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Production and Characterization of Lipopeptide Biosurfactant from *Bacillus subtilis* A8-8

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Abstract A biosurfactant-producing bacterial strain was selected from oil-contaminated soil because of its ability to degrade crude oil and tributyrin ($C_{4:0}$). The strain was identified as *Bacillus subtilis* A8-8 based on its morphological, biochemical, and physiological characteristics. When *B. subtilis* A8-8 was grown with crude oil as the sole carbon source, the biosurfactant from the strain emulsified crude oil, vegetable oil, and hydrocarbons. Soybean oil was the optimum substrate for the emulsifying activity and emulsion stability of the biosurfactant, both of which were superior to those of several commercially available surfactants. The biosurfactant was purified by a procedure including HCl precipitation, methanol treatment, and silica-gel chromatography. The partially purified biosurfactant was analyzed by TLC (thin-layer chromatography), SDS-PAGE, and HPLC and it reduced the surface tension of water from 72 mN/m to 26 mN/m at a concentration of 30 mg/l. Therefore, the purified lipopeptide biosurfactant has strong properties as an emulsifying agent and acts as an emulsion-stabilizing agent.

Key words: Lipopeptide biosurfactant, emulsification, *Bacillus subtilis* A8-8, CMC, surface tension

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding, such as oil/water or air/water interfaces [11]. Surfactants display various properties, for example, detergency, emulsification, foaming, and dispersion [2, 11, 14], that result from the presence of particular structural features.

Almost all surfactants in current use are chemically derived from petroleum; however, interest in microbial surfactants has been steadily increasing in recent years, as biosurfactants have numerous advantages compared with chemical surfactants, including a lower toxicity, higher biodegradability [43, 46], better environmental compatibility [3, 13], and higher specific activity at extreme temperatures, pH levels, and salinity [7, 27].

Biosurfactants are biologically surface-active agents produced by various microorganisms. On the basis of their molecular mass, biosurfactants isolated from microorganisms are generally classified into two groups: i) low molecular mass biosurfactants, such as glycolipids, lipopeptides, corynomycolic acids, and phospholipids, and ii) high molecular mass molecules, such as emulsans, alasan, liposan, polysaccharides, and protein complexes [36, 37, 43]. Lipopeptides with a low molecular mass, including bacillomycin, iturin, lichenysins, mycosubtilin, plipastatin, and surfactin, are well-known biosurfactants produced by *Bacillus* species [5, 13, 22] and exhibit surface-active properties, plus antifungal and antimicrobial activities [5, 17, 18, 23, 44, 45].

Since environmental compatibility is regarded as an increasingly important criterion for the selection of industrial chemicals, the use of biosurfactants in environmental applications, such as bioremediation and the dispersion of oil spills, has become increasingly frequent [2, 3, 13].

Accodingly, this study isolated *B. subtilis* A8-8 producing an effective biosurfactant. In addition, the biosurfactant was purified and characterized, and its properties compared with those of chemically synthesized surfactants.

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MATERIALS AND METHODS

Microorganism and Culture Conditions

B. subtilis A8-8 was used throughout this work and isolated from a soil sample polluted by oil constitutes. The basal medium used to cultivate the biosurfactant-producing bacterium was an M9 medium (6 g/l $NaHPO_4$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, pH 7.4, 2 g/l glucose, 0.0001 M $CaCl_2$, 1 mg/l casamino acid) containing 2% (v/v) crude oil [6, 26, 31]. The chemicals for the M9 medium were purchased from Sigma-Aldrich, Co. (St. Louis, MO, U.S.A.), and crude oil produced by the United Arab Emirates was obtained from S-Oil Refining Co. Ltd., Ulsan, Korea. The incubation was carried out at 37°C with shaking at 180 rpm for 4 days.

Isolation and Identification of Biosurfactant-Producing Bacterium

The soil samples were collected from oil-contaminated soil in Busan, Korea, and then appropriate amounts were suspended in sterile water, diluted, and spread on LB (Sigma-Aldrich, Co., St. Louis, MO, U.S.A.) agar plates containing 2% (v/v) crude oil and tributyrin ($C_{4:0}$, Sigma-Aldrich Co., St. Louis, MO, U.S.A.). The plates were incubated at 37°C for 2 days. After one day of incubation, the appearance of a halo zone around a colony spotted on a TB plate (LB agar plate containing tributyrin) was taken as evidence that a biosurfactant had been produced [17].

