16.67bc
3	50 (-1)	5 (1)	60 (-1)	90 (1)	35.90bc ± 3.45	35.89	14.72c
4	100 (1)	5 (1)	60 (-1)	30 (-1)	36.91ab ± 2.38	37.55	27.02a
5	50 (-1)	3 (-1)	180 (1)	90 (1)	30.37c ± 0.63	30.35	8.77e
6	100 (1)	3 (-1)	180 (1)	30 (-1)	43.07a ± 4.87	43.09	19.42b
7	50 (-1)	5 (1)	180 (1)	30 (-1)	20.74d ± 3.48	20.35	8.35d
8	100 (1)	5 (1)	180 (1)	90 (1)	34.17bc ± 3.12	34.45	24.80a

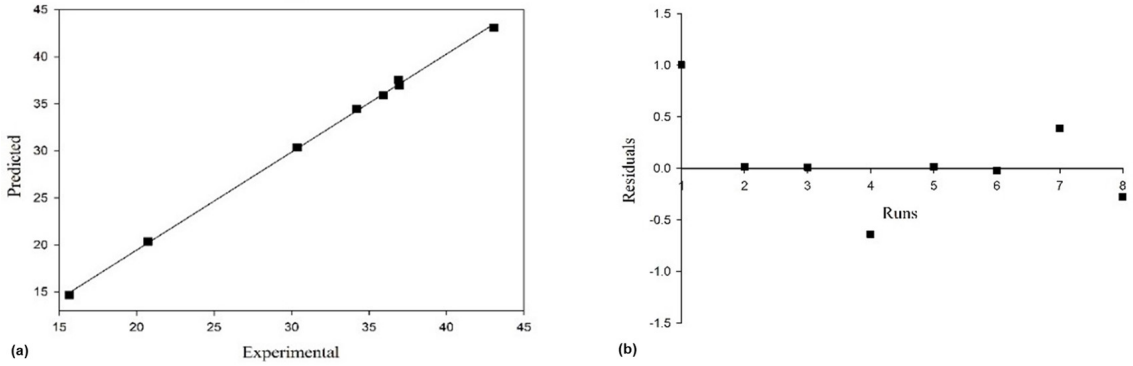


Fig. 1. Residue analysis of the fractional factorial design 2^{4-1}. (a) Experimental vs predicted values; (b) runs vs. residuals.

A 2^{4-1} design was used to select the most significant conditions for the biosorption process, and the highest DC value (43.07%) was obtained in run 6 with 100 mg/L dye concentration, pH 3, 180 rpm, and 30 min exposure time. The relationship between the four independent variables and the percentage of discoloration can be approximated by the following polynomial response model:

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \varepsilon \tag{4}$$

where y is the response variable DC (%); x_i's are the independent variables and ε is the residual term that represents the experimental error. The parameter β_0 is the overall mean of the response (discoloration), which is a constant of the model; β_i is a linear coefficient. The data obtained were adjusted to the general linear model and, by means of a linear regression, the coefficients were determined. From this analysis, the response polynomial resulted:

$$\begin{aligned} Dc(\%) =& 31.66 + 6.35x_1 + 0.4x_2 + 0.4x_3 + 2.75x_4 \\ & - 2.41x_1x_2 + 0.36x_1x_3 - 5.06x_1x_4 + \varepsilon \end{aligned} \tag{5}$$

where x_1 is the dye concentration, x_2 is the pH, x_3 is the agitation and x_4 is the exposure time. As seen in Fig. 1, the residual points estimated by the response polynomial generally fall on a straight line, indicating the error of a normal distribution and validating that the response polynomial fits appropriately with the experimental data (Montgomery, 2014).

An analysis of variance (ANOVA) and an estimation of parameters were performed (Table 4) to indicate the significance and adequacy of the regression, and a coefficient of determination of 0.91 and a coefficient of variation of 10.74 were obtained.

Table 4. Estimation of the parameters of the 2^{4-1} fractional factorial experimental design.

	DF	EP	ES	Value - t	Pr > |t|	SS
Interception	1	31.66	0.69	45.63	**<0.0001**	**a**
x_1	1	6.35	0.69	9.14	**<0.0001**	a
x_2	1	0.39	0.69	0.58	0.5729	b
x_3	1	0.39	0.69	0.57	0.5751	b
x_4	1	2.75	0.69	3.98	**<0.0011**	a
x_1x_2	1	-2.4	0.69	-3.47	**0.0032**	a
x_1x_3	1	0.35	0.69	0.52	0.6135	b
x_1x_4	1	-5.05	0.69	-7.29	**<0.0001**	a

DF, degrees of freedom; EP, estimation of parameters; ES, standard error; SS, statistical significance: a significant, b not significant.

