한국토양환경학회지 Vol.4, No.3 95~104, 1999
Journal of KoSES Vol.4, No.3 95~104, 1999

原著

Acinetobacter sp. 에 의한 액체, 고체 알칸의 미생물 분해특성 비교연구

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Comparative study on microbial degradation characteristics of liquid and solid n-alkanes by *Acinetobacter* sp.

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ABSTRACT

Comparative biodegradation studies of liquid and solid alkanes and of two different solid alkanes were conducted by an isolated *Acinetobacter* sp., which degraded crude oil alkanes simultaneously, for the determination of degradation mechanism of hydrophobic crude oil constituents. Also a model oil experimental system composed of a solid alkane, heneicosane, as a substrate and a non-degradable non-aqueous phase liquid, pristane, as an oil matrix was established and studied. It was proposed that the *Acinetobacter* sp. utilized hydrophobic substrates directly on the surface of them with no difference in the degradation rates between the liquid and solid alkanes. On the basis of the results from the heneicosane/pristane system which imitates crude oil matrix containing solid constituents, the crude oil matrix was considered to reduce the bioavailability of contained substrates by reducing the specific surface area of substrates to contact with microorganisms.

Key words : *Acinetobacter* sp., biodegradation, crude oil, hydrophobic substrates

요 약 문

소수성 오염물질인 원유성분의 분해메카니즘을 규명하기 위하여 원유중 알칸성분의 동시분해능을 지닌 *Acinetobacter* sp. 를 단리하여 액체/고체알칸, 서로다른 고체알칸을 단일 또는 복합기질로 하여 비교분해연구를 행하였다. 또한 비분해성 유기액상성분인 pristane을 원유매체, 고체알칸인 heneicosane 을 유효기질로 하는 실험계를 확립하여 원유성분의 분해모델로서 연구를 행하였다. 본 연구에 이용된 *Acinetobacter* sp. 는 기질의 물리적 상태가 다른 액체/고체 성분의 분해특성에 차이를 보이지 않고 소수성기질의 표면에 직접접촉하여 분해할수 있음을 시사하였으며, 서로 다른 고체알칸의 단일 또는 복합 분해연구와 탄화수소에 대한 미생물의 부착성 결과가 이를 믿바침하였다. 또한 고체성분이 원유중에서는 유기상내에 용존상태로 존재함에 착안한 heneicosane/pristane 계의 연구결과 원유매체는 미생물로의 유효기질 전달을 억제함을 밝혔으며, 단일성분으로 존재할때에 비하여 분해가 일어나기위한 미생물과의 접촉면적을 감소시키는 유효기질의 비표면적 저하가 주요 원인임을 알 수 있었다.

주제어 : *Acinetobacter* sp., 생분해, 원유성분, 소수성 오염물질

1. INTRODUCTION

Crude oil is an extremely complex mixture of hydrocarbons. Crude oil spilled in environment accepts chemically and biologically induced changes in the composition, which is known collectively as weathering. Microbial degradation plays a major role in the weathering process[1]. Oil spilled in water tends to spread and form a slick and as a result of wind and wave action, oil-in-water or water-in-oil (mousse) emulsions may form[2]. Dispersion of hydrocarbons in water in the form of oil-in-water emulsions increases the surface area of the oil and thus its availability for microbial attack. In terrestrial oil spill, infiltration of oil into the soil prevents evaporative losses of volatile hydrocarbons, which are toxic to microorganisms. Particulate matter can reduce, by absorption, the effective toxicity of the components of crude oil, but absorption and adsorption of hydrocarbons to humic substances probably contribute to the formation of persistent residues[3].

