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MODELING AND OPTIMIZATION OF REMOVAL OF CEFALEXIN FROM AQUATIC SOLUTIONS BY ENZYMATIC OXIDATION USING EXPERIMENTAL DESIGN

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Abstract - Antibiotics are used globally and, after use, they enter water sources in different ways. The presence of these compounds in the environment has created concerns about the toxicity of aquatic organisms and the emergence of antibiotic-resistant bacteria. The purpose of this study was to remove cefalexin from aqueous solutions by enzymatic oxidation using response surface methodology (RSM). For this purpose, batch experiments were performed to evaluate the effect of independent variables, including temperature, pH, contact time, enzyme activity, HBT mediator concentration, and antibiotic concentration. The residual cefalexin concentration was determined by HPLC. The Box-Behnken design of experiments and RSM were used to evaluate the overlap between variables. The results showed that the oxidation efficiency increased with increasing contact time and enzyme activity and decreasing antibiotic concentration. The highest and lowest removal percentages were 90.5% and 5.54%, respectively. Considering the value of R^2 (0.946) and adjusted R^2 (0.95) in the RSM model, one can state that the selected model is suitable for data analysis. Finally, the second-order polynomial analysis and the quadratic model were used as the best model for finding the relationship between the main variables and cefalexin removal efficiency. The Box-Behnken Design model can be effective for optimizing enzymatic oxidation of cefalexin, and laccase can be used to remove cefalexin.

Keywords: Enzymatic oxidation, laccase, cefalexin, Box-Behnken design.

INTRODUCTION

Medications are spreading into the environment and nature in many ways, including wastewater, and urban, medical, and industrial waste (Nazari et al., 2016). Currently, a wide variety of pharmaceutical products is very common in urban and factories wastewater (Azizi et al., 2017; Khetan and Collins, 2007; Martinez, 2009). The formation of antibiotic compounds in aquatic environments is an emerging subject, whose

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source is mainly the pharmaceutical industry, as well as the use of veterinary and human medicines (Fuoco, 2012; Le-Minh et al., 2010). About 30-90% of the dose consumed of these compounds and used by humans or animals can remain intact without degradation in their body, and is largely excreted as an active compound (Chee-Sanford et al., 2001; Jung, 2003). Many of these antibiotics have a toxic nature for smaller organisms in the environment, which can have a long-term indirect effect on environmental sustainability (Baquero et al., 2008; Martinez, 2009; Young et al., 2013). These compounds are highly resistant to biodegradation processes and remain in the environment for a long time due to their high stability. Their continued presence in the environment has caused many concerns for their long-term effects on human health (Fazlzadeh et al., 2016; Nazari et al., 2016).

Among the drugs, antibiotic isolation from sewage is of great importance due to its high utilization. Cefalexin (CEX) is one of the most used cephalosporin antibiotics, whose use and annual sales revenue in the early 21st century were 3,000 and 850,000,000 dollars respectively (Barber et al., 2004; Leili et al., 2018). Table 1 shows the main features of CEX (Estrada et al., 2012).

CEX is one of the antibiotics that is prescribed a lot and produced in large quantities (Elmolla and Chaudhuri, 2010; Sirés and Brillas, 2012). This antibiotic is widely used to treat a variety of human infections caused by Gram-positive and Gram-negative bacteria. Several serious environmental hazards from CEX in the environment have been a public health problem for many years (Ahmed and Theydan, 2012; Fazlzadeh et al., 2016; Vilt and Ho, 2009).

Removal of antibiotics from the environment involves high costs because the pharmaceutical industries should properly clean their wastewater before discharging into the environment. Therefore, the elimination of antibiotic residues from the environment is of particular importance and is an attractive subject for a case study (Kim et al., 2005; Košutić et al., 2007; Watkinson et al., 2007).

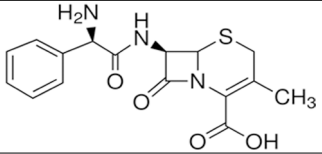
Typical routine separation methods of CEX from aqueous solution are liquid membrane separation, solid phase extraction, biological inoculation, electro-photon oxidation, Nano-filtration and Sono-chemical degradation (Estrada et al., 2012; Guo et al., 2010; Vilt and Ho, 2011; Zazouli et al., 2010).

The enzymatic oxidation of resistant pollutants is an environmentally friendly alternative method compared to conventional physicochemical methods (Hai et al., 2013). Over the past three decades, the use of biodegradation by enzyme has received a great deal of attention as an environmentally sustainable solution. The enzymatic oxidation method is an alternative to old methods because it can be efficient at different concentrations of contaminants. This method is easily controllable, requires low energy, and has the least impact on the ecosystem (Duran and Esposito, 2000; Karam and Nicell, 1997). Laccase is a multi-copper oxidase that is effective in the biodegradation process. Laccase performs selective oxidation catalysis of several natural and non-natural substrates using the air as a green oxidant. The only by product of this process is H_2O (Forootanfar et al., 2012). This enzyme is widely used in paper and paper pulp (Widsten and Kandelbauer, 2008), biological sensors (Madhavi and Lele, 2009), organic synthesis (Heidary et al., 2014), and especially in the removal of anti-inflammatory drug wastes (NSAIMs) (Lloret et al., 2013), benzodiazepines (Ostadhadi-Dehkordi et al., 2012), diclofenac (Xu et al., 2015) and carbamazepine (Hata et al., 2010).

