OPTIMIZATION OF REACTIVE BLUE 19 DECOLORIZATION BY *GANODERMA* SP. USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT
Synthetic dyes are extensively used in different industries. Dyes have adverse impacts such as visual effects, chemical oxygen demand, toxicity, mutagenicity and carcinogenicity characteristics. White rot fungi, due to extracellular enzyme system, are capable to degrade dyes and various xenobiotics. The aim of this study was to optimize decolorization of reactive blue 19 (RB19) dye using *Ganoderma* sp. fungus. Response Surface Methodology (RSM) was used to study the effect of independent variables, namely glycerol concentration (15, 20 and 25 g/L), temperature (27, 30 and 33 ^{o}C) and pH (5.5, 6.0 and 6.5) on color removal efficiency in aqueous solution. From RSM-generated model, the optimum conditions for RB19 decolorization were identified to be at temperature of 27^{o}C, glycerol concentration of 19.14 mg/L and pH=6.3. At the optimum conditions, predicted decolorization was 95.3 percent. The confirmatory experiments were conducted and confirmed the results by 94.89% color removal. Thus, this statistical approach enabled to improve reactive blue 19 decolorization process by *Ganoderma* sp. up to 1.27 times higher than non-optimized conditions.

Key words: Dye, Decolorization, Reactive blue 19, *Ganoderma* sp., Response Surface Method

INTRODUCTION
Synthetic dyes are extensively used in many industries. The problems associated with the discharge of colored effluents from various industries such as textile, paper, food, plastics and cosmetics have concerned both industrial and academic scientists (Mahmoodi *et al*., 2009). Approximately, 10000 different dyes and pigments are used industrially, and over 0.7-0.8 million tons of synthetic dyes are produced annually worldwide (Park *et al*., 2007; Revankar and Lele, 2007; Murugesan *et al*., 2007). All dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, many chemicals including oxidizing agents, and microbial attack. During processing, up to 15 percent of the used dyestuffs are related into the process water. Dye-containing effluents are hardly decolorized by conventional biological wastewater treatments. In addition to their visual effect and their adverse impact in terms of chemical oxygen demand, some synthetic dyes cause allergy, dermatitis and skin irritation and they are toxic, mutagenic and carcinogenic in humans (Wesenberg *et al*., 2003; Dos Santos *et al*., 2007; Ofomaja, 2009).

White rot fungi (WRF) are the most efficient ligninolytic organisms capable of degrading various types of dyes such as azo, heterocyclic,

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reactive and polymeric. This capability is due to extracellular non-specific enzyme systems composed of laccases, lignin peroxidases and manganese peroxidases. Laccase catalyze the oxidation of both phenolic and non-phenolic compounds. This ligninolytic system of WRF is directly involved in the degradation of various xenobiotic compounds and dyes. It has been frequently reported that laccase is the main enzyme of *Ganoderma* sp. (D'souza *et al*., 1999; Silva *et al*., 2005; Murugesan *et al*., 2007; Revankar and Lele, 2007; Sarnthima and Khammuang, 2008).
Use of WRF is the most unique technology of bioremediation as their ability to degrade structurally diverse xenobiotic organopollutants is higher. Thus, more technically advanced research efforts are required for searching, exploiting new fungal species and improvement of practical application to propagate the use of fungi for bioremediation of industrial effluents and contaminated soils (Fu and Viraraghavan, 2001; Wesenberg *et al*., 2003; Silva *et al*., 2005; Tavcar *et al*., 2006; Tachibana *et al*., 2007; Tripathi, 2007).
There are many variables or factors affecting enzyme production and decolorization that are expressed by different taxa and culture conditions. These features are important in the process design and optimization of fungal treatment of effluents (Wesenberg *et al*., 2003).
Previous studies about *Ganoderma* sp. has shown this fungus able to decolorize RB19 and fractional factorial design experiments has released that glycerol concentration, temperature and pH are effective variables on color removal efficiency. Thus, the objective of this study was to optimize decolorization of reactive blue 19 (RB19) dye by *Ganoderma* fungus using Response Surface Methology (RSM).
RSM is a very useful tool for this purpose as it provides statistical models which helps in understanding the interactions among the parameters that have been optimized. The advantages of using RSM have been reported to include reduction in number of experimental trials needed to evaluate multiple parameters and the ability of the statistical tool to identity interactions. In addition to analyzing the effects of the independent variables, the experimental methodology also generates a mathematical model that describes the overall process (Montgomery, 2001; Nurdiyana and Siti Mazlina, 2009).