Bioavailability of a chemical during bioremediation is determined by the rate of mass transfer relative to the intrinsic activity of microbial cells[4]. The rate at which microbial cells can convert chemicals during bioremediation depends on two factors : (i) the rate of uptake and metabolism (the intrinsic activity of the cell); (ii) the rate of transfer to the cell (mass transfer)[4]. Contact of cell with

substrate is obviously essential for substrate uptake by microorganisms. This condition is constantly maintained in the case of water-soluble substrate. However, in case of hydrocarbons which are essentially water-insoluble, the substrate must be transported through the aqueous phase in some way to achieve the cell substrate contact[5]. Two mechanisms have been proposed for the uptake of hydrocarbon by microbial cells. Hydrocarbon uptake takes place either through contact of the cells with large oil drops or from aqueous phase as solubilized or accommodated hydrocarbon, or through both[6,7]. For both mechanisms the degree of emulsification would be important although not necessarily rate limiting, as it will affect the interfacial area available for contact as well as the rate of substrate transfer into the aqueous phase thus increase the bioavailability of hydrocarbons. In sparingly soluble hydrocarbons, such as alkanes, substrate transfer into the aqueous phase is essential for the bioavailability of hydrocarbons[8~10].

In the present study, degradation characteristics of selected long-chain n-alkanes were examined for the investigation of the behavior of hydrophobic crude oil hydrocarbons in microbial degradation process. The examination of crude oil degradation characteristics by the isolate, *Acinetobacter* sp., in the previous study[11], was followed by the comparative studies, which were composed of liquid vs. solid alkane and two different solid alkanes. Finally, a solid alkane in non-degradable non-aqueous phase liquid (NAPL) was studied as a model system of crude oil.

2. MATERIALS AND METHOD

Preparation of microorganism

A Gram-type negative bacteria, *Acinetobacter* sp., was isolated from an activated sludge of a municipal wastewater treatment plant using Arabian light crude oil as a sole source of carbon and energy and was preserved on Luria-Bertani (LB) agar at 27℃. A colony was transferred to 20 mL of LB medium in a 100-mL Erlenmeyer flask and cultivated for 15 h at 27℃ with the constant shaking of 150 rpm. Cells were harvested by centrifugation at 5,000 g for 10 min and washed twice with mineral salts medium (MSM)[12]. Resuspended cells in MSM was used as an inoculum.

Degradation of selected *n*-alkanes by the isolate

To examine degradation characteristics of each alkane as sole carbon source by the *Acinetobacter* sp., degradations of heptadecane (C17) as a representative liquid alkane and of octadecane (C18) as a representative solid alkane at room temperature were conducted. Heneicosane (C21) and hexacosane (C26) were used for the comparative study of solid alkanes have different length of carbon chain and

different rate of degradation. Pristane was used as an oil matrix for the degradation of heneicosane dissolved in NAPL. The applied ratio of pristane/MSM was 1.0% (v/v).

For the preparation of sole carbon source medium, each 20 mL of MSM was supplemented with the desired amount of selected hydrocarbon in a 100-mL Erlenmeyer flask. After autoclaving at 121 ℃ for 15 min and abiotic shaking at 27℃ for 15 h, each flask was inoculated by the cells as 1 % of inoculum size. All the experiments were conducted at 27℃, 150 rpm shaker in triplicates.

Measurement

Cell growth was measured by the number of viable cells by counting colony forming unit (CFU) after the serial dilution in MSM and spreading in LB agar. Hydrocarbons were analyzed by capillary column (0.25 mm in diameter and 50 m in length) gas chromatography (GC-17A, Shimadzu, Japan) equipped with flame ionization detector (FID). After collecting 0.1 mL of culture broth for the measurement of cell growth, the whole culture volume was extracted with the same amount of chloroform for the analysis of hydrocarbons. Chloroform portion was carefully drawn by Pasteur pipette and injected by gas tight syringe into GC directly without concentration. Initial column temperature was kept at 150℃ for 5 minutes and increased to 300℃ by 10℃/min and then maintained for 20 minutes. Helium was supplied as carrier gas at the rate of 1.3 mL/min under the split ratio of 1/10. The temperatures of the injector and FID were 320℃ and 340℃, respectively.