One of the statistical models used in designing the experiments is the RSM method, which is a simple, effective way to optimize various processes. This method can be done using the central composite design (CCD) or Box-Behnken Design (BBD).

According to our best knowledge, there are no investigations about the removal of cephalexin (CEX) from aqueous solutions by the laccase enzyme until now. The current study is focused on the ability of laccase enzyme to remove cephalexin from aqueous solution. Moreover, the conversion of cephalexin catalyzed by laccase was optimized using the Box Behnken design of experiments, taking into account the major variables (6 variables) involved in enzymatic oxidation. In most studies, this has been done with a maximum of three to four parameters.

Table 1. Cefalexin properties

Solubility	Molecular Weight	Structure
	347.39 g/mol	1790 mg/L
$C_{16}H_{17}N_3O_4S$		

METHODS

Study method

This study is an experimental-practical study carried out on a laboratory scale. The study of CEX removal as a dependent variable against independent parameters such as initial antibiotic concentration, temperature, pH, residence time, HBT mediator concentration and enzyme activity was performed and optimization of the process and the interaction of variables were investigated using BBD.

Required chemicals

The enzyme used was laccase extracted from *Trametes versicolor* (EC1.10.3.2), CEX, hydroxybenzotriazole (HBT) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) mediators were from Sigma-Aldrich USA. Sodium citrate and citric acid with a purity of 99.5% and methanol (HPLC grade) were from Merck Germany. Distilled water prepared using a membrane method was used to prepare all solutions and buffers.

Sodium citrate and citric acid were used to prepare a citrate buffer with a specific pH, so that 0.1 mL citrate buffer was used with the pH required for the preparation of all solutions. To ensure the pH of the solution, the HACK pH meter was used.

To measure the enzyme activity, a Shimadzu UV-180 spectrophotometer was used. To measure CEX concentration, HPLC-UV (column C18 of internal diameter 4.6 mm and 250 mm long) and methanol-carrier phase (30 to 70%), and a detection wavelength of 263 nm were used. The devices used were calibrated and their calibration curves were plotted.

Determining the activity of the enzyme

ABTS was used as substrates to measure the activity of laccase enzyme. First, a 2 mM solution was prepared of ABTS in citrate buffer (0.1 m, pH = 4.5). After the reaction time, the absorbance of the reaction solution at 420 nm was measured using a spectrophotometer. Based on the molar coefficient, ABTS (ε_{420}=36000 $M^{-1}cm^{-1}$) was converted to enzyme units. The amount of enzyme that can oxidize in 1 minute 1 micromole of the substrate at pH = 4.5 and at 25 °C is equal to one laccase enzyme activity unit (Donati et al., 2015).

Optimization of enzymatic oxidation using the RSM method

The oxidation parameters include temperature, pH, time, enzyme activity, HBT concentration and CEX concentration. In this method, the 6 parameters intended were examined at three levels: high (+1), medium (0) and low (-1). Table 2 shows the factors and levels. Taking into account 6 variables, and using the Box-Behnken design of experiments (BBD), the number of experiments required for statistical analysis was determined to be 54 experiments with two replicates of each experiment; the number of experiments in this study was thus 108 experiments. Determining the appropriate range for the parameters studied was done by performing several oxidation experiments at the upper and lower levels of the parameter, as well as previous studies in this field. Experimental conditions and the results of enzymatic oxidation in cefalexin removal are shown in Table 2.

In this method, the experiments were performed according to the limits of BBD. For performing each experiment, 10 mL of a solution containing a specific concentration of CEX antibiotic was contacted at a specified temperature and pH over a specified period with a certain amount of enzyme and mediator. Samples were sampled at a volume of 0.5 cc after reactions and were filtered with a 0.22-micron filter. The antibiotic residue in the solution was measured by HPLC and the percentage of CEX removal calculated by Eq. (1):

$$R\% = \left((C_0 - C_t)/C_0\right) \times 100 \quad (1)$$

in which C_t and C_0 are the initial and final concentrations in ppm, respectively.

Table 2. Levels and selected codes of variables for designing the Box-Behnken Test

Variable	Unit	Factors	Range and level		
			Low (−1)	Middle (0)	High (+1)
pH	-	A	3	5	7
Temperature	ºC	B	30	45	60
Time	min	C	15	37.5	60
Activity	U/mL	D	0.25	1	1.75
Mediator concentration	mM	E	0.5	1	1.5
Concentration of cefalexin	mg/L	F	10	80	100

The results of the research were analyzed using Design Expert 7 software. The experiments were randomized to minimize system error. The interacting interference model coefficients are interpretations of the level of cefalexin removal (response) as the independent variable function. Data were analyzed by multivariate regression and the coefficients were analyzed by ANOVA with respect to the significance level ($P \leq 0.05$). 3D graphs (response surface curves) were plotted to examine the relationship between responses and independent variables.

