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Effects of Soil Types on the Biodegradation of Crude Oil by *Nocardia* sp. H17-1

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Abstract The degradation and mineralization of crude oil were investigated over 50-days in three soils, loamy sand, sand, and combusted loamy, which were artificially contaminated with crude oil (50 g kg^{-1}) and inoculated with *Nocardia* sp. H17-1. The degradation efficiency of total petroleum hydrocarbon (TPH) in sand was the highest at 76% among the three soils. The TPH degradation rate constants (k_{TPH}) in loamy sand, sand, and combusted loamy sand were 0.027 d^{-1}, 0.063 d^{-1}, and 0.016 d^{-1}, respectively. In contrast, the total amount of CO_2 evolved was the highest at 146.1 mmol in loamy sand. The CO_2 evolution rate constants (k_{CO2}) in loamy sand, sand, and combusted loamy sand were 0.057 d^{-1}, 0.066 d^{-1}, and 0.037 d^{-1}, respectively. Therefore, it seems that the degradation of crude oil in soils can be proportional to the soil pore space and that mineralization can be accelerated with the increase of organic substance.

Key words: Biodegradation, CO_2 evolution rate constant, *Nocardia* sp. H17-1, soil type, TPH degradation rate constant

Environmental contamination by petroleum and its derivatives is a problem, and appears to be increasing, with obvious ecological and economic implications. Biodegradation is one of the most significant processes for the reduction of hydrocarbons in soil. Biodegradation of oil in polluted soils is an efficient, economic, and versatile alternative to physicochemical treatment such as incineration, soil washing, and soil venting [2]. Many researchers have advocated the use of specially selected bacteria [12] or genetically engineered microorganisms to enhance the rate of biodegradation in soil containing crude oil [6]. In contrast, other reporters have shown that addition of microorganisms to environmental samples fail to enhance biodegradation [9].

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One possible reason for the failure of inoculation into contaminated sites is the lack of understanding of the environmental factors, which are necessary to enhance the survival and proliferation of any inoculants strain. The degradation of crude oil in soil is affected by several physicochemical and biological factors including the number and species of microorganisms present, the optimum environmental conditions for microbial degradation activity (e.g., oxygen and moisture content, temperature, pH, and nutrient levels), the chemical structure and type of oil, quantity and bioavailability of the contaminants, and the soil characteristics (e.g., pore size and distribution, and content of clay and organic materials) [4, 11]. In particular, fine particles and organic matter are mainly responsible for the adsorption of pollutants in soil [8].

We have previously demonstrated that a newly isolated *Nocardia* sp. H17-1 was able to degrade approximately 83% of Arabian light crude oil for 120 h under liquid culture [7]. The objective of the present study was to investigate the degradation of crude oil in three soils, loamy sand, sand, and combusted loamy sand, by *Nocardia* sp. H17-1. We examined the effects of soil texture and organic matter contents on the degradation of crude oil and the accompanying changes in the microbial activity with time in these soils.

Materials and Methods

Crude Oil and Soils

Arabian light crude oil was obtained from a petroleum company (Yu-Gong Petrol., Korea). The oil had an API (American Petroleum Institute) gravity of 33.4, and sulfur and nitrogen contents of 1.8% (w/w) and 1.7% (w/w), respectively. The main compositions of the oil were 13.4% asphaltene, 54.9% aliphatic hydrocarbons, 10.5% aromatic hydrocarbons, and 21.2% polar materials.

Two soil types used for the biodegradation of crude oil were collected from an agricultural area (Daejeon, Korea) with no history of hydrocarbon contamination. They were loamy sand and sand. Combusted loamy sand was prepared by heating the loamy sand at 700°C for 1 h to remove the organic matter. Soils were passed through a 2-mm sieve, and air-dried. An aliquot (1 kg) from each soil sample was artificially contaminated with 50 g of Arabian light crude oil.

Microorganism

An oil-degrading bacterium *Nocardia* sp. H17-1, which was isolated from oil-contaminated soil and previously reported by Lee *et al.* [7], was grown to the late exponential phase in Luria-Bertani liquid medium. Cells were centrifuged at 10,000 ×*g* for 20 min and the cell pellets collected were washed twice in 20 ml of sterile solution containing 0.2 g of NH_4NO_3 and 0.1 g of K_2HPO_4 per liter. The bacterial suspension was applied to the soil surface in droplets and then the soil was thoroughly mixed.

