The Journal of Microbiology, Dec. 1996, p.363-369

Vol. 34, No. 4

Emulsification of Crude Oil by *Acinetobacter* sp. SH-14

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(Received June 5, 1996/Accepted October 20, 1996)

As basic study to evaluate the treatability of oil-contaminated environment with bacteria, isolation and characterization of crude oil-degrading bacterium were carried out. A bacterial strain SH-14 capable of degrading crude oil was isolated from contaminated soils by enrichment culture technique and identified as *Acinetobacter* sp. by morphological, cultural and biochemical characteristics, and so named *Acinetobacter* sp. SH-14. The optimal medium composition and cultural conditions for the growth and emulsification of crude oil by *Acinetobacter* sp. SH-14 used were crude oil of 2.0%, KNO_3 of 0.2%, K_2HPO_4 of 0.05%, and $MgSO_4 \cdot 7H_2O$ of 1.0%, along with initial pH 7.0 at 30°C. *Acinetobacter* sp. SH-14 showed to be resistant to chloramphenicol and utilized various hydrocarbons such as dodecane, hexadecane, isooctane, cyclo-hexane etc., as a sole carbon source. *Acinetobacter* sp. SH-14 harbored a single plasmid. By agarose gel electrophoresis and curing experiment it was found that the genes for crude oil components degradation were encoded on the plasmid.

Key words: ***Acinetobacter* sp. SH-14, crude oil, hydrocarbon, biodegradation, emulsification**

Crude oil has been described as probably the most complicated natural mixture on earth (3). It consists mainly of hydrocarbons (50 to 98%), with variable amounts of oxygen, sulfur, and nitrogen. The composition of oil varies from field to field, between wells within a field, and even between samples from the same well. It is therefore not possible to give a precise constitution for crude oil, and no concise system has been developed for adequately classifying crude oil (5). It is recalcitrant due to such composition and act as pollutants when contaminated in the environment. Crude oil production in the world has increased from 278 million ton per year in 1938 to about 3,000 million ton per year in recent years (2). While accidental release may contribute to only a small percentage of the oil released into the marine environment, large accidental oil spills receive much attention and evoke considerable public concern.

There has appeared a large number of publications concerning the use of hydrocarbons as substrates for microbial growth (6, 9, 23). Such studies, however, usually involved the use of purified substrates, such as alkanes and aromatics. The potential of using microorganisms for the degradation of crude oil and its constitutes in minimizing contamination due to oil leaks and spills have prompted a number of investigators to study the process in laboratories (11, 12, 13, 19). Several microorganism, such as *Pseudomonas* sp. and *Acinetobacter* sp., have been reported to degrade hydrocarbons in the crude oil (22). In general, the degradation of crude oil by bacteria was accompanied by an emulsification, resulting in a oil-water interface. This is significant since there is evidence to indicate that the greater the oil-water interface, the faster the rate of oil decomposition by microorganisms (1). Namely, the growth on hydrocarbons of such microorganisms is associated with the production of surface-active compounds (biosurfactant) (10).

This report describes the isolation and partial characterization of an crude oil-degrading bacterium, *Acinetobacter* sp. SH-14, which was found to a plasmid.

Materials and Methods

Isolation and identification of bacteria

For the isolation of bacteria utilizing crude oil as a carbon source, enrichment cultures were carried in 250 ml Erlenmeyer flasks containing 50 ml of basal salts medium (BSM). The BSM consisted of 0.05% K_2HPO_4, 0.1% NH_4Cl, 0.2% Na_2SO_4, 0.0001% $CaCl_2 6H_2O$, 0.1% $MgSO_4 7H_2O$, and 0.0001% $FeSO_4$. This medium was supplemented with 1.0% crude oil as a carbon source. The crude oil

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used in this study was obtained from Kuwait. Samples collected from oil-contaminated soils in Pusan were inoculated in BSM broth and shaked at 200 rpm for 5-6 days at 30°C. After several transfers in BSM broth, morphologically distinct single colonies were screened for their ability to degrade crude oil. The bacterial strain SH-14 was selected for further study. The selected bacterial strain SH-14 was identified by investigating its morphological, cultural and biochemical characteristics according to the established method of Krieg and Holt (14).

