



# Response Surface Methodology to Optimize Degradation on Phenol by *Pseudomonas stutzeri* ZH-1

Yong Yan, Qingping Hu\*

College of Life Science, Shanxi Normal University, Linfen, China  
Email: \*hqp72@163.com

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## Abstract

In order to obtain the optimal conditions for the degradation of phenol by *Pseudomonas stutzeri* ZH-1, based on the single factor experiment, the Box Behnken design test of Response Surface Method was used. Four variables of glucose concentration (0.4, 0.6, 0.8 g/L), inoculation concentration (3%, 5%, 7%), pH (6, 7, 8) and temperature (35°C, 37.5°C, 40°C) were used to identify the significant effects and interactions in the batch studies. A second order polynomial regression model, has been developed using the experimental data. The experimental values were in good agreement with the predicted values, and the correlation coefficient was found to be 0.9901. Optimum conditions of the variables for the growth of *Pseudomonas stutzeri* ZH-1 and for maximum biodegradation of phenol are glucose concentration (0.7 g/L), inoculation concentration 5%, pH = 7 and temperature 37°C with maximum phenol degradation rate of 89.62%.

## Subject Areas

Biochemistry, Bioengineering

## Keywords

Response Surface Methodology, Box-Behnken, Phenol Degradation, *Pseudomonas stutzeri*

## 1. Introduction

Phenol compounds are among the most common pollutants. Phenol and its derivatives are the most representatives of the toxic organic pollutants, being produced in several industries and operations such as gas and coke oven industries,

polymeric resin production, petroleum refineries, fiber glass units, pharmaceuticals, explosive manufacturing, plastic and varnish industries, and textile industries, making use of organic dyes, smelting and related metallurgical operations, etc. [1] [2]. The U.S. Environmental Protection Agency (EPA) has listed phenol as a major pollutant with toxicity to aquatic microorganisms and malodors imparted at very low concentrations (0.005 mg/L) [3]. Phenol is toxic upon ingestion, contact or inhalation and it is recommended that human exposure to phenol not exceed 20 mg in an average day [4]. Also, phenol is toxic to fish and can be lethal at concentrations of 5 - 25 ppm. Additionally, concentrations as low as 0.1 ppm can taint the taste of fish. Due to their toxicity to microorganisms, phenol and its derivatives may often cause the breakdown of waste water treatment plants by inhibition of microbial growth [5]. Therefore, treatment of phenol effluents is critical to maintaining both human and wildlife environments.

Different chemical and biological treatment technologies have been employed for the reduction of phenol content in industrial waste water. The chemical technologies have proven to be costly and produce secondary toxic pollutants. Thus, the biological treatments become a favorable alternative because of their simplicity of operation, cost effectiveness, and it can potentially turn toxic materials into harmless products [6] [7]. Therefore, there is a considerable attention of many researchers in the isolation of microbes able to thrive on high concentrations of phenols [8]. *Pseudomonas* sp were screened and the species (*P. putida*) that degraded phenol in the shortest period of time and that which had high tolerance limit was selected for adaptation. Growth can be inhibited not by just the presence of toxic compounds but by the availability of micro and macro nutrients [9]. Therefore, it was thought worthwhile to study the effect of different media and to consider various environmental factors. Phenol biodegradation is sensitive to many factors such as pH, incubation periods, carbon and nitrogen sources, and enormous efforts in several studies have been made to obtain the optimal conditions that increase the efficiency of phenol-degrading bacteria [10].

The objective of the present study is to optimize the process parameters (temperature, pH, etc) for enhanced phenol degradation by *Pseudomonas stutzeri* ZH-1 using Response Surface Methodology. In the present investigation the parameters affecting the degradation of phenol were identified and Box-Behnken design model was performed to predict the (%) degradation of phenol. The Response Surface Methodology using the Box-Behnken Design of [11] experiments was used to develop a mathematical correlation between glucose concentration, inoculation concentration, pH and temperature and degradation of phenol.

## 2. Materials and Methods

### 2.1. Microorganism

*P. stutzeri* ZH-1 was originally isolated and identified from Fenhe River (in Shanxi Province, China). After being grown on the solidified Beef extract-peptone me-

dium containing (per liter): 5.0 g beef extract, 10.0 g peptone, 5.0 g NaCl, 18.0 g bacto-agar and 1000 mL tap water, the bacteria strain was stored at  $-80^{\circ}\text{C}$  in 30% (w/v) glycerol [12].

## 2.2. Culture Medium and Inoculum Preparation

The mineral salt medium (MSM) contained (g/L) [13]: study consisted of the following components (per liter):  $\text{K}_2\text{HPO}_4$  1.5 g,  $\text{NaH}_2\text{PO}_4$  1.5 g,  $(\text{NH}_4)_2\text{SO}_4$  2.0 g,  $\text{MgSO}_4$  0.2 g,  $\text{CaCl}_2$  0.01 g,  $\text{FeSO}_4$  0.001 g, the concentrations of phenol and culture conditions were adjusted following the subsequent experiments. The initial pH of all the mediums mentioned above was adjusted to 7.2 - 7.5.

