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Enhanced isolation and culture of highly efficient psychrophilic oil-degrading bacteria from oil-contaminated soils in South Korea

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Abstract

It is known that isolation of oil-degrading bacterial strains is difficult at low temperatures, and the biodegradation efficiency of oil-contaminated soil is significantly reduced in cold weather. In this study, 14 strains were isolated from oil-contaminated soil that grew well at 10°C by using a newly developed culture method.11 of the 14 isolates were successfully cultured in mineral salts medium containing 1,500 ppm of oil components, 500 ppm each kerosene, gasoline, and diesel as carbon sources, at 10°C for 2 weeks. The oil degradation efficiencies of these 11 isolates ranged from 36% to 100%, as measured by total petroleum hydrocarbon (TPH) degradation analyses. Three strains (*Pseudomonas simiae* G1-10, *P. taiwanensis* Y1-4, and *P. koreensis* Gwa2) displayed complete degradation (100%), and six others (*P. frederiksbergensis* G2-2, *P. arsenicoxydans* Y2-1, *P. umsongensis* Gwa3, *P. migulae* Gwa5, *Rhodococcus jialingiae* Y1-1, and *R. qingshengii* Y2-2) showed relatively high degradation efficiencies (>70%). This study suggests that these isolates can be effectively utilised in thetreatment of oil-contaminated soil in landfarming, especially during winter.

Key words

Oil-contaminated soil, Psychrophilic, *Pseudomonas*, *Rhodococcus,* Bioremediation

Introduction

Increased oil demand and consumption worldwide has led to serious environmental and human health issues. Oil contamination causes soil damage, and it percolates through the soil into groundwater, affecting water quality and the associated ecosystems. Thus, exploring methodologies to control the environmental risks caused by petroleum has been an active research topic in environmental and biological technology. Initially, chemical and/or physical treatments were utilised for environmental cleaning, which allowed effective degradation of petroleum products, but adverse effects such as hazardous by-products have rendered them unsuitable. In contrast, bio remediation is versatile and efficient for long-term use, in addition to being economically and environmentally viable (Margesin *et al*., 2001; Jain *et al*., 2011).

Many microbes are capable of utilising hydrocarbons as source of carbon and energy for growth. The chemical nature of petroleum mixture and environmental factors are determinants of microbial utilisation (Ward and Brock, 1976; Atlas, 1981; Jain *et al*., 2011). Some commonly occurring bacterial species with high degradation capacity for hydrocarbons, such as *Pseudomonas* spp., *Rhodococcus* spp., *Acinetobacter* spp., *Micrococcus* spp., *Sphingomonas* spp., *Gordonia* spp., *Microbulbifer* spp., *Alcanivorax* spp., *Marinobacter* spp., *Cellulomonas* spp., and *Dietzia* spp. have been characterised (Aislabie *et al*., 2000; Belousova and Shkidchenko, 2004; Brito *et al*., 2006). Recently, microbial degradation at low temperatures was studied and methods were found to eradicate soil contamination in cold areas such as the Arctic and Antarctic regions (Aislabie *et al*., 2000; Bej *et al*., 2000; Baraniecki *et al*., 2002; Belousova and Shkidchenko, 2004; Stallwood *et al*., 2005). Another study emphasised the significant role played by cold-adapted microorganisms in *in situ* biodegradation of hydrocarbons in cold environments. However, it is difficult to isolate efficient oil-degrading bacterial strains at low temperature, and this is particularly evident in samples from low-

temperature regions (Margensin *et al*., 2003).

In this study, bacteria from oil-contaminated soils were isolated by using a novel culture method at low temperatures, which was able to simulate the natural soil conditions, resulting in the isolation of a large number of highly effective psychrophilic oil degraders (nine strains with efficiency greater than 70%).

