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Microbial Remediation and Optimization of Oil Polluted Wetlands at Dalian Bay in China[1]

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Objective The wetland at Dalian Bay in the Northeast of China has been polluted by oil severely. The effect of various microbes and operation parameters on the bioremediation of oil-polluted wetlands at Dalian Bay was investigated and reported previously. In the study, other operation conditions related to the status of medium were investigated via statistical experimental design and analysis and a necessary information is involved to use micro-technology in the application. **Methods** The method used involved the direct inoculation of selected bacteria, which were capable of degrading oil. The operation conditions were further optimized and evaluated by gravimetrical assay. **Results** The optimal pH and temperature for the studied bacteria to degrade the existing oil pollutants were established as pH 8.0 and 27℃. The mixed of various bacteria showed better results in terms of oil degradation than any single one. Among the selected four factors, disturbance, oxidant, nutrients, and biosurfactant, the former two contributed more impacts on the oil degradation in the early stage of process, while the latter two became the limiting factors in the late stage. Three sets of optimal conditions were obtained for each individual stage, but no one was suitable for the overall process. **Conclusion** The study demonstrated the technical feasibility of using direct inoculation into the contaminated soil samples to remove oil pollutants. It suggested that the operation conditions should be monitored and adjusted during the different stages of bio-reactions in the process to achieve the best result of oil degradation.

Key words: Biodegradation; Micro-bioremediation; Oil pollution

INTRODUCTION

The wetlands are important natural resources, as they provide natural communities for various living creatures, and help maintain ecosystem equilibrium. The wetland at Dalian Bay in the Northeast of China is a typically salty wetland. It has been polluted severely at present. The main pollutants include petroleum oil and its derivatives such as phenols, and heavy metals such as mercury, lead, arsenic, cyanide, and chromium. Oil has been identified as the primary contaminant by the Environmental Protection Agency of China[1]. The attempts to remedy the Wetlands have been carried out[2].

There are many soil remediation techniques, including burying, emulsification, evaporation, solidification, dispersion, washing, thermal adsorption, nitrification, incineration and mechanical removal. However, most of these technologies are either costly or cannot lead to complete decomposition of contaminants. Biological treatment has attracted more attentions as a promising method for dealing with a wide range of wetlands polluted by various organic contaminants, particularly petroleum hydrocarbons. It involves the natural processes resulting in the efficient conversion of hazardous compounds into innocuous products. Thus, it is environmentally friendly and less expensive than other physical-chemical methods.

In terms of oil pollutant degradation, bioremediation provides a relatively fast approach by using various soil microorganisms to decompose petroleum hydrocarbons, also it reduces the risk of contaminating groundwater[3-4]. Microbial degradation by natural breeds is the primary option for environmental treatments, which can be enhanced by current bioremediation technologies[5]. The

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concept of microbial remediation appeared in the late 1980's. This technology involves appropriate microbes undergoing various physical and chemical reactions in the polluted soil system, and optimization of bioremediation conditions during the microbial metabolism the pollutants are degraded and removed[6-7].

The method used in the study involved the direct introduction of selected bacteria to the soil samples taken from the wetlands. The effect of these selected bacteria and operation conditions on oil biodegradation was investigated and reported previously[8]. The study clearly demonstrated that the direct soil inoculation was an effective method for bioremediation of the wetlands. Based on this, the attempts to optimize the operation parameters were further carried out in this study, which is supposed to provide the necessary information for future pilot and field studies.

MATERIALS AND METHODS

Soil

The soil samples were taken from the surface layer (0-15 cm) of the wetlands at Dalian Bay. The samples were then dried and sieved by a 1 mm screen, and stored at 4℃ in plastic bags prior to analyses. Physical-chemical characteristics of soil were: 10.3 g/kg of organic carbon, 1.751 g/kg of total nitrogen, 1.02 g/kg of total potassium, pH 8.07, 180 ± 20 mg/kg of mineral oil[2].

Bacteria

Bacteria presented in the soil samples and capable of degrading oils were isolated directly from the oil polluted soil samples. They were *Acinetobacter* sp. LT4, *Pseudomonas* sp. B2 , BJ8, CY11, and *Achromobacter* sp.BJ5.

Media

The Luria Bertani medium (LB) (pH7.0) containing 10 g of polypeptone, 5 g of yeast extract, and 5 g of NaCl per liter was prepared. The solid media were prepared by adding 1.8% agar based on the above formulation.

The mineral medium used throughout this study was prepared according to the description from Hareland[9]. The medium was sterilized prior to use.