RESULTS AND DISCUSSION

Verifying the model

Experiments were performed under the conditions specified by the BBD model in a standard manner. The results of the statistical plan and response procedures are summarized in Table 3. The results showed that the efficacy of CEX removal in different values of the six main variables was very different, which is the result of the effect of different levels of variables on the efficacy of CEX oxidation. According to the results of the study and Table 3, the highest and lowest percentages of CEX removal by the BBD method, respectively, were obtained in test experiments 15 and 26, which were about 90.5% and 5.54% respectively.

In addition, a small difference between the actual and predicted efficiency indicates the high accuracy of the model in estimating the response variable (CEX removal efficiency). Accordingly, second-order polynomial analysis and a quadratic model were used to find the relationship between the main variables and CEX oxidation efficiency as the best model. To validate the model and variance analysis, ANOVA was used, whose results are shown in Table 4. Accordingly, the efficacy of cefalexin removal for the significant variables is obtained according to the following Eq. (2):

$$\begin{aligned} R = & -511.6 + 8.8 * A + 93.858 * B + 2.4 * C + \\ & 52.5 * D + 144.93 * E - 0.013 * F - 0.086 * A^2 - \\ & 9.16 * B^2 - 2.38 * B * D - 2.51 * B * E - \\ & 0.0448 * B * F - 0.021 * C^2 - 13.19 * D^2 - \\ & 54.91 * E^2 - 0.00324 * F^2 \end{aligned} \quad (2)$$

wher: A is the reaction temperature, B is pH, a term with no metric, C is the reaction duration (min), D is the enzyme activity (U/mL), E is the HBT mediator concentration (mM) and F is the cefalexin concentration (mg/L).

The parameter F-value is the standard deviation of the data from the mean value. In general, for a model that predicts test results successfully, an F-value is very high and a p-value less than 0.05 means that the model is significant. For this model, the values of F-value and p-value were 78.88 and $P<0.0001$, respectively, indicating that the model was completely significant. In this equation, the linear parameters were E, D, C, B, A and F; second order parameters of the model were D^2, C^2, B^2, A^2, E^2 and F^2, along with the interaction parameters BD, BE and BF. As the values of R^2 and R^2adj are close to 1, it indicates a better relationship between laboratory and calculated results. In this model, the value of the parameter R^2 (0.946) is in accordance with R^2adj (0.95) that shows the model accuracy. The signal-to-noise ratio is measured using the Adequate Precision function, where a ratio more than 4 is desirable. For this model, the Adequate Precision was 30.5%, which indicates a high signal-to-noise ratio (Mourabet et al., 2012). The Durbin-Watson test was used to check the independence of the errors (the difference between the actual values and the values predicted by the regression equation) and the Durbin-Watson test statistic was 1.74. As it is 1.5 to 2.5, the assumption of the absence of correlation between the errors is not rejected and regression can be used.

In the analysis of experiments and the use of linear models, the validity of a model depends on some assumptions, including residuals that should have a normal distribution with mean zero, constant variance (∂^2), and residuals independent of each other. Fig. 1 shows these assumptions. Fig. 1 (a) shows the examination of residuals being normal, and given that no deviations were seen in the normality of the residuals, the assumption of the normality of the residuals was confirmed. Fig. 1 (b) is used to check the assumption of constancy of the variances of the residuals. If there is no particular trend in this graph, the assumption that the variance is constant is accepted. Given that in this diagram there is no particular trend indicating the increase or decrease of variance, the assumption of constant variance is accepted. Diagram 1c shows the independence between the residuals. If a trend like a sinusoid is not visible in this chart, then the assumption in question is accepted as well. In this chart, no particular trend shows, ruling out the independence of the residuals. Fig.1 (d) shows that the proposed model for CEX removal is well suited to the experimental data.

Table 3. Design and test results

Run	A	B	C	D	E	F	R (%)
	T(ºC)	pH	Time(min)	En(U/mL)	HBT(mM)	C(mg/l)	
1	45	5	15	0.25	1	10	37.1
2	45	5	60	0.25	1	100	39.2
3	45	7	37.5	1	0.5	10	16.35
4	30	5	60	1	1	10	64.6
5	45	7	37.5	1	1.5	10	25.1
6	45	5	15	0.25	1	100	17.4
7	30	3	37.5	0.25	1	55	7.6
8	60	3	37.5	0.25	1	55	14.5
9	45	3	37.5	1	0.5	10	33
10	60	7	37.5	0.25	1	55	7.32
11	45	3	15	1	0.5	55	8.9
12	45	7	60	1	0.5	55	18
13	30	5	37.5	0.25	1.5	55	29.76
14	45	3	60	1	1.5	55	37.5
15	45	3	37.5	1	0.5	100	5.54
16	60	3	37.5	1.75	1	55	31.4
17	30	5	37.5	1.75	0.5	55	24.6
18	45	3	60	1	0.5	55	20.6
19	45	5	37.5	1	1	55	77.1
20	45	5	15	1.75	1	10	75.5
21	45	5	37.5	1	1	55	79
22	45	7	15	1	1.5	55	8.5
23	60	5	37.5	1.75	0.5	55	54.7
24	30	5	15	1	1	100	15.95
25	60	5	15	1	1	100	17.3
26	45	5	60	1.75	1	10	90.5
27	30	5	37.5	1.75	1.5	55	51
28	45	7	37.5	1	0.5	100	7.3
29	45	3	15	1	1.5	55	16
30	45	7	60	1	1.5	55	20.8
31	30	5	60	1	1	100	47.33
32	45	7	15	1	0.5	55	7.65
33	60	5	15	1	1	10	47
34	45	5	15	1.75	1	100	31
35	60	7	37.5	1.75	1	55	13.1
36	45	7	37.5	1	1.5	100	13.6
37	45	5	37.5	1	1	55	78.5
38	45	3	37.5	1	1.5	100	22.8
39	30	7	37.5	1.75	1	55	10
40	60	5	37.5	0.25	0.5	55	27.6
41	45	5	37.5	1	1	55	78.3
42	30	3	37.5	1.75	1	55	28.3
43	60	5	60	1	1	100	49.4
44	45	5	60	1.75	1	100	66
45	45	5	37.5	1	1	55	78.85
46	30	5	15	1	1	10	30.5
47	60	5	37.5	1.75	1.5	55	58
48	60	5	60	1	1	10	63
49	45	5	60	0.25	1	10	73.6
50	30	7	37.5	0.25	1	55	6.45
51	60	5	37.5	0.25	1.5	55	43.6
52	45	3	37.5	1	1.5	10	49
53	45	5	37.5	1	1	55	77.45
54	30	5	37.5	0.25	0.5	55	15.06

