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Optimization of Horseradish Peroxidase Catalytic Degradation for 2-Methyl-6-Ethylaniline Removal Using Response Surface Methodology

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Received: 8 May 2019; Accepted: 22 May 2019; Published: 24 May 2019

Abstract: For optimizing the reaction conditions of 2-methyl-6-ethylaniline (MEA) degradation catalyzed by horseradish peroxidase (HRP), a response surface methodology with three factors and three levels was used in this research to establish a regression model, a ternary quadratic polynomial, in order to analyze temperature, H_2O_2 concentration and pH effects on MEA removal efficiency. The results showed that the regression model was significant ($p < 0.0001$), fitted well with experimental data and had a high degree of reliability and accuracy, and the data were reasonable with low errors. By analyzing interactions and solving the regression model, the maximum MEA removal efficiency was 97.90%, and the optimal conditions were defined as follows: pH 5.02, H_2O_2 concentration 13.41mM, and temperature 30.95 °C. Under the optimal conditions, the average MEA removal efficiency obtained from the experiments was 97.56%. This research can provide reference for the treatment of actual acetochlor industrial wastewater.

Keywords: horseradish peroxidase; 2-methyl-6-ethylaniline; removal efficiency; response surface methodology

1. Introduction

Acetochlor is a kind of systemic chloroacetamide herbicide, which has been widely used as the pesticides for control of some annual broadleaf and grass weeds in China [1]. Acetochlor industrial wastewater contains large amounts of highly toxic organic pollutants and heavy metals [2–4]. 2-methyl-6-ethylaniline (MEA) which belongs to the aniline compounds is the main metabolite of acetochlor in the wastewater [5]. On account of their high toxicity, persistence and bioaccumulation, aniline compounds are one of the priority pollutants listed by the United States Environmental Protection Agency (US EPA) [6]. Conventional removal methods of aniline compounds from wastewaters are mainly physical, chemical and biological methods. Through adsorption and desorption experiments, Tao et al. [7] found 13X molecular sieves synthesized from natural rock suitable for the purification of aniline-bearing wastewater. At pH 3.0, 40 °C and constant current 20 A, Brillas and Casado [8] found that degradation efficiency of the soluble total organic carbon gradually reached 61% after 2 h by the electro-Fenton process for aniline wastewater treatment in a flow reactor. Bacteria BA1, BA2 and BA3 were isolated from an aniline wastewater treatment reactor by Wang et al [9], and the removal efficiency of soluble COD in aniline wastewater reached 81% using the optimal combination of these three bacteria in the experimental process. These methods proved to be feasible and available, but they suffer from such shortcomings as low removal efficiency, high treatment cost, and secondary contamination. Thus, harmless treatment of aniline compounds in wastewater is urgent.

In fact, the use of natural enzymes in aniline compounds treatment has been proposed by many researchers [10–12]. Horseradish peroxidase (HRP) is a heme-containing enzyme that can catalyze the oxidation of certain organic pollutant molecules using hydrogen peroxide [13]. Most oxidation reactions catalyzed by HRP can be described [14] by the following reaction equations, Equations (1)–(3). In the equations, AH_2 and $AH\cdot$ represent a kind of reducing substrate and its free radical product, respectively. E is the original enzyme (i.e., HRP), E_I and E_{II} are its intermediates.

$$E + H_2O_2 \rightarrow E_I + H_2O \tag{1}$$

$$E_I + AH_2 \rightarrow E_{II} + AH^{\bullet} \tag{2}$$

$$E_{II} + AH_2 \rightarrow E + AH^{\bullet} + H_2O \tag{3}$$

In addition, certain indirectly degraded pollutants (e.g., polychlorinated biphenyls and metolachlor) could be adsorbed by the polymers of peroxidase catalytic reaction [15]. HRP is suitable for the treatment of refractory organic pollutants due to its extensive applicability [16], high catalytic activity [17] and mild reactive condition [18]. In order to maximize the removal efficiency of pollutants, how to optimize the reaction conditions needs to be explored in depth.