A primary culture was prepared by transferring two loops full of microorganisms from an agar slant culture into 100 mL of feed medium containing 20 ml of mineral salt medium and 80 ml of 50 mg phenol solution in a 250 mL erlenmeyer conical flask. This was then incubated in gyratory shaker for 48 h at a temperature of  $35^{\circ}\text{C}$  and agitated at a speed of 120 rpm. Thereafter, 10 mL of the primary culture was transferred into another 100 mL of feed medium in a 250 mL erlenmeyer conical flask and the incubation process was repeated. This was the secondary culture that was used as the inoculum for the degradation studies as this ensures that the organisms had fully adapted to growth on the phenol as sole source of carbon and energy.

## 2.3. Analysis Methods

Growth of the bacteria was monitored by measuring the optical density at 600 nm ( $\text{OD}_{600}$ ) of the culture broth using a spectrophotometer. Phenol concentrations were determined using the 4-aminoantipyrine method [14]. The phenol degradation efficiencies were calculated by the following equation:

$$R_p = (T_1 - T_2) / T_1 \times 100\% \quad (1)$$

Note that  $R_p$ ,  $T_1$  and  $T_2$  represent phenol degradation efficiency, the initial concentration of phenol in medium and the final concentration of phenol, respectively. All samples were analyzed three times to calculate an average value. Statistical analyses were performed by the SPSS and Design Expert, and differences were considered significant at  $p < 0.05$ .

## 2.4. Single-Factor Experiments to Study the Factors Influencing the Phenol Degradation of Strain ZH-1

Single-factor experiments were conducted for studying the phenol degradation of the strain ZH-1 under different culturing conditions: including glucose concentration, inoculation concentration, pH, and temperature. In inoculation concentration experiments, respectively, inoculate the bacteria suspension 1%, 3%, 5%, 7%, 9%, 11% of the inoculum into a mineral salt medium with glucose concentration of 0.6 g/L and phenol concentration of 200 mg/L,  $35^{\circ}\text{C}$ , pH = 7, 120 r/min shaking culture, samples were taken after 48 h to analyze the degradation rate of phenol, and three replicates were set for each experiment. Then, the same

scheme of different experimental conditions (pH, temperature, glucose concentration) was designed for the experiments.

## 2.5. Optimisation Studies

Box-Behnken design [11] was used for optimization of all the variables. A  $3^k$  factorial allows efficient estimation of a second degree quadratic polynomial. Suppose we code the levels in standardized units so that the values taken by each of the variables  $X_1, X_2, X_3, X_4, \dots, X_k$  are  $-1, 0$  and  $1$  and suppose also that the second degree quadratic polynomial fitted by the method of least square is:

$$Y = b_0 + \sum_{i=1}^k b_i X_i + \sum_{i=1}^k \sum_{j=1}^k b_{ij} X_i X_j \dots + \varepsilon. \quad (2)$$

A second order model is got such that the variance of  $Y$  is constant for all points equidistant from the center of the design. Where  $X_1, X_2, X_3, X_4, \dots, X_k$  are the input variables which influence the response  $Y$ ;  $b_0, b_i$  ( $i = 1, 2, 3, 4, \dots, k$ ),  $b_{ij}$  ( $i = 1, 2, 3, 4, \dots, k; j = 1, 2, 3, 4, \dots, k$ ) are known parameters, and  $\varepsilon$  is a random error.

Box-Behnken design for four independent variables was taken to obtain the combination of values that optimizes the response within the region of the three dimensional observation space, which allows one to design a minimal number of experimental runs. The model evaluates the effect of each independent variable to a response. The mathematical relationship connecting the variables and the response can be calculated by the quadratic polynomial equation:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_4 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{44} X_4^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{14} X_1 X_4 + b_{23} X_2 X_3 + b_{24} X_2 X_4 + b_{34} X_3 X_4. \quad (3)$$

where  $Y$  = predicted response;  $b_0$ -constant,  $X_1$  = glucose concentration (g/L),  $X_2$  = inoculation concentration (%),  $X_3$  = pH,  $X_4$  = temperature ( $^{\circ}$ C),  $b_1, b_2, b_3$  and  $b_4$ -linear coefficients,  $b_{12}, b_{13}, b_{14}, b_{23}, b_{24}$  and  $b_{34}$ -cross product coefficients. The low, middle and high levels of each variable (equally spaced) were designated as  $-1, 0$ , and  $1$ , respectively, and given in **Table 1**. The design experiments were carried out for analysis using Design Expert for the present study, a total of 29 experiments were necessary to estimate the coefficients for the degradation of phenol.

