



## Original Article

## Evaluation of Basic Blue 41 Removal from Aqueous Solutions by Laccase Mediated System Using Response Surface Methodology

Seyed Davoud Ashrafi<sup>1</sup>, Hossein Kamani<sup>2</sup>, Gholam Hossein Safari<sup>3\*</sup>

<sup>1</sup> Department of Environmental Health Engineering, Research Center of Health and Environment, School of Health, Guilan University of Medical Sciences, Rasht, Iran

<sup>2</sup> Health Promotion Research Center, Zahedan University of Medical Sciences, Zahedan, Iran

<sup>3</sup> Health and Environment Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

\* Corresponding author: Gholam Hossein Safari

Email: hsafari13@yahoo.com

## ABSTRACT

**Background:** Dyes are one of the most important pollutants found in the sewage of many industries, including; textile, printing, wood and paper industries, tanning, cosmetics and are often toxic, carcinogenic, mutagenic and non-biodegradable. This study aimed to investigate the removal of Basic Blue 41 (BB41) by the oxidation process by using laccase in presence of ABTS mediator.

**Methods:** The present study is an experimental in vitro study that investigated the main and interaction effects of three variables of pH, mediator concentration and laccase activity in three levels on BB41 removal efficiency by Response Surface Methodology (RSM), based on Box-Behnken design.

**Results:** According to the results of the dye removal experiments, in the solution pH of 5, 0.2 mM of ABTS and the 0.2 U mL<sup>-1</sup> of laccase, BB41 was not observed in the tested sample solution and the final solution was completely colorless. Also, the statistical analysis of the data obtained from the experiments and the results of the analysis of variance showed that the model was statistically significant (P-value < 0.0001) and could reliably predict BB41 removal efficiency by laccase enzyme.

**Conclusion:** This study found that laccase enzyme can be used to remove BB41 dye from aqueous solutions under optimum conditions designed by RSM.

**Keywords:** Enzyme, laccase, Dye, Basic Blue 41, Response Surface Methodology

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## Introduction

Dyes are one of the most important pollutants in the environment and water resources. Dyes are used in various industries such as textile, printing, wood and paper, tanning, cosmetics, food, plating and etc. As a result, wastewater produced in these industries contains varying concentrations of dyes. Among these industries, the textile industry has the most consumption of dye (1, 2). Discharging this type of wastewater into the environment can pollute the

groundwater and soil, consequently cause various diseases (3, 4). Almost one million tons of different dyes are synthesized worldwide each year. Recent surveys show that 15 to 20% of the consumed dyes in these industries enter sewage. One of these types of wastewater is the textile industry wastewaters and high pigmentation, suspended particles, high pH and Chemical Oxygen Demand (COD) are their most important characteristics (5-7).

Synthetic dyes, especially azo dyes, often have high

molecular weight, complex and aromatic structure. They are also toxic, mutagenic and carcinogenic as well as Highly resistant to sunlight and chemical degradation and disrupt the performance of conventional filtration systems (8, 9). The discharge of these wastewater not only affect the aesthetic aspect of the receiving water but also causes severe contamination of groundwater, soil and various diseases, preventing the transmission of sunlight into aquatic environments, resulting in reduced photosynthetic processes. As a result, the amount of dissolved oxygen in the river is reduced. These dyes increase the amount of consumed chlorine for chlorination of water and threaten the lives of aquatic animals and humans (10).

Various methods such as biological methods, chemical and physical processes or a combination of them have been used to remove dyes from aqueous solutions including coagulation or combination with flotation, filtration, sedimentation, electrocoagulation, ozone oxidation, irradiation and electrochemical processes, often referred to as electrochemical processes which majority of them are expensive (11-15). Adsorption is also one of the most widely used methods for removing pollutants including a variety of dyes from wastewater (16). In recent years, the bioaccumulation process has been extensively studied to remove industrial pollutants and has used microorganisms such as algae, fungi, bacteria, yeast, and actinomycetes (17, 18). Also, the population of microorganisms, which are naturally present in wastewater, can use soluble and colloidal biodegradable organic matter, including some dyes, as their food for growth and replication (19).

Enzymes are biocatalysts in the form of proteins that catalyze the chemical reactions of cells of living organisms. Different enzymes are used in process and non-process industries such as detergent, wood and paper industry, textile, leather, petroleum industry, wastewater treatment and detoxification (20-25).

