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Statistical Optimization and Decolourization of Methyl Violet Using Peroxisdase from Radish

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Abstract

Statistical optimization design based on Response surface methodology for the decolourization of methyl violet using peroxidase from radish (Raphanus sativus) was applied. Effect of four influencing parameters (pH, Temperature, Dye concentration, enzyme volume) on decolourization was studied using One Factor At a Time (OFAT) method. The factors were optimized using RSM based on Box- Behnken design and analyzed for its significance through Analysis of Variance. The optimized operating conditions for enzymatic degradation were found to be: Temperature 40°C; Dye concentration 100 mg/L; Incubation time 60 min; Enzyme volume 0.75 mL at pH 7, maximum decolourization efficiency of 72.46% was obtained at this optimized conditions. The predicted decolourization rate under the optimum conditions was 65.20%. Verification experiments carried out closely agreed with the predicted value. The determination coefficient of the model was R^2 0.9310. The results signify that RSM based Box-Behnken design is a dependable method to optimize the operating conditions of methyl violet decolourization using radish peroxidase.

Keywords*: Methyl violet, raphanus sativus peroxidase, response surface methodology, box behnken design*

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INTRODUCTION

Synthetic dyes are being extensively used in textile dyeing and printing processes. Dyes are generally very difficult to break down biologically, due to their highly structured organic compounds [1, 2]. In addition, many dyes are believed to be toxic, carcinogenic or to be prepared from known carcinogens such as benzidine or other aromatic compounds that might be formed as a result of microbial metabolism [3, 4].

Among the various dye classes, triphenyl methane dyes are largest and versatile, playing an important role in industrial applications [5]. Methyl violet, a triphenyl methane dye has been extensively used in medicine as a biological stain and in dyeing industries. Methyl Violet is classified as a recalcitrant dye as it remains in the environment for longer period; causes cancer, eye irritation and inflammatory response. *In vitro* and *in vivo* investigations further concluded that this dye is a mitotic poisoning agent and a potent clastogenes, which promote tumor growth in some species of fish [5–7]. Hence it is mandatory to degrade these dyes prior to effluent disposal.

Various physical/chemical methods have been used for the removal of dyes from wastewaters [8–10]. The most commonly used techniques for colour removal include chemical precipitation ion exchange, reverse osmosis, ozonization and solvent extraction etc. [11]. These methods have some drawbacks, such as being economically unfeasible; unable to completely remove the recalcitrant dyes and/or their organic metabolites [12]; generating a significant amount of sludge that may cause secondary pollution problems; substantially increasing the cost of these treatment methods; and involving complicated procedures [13, 14]. Physio-chemical methods of dye removal are effective only if the effluent volume is small and sometimes the degradation products are toxic [15].

Microbial decolourization associated with involvement of various enzymes such as lignin peroxidase, laccase, azoreductase and biotransformation enzymes are found to be

highly effective [16, 17]. However, microbial treatment would result in biomass accumulation, which will expand the treatment scale [18], and the decolourization process is usually slow. Hence, the recent focus has shifted towards enzyme based treatment of colored wastewater. Enzymatic decolourization and degradation have significant potential to address this problem due to their environmentally-friendly, inexpensive nature, and also they do not produce large quantities of sludge [10]. Several enzymes (peroxidases, manganese peroxidases, lignin peroxidases, laccases, microperoxidase-11, polyphenol oxidases and azoreductases) have been evaluated for their potential in decolourization and degradation of dyes [19].

Peroxidase is an oxidoreductase enzyme which contains an iron-porphyrin ring. It catalyzes the redox reactions between H_2O_2 as an electron acceptor and different kinds of substrates by means of O_2 liberation [20]. Reduction of peroxides at the expense of electron donating substrates, make peroxidases useful in a number of biotechnological applications such as biopulping and biobleaching in the paper industry [21]. The enzyme *horseradish peroxidase* (HRP) has been successfully used in diagnostic kits of medical interest. This enzyme is also well known for its effective capacity to oxidize a wide spectrum of aromatic compounds, as well as in the degradation of some important industrial dyes [22–24].

