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原著

활성슬러지에서 단리한 *Acinetobacter* sp. 에 의한 원유탄화수소분해

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Biodegradation of crude oil hydrocarbons by *Acinetobacter* sp. isolated from activated sludge

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ABSTRACT

A Gram-type negative bacteria that can utilize crude oil as the sole source of carbon and energy was isolated from an activated sludge of a local sewage treatment plant and identified tentatively as belonging to the genus *Acinetobacter*. The isolate could degrade *n*-alkanes and unidentified hydrocarbons in crude oil and utilize *n*-alkanes, hydrophobic substrates, as sole carbon and energy sources. *n*-Alkanes from tridecane (C13) to triacontane (C30) in crude oil were degraded simultaneously with no difference in degradation characteristics between the two close odd and even numbered alkanes in carbon numbers. The linear growth of the isolate and the degradation characteristics of *n*-alkanes suggested that the transport of substrates from the oil phase to the site where the substrates undergo the initial oxidation in microorganism might be the rate limiting in the biodegradation process of crude oil constituents. The remainder fraction of substrates after cultivation was considered to reflect the hydrocarbon inclusions in the cell mass, characteristics in *Acinetobacter* species, and to control the transport of substrates

from crude oil phase. On the basis of the results, the isolate was considered to play an important role in the degradation study of hydrophobic environmental pollutants.

Key words : *Acinetobacter*, crude oil hydrocarbons, hydrophobic substrates, biodegradation

요 약 문

원유를 탄소원으로 이용할수 있는 그람 음성 박테리아가 도시하수 처리장의 활성슬러지로부터 단리되어 *Acinetobacter*속으로 동정되었다. 이 균은 원유중의 알칸과 미확인된 다종의 탄화수소를 분해할수 있었으며 소수성 기질인 알칸을 단일 탄소원으로 이용하여 증식할수 있었다. 원유중 탄소수 13-30의 알칸은 동시에 분해가 진행되었으며 탄소수의 짝수, 홀수에 따른 분해특성의 차이는 보이지 않았다. 균의 선형적인 증식과 알칸 성분의 분해특성으로부터, 원유상에서 기질의 초기 산화가 진행되는 미생물부위까지 물질전달이 분해과정의 율속이 됨을 알수 있었다. 분해 진행후 반응계내 알칸의 잔류 현상이 관찰되었는데 이는 *Acinetobacter*속의 특성으로 알려져있는 세포내 탄화수소 함유체의 영향으로서 원유로부터의 물질전달을 제어하는 요인이 될수 있음을 시사하였다. 따라서 본 균은 환경중 잔류하는 소수성 오염물질의 분해메카니즘 연구에 중요한 역할을 한다고 사료된다.

중심어 : *Acinetobacter*, 원유탄화수소, 소수성 기질, 생분해

1. INTRODUCTION

Crude oil has been and is still used to cover the tremendous energy demand of industry and to produce synthetic organic chemicals. While accidental releases due to leakage, spills, improper disposal and accidents during transport may contribute to only a small percentage of the oil released into environment[1], large accidental oil spills receive much attention and evoke considerable public concern[2]. Crude oil is an extremely complex mixture of hydrocarbons. These compounds are mainly hydrophobic materials those can hardly be degraded or decomposed owing to their poor availability for microorganisms[3]. Factors which influence rates of microbial growth and enzymatic activities affect the rates of crude oil hydrocarbon biodegradation. The persistence of crude oil pollutants depends on the quantity and quality of the hydrocarbon mixture and on the properties of the affected ecosystem. In one environment crude oil hydrocarbons can persist indefinitely, whereas under another set of conditions the same hydrocarbons can be completely biodegraded within a few hours or days.

Techniques for dealing with oil pollution

include physical and chemical methods, which are fairly well established, and one strategy to remediate such polluted environments is to use the degradative capacity of microorganisms[4]. Biological methods include a number of different technologies; these include the use of absorbent for oil, biosurfactants to clean oily surfaces, biological polymers to coat surfaces, and the addition of materials to promote microbiological degradation of oil[5]. The last procedure is bioremediation, which has received the most attention, notably after the Exxon Valdez incident[6].

