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Optimizing laccase-mediated amoxicillin removal by the use of Box–Behnken design in an aqueous solution

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ABSTRACT

Abnormal use of antibiotics and discharging them into the sewage systems bring about serious and dangerous harms to the environment. The present study aims at the feasibility evaluation of amoxicillin removal from the aqueous solutions by means of enzymatic oxidation by taking advantage of response surface method based on Box–Behnken model. The present study is a library research. Therefore, the experiments are carried out in a laboratory for discontinuously evaluating the effects of independent variables such as temperature, pH, contact time, enzyme activity, hydroxybenzotriazole mediator concentration and the antibiotic concentration. The remaining amoxicillin's concentration has been determined by HPLC device. To implement the experiments, Box–Behnken model was used for measuring the variables' mutual effects. One-way analysis of variance was used for data analysis. The results showed that enzymatic oxidation output increases with the increase in contact time and enzymatic activity as well as with a decrease in the antibiotic's concentration. The highest and the lowest removal percentages were 91.5% and 5.36%, respectively. According to the high amounts of R^2 (0.974) and R^2adj (0.955), it can be said that the selected model is appropriate for data analyses. Finally, a quadratic polynomial model was applied as the best model of choice for figuring out the relationships between the main variables and amoxicillin elimination output. Response surface method can be effective in amoxicillin oxidation optimization and laccase can be used for amoxicillin elimination.

Keywords: Enzymatic degradation; Laccase; Amoxicillin; Box–Behnken design

1. Introduction

Nowadays, mass production of the medications worldwide (about 4,000 types of drug compounds with an annual volume ranging from 100 to 200,000 tons) is one of the major factors contributing to the increasingly high rate of the increase in water contaminations with drug wastes [1]. Since World War II, a large number of antibiotics were produced and consumed [2,3]. Such medications have been widely taken for promoting health and curing infections of humans as well as animals in veterinary fields of study [4,5]. A great deal of the medications that are consumed by the humans and animals are excreted and then they find their way to the urban sewage systems and refineries. Reducing the discharge of such contaminant is a must because they interfere in the environment even in low concentrations [6].

The presence of antibiotics in aquatic environments is among the major concerns of the environmentalists due to their low biodegradability, toxicity and instigation of drug resistance [7,8].

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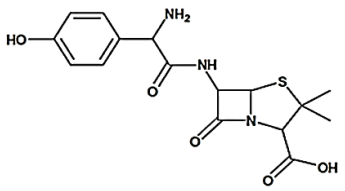
Amoxicillin (AMX) is considered as one of the 10 most frequently consumed antibiotics belonging to the penicillin family. It is a semi-artificial drug, the most predominant feature of which is the presence of a beta-lactam loop. Amoxicillin is very hardly absorbed in the human and animal bodies such that about 80%–90% of it, in case of being consumed orally, will not be broken down and it will be exuded to the environment in urine or solid waste [9–12]. Table 1 illustrates the main features of AMX [13].

Many of the active medications are made in such a way to remain stable and biologically active so as to be effective in their use for health care. Thus, preventing such drugs from polluting the environment by means of the old-fashioned methods used in the waste water treatment plants is excessively difficult and only a small fraction thereof is destroyed [2]. So far, chemical, physical and biological methods have been applied for the elimination of antibiotics, for instance, chemical oxidation and biological degradation (destructive methods), absorption, liquid extraction and substrate processes and techniques (nondestructive methods). Various methods can be selected depending on the contaminants' concentration in the sewage water and the costs they incur [14]. However, according to the low output of these methods and their inefficiency, it is necessary to develop novel methods for the elimination of beta-lactam antibiotics from the sewage [14]. AMX decomposition by photo-catalytic processes such as UV/H_2O_2/TiO_2 and UV/TiO_2.UV/ZnO [15], photo-Fenton [16,17] and Fenton oxidation process have been studied by taking advantage of experimental designs [11].

One method that is currently being used for the newly emergent contaminants' decomposition and degradation is the application of extracellular enzymes. Enzymatic degradation features high reactive characteristics and it is in need of relatively low dosages even for its use in industrial scale projects. In comparison with the chemical decomposition, chemical compounds enzymatic degradation consumes lower energies and produces lesser amount of residuals [18,19]. One of the most frequently applied enzymes for this purpose is laccase. Laccase, alone or with the help of some intermediating agents accelerates the oxidation of a wide spectrum of compounds such as phenol and its derivatives, benzenethiols, aromatic amines and polycyclic aromatic hydrocarbons [20–22]. The prominent feature of such a vital catalyst has made it to be applied as the original instrument in studies on the elimination of xenobiotics [23,24].

Table 1
Chemical properties of amoxicillin

Parameter	Character
Molecular formula	$C_{16}H_{19}N_3O_5S$
Molecular weight (g/mol)	365.4
Solubility in water (mg/L)	3,430 at 20°C
Appearance	Almost white powder
Molecular structure	

In recent decades, various approaches and models have been proposed for the determination of the number of the experiments and tests among which Box–Behnken design has demonstrated promising benefits including the reduction in the number of experiment stages, time, cost, material saving as well as the presentation of a statistical model for an ultimately precise description of the process [25–28]. According to the idea that amoxicillin removal from aqueous solutions by the use of laccase has not been studied up to the present point in time, the present study aims at measuring laccase ability in the elimination of amoxicillin from aqueous solution. Moreover, laccase-catalyzed amoxicillin transformation was optimized by the use of experimentation based on Box–Behnken design.

2. Materials and methods

2.1. Method

The present empirical study has been carried out in a laboratory scale for the purpose of surveying amoxicillin elimination, as the dependent variable, in contrast to the independent parameters such as primary antibiotics concentration, temperature, pH, retention time, hydroxybenzotriazole (HBT) mediator concentration and enzyme activity rate. Process optimization was accomplished via Box–Behnken design.

2.2. Chemicals

The enzyme used in the experiments was laccase which is produced from a fungus named *Trametes versicolor* (EC1.10.3.2). HBT and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) mediators and amoxicillin were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium citrate and citric acid, with a 99.5% degree of purity, were used for the preparation of 0.1 M buffer solution required for enzymatic reaction as well as methanol and acetonitrile solvents for the measurement of amoxicillin were procured from Merck (Darmstadt, Germany). Distilled water prepared based upon membrane methods was applied for the preparation of all the solutions and buffers.

2.3. Instruments

Shimadzu spectrophotometer device, model UV-180, was used to assay the enzyme activity. The AMX concentration was analyzed using HPLC-UV system, equipped with a reverse phase column (C18 column, with an annulus of 4.6 mm and a length of 150 mm). The mobile phase consisted of 0.5% acetic acid/acetonitrile (80:20, v/v) with an isocratic flow of 1 mL min^{-1}. AMX was detected at 254 nm [12]. All of the used devices were calibrated before doing experiments according to the corresponding catalogues and their calibration curves were drawn.

