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Efficient decolorization and detoxification of reactive orange 7 using laccase isolated from *Paraconiothyrium variabile*, kinetics and energetics

Shahla Rezaei[a], Hamed Tahmasbi[a], Mehdi Mogharabi[a,b], Sajad Firuzyar[a], Alieh Ameri[c], Mohammad Reza Khoshayand[d,#], Mohammad Ali Faramarzi[a,b,*]

[a] *Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, P.O. Box 14155-6451, Tehran 1417614411, Iran*
[b] *Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran*
[c] *Department of Medicinal Chemistry, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran*
[d] *Department of Drug and Food Control, Faculty of Pharmacy and Pharmaceuticals Quality Assurance Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran*

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ABSTRACT

The fungal laccase isolated from *Paraconiothyrium variabile* was applied to optimize the enzymatic decolorization and detoxification of the azo dye reactive orange 7 using response surface methodology. Initial screening using fractional factorial design was carried out to select important independent variables that significantly affect the yield of decolorization including enzyme activity, pH, and incubation time. Optimum condition for maximal dye decolorization obtained by central composite design and surface plots were laccase activity 1.25 U/mL, pH 6.0, and incubation time 12.5 min. In addition, the kinetic and energetic parameters for enzymatic decolorization of reactive orange 7 were studied and the calculated values for K_m, V_{max}, E_a, ΔH, and ΔS were 1.14 mM, 283.28 mM/min.mg, 40 kJ/mol, 89 kJ/mol, and 285 J/mol.K, respectively. Decolorization is endothermic and spontaneous at temperatures higher than 37.8 °C. Microtoxicity evaluation demonstrated a decrease in toxicity of the laccase-treated dye in comparison with the parent dye.

1. Introduction

Synthetic dyes with anthraquinone, phthalocyanine, triphenylmethane and azo-based chromophores are extensively used in many industries such as pulp and paper, textiles, printing, cosmetics, and pharmaceuticals. The discharge of industrial wastes containing such highly toxic pollutants into the environment, motivates researchers to develop environment-friendly dye removal methods [1–4]. Azo dyes, which contain at least one –N=N– group in their chemical structures, are known to be one of the most important groups of synthetic colorants that are extensively used in various industries [5–8]. Various physicochemical approaches have been employed to treat wastewaters containing synthetic dyes such as adsorption, chemical oxidation, membrane filtration, flotation, precipitation, and ultrasonic mineralization. Compared with physicochemical treatment methods which suffer from a number of limitations such as high cost, low efficiency, and production of secondary sludge, biological remediation processes are cost effective and environmentally benign techniques for eliminating dyes from industrial effluents [9–13].

Laccases, a member of enzymes known as multi-copper containing oxidases, are able to oxidize a broad range of aromatic and non-aromatic substrates via the simultaneous reduction of oxygen to water [14,15]. As a result, lacasses are employed in many industrial and biotechnological fields such as food, pulp, paper and textiles, biosensors, fuel cells, and organic synthesis [16]. Moreover, laccases are extensively used in the biodegradation of various chemical substrates such as lignin related structures, chlorophenols, organophosphorus compounds, and synthetic dyes [17]. The enzymatic decolorization of some azo dyes leads to the formation of non-specific free radicals without direct cleavage of the azo bonds, avoiding production of highly toxic aromatic amines [18]. Traditional methods for medium optimization that evaluate the effect of one variable while others variables are maintained at a certain level, suffer from several disadvantages such as time consuming, high cost, and ignoring total interactions between medium components [19]. Therefore, researchers are encouraged to develop statistical experimental approaches and response surface methodology (RSM), which provides a great amount of information based on only a small number of experiments [20].

* Corresponding author: Tel.: +98 21 66954712; fax: +98 21 66954712.
E-mail addresses: khoshayand@tums.ac.ir (M.R. Khoshayand), faramarz@tums.ac, ffaramarzi@yahoo.com (M.A. Faramarzi).
Correspondence for statistical experimental design: Tel.: +98 21 66494997.

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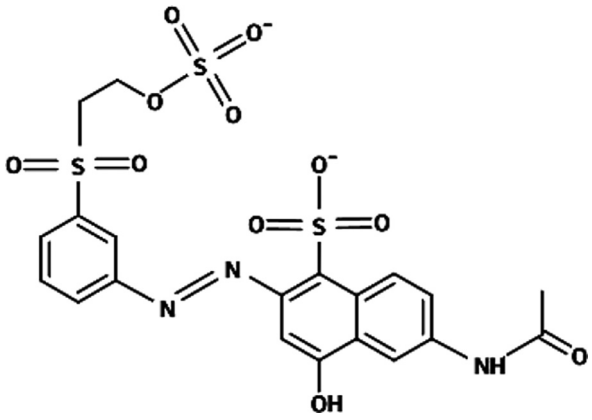

Fig. 1. Chemical structure of reactive orange 7.

In the present study, the purified laccase from a soil isolated ascomycete, *P. variabile*, was applied for decolorization of the synthetic dye reactive orange 7. Statistical experimental design was used to optimize the experimental conditions for enzymatic decolorization of reactive orange 7. In addition, the kinetic and thermodynamic parameters (K_m, V_{max}, E_a, ΔH, and ΔS) of the laccase-catalyzed decolorization process were determined and the toxic effect of untreated and laccase-treated dye solution was studied based on the growth inhibition of gram-positive bacterial strains.

