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Bioremediation of Crude Oil Contaminated Soil by Petroleum-Degrading Active Bacteria

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1. Introduction

Approximatelly 0.6 million tons of petroleum is poured into soil, groundwater, rivers and oceans every year in China. A large amount of the oil contaminated soil has not been remediated. The contaminated soil poses a severe threat to the environment and must be taken care of. Bioremediation has become one of the most popular and promising technologies with growing demand for the remediation of petroleum contaminated soils because pollutants can be completely removed at low cost.

Bioremediation uses microbes to degrade hydrocarbons in soil. In the absence of effective microbes, the effect of bioremediation on pollutants is limited because native microbes need long times to climate, and their low metabolic activities make a short term bioremediation difficult. Inoculation of petroleum-degrading active bacteria increases the number of effective microbes, and thereby accelerates the bioremediation process. Effective microbes can be found in oil contaminated soils.

This chapter summarizes the experimental outcome of the evaluation of the bioremediation of oil contaminated soil by several bacteria strains that were isolated from an oil contaminated soil from the north region of the Shaanxi province in China. This chapter includes the characterization of petroleum-degrading active bacteria and the effect of the oil environment on bacterial activities. Bioremediation depends on pH, temperature, oil concentration, nitrogen and phosphorous concentration, among others.

This chapter is structured in four sections as follows. The first and second sections present the separation, screening, and characterization of petroleum-degrading bacteria. The third and fourth sections presents the experimental observations obtained from the bioremediation of oil contaminated soil by the *Plesiomonas* SY_{23} bacteria.

2. Part I Separation and screening of petroleum-degrading bacteria

2.1 Summary

Seven bacteria strains were isolated from petroleum contaminated soil in the north of the Shaanxi province in China. These strains were studied and physiologically characterized. Preliminary results showed that the active strains were *Acinetobacter* SY_{21}, *Neisseria* SY_{22},

Plesiomonas SY_{23}, *Xanthomonas* SY_{24}, *Azotobacter* SY_{42}, *Flavobacterium* SY_{43}, and *Pseudomonas* SY_{44}. In addition, it was found that the biodegradation efficiency of total petroleum hydrocarbon (TPH) were higher than 80% after 8 days. The removal rates of TPH observed in this study were higher than the TPH removal rates previously reported (Truax et al, 1995; Ronald, 1996; Calabrese, et al, 1991). In addition, it was observed that more inoculums corresponding to a larger number of SY_{43} bacteria was present rendering a higher removal efficiency of TPH. Furthermore, the SY_{43} and SY_{23} strains adapted to the oil-contaminated soil setting establishing a local ecology, which rendered high removal efficiencies of TPH (88.4% and 73.4%, respectively), successfully remediating the contaminated soil.

2.2 Introduction

Areas of contaminated soil have increased rapidly in recent years due to the continuous growth and development of the oil industry. Meanwhile, the level of contamination becomes severe as time elapses.

Contaminated soils include many complex compounds such as alkanes, benzene, methylbenzene, and benzene, among others. These contaminants are toxic and usually categorized as carcinogenic substances. They can not be easily eliminated and eventually these contaminants will leach into the groundwater systems [1, 2]. Consequently, oil contamination is a serious environmental problem to our living ecosystem.

Bioremediation of oil contaminated soil requires low cost and does not lead to secondary pollution. In the last decades, relevant bioremediation techniques have been wildly studied. Most of these studies focused on enhancing bioremediation efficiency by increasing the activities of the native microorganisms by adding nutrients such as nitrogen (N) and phosphorous (P) [3]. These studies have shown that native microbes need a long time to domesticate due to slow growth rates. In addition, the low metabolic activities of these native microbes make a rapid decontamination difficult. Therefore, the application of bioremediation using indigenous microbes is restricted. Fortunately, petroleum-degrading active bacteria could be the solution to this problem. For instance, the Baltic General Investment (BGI) Corporation in America uses mixed microbes to improve bioremediation [4]. The research of Wilson (1993) presents bacteria strains which have shown high degradation rates of polycyclic aromatic hydrocarbons (PAHs) [5]. Bioremediation of petroleum contaminated soil is a complicated process, in which the pollutants characteristics, the ecological structure of microbes, and the environmental conditions must be considered. The adaptation characteristics of petroleum-degrading active bacteria can influence bioremediation. In this research, several strains were isolated from oil contaminated soils from the northern region of the Shaanxi province. The objectives of this work were to establish the characteristics of the strains and the kinetics of the bioremediation process.

2.3 Materials and methods

2.3.1 Crude oil and petroleum contaminated soil samples

Samples of the crude oil and the petroleum polluted soil used in this study were collected from oil wells located in the northern region of the Shaanxi province. The characteristics of the polluted soil samples are shown in Table 1.

No.	Total petroleum hydrocarbon (TPH) concentration (Conc.) (mg/kg dry matter)	pH	Water content (wt %)	Number of bacteria (CFU) (CFU/g dry matter)	
				Aerobic bacteria	Petroleum-degrading bacteria
Sample 1	209	8.89	8.3	$9.2x10^3$	$1.3x10^3$
Sample 2	148	8.66	15.5	$1.3x10^5$	$5.6x10^3$
Sample 3	28100	8.55	13.9	$4.4x10^4$	$2.2x10^4$
Sample 4	572	8.91	15.1	$3.4x10^5$	$1.0x10^5$

Table 1. Characteristics of Oil-Contaminated Soil Samples

2.3.2 Culture medium

The culture medium per unit used in this work was: 2 g of NH_4NO_3, 1.5 g of K_2HPO_4, 3 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7\ H_2O$, 0.01 g of anhydrous $CaCl_2$, 0.01 g of $Na_2EDTA \cdot 2H_2O$, 1 g of crude oil, 1000 mL of distilled water, with a pH ranging from 7.2 to 7.4.

2.3.3 Methodology

The procedure for bacteria gathering, separation, and purification was as follows. Ten grams (10 g) of crude oil contaminated soil were quantitatively placed into a 100 mL of culture medium (250 mL flask). This mixture was shaken using a shaker model THZ-82 shaker, manufactured by Changzhou Guohua Electronic Appliance Ltd, China, at a speed of 180 r/min for 7 days at 30ºC . Then, fifty milliliters (50 mL) of the medium were quantitatively added into a 100 mL of culture medium (250 mL flask). After which, the medium was shaken again at a speed of 180 r/min during 7 days at 30ºC. This procedure was repeated 3 times.

Then, the culture medium solution was taken and streaked repeatedly 3 times on the plate, after which the strains were added and the plate was introduced in the refrigerator using a slant culture.

In this work the concentration of petroleum hydrocarbons was determined using a non dispersive infrared oil instrument [6]. Bacteria count was performed by the plate counting method [7]. The operational and performance parameters used for the evaluation of bioremediation included the concentration of TPH, removal efficiency (RE), and number of bacteria (CFU).

2.4 Results

2.4.1 The degradation ability of petroleum-degrading active bacteria

In order to test the effect of oil-degrading active bacteria on oil removal, oil was added to the soil samples. After which, active bacteria were added to the contaminated soil samples. Two blank samples were needed here, one of which was sterilized and the other was non-sterilized. The sterilized blank sample was prepared by adding 0.2 wt% of mercury chloride into one of the oil contaminated soil samples. A control test was conducted, which consisted of a contaminated soil sample in the absence of active bacteria but containing the indigenous or native microorganisms samples.

Fig 1 shows the relationship between oil removal efficiency and bioremediation time. The oil removal efficiency in the sample containing mercury (sterilized blank sample) chloride was approximately 9%. The oil material lost in the sterilized blank sample containing mercury chloride is attributed to oil volatilization and aerial oxidation. In the case of the blank sample containing only native microorganisms, the oil removal efficiency increased linearly during the first week and then it leveled off around 37% of oil removal. This indicates that the indigenous microbes contributed to the removal of 25% of petroleum hydrocarbon, compared to the removal efficiency of the soil sample containing mercury chloride.

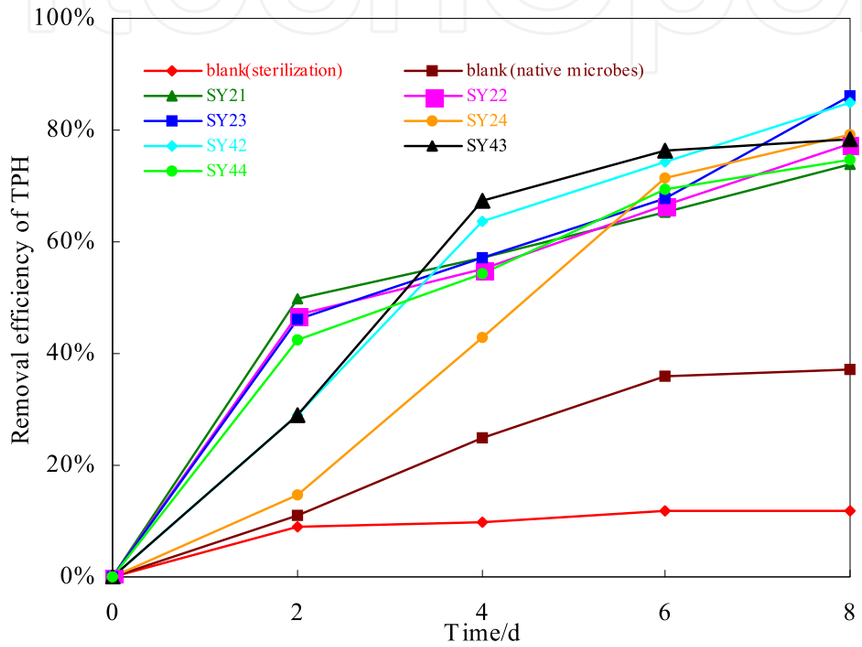


Fig. 1. The biodegradation efficiency of the strains evaluated

The soil samples inoculated with SY_{21}, SY_{22}, SY_{23}, and SY_{44} strains showed higher oil removal efficiencies. The removal efficiency of TPH reached up to 50% after 2 days of inoculation. The different strains inoculated in the contaminated soil showed the same removal rate up to 40%. The isolated strains rapidly adapted to the environment efficiently degrading hydrocarbons compounds. Fig. 1 shows that the biodegradation action of all the petroleum-degrading active bacteria tested follow the same trend. Thus the degradation efficiency of TPH increased gradually, which indicates that the contaminated soil provides an appropriated environment for bacterial growth. Eighty percent (80%) of hydrocarbons were degraded during the first 8 days of bioremediation while the degradation efficiency of the blank sample was only 37%. It is clear that the hydrocarbons were degraded mainly by the petroleum-degrading active bacteria. Fig.2 shows the variation of number of bacteria as a function of time. The number of bacteria increased gradually, as the bioremediation time progressed. The number of bacteria in the inoculated samples was higher that the number of bacteria in the blank sample. This indicates that the indigenous microbes were inefficient in degrading oil and that the inoculation of petroleum-degrading active bacteria shows potential in removing hydrocarbons contaminants.

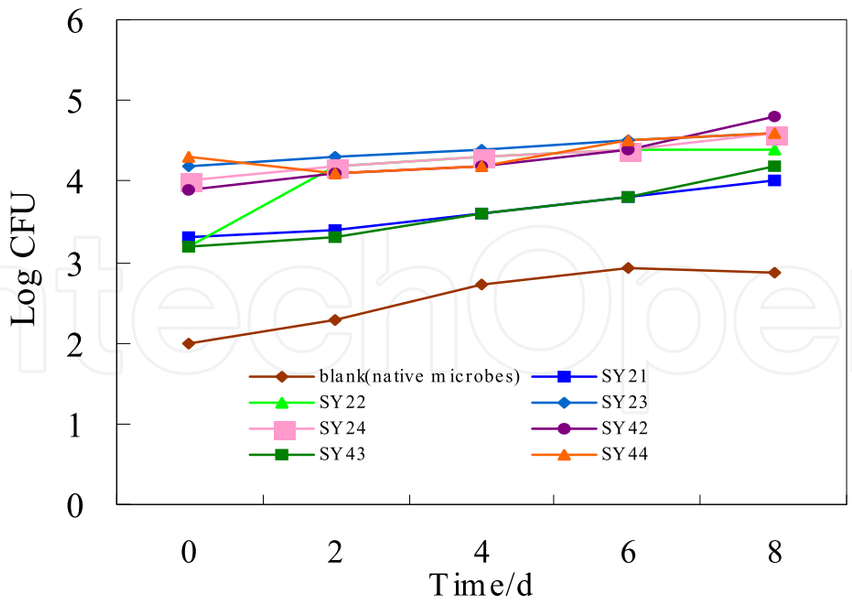


Fig. 2. The number change of 7 bacteria

The strains evaluated showed different oil degradation capacity and different patterns. Fig.3 presents the degradation rate of TPH by seven strains. It is observed that the rates of biodegradation of SY_{21}, SY_{22}, SY_{23}, and SY_{44} were higher in the second day, while the rates of SY_{42} and SY_{43} were higher in day 4, while in day 6, the highest degradation rate was shown by strain SY_{24}. The average degradation rates of TPH were within 0.01~0.1 $g \cdot kg^{-1} \cdot d^{-1}$, which are higher than degradation data previously reported [8-10], consequently these strains could degrade TPH more rapidly.