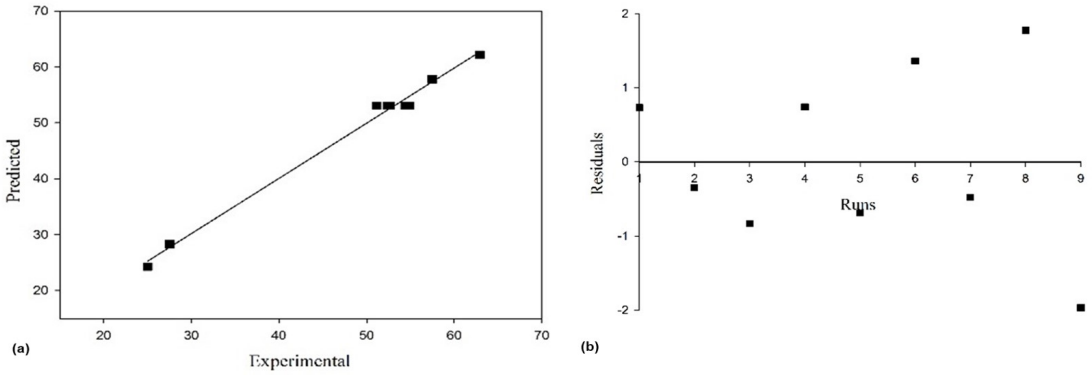


Fig. 2. Residual analysis of the first-order model. (a) Experimental vs predicted; (b) runs vs residuals.

The analysis of variance of the FFD demonstrated that the independent variables that are statistically significant [P <0.05 (variance of 95%)] in the biosorption of vat blue by *A. niger* were the dye concentration, x_1, and the exposure time, x_4, as well as the dye/pH and dye/ exposure time interactions, so x_1 and x_4 were used to obtain the first-order model.

The discoloration of dyes by biosorbents has been linked to agitation because it enhances the mass transfer and the oxygen levels of the cells and the medium (Ali *et al*., 2008). However, agitation in this study was not significant according to the analysis of variance, and this has already been reported for other processes involving the elimination of dyes by the biomes of *A. niger*. One case is the red azo dye, where it was found that agitation was not a significant element in its removal (Mahmoud *et al*., 2017).

The optimization of those variables was achieved using a complete factorial design, which generally describes the approximation of the real surface in a small region of x_i (Montgomery, 2014), which consists of a factorial design 2^2 with 5 central points, using as the central points 100 mg/L and 90 min, respectively. The matrix is shown in Table 5, where the coded values, the predicted values, and the adsorption capacity (Qe) are included. The values of x_2 and x_3 were kept constant according to the model obtained during the selection of variables, so the pH remained at its optimum value of 5 and the agitation at its optimum value of 180 rpm.

The highest percentage of DC was obtained in run 4, where the variables at their optimum values were used, reaching 62% of DC and a Qe of 60.64 mg/g.

Table 5. Matrix for obtaining the first-order model and experimental results.

Run No.	Dye (mg/L)	Time (min)	Experimental DC (%)	Predicted DC (%)	Qe (mg/g)
1	50 (-1)	30 (-1)	25d ± 2.42	24.27	9.23d
2	150 (1)	30 (-1)	57.5b ± 2.42	57.85	55.42b
3	50 (-1)	150 (1)	27.52d ± 3.04	28.35	10.15d
4	150(1)	150 (1)	62.91a ± 1	62.17	60.64a
5	100 (0)	90 (0)	52.4c ± 3.04	53.09	33.65c
6	100 (0)	90 (0)	54.45c ± 3.04	53.09	34.36c
7	100 (0)	90 (0)	52.61c ± 3.04	53.09	33.89c
8	100 (0)	90 (0)	54.86c ± 3.04	53.09	34.86c
9	100 (0)	90 (0)	51.12c ± 3.04	53.09	32.48c

Table 6. Estimation of first-order design parameters.

