Int. J. Environ. Res., 6(2):519-530, Spring 2012
ISSN: 1735-6865

Optimization of Garden radish (*Raphanus Sativus L.*) Peroxidase Enzyme for Removal of 2,4-dichlorophenol from 2,4- Dichlorophenoxyacetic Acid Wastewater

Riazi, Z.[1*], Ziai, S. A.[2], Shariat, S. M.[1], Farshchi, P.[1] and Khalili, F.[3]

[1] Department of Environmental Science, Graduate School of the Environment, Science and Research Branch, Islamic Azad University, Tehran, Iran

[2] Department of Pharmacology, Faculty of medicine, Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran

[3] Department of Environment, Institute of Iranian Mineral Processing Research, Kavosh research town, 9 km Qazvin / Karaj Freeway, Iran

Received 10 Jan. 2011; Revised 20 Aug. 2011; Accepted 10 Dec. 2011

ABSTRACT: Environmental pollution by 2,4-dicholorphenol (2,4-DCP), commonly found in industrial wastewater has been a concern for humans over the past 50 years. Garden Radish Peroxidase (GRP) can eliminate this poisonous pollutant. The aim of this study was to apply an experimental Response Surface Methodology (RSM) and Central Composite Design (CCD) to optimize GRP-based treatment in order to maximize the removal of 2,4-DCP from wastewater. The effects of four factors; pH, enzyme activity (U/mL), hydrogen peroxide (H_2O_2) concentration (mM), and substrate concentration (mg/L) and their interactions were investigated for 2,4-DCP removal using a second-order polynomial model. The suitability of the polynomial model was described using coefficient of determination (R^2 =90.7%) and the results were created by analysis of variance (ANOVA). A 3D response surface was made from the mathematical models and then applied to determine the optimal condition. These analyses exhibited that using a quadratic model was fitting for this treatment. Furthermore, desirability function was employed for the specific values of controlled factors for optimization and maximum desirability. Based on the desirability function results, the response predicted a 99.83% removal rate of 2,4-DCP from wastewater with 0.959 desirability. Under these conditions, the experimental removal percentage value would be 99.2%.

Key words: Radish peroxidase, Response surface methodology, Hydrogen peroxide and Environment

INTRODUCTION

Chlorinated Phenolic Compounds have long been recognized as a contributing to worldwide contamination because of their intrinsic chemical stability, high resistance to all types of degradation, and carcinogenic and genotoxic effects (Harayama, 1997). Chlorophenols are largely used for wide-spectrum biocides to control bacteria, fungi, algae, molluscs, and insects (Lu *et al.*, 1978). It should be noted that 2,4-dichlorophenl (2,4-DCP) and 4-chlorophenol (4-CP) are used as initial catalysts for the production of the herbicides 2,4,5-trichlorophenoxyacetic acid and 2,4dichlorophenoxyacetic acid (Kinzell *et al.*, 1979; Häggbolom, 1990; Häggbolom and Valo, 1995; Bae *et al.*, 2002). Treatment of these toxic contaminants should receive high priority (Munnecke, 1978). Munnecke (1977) has identified the adverse effects of these chlorinated compounds on human health as well as the environment. These compounds were classified as dangerous and resistant materials (El-Nabawi *et al.*, 1987; Harayama, 1997). They threaten human health with cancer and fetal mutation and the life of marine creatures (Exon, 1984; Hammel, 1989: Sakurai *et al.*, and Ping *et al.*, 2003). It is therefore important to find an effective new method to treat wastewater and to reduce concentrations of these poisonous compounds. Researchers have carried out many studies on various wastewater treatment methods such as physical (Estevinho *et al.*, 2007), chemical (Huston and Pignatello, 1996; Prousek, 1996; Barbusiński and Filipek, 2001) and biological (Kargi and Eker, 2005; Herrera *et al.*, 2008). These treatment methods are

