Bull Environ Contam Toxicol (2012) 89:680–685
DOI 10.1007/s00128-012-0723-7

Removal of Crude Oil by Microbial Consortium Isolated from Oil-Spilled Area in the Korean Western Coast

Su-Hee Cho · Kye-Heon Oh

Received: 12 March 2012 / Accepted: 29 June 2012 / Published online: 11 July 2012

Abstract The feasibility of using an indigenous microbial consortium for the removal of crude oil from an oil-spilled coastal area was explored with the ultimate aim of applying for bioremediation. Initially, we obtained the microbial consortium TK-2 that catalyzed the dispersion as well as the degradation of crude oil in supplemented sea water. GC and GC-MS were used to evaluate the removal patterns of crude oil during the incubation. The effective removal of crude oil by TK-2 occurred, and above 95 % of all aliphatic and aromatic compounds detected in this work was removed within 30 days of incubation. Two predominant crude oil-grown isolates derived from TK-2 revealed Gram-negative, rod-shaped cells. Both BIOLOG system and 16S rRNA sequencing were conducted to identify the strains, which were assigned to *Arthrobacter* sp. HK-2 and *Pseudoalteromonas* sp. HK-3, and registered in GenBank as [FJ477042] and [FJ477041].

Keywords Crude oil removal · Microbial consortium · Oil-spilled coastal area · Tae-An

Because oil spills can result in significant contamination of terrestrial, ocean, and shoreline environments, they receive a great deal of attention and evoke considerable public concern (Harayama et al. 1999). Several large accidental oceanic oil spills have occurred in recent history, most notably the *Amoco Cadiz* spill in the Brittany coastal area in 1978, the *Exxon Valdez* spill into Prince William Sound in 1989, the *Persian Gulf War* spill in 1991, and the *Gulf of Mexico* oil spill in 2010 (Atlas and Bragg 2009; Hazen et al. 2010).

The worst oil spill incident in Korea occurred on December 7, 2007, when a crane-carrying barge slammed into the *Hebei Spirit* oil tanker, resulting in the leakage of approximately 2.7×10^6 gallons of crude oil, a mix of Iranian Heavy, UAE Upper Zakum and Kuwait Export crude oils, into areas of the Tae-An National Maritime Park, located in the Western part of the Korean peninsula (ITOPF 2008). This incident was a great shock to the environment and to the economy, contaminating a substantial length of coastline and causing a considerable threat to the coastal aquaculture industry.

In general, spilled oil is removed by physical and chemical methods, which include treatment with absorbent pads, shore washing with high pressure water, mechanical tilling, removal of oiled sediments, and/or spraying with chemicals (Leacock 2005; Cunha et al. 2006). Microbiological methods have been the subject of a great deal of debate and several research efforts in recent years. In fact, it is bioremediation using microorganisms that has received the most attention, particularly after the Exxon Valdez incident (Swannell et al. 1996; Atlas and Bragg 2009).

Crude oil is a mixture of many different types of hydrocarbons, such as aliphatic and aromatic compounds. A number of studies have been published regarding the isolation and characterization of oil-degrading bacteria (Toledo et al. 2006; Lin et al. 2009; Mittal and Singh 2006). Although a wealth of information is now available regarding the ecology, biochemistry, and genetics of bacteria that degrade oil (Hamamura et al. 2001; Van Hamme et al. 2003), little information is currently available regarding possible treatment processes in situ, where oil spills present a serious problem.

S.-H. Cho · K.-H. Oh (✉)
Department of Life Science and Biotechnology, Soonchunhyang University, P.O.Box 97, Asan, Chung-Nam 336-600, Republic of Korea
e-mail: kyeheon@sch.ac.kr

This work describes the initial phase of a study in which the feasibility of microbial degradation of oil was explored for cleaning up a polluted environment. Because of the ultimate objective, application for bioremediation around oil-contaminated areas, the present work emphasized the development of the microbial consortium TK-2, which is capable of degrading oil as a carbon and energy source. In the present study, the results of nucleotide sequence analysis and scanning electron microscopy for the oil-degrading isolates are also provided.

