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Optimization of the enzymatic elimination of flumequine by laccase-mediated system using response surface methodology

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ABSTRACT

Response surface methodology (RSM) was applied to optimize the removal of flumequine (FLU) from aqueous solution by laccase from *Trametes versicolor*. Box–Behnken design (BBD) with four variables namely pH, temperature, FLU initial concentration, and 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) concentration was used to optimize these factors. The results showed that the predicted values for FLU removal were close to the experimental values, and the R^2 (0.9967) indicated that the regression was able to give a good prediction of response for the FLU removal process in the studied range. Optimization of the factors levels was carried out in two approaches. At first, all factors were set in the studied range. The selected optimal conditions for the maximum removal of FLU (98.27%) were predicted as: temperature of 39.64°C, pH of 4.06, FLU initial concentration of 90.74 mg L^{-1}, and ABTS concentration of 1.35 mM. Then, the optimization was carried out by minimizing the amount of ABTS, maximizing the FLU initial concentration, and maximizing the removal efficiency. The recommended optimum conditions are: temperature of 39.19°C, pH value of 4.32, FLU initial concentration of 125 mg L^{-1}, and ABTS concentration of 0.28 mM with the percentage FLU removal of 77.76%.

Keywords: Box–Behnken Design; Flumequine; Fluoroquinolone; Laccase; Optimization

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1. Introduction

During the recent years, it has become evident that the antibiotics widely used in human and veterinary medicine are being introduced into the environment [1]. Fluoroquinolone (FQ) is a group of strong antibiotics that are extensively used in human and veterinary medicines [2]. FLU is a synthetic FQ that is commonly used in aquaculture as prophylactics to prevent diseases [3], also it is very effective in treating urinary, pulmonary, and digestive tract infections, while activity against some Gram-positive and Gram-negative micro-organisms has also been reported [4,5]. The low bioavailability may result in high concentrations of FLU residues in the aquatic environment [3]. On the other hand, the removal of FLU in wastewater by conventional wastewater treatment plant is often incomplete [6] and residues of these compounds often appear in the environment with the potential of adversely affecting aquatic and terrestrial organisms.

Several treatment techniques involving photocatalyzed-doped TiO_2 [7], adsorption [2], electro-Fenton and photoelectro-Fenton [8], Fenton and photo-Fenton [6], and oxidation by chlorine dioxide [9] have been recently applied to the elimination of FLU from aqueous solution. Enzymatic treatment is an eco-friendly process for treating contaminants [10–12]. Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multi-copper blue oxidizes widely distributed in higher plants, fungi, insect, and bacteria. Laccases catalyze the oxidation of a wide range of substrates such as ortho- and para-diphenols, aromatic amines, phenolic acids, methoxy-substituted phenols, and several other compounds coupled to the reduction of molecular oxygen to water. The range of compounds oxidized by laccase can be expanded in the presence of appropriate redox mediators such as 1-hydroxybenzotriazole, violuric acid, and ABTS [10,13,14]. In recent studies, laccase was successfully used for the elimination of synthetics dye [15,16], antibiotics [13,17], triclosan [18–20], anthracene [21], benzodiazepines [22], pesticide [23], and several other contaminants from aqueous solution.

The enzymatic process like pollutant removal by laccase-mediated system has some limitations in its application such as relatively high production cost, and susceptibility to environmental factors like pH and temperature [24,25]. On the other hand, the ABTS is the most efficient laccase mediators; the drawbacks of ABTS are its high cost and the potential toxicity. In order to overcome these problems in this application, efforts must be made to optimize these factors that affect the process to maximize the removal efficiency.

Response surface methodology (RSM) can be utilized as a powerful statistical tool in order to optimize the contaminant removal, and reduce process variability, with few times, number of experiments, and overall cost. On the other hand, the statistical design is very useful to determine the main and interaction effects of variables [26]. Many studies concerning the laccase removal of pollutants have indicated that temperature, pH, initial pollutant concentration, mediator, and other experimental parameters influence the removal efficiency [14,19,22,27,28]. So, the aim of this work was to find out how pH, temperature, initial concentration of FLU, and ABTS influence FLU removal efficiency from aqueous solution by laccase-mediated system, and the optimization of FLU removal process and introducing a mathematical model for it, RSM based on BBD had been used.