3. RESULTS AND DISCUSSION

Comparative degradation of liquid alkane and solid alkane

The growth on and the degradation of heptadecane (C17, mp: 22~24℃) and

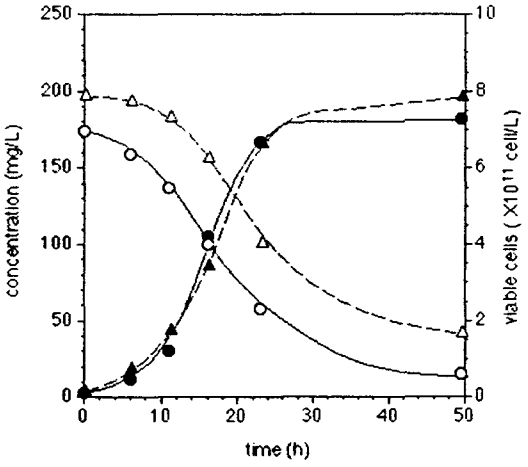

Fig. 1. Variations of viable cells and concentrations with time during the cultivation of *Acinetobacter* sp. on a liquid alkane, heptadecane (C17), and on a solid alkane, octadecane (C18), as sole carbon and energy source. Empty circle; C17, empty triangle; C18, solid circle; viable cells on C17, solid triangle; viable cells on C18, in flask, 150 rpm shaker, 27 C, in triplicates.

octadecane (C18, mp; 28~30℃) as the sole source of carbon and energy are shown in Fig. 1. The short exponential growth observed in the growth of *Acinetobacter* sp. on heptadecane was changed to linear growth, the transport limited growth. There was a little difference of growth between on heptadecane and on octadecane. The degradation rate of heptadecane observed in the crude oil degradation was slightly higher than that of octadecane[11]. Also a little difference in growth and degradation of those components was observed in Fig. 1. Though the applied concentrations of both heptadecane and octadecane were considerably larger than in the previous study of crude oil, their degradation characteristics were considered to reflect the effect of chain length on degradation rates, the shorter the higher. This degradation tendency was not changed significantly from the crude oil degradation to the single-substrate degradation, though the physical state of octadecane was changed from liquid, dissolution in crude oil phase, to solid, particulates in aqueous phase.

The physical state of crude oil hydrocarbons has a marked effect on their biodegradation. At very low concentrations hydrocarbons are soluble in water, but most oil spill incidents release petroleum hydrocarbons in concentrations far in excess of the solubility limits[13, 14]. Liquid hydrocarbons can be taken up and incorporated into the cell membrane, whereas the mechanism of utilization of solid substrates is not fully understood[15]. It has been suggested that microbial degradation of the insoluble phase of crystalline hydrocarbons, such as octadecane in this study, is difficult because of the large amount of energy needed to disperse the solid[16], and highly crystalline cellulose is degraded more slowly than amorphous forms of the polymer[17]. However, growth of microorganism on solid alkane was observed whether the bacterium was free or attached to the insoluble form of the solid hydrocarbon[16] and as in the case in the present study. This fact suggested that the isolated *Acinetobacter* sp. degraded the solid alkane directly on its surface rather than dissolved substrates, when considering the degradation difficulty of the insoluble phase of crystalline hydrocarbons due to large amount of energy required to disperse the solid might much lessen the degradation rate of octadecane than that observed in this study. On the basis of the results the isolated *Acinetobacter* sp. was expected to have high attachment ability to the surface of octadecane, which requires the high hydrophobicity of the cell surface. From this, the microbial degradation study of solid alkanes with different molecular weights was required to investigate the degradation characteristics of solid alkanes and the attachment ability of the isolate on the surface of solid alkane.

Comparative degradation of two different solid alkanes

To investigate whether the difference of the observed degradation rates between two different solid alkanes in the crude oil degradation[11] was depend on the property of a component itself or on the condition of a component, as sole source or one of the mixed source, comparative degradation study of solid alkanes was conducted using heneicosane (C21) and hexacosane (C26).