The traditional single-factor optimization tests one variable while keeping all other influence factors constant. When faced with systems of multi-variables, this method is cumbersome and time-consuming, or even leads to the drawing of wrong conclusions [19]. Response surface methodology (RSM) is a useful statistical tool. It can be used to optimize the response through analyzing and evaluating the effects of multiple variables and their interactions [20]. Based on experimental design and mathematical modeling, RSM establishes a regression equation for fitting function relation between influence factors and experimental result [21]. With this method, fewer experiments could provide sufficient information on the effects of variables [22]. This study was focused on the HRP catalyzed oxidative process of MEA removal. RSM was used to optimize three variables (temperature, pH and concentration of H_2O_2). The final objective was to discuss the possibility of HRP catalytic degradation for MEA, and to maximize the removal efficiency of MEA.

2. Materials and Methods

2.1. Experimental Materials

HRP (EC—Enzyme Commission 1.11.1.7) and catalase (EC 1.11.1.6) were purchased from Merck KGaA (Darmstadt, Germany). MEA was purchased from Ruize (Dalian, China). Acetonitrile (High performance liquid chromatography, HPLC grade) was purchased from Tedia (Fairfield, CA, USA). All other chemicals (e.g., hydrochloric acid, citric acid, potassium dihydrogen phosphate, and sodium hydroxide) purchased from Kemiou Chemical Reagent (Tianjin, China) were analytically pure.

2.2. Experimental Procedure

In order to resist the pH variety produced by the change of reaction solution quantity, various pH buffer solutions (200 mM phosphate buffer) were prepared at different temperatures. MEA was added into the buffer solutions for the preparation of 3.5 mM simulated water sample [23]. The centrifuge tube containing simulated water sample and a predetermined amount of HRP was preheated in a temperature-controlled incubator, shaking at 200 rpm. The reaction was initiated by addition of H_2O_2 whose concentration was changing in experiments, and inhibited by adding catalase after 6 h.

2.3. Analysis Methods

After being filtered by 0.22 µm aperture micropore film, the residual MEA of reaction solutions was detected through HPLC (Agilent 1100), containing a diode array ultraviolet detector and a reversed phase chromatographic column (YWG C-18, 4.6 × 250 mm, Dalian Elite Analytical Instruments Co., Ltd., Dalian, China). The mobile phase was composed of acetonitrile and water with volume fraction of 65/35, flow rate at 1.0 mL/min. The MEA with detective wavelength of 220 nm was eluted at 9.8 min. MEA removal efficiency was determined using Equation (4).

$$\text{MEAremovalefficiency}\ (\%) = \frac{C_i - C_f}{C_i} \times 100 \tag{4}$$

In this equation, C_i and C_f represent the initial and final concentrations of MEA, respectively.

2.4. Experimental Design and Optimization Method

Central composite design (CCD), a frequently used design of RSM, can be applied to the optimization of reaction conditions. Appropriate axial points in CCD can ensure the rotation and sequentiality of the experiments [24]. For a three-factor CCD, extreme values of axial points are ±1.68 ($\pm 2^{3/4}$). The levels of three experimental variables (pH, temperature and concentration of H_2O_2) are presented in Table 1.

Table 1. Experimental variables and levels for central composite design (CCD), $[H_2O_2]$: H_2O_2 concentration.

Coded Level	**Uncoded Level**		
	Temperature (°C)	**pH**	**$[H_2O_2]$ (mM)**
−1.68	23.59	2.11	4.59
−1	27.00	3.00	8.00
0	32.00	4.30	13.00
1	37.00	5.60	18.00
1.68	40.41	6.49	21.41

A three factor-three level CCD included 6 axial points, 6 center points and 8 star points, 20 runs in total. The data obtained from the CCD experiments were calculated by the software Design Expert 8.0.6 (Stat-Ease), and the response of MEA removal efficiency to three experimental variables was fitted to the general model a second order polynomial as Equation (5) below.

$$Y = B_0 + \sum_{i=1}^{n} B_i X_i + \sum_{i=1}^{n} B_{ii} X_i^2 + \sum_{1 \le i < j \le n}^{n} B_{ij} X_i X_j \tag{5}$$

In this equation, Y is the dependent variable (i.e., the MEA removal rate), X_i the ith independent variable (temperature, pH or H_2O_2 concentration), B_0 constant terms, B_i the linear coefficient, B_{ii} the coefficient of square term, and B_{ij} the interaction coefficient.

