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Isolation and Characterization of Psychrotrophic and Halotolerant *Rhodococcus* sp. YHLT-2

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Abstract A psychrotrophic bacterium was isolated from oil-contaminated groundwater and identified as *Rhodococcus* sp. YHLT-2. Growth was observed at the temperature of 4 to 30°C. This strain degraded various petroleum hydrocarbons such as crude oil, diesel oil, and gasoline over the whole range of temperatures tested. The *Rhodococcus* sp. YHLT-2 was capable of growing even at 4°C, exhibiting 90% of oil biodegradation after 20 days. Degradation of crude oil occurred at low temperature in nature. This strain was also able to grow at 7% NaCl, and utilized not only short chain alkenes (C_9 to C_{12}), but also a broad range of long chain alkenes (C_{19} to C_{32}) present in crude oil at 4°C. The *Rhodococcus* sp. YHLT-2 is expected to be of potential use in the *in situ* bioremediation of hazardous hydrocarbons under low-temperature and high-salt conditions.

Key words: Bioremediation, *Rhodococcus*, Psychrotroph, crude oil, Halotolerance

Petroleum contamination in soil and groundwater has become a widespread environmental problem. The petroleum hydrocarbons have adverse effects on ecosystems, and they may change the indigenous microbial population and/or the physicochemical properties of soil and groundwater [6, 9, 31]. A number of studies have reported the bioremediation of sites contaminated with petroleum hydrocarbons such as crude oil, diesel oil, and gasoline [2, 12, 13, 23, 30].

Many bacteria are capable of degrading petroleum hydrocarbons. More than seventy microbial genera are known to contain oil-degrading microorganisms (e.g., *Acinetobacter*, *Alchaligenes*, *Bacillus*, *Pseudomonas*, *Norcardia*, and *Rhodosporidium*) [1, 2, 4, 5, 8, 15, 16, 22, 27–29]. Most of these oil-degrading bacteria have the optimal microbial activity at medium temperatures; therefore, there is a limitation of bioremediation of gasoline-contaminated sites using mesophiles, which have very low activities at ≤10°C, in winter season or cold areas [1, 2, 4, 5, 8, 15, 16, 22, 27, 28]. The limitation of hydrocarbon biodegradation at low temperatures may be due to a lower mass transfer rate (e.g., high viscosity of oil) and changes in the physical properties of hydrocarbon (e.g., low aqueous solubility) at low temperatures [20], resulting in decreased bioavailability. Evidence for oil degradation by phychrophilic bacteria at low temperatures have been observed in sub-Arctic, Arctic, Antarctic, and alpine environments [1, 16, 19, 17]. However, little study has been reported on the bioremediation of petroleum-contaminated soil and groundwater using phychrophilic bacteria [18, 28, 29]. Phychrotrophic bacteria having oil-degrading activities at a wide range of temperatures (≤0 to 30–35°C) [18, 28, 29] are of significant importance in countries having four seasons, such as Korea, Japan, USA, and Europe.

In this study, a psychrotrophic and halotolerant petroleum oil-degrading bacterium was isolated from a gasoline-contaminated groundwater and identified. The effects of temperature and salt (sodium chloride) concentration on the growth rate of the isolated phychrotrophic bacterium were investigated.

Materials and Methods

Crude Oil and Media

The crude oil used was obtained from a gasoline company located in Incheon, Korea. The Basal Salt (BS) medium used to screen the oil-degrading bacteria contained the following: KH_2PO_4, 1.5 g/l; $Na_2HPO_4 \cdot 12H_2O$, 9 g/l; $(NH_4)_2SO_4$, 3 g/l; $CaCl_2 \cdot 2H_2O$, 0.01 g/l; $MgSO_4$, 0.15 g/l.

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Bushnell Hass (BH) medium contained the following: $MgSO_4 \cdot 7H_2O$, 0.406 g/l; $CaCl_2 \cdot 2H_2O$, 0.0265 g/l; KH_2PO_4, 1 g/l; NH_4NO_3, 1 g/l; $Na_2HPO_4 \cdot 12H_2O$, 6 g/l; and $FeCl_3 \cdot 6H_2O$, 6 g/l. BH medium with 1% (v/v) oil was used to estimate oil-degrading abilities at low temperature in an aerobic condition. The pH of the BH medium was adjusted to 7.0. The Luria Bertani (LB) medium used to estimate specific growth rate and dry cell weight contained the following: Tryptone, 10 g/l; Yeast extract, 5 g/l; NaCl, 10 g/l.