2.4. Laccase assay

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), one of the most commonly applied substrates, was used to measure laccase activity. First, a 2 mM of the substrate was prepared in citrate buffer (0.1 molar, pH = 4.5).

The changes in the maximum reactive mixture absorption were measured in 420 nm by spectrophotometer after giving it the sufficient time and the result was converted to enzyme units based on ABTS molar extinction coefficient (ε_{420} = 36,000 M^{-1} cm^{-1}). One unit of laccase activity is the amount of the enzyme that is capable of oxidizing the 1 micromole of the substrate in a pH = 4.5 in 25°C within 1 min [29].

2.5. Experimental design and statistical analysis

After the amoxicillin solutions were prepared through being solved in citrate buffer (0.1 M, pH in a range of 3–7), elimination experiments were carried out by adding laccase with the final activity rate of 0.25–1.75 U/mL, HBT between 0.5 and 1.5 mM and concentrations ranging from 10 to 150 mg/L [30,31] in 30°C, 45°C and 60°C and 50 rpm in a final volume of 5 mL. Samples were taken in 15, 37.5 and 60 min and passed through 0.45 μm PTFE filters and then amoxicillin concentration was measured in the samples by means of HPLC. Amoxicillin removal rate can be calculated via the following relation:

$$R\ (\%) = [C_0 - (C_t/C_0)] \times 100 \quad (1)$$

R = elimination rate in percent, C_0 = amoxicillin concentration before reaction in mg/L, C_t = amoxicillin concentration after reaction in mg/L.

All of the experiments were repeated twice and the removal output was reported in percentage.

2.6. Optimizing the enzymatic process by the use of response surface method

Response surface method was used for the evaluation of the independent variables' effect on the response performance (amoxicillin removal output) based on the Box–Behnken design. Independent variables in the present study were temperature (A), pH of the solution (B), time (C), enzyme activity (D), HBT concentration (E) and AMX preliminary concentration (F) in three levels, namely minimum, medium and maximum and in three forms, –1, 0 and +1 (Table 2).

Table 2
Independent variables and the range and levels of their experimental amounts

Variable	Factors	Range and level		
		Low (–1)	Middle (0)	High (+1)
pH	A	3	5	7
Temperature, °C	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 antibiotic, mg/L	F	10	80	150

In the present study, the entire array of the experiments, 54 tests with two replications resulting in a total of 108 tests, were conducted based upon Box–Behnken design. Empirical conditions and amoxicillin elimination results of the laccase enzyme have been given in Table 3 based on a factorial design.

Analyses were carried out by the use of Design Expert 7. To avoid the systematic errors, the experiments were undertaken randomly. Cross-sectional interaction model coefficients are interpretive of the amoxicillin removal rates (response) as the performance of the independent factors. The study data were analyzed by means of multiple regression analyses. Analysis of variance (ANOVA) was used for the data analyses considering a significance level of $P \leq 0.05$. In the end, variance analysis results were tabulated and diagrams of primary factors effects, Pareto chart, the surface response and factors interactions were drawn.

3. Results and discussion

3.1. Box–Behnken statistical analyses

In the present study, the experiments were carried out under specified conditions by Box–Behnken model. The results of the experiments are given in Table 3.

The first stage in results analysis is the selection of a proper model capable of predicting the system results with an appropriate precision. To do so in the current research paper, the proposed quadratic polynomial model was used. ANOVA is usually applied for the model assessment and testing the model's significance. The ANOVA results pertaining to amoxicillin removal by the use of laccase enzyme have been given in Table 4. Those factors with p-values greater than 0.05 for their individual or mutual effects are to be omitted from the model. According to the parameters remaining in the system, in the end, a model was offered for the prediction of the antibiotics elimination outputs. The model includes the factors' individual effects, interactive effects and the effects pertaining to the curvature. Eq. (2) indicates the proposed model:

$$\begin{aligned} R = {} & +81.63 + 2.1*A - 7.02 * B + 11.83 * C \\ & + 8.45 * D - 14.27 * F - 4.56 * AE + 3.54 * AF \\ & - 4.45 * BD - 3.45 * BE + 4.84* +4.87 * CF \\ & - 14.58 * A^2 - 41.86 * B^2 - 10.02 * C^2 \\ & - 6.85 * D^2 - 15.6 * E^2 - 3.21 * F^2 \end{aligned} \quad (2)$$

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

The ANOVA results confirm the model's accuracy. F is a scale of the data deviation from the mean values. Generally, for a model that successfully predicts the experiment results, F-value is usually very high and a probability value smaller than 0.05 is also interpreted as the model's statistical significance. F-value obtained for the proposed model is 110.37, indicating its perfect significance. In this equation, linear parameters A, B, C, D and F, quadratic parameters A^2, B^2, C^2, E^2 and F^2 along with interactive parameters AE, AF, BD, BE, BF and CF are the indicator parameters of the model.

Table 3
Experiment design and results

Run	A	B	C	D	E	F	*R* (%)
	T (°C)	pH	Time (min)	En (U/mL)	HBT (mM)	C (mg/L)	
29	45	5	37.5	1	1	80	81.4
36	60	5	60	1	1	10	74.5
47	45	7	60	1	0.5	80	19.2
7	45	3	15	1	1.5	80	20.5
14	30	5	15	1	1	10	58
20	30	5	15	1	1	150	17
49	45	7	60	1	1.5	80	21.3
17	30	5	37.5	0.25	1.5	80	48
8	45	3	60	1	1.5	80	39.48
32	45	5	15	0.25	1	150	16.5
5	45	3	15	1	0.5	80	9.7
35	60	5	15	1	1	10	53
34	45	5	60	1.75	1	150	70
21	45	5	15	0.25	1	10	59.8
40	60	5	37.5	1.75	1.5	80	69.3
23	45	5	15	1.75	1	10	83.4
39	60	5	37.5	0.25	1.5	80	51.7
3	45	3	37.5	1	0.5	10	46
4	45	3	37.5	1	1.5	10	55
33	45	5	15	1.75	1	150	37
12	60	3	37.5	1.75	1	80	37.5
41	60	5	15	1	1	150	18.7
48	45	7	15	1	0.5	80	10.3
50	45	7	15	1	1.5	80	7.6
2	30	3	37.5	1.75	1	80	32.7
10	45	3	37.5	1	1.5	150	26.5
11	60	3	37.5	0.25	1	80	19.1
15	30	5	37.5	0.25	0.5	80	22.4
16	30	5	37.5	1.75	0.5	80	39.1
22	45	5	60	0.25	1	10	79
42	60	5	60	1	1	150	68.5
18	30	5	37.5	1.75	1.5	80	66
25	45	5	37.5	1	1	80	81
19	30	5	60	1	1	150	52
26	45	5	37.5	1	1	80	79.8
27	45	5	37.5	1	1	80	81.2
13	30	5	60	1	1	10	80
28	45	5	37.5	1	1	80	80.7
45	45	7	37.5	1	0.5	10	22
1	30	3	37.5	0.25	1	80	8
52	45	7	37.5	1	1.5	150	14
53	60	7	37.5	0.25	1	80	8.6
30	45	5	37.5	1	1	80	80.7
51	45	7	37.5	1	0.5	150	6.3
37	60	5	37.5	0.25	0.5	80	31.9
38	60	5	37.5	1.75	0.5	80	65.7
9	45	3	37.5	1	0.5	150	5.36
43	30	7	37.5	0.25	1	80	8