Materials and Methods

Isolation of oil-degrading bacteria at low temperatures: Petroleum hydrocarbons used throughout this study were gasoline (~C_{10}), diesel (C_9–C_{23}), and kerosene (C_8–C_{18}), which were purchased from a gas station (GS Caltex, Suwon, Korea). Oil-contaminated soil samples were collected from three places in South Korea where US army was stationed: *viz*., Goyang, Gwacheon and Yongsan. Mineral salts medium (MSM) containing 1,500 ppm oil (500 ppm gasoline, 500 ppm diesel, and 500 ppm kerosene) was used as culture medium. Trans-well plates were used to isolate microorganisms from soil (Fig. 1). For isolation, 3 g of soil was added per well, in which each insert was placed. Subsequently, 3 ml of medium and 100 µl of inoculum (soil suspension) were added to the insert, the plate was capped, and then cultured in a shaking incubator for 2 weeks at 10°C.The liquid culture was serially diluted 1,000-fold, and approximately 100 µl of the diluted culture suspension was spread onto the MSM agar plate, with unique colonies selected after 1 week for further isolation on agar plates.

Assessment of oil-degradation efficiency: To examine the efficiency of oil degradation, 10 ml of MSM containing 1,500 ppm of the above mentioned oil components and 0.1 g of wet cells were transferred into a 50 ml screw-capped bottle (to prevent loss of hydrocarbon components due to volatility). For control, no bacterial cells were added. The bottles were then incubated in a shaking incubator for 2 weeks at 10°C.

Determination of total petroleum hydrocarbon: Total petroleum hydrocarbon (TPH) concentration was determined by gas chromatography (GC). TPH was extracted from 10 ml of each sample with an equal volume of dichloromethane (DCM) (Wako Chemical Co., Osaka, Japan) for 24 hr in a shaker. Next, 2 µl of DCM supernatant was transferred into a vial for GC analysis. TPH concentration in each extract was measured using gas chromatograph (HP 5890 Series II, Agilent, USA) equipped with a flame ionisation detector (FID), using an Ultra 2 capillary column (cross-linked, 5% phenylmethylsilicone; length, 25 m; internal diameter, 0.32 mm; and film thickness, 0.17 µm). The oven temperature was set at 50°C for the initial 2 min and increased by 8°C per min to a final temperature of 320°C, which was maintained for 10 min. The flow rate of carrier gas (N_2) was set at 1.0 ml min^{-1}, and the temperatures of injector and detector were 300°C and 320°C, respectively. The total sample volume injected was 2 µl. The percentage of degradation were calculated based on the differences between initial TPH concentration and final TPH concentration, determined by a standard TPH curve. Degradation can be considered as mineralisation because TPH degradation rates are usually similar to mineralisation rates.

Identification of microbial species by 16S rRNA sequencing: PCR-amplified 16S rRNA was sequenced to determine the 16S rRNA primary sequence. First, bacterial genomic DNA samples were extracted using an Instagene Matrix (BIO-RAD). Major part of 16S rRNA gene was amplified by PCR, using universal bacterial primer set: 518F, 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R, 5'-TAC CAG GGT ATC TAA TCC-3'. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing was performed using a PRISM Bigdye Terminator v3.1 Cycle Sequencing Kit. Primers used in these reactions were 518F and 800R. The mixture was incubated at 95°C for 5 min, cooled on ice for 5 min, and then analysed using an ABI Prism 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). The full sequence of 16S rRNA gene was compiled using the software SeqMan (DNASRAR Inc., USA). Using the EZtaxon server, phylogenetic neighbours were identified and the pairwise 16S rRNA sequence similarities were calculated (Kim *et al*., 2012).

Results and Discussion

Oil-contaminated soil samples were collected in March, when psychrophilic oil degraders may still dominate the soil. Fourteen bacterial strains were isolated from the samples by using the transwell plate liquid culture method. The method was modified to increase the diffusion and extraction effects of soil micronutrients into the culture medium as in the previous method, which did not use liquid medium with shaking and the agar plate spreading step (Svenning *et al*., 2003). This method was also simpler than the previous method because a light microscope or other specific tools are not needed. This method is more useful for obtaining diverse isolates than conventional methods, which led to no growth of three isolates (Table 1).

The strains grew well at 10°C and were grouped into the following four genera based on the criteria of 16S rRNA sequences: *Pseudomonas* spp., 9; *Rhodococcus* spp., 3; *Arthrobacter* sp., 1; and *Janthinobacterium* sp., 1 (Table 1). They were assumed to be oil degrading or oil tolerant because of their survival in oil-contaminated soil. This new method simulated the natural environment by using soil (Pham and Kim, 2014). The traditional culture medium (MSM) used in this study can include various carbon sources, complex compounds, and inorganic materials from soil, yielding higher numbers and more diverse isolates than similar media with only carbon sources with petroleum and simple nutrients (Pham and Kim, 2012).