2. Materials and methods

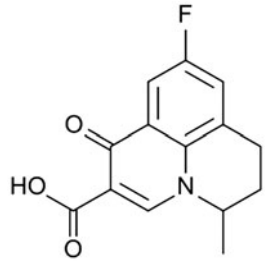
2.1. Chemicals

Laccase [EC 1.10.3.2, *p*-bezenediol:dioxygen oxidoreductases] from *Trametes versicolor* (>10 U mg^{-1}), 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and flumequine (see Table 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used which were in the highest available grades.

2.2. Activity assay

The laccase activity was assayed using a UV–visible spectrophotometer (UVD 2950, Labomed, Culver City,

Table 1
General characteristic of FLU

Parameter	Characteristic
Chemical name	Flumequine
CAS number	42,835−25−6
Class	Fluoroquinolone
Molecular weight	261.25 g mol^{-1}
Molecular formula	$C_{14}H_{12}FNO_3$
Maximum wavelength	248 nm
Chemical structure	

USA) with ABTS as a substrate (2 mM) in 0.1-M citrate sodium buffer at pH 4.5. To measure the laccase activity, 1 mL of the enzyme solution added to 1 mL of the ABTS solution and incubated at 40˚C. The change in absorbance at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1}) was recorded for 10 min and the catalytic activity was determined. One unit (U) was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min [29,30].

2.3. HPLC analysis of FLU

The concentrations of FLU were measured using a high-performance liquid chromatography (HPLC). HPLC consists of a Knauer LPG pump, an EZ-chrom HPLC system manager program with a UV–visible diode array detector (k-2500) set at the maximum absorption wavelength of 248 nm. A column of MZ-analysentechnik ODS-3 C18 (0.46 cm × 25 cm) packed with 5-μm spherical particles was used for separation. The samples were injected manually using a model SGE injection valve (SGE, Australia). The water, methanol, and acetonitrile, (40:30:30 v:v:v) mixture was used as a mobile phase at 30˚C with a constant flow rate of 1.0 mL min^{-1}. The FLU retention time was 7.8 min and the concentrations of the FLU were calculated based on the peak area of known standards.

2.4. Batch removal experiments

In order to study the ability of the laccase for removal of FLU, elimination experiments were carried out in a batch reactor. FLU stock (500 mg L^{-1}) was prepared in citrate sodium buffer (0.1 M) and appropriate dilutions (prepared by citrate sodium buffer) of this stock were used for elimination experiments. The removal studies were started by adding laccase (final activity 6 U mL^{-1}) to the reaction solutions (final volume of 3 mL) according to the pH, temperature, FLU initial concentrations, and ABTS, which are given in Tables 2 and 3, 150 rpm under dark for 2 h. The reaction tubes (Eppendorf 15 mL) were removed after 2 h and the reaction was stopped immediately by adding 3 mL of HPLC grade methanol, and stored at −20˚C for later HPLC analyze. Prior to determine the remained concentration of FLU, the samples filtered through 0.45-μm membranes and then measured using HPLC. Control samples were maintained with heat-inactivated laccase. The removal efficiency was calculated using the following Eq. (1):

$$\text{Removal efficiency } (\%) = 100 \times \frac{C_0 - C_t}{C_0} \quad (1)$$

where C_t is the FLU concentration (mg L^{-1}) at the end of the reaction and C_0 is the initial concentration of FLU.

2.5. Experimental design and data analysis

The experimental design was done using BBD according to Ferreira [26]. The experimental data (Table 3) were analyzed by the response surface regression procedure to fit the following quadratic polynomial model, (Eq. (2)):

$$R = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 AB + \beta_6 AC + \beta_7 AD + \beta_8 BC + \beta_9 BD + \beta_{10} CD + \beta_{11} A^2 + \beta_{12} B^2 + \beta_{13} C^2 + \beta_{14} D^2 \quad (2)$$

where R is the predicted percentage removal of FLU, β_0 is the intercept of quadratic polynomial model, β_{1-14} is the estimated coefficients, and A, B, C, and D is the coded variables in Table 2.

