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Oxidase-Peroxidase sequential polymerization for removal of a dye from contaminated water by horseradish peroxidase (HRP)/ glucose oxidase (GOx)/polyurethane hybrid catalyst

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Abstract−Horseradish peroxidase (HRP) and glucose oxidase (GOx) were co-immobilized on polyurethane, and the resulting HRP/GOx/polyurethane biocatalyst was characterized using scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDAX) mapping techniques. The prepared biocatalyst was used for removal of acid orange 7 as model azo dye. The required H_2O_2 for activation of HRP was in-situ produced using GOx to prevent deactivation of HRP in the presence of excess chemical H_2O_2. Central composite design (CCD) was applied for modeling and optimization of parameters affecting the activity of prepared biocatalyst. Under the optimum conditions, removal efficiency of the azo dye was predicted to be 87.47%, which was in good agreement with the experimental value (89.69%). In addition, the performance of the prepared biocatalyst for removal of two other dyes with different structure was investigated at the optimum conditions, and a removal efficiency of 91.56% and 95.25% was obtained for removal of methylene blue and malachite green, respectively. The results demonstrated that the resultant HRP/GOx/Polyurethane biocatalyst was able to decrease the chemical oxygen demand (COD) of a textile effluent from 740 mg/L to 96 mg/L, indicating that the prepared biocatalyst is an effective enzymatic system for treatment of real wastewater.

Keywords: Experimental Design, Horseradish Peroxidase, Immobilization, Glucose Oxidase, Response Surface Methodology

INTRODUCTION

Synthetic dyes, due to their accessibility and low cost, have gained increasing applications in different industries, such as textile, leather, cosmetic and food [1-3]. Annually, a large amount of these compounds are discharged in the environment through the effluents of these industries worldwide. There is a growing concern about the water streams and wastewater contaminated with synthetic dyes, because most of the synthetic dyes are toxic and mutagenic to living organisms [1,2,4]. So, their removal by a cost effective and efficient technology seems obligatory. Different physico-chemical methods, such as coagulation, adsorption, ozonation, photocatalysis, membrane treatment and biological technology, have been investigated for dye and pollutant removal from effluents [5-9]. In recent years, enzymatic-based methods have received great attention due to their eco-environmentally friendly, mild condition requirement, high selectivity, high efficiency and lack of toxic by-products formation during wastewater treatment [10]. Among the different peroxidase enzymes applied for dye decolorization and degradation, HRP seems to be a promising candidate for industrial effluents treatment owing to its stability, low cost and tolerance to wide ranges of temperature and pH [11,12].

Organic molecules of dyes can be oxidized and degraded by HRP in the presence of hydrogen peroxide. However, excess hydrogen peroxide in enzymatic reaction media leads to deactivation of HRP biocatalyst, which consequently results in a significant decrease in process efficiency [13,14]. An important strategy to solve this problem is the in-situ production of required hydrogen peroxide by enzymatic or electrochemical processes [15-17]. GOx is a promising enzyme which has been widely used for in-situ production of hydrogen peroxide in different enzymatic processes via oxidizing the β-D-glucose in the presence of oxygen [18]. Therefore, coupling the HRP as peroxidase enzyme and GOx as oxido-reductase enzyme seems to provide a promising enzymatic system with efficient performance for wastewater treatment.

Low stability, poor reusability and low recovery of enzymes limit the widespread application of this enzymatic system in different industries [19]. An important strategy for overcoming this limitation is co-immobilization of HRP and GOx enzymes on an appropriate support. Until now, many kinds of materials have been used as support for immobilization of enzymes including synthetic polymers, silica-based carriers, acrylic resins, and active membranes [7,8,19,20]. Among the different materials, polyurethane can be a suitable support for immobilization of enzymes owning to its remarkable advantages including cost-effectiveness and high stability of the obtained supported enzyme [21].

In the present research study, HRP and GOx enzymes were co-immobilized on polyurethane to prepare the HRP/GOx/polyurethane biocatalyst. The resultant biocatalyst was characterized using SEM and EDAX mapping analysis, and its performance was eval-

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uated by investigating the removal of acid orange 7 as a model azo dye. To prevent the deactivation of HRP owing to the presence of excess H_2O_2, the required H_2O_2 for activation of HRP was produced in-situ by GOx enzyme. CCD and response surface methodology (RSM) were applied for experiment design, modeling and optimization of parameters affecting the enzymatic activity of the prepared biocatalyst, including initial glucose concentration, HRP/GOx ratio, pH and temperature. In addition, the performance of the resultant biocatalyst was further evaluated through investigating the removal of two other dyes with different structures in the determined optimum conditions. Finally, the reduction of COD of a textile effluent (Hamkar rangrazan, Iran) was investigated in the presence of HRP/GOx/polyurethane enzymatic system in the optimum conditions to evaluate the performance of the resultant biocatalyst for treatment of real effluents.

EXPERIMENTAL

1. Chemicals

All chemicals were of analytical grade and used without further purification. Horseradish peroxidase (200 U/mg RZ=2.5) and hydrogen peroxide (30 w/v%) were purchased from Merck (Germany). Glucose oxidase from Aspergillus niger (EC.1.11.1.6, activity=700 U/mg) was purchased from local market (Arteen Shimi Co.). Acid orange 7, methylene blue and malachite green were obtained from Shimi Boyakhsaz Co. Iran. The characteristics of used dyes are presented in Table 1.