*Corresponding author E-mail: zahra.riazi@srbiau.ac.ir

often not suitable due to high cost, long treatment time, low efficiency, low degrees of purity, and the production of hazardous by-products. Some researchers have studied enzymatic treatment methodology by various herbal sources using peroxidase enzymes (Nicell *et al.,* 1992; Al-Kassim *et al.,* 1994; Buchanan and Han, 2000; Sakurai *et al.,* 2001; Sakurai *et al.,* 2003). Treatment by enzymes to remove aromatic compounds was first suggested by Klibanov et al. (1980). The peroxidase enzyme belongs to the oxidoreductases enzymatic group that already exists in the environment. In fact it is present in all plant cellular organisms such as figs, turnips, tobacco, soybean and root vegetables such as horseradish and garden radish (*Raphanus sativus* L. *var. sativus*). Horseradish peroxidase was discovered in 1885 and is among the first enzymes studied by biochemists (Bollag and Dec, 1998; Price and Stevens, 1989). Most research has focused on extraction of the enzyme from the horseradish plant (Aitken, 1993). Horseradish peroxidase is a plant glycohemoprotein and its enzymatic activity is due to cyclic reduction and the presence of iron atoms in the hematin group (Chance, 1951). In the presence of hydrogen peroxide, peroxidase enzymes catalyze the oxidation of various chlorinated phenols, anilines phenols and other aromatics to free radicals, which then combine to form insoluble polymers (Dunford and Stillman, 1976). These insoluble polymers can then be removed by sedimentation or filtration (Klibanov *et al.,* 1980). Research results show that 40 phenolic and aromatic compounds could be extracted from wastewater with an efficiency as high as 99% (Klibanov *et al.,* 1980).HRP enzyme treatment is one way to remove poisonous pollutants from the environment (Dec and Bollag, 1994; Klibanov *et al.,* 1983). Advantages of this method include the vast range of pH, temperature and substrate concentration that can be accommodated (Nicell *et al.,* 1992; Nicell *et al.,* 1993). Much research has been done on effective enzymatic treatment by peroxidase factors: Tong et al. (1999) studied the removal effect of HRP on 2,4-CP in real and experimental wastewater. Removal of hazardous aromatic waste using GRP was described by Ziai *et al.,* (2003), which compared the capabilities of pH variants; phenol, aniline, benzidine, acid red 88 and acid blue 62. GRP was introduced as a good substitute for HRP by Ziai *et al.,* (2003). Optimal conditions for pH, H_2O_2 to phenol molar ratio, HRP, as well as reaction times were determined to achieve at least 95% removal of phenols from synthetic wastewater (Wu *et al.,* 1998). Studies of pH, temperature, soybean peroxidase (SBP) enzyme activity, H_2O_2 concentration and substrate concentration have been investigated (Bollag and Dec, 1998; Kennedy *et al.,* 2002). And some research has used the RSM method with Central Composite Design (CCD) in physical (Oughlis-Hammache *et al.,* 2010), chemical (kasiri *et al.,* 2008) and enzymatic (Ghasempur *et al.,* 2007) treatment methods in order to optimize conditions for treating phenol and chlorinated phenol compounds.

This study used Response Surface Methodology (RSM), CCD and desirability function to optimize pH, enzyme activity, concentrations H_2O_2 and substrate to achieve optimum removal of 2,4-DCP, increase accuracy, to reduce cost, numbers of examinations and time, as well as to avoid errors (human and instrument).

MATERIALS & METHODS

Garden radish (*Raphanus Sativus L.*) roots used in this study were cultivated in Halijerd rejoin of Alborz province, Iran. Guaiacol solution, H_2O_2 solution (30%), 2,4-DCP (99%), Methanol HPLC grade, Potassium dihydrogen Phosphate, Potassium monohydrogen Phthalate, Boric acid, Hydrochloric acid and Sodium hydroxide were purchased from Merck Company. Chemicals of analytical grade were used.

Garden radish roots (500g) were smashed in a blender, suspended in water, mixed and compressed through cheesecloth; the slurry solution was then filtered by a Buchner funnel. 140 mg of crude enzyme powder was produced with a freeze dryer (Alberti and Klibanov, 1982).

The results of peroxidase activity were assayed according to a standard method (Putter, 1974). Initially, 0.05 ml of 20 mM guaiacol solution , 0.05 ml of H_2O_2 solution were added to 2.9 ml 0.1 M potassium phosphate buffer (pH7) then a 3ml aliquot of this mixture using 20 mg of crude enzyme powder was added to a glass cuvette. A Varian UV-Visible spectrophotometer was used to determine changes of "A at wavelength 436 nm. The unit of activity was calculated according to the formula below. The peroxidase activity was 1.24U/mL.

$$\text{Activity}(\text{U/mL}) = \frac{\Delta A}{\text{time}} \times \left[\frac{a \times b}{c \times 1 \times d}\right] \times 1000$$

a =Final volume of mixture b =4 c =25.5 d= 0.05

A primary standard stock solution containing 5000 mg/L 2,4-DCP in methanol was prepared and other working standard solutions were made from the stock solution in 100, 200, 300, 400 and 500 mg/L concentrations of pH values; 3.5, 5, 6.5, 8 and 9. The secondary solutions were prepared with pH 3 to 5 Phthalate buffer solution, pH 6 to 8 Phosphate buffer solution and pH 8 to 10 Alkaline borate buffer solution.

A GRP stock solution of 200 U/mL in milli-Q water was prepared and kept at 4 ºC. The secondary solution in various activities (0.1, 3.05, 6, 8.95, 11.9 U/mL) was prepared from the primary solution. Quantitative amounts of 2,4-DCP solution were poured into scaled cylinders as shown in Table 1 which had been designed by the CCD statistical method. Then the enzyme solution and H_2O_2 solution (according to Table 1) were added to the 2,4-DCP solution. Samples were shaken (50rpm) at room temperature for 3 hours and centrifuged for 20 minutes. After settlement, the samples were taken from the upper solution and filtered by 0.45 µm filters before they were analyzed (Bollag and Dec, 1998; Kennedy *et al.,* 2002).