Laccases are kind of enzymes that is capable of single-electron oxidation of many phenolic and non-phenolic organic substrates (26). The laccases are the most abundant extracellular polyphenol oxidases and also copper-containing oxidase. Although laccases preferentially oxidize phenolic compounds, they can oxidize a wide range of compounds including ortho-diphenols, para-diphenols aminophenol, benzo-thiols, polyphenols, polyamines and some diamines. They can also oxidize many organic and inorganic compounds (26, 27). In recent years, due to the advantages of enzymatic treatment of toxic and resistant contaminants over other conventional methods, it has attracted the attention of many researchers (28-30). Therefore, in this study, we investigated the removal of industrial azo dye BB41 as the most commonly used dyes from aqueous solutions using laccase enzyme extracted from white root fungus (*Trametes versicolor*). We also estimated the effect of parameters influencing the process of dye removal.

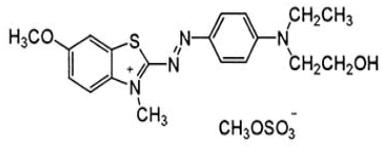
## Methods

### Chemicals

The enzyme used in this study was laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) extracted from

*Trametes versicolor* with activity levels of  $> 10 \text{ U mg}^{-1}$ . 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), laccase and BB41 dye were prepared from Sigma-Aldrich. The characteristics of BB41 used in this study are summarized in Table 1. Other chemical substances that applied in this study had the highest laboratory purity.

**Table 1.** Characteristic of BASIC BLUE 41 Dye Used in This Research

Parameter	Characteristic
Chemical name	BASIC BLUE 41
C.I. number	11105
Classification	Monoazo
Apparent colour	Blue
Molecular weight	482.57
Molecular formula	$\text{C}_{20}\text{H}_{26}\text{N}_4\text{O}_6\text{S}_2$
$\lambda_{\text{max}}$ (nm)	608
Chemical structure	

### Assay of laccase activity

The enzymatic activity of laccase is defined as the amount of enzyme that is required to produce one micromole of the oxidized ABTS, (2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)), within one minute and expressed in unit per milligram or ml (14-18). Since ABTS has a high molar absorption coefficient ( $36000 \text{ M}^{-1} \text{ cm}^{-1}$ ), meaning that it can have a high optical absorption at a lower concentration (31). Therefore, it is an accurate substrate for measuring enzyme activity showing more activity of enzyme. The standard method was used to measure laccase activity at pH level of 4.5 and ABTS substrate solution 2 mM at  $40^\circ \text{C}$ . Briefly, 1 ml of laccase solution was added to 1 ml of ABTS solution and ABTS oxidation was controlled for 10 minutes at 420 nm and then laccase activity was estimated (32).

### BB41 removal experiments

All experiments were performed in test tubes and in small volumes (5 ml). The reaction temperature variable was studied at different levels ( $20 - 60^\circ \text{C}$ ), and finally the optimum temperature of  $40^\circ \text{C}$  was used for other experiments. The retention time variable was studied at different levels (10-90 min), and finally the optimum time of 30 min was applied to achieve maximum efficiency for other experiments. PH variable evaluated at 3 different levels of 3, 5 and 7 and mediator concentration variable at 3 different levels of 0.05, 0.125 and 0.2 mM. In addition to this, the laccase at 3 different levels of 0.05, 0.125 and 0.2 (units per ml) were evaluated. After the reaction, the samples were used for BB41 spectrophotometry at 608 nm after smoothing and the residual dye concentration was read according to the calibration curve created. Finally, the dye removal efficiency was calculated in each phase of the experiment through the following equation:

$$\frac{(C_0 - C_e)}{C_0} * 100 = (\%) \text{ removal efficiency}$$

Where,  $C_0$  and  $C_e$  represent the initial and final concentrations of dye, respectively.

**Table 2.** Levels and Values of Independent Box Design Variables

Independent variables	Unit	Factors	Actual and coded values		
			-1	0	1
Initial pH	-	A	3	5	7
Laccase enzyme dose	U mL <sup>-1</sup>	B	0.05	0.125	0.2
ABTS dose	mM	C	0.05	0.125	0.2

*Design of experiments and statistical analysis of data*

In this study, the analysis of data and design of experiments were performed by response surface method based on Box-Behnken (33). Table 2 demonstrates three independent variables including pH, laccase dose, and ABTS mediator dose at three levels of high (+1), average (0) and low (-1) as determined by Design-expert version 7.0.0 (Stat-Ease, trial version).