2. Materials and methods

2.1. Chemicals and the enzyme

2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Reactive orange 7 (Fig. 1) was generously donated by Alvan Co. (Tehran, Iran). All other chemicals and reagents were of the highest purity available. Laccase was isolated from *P. variabile* [21]. The decolorization process was monitored using UV–Vis spectroscopy and the absorbance was scanned by a double-beam UV–Vis spectrophotometer (UVD 2950, Labomed, Culver City, CA, USA).

2.2. Laccase purification

The extracellular laccase from *P. variabile* was purified using the method noted in Forootanfar et al. [21]. In summary, after 10 days of incubation of large scale cultivation of *P. variabile* in sabouraud-2%-dextrose broth, the mycelia were removed using filtration and the extracellular liquid containing laccase was harvested. The extract filtrate obtained was saturated with $(NH_4)_2SO_4$ and then applied to an anion exchange column (Q-Sepharose XL). The collected enzyme was concentrated and saturated, and then applied to a Sephadex-G100 column. All purification steps were carried out at 4 °C (Table 1). Laccase activity and total protein amount were measured at each purification step. SDS–PAGE was finally used to check the purity of the isolated enzyme (Fig. 2).

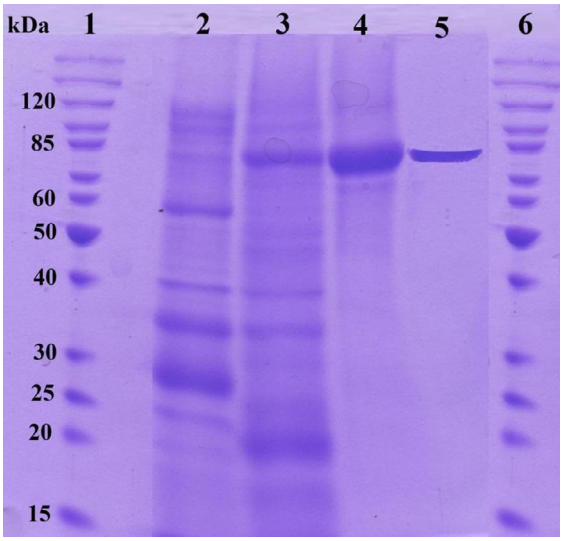

Fig. 2. SDS–PAGE of purification steps stained by Coomassie Blue.

2.3. Enzyme assay

The catalytic activity of laccase was determined using ABTS as a substrate [22]. Enzyme activity measurement was performed in a reaction mixture containing 100 mM citrate buffer (pH 4.5), 5 mM ABTS, and enzyme activity 0.5 U/mL. The oxidation rate of ABTS was monitored at room temperature by measuring the increase in absorbance at a specific wavelength of 420 nm ($\varepsilon_{420} = 36{,}000\ M^{-1}cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute.

2.4. Dye decolorization experiments

Decolorization experiments were performed using a reaction mixture containing citrate buffer (pH 3.0–7.0) and, various concentrations of dye (50–200 mg/L), laccase activity (0.5–1.5 U/mL), and incubation times (1–15 min) at an increasing temperature (30–70 °C). A decrease in the maximum absorbance of reactive orange (480 nm) was monitored to determine the percentage of decolorization. The following equation was applied to calculate the percentage of decolorization:

Decolorization (%) $= (A_{initial} - A_{final})/A_{initial} \times 100$; where $A_{initial}$ is the initial absorbance of reaction mixture and A_{final} is the absorbance after incubation time.

2.5. Experimental design and statistical analysis

2.5.1. Screening study

After preliminary study, the impact of effective variables on enzymatic dye decolorization was investigated by a fractional factorial design. In order to identify and select the significant factors, we used fractional factorial design, which is more feasible than other methods because it decreases the number of runs with suitable resolution [23]. Nevertheless, only a fraction of all possible levels of each variable are

Table 1
Purification of the extracellular laccase from the culture filtrates of *P. variabile*.

Purification step	Total protein (μg)	Total activity (mU)	Specific activity (mU/μg)	Yield (%)	Purification factor (fold)
Culture filtrate	155,500	132,760	0.854	100	1
$(NH4)_2SO_4$ (45–80%)	4268	43,710	10.24	32.9	11.99
Ion exchange	2437	50,110	20.56	37.7	24.07
Ammonium sulfate	1262	28,132	22.29	21.2	26.1
Gel filtration	308	6550	21.27	4.9	24.9

Table 2
Level of independent variables in fractional factorial design.

Variables	Symbol	Low level (−1)	High level (+1)
Enzyme activity	X_1	0.5	4
Dye concentration	X_2	50.0	200
pH	X_3	3.0	7.0
Temperature	X_4	30.0	70
Incubation time	X_5	1.0	15

Table 3
Fractional factorial design matrix and their observed response for reactive orange 7 decolorization.