(Continued)

Table 3 *(Continued)*

Run	A	B	C	D	E	F	*R* (%)
	T (°C)	pH	Time (min)	En (U/mL)	HBT (mM)	C (mg/L)	
31	45	5	60	0.25	1	150	46.7
6	45	3	60	1	0.5	80	22
24	45	5	60	1.75	1	10	91.5
44	30	7	37.5	1.75	1	80	10.6
46	45	7	37.5	1	1.5	10	29
54	60	7	37.5	1.75	1	80	13.6

Table 4
ANOVA results pertaining to amoxicillin elimination

Source	Sum of squares	df	Mean square	F value	p-Value Prob > F	
Model	75,914.25	27	2,811.64	110.37	<0.0001	Significant
A-T	178.58	1	178.58	7.01	0.0098	
B-pH	2,163.38	1	2,163.38	84.92	<0.0001	
C-Time	5,659.52	1	5,659.52	222.16	<0.0001	
D-En	2,885.45	1	2,885.45	113.27	<0.0001	
E-HBT	38.22	1	38.22	1.5	0.2242	
F-C	8,231.12	1	8,231.12	323.1	<0.0001	
AB	38.6	1	38.6	1.52	0.222	
AC	41.73	1	41.73	1.64	0.2043	
AD	17.18	1	17.18	0.67	0.4139	
AE	213.16	1	213.16	8.37	0.0049	
AF	200.51	1	200.51	7.87	0.0063	
BC	19.82	1	19.82	0.78	0.3803	
BD	317.29	1	317.29	12.45	0.0007	
BE	244.98	1	244.98	9.62	0.0027	
BF	376.26	1	376.26	14.77	0.0002	
CD	12.78	1	12.78	0.5	0.4808	
CE	33.5	1	33.5	1.31	0.2549	
CF	759.14	1	759.14	29.8	<0.0001	
DE	67.24	1	67.24	2.64	0.1082	
DF	15.8	1	15.8	0.62	0.4333	
EF	46.55	1	46.55	1.83	0.1803	
A^2	4,377.58	1	4,377.58	171.84	<0.0001	
B^2	36,052.82	1	36,052.82	1,415.22	<0.0001	
C^2	2,068.36	1	2,068.36	81.19	<0.0001	
D^2	966.36	1	966.36	37.93	<0.0001	
E^2	2,053.77	1	2,053.77	80.62	<0.0001	
F^2	211.77	1	211.77	8.31	0.0051	
Residual	2,038.01	80	25.48			
Cor total	77,952.26	107				

On the other hand, R^2, 0.974, complies with R^2adj, 0.955, which reflects the model's accuracy. The more the value approaches unity, the better the relationship between the calculated results and the laboratory results. Accuracy function (adequate precision) measures the signal to noise ratio and a ratio larger than 4 is generally considered optimum. The ratio is 34.53 for the proposed model and this is indicative of a high signal to noise ratio [25]. Durbin–Watson test was carried out to investigate the errors' independence (the difference between the real values and the predicted values calculated in regression equation) and because Durbin–Watson value was found to be 2.2 in the current research paper, that is, in a 1.5–2.5 range, the assumption of the absence of a correlation between the errors cannot be rejected and regression analysis can be used because such an assumption is a necessary hypothesis for accomplishing regression analyses.

Distributive sketching of the experimental data vs. the predicted values by the proposed model is shown in Fig. 1. The results therein are indicative of the model's acceptability. Residuals diagram analysis for figuring the model's goodness of fitness is based on three assumptions.

If these three assumptions are true, the selected model is credible otherwise another model should be chosen for the data analyses. Assessing the assumptions' accuracy is performed by means of the following diagrams [32].

Fig. 1(a) illustrates the residuals normality diagram. The middle line of the bisector for the first quarter designates the expected normal distribution values and dots, residual dots if they are close to the line, are expressive of the residuals' normality. There is no deviation seen in residuals' normality. Fig. 1(b) depicts the residuals' scattering diagram in contrast to the given fitted values used to investigate the residuals' variance invariability. In case that no special trend is found in the diagram, variance invariability assumption would be accepted. There is no specific trend seen in the diagram expressive of the variance getting higher or lower; therefore, variance invariability assumption is accordingly affirmed. Fig. 1(c) displays the residuals' scattering with respect to the data collection sequence and it is applied to evaluate the inter-residuals independence. In case that no trend is found, such as sinusoid trends, in the diagram, the corresponding hypothesis should, as well, be accepted. There is no special trend seen in the diagram with which the residuals' independence assumption can be rejected. Therefore, according to the analyses run over the above diagrams and the intended assumptions being accepted it can be stated that the selected model is appropriate for the data analyses. Fig. 1(d) is the proposed model for investigating amoxicillin removal