MATERIALS AND METHODS

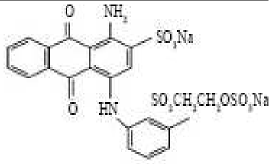
Microorganism

The organism used in this study, *Ganoderma* sp., was purchased from Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology. The stock cultures were maintained on potato dextrose agar (PDA) slants at 4 °C and subcultured at monthly intervals (Teerapatsakul *et al*., 2007).

Chemicals

Chemicals were purchased from Merck and Sigma-Aldrich companies. The characteristics of selected dye, reactive blue 19, are previously given (Rezaee *et al*., 2008), Table 1. It is an anthraquinone dye that constitutes the second most important class of textile dyes, after azo dyes (Dos Santos *et al*., 2007) and frequently used as starting material in the production of polymeric dyes and represents an important class of toxic and recalcitrant organopollutant (Palmieri *et al*., 2005).

Table 1: Main characteristics of RB19

Chemical structure	
C.I. generic name	C.I. Reactive Blue 19
Synonym	Remazol Brilliant Blue R
Molecular Formula	$C_{22}H_{16}O_{11}N_2S_3Na_2$
Molecular Weight	626.5 g/mol
λ_{max}	592 nm

Growth conditions

Precultures were prepared in 250mL flasks containing 150mL potato dextrose broth (PDB). Flasks were autoclaved at 121°C for 15min at 15psi, cooled and inoculated by fungal mycelia, which were grown on PDA for 12 days. Inocula were prepared by washing the mycelia from surface of a PDA slant, by addition of 15 mL sterile distilled water (Azin *et al*., 2008). Inoculated flasks were incubated at 28 ^{o}C for 4 days at 150 rpm to obtain fungal pellets with 1-3mm. 5mL of pellets under suspension condition were used to inoculate each main culture flask containing 45 mL Basal Salt medium and 150 mg/L RB19 (Park *et al*., 2007; Zanirun *et al*., 2009). The concentration of biomass was 1.5±0.165 g/L in each flask.

The basal medium for determination of fungal growth contained (g/L of distilled water): glucose: 20, yeast extract: 2.5, KH2PO4: 1, Na2HPO4: 0.05, MgSO4.7H2O: 0.5, CaCl: 2 0.01, FeSO4.7H2O: 0.01, MnSO4.4H2O: 0.001, ZnSO4.7H2O: 0.001 and CuSO4.5H2O :0.002. The pH was adjusted to 5.5 (Revankar and Lele, 2007).

Analytical methods

Color removal measurement

Color as American Dye Manufacturer Institute (ADMI) value was measured according to EPA Method 110.1. This method is an extension of the Tristimulus Filter Method. Tristimulus values are converted to an ADMI single number color difference, of the same magnitude assigned to platinum-cobalt standards, using the Adams Nickerson Color Difference (DE). Hach DR5000 spectrophotometer was used for ADMI values because standard curves and complex equations have been installed in this instrument (Kao *et al*., 2001). Percentage of decolorization was calculated as follows:

$$\text{Decolorization (\%)} = \left(1 - \frac{\text{ADMI}}{\text{ADMI}_0}\right) \times 100 \quad (1)$$

Where $ADMI_0$ and ADMI are initial and final solution colors.

Enzyme assay

Laccase activity was measured using 0.216 mM syringaldazine as the substrate. The assay mixture (3 mL) contained 2200 µL of phosphate buffer (pH=6.5), 500 µL supernatant, and 300 µL syringaldazine solution. The absorbance increase of assay mixture was monitored at 530 nm at environment temperature (Ride, 1980).

Biomass measurement

Dried-weight biomass was measured by gravimetric method through centrifuging submerged culture at 11000 rpm for 10 min, and then the pellets incubated at 65^{o}C for 48 h (Demain and Davis, 1999).

Experimental design

Response Surface Methodology (RSM) was used in this study to determine the optimum conditions for the color removal. The experimental design and statistical analysis were performed using MiniTab software. The experiments were based on a Box-Behnken design with a quadratic model in order to study the combined effects of three independent variables (glycerol concentration, temperature and pH).