Bioremediation is the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation process and biodegradation is known to be the principal natural process for the removal of the nonvolatile fraction of oil from the environment[7]. *In situ* bioremediation, which involves the use of indigenous microorganisms to degrade the target compounds, is receiving increasing attention due to its potential cost-effectiveness[8]. For this, the screening of potential crude oil degrading organisms is one of the key steps. A number of techniques have been evolved for screening of hydrocarbon degrading bacteria[9].

The present study aimed at isolation and characterization of a ubiquitous microorganism utilizing crude oil hydrocarbons as the sole carbon source without loss of generality for the possible application of an isolate to in situ bioremediation. From this point of view, a microorganism that can grow on the whole crude oil, with or without emulsification, was isolated from the environment. To investigate the behavior of crude oil hydrocarbons in microbial degradation process, degradation characteristics of crude oil hydrocarbons were examined using the isolate.

2. MATERIALS AND METHOD

Cultural media

Mineral salts medium (MSM) was used as the basal medium for all the studies of specified carbon sources[10]. For the screening of oil degraders, emulsified crude oil medium was used. It contained 0.5 % (v/v) of Arabian light crude oil and 0.5 % (v/v) of nonionic surfactant, Triton X-100, as an emulsifier in MSM. Crude oil agar medium was also prepared as a solid medium which contained 1 % (v/v) of crude oil, 0.24 mM of Triton X-100, and 1.5 % (w/v) of agar in MSM. Luria-Bertani (LB) medium and LB agar medium were used for the preparation of inoculum and for the counting of colony forming unit (CFU), respectively.

Isolation and identification of crude oil hydrocarbon degrader

Coarse particles were removed from the activated sludge from a municipal wastewater treatment plant by gentle sonication and centrifugation. A 100 μL of

broth free of coarse particles was inoculated in 100 mL of emulsified crude oil medium in 500-mL Erlenmeyer flask. After 2 weeks of cultivation at 27℃ with constant rotary shaking at 80 rpm, culture of 100 μL was transferred to crude oil agar medium and incubated for 3 days.

The microorganisms formed colonies on crude oil agar medium were candidate for the degradation of the crude oil hydrocarbons. Crude oil medium without Triton X-100 was prepared for working volume of 20 mL in 100-mL Erlenmeyer flask. Each flask was inoculated with 24-hr precultivated microorganisms in LB medium after washing twice with MSM. Cultivation was conducted for 4 days at 27℃, 80 rpm shaker.

Identification of the isolate was conducted using API 20 NE identification kit (bioMerieux) and Bergey's Manual of Determinative Bacteriology[11]. Morphological observations and physiological examinations were conducted.

Hydrocarbon metabolic capability of the isolate

Each 5 mL of MSM in a test tube was supplemented by the selected hydrocarbons (aromatic hydrocarbons, a sulfur containing aromatic hydrocarbon, pure *n*-alkanes, and a branched alkane) with the concentration of 1000 mg/L in case of solid materials, 0.1% (v/v) in case of liquid materials at room temperature and inoculated by the isolate precultivated in LB medium and washed twice with MSM. The number of viable cells and the concentration of hydrocarbons after 2 and 4 days of culture were monitored.

Degradation of crude oil hydrocarbons by the isolate

Crude oil degradation as sole carbon source was conducted. To obtain homogenized substrate, 1.5 mL of crude oil were dissolved in 13.5 mL of ethyl acetate and filtered by 0.22 μm filter unit (PVDF: polyvinylidene difluoride). The aliquots of 200 μL were put into 100-mL Erlenmeyer flasks. After evaporating ethyl acetate, 20 mL of MSM were added to each flask to get 0.1% (v/v) of crude oil as initial concentration, and autoclaved for 15 min at 121℃. The isolate cultivated on 20 mL of LB medium for 15 h were harvested by centrifugation at 5,000×g for 6 min, and washed twice with the same amount of MSM. The bacteria resuspended in 10 mL of MSM were inoculated into flasks as 1 % of inoculum size and cultivated at 27℃ with constant rotary shaking at 150 rpm. Cultivation was conducted in triplicates including abiotic controls.