**Table 1.** Factors and levels of response surface experiments.

Level	A Glucose concentration (g/L)	B Inoculation concentration (%)	C pH	D Temperature ( $^{\circ}$ C)
-1	0.4	3	6	35
0	0.6	5	7	37.5
1	0.8	7	8	40

### 3. Results and Discussion

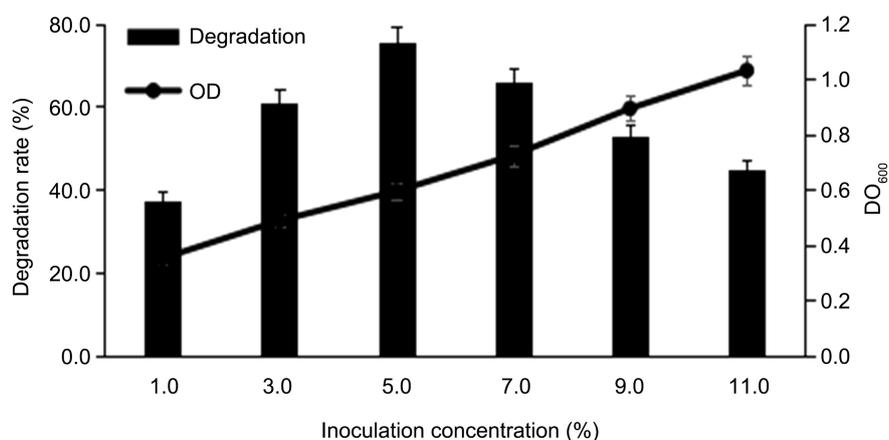
#### 3.1. Phenol Degradation Character of Strain ZH-1 under Various Conditions

##### 3.1.1. Effect of Inoculation Concentration

When the temperature 35°C, glucose concentration is 0.6 g/L, and the pH = 7, the effect of inoculation concentration on the degradation of phenol and cell growth by strain ZH-1 is shown in **Figure 1**. With the increase of the inoculation concentration, the degradation of phenol first increased and then decreased. When inoculating a small amount of microorganisms, the degradation effect may not be obvious, but as the amount of inoculation gradually increases, the degradation effect of microorganisms is gradually obvious. When the inoculation concentration was 5%, the degradation rate reached a maximum of 75.4%. When the inoculation amount exceeds 5%, the degradation effect gradually decreases. The possible reason is that with the excessive reproduction of the bacteria in the later stage, the competition of bacteria will lead to the shortage of nutrients, which will slow down the metabolism and growth rate of microorganisms, and affect the absorption and degradation of phenol by the bacteria.

##### 3.1.2. Effect of Glucose Concentration

When the temperature 35°C, inoculation concentration 5%, and the pH = 7, the effect of glucose concentration on the degradation of phenol and cell growth by strain ZH-1 is shown in **Figure 2**. Adding a certain amount of glucose to the mineral salt medium can obviously promote the ability of the strain to degrade phenol. When the glucose concentration is 0.6 g/L, the degradation of phenol by strain reaches to maximum (76.5%), which is much higher than the degradation of phenol without adding glucose. When the glucose concentration is more than 0.6 g/L, as the glucose concentration increases, the phenol degradation shows a downward trend. This may be due to the high glucose concentration, which causes strain ZH-1 to preferentially use glucose, thereby reducing the phenol utilization [15].



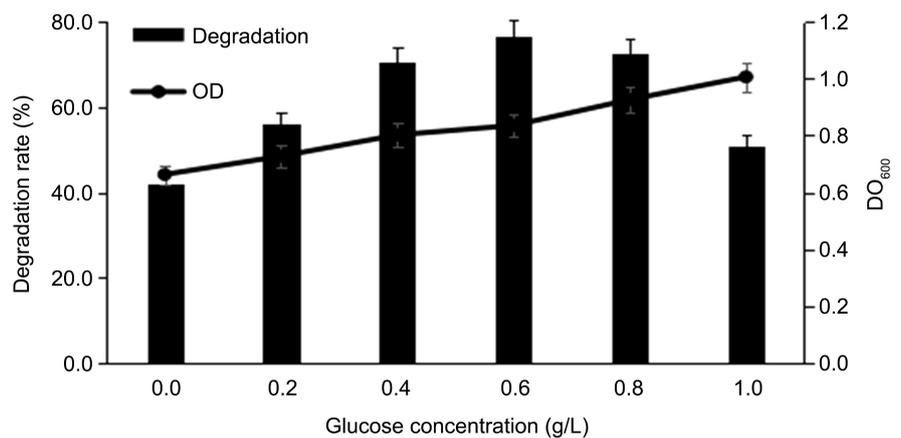
**Figure 1.** Effect of inoculation concentration on phenol degradation and cell growth by strain ZH-1.

### 3.1.3. Effect of pH

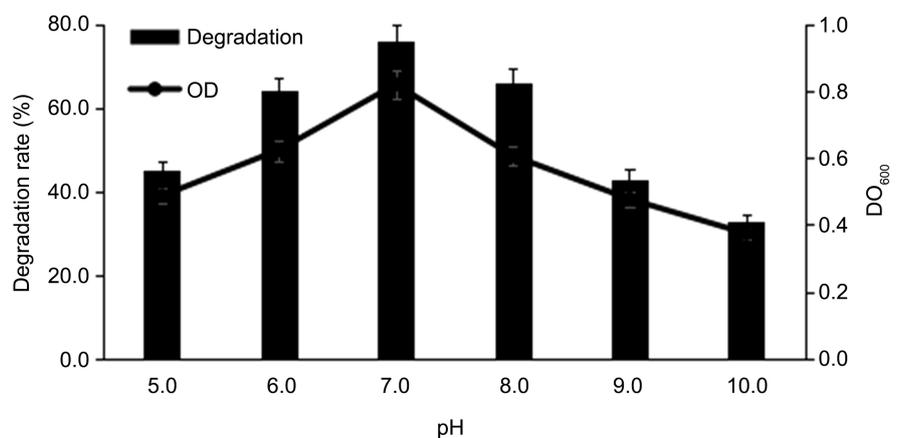
When the temperature 35°C and the glucose concentration 0.6 g/L, the effect of pH on the degradation of phenol and cell growth by strain ZH-1 is shown in **Figure 3**. The strain can degrade phenol better when the pH is 6 - 8, and the degradation rate of phenol is about 70%. However, under strong acid or strong alkali conditions, the strain biodegradation of phenol is inhibited. It may be that strong acid or strong alkali conditions affect the stability of enzymes in organisms and the chemical toxicity of phenol, which changes the binding of substrate molecules and enzymes and affects the degradation of phenol by strain ZH-1 [16].