The morphological, biochemical, and physiological characteristics of the isolates were used to identify them according to *Bergey's Manual of Systematic Bacteriology* [19] and the *Manual of Methods for General Bacteriology* [14]. For more accurate identification, the partial 16S rRNA gene and *gyrA* nucleotide sequences were also analyzed. As such, the 16S rRNA gene and *gyrA* fragments, corresponding to the *B. subtilis* 16S rRNA gene and *gyrA*, respectively, and numbering positions 9-1,553 [28] and 42-1,066 [8, 28], were PCR-amplified using four oligonucleotide primers; namely, p16Sf (5'-GAGTTTGATCCTGGCTCAG-3'), p16Sr (5'-AGAAAGGAGGTGATCCAGCC-3'), pGryAf (5'-CAGTCAGGAAATGCGTACGTCCTT-3'), and pGryAr (5'-CAAGGTAATGCTCCAGGCATTGCT-3'). The PCR products were then extracted using a Gel Extraction Kit (NucleoGen Inc., Daejeon, Korea) and the DNA cloned into a pGEM-T-easy vector (Promega Co., Madison, WI, U.S.A.) for sequencing. Phylogenetic trees were constructed using the Neighbor Joining and the Clustal X program.

Petroleum Products and Other Oils

The food-grade soybean oil was obtained from Ottogi Co., Busan, Korea, and the hydrocarbons and tributyrin ($C_{4:0}$) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, U.S.A.).

Surface Tension of Culture Broth

The surface tension of the culture broth without cells was measured by the Ring method [32] using a Du Nouy Tensiometer (Itoh Seisakusho, Ltd. Tokyo, Japan) every 6 h for 4 days.

Assay of Emulsification Activity and Stability

The emulsification activities of the biosurfactants were measured using a modified method of Cirigliano and Carman [9, 10]. The samples were diluted with distilled water to a final volume of 4 ml, and the solution mixed with 1 ml of a substrate (soybean oil, kerosene, crude oil, tributyrin, hydrocarbons). Thereafter, the mixture was shaken vigorously in a vortex mixer for 2 min, and then allowed to sit for 10 min before measuring the turbidity at 540 nm. The absorbance was expressed as the emulsification activity.

The emulsion stability was analyzed based on the emulsification activity [9, 10]. The emulsified solutions were allowed to stand for 10 min at room temperature, and then absorbance readings were taken every 10 min for 60 min. As such, the log of the absorbance was plotted versus time, and the slope (decay constant, K_d) calculated to express the emulsion stability.

Comparison of Emulsion Activity and Stability for Biosurfactants and Commercial Surfactants

The commercial surfactants were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), and their emulsification activities and stabilities measured, as described in Materials and Methods, with soybean oil as the substrate.

Purification of Biosurfactants

The bacterial cells were removed from the culture broth by centrifugation (13,000 ×*g*, 4°C, 15 min), and the supernatant acidified to pH 2.0 with 12 N HCl, after the formation of a precipitate at 4°C overnight [1, 44]. The precipitate collected by centrifugation was then extracted three times with methanol (100%) for 3 h. The crude biosurfactant was obtained as a brown-colored material using an EYELA rotary evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and then purified by silica gel 60 chromatography (Merck Co. Inc., Damstadt, Germany; 2.5×30 cm, 230–400 mesh).

Further purification was achieved using a reverse-phase HPLC system, SDS-PAGE [4, 29], and TLC.

Analysis and Identification of Biosurfactants

The homogeneity of the samples was checked, and the biosurfactants identified by TLC, SDS-PAGE, and HPLC. Precoated silica gel 60 F_{254} plates (Merck Co. Inc., Damstadt, Germany) were spotted with the samples and developed using the mobile phases chloroform/methanol/water (65:25:4, by vol.) [21, 40]. The plates that developed were then sprayed with water and dried with air [40],

