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Purification and Characterization of Biosurfactants Produced by *Pseudomonas* sp. SW1

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Abstract *Pseudomonas* sp. SW1 grew and produced biosurfactants on 3% hexadecane as the energy and carbon source. As a result of biosurfactant synthesis, the surface tension of the medium was reduced from 72 dyne/cm to 30 dyne/cm. The properties of biosurfactants that were purified from *Pseudomonas* sp. SW1 were investigated. The purification procedure included acid precipitation from culture supernatant, silica gel G60 column chromatography, and Sephadex G-150 gel filtration. The biosurfactants were separated into two different types, viz., types I and II. Biosurfactant type I significantly reduced the surface tension of water from 72 to 27 dyne/cm at concentration levels above 30 mg/l. The surface tension of water was reduced to a minimum of approximately 30 dyne/cm by biosurfactant type II at concentration levels over 80 mg/l. The biosurfactants were effective in a wide range of pHs, at NaCl concentrations of up to 4%, at $CaCl_2$ concentration up to 100 mM, and at temperatures up to 200°C for 8 h.

Key words: Biosurfactant, surface tension, emulsification, *Pseudomonas* sp. SW1

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 [3]. These properties render surfactants capable of reducing surface and interfacial tension and forming microemulsion where hydrocarbons are made soluble in water or where water is made soluble in hydrocarbons. Such characteristics result in excellent detergency, emulsifying, foaming, and dispersing traits [6, 7]. Therefore, surfactants are widely used in the petroleum, pharmaceutical, cosmetic, and food industries. Most of these compounds are chemically synthesized. Because of high production costs of synthetic surfactants, their hazard to human health, and increasing consumer demand for natural products, biosurfactants have become increasingly important [14, 19].

Microorganisms produce a variety of biosurfactants [15]. Biosurfactants have gained considerable interest in recent years due to their low toxicity, effectiveness under extreme conditions, and their role in preserving the natural ecosystem by microbially enhanced oil recovery during oil spills [21]. Because biosurfactants are easily biodegradable and can be produced from renewable and relatively cheaper resources, they may be able to replace synthetic surfactants [3].

Although there are many reports on microorganisms producing biosurfactants or bioemulsifiers, reports of microorganisms secreting physicochemically stable biosurfactants are rare. Previously, we reported the optimal conditions for biosurfactants production by a newly isolated *Pseudomonas* sp. SW1 [17]. The biosurfactant type I, produced by *Pseudomonas* sp. SW1, was determined to be a polymeric biosurfactant containing rhamnose, lipid, and protein. The biosurfactant type II was a rhamnolipid consisting of rhamnose and lipid [18].

In this report, we describe the purification process of biosurfactants from *Pseudomonas* sp. SW1 to apparent homogeneity and their physical and chemical properties.

MATERIALS AND METHODS

The Microorganism and Culture Conditions

The microorganism used in this study was *Pseudomonas* sp. SW1, which was isolated from oil-contaminated soil samples [17]. A mineral salt medium containing the following components (g/l) was used to cultivate the bacterial strain: KH_2PO_4, 0.3; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.4; and NH_4NO_3, 4.0. The medium also contained trace elements solution containing the following components

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(mg/l) $CaCl_2 \cdot 2H_2O$, 10; $MnSO_4 \cdot 5H_2O$, 10; $FeCl_3 \cdot 6H_2O$, 10; $ZnSO_4 \cdot 7H_2O$, 10. The pH of the medium was adjusted to 7.0 and sterilized by an autoclave. *Pseudomonas* sp. SW1 was grown for 6 days in 2 liters of mineral salt medium containing 3% (w/v) hexadecane as the sole carbon source at 30°C in a 5-liter fermentor. Cells were removed from the culture by centrifugation at 12,000 × g for 15 mins. A culture supernatant was used for the measurement of surface tension and emulsification activity.

Isolation and Purification of Biosurfactants

A culture broth was centrifuged for 20 min at 12,000 × g to obtain a cell-free supernatant. The pH of the supernatant was adjusted to 2.0 with 1 N HCl and allowed to stand for 12 h at 4°C. The precipitate was collected by centrifugation, and then dissolved in a mixture of dichloromethane and methanol (1:1, v/v). The solvent was evaporated and the residue was washed with 3 volumes of *n*-hexane to remove hexadecane. The crude material was collected for further purification by the chromatographic procedure described below.