Analytical Methods

Oil degradation rate The concentration of oil in the soil samples was determined gravimetrically by measuring the weight before and after dry extraction, and the level remained after biodegradation was evaluated by comparing the measurements with the control[10-11].

Biodegradation experiment design Biodegradation assays were carried out in 250-mL Erlenmeyer flasks containing 50 mL of mineral medium and 15 g contaminated soil as the sole source of carbon and energy. A direct inoculum was obtained from the environmental cultures in mineral media. An inoculum containing cells from enriched cultures was sub cultured in LB.

The direct inoculum together with samples were transferred to the enriched cultures and incubated for 5 or 10 days. The inoculum pre-grown in LB was obtained by sub culturing 500 μL of the above transfers to 50 mL of LB and incubated for overnight, 1 mL samples of the LB cultures were washed and used to inoculate the mineral medium with soil. The targeted concentration of cells was approximately 10^7 cfu m/L. Mixed bacteria was obtained by taking the same amount of each single strain and mixed completely with the inoculum at a size of 1% (v/v).

A negative control without inoculum was also included. Duplicates were made for all treatments. Specific environmental parameters were listed below. All parameters, other than specified, were maintained throughout the study.

Selection of optimal operation pH and temperature Reaction system pH was adjusted to 7.0, 8.0, 8.5, and 9.0 by adding different amounts of NaOH. After 27℃ and 120 r/min of incubation, oil residual concentration was determined.

The effect of temperature on microbial degradation was tested by incubation of the treated soil samples and the untreated control at 20, 23, 25, 27, and 30℃. The soil samples used in these experiments were pre-incubated for 3 days at the corresponding temperature.

Statistical design of experiments Four operation factors were selected, with three different levels of each factor chosen to follow the Latin Square experimental design. They are described in the following Table 1.

TABLE 1

Selection of Factors and Their Levels for the Experimental Design

Factors Levels	A (Disturbance)	B (Nutrients Ratio of oil/NH_4NO_3/K_2HPO_4)	C (Oxidant H_2O_2(/Soil))	D (Biosurfactant Tween-80 (/Soil))
1	No Shaking	75/1.6/0.6	0	0
2	Shaking Twice a Day	75/8/3	0.4%	0.3%
3	Shaking All the Time	75/24/9	0.85%	0.5%

RESULTS

Selection of Optimal pH

As shown clearly in Figs. 1 and 2, all studied bacteria displayed better performances at pH 8.0, in terms of degrading the presented oil in the soil (The initial oil content was 100%). In addition, the oil degradation rates increased over the operation time. After 8 days' cultivation, five microbes decomposed oil ranging from 28% to 45% at pH 8.0. After 12days, the microbes could be able to degrade the existing oil by 64 to 72%. In general, BJ8 and B2 performed better than other three.

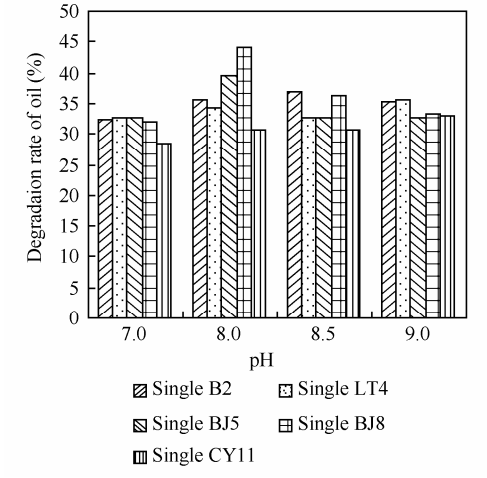

FIG. 1. Oil degradation after 8 d at different pH.

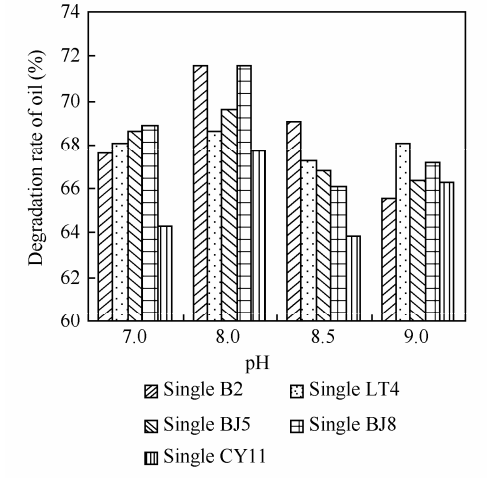

FIG. 2. Oil degradation after 12 d at different pH.

