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The optimization of reactive black 5 dye removal using *Coprinus cinereus* peroxidase (CIP)

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ABSTRACT

Coprinus cinereus (NBRC 30628) peroxidase was implemented to eliminate the diazo dye of reactive black 5 (RB5). The optimization was conducted in batch mode using three approaches, i.e., the one-factor-at-a-time (OFAT), factorial design, and response surface methodology (RSM). Based on the results of the OFAT method, the optimum conditions for decolorization of RB5 dye were at a temperature of 30°C, a pH of 9-10, an H_2O_2 concentration of 3.9 mM, and an RB5 concentration of 40 mg/L. In the first stage of statistical optimization, these factors plus enzyme activity were screened by the 2-factorial design, wherein enzyme activity, temperature, and hydrogen peroxide concentration were distinguished as the most significant parameters in the enzymatic decolorization of RB5. In the second stage, RSM was applied over three adopted factors through the central composite design (CCD), and a reduced cubic polynomial model was generated, which indicated an accurate regression (R^2 = 0.997, Adj.R^2 = 0.994) and no significant lack of fit (p-*value*> 0.05). The contour and surface plots suggested that the removal efficiency was enhanced by increased enzyme activity and decreased H_2O_2 concentration and temperature. The optimum condition was obtained at 1.0 mM H_2O_2, 6 U/mL enzyme activity, and 35°C for a maximum decolorization efficiency of 96.046%.

1. Introduction

Synthetic dyes are being widely implemented in the textile industry and discharged into the environment without appropriate treatment. The colorants produced are more than 8 × 10^5 tons per year and contain different types of chemicals, including chromophoric groups, such as azo, anthraquinone, triarylmethane, and reactive groups like vinyl sulphone, chlorotriazine, and trichloropyrimidine [1]. Nearly 65-75% of the

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industrial dyes produced worldwide are azo dyes [2]. However, based on the degree of fixation on the fiber, between 10 and 50% of the initial dye load is released into the spent dye bath effluent [3]. Many dyes and their intermediate aromatic products are believed to be toxic or carcinogenic [4,5]. Treatment of textile effluents using the conventional activated sludge process is difficult and ineffective because of their stability and resistance to degradation [6]. Currently, numerous physicochemical methods have been implemented for treating dye-containing wastewaters; these methods are often costly and have encountered problems such as incomplete dye degradation, sludge disposal issues, and the necessity for secondary treatments [7]. Other emerging techniques, such as ozonation [8,9], Fenton processes [10,11], electrochemical destruction [12], and photocatalysis [13], have also been applied, but they are usually complicated and economically unfeasible. Recently, biological methods have attracted a great deal of research attention for dye degradation due to their effectiveness and cost feasibility [4,14-16]. White-rot fungi could degrade versatile recalcitrant organic compounds, such as heavy metals, polycyclic aromatic hydrocarbons, chlorophenols, polychlorinated biphenyls, and dyes, using their extracellular enzymes [17-20]. Enzyme-catalyzed treatments have several advantages over other fungal or bacterial-assisted approaches, which use live or dead microorganisms: time-saving, absence of lag-phase delays, low sludge volume, capability of working in different contaminant concentrations, and ease and simplicity of process control [14]. The statistical optimization of the decolorization of mono-azo dye (acid orange 7) and anionic dye (reactive blue 221) in the batch system using the crude *Coprinus cinereus* peroxidase (CIP) enzyme has been performed previously [21,22]. Moreover, the decolorization of reactive black 5 (RB5) has been investigated in the continuous system using the conventional OFAT method [23]. Furthermore, a bi-substrate kinetic model based on the Ping-Pong mechanism was examined and well-fitted for the reaction rate of RB5 decolorization using the CIP enzyme [24].

This study tried to determine the optimal conditions for removing RB5 via the CIP enzyme. The optimization was conducted in the batch system using both OFAT and RSM approaches. RSM [25] is a well-known statistical approach for multivariable systems that has been vastly applied in the optimization of different lignolytic enzyme production and application for decolorization [26-28]. However, to the best of the authors' knowledge, this is the first research on the decolorization of RB5 by the CIP enzyme using the RSM technique, which would be useful to examine the effectiveness of this enzyme for future industrial applications.

2. Materials and methods

2.1. chemicals, microorganism and culture media

All chemicals were of commercially available analytical grade. *Coprinus cinereus* NBRC 30628 was prepared by the National Biological Resources Center, Japan. The fungal strain was stored on potato dextrose agar slants at 4 °C. The peroxidase preparation was accomplished in a liquid medium (pH=4) containing glucose (30 g/L) as a carbon source, peptone (10 g/L) as a nitrogen source, and yeast extract (5 g/L). One circular piece (1 cm in diameter) was cut out of a vigorously developing fungal culture on the PDA plates and utilized to inoculate 100 mL of sterilized medium in 250 mL Erlenmeyer flasks. The *C. cinereus* fungi were grown at 30 °C on a rotary shaker spinning at 150 rpm for about 21 days. The culture broth was filtered at the end of cultivation to eliminate the fungus pellets. The obtained clear solution, which has an approximate CIP activity of 25 U/mL, was stored at 4 °C and utilized for the dye decolorization experiments. A brief representation of enzyme production steps is exhibited in Figure 1A. The existence of other fungal enzymes, including lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, was assayed, and none of them were found in the crude enzyme solution [21,22]. The diazo dye was Sumifix Black B (Reactive Black 5), purchased from Sumitomo Chemical Co. Ltd., Japan. The chemical structure of this dye is depicted in Figure 1C.