Materials and Methods

Several indigenous microbial consortia, which are capable of degrading crude oil, were isolated from five different contaminated areas in the Tae-An National Maritime Park using an enrichment culture technique (Fig. 1). Among the consortia, the consortium used in this work, designated as TK-2, was derived from Manri-Po (site 3), and was chosen based on its ability to remove crude oil. Enrichment was carried out in 250 mL Erlenmeyer flasks containing 100 mL Bushnell-Hass (BH) medium (Difco, USA) and 10 g of marine sediment samples. BH medium contained (per liter of filtered natural sea water): 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 0.02 g $CaCl_2{\cdot}2H_2O$, 0.2 g $MgSO_4{\cdot}7\ H_2O$, 1.0 g NH_4NO_3, 0.05 g $FeCl_2$ and 1 % crude oil recovered from spilled areas as the sole carbon and energy source. After about 6 days of incubation at 20 ± 1°C on a rotary shaker at 150 rpm, the crude oil became evenly dispersed throughout the liquid cultures in the flasks. One milliliter of the culture was then transferred to the same medium containing 1 % crude oil. After one transfer, the oil became dispersed in only 2–4 days. The microbial consortium was maintained by serial transfers to fresh media at 3-day intervals before the biodegradation experiments. At intervals of 10 days, residual oil compounds were determined for each of the inoculated and control flasks. The flasks were tightly sealed with screw caps bonded with polytetrafluoroethane/silicone septa to minimize volatilization and abiotic loss of crude oil. Control flasks were used as abiotic controls. After 10, 20, and 30 days, the extent of crude oil removal was measured by gas chromatography.

Pure cultures, each capable of producing a clear zone on BH solid media covered with 1 % crude oil, were isolated by streaking the microbial consortium, thereby suggesting that this consortium was a mixture of crude-oil degrading bacteria. Five different colony types were isolated and found to grow both on nutrient agar and crude oil-containing solid media. The isolates were maintained by periodic transfer on nutrient agar. The isolates were grown in nutrient broth for 24 h, harvested, washed, and suspended in saline. Crude oil was added to the BH liquid culture medium at a concentration of 1 % (v/v). The flasks were incubated at 20 ± 1°C with constant shaking at 150 rpm and employed for further study.

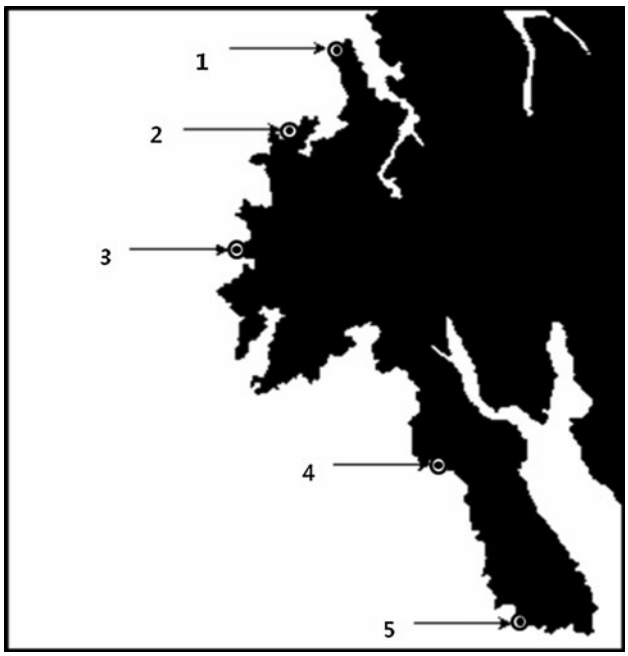

Fig. 1 Map showing the location of sampling sites around Tae-An; Sites *1*, Gurm-Po (36° 50′N, 126° 09′E); *2*, Hakam-po (36° 49′N, 126° 09′E); *3*, Manri-Po (36° 47′N, 126° 08′E); *4*, Baeksa-jang (36° 34′N, 126° 18′E); *5*, Baram-arae (36° 24′N, 126° 22′E)

