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Improvement of Heavy Oil Degradation by *Rhodococcus erythropolis* C2

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Rhodococcus erythropolis C2, which is able to degrade several kinds of fossil fuel, was isolated from soil samples. Oil consumption ratios of light oil, heavy oil (type-A), and heavy oil (type-C) of strain C2 were >80%, 80%, and 60%, respectively. The oil consumption ratio of the type-C oil increased by a maximum of 25% depending on the amount of light oil added. The oil consumption ratio of the heavy oil (type-C) was improved by decreasing the viscosity of the oil mixture. This indicated that the viscosity of the oil compound is an important factor to enhance fuel degradation.

Key words: bioremediation, oil degradation, *Rhodococcus erythropolis*, viscosity

The most widely distributed environmental pollution can be attributed to hydrocarbon contamination, caused by oil tanker accidents, storage tank ruptures, and transport accidents. The environment pollution by hydrocarbons at old petrol stations or factory sites is a serious problem as well, as not only does the pollution cause damages to the environment, but also the sales value of the land decreases significantly. Physical technologies, such as combustion and solidification, have been carried out to remove hydrocarbons from contaminated soils. Although physical techniques may shorten the work period with low costs, plants are not able to grow in these soils. It is well known that microbial degradation of spilled hydrocarbons is a major technique in the natural decontamination process[8]. Therefore, various bacteria degrading hydrocarbons have been investigated[3,4,6,9,11,12].

Fuels are classified into three classes according to their physical and chemical properties (Table1). Petroleum, a complex of individual compounds, and its components are generally grouped into four classes according to their differential solubility in organic solvents; (i) the saturates (n- and branched-chain alkanes and cycloparaffins), (ii) the aromatics (mono-, di-, and polynuclear aromatic compounds containing alkyl side chains and/or fused cycloparaffin rings), (iii) the resins (aggregates with a multitude of building blocks such as pyridines, quinolines, carbazoles, thiophens, sulfoxides, and amides), and (iv) the asphartenes (aggregates of extended polyaromatics, naphthenic acids, sulfides, polyhydric phenol, fatty acids, and metallopophyrins)[5]. Fuels also contain hundreds of complicated compounds (Table 1). An analysis of the hydrocarbon class composition was carried out by thin layer chromatography with flame ionization detection (TLC/FID, IATRON MK-6, Iatron, Tokyo, Japan)[13].

In our previous study, oil degradable bacteria (especially for fuels) were isolated from various places in Japan and assessed to their degradation characteristics for some types of fuel (Aoshima, H. *et al*., unpublished results). *R. erythropolis* C2, from Nishinomiya, Hyogo Pref., was found the most effective for degradation of several types of mineral oil of all isolated bacteria (Fig. 1). The oil consumption ratio

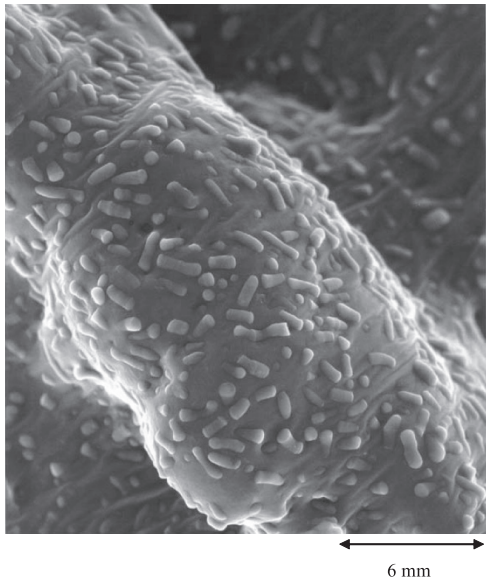

Fig. 1. Electron micrograph of *R. erythropolis* C2 (×5.0 K).

Table 1. Chemical properties of Light oils and Heavy oils.