Enrichment, Isolation, and Identification of Petroleum-Degrading Microorganisms

Samples of biofilm were obtained from a subway discharge system exposed to petroleum-contaminated groundwater in Seoul, Korea [7]. Two-hundred ml of BS medium was added to 500-ml flasks containing 10 g (as wet weight) of biofilm as inoculum and 1,600 mg crude oil as a carbon source. The flasks were sealed with silicon stoppers, and the cultures incubated at 15°C and 150 rpm. The enriched culture broth was diluted with sterilized water and spread on the LB plate, and then the cultures were incubated at 15°C for 2 to 3 days. Three representative colonies were selected and each colony was inoculated to a 50-ml flask containing 10 ml of BH medium. Crude oil was added to the flask to a final concentration of approximately 8,000 mg/l. The cultures were incubated at 15°C and 150 rpm.

Each single colony was resuspended to 30 µl of 0.5 N NaOH in a 1.5-ml microcentrifuge tube, boiled at 95°C for 30 min for lysis of the cell wall, and centrifuged at room temperature and 13,400 ×*g* for 15 min. The supernatant was used as the template. Fragments corresponding to nucleotide positions 341–926 of the *Escherichia coli* 16S-rDNA sequence were amplified with the universal eubacterial primers Bf341 (5'-CCT ACG GGA GGC AGC AG-3') and Br907 (5'-CCC CGT CAA TTC ATT TGA GTT T-3') [21]. PCR was performed in a GeneAmp^R PCR System 2700 (Applied Biosystems, Singapore) by touchdown PCR [11]. The cloned PCR fragments were sequenced with an ABI Prism model 373A automated DNA sequencer (Perkin Elmer Corp., Foster City, CA, U.S.A.). The obtained sequences were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm [7]. The calculation of sequence similarity and phylogenetic tree inference were carried out using the PHYDIT program (http://plaza.snu.ac.kr/~jchun/phydit/).

Effects of Temperature and NaCl Concentration on Growth Rate

Batch experiments were conducted to determine the effect of temperature on the specific growth rate of the cold-adapted crude oil-degrading bacteria; the strains YHLT-1 and YHLT-2 were inoculated in 100 ml of LB medium and incubated at 30°C, 15°C, and 4°C with shaking at 150 rpm. To determine the effect of NaCl concentration on the growth rate of oil-degrading bacteria, YHLT-2 was inoculated on 100 ml of LB medium containing 1%, 3%, 5%, 7%, and 9% (w/v) of NaCl and incubated at 15°C and 150 rpm. The optical density of culture broth was evaluated at 600 nm by using a spectrophotometer (Spectronic 20, Milton Roy Company, NY, U.S.A.).

Oil Degradation in Liquid Cultures

A 100 µl sample of pre-culture broth (v/v) of strain YHLT-2 at 30°C was inoculated into 10 ml of BH medium containing 1% crude oil (v/v, approximately 8,000 mg/l) in a 50-ml flask, and the culture was then incubated at 30°C, 15°C, and 4°C with shaking at 150 rpm. For each treatment, there were two replicates and a control. The average reading (OD value) of duplicates for each treatment was subtracted from that of its respective control, and OD was converted into dry cell weight.

Oil Degradation in Soil

To characterize strain YHLT-2 on soil medium containing crude oil, strain YHLT-2 was incubated in LB medium at 15°C and 150 rpm for 2 days and then centrifuged at 4°C and 8,000 rpm for 10 min. The pellet was resuspended with 10 ml of BS medium and centrifuged at 4°C and 8,000 rpm for 10 min. This step was repeated three times, and the pellet of the strain YHLT-2 was finally resuspended with 5 ml of BS medium. Cell broth was diluted with BS medium to 0.3 optical density at 600 nm. Four ml of diluted cell broth was inoculated on 5 g of soil amended with 10% crude oil (v/v, approximately 80,000 mg/l) and incubated at 15°C and 4°C with shaking at 150 rpm. Soil used was sieved at 860–600 µm and then dried at 50°C for 1 day. All samples were prepared in duplicates.

