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Statistical optimization for enhanced decolorization of Golden Yellow PRA by *Citrus reticulata* var. kinnow peroxidase and phytotoxicity evaluation of its degraded products

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ABSTRACT

In the present study, the decolorization of Golden Yellow PRA by *Citrus reticulate* var. kinnow peroxidase (GYPRA) was investigated. Optimization of important process parameters viz. pH, temperature, enzyme dose, concentrations of H_2O_2, and dye was done by applying one-factor-at-a-time and response surface methodology. Through OFAT analysis, maximum decolorization (91.77%) of the dye was observed with pH 2.0, temperature 40°C, enzyme dose 24 U/mL, H_2O_2 concentration 0.375 mM, and Golden Yellow PRA concentration of 100 mg/L. The effect of mediators was also investigated and 0.05 mM of vanillin caused 95.98% decolorization, within just 5 min. The presence of metal ions did not exert any drastic influence on the process of decolorization, ensuring it suitable for industrial applications. For statistical optimization, three factors of pH, enzyme dose, and dye concentration were selected. Then, response surface analysis was conducted through central composite design and a second-order polynomial model (0.9894) was generated. ANOVA analysis of this model indicated that the pH, enzyme dose, dye concentration and their interaction were the most significant in decolorization of GYPRA. Optimum condition through RSM was found to be the pH of 3, enzyme dose 26.77 U/mL, and dye concentration of 86.88 mg/L, for enhanced decolorization.

Keywords: Decolorization; Phytotoxicity; Golden Yellow PRA; Response surface methodology

1. Introduction

The ever-increasing world's population and its rapid adoption of an industrial-based lifestyle have certainly led to an enhanced anthropogenic impact on the environment. The textile industries are discharging lots of different toxic compounds to the environment at different operational stages. These effluents can percolate into the aquifer and deteriorate the quality of underground water or wherever it is thrown without proper pretreatment into the water bodies. Consequently, such hazardous compounds not only affect aquatic life but also become a reason of serious problems related to human health [1].

Synthetic dyes having complex aromatic molecular structure are classified as anionic direct, acid, and reactive dyes, cationic basic dyes, and non-ionic disperse dyes [2]. Among them, reactive dyes are

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typically azo-based chromophores combined with different types of reactive groups, e.g. vinyl sulfone, chlorotriazine, trichloropyrimidine, and difluorochloropyrimidine [3]. They have poor fixation rates and hence may be difficult to remove from wastewaters because of their low biodegradability and their weak absorption into activated sludge [4]. Therefore, innovative treatment technologies need to be investigated. Decolorization of dye wastewater by the action of oxidoreductive enzymes is the subject of many studies [5–8]. Among them peroxidase is the most important [9].

The application of peroxidase for treatment of textile dyes provides a more practicable system, with a number of advantages: (a) a very simple and applicable system with minimal requirements of compounds; (b) cost effective and eco-friendly system (c) stable enzyme, and (d) short treatment times [10]. Moreover, peroxidase can be used for treatment of wide spectrum aromatic dyes and can degrade/transform them either by precipitation or by opening the complex aromatic ring of dye structure [11].

Peroxidase has been isolated from different fruits including melon [12], peach [13], strawberry [14], apple [15], and papaya [16]. However, no promising results were obtained in terms of identifying peroxidases able to knock out HRP as the preferred plant peroxidase in biotechnology. The availability of highly stable and active peroxidases from sources other than horseradish would go a long way toward the development of a catalytic enzyme with broad commercial and environmental applications. In this regard, *Citrus reticulata* (CRP) would be a good option on the basis of its easy availability, high activity, and thermostable nature which is evident by our previous reports [17,18].

Citrus fruit is of high commercial value in Pakistani market and also the focus of most of the today's research is to explore economical sources; so in this paper for the first time, the potential of peroxidase extracted from peels of CRP has been investigated for degradation of Golden Yellow PRA by one-factor-at-a-time and response surface methodologies, and the promising results have been obtained. An important part of the project was to check the phytotoxicity of the dye and dye degraded samples, using *Zea mays* as bio indicator.

2. Materials and methods

2.1. Chemicals

All chemicals (purity > 98%) used in this work were purchased from Sigma–Aldrich Chemical Co. USA, while Golden Yellow PRA (GYPRA) was a sole gift from Haris Dyes and Chemical industries, Pakistan. Its molecular structure is shown in Fig. 1.

2.2. Isolation and partial purification of peroxidase from Citrus reticulate *var.* kinnow *peels*

Peels of *Citrus reticulate* var. kinnow were first thoroughly washed with distilled water. Then the enzyme was isolated, salt fractionated, and dialyzed [19]. After that, the enzyme activity and specific activity were also calculated.

2.3. Peroxidase assay

Peroxidase activity was determined colorimetrically using spectrophotometer (Cecil 7200) following the formation of tetraguaiacol (A_{max} = 470 nm, ε = 26.6 mM^{-1} cm^{-1}) with slight modification [20].

2.4. Optimization of decolorization conditions by OFAT methodology

First experiment was carried out at constant conditions of temperature (40°C), time (45 min), CRP dose (18 U/mL), GYPRA concentration (75 mg/L), and H_2O_2 concentration (0.25 mM) while varying the pH from 2.0 to 9.0. In the next experiment, temperature was optimized by different temperatures in the range of 25–70°C at a pH of 2.0, while fixing all the other parameters as described above. Similarly, subsequent series of experiments were carried out in order to optimize parameters like time, CRP dose, H_2O_2 concentration, and GYPRA concentration, within the ranges of 0–120 min, 3.0–36.0 U/mL, 0.0625–1.0 mM, and 25–250 mg/L, respectively [18].

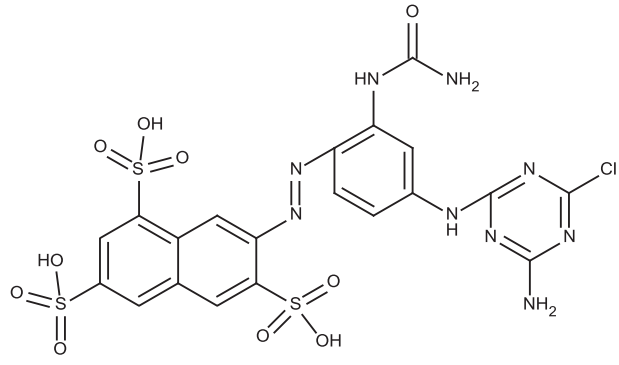

Fig. 1. Molecular structure of Reactive Golden Yellow PRA (λ_{max} 416).