**Results**

*Experimental design and dye removal*

According to the factors involved in the design of the experiment and having 3 replications at the central points, we had 15 runs and a total of 45 samples were tested (three replicates). Table 3 shows the design matrix with response surface method in three levels with for three studied variables based on the Box-Behnken, along with the obtained results from the experiments and predicted by the model and residuals.

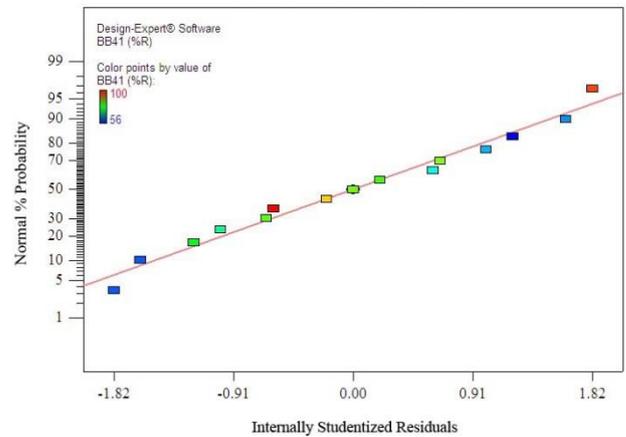
Figure 1 illustrates the results of the probability of the data being normal versus the studentized residual values. The analysis of the probability distribution of the residuals with a 95% confidence shows that the data obtained from the experiments have a normal distribution.

*Analysis of variance*

Analysis of variance was used to evaluate the effect of the studied variables on the removal of BB41 dye by laccase. According to the results of ANOVA (Table 4), the model obtained with F-value of 87.8 and P-value less than 0.0001, R<sup>2</sup> 0.9937 as well as Adjusted R-Squared = 0.9824 indicating a reliable model for the prediction of efficiency of BB41 removal by laccase enzyme. As depicted in Table 4, the direct effects of all three studied variables on BB41 dye removal efficiency were statistically significant (P-value < 0.05). While the interaction effects of AB and AC variables on dye removal efficiency were statistically

significant (P-value < 0.05). But, the interaction of BC variables on dye removal efficiency was not statistically significant (P-value = 0.0366). Also, the F-value of 5.06 associated with “lack of fit” indicates that lack of fit is not significant to pure error.

The results of data analysis on the relationship between BB41 removal percentages and the values predicted by the model are shown in Figure 2. Given that the removal efficiency values obtained from the experiments are close to the values calculated by the model and also the R<sup>2</sup> is high (0.9937), therefore, it can be concluded that the obtained model can predict the values of BB41 removal efficiency.



**Figure 1.** Normal probability of internally studentized residuals

Using response surface methodology by replacing the coefficients of the factors that are statistically significant in the quadratic polynomial model, the following model was obtained as a predictive model of BB41 removal efficiency.

$$(\%)R = +83-5.94A+5.69b+10.88C-2.62AB-5.25AC-15.44A^2-3.69B^2+5.69C^2$$

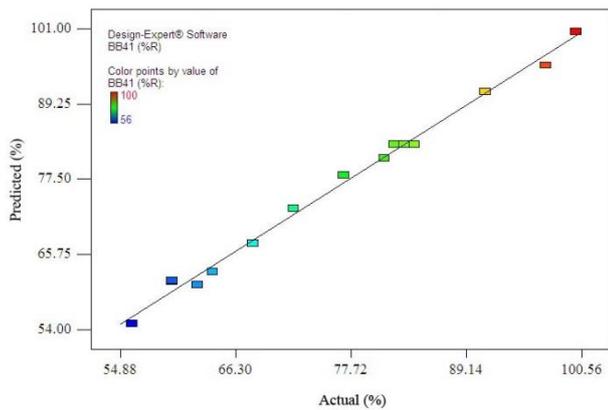
**Table 3.** Experimental Design Matrix at Different Levels of Variables in BB41 Removal