Via variance analysis, the fitting quality of the polynomial quadratic equation model was assessed by different criteria, such as F value, P value and R^2. The F value and the P value determined significances of the model and the quadratic equations items. The larger the F value while the smaller the P value, the more significant the terms accordingly. The model-fitting effect was evaluated by the coefficient of determination R^2 that the closer its value to 1, the higher model fitting degree. Combining with the analysis of contour plots of the response surface, the regression equation was solved and the optimal values of investigated variables were determined.

3. Results and Discussion

3.1. Model Fitting

Experiments were carried out to evaluate the effects of condition variables on the removal rate. It was based on the CCD design and repeated three times at each point. The CCD experimental schemes and results of removal efficiency were noted down. As shown in Table 2, the removal efficiency of MEA in the CCD experiments varied from 57.35% to 98.08%, and the average removal efficiency of MEA was 80.48% in different reaction conditions. Removal efficiency higher than 90% was only observed in center runs, which indicated that the CCD-based experimental schemes were reasonable.

Table 2. The CCD experimental schemes and results.

Run	Type	Uncoded Level			Removal Efficiency of MEA (%)
		Temperature (°C) X_1	pH X_2	$[H_2O_2]$ (mM) X_3	
1	Star	27.00	3.00	8.00	71.30
2	Star	37.00	3.00	8.00	64.35
3	Star	27.00	5.60	8.00	86.35
4	Star	37.00	5.60	8.00	75.21
5	Star	27.00	3.00	18.00	63.35
6	Star	37.00	3.00	18.00	64.58
7	Star	27.00	5.60	18.00	82.89
8	Star	37.00	5.60	18.00	82.08
9	Axial	23.59	4.30	13.00	79.15
10	Axial	40.41	4.30	13.00	62.84
11	Axial	32.00	2.11	13.00	57.35
12	Axial	32.00	6.49	13.00	88.15
13	Axial	32.00	4.30	4.59	75.97
14	Axial	32.00	4.30	21.41	83.33
15	Center	32.00	4.30	13.00	93.05
16	Center	32.00	4.30	13.00	95.83
17	Center	32.00	4.30	13.00	95.50
18	Center	32.00	4.30	13.00	98.08
19	Center	32.00	4.30	13.00	94.28
20	Center	32.00	4.30	13.00	94.78

MEA is the abbreviation of 2-methyl-6-ethylaniline.

The polynomial quadratic equation model for the removal efficiency of MEA was deduced through the Design-Expert software (Ver. 8.0.6, Stat-Ease, Minneapolis, MN, USA). The coefficients of the equation were calculated by the least squares method. After insignificant terms were excluded, the second order polynomial was presented in terms of the coded values for the removal efficiency of MEA. The results indicated that the polynomial quadratic model provided the best fit, and the ultimate equation was as Equation (6) below.

$$Y\% = -350.07 + 20.41X_1 + 47.60X_2 + 1.89X_3 - 0.14X_1{\cdot}X_2 + 0.09X_1{\cdot}X_3 + 0.24\,X_2{\cdot}X_3 - 0.34X_1^2 - 4.62X_2^2 - 0.21X_3^2 \quad (6)$$

where $Y\%$ is the removal efficiency of MEA, X_1, X_2 and X_3 were the temperature, pH and concentration of H_2O_2, respectively.

The response results of the removal efficiency of MEA were indicated through variance analysis, as shown in Table 3. From the whole model, lack of fit was not insignificant ($P > 0.05$) with the extremely low P value (<0.0001), and the corresponding F value being 45.02. All of these suggested that the model fitting was valid. The R^2 (coefficient of determination) was 0.9759, which indicated that 97.59% of the variations in the response could be explained by the fitting model within the variable ranges. The CV (coefficient of variation) of the repeated experiments was 3.44%, within the acceptable range of 10%, which showed that the experiments data had good reliability [25]. Adeq precision, the signal to noise ratio of the experiments, was 18.419 (greater than 4), which indicated that the model had enough signal intensity in response to the design [26]. In the model, p value of pH was less than 0.05, which indicated that pH was a significant factor of MEA removal. In the binomial, temperature, H_2O_2 concentration and pH were a significant factor of MEA removal. In the interaction terms, all the p value great than 0.05, which indicated that the interaction between temperature and pH, temperature and H_2O_2 concentration, and pH and H_2O_2 concentration was not obvious.