2.5. Effect of mediators

The effect of different mediators was checked using 0.05 mM concentration of each mediator (*p*-coumaric acid, 1, hydroxybenzotriazole (HOBT), syringaldehyde, vanillin, syringic acid, veratryl alcohol, and pyrocatechol). Then vanillin was used for further experiment to find out its optimum concentration within the range of 0.025–0.5 mM. While in the next experiment time of incubation (2.5–30 min) was also optimized at fixed concentration of mediator at its optimum level.

2.6. Effect of metal ions

For this experiment, 24 U/mL of the enzyme was first independently incubated with 1 mM concentration of different metal ions including Co^{2+}, Mg^{2+}, Mn^{2+}, Cd^{2+}, Cr^{3+}, Zn^{2+}, $Al^{3+,}$ Cu^{2+}, Pb^{2+}, Sr^{2+}, Ni^{2+}, Hg^{2+}, and Ca^{2+} for 60 min. Then 100 mg/L of GYPRA was treated independently with 100 µL of the above solution in the presence of 0.375 mM H_2O_2 under optimized conditions of pH, temperature, time of incubation, and mediator.

2.7. Calculation for dye decolorization

The efficiency of color removal was expressed as the percentage ratio of the decolorized dye concentration to that of initial one using the following formula:

$$\%\,\text{Decolorization} = [(A_i - A_f)/A_i) \times 100] \quad (1)$$

where A_i = absorbance of untreated dye at 416 nm, A_f = absorbance of CRP treated dye at 416 nm.

2.8. Optimization of decolorization conditions by RSM

Response surface methodology, used to identify the optimum conditions for a multivariable system, can predict the combined effect of some variables. RSM using central composite design (CCD) could efficiently be applied for the modeling and optimization of biological decolorization process, with the fewest number of experiments. CCD involves the following steps: performing the statistically designed experiments according to the design, factors, and levels selected; estimating the coefficients of the mathematical model to predict the response and check its adequacy [21,22].

On the basis of the classical study, three important parameters, pH (A), enzyme dose (B), and dye concentration (C) were chosen as the independent variables and percentage decolorization was the dependent response variable. A 2^3 full factorial CCD for the three variables each at five levels, consisting of 8 factorial points, 6 axial points, and 6 replicates at the centre points were employed, indicating that altogether 20 experiments were required.

Table 1 represents the experimental range and levels of independent variables, i.e. pH (2.0–3.0), enzyme dose (24–27 U/mL), and dye concentration (50–125 mg/L), while the other parameters like H_2O_2 concentrations (0.375 mM), temperature (40°C), and time of incubation (20 min) were kept as constant.

2.8.1. Statistical analysis

The Design Expert Software (Stat Ease, Trial version 7.0.0) was used for regression and graphical analysis of the data obtained.

Each variable was used to develop an empirical model which correlated the response to three variables using a second-degree polynomial equation as given by the following equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where Y is the predicted response, x_i, x_j, ..., x_k are the input variables, which affect the response (Y), x_i^2, x_j^2, ..., x_k^2 are the square effects, $x_i x_j$, $x_i x_k$, and $x_j x_k$ are the interaction effects, β_0 is the intercept term, β_i ($i = 1, 2, ..., k$) is the linear effect, β_{ii} ($i = 1, 2, ..., k$) is the squared effect, β_{ij} ($i = 1, 2, ..., k$; $j = 1, 2, ..., k$) is the interaction effect, and ε is a random error [23–25]. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) and the coefficient of determination (R^2) was calculated to find out the goodness of fit of the model.

2.8.2. Validation of the regression model

In order to verify the validity of regression equation, additional experiment was performed in triplicate under the optimum conditions obtained through Central composite RSM.

2.9. Phytotoxicity study

The toxicity of untreated and degraded dye was determined by measuring the phytotoxicity effect of these samples on seed germination of maize (*Zea*

Table 1
Levels of different process variables in coded and uncoded form for decolorization of Golden Yellow PRA

Variable	Code	Range and levels				
		−1.682	−1	0	+1	+1.682
pH	*A*	1.66	2.0	2.5	3.0	3.34
Enzyme dose (U/mL)	*B*	18.95	21	24	27	29.05
Dye concentration (mg/L)	*C*	24.43	50	87.50	125	150.57

mays) by following an earlier reported method [26]. Triplicate samples of 10 seeds were utilized for each test. After 72 h of incubation in the dark, seed germination percent and relative root elongation was noted for seeds placed in untreated dye samples, degraded dye samples, and control (distilled water) samples. The germination index (GI) was calculated as follows:

$$GI = GP \times La/Lc \quad (3)$$

where GP is the number of germinated seeds expressed as a percentage of control values, La is the average value of root elongation in the dye solutions, and Lc is the average value of root elongation in the control.

3. Results and discussion

3.1. Optimization of decolorization conditions by OFAT methodology

3.1.1. Effect of pH

pH is one of the influential factors in the expression of enzymatic activity as it changes the states of ionization of amino acid side chains or the ionization of the substrate [27].

The role of pH on decolorization (%) of GYPRA by CRP is demonstrated in Fig. 2.

The data showed that GYPRA was maximally decolorized in the acidic medium. The optimum pH reported for GYPRA was 2.0, above which a significant decrease in the extent of decolorization was observed such that a negligible % decolorization was recorded at pH 9.0, which might be due to the loss of enzyme activity at this extreme pH [28]. In the previous literature, Acid Black 10 BX was maximally decolorized at pH 2.0 [29]. Similarly, acidic medium (optimum pH 4.0) was considered suitable for maximum decolorization of synthetic recalcitrant dyes including Reactive Red 2 (85%), Reactive Black 5 (86%), Reactive Blue 4 (86%), Disperse Black 9 (89%), and Disperse Orange 25 (90%), mediated by cauliflower bud peroxidase [30]. Similarly, disperse dyes DR 17 and DB 1 were maximally decolorized at pH 3.0 by bitter gourd peroxidase [31]. However, the pH profile of HRP catalyzed decolorization of RB 19 showed maximum decolorization at pH 5.0 (96%), whereas no decolorization was observed at pH 3.0 and 8.0 [32].

