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Response surface analysis for enzymatic decolorization of Congo red by manganese peroxidase

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ABSTRACT

The enzymatic decolorization process of manganese peroxidase (MnP) is a complex system, which is greatly affected by the concentrations of H_2O_2, Mn^{2+}, dye and enzyme. This work aimed to study these factors and investigate the combined interactions between them by applying response surface methodology (RSM) for decolorization of Congo red with MnP from *Schizophyllum* sp. F17, meanwhile conventional one-factor-at-a-time analysis was carried out. Through the one-factor-at-a-time analysis the optimized H_2O_2, Mn^{2+}, Congo red and MnP extract was 0.2 mM, 0.5 mM, 50 mg/l and 0.8 ml, respectively, and the maximum decolorization attained under such conditions was 24.2%. Response surface analysis was conducted through Box–Behnken design and a second-order polynomial model ($R^2 = 0.8565$) was generated to describe the combined effect and the interactions quantificationally. ANOVA analysis indicated that the interactions between H_2O_2 and MnP, between dye and MnP were significant; the optimum condition through RSM was found to be 0.35 mM H_2O_2, 0.5 mM Mn^{2+}, 75 mg/l Congo red and 1.4 ml MnP extract, for maximum decolorization of 30.8%.

1. Introduction

Among industrial wastewaters, the treatment of dye wastewater from textile and dyestuff industries is one of the most challenging [1]. It is estimated that between 10 and 20% of about 0.7 million tons of dyestuff being manufactured each year and used in dyeing processed may be found in wastewater [2]. Many dyes are believed to be toxic carcinogenic or to be prepared from known carcinogens such as benxidine or other aromatic compound that might be reformed as a result of microbial metabolism [3]. The treatment of such dye-containing effluents is mostly based on physical and/or chemical methods, e.g. adsorption, concentration, chemical transformation and incineration. These methods for the removal of dyes are not suitable due to high cost, low efficiency, possibility of producing highly toxic byproducts, in-applicability to a wide variety of dyes [4,5]. Therefore, biodegradation attracts attention. Microbial decolorization has been claimed to be less expensive and less environmentally intrusive alternative [6].

By far the single class of microorganisms most efficient in breaking down synthetic dyes is the white-rot fungi (WRF) [7], which produces several types of oxidation enzymes. These extracellular enzymes lacking of substrate specificity enable white-rot fungi to degrade a wide range of dye compounds [8]. Manganese peroxidase (MnP) is an outstanding one of such enzymes. The catalytic cycle of MnP proceeds through an initial oxidation by H_2O_2 to an intermediary compound that, in turn, promotes the oxidation of Mn^{2+} to Mn^{3+}. Mn^{3+} is stabilized by organic acid, and the Mn^{3+} organic acid complex formed acts as the active oxidant. Excess H_2O_2 can oxidate the intermediary compound of MnP to an inactive form [9–11]. So, in the enzymatic decolorization system of MnP, there are four most important factors that decide the decolorization efficiency, which are the concentrations of H_2O_2, Mn^{2+}, dye and enzyme. The enzymatic decolorization process is a complicated system, thus it is important to analyze it as a whole and to understand the combination interactions between the four factors. Some reports that addressed decolorization of synthetic dyes by MnP optimized these four parameters [12–15], but all of these studies were carried out through the one-factor-at-a-time approach, in which only one factor was variable at a time while all others were kept constant. This approach is time-consuming and expensive. In addition, possible interaction effects between variables cannot be evaluated and misleading conclusions may be drawn. Response surface methodology (RSM) can overcome the difficulties of the one-factor-at-a-time approach, since it allows accounting for possible interaction effects between variables [16]. Response surface methodology is a collection of mathematical and statistical techniques useful for modeling and analysis of problems in which a response of interest is influenced by several variables, it provides

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important information regarding the optimum level of each variable along with its interactions with other variables [17,18].

The present study was performed to investigate the effects of the concentrations of H_2O_2, Mn^{2+}, dye and MnP on the decolorization of Congo red, a typical synthetic azo dye, through the one-factor-at-a-time approach and the RSM, to acquire deeper understanding on the enzymatic decolorization mechanism of MnP. *Schizophyllum* sp. F17, a local white-rot fungus, has previously shown potential for dye decolorization [19]. In this work, crude MnP extract used in the experiments was produced by *Schizophyllum* sp. F17 during solid-state fermentation (SSF) of rice hull, a natural lignocellulosic substrate.

