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

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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 16 S 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.

Keywords:

Bacterial consortia

Biodegradation

Crude oil

Culture revival

Glycerol stock

Turbine oil

Wastewater

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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

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 (Ciric et al. 2010; Wongsa et al. 2004; 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.

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% $CaCl_2$•$2H_2O$, 0.1% $MgSO_4$•$7H_2O$, 0.001% $MnSO_4$•$7H_2O$ and 0.0015% $FeSO_4$•$7H_2O$, pH 7.0. MSM was supplemented with TuO or another type of petroleum product (Ueno et al. 2006 a, b)

TuO-degrading consortia Tank-2 was 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

96 at 30°C with shaking at 160 rpm. After the first reviving culture (enrichment culture), a part
97 (200 μl) of the culture was inoculated into freshly prepared TuO-containing MSM and
98 cultured (second enrichment culture) as described above. This enrichment procedure was
99 repeated more than four times. These prepared consortia were designated as revived Tank-2.
100 TuO (type FBK turbine SH), a product of Nippon Oil Corporation (Tokyo, Japan),
101 was obtained from Hokkaido Electric Power Co., Inc. Car engine oil (Toyota Castrol motor
102 oil SN 5W-30; abbreviated EO) was purchased from a local market. Used car engine oil
103 (original Toyota Castrol motor oil SN 5W-30) was obtained from a local body shop. Arabian
104 light crude oil and Vityaz crude oil (Maki et al. 2008) were provided by Idemitu Kosan Co.,
105 Ltd. and Vityaz crude oil (Maki et al. 2008) from the Geological Survey of Hokkaido,
106 respectively. All oil types were autoclaved at 121°C for 20 min before use.
107
108 *2.2. Formulating a new microbial consortium to degrade TuO*
109
110 To formulate a new TuO-degrading consortium, wastewater samples that contained TuO were
111 collected from the oil-water separating tank at the Moiwa hydraulic plant, Hokkaido Electric
112 Power Co., Inc. located at Minami-ku, Sapporo in 2012. This was the same sampling site
113 where a wastewater sample was collected to prepare the original Tank-2 consortium in 2009.
114 Wastewater samples were processed as described previously (Hosokawa et al. 2010). Samples
115 were centrifuged at 7,000 rpm for 15 min to precipitate insoluble matters, after which the
116 pellets were suspended in 10 ml of the supernatant. This suspension was added as an
117 inoculum to 10 ml of MSM that contained TuO in a 50-ml flask. This was then incubated at
118 30°C on a rotary shaker (160 rpm) for two weeks. An aliquot of this culture (200 μl) was
119 transferred to another 50-ml flask containing 10 ml of the same medium and incubated as
120 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 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

146 method (Bligh and Dyer 1959), as described previously (Hosokawa et al. 2010). Total
147 petroleum hydrocarbons (TPHs) were separated into saturated, aromatic, resin and
148 asphalthene fractions and quantified by the thin-layer chromatography-flame ionization
149 detection method (TLC-FID) using an Iatroscan (Model MK-6), as described previously
150 (Goto et al. 1994; Ito et al. 2008; Hosokawa et al. 2010). When crude oils were analysed by
151 TLC-FID, the resin and asphalthene fractions were omitted from the calculations because of
152 their recalcitrant characteristics and the inclusion of cell-derived polar lipids in the
153 asphalthene fraction (Ito et al. 2008).
154
155 *2.5. Analysis of metals*
156
157 The concentrations of Al, Cr, Cu, Fe, Pb, Zn and other ions at >0.1 mg/L (ppm) in
158 TuO-containing wastewater were determined by an inductively coupled plasma-atomic
159 emission spectrometer (ICP-AES) (Model ICPE-9000). For preparations for hydride
160 generation analysis, 5 g of sample was mixed with 8 ml of nitric acid. This mixture was
161 subjected to microwave digestion first at 230°C for 40 min and then at 120°C for 15 min.
162 After cooling, the sample mixture volume was adjusted to 50 ml with distilled water. This
163 original sample solution was diluted with 2 M nitric acid at appropriate ratios and then
164 subjected to ICP-AES. Multi-element standard solution I (MERCK, Darmstadt, Germany)
165 was used as a standard.
166
167 *2.6. DNA procedures*
168
169 *2.6.1. Extraction of bacterial genomic DNA*
170 Bacterial cells were harvested by centrifugation at 7,000 rpm for 10 min at 4°C, after which

