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ORIGINAL ARTICLE

# Characteristics of Crude Oil Biodegradation by Biosurfactant-Producing Bacterium *Bacillus subtilis* JK-1

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**Abstract** The production of biosurfactant by *Bacillus subtilis* JK-1 was investigated under several conditions. In sea water inoculated with 10% (v/v) of seed culture, the surface tension decreased from 75.0 to 38.4 dyne/cm after 12 h of incubation, which was the highest reduction of surface tension (49%) among the conditions tested. Surface tension further decreased to 31.2 dyne/cm when grown in 1% (v/v) *B. subtilis* JK-1 inoculated into sea water containing 1% (v/v) crude oil. The decrease of surface tension was similar after 24 h in sea water, sea water containing 1% (v/v) crude oil, and sea water containing 10% (v/v) crude oil using a 10% (v/v) *B. subtilis* JK-1 inoculum. The biosurfactant produced by *B. subtilis* JK-1 displayed highest emulsification activity on soybean oil and crude oil. Maximum emulsification stability was obtained from hexane ($C_6$). Using crude oil as a substrate, the emulsification activity of the biosurfactant was much greater than those of chemically synthesized surfactants such as Tween 20 and sodium dodecyl sulfate (SDS). In addition, the bacterial biosurfactant possessed the best emulsification stability when hexane ($C_6$), and hexadecane ($C_{16}$) were utilized as substrates, as compared to Tween 20, Triton X-100, and SDS. The crude oil in the culture broth was degraded by *B. subtilis* JK-1, and the $C_1$-$C_{29}$ carbon chain was almost completely degraded during the 48 h incubation. These results suggest that the biosurfactant of *B. subtilis* JK-1 is an appropriate candidate for bioremediation of crude oil contaminant.

**Keywords** *Bacillus subtilis* JK-1 · biosurfactant · crude oil · emulsification · sea water · surface tension

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## Introduction

Oil pollution in terrestrial and aquatic environments is a common phenomenon that causes significant ecological and social problems. The Taean oil spill incident at the west coast of Korea during the winter of 2007 is a poignant example. Over 1,300,000 volunteers helped in the physical clean-up of the contaminated coastline of Taean. Physical collection methods such as booms, skimmers, and adsorbents typically recover no more than 10–15% of the spilled oil. Use of chemically synthesized surfactants as remediating agents is not favored due to their toxic effects on the existing biota in the polluted area (Thavasi et al., 2011).

Aquatic oil-based pollution poses a significant danger in Korea. The country is surrounded by the sea on three sides, and this sea proximity has fueled the development of an aquaculture and fishing industry that is an important contributor to the national economy. Most of the current oil emulsifiers are chemically synthesized surfactants. Their over-use can cause secondary contamination and toxic problems. To combat these problems, microbial bioremediation and non-toxic control techniques have been explored (Singh et al., 2008).

Bioremediation technology can be a non-disruptive, cost-effective, and highly efficient method of destroying many environmentally persistent toxic chemicals. Bioremediation technologies typically use in naturally occurring microorganisms to degraded hazardous wastes. Like all living creatures, microbes require nutrients, carbon, and energy to survive and multiply. Such organisms are capable of breaking down toxic chemicals to obtain food and energy, degrading them into harmless substances (United States Environmental Protection Agency, 1993). As well, the penetration power of the relatively small microbes enables the bioremediation of small cracks and crevasses in rocks and between sand grains and soil particles, allowing the remediation of oil contaminated environments.

More recently, the potential of biosurfactants has been recognized

and explored (Sekhon et al., 2012). Biosurfactants are natural surface-active compounds produced mainly by microorganisms that either adhere to cell surfaces or are secreted extracellularly. They have unique amphipathic properties derived from their complex structures, which consist of a hydrophobic and hydrophilic moieties. Their chemistry imparts the ability to reduce surface and interfacial tensions in aqueous solutions and hydrocarbon mixtures (Desai and Banat, 1997). Biosurfactants have been widely used in environmental protection, including oil spill and oil-contaminated tanker cleanup, removal of crude oil from sludge, enhanced oil recovery, bioremediation of sites contaminated with hydrocarbons, other organic pollutants and heavy metals (Rodrigues and Teixeira, 2008).