Biodegradation of Crude Oil

Degradation and mineralization of crude oil were studied using 15-g sterile soil samples (autoclaved at 121°C for 30 min). *Nocardia* sp. H17-1 was added to the three soils at levels of approximately 1×10^6 cells per g of soil. These soils were mixed with a sterilized spatula and vortex mixer, and the soil samples were aseptically transferred to a sterile glass tube. The water content of the soil samples was adjusted with sterile water to 50% of the maximum water holding capacity. These soils were kept in the dark at room temperature for 50 days.

To monitor the evolution of CO_2, a glass cup containing 5 ml of 1 N NaOH was anchored to the stopper of the soil test tube. The CO_2 trapped as carbonate in NaOH was analyzed with a total organic carbon analyzer (TOC 5000-A, Shimazdu, Japan). The growth of the microorganisms was estimated by periodic plate counts. All determinations were carried out in triplicate.

Analysis of Total Petroleum Hydrocarbons

At various intervals, soil samples were transferred to a 25-ml vial containing anhydrous sodium sulfate. Twenty ml of dichloromethane was added to the vial. Each vial was tightly capped, thoroughly mixed for 5 min with a vortex mixer and sonicated for 30 min in a water bath. The supernatant was passed through a 0.45 μm Teflon filter. One microliter of extract was analyzed using a gas chromatograph (Varian 3400CX, Varian, U.S.A.) equipped with a flame ionization detector. A DB-1 capillary column (30 m×0.32 mm with 0.25 μm film thickness, J&W Scientific, U.S.A.) was used and purified N_2 gas was used as a gas carrier at 50 ml/min. The operating temperature was started at 40°C for 5 min, increased to 170°C at a rate of 6°C/min, maintained at 170°C for 3 min, increased again to 300°C at a rate of 8°C/min, and maintained at 300°C for 10 min. The injector and detector were maintained at 250°C and 300°C, respectively.

Statistical Analysis

The biodegradation rate was fitted to the first-order degradation model:

$$\frac{C}{C_0}=e^{-k_{TPH}t}$$

where C is the substrate concentration at time t, C_0 is the initial substrate concentration, t is time (days), and k_{TPH} is the first-order rate coefficient (day^{-1}). The mineralization results were fitted to the first-order model:

$$P_t=P_{max}(1-e^{-kt})$$

where P_t is the amount of CO_2 produced at time t, P_{max} is the maximum amount of CO_2, and k is the first-order evolution rate coefficient (day^{-1}). Biodegradation and mineralization were fitted using Sigma Plot 5.0 (SPSS, U.S.A.) and statistical analysis was performed with a t-test.

Results and Disscussion

Characteristics of Soils

The properties of the investigated soils are presented in Table 1. Loamy sand, sand, and combusted loamy sand have 5.6%, 0.9%, and 0.8% of organic matter, and 12%, 3%, and 12% of clay, respectively. Combusted loamy sand has the same soil texture as loamy sand, except that organic matter has been removed.

Degradation of TPH in Crude Oil

The biodegradation of crude oil by *Nocardia* sp. H17-1 was investigated in three sterile soils over 50 days. After 20 days of incubation with *Nocardia* sp. H17-1, TPH in

Table 1. Characteristics of the three soil types used in the biodegradation experiments.

Soil type	Particle size (%)			Organic matter (%)	pH ($CaCl_2$)	Organic C (%)	Total N (%)
	Sand	Silt	Clay				
Loamy sand	72	14	12	5.58	4.5	3.38	0.09
Sand	91	6	3	0.89	6.8	0.30	0.02
Combusted loamy sand	72	14	12	0.78	6.7	0.10	0.01

crude oil had been reduced by 50±17% in loamy sand, 73±1% in sand, and 40±3% in combusted loamy sand (Fig. 1A). After 50 days, the fraction of the initial hydrocarbon remaining in the samples was 27±6%, 24±0%, and 42±7% in the loamy sand, sand, and combusted loamy sand, respectively. In all soil types, *Nocardia* sp. H17-1 degraded TPH most actively during the first 20 days of incubation. Subsequently, hydrocarbon degradation occurred only slowly. Results using uninoculated controls showed that for all soil types, approximately 30% of the added oil was eliminated abiotically during the 50-day incubation (Fig. 1B). This could be explained by an irreversible adsorption onto soil colloids, as well as evaporation of the most volatile crude oil fraction due to low CO_2 production and the absence of cell growth.

