Optimization of Degradation of Emulsified Oil by Microbial Missile Using Response Surface Methodology

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Abstract. Optimal conditions for microbial missile degradation of emulsified oil were predicted using the response surface method designed by Box-Behnken. The effects of the concentration of sodium alginate (SA), the concentration of calcium source (CaCO₃), the calcium ion chelator (CaCl₂), and the pH value on the degradation efficiency of oil pollution were investigated using calcium alginate gel as a matrix. The results show that the model fitting accuracy R²=0.9739, the concentration of SA, the concentration of calcium source, the concentration of calcium ion chelator and the pH value have a great influence on the response value of the degradation rate of emulsified oil. The model predicts that the optimal reaction conditions are SA concentration of 21.27 g/L, calcium source concentration of 29.71 g/L, calcium ion chelator concentration of 32.33 g/L, and pH is 8.56. The predicted degradation rate of emulsified oil reaches 71.50%. The model was further verified by experiments. The average value of the four experimental degradation rates under the optimal conditions was 71.61%, and the deviation was 0.11% compared with the predicted value. This proves that the method can be used to optimize the process parameters of microbial missiles to degrade the emulsified oil, provide optimized solutions for the treatment of actual oily wastewater.

Keywords: Response Surface Method, Microbial Missile, Calcium Alginate Gel

1. Introduction
Frequent marine spills in recent years have become an important source of pollution in the marine environment. For example, in 2010, a deep-sea oil well leak in the Gulf of Mexico in the United States and the Sanji collision in 2018 led to 136,000 tons of condensate oil spills. There are many technologies for cleaning petroleum hydrocarbon pollutants. Physical and chemical methods are the most commonly used methods. They have the advantage of being efficient and fast, but there is a risk of secondary pollution. Microbial method for pollution repair is a green and environmentally friendly technology without secondary pollution. Among them, in order to solve the problem of loss of free bacterial flora, immobilized microorganism technology has the advantages of high microbial density, rapid response, and low microbial loss[1,2]. Compared with the free bacterial agent, the immobilized bacteria can not only avoid population competition and the interference of environmental
conditions[3], but also form a higher bacterial agent concentration in local areas. This technology has been successfully applied to the remediation of petroleum hydrocarbon pollution many times.

Microbial missile technology treats oily sewage by using physical or chemical means to fix oleophilic bacteria on the carrier to make it dense and maintain biological activity[4]. It is combined with the internal driving action of bacteria under the impetus of wind and waves to track and adsorb oil pollution and achieve rapid degradation. Eliminate pollution. In the process of rapid adsorption and degradation of oil pollution by microbial missiles[5]. There are various influencing factors. In order to obtain a higher and optimal cleaning process, it is particularly important to balance the relationship between various factors. Response Surface Methodology (RSM) is also known as regression design. By analyzing the quantitative rule between the experimental index and each factor, the optimal combination of each factor level is obtained [6,7]. The response surface regression model is a combination of statistics, mathematics, and computer science. Through computer operations, the complex multi-dimensional space surfaces established by RSM are closer to reality [8]. Through the regression fitting of the process and the drawing of response surfaces and contour lines, the response values corresponding to the levels of each factor are obtained. Compared with the single-factor experimental design method and orthogonal experimental design, the response surface method has the advantages of reasonable experimental design, which can determine the optimal value of the experimental results, and can fit the obtained simulation equation with real values[9,10].

In order to simulate the calculation process of biodegradable emulsified oil, based on single-factor experiments. The effects of SA concentration, calcium source concentration, calcium ion chelator and pH value on the immobilized materials were studied. The response surface method was used to examine the influence of single factor and multiple factor interactions on the degradation process, and corresponding prediction equations were established to determine the optimal process.

2. Experimental Part

2.1. Experimental Materials and Instruments

SA, ethyl cellulose (EC), anhydrous ethanol, citric acid, calcium carbonate, and potassium bromide were of analytical grade and were purchased from Tianjin Fuchen Chemical Reagent Factory. Fourier Transform Infrared Spectrometer RAFFinity-1S WL, Shimadzu Corporation; Electric Blow Dryer G2-176, Beijing Keweiyongxing Instrument Co, Ltd; precision booster electric stirrer HHS-2S, thermostatic magnetic stirrer LKTC-B1-T, Shanghai Nanyang Instrument Co, Ltd.