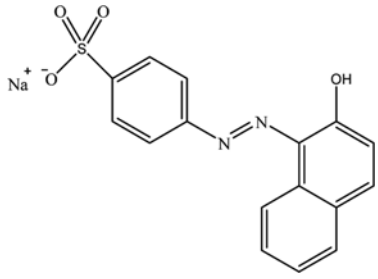
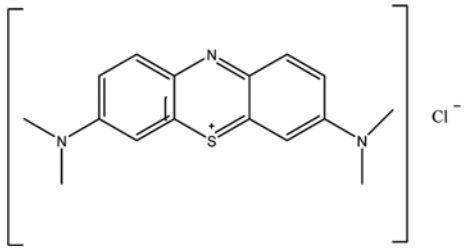
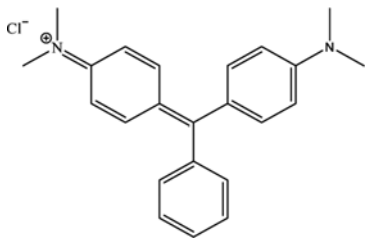
2. Immobilization of HRP and GOx Enzymes on Polyurethane Foam

The stability and the resistance of the enzymes to the denaturation can be significantly enhanced through immobilization [22]. Therefore, the HRP and GOx enzymes were immobilized on the polyurethane foam. For this purpose, five pieces of polyurethane foam (with the dimension of 1 cm×1 cm×1 cm) was immersed in 100 mL of phosphate buffer with the pH of 6, and subsequently it was submerged in glutaraldehyde solution as cross-linking agent with the concentration of 1% (V/V). After 1h, the treated polyurethane sponge was collected and washed with phosphate buffer for several times to remove excess glutaraldehyde molecules. In the next step, the resulted polyurethane sponge was submerged in 100 mL of enzyme solution containing 0.5 mg/L of HRP and GOx enzymes. To complete the co-immobilization process of enzymes on the sponge, the beaker containing sponge and enzymes solution was shaken gently at speed of 200 rpm for 1 h at 25 °C.

3. Activity Measurements of HRP and GOx Enzymes

The activity of free and immobilized HRP enzyme was measured spectrophotometrically applying 4-aminoantipyrene (4-AAP) method [23]. In this method, the H_2O_2 and phenol act as substrate and 4-AAP molecules act as chromogen. The decomposition rate of H_2O_2 is determined through color development measurement using spectrophotometer at 510 nm at 25 °C. For this purpose, the HRP solution is added into phosphate buffer solution with the pH of 7.4 containing 4-AAP (2.4×10^{-3} mol/L), phenol (1.0×10^{-2} mol/L) and H_2O_2 (2.0×10^{-4} mol/L). One unit (U) of HRP activity is defined

Table 1. Characteristics of the dyes

Dye	Chemical structure	Molecular formula	Mw (g/mol)	λ_{max} (nm)
Acid orange 7		$NaC_{16}H_{11}N_2NaO_4S$	350.32	365
Methylene blue		$C_{16}H_{18}ClN_3S$	319.85	668
Malachite green		$C_{23}H_{25}ClN_2Cl$	364.911	619

as quantity of enzyme that catalyzes the decomposition of 1 μmol of phenol within 1 min [24].

The enzymatic activity of the free and immobilized GOx was evaluated by determining the amount of H_2O_2 produced via oxidation of β-D-glucose by GOx enzyme in the presence of O_2 molecules. The GOx enzyme catalyzes the oxidation of β-D-glucose to H_2O_2 and β-D-glucono-γ-lactone in the presence of O_2. The quantity of generated H_2O_2 can be determined using I_3^- method, in which the H_2O_2 concentration is determined in the solution containing 0.1 mol/L of phosphate buffer and 20 mmol glucose [25]. One unit (U) of GOx activity is defined as the amount of enzyme which catalyzes the glucose for producing 1 μmol H_2O_2 within 1 min at 25 °C [26].

4. Experimental Procedure for Dye Removal and Analytical Methods

In this study, acid orange 7, methylene blue and malachite green were used as model dye pollutant to evaluate the biocatalytic activity of HRP/GOx/polyurethane enzymatic system. The concentration of dyes was determined using colorimetric method. At time intervals of 15 min, approximately 2.5 mL of solution was sampled and the residual concentration of dye in the reactor was determined using a UV-Vis spectrophotometer (WPA Lightwave S2000, England). The absorbance of acid orange 7, methylene blue and malachite green was measured at maximum wavelength of 483 nm, 668 nm and 619 nm, respectively. The decolorization efficiency of each dye was calculated using Eq. (1):

$$\text{Decolorization efficiency (DE \%)} = \frac{(A_0 - A_t)}{A_0} \times 100 \quad (1)$$

where, A_0 and A_t represent maximum absorbance of solution at initial time and after t min, respectively.