### 3.1.4. Effect of Temperature

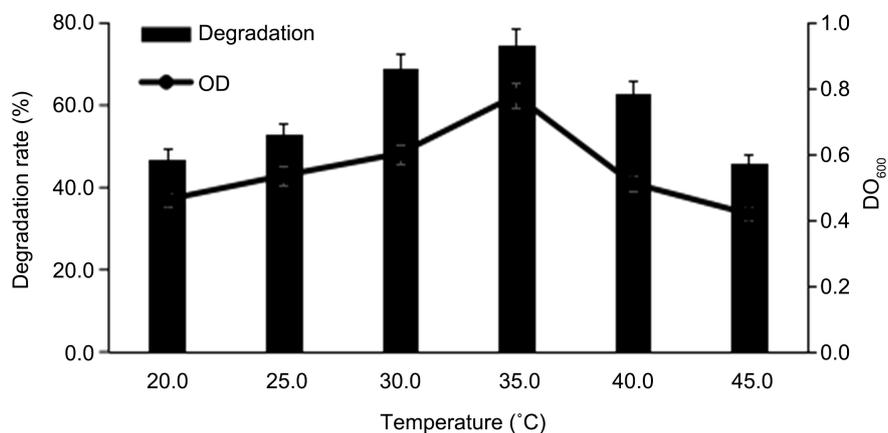
When the pH = 7, inoculation concentration 5% and the glucose concentration 0.6 g/L, the effect of temperature on the degradation of phenol and cell growth by strain ZH-1 is shown in **Figure 4**. When the temperature is 20°C - 45°C, the degradation of phenol is parabolic, and the degradation rate of phenol reaches to maximum (75%) at 35°C. When the temperature is lower or more than 35°C, the strain ZH-1 degradation ability of phenol decreased, indicating that ZH-1 can degrade phenol well at 35°C.



**Figure 2.** Effect of glucose concentration on phenol degradation and cell growth by strain ZH-1.



**Figure 3.** Effect of pH on phenol degradation and cell growth by strain ZH-1.



**Figure 4.** Effect of pH on phenol degradation and cell growth by strain ZH-1.

### 3.2. Optimization of Degradation Conditions of Phenol by Strain ZH-1

#### 3.2.1. Model Establishment and Significance Analysis

Response Surface Methodology is an empirical modelling technique involved in the evaluation of the relationship of a set of controlled experimental factors and observed results. The quantitative description of the process variables effects on phenol microbial degradation was performed [17]. The Response Surface Methodology results and variance analysis were tabulated in **Table 2** and **Table 3**.

The regression equation obtained after analysis of variance gives the level of degradation of phenol as a function of the different process variables: glucose concentration, inoculation concentration, pH and temperature [16] [18]. All terms regardless of their significance are included in the following equation:

$$Y = 90.97 + 0.14A + 1.26B - 0.11C - 1.64D - 0.065AB - 0.77AC + 0.080AD - 0.80BC - 1.15BD - 0.25CD - 4.04A^2 - 5.20B^2 - 6.13C^2 - 7.35D^2 \quad (4)$$

where  $Y$  = predicted response;  $A$  = glucose concentration (g/L),  $B$  = inoculation concentration (%),  $C$  = pH,  $D$  = temperature (°C)

It can be seen from **Table 3** that  $P < 0.0001$  of the coding equation model indicates that the model is extremely significant. The model lack-of-fit term  $P = 0.9957$ ,  $P > 0.05$ , and the model lack-of-fit term is not significant, indicating that the experimental results fit well with the mathematical type. The value of  $R^2$  indicates a high degree of correlation between the observed value and predicted values. The value of the determination coefficient is a measure of goodness of fit to the mode [17] [18]. The model determination coefficient is 0.9901 ( $R^2 = 0.9901$ ), indicating that the effect of pH, inoculation concentration, glucose concentration and temperature on the degradation of phenol by strain ZH-1 is 99.01%, indicating that the model has a good correlation and can be used to optimize the optimal degradation conditions for the strain to degrade phenol.

#### 3.2.2. Response Surface Analysis and Determination of Optimal Conditions

The influence of each factor on the response value may not be a simple linear

relationship but an extreme point. The response surface graph can directly reflect the interaction between the factors. The contour line can reflect whether the interaction between the two factors is significant. The oval shape of the contour line indicates that the effect between the two factors is significant, and the circular contour line indicates that the effect of the two factors is not significant [18].