Each collected sample was extracted with an equal volume of *n*-hexane, dehydrated with anhydrous sodium sulfate, filtered and concentrated on a rotary evaporator for 10 min, and then stored at 4°C in 2 mL glass vials sealed with screw-caps for analysis. Gas chromatography (GC) was carried out with an Agilent 6890 N gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a flame ionization detector. A DB-1 capillary column (30 m × 0.32 mm) with a film thickness of 0.25 μm was used, with helium as the carrier gas. The oven temperature program was as follows: increased from 40 to 170°C at 6°C/min and maintained for 3 min at 170°C, increased from 170°C to 300°C at 8°C/min, and held for 10 min at 300°C. The injector and detector temperature were maintained at 250 and 300°C, respectively. For calculation of residual oil compounds, peak areas (from triplicate samples) were quantified via integration. These values were expressed as the percentage removed relative to the amount of the corresponding compounds, which remained in the appropriate abiotic control samples. The samples were also analyzed via GC-MS. MS data were obtained with an Agilent 6973 mass selective detector equipped with an Agilent 6890 N gas chromatograph. The capillary column for GC-MS analysis was the same column used in GC analysis. The operation conditions used were as follows:

ionization energy, 70 eV; emission current, 200 lA; mass range m/z 50–500.

Genomic DNA was purified using a Clontech tissue kit. For the PCR amplification of 16S rRNA from two isolates, two universal primers, 27f (5′ AGA GTT TGA TCC TGG CTC AG) and 1522r (5′ AAG GAG GTG ATC CA(AG) CCG CA) were used as sense and anti-sense primers, respectively. PCR reactions were carried out for 1 min at 95°C, cycled 33 times (1 min at 95°C, 1 min at 55°C, 1 min 72°C), and then extended for 10 min at 72°C. The PCR products were inserted into pGEM-T vector, and transformed into *Escherichia coli* JM109. Two hundred nanograms of the double stranded DNA was employed as a template for sequencing together with both the T7 and SP6 primers. Nucleotide sequencing was conducted using an ABI 373A automated sequencer (Foster City, CA. USA). Sequence analysis was carried out using Lasergene software (DNA STAR, Inc., Madison, WI. USA) and BLAST database (NCBI, Bethesda, MD, USA) searches. The 16S rRNA sequences were aligned using Clustal X software (http://www.clustal.org), and the root phylogenetic tree was drawn via the neighbor-joining method in the MEGA4 package (Biodesign Institute, Tempe, AZ, USA). The 16S rRNA sequence data obtained in this study was deposited in the GenBank database (NCBI, Bethesda, MD, USA).

Colonies of oil-degrading bacteria grown on LB agar for 24 h were excised as small agar blocks. Subsequently, these colonies were pre-fixed, post-fixed, and dehydrated in an ascending graded series of ethanol. The cells were replaced with absolute hexamethyldisilazane (Electron Microscopy Sciences, Ft. Washington, PA, USA) and then air-dried. The cells were coated with gold and examined with a scanning electron microscope (Hitachi S-2500C, Japan).

Results and Discussion

The microbial consortium TK-2 was obtained from marine sediment samples collected from oil-spilled areas via enrichment with 1 % crude oil as the sole carbon and energy source. After 2–4 days of incubation, the oil fraction floating on the culture became visibly dispersed. Bacterial growth based on the turbidity increased during the first 2 days and then declined steadily. Removal patterns of 1 % crude oil were monitored every 10 days of incubation. TK-2 began to degrade all the aliphatic and aromatic compounds of crude oil simultaneously. The peaks indicated that the amounts of different compounds had decreased rapidly, and eventually all compounds were present only in extremely small amounts or were undetectable after 30 days of incubation, as shown by the gas chromatogram. Fourteen major compounds in crude oil were identified via GC-MS (Fig. 2). The mass spectra of the aliphatic and aromatic compounds are shown in Table 1. The crude oil used in this study contained an abundance of lower alkanes, including tridecane (C_{13}), pentadecane (C_{15}), and hexadecane (C_{16}). The aromatic contents in crude oil in order of 14.335, 19.836, and 29.244 min were naphthalene, fluorence, and pyrene, respectively. Both aliphatics (e.g., dodecane, tridecane, pentadecane, hexadecane, 2,6,10-trimethylpentadecane, 2,6,10,14-tetramethylhexadecane, *n*-nonadecane (C_{19}), eicosane (C_{20}), heneicosane (C_{21}), *n*-docosane (C_{22}), tricosane (C_{23}) and aromatics (e.g., naphthalene, fluorene, pyrene) were partially removed (approx. 60 %–90 %) during incubation, which lasted up to 10 days. The consortium TK-2 removed more of the short-chain alkanes than the long-chain alkanes when incubated up to 20 days, but >95 % of all the compounds detected in this work were removed within 30 days of incubation at 20°C.