whereas the other plates were sprayed with an ammonium molybdate-perchloric acid solution [a: 3 g ammonium molybdate in 25 ml H_2O; b: 1 N HCl; c: 60% $HClO_4$. Spray solution: mix total solution (a) with 30 ml (b) and 15 ml (c)] [41]. All the plates were then lightly heated to develop the spots formed by the different components in the samples. HPLC was performed using a reverse-phase liquid chromatography system, Waters 400 HPLC (Waters Co., Massachusetts, U.S.A.) equipped with a BDS C18 column (5 μ, 4.6×250 mm, Thermo Hypersil Ltd., Cheshire, U.K.) at room temperature. The system was operated at a flow rate of 1.0 ml/min with a solvent mixture of acetonitrile/water (60:40, by vol.) as the mobile phase. The purified biosurfactants were then subjected to 15% SDS-PAGE together with molecular mass markers (BSA, 66 kDa; albumin, 45 kDa; pepsin, 34.7 kDa; trypsinogen, 24 kDa; β-lactoglobin, 18.4 kDa; lysozyme, 14.3 kDa) according to standard protocols [30] using a Tris-glycine buffer as the gel buffer. After SDS-PAGE, the gel was stained with Coomassie blue R-250 and the biosurfactants extracted using the method of Kim and Jang [25]. The extracted biosurfactants were then confirmed by TLC using a solvent system (chloroform/methanol/water, 65:25:4, by vol.) and Iturin A was used as the standard.

CMC Value of Biosurfactant

The biosurfactant concentrations were determined by diluting the purified biosurfactant until reaching the critical micelle concentration (CMC), which was determined by plotting the surface tension as a function of the biosurfactant concentration, and then the surface tension at that point was designated as γcmc [24]. To ensure that the results were reproducible, the average of three independent measurements was taken.

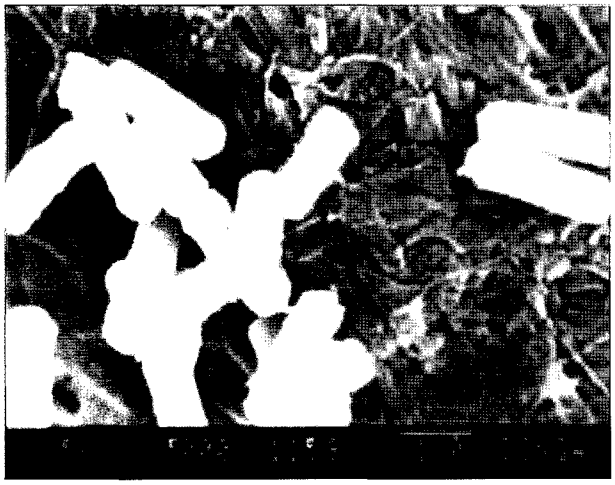

Fig. 1. Scanning electron microscopic (SEM) observation.
The colonies were fixed with glutaraldehyde and OsO_4, dehydrated by critical CO_2, and sputter-coated with platinum.

Results and Discussion

Characterization of Biosurfactant-Producing *B. subtilis* A8-8

The isolated strain shown in Fig. 1 was found to be a facultative anaerobic, Gram-positive, motile, rod-shaped bacterium with a length of 1.7 to 2.3 μm. The colonies were round and formed a mucoid gum, which was not swollen, yet oval and subterminally positioned. The strain used a variety of carbon sources for growth, including glucose, maltose, ribose, glycerol, mannitol, inositol, sorbitol, cellobiose, saccharose, and melibiose, but it did not use lactose, erythritol, rhamnose, or xylitol. It exhibited no arginin dehydrolase, lysine decarboxylase, ornithine decarboxylase, or tryptophan deaminase activity. In addition, it produced acetone, but did not produce indole or H_2S (Table 1).

Table 1. Morphological and physiological characteristics of biosurfactant -producing *B. subtilis* A8-8.

Characteristics	*Bacillus subtilis* A8-8
Morphological	rod
Gram stain	+
Mobility	+
Optimum temperature	37°C
Growth in air	+
Physiological	
Ortho-nitrophenylβ-D-galactopyranoside	-
Arginine dehydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Citrate utilization	-
Production of H_2S	-
Urease	-
Trytophan deaminase	-
Indole production	-
Acetone production	+
Proteolysis of gelatin	+
Glucose	+
Maltose	+
Lactose	-
Ribose	+
Glycerol	+
Erythritol	-
Mannitol	+
Inositol	+
Sorbitol	+
Xylitol	-
Cellobiose	+
Rhamnose	-
Saccharose	+
Melibiose	+
Amygdalin	-
L(T)arabinose	+
Adonitol	-
Inulin	+
Gluconate	-
2-Ketogluconate	-
5-Ketogluconate	-

+: Positive reaction; -: Negative reaction.