Measurement

Cell growth was measured by the number of viable cells by counting CFU. 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. Crude oil hydrocarbons were analyzed by capillary column (0.25 mm in diameter and 50 m in length, ULBON HR-1, Shinwa chemical Ind. Ltd., Japan) gas chromatography (GC-17A, Shimadzu, Japan) equipped with flame ionization detector (FID). The initial column temperature was kept at 80℃ for 5 minutes and increased to 200℃ by 10℃ /min, to 270 ℃ by 5℃/min, and then maintained for 7 minutes. The temperatures of injector and FID were 320℃ and 340℃, respectively. Helium was used as carrier gas at a flow rate of 1.5 mL/min. In case of crude oil measurement, chloroform portion of 1.0 mL volume was drawn to a vial, and then dried out using heat block at 60℃. After drying, the desired small amount of heptane was added into the vial to obtain proper concentrating ratio and injected into GC for measurement. In the studies of degradation capability of the isolate, selected hydrocarbons in chloroform portion was drawn and injected into GC, directly.

3. RESULTS AND DISCUSSION

Isolation of a crude oil hydrocarbon degrader

Eight different colonies were obtained and transferred to LB agar plates for temporal preservation at 4℃. Crude oil degradation as sole carbon and energy source was conducted by above 8 microorganisms for further selection. Fig. 1 showed the remained *n*-alkanes measured quantitatively after 2 weeks of cultivation. No detectable amount of *n*-alkanes was observed from the cultivation of a strain ACL04. In several cultures, accumulations of *n*-alkanes were observed in rather lower carbon numbered alkanes, 〈 C18. These accumulations were considered to be the intermediates or products of crude oil hydrocarbons other than *n*-alkanes. The strain ACL04 was picked up for the subsequent study of the degradation of crude oil *n*-alkanes.

Identification of the isolate

ACL04 was a Gram type-negative coccoid bacillus. It produced smooth pale yellowish colony on LB agar. It was negative in nitrate reduction, indole production, arginine dihydrolase, urease, protease, (*β*-galactosidase, (*β*-galactosidase, and citochrom oxidase. It could metabolize *n*-caprinic acid and malic acid, but could not do glucose, *L*-arabinose, *D*-mannose, *D*-mannitol, *N*-acetyl-*D*-glucosamine, maltose, potassium gluconate, adipionic acid, sodium citrate, and phenyl acetate (Table 1). On the basis of these characteristics the isolate was identified tentatively as belongs to the genus *Acinetobacter*. *Acinetobacter* is ubiquitous in the environment[12]. It has been estimated that acinetobacters may constitute 0.001% of the total heterotrophic aerobic population of soil and water[13]. The ability of crude oil