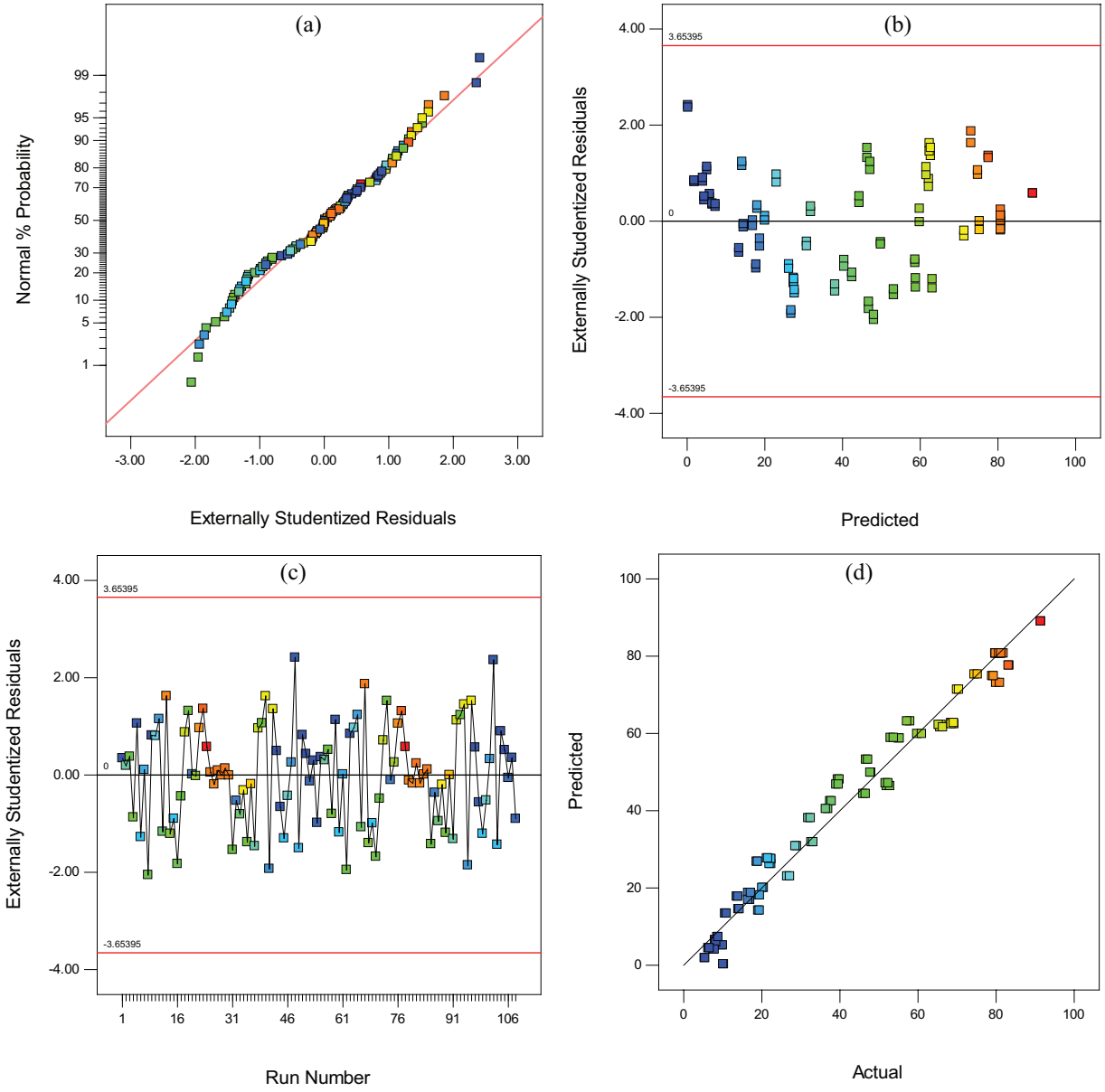


Fig. 1. Experimental data distribution vs. values predicted by the proposed model.

output, with R^2 and R^2adj values equal to 0.974 and 0.955, respectively, which are indicative of the model's compliance to the experimental data.

Fig. 2 demonstrates the effect of the preliminary factors on amoxicillin oxidation. According to the following diagram, it has been shown that the entire variables considered by the present article including pH, temperature, contact time, enzyme activity, HBT concentration and amoxicillin concentration possess an optimum point within the selected range. Therefore, it is evident that the domains are selected accurately.

Pareto chart is illustrative of the effects factors exert on oxidation process. Based on Fig. 3, pH, among the tested factors, is the most important and most significant factor in amoxicillin oxidation and amoxicillin concentration, time, temperature, enzyme activity and HBT concentration take the next ranks in importance. Also, it is clear from the diagram that pH and contact time have a reductive effect on amoxicillin concentration and enzyme activity and HBT concentration positively influences the oxidation process. It is also observed in the diagram that time and enzyme activity interaction is of a great importance in the experiment because of their positive effects on the oxidation.

3.2. Effect of solution's preliminary pH

According to the prior studies [33,34], pH exerts a considerable effect on enzyme activity and the amount of the radical produced, therefore, it is recounted as an important variable in enzymatic oxidation. It is seen, according to Figs. 2 and 4(a) in the present study, that the optimum pH value is 5 and with the decrease or increase of the pH, amoxicillin concentration increases as a result of which oxidation is reduced. This is due to the effect that pH has on enzyme activity as a result of which HBT radical production is reduced. Many of the enzymes' nature change irreversibly under acidic or basic conditions. The role of pH in enzyme's catalytic activity stems from the effect it has on the reacting groups in active enzyme positions (copper atoms in laccase enzyme). Optimum pH for a greater activity of the fungus laccase in oxidation processes under mildly acidic conditions ranges from 4 to 6 [35]. In a study conducted by Khlifi et al. [36], discoloration occurred in pH values of 4, 5 and 6 and the optimum pH was 5. In pH = 8, no blanching happened and in pH = 7 discoloration progressed slowly. These results are consistent with the findings obtained herein. Also, Fig. 4(a) shows the pH and contact time mutual effects on amoxicillin removal. Reaction time, as well, influences the enzymatic oxidation refining efficiency in such a way that amoxicillin oxidation increases with the increase in reaction duration.

3.3. Temperature effect

Temperature is an important and influential factor in biotechnological systems. Due to their protein-made composition, enzymes show particular sensitivity to temperature variations. Most of the enzymes slowdown in lower (10°C) and

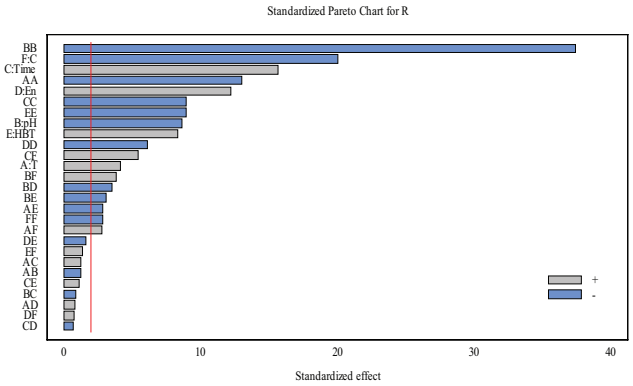


Fig. 3. Pareto chart for AMX removal (A: temperature, B: pH, C: time, D: En, E: HBT, F: amoxicillin concentration).