On the basis of their chemical structure, the major classes of biosurfactants include glycolipids, lipopeptides, lipoproteins, fatty acids, phospholipids, neutral lipids, polymeric compounds, and particulate matter (Desai and Banat, 1997). Biosurfactants have many advantages over their chemical similitude's such as easy degradation by the microorganisms, low toxicity, production using cheap raw materials, not easily affected by environmental factors such as temperature, pH, and ionic strength as well as have the unique property of biocompatibility and digestibility (Sekhon et al., 2012). Thus, biosurfactants can be utilized as an effective alternative for chemically synthesized surfactants that are not eco-friendly and whose manufacture, including by-products, can be hazardous to the environment (Maier and Soberon-Chavez, 2000).

Due to their effective physicochemical, surface-active, and biological characteristics, biosurfactants are widely used in different industries as functional ingredients in foods, microbiological, pharmaceutical, and therapeutical agents in the biological industries, as a biocontrol agent in agricultural applications, in downstream processing for bioprocessing applications, and in health and beauty cosmetic products (Brown, 1991; Banat et al., 2000; Cameotra and Makkar, 2004; Singh and Cameotra, 2004; Rodrigues et al., 2006; Nitschke and Costa, 2007; Joshi et al., 2008).

Bacteria are the main group of biosurfactant-producing microorganisms. *Bacillus* species are major producers of biosurfactants. Biosurfactant production by representatives of the *Bacillus* genus, including *Bacillus atrophaeus* (das Neves et al., 2007), *Bacillus licheniformis* (Gogotov and Miroshnikov, 2009), *Bacillus megaterium* (Thavasi et al., 2008), and *Bacillus subtilis* (Barros et al., 2008), has been reported. Among these, *B. subtilis* has been widely studied, with the first study of biosurfactant production published by Arima et al. (1968). *B. subtilis* is considered a suitable source for biosurfactant production owing to the absence of pathogenicity and its high surface activity (Sandrin et al., 1990; Besson and Michel, 1992). In general, biosurfactant production is influenced by the carbon source, nitrogen source, limiting nutrient(s), pH, amount of dissolved oxygen, temperature, agitation speed, supply of polyvalent ions, growth period, and inhibition due to the formed products. The amount of biosurfactant produced can be indirectly measured by testing the reduction ability of the surface tension or emulsification activity.

With particular relevance to Korea, tailoring microorganisms to degrade the oil-based components from the coastal sea water necessitates their ability to grow in water of a wide temperature range (1-28°C) and a salt concentration of about 3.5%.

The addition of biosurfactants to enhance the biodegradation of hydrocarbons has been the focus of several studies. Many reports have described the effect of exogenously added microbial biosurfactants in enhancing the bioremediation of crude oil-polluted soils by indigenous microbes (Abalos et al., 2004; Cubitto et al., 2004; Owsianiak et al., 2009; Satpute et al., 2010). Previously, we explored the optimization of culture media and conditions to increase the productivity of the biosurfactant produced by *B. subtilis* JK-1 (Joo and Kim, 2011; Kim, 2011). In the present study, we investigated the characteristics of crude oil biodegradation by *B. subtilis* JK-1 to verify its potential industrial application on hydrocarbon bioremediation.

## Materials and Methods

**Chemicals.** Crude oil was obtained from SK Energy, Ulsan, Korea. Hexane ($C_6$), hexadecane ($C_{16}$), paraffin ($C_nH_{2n+2}$, $n \geq 19$), toluene ($C_7H_8$), and benzene ($C_6H_6$) were purchased from Sigma Chemical Co. (USA) and used as substrate for emulsification activity. Soybean oil was obtained from commercial sources. To compare emulsification activity and stability of biosurfactant, we used Tween 20, Triton X-100, and sodium dodecyl sulfate (SDS) (all from Sigma, USA) as the chemically synthesized surfactants. Methanol was purchased from Bioshop Inc. (Canada).

**Microorganism.** *B. subtilis* JK-1 was previously isolated and characterized as a biosurfactant producing strain (Joo et al., 2007) and used for the present study due to its crude oil degrading characteristics. The strain was grown on Luria-Bertani (LB) agar containing 1% (w/v) NaCl (Bioshop Canada, Canada), 1% (w/v) tryptone (Difco, USA), 0.5% (w/v) yeast extract (Difco), 1.5% (w/v) agar, and was maintained in 40% (v/v) glycerol and stored below 0°C.

**Culture media and cultivation conditions.** The inoculum was prepared using the optimum medium for the biosurfactant produced by *B. subtilis* JK-1 (Joo and Kim, 2011), and seed culture was performed at 35°C and 200 rpm for 16 h. The optimum medium for the biosurfactant production contained 1% (w/v) soluble starch as an optimum carbon source, 0.5% (w/v) skim milk as an optimum nitrogen source, and 0.1% (w/v) $KNO_3$ as an optimum mineral source. To investigate the production of the biosurfactant under various conditions, four different sea water media were used: unaltered sea water, sea water with 0.5% (w/v) skim milk and 1% (w/v) soluble starch, sea water only with 0.5% (w/v) skim milk, and sea water with 0.1% (w/v) $KNO_3$.