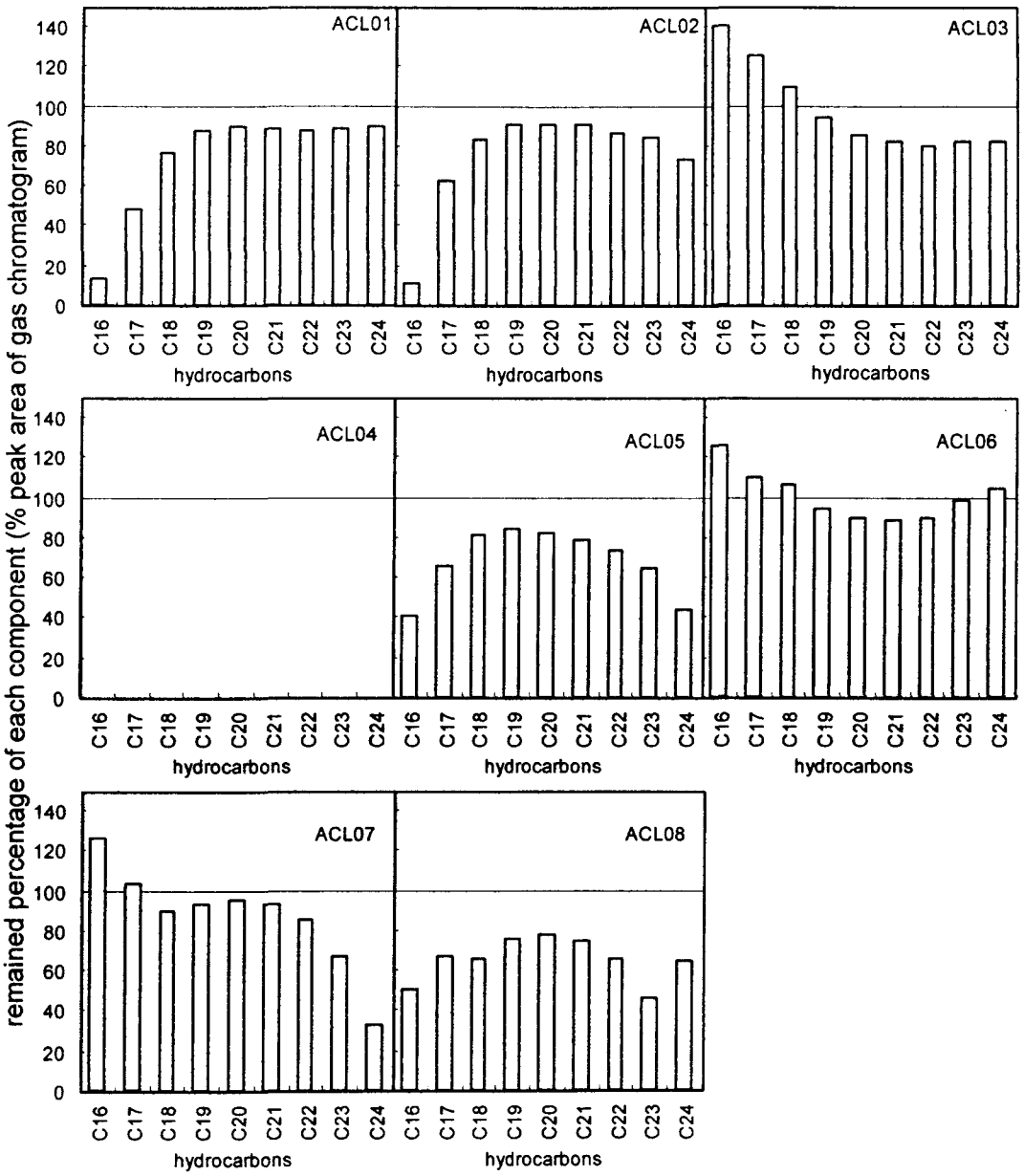

Fig. 1. Degradation of crude oil *n*-alkanes by the pure culture of each strain isolated from an activated sludge; after 2 weeks of cultivation.

component utilization was reported as a common feature of *Acinetobacter* species[14]. It would be rationale that the ACL04 was isolated from the activated sludge of a municipal wastewater treatment plant and had the ability of crude oil hydrocarbon

Table 1. Morphological and physiological characteristics of strain ACL04

Morphological aspects		Metabolism		Reaction	
Cell shape	spherical	Adipionic acid	−	Arginine dehydrolase	−
Cell size	<1 (m	D-Mannitol	−	Nitrate reduction	−
Colony	smooth	D-Mannose	−	Indole	−
Colony color	pale yellow	Glucose	−	Urease	−
Gram stain	negative	L-Arabinose	−	β-Glucosidase	−
Growth(30℃)	+	Malic acid	+	β-Galactosidase	−
Growth(37℃)	+	Maltose	−	Protease	−
		N-Acetyl-D-glucosamine	−	Cytochrome oxidase	−
		n-Caprinic acid	+		
		Phenyl acetate	−		
		Potassium gluconate	−		
		Sodium citrate	−		

degradation.

Hydrocarbon metabolic capability of *Acinetobacter* sp.

The degradabilities of benzene, toluene, *p*-xylene, anthracene, phenanthrene, and dibenzothiophene of the isolate were examined for the test of aromatic hydrocarbons and a sulfur containing aromatic hydrocarbon. Degradation of each pure *n*-alkane (hexadecane, C16; heptadecane, C17; octadecane, C18; nonadecane, C19; eicosane, C20; heneicosane, C21; hexacosane, C26; octacosane, C28) and a branched alkane (pristane) by the pure culture of the isolate in MSM were also conducted. The isolated *Acinetobacter* sp. could grow on each *n*-alkane as the sole carbon and energy source, while it failed to grow on pristane and the selected aromatic hydrocarbons. The isolate was considered to be lacking in ability to utilize mono- or poly-aromatic hydrocarbons and methyl-branched aliphatic hydrocarbons. However, when considering the growth of the isolate on highly hydrophobic, water insoluble, alkanes, the feasibility of the isolate on the pollution control of organic chemicals was suggested. It is also considered that the isolate could be used in the study of substrate uptake mechanism of hydrophobic materials.