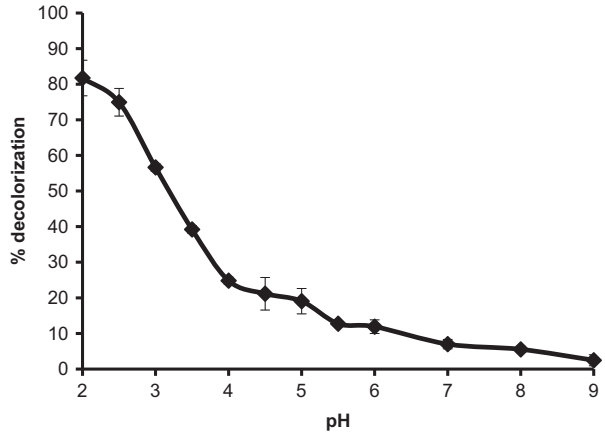

Fig. 2. Effect of pH on decolorization of GYPRA by CRP.

3.1.2. Effect of temperature

Temperature is one of the important parameters governing the action of enzymes. As the temperature increases, the enzyme activity increases because the more energy is available to speed up the reaction, until an optimum temperature value reaches at which the enzyme shows maximum activity and after that with further increase in temperature, the enzyme activity starts decreasing due to denaturation of enzyme which cause changes in active site that will no longer accept the substrate [33]. Thus, the effect of temperature on percent decolorization of GYPRA mediated by CRP was studied and the results are summarized in Fig. 3.

It is clear from the figure that in this case, temperature did not play a significant role on the process of decolorization and in the whole range, 79.67–84.82%

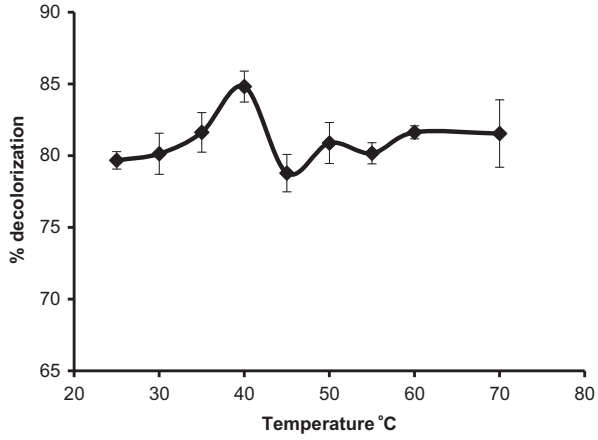

Fig. 3. Effect of temperature on decolorization of GYPRA by CRP.

decolorization was observed with maximum at 40˚C. It means CRP maintained its integrity in this broad range of temperature ranging from 25–70˚C. Almost 81.54% decolorization efficiency of CRP at an extreme temperature of 70˚C shows its thermostable nature. Previously, maximum decolorization of reactive dyes (DMBBLN 77%, DMBLR 99%, DMR 94%, and RBBR 97%) by HRP was reported at 35˚C [34]. For azo and anthraquinone dyes, the maximum decolorization by *Trichosanthes diocia* peroxidase was achieved at 40˚C [35].

3.1.3. Effect of time

Catalyst/substrate contact time is an important parameter which plays a significant role in dye degradation. So experiments were performed to notice the effect of time on both the dyes and absorption spectra were also recorded in this case (Fig. 4).

It is evident from the spectrum that first 5 min of contact time was very much important as almost 76% decolorization was monitored in this period. After which the process slowed down and almost 11% increase in % decolorization was obtained within next 15 min (optimum contact time) and then it became constant. The reason for slowdown of the reaction might be the simultaneous decrease in concentration of the reacting substances (CRP, dye, and H_2O_2). It can be concluded that the CRP performed quite efficiently with most of the decolorization in the first 5 min of contact. In similar studies, using HRP, Liu et al. [36] obtained 80 and 100% of decolorization, respectively, for methyl orange and bromophenol blue. However, Akhtar et al. [37] monitored that out of eight reactive dyes tested, only four (RR 120, RB

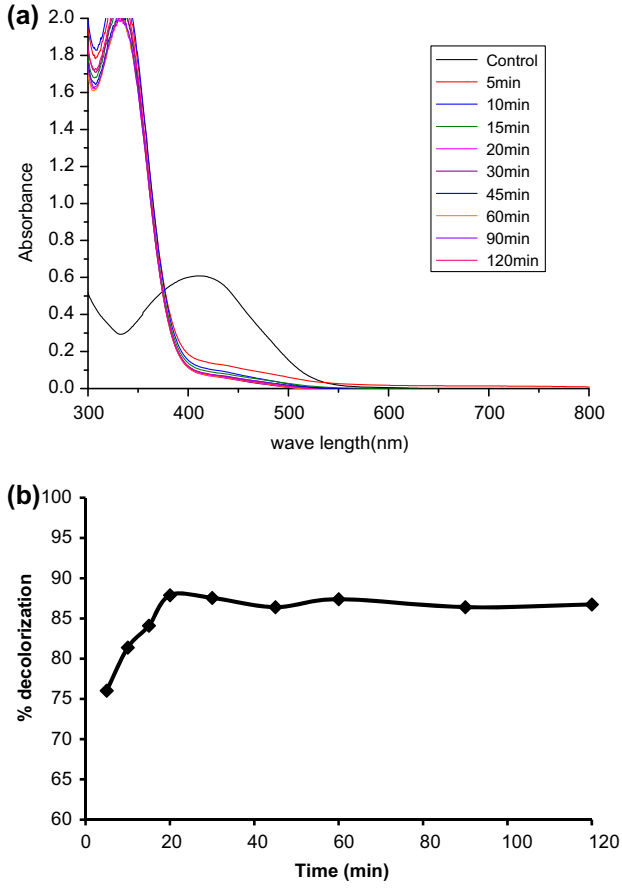

Fig. 4. Scanning spectrum of GYPRA with respect to time (a) and the effect of reaction time on decolorization of dye by CRP (b).