**Surface tension determination.** Biosurfactant production was routinely evaluated through the determination of surface tension. The surface tension of the culture broth supernatant and the biosurfactant solution was determined using a Surface Tensiomat®

21 tensiometer (Fisher Scientific Co., USA) at 20°C, according to the Du Nouy ring method (Zajic and Seffens, 1984). The measurements of surface tension in deionized water (DW) were used as controls. Surface tension values represent the average of three independent measurements performed.

**Isolation of the biosurfactant.** Biosurfactant was isolated as described previously (Ghojavand et al., 2008) with minor modifications. The bacterial cells were removed from the culture broth by centrifugation (10,000 rpm, 4°C, 15 min). The cell-free supernatant collected was acidified with 6 N HCl to pH 2.0. To obtain the precipitated biosurfactant, the cell-free supernatant was acidified with 6 N HCl to pH 2.0 and allowed to settle at 4°C overnight. The pellet was resuspended in alkaline water after adding 0.1 N NaOH to a final pH value of 8.0, and the solution was freeze dried. The dried biosurfactant was extracted three times using 10 mL methanol. The biosurfactant sample was obtained after the evaporation of the methanol.

**Characteristics of the biosurfactant on sea water.** Characteristics of the biosurfactant produced by *B. subtilis* JK-1 on sea water were investigated. The natural sea water [30‰ (‰, parts per thousand) salt content, and pH 8.0] was obtained from Imrang Beach, Korea. It was filtered to remove the precipitate and large particles, and was ultraviolet-treated. Experiments were conducted with the four aforementioned sea water media. The seed culture was added to each medium to a final concentration of 1 or 10% (v/v). Cultivations were carried out at 25°C with agitation (200 rpm). The control flask contained DW. Surface activity of the crude biosurfactant in each culture was measured every 12 h for 96 h of cultivation. As the initial value of surface tension differed according to the type of culture broth, the reduction of surface tension (%) was calculated.

**Shake flask experiments for the biosurfactant production on sea water containing crude oil.** This experiment was carried out to investigate the influence of biosurfactant on biodegradation of crude oil in natural sea water. Crude oil was used as the sole carbon source. Shake flask biodegradation experiments were carried out in 1 L Erlenmeyer flasks with 200 mL of the filtered sea water containing 1 or 10% (v/v) crude oil. Experiments were initiated by transferring 1 or 10% (v/v) of inoculum. Cultivation was carried out for 96 h at 25°C and 200 rpm. The surface tension of the crude biosurfactant produced in each culture broth was measured every 12 h for 96 h of cultivation.

**Assay of emulsification activity.** Emulsification activities of the biosurfactant and three chemically synthesized surfactants (Tween 20, Triton X-100, and SDS) were measured using the methods of Cirigliano and Carman (1984, 1985). The cell-free supernatant from culture was filter sterilized using a Millipore 0.2-μm membrane filter. The filtrate sample (2 mL) was placed in a screw-capped test tube (15 × 125 mm) and diluted with 2 mL of 0.1 M sodium acetate buffer (pH 3.0). After 1 mL of hydrophobic substrate was added, the tube was capped, and the mixture was vortexed for 2 min at 25°C at a rate of 220 strokes per min with a stroke length of 3 cm. The hydrophobic substrates tested for emulsification assay were soybean oil, crude oil, hexane, hexadecane, paraffin, toluene, and benzene. The resulting emulsion remained at rest for 10 min, and then the aqueous phase absorbance was measured at 540 nm. The blank used contained 2 mL of sterile optimum medium for the biosurfactant production. To compare emulsification activity of biosurfactant with chemically synthesized surfactant, 2 mL of chemically synthesized surfactant was used instead of filtrate sample. One unit of emulsification activity was defined as that amount of emulsifier that affected an emulsion with an absorbance at 540 nm of 1.0.

**Assay of emulsification stability.** Emulsion stability was analyzed with emulsification activity (Cirigliano and Carman, 1985). The biosurfactant and various chemically synthesized surfactants were compared for stability of emulsification activities. The emulsified solutions were allowed to stand for 10 min at room temperature, and the absorbance readings were taken every 10 min for 50 min. The log of the absorbance was then plotted versus time. The slope (decay constant, $K_d$) of the line was calculated, then expressed as emulsification stability.