Lai and Khanna (1996) reported that the mixed culture containing *Acinetobacter calcoaceticus* and *Alcaligenes odorans* degraded 40 %–58 % of crude oil, which was higher than that observed with individual cultures, and *A. calcoaceticus* degraded significantly more of the alkanes than the aromatics. In the abiotic controls, approximately 65 %–85 % of the compounds remained after 30 days of incubation, and these abiotic losses may be attributable to the volatilization or photooxidation of the compounds. Several recent studies have described the degradation of both aliphatics and aromatics by microbial consortia (Rahman et al. 2002; Tang et al. 2010). Sathishkumar et al. (2008) reported that a mixed bacterial consortium degraded a maximum of 77 % of the crude oil at 25°C. Additionally, Rahman et al. (2002) reported that the mixed bacterial consortium degraded a maximum of 78 % of the crude oil at 30°C. It is apparent that the removal of all aliphatics and aromatics by the indigenous microbial consortium TK-2 is more effective than has been previously reported.

Five isolates capable of utilizing crude oil as a sole carbon and energy source were isolated from the indigenous microbial consortium TK-2. In fact, our findings indicate that many kinds of bacterial strains may be involved in the degradation of crude oil. Among the isolates, two dominant bacterial strains (HK-2 and HK-3) were isolated from the microbial consortium, which was capable of growing on crude oil. The 16S ribosomal RNA gene obtained from the isolates was amplified via PCR using a universal bacterial primer set. Based on the BIOLOG system and phylogenetic analysis using 16S rRNA sequencing, two isolates obtained from the microbial consortium TK-2 were identified as *Arthrobacter* sp. HK-2 and *Pseudoalteromonas* sp. HK-3. The 16S rRNA sequence data have been deposited in the GenBank database under the accession numbers [FJ477042] for HK-2 and [FJ477041] for HK-3.

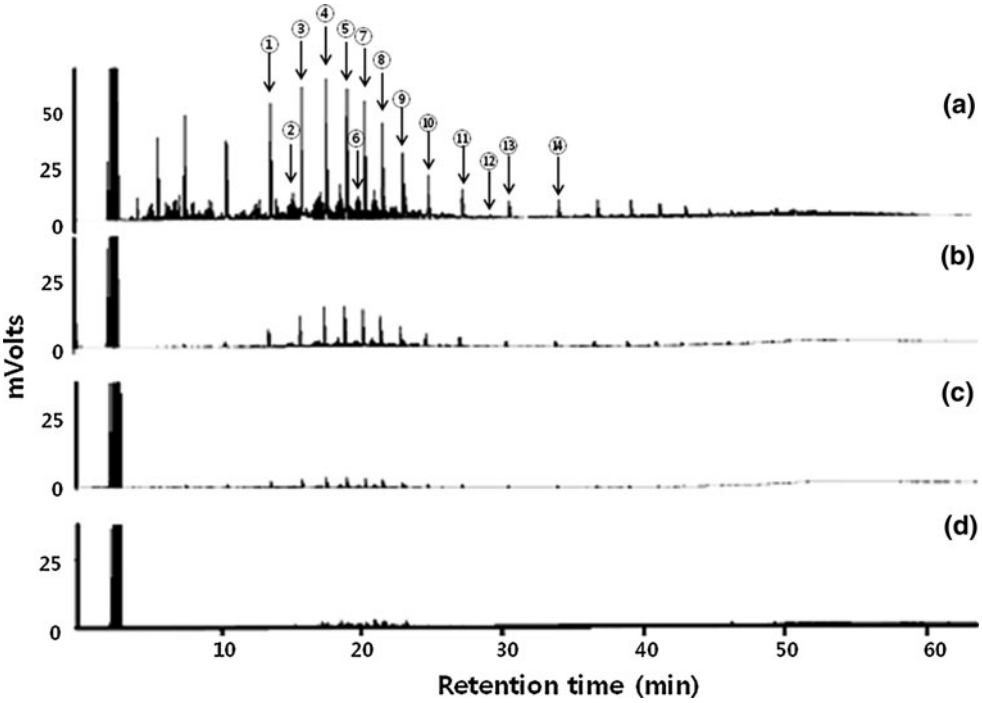

Fig. 2 Total ion chromatograms of supernatants of test culture TK-2 initially (**a**), after 10 days (**b**), 20 days (**c**), and 30 days of incubation (**d**). Major TIC peaks were identified via GC-MS analysis. Numbers for the peaks are shown in Table 1