Table 3. The variance analysis of the fitting model.

Source	Sum of Squares	DF	Mean Square	*F* Value	*P* Value
Model	3110.09	9	345.57	45.02	<0.0001
X_1	141.9	1	141.9	18.49	0.0016
X_2	945.73	1	945.73	123.2	<0.0001
X_3	3.56	1	3.56	0.46	0.5112
$X_1 \cdot X_2$	6.72	1	6.72	0.88	0.3716
$X_1 \cdot X_3$	37.95	1	37.95	4.94	0.0504
$X_2 \cdot X_3$	18.69	1	18.69	2.44	0.1497
${X_1}^2$	1024.32	1	1024.32	133.44	<0.0001
${X_2}^2$	878.93	1	878.93	114.5	<0.0001
${X_3}^2$	415.7	1	415.7	54.15	<0.0001
Residual	76.76	10	7.68		
Lack of fit	62.36	5	12.47	4.33	0.0667
Pure error	14.4	5	2.88		
Cor Total	3186.85	19			

The assumption of normality, the predicted removal efficiencies versus the actual ones, and the predicted responses versus the residual ones were listed in in Figures 1–3. As shown in Figure 1, the residuals were, in satisfying nearly linear distribution, suggesting that the errors were in bell-shaped normal distribution. As evident from the removal efficiencies of MEA in Figure 2, the data points were in distribution along diagonal lines, which implied that the predicted values of the model tallied with the actual experimental results. The points are in random distribution in Figure 3, which indicates that residuals did not contain any predictable information, and the neighboring residuals were not correlated. These illustrated that the polynomial quadratic model, with sufficient recommendations, was valid and the fitting of the equation was successful [27].

The perturbation plot for the removal efficiency of MEA in different temperature, pH and H_2O_2 concentrations is shown in Figure 4. The resulting plot illustrated the effect of temperature, pH and H_2O_2 concentration at central type in CCD (temperature 32 °C, H_2O_2 concentration 13 mM, pH 4.3 for 6 h). The effect of independent variable on the removal efficiency of MEA was in the order pH > temperature > H_2O_2 concentration. A relatively small change of the pH greatly impacted the removal efficiency of MEA. By contrast, the variety of the H_2O_2 concentrations could not lead a notable influence on the MEA removal efficiency. The influence of temperature, a modest factor, was neither too big nor too small.

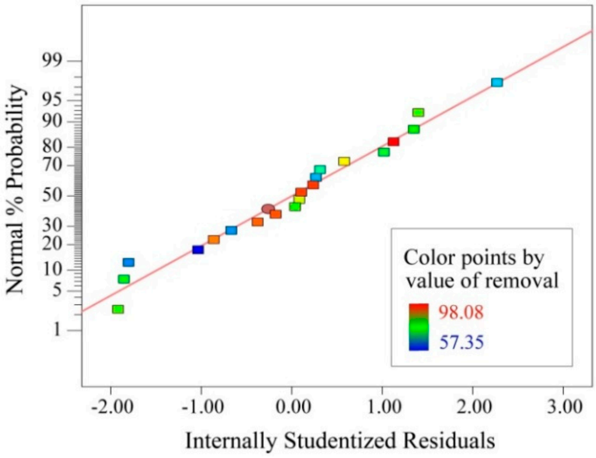


Figure 1. Normal probability of the residuals.

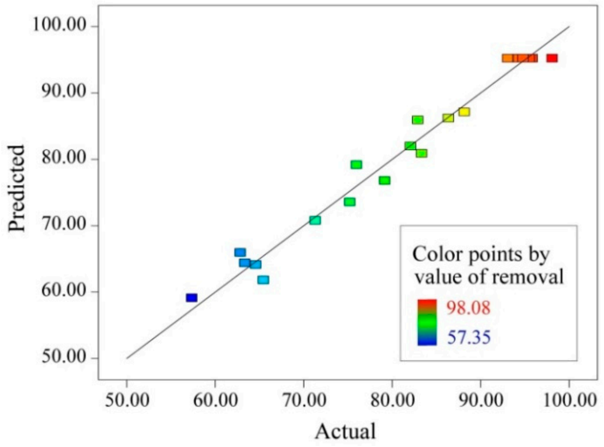


Figure 2. The predicted removal efficiencies versus the actual ones.

