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Purification and Characterization of Biosurfactant from *Tsukamurella* sp. 26A

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Abstract A biosurfactant produced by *Tsukamurella* sp. 26A was purified by procedures including acid precipitation, ethylacetate extraction, and adsorption chromatography. The purified biosurfactant reduced the surface tension of water from 72 mN/m to 30 mN/m at a concentration of 250 mg/l, whereas the minimum interfacial tension against *n*-hexadecane was lowered to 1.5 mN/m at a concentration of 40 mg/l. The compound stabilized oil-in-water emulsions with a variety of commercial oils and had strong emulsification and stabilization activities when compared to those of commercial emulsifiers and stabilizers. Surface tension was stable over a broad range of pH (2~12) and temperature (100°C, 3 h). The biosurfactant was identified as glycolipid having a hydrophilic moiety of trehalose.

Key words: Biosurfactant, glycolipid, *Tsukamurella* sp. 26A, surface tension, interfacial tension, bioemulsifier

Surface active compounds (surfactants, detergents, amphiphile) consist of both hydrophilic and hydrophobic moieties [11, 22, 32, 33]. They possess the capability to reduce the surface tension of aqueous media and to reduce the interfacial tension of liquid-liquid or liquid-solid systems [9]. Having these properties, surface active compounds are able to facilitate reactions and mass transfer at the interphase and can be applied to a variety of commercially important surfactant functions (emulsification, foaming, detergency, solubilization, wetting, and spreading) in industries as diverse as agriculture, building, elastomers, food, petrochemical production, and textiles [2]. They have also been used for the removal of oil sludges from oil storage tanks [3] and for the enhancement of oil recovery [16, 17]. Commercially available surfactants that are chemically synthesized seem to have certain limitations because many of them are toxic and biodegrade slowly [19]. In recent years, there has been a growing interest in the development of new microbial biosurfactants. The advantages of these biosurfactants are that new chemical types can be introduced, which could be difficult to chemically synthesize, and most of these biosurfactants are biodegradable [31]. Furthermore, sometimes, these compounds are less sensitive to extremes of temperature, pH, or salinity [30].

Recently, we isolated a microorganism from soil, designated as *Tsukamurella* sp. 26A, which produced a biosurfactant on liquid medium containing an *n*-hexadecane [5]. The biosurfactant not only reduced the surface and interfacial tension of the solution, but also stabilized oil-in-water emulsions with a number of water-immiscible compounds. This paper describes the purification and the partial characterization of the biosurfactant, trehalose lipid produced by *Tsukamurella* sp. 26A

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MATERIALS AND METHODS

Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories. *n*-Hexadecane was purchased from Sigma Co. (U.S.A). The thin layer chromatography (TLC) plate (Kieselgel 60 F254) was obtained from Merck Co. (Germany).

Microorganism and Culture Conditions

The strain used throughout this work, *Tsukamurella* sp. 26A, was isolated from soil samples [5]. Stock cultures of the organism were maintained on agar slants and transferred once a month. Inoculum was prepared by adding a loopful of cells from an agar plate to a test tube containing 10 ml nutrient broth and was grown at 30°C with gyratory shaking at 200 rpm. After 1 day, 2.5 ml of inoculum was added to 500-ml Erlenmeyer flasks, each containing 50 ml minimal medium (0.2% $NaNO_3$, 0.001% K_2HPO_4, 0.01% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02%

$CaCl_2 \cdot 2H_2O$, 0.02% yeast extract, and 0.02% tryptone, pH 7.0) supplemented with 7% *n*-hexadecane, and incubated at 30°C for 4 days.

Purification and Identification of Biosurfactant

The cells were removed from the culture broth by centrifugation at 12,000 rpm for 20 min. Crude biosurfactants were precipitated by lowering the culture supernatant to pH 2 with 1 N HCl and incubating at 4°C for 24 h and then separated by centrifugation at 12,000 rpm for 20 min. The precipitate was dissolved in a small volume of water and then extracted with one-half-volume of ethylacetate. The extract was evaporated to reduce volume, and loaded onto a silica gel 60 column for chromatography (column, 2.5×100 cm; solvent, chloroform: methanol= 4:1).

The purified biosurfactant was analyzed by the Diphenylamine reagent method [12], the α-naphtol-sulfuric acid method [12], the phenol-sulfuric acid method(4), the Anthron method [27], the Somogy-Nelson method [26], and the Elson-Morgan method [12] to detect carbohydrates, hydroxamine-ferric chloride reagent [12] for esterified fatty acids, the rhodamine 6G method [12] for lipids, and the ninhydrin method for amino acids.