The most direct way to measure bioremediation efficiency is to monitor hydrocarbon disappearance rates [3, 14]. During the 50 days, the k_{TPH} in loamy sand, sand, and combusted loamy sand was 0.027 d^{-1}, 0.063 d^{-1}, and 0.016 d^{-1}, respectively. The k_{TPH} of sand which contained similar organic matter to the combusted loamy sand was approximately 4 times greater than in combusted loamy sand. These results might reflect differences in soil type, the degree of sorption of petroleum hydrocarbons with soil particles, differences in the physicochemical properties of soils, organic matter content of the soils, and migration of microorganisms in the soil. Because sand has high air permeability, water movement, and low binding capacity [13], TPH in sand was initially more rapidly degraded than that in loamy sand and combusted loamy sand.

Mineralization

The mineralization of TPH in the three soil types inoculated with *Nocardia* sp. H17-1 showed a marked increase without a lag period when the bacterium was added to oil-contaminated soil (Fig. 2A). The total amounts of CO_2 evolved during 50 days were 146.1±0.2, 100.0±0.3, and 99.4±0.0 mmol in loamy sand, sand, and combusted loamy

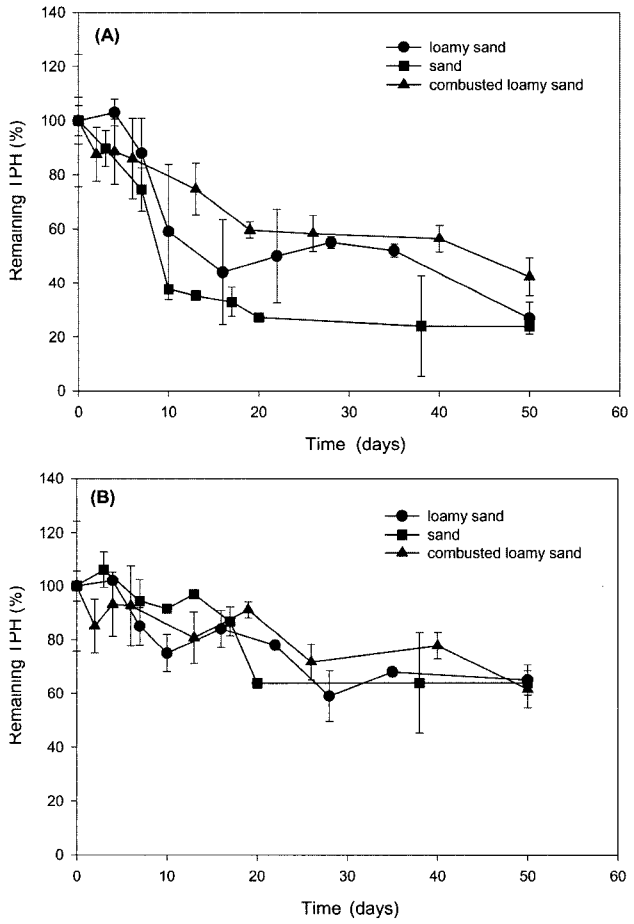

Fig. 1. Degradation of total petroleum hydrocarbons (TPH) by *Nocardia* sp. H17-1 (A) and uninoculated control (B) in three soil types.
Data points represent the average of triplicate cultures destructively sampled on each day. Bar is standard deviation.

