International Biodeterioration & Biodegradation 88 (2014) 77–82

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

The potential of glycerol in freezing preservation of turbine oil-degrading bacterial consortium and the ability of the revised consortium to degrade petroleum wastes

Kumiko Kurachi, Reia Hosokawa, Marina Takahashi, Hidetoshi Okuyama*

Graduate School Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

ARTICLE INFO

Article history:
Received 27 February 2013
Received in revised form
17 November 2013
Accepted 11 December 2013
Available online 7 January 2014

Keywords:
Bacterial consortia
Biodegradation
Crude oil
Culture revival
Glycerol stock
Turbine oil
Wastewater

ABSTRACT

The turbine oil (TuO)-degrading bacterial consortium Tank-2 (original Tank-2) was preserved as a glycerol stock at −80 °C from 2009 to 2012. Storage methods have been unavailable so far for any TuO-degrading bacterial consortia or isolates. To evaluate the usefulness of glycerol stock, the original Tank-2 consortium frozen in glycerol at −80 °C was thawed and then revived by repeated culture in mineral salts medium (MSM) containing 0.5% (w/w) TuO (revived Tank-2). The revived Tank-2 consortium exhibited a high activity to degrade TuO, which was equivalent to that of original Tank-2. It also degraded car engine oil, used car engine oil, Arabian light and Vityaz crude oils and TuO in wastewater. These results indicated that a glycerol stock at −80 °C was useful for storing Tank-2. PCR-denaturing gradient gel electrophoresis (DGGE) that targeted the V3 regions of 16S rRNA gene sequences showed that the DGGE band profiles of principal bacteria were significantly different between the original and revived Tank-2 consortia and between the revived Tank-2 culture grown in MSM containing TuO and that grown in MSM containing other types of petroleum products. This suggested that bacterial strains inherently residing in Tank-2 could adjust their compositions based on the storage and culture conditions.

1. Introduction

Turbine oil (TuO) is used for lubricating and controlling gas and steam turbine systems. TuO typically comprises 95–99.5% (w/w) of highly refined base oil (a mixture of branched alkanes and cyclic alkanes) and 0.5–5% (w/w) of additives (Hosokawa et al., 2010). The biodegradability of branched and cyclic alkanes is lower than that of aliphatic hydrocarbons (Gough and Rowland, 1990). According to Perry (1984), the susceptibility of hydrocarbons to microbial attack is in the following order: normal alkanes (*n*-alkanes) > isoalkanes > low molecular weight aromatics > cyclic alkanes. Thus, TuO, particularly cyclic alkanes, is assumed to be relatively recalcitrant to microbial degradation.

There have been numerous reports on bacteria and bacterial consortia that can degrade petroleum products, including gasoline (Wongsa et al., 2004; Lu et al., 2006), diesel oil (Wongsa et al., 2004; Ciric et al., 2010; Jung et al., 2010), car engine oil (Wongsa et al., 2004; Abioye et al., 2012), heavy oil (Wongsa et al., 2004; Aoshima et al., 2006; Hao and Lu, 2009) and crude oil (Razak et al., 1999; Rahman et al., 2002; Sathishkumar et al., 2008). However, there are only a limited number of bacteria that can degrade TuO.

Zvyagintseva et al. (2001) reported that *Rhodococcus erythropolis* and *Dietzia maris* are TuO degraders. Two types of TuO-degrading consortia, designated Atsuta (Ito et al., 2008) and Tank-2 (Hosokawa et al., 2010), which had been formulated from soil samples contaminated with crude oil and TuO-containing wastewater sampled at an electric power plant, respectively, efficiently degraded TuO. Their capacities to degrade TuO were consistently maintained as long as these consortia were continuously cultured in media that contained TuO.

Freezing and freeze drying are common means used for long-term storage of microbial cells (see: http://www.atcc.org/CulturesandProducts/tabid/167/Default.aspx). However, regardless of the isolated bacterial strains or consortia, appropriate reservations must be considered before their practical use, such as in bioaugmentation. To date, there have been no reports on whether a glycerol stock, the most commonly used storage method for bacterial cells, is useful for TuO-degrading bacteria or bacterial consortia.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; EO, engine oil; ICP-AES, inductively coupled plasma-atomic emission spectrometry; MSM, minimal salts medium; TLC-FID, thin-layer chromatography-flame-ionization detection; TPH, total petroleum hydrocarbon; TuO, turbine oil.

* Corresponding author. Tel.: +81 11 706 4523; fax: +81 11 703 2347.
E-mail address: hoku@ees.hokudai.ac.jp (H. Okuyama).

0964-8305/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibiod.2013.12.005

In this study, the availability and usefulness of a glycerol stock of the TuO-degrading consortium Tank-2, which had been stored at −80 °C for 3.5 years were evaluated. This revived consortium was tested for its ability to degrade TuO and types of petroleum products, including lubricating oils, crude oils and TuO in the wastewater from an electric power plant.

2. Materials and methods

2.1. Microbial consortia and culture media

To culture microbial consortia, we used minimal salts medium (MSM) that included 0.4% NH_4NO_3, 0.47% KH_2PO_4, 0.0119% Na_2HPO_4, 0.001% $CaCl_2{\cdot}2H_2O$, 0.1% $MgSO_4{\cdot}7H_2O$, 0.001% $MnSO_4{\cdot}7H_2O$ and 0.0015% $FeSO_4{\cdot}7H_2O$, pH 7.0. MSM was supplemented with TuO or another type of petroleum product (Ueno et al., 2006a,b).

