Optimization of reed-specific degrading bacteria by response surfaces for remediation of crude oil-polluted soil in Xinjiang, China

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Abstract: This paper discussed the optimization of conditions for remediation of crude oil-polluted soil based on pot experiment by applying reed-specific degrading bacteria, and using response surfaces methodology. We took the initial crude oil concentration, the amount of inoculation, the ratio of nitrogen and phosphorus, and the use of surfactant (Tween-80) as independent variables (factors), and the degrading ratio of crude oil as the dependent variable (response) after a 90-day experiment. The experiment explored the impacts of each independent variable and their interactions on the bioremediation of crude oil-polluted soil using the Box-Behnken design. Working with a simulated forecasting model the study obtained optimization values for the treatment parameters of 200 g/kg of the reed-specific degrading bacteria, a nitrogen to phosphorus ratio of about 6.0, and 0.2% surfactant. Under experimental conditions, for crude oil concentrations of 10, 30 and 50 g/kg, the optimal effects of the treatments achieved 71.87%, 66.61% and 54.52% degradation of the crude oil, respectively. The results can provide a basis for the technical development of plant-microorganism combined bioremediation of crude oil-polluted soil.

Keywords: crude oil pollution; plant-microorganism; response surfaces; soil remediation

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Xinjiang is an important oil producing region, yielding 1/8 of China's crude oil output (Yang, 2003). In the processes of crude oil exploration, storage and transportation, refining and machining, soil has suffered from serious oil pollution, resulting in huge economic losses (Guo, 2000; Sun and Yu, 2002). So, the remediation of crude oil-polluted soil has become an urgent issue to address. The search for remedial methods is at the core of the development of remediating technology.

Compared to physical and chemical remediation, bioremediation has less impact on human beings and the environment, and its cost is only 30%–50% of traditional physical and chemical remediation methods; it can be a highly efficient, economical and environ-

mentally-friendly clean technology (Qian and Liu, 2000; Liu et al., 2006; Cheng and Li, 2007; Mallavarapu et al., 2011). At present, bioremediation is mainly used for pollution control in soil, groundwater and oceans, and is *in situ* remediation (Rizzo et al., 2008; Beskoski et al., 2012). Plant-microorganism combined bioremediation is a valuable research direction, providing a synergistic effect between plants and special mycorrhizal fungi or rhizosphere microbes to increase the absorption and degradation of pollutants (Okoh and Trejo-Hernandez, 2006; Mohsenzadeh et al., 2010; Du et al., 2011; Liu et al., 2011). Zhang et al. (2009) and Chen et al. (2010), compared the effects of plant, microorganism and plant-microorganism combined remediation, respectively, and found that the

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impact of plant-microorganism combined remediation had the biggest beneficial effect. In pot experiments with ryegrass (*Lolium perenne*) and microorganisms, Tang et al. (2009, 2010) found that soil remediation by the combination of ryegrass and mixed microbial strains gave the best result, with a degradation rate of 58% after a 162-day treatment, which was significantly higher than that of the control. The experiment of Liu et al. (2009) showed that after a 120-day treatment, the degradation rate of petroleum in soil in treatment with adding microorganisms was 67.0%; the degrees of degradation were 38.23%, 36.57%, 40.67% and 38.67%, respectively, planted with sunflower, cotton, Bermuda grass, and Sudan grass; and the degree of petroleum degradation reached as high as 85.67% with cotton+microorganisms treatment. In a 2-year experiment to evaluate the remediation effect of plant-microorganism combinations on diesel-polluted soil, Lin et al. (2008) found that the removal rate of diesel oil using combined plant-microorganism was over 67%, which was 13%–30% higher than that achieved by plants alone. These results showed that an appropriate plant-microorganism synergy provides an approach of low cost, highly efficient remediation for oil-polluted soil.