The samples were analyzed for any residual 2,4-dichlorophenols by an Alliance Waters (Separation module 2690/5-quaternary gradient system) HPLC instrument equipped with a Dual UV Absorbance Detector (model 2487) and a Perfectsil target ODS-3 5.0 µm reverse phase C18 column (250*4.6 mm) maintained at 30ºC. The mobile phase was an isocratic (HPLC grade methanol 80% /HAc (Acetic acid) 1.0% in water 20%), pump flow rate of 1.0 mL/min, and wavelength 220 nm, the sample injected volume was 40 µL using an auto-sampler system and concentrations were automatically calculated by Millennium software (version 4.0). Blank samples without GRP and without H_2O_2 were prepared for all experiments. Standard solutions of 2,4-DCP were prepared with concentrations of 0.05 to 25 mg/L for the calibration curve.

CCD is one the primary design techniques in RSM used to build a second-order model (quadratic model) and commonly used for process optimization (Myers and Montgomery, 2002).

The CCD application accompanied by RSM works by showing the intrinsic value of the Response Surface in the experimented area and to show optimal values of independent variables.

Variable amplitudes were chosen based on previous studies which had been done by the traditional OVAT method (One Variable at a Time). The chosen amplitudes were as follows:

pH (3.5, 5, 6.5, 8, 9), substrate concentration (100, 200, 300, 400, 500 mg/L), H_2O_2 concentration (0.613, 1.226, 1.839, 2.452, 3.065 mM), enzyme activity (0.1, 3.05, 6, 8.95, 11.9 U/mL).

The CCD experiments were designed using four variables; H_2O_2 concentration, substrate concentration, enzyme activity and pH at 5 levels with 16 axial points (α =2). Fourteen repetitions were carried out in the central point to obtain estimates of error. This resulted in 31 experiments to examine variable stability and each experiment was repeated, overall there were 62 experiments. The selected runs were randomized. Results obtained in the laboratory were analyzed by regression instruction based on RSM (Montgomery, 2001). It was clear that the CCD was a good tool for describing curvature, which describes non-linear variation behavior. A second-order polynomial model equation was used in this study (Montgomery, 2001).

$$Y = b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+ b_{11}X_1^2+b_{22}X_2^2 +b_{33}X_3^2+b_{44}X_4^2+b_{12} X_1X_2 + b_{13}X_1X_3+ b_{14}X_1X_4+b_{23}X_2X_3 +b_{24}X_2X_4+ b_{34}X_3X_4 \qquad (a)$$

In the above equation, the predicted response is Y (removal percentage of 2,4-DCP) and X_1 (pH), X_2 (substrate concentration), X_3 (H_2O_2 concentration) and X_4 (enzyme activity) are the independent variable to influence the response Y.

b_1, b_2, b_3 and b_4 are linear effect coefficients of each variable, b_{11}, b_{22}, b_{33} and b_{44} are quadratic effect coefficients, b_{13}, b_{14}, b_{23} b_{24} and b_{34} are mutual effect coefficients of variables and b_0 is a constant coefficient of the central point. The study used Minitab software (Release14) for statistical design, data analysis, histogram chart, 3D curves and 2D plots. A regression model was made with analysis regression coefficients, a variance analysis table (ANOVA), p-values and F-values. A quality assessment of the polynomial model was expressed by the coefficient of determination R^2 and to obtain the maximum point of response, a mathematical method, observational assessment, 3D curves and 2D plots were used. Optimum condition was achieved to obtain maximum removal by desirability function.

RESULTS & DISCUSSION

The arrangement and the results of the 62 experiments were carried out as shown in Table 1. Using Minitab software the coefficients of the empirical model (Eq. a), and their statistical characteristics were evaluated (see Table 2). Table 2 also presents the results of estimation for the model regression coefficients.

A quadratic regression equation was developed to predict the removal of 2,4-DCP within selected conditions using RSM. The regression equation can be explained as follows:

$$Y=92.6906+1.0999X_1-0.0070X_2+1.4793X_3+0.6642X_4-0.0850X_1^2-0.0000X_2^2-1.1745X_3^2-0.0464X_4^2+0.0008X_1X_2-0.0251X_1X_3-0.0287X_1X_4+0.0098X_2X_3-0.0001X_2X_4+0.1510X_3X_4 \qquad (b)$$

Table 1. Design table showing the randomized run order of experiment, and uncoded values of the different variables in the experimental design for the determination of modelled response (Eq. a)