171, RB 4, RB 160) decolorized in 60 min, while the other took longer time by bitter gourd peroxidase. Actually, the reaction time has a direct relation to different structures of the dyes; this fact influenced the way of enzyme activity and as a result could cause variations in reaction time [38].

3.1.4. Effect of CRP dose

Enzyme dose plays an important role in decolorization of dyes and an optimal concentration level of enzyme is necessary to keep the reaction at a high and steady rate. So in order to analyze this important parameter, experiments were conducted by keeping substrate concentration (dye and H_2O_2) along with physical conditions as constant and changing the enzyme dose (3.0–36.0 U/mL) so that any variation in product formed was a function of enzyme dose. At

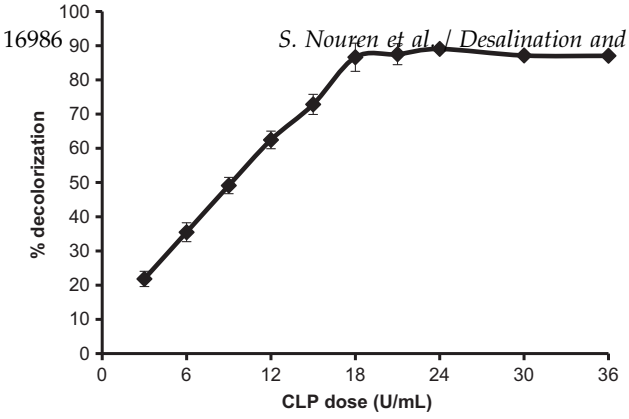

Fig. 5. Effect of enzyme dose on decolorization of GYPRA by CRP.

specified experimental conditions, the results are presented in Fig. 5.

It is quite clear from the data that initially decolorization percentage increased with increasing enzyme dose until optimum was achieved at 24 U/mL. After which there was no further increase in extent of decolorization was observed with increasing enzyme dose up to 36 U/mL. The reason might be that each peroxidase molecule catalyses fewer reactions under higher concentration of the enzyme hence decreasing the catalytic efficiency [32]. The similar trend was reported for decolorization of Reactive Blue 21 by horseradish peroxidase [39].

3.1.5. Effect of H_2O_2 concentration

H_2O_2 acts as a co-substrate of peroxidase and takes part in catalytic mechanism of peroxidase by oxidizing the enzyme into a reactive intermediate radical which accepts the aromatic substrates (dye molecules) and converts them into radicals which may further polymerize or degrade into small products. However, excess amount of this reagent in the reaction mixture inhibits the enzyme activity and when present in small quantity, limits the reaction rate [40,41].

Experiments were conducted in order to find out the optimum concentration of hydrogen peroxide for maximum decolorization of GYPRA by CRP and the results are shown in Fig. 6. As can be seen from the data that decolorization percentage increased with increasing concentration of hydrogen peroxide until an optimum was achieved at 0.375 mM. After the optimum concentration, a decline in decolorization percentage was reported with increasing concentration of hydrogen peroxide, which might be due to the inhibition of peroxidase activity possibly by causing irreversible oxidation of the enzyme ferriheme group which is essential for its activity. The result of this study was in agreement with previous reports of Vasantha et al. [42] and Osuji et al. [43], where also a

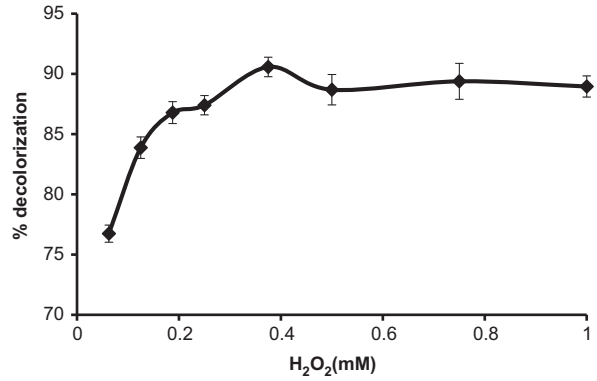

Fig. 6. Effect of H_2O_2 concentration on decolorization of GYPRA by CRP.

lower concentration of H_2O_2 was considered as optimum for various dyes treated.

3.1.6. Effect of dye concentration

Experiments were carried out by changing the dye concentration and the results obtained are reported in Fig. 7.

As depicted by the figure that in case of GYPRA, the optimum was obtained at a dye concentration of 100 mg/L. After which no further increase in percent decolorization was reported, but it gradually decreased until 250 mg/L of dye. It means that once the enzyme was saturated, any further addition of dye molecule were retaining in the solution thus increasing the absorbance and consequently lowering the percentage of decolorization. In a previous study similar trend was observed for decolorization of fluorescein dye by horseradish peroxidase [44].

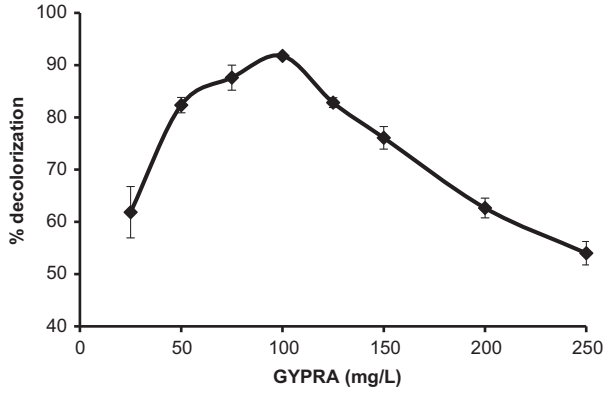

Fig. 7. Effect of dye concentration on decolorization of GYPRA by CRP.

3.2. Screening of compounds for mediating effect on decolorization of GYPRA

Experiments were performed in order to check the mediating potential of different compounds for improvement in the percent decolorization of GYPRA by CRP. The results are depicted in Fig. 8.