By choosing appropriate combinations of plants and microbes, polluted soil can be remediated. However, to obtain the expected result, the relationship between pollutants, plant species/variety and microorganism needs in-depth understanding, so that the remediation process can be effectively controlled. At present, the research in this field in China is mainly focused on microbial remediation, and there is less research on plant remediation and plant-microorganism combined remediation. In particular, the mechanisms and control factors for plant-microorganism combined remediation systems still lack in-depth understanding. Our study, based on the climate characteristics of Xinjiang, previous experiments and relevant information (Morgan and Watkinson, 1994; Sun and Song, 1999; Xia and Lin, 2000; Ren et al., 2004), found that reeds had a favorable effect on the remediation of crude oil-polluted soil, and that screened, mixed KL8-1 bacteria from the reed rhizosphere were effective in degrading oil. By pot experiment, the study optimized the amount of inoculation, surfactant,

nitrogen and phosphorus nutrients and soil water-retaining agent by using a response surfaces method to study the effect of reed-specific degrading bacteria remediation on crude oil-polluted soil. The aims were to research a rapid method for using reed-specific bacteria for degrading hydrocarbon pollutants of petroleum, and to provide support for establishing the technology of plant-microorganism combined remediation for crude oil-polluted soil of Xinjiang.

1 Materials and methods

1.1 Experimental design

The soil samples for the experiment were taken from the top 20 cm of unpolluted soil in the Karamy region. The mechanical composition of this soil is 18.2% sand, 58.4% silt and 23.4% clay. The soil samples were first sieved with a 2-mm mesh to remove large particles. The specific degrading bacteria KL8-1 used in the experiment were raised in the laboratory. One kilogram of soil was put into each of 35 cylindrical pots with the diameter of 10 cm and the height of 15 cm. The crude oil came from Karamy's second oil refinery. After 24 h of the soil mixing with the crude oil, nitrogen and phosphorus nutrient salts (using ammonium chloride and potassium phosphate) were added to the soil in the pots. The experiment contained 35 samples, of which 6 samples were controls. The samples 30–32 were controls with reed and samples 33–35 were controls without reed. The mixed bacteria KL8-1 were placed in pots of polluted soil, and then 20-cm reed seedlings were planted in the soil. During the experimental process, water was periodically irrigated to maintain the soil water content at around 20%. After 90 days the oil content in the soils was determined. The above steps were repeated 3 times using different amounts of the variables (crude oil, N:P ratios, KL8-1 bacteria and surfactant) (Table 1).

1.2 Measuring methods

The content of petroleum hydrocarbons in soil samples was determined by placing 2 g of dried soil into a 50-mL centrifugal tubes, adding 10-mL acetone/dichloromethane (1:1), conducting ultrasonic extraction for 15 min (minimum 300W) and then centrifuging the samples at 7,500 rev./min for 10 min. The result-

ing upper clear liquid was collected and filtrated by anhydrous sodium sulfate (dried for 2 h at 300°C). The crude oil contents in the pots were determined by spectrophotometry and using an infrared oil-measuring instrument (Model 510, ChinaInvent Instruments, China) (Morgan and Watkinson, 1994; Sun and Song, 1999; Qian and Liu, 2000; Ren et al., 2004).

1.3 Analysis method

Using the Box-Behnken method (Ferreira et al., 2007), the optimum balance of the 4 factors (different concentrations of crude oil-pollution, different inoculations of specific degrading bacteria, varied ratios of nitrogen and phosphorus, and different amounts of

surfactant) was established for the effect of the combined remediation of reed-specific degrading bacteria on crude oil-polluted soil (Tables 1 and 2). The results

Table 1 Code and actual values of variables used in the response surfaces

Level	Code A	Code B	Code C	Code D
	Crude oil concentration (g/kg)	Bio-mass*	N/P (v/v)	Surfactant (Tween 80; ‰)
Low level (−1)	10	0	5	0.2
Medium level (0)	30	1	6	0.6
High level (+1)	50	2	7	1.0

Note: *, the specific amounts of degrading bacteria (fixed by adding sterilized peat in which the quantity of microorganisms reached 10^{10}) were 0 g/kg (coded 0), 100 g/kg (coded 1) and 200 g/kg (coded 2).