The number of runs (n) necessary to obtain this quadratic polynomial equation is depend on the number of variables, which is given by the expression: $n = 2X(X-1) + Y$, where X is the number of variables and Y is the number of replicates at the center point [26]. So, this plan consists of 27 runs for four variables with three levels (−1, 0, and +1), as A, B, C, and D, are summarized in Table 2. The data were processed using the Design-Expert version 7.0.0 (Stat-Ease, trial version) and MINITAB 14 (PA, USA) computer

Table 2
Variable ranges and levels using BBD

Independent variables	Unit	Coded symbol	Range and level of actual and coded values		
			−1	0	1
pH	–	A	3	4.5	6
Temperature	˚C	B	25	40	55
FLU initial concentration	mg L^{-1}	C	25	75	125
ABTS concentration	mM	D	0.2	0.8	1.4

Table 3
BBD matrix of variables along with actual, predicted, and residual values

	Actual values of variables				Removal efficiency (%)		
Std.	A	B	C	D	Actual values	Predicted values	Residual values
1	3	25	75	0.8	65	65.45	−0.45
2	6	25	75	0.8	44	44.70	−0.70
3	3	55	75	0.8	72	70.87	1.13
4	6	55	75	0.8	57	56.12	0.88
5	4.5	40	25	0.2	79	77.79	1.21
6	4.5	40	125	0.2	76	74.87	1.13
7	4.5	40	25	1.4	98	98.70	−0.70
8	4.5	40	125	1.4	90	90.79	−0.79
9	3	40	75	0.2	57	57.83	−0.83
10	6	40	75	0.2	47	46.83	0.17
11	3	40	75	1.4	83.5	83.00	0.50
12	6	40	75	1.4	60	58.50	1.50
13	4.5	25	25	0.8	79	78.66	0.34
14	4.5	55	25	0.8	93.5	93.33	0.17
15	4.5	25	125	0.8	80	79.50	0.50
16	4.5	55	125	0.8	82	81.66	0.34
17	3	40	25	0.8	74	74.12	−0.12
18	6	40	25	0.8	57	57.87	−0.87
19	3	40	125	0.8	70	70.20	−0.20
20	6	40	125	0.8	50	50.95	−0.95
21	4.5	25	75	0.2	70	70.12	−0.12
22	4.5	55	75	0.2	73	74.54	−1.54
23	4.5	25	75	1.4	85	84.54	0.46
24	4.5	55	75	1.4	96	96.95	−0.95
25	4.5	40	75	0.8	93	95.13	−2.13
26	4.5	40	75	0.8	95.9	95.13	0.77
27	4.5	40	75	0.8	96.5	95.13	1.37

programs for performing the response surface studies and analysis of variance (ANOVA).

3. Results and discussion

3.1. RSM–BBD removal experiments

Table 3 shows the design matrix used in the four factors BBD, along with the percent of actual, predicted, and residual values for FLU removal by laccase-mediated system. The removal percent was found to range from 44 to 98% and the significant effect of each factor on removal percent was evaluated by a normal probability plot of residuals, parity plot, and interaction plots at 5% significance level using the Design-Expert version 7.0.0 (Stat-Ease, trial version) and MINITAB 14 (PA, USA) software.

Generally, it is important to assess the data from experiments coming from a normal distribution. The checking of the normality assumption can be made by plotting a normal probability plot of the residuals as given in Fig. 1(a). The normality assumption is satisfied if the residuals plot fairly close to a straight line and consequently the data are reliable. To validate the model further, the residuals were calculated (Table 3).

As shown in Fig. 1(a), all the values lie within the range of −2.13 and +1.5 (values between −3 and +3 being the acceptable limit), thereby validating the model. Also, the Fig. 1(b) shows the internally studentized residuals for all predicted data in this work and confirms that they are in the reliable range. This finding is supported by parity plot comparing the FLU removal data with the model predictions (Fig. 2). The predicted removal efficiency from the model at 95% confidence interval (CI) was also compared to the experimental removal efficiency. The high R^2 (0.9967) indicates the model is able to give a reasonably good estimate of response for the FLU removal by laccase-mediated system in the studied range. The maximum experimental response value was 98% removal, whereas its predicted value was 98.7% indicating a strong agreement between them (Table 3).