171 the pellets were freeze-dried overnight. Freeze-dried samples were suspended in 1 ml of 20
172 mM Tris–HCl buffer (pH 8.0) containing 250 mM EDTA. The suspensions were
173 homogenized in a sterilized mortar. A 200-µl aliquot was transferred to a 2-ml sterilized
174 screw-capped tube with a O-ring (Assist Co., Ltd., Tokyo) that contained 500 mg of sterilized
175 glass beads (105–150 µm in diameter; Polysciences, Inc., Warrington, PA), and the remainder
176 of the suspended cells was stored at –30°C.
177 A mixture of 400 µl of an organic solvent containing phenol, chloroform, and isoamyl
178 alcohol (25:24:1, by volume) and 800 µl of a stabilizing reagent [100 mM Tris–HCl (pH 8.0),
179 20 mM EDTA, 1.4 M NaCl and 2% (w/v) cetyltrimethyl ammonium bromide] were added to
180 the screw-capped tubes. This mixture was disrupted with a Mini-Beadbeater™ at 5,000 rpm
181 for 1 min, and then heated at 65°C for 15 min. After cooling on ice, the sample was
182 bead-beated at 5,000 rpm for 1 min, and centrifuged at 13,000 rpm for 10 min at 4°C.
183 Supernatants were transferred to 1.5 ml sterilized tubes and precipitated with ethanol.
184 Genomic DNA was finally dissolved in 50 µl of EDTA-containing 20 mM Tris-HCl buffer
185 mentioned above and stored at −30°C.
186
187 *2.6.2. PCR amplification of 16S rRNA gene sequences*
188 For denaturing gradient gel electrophoresis **(**DGGE), PCR reactions were carried out using
189 methods described by Hosokawa et al. (2010). A set of primers with a GC clamp, 338F-GC
190 (5′-CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC
191 TAC GGG AGG CAG CAG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′), which
192 corresponded to the V3 regions of the 16S rRNA gene sequence was used. PCR was run
193 using GC-338F and 518R primers. The PCR reaction mixture (final volume of 50 µl)
194 included 1 unit of Ex Taq DNA polymerase (TAKARA BIO, Kyoto, Japan), 200 nM dNTP
195 mixture, 25 pM of each primer, EX Taq buffer and genomic DNA as template. PCR was

196 carried out on a Mastercycler ep thermal cycler (Eppendorf AG) using the following
197 program: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 1 min at
198 94°C, 1 min at 55°C and 1 min at 72°C and finally a 5 min extension at 72°C to amplify 16S
199 rRNA gene fragments for DGGE. To amplify the whole 16S rRNA gene, the following
200 program was used: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 1
201 min at 94°C, 1 min at 55°C, 2 min at 72°C and finally a 5-min extension at 72°C.
202
203 *2.6.3. DGGE analysis*
204 DGGE used the D-code™ Universal Mutation Detection system, as described in Hosokawa
205 et al. (2010). PCR products (10 µl each) were mixed with 1 µl of alkaline gel-loading buffer
206 containing 300 mM NaOH, 6 mM EDTA, 18% (w/v) Ficoll and 0.25% (w/v) xylene cyanol
207 and then loaded on acrylamide gel containing a denaturing urea–formamide gradient ranging
208 from 20% to 60%. A 100% concentration of denaturant was defined as 7 M urea and 40%
209 (v/v) formamide (Muyzer et al. 1993). Electrophoresis was run at a constant voltage of 200 V
210 for 3 h at a constant temperature of 60°C. After DGGE, the gel was stained for 30 min in 0.5×
211 TAE containing 1 SYBR Green (BioWhittaker, Walkersville, MD, USA) and photographed
212 under UV illumination.
213

3. Results and Discussion

3.1. Reviving the glycerol-stocked Tank-2 consortium

The TuO-degrading consortium Tank-2 was formulated in 2009. This bacterial consortium exhibited a significantly and consistently high activity to degrade TuO (>90% of total TuO for two weeks at 30°C; Hosokawa et al. 2010). Since that time it has been preserved as a glycerol stock at −80°C.