The Response surface method (Box-Behnken), are new effective statistical methods that can be employed for optimization of the operational parameters, specifically to explore the relationship between a response and a set of design variables [25, 26]. BB design combines a two-level (full or fractional) factorial design and an incomplete block design, in which a certain number of factors are analyzed in all combinations for the factorial design; while other factors are placed at the central value in each block [27]. The RSM usually contains five steps: (i) to define independent input variables and the desired response adopting an experimental design, (ii) to perform regression analysis with quadratic response surface model, (iii) to find the significant parameters that affect the desired response by calculating the statistical analysis of variance (ANOVA) for independent input variables, (iv) to obtain optimum values of influencing parameters and (v) to conduct confirmation experiment and verify the optimal parameters [28].

In this paper, the effect of pH, temperature, dye concentration and volume of enzyme on dye decolourization was studied using One Factor at a Time (OFAT) approach. Response Surface Methodology (RSM) was applied to optimize the decolourization of the triphenyl methane dye methyl violet by the crude peroxidase from radish. Box Behnken design using 4 variables (pH, temperature, dye concentration and enzyme volume) was used to optimize the effect of these variables on dye decolourization.

MATERIALS AND METHODS

Chemical Reagents

Methyl violet of analytical grade was purchased from HI-Media (India). Stock standard solution of the dye (1%v/v) was prepared using double distilled water. This solution was diluted to appropriate concentrations before use.

Extraction of Peroxidase

Peroxidase was extracted from red radish pulp (*Raphanus sativus*). The vegetables were purchased from the local market. The extraction of the enzyme was carried out by chopping and blending 100 g of the vegetable with 200 mL of distilled water for 10 min into a homogenate, followed by filtration using a cheese cloth and centrifugation of the filtrate at 3000 rpm for 20 min [29]. The extract was then stored at 20°C. The enzyme was brought to room temperature before carrying out the experimental trials.

Enzyme Assay

Peroxidase activity was assayed using a UV-Visible spectrophotometer (Elico, India), based on the decomposition of hydrogen peroxide forming tetraguaiacol with guaiacol as the hydrogen donor, which can be measured at 436 nm with the colour development [30, 31]. Reaction media containing 2.8 mL of buffer, 0.05 mL of 18 mM guaiacol and 0.05 mL of H2O2 was prepared, to which 0.1 mL of the crude enzyme was added. The absorbance at 436 nm for 3 min at every 20

sec interval was read. One unit of peroxidase activity (U) was defined as the amount of enzyme catalyzing the oxidation of 1 μ mole of guaiacol in 1 min. The activity of the enzyme was calculated using the formula;

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

where, Vt is the final volume of reaction mixture (ml) = 3 mL, Vs is the sample volume (ml) = 0.1 mL, e is the micromolar extinction co-efficient of tetraguaiacol (cm^2/micro mol) = 25.5
4= derived from unit definition & principle.

Physiochemical Studies of the Enzyme
The effect of physiochemical parameters (pH, Temperature, Substrate concentration) on enzyme activity was studied. The procedure used for enzyme assay was repeated with the change of the necessary optimizing process variable. The effect of varying pH in the range of 4–9 was assayed at 30°C using Mcilvaine's buffer. The optimum temperature for maximum enzyme activity was determined by incubating the enzyme at different temperatures in the range of 30–70°C at the optimum pH. Effect of substrate guaiacol, was assayed by varying the volume (20–100 μL) of the substrate at 18 mM concentration. The enzyme concentration and activity was maintained as a constant throughout the enzyme kinetic studies and in the optimization experiments. Line Weaver Burk plot was plotted between inverse of substrate concentration on X-axis and inverse of enzyme activity on Y-axis, from the graph values of V_{max} and K_m (Michealis-Menton constant) were calculated [31].

Decolourization Studies
Decolourization studies were carried out using OFAT for four independent influencing variables (pH, Temperature, Dye concentration and Enzyme volume). The amount of every component varied with each optimization experiment. The response for all experiments was represented in the form of decolourization percentage which was a measure of the difference in the initial and final absorbance of the dye at the absorption maxima, 590nm. Crude enzyme extract was used for all decolourization experiments [32]. The reaction media was prepared containing 3 mL of the buffer, 0.5 mL of aqueous dye solution and 0.5 mL of the crude enzyme. The decolourization of dye, expressed as dye decolourization (%), was calculated according to the formula:

$$\text{Decolourization (\%)} = [(Ci - Ct)/Ci] \times 100 \quad (2)$$

where, Ci is the initial concentration of the dye and Ct is the dye concentration along the time [33].