Heneicosane was degraded more rapidly than hexacosane in the degradation of each alkane as sole carbon and energy source (Fig. 2). This tendency was not changed when heneicosane and hexacosane were supplemented as mixed sources. Their degradation were not diauxic but simultaneous in the degradation of mixed sources and the consumption ratios of C21/C26 for 24 h were nearly the same in the degradation of sole sources (Fig. 2) and of mixed sources (Fig. 3) as 1.6 and 1.5, respectively. The number of viable cells grown on each alkane was proportional to the amount of substrate consumed. Solid

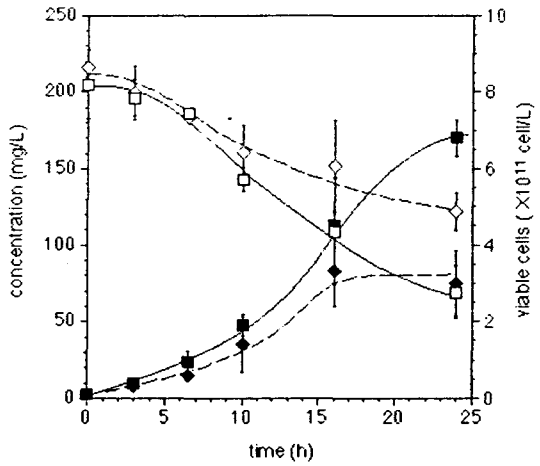

Fig. 2. Variations of viable cells and concentrations with time during the cultivation of *Acinetobacter* sp. on the sole substrate of solid alkane, heneicosane (C21) or hexacosane (C26). Empty square; C21, empty diamond; C26, solid square; viable cells on C21, solid diamond; viable cells on C26, in flask, 150 rpm shaker, 27 C, in triplicates.

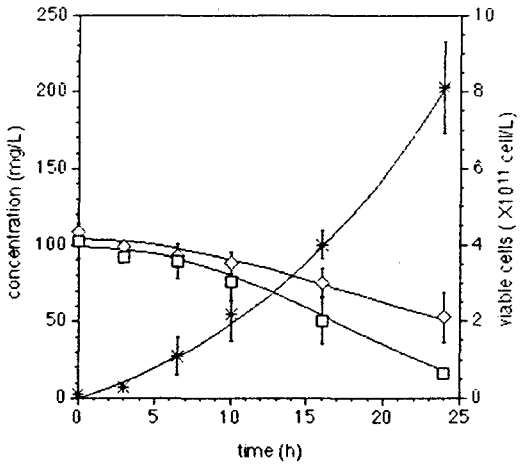

Fig. 3. Variations of viable cells and concentrations with time during the cultivation of *Acinetobacter* sp. on the mixed substrates of two solid alkanes, heneicosane (C21) and hexacosane (C26). Square; C21 in mixed substrates, diamond; C26 in mixed substrates, asterisk; viable cells on mixed substrates, in flask, 150 rpm shaker, 27 C, in triplicates.

particles of heneicosane and hexacosane were observed during cultivation in both culture types of sole source and mixed sources degradation. This observation also support the suggestion that the isolate could degrade the solid alkanes on their surfaces by direct contact with the solid particles, as expected from the comparative study of liquid vs. solid alkanes. In such circumstances, microbial cells attach to the surface of oil drops and uptake the hydrocarbons presumably through diffusion or active transport. The available surface area of oil drops for cell attachment and the hydrophobicity of cell surface would be the critical factors since the only attached microorganisms are responsible for the degradation of hydrocarbons.

Table 1 shows the result of microbial adhesion to hydrocarbon (MATH) assay grown on different carbon sources. The MATH assay[18] has been frequently used as a test of cell surface hydrophobicity and well reviewed[19]. When the *Acinetobacter* sp. grew on the hydrocarbons, the cell surface hydrophobicity by MATH assay was increased compared with the result of LB-grown cells. The increased cell surface hydrophobicity of the *Acinetobacter* sp. grown on hydrocarbons implied the increased attaching ability to the hydrophobic substrate and thus the increased transport of the substrates to the cell. And the growth ability on insoluble alkanes by direct contact is partially supported by the fact that the increased cell surface hydrophobicity is observed.