Extraction of Oil and Analytical Procedures

Samples were vigorously shaken for five minutes with an equal volume of hexane (typically 10 ml). To separate the emulsion, the mixture was held at room temperature for 1 h. The aqueous phase was used for an analysis of biomass and the hexane phase was collected for oil analysis. The optical density (OD) of the aqueous phase was monitored at 600 nm with a spectrophotometer (Spectronic 20, Milton Roy Company, NY, U.S.A.).

Crude oil and total petroleum hydrocarbon (TPH) concentrations in the hexane extracts, filtered by using a PTFE syringe filter (25 µm, 0.45 µm, Whatman), were measured on a gas chromatograph (HP 5890 II plus, Hewlett-Packard, Florida, U.S.A.) equipped with a flame ionization detector, using a HP-5 capillary column (60 m length, 0.23 mm internal diameter, and 2.5 µm film thickness). The oven temperature was set at 45°C for one minute and increased by 5°C per minute to a temperature of 100°C, and then increased by 8°C per minute to a final temperature of 320°C and a 5 min hold. The carrier gas (N_2) flow rate was

set at 1.5 ml/min, and the temperature of the injector and the detector were 260 and 250°C, respectively. The sample volume injected was 1 μl. External crude oil and TPH standards were used for calibration. External standards were prepared by dissolving crude oil in hexane, and a TPH standard was used (ASTM D5442 C12-C60 quantitative linearity standard, Supelco, U.S.A.). All samples were analyzed in duplicate.

RESULTS AND DISCUSSION

Isolation and Characterization of Oil-Degrading Microorganisms by 16S-rDNA Analysis

Figure 1 shows the phylogenetic tree of oil-degrading bacteria isolated from the biofilm. Three bacteria, strains YHLT-1, YHLT-2, and YH-1, showed the crude oil degradability. Three isolates, crude oil-degrading bacteria, were identified as *Rhodococcus* sp. and *Acinetobacter* sp. by a 16S-rDNA analysis. The grouping was separated into Actinobacteria and γ-subdivision. Strain YH-1 was grouped as γ-subdivision and strains YHLT-1 and YHLT-2 as Actinobacteria. Strain YHLT-1 was documented to be the cold-tolerant alkane-degrading *Rhodococcus* sp. from Antarctica with 99% similarity in BLAST. The strain YHLT-2 was also documented as the bacteria isolated from an oil-contaminated site. Previous studies on bacteria that are capable of degrading hydrocarbons include *Acinetobacter* sp., *Alcaligenes* sp. [15], *Alcanovorax* strains [25], *Pseudomonas* sp. [4, 22], and *Rhodococcus* sp. [5, 27, 28]. Some *Rhodococcus* sp. can grow at a low temperature, degrade petroleum hydrocarbons [5, 28, 29], and adhere to the oil-water interface, thereby indirectly increasing hydrocarbon availability [20].

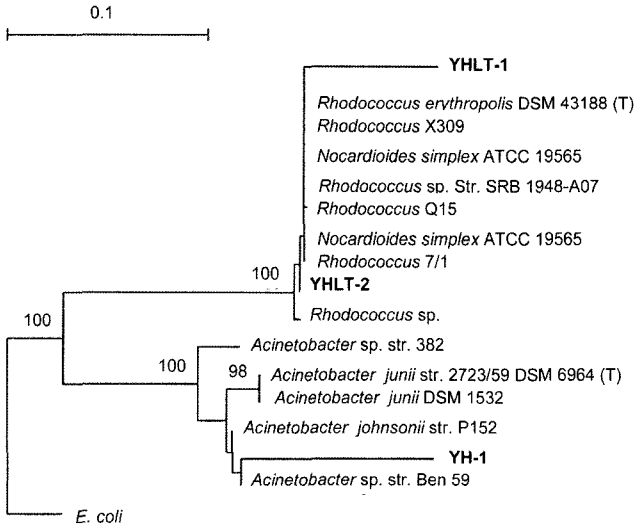

Fig. 1. The phylogenetic tree of oil-degrading bacteria isolated from the biofilm.