Run no.	X_1	X_2	X_3	X_4	X_5	Decolorization (%)
1	0.5	200	3	30	15	11.00
2	0.5	50	7	70	1	18.22
3	1.5	50	7	30	15	58.73
4	1.5	200	3	70	1	5.98
5	1.0	125	5	50	8	14.00
6	1.5	200	7	70	15	48.53
7	0.5	200	7	30	1	6.57
8	0.5	50	3	70	15	31.29
9	1.5	50	3	30	1	0.00
10	1.0	125	5	50	8	16.00

run due to time and financial constraints. Interactions higher than two-way are insignificant and are assumed to be zero. In this study, a resolution of $V\ 3^{(5-2)}$ was interpreted that there is no confounded relation between the main factors. Table 2 shows the factors that may affect decolorization (%) as a response using fractional factorial design. Table 3 lists the design matrix based on the statistical software package Design-Expert (version 7.0.0, Stat-Ease, Inc., Minneapolis, MN, USA). It suggests evaluation of five factors in 10 experiments at high (+1) and low (−1) levels, with two experiments at the center point and measurement of the corresponding response. Analysis of variance for screening variables is displayed in Table 4. All experiments in the current study were performed in triplicate, and the average is displayed.

2.5.2. Optimization study

Following the identification of the variables that influence decolorization significantly, RSM was applied as a statistical method to determine the optimal level of each factor. Central composite design is the most popular RSM method and was applied to optimize the decolorization. This design consists of a two-level fractional factorial and two other types of points: central and axial. Meanwhile, uniform precision and orthogonality are introduced as two prominent properties of this design. Enzyme activity (X_1), pH (X_3), and incubation time (X_5) were considered to be significant in the screening design and therefore were defined at five levels, which were coded as $-\alpha$, −1, 0, +1, and $+\alpha$. The value of α should be calculated the way it fulfills rotatability and orthogonality in the design. According to the central composite design generated by Design-Expert software, 20 experiments, including 8 factorial points, 6 axial points and 6 replicates at the center point for prediction variance near the center of design, were carried out. Table 5 lists the actual factor level design matrix and the corresponding experimental data in which the response value (Y) for each run is the average of the three responses. The following second-order polynomial function can be used to model the mathematical relationship between the response (Y) (synthetic dye decolorization) and the independent variables (X_i):

$$Y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Y, predicted response; β_0, intercept; β_1, β_2 and β_3, linear coefficients; β_{11}, β_{22} and β_{33}, squared coefficients; β_{12}, β_{13} and β_{23}, interaction coefficients of the equation and X_1, X_2 and X_3, independent variables). The linear, quadratic and interactive effects of the independent variables on the response were evaluated using this equation.

Design-Expert was used to perform the statistical analysis using regression modeling and response surface graphs. ANOVA was also used via Fisher's test to determine the effect of the independent variables on the response, and a p value < 0.05 was considered to be statistically significant. The fitness of the second-order polynomial model equation is indicated qualitatively by the multiple correlation coefficients (R^2) and adjusted R^2. Contour plots and three-dimensional surface plots illustrate the relationship and interaction between coded variables and the responses. The equation from the final quadratic model and a grid search of an RSM plot were used to determine the optimal points.

2.6. Decolorization kinetics and energetics

The steady-state kinetic studies of laccase-catalyzed decolorization of reactive orange 7 were performed over a dye concentration range of 0.025−5 mM. All decolorization experiments were carried out at the optimal temperature and pH. The effect of temperature on the rate of decolorization was investigated using a dye concentration range of 12.5–500 μmol/L and a temperature range of 10–50 °C. Percentage of decolorization was monitored as described in Section 2.4.

2.7. Microtoxicity studies

The toxic effect of untreated and laccase-treated dye solution was determined based on the growth inhibition of three gram-positive

Table 4
Analysis of variance for screening variables.

Source	Sum of squares	*df*	Mean square	*F* value	Prob > *F*
Model	3166.81	5	633.36	22.07	0.014
X_1-enzyme activity	266.34	1	266.34	9.28	0.060
X_2-dye concentration	163.44	1	163.44	5.69	0.100
X_3-pH	877.39	1	877.39	30.57	0.010
X_4-temperature	96.05	1	96.05	3.35	0.160
X_5-incubation time	1763.59	1	1763.59	61.44	0.004
Curvature	90.96	1	90.96	3.17	0.170
Residual	86.11	3	28.70		
Lack of fit	84.11	2	42.06	21.03	0.150
Pure error	2	1	2		
Cor total	3343.88	9			
R-squared	0.9735				
Adj. R-squared	0.9294				
Pred. R-squared	0.5952				

p value < 0.05 considered significant

Table 5
Central composite design matrix containing various condition and related responses.

Run	Enzyme activity (X_1)	pH (X_3)	Incubation time (X_5)	Actual response	Predicted response
1	0.5	3	4.00	5.72	6.21
2	1	5	9.50	99.67	61.97
3	1.5	3	4.00	24.39	26.30
4	1	5	9.50	99.00	98.20
5	1.5	7	15.00	100.00	99.30
6	1	5	0.25	15.75	16.70
7	1	1.64	9.50	3.16	4.90
8	1	5	9.50	99.50	100.00
9	0.5	3	15.00	8.69	9.34
10	1.5	7	4.00	93.17	100.48
11	1.5	3	15.00	23.89	33.00
12	0.5	7	15.00	52.52	54.55
13	0.5	7	4.00	22.61	38.90
14	1.84	5	9.50	47.08	48.44
15	0.16	5	9.50	100.00	95.40
16	1	5	18.75	100.00	98.50
17	1	5	9.50	99.67	86.87
18	1	5	9.50	100.00	99.60
19	1	8.36	9.50	25.59	32.00
20	1	5	9.50	99.33	86.87

Table 6
Effective size estimation of significant independent variables on decolorization of RO7 using the CCD design.