	DF	EP	ES	Value - t	Pr > |t|	SS
Interception	1	53.08	0.94	56.32	**<0.0001**	**a**
x_1	1	16.84	0.74	22.61	**<0.0001**	a
x_2	1	2.1	0.74	2.83	**0.0222**	a
x_1x_2	1	0.59	0.74	0.8	0.445	b
x_1^2	1	-9.72	1.2	-8.1	**<0.0001**	a

DF, degrees of freedom; EP, estimation of parameters; ES, standard error; SS, statistical significance: a significant, b not significant.

The data obtained were adjusted to the general linear model and, by means of a linear regression, the coefficients were obtained, where x_1 is the color concentration and x_2 is the exposure time and ε is the residual term. Under this analysis the response polynomial was obtained:

$$Dc(\%) = 53.08 + 16.85x_1 + 2.11x_2 - 9.73x_1^2 + \varepsilon \quad (6)$$

Based on the results obtained, a residual analysis was carried out in order to validate the response polynomial and to verify if the model is applicable to predicting DC with a minimum number of experiments. As seen in Fig. 2, the residual points estimated with the response polynomial generally fall on a straight line, indicating the error of a normal distribution and demonstrating that the response polynomial fits appropriately with the experimental data (Montgomery, 2014; Ozturk, 2014).

An analysis of variance (ANOVA) and the estimation of parameters were performed (Table 6) to justify the significance and adequacy of the developed regression, and a coefficient of determination of 0.9865 and a coefficient of variation of 4.47 were obtained.

The analysis of variance of the first-order model demonstrated that the two independent variables, x_1 and x_2, are statistically significant ($P < 0.05$ (variance of 95%)) in the DC of vat blue by the cells of *A. niger*, which is necessary to be able to apply a variable optimization methodology (Montgomery, 2014) such as the steepest ascent or ascent step methodology.

3.2 Method of steepest ascent

Once the first-order model was obtained and it was confirmed that the independent variables were significant for the response variable, the method of steepest ascent was applied, which is a procedure to move sequentially over the path of steepest ascent, i.e. in the direction of increasing response variable (discoloration).

In the linear model it was identified that the concentration of dye and time were significant, with an α of 0.05. The coefficients obtained for x_1 (16.85) and x_2 (2.11) in equation (6) indicated that we had to move 16.85 units in the direction of x_1 for each 2.11 in the direction of x_2 to move away from the center of the design (the point ($x_1 = 0$, $x_2 = 0$) along the path of maximum ascent with a slope of 2.11 / 16.85. Hence:

$$\frac{\alpha}{\alpha} = \frac{16.848}{16.848} = 1 \quad y \quad \frac{\beta}{\alpha} = \frac{2.108}{16.848} = 0.125 \quad (7)$$

For all of the experimental designs, a basic increment in color concentration (x_1) of 50 mg/L and time (x_2) of 60 min was used. Using the relationship between β_i and x_i it was observed that a color concentration of 50 mg/L is equivalent to an increase in the coded variable x_1 of $\Delta x_1 = 1$. Therefore, the increments along the trajectory of ascent were $\Delta x_1 = 1$ and $\Delta x_2 = 0.125 \sim \Delta x_2 = 7.5$ min. These values were used to generate the experimental matrix of the increments to apply to the steepest ascent trajectory in the process of dye DC. The experimental matrix and the results are shown in Table 7, where the increases in coded and natural variables are shown, in which the maximum increase was found in the seventh step, reaching 90% discoloration under the conditions of 450 mg/L of dye and 142.5 min of exposure time. Once the maximum points of the response variable were identified, a response surface methodology was performed as the central composite design.

Table 7. Results of the discoloration of vat blue by the fungus *A. niger* using the experimental design of the steepest ascent trajectory.

	Coded variables		**Naturals variables**		**Results**
Increments	Dye (mg/L)	Time (min)	Dye (mg/L)	Time (min)	DC (%)
0	0	0	100	90	41.65 ± 0.82
Δ	1	0.125	50	7.5	-
1	1	0.125	150	97.5	51.42 ±3.80
2	2	0.25	200	105	59.45 ± 1.48
3	3	0.375	250	112.5	71.62 ± 1.74
4	4	0.5	300	120	72.81 ± 0.96
5	5	0.625	350	127.5	77.34 ± 1.11
6	6	0.75	400	135	81.19 ± 1.48
7	7	0.875	450	142.5	90.85 ± 0.58
8	8	1	500	150	87.11 ± 0.88

3.3 Optimization of variables using the central composite design (CCD)

The CCD is a useful alternative to factorial designs and was originally developed by Box and Wilson and improved by Box and Hunter. This type of design generates as much information as a factorial design with three levels and requires less evidence than a complete factorial design (Aslan, 2008; Wang and Wan, 2009), so that the optimization of the DC process variables of vat blue by *A. niger* was achieved through the application of a response surface design, such as the CCD. This consisted of an experimental matrix of 12 runs, containing four central points (Table 8), which was devised based on the results obtained from the steepest ascent methodology plus the conditions of increments 6, 7, and 8.