**Gas chromatography-flame ionization detector (GC-FID) analysis of degraded residual crude oil.** Analysis of crude oil degradation was performed using gas chromatograph (Shimadzu GC-Q2010, Japan)-flame ionization detector (FID) analysis. The quantification of crude oil degradation was done by the calculation of peak area diminution. For the preparation of samples, grown cells (1%, v/v) were transferred to an optimum medium containing 1% (v/v) crude oil. Cultivation was conducted at 35°C, 200 rpm, for 48 h on a shaking incubator. Optimum medium containing 1% (v/v) crude oil without any inoculated cells and LB medium containing 1% (v/v) crude oil inoculated with *B. subtilis* JKC-15 (Joo et al., 2007) were used as controls. After cultivation for 48 h, the oil remaining in the medium was extracted using *n*-hexane. A DB-1MS capillary column (Agilent, USA) of 30 m length with an internal diameter of 0.25 mm wide bore of 0.25 μm film thickness were used. The oven temperature program was as follows: 60°C for 2 min and increased linearly at a rate of 6°C $min^{-1}$ to a final temperature of 300°C, which was maintained for 15 min. The operating temperatures of the injector and detector were 300 and 320°C, respectively. Helium gas was used as carrier gas. No internal standards were employed. Total time for one GC run was less than 35 min.

## Results and Discussion

**Characteristics of biosurfactant production during growth of *B. subtilis* JK-1 on sea water and sea water containing crude oil.** Biosurfactant production and application using microorganism in sea water has been poorly studied. The ability to reduce surface tension is an indication that the resident microorganisms are producing biosurfactant (Satpute et al., 2010). Seed culture was added to each different medium to a final concentration of 1% (v/v). The surface tension of culture supernatant dropped rapidly

after inoculation and reached its lowest value at 12 h of growth (Fig. 1). The biosurfactant biosynthesis by *B. subtilis* JK-1 occurred predominantly during the exponential growth phase (Kim, 2011). The increase in surface tension after 12 h of incubation showed that biosurfactant biosynthesis has been reduced, which is probably due to environmental factors and growth conditions in the culture medium. The surface tension reduction of sea water and sea water containing 0.1% (w/v) of $KNO_3$ were the highest (31–37%), in comparison with the other media tested (Fig. 1A). A 10% (v/v) seed culture was inoculated into the four different media (Fig. 1B). Experiments with a higher concentration (10%, v/v) of bacterial cells showed improvement in the biosurfactant production. Same as the result in Fig. 1A, the maximum biosurfactant production was observed when the strain was grown on sea water. The biosurfactant could reduce the surface tension of sea water from 75.0 to 38.4 dyne/cm (surface tension reduction 49%). During the growth of the strain on sea water containing 0.1% (w/v) $KNO_3$, the reduction value of surface tension was 28%. The biosurfactant production by *B. subtilis* JK-1 on sea water was proportional to the amount of the inoculated cells, and showed maximum biosurfactant producing capability only after 12 h of cultivation.

Associations between hydrocarbon biodegradation and biosurfactant production are of great interest in exploring the applications of bacteria in the bioremediation of hydrocarbon contaminated sites. Considering actual oil spills into sea water, biosurfactant production was studied using sea water containing crude oil. In addition, with the aim of conducting scale-up fermentor studies, the influence of the inoculation amount (1% and 10%, v/v) on biosurfactant production was studied. The biosurfactant production of *B. subtilis* JK-1 on crude oil (1 and 10%, v/v) was followed by measuring the surface tension every 12 h for 96 h of cultivation. The surface tension decreased after *B. subtilis* JK-1 was introduced to sea water containing crude oil. Inoculating 1% (v/v) *B. subtilis* JK-1 to the sea water containing 1% (v/v) crude oil resulted in the lowest values of surface tension (31.2 dyne/cm) following 24 h cultivation, and slightly increased to 41.9 dyne/cm by 96 h (Fig. 1C). On the contrary, in sea water containing 10% (v/v) of crude oil, the surface tension was slightly decreased at 36 h of incubation (40.5 dyne/cm). In this case, the surface tension was gradually increased with time and showed a similar surface tension as sea water. A 10% (v/v) seed culture solution was inoculated into each 1 and 10% (v/v) of crude oil containing sea water (Fig. 1D). After 24 h of cultivation, the surface tension values of all three media were significantly decreased, reaching a similar level in the range of about 33–34 dyne/cm. In the first 24 h, the surface tension continued to decrease at a fast rate and reached the lowest level. This was a crucial time for the bacteria to grow and excrete surface-active substance, as well as the time when the biosurfactant concentration in the culture medium was the highest. Presently, the biosurfactant that was produced was found to be highly