Effect of pH towards Dye Decolourization
To analyze the effect of pH towards dye decolourization, methyl violet dye solutions with initial pH of 4–9 were degraded with a constant dye concentration of 100 mg/L. Reaction media containing 3 mL of different pH Mcilvaine's buffer solutions, 0.5 mL of dye as aqueous solution of 100 mg/L concentration, was prepared [34]. 0.5 mL of the enzyme extract was added and incubated for one hour at room temperature and the absorbance was recorded.

Effect of Temperature on Decolourization
Reaction media containing 3 mL of the optimum pH buffer, 0.5 mL of the dye of 100 mg/L concentration was prepared. 0.5 mL of the crude enzyme extract was added and were incubated at various temperatures (30, 40, 50, 60, 70ºC) for one hour and the absorbance was recorded.

Effect of Dye Concentration on Decolourization
Varying dye concentration in the range of 100 to 500 mg/L was prepared maintaining the pH, temperature and crude enzyme extract constant and incubated at room temperature for one hour and the absorbance was recorded.

Effect of Enzyme Volume on Decolourization
Dye concentration of 100 mg/L was treated with varying volumes of crude enzyme extract

(0.25–1mL) at the optimum pH and incubated at room temperature.

Optimization of Process Variables

Using the Box-Behnken design, the most significant independent variables, were examined at three different levels, low (-), high (+) or basal (0). A four variable design was generated using the Design Expert Software 8.0.7.1. Four independent factors influencing the decolourization; temperature (°C), dye concentration (mg/L), incubation time (minutes) and volume of enzyme (mL) were selected and the decolourization percentage was considered as the response [35]. According to the applied design, 29 experimental runs were executed. For predicting the optimal operating conditions a second order polynomial model was fitted to correlate relationship between independent variables and response:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{12} X_1X_2 + \beta_{13}X_1X_3 + \beta_{14} X_1X_4 + \beta_{23} X_2X_3 + \beta_{24} X_2X_4 + \beta_{34} X_3X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \varepsilon \quad (3)$$

where, Y is response (decolourization), 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. β_{12}, β_{13}, β_{23} are the interaction coefficients. β_0 and ε are the constant and the random error, respectively. The results of the experimental trials were studied and analyzed by Design Expert version 8.0.7.1 Statistical software to estimate the response of the influencing variable. A quadratic model was fitted for the design; the fit quality was evaluated by coefficient of determination R^2. Table 1 displays the experimental range and levels of the influencing factors for decolourization.

Table 1: *Experimental Range and Levels of Independent Variables*.

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 volume (ml)	X_4	0.5	0.75	1.0

RESULTS AND DISCUSSION

Enzyme Studies

Raphanus sativus peroxidase activity was observed in the pH range of 4–9. Maximum activity was observed at pH 4, which is in accordance with the earlier reported results of Bhatti *et al*., 2012 [31], in which the peroxidase isolated from *Raphanus sativus* leaves exhibited maximum activity at pH 4. In order to check the effect of temperature, the enzyme was assayed at different temperatures (30–70°C) at the optimum pH (4). The enzyme exhibited maximum activity at 50°C. Our result was found to be closer to that reported by Duarte- Vaszquez *et al*., 2001 [36] for turnip peroxidase showing an optimum temperature of 55°C. The effect of substrate (guaiacol) concentration was studied at 18 mM concentration using varying volumes (20–100 µL) and the maximum activity was exhibited at 40µL which was closer to the standard assay volume (50 µL) reported by Putter J *et al.*, 1974 [30]. The concentration of the substrate present in the aqueous phase significantly influences enzyme-mediated reaction. If the amount of enzyme concentration is kept constant and the substrate concentration is gradually increased the reaction will increase until it reaches maximum. After obtaining the equilibrium state, further addition of the substrate will not change the rate of reaction [37].