2. Materials and methods

2.1. Microorganism and culture conditions

Schizophyllum sp. F17 was isolated from a decayed wood chip pile in the vicinity of Hefei, China [19]. The fungus was cultured on potato (*Solanum tuberosum*) dextrose agar (PDA) slants for 7 days at 28 °C. The grown mycelium mat was washed with sterile water by inoculating needle into 150 ml flask. The mycelia obtained were blended to mycelial suspension at 2000 rpm, and 100 ml liquid medium in 250 ml flasks were inoculated with 10 ml of the suspension. The liquid medium contained 20 g potato, 2 g dextrose, 0.3 g KH_2PO_4, 0.15 g $MgSO_4$ and 1 mg thiamine, and was autoclaved at 121 °C for 20 min. The mycelial pellets, forming in liquid medium after 3 days incubation at 28 °C on the rotary shaker with a speed of 130 rpm, were blended to mycelial suspension at 2000 rpm, 2.5 ml of which was used to inoculate 5 g SSF medium in 250 ml flasks. SSF medium containing 90% rice hull and 10% bean powder was humidified with 7.5 g water and autoclaved at 121 °C for 20 min. Solid-state culture was carried out at 22 °C for 7 days.

2.2. Preparation of crude enzyme extract

Hundred milliliter of sodium acetate buffer (10 mM, pH 6.0) was added to the SSF medium, the mixture was gently beaten and incubated on the rotary shaker at 130 rpm for 30 min. Then the mixture was filtered and the filtrate was used as crude enzymes for enzyme analysis and decolorization studies.

2.3. Enzyme assays

Manganese peroxidase and manganese-independent peroxidase (MIP) activities were assayed by oxidation of guaiacol to colored product using spectrophotometer at 465 nm. Reaction mixtures that contained 100 mM sodium lactate buffer (pH 4.5), 1 mM $MnSO_4$, 1 mM guaiacol and 0.1 mM H_2O_2 were for the total activities of MnP and MIP, reaction mixtures for MIP were similar to that but $MnSO_4$ was replaced by 1 mM EDTA, according to the method modified from that of Shrivastava et al. [5]. Lignin peroxidase (LiP) activity was determined as the oxidation rate of veratryl alcohol to veratraldyde in 200 mM sodium tartrate buffer pH 3.0 in the present of 0.2 mM of H_2O_2 at 310 nm [20]. Laccase (Lac) activity was followed spectrophotometrically through the oxidation of guaiacol [21]. One unit (U) was defined as the amount of enzyme required to produce 1 μmol product/min at 30 °C, and the enzymatic activities were expressed as U/l crude filtrate.

2.4. The one-factor-at-a-time analysis

Decolorization of Congo red with MnP filtrate was determined spectrophotometrically by measuring the decrease in A_{506}. The decolorization was carried out in 4 ml 100 mM sodium lactate buffer pH 4.5 containing 0.4 ml culture filtrate, 50 mg/l Congo red and 0.1 mM H_2O_2 with or without 1 mM $MnSO_4$, the mixtures without 1 mM $MnSO_4$ was added 1 mM EDTA. The decolorization in the medium with H_2O_2 and Mn^{2+} was due to MnP and MIP activities while the decolorization was contributed by MIP in the medium with H_2O_2 and EDTA. The reaction mixtures were incubated at 30 °C for 30 min. Control samples, without H_2O_2, were done in parallel identical conditions. The efficiency of decolorization by MnP was expressed as the percentage ratio of the decolorized dye absorbance to that of the control.

$$D_{MnP}(\%) = \frac{A_c - A_{MM} - A_{MIP}}{A_c} \times 100\% \quad (1)$$

where D_{MnP} = decolorization by MnP, A_c = absorbance of the controls at 506 nm, A_{MM} = absorbance of the decolorized mixtures with 0.1 mM H_2O_2 and 1 mM Mn^{2+}, A_{MIP} = absorbance of the decolorized mixtures with 0.1 mM H_2O_2 and 1 mM EDTA.

