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تحسين وتأثير متبادل لكمية الملح والمواد المغذية وكمية التلقيح على النفط القابل للتحلل الحيوي في البيئة البحرية

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الخـلاصـة

مع ازدياد استخراج النفط والنقل البحري، شهد ازديادا شديدا في حوادث فيضان النفط البحري أو تسرب المنتجات النفطية في كل أنحاء العالم. بالنسبة إلى تحلل الملوثات النفطية عند المنطقة البحرية، تعتبر تكنولوجيا الإصلاح البيولوجي طريقة اقتصادية وتحسن بيئي. على رغم كثير من البحوث يقرر تأثير عامل أحادي على التحلل البيولوجي، لكنها قليلة في التأثير المتبادل والتأثير المتراكم للعوامل البيئية على التحلل الحيوي. في هذه الرسالة، يتخذ أسلوب الاستجابة السطحية المنحنية في بحث التأثير المتبادل لكمية الملح والمواد المغذية وكمية التلقيح على النفط القابل للتحلل الحيوي لكي يزود بالسندات النظرية للإصلاح البيولوجي للمنطقة الملوثة البحرية بالنفط. تدل النتيجة على أنه للقيمة التجربية والقيمة المتوقعة توافق حسن. يحلل (variance ANOVA) وقيمة F في النموذج بشكل مزيد ويدل على أن النموذج بارز وتثبت الصيغة التربيعية المتعددة أيضا. إضافة إلى ذلك، يحلل القيمة المتوقعة ضد القيمة الفاعلية وقيمة الفرق ضد القيمة المتوقعة، تدل النتيجة على أن النموذج قابل للاستعمال. أخيرا يحلل التأثير المتبادل لثلاثة عوامل ويرتب العوامل: كمية الملح $>$ كمية التلقيح $\approx$ نسبة النيتروجين إلى الفوسفور.

العبارات المفتاحية: تكتيريا تحلل النفط؛ تكنولوجيا الإصلاح البيولوجي؛ الهيدروكربونات النفطية؛ التلوث النفطي البحري.

Optimization and interactive effects of salinity, fertilizer and inoculums concentration on biodegradation in marine environment

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ABSTRACT

With the speeding up of the exploitation and shipments of petroleum, the oil spill or off-shore oil production all over the world have dramatically increased. Bioremediation has been recognized as a cost-effective and environment-friendly approach for cleanup of the petroleum pollutants from marine environment. Though some studies have reported the effect of every factor on the biodegradation, there are few reports on the interactive and cumulative effect of environment factors on biodegradation. In this paper, interactive effects of salinity, fertilizer and inoculums concentrations on biodegradation were studied by response surface methodology, in order to provide a better theoretical basis for using biodegradation in the marine environment. The results indicated that there was good agreement between the predicted values and experimental values. Further analyzing the variance (ANOVA), the model F-value indicated the model was significant for biodegradation, and a second-order polynomial equation was consequently defined. Additionally, predicted value vs. actual value and residual value vs. predicted value were also analyzed and the results have shown that the model was effective. Lastly, according to the interactive effects of three factors, the factors were ranked by descending order: salinity＞inoculums concentration ≈ nitrogen and phosphorus ratio.

Keywords: Petroleum hydrocarbon-degrading bacteria; Bioremediation; Hydrocarbons; Marine oil pollution.

INTRODUCTION

With the speeding up of offshore oil development and transportation, there is a dramatic increase of the oil spill or off-shore oil production all over the world, which contribute to marine oil pollution (Poland JS, *et al*. 2003).These marine oil pollutions (e.g., ‘‘Deep Water Horizon’’ oil spill accident in the Gulf of Mexico and ‘‘Penglai 19-3’’ oil spill accident in 2011) have brought serious threat to the safety of the Marine ecological environment (Jenkins ME and Adams MA . 2011).