The crude material was dissolved in 5 ml of a mixture of chloroform and methanol (10:1, v/v) and subjected to a silica gel G60 column (2.5 × 100 cm) equilibrated with the same mixture. The loaded column was washed with 5 volumes of the equilibration mixture. The column was then eluted with the same mixture. The flow rate was 5 ml/min, and 5 ml each was fractionated, followed by biosurfactant detection. The active fractions containing biosurfactant types I and II were pooled and extracted with equal volumes of chloroform/methanol (1:1, v/v), and the solvents were evaporated to dryness. Biosurfactant type I was dissolved in 20 mM Tris-HCl buffer (pH 8.0) and loaded on a Sephadex G-150 column (1.5 × 80 cm) equilibrated with the same buffer. The column was then eluted with the equilibration buffer. Biosurfactant type II was dissolved in a mixture of chloroform/methanol/acetic acid (65:25:4, v/v/v) and applied to a silica gel G60 column (1.5 × 80 cm) equilibrated with the same mixture. The column was then eluted with the equilibration mixture.

The purity of the purified biosurfactant was examined by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). TLC was performed on a silica gel F_{254} TLC plate (Merck Co.) developed by various solvent systems before being stained using 50% sulfuric acid. HPLC was carried out using a Nova-Pak® C_{18} column. The mobile phase used was a mixture of acetonitrile and water (60:40, v/v), run at 15 ml/min and monitored by a differential refractometer. One μl of suitably diluted samples were injected.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a Mini-Protean™ II dual slab cell (Bio-Rad Labs, Hercules, U.S.A.). Electrophoresis was carried out as described in the manufacturer's instruction manual using 16.5% gel. The molecular weight of the purified biosurfactant was determined by gel filtration on a Sephadex LH-20 column (1.5 × 80 cm) equilibrated with 80% methanol.

Properties of the Biosurfactants

The effects of pH on the stability of the biosurfactants were tested after incubation in solutions of different pH for 24 h. Dry biosurfactant (20 mg) was dissolved separately in 10 ml of various acidic (pH 1~6) and alkaline (pH 7~12) solutions. The thermostabilities of the biosurfactant were measured after preincubation for 8 h at various temperatures (20~200°C) before the biosurfactant assay. The effects of various concentrations of sodium chloride (0~4.0%) and calcium chloride (10~100 mM) were also determined by adding sodium chloride and calcium chloride to the assay mixture. We also examined the abilities of purified biosurfactants to emulsify and stabilize oil-in-water emulsions using hydrocarbons and oils.

Analytical Methods

Dry cell weight was determined by washing the centrifuged cells once with distilled water and drying them to constant weight at 105°C. The surface tension of the culture supernatant and crude and purified biosurfactant was determined with a Tensiometer (Fisher Scientific, Surface Tensiomat® 21). The interfacial tension was also measured using the Tensiometer. Biosurfactant concentration was estimated by determining the factor for the culture supernatant necessary to reach the critical micelle concentration (CMC) [20]. If the concentration of the biosurfactant falls below the CMC, the surface activity depends only on the concentration of surface active molecules and, therefore, the values of surface tension increase. This dilution factor (F_{cmc}) is a direct measure of biosurfactant concentration. For the measurement of emulsifying activity, samples to be tested (0.5 ml) were introduced into a 50-ml flask containing 0.5 M NaCl in a TM buffer (20 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.0) to a final volume of 7.5 ml, and then 2 ml of a mixture of hexadecane/2-methylnaphthalene (1:1, v/v) was added. The samples were incubated with shaking at 30°C for 1 h. Turbidity was then determined at 540 nm [22]. Protein concentration was determined by the method of Lowry *et al.* [13] using bovine serum albumin as a standard protein. The total carbohydrate content was estimated by the phenol-sulphuric acid method [4].