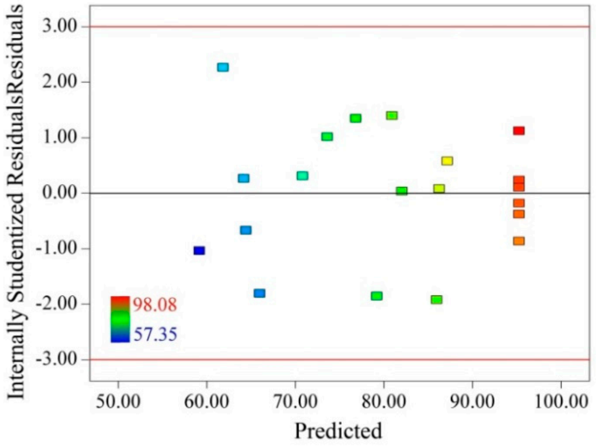


Figure 3. The predicted responses versus the residual ones.

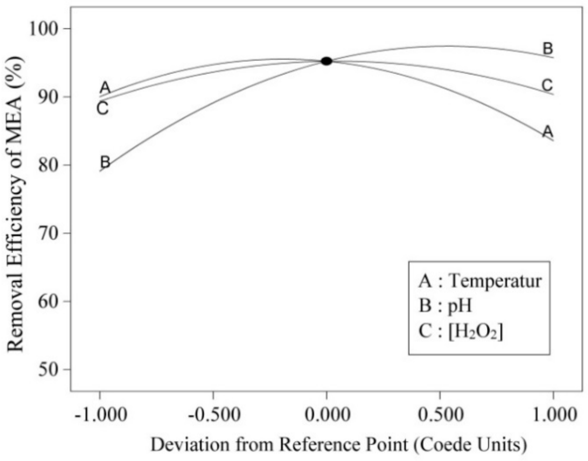
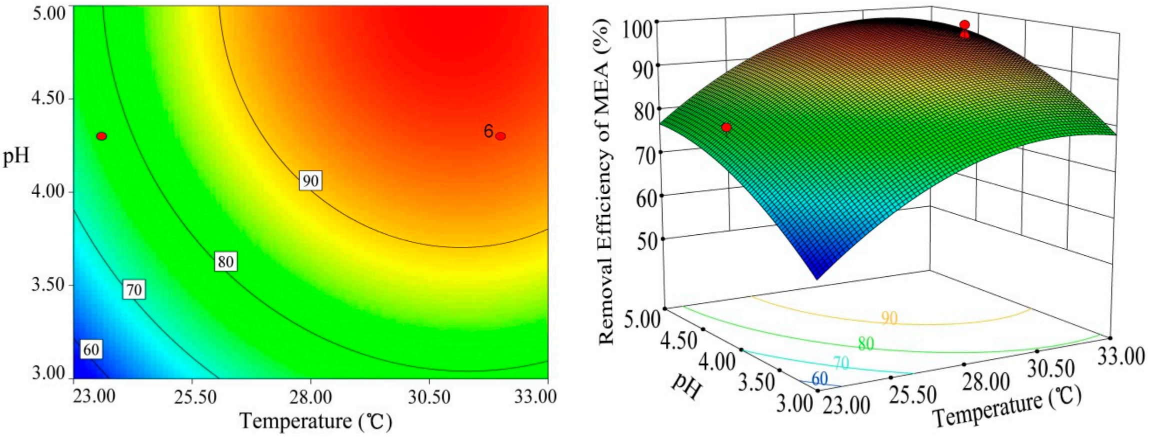


Figure 4. Perturbation plot for the removal efficiency of MEA. A: Temperature; B: pH; C: [H_2O_2] (mM).