At present, there are many cleanup and recovery methods for oil spills, such as physical, chemical and bioremediation treatments (Al-Mailem DM, *et al*. 2010; Bachmann RT, et al. 2014;Jiang WJ, et al. 2014;Jianliang Xue, *et al*. 2015).In these recovery methods, bioremediation treatment is considered as one of the important technologies (Jianliang Xue, *et al*. 2015). The mechanism of bioremediation is that the microorganisms transfer petroleum hydrocarbons into energy and certain organisms in the process of metabolism. Compared with physical and chemical methods, bioremediation is a low cost technology of strong adaptability and no secondary pollution characteristics for the marine ecological restoration are associated with it.

However, in contrast with other ecological systems such as soil environment and surface water environment, the effects of biodegradation in marine environment are limited. The reason is that some environmental factors (for example, temperature, salinity, fertilizer, etc.) in the marine environment are distinctly different from those in other environments, which limits the growth of microorganisms (Rengathavasi Thavasi, *et al*. 2007).

There were some reports on the effect of every factor on the biodegradation. For example, in the marine environment, salinity is an important factor, that significantly affects the activity of microbial ecology. Some studies have been indicative of better growth and biodegradation activity at the appropriate salinity (Mulherji S. *et al*. 2004). Rengathavasi T et al demonstrated that the maximum growth of P. aeruginosa is at 3.5% of salinity concentration (Rengathavasi Thavasi, *et al*. 2007). Similarly, the maximum growth of Rhodococcus erythropolis is at 2.5% of NaCl concentration (de Carvalho and da Fonseca. 2005). Meanwhile, once above 3.5% and below 0.5% of salinity concentration, the biodegradation activity of microorganism is significant (Mulherji S. *et al*. 2004). The other important factor influencing biodegradation is addition of fertilizer. Better results of biodegradation could be obtained by adding fertilizers in oil polluted sites(Gibbs C.F,et al. 1975). However, excessive fertilizers could have negative effect, which could inhibit the biodegradation activity (Chaillan, F. *et al*. 2006).

Although, the individual effects of environment factors on biodegradation have been indicated in some reports (Mukherji S., *et al*. 2002;Kriti Singh,et al. 2014; Bengtsson G., *et al*. 2003) there are few reports on the interactive and cumulative effect of different environmental factors such as salinity, fertilizer, and petroleum hydrocarbon concentration. Hence, in order to provide better theoretical basis for using biodegradation in the marine environment, optimization and interactive effects of salinity, fertilizer and inoculums concentration on biodegradation were studied.

MATERIALS AND METHODS

Isolation and enrichment of petroleum hydrocarbon -degrading bacteria

Sea water samples for isolated petroleum hydrocarbon -degrading bacteria was taken from an area near the kiaochow bay, Qingdao, Shandong province, China. The sea water samples were collected in sterile polythene bottles, and immediately sent to laboratory to be preserved at 4°C. A 3 ml sample was inoculated in liquid mineral medium (Coppotelli, B.M., *et al*. 2010), and 1 ml diesel was synchronously added in the medium. The medium with sample and diesel was incubated at 30°C, and 160 rpm for 7 days. Afterwards, the 3 ml of incubated sample was picked up from the medium, and was diverted to fresh liquid mineral medium. The same steps were repeatedly operated. Accordingly, the enrichment of petroleum hydrocarbon -degrading bacteria was gathered. Additionally, the petroleum hydrocarbon -degrading bacteria were further analyzed and showed that the preponderant microbes were Pseudomonas sp., Acinetobacter sp., and Rhodococcus sp.

Methods

(1) Response surface methodology

Response surface methodology is a tool for studying the interaction between two or more factors, which is based on the regression method as the tool of function estimation (Zahed M A, *et al*. 2012). The mechanism of response surface methodology is that the multifactor experiment factors and test results (response value) are estimated with polynomial approximation which establishes the functional relationship. According to the surface analysis, the individual and interactive effects of factors are analyzed (Li Lin, *et al*. 2014). Generally, two steps are applied in response surface methodology:

The first step is response surface design phase.

When test conditions depart from the surface of the optimal position, the first-order approximation model is adopted as follows:

$$Y = \beta_0 + \sum_{i=1}^{m} \beta_i \alpha_i + \varepsilon \qquad (1)$$

Where αi is encode variable, and βi is the slope or linear effect of αi.