Based on the above, pH 8.0 was selected as the optimal pH for all studied bacteria to degrade the oil in the soil samples.

Selection of Optimal Temperature

As shown in Figs. 3 and 4, all studied bacteria demonstrated better performances at 27℃. At this temperature, five bacteria could degrade 22% to 45% of oil after 8 days' operation. Over a time period of 12 days, oil degradation rates from all five microbes reached 70%. In general, BJ8, B2, and BJ5 were slightly better than other two bacteria.

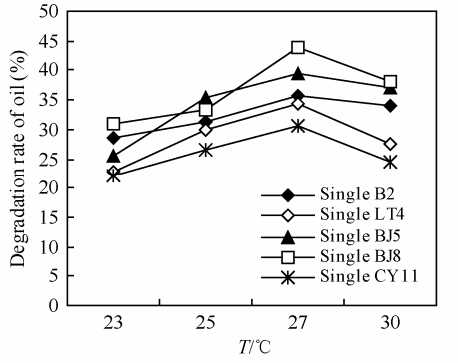

FIG. 3. Oil degradation after 8 d at diferent temperature.

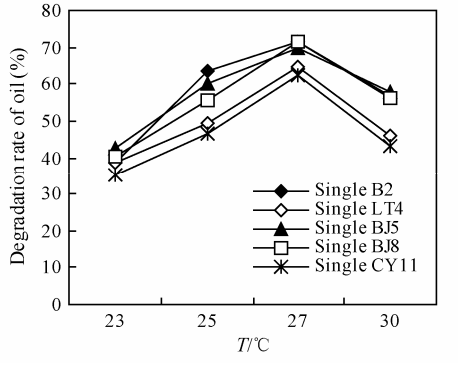

FIG. 4. Oil degradation after 12 d at different temperature.

Based on the above, 27℃ was determined as the optimal temperature for all studied bacteria to degrade the oil in the soil samples.

Comparison Between Different Single Strain and Mixed Bacteria Under the Optimal pH and Temperature

As shown in Fig. 5, the biodegradation rate of oil increased greatly by using mixed bacterial strains, even though it was noticed that the studied single strain degraded the oil quickly in the earlier days.

The operating conditions of mixed bacteria for oil degradation were further investigated and discussed below.

Optimization of Other Medium-related Parameters from the Statistical Experiment Design Using Mixed Bacteria

As shown in Table 2, during an operation time period of 15 days under 27℃ and pH 8.0 , the oil degradation

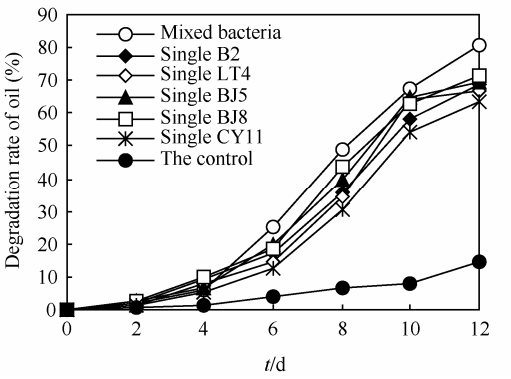

FIG. 5. Comparison of single and mixed microbes on oil degradation under the optimal pH and temperature.

profile of the mixed bacteria showed different patterns under the different operation conditions. For example, the rate of oil degradation increased most rapidly under the first condition, which was a combination of oil/NH_4NO_3/K_2HPO_4 at 75/1.6/0.6, no oxidant and biosurfactant involved, and without shaking. The slowest degradation occurred at the third condition of oil/ NH_4NO_3/K_2HPO_4 at 75/24/9, oxidant addition level at 0.85, biosurfactant at 0.5%, and without shaking. As it was just the direct observation, the detailed statistical analysis on the experimental data was needed to acquire the essential information regarding the major factor(s) and their appropriate levels.

TABLE 2

Percentage of Oil Degraded by the Mixed Bacteria Under the 9 Different Combinations of Operation Conditions Obtained From the Latin Square

Samples	Factors	5 Days Later (%)	10 Days Later (%)	15 Days Later (%)
1	A1B1C1D1	40.52	78.55	85.34
2	A1B2C2D2	40.54	70.53	82.18
3	A1B3C3D3	20.82	56.76	71.93
4	A2B1C2D3	29.71	64.20	76.28
5	A2B2C3D1	43.80	69.31	79.94
6	A2B3C1D2	41.50	64.19	80.40
7	A3B1C3D2	37.21	64.34	71.65
8	A3B2C1D3	39.07	67.71	76.22
9	A3B3C2D1	45.12	68.22	78.67

The statistical analysis was carried out based on three different operation stages: early stage (0-5 days), intermediate (6-10 days), and late stage (10-15 days).