For determination of H_2O_2 concentration, solutions A and B were prepared. Solution A with the volume of 250 mL consisted of 33 g of KI, 1 g of NaOH and 0.1 g of ammonium molybdate tetrahydrate, and solution B with the volume of 500 mL contained 10 g of potassium hydrogen phthalate (KHP). The reaction between H_2O_2 and I^- generated I_2, which subsequently react with I_2 to form I_3^-. The final product was measured by determining the absorbance of reaction mixture at maximum wavelength of 351 nm [25].

Table 2. Affecting parameters and their levels in the experimental design

Parameter	Levels				
	−2	−1	0	1	2
Glucose (mmol/L)	15	25	35	45	55
HRP/GOx ratio	0.50	0.87	1.25	1.63	2.00
Temperature (°C)	20.0	26.3	32.5	38.8	45.0
pH	5.0	6.3	7.5	8.8	10.0

5. Characterization of the HRP/GOx/Polyurethane Sponge Biocatalyst

The morphology of the polyurethane sponge which was used as support for HRP and GOx enzymes was investigated using a TESCAN SEM Model Vega (Czech Republic). To investigate the successful immobilization of HRP and GOx enzymes on the polyurethane sponge support, the EDAX analysis was performed using INCA (England) instrument.

6. Experimental Design and Optimization

In this research, CCD with four independent factors (HRP/GOx ratio, glucose, pH and temperature) at five levels was employed to design the experiments (Table 2). The experimental design resulted in 31 sets of experiment including 7 replications at the center point, 8 axial points and 16 factorial points.

7. Statistical Analysis

To reduce the error, enzyme activity measurements, dye removal experiments and H_2O_2 measurement were replicated for three times and average values were reported in this study. Minitab 16 was used for statistical analysis.

RESULTS AND DISCUSSION

1. Characterization

The SEM images of the bare polyurethane and HRP/GOx/polyurethane is represented in Fig. 1. As can be seen, the polyurethane foam used as support exhibits a honeycomb skeleton. Some protu-

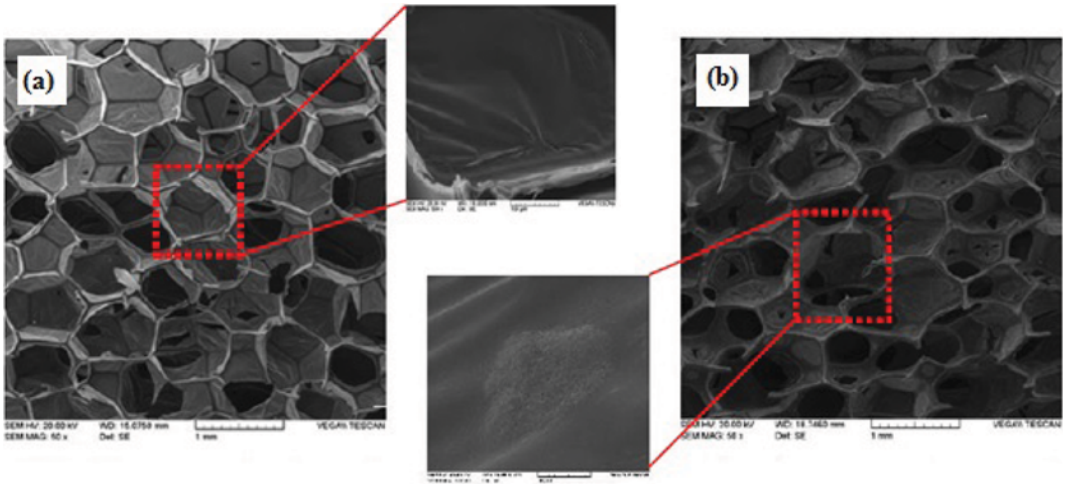


Fig. 1. SEM images of the (a) bare polyurethane and (b) HRP/GOx/polyurethane.

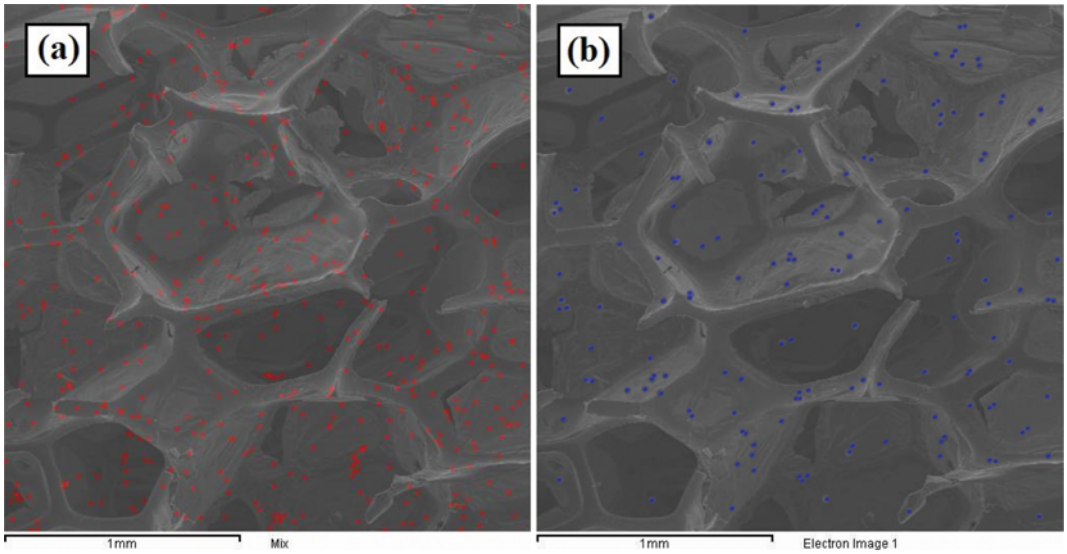


Fig. 2. EDAX mapping analysis of HRP/GOx/polyurethane (a) Ca mapping, (b) Fe mapping.