Run order	PtType	Blocks	pH	Substrate concentration (mg/L)	H_2O_2 concentration (mM)	Enzyme activity (U/mL)	Removal of 2,4-DCP (%)	
							Experimental	predicted
1	1	1	8	200	2.452	3.05	97.5	98.6
2	0	1	6.5	300	1.839	6	99.5	99.7
3	-1	1	5	200	2.452	8.95	99.4	99.3
4	0	1	9.5	300	1.839	6	98.5	98.7
5	1	1	9.5	300	1.839	6	98.7	98.7
6	-1	1	8	400	1.226	3.05	96.9	97.0
7	0	1	6.5	500	1.839	6	96.7	97.8
8	-1	1	5	400	2.452	3.05	98.0	98.5
9	-1	1	6.5	100	1.839	6	98.9	99.3
10	1	1	5	200	1.226	8.95	99.1	99.6
11	1	1	8	400	2.452	3.05	98.0	98.2
12	-1	1	5	200	2.452	3.05	98.0	98.6
13	1	1	5	200	1.226	3.05	98.7	99.3
14	1	1	6.5	300	1.839	6	99.4	99.7
15	1	1	6.5	300	1.839	0.1	96.0	96.2
16	0	1	5	400	2.452	8.95	99.5	99.8
17	0	1	6.5	300	3.065	6	98.4	98.5
18	1	1	6.5	300	0.613	6	96.0	96.0
19	-1	1	3.5	300	1.839	6	98.0	98.0
20	1	1	6.5	300	0.613	6	96.0	96.0
21	1	1	3.5	300	1.839	6	98.0	98.0
22	1	1	5	400	1.226	8.95	97.7	97.6
23	-1	1	5	400	2.452	8.95	99.8	99.8
24	1	1	6.5	300	1.839	6	99.3	99.7
25	0	1	8	200	2.452	3.05	97.7	98.6
26	1	1	5	400	1.226	3.05	96.0	96.1
27	0	1	8	400	1.226	3.05	97.1	97.0
28	1	1	6.5	300	1.839	6	99.4	99.7
29	1	1	8	200	1.226	8.95	98.3	98.5
30	-1	1	6.5	300	1.839	6	99.1	99.7
31	1	1	8	400	2.452	8.95	99.3	99.3

Table 1. Design table showing the randomized run order of experiment, and uncoded values of the different variables in the experimental design for the determination of modelled response (Eq. a)

Run order	PtType	Blocks	pH	Substrate concentration (mg/L)	H_2O_2 concentration (mM)	Enzyme activity (U/mL)	Removal of 2,4- DCP (%)	
							Experimental	predicted
32	0	1	6.5	300	1.839	6	99.6	99.7
33	0	1	5	200	2.452	3.05	97.3	98.6
34	-1	1	6.5	300	1.839	6	99.1	99.7
35	-1	1	6.5	300	1.839	6	99.5	99.7
36	1	1	6.5	300	1.839	6	98.8	99.7
37	1	1	6.5	300	1.839	6	99.3	99.7
38	-1	1	6.5	300	1.839	6	99.4	99.7
39	0	1	5	200	1.226	8.95	99.6	99.6
40	1	1	5	200	1.226	3.05	98.8	99.3
41	1	1	6.5	300	3.065	6	98.8	98.5
42	1	1	6.5	500	1.839	6	97.5	97.8
43	-1	1	6.5	300	1.839	11.9	98.9	99.0
44	-1	1	6.5	300	1.839	6	99.3	99.7
45	1	1	6.5	300	1.839	6	99.5	99.7
46	0	1	8	400	1.226	8.95	97.1	97.1
47	-1	1	5	400	2.452	3.05	98.6	98.5
48	0	1	6.5	300	1.839	6	99.5	99.7
49	1	1	6.5	300	1.839	11.9	98.9	99.0
50	0	1	8	200	2.452	8.95	99.5	99.4
51	1	1	5	400	1.226	3.05	96.1	96.1
52	0	1	6.5	300	1.839	0.1	96.0	96.2
53	-1	1	8	400	2.452	8.95	99.0	99.3
54	1	1	8	200	1.226	3.05	98.5	99.2
55	1	1	8	200	1.226	3.05	98.9	99.2
56	-1	1	8	200	2.452	8.95	99.2	99.4
57	1	1	8	400	1.226	8.95	97.2	97.1
58	1	1	5	400	1.226	8.95	95.9	97.6
59	1	1	8	400	2.452	3.05	98.1	98.2
60	1	1	6.5	100	1.839	6	98.6	99.3
61	1	1	8	200	1.226	8.95	98.1	98.5
62	1	1	5	200	2.452	8.95	99.0	99.3

Table 2. statistical evaluation of estimated regression coefficients for quadratic response (Eq.a)