Among all the compounds tested as mediator, no one brought a remarkable improvement in percent decolorization. However, vanillin showed its potential to act as a mediator, whereas all the remaining compounds decreased the decolorization percentage with the exception of HOBT and *p*-coumaric acid which did not affect the process. In a previous work, HOBT and vanillin were utilized for decolorization of reactive orange 15 and it was observed that HOBT was more efficient as a mediator than vanillin for *T. diocia* peroxidase [45].

It has been disclosed previously that the redox mediators have the ability to facilitate oxidation reaction between a substrate and an enzyme [46]. Actually, different redox mediators are having different efficiencies of mediation which is carried out by the mediator's redox potential and the substrate's oxidation mechanism [47]. The formation of free radicals may take place either via one-electron oxidation of the substrate or by abstraction of a H-atom from the substrate [48,49].

3.3. Effect of varying concentrations of vanillin on decolorization of GYPRA by CRP

Different experiments were conducted in order to find out the optimum concentration of vanillin for the enhancement in percent decolorization of GYPRA. The results thus obtained are represented in Fig. 9.

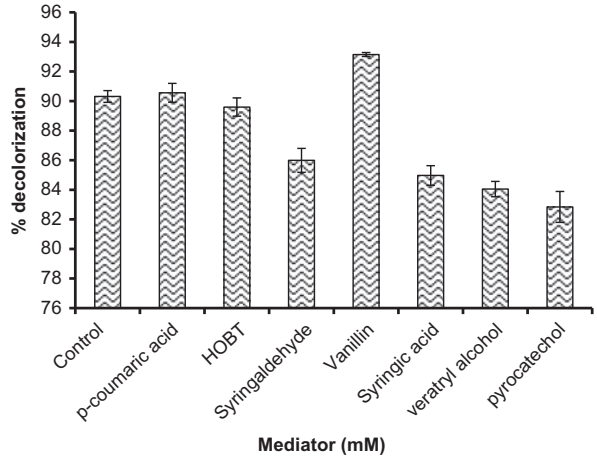

Fig. 8. Compounds investigated as redox mediators for mediating decolorization of GYPRA by CRP.

It can be noted from the figure that 0.05 mM of vanillin was sufficient to result in optimum decolorization for GYPRA. Although there was no improvement in percent decolorization reported with increasing amount of mediator after this optimum, but a continuous decreasing trend was observed. Similar kind of trend was observed in the previous literature for violuric acid which was used as a redox mediator for decolorization of disperse dyes by turnip peroxidase. The reason was found to be the dual role of the compound both as a mediator (at lower concentration) as well as a competitive inhibitor (at higher concentration), thus inhibiting enzyme activity [50].

3.4. Effect of time of incubation on decolorization of GYPRA by CRP in presence of vanillin

Fig. 10(a) showed the effect of time of incubation on decolorization of GYPRA by CRP in the presence of vanillin.

It is quite visible from the data that in the presence of mediator, the process of decolorization was efficient than that in its absence. Thus, the maximum decolorization was attained just within 5 min as indicated in Fig. 10(b). Although in the absence of mediator, the maximum decolorization was attained in 20 min.

3.5. Effect of metal ions on decolorization of GYPRA

As the effluents coming out from textile industries are also having heavy metals in it, so the experiments were performed in order to know their effect on extent of decolorization by CRP and the results are shown in Table 2.

The data showed that the metal ions did not show any significant effect on percent decolorization. Co^{2+},

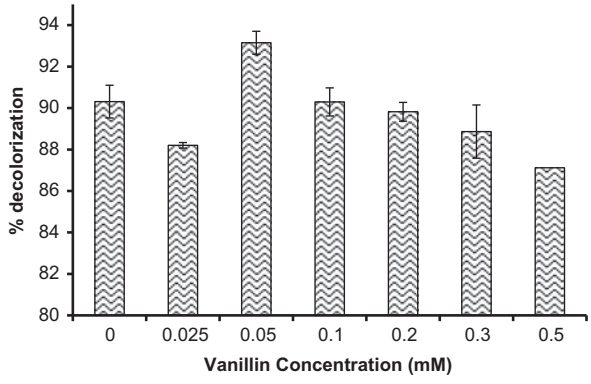

Fig. 9. Effect of different concentrations of vanillin on decolorization of GYPRA by CRP.

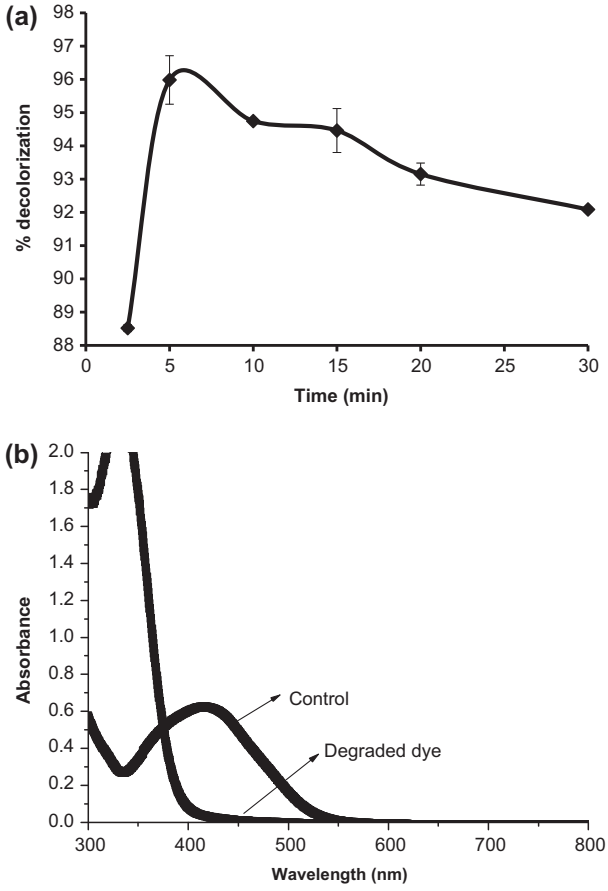

Fig. 10. Effect of time of incubation on decolorization of GYPRA in the presence of vanillin by CRP (a) and scanning spectrum of degraded GYPRA within 5 min (b).