Table 2 Box-Behnken experiment design and results

No.	Crude oil concentration (g/kg)	Biomass*	N/P (g/g)	Surfactant (Tween 80, ‰)	Degrading rate (%)	
					Measured value	Predicted value
1	30	0	6	1.0	51.15	51.69
2	50	1	7	0.6	60.64	59.98
3	30	0	6	0.2	45.57	46.96
4	30	2	7	0.6	62.68	61.64
5	50	1	5	0.6	52.16	54.11
6	30	1	7	0.2	56.28	56.31
7	30	2	5	0.6	63.76	62.17
8	10	1	7	0.6	60.16	59.96
9	30	1	5	0.2	57.32	56.10
10	30	1	6	0.6	50.47	50.63
11	30	2	6	0.2	65.36	66.67
12	10	1	5	0.6	59.80	61.39
13	30	0	7	0.6	43.19	43.63
14	30	1	6	0.6	61.35	61.35
15	30	1	6	0.6	61.35	61.35
16	10	1	6	0.2	64.21	63.22
17	50	1	6	1.0	56.29	56.13
18	50	0	6	0.6	46.96	45.36
19	10	0	6	0.6	53.41	52.72
20	50	2	6	0.6	60.44	60.50
21	30	0	5	0.6	48.11	48.00
22	30	2	6	1.0	63.92	64.28
23	30	1	6	0.6	61.35	61.35
24	30	1	7	1.0	54.28	54.87
26	30	1	6	0.6	56.08	55.63
27	10	2	6	0.6	61.35	61.35
28	10	1	6	1.0	68.78	69.75
29	30	1	5	1.0	65.86	65.16
30	10	0	0	0	12.50	-
31	30	0	0	0	11.80	-
32	50	0	0	0	10.30	-
33	10	0	0	0	5.21	-
34	30	0	0	0	4.36	-
35	50	0	0	0	4.98	-

Note: *, the specific amounts of degrading bacteria (fixed by adding sterilized peat in which the quantity of microorganisms reached 10^{10}) were 0 g/kg (coded 0), 100 g/kg (coded 1) and 200 g/kg (coded 2).

were fitted using quadratic multinomial regression. This was applied to predict the optimal point using differential calculations. SPSS statistical software was employed to conduct significance tests for the fitting equation and variance analysis. In the experimental process the crude oil concentration in the soil was 10–50 g/kg, the nitrogen content was between 0.2–3.0 g/kg, and the specific amounts of degrading bacteria (fixed by adding sterilized peat in which the quantity of microorganisms reached 10^{10}) were 0 g/kg (coded 0), 100 g/kg (coded 1) and 200 g/kg (coded 2).

2 Results and discussion

2.1 Results of remediation to crude oil-polluted soil using reed-specific degrading bacteria

After 60 days of plant-microorganism combined remediation, the crude oil contents in every treatment were reduced (Table 2). Because of the natural volatilization and light oxidation action, the rates of crude oil degradation in control pots were 10.3%–12.5%. The crude oil degradation in the soils with reed-specific degrading bacteria was significantly better than that of the control soils, recording crude oil degradation up to 43.19%–68.18%. In the soils with crude oil concentrations of 10, 30 and 50 g/kg, the degradation rates of petroleum hydrocarbons reached 68.78%, 65.86% and 60.64%, respectively, which indicated that crude oil concentration had a great impact on remediation by reed-specific degrading bacteria. Lower oil concentration allowed the reed-specific degrading bacteria to be more effective. The amount of surfactant had no remarkable impact on reed-specific degrading bacteria. This may be related to the fact that the specific degrading bacteria KL8-1 contains the surfactant which produces bacteria *Bacillus subtilis*. Reed-specific degrading bacteria were most effective at the nitrogen and phosphorus ratio of 6 and KL8-1 concentration of 200 g/kg.