Term	Coefficient	SE coefficient	T	P
Constant	92.6906	2.08192	44.522	0.000
X_1(pH)	1.0999	0.36855	2.984	0.004
X_2(Substrate concentration)	-0.0070	0.00499	-1.404	0.167
X_3(H_2O_2 concentration)	1.4793	0.81329	1.819	0.075
X_4(Enzyme activity)	0.6642	0.15906	4.176	0.000
X^2_1(PH* PH)	-0.0850	0.02283	-3.726	0.001
X^2_2(Substrate concentration * Substrate concentration)	-0.0000	0.00001	-6.403	0.000
X^2_3(H_2O_2 concentration * H_2O_2 concentration)	-1.1745	0.13668	-8.594	0.000
X^2_4(Enzyme activity * Enzyme activity)	-0.0464	0.00590	-7.863	0.000
X_1X_2(PH* Substrate concentration)	0.0008	0.00046	1.848	0.071
X_1X_3(PH* H_2O_2 concentration)	-0.0251	0.07467	-0.337	0.738
X_1X_4(PH* Enzyme activity)	-0.0287	0.01552	-1.848	0.071
X_2X_3(Substrate concentration * H_2O_2 concentration)	0.0098	0.00112	8.766	0.000
X_2X_4(Substrate concentration * Enzyme activity)	-0.0001	0.00023	-0.391	0.697
X_3X_4(H_2O_2 concentration * Enzyme activity)	0.1510	0.03797	3.978	0.000
R-Sq = 90.7%				
R-Sq(adj) = 88.0%				

The analysis was done using uncoded units

X_1 represents pH, X_2 is for the substrate concentration, X_3 is for H_2O_2 concentration, and X_4 is the enzyme activity. With regard to regression coefficients sign, the positive effects of pH, H_2O_2 concentration and enzyme activity were shown in the above mentioned equation, but substrate concentration had negative effect on 2,4-DCP removal (Y). 2,4-DCP removal was increased by pH, H_2O_2 concentration and enzyme activity increase, while it decreased with substrate concentration.

Kennedy, in optimizing enzymatic treatment of wastewater containing 2,4-DCP, found that by increasing substrate concentration from 100 to 300 mg/L, the removal percentage decreased from 83.5% to 71.5% (Kennedy *et al.*, 2002). Fang, in a study on the removal of aromatic compounds by HRP showed that H_2O_2 augmentation increased the removal of aromatic compounds (Fang and Barcelona, 2003). Research by Bollag and Fang on treatment based on HRP enzyme, stated that H_2O_2 and enzyme activity augmentation increased the percentage of 2,4-DCP that was effectively removed (Fang and Barcelona, 2003; Bollag and Dec, 1998).

This relatively high estimated value of the determination coefficient as a percentage (R^2 =90.7 %) shows a high correlation between data from the actual experiment and the predictions. The value of R^2 shows the model fitted 90.7 % of data from the experiment.
In Table 2 R^2(adj) value was also very near to the R^2 value, proof that there was no need for correction due to sample size and the number of factors in the model. The value of R^2 was also a confirmation of the model's accuracy in that only 9.3% of the total variations were not supported by a response.
The quality of the regression, estimated by the analysis of variance (ANOVA), is displayed in Table 3.

In the ANOVA table, the Fisher variance ratio is given by the equation, F-value $=Sr^2 / Se^2$ (Sr^2 is the ratio of the mean square due to regression and Se^2 is mean square due to error), and is a statistically valid measure of how well the factors explain the variation in the data about the mean. When the model is a suitable predictor of the experimental data, the calculated F-value can be greater than the tabular F-value. The evaluated values given in the ANOVA table, exhibit the model as highly important, as the calculated F-value (32.94) is much higher than the tabular F-Value (2.76) at a level of 5%. The significance of the model is also approved for the linear, square and mutual interaction factors.

Table 3. statistical analysis of variance (ANOVA) for the evaluated response

Source	DF	Seq. SS	Adj.SS	Adj.MS	F	P
Regression	14	69.5576	69.5576	4.96840	32.94	0.000
Linear	4	31.9606	4.6309	1.15773	7.67	0.000
Square	4	22.5473	22.5473	5.63682	37.37	0.000
Interaction	6	15.0497	15.0497	2.50828	16.63	0.000
Residual Error	47	7.0901	7.0901	0.15085		
Lack-of-Fit	10	6.6001	6.6001	0.66001	49.84	0.000
Pure Error	37	0.4900	0.4900	0.01324		
Total	61	76.6477				

The p-levels are a tool to control the important part of each of the regression coefficients.

