Testing Method of Degrading Heavy Oil Pollution by Microorganisms

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Abstract. With the development of human society, we are more and more relying on the petrochemical energy. The use of petrochemical energy not only brings us great convenience, but is also accompanied by a series of environmental pollution problems, especially oil pollution. Since it is impractical to restore all pollution problems, the proper use of some remedial measures, under the guidance of functional orientation, may be sufficient to minimize the risk of persistent and diffusing pollutants. In recent years, bioremediation technology has been gradually developed into a promising stage and has played a crucial role in the degradation of heavy oil pollution. Specially, microbes in the degradation of heavy oil have made a great contribution. This paper mainly summarizes the different kinds of microorganisms for degrading heavy oil and the detection method for degradation efficiency of heavy oil pollution.

1. Introduction
We begin to pay more and more attention to a variety of toxic organic compounds that have been brought into our life intentionally or unintentionally, among which petroleum hydrocarbons, as the most important energy resources and industrial raw materials, bring great convenience to our life, but produce a lot of environmental pollution problems. Currently, there are no better substitutes for petroleum hydrocarbons which make the solution of pollution problems very important. The bioremediation technology refers to the method [1] for eliminating or reducing the concentration of harmful substances in polluted areas by take advantage of the potency of biology, especially microorganisms. As a pollution-free and low-cost method, it is superior to other physical and chemical methods in degradation of heavy oil pollution.

The heavy oil pollution has seriously damaged the balance of the soil ecosystem- reducing the soil fertility, affecting the normal growth of plants, and even in filtrating underground and causing serious pollution to the groundwater. All of these have seriously affected our life. Currently, there are two kinds of main methods for controlling the heavy oil pollution in soil: physical-chemical methods and biological methods; physical-chemical methods involves in excavation and landfill, isolation control, oxidation, extraction, adsorption, and so on. However, all of these methods have drawbacks, like they are temporary relieving methods, and prone to bring secondary pollution. In the contrast, microbiological methods, such as plant cultivation, microbiological method, have become a hot topic in dealing with the problems on oil pollution in soil for their advantages, for example, low cost, better effective and free of secondary pollution [2-3]

Microorganisms have the following advantage on the degradation of heavy oil pollution: There are widely distributed numerous microorganisms with a strong reproductive capacity, adaptability and faster metabolic rate. The studies on degrading heavy oil pollution by microorganisms have shown that
the degradation efficiency of heavy oil pollution has been able to reach about 70% under certain conditions\cite{4-5}. In addition, it is possible to adjust the degradation ability of microorganisms to heavy oil pollution by adjusting the living environment of microorganisms, such as adjusting the pH, adding mineral elements, adjusting the temperature and so on\cite{6-7}. Currently, the microorganisms used for degrading heavy oil pollution are mainly from contaminated soils near the oil fields, oil-contaminated sea, forest soil, etc.\cite{8-10}. There are wide variety of microorganisms in different environment and different regions.

There are various testing methods for the evaluation of heavy oil pollution degradation by microorganisms. Most of these methods are mainly based on gas chromatography (GC) \cite{11-13}. GC is generally simple and accurate and can be combined with the use of mass spectrometry, flame ionization detector, and so on for more accurate results \cite{10,14}. Beside GC, there are also reports based on infrared absorption spectrum \cite{15}, UV spectro-photometer \cite{16}, acid-base titration \cite{17}, gravimetric analysis \cite{18}, and so on.