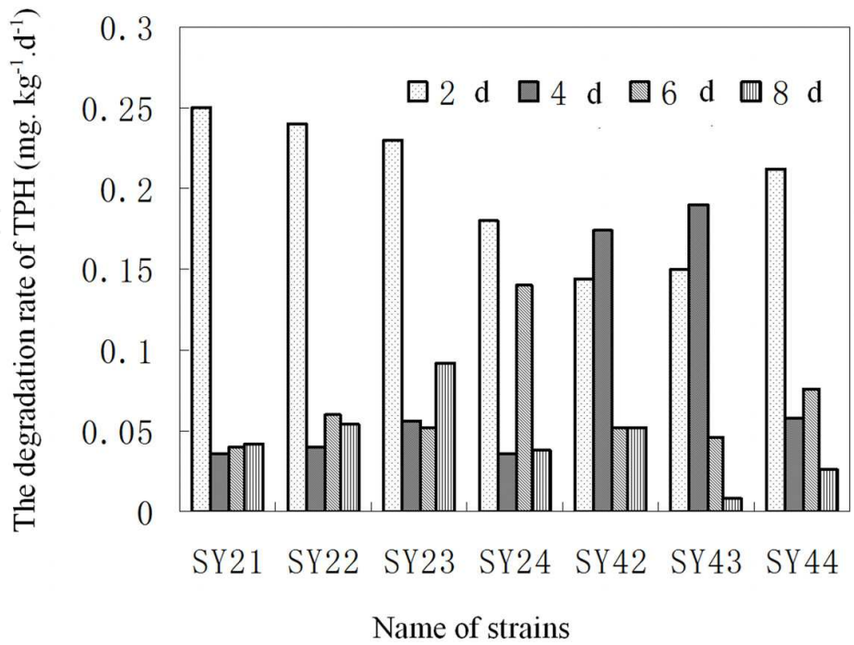


Fig. 3. The degradation rate of TPH by seven strains

Previous work [11] using marine filamentous bacteria reported rates of biodegradation in the range of 7.92×10^{-11} to 4.8×10^{-10} mg·cell^{-1}·d^{-1}. Similarly, other work[12] using pseudomonas showed that the highest oil degradation rate was from 1.44×10^{-10} to 3.77×10^{-9} mg·cell^{-1}·d^{-1}. Fig.4 presents the average degradation rate per cell of the seven strains used in this work. It shows that the highest degradation rate of 2.34×10^{-3} mg•cell^{-1}•d^{-1}was obtained for the strain SY_{43}, followed by strain SY_{23} with a degradation rate of 1.50×10^{-3} mg·cell^{-1}·d^{-1}. The degradation rate of the remaining strains ranged from 1.15×10^{-3} to 4.57×10^{-4} mg·cell^{-1}·d^{-1}. These results demonstrate that the rate of degradation shown by these strains is tens of thousands times higher than the degradation rate reported in previous work. Therefore, petroleum-degrading active bacteria show great potential for the bioremediation of TPH contaminated soils.

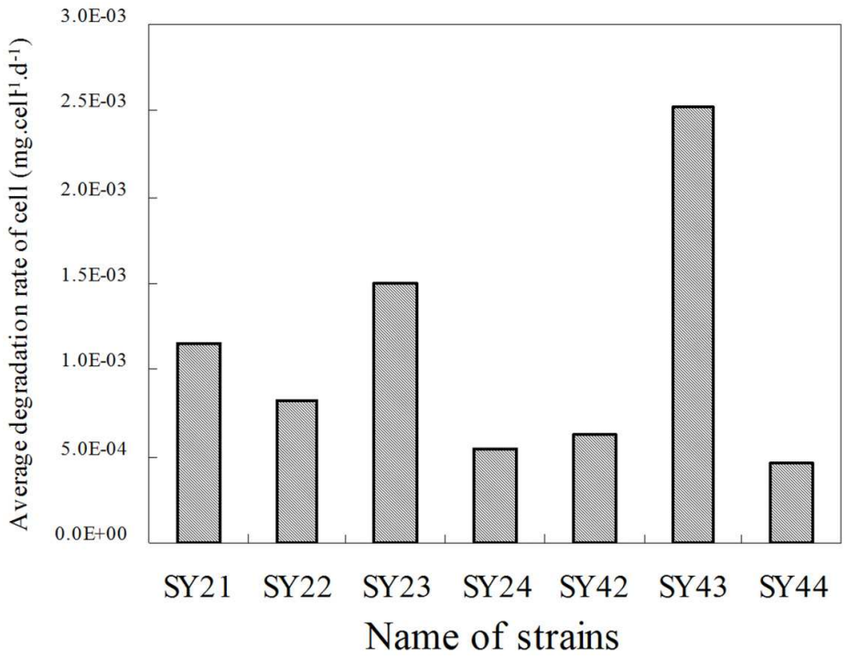


Fig. 4. Average degradation rate of cell by seven strains

2.4.2 Effect of concentration of TPH in contaminated soil on the biodegradation efficiency of petroleum-degrading active bacteria

Four oil samples containing different concentrations of TPH were prepared. The concentration of TPH in mud samples 1, 2, 3, and 4 were 125mg.L^{-1}, 56 mg L^{-1}, 3245 mg.L^{-1} and 209 mg.L^{-1}, respectively. In each sample the volume of inoculum was also varied as follows: 2 mL, 5 mL, and 10 mL. Fig.5 shows the number of bacteria and the removal efficiency (RE) of TPH observed in the mud samples during biodegradation. The number of bacteria increased as the volume of inoculum increased. Hence, the number of bacteria was enhanced by increasing the volume of inoculum. The removal efficiencies of TPH for sample 1 were 30%, 33%, and 70% for the corresponding inoculum volume of 2 mL, 5 mL, and 10 mL after 17 days of biodegradation. In addition, the RE of TPH of sample 2 was 20%, 42%, and 45% and the corresponding RE of TPH of sample 3 was 33%, 38%, and 45%.The

petroleum removal efficiency in sample 4 was 43%, 80%, and 81%. These results indicate that the higher the inoculum volume the higher the RE of TPH. The number of bacteria and RE of TPH for the soil samples were low when the inoculum volume was 2 mL. However, when the inoculum volume was increased from 2 mL to 5 mL the removal efficiency of TPH increased. The RE of TPH was similar when the inoculum volumes were 5 mL and 10 mL, respectively. This performance might indicate that a high inoculum volume enhances the competition ability of petroleum-degrading bacteria in relation to native microorganism. It could also aid the quick bacterial adaptation to the environment, which also increases the TPH degradation efficiency.

After 17 days of testing, the RE of TPH when the inoculum volume was 10 mL for sample 1, sample 2, sample 3, and sample 4 were 70%, 44%, 45%, and 80% respectively. The average biodegradation rate from sample 1, sample 2, sample 3, and sample 4 were 3.86×10^{-4} mg. $cell^{-1}\cdot d^{-1}$, 3.43×10^{-4} mg. $cell^{-1}\cdot d^{-1}$, 8.11×10^{-4} mg. $cell^{-1}\cdot d^{-1}$, and 5.86×10^{-4} mg. $cell^{-1}\cdot d^{-1}$, respectively.

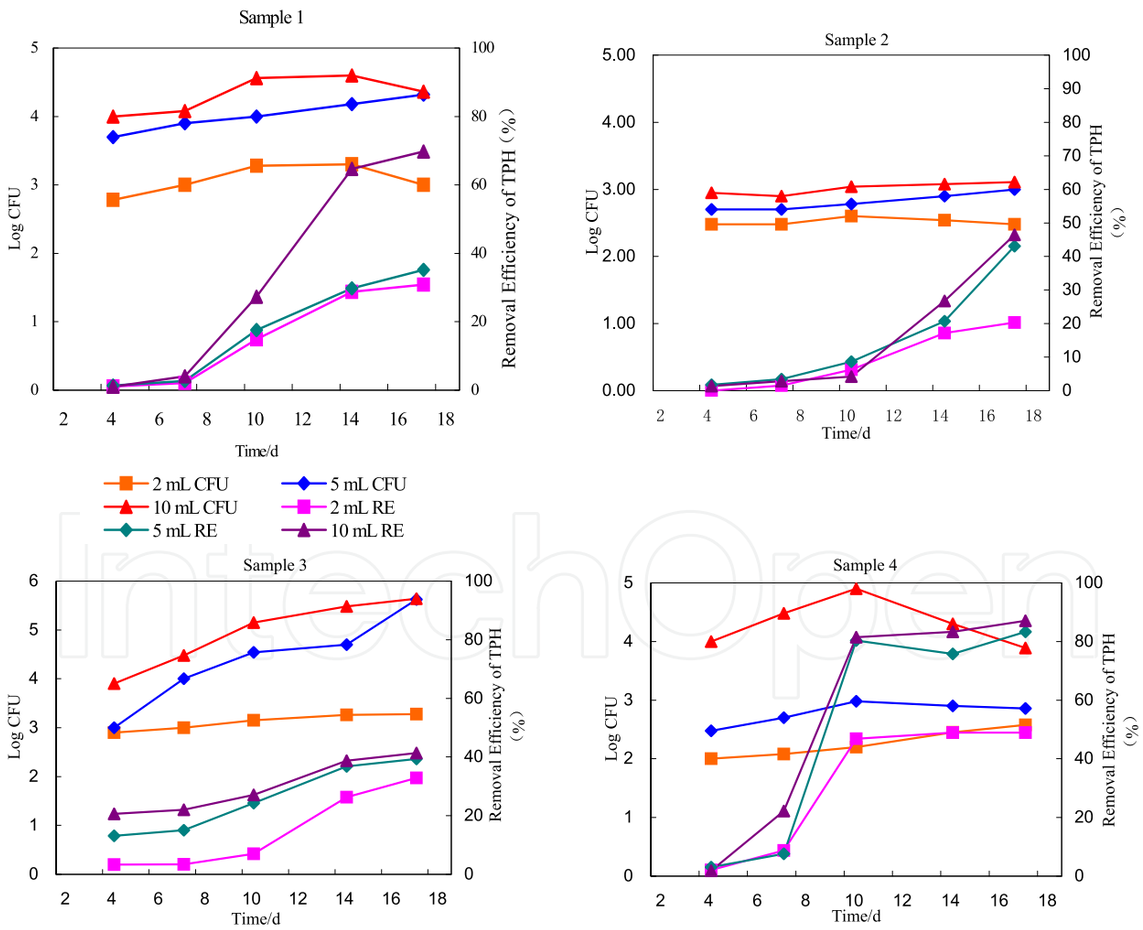


Fig. 5. The effect of inoculum volume and concentration of TPH in contaminated soil on biodegradation efficiency

2.4.3 Bioremediation of oil-contaminated soil by petroleum-degrading active bacteria

Figure 6 shows that significant removal of TPH took place in the soil samples inoculated with petroleum-degrading active bacteria compared with the removal of TPH in the blank sample. After 6 days of bioremediation, the percentage of TPH removal in the samples inoculated with bacteria was 17.2% and 19.2 % for SY_{43} and SY_{23} respectively; while the percentage of TPH removal in the blank sample was only 1.4%. At day 9, the removal efficiency of SY_{43} was 30.5%, which was higher than the removal efficiency observed for the strain SY_{23} (24.9%).

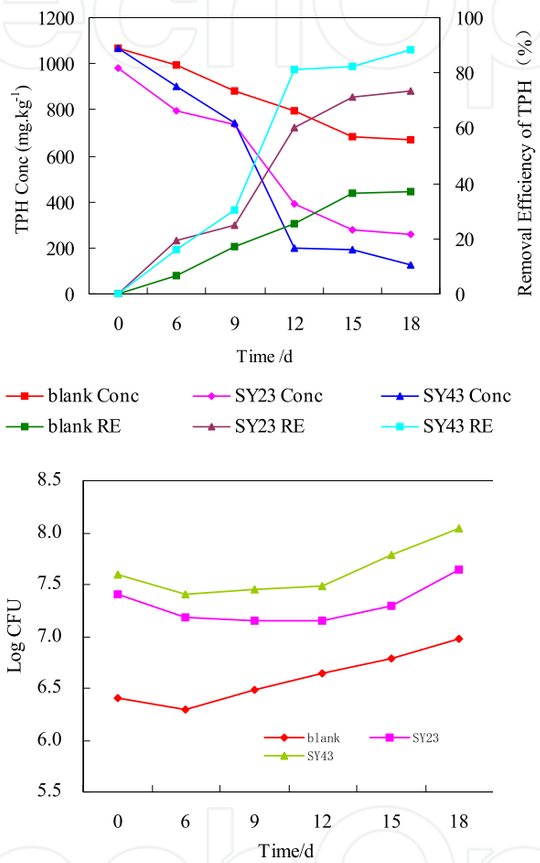


Fig. 6. TPH concentration and TPH removal efficiency (RE) as a function of bioremeditation time.

After 12 days of bioremediation, the TPH concentration of the sample having the strain SY_{43} declined to 213.3mg·kg^{-1} and the RE of TPH was 81.1%; while the TPH removal efficiency of the sample containing the strain SY_{23} and in the blank sample were 60.3% and 25.6%, respectively. After 18 days of inoculation, the removal efficiency in the three samples climbed up to 88.4%, 73.4%, and 37%, respectively. These observations indicate that the contaminated samples inoculated with petroleum-degrading active bacteria had higher oil removal efficiency than in the blank sample. The average degradation rates of TPH by SY_{43} and SY_{23} was 0.044 g·kg^{-1}·d^{-1}, 0.034 g·kg^{-1}·d^{-1} respectively; while the corresponding average degradation rate of the cells was 2.14x10^{-6} mg· cell^{-1}·d^{-1} and 1.64x10^{-6} mg· cell^{-1}·d^{-1} respectively. Figure 6 shows that the petroleum removal ability of SY_{43} is higher than that of SY_{23}. Fig.7 presents the number of bacteria during the bioremediation process for the strains

SY_{43}, SY_{23}, and the blank sample. The number of bacteria in the inoculated soil samples was 100 times bigger than the number of bacteria in the blank sample. The number of bacteria in the soil sample inoculated with SY_{43} was higher than the number of bacteria in the soil sample inoculated with SY_{23}. Thus, the number of petroleum-degrading active bacteria in the contaminated soil sample seems to be the main factor improving the bioremediation of the soil. The results show that both SY_{43} and SY_{23} could rapidly degrade TPH in the contaminated soil. The TPH degradation efficiency showed by the strain SY_{43} was better than the degradation efficiency achieved by the strain SY_{23}.

2.5 Conclusions

1. The bacteria strains isolated from petroleum contaminated soil in the north region of the Shaanxi province were *Acinetobacter* SY_{21}, *Neisseria* SY_{22}, *Plesiomonas* SY_{23}, *Xanthomonas* SY_{24}, *Azotobacter* SY_{42}, *Flavobacterium* SY_{43}, and *Pseudomonas* SY_{44}.
2. The TPH removal efficiency of these bacteria strains was 50% after 5 days of testing and 80% after 8 days of bioremediation. The average cell-degradation rate and the removal rate of TPH were thousand times higher compared to previously published results.
3. The number of active bacteria increases as the amount of inoculum increases, which enhances the competition ability of petroleum-degrading active bacteria against native bacteria. Simultaneously, this competition forces native bacteria to rapidly adapt to the environment improving their degradation ability, though, the bacteria strains show different petroleum removal trends even when the same inoculum amount was added, the average TPHs degradation rate was almost the same and the average degradation rate increases as the TPHs in contaminated soil increases.
4. Strains SY_{43} and SY_{23} show rapid degradation of TPH in contaminated soil. However, the degradation efficiency of SY_{43} was higher than the degradation efficiency of SY_{23}.