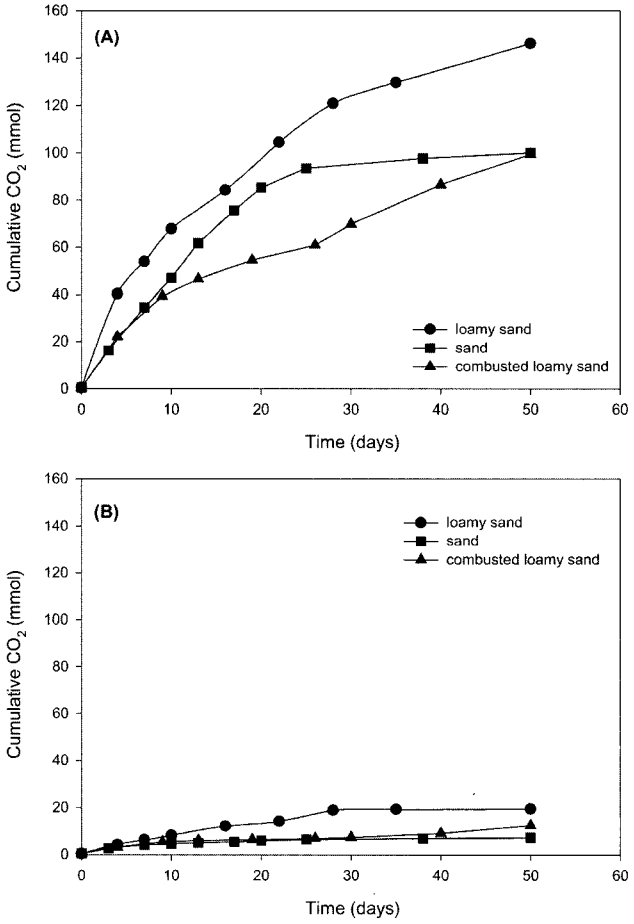

Fig. 2. CO_2 production by *Nocardia* sp. H17-1 (A) and uninoculated controls (B) during biodegradation of crude oil in three soil types.
Data points represent the average of triplicate cultures sampled on each day. Bar is standard deviation.

Table 2. First-order kinetic constants for the biodegradation of total petroleum hydrocarbon.

Treatment	k_{TPH} (day^{-1})	$t_{1/2}$	r^2	k_{CO2} (day^{-1})	P_{max} (mmol)	r^2
Loamy sand						
uninoculated	0.010	69.3	0.72	0.051	22.39	0.98
H17-1 inoculated	0.027	25.9	0.78	0.057	151.14	0.99
Sand						
uninoculated	0.007	99.0	0.84	0.115	6.93	0.96
H17-1 inoculated	0.063	11.1	0.84	0.066	108.28	0.98
Combusted loamy sand						
uninoculated	0.007	99.0	0.73	0.047	11.61	0.86
H17-1 inoculated	0.016	42.5	0.93	0.037	111.10	0.97

k_{TPH} is the first-order degradation rate constant.
k_{CO2} is the first-order evolution rate constant.

sand, respectively (Table 2). The estimated maximum amount of CO_2, P_{max}, was similar to the total amount of CO_2 evolved for 50 days (r^2=*0.99*, P<0.001).

The mineralization rates in loamy sand, sand, and combusted loamy sand were 0.19 mmolg^{-1}d^{-1}, 0.13 mmolg^{-1}d^{-1}, and 0.13 mmolg^{-1}d^{-1}, respectively. The mineralization rate in loamy sand was faster than that in sand and combusted loamy sand, but the first-order CO_2 evolution rate constant (k_{CO2}) was the greatest in sand among the three tested soils. The total amount of CO_2 was similar in sand, and combusted loamy sand, but k_{CO2} was 1.6 times greater in sand than that in combusted loamy sand. In the uninoculated controls, the amount of CO_2 evolved during the entire incubation period was 19.5, 7.3, and 12.4 mmol in loamy sand, sand, and combusted loamy sand, respectively (Fig. 2B).

Manilal and Alexander [8] found that the extent of pollutant sorption was directly related to the organic matter content of soils and that the degree of mineralization was lower in organic-rich soils. Weissenfels *et al.* [15] compared the degradation of tar oil in organic-poor soil with that in organic-rich soil; after 8 weeks of bioremediation, 62% of the hydrocarbons were degraded in organic-poor soil, whereas no significant degradation occurred in organic-rich soil. However, Mohn and Stewart [10] found that high total carbon concentrations were associated with high mineralization rate constants, and high sand contents were associated with longer time for half-maximal hydrocarbon mineralization. In this study, although the total amount of CO_2 produced was highest in loamy sand with high organic matter, the mineralization rate constant was highest in sand. These results could explain why TPH was readily bioavailable and rapidly converted to CO_2 by inoculum during the first 20 days, and the remaining TPH was no longer bioavailable to *Nocardia* sp. H17-1.