2. Microorganisms

There are many kinds of microorganisms that degrade oil pollution, and they are mainly screened from different oil-contaminated environment. Generally, several or dozen kinds of different bacterial species can be obtained in the same environment. Therefore, in the existing environment, multiple bacterial species are producing combined effects of degradation on oil pollution. Reports showed that Gram-negative bacteria perform better and dominate in the degradation process, \cite{19} when compared with Gram-positive bacteria. The most common bacteria are bacilli, of which bacillus and pseudomonas are found more in the process of study. The following table 2-1. shows several common microorganisms used for degrading heavy oil pollution.

| Bacterial strain          | References |
|---------------------------|------------|
| Pseudomonas               | 4, 6, 15, 18, 20, 21, 22 |
| Bacillus                  | 4, 15, 21, 23, 24 |
| Ochrobactrum              | 5, 16, 24   |
| Acinetobacter             | 4, 25       |
| Proteobacteria            | 19, 26      |
| Firmicutes                | 19, 26      |
| Alcaligenes               | 5, 19       |
| Microbacterium            | 5           |
| Chryseobacterium          | 15          |
| Micrococcus               | 27          |
| Nitratireductor           | 22          |
| Actinobacteria            | 26          |
| Penicillum                | 28, 29      |
| Aspergillus               | 28          |
| Ganoderma lucidum         | 7           |
| Candida bombicola         | 23          |

Bacteria have been found to be the microorganisms actively involved in the degradation of organic pollutants, and many bacteria mentioned above are capable of effectively reducing the polycyclic aromatic hydrocarbons. Benzopyrene in petroleum hydrocarbons is considered to be the most carcinogenic and toxic. Studies have shown that a variety of strains have the ability to degrade benzopyrene. \cite{30} For example, 10 strains of bacteria were isolated from various contaminated sites (oil, engine oil, wood), by Aitkin et al. \cite{30}, and there are at least three kinds of pseudomonas, as well as agrobacterium, bacillus and so on. It is generally idea that the common coordination effect of different bacterial species is better than that of single strain because different bacterial species have different sensitivity for different kinds of hydrocarbons. In the context that the heavy oil pollution is often a mixture of multiple hydrocarbons, the coordination between bacterial species can be more effective for
its degradation. Daane et al. [31-32] isolated and characterized the petroleum hydrocarbon degradation bacteria associated with the salt marsh plant roots. They divided the isolated bacteria into three major bacterial groups: Gram-negative pseudomonas, Gram-positive bacteria and bacillus. They found that the fraction with rich phenanthrene was able to take advantage of more polycyclic aromatic hydrocarbons than fraction with rich naphthalene. They confirmed that bacteria in the genus bacillus isolated from petroleum-contaminated sediments and salt-marsh roots could use naphthalene or phenanthrene as the sole carbon source and therefore could degrade polycyclic aromatic hydrocarbons. At present, the degradation efficiency of bacteria on oil pollution can be significantly improved by adjusting the living environment of microorganisms. Jingxiu Wang et al. [15] found that the optimum degradation conditions for crude oil with the concentration of 1000 mg/L by the three kinds of bacteria including chryseobacterium, bacillus and pseudomonas were 10 °C temperature, pH = 7 and salinity of 10g*L-1. The degradation efficiencies of these three kinds of bacteria were 62.3%, 61.6% and 60.9% after 8 days under these conditions.

In addition, some fungi microorganisms can also effectively degrade oil pollution. For example, Fuad Ameen et al. [28] extracted 45 kinds of fungi, mostly aspergillus, and other 9 species, from the beach near the Red Sea. The whole degradation efficiency on crude oil can reach 55.5%. There are many algae which also made a great contribution to the degradation of ocean oil pollution. In general, studies on degradation of heavy oil pollution by bacteria are most popular, and the kind of involved bacteria are diverse. In the current stage, the most challenge for bioremediation technology is that, microbial populations have their own living environment and limitation of survival time, thus a certain field of microorganisms cannot be applied in other fields. In other words, there are space limitation and application restrictions needed to be overcome.

3. Test method

There are various methods for testing the oil pollution degradation efficiency in bioremediation of heavy oil pollution. Currently, we mainly rely on spectrum, chromatography and other methods to examine the content of residual oil pollution and then to calculate the degradation efficiency. The most common test methods are as below.