RESULTS AND DISCUSSION

Growth and Biosurfactant Production

Figure 1 shows the pattern of biosurfactant formation by the bacterial culture used. Maximum biosurfactant production

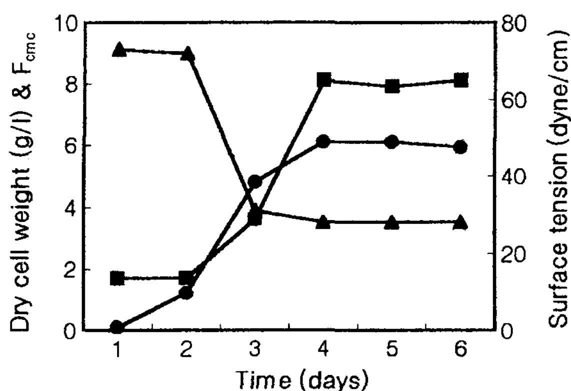

Fig. 1. Time course of cell growth and biosurfactant production by *Pseudomonas* sp. SW1.

Cells were cultivated in mineral salts medium containing 3% hexadecane for 6 days at 30°C and initial pH of 7.0. ●, dry cell weight; ■, dilution factor (F_{cmc}); ▲, surface tension.

took place during the late stationary phase of the culture. Reduction of the surface tension of the medium as a result of biosurfactant accumulation during the stationary phase was recorded to be maximum of 30 dynes/cm. *Pseudomonas aeruginosa* GS3 was reported to produce biosurfactant in growth-limiting conditions when the cells reached the stationary phase [14]. However, growth-associated production of emulsifiers has been reported from several other microorganisms [5, 8, 11].

Isolation and Purification of Biosurfactants

The biosurfactants were isolated from the culture supernatant of *Pseudomonas* sp. SW1 as described earlier in Materials and Methods. The crude biosurfactant recovered after acid precipitation was 4 g/l of the medium containing 3% hexadecane. It was dark brown in color and water soluble. The first silica gel G60 column chromatography of the crude product resulted in the separation of two products designated as biosurfactant types I and II (Fig. 2A).

The biosurfactant type I prepared in the previous stage was applied to a Sephadex G-150 column equilibrated with a 20 mM Tris-HCl buffer (pH 8.0). The surface tension reducing activity was eluted as a single peak with a constant ratio of protein to biosurfactant activity in each fraction (Fig. 2B). Biosurfactant type II was loaded on a secondary silica gel G60 column equilibrated with a mixture of chloroform/methanol/acetic acid (65:25:4, v/v). The surface tension reducing activity was eluted as a single peak with a constant ratio of sugar to biosurfactant activity in each fraction (Fig. 2C). Thus, the biosurfactants seemed to be nearly purified. The active fractions were pooled and saved for further analysis.

Biosurfactant types I and II isolated by TLC each gave a single spot in four different solvent systems. HPLC of the purified biosurfactants also resulted in one peak (data