2.2. Experimental Method

2.2.1. Preparation Method of Microbial Missile. A sharp-hole coagulation bath method was used to prepare microbial missiles immobilized with lyophilic bacteria. The experimental reagent is prepared as follows:

(1) Preparation of coating material: Weigh 3 g of ethyl cellulose (EC) and add it to 100 mL of absolute ethanol, and stir on a constant temperature magnetic stirrer for about 2 h.

(2) Preparation of cross-linking materials: Weigh 2 g of citric acid and 3 g of CaCl₂, add them to 100 mL of distilled water, and stir on a magnetic stirrer.

(3) The preparation process of the immobilized carrier is as follows: 3 g of SA and 3 g of CaCO₃ were added to 100 ml of water, and the solution was dissolved by magnetic stirring to obtain the stock solution A. The prepared CaCl₂ solution containing citric acid is placed under a 230 r/min electric stirrer, and a small amount of solution A is sucked with a syringe and dropped into it to form gel beads floating on the surface of the solution. After 30 minutes, the gel was filtered out with a strainer, washed twice with distilled water, dried at room temperature, and set aside.

(4) Immobilized oleophilic bacteria: The oleophilic bacteria are prepared by the laboratory, and the petroleum hydrocarbon-degrading bacteria are composed as follows: Rhodopseudomonas palustris. Pseudomonas grimmontii. Bacillus Subtilis. Bacillus cereus. Frankland. Altererythrobacter marinus.
Rhodococcus erythropolis, the immobilized oleophilic bacteria are fixed to gel beads by adsorption method.

(5) EC coating: Put gel beads in EC with a mass concentration of 30 g/L, stir on a magnetic stirrer for 20 min, filter, wash twice with absolute ethanol, and then wash twice with distilled water. The obtained microbial missile is put in a refrigerator for refrigerated use.

2.2.2. Measurement of degradation rate. The absorbance was measured with an ultraviolet spectrophotometer. The formula for calculating the degradation rate is:

\[
\text{Degradation rate(\%)=}(X_1 - X_2) / X_1 \times 100% \quad (1)
\]

Where \(X_1\) is the absorbance of the control supernatant, and \(X_2\) is the absorbance of the sample supernatant.

2.2.3. Single Factor Experimental Design. In order to determine the optimal selection range of response surface design factors, a single factor experiment was first performed to ensure the effectiveness of the response surface design. Table 1 shows the single factor synthesis condition parameters.

Table 1 Single factor experimental design

| Sample serial number | SA concentration /% | Calcium source concentration /g·L\(^{-1}\) | Calcium chelator concentration /g·L\(^{-1}\) | pH |
|----------------------|---------------------|------------------------------------------|------------------------------------------|----|
| A1/A2/A3/A4/A5       | 20/25/30/35/40      | 30                                       | 30                                       | 8  |
| B1/B2/B3/B4/B5       | 30                  | 10/20/30/40/50                           | 30                                       | 8  |
| C1/C2/C3/C4/C5       | 30                  | 30                                       | 10/20/30/40/50                          | 8  |
| D1/D2/D3/D4/D5       | 30                  | 30                                       | 30                                       | 0/4/8/12/14 |

2.2.4. Response Surface Optimized Design. Based on the results of the single-factor experiment, the influence of each factor on the degradation rate of the microbial missile was synthesized, and four factors affecting the preparation conditions were determined. Design Expert 8.0 software was used for Box-Behnken design. The four factors identified were A (SA concentration, g/L), B (calcium source concentration, g/L), and C (calcium ion chelator concentration, g/L), and D (pH), and the response value is \(Y\) (the degradation efficiency of microbial missiles to oil pollution). See Table 2 for specific experimental factor levels.