Growth of Strains YHLT-1 and YHLT-2 at Different Temperatures

Among the three stains isolated, strains YHLT-1 and YHLT-2 appeared to be psychrotrophic. Figure 2 shows the time profiles of the growth of *Rhodococcus* sp. YHLT-1 and YHLT-2 at different temperatures (30°C, 15°C, and 4°C) on LB medium. Both YHLT-1 and YHLT-2 exhibited a similar growth pattern on different temperatures. Specific growth rates of *Rhodococcus* sp. strain YHLT-1 were

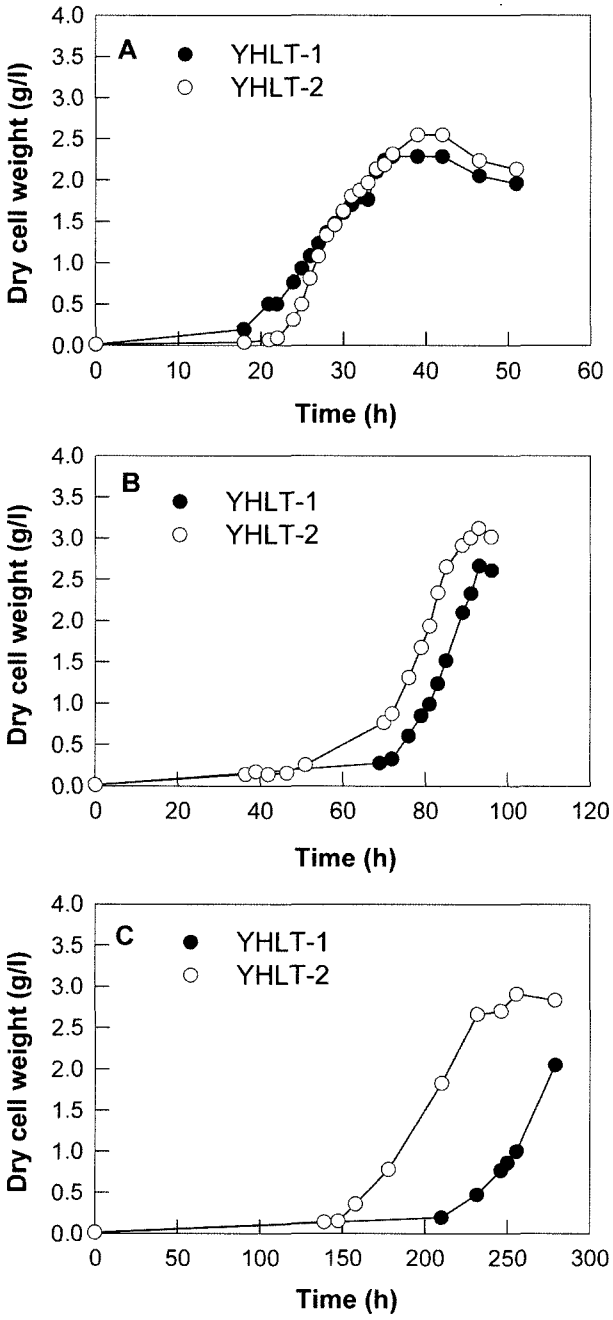

Fig. 2. Growth patterns of the YHLT-1 and YHLT-2 strains at (**A**) 30°C, (**B**) 15°C, and (**C**) 4°C, respectively.