Table 4. ANOVA results for CEX removal

	Sum of		Mean	F-value	p-value	
Source	**Squares**	**df**	**Square**		**Prob > F**	
Model	63384.21	27	2347.56	78.98	< 0.0001	**significant**
A	751.29	1	751.29	25.28	< 0.0001	**significant**
B	1290.22	1	1290.22	43.41	< 0.0001	**significant**
C	6452.43	1	6452.43	217.09	< 0.0001	**significant**
D	3857.10	1	3857.10	129.77	< 0.0001	**significant**
E	1447.57	1	1447.57	48.70	< 0.0001	**significant**
F	5983.55	1	5983.55	201.31	< 0.0001	**significant**
AB	11.61	1	11.61	0.39	0.5338	
AC	79.34	1	79.34	2.67	0.1063	
AD	12.54	1	12.54	0.42	0.5179	
AE	113.00	1	113.00	3.80	0.0547	
AF	33.15	1	33.15	1.12	0.2942	
BC	10.50	1	10.50	0.35	0.5541	
BD	202.85	1	202.85	6.82	0.0108	**significant**
BE	228.29	1	228.29	7.68	0.0070	**significant**
BF	260.02	1	260.02	8.75	0.0041	**significant**
CD	15.11	1	15.11	0.51	0.4779	
CE	59.06	1	59.06	1.99	0.1626	
CF	49.95	1	49.95	1.68	0.1986	
DE	2.025E-03	1	2.025E-03	6.813E-05	0.9934	
DF	38.91	1	38.91	1.31	0.2560	
EF	0.36	1	0.36	0.012	0.9126	
A^2	7621.83	1	7621.83	256.43	< 0.0001	**significant**
B^2	27602.78	1	27602.78	928.67	< 0.0001	**significant**
C^2	2395.80	1	2395.80	80.60	< 0.0001	**significant**
D^2	1114.87	1	1114.87	37.51	< 0.0001	**significant**
E^2	3915.03	1	3915.03	131.72	< 0.0001	**significant**
F^2	816.24	1	816.24	27.46	< 0.0001	**significant**
Residual	2348.10	79	29.72			
Cor Total	65732.31	106				

The effect of independent variables on CEX removal is shown in Fig. 2 The percentage of removal of CEX at pH 5, temperature 45°C, and HBT mediator concentration was 1 mM, and with increasing and decreasing pH, temperature and HBT from these values, efficiency decreased. By increasing the concentration of CEX from 10 to 100 mg/L, the removal rate was reduced. Moreover, the deletion percentage increased with increasing contact time from 15 to 60 minutes and enzyme activity from 0.25 to 1.75 U/mL. In this research, all of the variables considered have an optimal point in the selected domain, suggesting that the domains are properly selected.

In this study, the effect of effective factors on the oxidation process was investigated by a Pareto chart. As shown in Fig. 3, pH and temperature are the most important parameters for CEX oxidation. The importance of time, the concentration of CEC, HBT concentration and enzyme activity were in the following ranks. It is also observed that time, enzyme activity and HBT concentration have a positive effect and pH, temperature and CEX concentration have a negative effect on CEX oxidation.

The effect of temperature

One of the most important and influential factors in biotechnology is temperature. Enzymes show a specific sensitivity to temperature variations. The heat deforms the enzyme's 3D structure, destroying the active enzymes and disabling them. Enzyme activity reduces or deactivates at temperatures below 10 °C and above 60 °C (Fabbrini et al., 2002; Liu, 2006). In the study of Asgher et al. (2017), the highest activity of free laccase enzyme was observed at 45 ˚C.