When the test area approaches or lies within the optimal area of the response surface area, the second step is operated, the purpose of which is to obtain a precision approach value and to identify the optimal level of input variable factors in the optimal response surface area. The second-order approximation model is adopted as follows:

$$Y = \beta_0 + \sum_{i=1}^{m} \beta_i \alpha_i + \sum_{i<j}^{m} \beta_{ij} \alpha_i \alpha_j + \sum_{i=1}^{m} \beta_{ii} \alpha_i^2 + \varepsilon \qquad (2)$$

Where αi and α_j are encode variables, β_i is the linear effect of α_i, β_{ij} is the interactive effects of α_i and α_j, and β_{ii} is the secondary effect of αi.

(2)Design of Response surface methodology model

In order to investigate the interactive effects and optimize the factors, the three levels and three-factorial Box-Behnken Design (BBD) were applied. The three factors were nitrogen and phosphorus ratio (A), inoculums concentration (B) and salinity (C), respectively. The degradation efficiency of petroleum hydrocarbon (%) was considered as response value (Y). The BBD design is a method for evaluating and building nonlinear relations between indexes and factors and is especially suitable for small number of experimental designs. The independent factors and corresponding coded levels are shown in Table 1(Li Lin, et al.2014; Olawale O., *et al*.2015.).

Table 1. Factors and corresponding coded levels for the experimental design

Factors	**corresponding coded**	**coded levels**		
		-1	**0**	**1**
nitrogen to phosphorus ratio	A	1:15	1:10	1:5
inoculums concentration, g/L	B	20	30	40
Salinity	C	2.5%	3%	3.5%

Analysis

Diesel was the subject investigated, and the analysis method of diesel in the samples was as follows:

Initially liquid samples were extracted with n-butane three times. Then, the extraction liquid was centrifuged at 3000 rpm for 10 min. The diesel concentration was determined by ultraviolet spectrophotometry. The degradation efficiency of diesel was calculated using the following formula:

$$Y = \frac{C_0 - C_1}{C_0} \times 100\% \qquad (3)$$

Where, Y is the degradation efficiency of diesel, %; C_0 and C_1 are the initial and final concentration of diesel, respectively.

RESULTS AND DISCUSSION

Optimization of salinity, fertilizer and inoculums concentration on biodegradation using response surface methodology

(1) Result of experiment

According to Box-Behnken design, a total of 17 experiments were carried out to evaluate the effects of three factors at three different levels on the response. The design matrix of independent variables and the experimental results are presented in Table 2.

Table 2. Box-Benhnken experiment results of Petroleum Hydrocarbon biodegradation

No.	factors			Response values,Y		Absolute error	Relative error
	A	B	C	Real value	Predictive value		
1	-1	-1	0	82	84	-0.02	-0.02
2	1	-1	0	86	86	0	0
3	-1	1	0	87	87	0	0
4	1	1	0	91	89	0.02	0.02
5	-1	0	-1	88	88	0	0
6	1	0	-1	83	86	-0.03	-0.03
7	-1	0	1	82	80	0.02	0.02
8	1	0	1	87	86	0.01	0.01
9	0	-1	-1	84	81	0.03	0.03
10	0	1	-1	87	87	0	0
11	0	-1	1	80	80	0	0
12	0	1	1	77	80	-0.03	-0.03
13	0	0	0	78	79	0.08	0.08
14	0	0	0	78	79	-0.01	-0.01
15	0	0	0	78	79	-0.01	-0.01
16	0	0	0	76	79	-0.03	-0.03
17	0	0	0	77	79	-0.03	-0.03

As shown in Table 2, there is good agreement between the predicted values and experimental values.

(2)Further Analysis of variance (ANOVA)

According to the experiment results, the ANOVA of the experiment was further analyzed, and the result is presented in Table 3.