As shown in Fig. 3, the Box–Cox plot of a natural log (Ln) of the residual sum of square against lambda

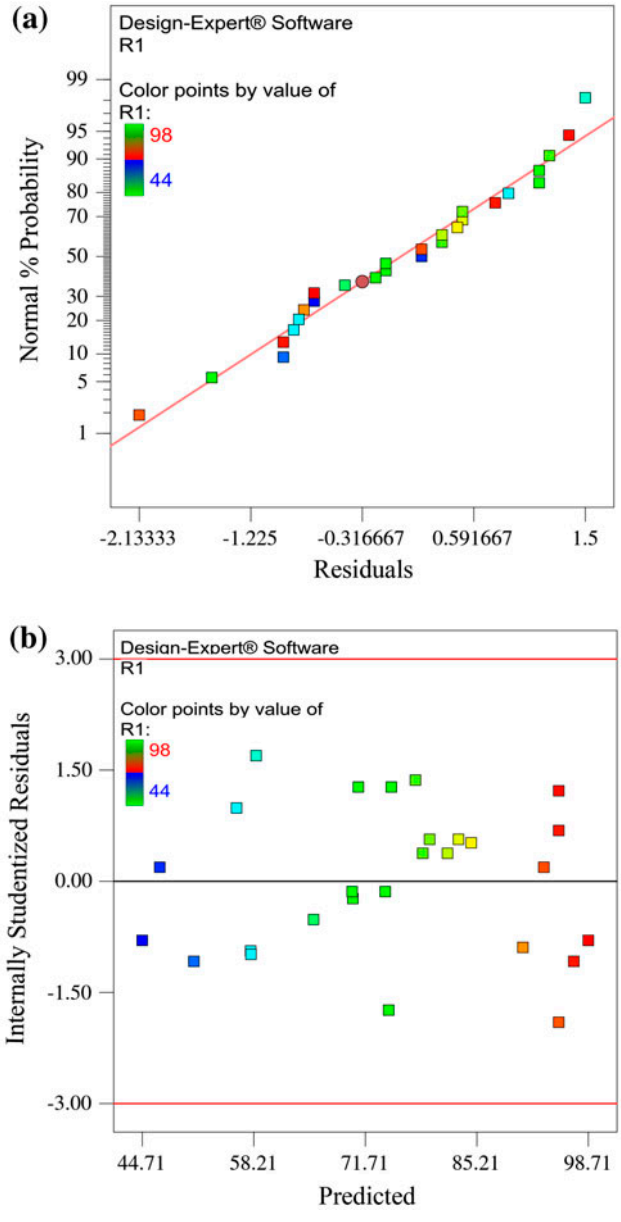


Fig. 1. Normal probability plots of residuals (a) and internally studentized residuals vs. predicted (b).

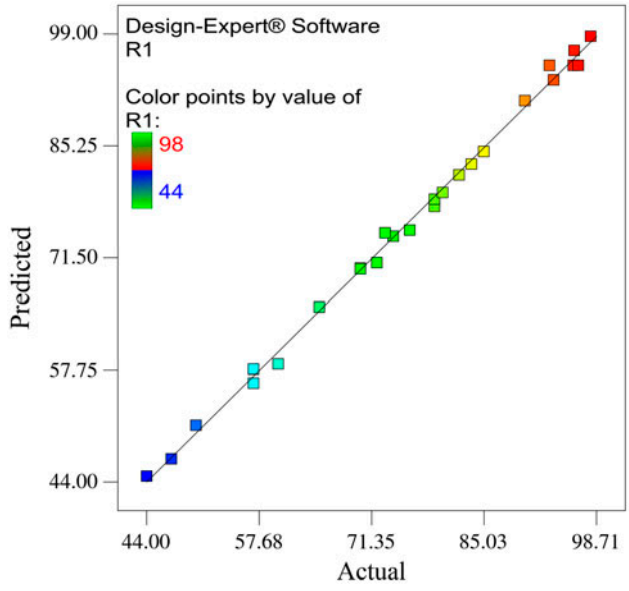


Fig. 2. Comparison of model predictions with the experimental data.

