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Studies on Decolorization Characteristics of Crude Peroxidase from *Raphanus sativus* Using Response Surface Methodology

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Abstract

Dye decolorization with the help of a plant based enzyme was investigated. Crude peroxidase (35.58 U/ml) extracted from the pulp of *Raphanus sativus* (radish) was used for decolorization of a basic dye, safranin. The effect of the influencing parameters of pH, temperature, dye concentration, and enzyme concentration on safranin decolorization was primarily studied using the traditional One Factor At a Time method (OFAT). The optimal values of each influencing parameter for maximum decolorization were obtained via the OFAT approach which can be extended to study factorial interactions by applying range levels statistically. To obtain the optimal operating conditions for decolorization, the influencing process parameters were optimized further by applying Response Surface Methodology based on Box-Behnken Design. A three level four factor design was generated using the Design Expert software version 8.0.7.1. A quadratic model was fitted for the experimental data obtained, and the significance of the model predicted was analyzed through Analysis of Variance (ANOVA) at the 95 % confidence interval. The determination coefficient, R^2, of the model was found to be 0.8993. The optimal process conditions were found to be a temperature of 30 °C, a dye concentration of 200 mg/l, an incubation time of 60 min, and an enzyme concentration of 26.69 U/ml, producing 30.07 % decolorization at an optimum pH of 7, which is in accordance with the predicted value of 29.24 %. Confirmatory experiments verified the adequacy of the model. The study provides a foundation for further research on enzyme based decolourization of safranin.

Keywords: Dye, radish, enzyme, safranin, decolorization, Box- Behnken design

Introduction

A huge amount of wastewater is generated in the process of dyeing; these effluents contain dye complexes of various aromatics, metals, chlorides, etc, which significantly affect plant photosynthesis, by reducing light penetration intensity, and are toxic to some aquatic fauna and flora when released into water bodies [1,2]., Moreover, several of the dyes employed are very stable to light, temperature and microbial attack, making them recalcitrant [3]. The confined toxicity of the effluent is due to the mutagenic and carcinogenic properties of the dyes or the degraded dye products present [4]. Also, the presence of these dyes in water causes allergy, dermatitis, and skin irritation in humans [5]. Hence, the treatment of the effluents containing these dyes is mandatory prior to discharge to the environment.

Various physico-chemical treatment methods, such as membrane technologies, ozonation, adsorption, chemical precipitation, flocculation, photolysis, and ion pair extraction, and biological processes, such as biodegradation and bioadsorption [6], are applied industrially for the treatment of wastewater contaminated with dye. The available methods involve high start-up costs, and in turn generate sludge, which adds to waste disposal problems; hence, they are not environment friendly [7].

Enzymatic decolorization and degradation methods have increasing importance these days, as they are an eco-friendly, cost-competitive alternative to conventional physico-chemical or microbial treatment

processes, which involves operational and costing limitations [8]. Enzymes obtained from plant sources are cheap to extract, and are safe for use compared to microbes, eliminating the chance of escape of mutant microbes into the environment [9].

Response Surface Methodology (RSM) is a statistical technique that is applied for designing experiments, building models, evaluating the effects of several variables, and optimizing the conditions for desired responses. In addition, the significance of these process parameters on the coupled responses could be established with a number of planned experiments [10]. Conventional optimization approaches vary one variable at a time, while keeping all others constant at specific conditions, which is time consuming, and also incapable of reaching true optimum process conditions, due to the elimination of eventual variable interactions [11].

The present work makes an attempt to study the degradation potential of peroxidase from a plant source, radish. The characteristic of the enzyme towards the decolorization of safranin was investigated. The effect of four process variables, pH, temperature, dye concentration, and enzyme concentration, were optimized initially by OFAT. The optimum operating process conditions were further optimized with the application of Response Surface Methodology based on Box-Behnken design. The model obtained was studied using regression analysis through Analysis of Variance (ANOVA).

Materials and methods

Materials

Safranin (dye content 85 %), guaiacol, and hydrogen peroxide (30 %) of analytical grade were procured from Hi-Media India. Dye stock solution of 1 g/l was prepared in distilled water and diluted into varying concentrations for our experiments.