Table 2 Impact factor levels and coding based on Box-Behnken response surface method

| Factor                        | Coding level |
|-------------------------------|--------------|
|                               | -1 | 0 | 1   |
| SA concentration,g/L         | 20 | 25| 30  |
| Calcium source concentration,g/L | 20 | 30| 40  |
| Calcium chelator concentration,g/L | 20 | 30| 40  |
| pH                            | 4  | 8 | 12  |

3. Results and Discussion

3.1. Single Factor Experiment Results

The single factor experimental results show that when the SA concentration is from 20 g/L to 25 g/L, the degradation rate of emulsified oil by microbial missiles increases. When the SA concentration is greater than 25 g/L, the degradation rate of microbial missiles by emulsified oil is the downward trend is because as the SA concentration increases, the more the cross-linking reaction between SA and Ca\(^{2+}\) occurs, the degradation rate reaches the maximum. When the SA concentration is higher than 25 g/L, the cross-linking reaction reaches the maximum. In order to obtain the best comprehensive performance of the microbial missile, the SA concentration of 25 g/L was selected as the optimal
condition. With the continuous increase of the calcium source concentration, the degradation rate of the microbial missile also continues to increase, until the calcium source concentration is greater than 30 g/L, the degradation rate of the microbial missile shows a downward trend. The reason is that when the calcium source concentration is less than 30 g/L, the cross-linking reaction between Ca\(^{2+}\) and SA occurs until the calcium source concentration is 30 g/L, the cross-linking reaction is the most sufficient, and then with the continuous increase of Ca\(^{2+}\), it will be in the microbial missile. The surface is covered with a layer of calcium film, which affects the release of bacteria in the microbial missile, and finally leads to a rapid decline in the degradation rate. In order to obtain the microbial missile with the best comprehensive performance, a calcium source concentration of 30 g/L was selected as the optimal condition.

The single factor experimental results show that when the concentration of calcium chelator is from 10 g/L to 30 g/L, the degradation rate of microbial missiles gradually increases, and then the degradation rate starts to decrease. The reason is that with the increase of calcium ion chelator, the cross-linking reaction between SA and Ca\(^{2+}\) is more sufficient. When the calcium ion chelator is 30 g/L, the degradation rate reaches the highest. The optimum condition was an ion chelator concentration of 30 g/L, when the pH is increased from 0 to 8, the degradation rate of microbial missiles increases, and when the pH is greater than 8, the degradation rate decreases, because the pH is increasing. The results show that too high and too low pH will affect the reaction between SA and Ca\(^{2+}\), leading to the degradation rate of microbial missiles. In order to obtain the best comprehensive performance of microbial missiles, pH 8 is selected as the optimal condition.

3.2. Response Surface Method Experimental Results

3.2.1. Analysis of Results. Based on the results of the single factor experiment, A (SA concentration, g/L), B (calcium source concentration, g/L), C (calcium ion chelator, g/L) and D (pH) were selected as 4 Independent factors, Y (degradation efficiency of microbial missiles to oil pollution) as the response value, the experimental design and experimental results based on the Box-Behnken response surface method are shown in Table 3. The established multiple linear regression equation is:

\[
Y = -259.34242 + 4.92533A + 6.12085B + 7.64393C + 18.05358D + 0.0271AB - 0.01415AC - 0.16725AD - 0.02418BC - 6.25000BD - 9.00000CD - 0.10153A^2 - 0.098134B^2 - 0.1092C^2 - 0.82418D^2
\]  

(2)

A credibility analysis is performed on the quadratic regression equation, where the correlation square of the regression equation is \(R^2 = 0.9739\) and the correlation coefficient of the model after correction is \(R^2_{adj} = 0.9478\), indicating that the model can explain 94.78% of the experimental data and prove that the response surface is applied. It is feasible to optimize the preparation conditions of microbial missiles.