3. Part II Growth characteristics of highly petroleum-degrading bacteria

3.1 Summary

The growth characteristics of 7 strains isolated from oil contaminated soil, as well as their respective degradation efficiency for various hydrocarbons were investigated. Factors that can impact biological oil degradation efficiency were revealed in a series of experiments. The results indicate that isolated strains could rapidly degrade crude oil, showing high activity in the first 13 h of bioremediation. These strains could grow in paraffin wax, which indicates that these strains could degrade long chain hydrocarbons. Some of them (SY_{22}, SY_{23}, SY_{24}, SY_{42}, and SY_{43}) were able to use short chain hydrocarbons and aromatic hydrocarbons as substrate, so these five strains are the preferred ones for the bioremediation of oil contaminated soil. Suitable pH for the growth of these five strains was in the range from 7 to 9. NH_4NO_3 and oil concentrations should range from 1000 mg/L to 1500 mg/L in order to achieve optimum conditions for petroleum hydrocarbon degradation. Adding organic matter such as starch and glucose accelerated oil and PAH degradation capability of the strains SY_{22}, SY_{42}, and SY_{23} strains. The presence of metal ions, such as Ni^{2+} and Co^{2+}, in soil decreased the crude oil degradation efficiency of these strains, while metal ions, such as Fe^{2+} and Mn^{2+}, did not affect the oil degradation activities.

3.2 Introduction

Eight million tons (Mt) of petroleum is spilled into the environment every year worldwide. In China, 0.6 Mts of petroleum enters into soil, groundwater, rivers and ocean every year. Oil contamination is a severe threat for our environment and thereby attracts general concern. Consequently, the remediation of oil polluted sites has become an important issue worldwide.

Bioremediation has become one of the most promising technologies for soil remediation, because the cost of remediation of petroleum contaminated soils by biological techniques is low; in addition to the fact that through bioremediation the complete removal of oil can be achieved; while no secondary pollution is introduced. Microorganisms used for bioremediation are usually grouped as indigenous and exogenous microbes. The addition of nutrients increases the activity of native microorganisms, however bioremediation is boosted with the addition of exogenous bacteria. Native microbes need a long time to domesticate, and thereby show low growth rates and low metabolic activity, which make decontamination slow and ineffective. Therefore, the application of bioremediation using indigenous microbes is restricted. Thus, the screening of petroleum-degrading active bacteria to remediate oil polluted soil is a necessary task. In this experimental work, several strains (seven) were isolated from oil contaminated soil in the north region of the Shaanxi province. The effect of pH, nutrition (nitrogen and phosphorous), and pollution intensity on the oil degradation efficiency of the isolated strains were investigated. Furthermore, based on the complexity of the soil systems, the degradation capacity of the isolated bacteria of different kinds of petroleum hydrocarbons and the effect of adding organic co-substrate and metal ions on the bioremediation were studied.

3.3 Experimental section

3.3.1 Experimental material

3.3.1.1 The Source of samples

The tested crude oil and petroleum polluted soil were collected at oil wells in the northern region of the Shaanxi province. The strains separated from the oil contaminated soil were SY_{21}, SY_{22}, SY_{23}, SY_{24}, SY_{42}, SY_{43}, and SY_{44} [13].

3.3.1.2 Culture medium

The recipes of the different culture media used in this work are provided as follows.

Recipe of liquid or solid beef grease & peptone cultivation medium: 10 g of peptone, 3 g of beef grease, 5 g of NaCl, 1000 mL of distilled water, pH 7.0. The medium can be solidified using 20 g of agar.

Recipe of Liquid inorganic salts cultivation medium: 2 g of NH_4NO_3, 1.5 g of K_2HPO_4, 3 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7\ H_2O$, 0.01 g of anhydrous $CaCl_2$, 0.01 g of $Na_2EDTA \cdot 2H_2O$, 1000 mL of distilled water, and pH 7.0.

Crude oil culture medium: addition of crude oil into liquid inorganic salts culture medium.

The medium above were all sterilized for 30 min under 121°C.

3.3.2 Experimental methodoloy

3.3.2.1 Strains separation

Crude oil was added into the soil collected in the north region of the Shaanxi province intermittently, to progressively increase the oil concentration in the soil, so as to obtain bacteria with high capacity for oil degradation. The process was carried out at aerobic conditions under continuous shaking. Temperature was controlled at 30°C. Pure strains were isolated 21 days later by streaking them repeatedly on a plate.

The separated strains were identified to the genus level depending on their morphological and physiological-biochemical characteristics in general ways.

The preparation of the bacterium suspension was carried out by inoculating the strains into liquid beef grease & peptone medium, which was pre-sterilized under 121°C for 30min. The mixture of medium and bacteria was shaken for 36 h (180 r/min) under 30°C. Later, the mixture was centrifuged (180 r/min) and the resulting suspension was discharged while the residual sediment was washed 3 times using phosphate buffer. Finally, the washed sediments were diluted using phosphate buffer in order to adjust the number of the cells in bacterium suspension to be 1×10^8.

3.3.2.2 Study on the growth characteristics of the strains

Under sterile conditions, strains were inoculated in 200 mL liquid beef grease & peptone medium, which had been previously sterilized. Then the mixture of bacteria and medium was shaken at 30°C and 180 r/min. Afterwards, optical density D_{460} of the bacterium liquid using light (460 nm wavelength) was measured at regular intervals.

3.3.2.3 Determination of petroleum hydrocarbons

Oil degrading strains were inoculated into the crude oil medium, which was previously sterilized. This mixture was shaken and the pH adjusted below 3. Then, the medium was placed into funnels, shook and the total volume brought to 100 mL after adding 20 mL of CCL_4 in order to extract the hydrocarbons present in it. This mixture was kept static for segregation to take place (layered). The under layer was filtered and dried using anhydrous sodium sulfate and then it was placed into a 50 mL volumetric flask. The upper-layer was extracted using CCL_4 twice, then filtered, and placed into a 50 mL volumetric flask. The concentration of petroleum hydrocarbon was determined using a non dispersive infrared oil analyzer and the biodegradation of petroleum hydrocarbon η was determined using the following equation (1).

$$\eta = \frac{c_0 - c_x}{c_0} \times 100\% \quad (1)$$

In this equation (1), c_0 and c_x represent the residual concentration of petroleum hydrocarbon in blank samples and the test samples, in mg/L; respectively.

3.3.2.4 Strains' hydrocarbon degradation efficiency tests

Six hydrocarbon compounds which included normal octane, paraffin wax, benzene, methylbenzene, phenol, and naphthalene were added to the inorganic medium that was

previously sterilized（121°C for30 min） using high pressure steam. Then, 5 mL of bacterium suspension were added to the medium. This mixture was shaken for 36 hours under 30°C and 180 r/min. After all the phenol was evaporated from the mixture, the bacterium suspension was added. Naphthalene mixed with acetone was also added into the bacterium suspension after all the acetone was evaporated from the mixture. Finally, the optical density D_{460} of the culture solution under 460nm was measured and the concentration of petroleum hydrocarbons was determined at regular intervals.

3.3.2.5 Evaluation of the factors influencing the activity of petroleum-degrading bacteria

Crude oil samples were mixed with petroleum ether to prepare a solution having a concentration of 60 g of crude oil/L of solution. Then, the mixture was filtrated using a 0.25 μm filter membrane. The filtrate was placed into a flask and the petroleum ether was completed evaporated from the flask. Then, pre-sterilized inorganic medium and 5 mL of bacterial suspension were added into the flask. Using this oil-contaminated soil samples as the starting point, several petroleum degradation experiments were performed at different conditions of pH, organic load, nitrogen source, carbon source and metal ions.

3.4 Results and discussions

3.4.1 Growth characteristic of petroleum-degrading active bacteria

3.4.1.1 Isolated strains cultivated in hydrocarbon medium

Table 2 shows bacterial growth, bacterial density, and the rate of oil biodegradation reached by each strain after 7 days of cultivation. The strains grew well in oil media and emulsified crude oil. The density of bacteria after 7 days of cultivation was observed to range between 1×10^7/mL and 1×10^9/mL. These results indicate that the strains used petroleum as the carbon source. The biodegradability (η) after 7 days of cultivation was between 43.8% and 58.9%, which exceeded the biodegradability of formerly reported petroleum-degrading bacteria B01(25.8%-32.8%) [11] and were close to that of O-8-3 *Pseudomonas*, marine bacteria SJ-06W, SJ-6, and SJ-16A-2 as previously reported [14].

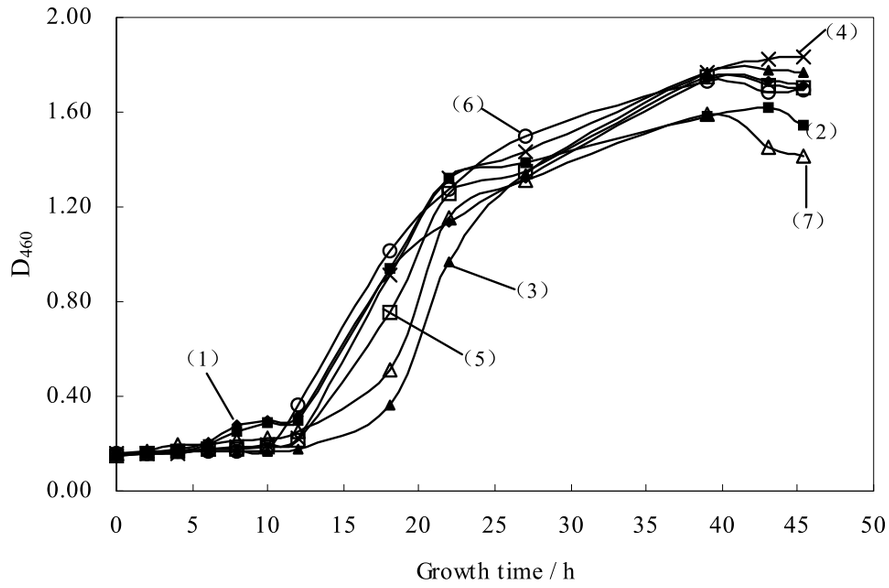
Strain	Growth and emulsification	Bacteria quantity (CFU.mL^{-1})	η (%)	Identification
SY_{21}	Complete emulsification and dense liquid	5.3×10^7	43.8	*Acinetobacter*
SY_{22}	Forming oil film and flock	2.4×10^7	46.7	*Neisseria*
SY_{23}	Complete emulsification and dense liquid	3.6×10^9	58.9	*Plesiomonas*
SY_{24}	Complete emulsification and forming flock	1.2×10^7	45.0	*Xanthomonas*
SY_{42}	Complete emulsification and dense liquid	3.2×10^8	47.6	*Azotobacter*
SY_{43}	Forming oil film and flock	6.7×10^8	53.3	*Flavobacterium*
SY_{44}	Forming dispersed flocks and dense liquid	9.2×10^7	45.8	*Pseudomonas*

Table 2. Growth And Identification of the Isolated Strains Cultivated in Hydrocarbon Medium After 7 Days of Cultivation

These strains of petroleum-degrading active bacteria were all gram-negative bacteria. The strains SY_{21}, SY_{22}, SY_{23}, SY_{24}, SY_{42}, SY_{43}, and SY_{44} were identified as *acinetobacter*, *neisseria*, *plesiomonas*, *xanthomonas*, *zoogloea*, *flavobacterium,* and *pseudomonas*, respectively. Previous research has shown that gram-negative bacterium dominate in microbes that can degrade petroleum hydrocarbon [15]. The *Xanthomonas*, *zoogloea*, *flavobacterium,* and *pseudomonas* strains have been extensively studied and used.

3.4.1.2 The growth trend in liquid medium

Figure 8 shows bacteria growth as a function of time. The curves in Fig. 8 indicate that bacteria grow rates were low during the first 13 hours, after which the bacteria grow rates followed a logarithm growth period during the next 13~23 hours; and then turned into a slow down growth period during the following 23~40 hours. Finally, bacteria began to die after 40 hours of activity. Thus, the strains showed the highest activity during the 13 to 23 hours of life.



(1)SY_{21}; (2)SY_{22};(3)SY_{23};(4)SY_{24}; (5)SY_{42};(6)SY_{43}; (7)SY_{44}

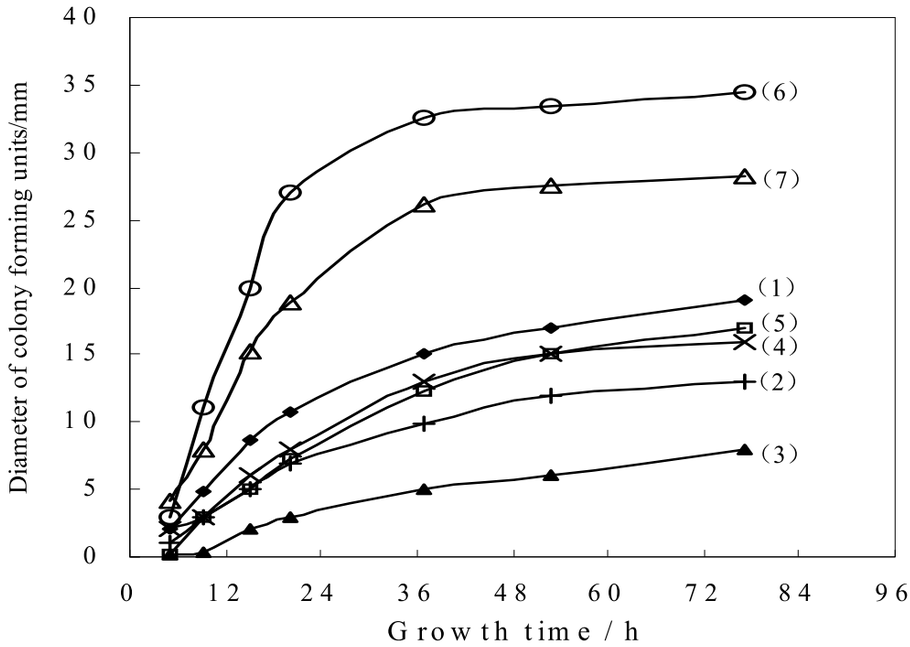
Fig. 8. Growth trend of the seven isolated strains in liquid cultivation medium

3.4.1.3 Growth trend in agar medium

Figure 9 shows the growth trend of the same 7 strains in agar media.