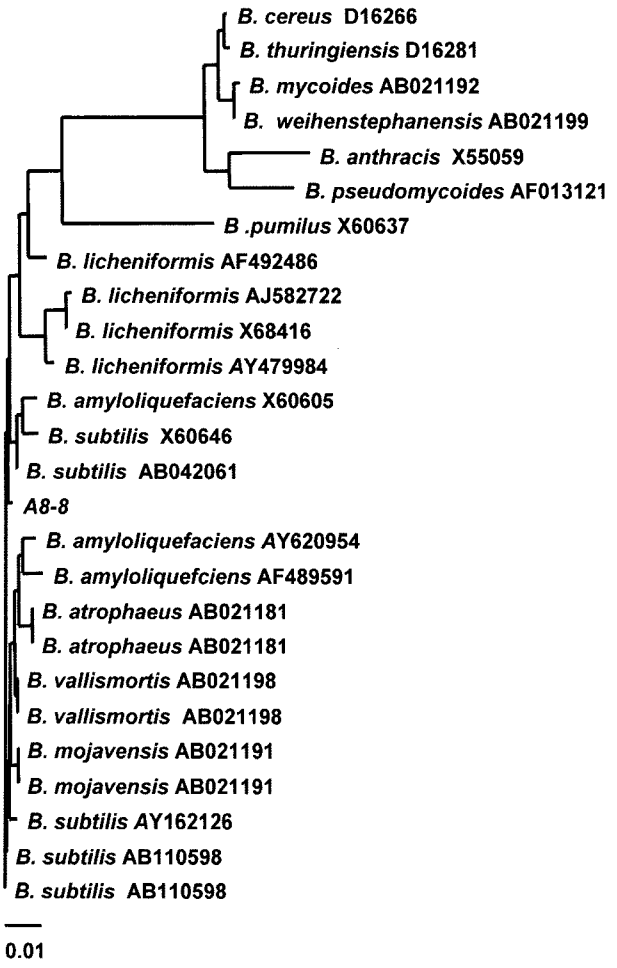

Fig. 2. Phylogenetic tree based on partial 16S rDNA nucleotide sequence.
The scale bar indicates 0.01 nucleotide substitutions per nucleotide position.

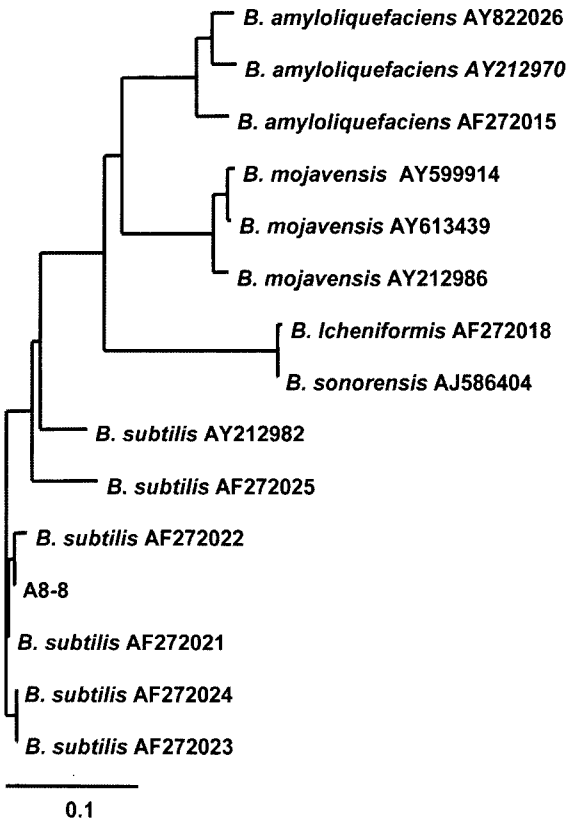

Fig. 3. Phylogenetic tree based on partial *gyrA* nucleotide sequence.
The scale bar indicates 0.1 nucleotide substitutions per nucleotide position.

The isolate was identified as *B. subtilis* A8-8 by Gram staining, API System (API 50 CHB ver. 3.0), and an analysis of the partial 16S rRNA and partal *gyrA* nucleotide (NT) sequences. The phylogenetic trees based on a neighbor-joining analysis of the 16S rRNA and *gyrA* NT sequences are given in Fig. 2 and Fig. 3, respectively. Several studies have recently been conducted on bacterial diversity in soil, marine, desert, and aqueous environments, utilizing extensive 16S rRNA sequence evaluations [12, 15, 29, 38, 42]. However, since the 16S rRNA sequences of diverse *Bacillus* species are highly similar to *Bacillus subtilis* (over 99%), partial 16S rRNA sequences provide insufficient resolution to distinguish various subspecies and close relatives of *B. subtilis* [35]. Thus, to obtain a more precise identification of *B. subtilis* A8-8, its partial *gyrA* sequences were compared with those of other *Bacillus* species, an approach that has proven useful for distinguishing *B. subtilis* from its close relatives [8].