The proposed factors and levels were obtained from screening experiments of 10 initial variables (i.e. type of carbon source, carbon source concentration, nitrogen source concentration, $CuSO_4$ concentration, temperature, ethanol concentration, inoculum volume, pH, shaker speed and dye concentration) using 2-level fractional factorial experimental design for color removal in preliminary stage of research.

The three selected variables were represented by X_1, X_2 and X_3, respectively. Each independent variable were coded in 3 levels which were -1, 0 and +1, as shown in Table 2. The optimization experiments were based on 15 combinations with two replicates. Table 3 represents the design matrix of the trials experiments. All experimental designs were randomized to exclude any bias.

Table 2: Independent variables and their coded levels used for the optimization of RB19 decolorization by *Ganoderma* sp.

Key	Factor	Levels		
		Low	Medium	High
		-1	0	+1
X_1	Glycerol concentration (g/L)	15	20	25
X_2	Temperature (°C)	27	30	33
X_3	pH	5.5	6.0	6.5

Table 3: Three factors in three levels Box-Behnken design used for the optimization of RB19 decolorization by *Ganoderma* sp.

Run	X_1	X_2	X_3	Decolorization (%)	Run	X_1	X_2	X_3	Decolorization (%)
1	-1	-1	0	95.1	16	0	-1	+1	94.3
2	0	0	0	92.9	17	-1	0	+1	92.7
3	0	-1	+1	94.4	18	0	+1	+1	89.1
4	+1	-1	0	94.6	19	0	+1	+1	88.2
5	+1	0	+1	93.5	20	+1	+1	0	89.9
6	+1	-1	0	94.9	21	0	0	0	93.4
7	+1	0	-1	89.8	22	-1	-1	0	94.4
8	-1	+1	0	89.0	23	0	+1	-1	87.1
9	+1	0	+1	92.8	24	0	0	0	93.0
10	0	-1	-1	92.6	25	0	0	0	92.6
11	0	+1	-1	87.9	26	-1	+1	0	89.4
12	0	0	0	92.1	27	+1	+1	0	89.1
13	-1	0	-1	90.0	28	0	-1	-1	92.3
14	-1	0	-1	88.8	29	0	0	0	92.3
15	+1	0	-1	88.3	30	-1	0	+1	93.3

RESULTS

Initial decolorization and fungal growth

To investigate growth rate and time course of color removal, 13 samples as two-replicated of basal medium incorporated to 150 mg/L RB19 dye were inoculated by 5 mL suspension fungal pellets as above-mentioned. Percent of color removal and laccase activity by *Ganoderma* sp. have are represented in Fig.1. In the basal medium, maximum color removal achieved 75.4% after 5 days. Fig.2 shows laccase production and growth curve of fungus. This figure represents that laccase production starts in secondary growth phase of fungus. Buswell, Heinzkill and Wesenberg have reported ligninolytic systems of WRF were mainly activated during secondary metabolic phase and triggered by nitrogen concentration or when carbon or sulfur became limiting (Merwe, 2002; Wesenberg, 2003).

The initial decolorization of RB19 was low compared to previous reports. Hence, in order to improve color removal by *Ganoderma* sp., a RSM experimental design was applied for investigation of the relationship between process variables to optimize decolorization efficiency.

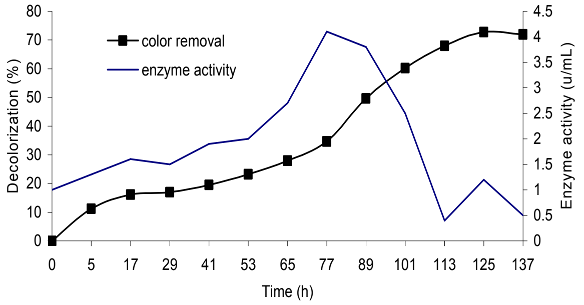


Fig. 1: Color removal and laccase activity in basal medium by *Ganoderma* sp.