berances can be observed in the HRP/GOx/polyurethane, which represents the immobilized enzymes. In addition, the successful immobilization of the enzymes was investigated using EDAX mapping analysis, and the results are shown in Fig. 2. The homogeneous mapping of Ca and Fe indicates that both HRP and GOx enzymes have successfully immobilized without any aggregation.

2. Azo Dye Removal Mechanism Using HRP/GOx/Polyurethane Enzymatic System

The Uv-Vis absorbance spectra variations of acid orange 7 in the presence of HRP/GOx/polyurethane enzymatic system are represented in Fig. 3. The intensity of the absorption peak at 483 nm decreases gradually during reaction time, indicating high efficiency of this enzymatic system for degradation of the acid orange 7.

According to the well-known ping-pong mechanism [27,28], the following steps (Eqs. (3)-(5)) can be proposed for the removal of the acid orange 7 in the presence of HRP and H_2O_2. In the first step, HRP is activated in the presence of H_2O_2, resulting in generating compound I and water molecule. The produced compound I contains an oxyferryl group in the active center, which is surrounded by porophyrin in the form of a Π radical cation [27]. In the next step, the resultant compound I can oxidize the dye molecules via oxidoreduction reaction, leading to generate dye radicals and compound II. Finally, the produced compound II interacts with other dye molecules to generate dye radicals and regenerated HRP.

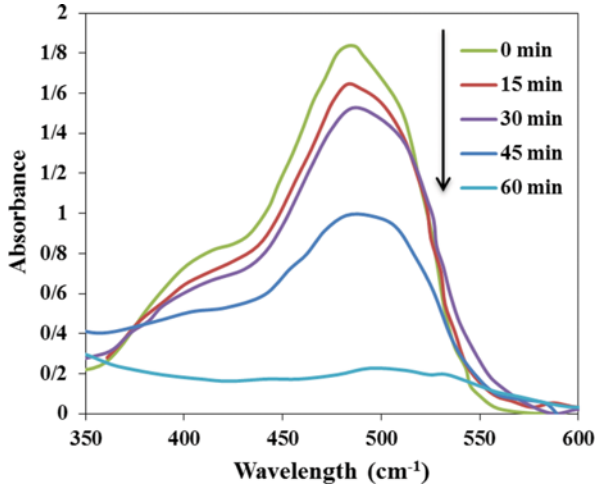


Fig. 3. UV-Vis absorbance spectra variations with reaction time for acid orange 7.

$$HRP + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (3)$$

$$\text{compound I} + \text{dye molecule} \rightarrow \text{compound II} + \text{dye radical} \quad (4)$$

$$\text{compound II} + \text{dye molecule} \rightarrow HRP + \text{dye radical} \quad (5)$$

In the studied HRP/GOx/polyurethane enzymatic system used for removal of acid orange 7, the required H_2O_2 is produced in-situ by immobilized GOx through oxidizing the β-D-glucose in the presence of O_2 as shown on Eq. (2) [29]:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{GOx} \text{gluconic acid} + H_2O_2 \quad (2)$$

The produced dye radicals can conjunct each other through the radical coupling reactions to form polymer molecules, which can be easily separated from the aquatic media via facile and cost-effective methods, e.g., precipitation and filtration. The schematic of the proposed mechanism is represented in Fig. 3.

3. Design of Experiments

To analyze and optimize the removal of acid orange 7 as azo dye using HRP/GOx/polyurethane enzymatic system, RSM was employed. The effect of the parameters influencing the removal efficiency of acid orange 7 including effect of glucose concentration, HRP/GOx ratio, pH and temperature were studied using experimental design techniques. In this study, the design was performed using Minitab 14 software, and RSM-CCD was implemented including 31 runs with 7 replicated points at the central point. The proposed design points are represented in Table 3.