Surface Tension of Biosurfactant-Producing Bacterium, *B. subtilis* A8-8

B. subtilis A8-8 was incubated in M9 media containing 2% crude oil at 37°C in a rotary shaker at 180 rpm for 96 h. Figure 4 shows the pattern of cell growth and surface tension of the resulting bacterial culture, where the cell growth was determined by measuring the absorbance of the culture at UV 600 nm. The surface tension of the culture filtrate decreased from 58 mN/m to 26 mN/m, and then remained stable at 26–27 mN/m after 36 h until 96 h (stationary phase). The lowest surface tension was 26 mN/m when the culture reached the stationary phase at 42 h. A reduction in the surface tension of media as a result of biosurfactant production and accumulation during the period between the exponential growth and stationary phases has already been reported for several other microorganisms [31, 39, 44].

Emulsifying Activity and Stability of Biosurfactant from *B. subtilis* A8-8

The emulsification activity of the biosurfactants from *B. subtilis* A8-8 was measured with various water-immiscible substrates (Table 2). The stabilization ability (Table 2) of the biosurfactant was described by the decay content, K_d

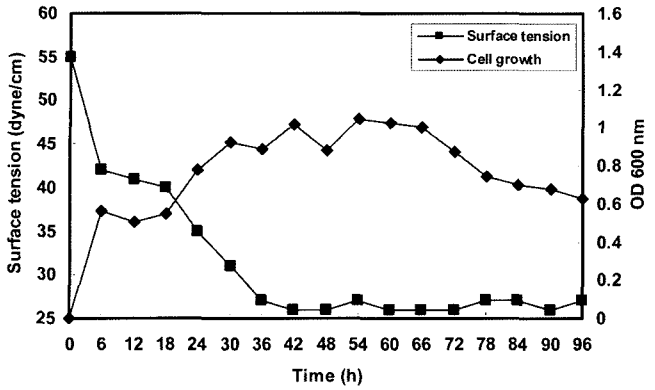

Fig. 4. Pattern of cell growth and decrease in surface tension with *B. subtilis* A8-8.
The *B. subtilis* A8-8 was grown at 37°C in an M9 medium supplemented with 6 g/l $NaHPO_4$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, pH 7.4, 2 g/l glucose, 0.0001 M $CaCl_2$, 1 mg/l casamino acid, and 2% (v/v) crude oil.

(the slope of the emulsion decay plot). Furthermore, emulsion decay plots were constructed for various emulsifying substrates in the presence of the biosurfactant from *B. subtilis* A8-8, and the respective K_d values were then calculated (Table 2). The results showed that the biosurfactant from *B. subtilis* A8-8 had a high emulsification activity and emulsion stability against tributyrin, kerosene, and crude oil as substrates, yet its highest emulsification activity and stability was against soybean oil as a substrate (Table 2). As such, the emulsification activity was similar to that of biosurfactant type I from *Norcardia* sp. L-417, although the emulsion stability of the biosurfactant from A8-8 was superior [24].

Comparison of Biosurfactant with Commercial Surfactants
Several commercial surfactants were investigated to compare their emulsification activities and emulsion stabilities with those of the biosurfactant from *B. subtilis* A8-8 (Table 3)

Table 2. Emulsification activity and stabilization of various substrates by biosurfactant.

Substrate	Emulsification activity ($OD_{540\ nm}$)[a]	Decay constant (K_d, 10^{-3})[b]
Soybean oil	2.75	-0.00
Tributyrin ($C_{4:0}$)	1.94	-1.88
Kerosene	1.46	-2.32
Crude oil	1.08	-5.89
Hexadecane (C_{16})	1.06	-2.54
Tetradecane (C_{14})	0.55	-4.48
Dodecane (C_{12})	0.71	-6.41
Decane (C_{10})	0.60	-6.11

[a]The emulsification assay was performed in the presence of the biosurfactant as described in the text. After an initial 10-min holding period, absorbance readings were taken every 10 min for 60 min.
[b]The log of the absorbance was plotted versus time and the slope (decay constant, K_d) calculated.

