

Breeding of in-situ Petroleum Degrading Bacteria in Hangzhou Bay and evaluating for the In-situ repair effect¹

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Abstract. In this paper, the restoration behaviour of the in-situ microorganisms in seawater and sediments to the marine accident oil spill was researched. The experimental study on the breeding of in-situ petroleum-degrading bacteria in the seawater and sediments of Hangzhou Bay and the restoration of oil spill were carried out. Making use of the reinforced microbial flora, combined with physical and chemical methods in field environment, petroleum degrading and restoration experiment were performed, the effect of the breeding of in-situ degrading bacteria was evaluated, and the standard process of in-situ bacteria sampling, laboratory screening, domestication and degradation efficiency testing were formed. This study laid a foundation for further evaluation of the advantages and disadvantages for the petroleum-degrading bacteria of Hangzhou Bay during the process of in-situ restoration. The results showed that in-situ microbes of Hangzhou Bay could reach the growth peak in 5 days with the suitable environmental factors and sufficient nutrient elements, and the degradation efficiency could reach 65.2% (or 74.8% after acclimation). And also the microbes could adapt to the local sea water and environmental conditions, with a certain degree of degradation. The research results could provide parameter support for causal judgment and quantitative assessment of oil spill damage.

1. Introduction

Maritime transport by ship is called "world trade carrier". With the rapid development of the world economy, marine transportation develops rapidly. However, the accompanying global major oil spill accidents occur frequently, which often brings catastrophic environmental destruction and property loss^[1]. How to deal with marine oil spill pollution has become the focus of research in the field of science and society at this stage^[2]. Hangzhou Bay is a semi-enclosed bay, in the south of Shanghai, and north of Zhejiang. Zhoushan Islands are located in the Baymouth. A large number of port terminals are distributed along the Hangzhou Bay. Natural reserves, marine fisheries grounds and other sensitive resources are in the inner Bay. In the event of an oil spill, serious environmental pollution and economic losses will be caused.

Microbiological restoration technology^[3] is a safe, efficient, environmentally friendly and no secondary-pollution treatment technology developed on the basis of biodegradation. Compared to other chemical reagents, microbial agents are gradually becoming a new type of environmentally friendly materials and are used in the treatment of marine environmental pollution^[4].



In recent years, many scholars are committed to screening the oil pollution degradation bacteria in land, sea water and sediment^[5-9]. Researchers have now found more than 100 genera and more than 200 kinds of petroleum-degrading bacteria^[10]. However, there are few petroleum degrading bacteria adapted to the environmental conditions of Hangzhou Bay. It is difficult to measure the degree of degradation contributed by petroleum degrading bacteria during in situ remediation. Lack of standardized test method and processes is the problem.

This study provides efficient in-situ petroleum degradation bacteria resources for the oil pollution in Hangzhou bay, provides a laboratory test method for degradation efficiency, and provides parameter support for the causality judgment and self-repairing capability assessment of oil spill.

2. Materials and methods

2.1. Experimental material

2.1.1. Bacteria source

Bacteria source was taken from the surface of sea water and sea sediment of the Hangzhou Bay which was at different distances from the natural shoreline.

2.1.2. Main culture medium

(1) Basal medium

Beef extract 5 g, peptone 10 g, NaCl 5 g, distilled water 1000 ml, pH 7.2-7.4, 121°C sterilization 20 min.

(2) Minimal medium

NaCl 10 g, NH₄Cl 0.50 g, KH₂PO₄ 0.50 g, K₂HPO₄ 1.0 g, MgSO₄ 0.50 g, CaCl₂ 0.2 g, KCl 0.10 g, FeCl₂·4H₂O 0.02 g, distilled water 1000 ml, pH 7.0, 121°C, sterilization 20 min.

2.1.3. Oil sample

180 # marine fuel oil Some text.

2.2. Experimental methods

2.2.1. Methods for determination of cell growth curve

The cell concentration was determined by diluting the bacterial solution to a certain multiple, and the absorbance value of the bacteria was measured at the absorption wavelength of 600 nm. Deionized water was used as a reference. This value reflects the amount of bacterial growth.

2.2.2. Method for determination of total petroleum hydrocarbon

(1) Determination of the maximum absorption wavelength of oil

Dilute the prepared oil sample stock solution into an 10 ml of oil solution with the concentration of 40mg/L. The maximum wavelength scan was performed with an ultraviolet spectrophotometer and petroleum ether as a reference, to determine the maximum absorption wavelength of the oil sample.