4. Model Fitting and Statistical Analysis

The mathematical relationship of azo dye removal efficiency and

Table 3. CCD matrix along with the obtained responses

STD order	Run order	Glucose (mmol/L)	HRP/ GOx ratio	Temperature (°C)	pH	Dye removal efficiency	
						Experimental	Predicted
4	1	45	1.56	27.5	5.5	43.76	47.21
26	2	30	1.13	35.0	7.0	81.18	79.86
30	3	30	1.13	35.0	7.0	79.65	79.86
10	4	15	1.56	42.5	5.5	35.03	36.04
24	5	30	1.13	50.0	7.0	39.11	37.21
12	6	45	1.56	42.5	5.5	52.15	55.25
3	7	45	0.69	27.5	5.5	20.09	20.08
31	8	30	1.13	35.0	7.0	80.12	79.86
8	9	45	1.56	27.5	8.5	58.16	62.42
11	10	45	0.69	42.5	5.5	14.11	16.38
25	11	30	1.13	35.0	7.0	79.16	79.86
22	12	30	1.13	35.0	10.0	69.17	65.86
5	13	15	0.69	27.5	8.5	34.13	33.58
27	14	30	1.13	35.0	7.0	80.17	79.86
2	15	15	1.56	27.5	5.5	32.29	31.25
19	16	0	1.13	35.0	7.0	0.09	0.21
23	17	30	1.13	20.0	7.0	39.31	34.38
20	18	60	1.13	35.0	7.0	34.28	27.38
28	19	30	1.13	35.0	7.0	81.01	79.86
13	20	15	0.69	42.5	8.5	28.13	28.38
15	21	45	0.69	42.5	8.5	36.98	39.58
29	22	30	1.13	35.0	7.0	79.15	79.86
14	23	15	1.56	42.5	8.5	43.93	45.75
1	24	15	0.69	27.5	5.5	15.99	19.38
9	25	15	0.69	42.5	5.5	14.01	12.42
21	26	30	1.13	35.0	4.0	38.11	34.71
7	27	45	0.69	27.5	8.5	39.93	41.54
18	28	30	2.00	35.0	7.0	76.98	72.54
17	29	30	0.25	35.0	7.0	30.01	28.04
6	30	15	1.56	27.5	8.5	38.98	39.21
16	31	45	1.56	42.5	8.5	73.01	72.21

the affecting parameters was determined through fitting the experimental data (reported in Table 3) with the suggested model using Minitab 16. Accordingly, the following regression equation (Eq. (6)) for azo dye removal efficiency is expressed:

$$Y\ (\text{Dye removal efficiency}\ (\%)) = -447.68 + 80.220\times A + 3.387\times B + 50.086\times C + 12.308\times D - 38.616\times A^2 - 0.073\times B^2 - 3.285\times C^2 - 0.196\times D^2 + 0.581\times A\times B + 0.895\times A\times D + 0.281\times B\times C \quad (6)$$

An analysis of variance (ANOVA) was performed to evaluate the obtained model, and the results are reported in Table 4. Fisher's F test (F-value), which is the ratio of mean square value due to the model variation to mean square value due to the error variance, is applied to assign the statistical significance of model [30]. At the significant level α=0.05 and for a certain number of freedom degrees in the model, the F-value should be greater than the value of F-tabulated [24,30]. In this study, the F-value of developed model is 89.36 and the chance that the "model's F value" resulted owing to the noise is only <0.01%. As can be seen in Table 4, a very low probably value (P-value) of <0.0001 is obtained for developed model, indicating that the model is significant (the P-value less than 0.05 imply the significance). The low F-value of the Lack of Fit (27.06) demonstrates that relative to the pure error the Lack of Fit is non-significance, which is favorable. The non-significance of lack of fit confirms the high ability of the developed model for prediction [31,32]. The

Table 4. ANOVA results for the fitted model

Source	Sum of squares	Degree of freedom	Mean square	F value	P-value
Regression	17510.8	14	1250.77	89.36	<0.0001
Lack of fit	219.1	10	21.91	27.06	<0.0001
Pure error	4.9	6	0.81		
Residual	223.9	16	14.00		
Total	17734.7	30			

R^2=0.9874, Adj R^2=0.9763

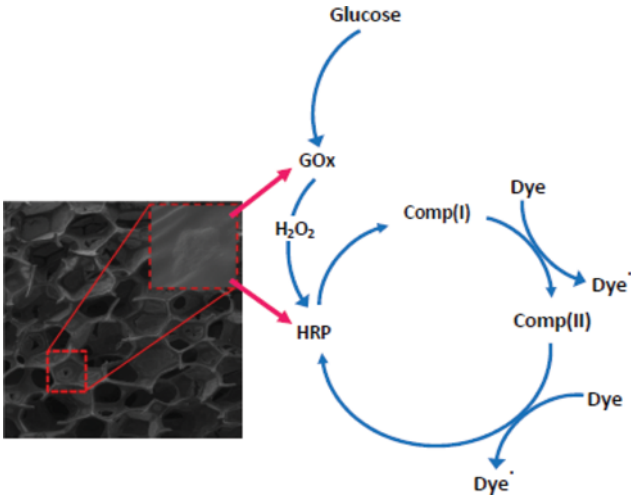


Fig. 4. The schematic presentation of the proposed mechanism for HRP/GOx/polyurethane enzymatic system.

results of ANOVA analysis demonstrate that there is only <0.01% chance that the F value of the Lack of Fit resulted due to the noise [31].

Fig. 4 represents the predicted azo dye removal efficiency (predicted using Eq. (6)) versus experimental values. The points are well distributed around the y=x line with high determination coefficients value (R^2=0.987). This high R^2 value indicates that the expressed regression equation can successfully predict the azo dye removal efficiency within the experimental range. Note that the R^2 is always increased by adding any variable, whether statistically significant or insignificant, to the model. Therefore, the adjusted-R^2 should be considered in evaluating the obtained model. Indeed, not only the value of the adjusted-R^2 is not necessarily increased by adding variables to the model, but also its value often decrease by adding unnecessary variables [24]. Thus, large differences between the R^2 and the adjusted-R^2 indicate that the model contains insignificant variables. As reported in Table 4, the adjusted R^2 value for the obtained model is 97.63%, which is close enough to the R^2 value. The significance and the standard error of each term in Eq. (6) are reported in Table 5. As mentioned, the p-value lower than 0.05 demonstrates the significance of the term. As can be seen, the coefficients for the linear effect of glucose concentration, HRP/GOx ratio, pH and temperature are significant. In addition, the p-values of the quadratic terms demonstrate that the coefficient of the all four affecting parameters is significant. However, among the interaction terms, the coefficients in the interaction terms for HRP/GOx ratio-glucose concentration and the HRP/GOx ratio-temperature are significant.