Fig.9 shows the variation of the diameter of colony forming of the different strains as a function of time. The colonies of SY_{21} were formed after 4 hours of activity. After 9.2 hours the diameter reached 4 mm. This colony expanded continuously in the first 20 hours during which the average growth rate was 12.84 mm/d. In addition the colony growth was circular having an ivory-opaque color with an arid and disordered surface. The SY_{22} strain formed a circular ivory and semitransparent colony after 4 hours of inoculation. The surface of the colony was wet and orderly with a diameter of 3 mm after 9.2 hour. Similar to the previous case, the colony expanded continuously within the first 20 hours with an average growth

rate of 8.41 mm/d. The colony formed by the strain SY_{23} was ivory and opaque with an arid and disordered surface. The SY_{23} colony was formed after 9.2 hours reaching a diameter of 2 mm after 15 hours. This colony also expanded continuously with an average growth rate at 2.49 mm/d. The SY_{24} formed an opaque and creamy yellow colony after 4 hours of inoculation. Its surface was flat and disordered, and the colony expanded continuously during the first 37 hours at a growth rate at 8.43 mm/d. After inoculation the strain SY_{42} formed a white transparent and circular colony. The surface of the colony was wet and orderly. The average growth rate of this colony was 5.30 mm/d. The colony made up by the SY_{43} strain was white-transparent and disordered with a wet and smooth surface. The average growth rate of this colony was 5.30 mm/d. The SY_{44} strain formed an ivory semitransparent circular colony. Its surface was wet, smooth, and orderly and reaching a diameter of 4.2 mm after 4 hours. The colony expanded continuously during the first 15 hours with an average growth rate of 24.32 mm/d. The average growth rate of the 7 isolated strains ranged from 2.49 to 32.4 mm/d.



(1)SY_{21}; (2)SY_{22};(3)SY_{23};(4)SY_{24}; (5)SY_{42};(6)SY_{43}; (7)SY_{44}

Fig. 9. Growth tendency of seven isolated strains in agar cultivation medium

3.4.2 The degradation ability of petroleum-degrading active bacteria toward different types of hydrocarbons

The majority of petroleum-degrading bacteria can degrade only few kinds of hydrocarbons [15-16]. The middle-chain and long-chain normal alkane can be degraded by most petroleum-degrading bacteria. However, the short-chain hydrocarbons and aromatic hydrocarbons can only be degraded by few petroleum-degrading bacteria. For the majority of bacteria it is difficult to digest short-chain and aromatic hydrocarbons, which can even be toxic.

In this work, the hydrocarbon degradation capability of the 7 strains was evaluated using the following hydrocarbon compounds: octane, paraffin wax, benzene, methylbenzene, phenol, and naphthalene. The initial concentration of these hydrocarbon compounds were 125 mg/L, 64800 mg/L, 200 mg/L, 14.4 mg/L, 200 mg/L, and 330 mg/L, respectively. During these tests the temperature and pH were set at 30°C and 7, respectively.

Table 3 shows that the seven strains grew in the paraffin wax media (The optical density D_{460} measured range from 0.117 to 0.450). The degradation efficiency of paraffin wax shown by the SY_{43} strain was 81.3%, which was the highest degradation efficiency observed, while the strain SY_{21} showed the lowest efficiency at 43.7% degradation. The degradation efficiencies of the other strains were between SY_{43} and SY_{21}. All of these 7 strains showed a high degradation capability toward middle and long-chain alkane, as the 90% of paraffin wax consisted of C_{18}~C_{61} normal and isomeric alkanes [17]. The degradation efficiencies of naphthalene by the 7 strains were about 40%. The SY_{23} and SY_{24} strains showed high abilities to degrade benzene, methylbenzene, and phenol as the degradation efficiency reached from 80% to 90%. The majority of these strains showed low degrading efficiency toward normal octane, with the exception of strains SY_{24} and SY_{43}, which showed degradation rates of 54.4% and 56.8% respectively. These observations indicate that the following strains SY_{22}, SY_{23}, SY_{24}, SY_{42}, and SY_{43} are capable of degrading more than one hydrocarbon, which makes them potential candidate strains for the bioremediation of petroleum contaminated soil.

Hydrocarbon medium	D_{460}							η (%)						
	SY_{21}	SY_{22}	SY_{23}	SY_{24}	SY_{42}	SY_{43}	SY_{44}	SY_{21}	SY_{22}	SY_{23}	SY_{24}	SY_{42}	SY_{43}	SY_{44}
C_8H_{18}	0.103	0.013	0.011	0.116	0.017	0.249	0.015	35.2	12.8	12.0	54.4	21.6	56.8	20.8
Paraffin wax	0.300	0.322	0.132	0.117	0.320	0.409	0.450	43.7	60.1	47.3	47.3	66.6	81.3	62.8
Benzene	0.023	0.011	0.120	0.08	0.036	0.056	0.035	21.0	10.0	90.5	80.9	46.0	71.2	63.9
Naphthalene	0.073	0.034	0.032	0.048	0.030	0.030	0.040	44.7	42.6	35.0	34.4	40.8	43.5	42.6
Phenol	0.033	0.017	0.112	0.104	0.058	0.085	0.067	21.0	10.0	90.5	80.9	46.0	71.2	63.9
Xylene	0.014	0.052	0.075	0.090	0.023	0.007	0.007	8.3	11.1	84.7	93.8	9.7	6.9	4.2

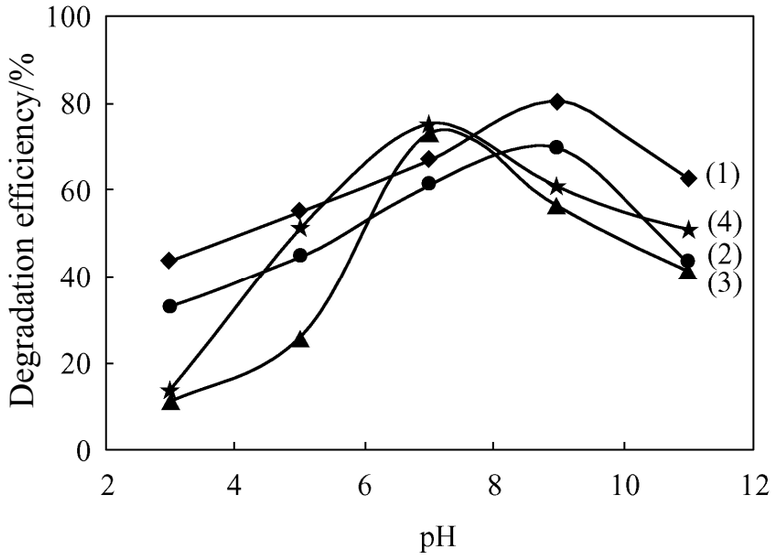
Table 3. Growth Tendency And Degradation Efficiency of the Seven Isolated Strains in Different Hydrocarbon Medium

3.4.3 Factors influencing the hydrocarbon degradation efficiency of petroleum-degrading active bacteria

3.4.3.1 The effect of pH

In microorganisms, biochemical reactions are catalyzed by enzymes. It is well known that enzymatic reactions occur within a suitable pH range and microorganisms are sensitive to the alteration of pH. Thus, it is necessary to determine the optimum pH value suitable for petroleum degradation by bacteria. The pH value of soil normally ranges between 2.5 to 11.0. Thus, before the inoculation of strains into the crude oil media (petroleum concentration was fixed at 600 mg/L), the pH value was adjusted to 3, 5, 7, 9, and 11 for each medium. The experiments were carried out at a rotation speed of 180 r/min for 96 hours at 30°C. After which, the concentration of petroleum hydrocarbon was determined and the degradation efficiency was calculated. Figure 10 shows the degradation efficiency of 4 of the strains.

Fig. 10 indicates that SY_{22} and SY_{23} strains could degrade oil at a pH=9.0 with degradation efficiencies of 80% and 69.4%, respectively. Meanwhile, strains SY_{24} and SY_{42} had the ability of degrading oil at a pH value of 7.0 with degradation efficiencies of 73.1% and 74.9%, respectively.



(1)SY_{22}; (2)SY_{23}; (3)SY_{24}; (4)SY_{42}

Fig. 10. Effect of pH value on the hydrocarbon degradation efficiency of some of the isolated strains

3.4.3.2 Effect of petroleum hydrocarbon concentration

Five (5) mL of bacterium suspension was inoculated in the crude oil media. The concentration of hydrocarbon was varied as follows: 200, 600, 1000, 1500, 3000 mg/L. The Bioremediation tests were conducted at 30°C and the pH was adjusted to a value of 8.0. The rotation speed was set at 180r/min for 96 hours. Table 4 summarizes the degradation efficiencies of the strains. The observations in Table 4 indicate that the hydrocarbon degradation efficiency shown by these strains exceeded 60% when the mass concentration of petroleum hydrocarbon was 1000 mg/L. The hydrocarbon degradation efficiency shown by the SY_{24} strain was reduced when the mass concentration of petroleum hydrocarbon increased to 1500 mg/L. The hydrocarbon degradation efficiency of all the strains was reduced when the mass concentration of petroleum hydrocarbon was increased to 3000

TPH concentration	**η (%)**			
(mg.L^{-1})	**SY_{22}**	**SY_{23}**	**SY_{24}**	**SY_{42}**
200	35.3	61.5	57.5	49.2
600	57.8	62.0	60.5	60.6
1000	61.6	64.8	63.5	63.5
1500	63.2	66.8	50.2	65.8
3000	46.0	56.2	43.0	37.4

Table 4. Effect of Petroleum Hydrocarbon Concentration on the Strains Hydrocarbon Degradation Efficiency

mg/L. These results indicate that excessive concentration of petroleum hydrocarbon restricted the growth of the strains and consequently reduced the hydrocarbon degradation efficiency. The SY_{23} strain showed the highest TPH degradation efficiency at all TPH concentrations, which indicates its endurance to the TPH toxicity.

3.4.3.3 Effect of different nitrogen sources

In order to determine the impact of the nitrogen source on the strain degradation efficiency, a series of different nitrogen sources were used in addition to NH_4NO_3 (200 mg/L) in the inorganic media. The concentration of the different nitrogen sources was set as 350 mg/L. The rotation speed was set at 180r/min for 96 hours at 30°C and at pH of 8.0. After 96 hours of inoculation the concentration of the residual petroleum was determined. Table 5 presents the hydrocarbon degradation efficiency as a function of nitrogen source. Table 5 indicates that all the strains showed the highest degradation efficiency when NH_4NO_3 was used as the nitrogen source, while the lowest degradation efficiencies were observed when $NaNO_3$ was used as the nitrogen source. This indicates that NH_4NO_3 is the best nitrogen source for the strains under evaluation, which is in agreement with the results presented by other researchers [18]. It is important to mention that the SY_{23} strain showed a high degradation efficiency when $(NH_4)_2SO_4$ and Urea were used as nitrogen sources, which points out that SY_{23} could be used for remediation situations were these nitrogen sources are readily available.

Nitrogen source	η (%)			
	SY_{22}	SY_{23}	SY_{24}	SY_{42}
NH_4NO_3	46.7	48.5	52.5	42.6
$(NH_4)_2SO_4$	20.2	47.1	30.0	30.9
$NaNO_3$	13.3	10.9	28.6	21.2
Urea	28.9	39.2	33.0	39.9

Table 5. Effect of Nitrogen Sources on the Petroleum Hydrocarbon Degradation Efficiency

3.4.3.4 Effect of different carbon sources

Glucose and starch were used as co substrate in the inorganic medium in which petroleum concentration was fixed at 1000 mg/L. These experiments were carried out at a rotation speed of 180 r/min for 96 hours at a temperature and pH of 30°C and 8.0, respectively.

Table 6 shows the effect of dosing co substrate on the removal efficiency of petroleum. Table 6 shows that the degradation efficiency of petroleum hydrocarbon by the SY_{22} and SY_{23} strains increased from 43.8% and 17.6% to 71.5% and 70.2%, respectively. These results show that the strains degradation efficiency was enhanced by the use of glucose and starch as carbon sources. The explanation is that glucose and starch can be used as co-metabolism medium during the petroleum degradation process [19]. The SY_{22} and SY_{42} strains had high degradation efficiency of naphthalene (42.6% and 40.8% as showed in Table 3), these efficiencies were improved after adding glucose and starch. The SY_{23} strain also showed high degradation efficiency of benzene, methylbenzene, and phenol with corresponding efficiencies of 90.5%, 84.7% and 90.5%.These observations indicate that the SY_{23} strain can degrade PAH in crude oil and that the degradation efficiency can be increased to a large extent after adding starch. Thus, bacteria activity can be improved by adding the

appropriate carbon sources, in this case glucose and starch. It has been previously reported that using glucose as carbon source improves the degradation efficiency of PAH and if glucose is fed intermittently the abilities of bacteria to degrade petroleum hydrocarbon could be maximized [20].

Carbon source	η (%)			
	SY_{22}	SY_{23}	SY_{24}	SY_{42}
Oil	43.8	36.9	35.0	17.6
Oil+Starch	71.5	46.7	41.5	70.2
Oil+Glucose	58.2	60.8	30.7	35.6

Table 6. Effect of Carbon Sources on the Strains Petroleum Hydrocarbon Degradation Efficiency

3.4.3.5 Effect of different metal ions

The concentration of metal ions increased in oil fields due to the aging and mineralization of soil during the weathering process of petroleum contaminated soil. In order to find the impact of the presence metal ion on the biological removal of petroleum, metal ions of Fe^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+} were added into the inorganic salt liquid media in which the petroleum hydrocarbon concentration was 1000 mg/L.

The experiment was carried out at rotation speed of 180 r/min for 96 hours at 30°C and a pH of 8.0. Table 7 shows the calculated petroleum removal efficiencies and it clearly shows that the degradation efficiency of the strains declined significantly after adding Ni^{2+}. It seems that a high concentration of Ni^{2+} restricted the activities of the microorganisms. For instance, the degradation efficiency of SY_{43} strain was decreased from 53.3% to 10.9%. In contrast, adding Fe^{2+} improved the degradation efficiency of petroleum hydrocarbon (SY_{21} and SY_{23} strain). However, the activity of SY_{43} strain was restricted. After adding Mn^{2+} the degradation efficiency of oil by SY_{23} strain was enhanced by 12% while the degradation efficiency by SY_{21} and SY_{43} strain was not affected. The degradation efficiency of oil achieved by the SY_{21} and SY_{43} strains decreased 16% and 12% respectively after adding the metal ions of Co^{2+}. These results point out that the addition of metal ions such as Fe^{2+} and Mn^{2+} has a favorable influence on the oil degradation efficiency. On the contrary, the degradation efficiency of petroleum hydrocarbon was decreased after adding metal ions such Ni^{2+} and Co^{2+}.