Media and culture conditions

Optimization of culture conditions was investigated using BSM containing appropriate concentration of crude oil as the carbon source. Different levels of aeration were obtained by varying the amount of medium in the 250 ml Erlenmeyer flasks and keeping the agitation constant, i.e., 200 rpm. The ability to grow on various hydrocarbon substrates, such as hexadecane, isooctane, cyclopentane, benzene etc., was also determined on the BSM. The BSM adjusted to pH 7.0 was used with an appropriate hydrocarbon substrate at concentration of 0.1 to 0.5% as the sole carbon source. The cells were grown at 30 with shaking (200 rpm) for 5-6 days. On BSM agar plate, hydrocarbons were put in small tubes sealed at one end and placed on the lids of BSM agar plates in vapor phase (27). The plates were incubated for 10 day at 30°C. Luria broth (LB) containing 1.0% tryptone, 0.5% yeast extract and 1.0% NaCl was adjusted to pH 7.0 and used for the determination of minimal inhibitory concentration (MIC) of antibiotics.

Isolation and detection of plasmid

Acinetobacter sp. SH-14 was examined for the presence of plasmid. Bacterial strain was grown overnight in 10 ml of either BSM containing hexadecane as the sole carbon source or in LB. Alkaline lysis method (21) was used to isolate the plasmids. For the detection of plasmid band, samples of 10 to 20 μl of plasmid DNA preparation were subjected to agarose gel electrophoresis (21). A horizontal gel of 0.7% agarose in TBE buffer was run at 50 V for 1 hour. Gels were stained in a solution of ethidium bromide (1 μg/ml) for 30 min, rinsed and photographed under UV illumination.

Plasmid curing experiment

Curing experiments were carried out by treating with mitomycin C as described by Chakrabarty *et al.* (4). Single colony from BSM agar plate was inoculated into culture tubes containing 10 ml LB. After overnight growth at 30°C, 50 μl of a 10^3 dilution of the culture was inoculated into 10 ml of LB containing various concentrations of mitomycin C (0-60 μg/ml), and incubated with shaking for 24 hours until cells grew well. Then appropriate dilutions were made and 0.1 ml was spread on LB agar plates and replicated to the BSM agar plates containing appropriate carbon source for scoring; and selective individual clones for the phenotype were obtained.

Analytical methods

Cell growth was monitored by measuring the culture supernatant turbidity at 600 nm with a spectophotometer after residual crude oils were extracted from cultures by using chloroform as the solvent (7). The evidence of microbial degradation on crude oil is a quick and extensive emulsification of the oil (12). So, degradation of crude oil was expressed as emulsification. For the measurement of emulsifying activity, samples were removed and centrifuged at 10,000 g for 20 min, and the turbidities of the supernatant fluids were measured at 540 nm. The biodegradation of hydrocarbons in the culture medium were determined by measuring the decrease in maximal absorbance using clear supernatant of cultures after centrifugation at 10,000 g for 10 min.

Results and Discussion

Isolation and identification of crude oil-degrading bacterium

Twenty strains capable of degrading crude oil were isolated from the soil samples collected in Pusan. Petroleum-degrading bacteria were divided into three types according to degradation activity, i.e., (i) assimilation process without biosurfactant production, (ii) emulsification process with biosurfactant production, (iii) assimilation plus emulsification (18). As a result of observation of culture fluid, our strains were also divided into three types (Fig. 1). Of them, the strain SH-14 was found to possess

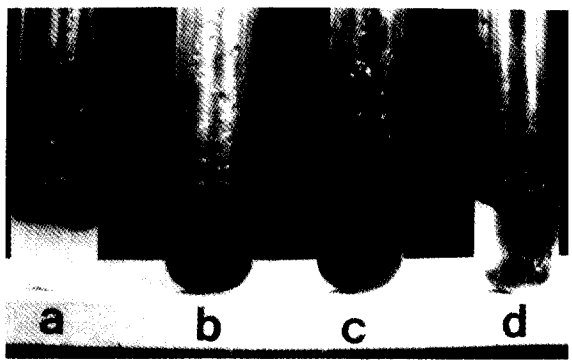

Fig. 1. Patterns of crude oil degradation by isolated strains. a, assimilation process without biosurfactant production; b, emulsification process with biosurfactant; c, assimilation plus emulsification; d, control (no degradation).