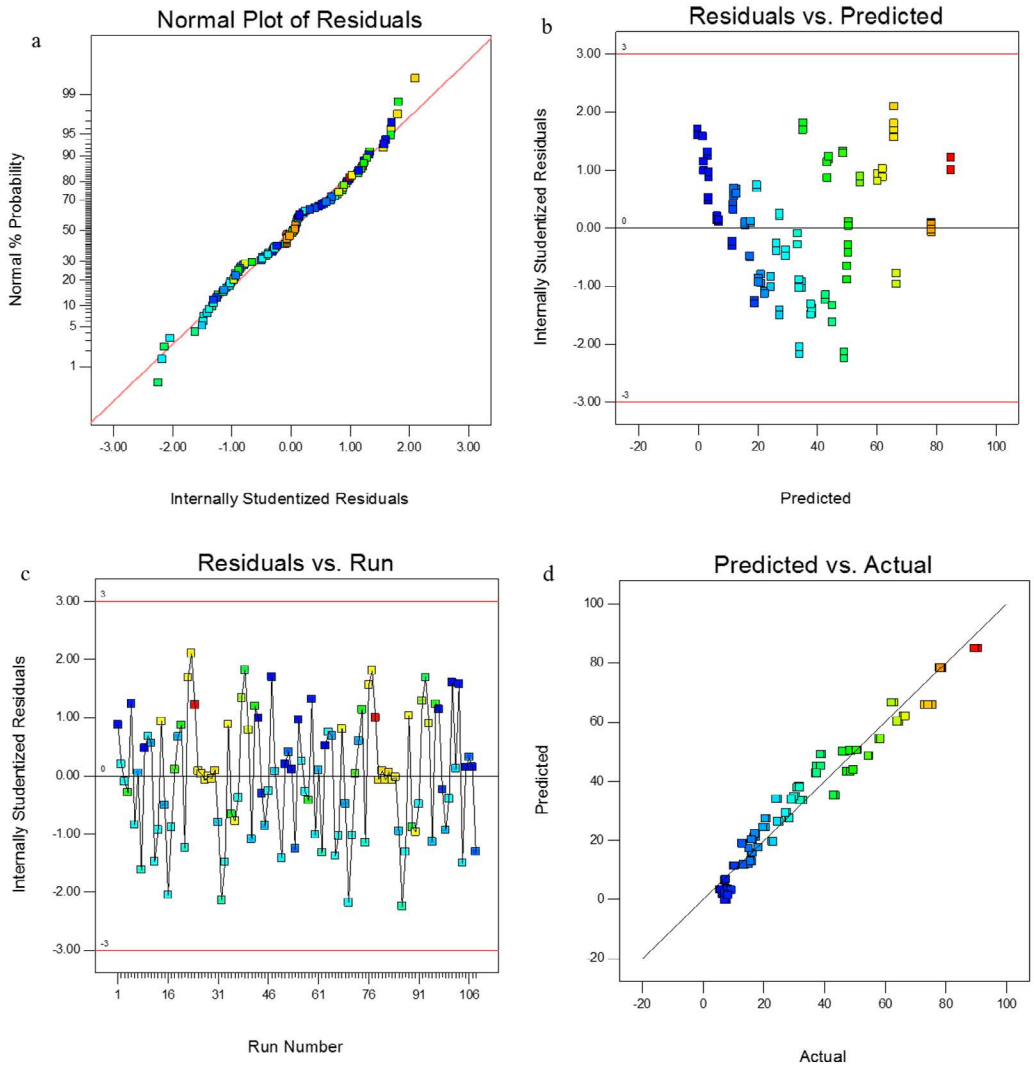


Figure 1. Distributive plotting of experimental data against the predicted valuesfor CEX removal.

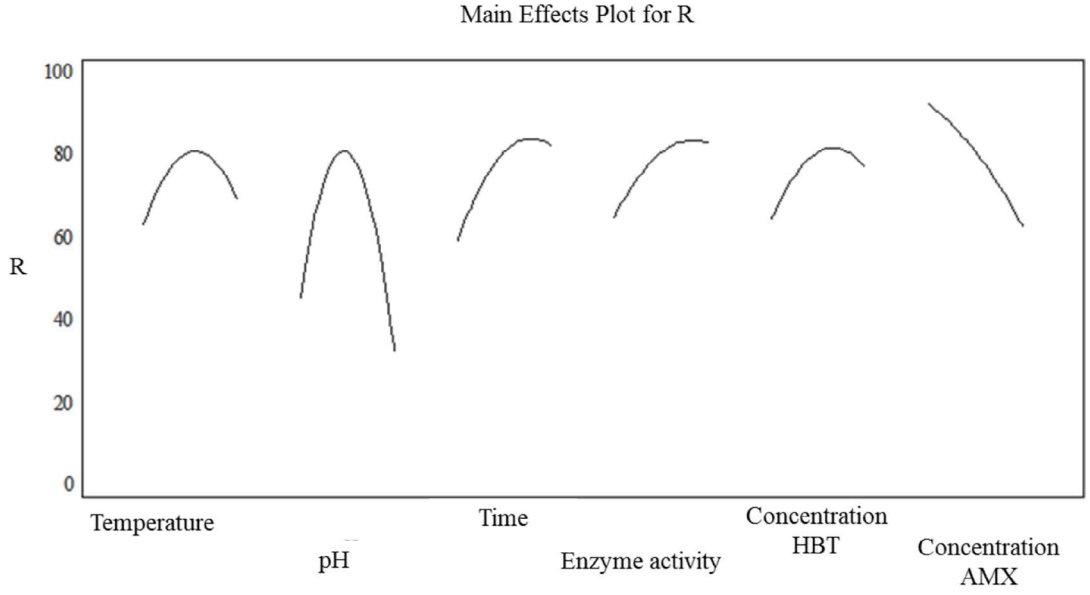


Figure 2. Chart of the Effect of Initial Factors.