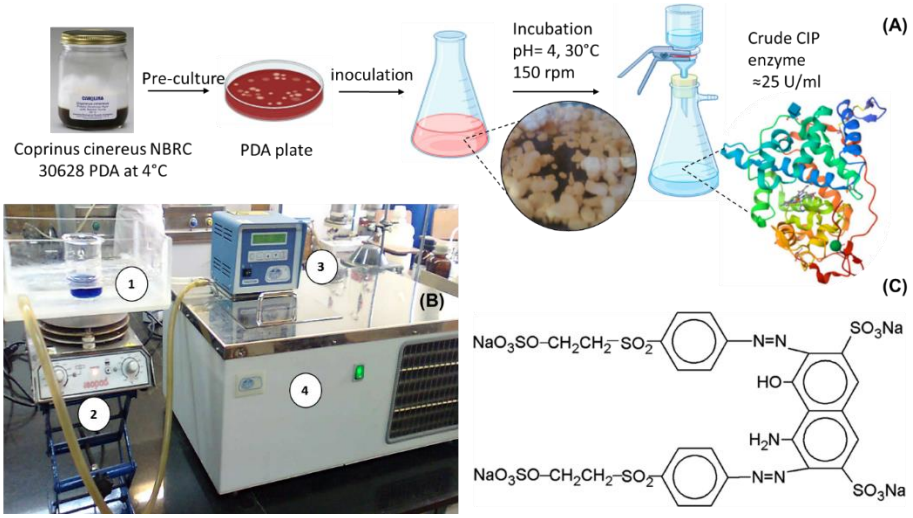

Fig.1. (A) The steps of enzyme production include pre-culture, incubation, and vacuum filtration. The first inset shows a figure of fungal pellets in the liquid medium, and the second inset indicates the structure of CIP determined to be 2.0 A by the X-ray diffraction method (the heme group (light purple) surrounded by different alpha helices) [29]; and (B) The experimental setup used for batch decolorization of RB5 included (1) a hand-made water container with an inlet and outlet for water recirculation, (2) a magnetic stirrer, (3) a recirculation pump, and (4) a water bath equipped by a digital temperature controller; and (C) the chemical structure of reactive black 5 dye (RB5) (C.I. 20505; M.W = 991 Da).

2.2. Enzyme assay

The enzyme activity assay was similar to previous works [21,22]. A reaction mixture was prepared using a phosphate buffer (pH 7.0, 65 mM), 4-Aminoantipyrine (0.63 mM), phenol (10 mM), hydrogen peroxide (3.1 mM), and Triton X-100 (0.97 g/L) in a total volume of 3.1 mL. The mixture was then incubated at 37 °C for 10 minutes. The reaction commenced with the addition of 0.1 mL of enzyme solution, and the initial rise in absorbance was observed at 500 nm for 1 minute. One unit of CIP activity (U) is defined as the quantity of the enzyme that can oxidize 1 µmol of H_2O_2 per minute [30]. The enzyme activity was calculated using Eq. 1:

$$\text{Enzyme activity [U/ml]} = (\Delta A_{bs} / (V_e \times K \times \varepsilon)) \times D_m \times V \quad (1)$$

where ΔA_{bs} is the absorption difference between the enzyme and blank samples. The blank solution was totally similar to the enzyme solution, but with the same volume of phosphate buffer (pH = 7) instead of H_2O_2. V_e, K, and ε represent the volume of enzyme (0.1 mL), the stochiometric ratio of product to reactant (12.88), and the distinction coefficient (0.5), respectively. D_m and V are the dilution factor and total volume of solution (including enzyme volume), respectively. The peroxidase-catalyzed degradation of azo dyes could be described by the following generally accepted cyclic reactions [24,31]:

$$E + H_2O_2 \xrightarrow{K_1} E1 + H_2O \quad (2)$$

$$E1 + RB5 \xrightarrow{K_2} E2 + RB5^* \quad (3)$$

$$E2 + RB5 \xrightarrow{K_3} E + RB5^* + H_2O \quad (4)$$

$$RB5^* + RB5^* \xrightarrow{K4} RB5^* - RB5^* \quad (5)$$

In this simplified form of the bi-substrate ping-pong mechanism, the active site of the enzyme (*E*) interacts with H_2O_2 in a two-electron pathway, followed by the transformation of active sites and the production of the first active form of the enzyme (*E1*) (Eq. 2). *E1* could oxidize *RB5,* generating the second active form of enzyme (*E2*) and a free radical of the azo dye (*RB5**) in the one-electron path reaction (Eq. 3). In a similar reaction, *E2* reacts with *RB5* and produces the resting form of the enzyme (*E*) and a free radical (Eq. 4). Free radicals could bond with each other and terminate the catalytic cycle (Eq. 5). In the case of the presence of extra H_2O_2 in the solution, it could oxidize *E2* to an inactive form of the enzyme (*E3*) (Eq. 6), which could spontaneously break down to the native form of the enzyme with a very slow rate of reaction (Eq. 7).

$$E2 + H_2O_2 \xrightarrow{K_{app}} E3 + H_2O \quad (6)$$

$$E3 \rightarrow E + O_2^- \quad (7)$$

2.3. Dye decolorization

The dye concentration was measured photometrically at the maximum absorption wavelengths for RB5, i.e., 595 nm, using a UV-Vis recording double-beam spectrophotometer. The decolorization efficiency (DE) was defined according to Eq. (8):

$$DE(\%) = \frac{C_0 - C}{C_0} \times 100 \quad (8)$$

where C_0 (mg/L) and C (mg/L) are respectively the initial concentration and the concentration of *RB5* at a given time. The initial decolorization rate (IDR) was defined as the linear section slope of the dye concentration versus time plot. During the first stage of the study, i.e., OFAT optimization, batch tests were carried out in a glass reactor with 25 mL of the reaction mixture in a water bath at a certain temperature. The reactor contents were agitated using a stir bar and a magnetic stirrer. A photograph of the experimental set-up used for the OFAT optimization step of *RB5* decolorization is shown in Figure 1C. During the second stage of optimization using statistical optimization, the experiments were conducted in test tubes with 2.5 mL of the reaction mixture. The tubes were incubated for 15 minutes in a water bath at the specified temperature and intermittently mixed using a tube vortex [21,22]. All the experiments of the OFAT and factorial design optimization steps were duplicated, while the experiments of RSM optimization were performed in triplicate for more accuracy.

2.4. OFAT optimization

The OFAT optimization implies that only one independent influencing factor is changed while the others remain fixed at certain levels. The choice of an appropriate pH, temperature, H_2O_2, and dye concentration is vital for optimal performance and future development of the process. Hence, the following influencing parameters were first optimized using the OFAT method: initial dye concentration (20-100 mg/L), H_2O_2 concentration (0.65-19.58 mM), temperature (22-60 °C), and pH (4.0-10.0). Different buffer solutions were used to study the pH effect. For pH 2, 40 mM potassium chloride/HCl was used; for pH 4, 27.78 mM citrate/phosphate; for pH 6, 7, and 8, 40 mM phosphate; for pH 9, 20 mM borax/boric acid; and for pH 10, 17.5 mM borax/NaOH. Since it is well known that the rate and performance of an enzymatic reaction are enhanced by increasing enzyme activity [23], the effect of this parameter was not considered in OFAT optimization. Hence, enzyme activity was kept constant at 5 U/mL in all experiments in this section. Different experimental conditions for OFAT optimization are summarized in Table 1. As stated before, the decolorization assays in this step of optimization were conducted in duplicate, and their average values and standard deviations are reported.