The p-values <0.05 are the more important. Table 2 shows all the linear, square, and interaction factors (except X_2, Substrate concentration with p-value =0.167), X_3 (H_2O_2 concentration) with p-value =0.075, X_1X_2 (pH*Substrate concentration) with p-value =0.071، X_1X_3 (pH*H_2O_2 concentration) with p-value =0.738 ،X_1X_4 (pH*Enzyme activity) with p-value =0.071, X_2X_4 (Substrate concentration*Enzyme activity) with P-value =0.697 are significant (at α <0.05 level). Therefore, the model confirms the attendance of curvature in the response surface. Once again the p-values for the regression (Table 2) matched the model. Considering that some of the main and mutual effects in the model were not important, they were ignored. Furthermore, because factors such as substrate concentrationand H_2O_2 concentration were important for some of the parameters, these main factors were not in themselves important so they were kept in the model and three mutual effects X_1X_2 (pH*Substrate concentration), X_1X_3 (pH*H_2O_2 concentration) and X_1X_4 (pH*Enzyme activity) were ignored, and then regression coefficients were estimated and the variance analysis table (ANOVA) was evaluated repeatedly. More assessment of regression analysis gave the regression coefficients and these were substituted in equation (a) and the predicted response (Y) was cleared which was close to the experimental results, fitting the model.

Due to new analysis, all main and mutual effects were significant (p-value <0.05) and R^2 for this model was 89.4%. Analysis showed that the model was suitable and changes in the response can be assessed by 4 factors. The variance analysis table showed that because p-value =0.000 and F-values were large the second-order model was completely suitable.

It should be noted that the computation executed after omission of the non-significant factors did not perceptibly enhance the quality of the quadratic adequacy (e.g. initial R^2 =90.7% changed to R^2 =89.4% and initial R^2 (adj) =88% changed to R^2 (adj) =87%). It is clear that such omission cannot always assure the enhancement of the model (Mason *et al.*, 2003).

The model was certified after further statistical modeling and 3 theories were assessed: 1) error normality 2) error variance stability 3) independence of error. In order to assess error normality, a normal probability plot was used. Results of the plot of residuals displayed that data were stable and unusual points were not significant. It also displayed that the residuals usually fall on a straight line implying that the errors were divided normally.

In order to assess variance stability, a plot showing residuals versus fitted values was used. Due to the none-cyclic and specific behavior, residuals' stability was confirmed and residuals versus order of the data plot showed there was no coordination between residuals.

It can be concluded from these analyses that the suggested model was suitable and there was no reason to doubt the independence or constant variance assumptions.Optimization may be carried out using mathematical (numerical) or graphical (contour plot) approaches.

Figs. 1a to 1f show the various three-dimensional plots of the model. These plots are useful for visualizing the response surface generated by the model.
In this study, plots expressed by the regression model shown in Fig.s 1a to 1f that the 2,4-DCP quantity (Y) was affected by two factors (in each figure two other factors were considered constant).

Eventually, after making the regression model, a numerical optimization method by desirability function was implied to optimize the response.A useful approach to optimization of multiple responses was to use the simultaneous optimization technique popularized by Derringer and Suich (1980).

The general approach is to first change each response y_i, i =1,2,…,m into an individual desirability function di that varies over the range :

$$0d \le d_i d \le 1$$

If the response y_i, is at its target, Then d_i =1, and if the response is outside a tolerable area, d_i =0. The responses to H_2O_2 concentration, substrate concentration, enzyme activity and pH were transmuted into an appropriate desirability scale d_1, d_2, d_3 and d_4.

Once function d_i was explained for each of the m responses of interest, an overall impartial function (D), representing the global desirability function, was