| No. | A   | B   | C   | D   | Y    |
|-----|-----|-----|-----|-----|------|
| 1   | 20.00 | 30.00 | 30.00 | 12.00 | 63.25 |
| 2   | 25.00 | 30.00 | 30.00 | 8.00  | 65.42 |
| 3   | 25.00 | 30.00 | 20.00 | 4.00  | 45   |
| 4   | 25.00 | 30.00 | 30.00 | 4.00  | 44   |
| 5   | 25.00 | 40.00 | 40.00 | 4.00  | 44.22 |
| 6   | 20.00 | 40.00 | 30.00 | 8.00  | 61.3 |
| 7   | 25.00 | 20.00 | 30.00 | 12.00 | 44   |
| 8   | 25.00 | 30.00 | 30.00 | 8.00  | 71.25 |
| 9   | 25.00 | 30.00 | 20.00 | 12.00 | 47   |
| 10  | 25.00 | 30.00 | 40.00 | 12.00 | 44.78 |
Figure 1 shows the comparison between the predicted value and the actual value of the degradation rate of emulsified oil by microbial missiles. As can be seen from Figure 1, the experimental values are in good agreement with the predicted values of the model, indicating that the selected model can reflect the actual relationship between the independent variable and the dependent variable.

![Predicted vs. Actual](image)

**Figure 1.** Comparison of model predictions and experimental results

The analysis of variance and significance test is performed on the model. The results are shown in Table 4. The results in Table 4 show that the F value of the model is 37.3, and the Pr>F value is less than 0.05, indicating the model's significance and reliability.
than 0.0001 (less than 0.05 indicates a better regression effect), indicating that the concentration of SA, the concentration of calcium source, the concentration of calcium ion chelator, and the pH The degradation rate of microbial missiles has a large impact. The significant order of the four influence factors is: SA concentration> calcium source concentration> pH> calcium chelator concentration. In addition, there are certain interactions between the four influence factors, of which A (SA concentration) and D (pH value), B (calcium source concentration) and C (calcium ion chelator concentration) interacted significantly.

Table 4 Anova response surface quadratic model

| Source                        | Sum of Squares | df | Mean Square | F     | Pr>F  |
|-------------------------------|----------------|----|-------------|-------|-------|
| Model                         | 2361.25        | 14 | 168.66      | 37.30 | < 0.0001 |
| A-SA concentration            | 363.33         | 1  | 363.33      | 80.35 | < 0.0001 |
| B-Calcium source concentration| 21.84          | 1  | 21.84       | 4.83  | 0.0453 |
| C- Calcium chelator concentration| 4.18          | 1  | 4.18        | 0.92  | 0.3526 |
| D-pH                          | 9.97           | 1  | 9.97        | 2.21  | 0.1597 |
| AB                            | 7.34           | 1  | 7.34        | 1.62  | 0.2233 |
| AC                            | 2.00           | 1  | 2.00        | 0.44  | 0.5166 |
| AD                            | 44.76          | 1  | 44.76       | 9.90  | 0.0071 |
| BC                            | 23.39          | 1  | 23.39       | 5.17  | 0.0392 |
| BD                            | 0.25           | 1  | 0.25        | 0.055 | 0.8175 |
| CD                            | 0.52           | 1  | 0.52        | 0.11  | 0.7399 |
| A²                            | 41.79          | 1  | 41.79       | 9.24  | 0.0088 |
| B²                            | 624.67         | 1  | 624.67      | 138.15| < 0.0001 |
| C²                            | 773.44         | 1  | 773.44      | 171.06| < 0.0001 |
| D²                            | 1127.97        | 1  | 1127.97     | 249.46| < 0.0001 |
| Residual                      | 63.30          | 14 | 4.52        |       |       |
| Lack of Fit                   | 43.10          | 10 | 4.31        | 0.85  | 0.6201 |
| Pure Error                    | 20.20          | 4  | 5.05        |       |       |
| Cor Total                     | 2424.55        | 28 |             |       |       |