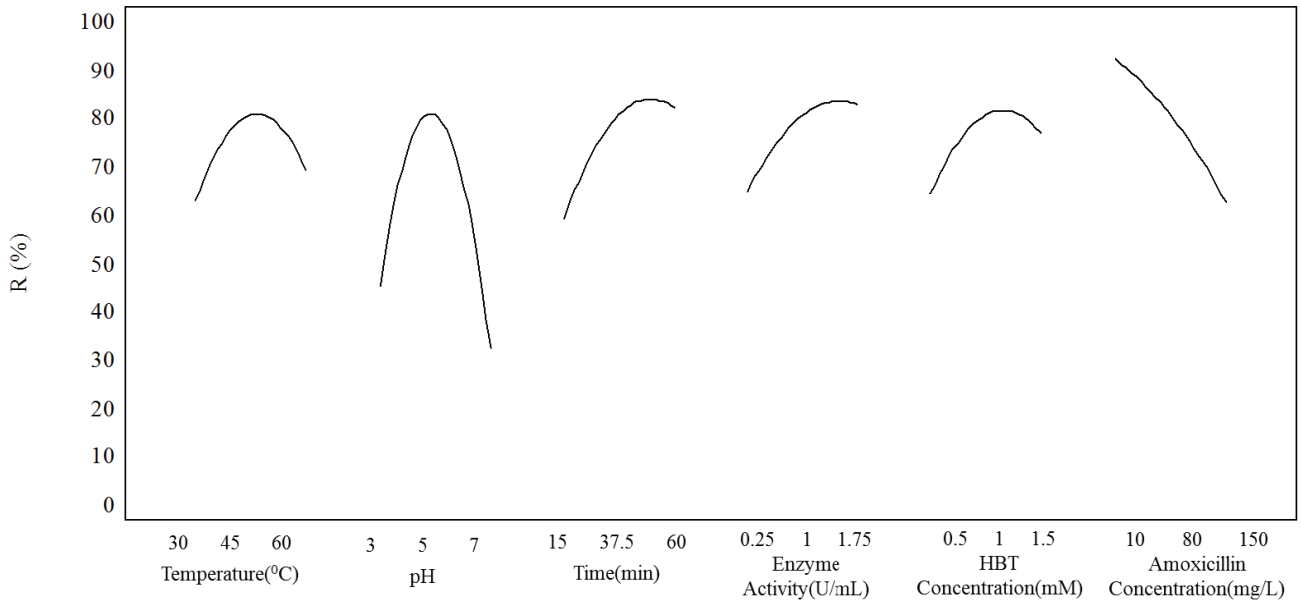


Fig. 2. Preliminary factors effects diagram.

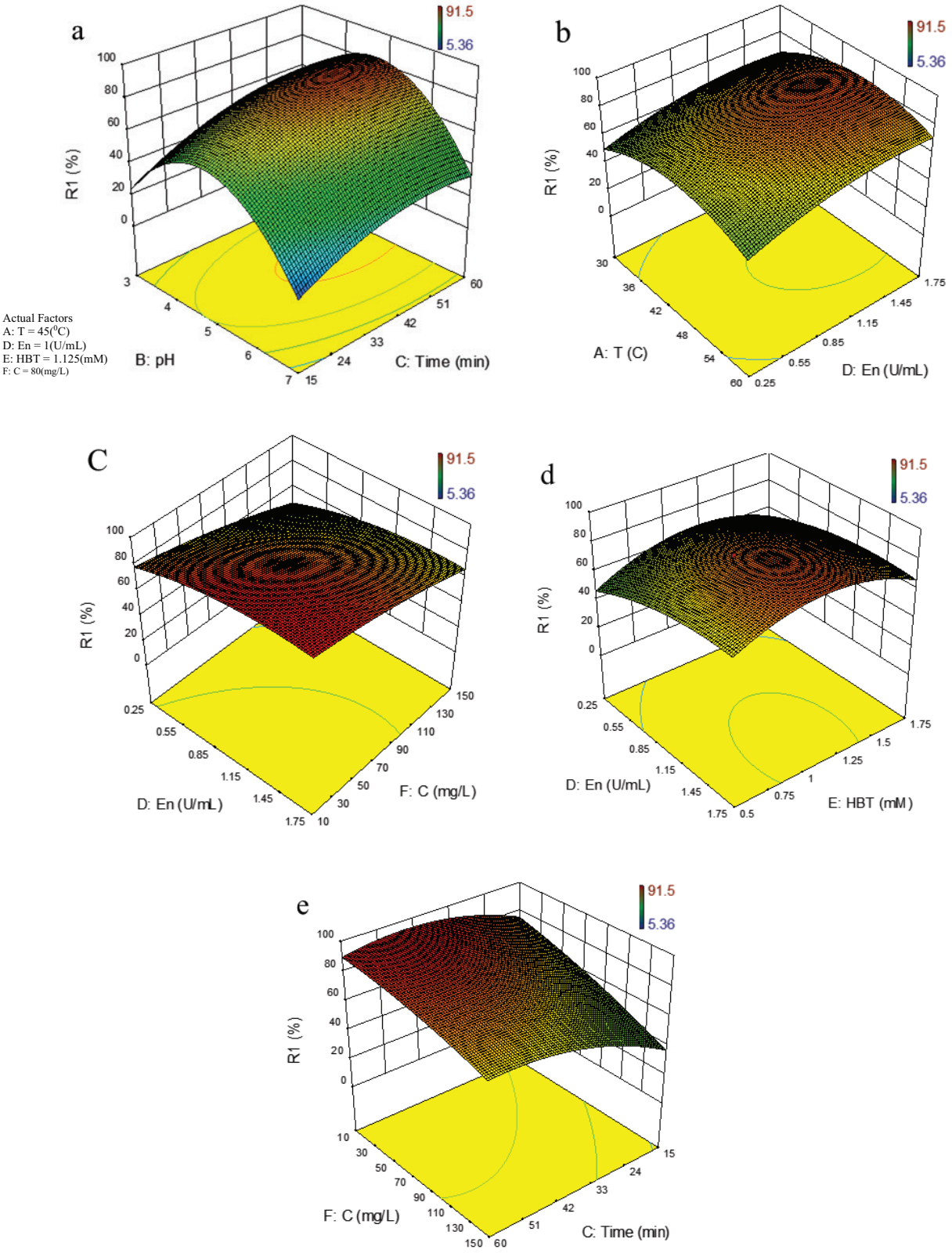


Fig. 4. Overlap diagram of the independent variables effects on amoxicillin removal efficiency. (a) pH and time (T = 45°C, En = 1 U/mL, HBT = 1.125 mM, C = 80 mg/L); (b) temperature and enzyme activity (pH = 5, time = 37.5 min, HBT = 1.125 mM, C = 80 mg/L); (c) enzyme activity and amoxicillin concentration (T = 45°C, pH = 5, time = 37.5 min, HBT = 1.125 mM); (d) enzyme activity and HBT concentration (T = 45°, pH = 5, time = 37.5 min, C = 80 mg/L) and (e) amoxicillin preliminary concentration and time (T = 45°C, pH = 5, En = 1 U/mL, HBT = 1.125 mM).