Metal ion	η (%)		
	SY_{21}	SY_{23}	SY_{43}
-	58.2	38. 9	53.3
Fe^{2+}	64.1	46.7	40.2
Mn^{2+}	54.3	50.6	54.6
Ni^{2+}	33.3	20.0	10.9
Co^{2+}	42.5	43.0	45.1

Table 7. Effect of Metal Ions on the Strains Degradation Efficiency

3.5 Conclusions

1. Seven strains were isolated from petroleum contaminated soil. The degradation efficiency of oil after seven days of cultivation ranged from 43.8% to 58.9%, which

indicates high strains activity. These strains show a logarithm growth trend after 12 to 13 hours of inoculation with an average growth rate of the respective colonies between 2.49 to 2.4 mm/d.

2. The strains SY_{21}, SY_{22}, SY_{23}, SY_{24}, SY_{42}, SY_{43}, and SY_{44} were categorized as *acinetobacter, neisseria, plesiomonas, xanthomonas, zoogloea, flavobacterium* and *pseudomonas* respectively.
3. These strains are capable of using normal octane, paraffin wax, benzene, methylbenzene, phenol, and naphthalene as the solo carbon source. Five of these strains: SY_{22}, SY_{23}, SY_{24}, SY_{42} and SY_{43} show the ability of degrading more than one hydrocarbon, which make them potential candidates for the bioremediation of petroleum contaminated soil.
4. A pH value of 7.0 was optimum for the growth of strains SY_{21} and SY_{42} while a pH value of 9.0 was optimum for the development of strains SY_{22} and SY_{23}.
5. The strains show an optimum degradation of petroleum hydrocarbon when NH_4NO_3 was used as a nitrogen source in contaminated soil containing oil concentrations ranging from 1000 mg/L to 1500mg/L.
6. The degradation efficiency of strains SY_{22}, SY_{42}, and SY_{23} is significantly enhanced by the addition of starch and glucose.
7. The presence of metal ions such as Ni^{2+} and Co^{2+} in the oil contaminated soil decreases the strains degradation efficiency, while the presence of Fe^{2+} and Mn^{2+} does not affect the oil degradation by the strains, on the contrary might improve it.

4. Part III Bioremediation of oil contaminated soil by SY23 petroleum-degrading active bacteria - Impact of bulking agent and tillage on bioremediation of petroleum-contaminated soil

4.1 Summary

The impact of adding bulking agent and tillage on bioremediation of petroleum contaminated soil was studied. The results showed that the strain degradation efficiency of TPH was 76% in 48 days after adding bulking agents and tillage. This degradation efficiency was 15% higher than the degradation efficiency in the control experiment in which bulking agent was not added. Meanwhile, the degradation rate was 2.34 times higher in the sample containing bulking agent compared to the control sample. The addition of bulking agent increased water content in the soil as the bulking agent absorbed water, which improved the degradation of TPH. Gas chromatography-Mass Spectrometer (GC-MS) analysis indicated that the peak numbers of the GC profile decreased from 32 to 14 in 64 days after adding bulking agent. This result indicated that branched alkanes, alkene, carotane, and alkylnaphthalenes were thoroughly degraded. The peak numbers of the GC profile decreased 10 times. Furthermore residual hopanes and steranes were also thoroughly degraded after the combined treatment of the oil contaminated soil with bulking agents and tillage. The degradation efficiency of TPH decreased as tillage times decreased, the optimum tillage frequency was once per day with a shovel.

4.2 Introduction

Petroleum could lead to the contamination of ground water after petroleum sprays into soil. Bioremediation of petroleum hydrocarbon contaminated soil is considered a new technology with a broad prospect because of its low-cost [9]. During in situ bioremediation,

sufficient dissolved oxygen (DO) is necessary to keep the degradation activity of bacteria. It has been reported that the mineralization of TPH in soil will be restrained when the DO concentration is lower than 15% (dry soil) or 5% (wet soil) [21]. Tillage is a mechanical way to improve the local environment in the soil. This method can change the physico-chemical characteristic of soil and stimulate the activity of bacteria by increasing the oxygen content in the soil and aiding the release of carbon dioxide [22, 23]. Bulking agents are cheap low density materials. The addition of bulking agents into the soil reduces the density, increases the porosity, and enhances the spread rate of oxygen in the soil [24]. This research evaluates the impact of adding a bulking agent and tillage on the bioremediation of petroleum-contaminated soil.

4.3 Materials and methods

4.3.1 Materials

4.3.1.1 Samples source

Oil contaminated soil samples were collected at contaminated sites surrounding oil wells from an oil field located in the northern region of the Shaanxi province, China. The samples were homogenized, sieved (pore size 2 mm), and stored in a dark and ventilated fridge at 4°C until use.

4.3.1.2 Microbial inoculations

Petroleum degrading active bacteria were collected from oil contaminated soil samples [25]. The solid SY_{23} strain was obtained as a concentrated bacteria suspension that was separated by centrifuge. The solid bacterial product was prepared after the solid SY_{23} strain was dried on sterilized condition at 37^{o}C for 48 hours.

4.3.2 Experimental methods

4.3.2.1 Processing methods

In order to prepare a bacterial-rich suspension, certain amount of the solid bacterial product was added into a bacteria-free buffer. A substrate medium was added into the bulking agent to prepare a mixture. Later, the mixture was sterilized. Then, the bacterial-rich suspension was mixed with the bulking agent mixture which was cultured for 24 hours. The final product was ready for addition into the petroleum contaminated soil.

4.3.2.2 Analytical methods

The petroleum hydrocarbon (TPH) composition was determined using an OCMA - 350 non-dispersive infrared oil analyzer. Oil components were measured using a gas chromatograph (Model Trace 2000) and a Mass Spectrometer (Ms) model Voyager 5975B, manufactured by Agilent USA. The GC was equipped with a 30 m long capillary (DB-5) in which the stationary phase was 0.25 µm thick. The measurable range of relative molecular weights was 30 to 450. GC conditions for analysis were as follows: carrier gas: helium (0.8 $mL.min^{-1}$); Flame Ionization Detector (FID) temperature: 320^{o}C; the initial temperature was 40^{o}C and it was maintained for 5 minutes. Then, the temperature was increased to 300^{o}C at a speed of 10 ^{o}C /min. Conductivity meter was used to measure conductivity and pH test paper was used to measure pH values. The conductivity and pH values must be analyzed under the

conditions where the ratio of bulk factor of soil to water was 1:5(W/V)[8]. Finally, constant weight method was used to measure moisture content.

4.3.3 Design of the experiments

The objective of the experimental design was to investigate the bioremediation of petroleum hydrocarbon contaminated soil by bioaugmentation products through bioremediation simulation tests with the addition of bulking agent and tillage. The experiments were carried out in basin ports having diameters of 15 cm and depths of 15cm. A mass of 1000 g of soil was used in each group. Table.8 summarizes the experimental conditions. Each experimental group was conducted in triplicate. Experiments were carried out at room temperature, which varied in the range of 16 to 20^{o}C. Samples were taken for analysis at time intervals to measure pH, conductivity, TPH concentration, and moisture content. The sample interval was increased when the rate of petroleum-degradation started to decrease.

Items	Sample number	Dose of bacteria agent(mg.kg^{-1})	Dose of Sawdust (g)	Treatment
Tillage and bulking agent	**CK**	0	0	Stagnant
	AJ	0.6	0	Stagnant
	BJ	0.6	80	Stagnant
	AF	0	0	Tilled once every day
	BF	0.6	0	Tilled once every day
	CF	0.6	80	Tilled once every day
Tillage frequency	**F1**	0.6	80	Tilled once every day
	F2	0.6	80	Tilled once every three days
	F3	0.6	80	Tilled once every five days
	F0	0.6	80	Stagnant

Table 8. Experimental Design

4.4 Results

4.4.1 The influence of bulking agent on the degradation of petroleum hydrocarbons

Figure 11 shows the experimental observations, and it indicates that the addition of bulking agent and tillage aid the reduction of petroluem concentration. Samples subjected to tillage had the lowest remaining TPH concentration. TPH concentration declined from 4.5 g/kg to 1.737 g/kg (57%) after tilling for 19 days with bulking agent. While, for sample BF without bulking agent only 23% of the TPH was degraded. The degradation rate in sample BF was only 0.968 g/kg. Thus, the removal rate of TPH was 2.34 times higher when bulking agent was added. The concentration of TPH in sample CF with bulking agent after 48 days of bioremediation had declined 3.044 g/kg (76%). Meanwhile the sample without bulking agent (BF) only declined 2.611 g/kg (61%). For the Stagnant samples with added bulking agent, the removal efficiency of TPH was 66.4% while the removal efficiency of the samples without bulking agent was only 50.9% after 48 days. These observations indicate that the addition of bulking agent accelerates the removal rate of TPH by bacteria. The bulking agent

increases the permeability of the contaminated soil so that the oxygen transfer rate and the water-holding capacity are also enhanced. This provides an advantageous condition for bioremediation of oil contaminated soil. Research conducted by Xiaomei Ye [26] showed that the concentration of TPH declined 70% after 120 days of degradation which is in agreement with this study.

Figure 11 indicates that the removal efficiency of samples AF, BF, and CF were 22%, 33%, and 67% respectively after tilling for 26 days. Meanwhile, the removal efficiency in those samples without tilling was 2%, 22%, and 56% respectively. It is obvious that tillage has increased the removal efficiency of TPH. Tillage not only raises the content of oxygen in soil but also accelerates the speed of substrate transfer from outside into the biomembrane. The oxygen concentration in soil has a significant effect on the degradation of TPH. During pure oxygen aeration, the release rate of CO_2 is 0.013 mol/d. However, the production of CO_2 is reduced to 0.004 mol/d during air aeration [27]. In addition, tillage separate the soil block into small ones and mixes the soil. Therefore, tillage causes redistribution of C, N, and water in the contaminated soil. It has been reported [28] that tillage improves the removal efficiency of tetrachloroethylene and trichloroethylene in contaminated soil.

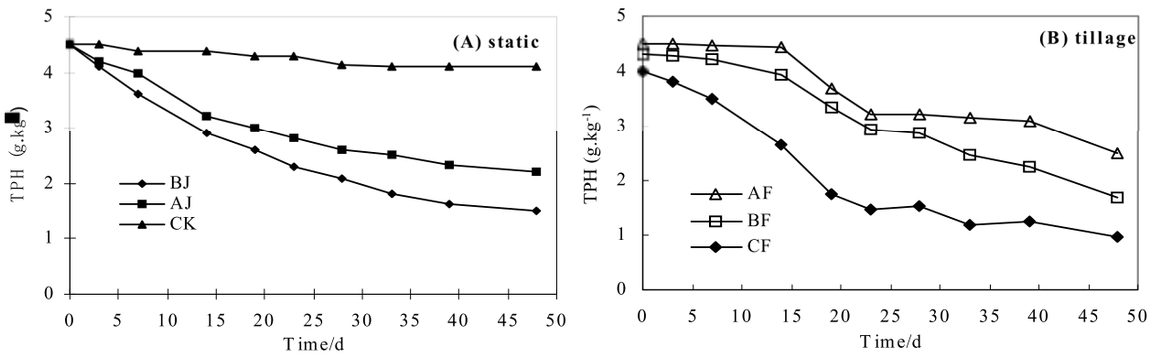


Fig.11. Influence of bulking agents on the degradation of TPH from oil-contaminated soil that was (A) left stagnant, (B) tilled once every day

4.4.2 Changing patterns of the oil component with bulking agent

In order to understand the way bulking agents work, extractions of soil samples after degradation for 64 days were analyzed by GC-MS. Table 9 and Figure 12 summarizes the results of the GC-MS. Fig.12 indicates that the removal efficiency of oil components in the samples with bulking agent were higher than in the blank sample. Comparing the GC-MS spectrum for sample AJ (bulking agent-free) with sample BJ (with bulking agent added), which were left stagnant for 64 days, 32 peaks were shown in the AJ soil sample (bulking agent-free) spectrum while the BJ showed only 14 peaks in the GC-MS. The branched alkanes, alkene, carotane and alkylnaphthalenes in sample BJ (containing bulking agent) were thoroughly degraded. But the removal rate of branched alkanes, alkene, carotene, and alkylnaphthalenes in sample AJ (bulking agent-free) were 57.2%, 67.8%, 68.8%, and 79.7% respectively. The addition of bulking agent promotes the growth rate of the active bacteria because bulking agent keeps water in the soil and aids oxygen transfer. So the removal rate of normal paraffin, hopanes, and steranes could also be promoted under these conditions.

After combined treatment of addition of bulking agent and tillage, 10 peaks in the GC-MS spectrum disappeared and residual branched alkanes, alkene, carotene, alkylnaphthalenes, hopanes, and steranes were also thoroughly degraded. As well as the removal of 99.7% of normal paraffin. Adding bulking agent promotes the interaction between bacteria and TPH. Likewise, the combined treatment with bulking agent and tillage demonstrate improved bioremediation.