As shown in Figure 1a, TuO degradation by revived Tank-2 gradually increased up to approximately 90% of the total for the third enrichment culture and this activity was consistently maintained after the fourth enrichment culture (data not shown). Considering that TuO degradation by the original Tank-2 consortium was also consistently approximately 90% (Hosokawa et al. 2010), it was concluded that Tank-2 could be completely revived after storage at −80°C for more than three years. The revived Tank-2 consortium that was enriched more than three times was used for further experiments.

Figure 1a also shows the time-dependent increase in TuO degradation by a newly prepared TuO-degrading consortium (Moiwa-KK). After the third enrichment culture, TuO degradation by the revived Tank-2 and Moiwa-KK consortia was consistently approximately 90% and approximately 80%, respectively, of total TuO (Fig. 1b).

3.2. Degradation of different petroleum products by revived Tank-2

The original Tank-2 consortium could degrade not only TuO, but also different types of petroleum products, such as diesel oil, car engine oil and Arabian light crude oil (Fig. 2a and Hosokawa et al. 2010). In this study, in addition to these petroleum products, used car engine

239 oil and Vityaz crude oil were also used as the sole carbon and energy sources for the revived
240 Tank-2 consortium. Revived Tank-2 degraded TuO (63% of total), car engine oil (64% of
241 total), used car engine oil (81% of total), Arabian light crude oil (72%) and Vityaz crude oil
242 (28% of total) after one week (Fig. 2b). Except for Vityaz crude oil, > 80% of each petroleum
243 product was degraded by revived Tank-2 after two weeks (Fig. 2b). The relatively high
244 degradation activity for engine oil by the revived Tank-2 consortium was probably because
245 the main components of car engine oil, as well as TuO, are n-alkanes (Koma et al. 2003).
246 Thus, the bacterial community structure of the Tank-2 consortium may be readily adaptable
247 to a medium that contains car engine oil.
248 Arabian light crude oil was completely degraded by revived Tank-2 after two weeks.
249 However, Vityaz crude oil degradation was less than that for Arabian light crude oil (Fig. 2b).
250 This longer time-dependent degradation of Vityaz crude oil by revived Tank-2 implies that a
251 much longer culture time in MSM supplemented with Vityaz crude oil would be needed for
252 Tank-2 to adjust its microbial structure to be more adaptable to this crude oil, which contains
253 more toxic, volatile and aromatic hydrocarbons than Arabian light crude oil (Maki et al.
254 2008).
255 The original Tank-2 consortium was known to form cell aggregates during culture in
256 TuO-containing MSM and TuO degradation was considerably reduced when these bacterial
257 aggregates were not inoculated along with a liquid aliquot that contained free-living bacteria
258 (Hosokawa et al., 2010). These results suggested that free-living bacteria and bacteria
259 comprising cell aggregates were both involved in TuO degradation in the original Tank-2
260 culture. Cell aggregates were also formed in the revised Tank-2 culture with TuO-containing
261 MSM, which indicated that bacteria in cell aggregates and liquid culture were also both
262 involved in TuO degradation by revised Tank-2. In contrast, during the degradation of fresh
263 and used car engine oils, Arabian light crude oil and Vityaz crude oil by revived Tank-2, cell

aggregates did not form and thus, the bacterial strains involved in degrading these oil products may be different from those involved in degrading TuO (see below).