5. The Effect of Parameters on the Azo Dye Removal Efficiency Using HRP/GOx/Polyurethane Enzymatic System

The interaction between the variables, which is an important parameter for optimizing the dye removal efficiency, can be investigated using surface and contour plots [24,33]. For preparing the surface and contour plots with good quality, MATLAB software was applied. For this purpose, the obtained model from RSM was introduced to the MATLAB software, and the surface and contour plots were derived. In the surface and contour plots, representing the interaction of two variables, while two of the variables were varied within the experimental range, the other two were maintained constant at their zero levels. The interaction between the glucose concentration and the HRP/GOx ratio is shown in Fig. 5(a). As can be seen, the dye removal efficiency enhanced significantly by increasing the concentration of glucose up to 35.15 mmol/L, and then decreased by further glucose concentration enhancement. Low removal efficiency of the azo dye at the low glucose concentration can be attributed to the limited H_2O_2 production. In this enzymatic system, the glucose is a primary substrate for production of H_2O_2 using GOx enzyme, so the low concentration of glucose results in limiting the production of the H_2O_2, which consequently results in low dye removal efficiency. On the other hand, high concentration of the glucose results in producing large amount of H_2O_2. So, the low removal efficiency of the azo dye at the high concentration of the glucose can be attributed to the de-

Table 5. ANOVA results for the coefficients of the model

Model		Coefficient	Standard error	T	P
Constant	-	−447.68	37.624	−11.899	<0.0001
HRP/GOx ratio	A	80.220	16.9711	4.727	<0.0001
Glucose	B	3.387	0.4821	7.025	<0.0001
pH	C	50.086	5.6393	8.882	<0.0001
Temperature	D	12.308	1.1279	10.912	<0.0001
(HRP/GOx)×(HRP/GOx)	A^2	−38.616	3.6551	−10.565	<0.0001
Glucose×Glucose	B^2	−0.073	0.0031	−23.60	<0.0001
pH×pH	C^2	−3.285	0.3109	−10.565	<0.0001
Temperature×Temperature	D^2	−0.196	0.0124	−15.746	<0.0001
(HRP/GOx)×Glucose	A×B	0.581	0.1425	4.076	0.001
(HRP/GOx)×pH	A×C	−2.381	1.4252	−1.671	0.114
(HRP/GOx)×Temperature	A×D	0.895	0.2850	3.141	0.006
Glucose×pH	B×C	0.281	0.0416	1.938	0.040
Glucose×Temperature	B×D	0.007	0.0083	0.869	0.398
pH×Temperature	C×D	0.039	0.0831	0.468	0.646

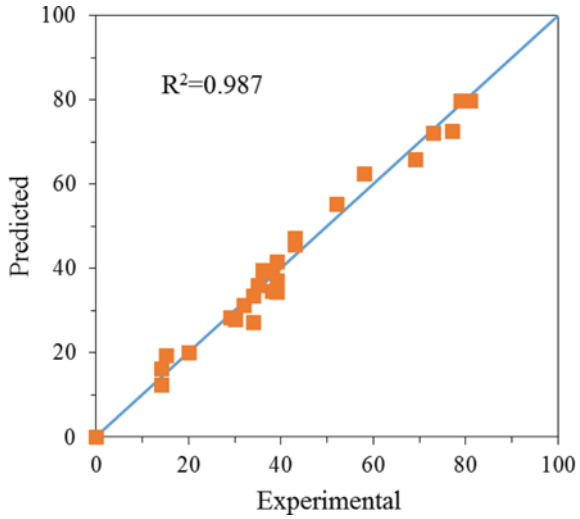


Fig. 5. Predicted versus experimental plot for acid orange 7 removal efficiency.