Decolourization Studies

Effect of pH towards Decolourization

Enzymes have a characteristic pH at which they show maximum activity, the pH optimum being dependent on the nature of substrate used [38, 39]. The decolourization was found to increase constantly in the range of 7–9, showing maximum activity at pH 7. Methyl violet was decolorized to about 16.67% by RSP at this pH after one hour of incubation.

Effect of Temperature on Enzyme Activity

The dye decolourization was not significantly affected by changing the temperature from 40–

60°C. However, it was decolorized maximally at 40°C above which the decolourization was decreased consistently in accordance to the known relationship between thermal deactivation of enzymes. Below the optimum temperature, the enzyme might not be able to achieve its energy of activation for reaction with the dyes; hence again low decolourization was observed [40]. The maximum decolourization observed at 40°C was found to be 17.12% for RSP. Bhatti *et al*., 2012 [31] reported an optimum temperature of 50°C for RSP towards decolourization of direct dyes Solar BlueA and Solar Flavine5G. Similarly maximal decolourization of RR120 and RB 171 was observed at 40°C for white radish peroxidase (WRP) [41].

Effect of Dye Concentration on Enzyme Activity

The maximum decolourization shown by RSP was with 100 mg/L of dye concentration producing 8.1%. Concentrations below and above this range showed decrease in decolouri zation. The crude enzyme was able to tolerate the dye concentration up to 300 mg/L at optimum pH.

Effect of Enzyme Volume on Decolourization

The effect of enzyme volume showed exponential increase in decolourization rate with increase in volume. The maximum decolourization was observed at 1.0 mL of enzyme and was found to be 28.6%.

Optimization of Process Variables

The 29 runs designed by the software were carried out and the experimental results in the form of decolourization percentage were analyzed through RSM to obtain an empirical model for the best response. Table 2 represents the BBD runs for decolourization of methyl violet. The response was fitted using a quadratic model explaining the mathematical relationship between the independent variables and the dependent response, and the significance of the model was 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 [42]. The actual values obtained from the experimental trials were found to be in good agreement with predicted values evaluated by the software. Eq. (4) represents the predicted equation for the model,

$$Y = 22.47 + 2.67X_1 - 5.59\ X_2 - 12.78X_3 + 1.25X_4 - 1.76\ X_1X_2 + 6.86X_1X_3 - 0.57\ X_1X_4 + 9.71\ X_2X_3 - 7.07\ X_2X_4 + 0.18\ X_3X_4 - 4.08X_1^2 + 0.95\ X_2^2 + 13.69\ X_3^2 - 3.35\ X_4^2 \quad (4)$$

Table 3 shows the results of regression analysis using ANOVA. The model was found to be significant at the 5% level this is evident from the F-value of 13.49 and probability value ($P<0.05$) of < 0.0001 for RSP. The values of "Prob>F" less than 0.05 indicate that the model terms are significant. The model adequacy was further confirmed by a satisfactory value of determination coefficient (R^2), which was calculated to be 0.9310 for RSP. The value suggests that the model could predict 93.1% of the variability in the response. The model also revealed a statistically insignificant lack of fit at 5% level with P= 0.4909 for RSP. In this study, X_2, X_3, X_1X_3, X_2X_3, X_2X_4 and X_3^2 are found to be significant model terms.

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 [25]. Figures 1, 2 and 3 represent the 2D contour plots representing the statistical two variable interactions, indicating that dye concentration plays a vital role along with enzyme volume and incubation time towards enzyme mediated dye decolourization, while the interaction between other investigated factors were found to be insignificant. The optimum values for temperature, dye concentration, incubation time and enzyme volume was found to be 40°C, 100 mg/L, 60 min and 0.75 mL, respectively, producing a decolourization rate of 72.46% experimentally found closer to the predicted values of 65.20%.