	Class	Light oil					Heavy oil					
		S1	1	2	3	S3	Type-A		Type-B	Type-C		
							1	2		1	2	3
Chemical Properties	Flash Point	50<	50<	50<	45<	45<	60<			70<		
	Distilled Attribution (°C) (90% distilled Temp.)	360>	360>	350>	330>	330>	—					
	Pour Point (°C)	+5	–2.5	–7.5	–20	–30	5>		10>	—	—	—
	Setane Index	50<	50<	50<	45<	45<	—					
	Viscosity (mm^2/s)	2.7<	2.7<	2.5<	2.0<	1.7<	20>		50>	250>	400>	400~1000
	Sulfur (wt%)			0.20>			0.5>	2.0>	3.0>	3.5>	—	—
	Water (vol%)			—			0.3>		0.4>	0.5>	0.6>	2.0>
	Ash (wt%)			—			0.05>			0.5>		
TLC/FID	Saturates	—	68.5	—	—	—	62.2	—	—	37.2	—	—
	Aromatics	—	27.5	—	—	—	34.9	—	—	55.4	—	—
	Resins	—	3.6	—	—	—	2.6	—	—	3.7	—	—
	Asphartens	—	0.4	—	—	—	0.4	—	—	3.7	—	—

of strain C2 was almost 80% for both light and heavy oil (type-A). However, the oil consumption ratio for heavy oil (type-C) of *R. erythropolis* C2, had a maximum of 60%, and was lower than that for light oil and type-A oil. In order to improve the biodegradability of *R. erythropolis* C2 for heavy oil (type-C), we examined the effects of detergents such as food additives and light oil on the biodegradation of heavy oil (type-C).

R. erythropolis C2 was pre-cultured in 0.5% yeast extract at 35°C for 24 h. The pre-culture solution (0.2 ml) was added to 20 ml of W medium (2.0 g $(NH_4)_2SO_4$, 0.2465 g $MgSO_4$, 2.78 g $FeSO_4$, 14.7 g, $CaCl_2$, 0.5 g NaCl, 14.3 g Na_2HPO_4, 5.44 g KH_2PO_4, 2.01 g $ZnSO_4$, 0.15 g $(NH_4)_6Mo_7O_{24}$, 0.2 g $CuSO_4$, 0.4 g $CoCl_2$, 1.49 g $MnSO_4$ per 1 L) containing 20,000 ppm heavy oil (type-C) and 0 to 20,000 ppm of light oil as sole carbon and energy source or 0.0005% to 0.05% of detergent, and cultured at 120 rpm, at 35°C for 72 h. In order to examine the effectiveness of the detergents, various types of Triton-X, sodium dodecyl sulphate (SDS), fatty acid esters, polymer detergents such as casein and carboxymethyl cellulose were used to enhance the degradability of heavy oil (type-C) by *R. erythropolis* C2.

The oil consumption ratio by strain C2 was estimated by the weight measurement method after the chloroform-methanol (=3 : 1 v/v) extraction. Chloroform-methanol (18 ml) was added to the culture (20 ml) to extract the remaining oil component, and the mixture of solvent and culture was stirred at 120 rpm for 15 min at room temperature. The mixture was transferred to a centrifuge tube and centrifuged at 6,000×*g* for 10 min at room temperature. The weight of the extracted oil was measured after the lower-layer was collected and the solvent had volatilized for 3 days. Oil consumption ratio was calculated from residual oil components.

