



Study on fungi-bacteria augmented remediation of petroleum contaminated soil from Northwest of China

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Abstract

A new bioremediation method for petroleum contaminated soil (PCS) using both fungi and bacteria was proposed and investigated using *Glomus caledonium* NW03 and *Bacillus subtilis* NW08, both of which were isolated from PCS of Petro China of Changqing (PCOC), Shaanxi, China. The removal of total petroleum hydrocarbon (TPH) by the mixture was higher than the sum of the individual removal obtained in the pure culture. It was demonstrated that the growth behavior of the inocula and the degradation of TPH were enhanced by the mix culture of both fungi and bacteria. The remediation via inoculating the fungal-bacterial consortium removed 92.6% of TPH in 60 days while the control experiment with the indigenous microorganisms removed 21.9%. Significant reductions of each petroleum composition were shown by chromatogram analysis in treated soil, while the alkane fraction with molecule chains longer than 28 C almost disappeared.

Key words: Bioaugmentation, bioremediation, fungi, bacteria, petroleum contaminated soil.

Introduction

Oil pipeline leakage and accidental oil spills are common problems in petroleum industry resulting into contamination of soils. Toxic heavy metals and petroleum hydrocarbons present in such contaminated soils can leach into the surrounding subsurface and groundwater, posing a threat to the environment and to human health ¹⁻³. Petroleum contaminated soil (PCS) is a mixture of sand, silt, clay and petroleum products. In north Shaanxi Province of China, Petro China of Changqing (PCOC) generates approximately 86,000 tons/year of PCS which raises a real disposal problem. Many of the standard treatment processes used to decontaminate soil and groundwater have been limited in their application to PCS, for prohibitively expensive or potential threats to the environment ⁴⁻⁷.

Microbial degradation represents the major route responsible for the ecological recovery of PCS ⁸. Many species of bacteria have been shown in recent years to have the abilities to use petroleum hydrocarbon as sole source of carbon and energy. PCS bioremediation techniques have been carried out either by adding nutrients (biostimulation) or degrading microorganisms (bioaugmentation) to the contaminated soil ⁹. Bioaugmentation may be carried out by adding either pure culture or microbial consortium. The introduction of microbial consortium has the advantage over pure culture because the consortium can resist wide variations in natural environment ¹⁰. However, the isolation and maintenance of microbial consortium is difficult.

The objective of this study was to explore the efficiency of bioaugmentation through applying microbial consortium to PCS. Strains of *Glomus caledonium* NW03 and *Bacillus subtilis* NW08, isolated from petroleum contaminated soil sample of PCOC oil

field, were inoculated as fungal-bacterial consortium. Each fraction of petroleum hydrocarbon in PCS was monitored during bioaugmentation by GC. Since this bioaugmentation was performed by only two kinds of strains, the more complicated microbial consortium could be developed using the similar approach.

Materials and Methods

Chemicals: Aliphatic hydrocarbons (octane, dodecane, hexadecane, octadecane) were purchased from Merck-Schuchardt, Germany. Acetone and hexane were purchased from Sigma-Aldrich, USA. Petroleum contaminated soil sample was collected at a depth of 0.3 m below the surface from a waste pit of PCOC, Shaanxi Province, China.

Microorganisms: Strains of *G. caledonium* NW03 and *B. subtilis* NW08 were isolated from petroleum contaminated soil sample of PCOC oil field.

Biodegradation of petroleum hydrocarbons in soil: The ability of the strains to remediate the petroleum contaminated soil sample was investigated by carrying out the biodegradation experiment in soil for 60 d under indoor laboratory condition. One kilogram of PCS sample (level of TPH contamination was 106.6 g/kg soil), was layered in each of 5 bioreactors. Prior to starting the biodegradation experiment, the soil samples were treated with sterilization. The soil was then inoculated by 1×10^7 cfu/g of either *G. caledonium* NW03, *B. subtilis* NW08 or NW0308 fungal-bacterial consortium (mixed in a proportion of 1:1). The soil was thoroughly trilled and M9 medium was added at every 2 weeks interval to maintain the

moisture level in the soil ¹¹. To determine the extent of biodegradation, the soil-phase TPH concentrations were analyzed during 60 d. A control and an unsterilized soil sample were run in parallel where the soil was treated with un-inoculated medium. The bioreactor and experiment design are presented in Table 1.

Table 1. Bioreactors and experimental design.