Table 1 Total removal of major compounds in 1 % crude oil by the microbial consortium TK-2 based on GC-MS analysis data

Peak No	Retention time (min)	Compounds	Percentage of crude oil removed[a]				
			Total removal			Abiotic loss[c]	Biodegradation[d]
			10 days	20 days	30 days[b]		
1	13.703	Dodecane	90.3	94.7	99.6	34.7	64.9
2	14.335	Naphthalene	91.8	95.9	99.3	35.1	64.2
3	15.883	Tridecane	80.9	89.2	99.1	32.6	66.5
4	17.609	Pentadecane	76.2	87.2	98.2	31.4	66.8
5	19.303	Hexadecane	75.7	87.4	97.5	29.2	68.3
6	19.836	Fluorene	79.0	91.7	99.2	30.7	68.5
7	20.351	2,6,10-trimethyl-pentadecane	73.5	91.3	99.4	28.2	71.2
8	21.567	2,6,10,14-tetramethyl-hexadecane	61.2	79.6	98.2	24.7	73.5
9	22.991	*n*-Nonadecane	75.8	87.1	99.1	26.8	72.3
10	24.800	Eicosane	75.2	86.2	98.7	15.3	83.4
11	27.204	Heneicosane	73.5	85.8	97.9	21.7	76.2
12	29.244	Pyrene	72.6	85.2	99.3	30.3	69.0
13	30.488	*n*-Docosane	71.6	85.0	99.8	25.2	74.6
14	33.963	Tricosane	68.4	84.3	96.3	17.5	78.8

[a] The results are an average of two separates experiments each having three replicates. The standard deviation was around 6 %

% total crude oil remove after 30 days of incubation; b = c + d

It has been demonstrated that several bacteria are capable of utilizing crude oil as a sole source of carbon and energy. In particular, two representative genera, *Arthrobacter* and *Pseudoalteromonas*, have been reported to degrade crude oil effectively (Kato et al. 2001; Deppe et al. 2005; Lin et al. 2009). The growth of bacteria on crude oil presents particular problems because crude oil is immiscible in water. Many bacteria are able to emulsify crude oil by producing biosurfactants that increase the adhesion of cells to the oil droplet. Biosurfactants reduce the surface tension by accumulating at the crude oil-water interface, increasing the surface area of insoluble