(2) Protract of Standard Curve^[11]

Dilute the prepared oil sample stock solution into an oil solution with the concentration of 100mg/L. Take 8 10mL-colorimetric tubes, add 0, 0.4 ml, 0.8 ml, 1 ml, 1.5 ml, 2 ml, 3 ml, 4 mL oil diluents in each tube, which was fixed volume with petroleum ether. At the maximum absorption wavelength of the oil sample, the absorbance values were measured with a 10 mm quartz colorimetric ware by using petroleum ether as a reference to draw oil standard curve.

(3) Transfer the oil diluents sample which volume has been measured into the 1000 mL separating funnel. Clean the sample bottle with 20 mL petroleum ether and transfer into the separating funnel. Fully shake for 3 minutes, and wait for statically layered. Move the water layer into the sample bottle.

- (4) Filter the petroleum ether extract into a 50 mL volumetric flask.
 - (5) Remove the water layer into the separatory funnel, and repeat extraction with 20 mL petroleum ether. And then wash the separatory funnel with 10 mL petroleum ether funnel, collect the washing liquid in the same volume bottle, and use petroleum ether to fix volume.
 - (6) At the selected wavelength, measure the absorbance with a 10 mm quartz cuvette and use petroleum ether as a reference.
 - (7) Take the same volume of pure water, and operate the same as the oil diluent. Perform the blank test to measure the absorbance.
 - (8) Use the absorbance determined by the oil diluent sample, minus the absorbance of the blank test, to figure out the corresponding oil content from the standard curve.
 - (9) Calculation of petroleum degradation efficiency
- Petroleum degradation efficiency (%) = (Initial concentration C_0 - Determination concentration C) / Initial concentration $C_0 \times 100\%$

2.2.3. Method for preserving bacteria

There are many commonly used methods of microbiological preservation: periodic transplantation preservation, liquid wax preservation, carrier preservation, freeze-drying /vacuum-drying, liquid nitrogen cryopreservation, glycerol suspension preservation and glycerol cryopreservation^[12]. In this study, the methods of periodic transplantation preservation and glycerol cryopreservation were used.

3. Screening process of the in-situ petroleum degrading bacteria in Hangzhou Bay

3.1. Sampling and sample pretreatment

The sampling sites were different from the coastline. Sampling site 1# was near the levee, root of a trestle oil wharf in Hangzhou Bay. The sampling site 2# was located at a distance of 200-300 m from the levee. The sampling site 3# was located at a distance of 600-700 m from the levee. For the 3 sampling sites, each sampling site was taken from the sediment and surface of seawater, and each sample was taken with 2 parallel samples.

The retrieved samples were pretreated to remove fine solids. Different separation methods have been developed for different samples. For solid samples: take 1g of solid sample, and add to a conical flask containing 99 ml sterile water or sterilized saline water and glass beads. Stir with a magnetic stirrer for 15 min quickly to isolate the microorganisms from the solid particles to the water phase. Then, stop stirring and let stand for a while, and take 10 ml supernatant as a refined sample. Under aseptic operation, it was inoculated to a conical flask containing a medium for enrichment culture. For liquid samples: take a liquid sample and add to a conical flask. Stirred with a magnetic stirrer for 15 min quickly. Then, stop stirring and let stand for a while, and take the supernatant as a refined sample. The work flows were shown in Figure 1,2, respectively. The samples were pretreated and preserved in a refrigerator at 4 °C.

Take 2.5 mg oil samples carefully, transfer to the 25ml volumetric flask carefully, and add the petroleum ether solution with a boiling range of 60-90 °C. Dissolve the oil and make it to the calibration line to get the standard oil solution of 100 mg/L. Put it in a dark reagent bottle and preserved it in a unoxidized state.

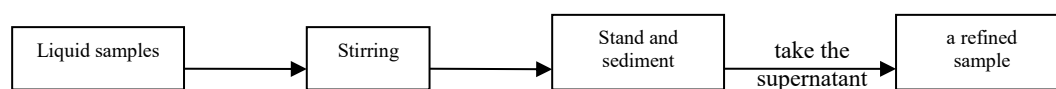


Fig. 1 pretreatment of liquid sample

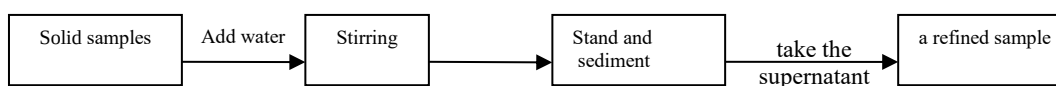


Fig. 2 pretreatment of solid sample

3.2. Protract of standard curve of the oil solution

(1) Standard oil solution series

Take 8 10mL-colorimetric tubes, add 0, 0.4 ml, 0.8 ml, 1 ml, 1.5 ml, 2 ml, 3 ml, 4 mL standard oil diluents with the concentration of 100mg/L in each tube. Fix volume each with petroleum ether and shake up, which could be considered as a series of different concentrations of standard oil solution, as shown in Table 1.