Bioreactors	Sterilization	Strains	Inoculation
A	Y	B.NW08	1×10 ⁷ cfu/g
B	Y	G.NW03	1×10 ⁷ cfu/g
C	Y	NW0308 Con.	1×10 ⁷ cfu/g
D	N	Indigenous	-
E	Y	-	-

Chemical analysis of TPH: Total petroleum hydrocarbon (TPH) was extracted from 10 g of soil by sequential extraction with 100 ml of hexane, methylene chloride, and chloroform. All the three extracts were pooled and dried at room temperature by evaporation of the solvents under a gentle nitrogen stream in a fume hood. After evaporation of the solvent, the amount of residual TPH was determined gravimetrically ¹².

Analysis of crude petroleum composition by GC-MS: The crude petroleum composition in PCS was analyzed by gas chromatography/mass spectrometry (GC-MS) using GC-MS spectrometry (GCMS-QP2010; Shimadzu Corp. Japan). The relative content of each fraction was obtained by surface integral of total ion current spectrum with MS.

Analysis of alkane fraction by gas chromatography: The alkane fraction was analyzed by gas chromatography (GC) using a FID detector (GC2201; Shimadzu Corp. Japan). The column was CPSil 8 low bleed (30 m×0.25 mm×0.25 μm) coupled with a CP-Sil 5 CB low bleed/MS (30 m×0.25 mm×0.25 μm) column with helium as a carrier gas. The column temperature was 80–240°C for 30 min with 5°C/min increment and hold at 240°C for 30 min, the injector temperature was 240°C and the transfer line temperature was 300°C. Individual components present in the alkane fraction were determined by matching the retention time with authentic standards.

Results

The analysis of TPH degradation: Soils from different bioreactors exhibited differences in both rate and extent of degradation. Bioaugmentation of *G. caledonium* NW03 with *B. subtilis* NW08 showed a significant decline of total petroleum hydrocarbon (TPH) content in petroleum contaminated soil, compared to their individual remediation. Bioremediation with *G. caledonium* NW03, *B. subtilis* NW08 and NW0308 consortium showed that TPH levels were reduced by 53.1%, 38.1% and 92.6 % at the end of 60 d respectively. The removal of TPH by the mixture was higher than the sum of the individual removal obtained in the pure culture. In contrast, the TPH level was reduced by only 1.99 % in control soil (Bioreactor E) (Fig. 1). Further, degradation of TPH by indigenous soil microbe (Bioreactor D) was at a quite low level compared with the biodegradation of TPH by NW0308 consortium. It was demonstrated that the consortium was more efficient in both growth behavior and the degradation of TPH than that of the indigenous microorganisms.

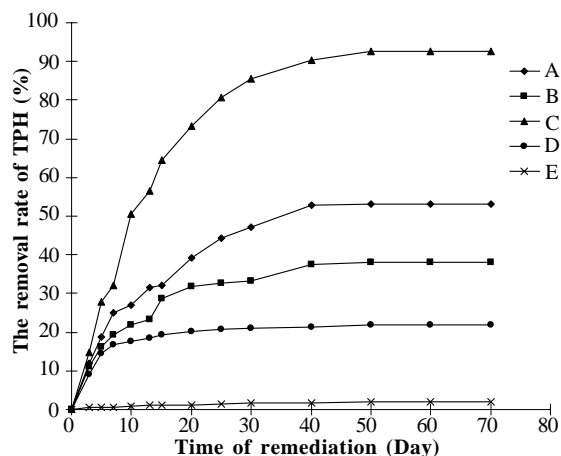


Figure 1. Degradation of TPH in each bioreactor.

The analysis of crude petroleum composition: GC/MS analysis showed that TPH in the PCS contains series of hydrocarbons. The level of alkanes (70.28%) represented the largest constituent of the crude petroleum, followed by aromatic fraction (11.52%) and then NSO fraction (nitrogen, sulfur and oxygen-containing compounds) (18.20%) (Fig. 2).

The GC analysis of alkane fraction: GC analyses of alkane fraction obtained from the bioreactor C before (0 d), in the middle of (20 d) and at the end (60 d) of bioremediation experiment is depicted in Fig. 3. The results showed that alkanes level was significantly reduced in PCS seeded with NW0308 consortium in 60d.

Further quantitative analysis compared with n-alkane standards showed the content and removal rate of different alkane fraction in each bioreactor (Table 2). The alkane fraction was divided into 5 parts (<C14, C14-C16, C16-C24, C24-C28, >C28) for easier analyses. Augmented remediation enhanced the efficiency of biodegradation process in each alkane fraction part. The content of alkane fraction >C28 in bioreactor C was decreased gradually and reached the nearly undetectable level within 60 days, meanwhile 86.9% of which was remained at the end of treatment under the degradation of indigenous microorganisms.