Table 1. Taxonomical characteristics of the isolated strain SH-14

Characteristics	Result	Characteristics	Result
Morphological characteristics		Voges-Proskauer	negative
Cell shape	coccobacillus	reaction	
Cell size(μm)	0.8~1.4×1.5~1.9	Methyl red reaction	negative
Gram stain	negative	Nitrate reduction	negative
Motility	negative	Growth on	growth
Spore formation	negative	MacConkey agar	
Cultural characteristics		Carbohydrate	
Colony shape	circular, entire, convex	fermentation:	negative
Colony surface	smooth	Glucose	negative
Colony color	cream	Sucrose	negative
Colony opacity	opaque	Lactose	negative
Biochemical characteristics		Xylose	negative
Cytocharome oxidase	negative	Maltose	negative
Catalase	positive	Galactose	negative
Oxidation/fermentation	inert	Fructose	negative
β-Galactosidase	negative	Rhamnose	negative
Arginine dehydrolase	positive	Sorbitol	negative
Lysine decarboxylase	negative	Inositol	negative
Ornithine decarboxylase	positive	Mannitol	negative
Citrate utilization	negative	Melobiose	negative
H_2S production	negative	Inuline	negative
Urease	negative	Arabinose	negative
Gelatin liquefaction	negative	Raffinose	negative
Tryptophane deaminase	negative	Sorbose	negative
Indole production	negative	Trehalose	negative

emulsifying activity and had the highest degradation activity for crude oil. For this reason, isolated SH-14 was selected for further investigation. The morphological, cultural and biochemical properties of the isolated bacterial strain SH-14 are listed in Table 1. It was a Gram-negative bacterium that formed nonspore-forming short rod-shaped cells without flagella. It gave negative reaction to β-galactosidase and H_2S production. It was positive to citrate utilization. It also reacted negatively to fermentation of glucose, sucrose and mannitol. According to *Bergey's Manual Systematic Bacteriology* (14), the isolated strain SH-14 was identified as a strain of the genus *Acinetobacter*. Thus, the isolated strain was tentatively named as *Acinetobacter* sp. SH-14. Petroleum hydrocarbons in subsurface environments are often difficult to remove because they will adsorb to surfaces and can be retained by capillary action as an immiscible, separate phase. To counter these effects, emulsifying agent, biosurfactant, have been proposed for the removal of adsorbed compounds from soils and the mobilization of separate-phase, immiscible petroleum hydrocarbons (24). It has been reported that *Acinetobacter calcoaceticus* RAG-1 can grow on crude oil by producing an extracellular emulsifying agent termed emulsan (17). It suggests that *Acinetobacter* sp. SH-14 probably produces an emulsifying material similar to emulsan produced by *Acinetobacter calcoaceticus* RAG-1.

Optimization of culture conditions

In order to increase the degradation of crude oil, the medium compositions and growth conditions were investigated. Maximum growth and emulsification had occured at 30°C, which is 2.5 times as high as that at 20°C. Although the culture grew slowly at 20 and 37°C, there was little growth when the culture was below 10 and above 45 (dara not shown). The effects of the initial pH of media on the growth and emulsification of *Acinetobacter* sp. SH-14 was also studid. The cell growth and the emulsification followed a similar pattern. *Acinetobacter* sp. SH-14 showed the maximum growth and emulsification at pH 7.0. The growth and emulsification at pH 10.0 was seriously inhibited. There was little growth when the pH of the medium was below 5.0 (data not shown).

To confirm the effect of crude oil concentration on the growth and emulsification, *Acinetobacter* sp. SH-14 was cultured in different media ranging from 0.1% (v/v) to 3.0% (v/v) of crude oil. As shown in Fig. 2, the growth and emulsification of *Acinetobacter* sp. SH-14 were direct-

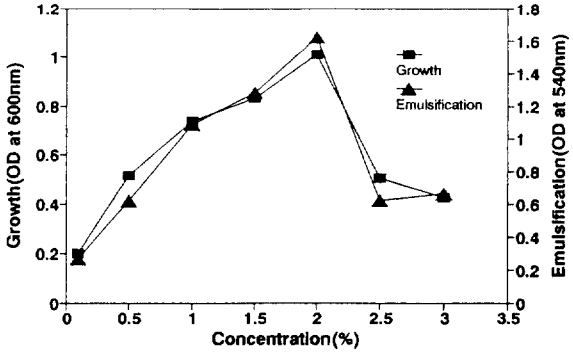

Fig. 2. Effect of crude oil concentration on the growth and emulsification of *Acinetobacter* sp. SH-14.