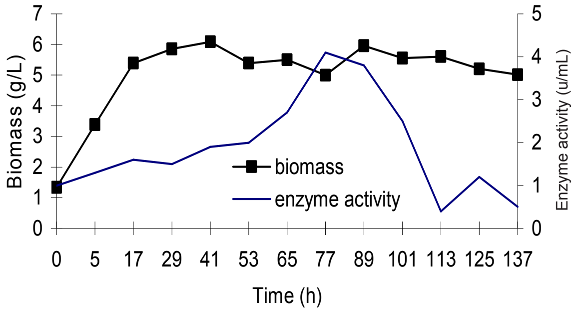


Fig. 2: *Ganoderma* sp. growth curve and laccase activity in basal medium

Box-Behnken experimental design

By the design of Box-Behnken (Table 2) followed by 30 trial experiments (Table 3), color removal efficiency varied from 87.1% to 95.1% in the 15 different combinations with duplication. Thus, the best conditions in conducted experiments were the process with 15 g/L glycerol, 27°C and pH=6.0. The maximum decolorization achieved was with 100 mg/L dye after 5 days.

Table 4 shows the analysis of variance (ANOVA) of the results for the decolorization. The linear and quadratic effects of variables was significant ($p<0.0001$), while there was no significant interaction ($p<0.798$).

In order to analyze optimum and statistically significant factors and interactions, a second order (quadratic) polynomial equation fitted the experimental data for decolorization by *Ganoderma* sp., was constructed with a multiple correlation coefficient (R^2) of 0.94.

Table 4: The analysis of variance of optimization experimental design for decolorization of RB19 by *Ganoderma* sp.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	162.842	162.842	18.0935	34.73	0.000
Linear	3	145.262	145.262	48.4208	92.95	0.000
Square	3	17.045	17.045	5.6818	10.91	0.000
Interaction	3	0.534	0.534	0.1779	0.34	0.796
Residual error	20	10.418	10.418	0.5209		
Lack-of-fit	3	5.335	5.335	1.7783	5.95	0.006
Pure error	17	5.083	5.083	0.2920		
Total	29	173.260				

Decolorization (%) =

$$92.717 - 2.7 X_2 + 1.338 X_3 - 0.115 X_1^2 - 0.515 X_2^2 - 1.465 X_3^2 + 0.113 X_1 X_2 + 0.138 X_1 X_3 - 0.188 X_2 X_3. \quad (2)$$

Where X is the coded value (between -1 and +1) for the factor indicated by attached subscript in Table 2. The coefficients of temperature (linear), pH (linear and quadratic) were statistically significant at a level of $p<0.0001$; however, no interactions were statistically significant.

Fig. 3 shows the response surface plots described by the regression model and drawn to illustrate relationships between factors on decolorization efficiency under the sets of conditions and treatment levels tested. Results of using different glycerol concentrations at three levels of 15, 20 and 25 g/L, with temperatures of 27, 30 and 33°C, at which the pH was controlled at 5.5, 6.0 and 6.5, showed that glycerol concentration, 15 to 20 g/L, temperature at 27 °C and the pH at 6 to 6.5 were efficient values for color removal by fungus.

Comparison of the observed versus predicted yields is shown in Fig.3. The points above or below the diagonal line represent areas of over- or under-prediction of the model. This showed that no significant violations of the model were found in the analysis, with 94% correlation of the model with the experimental data obtained.

Optimal condition for color removal by *Ganoderma* sp. suggested by the Box-Behnken design was glycerol concentration of 19.14 g/L as carbon source, temperature=27 °C and pH=6.26 with 95.3% color removal. To confirm the optimal condition predicted for the decolorization of RB19, a set of duplicated experiments using the optimal combination of the independent variables was conducted. The highest decolorization efficiency by fungus was as high as 94.89% (Fig. 4) which was 1.26 times higher than the non-optimized conditions. Fig. 4 also shows that laccase activity in optimal combination has improved and was as high as 5.1 u/mL.