0.14 h^{-1}, 0.09 h^{-1}, and 0.03 h^{-1} at 30°C, 15°C, and 4°C, respectively. Specific growth rates of strain YHLT-2 were 0.42 h^{-1}, 0.09 h^{-1}, and 0.03 h^{-1} at 30°C, 15°C, and 4°C, respectively. Overall, the specific growth rates decreased with decreasing temperature; however, Figs. 2B and 2C showed that both strains could grow at low temperatures. It indicates that the strains YHLT-1 and YHLT-2 are psychrotrophic bacteria, which grow at low temperature but display a maximum growth in the mesophile range [18, 28, 29]. This result suggested that strain YHLT-2 might be a cold-adapted bacterium or psychrotrophic bacterium. The strain YH-1 did not grow at low temperature. Although the specific growth rate of the strains YHLT-1 and YHLT-2 were almost the same at 15°C and 4°C, the strain YHLT-2 had a shorter lag period. Hence, the stain YHLT-2 was selected for further study.

Growth of Strain YHLT-2 at Different NaCl Concentrations

Table 1 lists the specific growth rate of the strain YHLT-2 in LB medium supplemented with different concentrations of NaCl at 15°C. The strain YHLT-2 could grow at 15°C on LB medium containing up to 7% (w/v) NaCl. Its specific growth rates at 1, 3, 5, and 7% (w/v) NaCl were 0.11, 0.10, 0.05, and 0.04 h^{-1}, respectively. The growth of the strain YHLT-2 was completely inhibited at 9% (w/v) NaCl, indicating that it is a halotolerant bacterium, which can grow at moderate salt concentrations, even though it grows best in the absence of salt. There are few studies on the relationship between *Rhodococcus* sp. and halotolerance. Zvyagintseva *et al.* [32] reported that an oil-degrading bacterium, *R. erythropolis* strain INMI 100, exhibited low halotolerance. The halotolerant *Rhodococcus* sp. strain YHLT-2 is expected to be of potential use for *in situ* bioremediation in marine environment.

Crude Oil Degradation in Liquid Cultures by *Rhodococcus* sp. YHLT-2

Figure 3 shows that that the *Rhodococcus* sp. YHLT-2 was capable of using crude oil as carbon and energy sources, indicating that they are oil-degrading bacteria. Figure 3A shows that the lag period increased with decreasing temperature. There was no lag phase in the YHLT-2 growth on BH medium containing crude oil at 30°C; however, there were

Table 1. Specific growth rate of strain YHLT-2 in LB medium supplemented with different concentrations of NaCl at 15°C.

NaCl concentration (g/l)	Specific growth rate (/h)
10	0.11
30	0.10
50	0.05
70	0.04
90	0

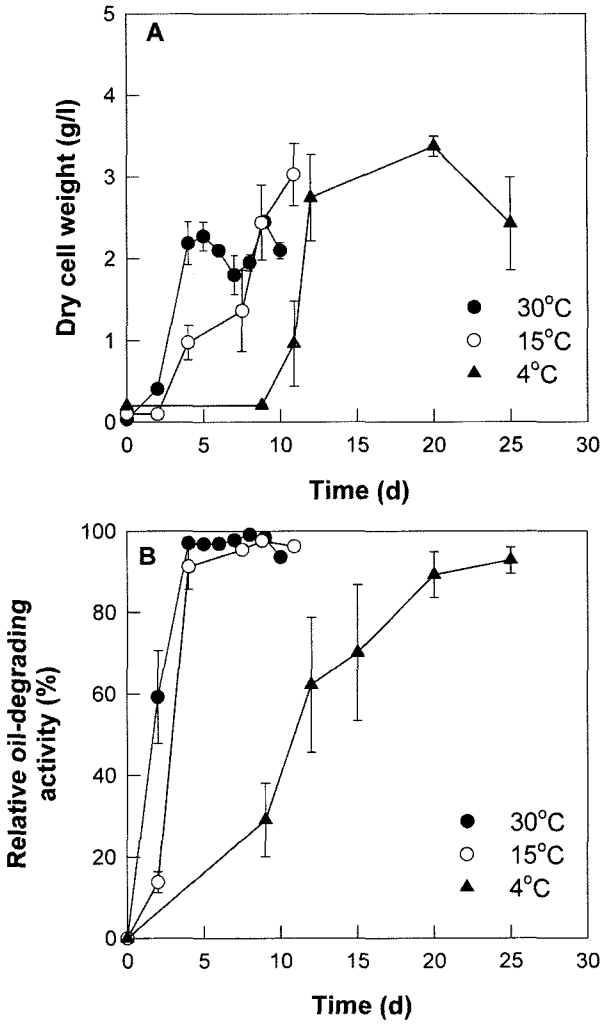

Fig. 3. (**A**) Growth pattern and (**B**) relative oil-degrading activity of strain YHLT-2 in liquid culture at different temperatures.