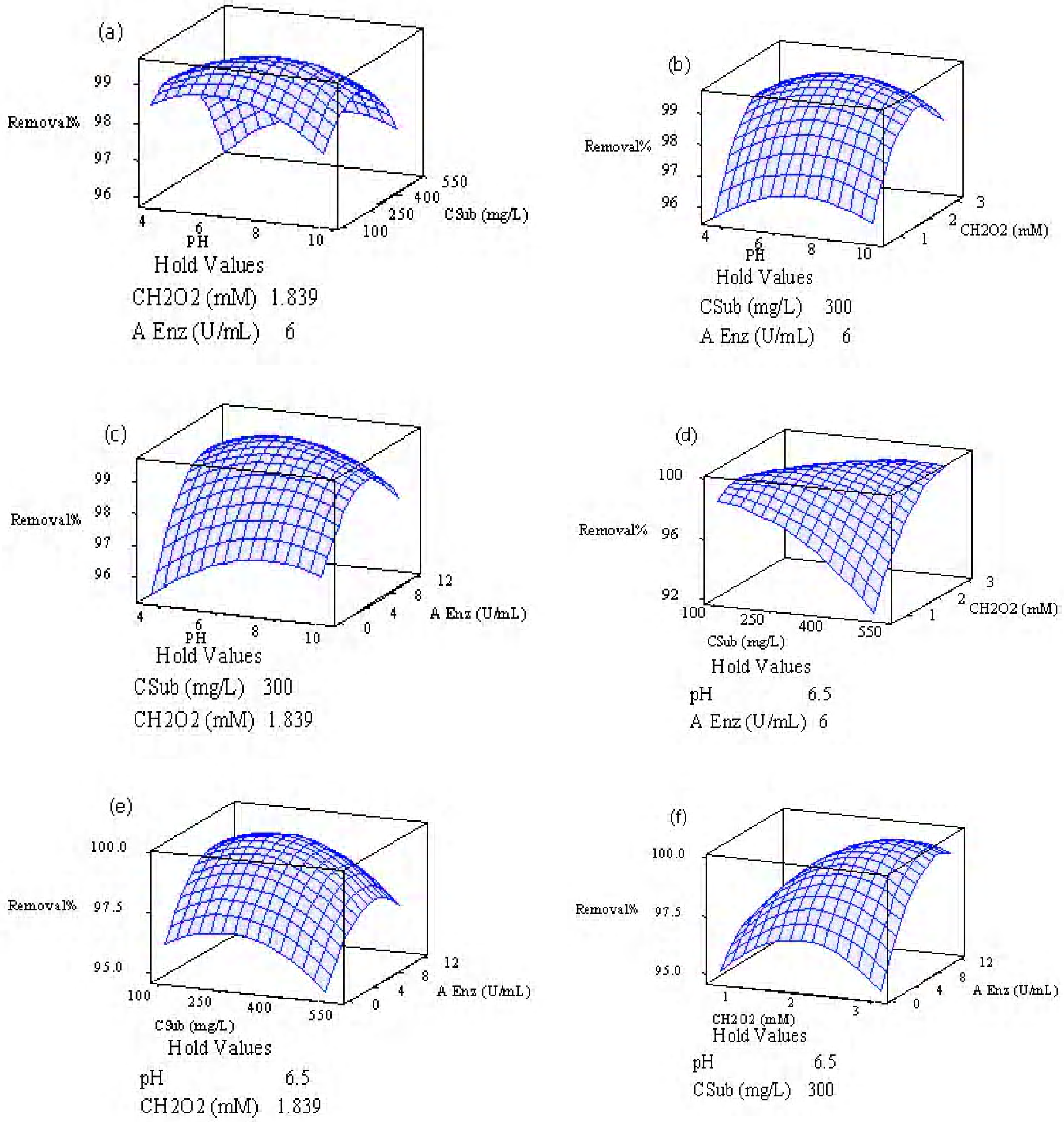

Fig. 1a-1f. Three -dimensional plot of response surface plots (Y). (a) response plot of substrate concentration (mg/L) vs. pH; (b) response plot of H_2O_2 concentration (mM) vs. pH; (c) response plot of enzyme activity (U/mL) vs. pH; (d) response plot of H_2O_2 concentration (mM) vs. substrate concentration (mg/L); (e) response plot of substrate concentration (mg/L) vs. enzyme activity (U/mL); (f) response plot of H_2O_2 concentration (mM) vs. enzyme activity (U/mL)

computed. Then the design variables were selected to maximize the overall desirability, where m was the number of responses to be optimized (Myers and Montgomery, 2002).

$$D = (d_1 d_2 \ldots d_m)1/m$$

Table 4 is a summary of desirability function parameters.

Table 4. Parmeters of function desirability

Goal	Lower	Target	Upper	Weight	Import
Maximum	96	100	100	1	1

Optimized quantities were achieved for a global solution (Table 5).
Fig. 2 indicates the recommended values for each factor to achieve optimal response.

The graph in Fig. 2 indicates how individual factors in each column influence the response while the other factors are held constant. The values between Hi and Lo values optimal parametric setting were recommended by the software to obtain the most suitable responses. In Fig. 2, D is the composite desirability and d is the individual desirability. The maximum values for D and d are 1.000 (Myers and Montgomery, 2002). Fig. 2 shows values for D and d in optimal conditions as 0.959, confirming that the model proposed is suitable .

Table 5. Optimum values of global solution

Parameter	Value
pH	6.5
Substrate concentration (mg/L)	430.80
H_2O_2 concentration (mM)	3.06
Enzyme activity (U/mL)	9.74

In the recommended optimal model, parametric settings of pH (6.5), substrate concentration (430.8 mg/L), H_2O_2 concentration (3.06 mM) and enzyme activity (9.74 U/mL) were set. The response for this set of values for the removal of 2,4-DCP with desirability of 0.959 was 99.83%. Therefore, the predicted optimum condition was taken as (pH 6.5, H_2O_2 concentration 3.06 mM, substrate concentration 430.8 mg/L and enzyme activity 9.74 U/mL) for application for the measurement of assay 2,4-DCP in the sample for the experiment. Tests for optimization using the linear range method were determined by a calibration curve (2,4-DCP area against 2,4-DCP concentration) see Fig. 3. The determination of an optimal condition was by the measurement of 2,4-DCP in each sample; its 2,4-DCP content was calculated, the sample response for maximum removal was %99.2 (see Fig. 3).