**Table 2.** The actual experimental design and results.

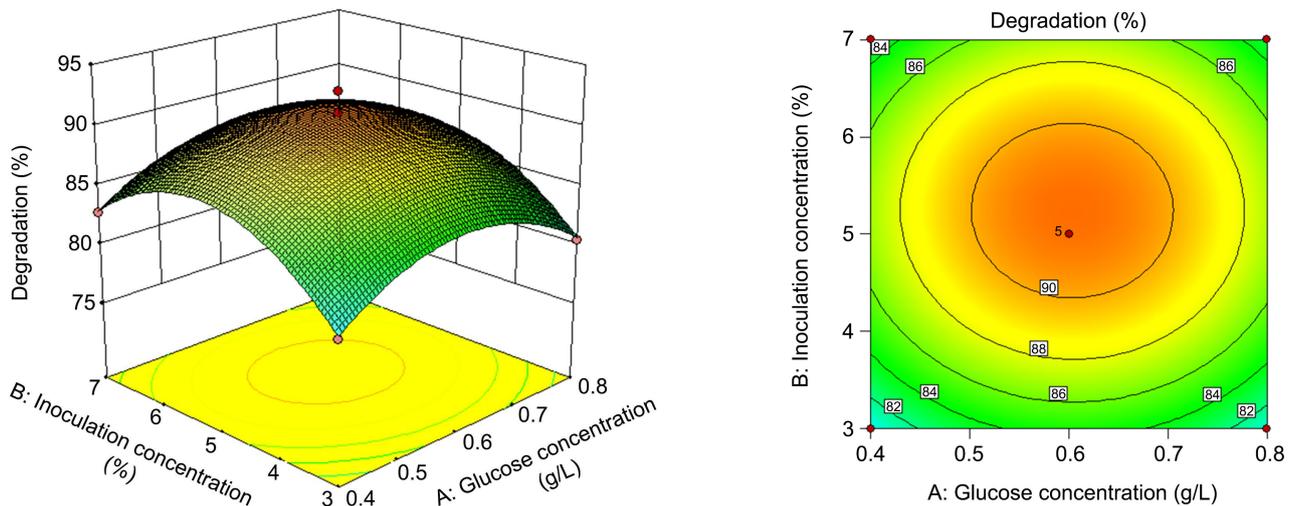
Treatment	A	B	C	D	Y (%)
1	0.4	3	7	37.5	80
2	0.8	3	7	37.5	80.44
3	0.4	7	7	37.5	82.82
4	0.8	7	7	37.5	83
5	0.6	5	6	35	78.65
6	0.6	5	8	35	79.19
7	0.6	5	6	40	75.97
8	0.6	5	8	40	75.5
9	0.4	5	7	35	81.5
10	0.8	5	7	35	81.08
11	0.4	5	7	40	77.85
12	0.8	5	7	40	77.75
13	0.6	3	6	37.5	77.56
14	0.6	7	6	37.5	81.61
15	0.6	3	8	37.5	79.2
16	0.6	7	8	37.5	80.05
17	0.4	5	6	37.5	80.17
18	0.8	5	6	37.5	82.53
19	0.4	5	8	37.5	81
20	0.8	5	8	37.5	80.27
21	0.6	3	7	35	77.83
22	0.6	7	7	35	82.58
23	0.6	3	7	40	76.95
24	0.6	7	7	40	77.1
25	0.6	5	7	37.5	90.32
26	0.6	5	7	37.5	91.04
27	0.6	5	7	37.5	90.32
28	0.6	5	7	37.5	90.32
29	0.6	5	7	37.5	92.86

**Table 3.** Results of regression analysis of the Box-Behnken experiment.

Source	Sum of squares	Degree of freedom	Mean square	F value	P value	Significance
Model	644.07	14	46	100.22	<0.0001	**
A	0.25	1	0.25	0.54	0.4732	
B	19.20	1	19.20	41.83	<0.0001	**
C	0.14	1	0.14	0.30	0.5941	
D	32.37	1	32.37	70.53	<0.0001	**
AB	0.017	1	0.017	0.037	0.8506	
AC	2.39	1	2.39	5.20	0.0388	*
AD	0.026	1	0.026	0.056	0.8167	
BC	2.56	1	2.56	5.58	0.0332	*
BD	5.29	1	5.29	11.52	0.0044	**
CD	0.26	1	0.26	0.56	0.4684	
A <sup>2</sup>	106.03	1	106.03	230.99	<0.0001	**
B <sup>2</sup>	175.52	1	175.52	382.36	<0.0001	**
C <sup>2</sup>	243.89	1	243.89	531.30	<0.0001	**
D <sup>2</sup>	350.47	1	350.89	763.49	<0.0001	**
Residual	6.43	14	0.46			
Lack of fit	1.58	10	0.16	0.13	0.9957	
Pure Error	4.84	4	1.21			
Cor total	650.49	28				
R <sup>2</sup> <sub>adj</sub> = 0.9802 R <sup>2</sup> = 0.9901						

Note: \*, \*\* respectively indicate significant differences at the P < 0.05, P < 0.01 levels.