Table 8. Experimental design and results of the central composite design.

	Naturals		**Results**		
Run No.	Dye (mg/L)	Time (min)	DC (%)	Predicted	Residuals
1	400 (-1)	136 (-1)	87.24 ± 0.3	86.81	0.42
2	400 (-1)	150 (1)	85.98 ± 0.79	85.55	0.42
3	500 (1)	136 (-1)	90.74 ± 0.5	90.31	0.43
4	500 (1)	150 (1)	92.47 ± 0.59	92.05	0.41
5	450 (0)	143 (0)	91.26 ±1.49	89.77	1.49
6	450 (0)	143 (0)	88.99 ±0.39	89.77	-0.78
7	450 (0)	143 (0)	89.5 ±0.23	89.77	-0.26
8	450 (0)	143 (0)	89.32 ±0.07	89.77	-0.44
9	520.7 (α)	143 (0)	94.06 ±0.43	94.48	-0.42
10	379.3 (-α)	143 (0)	89.39 ±0.48	89.82	-0.42
11	450 (0)	153 (α)	89.85 ±0.14	90.27	-0.42
12	450 (0)	133 (-α)	79.72 ±0.33	80.15	-0.42

Table 9. Estimation of parameters of the CCD.

	SS	DF	MS	Value - F	Pr > F	SE
Model	140.76	7	20.11	17.65	0.0073	a
x_1	10.9	1	10.9	9.57	0.0364	a
x_2	51.31	1	51.31	45.03	0.0026	a
x_1x_2	2.24	1	2.24	1.96	0.2339	b
x_1^2	9.09	1	9.09	7.98	0.0476	a
x_2^2	33.22	1	33.22	29.15	0.0057	a
$x_1^2x_2$	24	1	24	21.06	0.0101	a
$x_1x_2^2$	1.43	1	1.43	1.26	0.3249	b
Residual	4.56	4	1.14			
Lack of fit	1.45	1	1.45	1.4	0.3212	b
Pure Error	3.1	3	1.03			
Cor. Total	145.32	11				

SS, sum of squares; DF, degrees of freedom; MS, mean squares; SE, statistical significance: a significant, b not significant.

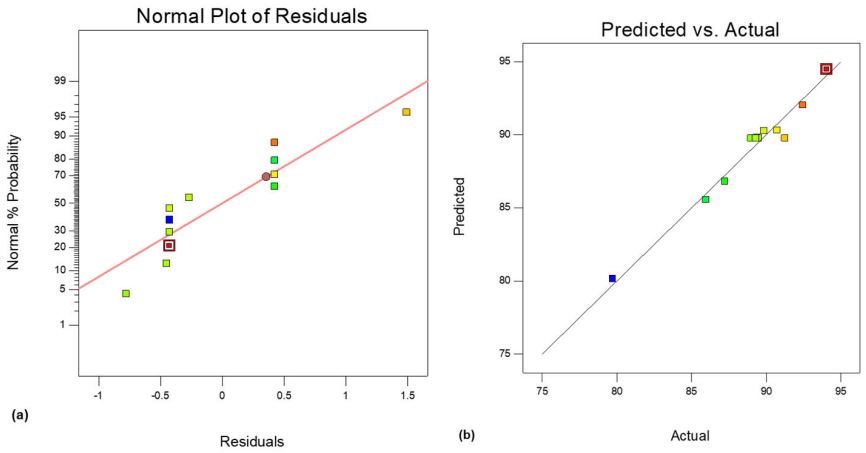


Fig. 3. Analysis of the residuals of the DC. (a) Normal probability of the residuals; (b) predicted vs. actual.

The factors were selected based on the FFD, where the dye concentration and the exposure time were statistically significant, and improved by the steepest ascent methodology, where 90% (DC) was reached for the response variable. The CCD was applied in order to characterize the response surface. This is understood as determining if the maximum point found in the steepest ascent methodology is a stationary point of maximum or minimum response or a saddle point, and the relative sensitivity of the response to the variables x_1 and x_2 (Montgomery, 2014).