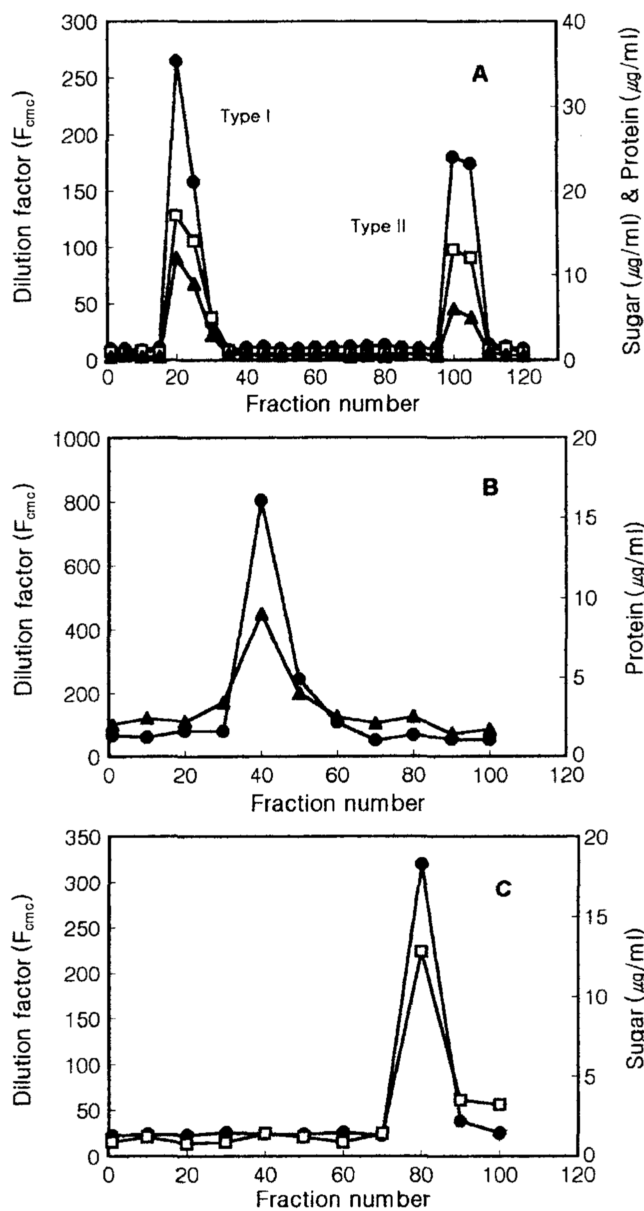

Fig. 2. Elution profiles of column chromatography for purification of biosurfactants.

A, Elution diagram of first silica gel G60 column chromatography; B, Elution diagram of biosurfactant type I from Sephadex G-150 gel filtration column chromatography; C, Elution diagram of biosurfactant type II from secondary silica gel G60 column chromatography. ●, dilution factor (F_{cmc}); □, sugar (μg as glucose); ▲, protein (μg as BSA).

not shown). Biosurfactant type I had a marked protein band with a molecular weight of 11,000 in gel electrophoresis (Fig. 3). The molecular weight of biosurfactant type II was estimated to be approximarely 5,000 from the elution volume of gel filtration on a Sephadex LH-20 (Fig. 4).

Properties of the Biosurfactants

One of the characteristic properties of surfactants is their ability to lower the surface tension of aqueous solutions.

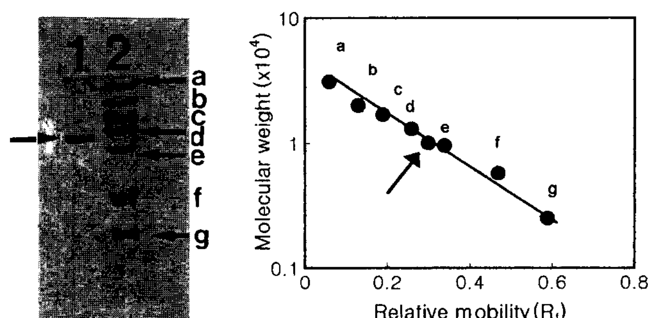

Fig. 3. SDS-PAGE of biosurfactant type I.

Lane 1 and ↑, purified biosurfactant type I; Lane 2, molecular standards containing (a) carbonic anhydrase (31,000), (b) soybean trypsin inhibitor (19,700), (c) horse heart myoglobin (16,900), (d) lysozyme (14,400), (e) myoglobin fragment (F1) (8,100), (f) myoglobin fragment (F2) (6,200), and (g) myoglobin fragment (F3) (2,500).

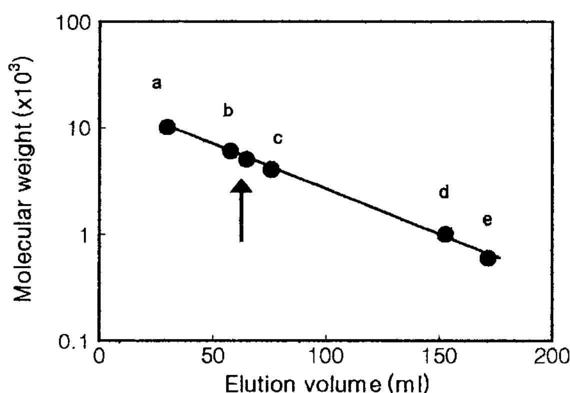

Fig. 4. Estimation of molecular weight of biosurfactant type II by using Sephadex LH-20 gel filtration.

a, polyethylene glycol (10,000); b, polyethylene glycol (6,000); c, polyethylene glycol (4,000); d, polyethylene glycol (1,000); e, polyethylene glycol (600). ↑, Biosurfactant type II.