Of the 14 strains isolated, 11 strains could degrade oil (mixture of gasoline, diesel, and kerosene) at 10°C (Table 1 and Fig. 2). These could be divided between two genera-*Pseudomonas* and *Rhodococcus* with high or low TPH-degrading rates. Three

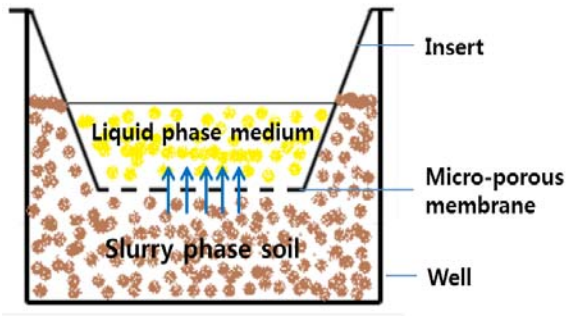

Fig. 1 : Schematic transwell system used for isolating bacteria from oil-contaminated soil. Arrows indicate direction of soil nutrient diffusion into liquid medium through a micro-porous membrane

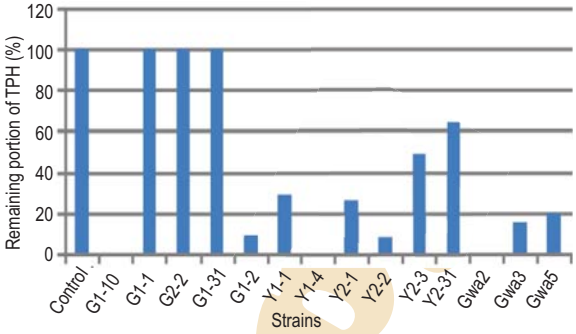

Fig. 2 : Residual TPH for individual isolates grown in liquid medium at 10°C for 2 weeks. Note: control (no cells) indicates 100% remaining as well as three isolates that did not grow

Table 1 : Psychrophilic bacterial strains isolated from oil-contaminated soils, including their closest species and GenBank accession numbers, and oildegradation efficiencies

No.	Strain	Closest species	Similarity (%)	Accession no.[1)]	Degradation efficiency (%)	Accession no.[2)]
1	G1-10	*Pseudomonas simiae*OLi^T	99.48	AJ936933	100.0	KF578422
2	G1-1	*Janthinobacterium lividum* DSM 1522^T	99.31	Y08846	(-)[3)]	KF578420
3	G1-31	*Arthrobacter nitroguajacolicus* G2-1^T	98.97	AJ512504	(-)	KF578423
4	G1-2	*Rhodococcus baikonurensis* GTC 1041^T	100.00	AB071951	(-)	KF578421
5	G2-2	*Pseudomonas frederiksbergensis* JAJ28^T	99.93	AJ249382	90.6	KF578424
6	Y1-1	*Rhodococcus jialingiae* djl-6-2^T	100.00	DQ185597	71.0	KF604730
7	Y1-4	*Pseudomonas taiwanensis* BCRC 17751^T	99.72	EU103629	100.0	KF578428
8	Y2-1	*Pseudomonas arsenicoxydans* VC-1^T	99.09	FN645213	73.4	KF604731
9	Y2-2	*Rhodococcus qingshengii* djl-6^T	100.00	DQ090961	91.5	KF578429
10	Y2-3	*Pseudomonas mandelii* CIP 105273^T	99.45	AF058286	51.4	KF578430
11	Y2-31	*Pseudomonas arsenicoxydans* VC-1^T	99.86	FN645213	36.0	KF578431
12	Gwa2	*Pseudomonas koreensis* Ps 9-14^T	99.96	AF468452	100.0	KF578425
13	Gwa3	*Pseudomonas umsongensis* Ps 3-10^T	100.00	AF468450	84.5	KF578426
14	Gwa5	*Pseudomonas migulae* CIP 105470^T	99.86	AF074383	80.5	KF578427