3.2. Influence Factor of 2-Methyl-6-Ethylaniline (MEA) Removal Efficiency

For the sake of an intuitive sense on the interactions among temperature, pH and H_2O_2 concentration, the three-dimensional plots of these factors were drawn for two variables at a time, keeping the third factor unchanged (at center level). The combined effects of the pH and the temperature on the removal efficiency of MEA are shown in Figure 5. We could see that the removal efficiency of

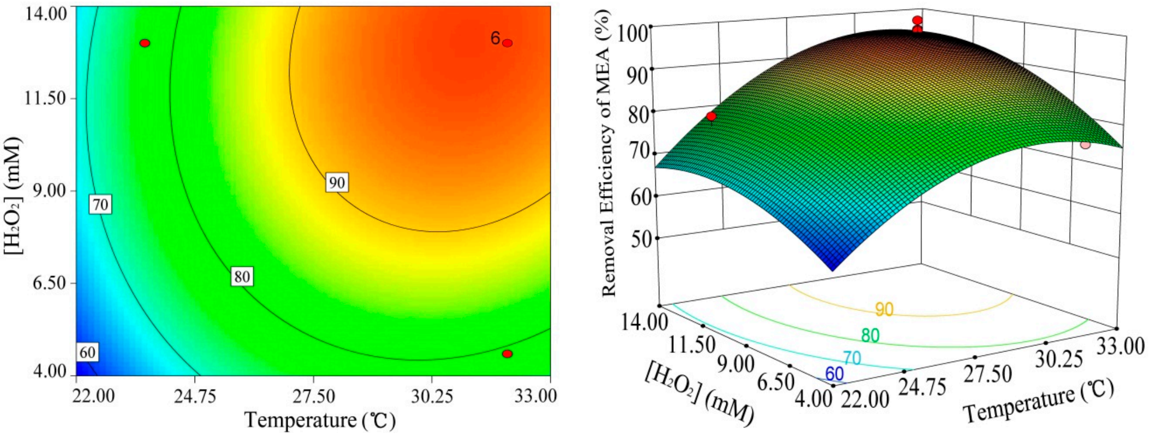
MEA slowly increased at a high level given a high pH, as temperature increased. Similarly, at a high given temperature, with the increase of the pH, the removal efficiency of MEA increased slowly at a high level.



(a) **(b)**

Figure 5. Response surface plots for the temperature and pH at $[H_2O_2]$ = 13.00 mM. (**a**) Contour, (**b**) 3D surface.

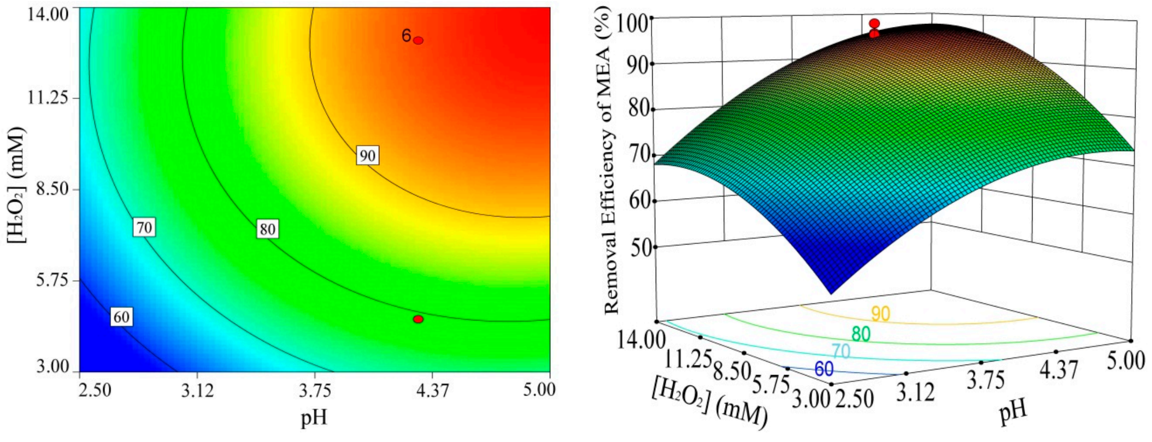
The joint effects of the H_2O_2 concentration and temperature on the removal efficiency of MEA were shown in Figure 6. It could be seen that the removal efficiency of MEA slowly increased at a high level given a high H_2O_2 concentration, as temperature increased. Similarly, at a high given temperature, with the increase of the H_2O_2 concentration, the removal efficiency of MEA increased slowly at a high level.