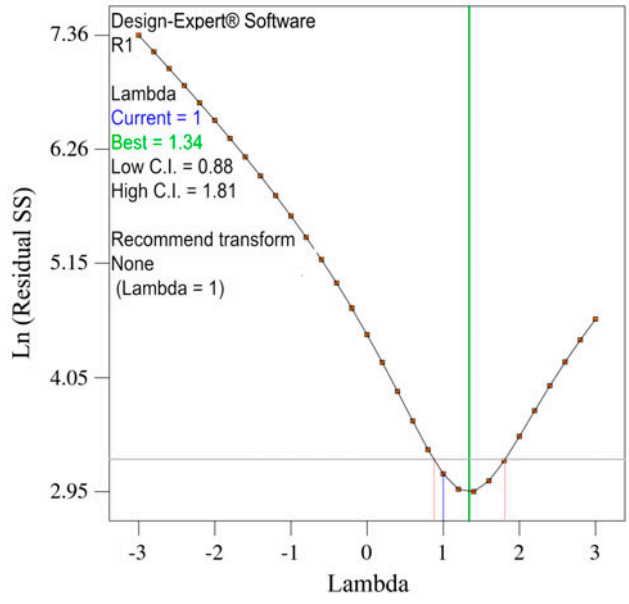


Fig. 3. Box-Cox plot of model transformation for FLU removal by laccase-mediated system.

was plotted. In ideal conditions, the value of current lambda must be between low and high CI [31]. According to this figure, the low CI value is 0.88 and the high value is 1.81, and the current point of CI ($\lambda = 1$), lies close to model design value (1.34), indicating no transformation of the model required.

3.2. Analysis of variance (ANOVA)

Determination of the significant main and interaction effects of variables affecting the removal efficiency was performed by applying an ANOVA. The main and interaction effects of each variable having p-values <0.05 are considered as statically significant. As shown in Table 4, the main effects of all variables and the interaction effects of all factors except AC and CD are statically significant. So by substituting the coefficients of significant factors by their values in Eq. (2) we get Eq. (3):

Table 4
Analysis of variance (ANOVA) for the fitted quadratic model of FLU removal

Source	Degrees of freedom	Sum of squares	Mean square	*F*-value	*p*-value Prob. > *F*	Status
Model	14	6,747.87	481.99	256.03	<0.0001	Significant
A	1	945.18	945.18	502.09	<0.0001	Significant
B	1	212.52	212.52	112.89	<0.0001	Significant
C	1	88.02	88.02	46.75	<0.0001	Significant
D	1	1,017.52	1,017.52	540.51	<0.0001	Significant
AB	1	9.00	9.00	4.78	0.0493	Significant
AC	1	2.25	2.25	1.19	0.2957	Insignificant
AD	1	45.56	45.56	24.20	0.0004	Significant
BC	1	39.06	39.06	20.75	0.0007	Significant
BD	1	16.00	16.00	8.50	0.0130	Significant
CD	1	6.25	6.25	3.32	0.0934	Insignificant
A^2	1	4,157.72	4,157.72	2,208.61	<0.0001	Significant
B^2	1	334.61	334.61	177.74	<0.0001	Significant
C^2	1	81.98	81.98	43.55	<0.0001	Significant
D^2	1	171.51	171.51	91.10	<0.0001	Significant
Residual error	12	22.59	1.88			
Lack of fit	10	15.58	1.55	0.44	0.8438	Insignificant
Pure error	2	7.00	3.50			
Core total	26	6,770.46				

$$R = +95.13 - 8.87A + 4.21B + 2.71C + 9.21D + 1.50AB - 3.38AD - 3.13BC + 2BD - 27.92A^2 - 7.92B^2 - 3.92C^2 - 5.67D^2 \quad (3)$$

where R is the predicted percentage of FLU removal, A, B, C, and D is the coded variables in Table 2.

As shown in Table 4, the results showed that the quadratic polynomial model, Eq. (3), is statistically significant and sufficient to represent the actual relationship between the efficiency of FLU removal and the significant variables, with p-value < 0.0001 and $R^2 = 0.9967$. For additional validation of the model, adjusted R^2 was used for confirming the model adequacy. The adjusted R^2 was calculated to be 0.9928. The values of R^2 and adjusted R^2 are close to 1 that advocates a high correlation between the actual and the predicted values. The p-value (0.8438) of the lack of fit which is greater than 0.05 indicates that the lack of fit for the regression is insignificant. Adequate precision as a measurement of signal to noise ratio greater than 4 is desirable [32]. According to ANOVA, it is 52.803 for this regression. These values of lack of fit, p-value, and adequate precision verified that model is significant for the removal of FLU.