3.3. Degradation of TuO in wastewater by revived Tank-2

The capacity to degrade TuO by revived Tank-2 was evaluated using TuO-containing wastewater collected at a hydraulic plant where TuO was routinely used. The concentration of TuO in this wastewater was estimated to be 3% (w/v) by TCL-FID. For the degradation test, the concentration of TuO was adjusted to 1.5% (see Table 1).

Approximately 70% of the total TuO in wastewater was degraded by revived Tank-2 after one week (Table 1). This was much higher than the 37% of the total in the degradation test when fresh (unused) TuO was used as a substrate. Interestingly, TuO degradation in the wastewater inoculated with no revived Tank-2 was approximately 64%. These results demonstrated that revived Tank-2 could utilize TuO components in wastewater as carbon sources and that these components could be preferentially utilized by bacterial strains native to the wastewater.

The relatively low TuO degradation rate by revived Tank-2 in freshly prepared MSM could be explained by: (1) revived Tank-2 may inherently consist of bacteria that can degrade TuO at its relatively low concentration (i.e. 1.5% TuO inhibits the growth of TuO degraders in the revived Tank-2 consortium); (2) bacteria native to TuO-containing wastewater used in this study can degrade TuO at concentrations as high as 1.5%; and (3) TuO wastewater may contain a compound(s) that stimulates the growth of TuO degraders.

Because this wastewater might contain some metals derived from turbine facilities and these metal contaminants might affect bacterial growth, the major metal elements in the TuO-containing wastewater were analysed by ICP. Al (11 ppm), Cr (0.06 ppm), Cu (0.3 ppm),

289 Fe (17 ppm), Pb (0.16 ppm) and Zn (0.30 ppm) were the principal components detected in the
290 TuO-containing wastewater, and all of these metals were at lower than detectable levels in
291 fresh (unused) TuO. Although it has not been formulated whether elements contained in the
292 wastewater affected TuO degradation by revived Tank-2, one or more elements in this
293 wastewater may enhance the growth of TuO-degrading bacteria in this consortium. This
294 speculation is supported in that revived Tank-2 preferentially degraded used car engine oil as
295 compared to fresh car engine oil (Fig. 2b), although the metal components in the used car
296 engine oil were not analysed.
297 All of these findings suggest that bacterial growth in revived Tank-2 would be
298 enhanced rather than inhibited by elements and/or unidentified substances in the
299 TuO-containing wastewater and in used car engine oil. TuO degradation in a culture with no
300 inoculums was higher than that in the culture inoculated with the revised Tank-2 consortium
301 (Table 1). This suggested that bacteria native to the TuO-containing wastewater used in this
302 study could have adapted to this TuO-containing wastewater environment and that these
303 bacteria exhibited a higher degradation activity towards TuO than the bacteria in revived
304 Tank-2.
305
306 *3.4. Microbial community structure of Tank-2 consortia*
307
308 Based on DGGE analysis that targeted 16S rRNA gene sequences, the original Tank-2
309 consortium consisted of at least 14 principal bacterial strains (Fig. 3, bands 1–14) and its
310 DGGE band profile was consistently maintained when it was continuously cultured in MSM
311 containing TuO (Hosokawa et al. 2010). The number of DGGE bands for the freeze–thawed
312 sample of Tank-2 decreased to 10 (bands B, C, D, F, H, J, K, N, O, P and S) and this band
313 profile was significantly different from that of original Tank-2 (Fig. 3a). The intensity of each

band for freeze–thawed Tank-2 was low, even though a whole DNA sample extracted from 1 ml of frozen Tank-2 was used as a template for PCR when amplifying the 16S rRNA gene. This suggested that some of the bacteria cells in the frozen Tank-2 culture might have died and that their genomes had decayed during thawing.