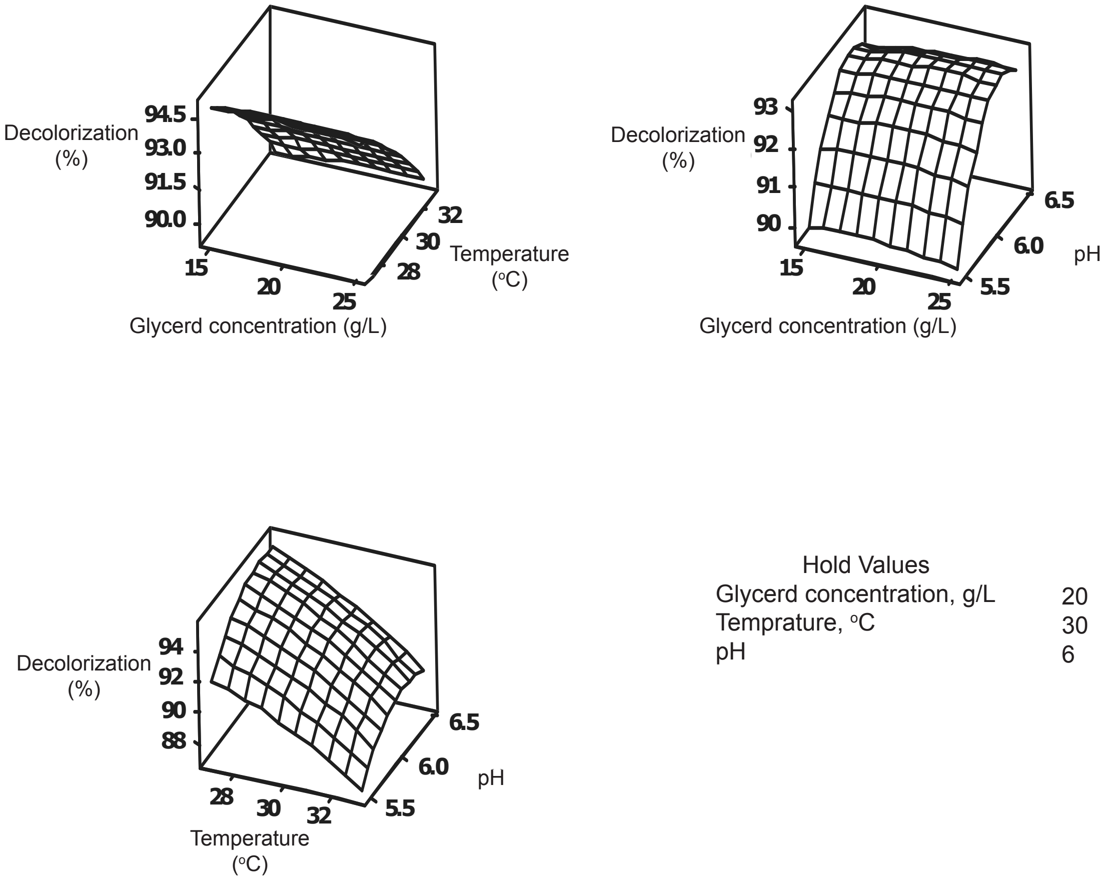


Fig. 3: Response surface plots showing the effect of independent variables on RB19 decolorization (response) by *Ganoderma* sp.

DISCUSSION

Most of the previous studies have focused on *Phanerochaete chrysosporium* and *Trametes versicolor*. There has been a growing interest in studying ability of a wide array of white rot fungi for use in various biotechnological applications. Hence, in the present research, *Ganoderma* sp. was explored for its color removal ability.

This study considered three independent variables with three levels on RB19 decolorization of *Ganoderma* sp.. To achieve the results obtained in this study using a full factorial design would have required $3^3 \times 2$ replicates experiments taking into account all the factors involved. By using Box-Behnken design, a significantly smaller combination of factors and levels could be used for effectively examining the effect of interacting factors on color removal. Thus, only a limited number of experiments (30) were suggested. Optimal conditions showed 1.26 times increase in decolorization efficiency and laccase activity compared to the non-optimized conditions. The color removal and enzyme activity of fungus achieved in this work were upto 94.89% and 5.1 u/mL, respectively, that represented a significant improvement and demonstrating success in using statistical design of Box-Behnken.

Results of this study led us to consider pH and temperature of culturing. Both had significant effect on decolorization. The first had linear and quadratic effect, and second had linear effect. Effect of glycerol concentration as carbon source was not statistically significant. Also, there was no significant interaction between factors.