Table 3. Comparison of emulsification and stabilization properties of biosurfactant solution and commercial surfactants.

Surfactant	Emulsification activity ($OD_{540\ nm}$)	Decay constant (K_d, 10^{-3})
Biosurfactant	2.75	-0.00
Tween 20	2.71	-0.14
Tween 40	2.63	-0.12
Tween 80	2.60	-0.14
Span 40	1.80	-0.43
Span 85	0.88	-5.68
Triton X-100	2.36	-0.18
SDS	1.51	-16.05

The emulsification activity of the stabilizer was analyzed using soybean oil as described in the text. The decay constant (K_d) was calculated as described in footnote a, Table 2.

when using soybean oil as the substrate. The results indicated that the emulsification activity of the biosurfactant from *B. subtilis* A8-8 was better than that of several commercially available surfactants, except for the Tween detergents (20, 40, 80); however, its emulsion stability was far superior to that of all the commercial surfactants tested, indicating that the biosurfactant from A8-8 could be useful as an emulsifying and emulsion-stabilizing agent.

Isolation and Purification of Biosurfactant
The biosurfactant was purified from the culture filtrate of *B. subtilis* A8-8 grown in an LB-medium, as described in

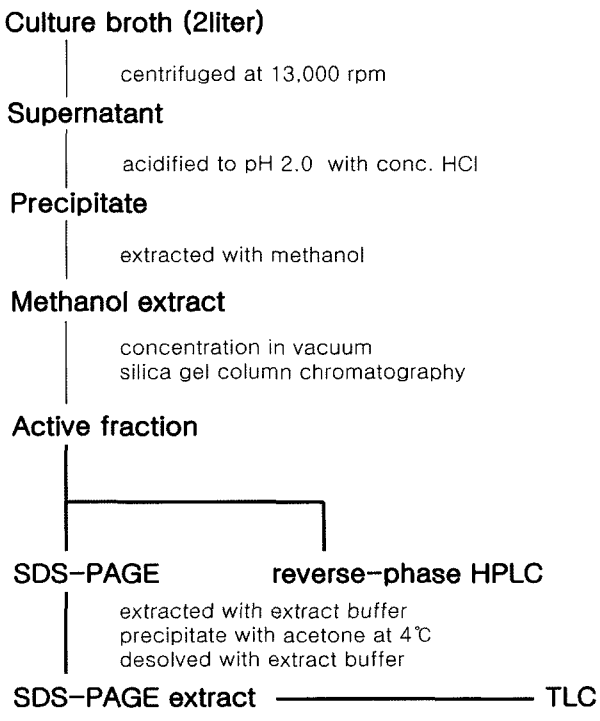

Fig. 5. Purification scheme for biosurfactant produced by *B. subtilis* A8-8.

Materials and Methods, and the purification procedure consisted of four steps, as shown in Fig. 5: HCl fractionation, methanol treatments, silica gel column chromatography, and SDS-PAGE extraction. The brown-colored precipitate containing the biosurfactant was obtained by 12 N HCl treatment and precipitation at 4°C overnight. The crude biosurfactant was extracted three times with methanol and concentrated in a rotary evaporator. It was then identified as several spots on a TLC plate developed using a one-solvent system (chloroform/methanol/water, 65:25:4, by vol.). Thereafter, the crude biosurfactant was concentrated in a rotary evaporator, dissolved in butanol (100%), and loaded onto a silica gel 60 column equilibrated with butanol (100%). The active fraction was then collected, concentrated, and dissolved in methanol to estimate its purity by reverse-phase HPLC. The HPLC chromatogram showed 4 peaks (data not shown), and the sample was detected as a broad band on the SDS-PAGE gel (Fig. 6A). Although the broad band on the SDS-PAGE gel was not expected to be the antibiotic reported earlier by Bechard *et al.* [5], the structure and molecular weight did resemble those of an antibiotic (data not shown). Thus, to confirm the accuracy, the band-positioned biosurfactant was extracted from the SDS-PAGE gel and developed by TLC. The purified biosurfactant was identified as one spot (R_f, 0.71) on the TLC plate sprayed with ammonium molybdate-perchloric acid solution, and displayed a white color on the inside and blue-black color on the outside of the spot.