2.2 Establishment of fitting model for reed-specific degrading bacteria remediation

Using the Box-Behnken design method, the 4 factors (the concentrations of crude oil, the amount of inoculation, the ratio of nitrogen and phosphorus, and the amount of surfactant added) optimized the effect of

combined remediation of reed-specific degrading bacteria on crude oil-polluted soil. A prediction model of the quadratic multinomial regression equation is as follows:

$$Y = 61.35 - 4.15A + 8.05B - 1.23C + 0.61D - 0.47AB - 0.51AC - 0.36AD + 0.96BC - 1.75BD - 1.33CD - 0.80A^2 - 3.46B^2 - 4.02C^2 - 0.51D^2.$$

Where, Y is the predicted petroleum degrading rate; A, B, C and D represent the concentrations of crude oil, the amount of inoculation, the ratio of nitrogen and phosphorus, and the amount of surfactant added (Tween-80), respectively.

The variance analysis of the prediction model showed that the experimental results were consistent with the prediction model of the quadratic multinomial regression equation, with $R^2=0.9819$ (Table 3). The equation can well predict the effect of remediating crude oil-polluted soil using reed-specific degrading bacteria. In addition, the results show that the 4 factors A, B, B^2 and C^2 had significant impact on the effect of combining plant-microorganism remediation.

The response surfaces of the different factors which impacted the remediation of crude oil-polluted soil using reed-specific degrading bacteria and their interaction (Fig. 1a) show that the higher the amount of microbial inoculation, the more the reducing crude oil concentration, and the better is the remediation effect. This indicates that microorganisms for degrading crude oil are one of the key factors in the biological remediation process. In addition, while crude oil concentration is low, the ratio of toxic substance to microorganisms is low and the degradation by the microorganisms is effective. When crude oil concentration increases, the concentration of toxic and harmful substances in the crude oil will increase, and then the effectiveness of bioremediation declines. The study of Zhu et al. (2001) found the same results.

The addition of nitrogen-phosphorus nutrient salts and surfactant can accelerate crude oil degradation. The actions of nutrient salts are mainly to promote microbial development and reproduction which benefits the process of degradation (Walworth et al., 2001). The study of He et al. (2004) found that excessive nitrogen inhibited crude oil degradation, and it is more suitable that the ratio of nitrogen and phosphorus is

Table 3 Variance analysis for prediction model of response surfaces' quadratic multinomial

	The degree of freedom	Standard deviation	F value	Prob>F	The degree of significance
Prediction function	14	84.97	54.13	<0.0001	Significant
A	1	206.84	131.76	<0.0001	Significant
B	1	776.83	494.87	<0.0001	Significant
C	1	18.08	11.52	0.0044	Non-significant
D	1	4.47	2.84	0.1138	Non-significant
AB	1	0.89	0.57	0.4632	Non-significant
AC	1	1.05	0.67	0.4270	Non-significant
AD	1	0.52	0.33	0.5746	Non-significant
BC	1	3.69	2.35	0.1477	Non-significant
BD	1	12.32	7.85	0.0141	Non-significant
CD	1	7.08	4.51	0.0521	Non-significant
A ²	1	4.16	2.65	0.1258	Non-significant
B ²	1	77.75	49.53	<0.0001	Significant
C ²	1	104.93	66.85	<0.0001	Significant
D ²	1	1.68	1.07	0.3190	Non-significant
Residual standard deviation	14	1.57			
$R^2=0.9819$		$R_{Adj}^2=0.9637$			

Note: A, B, C and D represent the concentrations of crude oil, the amount of inoculation, the ratio of nitrogen and phosphorus, and the amount of surfactant added (Tween-80), respectively.