Only three DGGE bands (bands 5 and C, bands 6 and D, bands 8 and F) were common to the original and freeze–thawed Tank-2 consortia. The number of bands for revived Tank-2 was 13 (bands A, B, D, E, G, I, L, M, N, P, Q, R and S), but its DGGE band profile differed significantly from that of original Tank-2. Only bands 10 and I appeared to be common to these two consortia. Interestingly, relatively dense bands, such as bands E, G, I, L and M, appeared only for revived Tank-2, which suggested that bacteria corresponding to some of these DGGE bands could be simply freeze–thaw-resistant, but not TuO-degrading. Although TuO-containing-wastewater sampling to formulate Tank-2 and Moiwa-KK was performed at the same hydraulic plant of the electric power company and the enrichment processes used were also the same, the microbial community structures of these two consortia were entirely different.

The DGGE band profiles for the cultures grown in MSM supplemented with TuO, engine oil, and Arabian light and Vityaz crude oils were compared (Fig. 3b). Some bands (bands B, E, G, M, N, P, Q, and R) were common to all four cultures. However, band A was common and band G was dominant for the consortia containing lubricating oils (TuO and car engine oil) and bands M, N, and P were commonly dominant for the consortia containing crude oils (Arabian light and Vityaz). Because cycloalkanes are the major components only in lubricating oils, bacteria corresponding to bands A and G may be cycloalkane degraders. Similarly, bacteria corresponding to bands M, N and P in the cultures of crude oil may be degraders of polycyclic aromatic hydrocarbons (PAHs), which are the major components only in crude oils.

Figure 3c shows the DGGE band profiles for the microbial consortia cultured in TuO-containing wastewater. Ten bands (bands B, C, D, F, G, M, N, P, R, and S) were detected for revived Tank-2-inoculated wastewater culture that contained 1.5% TuO (lane 3 in Fig. 3c). Relatively dense bands (B, D, M, and P) were detected for Tank-2 culture supplemented with fresh TuO at 1.5% (lanes 2 and 3 in Fig. 3c), which suggested that bacteria corresponding to these DGGE bands were native in revived Tank-2 and that these bacteria would be mainly responsible for degrading a high concentration (1.5%) of TuO. Bands b, g, h, and s were detected only for the 1.5% TuO-containing wastewater culture inoculated with no Tank-2 (lane 4 in Fig. 3c), which indicated that bacteria corresponding to these bands originated from the wastewater.

Nine DGGE bands (bands a, c, e, f, j, l, n, p, and r in lane 5, Fig. 3c) were detected for the Moiwa-KK consortium, which had been formulated in TuO (0.5%)-containing MSM. Although the same TuO-containing wastewater was used, no common bands were detected between the Moiwa-KK consortium and the culture containing 1.5% TuO-containing wastewater. These results again suggested that the bacterial species capable of degrading 0.5% TuO and 1.5% TuO were different.

4. Conclusions

A TuO-degrading bacterial consortium, Tank-2, maintained its high capacity to degrade TuO after storage for 3.5 years at −80°C and could utilize various types of petroleum products as substrates without enrichment culture. TuO in wastewater and used EO were degraded well by revived Tank-2. However, most of the bacterial strains in the original and the revived TuO-degrading microbial consortia were different form one another. Bacterial strains inherently residing in Tank-2 had the potential to flexibly adjust their composition in

accordance with storage and culture conditions. Tank-2, and probably the new consortium Moiwa-KK, even after freeze-storage can be used as inocula for bioaugmentation to remediate waters that are polluted with various types of lubricating and crude oils.

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Figure legends

Figure 1. Degradation of turbine oil (TuO) in TuO-containing mineral salts medium by the revived Tank-2 consortium and a newly prepared TuO-degrading consortium, Moiwa-KK. (a) Time-dependent increases in TuO degrading activity of revived Tank-2 and Moiwa-KK. (b) TuO degradation by the original and revived Tank-2 consortia and Moiwa-KK. Culture was carried out for two weeks at 30°C with shaking at 160 rpm. TuO was extracted and analysed as described in the text.