The coefficient of the model and the corresponding P-values (**Table 3**) suggest that among the test variables, inoculation concentration and temperature are highly significant. These observations can be interpreted as a consequence of proportional relationship between the variables and phenol degradation. The mutual effects of temperature, pH, glucose concentration and inoculation concentration are of equal significance. This data analysis also substantiates the inference that can be drawn from three-dimensional contour plots (3-D graphics) as shown in **Figures 5-10**, respectively. The interactions among temperature, pH, glucose concentration and inoculation concentration are quite prominent from the elliptical nature of the respective contour plots. These figures also suggest the optimum range of the process variables.



**Figure 5.** Response surface (left) and contour map (right) of glucose concentration and inoculation concentration.

**Figure 5** shows the response surface and contour map of the interaction effect between the glucose concentration and inoculation concentration on degradation of phenol. From the contour map it can be observed that the graphic is close to round. Therefore, the interaction effect of glucose concentration and inoculation concentration is not significant.

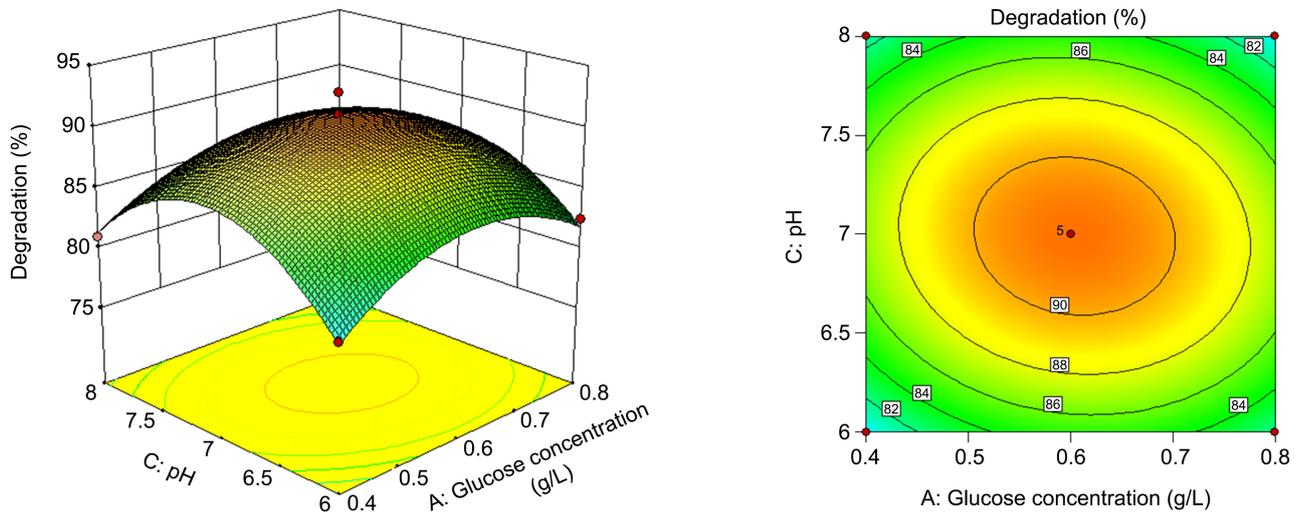
**Figure 6** shows the response surface and contour map of the interaction effect between the glucose concentration and pH on degradation of phenol. It is obvious that the AC interaction curve of the response surface is steep, and the contour map is nearly elliptical, indicating that the impact on phenol degradation is significant.

**Figure 7** shows the response surface and contour map of the interaction effect between the glucose concentration and temperature on degradation of phenol. From the contour map it can be observed that the graphic is close to round. Therefore, the interaction effect of glucose concentration and temperature is not significant.

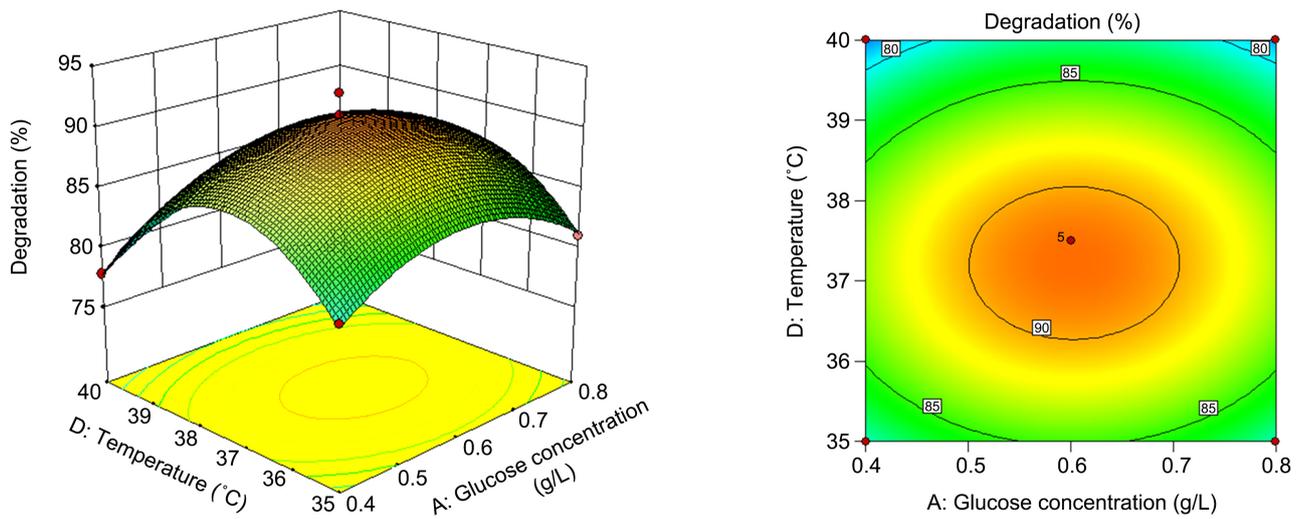
**Figure 8** shows the response surface and contour map of the interaction effect between the inoculation concentration and pH on degradation of phenol. It is obvious that the BC interaction curve of the response surface is steep, and the contour map is nearly elliptical, indicating that the impact on phenol degradation is significant.

