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Biodegradation of A-fuel oil in soil samples with bacterial mixtures of *Rhodococcus* and *Gordonia* strains under low temperature conditions

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Bioaugmentation is an effective treatment to clean up polluted sites using contaminant-degrading bacteria. However, this treatment is influenced by various environmental conditions, including temperature. In this study, an effective bioaugmentation system under low temperature condition was developed with three *Rhodococcus* (strains A, C, and D) and one *Gordonia* (strain B) oil-degraders, which are officially permitted for bioaugmentation applications in Japan. The oil-degrading ability of each strain and mixture was assessed in liquid culture and in model soils supplemented with A-fuel oil. In liquid culture, *Rhodococcus* strains A and C degraded the A-fuel oil in cold temperature conditions (15°C and 10°C) as well as in mesophilic condition (30°C). In the model soil samples, the mixture of four degraders was the most effective at removing the A-fuel oil under mesophilic condition (>90%), suggesting that strains B and/or D might have factors that promote degradation. In contrast, A-fuel oil was efficiently removed (>80%) in the soil samples inoculated with A or C as well as that with mixture in cold temperature condition, suggesting that strains A and C were the major degraders under cold condition. Our results indicate that the four degraders could be applied to the bioaugmentation in cold areas.

[**Key words:** Biodegradation; Bioaugmentation; A-fuel oil; Soil; Low temperature; *Rhodococcus*]

Although bioaugmentation has been considered an effective treatment to solve environmental pollution in contaminated soils, several challenges remain. This treatment is influenced by various environmental conditions of the contaminated sites, including the soil particle size, pH, water content, temperature, and indigenous microbes (1). Among these, temperature is one of most important factors affecting the feasibility of bioremediation (1,2). As the temperature increases, the solubility of oils and polyaromatic-hydrocarbons (PAHs) increases, which in turn increases the bioavailability of PAH molecules. On the other hand, dissolved oxygen level decreases with increasing temperature, resulting in reduced metabolic activity of aerobic mesophilic microorganisms (2). Development and optimization of bioaugmentation systems under the different temperature conditions is required for successful removal of contaminants in the environment. A large number of studies have reported on the degradation of oils in cold areas and their psychrotrophic degraders (3–8). It has been known that bacteria can respond quickly to crude oil in near-freezing seawater (9). Low temperature crude oil degradation has also been observed in polar and alpine soil (6) and by several bacterial strains in culture (7,8). *Rhodococcus*, a gram-positive bacterium, has been known as one of the major oil- and PAHs-degraders (10). Some strains of the genus can metabolize oils and PAHs in low temperatures (0°C–5°C) (3–5); however, it is still unclear whether these bacteria can be effective in bioaugmentation. In the present study, the effect of temperature on the biodegradation of A-fuel oil were assessed in liquid culture and in soil samples with four degraders, including three *Rhodococcus* strains and one *Gordonia* strain, which have been permitted for bioaugmentation by the Environment Agency in Japan based on the guidelines for environmental risk assessment on bioremediation (bioaugmentation) to prefectural governments.

MATERIALS AND METHODS

Bacterial strains and culture conditions Bacterial strains used in this study are *Rhodococcus erythropolis* strain A, *Gordonia polyisoprenivorans* strain B, *R. erythropolis* strain C, and *Rhodococcus rhodochrous* strain D. These strains were permitted as microbial degraders for use in bioaugmentation by the Japanese ministry of environment in 2011 (Guidelines for Environmental Risk Assessment on Bioremediation). They were grown overnight at 30°C in 3-fold diluted Lysogeny Broth (1/3 LB) (tryptone 3.3 g/L, yeast extract 1.7 g/L and NaCl 5.0 g/L) (11) or phosphate-buffered minimal salt medium [W medium: KH_2PO_4 (1.7 g/L), Na_2HPO_4 (9.8 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L), $FeSO_4 \cdot 7H_2O$ (0.95 mg/L), MgO (10.75 mg/L), $CaCO_3$ (2.0 mg/L), $ZnSO_4 \cdot 7H_2O$ (1.44 mg/L), $CuSO_4 \cdot 5H_2O$ (0.25 mg/L), $CoSO_4 \cdot 7H_2O$ (0.28 mg/L), H_3BO_3 (0.06 mg/L), and concentrated HCl (51.3 μL/L)] (11) with A-fuel oil (a Japanese heavy oil with the lowest viscosity) as a sole carbon source.