In this section, the single factor effects of H_2O_2, Mn^{2+}, dye and MnP concentrations on enzymatic decolorization of Congo red were investigated. Different H_2O_2 dosages (0, 0.025, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, and 0.4 mM), Mn^{2+} concentrations (0, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 mM), Congo red concentrations (25, 50, 75, 100, 125, and 150 mg/l) and amounts of MnP filtrate (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 ml) were used according to preliminary studies.

Table 1
Levels and actual values of the variables tested

Factor	Variable	Unit	Range and levels of coded		
			−1	0	1
A	H_2O_2	mM	0.05	0.2	0.35
B	Mn^{2+}	mM	0.5	1.5	2.5
C	Dye	mg/l	25	50	75
D	Enzyme	ml	0.2	0.8	1.4

2.5. Response surface analysis

RSM, an experimental approach to identify the optimum conditions for a multivariable system, can predict the combined effect of some variables. In present work, response surface methodology was employed to probe and describe quantificationally the interactions between H_2O_2, Mn^{2+}, Congo red and MnP concentration by constructing mathematical model. A class of three level complete factorial design for the estimation of the parameters was developed by Box–Behnken [22]. We selected Box–Behnken design for the study on the interactions. Three different concentrations of H_2O_2 (0.05, 0.20, and 0.35 mM), Mn^{2+} (0.5, 1.5, and 2.5 mM), dye (25, 50, and 75 mg/l) and enzyme (0.2, 0.8, and 1.4 ml) were chosen as the critical variables and designated as A, B, C and D respectively, as shown in Table 1. Dye decolorization experiments were carried out according to the arrangement presented in Table 2. Data were analyzed using Design Expert 7.0 program including ANOVA to find out the interaction between the variables and the response. The quality of the fit of the model was expressed by the coefficient of determination (R^2) in the same program. The response surface plots were built using Matlab 7.0 program.

3. Results and discussion

Schizophyllum sp. F17 grew on rice hull medium under solid-state condition, after 5 days of culture, MnP activity reached maximum 133.3 U/l (Fig. 1) and it was the main enzyme produced, since low MIP (7 U/l) and no LiP or laccase was detected. This crude extract of 5-day-old medium was used as the MnP source for the analysis of the enzymatic decolorization system.

Table 2
The Box–Behnken design matrix with response

Run	Factor 1 (*A*: H_2O_2)	Factor 2 (*B*: Mn^{2+})	Factor 3 (*C*: Dye)	Factor 4 (*D*: Enzyme)	Decolorization (%)
1	0	0	0	0	22.4
2	1	0	1	0	24.6
3	0	0	−1	1	1.4
4	0	0	1	−1	5.2
5	−1	0	0	−1	8.2
6	0	1	0	−1	7.9
7	0	0	0	0	22.6
8	0	−1	1	0	23.6
9	0	−1	0	−1	5.9
10	1	1	0	0	23.9
11	1	0	0	1	21.7
12	1	−1	0	0	25.4
13	0	1	0	1	20.6
14	0	1	−1	0	2.5
15	−1	−1	0	0	8.3
16	−1	0	1	0	8.6
17	0	0	0	0	23.2
18	0	−1	0	1	21.9
19	1	0	0	−1	9.2
20	0	0	0	0	19.3
21	1	0	−1	0	7.7
22	0	1	1	0	23.9
23	−1	0	−1	0	2.0
24	0	0	−1	−1	14.6
25	−1	0	0	1	22.5
26	0	−1	−1	0	8.3
27	−1	1	0	0	5.2
28	0	0	0	0	19.5
29	0	0	1	1	22.5

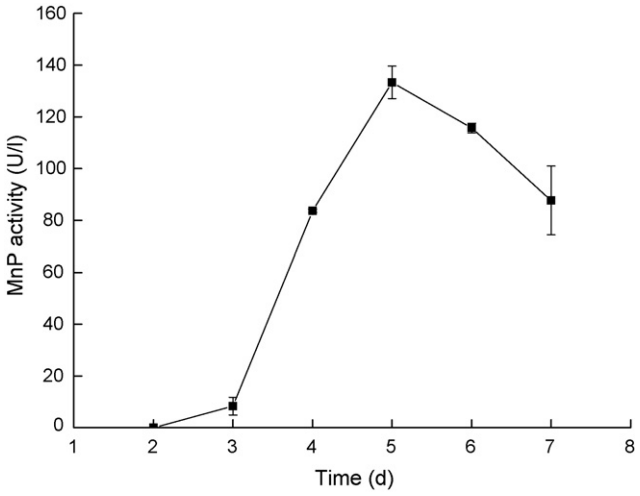

Fig. 1. MnP production by *Schizophyllum* sp. F17 on rice hull medium.