Table 1. Investigating the effect of different influencing parameters on decolorization of reactive black 5, using OFAT method.

Step	Investigated variable	Dye conc. (mg/L)	Buffer	H_2O_2 conc. (mM)	CIP activity (U/mL)	Temperature (°C)
1	H_2O_2	40	80 mM Phosphate Buffer, pH 7	0.65-19.58	5	22 ± 1
2	pH	40	pH 2-10	3.91	5	22 ± 0.5
3	Temperature	40	62 mM Borax/boric acid Buffer, pH 9	3.91	5	(22-60) ± 0.5
4	RB5 conc.	20-100	64 mM Borax/boric acid, pH 9	3.91	5	30 ± 0.5

2.5. The Statistical Optimization

2.5.1. Screening step using the two-level factorial design

As the first stage of statistical optimization, the two-factorial design method was used to screen the significant factors. This design permits the estimation of the main and interaction effects of parameters simultaneously. The two-level factorial design for five factors was employed. The adopted factors and their levels are indicated in Table 2. The designed experiments for the first stage of

optimization are presented in Table 3. The experiments for this step were performed twice, and the average values of decolorization efficiency are reported as the response in Table 3. Design Expert 7.0, Stat-Ease, Inc. (trial version), was used to analyze the data.

2.5.2. Final optimization step using the response surface methodology

During the second stage of statistical optimization, factors recognized as significant in the first stage, using factorial design, were more accurately optimized using RSM. In the present study, the central composite design model was implemented for three significant variables: temperature (20-50°C), hydrogen peroxide concentration (1-10 mM), and peroxidase activity (5-30 U/mL). In this stage of optimization, all the experiments were performed in triplicate, and the average values of dye decolorization efficiency were stated as the response. Other parameters, including the dye concentration and pH, were kept constant during the tests at 40 mg/L and 9.0, respectively.

Table 2. Levels and actual values of variables tested in two-factorial design.

Factors	Range and Levels Coded	
	-1	+1
A- Enzyme activity (U/mL)	1	6
B- H_2O_2 Concentration (mM)	1	10
C- Temperature (°C)	22	50
D- Dye concentration (mg/L)	20	100
E- pH	4	10

Table 3. The actual two-factorial design of experiments and response for RB-5 decolorization.

Run	Factor 1 A-Enzyme (U/mL)	Factor 2 B-H_2O_2 concentration (mM)	Factor 3 C-Temperature (°C)	Factor 4 D-Dye concentration (mg/L)	Factor 5 E-pH	Decolorization Efficiency (%)
1	6	10	50	100	10	3.27
2	1	1	22	20	10	10.89
3	6	10	22	100	4	11.64
4	1	10	22	20	4	10.96
5	1	1	50	20	4	0.50
6	6	1	22	100	10	44.11
7	1	10	22	100	10	7.95
8	6	1	22	20	4	43.66
9	1	10	50	20	10	9.03
10	6	10	50	20	4	20.85
11	6	1	50	100	4	31.45
12	1	1	50	100	10	4.81
13	6	10	22	20	10	8.43
14	1	10	50	100	4	10.45
15	6	1	50	20	10	9.15
16	1	1	22	100	4	17.96

3. Results and discussion

3.1. The OFAT optimization results

3.1.1. H_2O_2 concentration effect

The effect of H_2O_2 concentration on decolorization was explored in the concentration range of 0.65 to 19.59 mM. Figure 2 (A) and (B) exhibit the time course of decolorization at different H_2O_2 concentrations and the dependence of decolorization efficiency (DE) and initial decolorization rate (IDR) on the initial H_2O_2 concentration, respectively. According to the peroxidase-catalytic cycle, the addition of H_2O_2 is necessary for the generation of the active forms of enzymes (E1 and E2) and the commencement of

the decolorization process (Eqs. 2 and 3). In very low peroxidase concentrations (less than 1 mM), decolorization was not performed completely due to the deficiency of H_2O_2 after five minutes; therefore, the initiating reactions of the catalytic enzymatic cycle (Eqs. 2 and 3) were not carried out to generate sufficient E1 and E2 compounds. In the H_2O_2 concentrations between 1 and 2 mM, the decolorization reaction started with a satisfactory speed and progressed to an acceptable extent (72.38% color removal in 1.96 mM H_2O_2) but reached a plateau after 20 minutes, likely due to the termination of H_2O_2 in the solution. For the peroxidase concentrations in the range of 2.5 to 4 mM, the IDR showed an obvious decrement; nonetheless, considering the fact that decolorization proceeds well in a wider time interval, the overall DE was enhanced, and the peak color removal efficiency of 75.44% was achieved at the peroxidase concentration of 3.91 mM. However, both the DE and IDR obviously decreased in concentrations higher than 4 mM, owing to the formation of the inactive form of enzyme (E3) following the reaction of Eq. 5 in the presence of excess peroxidase, which could be very slowly converted to the resting form of enzyme as described in Eq. 7. In this context, Ikehata [32] reported that the presence of excess hydrogen peroxide in the reaction mixture resulted in CIP inactivation. We also observed similar trends in the decolorization of acid orange 7 (monoazo dye) and reactive blue 221 (anionic dye) by crude CIP at high concentrations of hydrogen peroxide [21,22]. However, in the decolorization of RB221, the inactivation of the CIP enzyme and reduction in decolorization were not evident except for the extremely high concentration of hydrogen peroxide, which corresponded to a very high decolorization rate of this dye. Similarly, other researchers confirmed the inhibitory effect of the high H_2O_2 concentrations on the performance of MnP [33-35]. However, decolorization by LiP did not exhibit an analogous trend [33,36], presumably due to the presence of veratryl alcohol in the LiP reaction that may impede the formation of compound III (E3) by speeding up the catalytic cycle and keeping the enzyme from deactivation [33]. Likewise, Young and Yu [37] reported different methods for different structures of dyes at high concentrations of H_2O_2. They observed that the decolorization of some dyes, such as acid orange-74 and reactive blue-15, was performed without any inhibition, while the other dyes, like acid violet-7 and acid blue-25, could not degrade adequately at high H_2O_2 concentrations. M. Moreira *et al.* [34] inspected the effect of the H_2O_2 supply strategy on the removal of one polymeric dye by MnP from *Bjerkandera* sp. BOS55 and *Panerochaete chrysosporium*. They concluded that keeping the H_2O_2 supply rate lower than the maximum H_2O_2 decomposition rate prevented the accumulation of excess H_2O_2. Ivanec-Goranina [29] studied the proficiency of recombinant *Coprinus cinereus* (rCiP) peroxidase for degradation of azo dye, 2,2'-dihydroxyazobenzene (DHAB), in different conditions. Their results indicated that the relationship between the initial reaction rate and H_2O_2 concentration could be accurately described by the Michaelis-Menten equation. The H_2O_2 content was explored in the range of 5 to 150 μM, and a concentration of 100 μM provided the saturation level without any restriction of biocatalytic oxidation reactions of DHAB [29]. Therefore, the optimum H_2O_2 concentration depends on both the kinetics of the enzymatic reaction and the H_2O_2 decomposition rate.