Table 3. ANOVA of experimental data of biodegradation

Source	Coefficient estimates	Standard Error	Sum of squares	Mean square	F-value	p-values	
model	—	—	279.24	31.03	4.75	0.026	significant
Lack of fit	—	—	44.50	14.82	49.44	0.0013	significant
intercept	77.40	1.14	—	—	—	—	
A	1.0	0.90	8.0	8.0	1.23	0.3049	
B	0.50	0.90	2.0	2.0	0.31	0.5972	
C	-1.25	0.90	12.5	12.5	1.91	0.209	
AB	0.0	1.28	7.390×10^{-13}	7.390×10^{-13}	1.1320×10^{-13}	1.000	
AC	2.50	1.28	25.0	25.0	3.83	0.0912	
BC	0.0	1.28	7.390×10^{-13}	7.390×10^{-13}	1.1320×10^{-13}	1.000	
A^2	6.80	1.25	194.69	194.69	29.82	0.0009	
B^2	2.30	1.25	22.27	22.27	3.41	0.1072	
C^2	0.80	1.25	2.69	2.69	0.41	0.541	
R^2			0.9852				

It is seen from Table 3, that the model F-value was 4.75, which implied that the model was significant for biodegradation. And, there is only a 2.60% chance that a «Model F-Value» this large could occur due to noise. The Lack of Fit F-value was 49.44, which also implied that the Lack of Fit is significant. There is only a 0.13% chance that a «Lack of Fit F-value» this large could occur due to noise. Additionally, the R2 of the model was 98.31%, which indicated a good relation between the experimental values and the predicted values. The high R2-value indicates that the regression model accounts well for the relationship between the independent variables and the response.

(3) Second-order polynomial equation

The multiple regression analysis was performed based on the RSM results. According to the ANOVA result of the experiment, the second-order polynomial equation was defined as:

$$Y=77.4+1.0A+0.50B-1.25C+0.0AB+2.5AC+0.0BC+6.8A2+2.30B2+0.8C2 \quad (4)$$

Where, Y is the degradation efficiency of diesel, %; A, B, C are the nitrogen to phosphorus ratio, inoculums concentration and salinity, respectively.

Analyzing the second-order polynomial equation, the optimum conditions of biodegradation were: nitrogen to phosphorus ratio of 1:5, inoculums concentration of 30g/L, and salinity at 2.5%. Under these conditions, the degradation efficiency of predicted values and the experimental values were 84.27% and 84.21%, respectively.

The residual normal, predicted value vs. actual value, and residual value vs. predicted value were also analyzed and the results are shown in Figs1, 2, and 3.

It is seen from Fig.1, that each point formed a straight line, which indicated that the residual normal followed a normal distribution (Jadhav S B, et al.2013). Fig.2 depicted the predicted value vs. actual value, which indicated that the difference between these values was small. Fig. 3 shows that the residual values are distributed on both sides of a central line, which indicates that the residual value was not dependent on the predicted value. In conclusion, the model was effective.