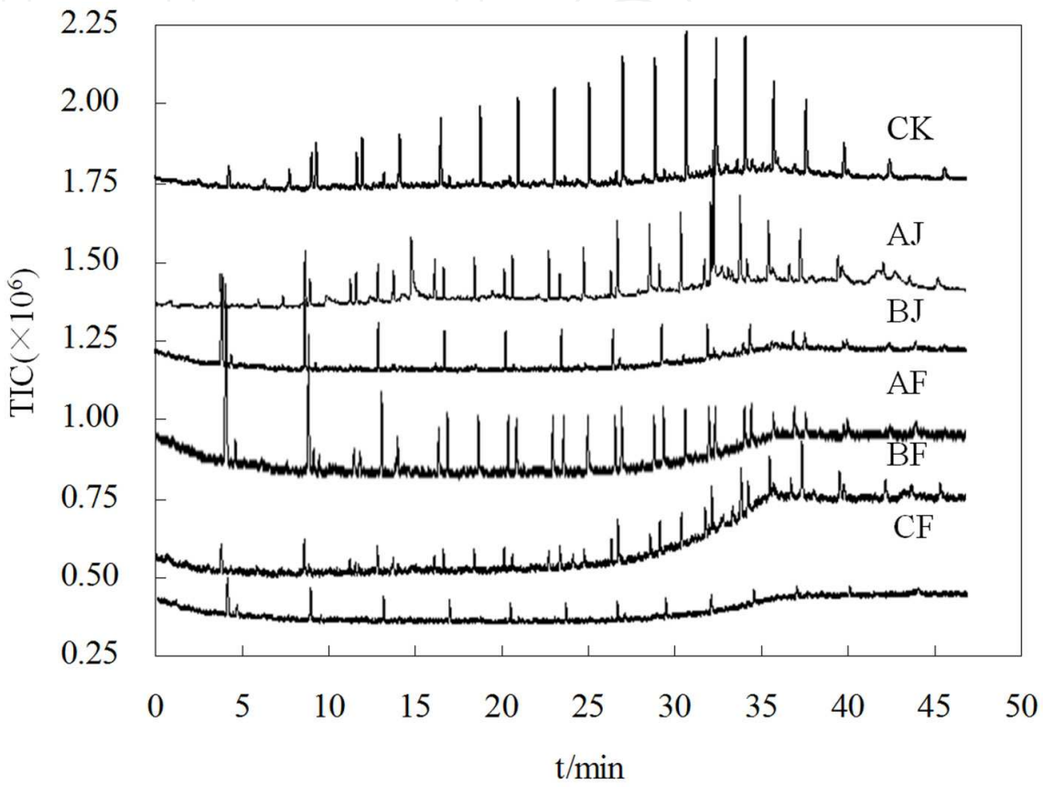


Fig. 12. TIC of remaining TPH concentration in soil on different treatment condition

Number	Peak area A(×10³) and Degradation efficiency η (%)													
	Alkanes				Alkene		Crotane		Alkyl-naphtha-lenes		Hopanes		Steranes	
	n-Alkanes (m/z=57)		Branched alkanes (m/z=97)		(m/z=55)		(m/z=125)		(m/z=128)		(m/z=191)		(m/z=217)	
	A	η	A	η	A	η	A	η	A	η	A	η	A	η
Control	39502		3466		8074		845		251		542		156	
CK	13992	64.6	1684	51.4	2645	67.2	332	60.7	83	66.9	46	91.5	49	68.6
AJ	10875	72.5	1484	57.2	2601	67.8	264	68.8	51	79.7	261	51.8	27	82.7
BJ	627	98.4	9	99.7	33	99.6	0	100.0	0	100.0	78	85.6	6	96.2
AF	2139	94.6	143	95.9	341	95.8	29	96.6	0	100.0	20	96.3	0	100.0
BF	1409	96.4	75	97.8	137	98.3	9	98.9	0	100.0	127	76.6	13.7	91.2
CF	110	99.7	0	100.0	0	100.0	0	100.0	0	100.0	0	100.0	0	100.0

Table 9. GC-MS Analysis of Petroleum Hydrocarbon after Adding Bulking Agents

4.4.3 Influence tillage times on the degradation of TPH

Figure 13 shows the experimental matrix designed to explore the influence of tillage time and the addition of bulking agent on the degradation of TPH. In the samples evaluated the change of TPH followed the same trend. TPH concentration decreased as a function of time with the removal rate increasing gradually. TPH content in sample F1 (tilled once a day) was the lowest since day 3, followed by samples F2 and F3. The biodegradation level in sample F0 (without tillage) was the worst. The removal efficiency of TPH in F1 was up to 92.64% while the removal efficiency in samples F2, F3, and F0 was 80.46%, 69.14%, and 60.26% after 36 days of biodegradation. Likewise, the removal efficiency of TPH decreased as tillage times decreased. The optimum tillage frequency was once a day every day using a shovel. If economic factors are considered, then a minimum of once tillage each 3 days would be acceptable.

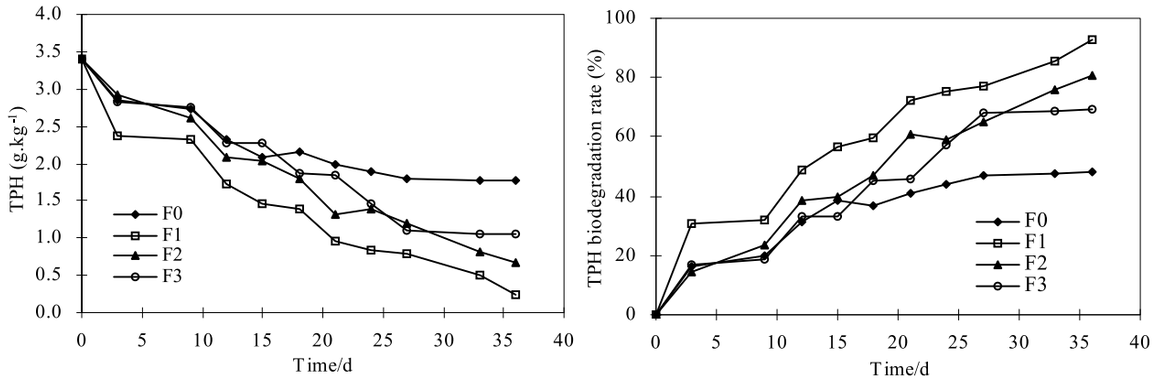


Fig. 13. Influence of tillage times on the degradation of TPH in oil-contaminated soil

Previous reports, including EPA [29], indicate that the degradation of oil contaminants by indigenous bacteria follows a first order reaction, that is expressed by the following equation:

$$\ln C = \ln C_0 - K_T.t \quad (2)$$

In equation (2), C represents concentration of TPH in the soil in mg • kg^{-1}; K_T represents substrate removal constant in d^{-1}; and t represents degrading time in days.

A plot of lnC as a function of time results in a linear regression with $\ln C_0$ as the intercept and K_T as the slope. Then, the half-life time of the petroleum contaminant in the soil can be calculated as $t_{1/2}=\ln 2/K_T$, as shown in Table 10. These data indicates that the removal rate increases with tillage times.

Sample Number	Tillage frequency	Equation	R^2	K_T (d^{-1})	Half-life (d)
F1	Tilled once every day	$c=3402e^{-0.0636t}$	0.95	0.0636	11
F2	Tilled once every three days	$c=3402e^{-0.0441t}$	0.97	0.0441	16
F3	Tilled once every five days	$c=3402^{-0.0352\,t}$	0.95	0.0352	20
F0	Stagnant	$c=3402e^{-0.0177t}$	0.90	0.0177	40

Table 10. Half-life of the Petroleum Contaminant in Soil at Different Tillage Times

4.5 Discussion

To explain how the removal efficiency of TPH is increased by the addition of bulking agents, pH, electric conductivity, and soil moisture were analyzed. The samples under evaluation had almost the same pH ranging from 6.0 to 6.7 during the evaluation period. Similarly, the electric conductivity in the samples ranged from 200 to 300 μs/cm during testing, with the exception of water content, which was different for the samples tested. Figure 14 summarizes the results.

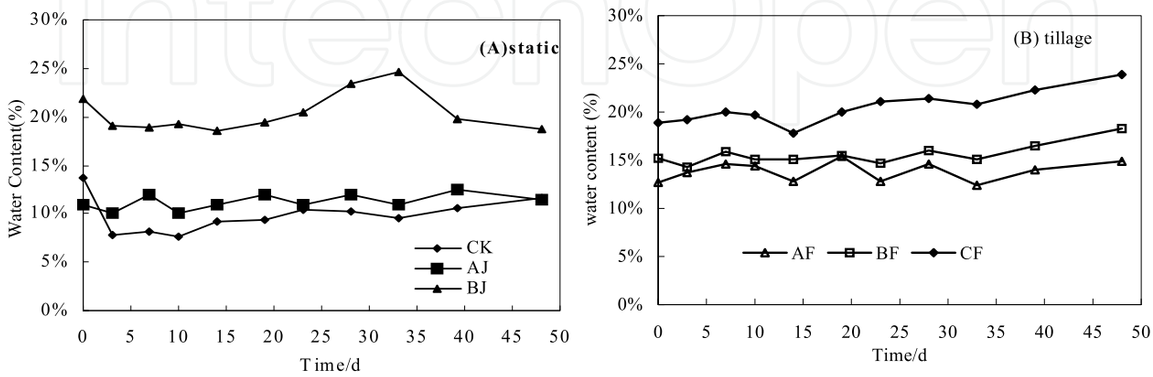


Fig. 14. Influence of bulking agents on water content of oil-contaminated soil that was (A) left static, (B) tilled once every day

Fig.14 displays the curves of water content of the oil-contaminated soil sample that was left stagnant and for the samples that were tilled once every day. The experimental observations revealed that although the dose of water was the same for all the samples, the water content in the samples was quite different. The water content of the samples containing bulking agent was kept in 20% while the content of water in the blank sample was only about 15%. The bulking agent or saw dust shows high capacity for water absorption. The water saturation rate in these samples is very fast, with saturation times of just 3 min. The water absorption capacity of saw dust is 4.45 times bigger than its own weight [30]. In the samples containing saw dust, it was observed that the saw dust absorbs the water as soon as it is dosed into the sample. However in the case of the samples free of saw dust, water added would transfuse along the holes in soil. So that the water content in the blank sample (saw dust-free) is lower than in the samples containing saw dust. Furthermore, Figure 14(B) indicates that during the evaluation period, in the tilled samples without saw dust, the water content is unstable, which can be explained by the fact that in this sample, tillage accelerates the speed of water evaporation. Saw dust retains the water in the soil, in addition to enhancing the air permeability in the contaminated soil sample which benefits the degradation of TPH by bacteria.

4.6 Conclusions

1. Adding bulking agent enhances the retention of water and the air transfer in the soil. Experimental results show that the degradation efficiency of TPH was 76% in 48 days after adding bulking agents and tillage, which was 15% higher than the degradation efficiency in the samples without bulking agent. Likewise, the degradation rate is 2.34 faster in the samples containing bulking agent that in the samples without bulking

agent. GC-MS analysis indicates that the peaks (concentration) of the pollutants in the GC spectrum decreased from 32 to 14 in 64 days after bioremediation of adding the bulking agent.

2. Tillage not only raises the content of oxygen in the soil but also accelerates the speed of transfer of substrates from outside into the biomembrane. The removal rate of TPH in samples that were tilled was 1.2 times higher than in the samples without tillage. Especially the concentration peaks of the pollutant components in the GC spectrum decreased 10 times after 64 days of degradation. Similarly, residual branched alkanes, alkene, carotene, alkylnaphthalenes, hopanes, and steranes were also thoroughly degraded after the application of the combined treatment with bulking agent and tillage. The degradation efficiency of TPH decreased as tillage times was decreased; the optimum tillage frequency is once every day with a shovel.

5. Part IV Impact of bioaugmentation dosage and temperature on bioremediation of petroleum-contaminated soil

5.1 Summary

The influence of bioaugmentation products dosage and temperature on bioremediation was studied. The results showed that the degradation rate was related positively to the amount of inoculation. When the inoculums dose was increased to 0.6 $mg.kg^{-1}$ the degradation efficiency of petroleum hydrocarbon (TPH) increased to 87% in 48 days. The results of GC-MS indicated that the petroleum constituents in the oil-contaminated soil were 82.7% *n*-alkane, 16% alkene and the balance corresponded to others hydrocarbons, such as carotane, alkylnaphthalenes, hopanes, and steranes. The concentration peaks in the GC spectrum decreased from 32 to 14 within 40 days of bioremediation. This result indicated that branched alkanes, alkene, and alkylnaphthalenes were thoroughly degraded. However, linear alkanes chains, hopanes, and steranes were still remaining in the soil. In addition, the longer *n*-alkane chains were degraded with relatively higher rate, leaving the shorter part of the n-alkane as the residual fraction at the end of the bioremediation test. Moreover, it was observed that the concentration of residual *n*-alkane (short chains) decreased as the inoculation volume increased. This indicates that an increase in the amount of bioaugmentation products into the contaminated soil clearly improves the biodegradation efficiency. It was also determined that temperature has an important effect on degradation efficiency. For instance it was observed that at 30°C, the concentration of TPH in the soil sample was reduced by 80%, whereas at a temperature of 20°C, the removal efficiency of TPH was only 60%, which indicated that higher temperatures favors TPH degradation and accelerates bioremediation.

5.2 Introduction

As the development of the petroleum industry in China increases, soil contamination by petroleum products also increases in both: areal extension and concentration. Common pollutants in the contaminated soil are hydrocarbon compounds such as: benzene, methylbenzene, and dimethylbenzene, which are highly toxic and some of them may be carcinogenic. These hydrocarbons could be difficult to degrade after entering the soil [1]. Furthermore, once these pollutants are established in the soil, they could enter into the surrounded underground water and river basins. Bioremediation of petroleum

hydrocarbon contaminated soil is considered a new technology with a broad prospect because of its low-cost and pollution-free characteristics. Previous research has focused on the stimulation of the activity of the indigenous microorganisms by adding nutrient substances such as nitrogen (N) and phosphorous (P) [3]. However, bioremediation would be restricted by the slow-growth rate and limited amount of the indigenous microorganisms, which can be overcome through the addition of active microorganisms that can easily adapt to the contaminated soil environment. Several studies on this issue have been published. For example, a patented technology by the BGI (Baltic General Investment Corporation, USA) promotes the degradation rate of petroleum hydrocarbons by using mixed microorganisms [4]. Wilson (1993) [31] also reported some strains which quickly degrade PAHs. Xiao-Fang (2007) [32] found that exogenous microbes increase the degradation of petroleum contaminated soil. Another research [33] showed that by splashing bacteria into the petroleum contaminated soil, with oil content ranging from 5% to 45%, degradation rates reached up to 90% after 150 days of testing. The addition of petroleum-degrading active bacteria and microbial inoculation into petroleum contaminated soil is an in-situ bioremediation technology which has been wildly investigated. Efficient bioremediation have been obtained by the application of microbial inoculation-formulations, which have been recently developed, into actual bioremediation projects [34]. This section of the chapter summarizes the influence of bioaugmentation dosage, dosage sequence, and temperature on the bioremediation of petroleum hydrocarbon contaminated soil and provides a theoretical foundation for practical applications.

5.3 Materials and methods

5.3.1 Materials

5.3.1.1 Soil samples

The oil contaminated soil samples used in these experiments were collected from areas surrounding producing wells from an oil field located in the northern part of the Shaanxi province In China. The soil samples were free from additional dirt, pulverized, and well mixed before the experiments.

5.3.1.2 Microbial inoculations

Oil contaminated soil around oil wells was used to isolate bacteria which utilized crude oil as the only carbon source. Bacteria at hand in this oil contaminated soil samples were screened and separated and ranked as highly active petroleum-degrading bacteria [14]. The strain SY_{23}, which is commonly reported in the literature [14] was separated under aseptic conditions that was then dried and sterilized at 37^{o}C for 48 hours. At this stage, the bioaugmentation product was ready for use.