[1)]accession number of closest species in GenBank; [2)]accession number of isolated strains in GenBank; [3)](-) indicates no bacterial growth

isolates showed 100% degradation of TPH at 1,500 ppm hydrocarbon at 10°C during 2 weeks (Fig. 3):*Pseudomonas* sp. G1-10, Y1-4, and Gwa2. They were similar to *P. simiae* OLi^T, *P. taiwanensis* BCRC 17751^T, and *P. taiwanensis* BCRC 17751^T, respectively. *P. simiae* OLi^T was isolated from clinical specimens from monkeys (Vela *et al*., 2006), *P. taiwanensis* BCRC 17751^{T}from soil (Wang *et al*., 2010) and *P. koreensis* Ps 9-14^T from farm soil (Kwon *et al*., 2003).

The other effective degraders were *Rhodococcus* sp. Y2-2 (91.5% efficiency), *Pseudomonas* sp. G2-2 (90.6%), *Pseudomonas* sp. Gwa3 (84.5%), *Pseudomonas* sp. Gwa5 (80.5%), *Pseudomonas* sp. Y2-1 (73.4%), *Rhodococcus* sp. Y1-1 (71.0%), *Pseudomonas* sp. Y2-3 (51.4%), and *Pseudomonas* sp. Y2-31 (36.0%). The strains closest to them were *R. qingshengii* djl-6^T, isolated from carbendazim-contaminated soil (Xu *et al*., 2007); *P. frederiksbergensis* JAJ28^T, isolated from soil at coal gasification site and closely related to phenanthrene-degrading bacteria (Andersen *et al*., 2000); *P. umsongensis* Ps 3-10^T (Kwon *et al*., 2003); *P. migulae* CIP 105470^T (Verhille*et al*., 1999); *R. jialingiae* djl-6-2^T, isolated from sludge of carbendazim wastewater treatment facility (Wang *et al*., 2010); *P. mandelii* CIP 105273^T (Verhille*et al*., 1999) and *P. arsenicoxydans* VC-1^T (Campos *et al*., 2010).

Collectively, this study led to successful identification of nine *Pseudomonas* members and two *Rhodococcus* members as effective psychrophilic oil degraders. Previous studies have found that members of genus *Pseudomonas* were active degraders at low temperature (Margensin *et al*., 2003; Belousova and Shkidchenko, 2004; Stallwood *et al*., 2005). Several studies have also reported that many *Rhodococcus* strains may effectively

degrade oil components in cold environments (Whyte *et al*., 1998; Bej *et al*., 2000; Margensin *et al*., 2003; Belousova and Shkidchenko, 2004). However, the number of isolates described here in were greater and the TPH degradation efficiencies appeared higher than previously reported (Whyte *et al*., 1998; Bej *et al*., 2000; Belousova and Shkidchenko, 2004), although they were tested under different conditions. For examples, Whyte and colleagues (1998) showed that *Rhodococcus* sp. Q15 was able to degrade 15–53% alkanes at 5°C in MSM containing 0.1% (v/v) diesel for 2 weeks, and Bej *et al.* (2000) showed that *Rhodococcus* spp. mineralised approximately 33% dodecane (C_{12}, 0.07% (v/v)), as a representative of alkanes, at 16°C in Bushnell Haas media for 15 days. In addition, the maximum petroleum degradation (40%) was observed in *Rhodococcus* sp. DS-07, cultivated at 4–6°C for 10 days in liquid mineral nutritive medium containing 8.16 g l^{-1} petroleum (Belousova and Shkidchenko, 2004).

While 11 strains grew rapidly within 3 days at 10°C and 1,500 ppm oil, three isolates, namely, *Janthinobacterium* sp. G1-1, *Arthrobacter* sp. G1-31 and *Rhodococcus* sp. G1-2, did not grow well in artificial media (Pham and Kim, 2012) or did not use oil components as carbon sources. For future study, to make the continuous growth of these three strains not grown, we may develop different media and / or different culture systems from the one developed in this study.

In conclusion, using a novel culture technique, this study isolated a number of highly efficient psychrophilic oil degraders that may help in achieving better *in situ* and *ex situ* bioremediation for cleaning oil-contaminated soil during winter.

Acknowledgments

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