TuO-degrading Tank-2 consortia were used throughout this study. The original Tank-2 was formulated in 2009 (Hosokawa et al., 2010) and had been preserved at −80 °C as a glycerol stock. In this study, frozen original Tank-2 was revived from storage, subjected to re-testing for its capacities to degrade various types of petroleum products and these results were compared with those for the original Tank-2 consortium.

Glycerol-stocked original Tank-2 was first thawed at room temperature. The thawed Tank-2 culture (1 ml) was then transferred to a 50-ml flask containing 10 ml of MSM supplemented with 0.5% (w/w) of TuO (TuO-containing MSM) and cultured for two weeks at 30 °C with shaking at 160 rpm. After the first reviving culture (enrichment culture), a part (200 μl) of the culture was inoculated into freshly prepared TuO-containing MSM and cultured (second enrichment culture) as described above. This enrichment procedure was repeated more than four times. These prepared consortia were designated as revived Tank-2.

TuO (type FBK turbine SH), a product of Nippon Oil Corporation (Tokyo, Japan), was obtained from Hokkaido Electric Power Co., Inc. Car engine oil (Toyota Castrol motor oil SN 5W-30; abbreviated EO) was purchased from a local market. Used car engine oil (original Toyota Castrol motor oil SN 5W-30) was obtained from a local body shop. Arabian light crude oil and Vityaz crude oil (Maki et al., 2008) were provided by Idemitu Kosan Co., Ltd. and Vityaz crude oil (Maki et al., 2008) from the Geological Survey of Hokkaido, respectively. All oil types were autoclaved at 121 °C for 20 min before use.

2.2. Formulating a new microbial consortium to degrade TuO

To formulate a new TuO-degrading consortium, wastewater samples that contained TuO were collected from the oil-water separating tank at the Moiwa hydraulic plant, Hokkaido Electric Power Co., Inc. located at Minami-ku, Sapporo in 2012. This was the same sampling site where a wastewater sample was collected to prepare the original Tank-2 consortium in 2009. Wastewater samples were processed as described previously (Hosokawa et al., 2010). Samples were centrifuged at 7000 rpm for 15 min to precipitate insoluble matters, after which the pellets were suspended in 10 ml of the supernatant. This suspension was added as an inoculum to 10 ml of MSM that contained TuO in a 50-ml flask. This was then incubated at 30 °C on a rotary shaker (160 rpm) for two weeks. An aliquot of this culture (200 μl) was transferred to another 50-ml flask containing 10 ml of the same medium and incubated as described above. Because bacterial cultures, including cultures of the original Tank-2, formed aggregates during culture, 200 μl of each liquid culture that contained small cell aggregates were inoculated directly into 10 ml of fresh TuO-containing MSM. These cultures were incubated at 30 °C with shaking (160 rpm) for two weeks. The formulated TuO-degrading consortium was designated as Moiwa-KK.

2.3. Degradation test

To estimate the degradation of TuO and other types of petroleum products, 200 μl of the pre-culture for revived TuO was transferred to a 50-ml flask that contained 10 ml of MSM supplemented with either TuO, EO, Arabian light crude oil or Vityaz crude oil at 0.5% (w/v). Culture was carried out for one or two weeks at 30 °C with shaking at 160 rpm. When the thawed Tank-2 consortium was used as the inoculum, the total volume (1 ml) of the original Tank-2 culture in a microfuge tube that had been frozen for 3.5 years at −80 °C was thawed at room temperature and then transferred into the same TuO-containing medium as described above.

TuO in wastewater was also used as the carbon source for revived Tank-2. Culture media were prepared as shown in Table 1. For this test, the concentration of TuO in the medium was adjusted to 1.5%, which was three times higher than that of normal TuO-containing MSM. A 200-μl aliquot of the culture grown in TuO-containing MSM was inoculated directly into 10 ml of MSM supplemented with 0.5% (w/v) of the various petroleum products. Culture was performed as described above.

2.4. Extraction and analysis of hydrocarbons

Petroleum product extraction was performed with chloroform using the modified Bligh-Dyer method (Bligh and Dyer, 1959), as described previously (Hosokawa et al., 2010). Total petroleum hydrocarbons (TPHs) were separated into saturated, aromatic, resin and asphalthene fractions and quantified with the thin-layer chromatography-flame ionization detection method (TLC-FID) using an Iatroscan (Model MK-6), as described previously (Goto et al., 1994; Ito et al., 2008; Hosokawa et al., 2010). When crude oils were analysed by TLC-FID, the resin and asphalthene fractions were omitted from the calculations because of their recalcitrant characteristics and the inclusion of cell-derived polar lipids in the asphalthene fraction (Ito et al., 2008).

Table 1
Degradation of TuO in wastewater by revived Tank-2.

Culture[a]	Media component[b]			Degradation of TuO
	MSM and wastewater (WW)	Revived Tank-2	Unused TuO	
Culture 2	10 ml MSM (×1) plus no WW	200 μl	1.5% added	36.7% ± 3.0%
Culture 3	5 ml MSM (×2) plus 5 ml WW	200 μl	Not added	70.7% ± 2.0%
Culture 4	5 ml MSM (×2) plus 5 ml WW	Not added	Not added	63.7% ± 3.2%
Culture 5	10 ml MSM (×1) plus no WW	Not added[c]	Not added	53.5% ± 6.9%

[a] All cultures contained 1.5% (w/w) TuO.
[b] MSM (×1), mineral salts medium (MSM) at its normal concentration; MSM (×2), MSM at two-times concentration; WW, original wastewater containing TuO (3%).
[c] 200 μl of Moiwa-KK was added instead of Revived Tank-2.