Cu^{2+}, and Pb^{2+} somewhat enhanced, whereas Cd^{2+}, Cr^{3+}, and Hg^{2+} slightly reduced the decolorization percentage while all the remaining metals did not show any effect. Actually metal ions may inhibit the enzymatic reactions by changing the conformation of active site or speed up/fasten the reaction if employed as cofactor of the enzyme [51].

3.6. Optimization of decolorization conditions by response surface methodology

Using the rotatable CCD method, a total of 20 experiments with different combinations of pH (factor *A*, 2–3), enzyme dose (factor *B*, 24–27), and dye concentration (50–125 mg/L) were performed. Dye decolorization was used as the response (*Y*, %). The response obtained for all the experiments along with the predicted values is represented in Table 3. It is obvious from the data that % decolorization varied from 69.20–93.17%.

Table 2
Effect of metal ions on decolorization of GYPRA by CRP

Metal ions	Decolorization (%) for GYPRA
Control	92.04 ± 0.67
Co^{2+}	93.23 ± 1.04
Mg^{2+}	92.12 ± 0.72
Mn^{2+}	92.37 ± 0.76
Cd^{2+}	89.62 ± 1.80
Cr^{3+}	90.08 ± 0.55
Zn^{2+}	92.07 ± 0.51
Al^{3+}	92.64 ± 0.23
Cu^{2+}	93.17 ± 0.27
Pb^{2+}	93.26 ± 0.58
Sr^{2+}	92.09 ± 0.35
Ni^{2+}	91.06 ± 0.33
Hg^{2+}	90.47 ± 0.99
Ca^{2+}	92.11 ± 0.20

By applying the multiple regression analysis, following second-order polynomial equation was obtained:

$$Y(\%) = 86.44 - 4.32A + 1.92B - 1.72C + 1.59AB - 0.73AC + 1.52BC - 0.67A^2 - 0.27B^2 - 4.95C^2 \quad (4)$$

where *Y* was the predicted response, and *A*, *B*, and *C* were the coded values of the test factors. The statistical significance of the model equations was evaluated by Fisher's *F*-test for ANOVA.

For the purpose of testing the hypothesis on the parameters of the model, ANOVA, a statistical technique is applied which subdivides the total variation present in a set of data into its component parts associated with specific sources of variation [52,53]. The ANOVA results are represented in Table 4.

In this case, all the model terms (*A*, *B*, *C*, *AB*, *AC*, *BC*, A^2, C^2) were significant ($p < 0.05$) with the exception of a quadratic term, B^2 which was insignificant. The coefficient of determination, i.e. R^2 (0.9894) and adjusted R^2 (0.9799) were closer to 1 depicting a high correlation between the observed and predicted values [54–56]. The predicted R^2 value (0.9340) was also in close agreement to adjusted R^2 value. Besides this, relatively very low value of coefficient of variation (1.08%) as well as standard deviation (0.89) depicted the high reliability of the experiments performed [57]. The non-significant lack of fit test ($p > 0.05$) also confirmed the model adequacy. The adequate precision (36.617) was reported to be desirable as it was greater than 4.

Table 3
CCD matrix for three coded independent variables along with experimental and predicted values for decolorization (%) of Golden Yellow PRA by CRP

	Coded values			Decolorization (%) Golden Yellow PRA	
Std. order	*A*	*B*	*C*	Experimental	Predicted
1	−1	−1	−1	86.87	87.05
2	+1	−1	−1	76.86	76.69
3	−1	+1	−1	83.61	84.65
4	+1	+1	−1	80.87	80.66
5	−1	−1	+1	81.24	82.03
6	+1	−1	+1	69.20	68.75
7	−1	+1	+1	84.97	85.72
8	+1	+1	+1	78.42	78.82
9	−1.682	0	0	93.17	91.80
10	+1.682	0	0	76.75	77.29
11	0	−1.682	0	82.38	82.45
12	0	+1.682	0	89.79	88.90
13	0	0	−1.682	75.55	75.32
14	0	0	+1.682	70.16	69.55
15	0	0	0	86.84	86.44
16	0	0	0	87.13	86.44
17	0	0	0	85.94	86.44
18	0	0	0	85.54	86.44
19	0	0	0	86.77	86.44
20	0	0	0	86.26	86.44

Notes: *A*: pH, *B*: enzyme dose (U/mL), and *C*: dye concentration (mg/L).

Table 4
ANOVA results of quadratic model for decolorization (%) of Golden Yellow PRA by CRP

Source	Sum of squares	df	Mean Square	*F*-value	*p*-value Prob. > *F*
Model	742.4	9	82.49	104.1	<0.0001[a]
A-pH	254.4	1	254.4	321.0	<0.0001[a]
B-Enzyme dose	50.14	1	50.14	63.26	<0.0001[a]
C-Dye concentration	40.24	1	40.24	50.76	<0.0001[a]
AB	20.32	1	20.32	25.64	0.0005[a]
AC	4.26	1	4.26	5.37	0.0430[a]
BC	18.59	1	18.59	23.45	0.0007[a]
A^2	6.45	1	6.45	8.14	0.0172[a]
B^2	1.05	1	1.05	1.32	0.2765[a]
C^2	353.1	1	353.1	445.5	<0.0001[a]
Residual	7.93	10	0.79		
Lack of fit	6.10	5	1.22	3.34	0.1061[a]
Pure error	1.83	5	0.37		
Cor total	750.3	19			

[a]Values of "Probability > *F*" less than 0.05 indicate model terms are significant.

3.6.1. Normal plot of residuals

Normal plot of residual is a graphical way in order to check the normality of the residuals such that they should roughly follow a straight line. Fig. 11 represented the normal % probability for GYPRA. It is obvious from figures that almost all the points were normally distributed along the line. In practice, for a large data, moderate departures or deviations from

normality may occur which do not affect the results quite seriously [58]. The normal distribution is also indicator of the fact that the model satisfied the assumptions of the ANOVA and no additional transformation of the response (% decolorization) was required.