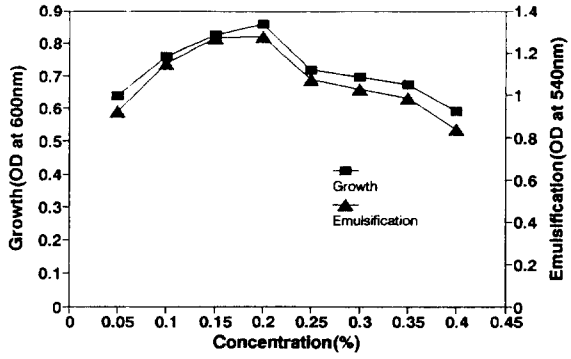

Fig. 3. Effect of KNO_3 concentration on the growth and emulsification of *Acinetobacter* sp. SH-14.

Table 2. Effect of nitrogen source on the growth and emulsification of *Acinetobacter* sp. SH-14

Nitrogen source (0.1%)	Growth (OD at 600 nm)	Emulsification (OD at 540 nm)
None	0.276	0.310
NH_4Cl	0.673	0.984
$(NH_4)_2SO_4$	0.518	0.743
NH_4NO_3	0.980	1.560
KNO_3	1.040	1.671
$NaNO_3$	0.910	1.624
Tryptone	0.734	1.319
Yeast extract	0.647	1.209
Beef extract	0.314	0.317
Peptone	0.396	0.474

Various nitrogen sources were added at the final concentration of 0.2% to the BM containing 2.0% crude oil as a carbon source.

ly proportional to crude oil concentrations up to 2.0%. At higher crude oil concentrations, growth and emulsification of *Acinetobacter* sp. SH-14 were greatly inhibited. A concentration of 2.0% crude oil was optimal for the cell growth and emulsification.

Various organic or inorganic nitrogen sources were investigated to determine the most suitable nitrogen source for growth and emulsification, where 0.2% of each nitrogen source was added as a sole nitrogen source to BSM containing 2.0% of crude oil. As shown in Table 2, KNO_3 was the best nitrogen source for the growth and emulsification. NH_4NO_3 and $NaNO_3$ were good, too. It can be assumed that *Acinetobacter* sp. SH-14 have nitrite and nitrate reductase, and more requirement of potassium ion than other ions (chloride, sulfate), but further study needs to be conducted. Some organic nitrogen sources, such as beef extract and peptone were not found to be effective for growth and emulsification. The optimal concentration of KNO_3 for growth and emulsification was 0.2% (Fig. 3). Optimal concentrations of K_2HPO_4, $CaCl_2$ $6H_2O$, and $MgSO_4$ $7H_2O$ were 0.01%, 0.0005%, and 1.0%, respectively (data not shown).

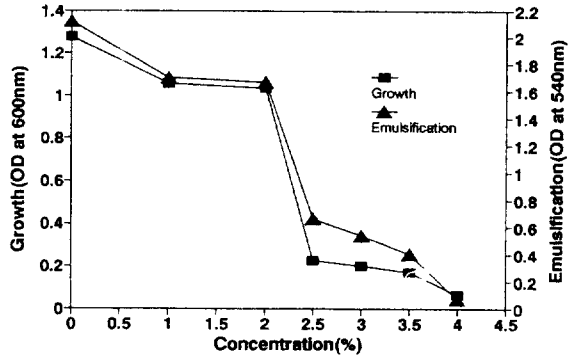

Fig. 4. Effect of NaCl concentration on the growth and emulsification of *Acinetobacter* sp. SH-14.

Table 3. Effect of aeration level on the growth an emulsification of *Acinetobacter* sp. SH-14

Medium volume* (ml)	Growth (OD at 600 nm)	Emulsification (OD at 540 nm)
50	0.834	1.217
50	1.119	1.766
70	0.976	1.554
100	0.924	1.455
120	0.777	1.179

*Obtained by varying the amount of medium in the 250 ml Erlenmeyer flasks and keeping the agitation constant, i.e., 00 rpm.