3.1. Gas chromatography-Mass spectrometry (GC-MS)

Gas chromatography - Mass spectrometry (GC-MS) is a method which combines the features of gas chromatography and mass spectrum for identifying different substances in the samples, and it is the most widely used method for testing oil pollution degradation. The main components of petroleum hydrocarbon are alkenes and polyaromatic hydrocarbon. Firstly, alkenes fraction and polycyclic hydrocarbons fraction are separated roughly through column chromatography. Then, both fractions can be detected effectively and respectively by GC-MS. By this way, the content and degradation efficiency of each component can be determined; the total oil pollution degradation efficiency can also be deduced. For example, Jingxiu Wang et al. [15] conducted research on the degradation of oil pollution by three bacterial strains in cold climate with GC-MS. According to their research, the small and medium molecular weight in the oil pollution after microbial action increases significantly and large molecular weight decreases obviously, which means that macromolecules are gradually degraded into small molecules after microbial degradation.

Besides, by detecting degradation process with GC-MS, it can be found out that which hydrocarbon compounds microorganism is more sensitive to and has more degradation ability and so on. These series of detailed information could greatly help to further study of microorganism’s degradation ability. For example, SERGI DI’EZ et al. [33] conducted a research on the bioremediation of two microbiomes with GC-MS. The results show that, n-alkane, alkyl cyclohexane, alkylbenzene, diaromatic hydrocarbon and triaromatic hydrocarbon are degraded obviously, and the biodegradation efficiency of higher polycyclic hydrocarbon is limited under the initial action. In addition, by analyzing and comparing the molecular species at different time quantum, the degradation tendency of oil pollution is given: non-ring isoprene > disilane > C27- gonane > bb- gonane > pentacyclic triterpene > monoaromatics> gonane > triarylstane.
3.2. Gas chromatograph - Flame ionization detector (GC-FID)
Similar to GC-MS, the concentrations of different components, such as alkanes, aromatic compound, can be detected by using gas chromatograph (GC) and flame ionization detector (FID) jointly, as well as the overall petroleum hydrocarbon and the degradation efficiency can be determined finally. Since FID has a high sensitivity, good stability and rapid response to organic compounds and tolerance to a wider temperature range, volatile hydrocarbon can be detected more conveniently. FID is widely used in organic compounds detection. Eman Koshlauf et al. [14] detected the impact of peas on the degradation of petroleum hydrocarbon in polluted soil through GC-FID. Their results showed that the optimum degradation efficiency could reach 96.1%.

3.3. Microbiological respiration (output of carbon dioxide)
The degradation of oil pollution by microorganism mainly relies on metabolism process using hydrocarbon compound as the carbon source. Large quantity of carbon dioxide will be produced during the metabolism. The determination of CO$_2$ content can be used for indication of the metabolism activity of microorganism indicated, and further used for the determination of degradation efficiency of oil pollution. Acid-base titration, gas chromatographic method, barium peroxide absorption method and other methods can be adopted for the determination of CO$_2$ content. For example, OBIRE, O et al. [34] determined the degradation efficiency of microorganism by detecting the output of CO$_2$ with Cornfield (1961) method. Cornfield method was firstly reported in 1961. In this method, barium peroxide solution is added in airtight reactor producing barium carbonate after absorbing CO$_2$. Then the content of absorbed CO$_2$ is determine by acid base titration. Cornfield method is relatively complicated. With the development of detection method the method of detecting CO$_2$ output becomes convenient and rapid now. For example, Samih Al Kharusiet al. [35] made the analysis of CO$_2$ contents in the 250 milliliter gas absorbed from airtight reaction bulb in different time stages with gas chromatographic method, the degradation ability of microorganism can be determined by comparing the CO$_2$ output produced at different time stages or under different conditions.

Though it is easy to determine the degradation ability of microorganism by detecting CO$_2$ output with microbiological respiration, the microbe activity and degradation can only be concluded by comparison, the degradation efficiency cannot be obtained clearly, which is the most disadvantage of this method.