Revankar and Lele (2007) reported 73% decolorization of RB19 in 100 mg/L dye in 8 hours by *Ganoderma* sp. WR-1 that was less efficiency (but higher rate) than this study. They optimized

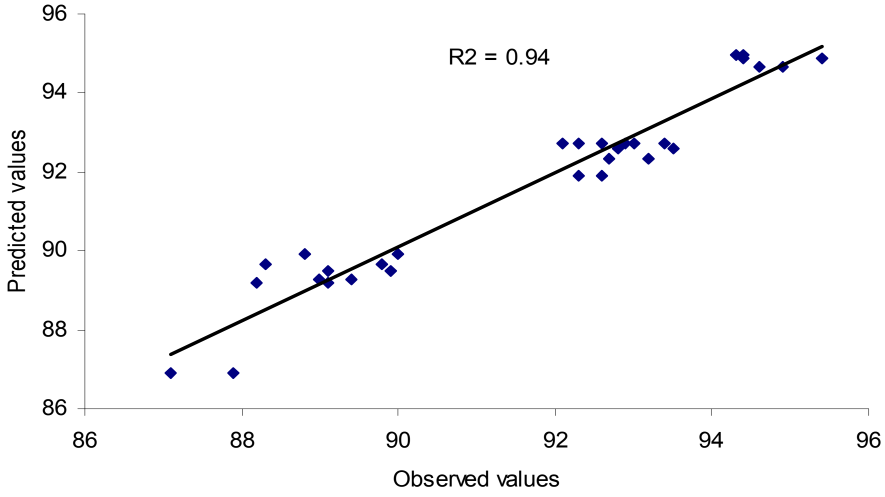


Fig. 4: Observation and prediction of RB19 decolorization by *Ganoderma* sp. calculated with the model

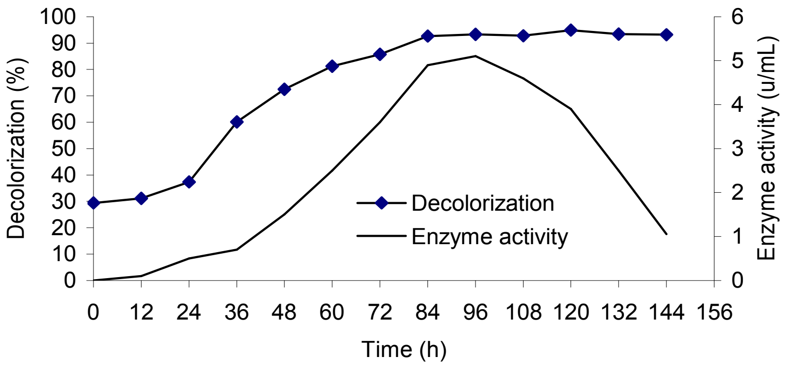


Fig. 5: Confirmatory run using the optimal conditions for RB19 decolorization by *Ganoderma* sp., suggested by experimental design

process conditions using Taguchi orthogonal array experimental design and obtained the best conditions as 2% starch (carbon source), 0.125% yeast extract (nitrogen source) and 0.0001% $CuSO_4$ (inducer) for decolorization. The pH and temperature were 5.5 and 28 °C, respectively.

Palmieri *et al.,* (2005) reported decolorization of RBBR (RB19) obtained in optimized liquid culture of *Pleurotus ostreatus*.

In their study, total color removal was achieved after nine days that was longer than 5 days of this study.

Novotny *et al*., (2004) have shown dye decolorization with *Irpex lactus* in 3 to 5 days of incubation. Mazmaci *et al*., (2002) have reported the decolorization of methylen blue in 8 days. Thus, Ganoderma in this work was better than other strains in the extent of decolorization.

Asgher *et al* (2008) referred to many reports that decolorization of RB19 was carried out by a variety of white-rot fungi. In most cases laccase was responsible for color removal.

According to the results of this study and other reports, it is clear that decolorization ability of white rot fungi can be substantially increased by carefully optimizing the operational conditions such as nutrient content of the media culture, age of fungus and environmental/operational conditions. Toh *et al* (2003) have also clearly shown the difference in decolorization ability of different fungi for different dyes (Erkurt *et al*., 2007).

This study showed the use of statistical optimization tools and response surface methodology (RSM) enables and helps finding the optimum levels of the most significant variables for color removal with minimum effort and time. By this method, decolorization of RB19 dye achieved as high as 95% after 5 days, while, it has been reported that the half-life of hydrolyzed RB19 is about 46 years at pH=7 and 25°C (Dos Santos *et al*., 2007).

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