3.1. The one-factor-at-a-time analysis

In this section the single factor effects of H_2O_2, Mn^{2+}, Congo red concentrations and MnP dosage on enzymatic decolorization were investigated.

The decolorization rate increased with the H_2O_2 concentration increasing from 0 to 0.2 mM, the maximum decolorization 22.8% was observed when the H_2O_2 was 0.2 mM, and higher H_2O_2 concentration decreased the decolorization tardily (Fig. 2a). Previously, Mielgo et al. [12] optimized conditions for degradation of orange II by crude MnP of *Bjerkandera* sp. BOS55. They reported that, with the increasing amount of H_2O_2 added, decolorization was reduced and MnP was rapidly inactivated. Yu et al. [15] reported the sharply decrease of decolorization from maximum to zero with the H_2O_2 changing from 0.1 to 0.4 mM when decolorizing reactive brilliant red K-2BP by crude MnP of *P. chrysosporium*. Comparing the two MnPs, the MnP of *Schizophyllum* sp. F17 was not so sensitive to high concentration of H_2O_2 that the decolorization did not change acutely when H_2O_2 increased from 0.1 to 0.4 mM.

Low Mn^{2+} concentration was infaust for enzymatic decolorization of Congo red, but high concentration of Mn^{2+} (>0.5 mM) did not markedly influence the decolorization (Fig. 2b). Mielgo et al. also proved that only a very small concentration of Mn^{2+} (0.033 mM) was required for almost completed decolorization and higher concentration (to 1 mM) did not improve decolorization, which is similar to our result. But, Moreira et al. [13] reported that the increase in the Mn^{2+} concentration (from 0.033, to 1 mM for crude MnP of *P. chrysosporium*, to 5 mM for *Bjerkandera* sp. BOS55) led to a significant decrease in decolorization of Poly R-478 by crude MnP. Yu et al. indicated that decolorization gained its maximum at the background concentration of Mn^{2+} (0.137 mM) in the crude enzyme (from liquid medium) and decreased rapidly with the addition of additional Mn^{2+}. All these works proved the satisfaction of low concentration of Mn^{2+} for decolorization, but the effects of high concentrations of Mn^{2+} were different, possibly because of the variation of the strains.

Different Congo red concentrations were used to determine the effect of dye concentration on enzymatic decolorization of Congo red in the reaction mixture containing 0.4 ml crude enzyme. Dye decolorization rate was improved from 16.4 to 20.7% by increasing Congo red concentration from 25 to 50 mg/l, but higher dye concentration inhibit the decolorization (Fig. 2c). This phenomenon, the decolorization increased and then decreased with the increase of dye concentration, is similar to the result of Congo red decolorization by *Schizophyllum* sp. F17 in bio-contact reactor [20] and the result of RBBR decolorization by laccase [6]. Many paper reported that various dyes exhibited inhibition effects on decolorization by ligninolytic enzymes [12,15,23,24], and according to Yu et al., high dye concentration would give rise to a slower decolorization during which enzyme stability can be easily hurt. But, the reason for decolorization increased with dye concentration need further researches to explain.