Purified biosurfactants from *Pseudomonas* sp. SW1 were dissolved in aqueous solutions at concentrations ranging from 0 to 100 mg/l (w/v). As seen from Fig. 5, the CMC for biosurfactant type I is 30 mg/l, and the corresponding minimum surface tension at the CMC is 27 dyne/cm, while the CMC for biosurfactant type II is 80 mg/l at a corresponding surface tension of 30 dyne/cm. The minimum interfacial tension towards hexadecane was lowered to values smaller than 4.5 dyne/cm at a concentration of 12 mg/l of biosurfactant type I.

Figure 6A shows the effects of pH on the biosurfactants' properties. The biosurfactants were stable and active in a wide pH range of 2-12. The effects of pH on surface tension reducing activity has been reported for biosurfactants for different microorganisms. Maximum activity of liposan from *Candida lipolytica* is between pH 2.0 and pH 5.0 [2]. For emulsan, sharp maximum activity was obtained between pH 5.0 and pH 6.0; above pH 7.0, activity was completely lost [10]. The chemically synthesized surfactants such as sodium dodecyl sulfate (SDS) and linear alkylbenzene sulfonate (LAS) showed stable surface tension from pH 4.0 to 9.5, but an increase in surface tension from 27 to 32 dyne/cm at pH 10.3 [12].

Thermostability of the biosurfactants is shown in Fig. 6B. Surface tension was measured using the biosurfactant solution after incubation at each temperature for 8 h. The biosurfactants were shown to be stable at temperatures ranging from 20 to 200°C for 8 h. Liposan has been reported to be stable only up to 70°C [2]. Both biosurfactant types of *Pseudomonas* sp. SW1 exhibited a higher level of thermal stability than other bacterial and chemically synthesized surfactants reported by Kim *et al.* [12].

Sodium salt is a major component of sea water, and calcium salt, included in industrial water, frequently breaks the emulsion between oil and water in the practical process [9, 12]. The effects of NaCl and $CaCl_2$ concentrations on the stability of surface tension reducing activity are shown in Figs. 6C and 6D. Biosurfactant type I was unaffected by NaCl up to 4% and $CaCl_2$ up to 100 mM, although surface tension increased slightly with increasing salt concentration.

In addition to surface and interfacial tension, stabilization of an oil and water emulsion is commonly used as a surface activity indicator [1]. Table 1 presents experimental results on the emulsifying activity of the biosurfactants

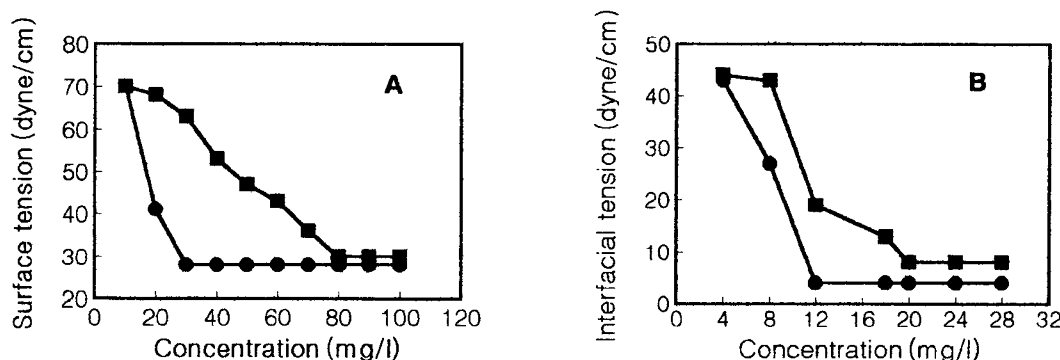

Fig. 5. Surface tension (A) and interfacial tension (B) measurements in various concentrations of purified biosurfactants.

The interfacial tension was measured against hexadecane; ●, biosurfactant type I; ■, biosurfactant type II.