Figure 2. Degradation of different types of petroleum hydrocarbons by original and revived Tank-2. (a) Original and (b) revived-Tank 2 consortia were cultured in MSM containing 0.5% (w/v) of different petroleum products. The results for original Tank-2 were obtained using the consortium maintained by continuous culture in MSM containing 0.5% TuO (see Hosokawa et al. 2010). Culture was for two weeks at 30°C, after which any remaining petroleum products were extracted and analysed as described in the text.

Figure 3. PCR-denaturing gradient gel electrophoresis (DGGE) analysis targeting the 16S rRNA gene sequences in different culture types. (a) DNA for templates was extracted from freeze–thawed Tank-2 (Freeze–thawed) and revived (Revived) Tank-2. Capital letters indicate DGGE bands for freeze–thawed and revived Tank-2 cultures. Bands D, N, P and S were commonly detected for freeze–thawed and revived Tank-2. Lane for Original indicates the DGGE profile for the original Tank-2 culture by Hosokawa et al. (2010). (b) Template DNA was extracted from revived Tank-2-inoculated cultures in MSM that contained either TuO (TuO), unused car engine oil (Engine oil), Arabian light crude oil (Arabian light) or Vityaz crude oil (Vityaz). Capital letters indicate DGGE bands corresponding to the DGGE bands

456 for freeze–thawed and revived Tank-2 cultures shown in Fig. 3a. Bands indicated by arrows
457 at the same distance from the top of gel have the same denotation. The band indicated by *
458 was detected only in the Arabian light crude oil culture. (c) Revived Tank-2 grown in MSM
459 containing 0.5% TuO was inoculated in MSM that contained either 1.5% unused TuO
460 (culture 2) or 1.5% TuO-containing wastewater (culture 3). For culture 4, no revived Tank-2
461 was inoculated into 1.5% TuO-containing wastewater. Culture 5 was a newly formulated
462 TuO-degrading Moiwa-KK consortium that was cultured in MSM containing 0.5% TuO.
463 Culture was for one week at 30°C with shaking at 160 rpm. Lane shown is the same as the
464 lane for Revived in Fig. 3a. Capital letters for lanes 1, 2 and 3 indicate DGGE bands for
465 freeze–thawed and revived Tank-2 cultures. Small letters for lanes for cultures 4 and 5
466 indicate DGGE bands arising from the TuO-containing waste water collected for this study.
467
468

1 Fig. 1

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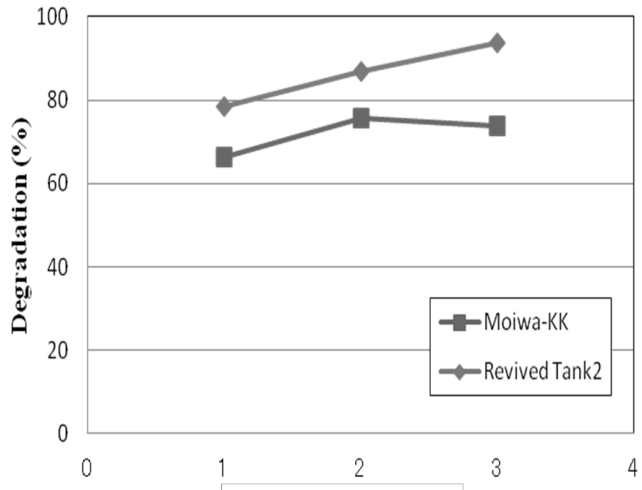
(a)



(b)