3.1.2. pH effect

The effect of pH on the removal of RB5 was inspected using different buffer types (section 2.4). Figure 3 depicts the effect of pH on the decolorization of RB5. The time course of decolorization at different pH values is presented in Figure 3A, and an overall comparison of DE and IDR values is shown in Figure 3B. DE augmented sharply with the increase in pH from 2 to 6, and the rate of change became slower thereafter. Also, IDR was boosted rapidly by increasing the pH from 4 to 9, and then it had a slight increase when the pH changed from 9 to 10. Although IDR was enhanced slightly as pH increased from 9 to 10, according to Figure 3A, the color removal efficiency reached a plateau after 6 minutes at pH 10. Therefore, the elimination of RB5 performed well in a broad range of pH (6 to 10), with the maximum DE value of 83.54% at the pH of 9.

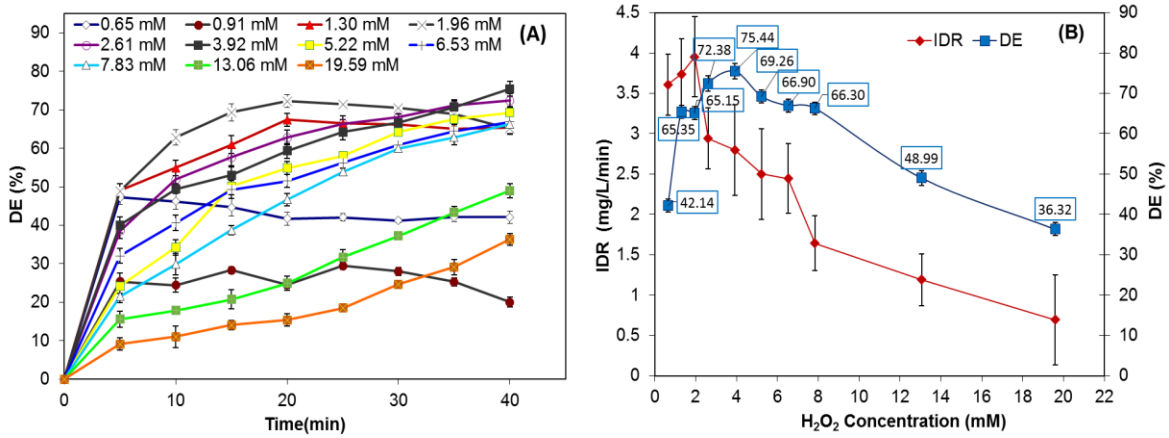

Fig. 2. H_2O_2 effect: (A) time course for decolorization efficiency (DE) of RB5 in different H_2O_2 concentrations, and (B) the effect of H_2O_2 concentration on decolorization efficiency (DE) and initial decolorization rate (IDR), data labels indicate the maximum DE values at each peroxide concentration. (Constant variables: Dye concentration= 40 mg/L, pH=7, CIP enzyme activity=5 U/mL, and temperature= 22ºC).

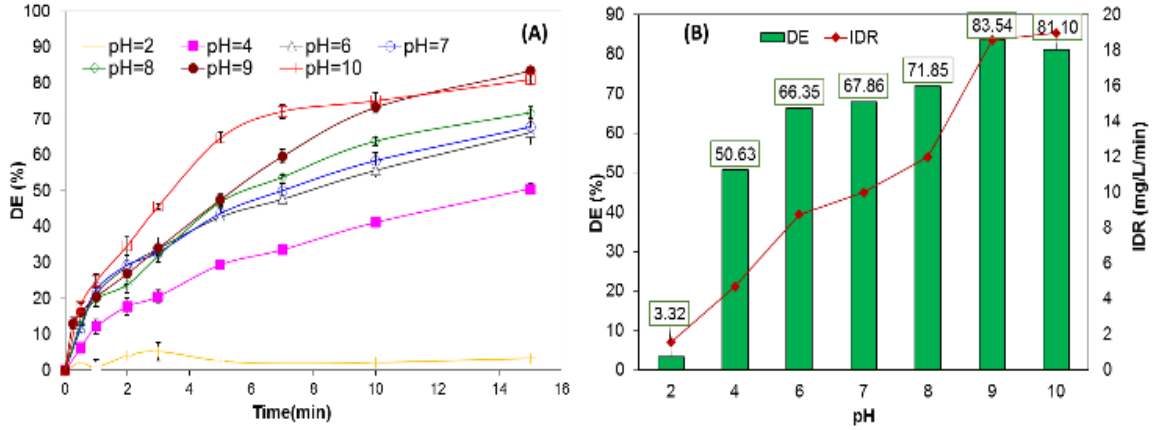

Fig. 3. pH effect: (A) time course for decolorization of RB5 at different pH values, and (B) the effect of pH on DE and IDR, data labels show the maximum DE at each pH value. (Constant variables: Dye concentration= 40 mg/L, *H_2O_2 concentration=3.91 mM,* CIP enzyme activity=5 U/mL, and temperature= 22 ºC).