On the other hand, the percentage of alkane fraction degradation in each bioreactor showed that fractions with longer molecular chains (>C28) obtained significant higher removal rate (average 52.4%) compared to these with shorter (<C14) (average 4.8%).

Further, lower amount of alkane fraction >C28 (5.81 g/kg) remained in soil inoculated with *G. caledonium* than in which inoculated with *B. subtilis*. However, the remained amount of alkane fraction >C28 in both bioreactors was still higher than that in soil inoculated with consortium (0.69 g/kg) at Day 60.

For alkane fraction <C14, relatively low removal rate was shown during the remediation compared to the long-chain alkane fraction. Removal rate of fractions <C14 only increased at the decrease of degradation efficiency on long-chain alkane fractions. This should be attributed to that the rapid degradation of the long-chain alkane fractions made a content increase of the short-chain alkane fractions. As remediation time expanded, the removal rate of fractions <C14 would increase to a stable level.

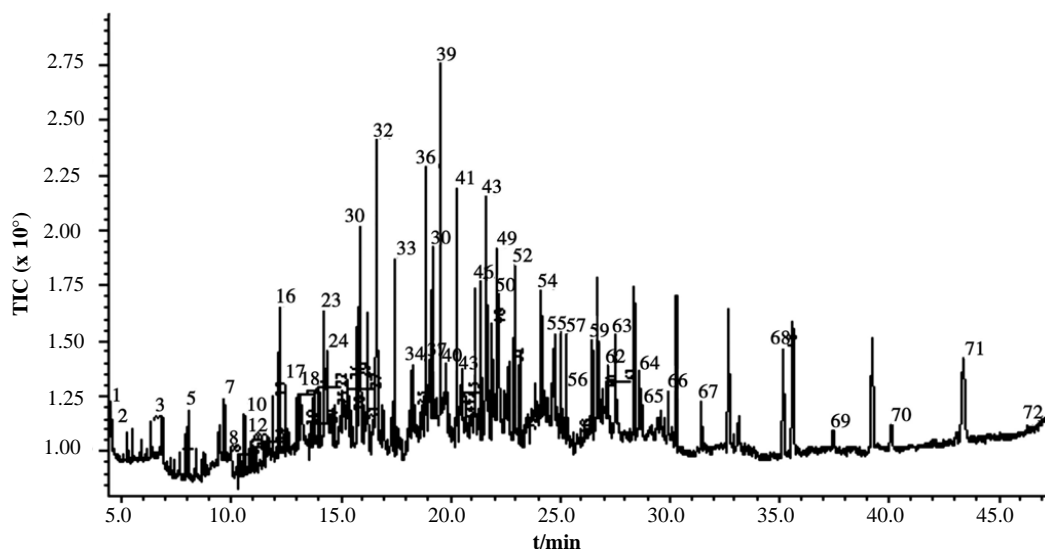


Figure 2. GC/MS analysis of crude petroleum in PCS before degradation.

Peaks labeled 8, 17, 22, 26, 36, 38, 47, 55, 57, 59, 63, 72 were n-alkane (C12, C14, C16, C17, C21, C22, C23, C24, C34 etc.), relative content before degradation was 24.82%; 5, 9, 10, 11, 12, 13, 61, 4, 15, 16, 18, 25, 30, 33, 34, 35, 37, 41, 43, 64, 66, 67, 68, 69, 70, 71, 1, 20, 53, 23, 56, 27, 28, 49, 52, 54, 62 were isoparaffin and cyclane, relative content before degradation was 45.46%; 2, 6, 7, 19, 29, 40, 44, 51, 60 were dretux (naphthalene, PAHs etc.), relative content before degradation was 11.52%; 3, 4, 65, 48, 46, 21, 50, 45, 42, 39, 32, 31, 24, 58 were NSO (nitrogen, sulfur and oxygen-containing compounds), relative content before degradation was 18.20%.

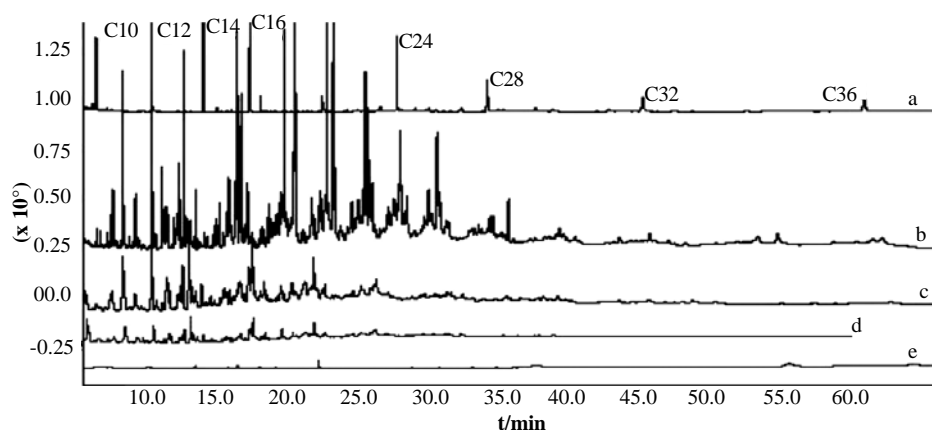


Figure 3. GC fingerprinting of alkane fraction of soil.