Degradative assays in liquid culture For each degrader, nine 200-mL flasks were prepared with 100 mL of W-medium containing A-fuel (500 mg/L). One mL of each pre-cultured strain was diluted with 5 mL of 1/3 LB and incubated for 2 d (30°C)

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and then transferred into one of the prepared flasks. Three flasks were used as 0-day samples and the remaining six were incubated at 30°C with 130 rpm for 6 and 12 days. Afterwards, 30 mL hexane was added to each sample (0-, 6-, and 12-day samples) and the remaining A-fuel oil was extracted by shaking the flask at 250 rpm for 25 min. After standing still for 30 min, 1 mL of the supernatant was subjected to a gas chromatograph system equipped with FID detector (GC-2014, Shimadzu, Kyoto, Japan) and autosampler (AOC-20i, Shimadzu) after removing water and debris by Na_2SO_4 addition and centrifugation. The GC column was Agilent J&W DB-1 (Agilent Technologies, Santa Clara, CA, USA) and helium was used as carrier gas at a flow rate of 3.58 mL/min, with a temperature increase of 20°C/min from 40°C (initial temperature) to 250°C and 10°C/min from 250 to 320°C (final temperature), with holds of 5 min at the initial and final temperatures. CDS-Lite ver. 5 (LAsoft, Chiba, Japan) was used to analyze the chromatography data.

Preparation of model soil samples Model soil samples were prepared by mixing two different commercial horticultural soils: Akadama soil, which is a volcanic ash soil in Kanto area in Japan (non-heat-treated), and Sibame sand, which is a dried sand (heat-treated). Both soils are from Fujimi group Japan. Akadama soil (240 g) and Shibame sand were mixed in a stainless tray. The percentages of sand, silt, and clay of the model soil were 85.33%, 1.39%, and 13.26%, respectively. The texture of the model soil is therefore considered loamy sand, according to the Soil Survey Manual of the United States Department of Agriculture (12). The soil pH was 5.9 (H_2O), and its total carbon and total nitrogen were 3.0 g/kg and 0.30 g/kg, respectively. Weight of dry soil was measured and 100 mL distilled water was added to a final water content of 38%. Afterward, the trays were covered by a plastic wrap with holes and incubated at 15°C for one week. The evaporated water amount was measured daily and fresh water (0.7–3.6 mL each time) was added to the samples and mixed well. A-fuel oil was added to the resultant soil samples (500 mg/kg-soil final concentration) with 10 mL sterilized distilled water and mixed well.

Degradative assays in model soil samples Two mL of each of the four precultured A-fuel degrader strains in 1/3-LB (incubated at 30°C, 120 rpm for 48 h) was transferred to 200 mL of fresh 1/3 LB in a 500-mL Sakaguchi flask and incubated at 30°C with 135 rpm for 48 h. Three flasks were prepared for each strain. The cells were harvested and resuspended in 10 mL sterilized distilled water (corresponding to 1.2×10^7 cells) and added to the soil samples. Four different soil samples were prepared: non-inoculated sample, inoculated with strain A, with strain C, or with a mixture of the four strains. The soil samples were incubated at 15°C for 24 d and of 1 g of soil was sampled in triplicate after 0, 3, 6, 12, 18, and 24 d. The extraction of the remaining A-fuel oil from the samples were performed with 3 mL hexane after addition of 3 mL PBS to the soil samples in capped 15-mL glass test tubes by shaking them at 250 rpm for 60 min. After left standing for 60 min, 1 mL of the supernatant was subjected to a gas chromatograph system as described above.

RESULTS AND DISCUSSION

Biodegradation of A-fuel oil by the four degraders under different temperature conditions in liquid media Four degraders, *R. erythropolis* strain A, *G. polyisoprenivorans* strain B, *R. erythropolis* strain C, and *R. rhodochrous* strain D, are permitted for bioaugmentation by the Japanese Environment Agency. Their degrading abilities were assessed in liquid W medium containing 500 mg/L A-fuel oil at the mesophilic temperature 30°C and the cold temperatures 15°C and 10°C. We could detect statistically significant differences between control and samples inoculated with A, B, C, or a mixture of all four strains, by the Kruskal–Wallis test ($p < 0.05$) and Tukey's test ($p < 0.05$) (Fig. 1). After 12-day treatments, the residual A-fuel oil contents were below 30% under 30°C in the samples treated with strains A (26%), B (21%), C (22%), and mixture (17%), whereas those of the control and the sample inoculated with D was more than 90% (Fig. 1). Notably, the residual oil contents for strains A and C at 15°C were comparable (10–20%) to those at 30°C (Fig. 1). Even at 10°C, the residual oil was 18% in the mixture (Fig. 1). As for the strain D samples, significant reduction of A-fuel oil was not detected under any temperature conditions, suggesting that the oil-degradation rate of strain D is slower than the other strains (Fig. 1). The effect of mixing these degraders was not detected in liquid culture under the different temperature conditions (Fig. 1).