Peroxidase extraction and assay

Peroxidase was extracted from the pulp of *Raphanus sativus* (radish). For extraction, 100 g of the vegetable pulp was chopped and finely blended with 200 ml of distilled water for 15 min, after which it was filtered, and the filtrate was centrifuged at 6,000 rpm for 20 min. The supernatant obtained was filtered again and used as the source of crude enzyme [12]. This crude enzyme extract was employed for all decolorization experiments [13]. The enzyme activity was assayed according to the method of Putter *et al.* [14], based on the formation of tetraguaiacol. Reaction mixture consisting of 2.8 ml of Mcilvaine's buffer, 0.1 ml of crude enzyme, 0.05 ml of 18 mM guaiacol, and 0.05 ml of H_2O_2 was prepared, and the absorbance was recorded at 436 nm over a one minute interval. The activity of the enzyme was calculated using the formula;

$$Enzyme\ activity\ (u/ml) = \frac{(DA436/min \times 4 \times Vt \times dilution\ factor)}{(e \times Vs)} \quad (1)$$

where $DA436$/min is the change in absorbance over a one min interval ($Abs_{60sec} - Abs_{0sec}$)
V_t is the total volume of the reaction mixture = 3 ml
V_s is the enzyme volume = 0.1 ml
e is the micromolar extinction co-efficient of tetraguaiacol (cm^2/micro mol) = 25.5
dilution factor = 30
4 is derived from unit definition & principle

One unit of peroxidase activity (u) was defined as the amount of enzyme catalyzing the oxidation of 1 μ mole of guaiacol over 1 min.

Decolorization studies

The decolorization of safranin using the crude peroxidase extract was studied, considering four influencing process variables, pH, temperature, dye concentration, and enzyme concentration, primarily using the traditional One Factor At a Time approach (OFAT). The reaction mixture, consisting of 3 ml of Mcilvaine's buffer, 0.5 ml of dye solution, and 0.5 ml of enzyme was used for study [15], and the

necessary process variable under examination was varied accordingly. The effect of pH in the range of 4 - 9; the effect of temperature from 30 - 70 °C; the effect of dye concentration in the range of 100 - 500 mg/l, and the effect of enzyme concentration in the range of 8.89 - 35.58 U/ml was studied. The decolorization was monitored after 1 h of incubation in the dye λmax (520 nm), using a UV-Vis Spectrophotometer (Elico, India). The maximum absorbance of untreated dye solution as a control was also measured [16]. Each test was carried out in triplicate, and the average of the values were plotted to obtain the optimal value. Decolorization was expressed as % decolorization, which was calculated using the following formula;

$$Decolorization\ (\%) = \left[\frac{(Ci-Ct)}{(Ci)}\right] \times 100 \quad (2)$$

where *Ci* is the initial concentration of the dye, and *Ct* is the dye concentration over time [17].

Optimization of process variables using Box-Behnken design

Optimization of the operating conditions for the decolorization of safranin and the significant variable interactions were studied by applying RSM, based on Box-Behnken design. A four factor three level design was generated using the Design Expert software version 8.0.7.1. The factors considered for the study includes; Temperature (°C), Dye concentration (mg/L), Incubation time (Enzyme reaction time) (min) and Enzyme concentration (U/ml) keeping the pH of the solution constant. A 29 trial combination experiment proposed was carried out in duplicate and the response in form of % decolourization was analyzed [18]. **Table 1** represents the range and levels of process variables.

Table 1 Experimental ranges and levels of independent variables for safranin.

Independent variable	Factors	Range levels		
		–1	**0**	**1**
Temperature (°C)	X_1	30	40	50
Dye concentration (mg/l)	X_2	100	200	300
Incubation time (min)	X_3	60	90	120
Enzyme concentration (U/ml)	X_4	17.79	26.69	35.58

Results and discussion

Decolorization studies

The decolorization study was carried out using crude enzyme extract (35.8 U/ml). The traditional OFAT approach studies the effect of each influencing parameter separately, varying one factor at a time, and keeping the others constant. The effect of pH in the range of 4 - 9 was monitored, which showed an optimum at pH 7, producing 17.18 % decolorization for 100 mg/l initial dye concentration at 30 °C after 60 min of incubation. pH has a major effect on decolorization, and the optimal pH for color removal often lies in the range of 6.0 to 10.0 for most dyes [19] .The temperature study showed an optimum at 40 °C, producing 19.51 % decolorization for 100 mg/l initial dye concentration at optimum pH after 60 min of incubation. Similar results were reported by Bhatti *et al*. [20] for radish leaf peroxidase, with an optimum of 50 °C towards decolorization of two direct dyes, Solar Blue A and Solar Flavine5G, and Satar and Hussain [21], for white radish peroxidase (WRP), at 40 °C for maximal decolorization of RR120 and RB 171. The optimum dye concentration was found to be 300 mg/l, producing 11.5 % decolorization at optimum pH and temperature after 60 min of incubation. The effect of enzyme concentration showed exponential increase in decolorization rate, with increase in concentration showing an optimum of 35.58 U/ml, producing 27.4 % decolorization for 100 mg/l initial dye concentration at the optimum pH and

temperature after 60 min of incubation. **Figure 1** represents the effect of pH, temperature, dye concentration, and enzyme concentration on decolorization, respectively.