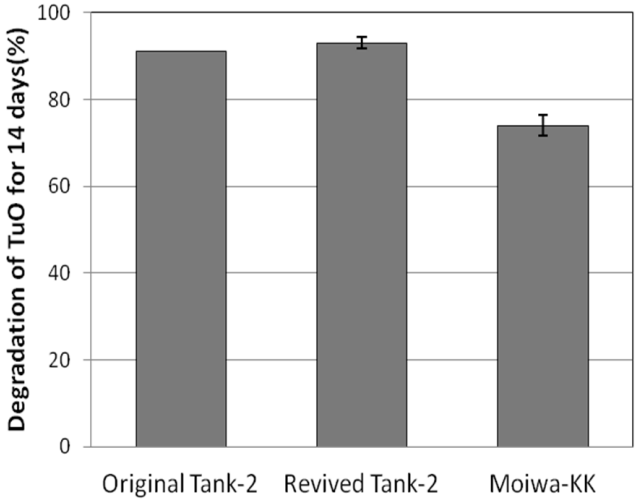


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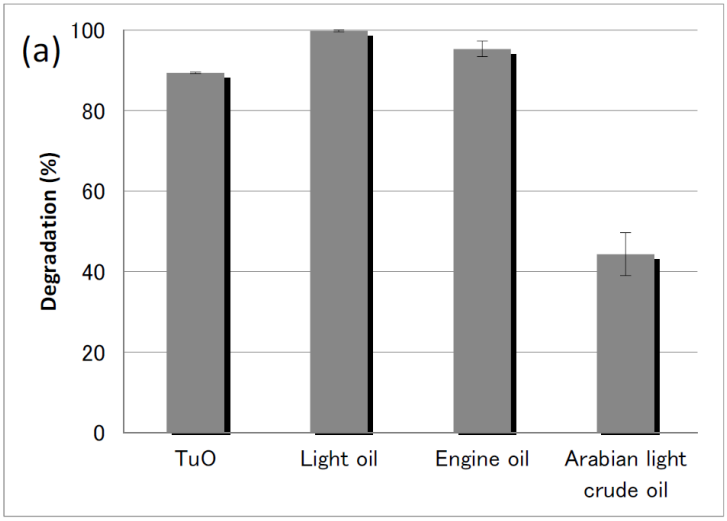
Figure2

Fig. 2.