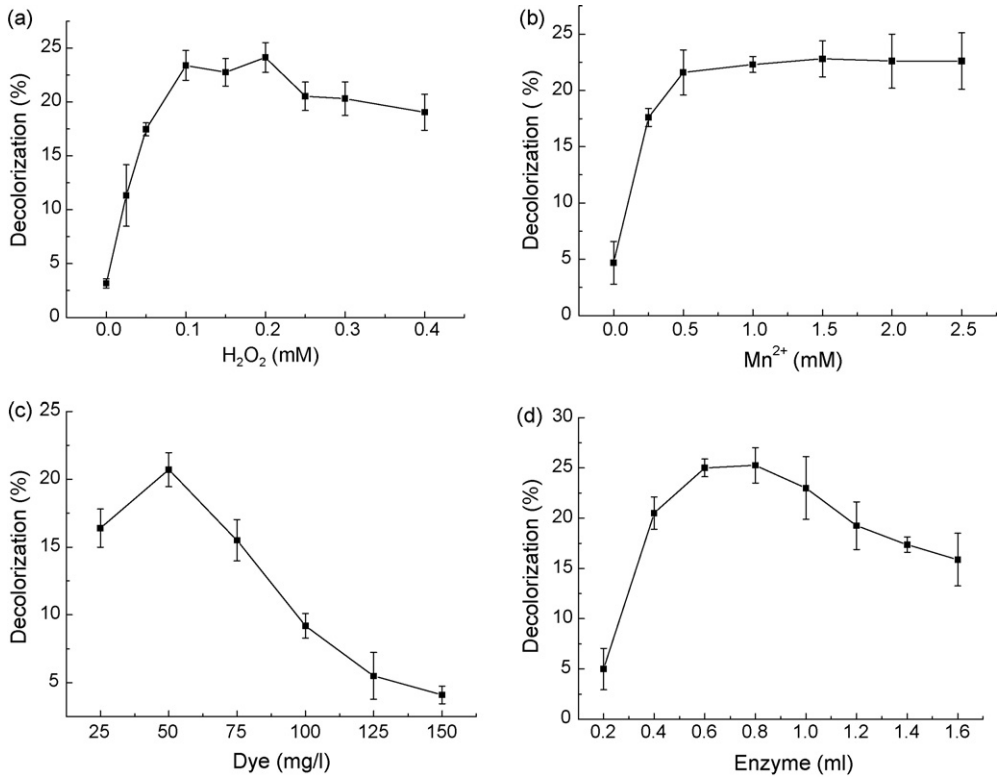

Fig. 2. (a–d) The single factor effects of concentrations of H_2O_2, Mn^{2+}, dye and on decolorization of Congo red.

To test the influence of enzyme dosages on decolorizing Congo red of 50 mg/l, the decolorization rate of Congo red increased with the increase in amount of crude enzyme used up to 0.8 ml, however amounts above 0.8 ml decreased the decolorization (Fig. 2d), this result conflicted with that of many previous papers reporting that higher concentration (than a certain value) of enzyme neither increased nor inhibited decolorization [6,12,15]. This interesting result was rarely reported and should be studied in future work.

The conditions optimized through the one-factor-at-a-time analysis was H_2O_2 0.2 mM, Mn^{2+} 0.5 mM, Congo red 50 mg/l and MnP extract 0.8 ml, and the maximum decolorization attained under such conditions was 24.2%.

3.2. Response surface analysis

Sophisticated combination and interactions exist between the four factors; therefore each one's suitable concentration required would change with others'. In previous reports, most studies on decolorization conditions were carried out through single factor analysis, which could not exactly reflect the enzymatic reaction as a complicated system. In this study, we aimed to investigate the effects of combination of H_2O_2, Mn^{2+}, dye and enzyme concentration and the interactions between them on decolorization of Congo red by crude MnP. For this purpose, Box–Behnken design was chosen in the experiment.

Levels of the four variables are presented in Table 1; Table 2 shows the data resulting from the experiment of the effect of the four variables H_2O_2 concentration (A), Mn^{2+} concentration (B), dye concentration (C) and MnP amount (D) on decolorization of Congo red. The experimental results were analyzed through RSM to obtain an empirical model for the best response. The model was used to explain the mathematical relationship between the independent variables and the dependent response. The mathematical expression of relationship to the decolorization of Congo red with variables A, B, C and D is shown below:

$$\begin{aligned}\text{Decolorization} \quad =& +21.40+6.68\times A-1.17\times B+5.61\times C+3.30\times D\\ &-5.21\times A^2-0.87\times B^2-4.94\times C^2-5.93\times D^2\\ &+0.40\times A\times B+2.58\times A\times C+4.55\times A\times D\\ &+2.67\times B\times C-0.82\times B\times D+7.63\times C\times D\end{aligned} \tag{2}$$