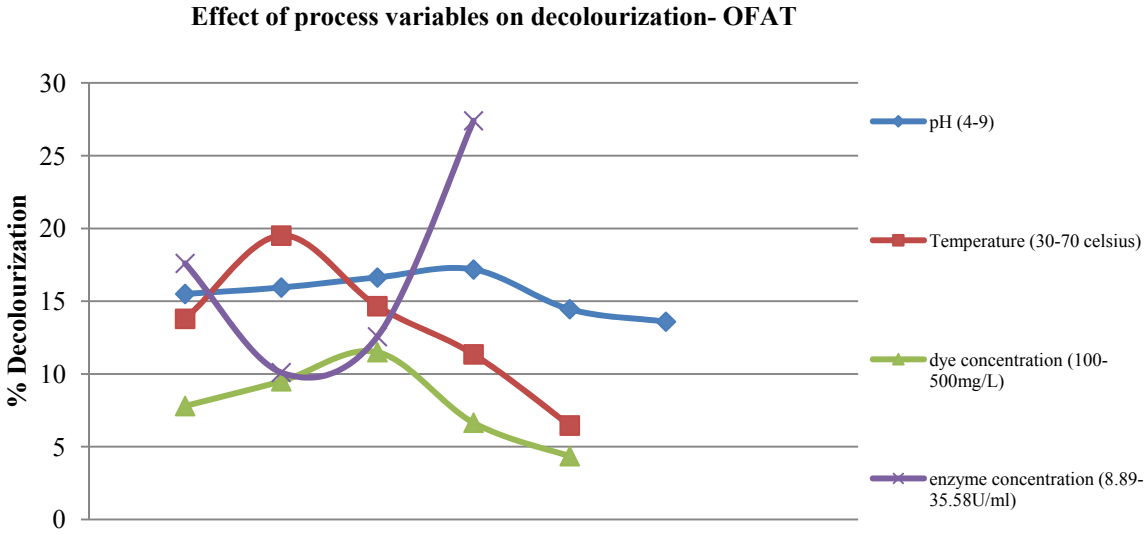

Figure 1 Effect of pH, temperature, dye concentration and enzyme concentration on decolorization of safranin using OFAT.

Optimization of process variables using Box-Behnken design

Experimental runs were designed and performed, and responses in the form of % decolorization were analyzed using software. **Table 2** shows experimental and predicted responses for the designed trials. A quadratic model was fitted for the response, and the significance of the variables and the nature of variable interactions were analyzed through Analysis of Variance (ANOVA). ANOVA is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation, for the purpose of testing hypotheses on the parameter of the model [22]. A regression analysis applying a second order polynomial equation for the predicted model was studied. The predicted polynomial equation for safranin decolorization in terms of coded factors is shown in Eq. (3).

Table 2 Experimental conditions of Box-Behnken Design run for safranin decolorization by *Raphanus sativus* peroxidase.

Std	Temp (°C) (X_1)	Concentration (mg/l) (X_2)	Incubation time (min) (X_3)	Enzyme concentration (U/ml) (X_4)	Response (%) Decolorization	
					Experimental	Predicted
1	30	100	90	26.69	22.48	21.95
2	50	100	90	26.69	9.45	9.53
3	30	300	90	26.69	17.79	17.35
4	50	300	90	26.69	20.57	20.74
5	40	200	60	17.79	17.63	16.18
6	40	200	120	17.79	10.19	9.51
7	40	200	60	35.58	4.94	5.26
8	40	200	120	35.58	16.51	17.60
9	30	200	90	17.79	15.43	18.97
10	50	200	90	17.79	12.14	12.69
11	30	200	90	35.58	16.19	15.79
12	50	200	90	35.58	16.43	13.05
13	40	100	60	26.69	12.06	11.15
14	40	300	60	26.69	13.54	14.84
15	40	100	120	26.69	15.5	14.36
16	40	300	120	26.69	16.22	17.29
17	30	200	60	26.69	30.07	29.24
18	50	200	60	26.69	8.72	10.27
19	30	200	120	26.69	18.98	17.61
20	50	200	120	26.69	26.56	27.56
21	40	100	90	17.79	6.56	6.72
22	40	300	90	17.79	13.55	11.41
23	40	100	90	35.58	4.37	6.69
24	40	300	90	35.58	8.61	8.62
25	40	200	90	26.69	10.19	12.88
26	40	200	90	26.69	13.19	12.88
27	40	200	90	26.69	14.31	12.88
28	40	200	90	26.69	17.84	12.88
29	40	200	90	26.69	8.89	12.88