higher temperatures (60°C), or they become inactive. Thermal inactivation is predominantly due to the changes in the 3D structure of the enzyme because of the enzymes' active sites being destroyed [37]. Asgher et al. [38] investigated the effect of various variables on enzyme activity in a study. It was determined that free laccase enzyme is most active at 45°C [38]. The effect of temperature variations on laccase efficiency in removing amoxicillin has been found in temperatures 30°C, 45°C and 60°C. The results of the current part of the present research paper have been shown in Figs. 2 and 4(b). In enzymatic oxidation process, as shown in Fig. 2, amoxicillin elimination output shows increasing trends from 30°C to 45°C and the highest output is attained at 45°C; moreover, it was indicated that with the increase in the temperature further above this value, the elimination output sustains a reduction. Fig. 4(b) shows the mutual effects of the temperature and enzyme activity and as it can be seen the highest output is obtained at 45°C and enzyme activity equal to 1.75 U/mL. In the study carried out by Forootanfar et al. [33], the optimum temperature for laccase in discoloring the wastewater is reported to be 45°C. Also, in a study performed by Kim and Nicell [39] on the bisphenol A elimination by the use of laccase, it was figured out that the best elimination output can be achieved at 45°C. These latter findings, as well, correspond to the results obtained herein. Fig. 4(b) shows the mutual effects derived from temperature and enzyme activity on the enzymatic oxidation processes and as it can be discerned, the highest efficiency is obtained at a temperature of 45°C and enzyme activity of 1.75 U/mL.

3.4. Enzyme activity effect

To measure the optimum amount of laccase enzyme, the experiments were carried out in 0.25, 1 and 1.75 U/mL amounts of laccase and the results have been given in Figs. 2 and 4(c). As it can be observed from the diagrams, the enzymatic oxidation and, subsequently, amoxicillin elimination rate increases with the increase in enzyme activity. The highest output in enzyme activity was observed in 1.75 U/mL. Benzina et al. [40] demonstrated that with the increase in laccase concentration from 0.6 to 0.8 U/mL, (pH = 5, a retention time of 20 h, HBT mediator concentration of 0.55, a temperature of 30°C), discoloration output increases from 0.77 to 0.88 [40]. Also, in a study undertaken by Forootanfar et al. [33], it was shown that with the increase in enzyme activity up to 2 U/mL, the discoloration rate increases as well and with the increase in the enzyme activity further up the aforesaid value, the elimination variation rates are negligible. These findings are consistent with the results obtained herein.

3.5. HBT mediator effect

HBT, in concentrations equal to 0.5, 1 and 1.5 mM, has been used as a mediator in the present study. HBT's mediatory activity is attributed to the effect of N–O group in laccase, especially in higher concentration [41,42]. Due to high redox potential (1,084 mV) and the catalytic role of N–OH, HBT is one of the most effective mediators [43]. There are reports of the mediated laccase systems applicability in refining the hazardous organic contaminants and artificial paints [23]. The results of this section of the study demonstrated that enzymatic oxidation output in the presence of HBT mediator increases in 0.5–1 mM and with further increasing the mediator concentration up the aforementioned value the output decreases. The results pertaining to the effect of mediator concentration can be observed in Figs. 2 and 4(d). Also, Fig. 4(d) is illustrative of the mutual effects of enzyme activity and HBT mediator on amoxicillin oxidation and, as seen, the highest elimination rate is found in an enzyme activity of 1.75 U/mL and HBT concentration of 1 mM. In a study carried out by Rahmani et al. [22] on sulphonamide antibiotic removal by the use of laccase enzyme, it was concluded that increasing HBT concentration to 1 mM causes an increase in enzyme efficiency but the increase in HBT further up this value causes a reduction in oxidation efficiency. Also, Yousefi-Ahmadipour et al. [44] obtained an optimum concentration equal to 1 mM for HBT in enzyme-aided removal of ketoconazole which is deemed confirming the results obtained herein.

3.6. Amoxicillin concentration effect

Mediatory enzyme reaction largely depends on the concentration of the substrate existing in the liquid phase in such a manner that the reaction speeds up with the increase in substrate concentration in respect to the enzyme activity as well as to mediator concentration and the increase in substrate concentration does not have any effect on the reaction speed after reaching equilibrium. Also, the more the enzyme to substrate ratio is increased the greater the oxidation. An experiment was conducted in 10, 80 and 150 mg/L amoxicillin. The results are shown in Figs. 2, 4(d) and 4(c). Figs. 4(b) and (c), respectively, illustrate the mutual effects of enzyme activity, amoxicillin concentration and reaction time with concentration. Enzymatic oxidation rate decreases with the increase in amoxicillin concentration, as the substrate, against fixed enzymatic activities such that the highest and the lowest efficiency rates are observed in amoxicillin concentrations of 10 and 150 mg/L, respectively. Tahmasbi et al. [45] applied laccase enzyme for the removal of imipramine from an aqueous environment to show that the elimination output decreases with the increase in contaminant's concentration from 0.1 to 0.3 mg/mL. In a study that was undertaken by Rezaei et al. [46] in removing Acid Blue 92 paints by the use of laccase enzyme, the highest and the lowest removal rates were observed in 75 and 200 mg/L, respectively. These findings conform to the results obtained herein.

3.7. Process optimization

In the current research paper, optimization, temperature independent variables, pH, retention time, enzyme activity rate, HBT mediator concentration, antibiotic's preliminary concentration and maximum amoxicillin removal output were taken into consideration within the area of study case. The study aimed at the determination of optimum temperature, pH, retention time, enzyme activity rate, HBT mediator concentration and antibiotic preliminary concentration for acquiring better amoxicillin removal outputs. Various modes were investigated corresponding to the experiments for finding out the optimum conditions and finally the optimum conditions were concluded and presented in Table 5. Amoxicillin removal output was predicted to be 95.3% under such optimized conditions. This is the result that has been confirmed in empirical tests under the same conditions.