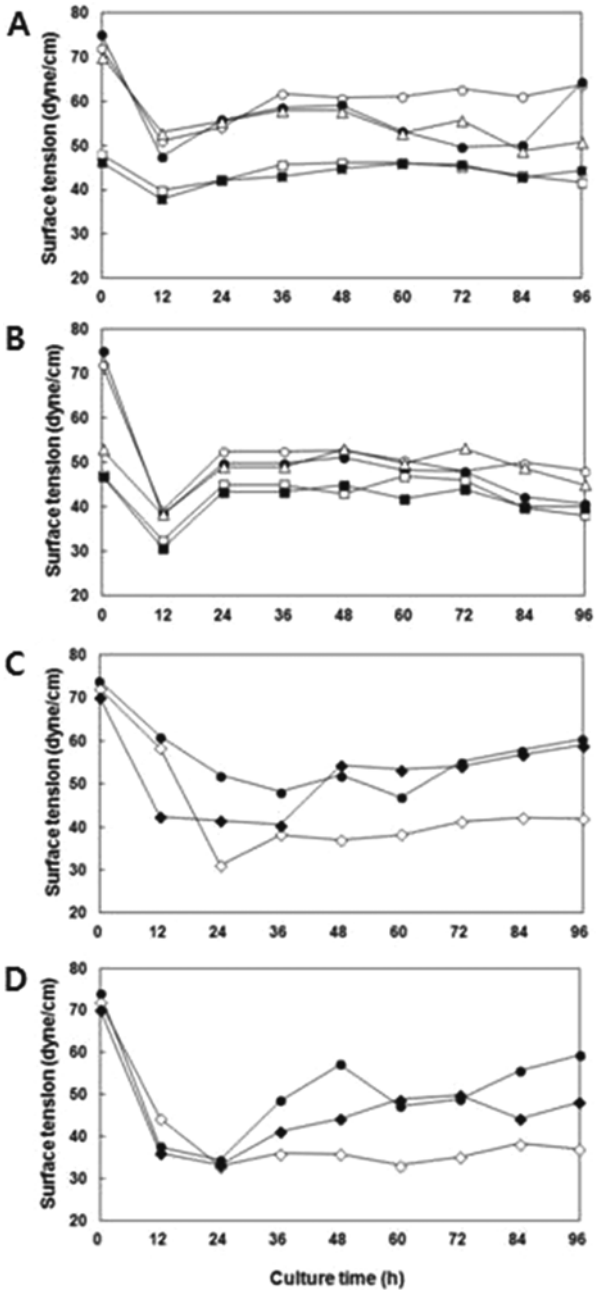


**Fig. 1** Surface tension decrease by cultures of *B. subtilis* JK-1. Each culture of the strain was grown at 25ºC and 200 rpm for 96 h. (A) Seed culture (2 mL) was inoculated into 200 mL of different media [1% (v/v) inoculation]. (B) Seed culture (20 mL) was inoculated into 200 mL of different media [10% (v/v) inoculation]. (C) Seed culture (2 mL) was inoculated into 200 mL of medium [1% (v/v) inoculation]. (D) Seed culture (20 mL) was inoculated into 200 mL of medium [10% (v/v) inoculation]. Symbols: ○, DW; ●, sea water; □, sea water containing 1% (w/v) of soluble starch and 0.5% (w/v) of skim milk; ■, sea water containing 0.5% (w/v) of skim milk; △, sea water containing 0.1% (w/v) of $KNO_3$; ◇, sea water containing 1% (v/v) of crude oil; ◆, sea water containing 10% (v/v) of crude oil