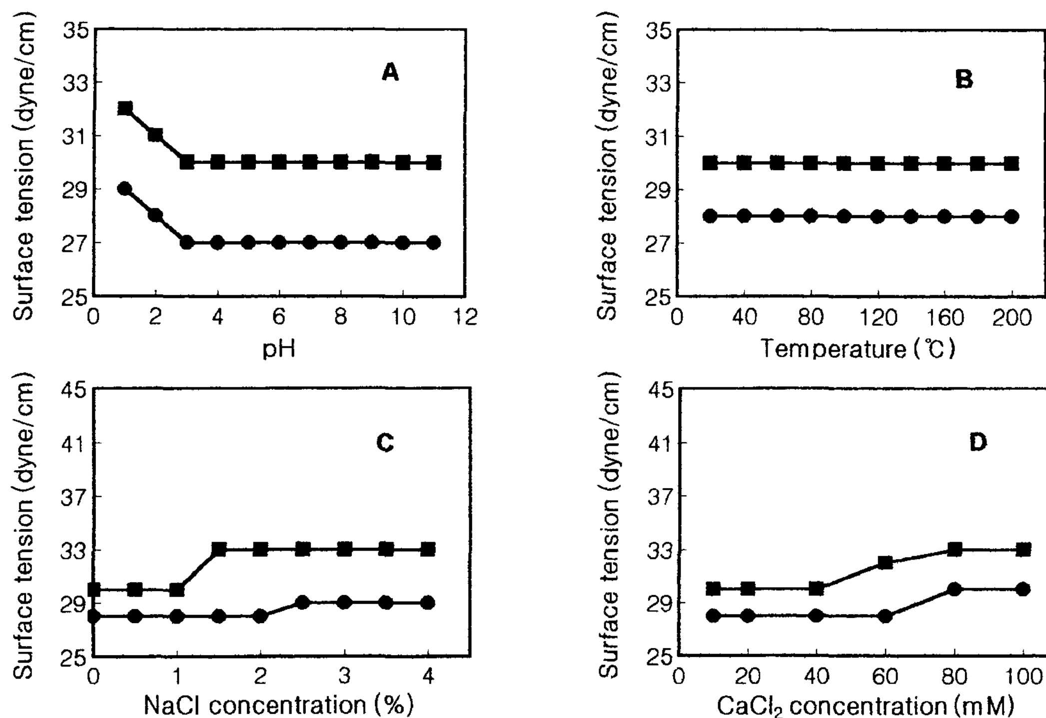

Fig. 6. Effects of pH (A), temperature (B), NaCl concentration (C), and $CaCl_2$ conentration (D) on the stability of purified biosurfactants.

Surface tension was measured using a biosurfactant solution (2 mg/ml); ●, biosurfactant type I; ■, biosurfactant type II.

Table 1. Emulsifying activity and stabilization of emulsions by biosurfactants.

Substrates	Emulsifying activity		Decay constant (K_d, 10^{-3})*	
	Type I	Type II	Type I	Type II
n-Dodecane	2.234	25.341	-2.46	-12.04
n-Tetradecane	2.564	28.922	-6.52	-30.97
n-Hexadecane	2.786	29.852	-10.42	-32.85
n-Octadecane	2.554	29.154	-4.67	-28.62
Paraffin	1.065	23.422	-0.72	-1.13
Olive oil	1.242	21.340	-1.0	-2.04
Soybean oil	1.614	25.437	-8.4	-24.97
Corn oil	1.316	23.741	-0.86	-0.68
Peanut oil	1.143	20.095	-0.72	-4.13
Castor oil	1.442	18.761	-0.51	-3.76
Crude oil	1.514	27.425	-7.98	-43.92
Bunker-A oil	1.132	26.242	-1.24	-22.12
Bunker-B oil	1.096	25.762	-0.97	-109.05
Bunker-C oil	1.116	26.157	-0.68	-20.45
2-Methylnaphthalene	1.419	26.420	-0.57	-8.6

*Emulsification assay was performed in the presence of a purified biosurfactant (50 mg). After the initial 10-min holding period, 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.

with various oils and hydrocarbons. Emulsification assay was performed in the presence of a purified biosurfactant (50 mg). Biosurfactant type I has a high emulsification specificity towards straight-chain alkanes, and a rather low efficiency with edible and petroleum oils, whereas biosurfactant type II has very high emulsification activity in the compounds tested. Biosurfactant type II also has high emulsion stability. This result is similar to that of Kaplan and Rosenberg's [10]. Thus, the broad range of pH stability, thermostability, and salt tolerance suggest that our biosurfactants could be useful in oil tank clean-up and in microbial-enhanced oil recovery.

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REFERENCES

1. Abu-Ruwaida, A. S., I. M. Banat, S. Haditirto, A. Salem, and M. Kadri. 1991. Isolation of biosurfactant-producing bacteria, product characterization, and evaluation. *Acta Biotechnol.* **11:** 315–324.
2. Cirigliano, M. C. and G. M. Carman. 1984. Isolation of a bioemulsifier from *Candida lipolytica*. *Appl. Environ. Microbiol.* **48:** 747–750.