Statistical analysis in the early stage (0-5 days) of the experiment According to the statistical principle that the higher the R value is, the more significant the effect is from the correlated factor (herein the operation conditions) on the tested parameter (herein the oil degradation rate). Therefore, the major factor, which affected the oil degradation most significantly in the early stage, was the disturbance condition (A), followed by oxidant (C), biosurfactant (D), and the ratio of nutrients (B). Namely, the sequence of the studied four factors was A>C>D>B, which reflected their importance on the oil degradation (Table 3).

TABLE 3

Statistical Analysis Based on the Oil Degradation During the Early Stage From 0-5 Days

Sample Number	A	B	C	D	Degradation Rate（%）
1	1	1	1	1	60.52
2	1	2	2	2	60.54
3	1	3	3	3	40.83
4	2	1	2	3	59.71
5	2	2	3	1	63.80
6	2	3	1	2	61.50
7	3	1	3	2	57.21
8	3	2	1	3	59.07
9	3	3	2	1	61.12
I	162	177	181	181	
II	185	183	183	179	
III	177	163	161	160	
i	54.0	59.0	60.3	59.7	
ii	61.7	61.0	61.0	60.3	
iii	59.0	54.3	53.7	53.3	
R	7.7	6.7	7.3	7.0	

Note. Row I to III represents the sum of the oil degradation rate for each factor at their individual levels 1-3. Row i-iii are the average data correlated to Row I-III. Row R displays the difference between the maximum and minimum in each column of i-iii.

Using the data from Row i-iii to plot Fig. 6, the best level of each factor was obtained. All the factors displayed great effect on oil degradation at their second levels. This contributed the optimal operation condition as A2C2D2B2, namely, under a shaking condition of twice a day, a medium containing three nutrients at a ratio of oil/NH_4MO_3/K_2HPO_4 (75/1.6/0.6), and 0.4% (soil) H_2O_2 and 0.3% biosurfactant would aid the mixed bacteria to degrade oil most significantly.

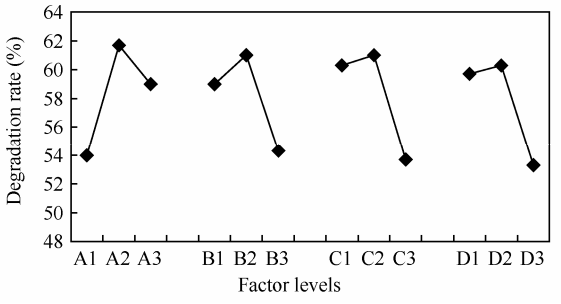

FIG. 6. Trendence of factor levels.

Statistical analysis in the intermediate (6-10 days) and late (10-15 days) stages Similar analysis was carried out based on the experiment data collected in the intermediate and late stages. The optimal conditions were obtained, D3B2A3C3 (Biosurfactant Tween-80 (/soil) 0.5%, Nutrients ratio of oil/NH_4NO_3/K_2HPO_4 75/8/3, Disturbance 120 rpm, Oxidant H_2O_2 (/soil) 0.85%) in the middle stage, and D1B2A3C3 (Biosurfactant Tween-80 (/soil) 0, Nutrients ratio of oil/NH_4NO_3/K_2HPO_4 75/8/3, Disturbance 120 rpm, Oxidant H_2O_2 (/soil) 0.85%) in the late stage.

The R values (the difference between the best and worst levels for each factor) were plotted in Fig. 7.

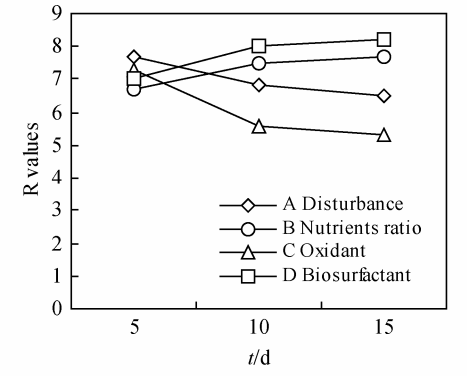

FIG. 7. The significance of the different factors' impact on oil degradation.

As shown in Fig. 7, the significance of the different factors' impact on oil degradation changed over time.

DISCUSSIONS

Biodegradation of oil was affected by the background pH[12]. The optimal pH range matched the natural condition of the wetland where was salty and slightly basic. Under the condition of lower than 27℃ or higher than 30℃, all bacteria could not work effectively in terms of degradation of oil. The optimal temperature range was accorded with the practical environment.