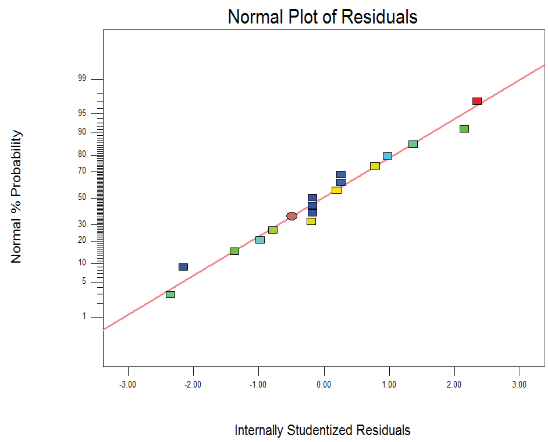

Fig.1 Probability plot of residual normal

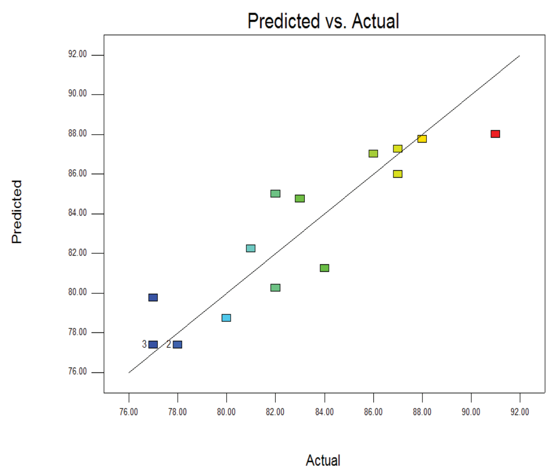

Fig.2 Difference between predicted value vs. actual value

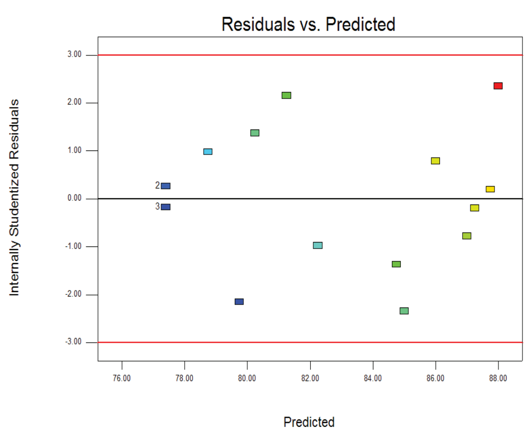

Fig.3 Residual value vs. predicted value

Interactive effects of salinity, fertilizer and inoculums concentration on Biodegradation

To evaluate the interactive effect of two factors, three dimensional plots and contour plots are given in Figs.4, 5 and 6.

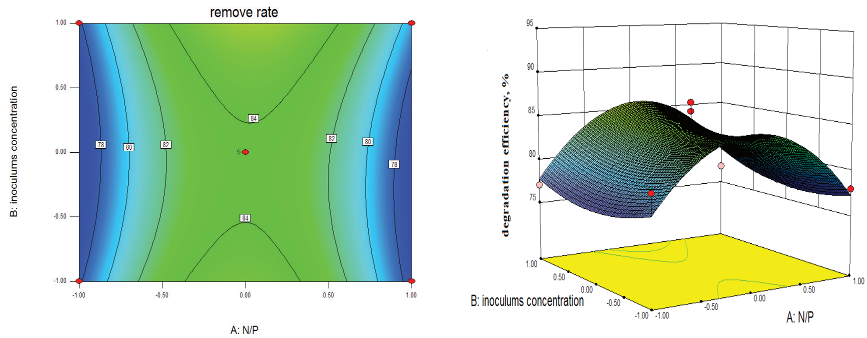

Fig. 4 Contour and three-dimensional corresponding response surface plots for N:P ratio and inoculums concentration

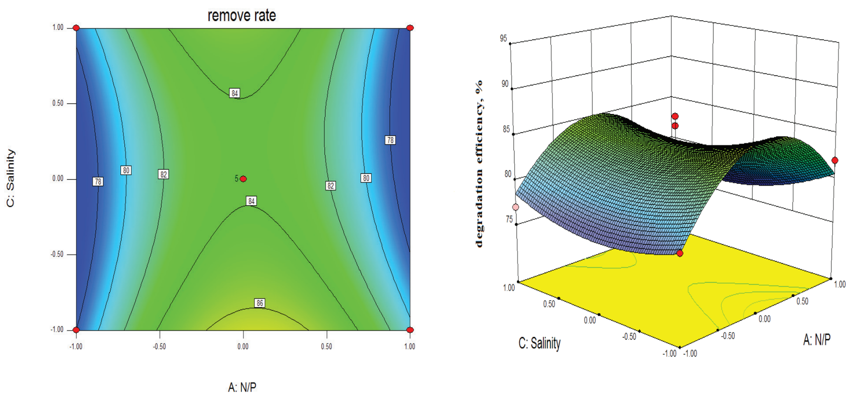

Fig. 5 Contour and three-dimensional corresponding response surface plots for N:P and salinity plots