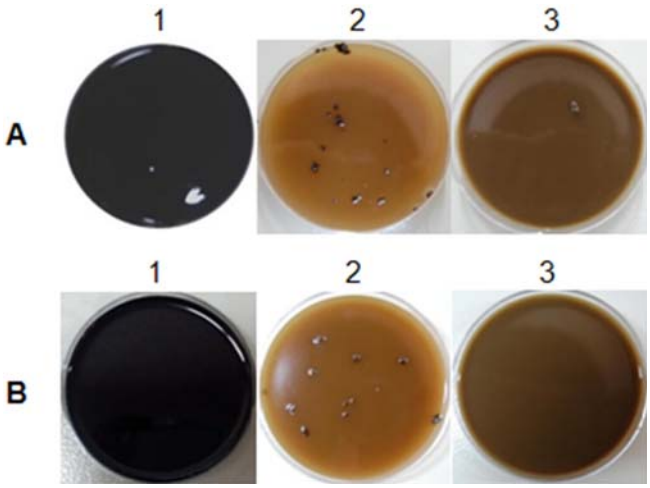


**Fig. 2** Photographs of the degradation pattern of *B. subtilis* JK-1 [(A) 1% (v/v) inoculation (B) 10% (v/v) inoculation] grown on crude oil containing sea water. Cultivation was carried out for 96 h at 25°C. Control sample with crude oil at 1% (v/v) in the absence of bacteria (1) as a control, (2) 1% (v/v) of crude oil, and (3) 10% (v/v) of crude oil

effective, because its crude solution could lower the surface tension of the culture broth to 31–34 dyne/cm. In sea water containing 1% (v/v) crude oil, no significant change in surface tension was observed until the end of cultivation. However, the surface tension of sea water and sea water containing 10% (v/v) of crude oil increased gradually from 24 to 96 h. Biosurfactant production could be influenced by amount of crude oil in sea water to some extent (Fig. 1C and D). Thus, it can be inferred that *B. subtilis* JK-1 excreted biosurfactant more effectively when cultured in sea water containing 1% (v/v) crude oil. Although the production of biosurfactant could be somewhat affected by the inoculum volume, inoculation of 1% (v/v) *B. subtilis* JK-1 resulted in a similar reduction capacity of surface tension compared to that of 10% (v/v), implying that the addition of even a small amount of *B. subtilis* JK-1 suspension can efficiently improve biosurfactant production. *B. subtilis* JK-1 also had a crude oil degrading activity, as visualized by the clear color that developed after 96 h of incubation at 25°C (Fig. 2). Biosurfactant production was detected based on the ability of the strain to emulsify the dark layer of crude oil present in the plate culture. The differences in plate color likely reflect the amount of crude oil included in the sea water. These results indicate that crude oil have an appropriate substrate for biosurfactant production. The induction of biosurfactant production by *B. subtilis* JK-1 would be useful in managing marine environments contaminated by crude oil.

**Evaluation of emulsification activity of the biosurfactant produced by *B. subtilis* JK-1.** Emulsification of tested oil and hydrocarbons by the biosurfactant indicated the possibility of its applicability against different types of hydrocarbon pollution. Emulsification activities of the biosurfactant produced by *B. subtilis* JK-1 were measured with different substrates (Table 1). Emulsification activity of different substrates by the biosurfactants was in the order of soybean oil > crude oil > hexane > hexadecane > benzene > paraffin > toluene. In particular, the emulsification activity against crude oil was 2.22, which is higher than 1.96 of *B. subtilis* TBH40-3 reported by Kim et al. (2004). Therefore, crude oil was the second substrate that has been highly emulsified by the biosurfactant used in the present study, which coincided with the

**Table 1** Emulsification activity for different emulsifying substrates by biosurfactant from *B. subtilis* JK-1 and chemically synthesized surfactants

| | Emulsification activity ($OD_{540\ nm}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Soybean oil | Hexane ($C_6$) | Hexadecane ($C_{16}$) | Crude oil | Paraffin ($C_nH_{2n+2}$) | Toluene ($C_7H_8$) | Benzen ($C_6H_6$) |
| Biosurfactant of *B. subtilis* JK-1 | 2.52 | 1.72 | 1.06 | 2.22 | 0.82 | 0.78 | 0.91 |
| Tween 20 | 2.74 | 1.82 | 1.21 | 2.11 | 0.92 | 0.90 | 1.02 |
| Triton X-100 | 2.42 | 1.79 | 1.10 | 2.36 | 1.21 | 0.65 | 1.00 |
| Sodium dodecyl sulfate (SDS) | 1.72 | 1.66 | 1.01 | 2.13 | 1.02 | 0.72 | 0.99 |

Results represent the average of three independent experiments.