The value of α, which gives the response polynomial the property of rotatability, depends on the number of points of the factorial portion of the design, which means how many factors and the factorial portion are involved in the design. So that:

$$\alpha = (nf)^{1/4} = (4)^{1/4} = \sim 1.414 \qquad (8)$$

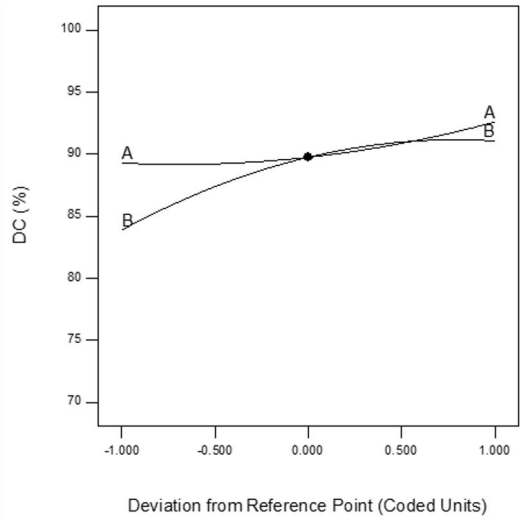


Fig. 4. Effects of the significant parameters on DC in the function of coded values.

Therefore, in the model obtained, the value of α needed to achieve invariability before the rotation is ~1.414, allowing rotatability of the design around the axial points and ensuring that the variance of the prediction of the model is constant for all points equidistant from the center of the design (Aslan, 2008; Wang and Wan, 2009). By means of a multiple linear regression of the experimental data the coefficients were obtained, where x_1 is the dye concentration and x_2 is the exposure time. Under this analysis, a response model was obtained that was of quadratic order including interactions between x_1 and x_2, conferring greater adjustment to the experimental data:

$$Dc(\%) = 89.77 + 1.65x_1 + 3.58x_2 + 0.75x_1x_2 + 1.19x_1^2 - 2.28x_2^2 - 3.46x_1^2x_2 + 0.85x_1x_2^2 + \varepsilon \quad (9)$$

Based on the results obtained, a residual analysis was performed in order to validate the response polynomial obtained and to verify if the model is applicable to the prediction of DC. Although the adjusted model has a correlation coefficient of 0.9686 it was necessary to analyze its normal probability graph for residuals, which exhibits a linear behavior (Fig. 3a). This gives an indication of the error in the normal distribution and validates that the polynomial of response fits appropriately with the experimental data (Sahan and Ozturk, 2014). Figure 3b displays a linear trend for the relationship between the predicted values and the experimental values, confirming non-bias in the measurements; that is, there is no indication of an appropriate correlation in the residuals with a level of significance of 5%.

An analysis of variance (ANOVA) and estimation of the parameters were performed (Table 9) to justify the significance and adequacy of the developed regression, where a coefficient of determination of 0.9686 and a coefficient of variation of 1.2 were obtained when the variables dye concentration and exposure time and their interactions were statistically significant.

In the disturbance diagram obtained (Fig. 4), the effects of the parameters on the DC of vat blue by *A. niger* are shown concurrently. This diagram represents an overview of the effects of the parameters on the results (Najib *et al*., 2017). The slope of line B (exposure time) shows that this parameter had a lesser effect than line A (dye concentration) on the DC response variable.

The variable with the greatest impact on the DC of vat blue by *A. niger* is the dye concentration, where higher dye concentrations are associated with the major percentage of discoloration. In Figs. 5a and b the contour and the surface of the response variable (DC) as a function of the dye concentration (mg/L) and the exposure time (min) are displayed. Very high percentages of discoloration, greater than 90%, were reached when the dye values were high (above 450 mg/L) and the exposure times were short. However, longer exposure times did not increase the percentage of discoloration because the concentration of the dye had a more significant effect.

The DC of vat blue by *A. niger* was adjusted via equation (9), which is valid for a concentration range of 379.3 to 520.7 mg/L and a exposure time of 133 to 153 min at a pH of 5, 180 rpm and room temperature (28 °C ± 1). The stationary point or optimal value for the variable response (DC) was 94% with a dye concentration of 520.78 mg/L and a exposure time of 143 minutes.