Table 5
Independent variables' optimum conditions for amoxicillin removal

Factor	Low	High	Optimum
Temperature (°C)	30	60	48.8
pH	3	7	4.8
Time (min)	15	60	43.5
En (U/mL)	0.25	1.75	1.35
HBT (mM)	0.5	1.5	1.04
Amoxicillin concentration (mg/L)	10	150	11.3
Optimum value (%)		95.3	

4. Conclusion

According to the results obtained from the present study, the most important parameter having a large effect on the process was pH; the optimal value of which was 5 in the present study. It was also showed that removal efficiency decreases with the increase or decrease in the aforesaid pH value. Optimum conditions for amoxicillin removal as pointed out by the process adopted in the current research in terms of pH, temperature, enzyme activity, HBT concentration and amoxicillin concentration were 5, 45°C, 1.75 U/mL, 1 mM and 10 mg/L, respectively. Under optimized conditions, laccase enzyme efficiency was found to be 91.5% at the end of a 60-min experiment period. Based on the obtained results and according to the acceptable performance and appropriate removal of amoxicillin by laccase, enzymatic oxidation can be applied as an appropriate and highly efficient method for treating the wastewaters containing amoxicillin. Furthermore, based on the findings, it can be concluded that experimental design methods are efficient means for reducing the costs and the quantity of the tests. Additionally, running surveys on variables' mutual effects can assist us in gaining a better insight regarding the way and the extent to which the independent variables influence the dependent variables.

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References

[1] L.M. Pastrana-Martínez, J.L. Faria, J.M. Doña-Rodríguez, C. Fernández-Rodríguez, A.M. Silva, Degradation of diphenhydramine pharmaceutical in aqueous solutions by using two highly active TiO_2 photocatalysts: operating parameters and photocatalytic mechanism, Appl. Catal., B, 113 (2012) 221–227.
[2] A. Eslami, H. Bahrami, A. Asadi, A. Alinejad, Enhanced sonochemical degradation of tetracycline by sulfate radicals, Water Sci. Technol., 73 (2016) 1293–1300.
[3] N.F. Moreira, C.A. Orge, A.R. Ribeiro, J.L. Faria, O.C. Nunes, M.F.R. Pereira, A.M. Silva, Fast mineralization and detoxification of amoxicillin and diclofenac by photocatalytic ozonation and application to an urban wastewater, Water Res., 87 (2015) 87–96.
[4] H. Dong, X. Yuan, W. Wang, Z. Qiang, Occurrence and removal of antibiotics in ecological and conventional wastewater treatment processes: a field study, J. Environ. Manage., 178 (2016) 11–19.
[5] A. Widyasari-Mehta, S. Hartung, R. Kreuzig, From the application of antibiotics to antibiotic residues in liquid manures and digestates: a screening study in one European center of conventional pig husbandry, J. Environ. Manage., 177 (2016) 129–137.
[6] R. Mohammadi, B. Massoumi, M. Rabani, Photocatalytic decomposition of amoxicillin trihydrate antibiotic in aqueous solutions under UV irradiation using Sn/TiO_2 nanoparticles, Int. J. Photoenergy, 2012 (2012) 1–11.
[7] G.Z. Kyzas, J. Fu, N.K. Lazaridis, D.N. Bikiaris, K.A. Matis, New approaches on the removal of pharmaceuticals from wastewaters with adsorbent materials, J. Mol. Liq., 209 (2015) 87–93.
[8] C. Cai, L. Wang, H. Gao, L. Hou, H. Zhang, Ultrasound enhanced heterogeneous activation of peroxydisulfate by bimetallic Fe-Co/GAC catalyst for the degradation of Acid Orange 7 in water, J. Environ. Sci., 26 (2014) 1267–1273.
[9] Z. Shaykhi, A. Zinatizadeh, Statistical modeling of photocatalytic degradation of synthetic amoxicillin wastewater (SAW) in an immobilized TiO_2 photocatalytic reactor using response surface methodology (RSM), J. Taiwan Inst. Chem. Eng., 45 (2014) 1717–1726.
[10] E.S. Elmolla, M. Chaudhuri, Photocatalytic degradation of amoxicillin, ampicillin and cloxacillin antibiotics in aqueous solution using UV/TiO_2 and $UV/H_2O_2/TiO_2$ photocatalysis, Desalination, 252 (2010) 46–52.
[11] V. Homem, A. Alves, L. Santos, Amoxicillin degradation at ppb levels by Fenton's oxidation using design of experiments, Sci. Total Environ., 408 (2010) 6272–6280.
[12] Y.J. Jung, W.G. Kim, Y. Yoon, J.-W. Kang, Y.M. Hong, H.W. Kim, Removal of amoxicillin by UV and UV/H_2O_2 processes, Sci. Total Environ., 420 (2012) 160–167.
[13] K. Yaghmaeian, G. Moussavi, A. Alahabadi, Removal of amoxicillin from contaminated water using NH_4Cl-activated carbon: continuous flow fixed-bed adsorption and catalytic ozonation regeneration, Chem. Eng. J., 236 (2014) 538–544.
[14] V. Homem, L. Santos, Degradation and removal methods of antibiotics from aqueous matrices–a review, J. Environ. Manage., 92 (2011) 2304–2347.
[15] E.S. Elmolla, M. Chaudhuri, Degradation of amoxicillin, ampicillin and cloxacillin antibiotics in aqueous solution by the UV/ZnO photocatalytic process, J. Hazard. Mater., 173 (2010) 445–449.
[16] E.S. Elmolla, M. Chaudhuri, Degradation of the antibiotics amoxicillin, ampicillin and cloxacillin in aqueous solution by the photo-Fenton process, J. Hazard. Mater., 172 (2009) 1476–1481.
[17] A.G. Trovó, R.F.P. Nogueira, A. Agüera, A.R. Fernandez-Alba, S. Malato, Degradation of the antibiotic amoxicillin by photo-Fenton process–chemical and toxicological assessment, Water Res., 45 (2011) 1394–1402.
[18] G. Rios, M. Belleville, D. Paolucci, J. Sanchez, Progress in enzymatic membrane reactors–a review, J. Membr. Sci., 242 (2004) 189–196.
[19] F.I. Hai, K. Yamamoto, F. Nakajima, K. Fukushi, Application of a GAC-coated hollow fiber module to couple enzymatic degradation of dye on membrane to whole cell biodegradation within a membrane bioreactor, J. Membr. Sci., 389 (2012) 67–75.
[20] H. Forootanfar, M.M. Movahednia, S. Yaghmaei, M. Tabatabaei-Sameni, H. Rastegar, A. Sadighi, M.A. Faramarzi, Removal of chlorophenolic derivatives by soil isolated ascomycete