5.3.2 Experimental methods

5.3.2.1 Bioremediation methods

In order to prepare a bacterial-rich suspension, certain amount of the solid bacterial product was added into a bacteria-free buffer. A substrate medium was added into the bulking agent to prepare a mixture. Later, the mixture was sterilized and the bacterial-rich suspension was

mixed with the bulking agent mixture, which was cultured for 24 hours. The final product was ready for addition into the petroleum contaminated soil.

5.3.2.2 Analytical methods

The composition of the petroleum hydrocarbon (TPH) was determined by OCMA-350 non-dispersive infrared oil analyzer. The concentration of the petroleum components was determined by GC-MS. A CG 7890, Model Trace 2000, was manufactured by Agilent was used and the mass spectrometer 5975, model Voyager, manufactured by Agilent was employed for the analyses. The GC was equipped with a 30m long DB-5 capillary, with a stationary phase of 0.25μm. The range of relative molecular mass was from 30 to 450, and the first 6.10 min corresponded to the solvent peak. The conditions of the GC analysis were: temperature was increased at a rate of 10^{o}C/min from 100^{o}C to 200^{o}C, and then the temperature was increased at rate of 5^{o}C/min up to 280^{o}C. The contact temperature between the Ms and GC was 250oC. Conductivity was determined using a conductivity meter and pH was determined using pH test paper. Finally, moisture content was determined by the constant weight method.

5.3.3 Design of experiments

An experimental matrix was designed to evaluate the effect of different inoculation schemes and dosage of bioaugmentation products on the bioremediation of petroleum hydrocarbon contaminated soil. The experiments were carried out in basin ports having a diameter of 15cm and depth of 15cm. In every group, 1000g of soil was used. Table.11 summarizes the experimental observations. Each group was conducted by duplicate. Every group was turned over once a day and 20mL of water were added every day [35]. During this set of experiments, the moisture content in the environment was maintained at 18% using a humidifier. Samples were taken at constant time intervals for pH, conductivity, petroleum concentration, and moisture content analyzes. The sampling intervals were increased when the petroleum-degradation rate started to decrease.

Number	Bioaugmentation products dose (mg.kg^{-1})	Temperature (^{o}C)	Moisture (%)	Bioaugmentation products inoculations	Treatment measures
Experiments of bioaugmentation products dosage	0(CK)	20	18	No addition	Turn over
	0.01	20	18	Addition one time	Turn over
	0.2	20	18		Turn over
	0.4	20	18		Turn over
	0.6	20	18		Turn over
Experiments of bioaugmentation products inoculations	0.2	20	18	Stepwise addition (two times)	Turn over
	0.4	20	18		Turn over
	0.6	20	18		Turn over
Experiments of temperature	0.2	20	18	Addition one time	Turn over
	0.2	37	18		Turn over

Table 11. Experimental Observations

5.4 Results and discussions

5.4.1 Influence of bioaugmentation products dosage on the degradation of petroleum hydrocarbons in soil

Figure 15 and Figure 16 show the residual TPH concentration and the TPH degradation rate in soil as a function of bacteria dosage and time. The initial TPH concentration in soil was 4.3 g•kg^{-1}. The dosage of bioaugmentation products was 0.01, 0.2, 0.4, and 0.6mg•kg^{-1} respectively. Figure 16 indicates that the TPH degradation rate after 8 days of biodegradation for the corresponding bacteria dosage conditions were 2%, 10%, 25%, and 40% respectively. While, the degradation rate of the blank sample without bacteria (CK) was only 3%. The TPH degradation efficiency during 48 days of testing was 47%, 68%, 79%, and 87%; while the degradation efficiency in the blank sample (CK) did not achieve even 20%.

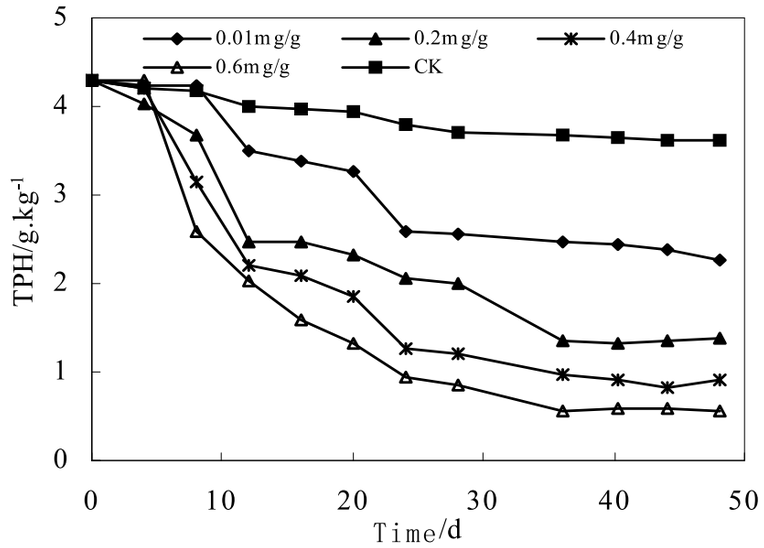


Fig. 15. Residual TPH concentration in soil as a function of bacteria dosage and time.

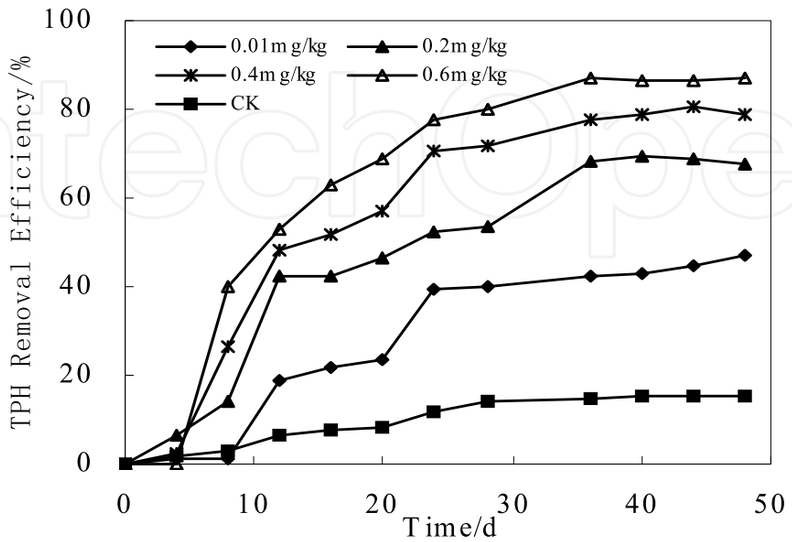


Fig. 16. TPH degradation rate as a function of bacteria dosage and time.

Previous research [36] showed that the key problem of bioremediation by adding exogenous bacteria is how to solve the competition between exogenous and indigenous bacteria. Experimental observations show that exogenous bacteria survive well with the indigenous flora. As demonstrated through this experimental study, the TPH degradation rate in soil is positively related to the dosage of bioaugmentation products. The highest degradation rate was achieved when the bioaugmentation products dosage was 0.6 mg•kg^{-1}. Furthermore, at low dosage (0.01 mg•kg^{-1}) of bioaugmentation products did rise significantly the degradation rate after 20 days of testing; due to exogenous bacterial growth.

Previous reports [37] indicate that the degradation of petroleum contaminated soil by bacteria can be described by a first order reaction: $\ln C=\ln C_0-K_T.t$

Where : C is petroleum content in soil mg•kg^{-1}; K_T is substrate removal constant d^{-1}

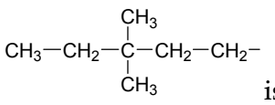
A plot of ln$C$ as a function of time renders a straight line with intercept lnC_0 and slope K_T. The dynamic equations and the half-life of petroleum contaminants in soil were calculated using the following expression: $t_{1/2}=\ln 2/K_T$. Table 12 shows the half-life of the petroleum contaminants in soil as a function of bacteria dosage.Table 12 also shows that the TPH remediation cycle in soil is 300 days shorter than in the blank or control sample (CK). The substrate removal constant K_T indicates that biodegradation increases as the dosage of bacteria increases while the half-life of the petroleum contaminants is reduced. The optimum dosage was 0.6 mg•kg^{-1}.

Number	Dose (mg•kg^{-1})	Dynamic equations	R^2	K_T	Half-life (d)
1	CK	$c=4246e^{-0.00225t}$	0.96	0.00225	308
2	0.01	$c=4023e^{-0.0302t}$	0.96	0.0302	23
3	0.2	$c=4318e^{-0.0373t}$	0.96	0.0373	19
4	0.4	$c=4203^{-0.0454\ t}$	0.96	0.0454	15
5	0.6	$c=4299e^{-0.0604t}$	0.97	0.0604	11

Table 12. Half-life of the Petroleum Contaminants in Soil at Different Dosage Conditions

To investigate the effect of microbial inoculums on the degradation of hydrocarbons, samples were subjected to extraction after 48 days of degradation. The composition of the extract liquors were analyzed through GC-MS. Fig.17 shows the GC-MS spectra. The GC-MS spectra confirm that most of the petroleum constituents in soil were degraded by the microbial inoculums. When the dose was increased to 0.6 mg•kg^{-1}, the peak lines were almost parallel with the base line. Fig.17 clearly shows that increasing bacteria dose improves TPH degradation and accelerates bioremediation.

GC-MS indicated that the dominant petroleum constituents in the oil-contaminated soil were 82.7% n-alkane, 16% alkene, and the balance corresponded to other hydrocarbons, such as carotane, alkylnaphthalenes, hopanes, and steranes. Monocyclic benzene series were not found in the hydrocarbon analyzed, which is in agreement with Xiao-Fang's study (2007)[32]. Table 13 presents the (TPH) degradation rate as a function of bacteria dosage for a period of 48 days. Specific charge (m/z) measurement was used to determine chemical

structure. The specific charge (m/z) for $[CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2]^-$ is 57, for  is 97, and for $[CH_3\text{-}CH{=}CH\text{-}CH_2]^-$ is 55. So that, Table 13 indicates that branched alkanes and alkenes are easier to degrade than linear alkanes. The content of linear alkanes decreases as bacteria dosage increases. The degradation rate of linear alkanes was increased to 97.4% when the dose of bacteria was increased to 0.6mg•kg^{-1}. The residual components shown in Table 13 were mainly linear alkanes.

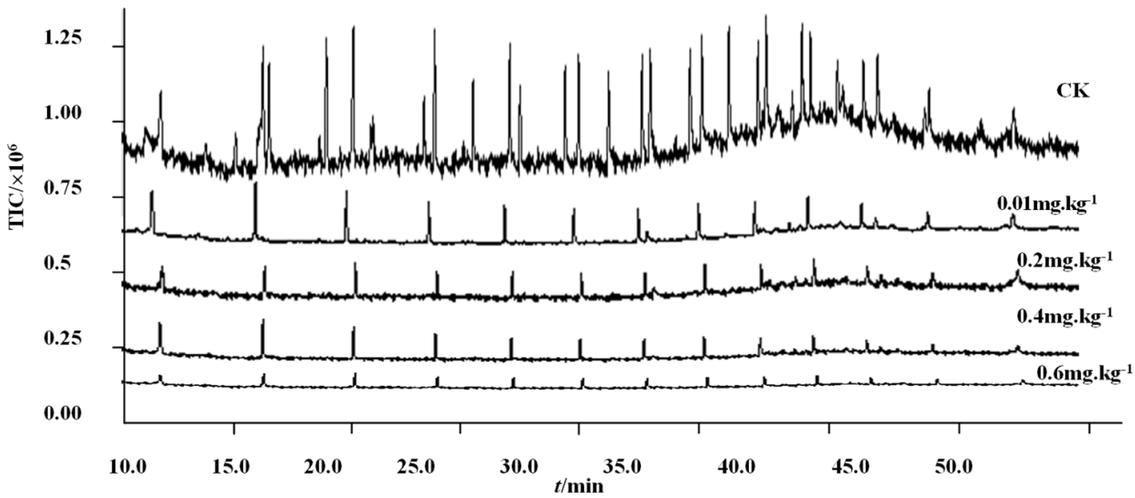


Fig. 17. TIC of remaining TPH concentration in soil at different dose condition

Bacteria dose (mg•kg^{-1})	Degradation rate of different HC in 48 days (%)					
	Alkanes				Alkenes (*m/z* =55)	
	Linear alkanes (*m/z* =57)		Branched alkanes (*m/z* =97)			
	$A\times10^3$	η(%)	$A/\times10^3$	η(%)	$A(\times10^3)$	η(%)
CK	2303.0		142.0		410.0	
0.01	278.0	87.9	0.0	100.0	6.0	98.5
0.2	261.0	88.7	0.0	100.0	5.0	98.7
0.4	81.0	96.5	0.0	100.0	0.0	100.0
0.6	61.0	97.4	0.0	100.0	0.0	100.0

Table 13. Peak Area and Removal Rate of Alkane & Alkene in Soil at Different Dosage of Bacteria

The hydrocarbon degradation rate was monitored when bacteria dosage was 0.2mg•kg^{-1}. Samples were taken for GC-MS analysis at the following degradation times: 0d, 4d, 8d, 40d, and 48d respectively. Fig.18 and Table 14 show the experimental observations. Table 14 indicates that the peak values on the GS-MS spectrum are reduced after 4 days of bioremediation, which indicates that hydrocarbon biodegradation is taken place GC-MS analysis shows that 98.5% of alkenes were degraded, followed by 87.9% degradation of

linear alkanes and 41 % degradation of branched alkanes. The degradation rate of branched alkanes, carotene, and alkylnaphthalenes was increased up to 74.9%, 91.0%, and 100% respectively in 8 days of testing. At the same time the peak of the phytan (Ph, 2, 6, 10, 14-tetramethylhexadecane) in the GC-MS spectrum, (shown by the arrows in Figure 18) almost disappeared. The Ph and Pristane (Pr, 2, 6, 10, 14-Tetramethylpentadecane) were not detected after 48 days of bioremediation. Thus, the presence of these components in the contaminated soil sample is taken as an indication of the biodegradation of the petroleum hydrocarbon in the contaminated soil. In Fig. 18, the peak of Ph could be seen in the CK line after 48 days of biodegradation. However, the Ph peak disappeared after inoculating bioaugmentation products. This is strong evidence that bioaugmentation products could degrade petroleum. The concentration peaks from the GC-MS spectra decreased from 32 to 14 after 40 days of bioremediation. This result points out that branched alkanes, alkene, and alkylnaphthalenes were thoroughly degraded. However, linear alkanes, hopanes, and steranes were left in the soil as pollutants. The residual part of the n-alkane corresponds to the short chains because bacteria degraded the longer n-alkane chains, which is in agreement with previous studies[37], which have reported that the degradation rate of n-alkanes is reduced as the chain length increases. Previous work [37, 38] claims that marine filamentous fungi have higher degradation capacity for eicosane, tetracosane, and hexacosane than bacteria. So, finding bacteria capable of degrading long chains of n-alkane is a key issue in bioremediation of petroleum hydrocarbon pollutants. The strain SY_{23} in this experiment is a plesiomonas. Hydrocarbon biodegradation tests have shown that SY_{23} bacteria have high capacity for degrading benzene, methylbenzene, phenol, and long chains of n-alkanes. During these experiments it was observed that the color of the raw contaminated soil changes from brown to yellow after the addition of bacteria; which could also indicate that biodegradation of petroleum hydrocarbon is taken place. Bioremediation rate could be increased if inoculation volume is increased. In addition, it was observed in