Comparable results were observed for the removal of Acid Orange7 and Reactive Blue 221 by *C. cinereus* peroxidase (IFO 30628), and dye removal was performed satisfactorily in the alkaline pH range, with the best pH being 9. Nevertheless, the decolorization of RB221 occurred almost completely in the pH range of 4 to 10 [21,22]. Mansouri and Kariminia [23] found that RB5 decolorization using a similar CIP type performed best at a pH range of 8 to 9. They concluded that dye removal's dependency on pH was the result of different reaction rates of E1 and E2 generation and decomposition at distinct pH values. The reaction rate of E1 generation (k_1) was high (6–10 $\times 10^6$ $M^{-1}s^{-1}$) at neutral and alkaline pH values, but it decreased sharply at acidic conditions. The k_2 and k_3 dependencies on pH had an inverse parabola shape with peak values of 120×10^6 $M^{-1}s^{-1}$ and 48×10^6 $M^{-1}s^{-1}$ at a pH of 8, respectively. Both k_2 and k_3 for the CIP enzyme decreased in the pH range of 5 to 6, nearly reaching the same value, while k_2 was normally 10 times greater than k_3 for the other peroxidases in this pH range. As a result, the CIP enzyme's activity abruptly decreased in acidic conditions due to the high k_3 value. Similarly, Sakurai *et al.* [30] reported the optimal pH between 9 and 10 for the removal of bisphenol A by *C. cinereus* peroxidase (IFO 30628). However, Ikehata [32] observed that the optimum value of pH for phenol removal using peroxidase produced from two different species of *C. cinereus* was around 7.0. The optimum pH for CIP implementation is seemingly interconnected with its *C. cinereus* source type.

3.1.3. Temperature effect

The time course of RB5 elimination at different temperatures and the relation of IDR and DE with temperature are presented in Figure 4A and B, respectively. The IDR was enlarged by increasing the temperature to 35°C and then decreased linearly afterward. The decolorization efficiency was maintained at nearly 85% from 20°C to 40°C but sharply dropped to 14% at 45°C, and finally at 60°C reached zero. The dramatic diminishment of DE was attributed to the deactivation of CIP at higher temperatures. Altogether, the best temperature for the decolorization of RB5 was found to be around 35°C; this was consistent with the results of the decolorization of AO7 using CIP [21], while the decolorization of RB221 by the CIP enzyme was performed efficiently between 20°C and 50°C due to the high rate of the enzymatic reaction [22]. Hadibarata et al. (2013) investigated the decolorization of RB5 using the White-Rot Fungus *Pleurotus eryngii* F032 and observed a notable enhancement of decolorization efficiency from 46.37 to 96.77% when the temperature increased from 15 to 40°C. They concluded that increasing the temperature could improve the solubility of enzymes (*Laccase*, *MnP*, and *LiP*), thereby boosting the enzymatic activity of fungi for dye degradation [38]. Lei et al. (2017) compared the performance of the wild-type and the genetically modified type (CIPmt5) of *Coprinus cinereus* peroxidase on the decolorization of seven different textile dyes. CIPmt5 was synthesized by substituting five amino acids in the wild-type CIP. The results showed that the optimal temperature and pH of decolorization changed from 25°C and 5.0 for wild-type CIP to 45°C and 6.5 for CIPmt5, respectively [39]. Overall, the optimal range of temperature for enzymatic degradation could be influenced by many factors, including dye type and structure; however, it is strongly determined by the catalytic properties of the enzyme.

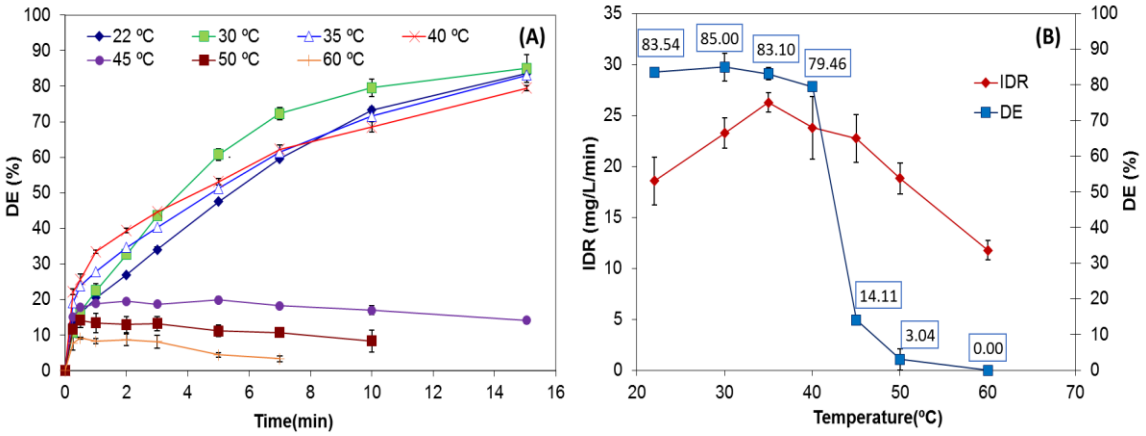

Fig. 4. Temperature effect: (A) time course for decolorization of RB5 at different temperatures, and (B) the effect of temperature on DE and IDR. Data labels exhibit the maximum DE at each temperature. (Constant variables: Dye concentration= 40 mg/L, H_2O_2 *concentration=3.91 mM,* CIP enzyme activity=5 U/mL, and pH=9).

3.1.4. Initial dye concentration effect

The initial dye concentrations in the range of 20 to 100 mg/L were examined. Fig. 5A and B show the variation of DE versus time in different dye concentrations and the effect of initial dye concentration on IDR and DE, respectively. IDR had a direct relationship with dye concentration and improved linearly with the increase in dye concentration. At lower dye concentrations, however, DE had a higher percentage. As the concentration of RB5 rose from 20 mg/L to 40 mg/L, DE was enhanced slightly. Then, the efficiency of dye removal decreased sharply at higher dye concentrations. Similarly, the decolorization efficiency was diminished at high dye concentrations using other enzymes [33, 36,37]. This phenomenon might occur due to the probable damage of enzymes at high dye concentrations, or it could be a sign of a higher demand for H_2O_2 or enzymes to perform decolorization efficiently at higher dye concentrations. Similar results were obtained when the decolorization of RB5 using CIP in a continuous system was investigated. Although a relatively similar maximum DE was obtained in 20 and 40

mg/L concentrations of RB5, the peak DE remained stable in higher time intervals for the 40 mg/L concentration. The DE was obviously decreased by higher dye contents during the decolorization process, likely due to the deactivation of enzymes at high dye concentrations [23].