$$Y_{safranin} = 12.88 - 2.25\ X_1 + 1.65\ X_2 + 1.42X_3 - 0.704X_4 + 3.95\ X_1X_2 + 7.23X_1X_3 + 0.88X_1X_4 - 0.91X_2X_3 - 0.68X_2X_4 + 4.75X_3X_4 + 5.64X_1^2 - 1.12X_2^2 + 2.65X_3^2 - 3.39\ X_4^2 \quad (3)$$

where Y is response (decolorization), X_1, X_2, X_3, and X_4 are coded variables, X_1^2, X_2^2, X_3^2,and X_4^2 are the square effects, X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, and X_3X_4 are the interaction effects, β_1, β_2, and β_3 are the linear coefficients, β_{11}, β_{22}, and β_{33} are the squared coefficients, and β_{12}, β_{13}, and β_{23} are the interaction coefficients. β_0 and ε are the constant and the random error, respectively [23].

Table 3 shows the results of regression analysis using ANOVA for safranin decolorization. The predicted model was found to be significant at the 5 % level; this is evident from the F-value (8.93) > 1 and the probability value ($p < 0.05$) of 0.0001. The values of "Prob > F" less than 0.05 indicates that the model terms are significant [24]. Integrity in the model can be evaluated by the determination coefficient (R^2); it measures the proportion of variation explained by the model [25]. The value of determination coefficient (R^2) was calculated to be 0.8993, indicating that 89.93 % variations in the response can be explained by the model.

The model also revealed a statistically insignificant lack of fit at the 5 % level, with P = 0.8915 for RSP. The lack-of-fit of the model signifies the failure of the model to represent the experimental data at the domain points not included in the regression [24]. In this study, X_1, X_2, X_1X_2, X_1X_3, X_3X_4, X_1^2, X_3^2, and X_4^2 were found to be significant model terms. The optimum values for temperature, dye concentration, incubation time, and enzyme concentration was found to be 30 °C, 200 mg/l, 60 min, and 26.69 U/ml, respectively, producing a decolorization rate of 30.07 % found experimentally, close to the predicted value of 29.24 %.

A circular contour plot of response surfaces suggest that the interaction is negligible between the corresponding variables, while an elliptical or saddle contour plot indicates significance in the interactions between the corresponding variables [26]. **Figures 2 - 4** shows the 3D contour plots representing the significant statistical interaction between the process variables for safranin decolorization.

Table 3 ANOVA for safranin decolorization.

Source	Sum of squares	Degrees of freedom	Mean square	F-Value	Probability > F
Model	890.36	14	63.59	8.93	0.0001*
Residual	99.61	14	7.11		
Lack of fit	49.71	10	4.97	0.39	0.8915
Pure error	49.89	4	12.47		
R - squared - 0.8993		Adjusted R squared - 0.7987			

*Statistically significant at 95 % confidence limit

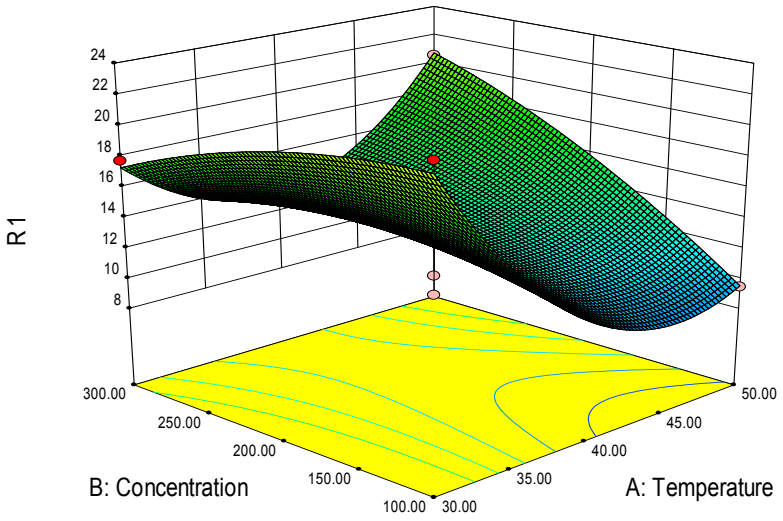

Figure 2 3D Contour plot showing factor interactions between temperature and dye concentration for safranin decolorization.