Factor	Coefficient estimate	*df*	Standard error	95% CI low	95% CI high
Intercept	99.64	1	9.82	77.77	121.51
X_1	4.55	1	6.51	−9.96	19.06
X_3	18.83	1	6.51	4.32	33.34
X_5	13.07	1	6.51	−1.44	27.58
X_1X_5	−4.65	1	8.51	−23.61	14.31
X_3X_5	3.58	1	8.51	−15.38	22.54
X_1X_3	8.07	1	8.51	−10.89	27.03
${X_1}^2$	−8.75	1	6.34	−22.88	−5.37
${X_3}^2$	−28.80	1	6.34	−42.92	−14.67
${X_5}^2$	−14.99		6.34	−29.11	−0.86

Confidence interval = CI; p value < 0.05 considered significant

bacterial strains (*Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* ATCC 10240, and *Bacillus subtilis* ATCC 6633) and three gram-negative bacterial strains (*Salmonella typhi* ATCC 19430, *E. coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 9027). First, each bacterial strain was cultivated in Muller–Hinton broth medium to reach the optical density (OD_{600}) of 0.2. Thereafter, dye (final concentration of 100 mg/L) and its related laccase-treated solution was separately inserted into the prepared culture media and incubated at 37 °C followed by measuring the OD_{600} of each bacterial strain every 2 h for 10 h. The growth inhibition percentage (GI%) was then calculated based on the equation of $[(1 - D_{600S}/OD_{600C}) \times 100]$; where OD_{600S} is the OD_{600} of the sample and OD_{600C} is the OD_{600} of the control. All experiments were carried out in triplicate and means of the results were reported.

3. Result and discussion

3.1. Fractional factorial design for screening of important variables

A fractional factorial design was used to screen the effect of independent variables involving enzyme activity (X_1), dye concentration (X_2), pH (X_3), temperature (X_4), and incubation time (X_5). The coded range (high and low) of independent variables and the related responses are presented in Table 2 and Table 3, respectively. Statistical analysis of data via one way ANOVA showed that enzyme activity (X_1), pH (X_3), and incubation time (X_5) evidencing P-value less than 0.05 were determined as significant variables affecting the decolorization (Table 4). The multiple correlation coefficient (R^2) showed that 97% of the variations for dye decolorization efficiency are explained by the independent variables. In addition, the difference between adjusted R^2 (93%) and the predicted R^2 (59%) showed that the first-order model is not able to evaluate the relationship between independent factors and the response. Therefore, a second-order model was employed as an adequate mathematical equation for further investigation.

3.2. Optimization of dye decolorization conditions

Central composite response surface methodology was employed efficiently for the optimization of the selected significant variables including enzyme activity (X_1), pH (X_3), and incubation time (X_5). Table 5 lists the complete design matrix and the corresponding results of performing a total 20 runs to determine the effect of three independent variables. The coefficient of significant independent variables and 95% confidence intervals for each model parameter are presented in Table 6. Some of confidence intervals in Table 6 include zero which correspond to terms in the model that are not significant. In this model, enzyme with quadratic term, pH with linear and quadratic and incubation time with quadratic term on decolorization are significant. The plot of externally studentized residual *versus* the observed values for decolorization of reactive orange 7 showed no trend which proves the homogeneity of variance in the data (Fig. 3). Thus, for modeling decolorization the quadratic second-order polynomial equation was determined to be adequate over the range of independent variables in this study and the equation fitted to the data is as follows:

$$Y = 99.64 + 4.55X_1 - 18.83X_3 + 8.07X_1X_3 - 4.65X_1X_5 + 3.58X_3X_5 - 8.75{X_1}^2 - 28.8{X_3}^2 - 14.99{X_5}^2 \quad (2)$$

The Y indicates the yield of decolorization, and, X_1, X_3, and X_5 represent enzyme activity, pH and incubation time, respectively.

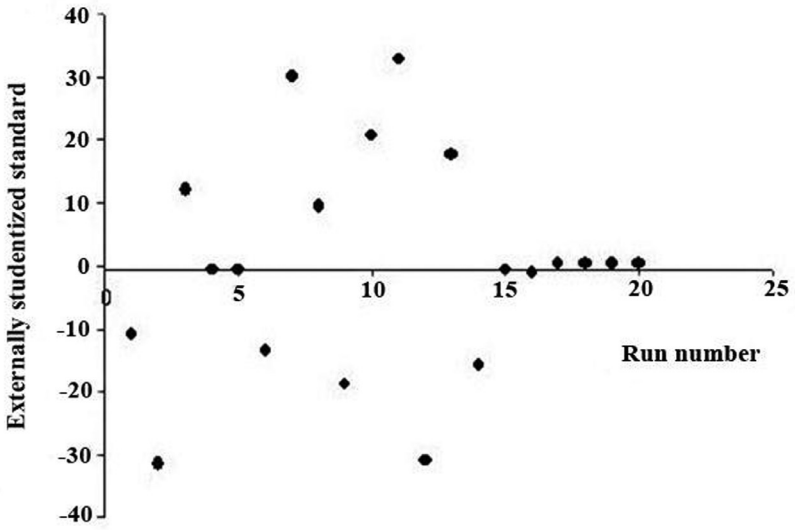

Fig. 3. Residual plot; Standardized residuals *versus* fitted values.