In liquid media under low temperature conditions (15°C and 10°C), flocculation of putative bacterial aggregates was found in the

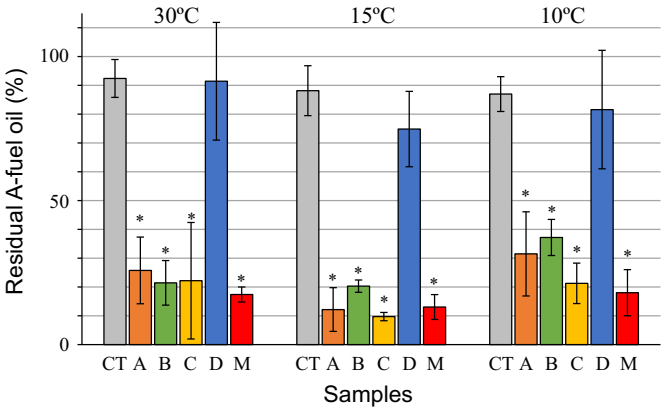

FIG. 1. Residual A-fuel oil after the treatment with each of four degraders and/or a mixture of them for 12 days at different temperatures (30°C, 15°C, and 10°C). 'A' to 'D' indicates the sample treated with *Rhodococcus erythropolis* strain A, *Gordonia polyisoprenivorans* strain B, *R. erythropolis* strain C, and *R. rhodochrous* strain D, respectively. 'M' indicates the sample treated with a mixture of all four strains, while 'CT (control)' indicates the sample without bacterial inoculation. Each experiment was performed at least three times and average values and standard deviations of triplicate data are shown. Asterisks indicate statistically significant differences detected between control and the sample by Tukey's test ($p < 0.05$).

samples with strain A or C and mixture (data not shown). This phenomenon was reported in the case of other *Rhodococcus* strains under low temperature (13). The observation of the flocs under fluorescence microscopy after SYBR Green staining confirmed that they contained bacterial cells (data not shown). To exclude the possibility that these flocs absorbed the A-fuel oil, oil extraction and detection from the flocs was carried out with hexane and GC analyses. As a result, no A-fuel oil was detected from the floc, indicating that the oil was not absorbed by the bacterial flocs but metabolized by the degraders.

Biodegrading ability of four bacteria for A-fuel oil under different temperature conditions in model soil samples Because the effective degradation of A-fuel oil was observed even in the lower temperatures with strains A and C, model soil samples were prepared to assess whether these degraders could remove oil from soil under low temperature conditions. Changes in residual A-fuel oil (500 mg/L at the initial point) in the soil samples were monitored under 30°C and cold temperature (15°C). Degradation of the oil was observed in the soil samples inoculated with strain A, C, and mixtures of the four degraders under 30°C, although the residual A-fuel oil content in the sample inoculated with the mixture was lower than that with A or C after 12 days incubation (Fig. 2A). This result implies that the four degraders could cooperatively degrade the oil in the soil under mesophilic condition. On the other hand, the residual A-fuel oil levels were similar in the samples inoculated with A, C, and mixture after 12 days of incubation at 15°C (Fig. 2B). Their degradation rates were higher in the samples inoculated with four strains in both temperature conditions, although those with strains A and C were as high as that of mixture under low temperature condition (Table 1). These results showed that the major degraders in the mixture could be strains A and C under the low temperature conditions.

At both temperatures, the A-fuel oil was removed even in the control samples (without inoculation of degrader(s)) (Fig. 2A, B), probably due to the volatilization of the A-fuel oil and/or degradation of the oil by indigenous bacteria in the soil samples. The total number of bacteria in the model soils did not change (around 10^8 cells/g soil, less than 10-fold difference during the incubation) in any sample (data not shown). This fact suggests the presence of a