3.6.2. Optimization and effects of independent variables on the process of decolorization

In order to understand the optimizations and effects of the independent variables on the process of decolorization, contour plots were used, which show the behavior of the response with respect to simultaneous changes in two independent variables [59]. Actually, in these plots, one variable is held constant at its zero level, while the other two are varied within their experimental ranges [60]. So in short, these plots are very effective for the interpretation of both linear as well as interaction effects of two variables at the same time.

3.6.2.1. Combined effect of pH and enzyme dose. In order to find out the combination effect of pH (*A*) and enzyme dose (*B*) on the % decolorization, the response surface methodology was used and the results are represented in the form of contour plot (Fig. 12). In this plot dye concentration (*C*) was held constant (87.50 mg/L) while pH and enzyme dose were changed within their specified experimental ranges. Actually, the combined effect was significant with *p* value of 0.0005.

Fig. 12 represented that % decolorization increased with increasing enzyme dose and decreasing pH and the optimum point seem to be located toward the lowest level of pH and enzyme dose between 24 and 27 U/mL.

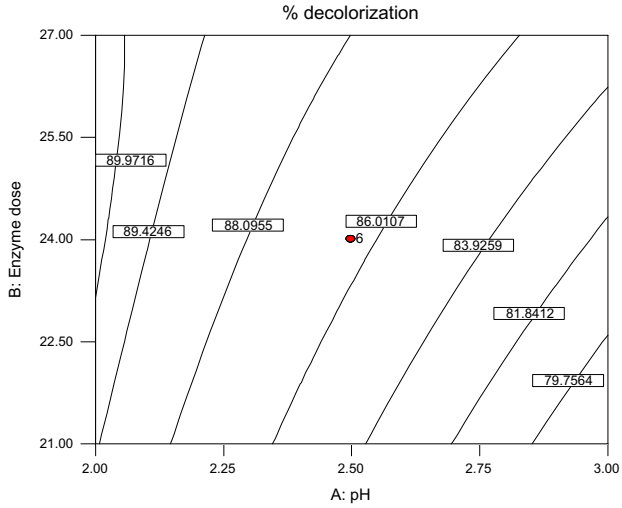

Fig. 12. Contour plots showing the combined effects of pH and enzyme dose.

3.6.2.2. Combined effect of pH and dye concentration. Fig. 13 revealed the mutual effect of pH (*A*) and dye concentration (*C*) for decolorization of GYPRA, at a fixed enzyme dose of 24 U/mL. The maximum response was obtained when the dye concentration values were between 75.50 and 91.50 mg/L and pH around 2.0. The significant *p* value ($p < 0.0430$) showed the importance of this interaction effect in the decolorization percentage of GYPRA by CRP.

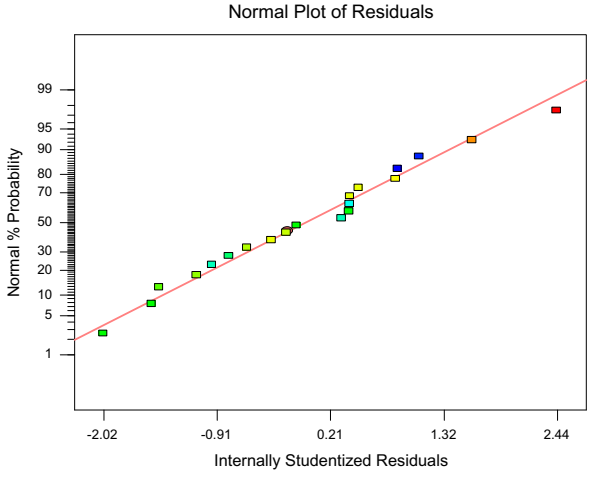

Fig. 11. The studentized residual and normal % probability plot for decolorization of GYPRA.

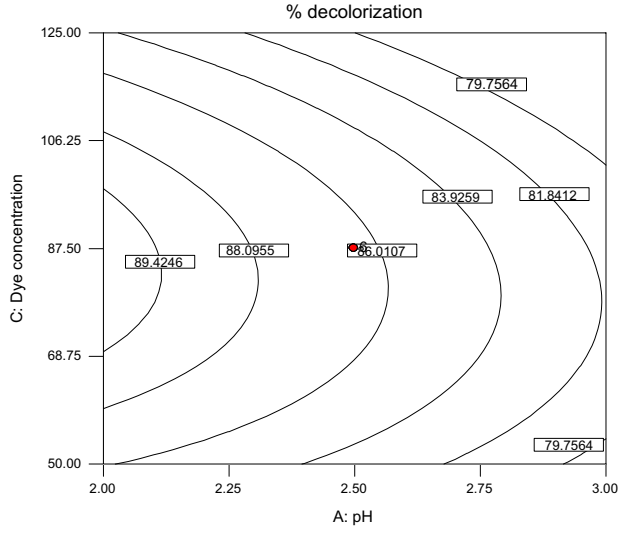

Fig. 13. Contour plots showing the combined effects of pH and dye concentration.

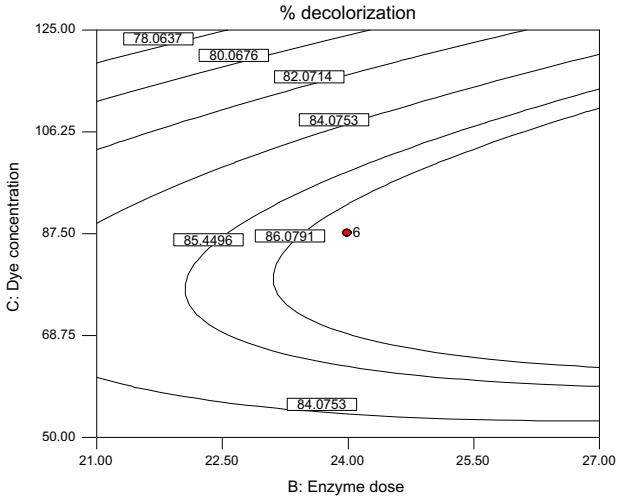

Fig. 14. Contour plots showing the combined effects of enzyme dose and dye concentration.