2 and 5 days of lag phase at 15°C and 4°C, respectively. This indicates that the YHLT-2 might require some time to adapt at low temperatures. Figure 3B shows the relative oil-degrading activity of the *Rhodococcus* sp. YHLT-2 measured at 30°C, 15°C, and 4°C, respectively. The relative activity was greater than 90% after 4 days at 30 and 15°C, while the relative activity at 4°C was about 30% after 10 days. Although the activity of the YHLT-2 was the greatest at 30°C followed by 15°C, the bacterium still showed the oil-degrading activity at as low as 4°C, confirming that the *Rhodococcus* sp. YHLT-2 is a phychrotrophic bacterium capable of degrading oil at temperatures ranging from 4 to 30°C. Psychrotrophic *Rhodococcus* or *Pseudomonas* species has previously been reported to degrade alkanes at low temperatures [28, 29].

Figure 4 shows residual crude oil by the strain YHLT-2 at 4°C. Main peaks of residual crude oil were not detected after 20 days, indicating that the YHLT-2 degraded a broad

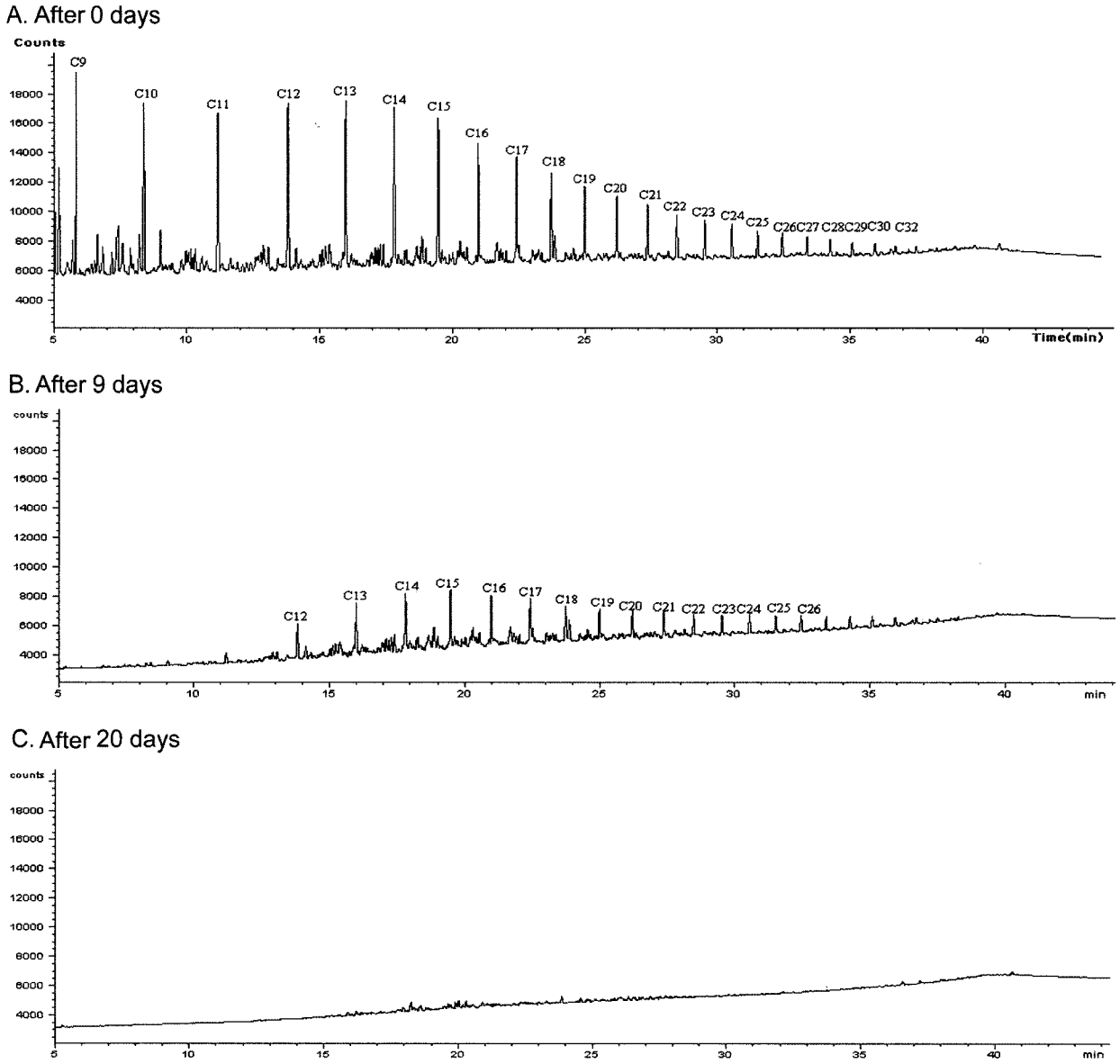

Fig. 4. Gas chromatograms of petroleum hydrocarbons extracted from the liquid culture broths incubated at 4°C.