**Table 2** Emulsification stability for different emulsifying substrates by the biosurfactant from *B. subtilis* JK-1 and chemically synthesized surfactants

| | Emulsification stability ($K_d$, $10^{-3}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Soybean oil | Hexane ($C_6$) | Hexadecane ($C_{16}$) | Crude oil | Paraffin ($C_nH_{2n+2}$) | Toluene ($C_7H_8$) | Benzen ($C_6H_6$) |
| Biosurfactant of *B. subtilis* JK-1 | −6.50 | −1.92 | −9.20 | −7.50 | −6.56 | −7.01 | −6.45 |
| Tween 20 | −0.12 | −7.69 | −9.92 | −2.88 | −7.21 | −5.82 | −5.08 |
| Triton X-100 | −0.15 | −10.88 | −11.21 | −0.19 | −5.25 | −7.11 | −10.25 |
| Sodium dodecyl sulfate (SDS) | −15.86 | −15.98 | −19.30 | −14.72 | −10.96 | −19.21 | −15.70 |

For measurement of the decay constant, after the initial 10 min holding period, the absorbance readings were taken every 10 min for 50 min. The log of the absorbance was then plotted versus time, and the slope (decay constant, $K_d$) of the line was calculated. Results represent the average of three independent experiments.

biodegradation of crude oil shown in Fig. 3. Three chemically synthesized surfactants (Tween 20, Triton X-100, and SDS) were examined for their emulsification activities by using different substrates, and were then compared with the biosurfactant produced by *B. subtilis* JK-1 (Table 1). The biosurfactants from *B. subtilis* JK-1, Tween 20, and Triton X-100 showed maximum emulsification activity against soybean oil. In this case, the emulsification activity of the biosurfactant [optical density at 540 nm ($OD_{540}$)=2.52] was comparatively lower than the emulsification activity recorded with Tween 20 ($OD_{540}$=2.74). These results are superior to those described for *B. subtilis* LAMI005 and *B. subtilis* LAMI009 by Sousa et al. (2012). Interestingly, the biosurfactant was found to have similar capacity to emulsification activity with hexane as compared with the chemically synthesized surfactant. Furthermore, the emulsification activities showed that the biosurfactant possessed similar capacity with aromatic hydrocarbons (paraffin, toluene, and benzene) as compared to the chemically synthesized surfactant. The highest emulsification activities were observed with Triton X-100 ($OD_{540}$=2.36), followed by the biosurfactant ($OD_{540}$=2.22) when crude oil was used as substrate. These results suggest that, under the current experimental conditions, the biosurfactant produced by *B. subtilis* JK-1 can replace the chemically synthesized surfactant in degrading hydrocarbons.

**Evaluation of emulsification stability of the biosurfactant produced by *B. subtilis* JK-1.** Emulsification stability of the biosurfactant produced by *B. subtilis* JK-1 (Table 2) was measured. Stabilization ability of the biosurfactant was described by the decay constant, $K_d$ (the slope of the emulsion decay plot). Emulsion decay plots were constructed for various emulsifying substrate emulsions in the presence of the biosurfactant, and their respective $K_d$ values were calculated. The biosurfactant was the most stable on hexane with a $K_d$ of –1.92. The biosurfactant produced by *B. subtilis* JK-1 was superior to those described to the biosurfactant type II from *Nocardia* sp. L-417 (Kim et al., 2000). Table 2 presents data on the comparison of emulsifying stability of the biosurfactant of *B. subtilis* JK-1 with the chemically synthesized surfactants. For Tween 20 and Triton X-100, higher and more emulsification stabilities were detected when soybean oil was used as substrate. The biosurfactant was more stable on hexane than the tested chemically synthesized surfactants. Moreover, on hexadecane, the biosurfactant was comparatively more stable as compared to the chemically synthesized surfactants. Triton X-100 was the most stable on crude oil (–0.19) followed by Tween 20 (–2.88), biosurfactant (–7.50), and SDS (–14.72). In addition, the biosurfactant showed higher stability on the tested substrates than SDS. The emulsification stability of the biosurfactant observed in this study was comparatively similar to the emulsification stability recorded with the chemically synthesized surfactants.

**Crude oil degradation capacity of *B. subtilis* JK-1 by GC-FID analysis.** The biodegradation of aliphatic hydrocarbons present in crude oil by *B. subtilis* JK-1 was confirmed by GC–FID analysis (Fig. 3). GC–FID analysis showed that the aliphatic fraction of the abiotic control of crude oil contained simple aliphatic hydrocarbons