activation of the HRP enzyme in the presence of high concentration of H_2O_2. A similar trend can be seen for the effect of the HRP/GOx ratio. To prepare enzymatic system with different HRP/GOx ratio, the amount of the HRP was maintained constant and the amount of the GOx was varied. As can be seen in the contour and surface plots of Fig. 5(a), the azo dye removal efficiency enhanced by increasing the ratio of the HRP/GOx up to the value of the 1.48, and then it decreased. Low value of the HRP/GOx ratio equals to large amount of GOx, which leads to generate large amount of the H_2O_2. So, the low removal efficiency of the azo dye in the presence of enzymatic system with low HRP/GOx ratio can be attributed to the deactivation of the HRP in the presence of large amount of the H_2O_2, resulted due to the presence of large amount of GOx. On the other hand, high value of the HRP/GOx ratio results in producing limited amount of H_2O_2 which leads to low removal efficiency of the azo dye. As can be seen, the optimum value for the HRP/GOx was predicted to be 1.48, in which the optimum amount of the H_2O_2 can be produced. The interaction between the temperature and the HRP/GOx ratio is presented in the Fig. 5(b) by related surface and contour plots. As can be seen, the removal efficiency of azo dye enhanced by increasing the reaction temperature from 20 °C to 38 °C in the presence of HRP/GOx/polyurethane enzymatic system. Indeed, temperature increment leads to an enhancement in the rate of H_2O_2 production and the rate of removal process, which consequently results in an increase in the removal efficiency of azo dye. However, the removal efficiency of azo dye decreases beyond the 38 °C, which can be attributed to the destruction and the deactivation of the HRP and GOx enzymes [34]. Fig. 5(c) represents the interaction of the glucose and pH. The removal efficiency of the azo dye increased with increasing the pH up to ~7.5 and then decreased at higher pH. Similar results have been reported in many investigations [35-37]. The observed low removal efficiency of azo dye in very acidic and basic media can be ascribed to the denaturation of the HRP and GOx enzymes in these media.

Normal probability of the residuals is shown in Fig. 6. As can be seen, the obtained data points are distributed normally on a straight

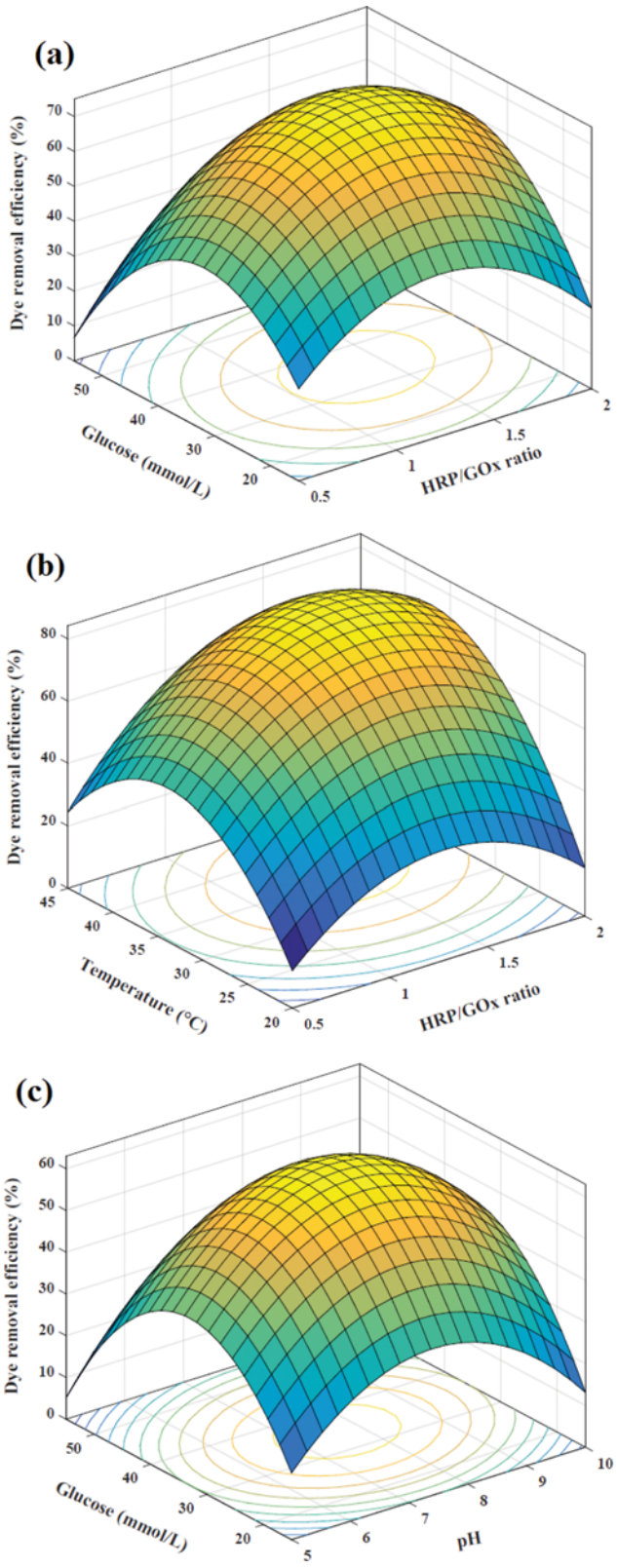


Fig. 6. The response surface and contour plots representing the simultaneous influence of (a) glucose concentration (mmol/L) and HRP/GOx ratio (pH=7 and T=35 °C), (b) temperature (°C) and HRP/GOx ratio (pH=7 and [Glucose]=35 mmol/L), (c) glucose concentration (mmol/L) and pH (T=35 °C and HRP/GOx=1.5) on the acid orange 7 removal efficiency.