Degradation of a solid alkane dissolved in a non-aqueous phase liquid

The actual crude oil contains lots of solid alkanes as dissolved form in oil matrix. To imitate crude oil matrix containing solid constituents, MSM was supplemented by a non-degradable NAPL, pristane, delivering desired amount of heneicosane. Pristane was a nondegradable hydrocarbon to the *Acinetobacter* sp. and was able to dissolve the desired amount of heneicosane. Used pristane had ca. 2% of impurities, and one of the major impurities was identified as

Table 1. Cell Hydrophobicity of *Acinetobacter* sp. by MATH* Assay after 50 h of Cultivation on Different Media

Growth media	LB**	C21	C26	C21 + C26
Hydrophobicity (%)	35	80	50	70

* Microbial adhesion to hydrocarbon; Each 4 mL of cell suspension adjusted to OD400 of 1.0 was added by 1 mL of hexadecane and mixed vigorously for 60 sec. After 30 min of settling, the OD400 of aqueous phase was measured. The reduced percentage of OD400 was referred to cell surface hydrophobicity.

** after 15 h of precultivation

heneicosane. One of it, identified as heneicosane, was degraded, while another two components were persistent to the end of cultivation[20]. The degradation of dissolved heneicosane in pristane was reduced when compared with that of solid form in the above section. The degradation rate was not proportional to the substrate concentration, which could be observed in the range of 25~100 h where the number of viable cells were constant (Fig. 4). The globules of pristane were observed during cultivation. The oil phase of pristane was rather a superficial drifting phase than minute droplets, whereas heneicosane was

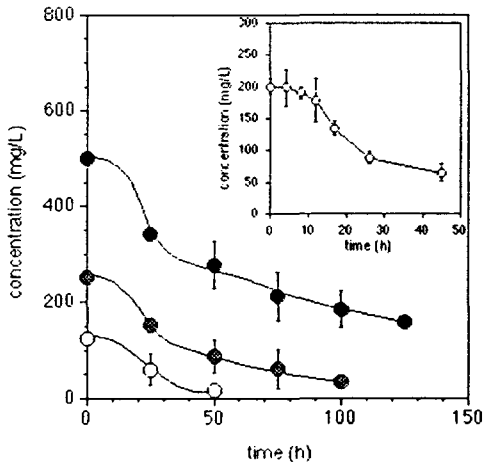

Fig. 4. Microbial degradation of heneicosane (C21) dissolved in 1.0% (v/v) of pristane by *Acinetobacter* sp. as a model crude oil system, in flask, 150 rpm shaker, 27 C, in triplicates. The encapsulated plot shows the details of initial degradation tendency in short-term intervals.

tiny particles without pristane. This might lead up to the reduced surface area of oil phase thus the mass transfer limitation. The portion of oil phase, pristane, which contained desired amount of heneicosane in the system was set as 1% (v/v). This value was selected from the consideration of available maximum value, where the oil phase could be dispersed in stable fully enough to confirm there exist no coalescence of the oil drops[21]. Thus this volume fraction of 1% of oil was considered to be rational to maintain hydrodynamic constant interfacial area between oil and aqueous phase. But the agitation in the flask with 150 rpm shaker was found too weak to obtain fully dispersed droplets of pristane. However, this might be the case in the actual oil spill where large masses or plates of mousse establish unfavorably low surface-to-volume ratios, inhibiting biodegradation[22] and tar-balls, large aggregates of weathered and undegraded oil, also restrict access by microorganisms because of their limited surface area[23]. On the basis of the results, the crude oil matrix was considered to reduce the bioavailability of contained alkanes, whereas they could be utilized directly by the isolated *Acinetobacter* sp. on their surfaces.

4. CONCLUSIONS

An isolated *Acinetobacter* sp. degraded crude oil alkanes simultaneously with no difference in the degradation rates between

liquid and solid alkanes. A model oil system composed of heneicosane and pristane suggested that the oil matrix could reduce the biodegradability of its constituents by reducing the available contact area between microorganisms and substrates.

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