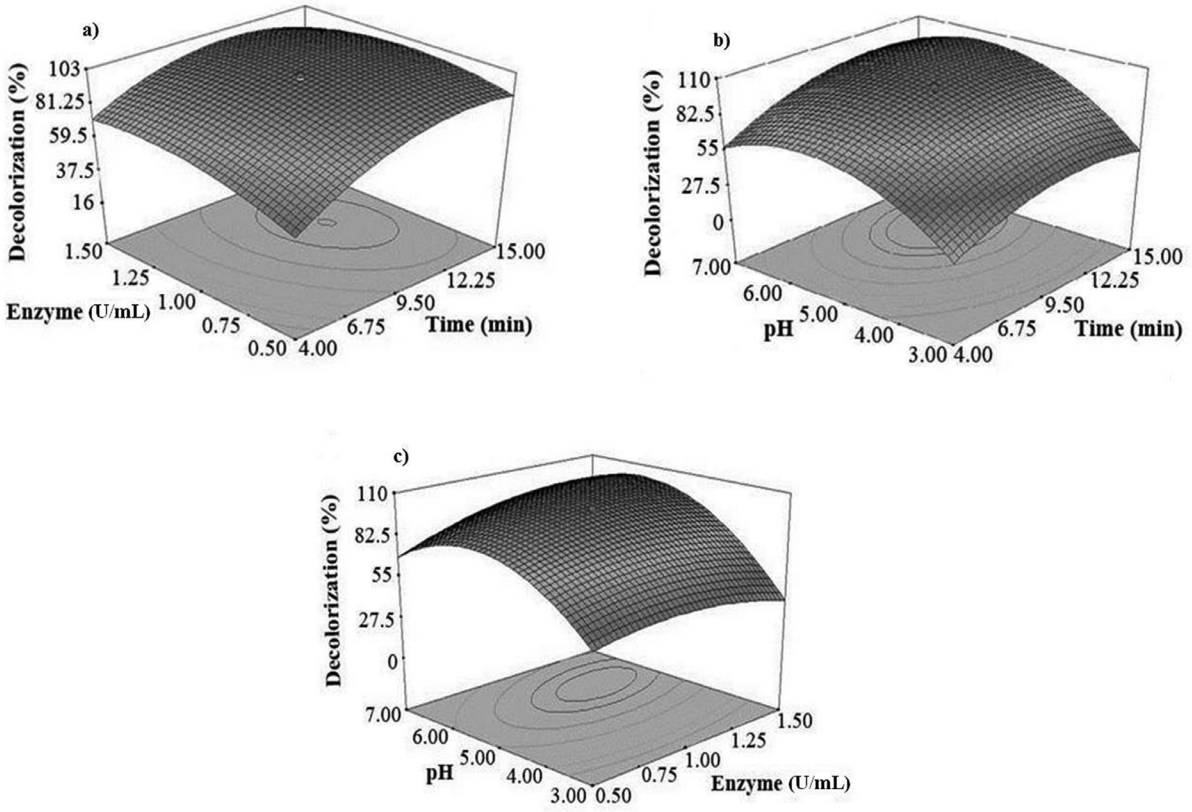

Fig. 4. Response surface plot representing the effects of interactions between (a) enzyme activity and time, (b) pH and time, (c) enzyme activity and pH.

Contour and three-dimensional response surface plots are two graphically powerful tools for studying the pattern of interactions between variables. These plots, which were applied using Design-Expert software, were employed in this study. The 3D response surface plots presented in Fig. 4(a–c) show the interactions between independent variables which are significantly important for the decolorization of reactive orange 7. The surface plot shows increase in dye decolorization with increase in incubation time. Maximum decolorization was obtained when incubation time exceeded 10 min, which can be explained by the time necessary for the interaction between the dye and the enzyme (Figs. 4a and b) [24]. Given an acidic pH, increasing the incubation time affected slightly the yield of decolorization (Fig. 4b). The enzymatic decolorization of reactive orange 7 strongly was influenced by changes in pH and dye degradation improved over a wide range of pH 3.0–6.0 (Fig. 4c). Similar result was reported by Khouni et al. [25] using laccase for the decolorization of two synthetic dyes including black novacron R and blue bezaktiv S-GLD150. Bacterial decolorization has been associated with oxidoreductase