3.4 Nature of the discoloration interaction between the dye and the fungus

Dyes are usually degraded by breaking the bonds of the chromophore or auxochromic group. Several physical or chemical treatments have been used to remove dyes in wastewater, but they have not been used in the textile industry, due to their high operating costs and problematic management of their waste (Mahmoud *et al*., 2017).

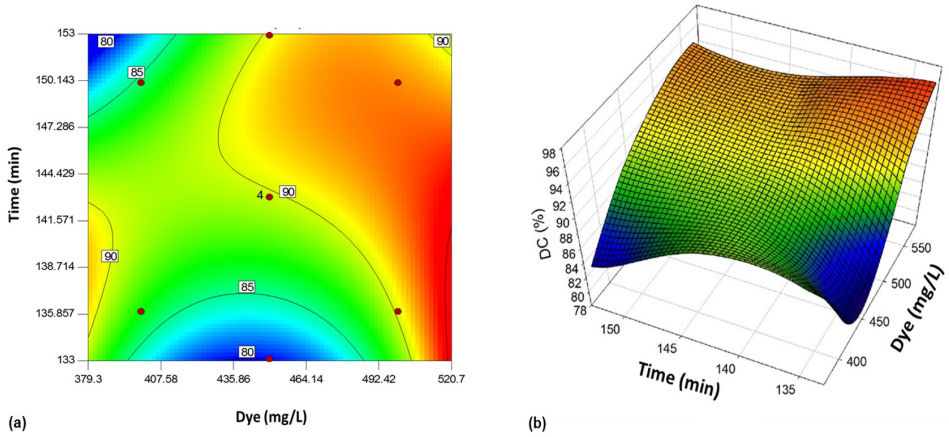


Fig. 5. (a) Contour plot and (b) surface response for vat blue discoloration, as a function of exposure time and dye concentration derived from the central composite experimental design analysis.

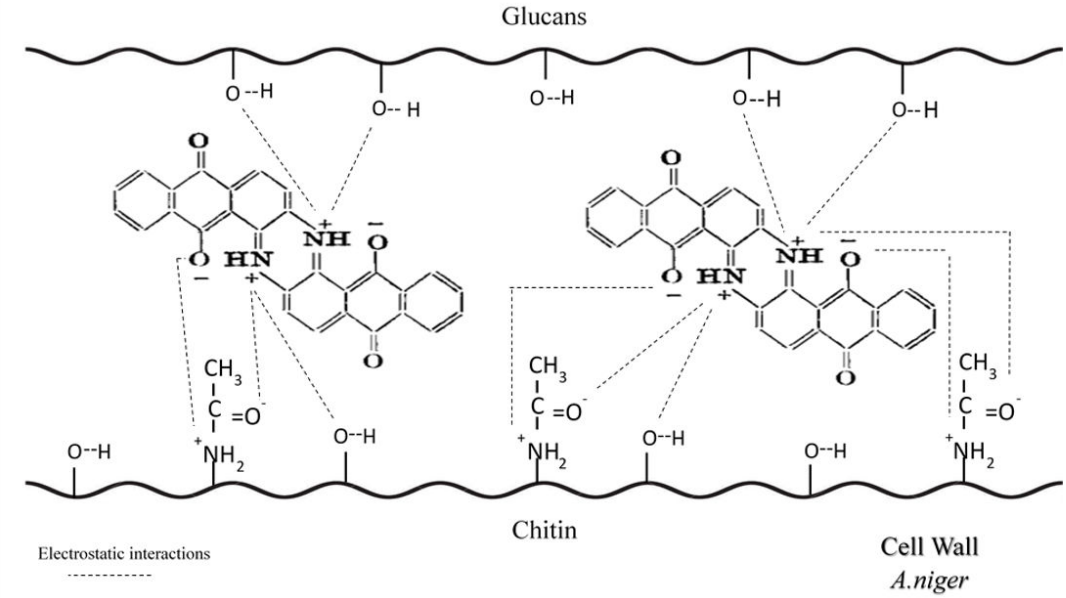


Fig. 6. Proposed process of physisorption of blue vat to *A. niger*.

The mechanisms of biosorption of dyes on biomaterials such as a fungal biomass depend to a large extent on the chemical structure and the functional groups of the type of dye as well as the functional groups on the cell wall of the microbe. These factors play different roles in the biosorption, which can be attributed to a physical or chemical adsorption process or both (Fu *et al*., 2002; Srinivasan *et al*., 2010). There is little information available on the types of interactions between a microbial biomass and dyes, such as surface adsorption, ion exchange, complexation (coordination), complexation/chelation and micro precipitation, that can specifically explain the adsorption mechanisms.