(a) **(b)**

Figure 6. Response surface plots for the temperature and $[H_2O_2]$ at pH = 4.30. (**a**) Contour, (**b**) 3D surface.

The combined effects of the pH and the H_2O_2 concentration on the removal efficiency of MEA are shown in Figure 7. We can see that the removal efficiency of MEA slowly increased at a high level given a high H_2O_2 concentration, as pH increased. Similarly, at a high given pH, with the increase of the H_2O_2 concentration, the removal efficiency of MEA increased slowly at a high level.



(a) **(b)**

Figure 7. Response surface plots for the t pH and [H_2O_2] at 32.00 °C. (**a**) Contour, (**b**) 3D surface.

As shown in Table 4, by solving the extremum of the ternary quadratic polynomial model, the maximum MEA removal efficiency was 97.90%, and the optimal conditions were defined as follows: pH 5.02, H_2O_2 concentration 13.41 mM, and temperature 30.95 °C. Under the optimal conditions, the average MEA removal efficiency obtained from the experiments was 97.56%. HRP catalytic degradation for MEA removal is the typical enzyme-catalyzed reaction, and the enzyme activity and stability are important factors affecting removal efficiency [28]. In the research on the removal of pentachlor ophenol, Ye et al. [29] suggested that the optimal pH for HRP was 5–6. Studying the inactivation and catalytic pathways of horseradish peroxidase with m-chloroperoxybenzoic acid, Rodriguez-Lopez et al. [30] found that horseradish peroxidase lost its activity in too acid or alkaline conditions. Arnao et al. [31] proposed the two competitive (catalytic and inactivating) routes reaction mechanism in research on the inactivation of peroxidase by hydrogen peroxide. An over-high H_2O_2 concentration inhibited the activity of HRP. Unlike pH and H_2O_2 concentration, the inactivation was mainly caused by HRP denaturation under a high-temperature condition [32]. This was in good agreement with the experimental study on dyestuff degradation catalyzed by HRP [33].

Table 4. The removal efficiency of MEA under the optimal conditions.

Optimum Conditions			**MEA Removal Efficiency (%)**	
pH	H_2O_2 (mM)	Temperature (°C)	Experimental	Predicted
5.02	13.41	30.95	97.56	97.90

4. Conclusions

In this research for optimizing the reaction conditions of MEA degradation catalyzed by HRP, RSM was used to analyze temperature, H_2O_2 concentration and pH effects on MEA removal efficiency. Some conclusions can be drawn as follows:

(1) A regression model for the removal efficiency of MEA, the ternary quadratic polynomial, was established. The variance analysis showed that the regression model was significant ($p < 0.0001$), fitted well with experimental data and had a high degree of reliability and accuracy, and the data were reasonable with low errors.

(2) By analyzing interactions and solving the regression model, the maximum MEA removal efficiency was 97.90%, and the optimal conditions were defined as follows: pH 5.02, H_2O_2 concentration 13.41 mM, and temperature of 30.95 °C. Under the optimal conditions, the average MEA removal efficiency obtained from the experiments was 97.56%.

The results can provide a reference for the treatment of acetochlor industrial wastewater, and the stabilization and reutilization of HRP in the process of the pollutant's degradation are worth further study.

Author Contributions: S.S. and Q.W. designed and directed the experiments; S.S., L.C. and L.M. conducted the experiments; S.S. and J.S. processed and analyzed the data; S.S. and Q.W. wrote the paper; J.S. and Y.X. revised and adjusted the paper. All authors have confirmed the final manuscript.

Funding: This study was financially supported by the sub topic of the National Key Technology R&D Program (No. 2015BAC05B05).

Acknowledgments: The authors express our gratitude and respect to the reviewers and the editors for their hard work and valuable suggestions, which improved the academic level of this paper.

Conflicts of Interest: All authors solemnly declare no conflict of interest.

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