Most detergents did not significantly improve oil degradation. The growth of strain C2 was inhibited by the addition of Triton-X series and SDS. While strain C2 was able to grow in the fatty acid ester better than that in other detergents, fatty acid esters consisting of trehalose slightly improved oil degradation. This result suggest that strain C2 might be degrade the fatty acid as a carbon source before the heavy oil compound. The fatty acid ester is structurally similar to the biosurfactant such as trehalose 6, 6'-dimycolate secreted by *Rhodococcus* sp[1]. Therefore, the fatty acid ester may slightly contribute in forming stable oil-in-water emulsions of heavy oil to promote the indigenous biosurfactant of C2. In contrast to detergents, light oils enhanced the heavy oil consumption ratio according to the increased amount of light oil (Table 2). Most of the contents of light oil were saturates and aromatics, which are easily degraded by strain C2. Therefore, the amount of bacteria in a liquid culture containing light oil as a sole carbon source was more numerous than that containing heavy oil (type-C). As a result, light oil increased the production capacity of the biosurfactant. Simultaneously, the biosurfactant was secreted into the culture solution and was able to stabilize a heavy oil-in-water emulsion. In addition, this phenomenon can be ascribed to co-oxidation, in which persistent hydrocarbons are oxidized in the presence of hydrocarbons which can serve as growth substrate such as light oil. Evidence for co-oxidation of recalcitrant substrates was provided by asphaltenes and other aromatic hydrocarbons[2,7]. In addition, light oil could be helpful to reduce the viscosity of heavy oil, and increase the affinity of heavy oil for the culture solution. Sugiura *et al.* have investigated the biodegradation of four different crude oil samples, and demonstrated the

Table 2. Consumption ratios of heavy oil (type-C) under various conditions.

Light oil (ppm)	C-heavy oil (ppm)	Total oil conc. (ppm)	Consumption ratio of total oil mixture (%)	Consumption ratio of C-heavy oil (%)
0	20,000	20,000	43.4 (±17.5)	43.4
5,000	20,000	25,000	62.9 (±4.5)	56.1
10,000	20,000	30,000	80.3 (±11.2)	75.1
15,000	20,000	35,000	85.3 (±2.4)	81.7
20,000	20,000	40,000	87.9 (±3.2)	85.8
20,000	0	20,000	93.0 (±0.5)	—

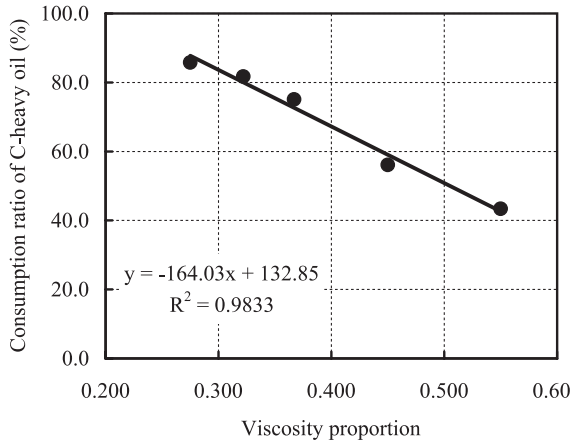

Fig. 2. Relationship between the viscosity proportion of oil compounds and consumption ratio of heavy oil (type-C) consumption by *R. erythropolis* C2. Viscosity proportion showed values of viscosity at 5 rpm per viscosity at 1 rpm.

biodegradability of crude oil was negatively proportional to viscosity[10].

The viscosity of each oil sample was determined by a rotary viscometer (type; Programmable DV-III+ Rheometer, Brookfield, USA) at 22°C at a shearing rate of 2/sec^2. The oil consumption ratio of heavy oil was improved by the decrease in the viscosity of the oil mixture (Fig. 2). Oil viscosity and the consumption of heavy oil had a negative proportional relationship. The equation of the calibration curve was $Y=-164.03X+132.85$ and the correlation coefficient (r^2) was 0.9833. Hence, a reduction in oil viscosity accelerated the degradation ability of heavy oil by strain C2. However, in the case of soil contamination, it is difficult for bacteria to attach to the solidified oil components after weathering. In order to improve the effectiveness of the contact between solidified oil and bacteria, dissolution of solidified oil by light oil will be the effective method to encourage the oil degradation in soil environment.

In this study, the relationship between fuel degradation and viscosity was demonstrated[14]. It is highly possible that the reduction of viscosity of polluted oils contributes to accelerate degradation of contaminated soil environments. To further elucidate the advantage of lower oil viscosity for bacteria, we started to analyze the amount of bacteria under various viscosities.

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