**Figure 9** shows the response surface and contour map of the interaction effect between the inoculation concentration and temperature on degradation of phenol. It is quite clear that the BD interaction curve of the response surface is steep, and the contour map is nearly elliptical, indicating that the impact on phenol degradation is the most significant.

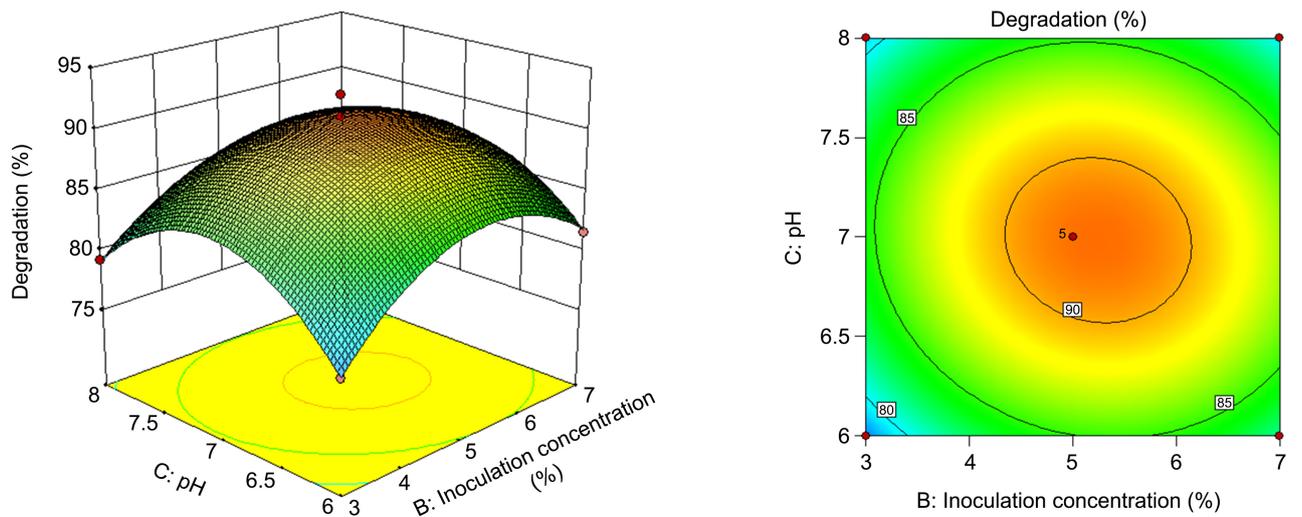
**Figure 10** shows the response surface and contour map of the interaction effect between temperature and pH on degradation of phenol. From the contour map it can be observed that the graphic is close to round. Therefore, the interaction effect of temperature and pH is not significant.



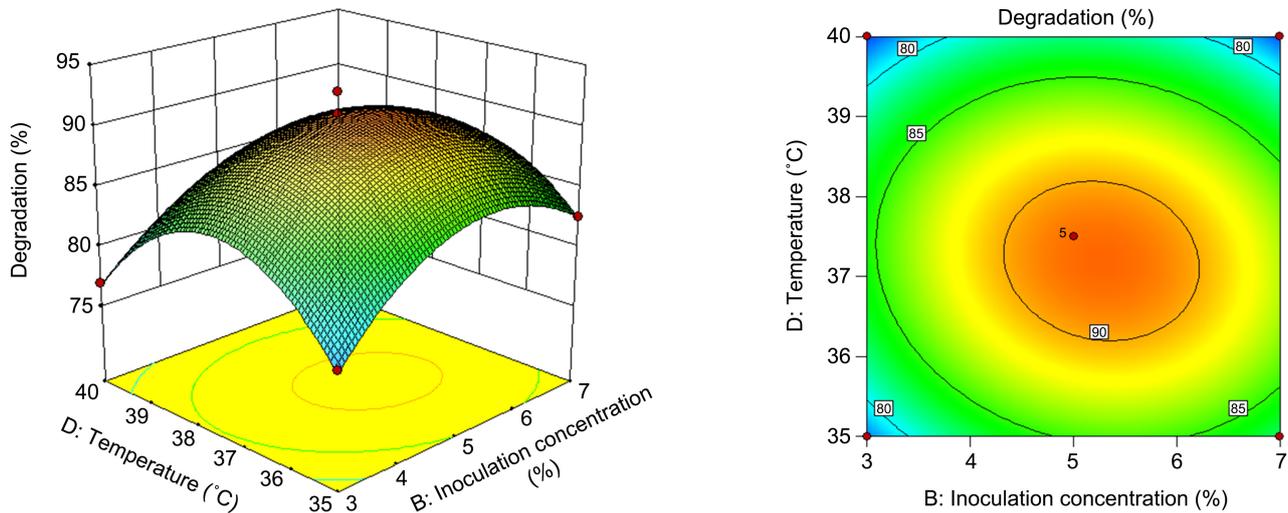
**Figure 6.** Response surface (left) and contour map (right) of glucose concentration and pH.