enzymes such as laccase, azoreductase, NADH dehydrogenase, and cytochrome C oxidase. The decolorization efficiency of azo dye reactive blue 160 (200 mg/L) by *Proteus hauseri* ZMd44 was more than 90% after 24 h, showing that intracellular protein NADH dehydrogenase (257.2 U/mg) plays a significant role in the enzyme-catalyzed decolorization process [26]. The *Bacillus subtilis* strain ORB7106 gene azoR1 as a putative azoreductase used to decolorize methyl red on agar plates and in liquid cultures over a wide range of pH (5–9), temperatures (25–45 °C), and dye concentrations (10–200 mg/L) [27]. Jadhav et al. [28] reported a consortium-GB containing two microorganisms *Bacillus* sp. and *Galactomyces geotrichum* MTCC 1360 to decolorize synthetic dye brilliant blue G under optimal conditions (pH 9 and temperature 50 °C). Lignin peroxidase, laccase, and riboflavin reductase have been reported to be dye-degrading enzymes in consortium-GB [28].

Some studies have shown that the stability of laccase decrease in highly acidic condition (pH 3.0) [25,29]. While the activity of laccase decreases dramatically at acidic pH, no significant loss of enzyme activity was observed at pH 5.0 [30]. However, the increase in enzyme activity slightly influenced the yield of decolorization and the maximum decolorization observed at enzyme activity 1.25 U/mL (Fig. 4a). Balan et al. [31] reported that increasing the activity of laccase from 0 to 2.16 U/mL increased the rate of decolorization of malachite green by laccase isolated from *Pleurotus florida*. Hadibarata et al. [32] demonstrated the efficiency of laccase catalyzed decolorization of a variety of synthetic dyes, including anthraquinone, azo, and triphenylmethane dyes, was enhanced with an increase in enzyme activity and incubation time. Moreira et al. [33] reported real effluent decolorization at 50 °C and pH 4 by laccase isolated from *Peniophora cinerea*. Recently, recombinant CotA-laccase was applied at 40 °C and pH 10 for efficient decolorization (95%) of indigo carmine after 10 min [34]. Kalaiarasan et al. [35] reported optimized experimental parameters, e.g., enzyme concentration (10 U/mL), dye concentration (10 mg/L), temperature (25 °C) and reaction time (150 min) to achieve maximum decolorization of acid black 10BX. A novel bioelectricity-generating bacterium (*Proteus hauseri* ZMd44) has been reported to produce McoA-laccase with stability in acidic pH and potential applications in dye-containing wastewater treatment [36]. Han et al. [37] showed that bacterial decolorization by strains of *Rahnella aquatilis*, *Acinetobacter guillouiae*, *Microvirgula aerodenitrificans*, and *Pseudomonas* sp. was mainly catalyzed via the enzymatic expression of riboflavin reductase and azoreductase.

3.3. Validation of the model

In order to validate the model, the aforementioned optimal conditions for decolorization were used, and the practical results in the laboratory were measured. The optimum condition for enzymatic decolorization of reactive orange were found as enzyme activity 1.25 U/mL, pH 6.0, and incubation time 12.5 min. Under these optimal conditions, 99% dye decolorization was obtained, which agrees with the value predicted by the model This is a proof of concept that in this study mathematical modeling predicted the response accurately.

3.4. Kinetics and energetics of decolorization

Kinetic analysis of dye decolorization was performed based on the interpretation of the Michaelis–Menten and Eadie–Hofstee plots. The experimental data can fit quite well with Michaelis–Menten equation and the obtained results suggest the first order reaction (Fig. 5a). In addition, the Eadie–Hofstee diagram was used to estimate V_{max} and K_m which were 283.28 mM/min mg and 1.14 mM, respectively (Fig. 5b). The activation energy (E_a = 40 kJ/mol) was calculated from the slope of Arrhenius plot. The activation energy of laccase catalyzed degradation of 2,4-dichlorophenol at pH 5.5 is