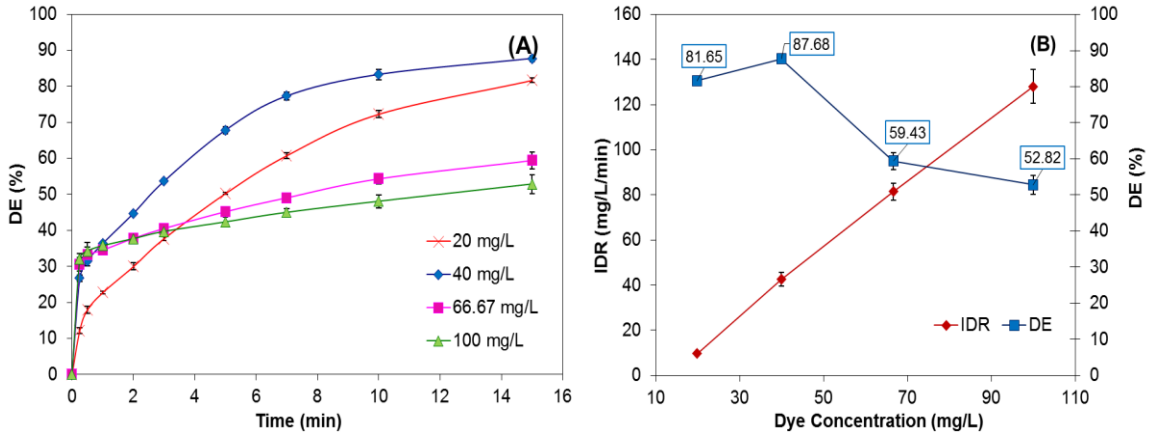

Fig. 5. Dye concentration effect: (A) time course for decolorization of RB5 with different dye concentrations, and (B) the effect of initial dye concentration on DE and IDR. Data labels indicate the highest DE at each dye concentration (Constant variables: *H_2O_2 concentration=3.91 mM,* CIP enzyme activity=5 U/mL, temperature=30 ºC, and pH=9).

Therefore, the optimum conditions for RB5 decolorization based on the OFAT optimization method were an H_2O_2 concentration of 3.91 mM, a temperature of 30 ºC, a pH of 9, and the highest available CIP enzyme activity of 5 U/mL. In these conditions, the maximum decolorization efficiency was 87.68 %.

3.2. Statistical optimization

3.2.1. Two-level factorial design

In the OFAT optimization method, the experiments were carried out through single-factor investigation and did not include the interactions between the parameters. The complicated nature of enzymatic reactions could be better reflected using statistical optimization, which embraces both the singular and interactive effects of the parameters. In the first step of statistical analysis, the effect of five parameters, including enzyme activity (*A*), H_2O_2 concentration (*B*), temperature (C), dye concentration (D), and pH (E), on the decolorization process was investigated using the 2-factorial design method.

Table 2 presents the levels of five independent variables, and Table 3 shows the designed experiments accompanied by DE as the response. The results of the analysis of variance (ANOVA) are exhibited in Table 4. Accordingly, the variables A, B, and C and the interactions of AB and BC are statistically significant (P-value < 0.05). The low P-value of the model confirms its significance. The obtained mathematical expression of the model is shown in Eq. (9).

$$DE\ (\%)=15.48 + 6.33 \times A - 5.03 \times B - 4.17 \times C - 5.58 \times A\times + 4.78 \times C \times D \quad (9)$$

The R^2 and adjusted R^2 values of 0.9975 and 0.9942 were respectively obtained for this model, which confirmed the reliability and adequacy of the model and approved the acceptable correlation between the experimental and predicted values of response, as depicted in Table 6. Also, the lack-of-fit of the model was not significant (>0.05). Moreover, the residual analysis results revealed that residuals have a normal distribution, and except for one, all the residuals were lower than two. Also, the externally studentized residuals lie within the acceptable range of (–3) and (+3), which authenticated the model's adequacy. Furthermore, a high adequate precision value of 63.967 corroborated the sufficient signal-to-noise ratio and significance of this model for the prediction of decolorization performance.

3.2.2. Response surface methodology

The results of the screening step of optimization using the factorial method revealed that peroxidase activity, hydrogen peroxide concentration, and temperature were more important among the others. Hence, these factors were adopted for RSM optimization, as presented

in Table 5. The RSM approach was performed based on the CCD model. The designed experiments and the experimental and predicted values of DE (response) are shown in Table 6. The results of the ANOVA analysis for the RSM model are presented in Table 7. Moreover, the correlation between DE and significant parameters of enzyme activity (A), H_2O_2 concentration (B), temperature (C), and their significant interactions are exhibited in Eq. (10).

$$DE\ (\%) = 17.31 + 11.85 \times A - 14.44 \times B - 5.40 \times C - 4.44 \times A \times B - 4.20 \times A \times C + 3.68 \times B \times C + 4.78 \times A^2 + 6.15 \times B^2 - 2.58 \times C^2 + 1.88 \times A^2 \times B - 2.03 \times A^2 \times C \quad (10)$$

Table 4. Analysis of variance (ANOVA) for 2-factorial model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2185.85	5	437.16	8.29	0.0025	significant
A-Enzyme	640.36	1	640.35	12.15	0.0059	
B-H_2O_2 concentration	404.60	1	404.59	7.68	0.0198	
C-Temperature	278.62	1	278.62	5.29	0.0443	
AB	497.31	1	497.31	9.43	0.0118	
BC	364.96	1	364.95	6.92	0.0251	
Residual	527.12	10	52.71			
Total	2712.97	15				

R^2=0.8057 Adj.R^2=0.7086

Table 5. The level and range of independent variables chosen for RSM design.

Factors	Range and Levels Coded				
	-1.682	-1	0	+1	+1.682
A- Enzyme (U/mL)	1	2.01	3.5	4.99	6
B- H_2O_2 Concentration (mM)	1	2.82	5.5	8.17	10
C- Temperature (°C)	20	26.08	35	43.92	50

3.2.3. Effect of interactive variables

Fig. 6 depicts the combined effect of H_2O_2 and CIP enzyme concentrations on RB5 decolorization at a fixed average temperature of 35°C. As expected, the decolorization efficiency was boosted with the increase in enzyme activity. DE was diminished by the increase in H_2O_2 concentration, even in the presence of high enzyme activity. This is consistent with results obtained from OFAT optimization, which indicated the deactivation of peroxidase at a higher concentration of H_2O_2. The highest decolorization performance (96.046 %) was obtained at a low H_2O_2 concentration (1.0 mM) and high activity of the crude enzyme (6 U/mL). The lower optimum value for H_2O_2 concentration compared with that obtained by the OFAT method (3.9 mM) might be attributed to the different experimental conditions in these two methods. In OFAT optimization, continuous mixing might result in improving the accessibility of H_2O_2 and enzymes in reactions and increasing the H_2O_2 decomposition rate. Therefore, a higher concentration of H_2O_2 could be applied in the case of OFAT experiments. Figure 7 indicates the combined effect of enzyme activity and temperature. DE was raised by increasing enzyme activity and decreasing temperature. Moreover, in higher enzyme activities, the efficiency was highly dependent on temperature, but at lower enzyme activities, decolorization efficiency became less sensitive to temperature.