Statistical analysis shows that the coefficients of B, B^2, $A\times B$, $A\times C$, $B\times C$ and $B\times D$ were statistically insignificant at 10% level, which were deleted from the equation and added to the lack of fit. As a result, a new regression model was obtained below:

$$\begin{aligned}\text{Decolorization} = \quad &+20.83+6.48\times A+5.61\times C+3.30\times D\\ &-5.05\times A^2-4.77\times C^2-5.76\times D^2\\ &+4.55\times A\times D+7.63\times C\times D\end{aligned} \tag{3}$$

The results of analysis of variance (ANOVA) for Eq. (3) are shown in Table 3. The model of the equation was significant at 1% level and each term was significant at 5% level. A coefficient of determination (R^2) value of 0.8565 showed that the equation was highly reliable, the model also revealed statistically insignificant lack of fit ($P=0.0636$) at 5% level. The model was found to be adequate for prediction within the ranges of variables.

From Eq. (3) and the ANOVA analysis, the influence of Mn^{2+} concentration was not significant at 10% level in the range of 0.5–2.5 mM; this result indicated that high concentration of Mn^{2+} (above 0.5 mM) did not improve the decolorization of Congo red, which was corresponding with the result of single factor analysis.

The combined effect and the interactions between the four variables can be described quantificationally using RSM, which are difficult to be observed in conventional methods. From Eq. (3) and Table 3 the interactions between variable H_2O_2 (A) and MnP (D), dye (C) and MnP (D) on decolorization of Congo red were significant at 5% level, which are shown through Figs. 3 and 4.

Table 3
ANOVA results for the equation of Design Expert 7.0

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*P*
Model	1743.6	8	217.95	15.41	<0.0001
A	503.11	1	503.11	35.56	<0.0001
C	377.44	1	377.44	26.68	<0.0001
D	130.68	1	130.68	9.24	0.0072
$A \times D$	82.81	1	82.81	5.85	0.0273
$C \times D$	232.56	1	232.56	16.44	0.0007
A^2	171.36	1	171.36	12.11	0.0027
C^2	153.19	1	153.19	10.83	0.0041
D^2	223.15	1	223.15	15.77	0.0009
Residual	292.13	20	14.61		
Lack of fit	278.43	16	17.4	5.08	0.0636
Pure error	13.7	4	3.42		
Total	2035.73	28			

R^2: 0.8565; adj R^2: 0.7991.

Fig. 3 represents the effect of varying H_2O_2 concentrations and MnP amounts at fixed concentration of Mn^{2+} 1.5 mM and dye 50 mg/l. The Congo red decolorization increased with H_2O_2 concentration and enzyme amount, the best decolorization (24.4%) was observed at high H_2O_2 concentration (0.35 mM) and large amount of crude enzyme (1.4 ml). This result informed that high concentration of MnP must be activated by high concentration H_2O_2 for efficient enzymatic reaction.

Fig. 4 shows the combined effect of varying concentrations of Congo red and amounts of MnP on decolorization. The response surface plot reveals that increase in dye concentration and enzyme amount, keeping the Mn^{2+} and H_2O_2 concentration at fixed level of 1.5 and 0.2 mM, increased the decolorization activity. From the surface plot, at 75 mg/l Congo red and 1.4 ml crude MnP the dye decolorization attained maximum (26.8%).

Eq. (3) describes the mathematical relationship between the decolorization of Congo red and the concentrations of H_2O_2, dye and MnP. Figs. 3 and 4 show the interactions between H_2O_2 and MnP, between dye and MnP, quantificationally. The second-order polynomial regression Eq. (3) was solved by the sequential quadratic program MATLAB 7.0. The maximum response observed in response plots was 32.8% at 0.35 mM H_2O_2, 75 mg/l Congo red and 1.4 ml MnP extract, which was corresponding with the experimentally obtained decolorization of 30.8% under such conditions (with 0.5 mM Mn^{2+}).