(2) Determination of standard curve^[13]

In the maximum absorption wavelength $\lambda = 230\text{nm}$ from the test, determine different concentrations of oil solution absorbance value, and according to the test results draw a standard curve, as shown in Figure 3.

The standard curve correlation equation is $A=0.0222C+0.0300$, $R^2=0.9969$. Absorbance A and concentration C is linear correlation significantly, which could be measured oil degradation rate.

Table 1. Determination results of standard curve of oil solution

Serial number	1	2	3	4	5	6	7	8
concentration (mg/l)	0	4	8	10	15	20	30	40
absorbance (A)	0	0.112	0.221	0.271	0.369	0.487	0.692	0.902

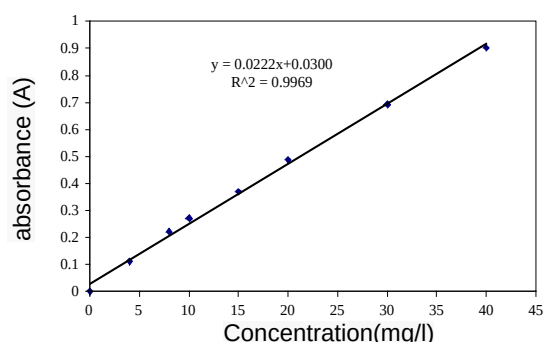


Fig.3. Standard curve of the oil solution

3.3. Screening, domestication and separation of petroleum-consuming bacteria

3.3.1. Bacterial strains primary screening

The screening process of degrading bacteria was the enrichment of petroleum degrading bacteria with petroleum hydrocarbon as the sole carbon source. Specific process was:

(1) A refined sample of 10 mL was added to a conical flask containing 100 mL basic medium (oil content was 0.1%), the temperature of the shaking table 30°C, rotating speed 140 r/min, enrichment culture for 3 d.

(2) The above enrichment medium of 10 ml was added to a conical flask containing 100 ml mineral salt medium (oil content was 0.1%), the temperature of the shaking table 30°C, rotating speed 140 r/min, enrichment culture for 5 d.

(3) Determining the oil content of the above mineral salt medium, the degradation rate of the petroleum-consuming bacteria was calculated.

3.3.2. Domestication and secondary screening

The above two type of petroleum-consuming bacteria samples with high oil degradation efficiency were further domesticated and re-screened. The specific process was: Take the culture medium which was enriched in two samples with high oil degradation efficiency, and add to a conical flask which containing 100 ml mineral salt medium (oil content was 0.1%), the temperature of the shaking table 30°C, rotating speed 140 r/min, culture for 5 d. The medium oil content was measured every 1 day from the time of inoculation. Since then, transfer two or three generations. The concentration of oil in the medium was increased by 0.1%, 0.3%, 0.5%. Determine the oil content of the above mineral salt medium, the degradation rate of the petroleum-consuming bacteria was calculated again.

3.3.3. Plate isolation

The domesticated medium was diluted with sterile water, in which the petroleum-consuming bacteria has growth for 24 hours. Then, take 0.2 ml of dilutions to the beef extract peptone plate (oil content was 0.1%), and coating uniformly with sterile spatula. Spread it evenly across the surface of the plate. Each dilution is repeated three times. And incubated in a thermostatic incubator at 30°C for 5 days. Pick a single colony deposit.

4. Laboratory test analysis of the degradation property

4.1. Analysis of degradation efficiency of the primary screening bacteria

Seven samples were obtained by primary screening (1-3 water samples and 1-4 mud samples were No.1-7 in turn), to determine the efficiency of oil degradation respectively. The test results are shown in Table 2 below.

Table 2. The result of the degradation rate of each sample bacteria on oil

Sample number	1	2	3	4	5	6	7
The degradation rate (%)	52.7	18.1	65.2	37.8	41.3	20.6	50.8

According to the measurement results, the degradation rate of the sample bacteria was compared. The degradation rate of petroleum hydrocarbons was higher in samples 1# and 3#, reaching 52.7% and 65.2%. In this way, the strains in 1# and 3# samples were selected for domestication and culture, which were named as bacteria-1# and bacteria-3#.