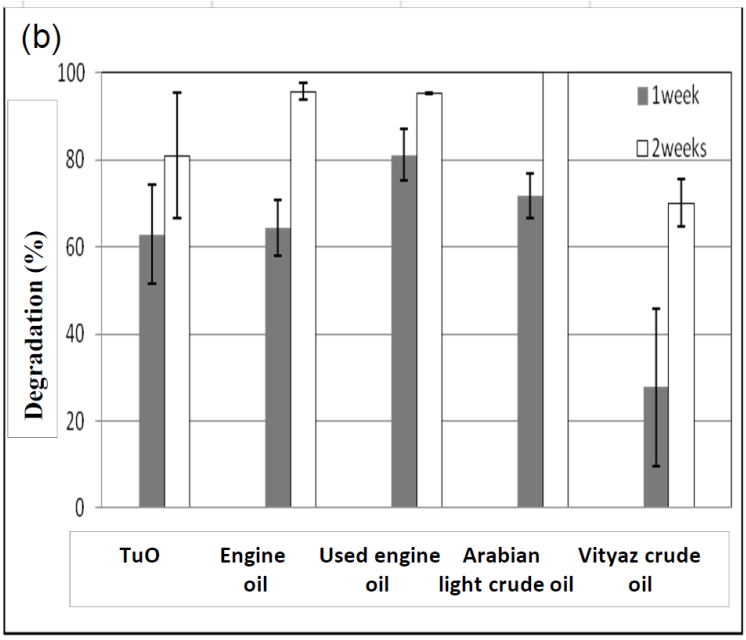


Fig. 3

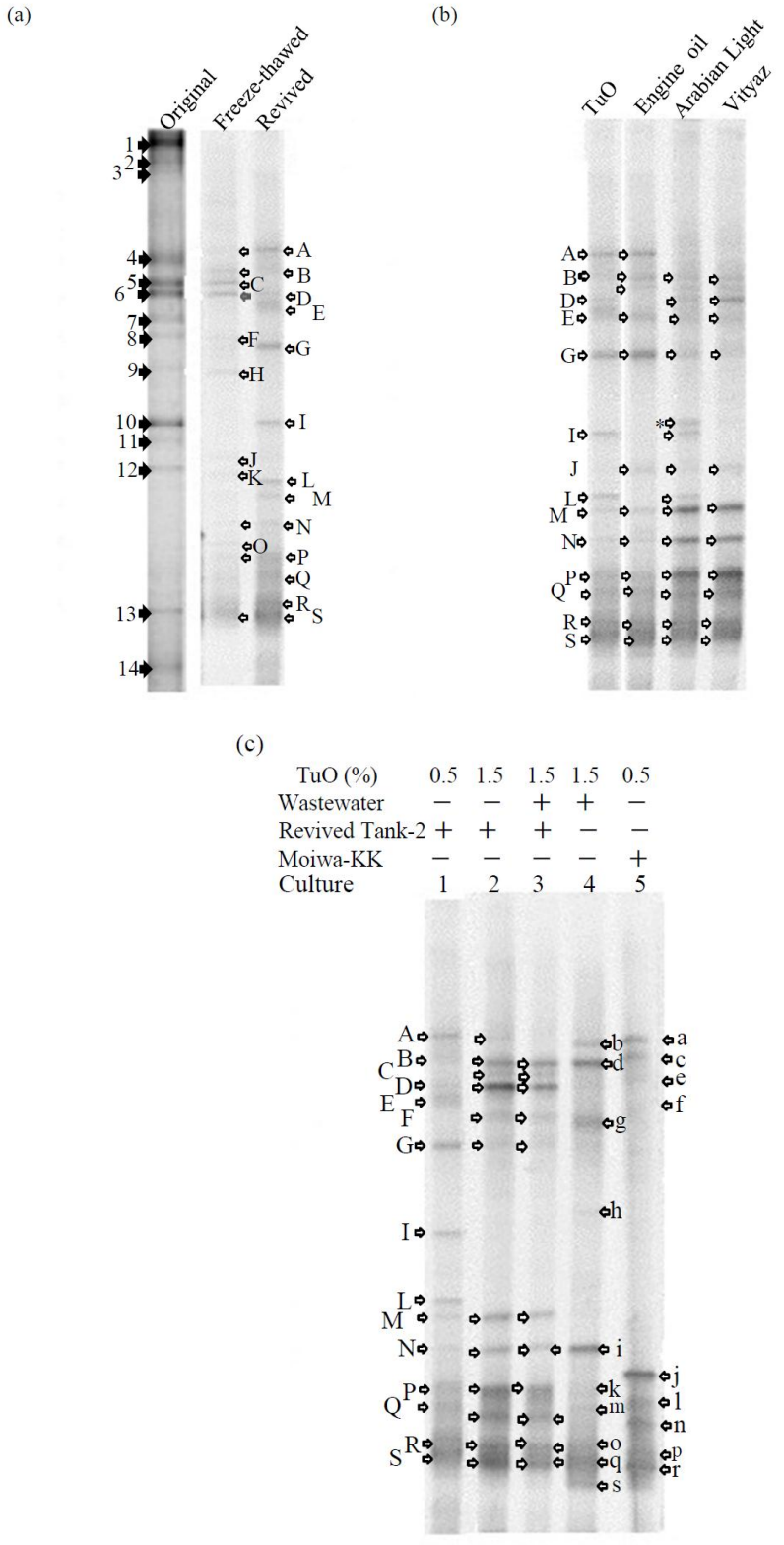

(a)
Original
Freeze-thawed
Revived
(b)
TuO
Engine oil
Arabian Light
Vityaz
(c)
TuO (%) 0.5 1.5 1.5 1.5 0.5
Wastewater − − + + −
Revived Tank-2 + + + − −
Moiwa-KK − − − − +
Culture 1 2 3 4 5

Table 1

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

Culture*	Media component**			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***	Not added	53.5% ± 6.9%

*, All cultures contained 1.5% (w/w) TuO.

**, MSM (×1), mineral salts medium (MSM) at its normal concentration; MSM (×2), MSM at two-times concentration; WW, original wastewater containing TuO (3%).

***, 200 µl of Moiwa-KK was added instead of Revived Tank-2.