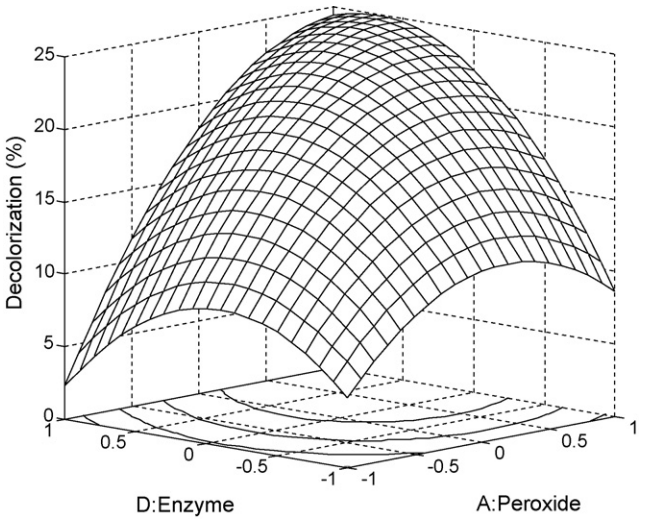

Fig. 3. Response surface plot showing the interaction between H_2O_2 dosage (*A*) and Enzyme amount (*D*) at fixed concentration of Mn^{2+} 1.5 mM and dye 50 mg/l.

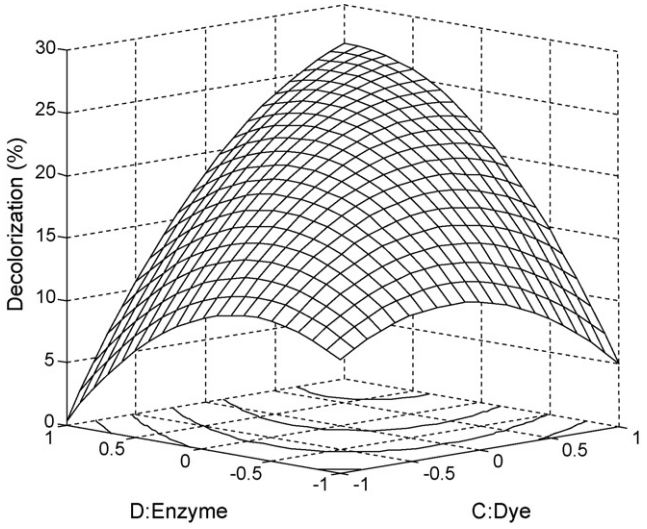

Fig. 4. Response surface plot showing the interaction between dye concentration (*C*) and Enzyme amount (*D*) at fixed concentration of H_2O_2 0.2 mM and Mn^{2+} 1.5 mM.

In previous studies, RSM has been applied in optimization of mediums for ligninolytic enzyme production and decolorization with fungal cultures [25–29]. For enzymatic decolorization with ligninolytic enzymes, Murugesan et al. [30] first utilized RSM to optimize factors of decolorization of reactive black 5 by laccase. But to our knowledge there has been no papers focused on statistical study of enzymatic decolorization with MnP. As the main purpose of this work, the interactions between H_2O_2, Mn^{2+}, dye and MnP concentration were investigated to acquire deeper understanding on the enzymatic decolorization mechanism of MnP. Throng RSM, two interactions between H_2O_2 and MnP, dye and MnP were proved statistical significant in the studied ranges, while there were six probable interactions between the four factors. This results would supplement the previous understand on enzymatic decolorization of MnP, and help to efficiently decolorize synthetic dyes by MnP.

It is noteworthy about the concentration of H_2O_2, dye and MnP optimized by RSM, such results reflected the enzymatic decolorization mechanism when interactions existing in the decolorization system. Based on the results, we can summarize that, using MnP to decolorize Congo red, the best decolorization was obtained at high concentration (75 mg/l) of dye that needed large amount of MnP to decolorize, however such enzymatic reaction required high concentration of H_2O_2 to activate. This trend was clearly displayed by the response surface plots, and was quantified by the mathematic model, but, it could not be observed in previous reports employing one-factor-at-a-time approach [12–15].

4. Conclusions

Congo red was decolorized in vitro by crude MnP from *Schizophyllum* sp. F17 grown on rice hull medium. Four important factors H_2O_2, Mn^{2+}, dye and MnP concentrations were studied through one-factor-at-a-time approach and RSM. The single factor effects of the four parameters were studied through one-factor-at-a-time approach, and the results showed that moderate concentrations of them were propitious for Congo red decolorization. The response surface analysis employing Box–Behnken design constructed a regression model to describe the combined effect and the interactions between the factors and the results indicated that the interactions between H_2O_2 and MnP, between dye and MnP were statistical significant.

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