To study the effect of NaCl on the growth of *Acinetobacter* sp. SH-14, various concentrations of NaCl were added to the BSM (Fig. 4). The maximal growth was obtained from no addition of NaCl. The addition of NaCl inhibited growth and emulsification. This indicate that *Acinetobacter* sp. SH-14 was isolated from soil.

The effect of aeration on the growth of *Acinetobacter* sp. SH-14 is presented in Table 3. Maximal growth and emulsification were obtained with medium volume of 50 ml in 250 ml Erlenmeyer flask. Increasing the medium volume to 100 ml did slightly reduce the growth and

Table 4. Utilization of hydrocarbons by *Acinetobacter* sp. SH-14

Substrates[a]		Growth
Oils (2.0%)[b]	Crude	+++[c]
	Bunker-A	+++
	Bunker-B	++[d]
	Bunker-C	+++
n-Alkanes	Dodecane	+++
	Tetradecane	+++
	Hexadecane	+++
Branched alkanes	Isooctane	++
	Isopropyl alcohol	+[e]
	Isopropyl ether	+
Cyclic alkanes	Cyclopentane	+
	Cyclohexane	+
Aromatic hydrocarbons	Benzene	-[f]
	Toluene	-
	Phenol	-

[a]: Hydrocarbons were put in small tubes sealed at one end and placed in the lids of BSM agar plates with vapor phase.
[b]: Oils were added at the final concentration 2.0%(w/v) to the BSM.
[c]: good growth, [d]: growth, [e]: poor growth, [f]: no growth

emusification. However, it would appear that the growth and emulsification are much more reduced under 30 ml or 120 ml of medium volume. It suggests that critical concentration of dissolved oxygen (DO) determing the growth and emulsification of *Acinetobacter* sp. SH-14 exists. In case of 30 ml of medium volume, crude oil was attached on the wall of shaking flask, and thus the growth and emulsification may decrease.

Hydrocarbon utilization

Crude oil is composed of straight-chain, branched and cyclic alkanes, and aromatic hydrocarbons (1). In such a complex mixture many compounds are likely to inhibit the growth of some microorganisms. To ascertain this, therefore, *Acinetobacter* sp. SH-14 was examined to degrade various hydrocarbons consisting crude oil as shown in Table 4. *Acinetobacter* sp. SH-14 was able to grow on various hydrocarbon components in crude oil. Namely, the substrate specificity of this bacterium was relatively broad. *Acinetobacter* sp. SH-14 utilized crude oil, bunker oil-A, B, n-alkanes, branched alkanes, and cyclic alkanes as a sole carbon source, while it did not utilize aromaic hydrocarbons such as benzene, toluene and phenol. The strain was found to be retarded in its growth in cyclic alkanes-containing media, requiring at least 7 days of incubation. On the other hand, growth in crude oil and n-alkanes appeared within 3 days. It has been reported that, during the oxidation of crude oil by microorganisms, monocyclic and dicyclic aromatic compounds are preferentially degraded (25). *Pseudomonas* sp. HL7b was found to be capable of degrading a wide range of aromatic hydrocarbons, but not aliphatic hydrocarbons (8). *Acinetobacter* sp. SH-14 used in this study showed that alkanes were preferentially utilized as a carbon and an energy source. In other words, *Acinetobacter* sp. SH-14 utilized various alkane components in crude oil except for aromatic components. This may be related to the differences in uptake mechanisms of aromatics from those of alkanes. However, the pattern of alkane utilization does depend on the chemical structure of alkanes, that is cyclic alkanes tending to be more resistant to microbial degradation. This suggests that factors other than solubility determines the utilization of alkanes by microorganism (15).

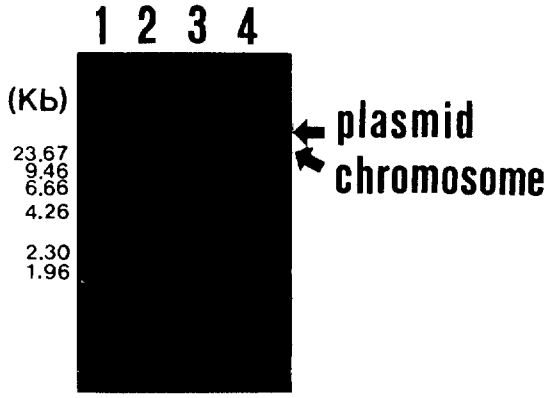

Fig. 5. Agarose gel electrophoresis of the plasmid DNA from wild strain and cured strain of *Acinetobacter* sp. SH-14. lane 1, λDNA digested with *Hin*dIII; lane 2, cured strain; lane 3, wild strain from hexadecane; lane 4, wild strain from LB.