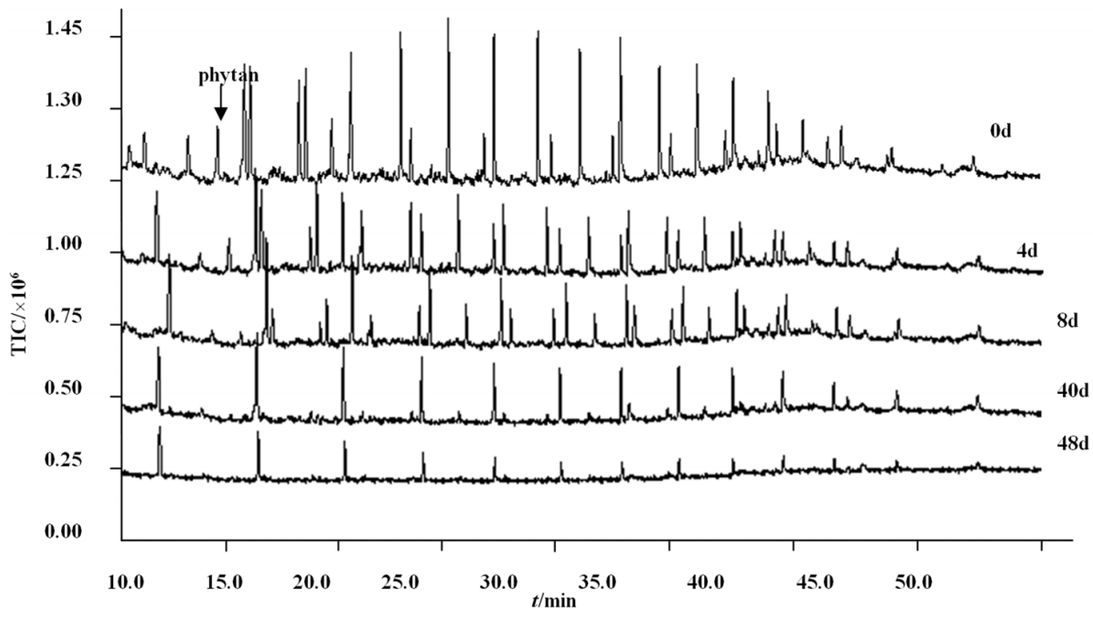


Fig. 18. TIC of TPH concentration in soil during bioremediation

this study that hopanes were not degraded during the first 8 days of testing, however it reaches 53% degradation after 40 days. This shows that hopanes are stabilized in the contaminated soil. In general, hopanes are hardly degraded if their concentration in the contaminated soil is too high. In this work, the concentration of hopanes was low (58mg/kg) and 60% of this concentration in the soil was removed.

Testing Time	Peak area $A\times10$ and removal rate η(%)of different kind of alkenes													
	Alkanes													
	Linear alkanes (m/z =57)		Branched alkanes (m/z =97)		Aalkenes (m/z =55)		Carotane (m/z =125)		Alkylnaphthalenes (m/z =128)		Hopanes (m/z =191)		Steranes (m/z=217)	
	A	η	A	η	A	η	A	η	A	η	A	η	A	η
0d	9255.0		981.0		1998.0		119		26.0		84.0		11.2	
4d	6524.0	87.9	577.0	41.0	6.0	98.5	113	5.0	4.0	86.4	82.0	2.3	9.53	14.9
8d	3489.0	88.7	246	74.9	5.0	98.7	10.7	91.0	0.0	100.0	82.0	2.3	5.7	49.1
40d	981.0	96.5	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	39.0	53.0	3.0	73.2
44d	242.0	97.4	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0

Table 14. Analysis of Petroleum Hydrocarbon after Inoculation of Microorganisms

5.4.2 The Influence of bioaugmentation product injection scheme on the degradation of petroleum hydrocarbon

Samples were divided into two groups A and B. In group A, the bioaugmentation products were injected all at once in one application. In group B, half of the bioaugmentation products were injected at the beginning of the test, and the other half was injected after 20 days of testing. The total bioaugmentation products in the two groups were the same. Three series of experiments were conducted for each group having a different concentration of the bioaugmentation products in each series as follows: 0.2 mg•kg^{-1},0.4 mg•kg^{-1}, and 0.6 mg•kg^{-1} respectively. Figure 19 summarizes the GC-MS results after 48 days of degradation. Figure 19 indicates that the value of concentration peak in the GC-MS spectra for group A was lower than for group B. The CG-MS results show that the main residual components were linear alkanes and hopanes. The peak area of the linear alkanes in group A was 170000, while the peak area of the linear alkanes in group B was 261000 when the bioaugmentation products dose was 0.2 mg•kg^{-1}. The peak area of the linear alkanes in group A was 81000 while the peak area of the linear alkanes in group B was 175000 when the bioaugmentation products dose was 0.4 mg•kg^{-1}, and finally the peak area of the linear alkanes in group A was 61000 while the peak area of the linear alkanes in group B was 142000 for a dose of 0.6mg•kg^{-1}. These observations indicate that the best sequence of bioaugmentation products application is to inject all products at once, which is in agreement with previous studies [39]. Thus, the addition of the total amount of bioaugmentation products aids the quick adaptation of the bacteria to the environment, that results in petroleum components removal efficiency. Injecting the bioaugmentation products in only one application degraded about 50% of TPH in the soil after 20 days of testing; while only 30% of TPH were degraded in group B. Degradation rates were 68%, 79%, and 87% when the concentration of the bioaugmentation products were 0.2 mg•kg^{-1}, 0.4 mg•kg^{-1}, and 0.6 mg•kg^{-1} respectively. In Group B, the late injection of the remaining half of the bioaugmentation products after 20

days of testing did not increase the degradation of TPH. Finally, in group A the degradation rates after 48 days of testing were 54%, 63% and 74%.

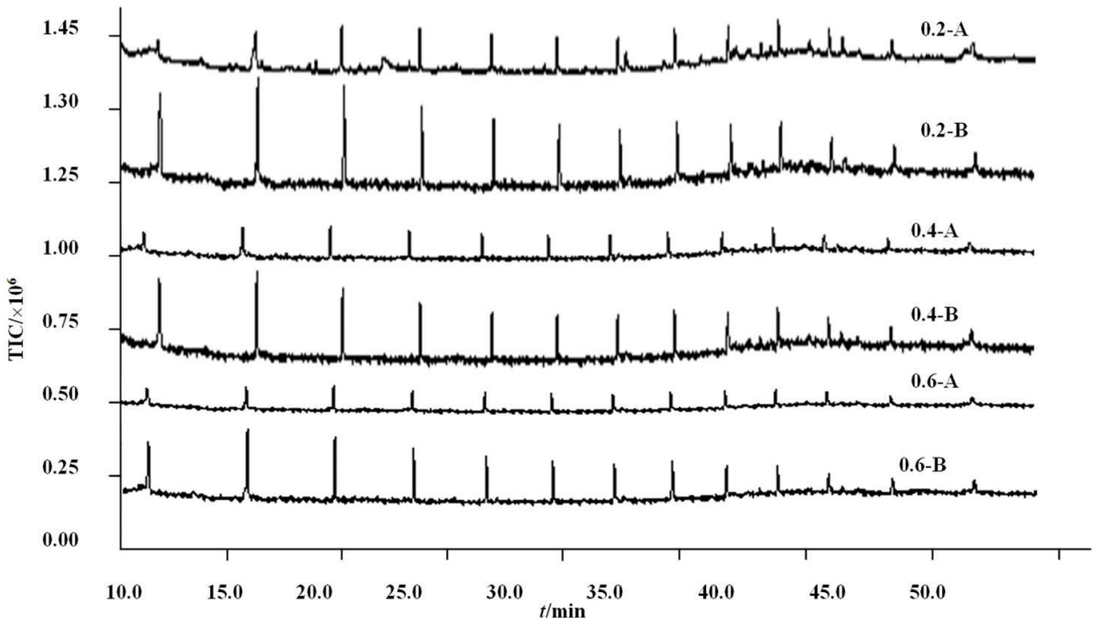


Fig. 19. TIC TPH concentration in the soil at different injection schemes of the bioaugmentation products

5.4.3 Influence of temperature on the biodegradation of petroleum hydrocarbon

Several studies [30] indicate that temperature is an important variable that determines the efficient degradation of petroleum hydrocarbon in contaminated soil. Two groups of experiments were carried out at two different temperature conditions. In the first group, the

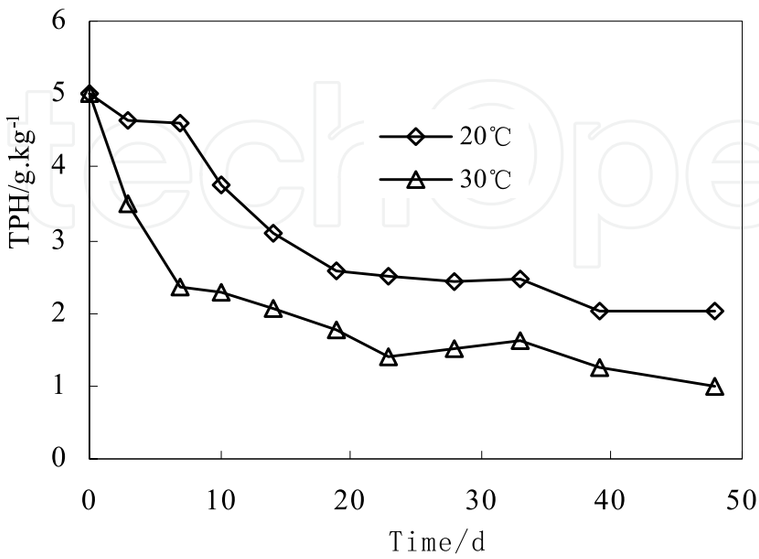


Fig. 20. TPH concentration in the soil as a function of temperature and degradation time

average temperature was 20°C while in the second group of experiments the average temperature was 30°C. The experiments were conducted between May 1st to June 27th in 2006. Figure 20 presents the residual TPH concentration in the soil as a function of temperature and testing time. These experiments indicatd clearly the effect of temperature on TPH degradation. At 30°C, the degradation of TPH reached 29.8% after 3days of treatment, whereas at 20°C, the degradation of TPH reached only 7%. This indicates, that at higher temperatures, bacteria grow faster, which accelerates bioremediation. For the experiments conducted at 20°C, the degradation rate of TPH increased rapidly after 10 days, which points out that the adaptation of bacteria in the low temperature environment took longer, which resulted in a total TPH degradation of only 60% at the end of the testing period. This overall degradation percentage (at 20°C) was 20% lower than the total degradation of TPH reached in the experimental group conducted at 30°C.

5.5 Conclusions

1. Exogenous bacteria survive quite well in the presence of indigenous flora. The degradation rate of TPH contained in the soil was related to the dosage of bioaugmentation products. In this work, the highest degradation rate was achieved when the dose of bioaugmentation products was 0.6 $mg \bullet kg^{-1}$.
2. GC-MS analysis indicates that the dominant petroleum constituents in the oil-contaminated soil were 82.7% n-alkane, 16% alkene, and the balance corresponded to of others hydrocarbons, such as carotane, alkylnaphthalenes, hopanes, and steranes. The height of the GC spectrum peaks decreased from 32 to 14 after 40 days of bioremediation; this indicates that branched alkanes, alkene, and alkylnaphthalenes were thoroughly degraded. However, linear alkanes, hopanes and steranes were left in the soil. Furthermore, long chains of n-alkane were degraded, while short chains of n-alkane were left as residual hydrocarbon in the soil. It was determined that increasing inoculation increases the capacity of the bacteria to attack short chains n-alkanes. The application of bioaugmentation products into soil in one dosage significantly improved the biodegradation efficiency rather than the application of bioaugmentation products in several dosages.
3. Temperature is the dominant variable that determines the efficiency of bioremediation of petroleum hydrocarbon contaminated soil. At higher temperatures, bacteria grow and metabolize faster. At 20°C, the degradation of TPH increased rapidly after 10 days of testing, which indicates that the bacteria slowly adapted to the low temperature environment. The total degradation percentage of TPH rate was 60% at 20°C, which was 20% lower than the degradation percentage of the experimental group tested at a higher temperature (30°C).

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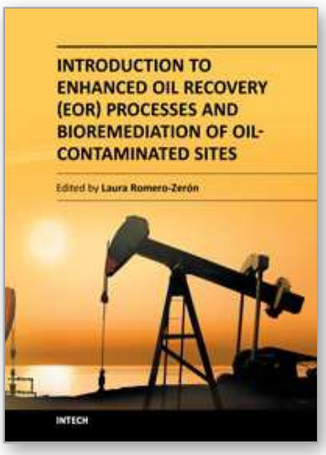

Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites

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This book offers practical concepts of EOR processes and summarizes the fundamentals of bioremediation of oil-contaminated sites. The first section presents a simplified description of EOR processes to boost the recovery of oil or to displace and produce the significant amounts of oil left behind in the reservoir during or after the course of any primary and secondary recovery process; it highlights the emerging EOR technological trends and the areas that need research and development; while the second section focuses on the use of biotechnology to remediate the inevitable environmental footprint of crude oil production; such is the case of accidental oil spills in marine, river, and land environments. The readers will gain useful and practical insights in these fields.

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