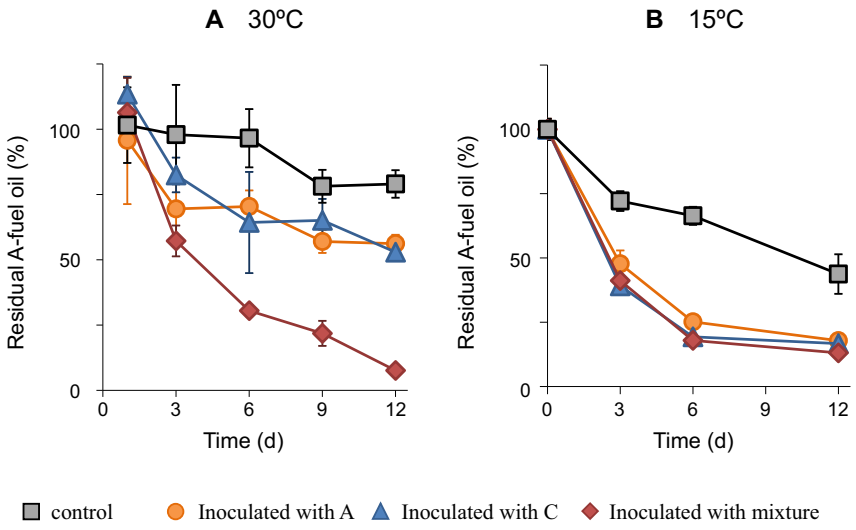

FIG. 2. Changes in residual A-fuel oil in model soil samples inoculated with *R. erythropolis* strain A, C, and/or a mixture of all four strains at 30°C and 15°C. Control indicates the sample without bacterial inoculation. Each experiment was performed at least three times and average values and standard deviations of triplicate data are shown.

TABLE 1. Degradation rates of A-fuel oil in the model soil samples during 12 d (288 h).

Temperature (°C)	[a]Degradation rate of A-fuel oil (mg/kg-soil/h) in the model soils inoculated with:			
	A	C	Mix (A, B, C, D)	Control (no degraders)
30	0.76 (0.059)	0.82 (0.056)	1.6 (0.015)	0.36 (0.092)
15	1.4 (0.031)	1.4 (0.023)	1.5 (0.014)	0.98 (0.13)

[a] Numbers in parentheses are standard deviations of the rates.

large number of indigenous bacteria in the model soil samples, which was maintained even in the control samples probably because they could metabolize the A-fuel oil.

Surprisingly, the amount of residual A-fuel oil in the soil sample with A or C at the cold temperature was lower than that under the mesophilic temperature (Fig. 2A, B), while large differences in A-fuel degrading ability of strain A and C in liquid culture were not observed between 30°C and 15°C (Fig. 1). This was probably due to the differences in the accessibility to the substrate (A-fuel oil) for the degraders between liquid and soil conditions. Considering that strains A and C underwent flocculation in liquid culture under lower temperature conditions, they might produce a biosurfactant, bioflocculant, or extracellular polysaccharides, as has been known to occur in other *Rhodococcus* strains (14–16). It was reported that the cell flocculation of degraders was important for oil degradation because it increased the accessibility of cells to the hydrophobic (insoluble) substrate (16). Because the flocculation of strain A or C was not detected under the mesophilic conditions, it might not be easy for these strains to access the substrate in model soil at 30°C. In contrast, these strains could undergo flocculation under cold temperature, thus potentially increasing the accessibility to the substrate in the soil samples.

As for the mixture samples, substrate accessibility may be improved by the presence of strains B and/or D at 30°C. Although the reason for this phenomenon remains unclear, there are some possibilities. *R. rhodochrous* S-2, which belongs to the same species as strain D, is known to produce extracellular polysaccharide (EPS) and is effective at promoting the biodegradation of aromatic compounds in oil-contaminated marine environments (14). This EPS is important for the emulsification of the oil in the water environments and increasing the biodegradation of PAHs (14,17). Strain D might therefore possibly also produce EPS to help the degradation of the A-fuel oil at 30°C. Strains B and D might also possibly be better at degrading high molecular weight (HMW) alkanes than the other degraders, although their degrading rates were lower. This possibility was supported by the changes in GC chromatograms with the peaks of the derived from HMW alkanes decreasing faster in B and D strains than in A and C strains (data not shown).

Conclusions In this study, inoculation of mixtures of the four degraders into model soils under different temperature conditions successfully removed the contaminants. It was also shown that strains A and C were the major degraders in the lower temperature conditions. Since the four degraders are officially allowed to be used in bioaugmentation by the Japanese Ministry of the Environment, they could be applied to real contaminated fields in the next step. For a more effective application of these degraders, their in-depth analyses at the genomic level will be important to understand how they degraded the A-fuel oil in different environmental conditions.

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