The result obtained by RSM was quite consistent with the OFAT optimization and maximum decolorization percentage was obtained within the experimental range [61], further confirming the suitability of the ranges of variables selected for performing RSM experiments.

3.6.2.3. Combined effect of enzyme dose and dye concentration. The contour plot for the interactive effect of enzyme dose (*B*) and dye concentration (*C*) represented that % decolorization increased with increasing dye concentration up to the region of 87.50 mg/L which increased with increasing enzyme dose, at a fixed pH of 2.5 (Fig. 14). The significance of this combined effect was evident by a *p*-value of 0.0007.

3.6.3. Verification of the regression model

The experimental model was verified by performing experiments in triplicate at the numerically optimized conditions predicted by the regression model for three independent variables viz. pH, enzyme dose, and dye concentration which were as follows: 3.0, 26.77 U/mL and 86.88 mg/L with 84.47% decolorization for GYPRA, respectively. The experimental value of 84.04 ± 0.34% was reported to be in reasonable agreement with the predicted value.

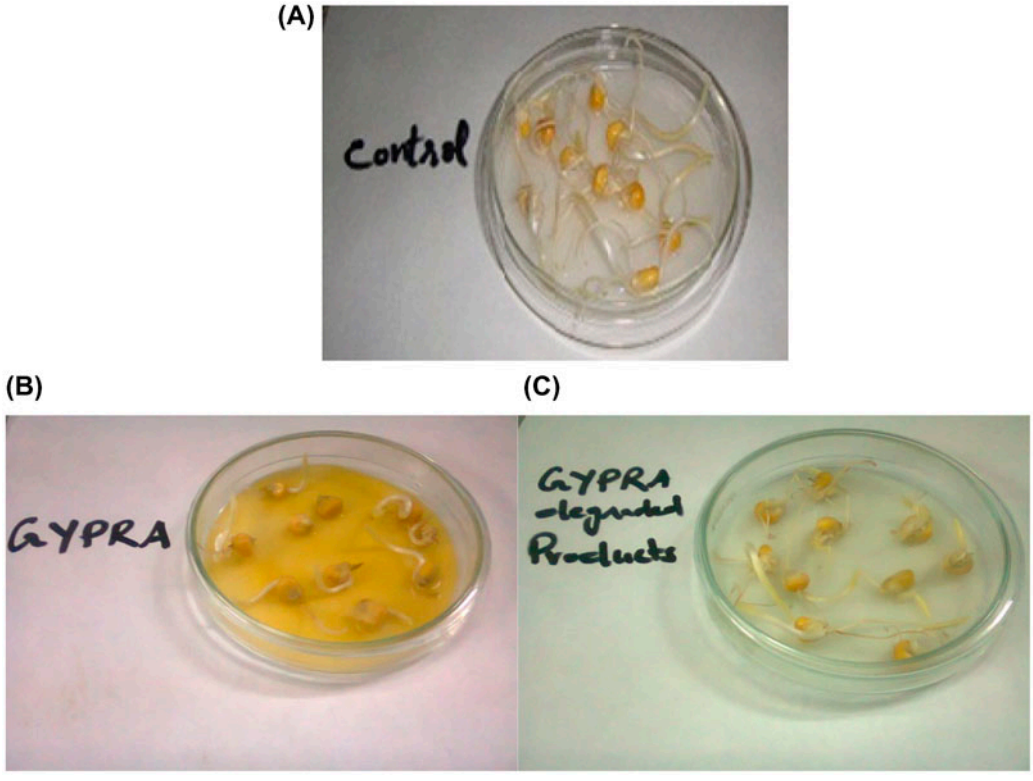

Fig. 15. Photograph showing growth of maize after 72 h of incubation in: (A) distilled water (control), (B) GYPRA, and (C) degraded products of GYPRA.

As a result, it can be said that RSM was used successfully as a fast and error-free approach for the optimization of conditions for decolorization of GYPRA by CRP peroxidase with respect to the parameters of pH, enzyme dose, and dye concentration. Besides, the interaction study between these components provided an additional advantage of employing RSM. Moreover, decolorization of GYPRA at intermediate levels, which were not experimentally studied, or the system performance at any experimental point with different combination of the variables may be estimated by using this method.

3.7. Phytotoxicity evaluation

The mean value for the root length of 30 maize seeds in distilled water (control) was reported to be 4.63 ± 0.36 cm with 100% seed germination. Whereas the mean values of root lengths for GYPRA and its degraded products were evaluated to be 0.796 ± 0.35 cm (82% decreased root length than control) and 1.40 ± 0.19 cm (69% decreased root length than control) with 50 and 66% germination, respectively. So the seeds grown in GYPRA and its degraded products showed a GI of 8 and 20%, respectively. It means that the degraded products of GYPRA are somewhat less toxic than the original dye but are still falling in high phytotoxic limit.

Fig. 15 shows the growth of maize seeds after 72 h of incubation in distilled water (Fig. 15(A)), in GYPRA sample (Fig. 15(B)) in GYPRA degraded products (Fig. 15(C)).

Earlier, some extent of detoxification was also reported for peroxidase treated samples of acidic and reactive dyes performing *Allium cepa* test [62,63]. The toxicity of a number of dyes, especially those including azo groups was reduced after treatment with enzymes, although there was no correlation existed between decolorization and detoxification [64,65].

4. Conclusion

In this study, decolorization of Golden Yellow PRA using CRP enzyme extracted from peels of kinnow was investigated. One-factor-at-a- time approach was applied first in order to investigate the effect of influencing parameters on decolorization. Then, response surface methodology was employed through CCD in order to investigate the effect of influencing factors (pH, enzyme dose, dye concentration) and their combined interactive effect on decolorization efficiency. A second-order polynomial equation with a coefficient of determination (R^2) equal to 0.9894 was generated for decolorization by CRP. Optimum values for the significant parameters were: pH 3.0; enzyme dose, 26.77 U/mL, and dye concentration, 86.88 mg/L. Besides this, phytotoxicity analysis was also conducted on *Zea mays* showing less toxicity of the degraded dye as compared to the original one. So this eco-friendly, economical, and efficient method can be further employed for the degradation of synthetic dyes from industrial effluents.

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