4.2. Analysis of degradation efficiency of the domestication and secondary screening bacteria

The above enrichment cultivation solution (1# and 3#) of 10 ml were added separately to conical flasks containing 100 ml mineral salt medium (oil content was 0.1%), the temperature of the shaking table 30°C, rotating speed 140 r/min, enrichment culture for 5 d. The medium oil content was measured every 1 day from the time of inoculation.

From Figure 4, ①The first passage bacteria: the degradation rate of the bacteria-1# was the highest after 3 days, and the degradation rate reached 53.4% after 5 days. The degradation rate of the bacteria-3# increased rapidly after 2 days, and the degradation rate reached 62.6% after 5 days. ②The second passage bacteria: the degradation rate of the bacteria-1# and the bacteria-3# increased rapidly after the second day, and the growth rate slowed down after the 4th day, and entered the steadily increase period. After 5 days of culture, the degradation rate of the bacteria-1# and the bacteria-3# were 61.2% and 68.7% respectively. ③The third passage bacteria: the degradation rate of the bacteria-1# increased rapidly after the second day, and the growth rate slowed down after the 4th day, and entered the steadily increase period. The degradation rate of the bacteria-3# increased rapidly after the first day, and entered the steadily increase period after the third day. After 5 days of culture, the degradation rate of the bacteria-1# and the bacteria-3# were 68.5% and 74.8% respectively.

The degradation efficiency of the bacteria-1# and the bacteria-3# under different oil content conditions after 5 d culture were compared. The results showed that the degradation rate increased

obviously after domestication. The degradation rate of bacteria-1# increased from 53.4% to 68.5% after culture. The degradation rate of bacteria-3# increased from 62.6% to 74.8% after culture.

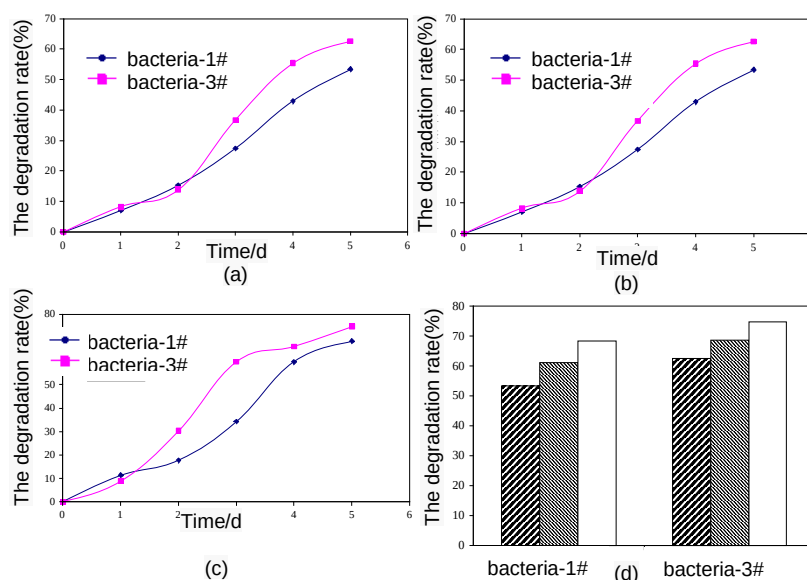


Fig.4. Degradation rate of the domestication and secondary screening of bacteria from each generation

4.3. Determination of the bacteria growth curve after domestication

The above enrichment cultivation solution of 2 ml was added to a conical flask containing 100 ml medium which was close to the composition of Hangzhou Bay sea water (oil content was 0.5%), pH 7.5, the temperature of the shaking table 30°C, rotating speed 140 r/min, enrichment culture for 7 d. The value of OD600 which reflected growth of the bacteria was measured every 1 day from the time of inoculation. The measurement results were shown in Figure 5.

It was seen from Figure 5, the bacteria-1# reached the growth peak in 3 days, and entered the steadily increasing period after the 6th day. The bacteria-3# reached the growth peak in 2 days, and entered the steadily increasing period after the 3th day. Further showed that the two types of bacteria could make good use of the hydrocarbons from the oil polluted sea water as a carbon source, with the ability to degrade petroleum hydrocarbons.