3.3. Main and interaction effects of variables

It can be seen from Table 4, the main effect of all four factors is statically high significant with <0.0001 of p-value, and the ABTS concentration found to have the greatest effect on the removal efficiency of FLU, with the highest F-value of 540.51, followed by pH with the F-value of 502.09. The third effective factor on removal efficiency was the temperature with F-value of 112.89, and the FLU initial concentration was the latest effective factor with lower F-value (46.75) on the FLU removal efficiency.

The interaction effect between the four variables on the percentage of FLU removal studied in this work can be described using RSM. To investigate the interaction effect of pH and other factors, the RSM was used and results were shown in the form of 3D surface plots (see Fig. 4(a–c)). According to Table 4, the interaction effect between pH and temperature was significant due to their p-value lower than 0.05 (0.0493). Fig. 4(a) represents the effect of pH and temperature in fixed average FLU concentration (75 mg L^{-1}) and ABTS (0.8 mM) on the removal efficiency. As there was an increase in pH, the removal efficiency increased with an increase in temperature up to the optimum level. These figures indicate that the best condition for the removal of FLU by laccase-mediated system was at temperatures between 40 and

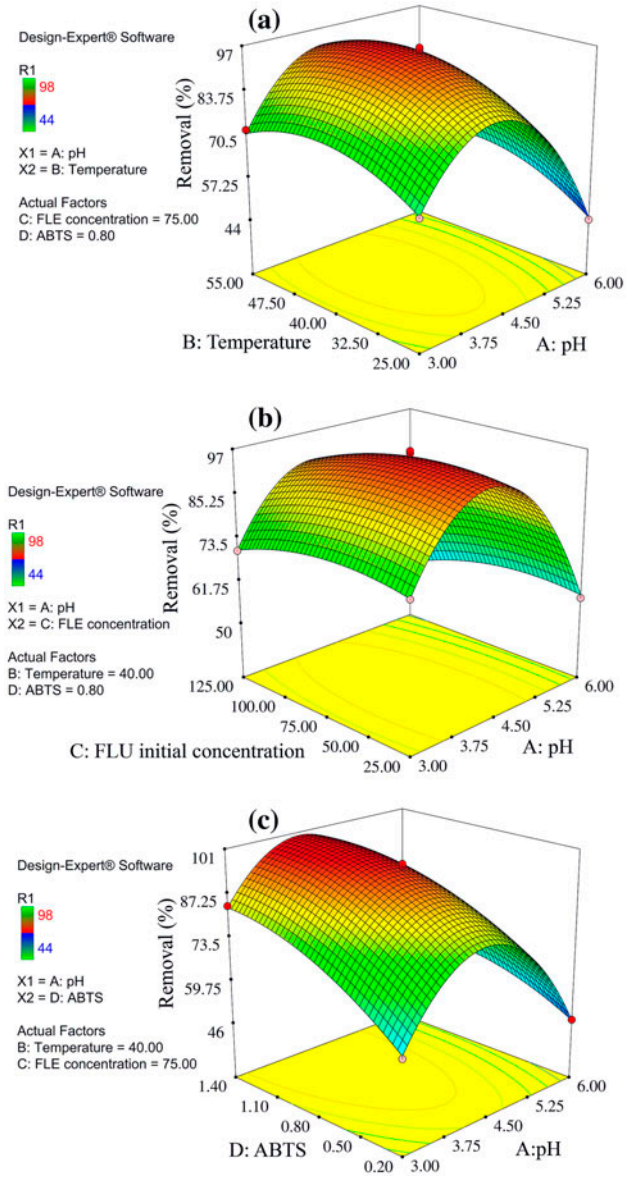


Fig. 4. Three dimensional surface plots from BBD showing the interaction effects of (a) pH and temperature, (b) pH and FLU initial concentration, and (c) pH and ABTS on removal of FLU.

45°C and pH values between 4.1 and 4.4. The results showed that the optimal pH for the FLU removal is under mild acidic conditions and the FLU removal was highly sensitive to changes in pH. These results were in agreement with the findings of Daâssi et al. [32], in dye removal. A description might be that laccase activity at higher pH is decreased by the binding of OH anion to the copper of laccase, which interrupts the internal electron transfer pathway [33]. On the other hand, in acidic condition, H^+ ion coupled with nitrogen atom of laccase and changes in the ionization state of the active site which lead to loss in activity [34]. Also, the changes in pH values can alter the chemical reactions of ABTS in coupling with laccase, and thus results in various oxidation rates. In addition, the stability of laccase is generally higher at mild acidic condition, and it loses at lower pH [32,33]. According to Table 4, the elimination of FLU was significantly affected by the temperature. Temperature is one of the significant factors which affect and control enzyme stability and activity. Most of the laccases were stable and retained its activity up to the temperature 60°C, and loses rapidly its activity at higher temperature due to thermal denaturation [33,35]. According to the results, the maximum removal efficiency of FLU achieved near the temperature optimum of the laccase from *Trametes versicolor* [33,35].