Table 6. The central composite design (CCD) matrix for three independent variables along with the observed and the predicted values of response.

Run	Factor 1 A-Enzyme (U/mL)	Factor 2 B-H_2O_2 Concentration (mM)	Factor 3 C-Temperature (°C)	DE (%) Actual Value	Predicted Value
1	2.01	2.82	26.08	29.75	28.84
2	4.99	2.82	26.08	68.76	69.83
3	2.01	8.18	26.08	5.64	5.25
4	4.99	8.18	26.08	27.92	28.47
5	2.01	2.82	43.92	15.40	15.01
6	4.99	2.82	43.92	38.65	39.20
7	2.01	8.18	43.92	7.03	6.13
8	4.99	8.18	43.92	11.49	12.56
9	1.00	5.50	35.00	9.28	10.89
10	6.00	5.50	35.00	52.61	50.76
11	3.50	1.00	35.00	59.10	58.99
12	3.50	10.00	35.00	10.52	10.41
13	3.50	5.50	20.00	19.23	19.12
14	3.50	5.50	50.00	1.05	0.94
15	3.50	5.50	35.00	16.82	17.31
16	3.50	5.50	35.00	16.23	17.31
17	3.50	5.50	35.00	18.54	17.31
18	3.50	5.50	35.00	16.51	17.31
19	3.50	5.50	35.00	17.41	17.31
20	3.50	5.50	35.00	18.31	17.31

Table 7. Analysis of variance (ANOVA) for RSM parameters fitted to the polynomial equation.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	6359.082	11	578.0984	299.0312	< 0.0001	significant
A-enzyme	1918.962	1	1918.962	992.6158	< 0.0001	
B- H_2O_2 Concentration	1179.785	1	1179.785	610.2641	< 0.0001	
C-Temperature	165.2562	1	165.2562	85.48157	< 0.0001	
AB	157.7959	1	157.7959	81.62259	< 0.0001	
AC	141.0097	1	141.0097	72.93964	< 0.0001	
BC	108.2415	1	108.2415	55.98976	< 0.0001	
A^2	329.1655	1	329.1655	170.2664	< 0.0001	
B^2	544.5498	1	544.5498	281.6776	< 0.0001	
C^2	95.62372	1	95.62372	49.46299	0.0001	
A^2B	11.74155	1	11.74155	6.073518	0.0390	
A^2C	13.67391	1	13.67391	7.07306	0.0288	
Residual	15.4659	8	1.933238			
Lack of Fit	10.89437	3	3.631458	3.971819	0.0859	not significant
Pure Error	4.571529	5	0.914306			
Total	6374.548	19				

R^2=0.9975 Adj.R^2=0.9942

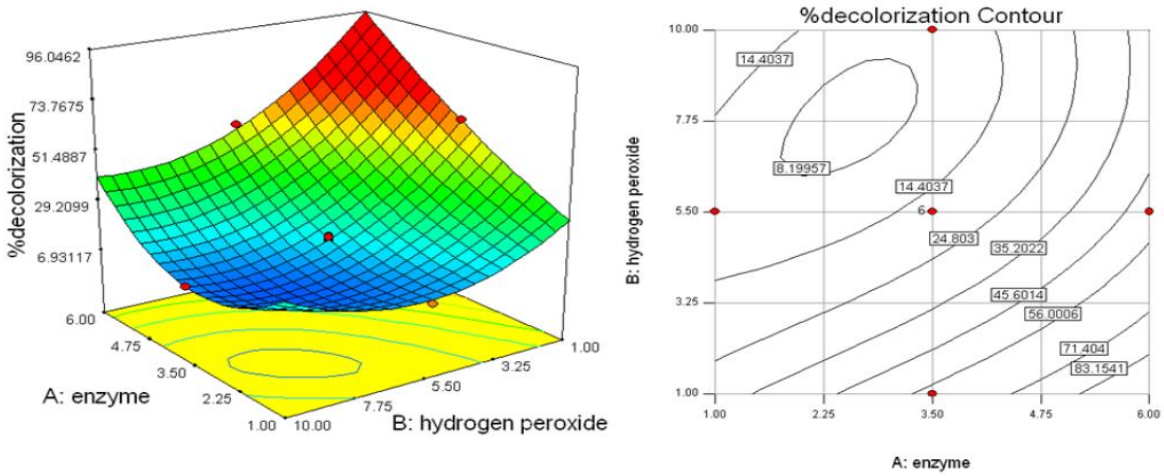

Fig. 6. The interactive effect of H_2O_2 and CIP enzyme concentrations on decolorization efficiency at a fixed temperature of 35 °C.

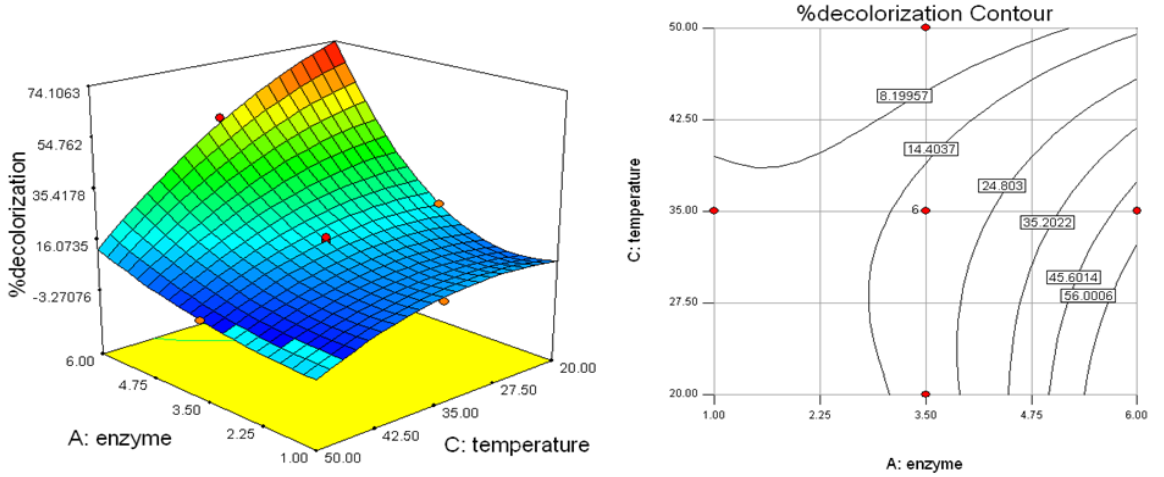

Fig. 7. The combined effect of enzyme activity and temperature on RB5 decolorization efficiency.