3. Desai, J. D. and I. M. Banat. 1997. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* **61:** 47–64.
4. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Reberas, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Biochem.* **28:** 1–6.
5. Duvnjak, Z. and N. Kosaric. 1985. Production and release of surfactant by *Corynebacterium lepus* in hydrocarbon and glucose media. *Biotechnol. Lett.* **7:** 793–796.
6. Greek, B. F. 1990. Detergent industry ponders products for new decade. *Chem. Eng. News* **68:** 37–38.
7. Greek. B. F. 1991. Sales of detergents growing despite recession. *Chem. Eng. News* **69:** 25–52.
8. Johnson, V., M. Singh, V. S. Saini, D. K. Adhikari, V. Sista, and N. K. Yadav. 1992. Bioemulsifier production by an oleaginous yeast *Rhodotorula glutinis* IIP-30. *Biotechnol. Lett.* **14:** 487–490.
9. Jung, H.-K., J.-B. Lee, G.-B. Yim, and E.-K. Kim. 1995. Properties of microbial surfactants S-acid. *Kor. J. Biotechnol. Bioeng.* **10:** 71–77.
10. Kaplan, N. and E. Rosenberg. 1982. Exopolysaccharide distribution of and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. *Appl. Environ. Microbiol.* **44:** 1335–1341.
11. Kim, H.-S., C.-H. Lee, H.-H. Suh, K.-H. Ahn, H.-M. Oh, G.-S. Kwon, J.-W. Yang, and B.-D. Yoon. 1997. A lipopeptide biosurfactant produced by *Bacillus subtilis* C9 selected through the oil film-collapsing assay. *J. Microbiol. Biotechnol.* **7:** 180–187.
12. Kim, H.-S., B.-D. Yoon, C.-H. Lee, H.-H. Suh, H.-M. Oh, T. Katsuragi, and Y. Tani. 1997. Production and properties of a lipopeptide biosurfactant from *Bacillus subtilis* C9. *J. Fermen. Bioeng.* **84:** 41–46.
13. Lowry, O. H., N. H. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
14. Patel, R. M. and A. J. Desai. 1997. Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses. *Lett. Appl. Microbiol.* **25:** 91–94.
15. Pruthi, V. and S. S. Cameotra. 1997. Rapid identification of biosurfactant-producing bacterial strains using a cell surface hydrophobicity technique. *Biotechnol. Tech.* **11:** 671–674.
16. Rogenberg, M. and A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick. 1979. Emulsifier of *Athrobacter* RAG-1: Isolation and emulsifying properties. *Appl. Environ. Microbiol.* **37:** 402–408.
17. Son, H.-J., W.-S. Suk, G. Lee, and S.-J. Lee. 1997. Production of biosurfactant by *Pseudomonas* sp. SW1 for microbial remediation of oil pollution. *Kor. J. Microbiol.* **33:** 193–198.
18. Suk, W.-S., E.-G. Lim, H.-J. Son, G. Lee, and S.-J. Lee. 1999. Compositional analysis and some properties of biosurfactant from *Pseudomonas* sp. SW1. *Kor. J. Appl. Microbiol. Biotechnol.* **27**(1):.
19. Torabizadeh, H., S. A. Shojaosadati, and H. A. Tehrani. 1996. Preparation and characterisation of bioemulsifier from *Saccharomyces cerevisiae* and its application in food products. *Food Sci. Technol.* **29:** 734–737.
20. Zajic, J. E. and W. Seffens. 1984. Biosurfactants. *CRC Crit. Rev. Biotechnol.* **1:** 87–107.
21. Zinjardes, S., A. H. Chinnathambi, A. H. Lachke, and A. Pant. 1997. Isolation of an emulsifier from *Yarrowia lipolytica* NCIM 3589 using a modified mini isoelectric focusing unit. *Lett. Appl. Microbiol.* **24:** 117–121.
22. Zucherberg, A., A. Diver, and Z. Peeri. 1979. Emulsifier of *Athrobacter* RAG-1: Chemical and physical properties. *Appl. Environ. Microbiol.* **37:** 414–420.