From Table 6, the difference between the predicted result and the values demonstrated by the experiment, show that under an optimal condition for 2,4-DCP the

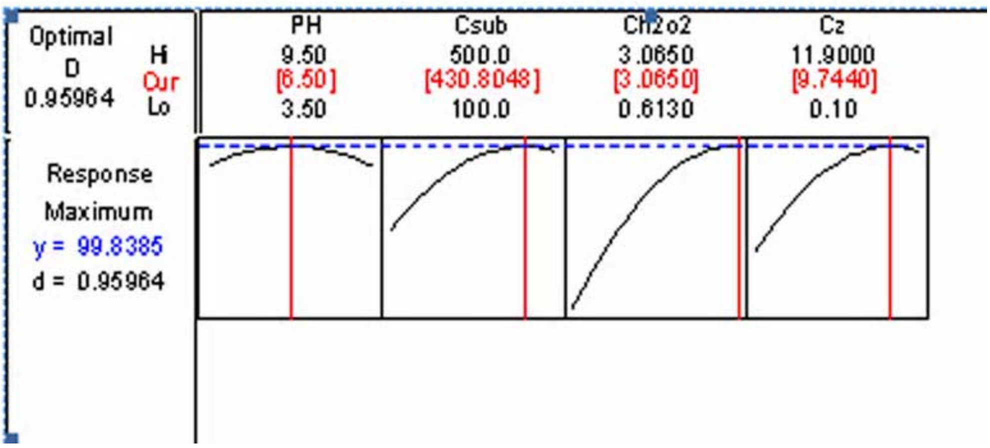

Fig. 2. recommended input variables

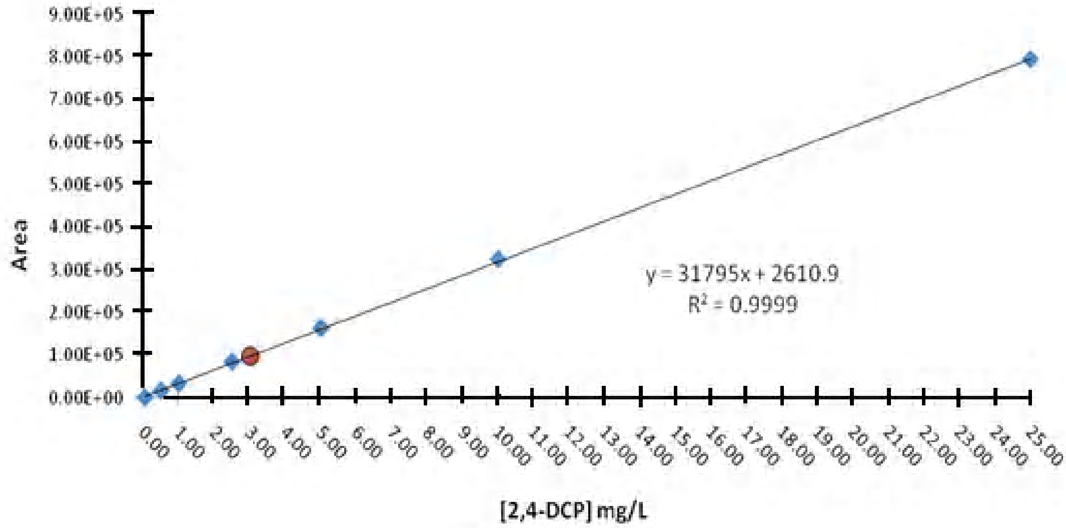

Fig. 3. calibration curve (area vs.concentration) using standard 2,4-DCP solution(■) and the resulting experimental area value (●) obtained for sample using the optimized conditions method

Table 6. Optimal removal conditions and the predicted and experimental value for 2,4-DCP

Optimum conditions		Removal of 2,4-DCP (%)		
Parameter	Value	Experimental	predicted	Difference (%)
pH	6.5	99.2	99.83	0.63
Substrate concentration (mg/L)	430.80			
H_2O_2 concentration (mM)	3.06			
Enzyme activity (U/mL)	9.74			

difference was 0.63%. This was presented to demonstrate that the response model was a suitable tool to display the predictions.

CONCLUSION

In this study, RMS combined with CCD was successfully used to optimize the four factors; pH, enzyme activity, substrate concentration and H_2O_2 concentration, in order to remove the optimum amount of 2,4-DCP from wastewater. The non-linear nature of the model response for this system was explained by a second–order polynomial equation. It was shown how this method was suitable for process design and to determine the importance of the various factors, and their mutual effects to obtain optimized quantities. Conditions were optimized in pH (6.5), with enzyme activity (9.74 U/mL), substrate concentration (430.8 mg/L) and H_2O_2 concentration (3.06 mM) and maximum removal of 2,4-DCP under these conditions. By using desirability function outcomes (99.83%) of those predicted the optimum quality assay result for 2,4-DCP was accomplished in one sample under the optimum condition. The result was that 99.2 % of 2,4-DCP was removed, confirming the optimization model.

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