Most *Acinetobacter* strains tested for the ability to utilize *n*-alkanes have been reported to assimilate only compounds with more than 10 to 12 carbon atoms[15]. Though there has been reported[16] the oxidation of the terminal methyl group from the formation of pristanol and pristanediol from pristane by *Acinetobacter*, the isolate could not utilize pristane thus do terminal methyl

group. Growth on or respiration with diterminally branched alkanes have not been reported while alkyl chains are rapidly oxidized beyond the carboxylic acid stage to intermediary metabolites and carbon dioxide[14].

Degradation of crude oil hydrocarbons by *Acinetobacter* sp.

Gas chromatograms of crude oil hydrocarbons with the identified *n*-alkanes after cultivation are shown in Fig. 2. The isolate grew on crude oil of the initial concentration of 0.1% (v/v) and degraded *n*-alkanes simultaneously (Figs. 3 and 4). No significant pH change was observed. No abiotic loss occurred during cultivation by conducting inoculation after 15 h of abiotic shaking, which was determined by pre-experiments (data were not shown) for excluding any abiotic loss due to physical and chemical actions presented in other reports[17]. Therefore, any decrease in the result was attributed strictly to biodegradation.

After 25 h of culture, the concentrations of *n*-alkanes from C13 to C30 decreased nearly to the values below detection limits. Fig. 2 revealed the possibility of the existence of another components, excluded in the quantitative analysis in this measurement, that could be degraded and attributed to microbial growth. Unidentified components served to microbial growth was considered to include long chain alkyl-

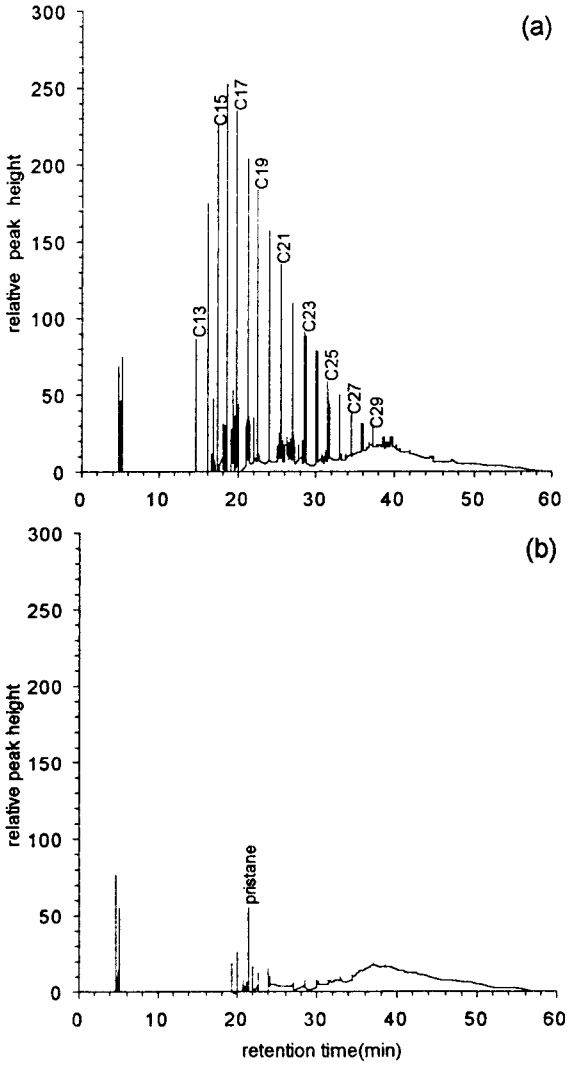

Fig. 2. Gas chromatogram of crude oil components analyzed by GC-FID after cultivation; (a) abiotic control, (b) pure culture of *Acinetobacter* sp. Mineral salts medium including 0.1% (v/v) of crude oil was autoclaved and incubated aseptically for 15 hours prior to inoculation for eliminating any abiotic loss during cultivation.