Table 2: *Experimental Conditions of BBD Runs of Design Expert 8.0.7.1. for Methyl Violet Decolourization by Raphanus sativus Peroxidase.*

Std.	**Temp (°C) (X_1)**	**Concentration (mg/L) (X_2)**	**Incubation Time (min) (X_3)**	**Enzyme (mL) (X_4)**	**Response (%) Decolourization**		**Residual**
					Experimental	Predicted	
1	30	100	90	0.75	22.14	20.50	1.64
2	50	100	90	0.75	23.03	29.35	-6.32
3	30	300	90	0.75	19.46	12.84	6.62
4	50	300	90	0.75	13.33	14.66	-1.33
5	40	200	60	0.5	41.61	44.54	-2.93
6	40	200	120	0.5	18.7	18.59	0.105
7	40	200	60	1	46.87	46.67	0.198
8	40	200	120	1	24.71	21.48	3.23
9	30	200	90	0.5	9.18	10.55	-1.36
10	50	200	90	0.5	19.72	17.02	2.69
11	30	200	90	1	13.32	14.19	-0.87
12	50	200	90	1	21.57	18.39	3.18
13	40	100	60	0.75	72.46	65.20	7.26
14	40	300	60	0.75	33.6	34.61	-1.01
15	40	100	120	0.75	23.04	20.21	2.83
16	40	300	120	0.75	23.02	28.46	-5.44
17	30	200	60	0.75	45.36	49.06	-3.70
18	50	200	60	0.75	40.86	40.67	0.18
19	30	200	120	0.75	7.46	9.77	-2.31
20	50	200	120	0.75	30.41	28.83	1.58
21	40	100	90	0.5	16.44	17.33	-0.89
22	40	300	90	0.5	22.7	20.31	2.38
23	40	100	90	1	29.48	33.99	-4.51
24	40	300	90	1	7.44	8.67	-1.23
25	40	200	90	0.75	16.03	22.47	-6.44
26	40	200	90	0.75	19.2	22.47	-3.27
27	40	200	90	0.75	28.19	22.47	5.72
28	40	200	90	0.75	23.54	22.47	1.06
29	40	200	90	0.75	25.4	22.47	2.93

Table 3: *Analysis of Variance for Methyl Violet Decolourization by Raphanus sativus Peroxidase.*

Source	Sum of Squares	Degrees of freedom	Mean Square	F Value	Prob > F
Model	4900.74	14	350.05	13.49	< 0.0001*
X_1-temperature	85.33	1	85.33	3.28	0.0912
X_2-dye concentration	374.53	1	374.53	14.44	0.0020*
X_3-incubation	1961.47	1	1961.47	75.62	< 0.0001*
X_4-enzyme	18.85	1	18.85	0.73	0.4083
X_1X_2	12.32	1	12.32	0.47	0.5020
X_1X_3	188.37	1	188.37	7.26	0.0174*
X_1X_4	1.31	1	1.31	0.05	0.8254
X_2X_3	377.13	1	377.13	14.54	0.0019*
X_2X_4	200.22	1	200.22	7.72	0.0148*
X_3X_4	0.14	1	0.14	0.005	0.9423
X_1^2	108.27	1	108.27	4.17	0.0603
X_2^2	5.87	1	5.8777	0.23	0.6414
X_3^2	1216.9	1	1216.90	46.91	< 0.0001*
X_4^2	72.71	1	72.71132	2.80	0.1163
Residual	363.15	14	25.94		
Lack of Fit	268.53	10	26.85	1.13	0.4909
Pure Error	94.61	4	23.65		
Cor Total	5263.89	28			

**Statistically significant at 95% confidence limit*

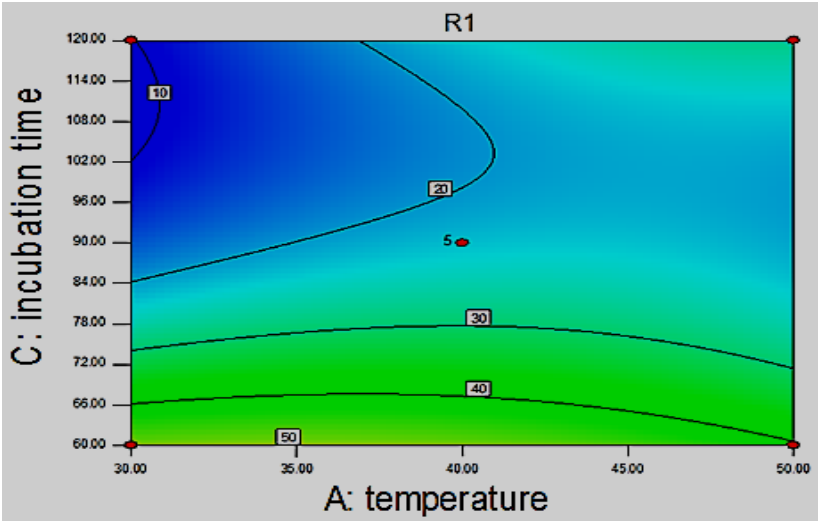

Fig. 1: *2D Contour Plot Showing the Interaction between Temperature and Incubation Time.*