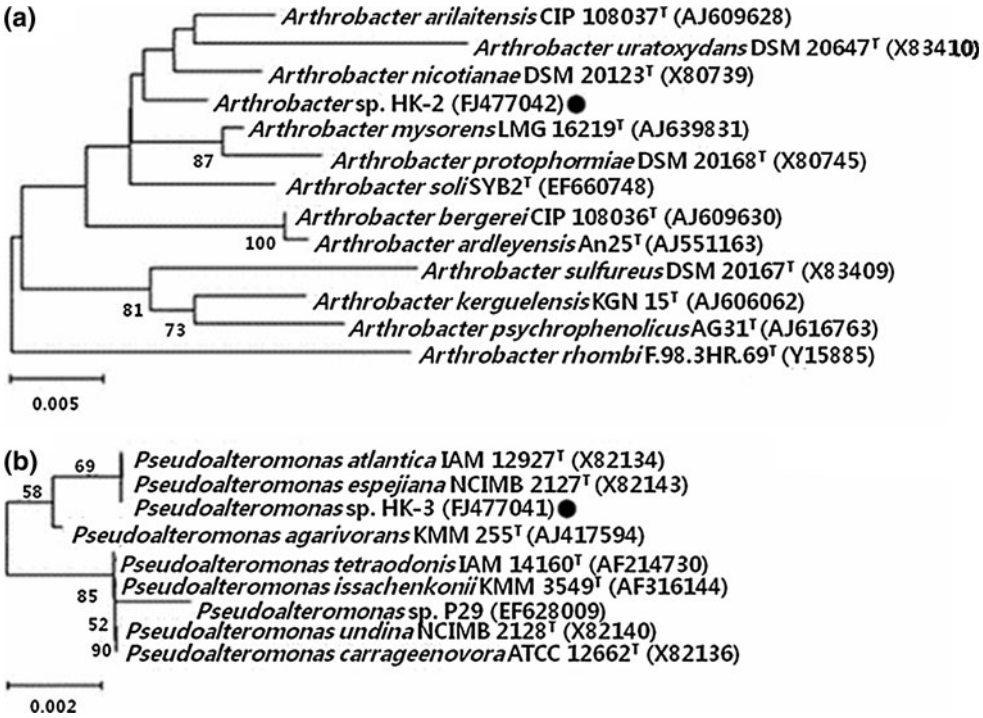

Fig. 3 Phylogenetic relationships between *Arthrobacter* sp. HK-2 (**a**) and *Pseudoalteromonas* sp. HK-3 (**b**), and their related species based on 16S rRNA gene sequence analysis. Bootstrap percentages (based on 100 replicates) >50 % are shown at branch points. Bar, 0.005 substitutions per nucleotide position

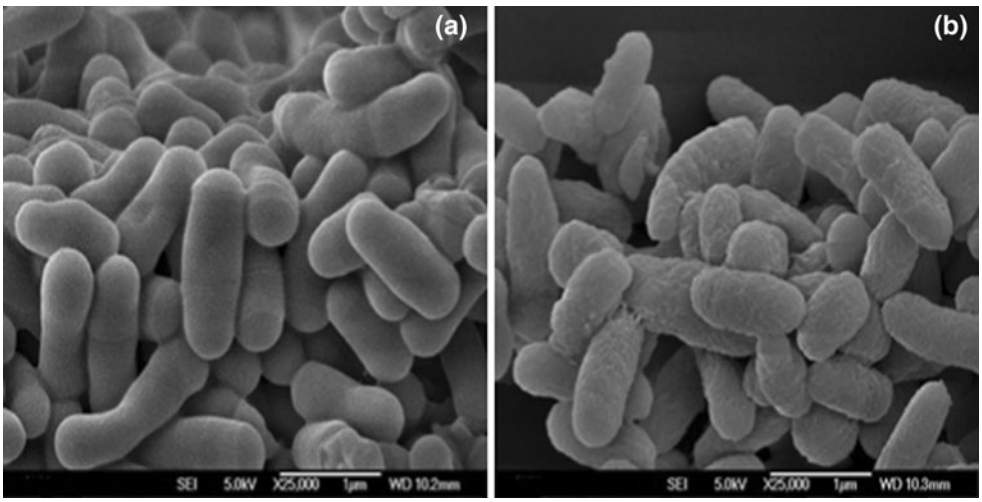

Fig. 4 Scanning electron micrographs of the oil-degrading bacteria, *Arthrobacter* sp. HK-2 (**a**) and *Pseudoalteromonas* sp. HK-3 (**b**), isolated from the enriched microbial consortium TK-2

compounds, which leads to increased bioavailability and biodegradation of crude oil (Batista et al. 2006). Our previous work reported the optimized production of biosurfactant from strain HK-3 (Cho et al. 2011). In these respects, data obtained from strains HK-2 and HK-3 were consistent with previous information regarding crude oil-degrading bacteria. The phylogenetic trees of the HK-2 and HK-3 isolates were prepared as shown in Fig. 3. Two strains were examined under scanning electron microscopy. Morphological examination of these isolates showed that all were Gram-negative, typical rod-shape cells; *Arthrobacter* sp. HK-2 has a smooth surface (Fig. 4a), whereas *Pseudoalteromonas* sp. HK-3 displays an irregular and undulated morphology (Fig. 4b). Morphological, phylogenetic, and physiological characteristics of the bacterial strain HK-3 have been previously reported (Cho and Oh 2010).