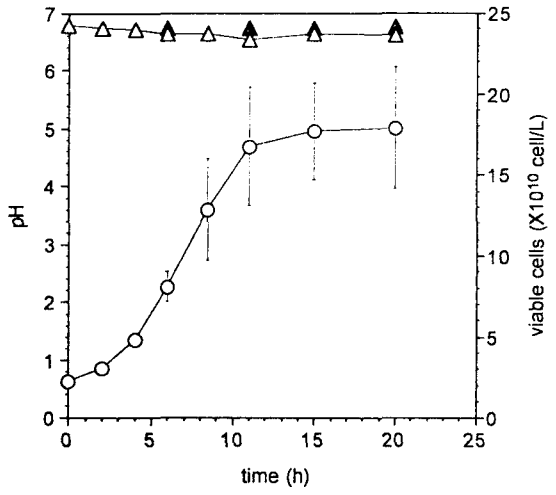

Fig. 3. Variations of pH and viable cells with time during cultivation. Empty circles; viable cells, empty triangles; pH in cultivation, solid triangles; pH in abiotic control. MSM including 0.1% (v/v) of crude oil in flask was inoculated by *Acinetobacter* sp. and cultivated on 150 rpm shaker, 27℃, in triplicates. Cells grown on LB medium for 15 hours were harvested by centrifugation at 5,000 × g for 6 min and washed twice with MSM prior to inoculation. The inoculum size was 1% (v/v).

branched hydrocarbons. Remaining large three peaks in Fig. 2 after 25 h of cultivation around 20 min of retention time were not correspond to the *n*-alkanes. These three peaks were considered to be methyl-branched hydrocarbons and one of them was considered to be pristane.

Rather a linear growth than an exponential growth was observed (Fig. 3). This phenomenon is to be discussed from the mass transfer point of view. When consider the crude oil droplet as a matrix which contained small portion of available components we can suppose the importance of transport of substrates to the surface of microorganism and of penetration of them to the site where the substrates undergo the initial oxidation. This transport rate was considered to control the overall rate of growth and substrate uptake, by lowering the saturation coefficient in Monod type microbial reaction kinetics. The transport limitation of substrates in bulk oil matrix to the surface of oil droplets, where the microorganisms can contact with substrates and thus uptake the exogenous substrates, was also supposed from the observed oil slick during cultivation which might have high viscosity to inhibit transportation. The viscosity of oil reduces the degree of spreading of the oil in soil and aquatic matrices[18], thus diminishes the availability of the oil constituents to microbial utilization. The ability of microorganism to utilize the crude oil hydrocarbons was reported to depend not only on the composition of the unsaturated fraction but also on that of the asphaltic fraction[19]. This might explain the possibility of retarded transportation of utilizable compounds due to the existence of refractory constituents to microorganisms in the oil droplets.