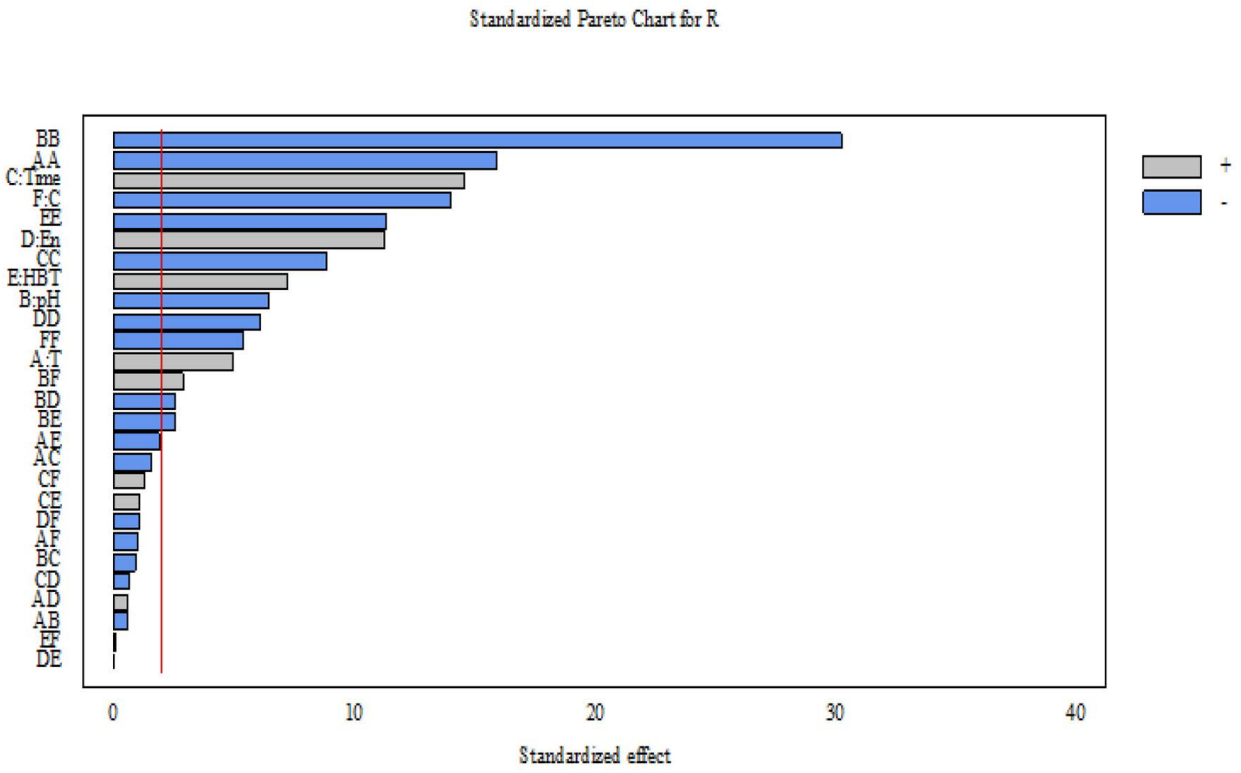


Figure 3. Pareto chart. A is the reaction temperature, (B) is the pH, C is the reaction duration, D is the enzyme activity, E is the HBT mediator concentration and F is the cefalexin concentration.

The effect of temperature changes on the efficacy of laccase enzyme in CEX oxidation was performed at 30, 45 and 60°C. The results are shown in Figs. 2 and 4 (a). In this process, the efficacy of CEX removal increased from 30 to 45°C and the highest efficiency was observed at 45°C. As the temperature rises from 45°C to 60°C, the removal efficiency decreases. Fig.4 (a) shows the interaction between temperature and pH, with the highest removal efficiency at 45 and pH 5. In the studies of Forootanfar and Kim, which were performed, respectively, on wastewater and removal of bisphenol A using leccase, the best efficiency was obtained at 45 ˚C (Forootanfar et al., 2016; Kim and Nicell, 2006), which is consistent with the results of this research. Fig.4 (b) shows the interaction of temperature and time in enzymatic oxidation. As is evident from the figure, the efficacy of CEX removal increased upon increasing the reaction time from 15 to 60 minutes.

The effect of soluble pH

The pH is an important variable in enzymatic oxidation and has a great effect on the activity of the enzyme and the value of HBT radical produced (Asadgol et al., 2014; Forootanfar et al., 2016). In this study, according to Fig. 2 and 4 (a), the optimum pH value is 5 and, by decreasing and increasing the pH away from this amount, CEX concentration is increased and, as a result, the amount of oxidation decreases. Enzymes change in nature in an acid or alkaline environment. The role of pH in the catalytic activity of the enzyme is due to the effect on the reactive groups of copper atoms in the laccase enzyme. The optimum pH for the activity of most of the fungal Laccase for oxidation is in the range of 4-6 (Baldrian, 2006). In the study of Tahmasbi et al. (2016), the enzymatic degradation of imipramine was observed at pH 3 to 6 and an optimum pH of 4.9. In addition, in the study of Asadgol et al. (2014), in the elimination of bisphenol A, an optimum pH of 5 was obtained for laccase enzyme. These studies confirm the results of this study. In addition, Fig. 4 (a) shows the mutual effect of pH and temperature on CEX removal, with the highest efficiency at pH 5 and temperature of 45°C.