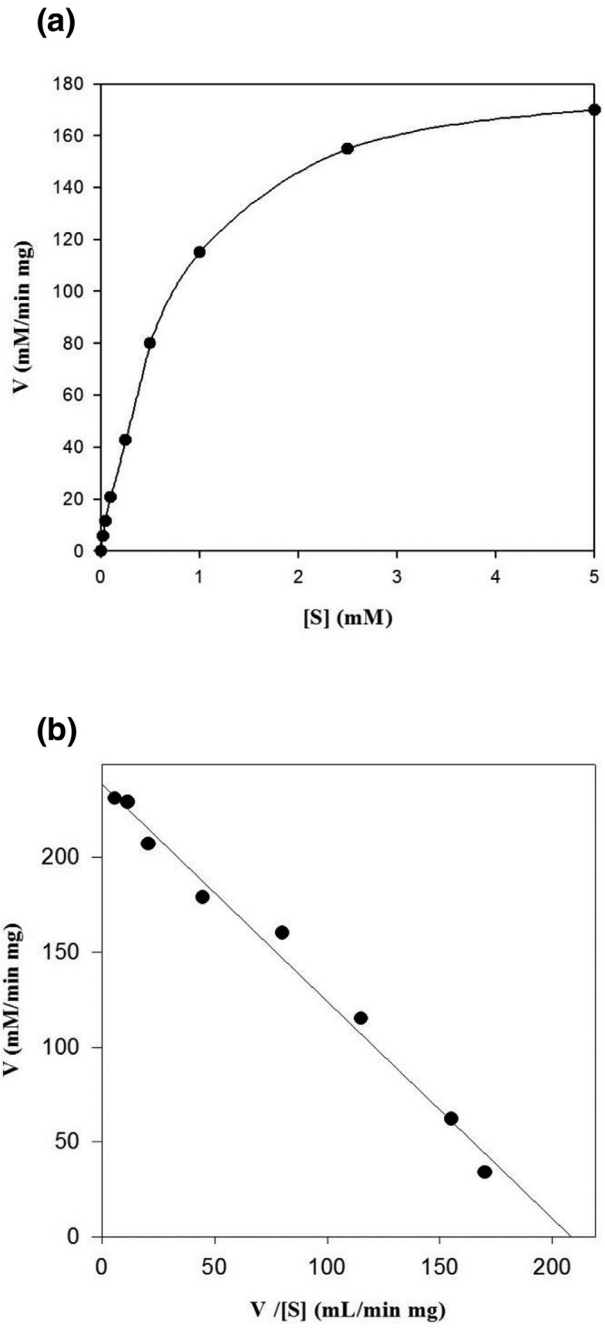

Fig. 5. Kinetic study. (a) Michaelis–Menten plot, (b) Eadie–Hofstee plot.

reported to be approximately 44.8 kJ/mol [38]. Aktaş et al. [39] reported the activation energy 47 kJ/mol for 1-naphthol enzymatic polymerization in the presence of laccase. Thermodynamic and kinetic studies of free laccase and fungal biomass from white-rot fungi were compared in the laccase-catalyzed decolorization of trypan blue and the activation energy of decolorization was determined to be 22 kJ/mol for both systems [40]. ΔH and ΔS of the dye decolorization process were calculated using van't Hoff analysis and found to be 89 kJ/mol and 285 J/mol K, respectively (Fig. 6). Annuar et al. [41] reported that the kinetics of the enzymatic decolorization of trypan blue followed a first-order behavior. The decolorization process was endothermic with enthalpy and entropy values of 45.6 kJ/mol and 146 J/mol K, respectively. Ramsay et al. [42] studied the first-order decolorization of synthetic dyes including amaranth, reactive black 5, reactive blue 19, and direct black 22 using *Trametes versicolor* laccase.