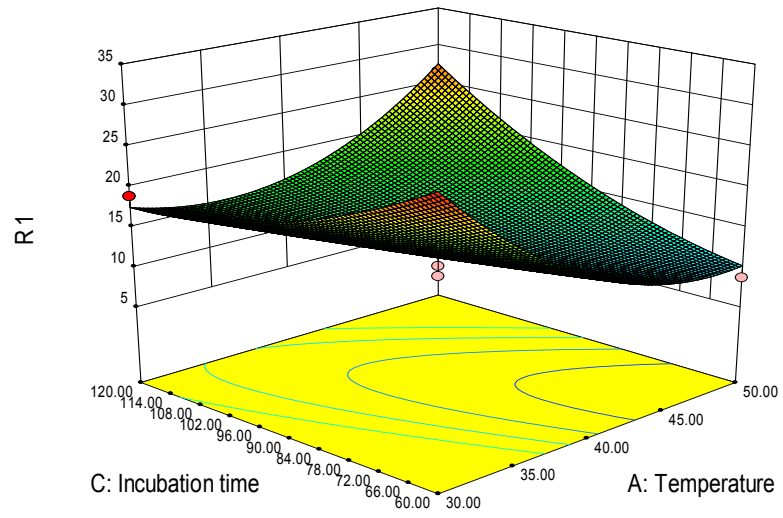

Figure 3 3D Contour plot showing factor interactions between temperature and incubation time for safranin decolorization.

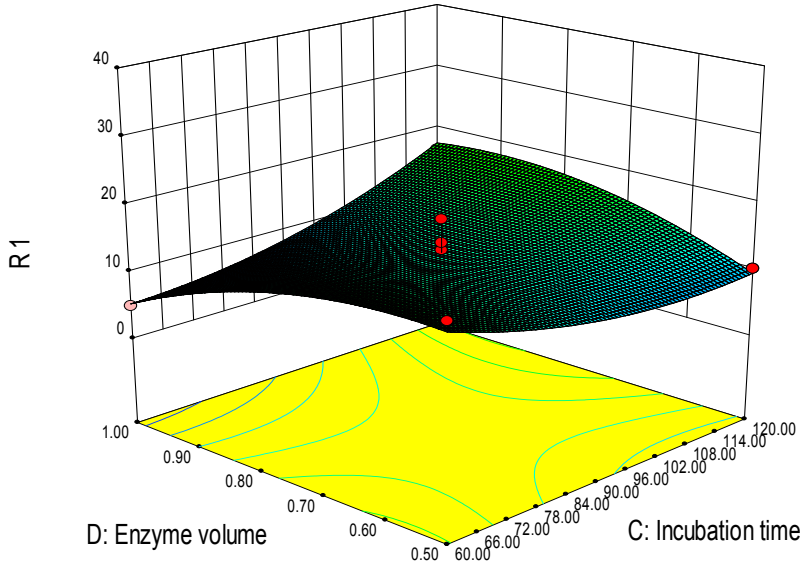

Figure 4 3D Contour plot showing factor interactions between incubation time and enzyme concentration in terms of volume for safranin decolorization.

Conclusions

Crude peroxidase enzyme was used as a biocatalyst for the decolorization of safranin. RSM was successfully applied to determine the optimal operational conditions. The optimum values for temperature, dye concentration, incubation time, and enzyme concentration was found to be 30 °C, 200 mg/l, 60 min, and 26.69 U/ml, respectively, producing a decolorization rate of 30.07 % found experimentally, close to the predicted value of 29.24 % for safranin.

Quadratic models, developed in terms of temperature, dye concentration, incubation time, and enzyme concentration to represent the decolorization percentage and the corresponding coefficients of independent variables, were estimated by the application of Design Expert version 8.0.7.1. The application of Response Surface Methodology for optimization of decolorization process variables enhanced the decolorization efficiency of the enzyme, producing higher rates of decolorization compared to that obtained by the OFAT method.

The enzymatic treatment method proposed provides a base for effluent treatment methods in dyeing industries, aiding in the disposal of treated effluents, which is a major topical environmental issue. Further expansion of research works in this field can provide a better solution to related issues.

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