Figure 8 illustrates the interaction between hydrogen peroxide concentration and temperature on DE. As seen from Figure 8, the increase in both hydrogen peroxide concentration and temperature resulted in a decrease in decolorization efficiency. The highest DE was obtained at the lowest concentration of H_2O_2 and the deepest temperature. In addition, a comparison between Figures. 6, 7, and 8 suggests that the effect of temperature was less important than the effect of H_2O_2 concentration and enzyme activity. Finally, according to the defined model, the optimum conditions could be predicted as pproximately 6 U/mL CIP concentration, 1.0 mM H_2O_2 concentration, and a temperature of 35°C for 96.046 % decolorization efficiency. Table 8 compares the performance of the present study for the decolorization of Reactive Black 5 using different bioremediation methods. Biosorption using live or dead cells of fungal strains is another possible methodology other than enzymatic degradation. The present study achieved the highest removal efficiency of RB5.

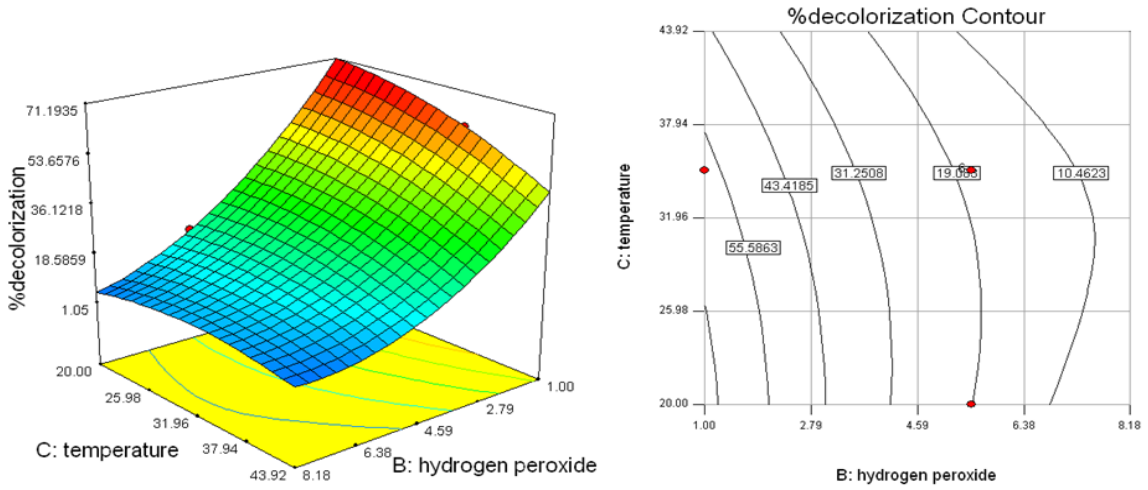

Fig. 8. The interactive effect of hydrogen peroxide concentration and temperature on decolorization efficiency of RB5.

Table 8. The comparison of reactive black 5 (RB5) decolorization by different fungal strains.

Mechanism	Microbial strain	Microbial type	Dye conc. (mg/L)	Enzyme activity (U/mL)	Redox mediator	pH	Temp. (°C)	DE / time	Ref.
biosorption	*Aspergillus tamari*	Fungi	100			7	30	95.96%/ 120 h	[40]
biosorption (Dead cells immobilized on SA[a])	*Gloeophyllum trabeum*	brown-rot fungus	50			7	40	53.08/7 days	[41]
biosorption (Living cells immobilized on SA[a])	*Gloeophyllum trabeum*	brown-rot fungus	50			3	25	13.35%/7 days	[41]
biosorption	*Phanerochaete chrysosporium*	White rot fungus	50			6	25	92.4%/2 h	[42]
biosorption	*Aspergillus flavus*[c]	fungi	100	300 mg/L	----	5	30	91%/2 h	[43]
Biosorption+ enzymatic	*Pleurotus eryngii F032*	White rot fungus	10	----	----	3	40	93.56%/72 h	[38]
Laccase enzyme	*Trametes trogii*	white rot fungus	25	1	0.7 mM	5	AT[d]	92%	[44]
laccase-like enzyme	*Coriolopsis gallica*	white rot fungus	25	0.5	4.5 mM	4.2	55	82%/2 h	[45]
PEDMT–HRP[b]	*Armoracia rusticana*	horseradish plant root	25	1	3% (v/v)	6	45	47.99%/0.5 h	[31]
CIP	*Coprinus Cinereus*	white rot fungus	40	5	96.1 mM	8	AT[d]	73%/12 min	[23]
CIP	*Coprinus Cinereus*	white rot fungus	40	6	1.0 mM	9	35	96.046%	This study

[a]SA: sodium alginate
[b] Horseradish Peroxidase (HRP) Immobilized on poly (ethylene glycol dimethacrylate-N-methacryloyl-amido-L-tryptophan methyl ester) [PEDMT] microbeads
[c] pretreated by NaOH
[d] ambient temperature

4. Conclusions

The performance of reactive black 5 (RB5) removal using crude *Coprinus cinereus* peroxidase was investigated by three different methods. Firstly, the effect of four well-known important parameters, namely temperature, pH, H_2O_2 concentration, and dye concentration, was explored separately, and the optimum value of these parameters was estimated using the traditional OFAT method. Then, a two-step statistical optimization was performed to find the optimum conditions for the decolorization of RB5. A two-factorial design and RSM methodology were implemented for screening and the final optimization step. In the screening step, five parameters were investigated and ANOVA analysis results revealed that pH and dye concentration were of lesser importance than the three other parameters. Therefore, the remaining parameters of H_2O_2 concentration, enzyme activity, and temperature were selected as the most significant parameters to optimize using the central composite design of RSM optimization. Finally, the optimum conditions of 1.0 mM H_2O_2, 6 U/mL enzyme activity, and 35°C were obtained for a maximum decolorization efficiency of 96.046 %.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that would influence this paper.

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