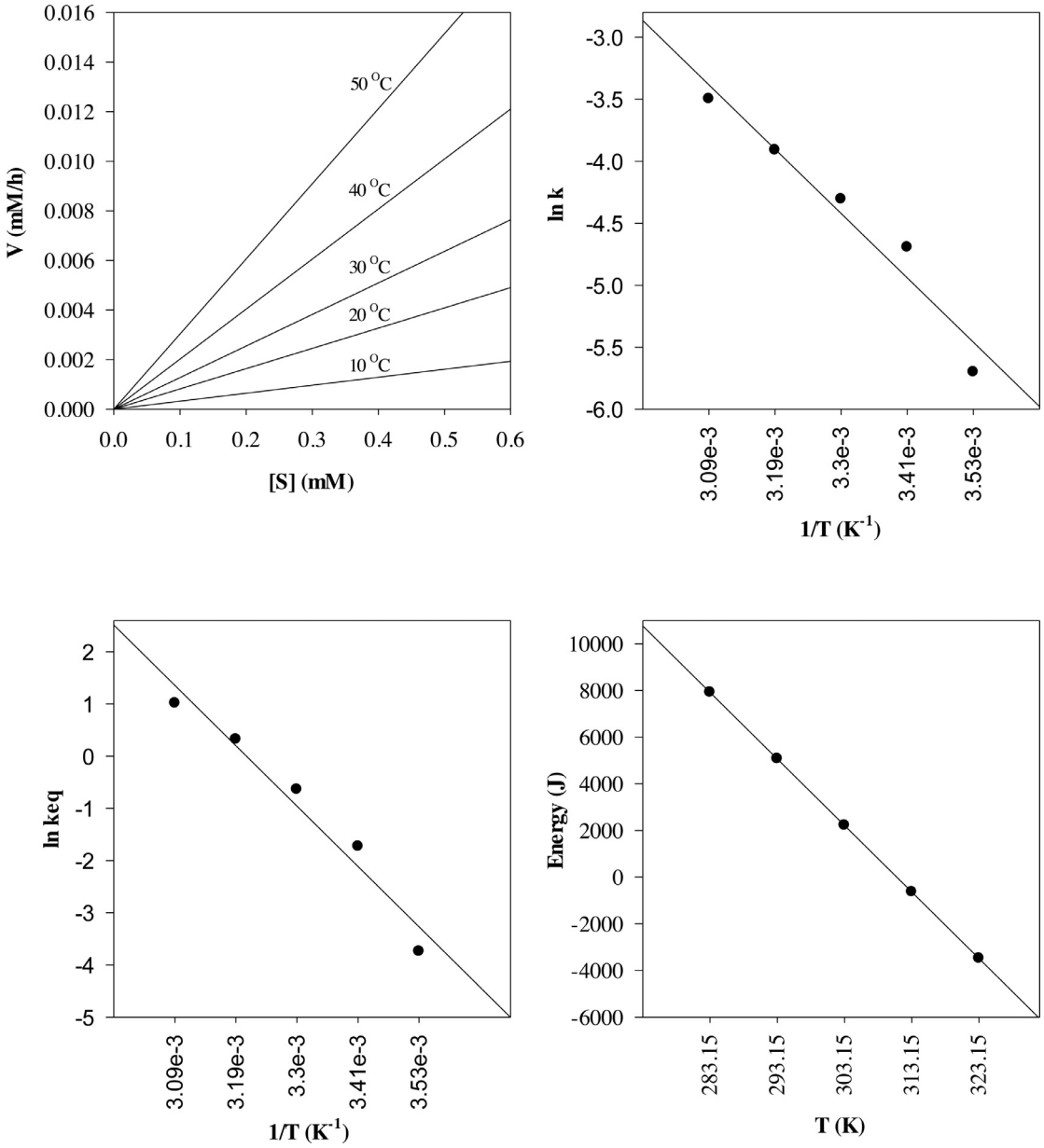

Fig. 6. Energetic study. (a) dependence of decolorization rate on temperature (10–50 °C), larger line slope values observe at higher temperatures, (b) Arrhenius plot, (c) van't Hoff plot, (d) Gibbs free energy changes plot.

Based on the value of the Gibbs free energy change, the decolorization reaction was spontaneous at temperatures higher than 37.8 °C. Razak and Annuar [40] reported that the positive values of ΔH and ΔS for the laccase catalyzed decolorization indicate the endothermic nature of the process with increasing disorder as the temperature rises. Thermodynamic studies revealed enthalpy 93.03 kJ/mol and the Gibbs free energy 90.19 kJ/mol for the degradation of procion red HE7B via the fungus *Pleurotus ostreatus* [43].

3.5. Dye toxicity

Due to the probable toxicity activity of produced metabolite(s) of synthetic colorants after physicochemical or biological treatment [44], various bioassays have been developed such as evaluating cytotoxicity on mammalian cell lines [45] and the growth inhibition of microbial and yeast strains (for example, *Bacillus megaterium*, *E. coli*, and *Saccharomyces cerevisiae*), as well as phytotoxicity investigations on plant seeds of *Oryza sativa* or *Triticum aestivum* [17]. The obtained results of growth inhibition of bacterial strains in the present study revealed GI% of 66 ± 1.3, 59 ± 0.4, 57 ± 1.3, 48 ± 0.9, 37 ± 0.5, and 41 ± 0.8 in the presence of *M. luteus*, *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *S. typhi*, respectively, when reactive orange 7 was inserted into their culture medium. However, the GI% for the laccase-treated dye solution was found to be 43 ± 2.3, 39 ± 1.8, 30 ± 0.5, 36 ± 1.3, 26 ± 1.2, and 28 ± 0.7 in the presence of *M. luteus*, *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *S. typhi*, respectively, which was significantly lower for all bacterial strains. Bibi et al. [46] reported a significant reduction in mycelial growth inhibition of the white-rot fungus *Phanerochaete chrysosporium* after the addition of a sample obtained from laccase-treated of malachite green (a triphenylmethyl dye) compared with that of the control. Palmieri et al. [47] determined 95% viability for *B. cereus* in the presence of a sample obtained from the enzymatic elimination of remazol brilliant blue R (an anthraquinone dye) using the purified laccase *Pleurotus ostreatus*. Microtoxicity studies performed before and after decolorization by free or immobilized laccase using *Vibrio fischeri* showed a

significant decrease in the toxicity of laccase-treated dyes [48]. Phytotoxicity studies of the laccase-treated reactive dye remazol brilliant blue R (RBBR) showed root elongation and seed germination 11.3 mm and 82%, respectively, when seeds were immersed in RBBR solution. However, in the case of laccase-treated dye, root elongation and seed germination were 21.3 mm and 100%, respectively [49]. Treatment of reactive dye effluent with laccase reduced effluent toxicity to the yeast *S. cerevisiae* BY4741 and the bacterium *B. cereus* with a decrease in growth inhibition of 48 and 18%, respectively [50]. The azoreductase PpAzoR from *Pseudomonas putida* demonstrated a wide specificity for decolorization of azo dyes, but the final products exhibited higher toxicity than the intact dyes [51]. Azoreductase enzyme isolated from *Bacillus velezensis* degraded azo dye direct red 28 to benzidine and 4-aminobiphenyl, which are potent mutagens [52].

4. Conclusions

The optimization of the laccase-catalyzed decolorization of the reactive orange 7 was achieved through RSM. Incubation time, pH, and enzyme activity were the most important factors affecting the yield of decolorization. RSM was efficiently used to determine the optimum level of the factors noted above using central composite design. The optimum conditions for maximum decolorization of reactive orange 7 were found to be enzyme activity 1.25 U/mL, pH 6.0, and incubation time 12.5 min. The kinetic study of decolorization with respect to temperature and dye concentration approximated first-order reaction. The V_{max} and K_m were found to be 283.28 mM/min.mg and 1.14 mM, respectively using Eadie–Hofstee kinetics. The obtained results show that the use of the laccase has a valuable potential to remove toxic dyes and additional reactor-scale studies are required to determine actual industrial applications.

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