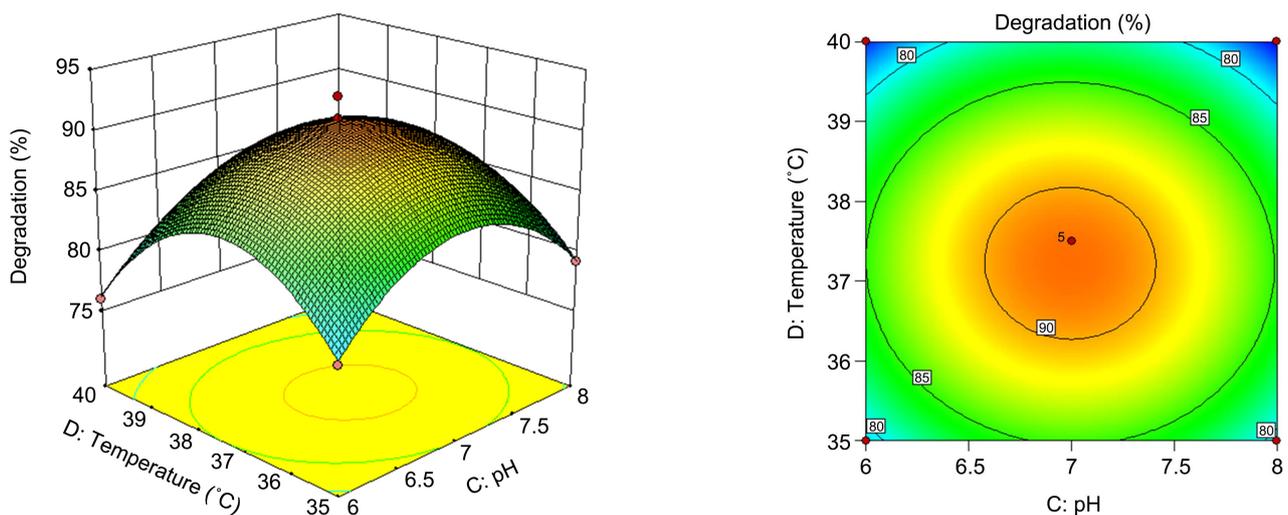
**Figure 7.** Response surface (left) and contour map (right) of glucose concentration and temperature.



**Figure 8.** Response surface (left) and contour map (right) of inoculation concentration and pH.



**Figure 9.** Response surface (left) and contour map (right) of inoculation concentration and temperature.



**Figure 10.** Response surface (left) and contour map (right) of pH and temperature.

Through the test model and response surface analysis, the best conditions for strain ZH-1 to degrade phenol are temperature 37.19°C, inoculation concentration 5.098%, pH = 6.810 and glucose concentration 0.688 g/L. Under these conditions, the predicted value of phenol degradation rate is 90.267%.

### 3.3. The Experiment of Model Validation

In order to test the accuracy of the model prediction, a phenol degradation experiment was carried out under the best predicted conditions: temperature 37°C, inoculation concentration 5%, pH = 7 and glucose concentration 0.7 g/L. Strain ZH-1 was inoculated in mineral salt medium containing 200 mg/L of phenol and cultured at 120 r/min for 48 hours. The degradation rate of phenol was obtained 89.62%, with little deviation from the predicted value of 90.267%, indicating that the model can effectively optimize and predict the conditions for the strain to degrade phenol.

## 4. Conclusion

The degradation condition of phenol by *P. stutzeri* ZH-1 was originally isolated and identified from Fenhe River was optimized in laboratory. Single-factor experiments showed that when the pH = 7, temperature 35°C, glucose concentration 0.6 g/L, and inoculation concentration 5%, as a matter of fact, the degradation effect of phenol by the strain ZH-1 was the best. Response Surface Methodology experiment results in that the optimal conditions for phenol degradation were: temperature 37°C, inoculation concentration 5%, pH = 7 and glucose concentration 0.7 g/L; under the conditions, the strain ZH-1 exhibited efficient phenol degradation ability with maximum phenol degradation rate of 89.62%, with little deviation from the predicted value of 90.267%. Therefore, strain ZH-1 was a promising candidate in the extensive application of various pollution control systems including industrial waste water, pharmaceuticals, explosive manufacturing, plastic, petroleum refineries, etc.

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## Authors' Contributions

This work was carried out in collaboration between both authors. Author YY performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author QPH designed the study, managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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