With increasing the number of carbons, their degradation rates decreased in

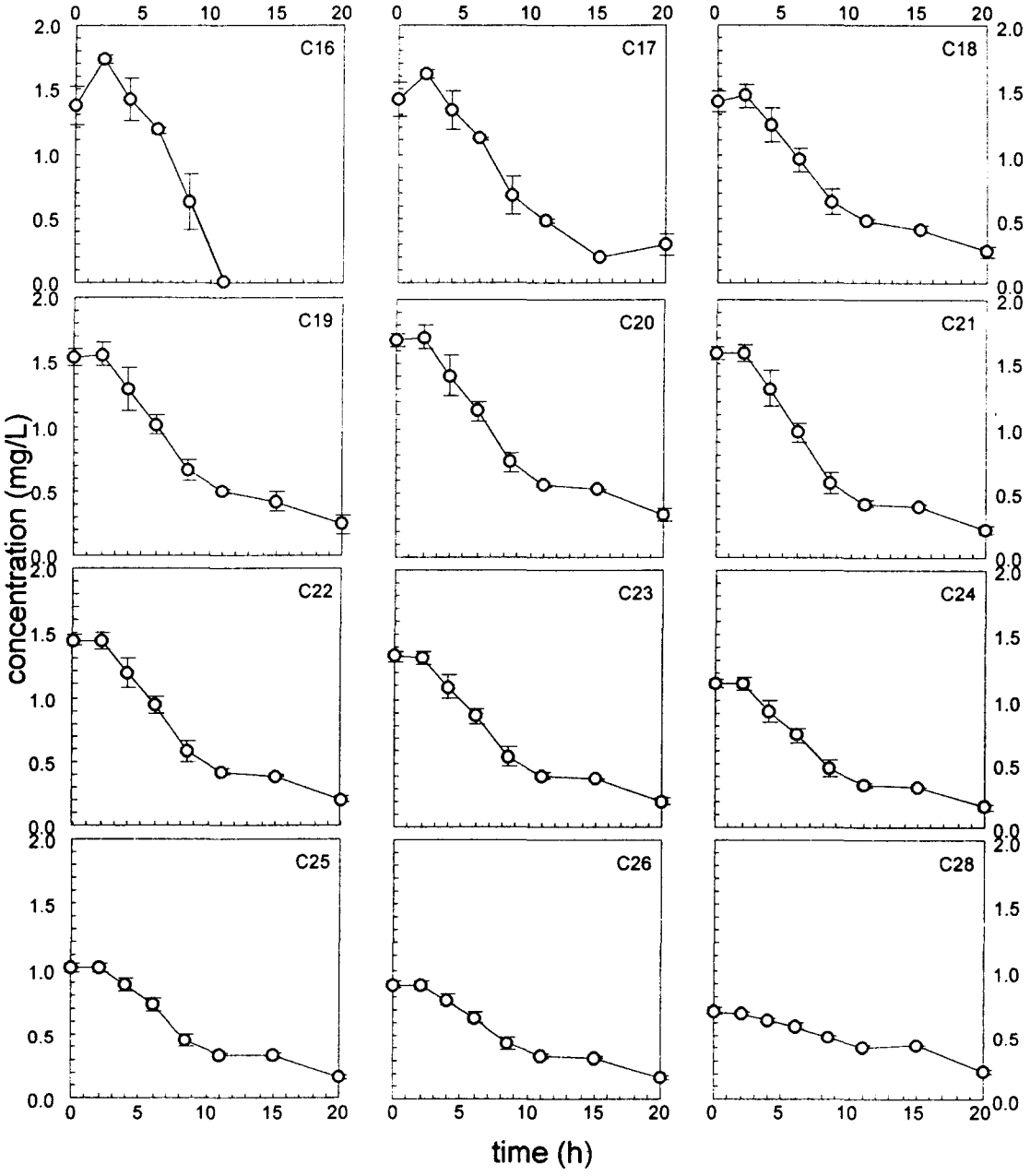

Fig. 4. Degradation of crude oil *n*-alkanes by the pure culture of *Acinetobacter* sp. on 0.1% (v/v) of crude oil in MSM; 150 rpm shaker, 27℃, in triplicates.

relatively low molecular weight alkanes (C17(≥), whereas this phenomenon was not cleared in the range of C18~C26, those are solid alkanes at room temperature when they exist as sole hydrocarbons (Fig. 4). Relatively low molecular weight *n*-alkanes in the range of C13~C16 were degraded rapidly. Accumulation observed in this range of *n*-alkanes at the initial stage of degradation was considered as the result of the cleavage of long chain branches from alkyl-branched hydrocarbons. There was no difference in degradation characteristics by the isolate between the two close odd and even numbered alkanes in carbon numbers.

The degradation of the observed *n*-alkanes diminished from the beginning of the stationary phase, possibly owing to transport limitation in providing substrates to the existing microorganisms. The diminished degradation of the observed *n*-alkanes caused tailing in the remainder fraction of *n*-alkanes. This remainder fraction was considered to rise from hydrocarbon inclusions. The detection of intracellular membrane-surrounded alkane inclusions and intracytoplasmic membranes including unmodified hydrocarbon growth substrate in alkane-grown *Acinetobacter* have been reported[20~22]. The hydrocarbon inclusions in microorganisms were considered to control the transport of hydrocarbons from the bulk oil phase. It was considered that the transport of hydrocarbons would stop when the hydrocarbon concentration of bulk oil reached that of cell surface. And this hydrocarbon concentration of cell surface likely to be in the equilibrium with that of intracellular or intracytoplasmic membrane.

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