Run	Levels			Observed	Response	
	A	B	C		Predicted	Residuals
1	3	0.05	0.125	60	61.500	-1.500
2	7	0.05	0.125	56	54.875	1.125
3	3	0.2	0.125	77	78.125	-1.125
4	7	0.2	0.125	62.5	61.000	1.500
5	3	0.125	0.05	64	63.062	0.937
6	7	0.125	0.05	60	61.687	-1.687
7	3	0.125	0.2	97	95.312	1.687
8	7	0.125	0.2	72	72.937	-0.937
9	5	0.05	0.05	68	67.437	0.562
10	5	0.2	0.05	81	80.812	0.187
11	5	0.05	0.2	91	91.187	-0.187
12	5	0.2	0.2	100	100.562	-0.562
13	5	0.125	0.125	82	83.000	-1.000
14	5	0.125	0.125	84	83.000	1.000
15	5	0.125	0.125	83	83.000	0.000

**Table 4.** Analysis of Variance of Data Obtained from BB41 Removal by Laccase Enzyme

Source	df	SS	MS	F Value	p-value
Model	9	2717.6	301.9	87.8	< 0.0001
A	1	282.0	282.0	82.0	0.0003
B	1	258.7	258.7	75.2	0.0003
C	1	946.1	946.1	275.2	< 0.0001
AB	1	27.5	27.5	8.0	0.0366
AC	1	110.2	110.2	32.0	0.0024
BC	1	4	4	1.1	0.3300
A <sup>2</sup>	1	879.9	879.9	255.98	< 0.0001
B <sup>2</sup>	1	50.2	50.2	14.6	0.0124
C <sup>2</sup>	1	119.4	119.4	34.7	0.0020
Residual	5	17.1	3.4		
Lack of fit	3	15.1	5.0	5.0	0.1694
Pure error	2	2	1		
Cor total	14	2734.8			

R-Squared = 0.9937; Adjusted R-Squared = 0.9824; Adequate precision = 30.1

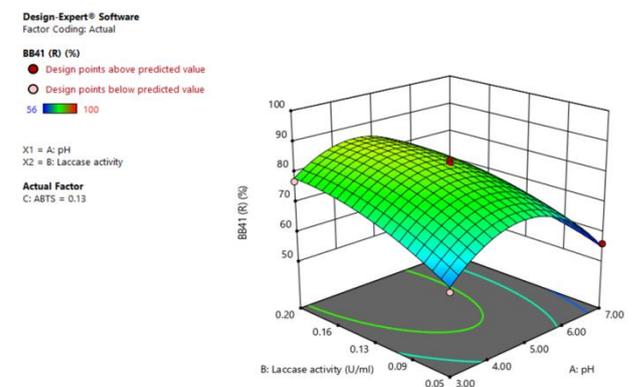


**Figure 2.** Plot of BB41 removal values obtained from the experiments and the values predicted by the model

This equation is based on the coded values of the factors in which R is the removal efficiency predicted by models and A, B and C, are the initial pH of the solution, the laccase enzyme activity, and the ABTS mediator dose, respectively. Figure 3 shows the response surface plot of the interaction effect of the pH and laccase enzyme activity on BB41 dye removal efficiency. As can be observed in this figure, increasing the pH of the solution until about 5 the removal efficiency of BB41 dye will increase. However further pH increasing, the dye removal efficiency will decrease. The result that shows dyes removal or other organic substance by the enzyme at relatively acidic pH has the highest efficiency than alkaline or acidic pH has been further confirmed in other studies (34, 35). It can also be concluded that increasing the laccase activity at pH 5 raises the removal efficiency of BB41 dye more strongly than when the pH is below or above 5. According to the results of ANOVA (Table 4), the interaction effect of these two parameters on BB41 dye removal was statistically significant (p-value, 0.0366).

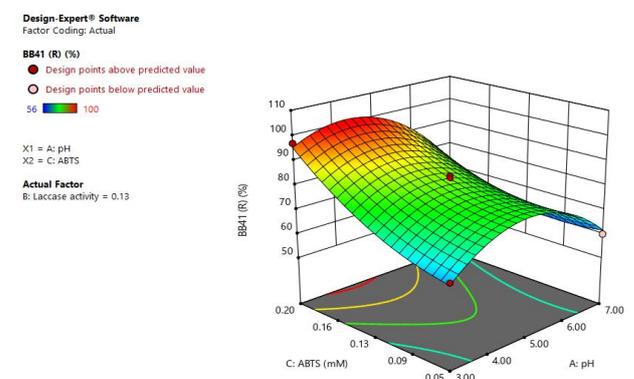
Figure 4 shows the response surface plot of the interaction effect of the pH factor and ABTS mediator on BB41 removal efficiency. As can be observed in this figure, the highest dye removal efficiency is obtained at pH 5, and by increasing or decreasing the pH of this amount, the dye removal efficiency decreases. It can also be concluded that at pH 5 the BB41 dye removal efficiency is more affected by ABTS and the dye removal efficiency is increased to a greater extent. According to the results of ANOVA (Table 4),

since the P-value of interaction of these two parameters is less than 0.05 (0.0024), the interaction of these two parameters on BB41 removal was statistically significant.