All the studied strains were selected from the contaminated wetland, and they were identified with capability to degrade certain pollutants. The soil may contain various oil derivatives, such as monoaromatic, linear and highly branched alkene hydrocarbons, polycyclicaromatic hydrocarbons. Each single strain might be only capable to degrade one or some of these components. The mixed bacterial community apparently showed a better result due to their synergetic effects, and this also agreed to the statements found in literature[13-14]. There also were a number of intermediates with unknown persistence and toxicity during the degradation as a result of co-metabolism and the mixed bacteria were helpful to keep a balanced micro-ecosystem[15].

Based on the statistical analysis, the factor A, disturbance, contributed the major impact on oil degradation in the early stage. At its second level, shaking twice a day, oxygen was supplemented sufficiently into the culture medium, which was also mixed well by shaking at meanwhile. Thus, it greatly favored the growth of biomass and further enhanced the oil degradation rate. The factor C, H_2O_2, also showed its importance. At its addition level of 0.4%, it promoted the oil degradation probably by two ways, 1. partially decomposing the existing oil hydrocarbons and making them more available to the bacteria; 2. increasing the oxygen concentration in the medium and thus enhancing the microbe activities. Generally, these two factors showed greater impacts than other two factors, nutrients and biosurfactant, in the early stage of the experiment.

The impact from the disturbance and oxidant decreased over time, while that of biosurfactant and nutrients increased. Initially, the disturbance had the most significant impact, while it fell to the third place in the middle and late stage. This is probably due to the fact that in the early stage, the growth of biomass dominated the process occurring in the system, and this tendency slowed down when the bacteria accumulated to certain amount over time. In this situation, the sufficient amount of oxygen in the system was the limiting factor in the early stage, which was achieved by proper disturbing and external oxygen supplement from the oxidant.

Similar situation happened to the impact of oxidant, which was supposed to help decompose the oil hydrocarbon substrates and provide external oxygen.

With the operation continued, oil degradation dominated the process in the system in the late stage. At this moment, the capability of the bacteria to degrade oil was relatively more dependent on the levels of the biosurfactant and nutrients rather than the oxygen level. The biosurfactant became the limiting factor for oil degradation. This is probably because that the selected biosurfactant (TWEEN 80) enhanced the hydrophilic property of the hydrocarbon pollutants in the system, and thus made the oil content more available for the bacteria. However, in the last stage, the best addition level of the biosurfactant reduced to 0 (level 1), which was probably due to the toxic property of the biosurfactant that would poison the biomass and further inhibit the oil degradation. This observation agreed to the result found by *Patrick P.E. Carrier* in the study on the effect of non-ionic surfactant triton-x100 on the bioremediation of phenolic oil pullulated soil[16].

CONCLUSION

The present study optimized operation parameters for the mixed bacteria to degrade the oil pollutants in the laboratory scale were investigated and established, which would be useful for the future pilot and/or field studies. The best pH and temperature were pH 8 and 27℃, respectively. Other operation parameters related to the composition of the media, such as disturbance, nutrient ratio, oxidant, and biosurfactant, were studied and the optimal combinations were established via the statistical experimental design and analysis. In the early stage of process, disturbance and the oxidant contributed relatively more significant impacts on the oil degradation rate. At this point, the optimal conditions were A2C2D2B2 (Disturbance Shaking twice a day, Oxidant H_2O_2 (/soil) 0.4%, Biosurfactant Tween-80 (/soil) 0.3%, Nutrients ratio of oil/NH_4NO_3/K_2HPO_4 75/8/3,). While the operation continued, the limiting factors changed to the biosurfactant and nutrients. The optimal conditions changed to D3B2A3C3 (Biosurfactant Tween-80 (/soil) 0.5%, Nutrients ratio of oil/NH_4NO_3/K_2HPO_4 75/8/3, Disturbance 120 rpm, Oxidant H_2O_2(/soil) 0.85%) in the middle stage, and D1B2A3C3 (Biosurfactant Tween-80 (/soil) 0, Nutrients ratio of oil/NH_4NO_3/K_2HPO_4 75/8/3, Disturbance 120 rpm, Oxidant H_2O_2(/soil) 0.85%) in the late stage. Apparently, the optimal combinations of four factors varied during the process. No single setting of optimal conditions was suitable for the overall process. Thus, to achieve the best result of oil degradation, the operation conditions should be monitored and adjusted during the different stages of bio-reactions in the process.

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