- Design points above predicted value
- Design points below predicted value

Fig. 1 Response surfaces of impacts of different factors and their interaction. (a) crude oil concentration and biomass of bacteria; (b) crude oil concentration and N/P ratio; (c) crude oil concentration and surfactant; (d) N/P ratio and biomass of bacteria.

between 5:1 and 6:1. From Fig. 1b it can be seen that when the ratio of nitrogen and phosphorus is 6, the degradation is the most complete. The effect on crude oil degradation through adding nutrient salts into reed-specific degrading bacteria was better than that of reed-specific degrading bacteria. This may be because the nutrient salts promote the coordinated action between reed and specific degrading bacteria KL8-1. The effect of adding surfactant into the reed-specific degrading bacteria was not significant to the degradation effect of crude oil. When the surfactant concentration was increased, the rate of crude oil degradation showed almost no change (Fig. 1c). From the response surfaces of nutrient salts (the ratio of nitrogen and phosphorus) and their interaction (Fig. 1d), it can be seen that when the ratio of nitrogen and phosphorus is 6 and the biomass of bacteria is the largest, the effect on hydrocarbon degradation is the best. However, adding extra nitrogen (the ratio of nitrogen to phosphorus was 7) did not improve the effect of crude oil degradation.

2.3 Validation of prediction model

Under different conditions of crude oil concentration, the validation to the optimized results of the prediction model showed that under the optimum condition, when crude oil concentrations were 10, 30 and 50 g/kg, the effects of crude oil remediation were 71.87%, 66.61% and 54.52% degradation, respectively, which is very near to the predicted values with the standard deviation of less than 2%. This indicates that the prediction model can be used to predict the effect of reed-specific degrading bacteria remediation on crude oil-polluted soil. When crude oil concentrations were 50 g/kg, the removing rate of crude oil decreased to 53.52% (Table 4). Crude oil in high concentrations

can restrain the growth of reeds and specific degrading bacteria, resulting in the drop in the removal rate of crude oil.

4 Conclusions

Using the Box-Behnken design, taking the initial crude oil concentration, the amount of inoculation, the ratio of nitrogen and phosphorus, and surfactant (Tween-80) as independent variables (factors), and taking the degrading ratio of crude oil after a 90-day experiment as a dependent variable (response), we studied the impacts of each independent variable and their interactions on the bioremediation of crude oil-polluted soil. The results show that the concentration of crude oil, the amount of microbial inoculation, and nutrient salts (the ratio of nitrogen and phosphorus) are the key factors affecting plant-specific degrading bacteria remediation in crude oil-polluted soil, and the best biological remediation conditions occurred with immobilized specific degrading bacteria of 200 g/kg, N/P ratio of about 6, and surfactant dosage of 0.2%.

Taking the degradation rate of petroleum as the response value, the prediction model of the quadratic multinomial was obtained by conducting a simulation using the central combination of response surfaces. On the basis of the quadratic multinomial regression equation, the study calculated the optimal remediation effects on crude oil-polluted soil with crude oil contents of 10, 30 and 50 g/kg, and generated crude oil degradation rates of 71.87%, 67.61% and 54.52%, respectively. These are higher than the experimental values of 68.78%, 65.86% and 60.64% for each single factor. The response surfaces method is an effective approach for optimizing combinations of treatments for bioremediation of crude oil-polluted soil.

Table 4 Validation of model optimization for crude oil-polluted bioremediation

Crude oil concentration (g/kg)	Biomass*	N/P (g/g)	Surfactant (‰)	Degrading rate (%)		SD
				Measured value	Predicted value	
10	2	6.19	0.21	71.87	71.12	0.33
30	2	6.11	0.22	66.61	67.39	1.56
50	2	6.07	0.20	54.52	54.57	0.12

Note: *, the specific amount of degrading bacteria (fixed by adding sterilized peat in which the quantity of microorganisms reached 10^{10}) was 200 g/kg (coded 2); SD=standard deviation.

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