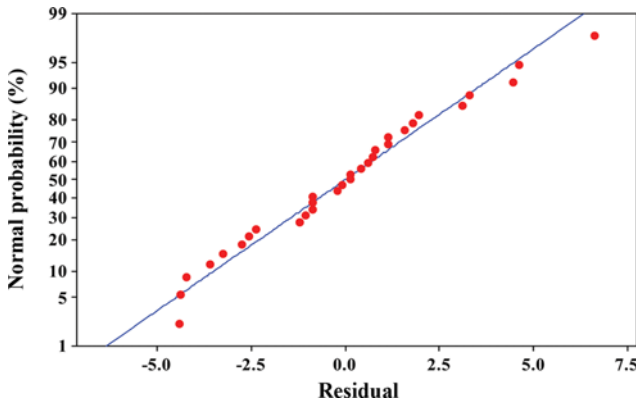


Fig. 7. Normal probability plot of the experimental results for acid orange 7 removal efficiency.

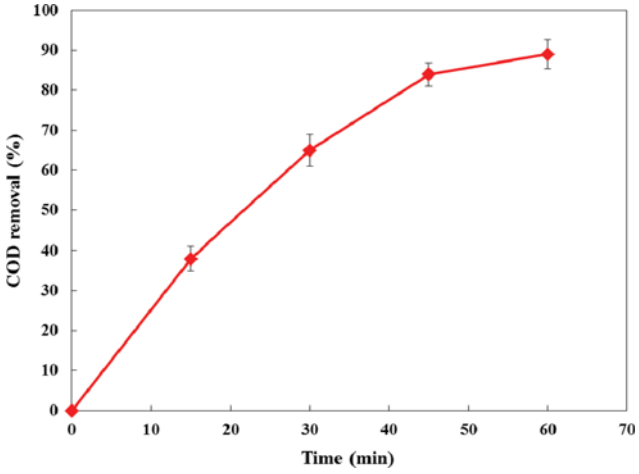


Fig. 8. COD removal of the acid orange 7 solution in the presence of HRP/GOx/polyurethane enzymatic system. Experimental conditions: [acid orange 7]=20 mg/L, [glucose]=35.15 mmol/L, HRP/GOx=1.48, temperature=36.36 and pH=7.75.

trend line, indicating the good adequacy of the obtained model.

6. Optimization and Validation

To determine the optimum amount of affecting parameters which lead to the highest azo dye removal, the optimization tool of Minitab 16 software was applied. For this purpose, the desirable goal in removal efficiency of azo dye was set on the maximum value and all the affecting parameters were placed within the studied range. Accordingly, the maximum removal efficiency of azo dye was predicted to be 87.47% under optimum conditions (initial glucose concentration of 35.15 mmol/L, HRP/GOx of 1.48, pH of 7.75 and temperature of 36.36 °C). To validate the accuracy of the optimization, an experiment under optimized conditions was carried out. The azo dye removal efficiency was found to be 89.69%, which is in excellent agreement with the predicted value, confirming that RSM can be applied as a powerful tool for modeling and determining the optimum values of affecting parameters in enzymatic system.

7. Changes of COD with Reaction Time

As shown in Fig. 7, the COD of the solution containing 20 mg/L of acid orange 7 as azo dye was significantly decreased 89% after 60 min of treatment in the presence of HRP/GOx/polyurethane in the determined optimum conditions (initial glucose concentration of 35.15 mmol/L, HRP/GOx ratio of 1.48, pH=7.75 and temperature=36.36 °C). This result indicates the effective performance of the obtained enzymatic system for treatment of wastewater containing azo dye.

In addition, the COD of a textile effluent (Hamkar rangrazan, Iran) significantly decreased from 740 mg/L to from 96 mg/L in the presence of HRP/GOx/polyurethane biocatalyst in the determined optimum conditions after 90 min of treatment. The observed 87.07% COD reduction confirmed that the prepared biocatalyst is an effective enzymatic system for treatment of real wastewaters.

8. HRP/GOx/Polyurethane Biocatalyst Performance Study for Removal of Methylene Blue and Malachite Green

To investigate the capability of the resultant HRP/GOx/polyurethane biocatalyst for removal of the different dyes with different structure, the removal efficiency of the methylene blue and malachite green was studied in the determined optimum conditions. After 60 min of treatment, the removal efficiency was found to be 91.56% and 95.25% for methylene blue and malachite green, respectively, indicating the excellent capability of the biocatalyst for removal of dyes with different structures.

CONCLUSION

HRP and GOx enzymes were co-immobilized on polyurethane foam, and the resulting biocatalyst was characterized by using SEM/EDAX. The performance of obtained biocatalyst was evaluated by investigating the removal of acid orange 7 as a model azo dye. CCD and RSM were applied for experiment design, modeling and optimization of prepared biocatalyst. The optimum conditions were found to be as follows: initial glucose concentration=35.15 mmol/L, HRP/GOx=1.48, pH=7.75 and temperature=36.36. Under the optimum conditions the removal efficiency of the azo dye was predicted to be 87.47%, which was in good agreement with the experimental value (89.69%), indicating the powerful ability of the RSM for modeling and optimization of enzymatic systems. In addition, the performance of the obtained biocatalyst at optimum conditions was tested for removal of two other dyes with different structures, and the removal efficiency of 91.56% and 95.25% was obtained for removal of methylene blue and malachite green, respectively. Moreover, the resultant biocatalyst was able to decrease the COD of a real wastewater from 740 mg/L to 96 mg/L in optimum conditions, indicating that the prepared biocatalyst is an effective enzymatic system for treatment of real wastewater.

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