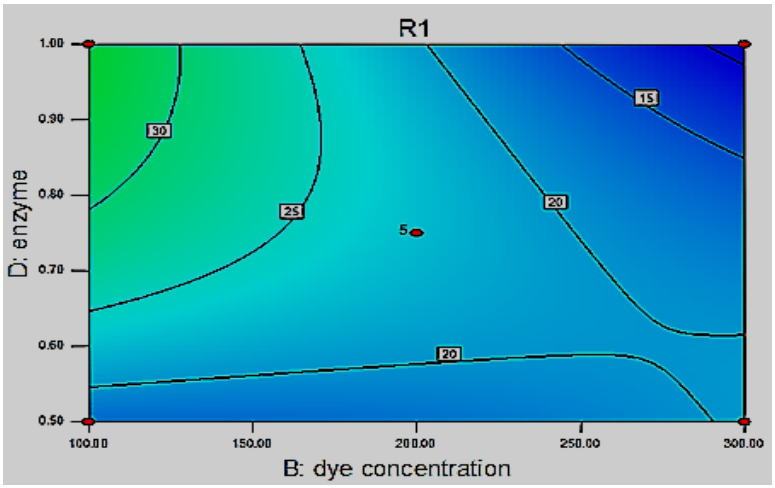

Fig. 2: *2D Contour Plot Showing the Interaction between Dye Concentration and Enzyme Volume.*

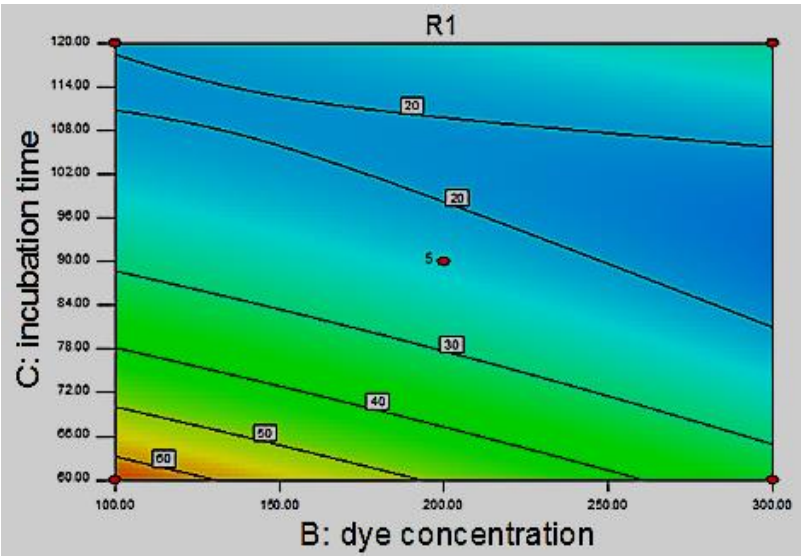

Fig. 3: *2D Contour Plot Showing the Interaction between Dye Concentration and Incubation Time.*

CONCLUSIONS

Crude peroxidase enzyme was used as a biocatalyst for the decolourization of the triphenyl methane dye, Methyl violet. Response surface methodology (RSM) was successfully applied to determine the optimal operational conditions for maximum decolourization, which were found to be temperature of 40°C, dye concentration 100 mg/L, incubation time 60 min and enzyme volume 0.75 mL.

A quadratic model, developed in terms of temperature, dye concentration, incubation time and enzyme volume, to represent the decolourization percentage and the corresponding coefficients of independent variables was estimated by the application of Design Expert version 8.0.7.1.

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