According to ANOVA results (Table 4), the interaction effect between pH and FLU initial concentration was insignificant due to their *p*-value higher than 0.05 (0.2957). As shown in Fig. 4(b), as there was an increase in pH, the removal efficiency increased with a decrease in FLU initial concentration up to the optimum level, but this is statically insignificant. The highest removal efficiency (98%), achieved when the initial concentration of FLU is in the lowest level, and in this situation the actual amount of the FLU removal is 12.25 μg FLU per 1U laccase. But the highest actual removal of FLU (56.25 μg FLU per 1U laccase) achieved when the initial concentration of FLU is in the highest level, although the removal percent is not in maximum (90%). So, it indicates that the initial concentration of FLU has a positive relation with the actual removal of FLU and negative relation with the removal percent of FLU. This is in agreement with the finding of Katuri et al. [36].

Fig. 4(c) shows the interaction effect of pH and ABTS concentration on the percentage of FLU removal. According to their *p*-value (0.0004), there was a significant effect between pH and ABTS concentration. This graph indicates that as there was an increase in pH, the removal efficiency increased with an increase in ABTS concentration up to the optimum level.

It has been revealed from ANOVA results (Table 4), the interaction effect between temperature and FLU initial concentration (*p*-value = 0.0007) also temperature and ABTS (*p*-value = 0.0130) were statically significant. Fig. 5(a) shows that as there was an increase in temperature, the removal efficiency increased with a decrease in FLU initial concentration, but, as shown in Fig. 5(b), as there was an increase in temperature up to the optimum level, the removal efficiency increased with an increase in ABTS concentration. According to the *p*-value 0.0934 (Table 4), the

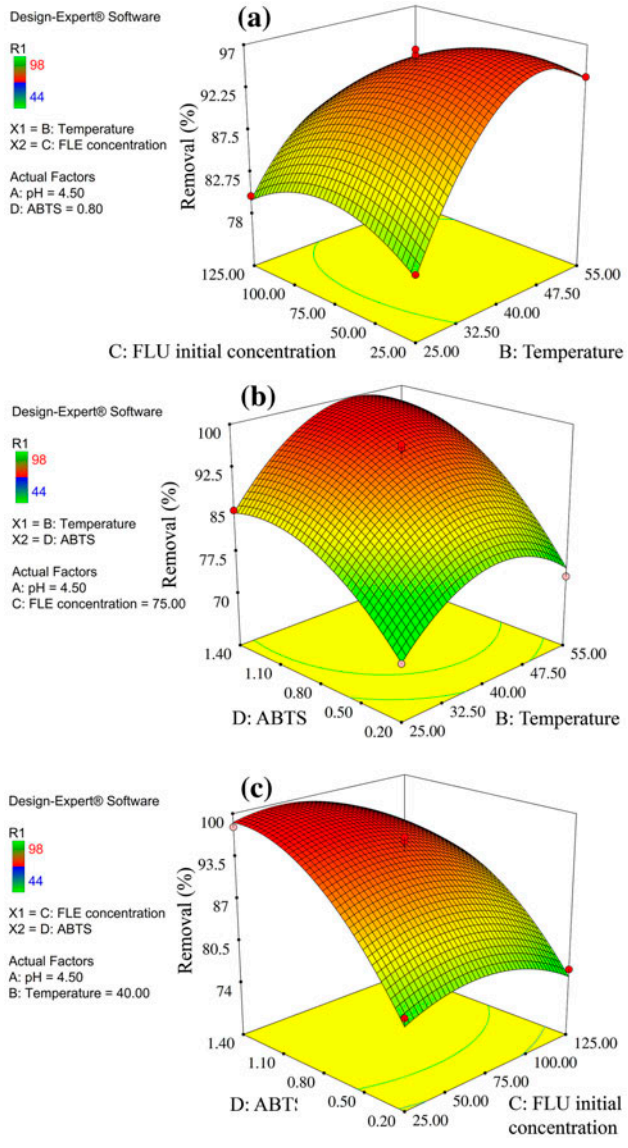


Fig. 5. Three dimensional surface plots from BBD showing the interaction effects of (a) temperature and FLU initial concentration, (b) temperature and ABTS, and (c) FLU initial concentration and ABTS on removal of FLU.