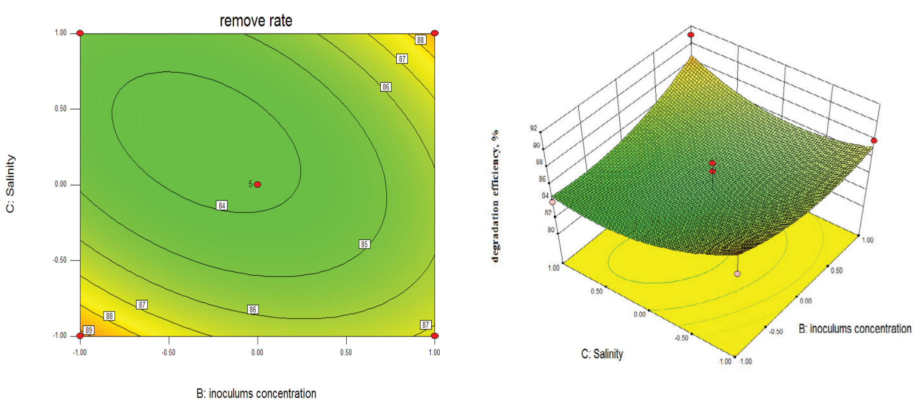

Fig.6 Contour and three-dimensional corresponding response surface plots for inoculums concentration and salinity

(1)Interactive effects of N:P and inoculums concentration

In Fig.4, the interactive effects of N:P and inoculums concentration were shown. With the increase of N:P in a certain range (from 1:15 to 1:10), the degradation efficiency of diesel decreased, while, the degradation efficiency increased with the unceasingly increase of N:P and, the maximum degradation efficiency could be above 86%. When the inoculums concentration changed, the same trend of degradation efficiency was demonstrated. As seen from the contour plot, the effect of N:P level on degradation efficiency was the same as that of inoculums concentration. This is probably due to the fact that the higher inoculums concentration can enhance the degradation efficiency within a suitable fertilizer. In conclusion, the N:P ratio and inoculums concentration have the same effect on biodegradation of diesel.

(2)Interactive effects of N:P and salinity

As seen from the Fig.5, the effect of salinity on degradation efficiency was obviously different from that of N:P. For a certain N:P, an increased degradation efficiency of diesel was observed with a decrease in salinity. This is probably due to the fact that the higher salinity limits the biological activity (de Carvalho and da Fonseca,2005). However, the degradation efficiency was obvious for the studied ranges of N:P and salinity. Especially, when salinity was between 2.8% to 3.5%. The degradation efficiency firstly decreased, and then increased, with the increase of N:P. But, when salinity ranged from 2.5% to 2.75%, the degradation efficiency slowly changed. Therefore, salinity was the prominent factor, owing to the interaction between N:P and salinity.

(3)Interactive effects of inoculums concentration and salinity

As seen from Fig.6, the effect of inoculums concentration on biodegradation was different for different ranges of salinity. For example, when salinity ranged from 2.75% to 3.5%, the degradation efficiency decreased rapidly, and then increased slightly with increase in inoculums concentration. When salinity ranged from 2.5% to 2.75%, the removal decreased. Therefore, salinity was the prominent factor, owing to the interaction between inoculums concentration and salinity.

In conclusion, according to the interactive effects of the three factors and p-values (in Table.3), the factors were in several groups by descending order: salinity＞inoculums concentration≈ nitrogen and phosphorus ratio.

CONCLUSIONS

In order to analyze interactive effects and optimization of factors on biodegradation in marine environment, the response surface methodology was successfully employed. The results were as follows:

(1)Three factors were selected to analyze how they influence the degradation efficiency of diesel by Box-Behnken Design.There was the good agreement between the predicted values and the experimental values.

(2) Analyzing the variance (ANOVA), the model F-value indicated the model was significant for biodegradation. Multiple regression analysis was performed based on the RSM results. The optimum biodegradation conditions were found to be nitrogen to phosphorus ratio of 1:5, inoculums concentration of 30g/L, and salinity at 2.5%. Under these conditions, the degradation efficiency of predicted and experimental values were 84.27% and 84.21%, respectively.

(3) Residual normal, predicted value vs. actual value, and residual value vs. predicted value were also analyzed and the results showed that the model was effective.

(4) The level of interaction of the three factors can be ranked by descending order: salinity＞inoculums concentration ≈ nitrogen and phosphorus ratio.

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