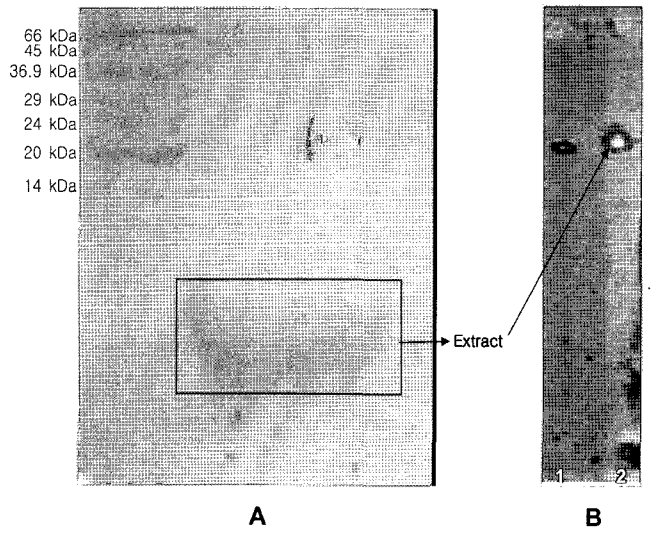

Fig. 6. SDS-PAGE patterns and TLC showing purified biosurfactant.

A. The biosurfactant was loaded at 30 µl (14.4 µg) and the standard markers (BSA, 66 kDa; albumin, 45 kDa; pepsin, 34.7 kDa; trypsinogen, 24 kDa; β-lactoglobin, 18.4 kDa; lysozyme, 14.3 kDa) at 10 µl. **B.** 1) Iturin A was used as the standard marker; 2) the spot indicates the biosurfactant extracted from the SDS-PAGE gel.

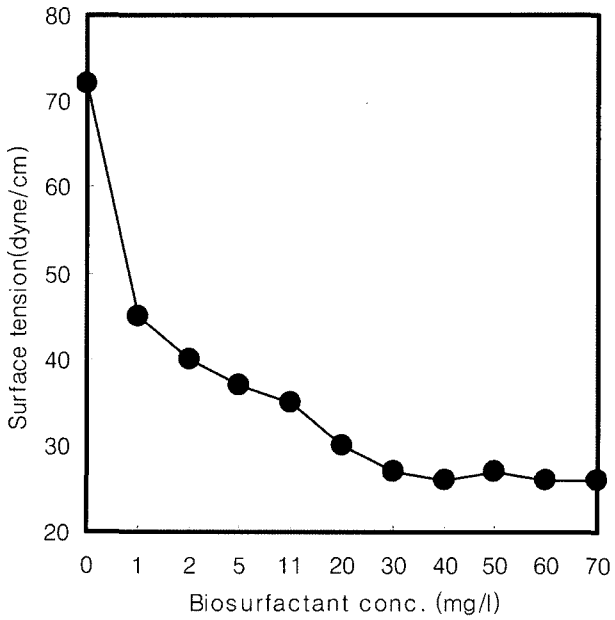

Fig. 7. Decrease in surface tension with purified biosurfactant.
The surface tension was measured by the Ring method using a DeNouy Tensiometer at room temperature.

Touchstone [41] previously mentioned that lipids and fatty acids produce blue-black spots. The R_f values and color of the purified biosurfactant were similar to those of iturin A (Sigma-Aldrich, U.S.A.) (Fig. 6B). Hence, it is believed that the purified biosurfactant was a lipopeptide with a similar structure to iturin A. Furthermore, this would seem to be the first time an ammonium molybdate-perchloric acid solution has been used to visualize a lipopeptide biosurfactant.

CMC of Purified Lipopeptide Biosurfactant

The purified lipopeptide biosurfactant was dissolved in distilled water, and the surface tension of the water was measured with various concentrations of the biosurfactant. As shown in Fig. 7, the CMC for the lipopeptide biosurfactant was approximately 30 mg/l, and the biosurfactant reduced the surface tension of the water from 72 mN/m to 26 mN/m. Generally, the surface tension at the CMC for various purified biosurfactants has been reported to range from about 27 to 35 mN/m [24, 31, 39]. For practical purposes, it is important to distinguish between an efficient surfactant and an effective surfactant, where the efficiency is measured by the surfactant concentration required to produce a significant reduction in the surface tension of water, whereas the effectiveness is measured as the minimum value to which the surface tension can be reduced [24, 34]. Therefore, the important characteristics of a potent biosurfactant are its ability to lower the surface tension of an aqueous solution and a low CMC [24].

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