(a) The GC spectrum of n-alkane standards; (b) at 0 d of bioremediation in bioreactor C; (c) at 20 d of bioremediation in bioreactor C; (d) at 60 d of bioremediation in bioreactor C; (e) solvent blank.

Table 2. GC analysis results of crude oil degradation process in bioreactor (A, B, C, D).

R	alkane	0d		10d		20d		30d		60d	
		C	C	R%	C	R%	C	R%	C	R%	
A	<C14	3.48	3.25	6.5	3.82	-9.8	2.37	31.7	3.43	1.3	
	C14-C16	9.55	7.46	21.8	6.16	35.4	5.36	43.8	2.90	69.6	
	C16-C24	44.35	34.42	22.4	26.25	40.8	22.93	48.3	20.49	53.8	
	C24-C28	35.74	27.77	22.3	24.80	30.6	20.55	42.5	12.83	64.1	
	>C28	13.45	11.82	12.1	10.33	23.2	9.31	30.8	7.44	44.7	
B	<C14	3.48	3.83	-10.0	3.37	3.2	3.27	5.6	3.79	-8.9	
	C14-C16	9.55	8.05	15.7	7.54	21.0	6.92	27.5	6.65	30.4	
	C16-C24	44.35	36.01	18.8	33.97	23.4	31.13	29.8	27.70	37.1	
	C24-C28	35.74	28.56	20.1	27.09	24.2	24.34	31.9	21.26	40.5	
	>C28	13.45	10.18	24.3	8.54	36.5	7.77	42.2	5.81	56.8	
C	<C14	3.48	4.06	-16.5	3.71	-6.4	1.34	61.7	2.63	24.6	
	C14-C16	9.55	7.47	21.8	5.20	45.6	2.45	74.4	1.95	79.6	
	C16-C24	44.35	31.33	29.4	26.68	39.8	10.39	76.6	7.19	83.8	
	C24-C28	35.74	20.62	42.3	14.10	60.6	6.12	82.9	2.11	94.1	
	>C28	13.45	6.82	49.3	4.19	68.8	0.98	92.8	0.69	94.9	
D	<C14	3.48	3.58	-3.5	3.49	-0.4	3.32	4.4	3.39	2.4	
	C14-C16	9.55	8.11	15.1	7.52	21.3	7.17	24.9	6.67	30.2	
	C16-C24	44.35	40.00	9.8	38.10	14.1	36.28	18.2	34.81	21.5	
	C24-C28	35.74	31.45	12.0	30.24	15.4	29.34	17.9	28.66	19.8	
	>C28	13.45	12.93	3.9	12.43	7.6	11.96	12.1	11.69	13.1	

C=content of alkane fractions (g/kg); R% = Removal rate of alkane fractions (%) (m:m).

Discussion

GC/MS analyses and other study demonstrated that n-alkanes (C14–C28) were preferentially degraded compared to other fractions present in crude petroleum oil by microorganisms used in this study. Crude petroleum oil is a complex mixture of hydrophobic components like n-alkanes, aromatics, resins and asphaltenes, and microorganisms are known to attack and degrade a specific component as compared with other components of oil. It has been observed that the same compounds in different crude oil samples were degraded to different extent by the same organisms, implying that the bioavailability of a particular compound in a crude oil sample and not its chemical structure may be a sole determining factor for effective biodegradation of the compound¹³. Key factors such as presence of a specific and/or higher amount of inducible enzyme(s), substrate specificity of hydrocarbon degrading enzymes and presence of sphingolipids or other specific molecule(s) in the outer membrane structure of bacteria may be responsible for higher metabolism of TPH by *G. caledonium* NW03 strains compared to *B. subtilis* NW08 strain. In conclusion, the findings in this study showed that *G. caledonium* NW03 and *B. subtilis* NW08 strains from Northwest China could be useful in bioremediation of soils highly contaminated with crude petroleum oil hydrocarbons. The nature of these bacteria could add further advantage for their use in bioremediation of petroleum contaminated soils in China.

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