range of petroleum hydrocarbons after 20 days, including by-products. Even though the production of biosurfactants by strain YHLT-2 was not confirmed, the strain YHLT-2 may enhance the rates of uptake and biodegradation by producing biosurfactants. Biosurfactants facilitate the transport of hydrocarbons to the cell surface by making the surface hydrophobic, and increase the aqueous solubility of hydrophobic hydrocarbons by partitioning hydrocarbons into micelles. Many crude oil-degrading bacteria have been reported to produce biosurfactants, and biosurfactant-producing bacteria are found in higher concentrations in hydrocarbon-contaminated soils [24]. These bacteria include *Arthrobacter* sp., *Bacillus* sp., *Candida* sp., *Corynebacterium* sp., *Pseudomonas* sp., and *Rhodococcus* sp. [3].

Figure 5 shows the relative TPHs (C_{12} to C_{32}) degradation activity of the strain YHLT-2 at different temperatures. The strain YHLT-2 showed similar degradation patterns of TPHs at 30°C and 15°C. As expected, the degradation of hydrocarbons decreased with increasing molecular weight of hydrocarbons: the YHLT-2 could utilize not only short-chain alkanes (C_5 to C_{12}), but also a broad range of long-chain alkanes (C_{19} to C_{30}) present in petroleum at various temperatures.

Crude Oil Degradation in Soil by *Rhodococcus* sp. YHLT-2

Figure 6 shows the relative degradation activity of petroleum by the strain YHLT-2 at 15°C and 4°C in soil. The relative