Growth

During the first 20 days of incubation in loamy sand, the amount of *Nocardia* sp. H17-1 in the soil quickly increased from an initial inoculation level of 1.0×10^6 CFU per g of soil until it reached a value of over 10^8 CFU per g of soil (Fig. 3). This value was maintained over the rest of the incubation period. In sand and combusted loamy sand, however, the number of *Nocardia* sp. H17-1 cells remained almost unchanged or slightly decreased over the 50-day period.

Sterile soils offer an opportunity for the growth of inoculums because carbon derived from the killed biomass is readily available to the inoculums [1, 5]. In our results, the initial increasing growth of *Nocardia* sp. H17-1 in loamy sand containing high organic matter may have been affected by carbon sources present in the soil other than those associated with the pollutant. However, *Nocardia* sp. H17-1 in sand and combusted loamy sand that contained low organic carbon was able to utilize crude oil as its sole carbon source.

Correlation Between Degradation of TPH and CO_2 Evolution

The total amount of evolved CO_2 showed a significant correlation with the extent of TPH degradation in the three

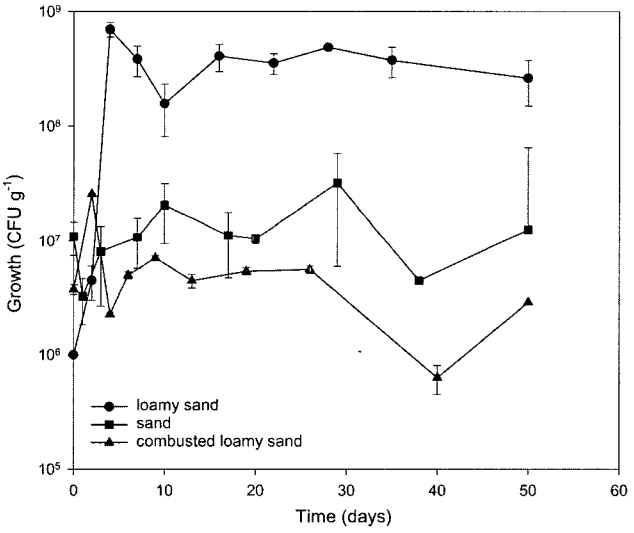

Fig. 3. Growth of *Nocardia* sp. H17-1 during biodegradation of crude oil in three soil types.
Data points represent the average of triplicate cultures sampled on each day. Bar is standard deviation.

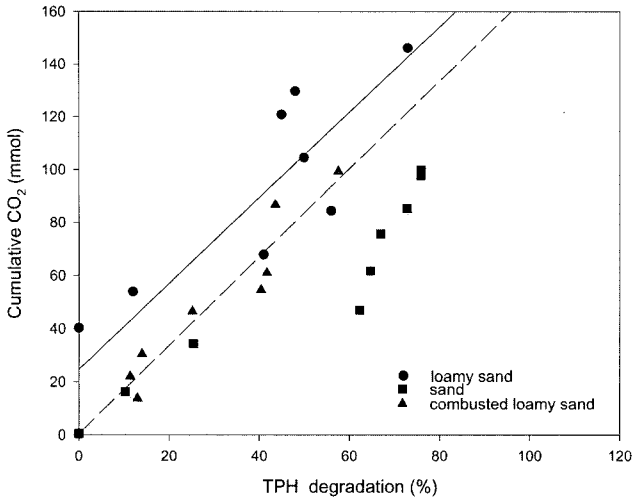

Fig. 4. Relationship between the extent of TPH degradation and total amount of CO_2 produced.

soils; y=1.616x+24.78, $P<0.01$, y=1.121x+1.065, $P<0.0001$, and y=1.662x+0.531, $P<0.0001$ for the loamy sand, sand, and combusted loamy sand, respectively (Fig. 4). Interestingly, CO_2 production per TPH degradation in loamy sand and combusted loamy sand showed similar slopes, whereas that in sand and combusted loamy sand showed the same y-intercept.

As shown in Fig. 4, the extent of crude oil degradation by *Nocardia* sp. H17-1 could be indirectly explained by the evolved CO_2 in the different soils which had the same soil texture but contained different organic matter contents. As in the cases above, this could apply to soils with similar organic matter contents but with different soil texture. The y-intercept reflects the mineralization of indigenous organic matter in soils, and the two soils with low indigenous organic matter have the same intercept close to zero, whereas the sample with higher amounts of organic matter shows CO_2 evolution even when no oil has been degraded, presumably because of mineralization of the indigenous organic matter.

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