of *Paraconiothyrium variabile* and studying the role of its extracellular laccase, J. Hazard. Mater., 209 (2012) 199–203.
[21] E. Dehghanifard, A.J. Jafari, R.R. Kalantary, A.H. Mahvi, M.A. Faramarzi, A. Esrafili, Biodegradation of 2, 4-dinitrophenol with laccase immobilized on nano-porous silica beads, J. Environ. Health Sci. Eng., 10 (2013) 25.
[22] K. Rahmani, M.A. Faramarzi, A.H. Mahvi, M. Gholami, A. Esrafili, H. Forootanfar, M. Farzadkia, Elimination and detoxification of sulfathiazole and sulfamethoxazole assisted by laccase immobilized on porous silica beads, Int. Biodeterior. Biodegrad., 97 (2015) 107–114.
[23] A.I. Cañas, S. Camarero, Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes, Biotechnol. Adv., 28 (2010) 694–705.
[24] S. Ashrafi, S. Nasseri, M. Alimohammadi, A. Mahvi, M. Faramarzi, Optimization of the enzymatic elimination of flumequine by laccase-mediated system using response surface methodology, Desal. Wat. Treat., 57 (2016) 14478–14487.
[25] M. Mourabet, A. El Rhilassi, H. El Boujaady, M. Bennani-Ziatni, R. El Hamri, A. Taitai, Removal of fluoride from aqueous solution by adsorption on Apatitic tricalcium phosphate using Box–Behnken design and desirability function, Appl. Surf. Sci., 258 (2012) 4402–4410.
[26] K. Yetilmezsoy, S. Demirel, R.J. Vanderbei, Response surface modeling of Pb (II) removal from aqueous solution by *Pistacia vera* L.: Box–Behnken experimental design, J. Hazard. Mater., 171 (2009) 551–562.
[27] M. Khajeh, M. Kaykhaii, A. Sharafi, Application of PSO-artificial neural network and response surface methodology for removal of methylene blue using silver nanoparticles from water samples, J. Ind. Eng. Chem., 19 (2013) 1624–1630.
[28] S. Dutta, Optimization of Reactive Black 5 removal by adsorption process using Box–Behnken design, Desal. Wat. Treat., 51 (2013) 7631–7638.
[29] E. Donati, C.M. Polcaro, P. Ciccioli, E. Galli, The comparative study of a laccase-natural clinoptilolite-based catalyst activity and free laccase activity on model compounds, J. Hazard. Mater., 289 (2015) 83–90.
[30] A. Eslami, A. Asadi, M. Meserghani, H. Bahrami, Optimization of sonochemical degradation of amoxicillin by sulfate radicals in aqueous solution using response surface methodology (RSM), J. Mol. Liq., 222 (2016) 739–744.
[31] X. Weng, Q. Sun, S. Lin, Z. Chen, M. Megharaj, R. Naidu, Enhancement of catalytic degradation of amoxicillin in aqueous solution using clay supported bimetallic Fe/Ni nanoparticles, Chemosphere, 103 (2014) 80–85.
[32] M. Zazouli, M.A. Ebrahimzadeh, J. Yazdani Charati, A. Shiralizadeh Dezfoli, E. Rostamali, F. Veisi, Effect of sunlight and ultraviolet radiation in the titanium dioxide (TiO_2) nanoparticles for removal of furfural from water, J. Mazandaran Univ. Med. Sci., 23 (2013) 126–138.
[33] H. Forootanfar, S. Rezaei, H. Zeinvand-Lorestani, H. Tahmasbi, M. Mogharabi, A. Ameri, M.A. Faramarzi, Studies on the laccase-mediated decolorization, kinetic, and microtoxicity of some synthetic azo dyes, J. Environ. Health Sci. Eng., 14 (2016) 7.
[34] Z. Asadgol, H. Forootanfar, S. Rezaei, A.H. Mahvi, M.A. Faramarzi, Removal of phenol and bisphenol-A catalyzed by laccase in aqueous solution, J. Environ. Health Sci. Eng., 12 (2014) 93.
[35] P. Baldrian, Fungal laccases–occurrence and properties, FEMS Microbiol. Rev., 30 (2006) 215–242.
[36] R. Khlifi, L. Belbahri, S. Woodward, M. Ellouz, A. Dhouib, S. Sayadi, T. Mechichi, Decolourization and detoxification of textile industry wastewater by the laccase-mediator system, J. Hazard. Mater., 175 (2010) 802–808.
[37] M. Fabbrini, C. Galli, P. Gentili, Radical or electron-transfer mechanism of oxidation with some laccase/mediator systems, J. Mol. Catal. B Enzym., 18 (2002) 169–171.
[38] M. Asgher, S. Noreen, M. Bilal, Enhancing catalytic functionality of *Trametes versicolor* IBL-04 laccase by immobilization on chitosan microspheres, Chem. Eng. Res. Des., 117 (2017) 1–11.
[39] Y.-J. Kim, J.A. Nicell, Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A, Bioresour. Technol., 97 (2006) 1431–1442.
[40] O. Benzina, D. Daâssi, H. Zouari-Mechichi, F. Frikha, S. Woodward, L. Belbahri, S. Rodriguez-Couto, T. Mechichi, Decolorization and detoxification of two textile industry effluents by the laccase/1-hydroxybenzotriazole system, Environ. Sci. Pollut. Res., 20 (2013) 5177–5187.
[41] L. Papinutti, P. Dimitriu, F. Forchiassin, Stabilization studies of *Fomes sclerodermeus* laccases, Bioresour. Technol., 99 (2008) 419–424.
[42] S.D. Ashrafi, S. Rezaei, H. Forootanfar, A.H. Mahvi, M.A. Faramarzi, The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, *Paraconiothyrium variabile*, Int. Biodeterior. Biodegrad., 85 (2013) 173–181.
[43] S. Ostadhadi-Dehkordi, M. Tabatabaei-Sameni, H. Forootanfar, S. Kolahdouz, M. Ghazi-Khansari, M.A. Faramarzi, Degradation of some benzodiazepines by a laccase-mediated system in aqueous solution, Bioresour. Technol., 125 (2012) 344–347.
[44] A. Yousefi-Ahmadipour, M. Bozorgi-Koshalshahi, M. Mogharabi, M. Amini, M. Ghazi-Khansari, M.A. Faramarzi, Laccase-catalyzed treatment of ketoconazole, identification of biotransformed metabolites, determination of kinetic parameters, and evaluation of micro-toxicity, J. Mol. Catal. B Enzym., 133 (2016) 77–84.
[45] H. Tahmasbi, M.R. Khoshayand, M. Bozorgi-Koushalshahi, M. Heidary, M. Ghazi-Khansari, M.A. Faramarzi, Biocatalytic conversion and detoxification of imipramine by the laccase-mediated system, Int. Biodeterior. Biodegrad., 108 (2016) 1–8.
[46] S. Rezaei, H. Tahmasbi, M. Mogharabi, A. Ameri, H. Forootanfar, M.R. Khoshayand, M.A. Faramarzi, Laccase-catalyzed decolorization and detoxification of Acid Blue 92: statistical optimization, microtoxicity, kinetics, and energetics, J. Environ. Health Sci. Eng., 13 (2015) 31.