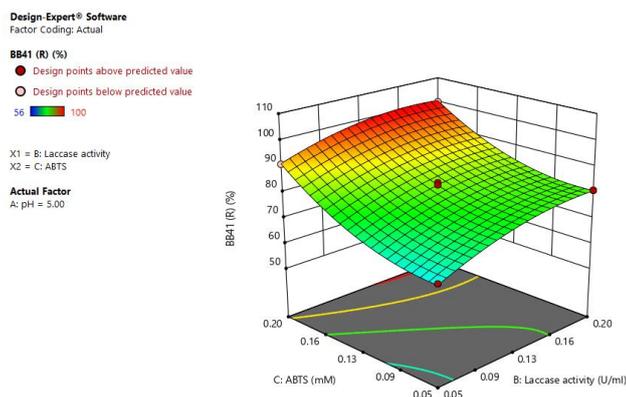


**Figure 3.** Response surface plot of interaction effect of pH and laccase activity on BB41 removal efficiency

Figure 5 shows the response surface plot of the interaction effect of the studied factors of Laccase enzyme and ABTS on BB41 removal efficiency. As can be observed in this figure, increasing the amount of laccase enzyme increases BB41 removal efficiency, whereas this increase does not raise significantly with increasing ABTS. According to the results of the ANOVA (Table 4), since the P-value of the interaction of these two parameters was greater than 0.05 (0.3300), the interaction of these two parameters on BB41 removal was not statistically significant.



**Figure 4.** Response surface plot of interaction effect of pH and ABTS mediator on BB41 removal efficiency



**Figure 5.** Response surface plot of interaction effect of laccase and ABTS mediator on BB41 removal Efficiency.

### Discussion

A wide variety of different processes have been used to remove dyes from aqueous solutions. Each of them can have its advantages and disadvantages. Laccase, through its Type 1 copper, oxidases BB41 and transfers the electron to its other copper and reduction of  $O_2$  to  $H_2O$  is done (36). In this study, the response surface methodology based on Box-Behnken model was used and the effect of studied variables on dye removal by laccase enzyme was evaluated. The advantages of this method of study is the need to fewer sample number for the survey and consequently fewer study time and low cost are needed for this method of investigation. In addition to evaluating the main effects of factors on dye removal efficiency, the interaction effects of factors were also investigated. The results and statistical analysis revealed that all three studied variables of initial pH, amount of laccase enzyme and ABTS had a significant main effects on the removal of BB41 by laccase enzyme. From the results of ANOVA, it can be seen that the factor of C (ABTS dose) have the greatest effect on the removal of BB41, with the highest F-value of 275.2. So, it indicates that the ABTS dose has a positive and great effect on the removal of BB41. This is in agreement with the finding of Ashrafi et al (34). Furthermore, the interaction effects between the studied pH and activity of laccase enzyme as well as ABTS mediator on BB41 removal efficiency was statistically significant. However, the interaction between laccase enzyme activity and ABTS mediator was not statistically significant. The results of various studies show that Laccases are capable of eliminating a wide range of dyes (9, 37, 38).

### Conclusion

Removal of dyes from aqueous solutions using laccase enzyme represents a suitable, effective and environmentally friendly method compared to other methods. This study investigated the ability of laccase enzyme for BB41 removal. Experiments were made as a function of initial pH (3-7), laccase enzyme dose (0.05-0.2) and ABTS dose (0.05-0.2). Response surface methodology based on Box-Behnken model was used to survey the role of factors on BB41 removal. The Response surface methodology based on Box-Behnken design can be used to develop mathematical models for estimating BB41 removal by

laccase enzyme. The present study found that laccase enzyme is capable of BB41 removing from aqueous solutions. It was shown that quadratic regression model could properly interpret the experimental data with  $R^2$  value of 0.9937 and this value of  $R^2$  indicates the high correlation between observed and predicted values.

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### Ethical consideration

The study protocol has been approved by institutional Review Board of Guilan University of Medical Sciences, Rasht, Iran.

### Conflicts of interests

Authors declared no conflict of interest.

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