The effect of enzyme activity

The effect of laccase enzyme activity on CEX removal was evaluated at 1 and 0.25 and 1.75 U/mL. The CEX oxidation pattern with increasing enzyme activity is observed in Fig. 2 and 4 (c). In this study, CEX removal is increased by increasing enzyme activity.

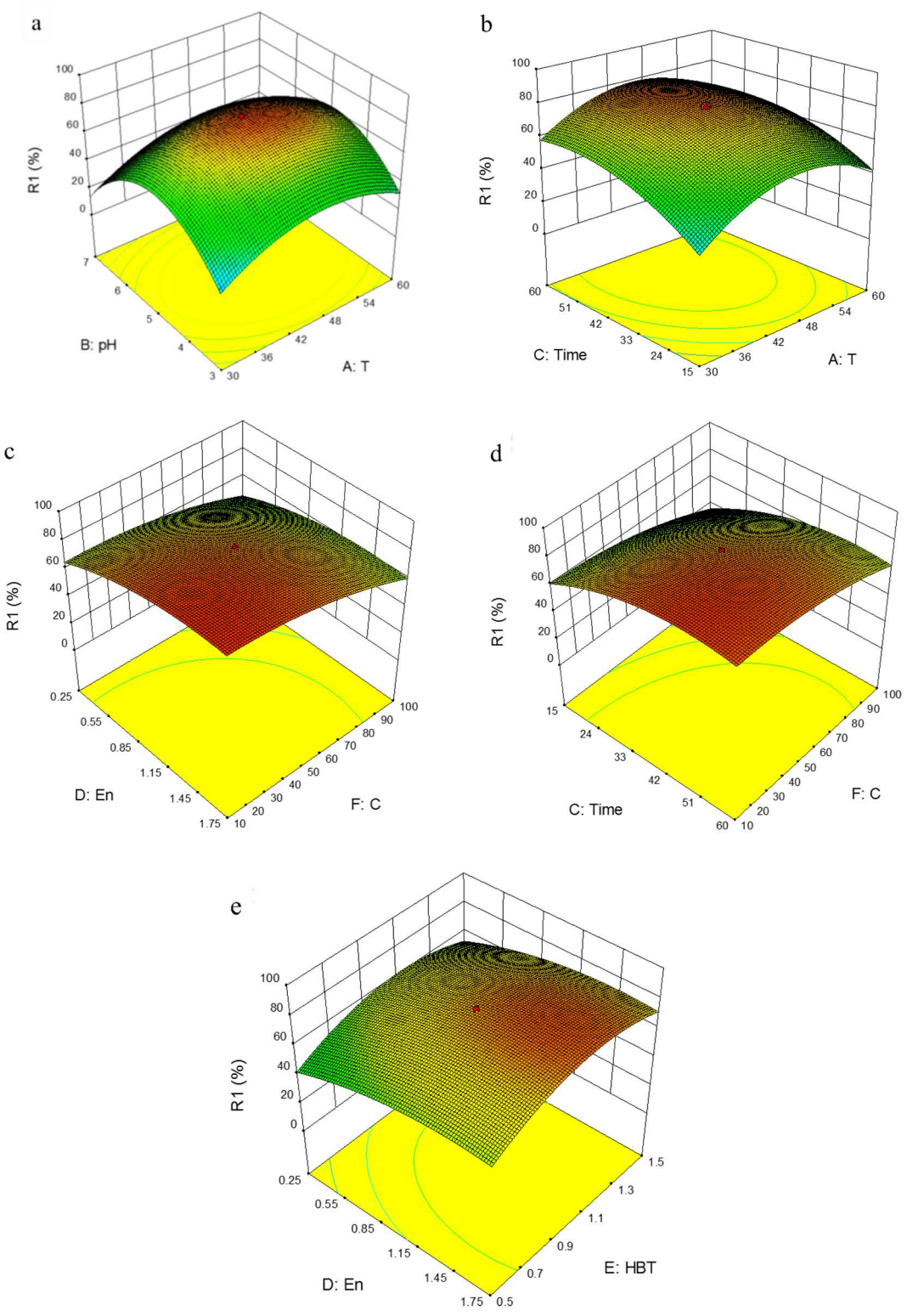


Figure 4. Responses surfaces forthe interactions of independent variables on the efficacy of CEX removal: (a) temperature and pH; (b) temperature and reaction time; (c) enzyme activity and initial concentration of cefalexin; (d) initial concentration of CEX and time; and (e) enzyme activity and HBT concentration)

The highest efficiency of enzyme activity is 1.75 U/mL. Rezaei et al. (2015) showed that with increasing activity of laccase enzyme from 0.75 U/mL to 2.5 U/mL, Acid Blue 92 removal efficiency increased. In the study of Forootanfar et al. (2016), it was observed that an increase in enzyme activity up to 2 U/mL increased the removal rate of stain, but an increase in enzyme activity did not have much effect on stain removal. In addition, in the study by Liu et al. (2012), it was found that increased enzyme activity increases the amount of phenol removal. This is consistent with the present study.