The cell wall of *A. niger* consists mainly of chitin, polysaccharides, carboxyl groups, lipids and amino acids. Electrostatic attractions between the negative charge of the dye and the positive charge of functional groups on the cell wall will promote the biosorption of the dyes (Ali *et al*., 2008). Fu and Viraraghavan in 2002 confirmed that amino and carboxyl groups are the main binding sites, while phosphate groups and lipid fractions are not the main binding sites. It has been reported that, in the process of biosorption to *A.*

niger, the adsorption of dyes can occur in and on the hyphae (Mahmoud *et al*., 2017; Ali *et al*., 2008; Fu, and Viraraghavan, 2001).

Johnston in 1965 conducted a comprehensive study of the composition of the cell wall of *A. niger*, showing that aspartic acid, glutamic acid, glycine, alanine, serine, threonine, leucine, isoleucine, valine, tyrosine, phenylalanine, proline and cysteine were detected. arginine, lysine and histidine; The main sugars found were D-glucose, D-arabinose, D-mannose, D-glucosamine, D-galactosamine and galactose, and acetyl groups made up 3.4% of the cell wall. Evidence was obtained that, as the cells of *A. niger* physiologically aged, the percentages of mannose and galactose decreased, in contrast to glucose, which increased to account for over 90% of the carbohydrate portion of the cell wall. This could be related to the dye adsorption sites corresponding to carboxyl groups (Mahmoud *et al*., 2017; Ali *et al*., 2008; Fu and Viraraghavan, 2001). In this investigation the *A. niger* cells had a physiological age of approximately 80 hours, which meant that the functional groups at this physiological age would not be expected to play an important role in the dye biosorption process.

As shown in Fig. 6, a process of physisorption of the dye to the cell wall is proposed, in which the positive charges of the interacting chitin components of the cell wall are involved and present a possible attraction to the negative charges of the blue dye structure. In the same way the negative charges of the glucans, which are the main components of the cell wall of *A. niger*, might also interact with the positive charges of the blue vat. Both these interactions would be expected to promote the process of physisorption to the cell wall and increase the discoloration of the dye in the liquid phase.

3.5 GOX activity

Once the process of vat blue DC by the biomass of *A. niger* was optimized, the enzymatic activity was quantified in orden to check whether the DC response variable was proportional to the production of hydrogen peroxide (H_2O_2) generated by the activity of GOX in the active biomass or due to interactions of the dye molecules with the structural components of the fungal biomass. The enzymatic activity and the production of H_2O_2 were quantified by the iodide iodate method (Klassen, 1994). Subsequently, the conditions generated for obtaining the first-order model and finally measuring the DC were applied, in which a GOX activity of 1.98 ± 0.16 U/mL and an H_2O_2 concentration of 1.43 ± 0.25 mg/L were obtained in the active biomass, conditions possibly related to the dye DC.

Conclusions

The process of DC of vat blue by the active biomass of *A. niger* is possibly generated by two phenomena: first, the presence of H_2O_2 in the mycelium and, second, biosorption on the cell wall due to structural compounds that might have a fundamental role in the biosorption process of the dyes, causing *A. niger* to have a greater affinity for DC of anthraquinones-type dyes, such as vat dyes.

The FFD allowed an adequate selection of the most significant variables affecting the DC, which were the dye concentration and exposure time. Optimization of the response variables was successfully achieved by obtaining a first-order model that was used in the steepest ascent step methodology and optimizing it using response surface methodology with a rotatable central composite design. The optimal conditions of this model were high concentrations of vat blue (450-500 mg/L) and prolonged exposure times (133-150 min), which gave DC values above 90%, with a maximum of 94%. Of the 64% removed during the application of the first-order model, a contribution of 28% of DC was attributed to glucose oxidase activity, which added to the process of physisorption to the cell wall of the biomass. A GOX activity of 1.98 ± 0.16 U/mL and high concentration of H_2O_2 (1.43 ± 0.25 mg/L) were measured in the active biomass.

The *A. niger* biomass demonstrated a high efficiency for vat blue anthraquinone dye removal, indicating its appropriateness as an alternative adsorbent for the treatment of colored effluents composed of vat dyes.

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