A variety of biochemical characteristics of these isolates were examined, but no further efforts were made to characterize the pure cultures and their potential interactions in

the present work. A close examination of the time-course data revealed that more than 90 % of the crude-oil was removed within 10 days. Based on the data, the indigenous microbial consortium TK-2 has the potential to be used in the bioremediation of oil spills. An indigenous microbial consortium approach was essential for this objective because a foreign culture approach for the improved clean-up of spilled oil may disturb the marine ecosystem. Future studies will emphasize the possible bioremediation process in situ, where spilled oil represents a serious problem, using the indigenous microbial consortium TK-2.

Acknowledgment This work was supported in part by the Soonchunhyang University Research Fund.

References

Atlas R, Bragg J (2009) Bioremediation of marine oil spills: when and when not-the *Exxon Valdez* experience. Microbial Biotechnol 2:213–221

Batista SB, Mounteer A, Amorim FR, Totola MR (2006) Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. Bioresour Technol 97:868–875

Cho SH, Oh KH (2010) Characterization of the biosurfactant-producing bacterium, *Pseudoalteromonas* sp. HK-3 isolated from the crude-oil contaminated areas. Kor J Microbiol 46: 346–351

Cho SH, Ma CW, Oh KH (2011) Optimized production of biosurfactant by the indigenous bacterium, *Pseudoalteromonas* sp. HK-3 originating from oil-spilled areas. KSBB J. 26:57–61

Cunha CD, Rosado AS, Sebastian GV, Seldin L, von der Weid I (2006) Oil biodegradation by *Bacillus* strains isolated from the rock of an oil reservoir located in a deep-water production basin in Brazil. Appl Microbiol Biotechnol 73:949–959

Deppe U, Richnow HH, Michaelis W, Antranikian G (2005) Degradation of crude oil by an arctic microbial consortium. Extremophiles 9:461–470

Hamamura N, Yeager CM, Arp DJ (2001) Two distinct monooxygeneases for alkane oxidation in *Nocardioides* sp. strain CF8. Appl Environ Microbiol 67:4992–4998

Harayama S, Kishira H, Kasai Y, Shutsubo K (1999) Petroleum biodegradation in marine environments. J Mol Microbiol Biotechnol 1:63–70

Hazen TC, Dubinsky EA, DeSantis TZ, Anderson GL, Piceno YM, Singh N et al (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 300:204–2008

International Tanker Owners Pollution Federation Ltd (ITOPF) (2008) The environmental impact of the *Hebei Spirit* oil spill, Taean, South Korea. http://www.itopf.com/news-and-events/documents/HEBEISPIRIT-Environmentalimpact.pdf (07.12.07)

Kato T, Haruki M, Imanaka T, Morikawa M, Kanaya S (2001) Isolation and characterization of psychrotrophic bacteria from oil-reservoir water and oil sands. Appl Microbiol Biotechnol 55:794–800

Lai B, Khanna S (1996) Degradation of crude oil by *Acinetobacter calcoaceticus* and *Alcaligenes odorans*. J Appl Bacteriol 81: 355–362

Leacock E (2005) The *Exxon Valdez* oil spill. Facts on file Inc, New York, pp 34–46

Lin X, Yang B, Shen J, Du N (2009) Biodegradation of crude oil by an arctic psychrotrophic bacterium *Pseudoalteromonas* sp. P29. Curr Microbiol 59:341–345

Mittal A, Singh P (2006) Isolation of hydrocarbon degrading bacteria from soils contaminated with crude oil spills. Ind J Exp Biol 47:760–765

Rahman KSM, Thahira-Rahman J, Lakshmanaperumalsamy P, Banat IM (2002) Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresour Technol 85:257–261

Sathishkumar M, Binupriya AR, Baik SH, Yun SE (2008) Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas. Clean 36(1):92–96

Swannell RPJ, Lee K, McDonagh M (1996) Field evaluations of marine oil spill bioremediation. Microbiol Rev 60:342–365

Tang X, He LY, Tao XQ, Dang Z, Guo CL, Lu GN, Yi XY (2010) Construction of an artificial microalgal-bacterial consortium that efficiently degrades crude oil. J Hazard Mater 181:1158–1162

Toledo FL, Calvo C, Rodelas B, Gonzalez-Lopez J (2006) Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Syst Appl Microbiol 29:244–252

Van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Molec Biol Rev 67:503–549