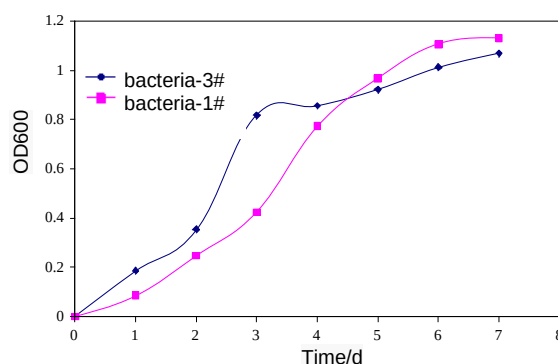


Fig. 5. Growth curve of degrading bacteria

4.4. Analysis of morphology of the bacteria

For the screened bacteria-1# and the bacteria-3# which were isolated by streak plate method, the color of the bacteria-1# was ivory, the colony texture was smooth and the colony diameter was about 3 mm. The color of the bacteria-3# was light yellow, the colony texture was smooth and the colony diameter was about 1 mm.

4.5. Analysis of degradation efficiency of different environmental conditions

The above enrichment cultivation solution was added to a conical flask containing 100 ml medium which was close to the composition of Hangzhou Bay sea water (oil content was 0.5%). The degradation efficiency and the growth curve of degrading bacteria were tested for different pH and temperature respectively.

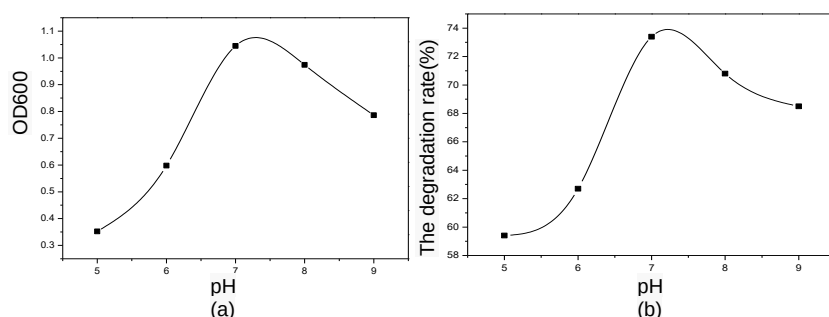


Fig. 6. The results of effect of pH on the growth of the strain and the oil degradation efficiency

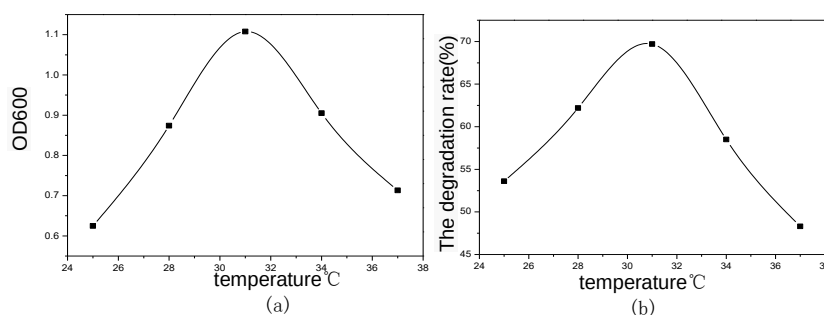


Fig.7. The results of effect of the temperature on the growth of the strain and the oil degradation efficiency

The results showed that, the temperature of the shaking table 30°C, 140 r/min, the bacteria grew well in the range of pH5.0 to pH9.0, and the degradation efficiency was 59.5-74%, as shown in Fig.6. The rotating speed of the shaking table 140 r/min, pH 7.5, the bacteria grew well in the range of the temperature of 25°C to 37°C, as shown in Fig.7.

5. Conclusion

(1) In this study, the in-situ petroleum degrading bacteria were bred from seawater and sediments of Hangzhou Bay. The standard flow of in-situ bacteria sampling, laboratory screening, domestication and degradation efficiency testing were formed.

(2) The results showed that the in-situ bred microbes of Hangzhou Bay reached the growth peak in 5 days under the suitable environmental factors and sufficient nutrient elements, and the degradation efficiency reached 65.2% (or 74.8% after acclimation). The bacteria have a certain adaptability on the local Hangzhou Bay of seawater composition, different pH, and temperature conditions, with the ability to degrade petroleum hydrocarbons.

(3) This study laid a foundation for further evaluation of the advantages and disadvantages of petroleum-degrading bacteria of Hangzhou Bay in the process of in-situ restoration and provided parameter support for causal judgment and quantitative assessment of oil spill damage.

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