The Effect of HBT Mediator

This study used N-hydroxybenothiazole (HBT) as a mediator for cephalexin enzymatic oxidation at concentrations of 0.5, 1, and 1.5 mM. The interfacing activity of HBT is in enzymatic oxidation is proportional to the effect of the N-O$^{\bullet}$ group on laccase activity, especially at high concentrations (Ashrafi et al., 2013; Cañas and Camarero, 2010; Papinutti et al., 2008). HBT has a high redox potential (1084 mV) (Ostadhadi-Dehkordi et al., 2012) and the laccase-HBT system is one of the most successful laccase-mediator systems for use in the removal of artificial contaminants and stains (Khlifi et al., 2010).

The results showed that the enzymatic oxidation efficiency in the presence of HBT had an increasing trend up to 1 mM concentration, but the efficiency decreased with further increase in the concentration of HBT. The results of the effect of HBT concentrations are presented in Figs. 2 and 4 (e). The interaction between the activity of the enzyme and HBT in cefalexin oxidation is shown in Fig. 4 (e). The removal is the most at the enzyme activity of 1.75 U/mL and 1 mM concentration of HBT. In a study, Mirzadeh et al. (2014) used laccase enzyme to remove Acid Blue 25 and Acid Orange 7. The results of this study showed that an increase of HBT concentration up to 1 mM increased the enzyme efficiency, but further increase in HBT had no effect on the oxidation efficiency. In addition, Khlifi et al. (2010) obtained an optimal concentration of HBT of 1 mM in enzymatic treatment of tissue sewage, which confirms the results of this study.

The Effect of Cephalexin Concentration

The concentration of the substrate in the liquid phase has a great influence on the enzyme-mediator reaction. By increasing the concentration of the substrate proportional to the enzyme activity and the concentration of the mediator, the reaction rate increases to a certain extent, but after reaching the balance, the increase of the substrate does not affect the reaction rate. Moreover, the higher the ratio of the enzyme to the substrate, the higher the oxidation is. The study was performed at 10, 80 and 100 mg/L concentrations of cefalexin, the results of which are shown in Figs. 2, 4 (d, c). Fig. 4 (c) and 4 (d), respectively, show the interaction of enzyme activity with cefalexin concentration and reaction time with concentration. By increasing the concentration of cefalexin as a substrate against the enzyme activity, the enzymatic oxidation rate constant decreases. The highest and lowest efficacy was observed in 10 and 100 mg/L concentration of cefalexin. In the removal of 2,4-dinitrophenol using laccase enzyme from the aquatic environment, Dehghanifard et al. (2013) showed that, with increasing concentrations of pollutant from 0.05 to 0.15 mM, the removal efficiency reduced, which confirms the results of this research.

Process optimization

In this study, the optimization of enzymatic oxidation was performed for the independent variables of temperature, pH, residence time, enzyme activity, HBT mediator concentration and antibiotic concentration. The aim of this study was to determine the optimal amount of reaction time, temperature, pH, enzyme activity, HBT mediator concentration and antibiotic initial concentration in order to achieve maximum efficacy of cefalexin removal. Different modes were tested based on the experiments using the BBD method to determine optimal conditions. Finally, the optimum conditions were determined according to Table 5. In optimal conditions, the efficacy of CEX removal was predicted to be 91%. Experimental tests in similar optimized conditions with 88.8% removal efficiency confirmed this issue.

Table 5. Optimal Conditions of the Independent Variables for Cefalexin Removal

Factor	Low	High	Optimum
T	30	60	46.6
pH	3	7	4.6
Time	15	60	48
En	0.25	1.75	1.54
HBT	0.5	1.5	1.1
C	10	100	11.8
Optimum value		91	

CONCLUSION

The results showed that the pH parameter had the most effect on CEX enzymatic oxidation with an optimum value of 5. By increasing and decreasing pH away from this value, oxidation and cefalexin removal reduced. The optimal enzyme oxidation conditions for CEX removal for pH, temperature, enzyme activity, HBT concentration and CEX concentration were determined as 5, 45 ˚C, 1.75 U/mL, 1mM, and 10 mg/L, respectively. In these conditions, the efficiency of Laccase enzyme after 90 minutes of reaction was 90.5%. Considering the results and paying attention to the acceptable performance and the appropriate removal of cefalexin by the enzyme Laccase, enzyme oxidation can be used as a suitable and high-performance process for the treatment of industrial and septic sewage containing CEX antibiotic. The findings also showed that the design of the experiment could be used to reduce costs and the number of experiments and to examine the interactions of variables and determine the optimal conditions. The cephalexin concentration range of 10-60 mg/l or less can be found in the environment (wastewater effluent of clinical centers). According to research findings, with decreasing cephalexin concentration, the removal efficiency increases. Low concentrations of cephalexin in the environment can facilitate removing this antibiotic.

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