So far it has been impossible to degrade a crude oil completely under laboratory conditions (16). Our *Acinetobacter* sp. SH-14 was also appeared the same result. Therefore, in order to cleanse of oil-contaminated environment with bacteria, isolation of various microorganisms which can utilize a significant proportion of the alkanes and possibly some of the aromatic compounds had need to carry out.

Detection of plasmid DNA and Curing of cells harboring plasmid

The agarose gel electrophoresis of plasmid DNA isolated from *Acinetobacter* sp. SH-14 is shown in Fig. 5. Lane 1 of Fig. 5 shows DNA of bacteriophage digested by Hind III endonuclease as a size marker. Lane 3 of Fig. 5 shows that *Acinetobacter* sp. SH-14 carries a large plasmid.

To determine the relationship between degradability of crude oil and plasmid, curing experiments were carried out under sublethal concentration of mitomycin C (Table

Table 5. Curing of *Acinetobacter* sp. SH-14 by mitomyin C

Mitomycin conc. (μg/ml)	Growth (OD at 600 nm)	Frequency of curing (%)*
0	0.616	ND
10	0.037	ND
20	0.937	ND
30	0.686	ND
40	0.496	90.2

*(Number of cured colonies/number of total colonies)×100
ND; not determined.

5). When treated with various concentration of mitomycin C, growth appeared after 1 day in culture containing concentrations below 30 μg/ml, but in case of 40 μg/ml, growth appeared after prolonged incubation. In the culture containing above 50 μg/ml, no growth occurred. Thus 40 μg of mitomycin C/ml was considered as sublethal concentration in *Acinetobacter* sp. SH-14. Then the loss of the ability to grow on crude oil and various hydrocarbons at this mitomycin C concentration was investigated. At this concentration, occurrence of cured cells was at a rate of 90.2%. In general, mitomycin C is known as a effective curing agent in *Pseudomonas* spp. (26). For example, growth of *Psudomonas putida* PpG6 in the presence of 10, 20, and 27 μg mitomycin C/ml resulted in the appearance of OCT$^-$ strains at frequencies of 1, 3,, and 7%, respectively (22). In comparison with *Pseudomonas putida* PpG6, our data led us to conclude that mitomycin C is a proper general agent for plasmid curing in *Acinetobacter* sp. As shown in Fig. 5, we made a cured strain that does not carry the plasmid by curing. These cured cells lost the ability to grow on crude oil, n-alkanes, branched alkanes and cyclic alkane. Therefore, the use of various alkanes by *Acinetobacter* sp. SH-14 depends on whether the strain has a plasmid or not. Whereas *Acinetobacter* sp. SH-14 showed to be resistant to chloramphenicol (data not shown), the cured strain of *Acinetobacter* sp. SH-14 was sensitive to chloramphenicol. This could mean that the genes for alkane degradation and antibiotic resistance are locate on the plasmid in *Acinetobacter* sp. SH-14. It was reported that the most ability degrading hydrocarbon was exist on the genes of plasmid (4). *Acinetobacter calcoaceticus* RA57 was found to contain four plasmids: pSR1 (5.1 kb), pSR2 (5.4 kb), pSR3 (10.5 kb), and pSR4 (20 kb). Among them, pSR4 was related to the physical interaction of the cells with the hydrocarbon substrate, rather than to its metabolism (20). On the other hand, all activities necessary for the growth of *Acinetobacter calcoaceticus* BD413 and *Acinetobacer* sp. HO1-N on alkanes appeared to be coded by chromosomal DNA (22). We are now investigating the transformation experiment by electroporation in order to confirm the relationship between isolated plasmid and alkane degradation.

Although no biological function of this plasmid is known so far, it may be useful as a vector in *Acinetobacter* spp., if genetic characteristics is more exactly demonstrated. This study suggests that to enhance mobility and biodegradation of crude oil and alkanes, application of *Acinetobacter* sp. SH-14 capable of producing emulsifying agent is useful.

Acknowledgement

This work was supported by a grant of Genetic Engineering Research Program (1995) from Korean Ministry of Education.

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