interaction effect between FLU initial concentration and ABTS concentration is statically insignificant, and it can be realized from Fig. 5(c) that as there was a decrease in FLU initial concentration, removal efficiency increased with an increase in ABTS concentration. According to the results, the FLU removal percentages were significantly increased by increasing the ABTS concentration in the studied range (Figs. 4(c), 5(b) and (c)). Due to the high redox potential (1.8 V), ABTS is one of the most efficient mediators that has been successfully applied for elimination of 2,4-dinitrophenol [37], sulfonamide antibiotics [13,14], benzodiazepines [22], and synthetic acid dye [38]. The result of ABTS influences on the removal efficiency is in agreement with those obtained by other researchers [13,14,22]. As shown in Figs. 4(c), 5(a) and (c), the removal efficiency of FLU was increased up to certain concentration of FLU. However, the removal rate decreased with further increasing FLU initial concentration. It indicates that after obtaining the equilibrium state, any further addition of FLU did not alter the rate of reaction. A few studies also indicated that the effect of laccase oxidation was correlated to the initial concentration of substrate. For example, the decolorization of Remazol Brilliant Blue R and Remazol Black 5 by laccase, decreased with increasing dye concentration [39], and the decolorization rate was found to increase up to 20 mg L^{-1} of the dye; however, the decolorization rate decreased above 20 mg L^{-1} of dye concentration [36].

3.4. Optimization

The optimization of FLU removal by laccase-mediated system was carried out by a multiple response method called desirability (D) function to optimize different combinations of all four factors and response. For this object, numerical optimization approaches with the software Design-Expert version 7.0.0 (Stat-Ease, trial version) were adopted by setting the optimization criteria in two approaches. Once response was set in maximize and all the factors were set in a range which coded as −1 and +1 with 30 cycles per optimization. The selected optimum conditions for FLU removal were predicted as follows: desirability of 1, temperature of 39.64˚C, pH value of 4.06, FLU initial concentration of 90.74 mg L^{-1}, and ABTS concentration of 1.35 mM for removal efficiency of 98.27%. Then, response and FLU initial concentration were set in maximize, pH and temperature were set in the studied range, whereas ABTS concentration was set in minimize. The selected optimum conditions for FLU removal were predicted as follows: desirability of 0.836 temperature of 39.19˚C, pH value of 4.32, FLU initial concentration of 125 mg L^{-1}, and ABTS concentration of 0.28 mM for removal efficiency of 77.76%. Finally, two experiments were carried out at the two predicted optimized condition, 99.4 and 78.6% removal efficiency were achieved, which are close to that of the model prediction.

4. Conclusions

Box–Behnken statistical experimental design and RSM was used to investigate the effect of influencing

parameters on FLU removal by laccase-mediated system. Four factors have been examined. After applying ANOVA, the results showed that the main effects of all the studied factors were significant and interaction effect of all the factors were significant, except interaction between pH and FLU initial concentration. The ABTS concentration and pH were found to have the greatest effect on the removal efficiency, while the temperature and FLU initial concentration showed lesser effects than them. The proposed quadratic polynomial model obtained by RSM agrees well with the experimental data, with correlation coefficients (R^2) of 0.9967. When all the factors were set in the studied range, the optimal conditions for the maximum removal of FLU (98.27%) were predicted as follows: temperature of 39.64˚C, pH of 4.06, FLU initial concentration of 90.74 mg L^{-1}, and ABTS concentration of 1.35 mM. On the other hand, when the pH and temperature were set in the studied range, FLU initial concentration in maximize, and ABTS concentration in minimize, the optimal conditions were predicted as follows: temperature of 39.19˚C, pH value of 4.32, FLU initial concentration of 125 mg L^{-1}, and ABTS concentration of 0.28 mM for removal efficiency of 77.76%.

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