3.4. Quality method
Quality method is a simple and convenient method for determining the degradation efficiency of microorganisms, that is, to determine the quality of reaction samples before the degradation, and then to determine the quality of remaining reaction samples after a period of degradation. The quality of the degraded oil pollution can be obtained by comparing the difference between the two qualities, and the degradation efficiency can be determined by simple calculations. However, this method can be only applied to the preliminary determination, with a relatively large error. And the quality method can only be used for figuring out the changes of total oil pollution, but not the determination of degradation efficiency of each component. Besides, the quality method cannot be used for detection the degradation process of long-chain alkenes to short-chain alkenes. By using this method, Qu Lina et al. [36] determined the degradation efficiency of microorganisms for oil pollution in soil.

3.5. Ultraviolet spectrophotometer method
Similar to the quality method, the determine the degradation efficiency by ultraviolet spectrophotometer analysis is also based on determination of the total oil pollution content. This method determines the degradation efficiency of the microorganism for oil pollution by detecting the change of absorbance of the mixed oil pollution solution. For example, Chen Libua et al. [37] applied this method to study the degradation efficiency of microorganisms in heavy oil pollution soil. Firstly, they used the absorbance of oil pollution solution with known concentration at 225nm to draw the standard curve, and then they determined the absorbance of oil pollution solution before and after degradation and calculated the
concentration of oil pollution solution before and after degradation. Finally, the degradation efficiency was obtained. The issue with this method is that the standard curve of oil pollution containing different components is different. Therefore, it is necessary to draw a different standard curve for different oil pollution degradation, which is slightly cumbersome.

3.6. Infrared spectroscopy
The main component of oil pollution is hydrocarbon. The C-H bond of the alkanet compound has tensile vibration at 2930 cm⁻¹. The concentration of the alkanet compound can be obtained by determining the absorption spectrum of the infrared spectroscopy, and then the degradation efficiency can be determined by comparing the oil pollution concentration. Compared with the UV spectrophotometer, this method is easier to operate. A. M. Zyakun et al. [38] determined that the degradation efficiency of microorganisms is in the range of 38±5% by this method. It was found that the results were consistent with that measured by GC-MS.

There are a wide variety of methods for determination of the oil pollution degradation efficiency. For the moment, gas chromatography is the most widely used method. Gas chromatography is mainly used with the combination of a series of other detection methods. Among these methods, gas chromatography-mass spectrometry is most widely used. This method featured with the advantages of high precision, high sensitivity and simple operation steps. Most importantly, oil pollution, as a series of mixtures of hydrocarbon compounds, can be effectively separated by this method, and then the degradation efficiency of different components can be determined. In addition, UV-visible absorption, infrared-visible absorption, quality method, microorganisms' respiration method and other methods are generally used as an auxiliary method together with GC-MS method and can also be used as the verification of result. For example, Martínez ÁlvarezLM et al [39] determined the concentration of hydrocarbons by GC-FID and IR, and have determined the oil pollution degradation efficiency of microorganisms by these two methods.

4. Conclusion
Large number of organic chemicals, such as petroleum hydrocarbons, halogenated and nitro aromatic compounds, phthalates, etc., have seriously polluted the soil and aquatic environment. The petroleum hydrocarbon contaminants pose a serious threat to human health and the environment because of its low reactivity and resistance to degradation. The conventional restoration method for these contaminated sites based on either physical or chemical methods is technically and economically challenging. In contrast, bioremediation is relatively effective, cost-effective and the most promising technology. Therefore, bioremediation technology is deemed as an effective and economical alternative over physical and chemical methods. We use microorganisms for the degradation of heavy oil pollution, which can avoid the secondary pollution and be at lower costs for its treatment. However, the existing bioremediation method is subject to many limitations, including poor ability of microbial communities, low bioavailability in space and time scales, and no reference values for functional test of bioremediation. At present, the lower microorganism degradation efficiency of heavy oil pollution under natural conditions is still the most serious problem to be overcome. It is of particular importance to use microorganisms to achieve efficient degradation of oil pollution and to determine its degradation efficiency in a simple and convenient manner.

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