3.2.2. Response Surface Analysis. Figure2 and Figure3 are the response curves between A (SA concentration) and D (pH value), B (calcium source concentration), and C (calcium ion chelator concentration). It can be seen from Figure 2 that the interaction between the SA concentration and the amount of pH value is very obvious. When the pH value remains the same, the degradation rate rises to the highest point and then decreases with the continuous increase of the SA concentration. The main reason is that SA reacts more fully with Ca²⁺ with increasing concentration. When Ca²⁺ is completely reacted, the remaining SA will affect the degradation rate of the microbial missile and reduce the degradation rate. It can also be seen from the Figure that when the concentration of the calcium ion chelator remains unchanged, the degradation rate increases first with the increase of the concentration of the calcium source, and then decreases. The main reason is that the reaction between a large amount of Ca²⁺ and SA has reached saturation, and then increasing the concentration of the calcium source will only introduce a large amount of Ca²⁺, which is not good for the improvement of the microbial missile degradation rate. It can be seen from Figure 2 and Figure 3 that the above four factors have the greatest influence on the degradation rate of microbial missiles, followed by the SA concentration, the calcium source concentration, the pH value, and the calcium ion chelator concentration.
As four factors are selected, the best conditions for preparing microbial missiles are obtained according to the regression model and response surface analysis (see Figure 4 for the optimization
results of response surface): When the SA concentration is 21.27 g/L, the calcium source concentration is 29.71 g/L, the calcium ion chelator concentration is 32.33 g/L, and the pH is 8.56, the predicted degradation rate reaches 71.50%. In order to verify the accuracy of the predicted optimal conditions 4 parallel experiments were performed. Parallel test results showed that the average degradation rate was 71.61%, slightly higher than expected. The deviation from the predicted value was 0.11%, indicating that the model optimizes and degrades the preparation conditions of microbial missiles. The prediction of the rate is more accurate and reliable.

3.3. Sample Characterization.
In order to verify the structural formula of the compound, the infrared characterization of the microbial missile synthesized under the optimal process conditions was performed, and the results are shown in Figure 5. There is a hydroxyl (-OH) stretching vibration peak of SA at a wavelength of 3433 cm\(^{-1}\), a carbonyl (C=O) stretching vibration peak of SA at 1058 cm\(^{-1}\), and a carboxylic acid functional group of SA at 1423 cm\(^{-1}\) (-COOH) symmetrical stretching vibration peak, There is an asymmetric stretching vibration peak of the functional group (=C-H) of EC at 609 cm\(^{-1}\), and a stretching vibration peak of the functional group (C=C) of EC at 1604 cm\(^{-1}\). The microcapsule carrier has a wide absorption band at 3200 ~ 3400 cm\(^{-1}\). This is a hydroxyl stretching vibration associated with the formation of a hydrogen chain. A series of absorption peaks overlap and overlap with CH absorption (3000~3100cm\(^{-1}\)) This indicates that there is a hydrogen chain interaction force between SA and EC. It can be proven that SA and Ca\(^{2+}\) are combined.

![Figure 4. Optimized response surface](image-url)
Figure 5. Infrared spectrum of microbial missile

4. Conclusion

1) The analysis of the response surface method designed by Box-Behnken shows that SA concentration, calcium source concentration, calcium ion chelator concentration, and pH have important effects on the degradation rate of emulsified oil by microbial missiles. The regression equation is:

\[ Y = -259.34242 + 4.92533A + 6.12085B + 7.64393C + 18.05358D + 0.0271AB - 0.01415AC - 0.16725AD - 0.02418BC - 6.25000BD - 9.0000CD - 0.10153A^2 - 0.098134B^2 - 0.1092C^2 - 0.82418D^2 \]

The results of analysis of variance show that the fitting test is very significant, and the coefficient of determination reaches 0.97. This equation can better predict the variation law of mullite whisker aspect ratio with various parameters.

2) According to the response surface optimization, the optimal process conditions for the preparation of microbial and microbial missiles for the treatment of marine oil wastewater were: SA concentration was 21.27 g/L, calcium source concentration was 29.71 g/L, calcium ion chelator concentration was 32.33 g/L, pH is 8.56.

3) Under the optimal preparation conditions, four parallel experiments were carried out. The degradation rate of the emulsified oil by the microbial missile was 71.61%, which was 0.11% deviation from the predicted value, indicating that the model was optimized for the preparation conditions of the microbial missile and the degradation rate was predicted more accurate and reliable.

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