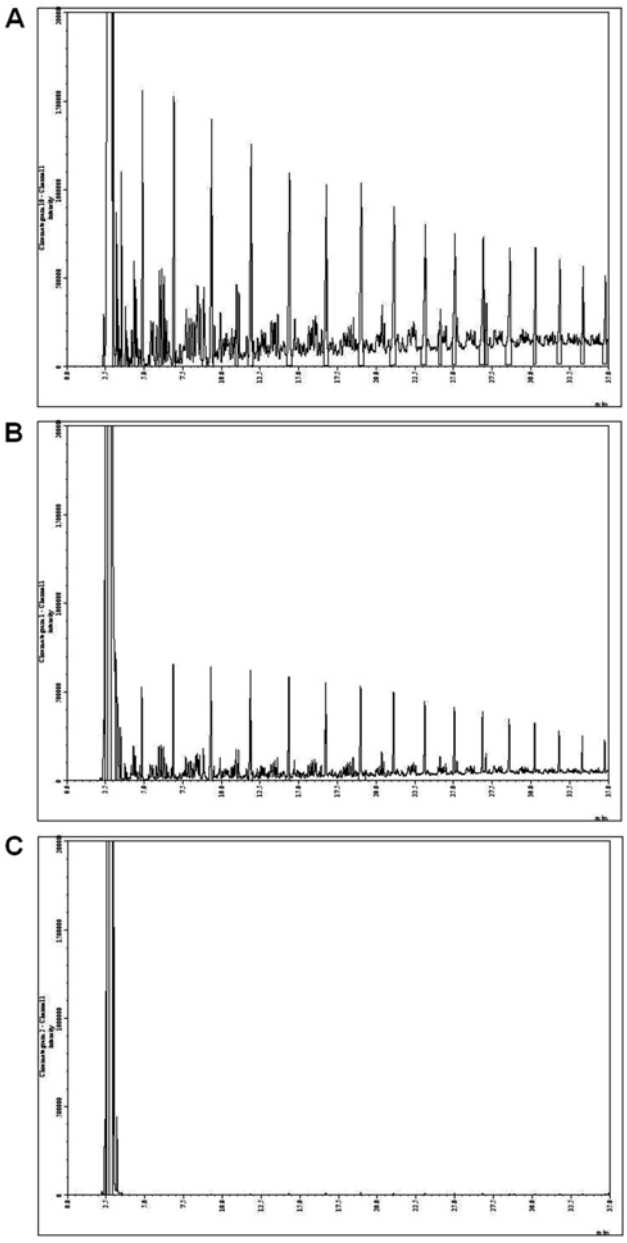


**Fig. 3** GC-FID analysis of crude oil degradation pattern after 48 h of incubation. (A) without inoculation (abiotic control of crude oil) (B) with *B. subtilis* JKC-15 (as a control) (C) with *B. subtilis* JK-1. All traces are representative of triplicate cultures.

(Fig. 3A) (Abdel-Mawgoud et al., 2008). The strain JK-1 showed a much more enhanced biodegradation when compared with those of the strain JKC-15. The peaks of *n*-alkanes significantly decreased, and the *n*-alkanes were almost completely degraded after 48 h of cultivation (Fig. 3C). Furthermore, in the GC-FID experiments, the culture broth mixed with crude oil was coated on the internal wall of flask when collecting the sample in every designated time after inoculating *B. subtilis* JK-1, and the crude oil was easily removed without using chemically synthesized commercial surfactants

(data not shown).

The collective results indicate that the biosurfactant from *B. subtilis* JK-1 is able to break apart the compounds present in crude oil. *B. subtilis* JK-1 may be a suitable candidate microorganism for practical field application to achieve effective *in situ* bioremediation of crude oil-contaminated sites.

In the present study, the characteristics of crude oil degradation of the biosurfactant produced by *B. subtilis* JK-1 was investigated. The strain was able to grow and produce the biosurfactant at 15–45°C, at a pH of 6–10, and at 0–10% (w/v) NaCl (Kim, 2011). Taking into consideration results of this previous study, sea water is a suitable environment for biosurfactant production by *B. subtilis* JK-1, because the strain readily degrades crude oil present in sea water. This strain was able to grow or produce the biosurfactants when crude oil was used as a carbon source. Furthermore, the biosurfactant of *B. subtilis* JK-1 has emulsification activity and stability against crude oil, and has a similar emulsification capacity compared to three chemically synthesized surfactants tested. This indicates that the biosurfactant may be capable of replacing the existing chemically synthesized surfactants. An emulsification assay with the biosurfactant against different hydrocarbons indicated the possibility of the application of biosurfactants against diverse forms of hydrocarbon pollution. In addition, we confirmed that the biosurfactant of *B. subtilis* JK-1 has an outstanding ability of degrading crude oil components. This is of particular importance for marine habitats, as South Korea does not approve the introduction of foreign species and genetically modified microorganisms to environment, but allows the use of indigenous strains such as *B. subtilis* JK-1 (Joo et al., 2007). In the case of any oil spills, the strain could be introduced in large numbers as a part of bioremediation efforts in locales such as gaps in rocks or on beaches.

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