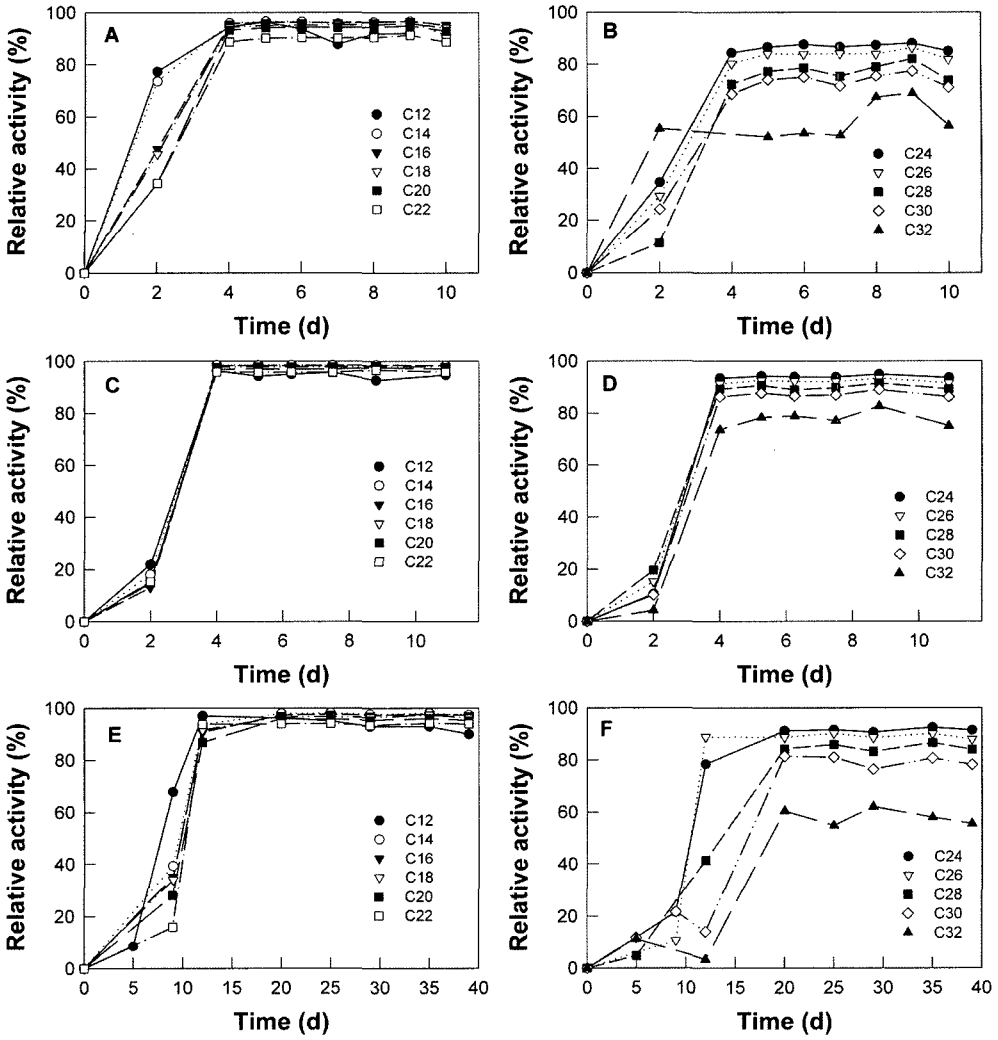

Fig. 5. Relative TPHs degradation activities of the strain YHLT-2 at (**A**, **B**) 30°C, (**C**, **D**) 15°C, and (**E**, **E**) 4°C, respectively, in liquid culture.

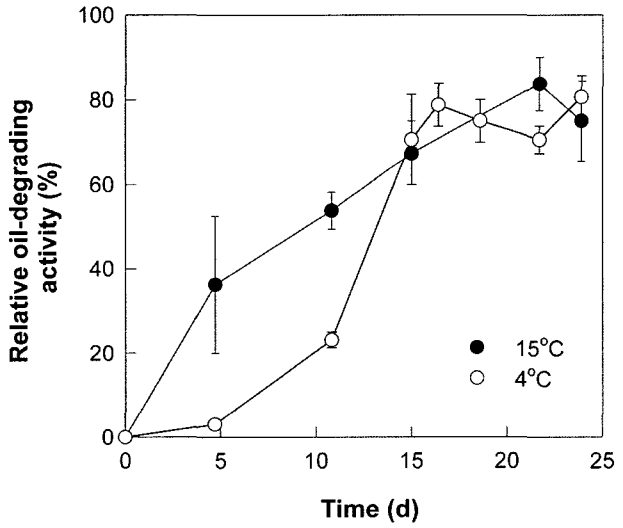

Fig. 6. Relative petroleum degradation activity of the strain YHLT-2 in soil.

activity up to 15 days was greater at 15°C than at 4°C, but the activity was comparable at both temperatures after 15 days. The strain YHLT-2 showed a lower degradation activity in soil than in liquid culture. Margesin and Schinner [18] also reported that the relative activity of oil degradation decreased in soil, compared with aqueous medium. The reasons could be competition with indigenous soil microorganisms for limiting nutrients and the sorption of microorganisms to soil particles [10, 14, 26].

In conclusion, we demonstrated that the *Rhodococcus* sp. YHLT-2 is a phychrotrophic and halotolerant bacterium that can be applied to the bioremediation of oil-contaminated soil and groundwater in temperature-fluctuating areas as well as cold marine environments.

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