TLC and HPLC Analysis

The homogeneity of the purified biosurfactant was identified by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Precoated TLC-plate (Merck) spotted with samples were developed with the following 4 solvent systems; chloroform:methanol = 80:20, chloroform:methanol:water = 80:20:2, ethylacetate: butanol:methanol:water =16:3:2:1, and isopropylalcohol: water:ammonia water = 8:1.9:0.3. The components on the plates were located by heating after spraying 50% sulfuric acid. HPLC was analyzed with a reverse-phase liquid chromatography system (Waters HPLC, M610E/M410) equipped with a Nova-pak C_{18} column (Waters, 3.9×150 mm) at room temperature. The system was operated at a flow rate of 0.5 ml/min with a solvent mixture, CH_3CN: water = 50:50.

Assay of Emulsification Activity

Emulsification activities of the biosurfactant and other commercial emulsifiers were measured by the method of Cirigliano and Carman with modification [7, 8]. The sample was diluted with distilled water to a final volume of 4 ml and 1 ml of *n*-hexadecane was added. The mixture was shaken vigorously on a vortex mixer for 2 min. The resulting uniform oil-in-water emulsion was allowed to sit for 10 min, after which its turbidity was measured at 540 nm. The absorbance was multiplied by the dilution factor and expressed as the emulsification activity.

Measurement of Biosurfactant Concentration

Biosurfactant concentration was estimated by determining the dilution factor of the culture broth necessary to reach the critical micelle concentration (CMC) [33]. If the concentration of surfactant falls below the CMC, the surface activity depends only on the concentration of monomeric surface active molecules and therefore, the values of the surface tension increase. This dilution factor is termed Fcmc which is a direct measure of biosurfactant concentration.

Surface and Interfacial Tension

The surface tension and interfacial tension of the biosurfactant were measured using the Ring Method [6, 20] of a CSC-DuNouy Tensiometer (Cole-Parmer Instrument Co., U.S.A.). The measurements of interfacial tension were made against *n*-hexadecane.

Infrared (IR) Spectroscopy

The biosurfactant (2 mg) was completely ground with potassium bromide (400 mg) in a mortar and the mixture was pressed to form a disk. The spectrum was obtained by using a Polaris FT-IR spectrometer (Matson, U.S.A.).

Saponification

The biosurfactant was dissolved in diethyl ether (10 ml) and treated with 0.5 M NaOH in 90% ethanol (15 ml) for 12 h at 60°C for hydrolysis of the ester bond [24]. After saponification, water (10 ml) was added and mixed with the hydrolysate. The resulting ether-extractable and water-soluble fraction was separated. The ether-extractable fraction was analyzed by gas chromatography (Hewlett Packard 5890A, column, 5% OV-101 packed glass column; oven temperature, 80~150°C; carrier gas, N_2; flow rate, 10 ml/min; detector, flame ionization detector). The aqueous solution containing sugar was evaporated and desalted by gel filtration chromatography (1.5×80 cm) using a Sephadex G-10, and then analyzed by HPLC (Carbohydrate analysis column, 4.6×250 mm; flow rate, 1.0 ml/min; mobile phase, acetonitrile:water = 70:30; detector, Waters 410 differential refractometer) for evaluation of its structure.

Detection of Sugar Moiety

Water-soluble fraction after saponification of biosurfactant was hydrolyzed with 1 ml of 2 N H_2SO_4 in a screw-capped tube at 90°C for 4 h [29]. After the hydrolysis, $CaCO_3$ was then added to neutralize the acid and the resulting solution was desalted by gel filtration chromatography, as described for saponification.

A portion of sugar after desalting was trimethylsilylated and analyzed with GC-MSD. For the preparation of trimethylsilyl (TMS) derivatives, the acid hydrolysis product (10 mg) was treated with anhydrous pyridine (0.1 ml),

Table 1. Identification of biosurfactant.

Component	Detection reagent	Native biosurfactant	Hydrophilic component of biosurfactant[a]	Acid hydrolysis product of hydrophilic component[b]
Carbohydrate	Diphenylamine reagent	+	+	+
	α-Naphtol-sulfuric acid	+	+	+
	Phenol-sulfuric acid	+	+	+
	Anthron	+	+	+
	Somogyi-Nelson arsenomolybdate	-	-	+
	Elson-Morgan reagent	-	-	-
Esterified fatty acid	Hydroxylamine-ferric chloride reagent	+		
Lipid	Rhodamine 6G	+		
Amino acid	Ninhydrin	-		

[a]Biosurfactant (10 mg) was dissolved in ethyl ether (10 ml) and treated with 0.5 M NaOH in 90% ethanol (15 ml) for 12 h at 60°C. After saponification, water (10 ml) was added and the hydrophilic fraction was separated.
[b]The hydrophilic component of biosurfactant was hydrolyzed with 1 ml of 2 N H_2SO_4 in the screw-capped tube at 100°C, 4 h, and neutralized with calcium carbonate.

hexamethyldisilazane (0.2 ml), and trimethylchlorosilane (0.1 ml). The reaction was carried out in a plastic-stoppered vial. The reaction mixture was shaken vigorously for about 30 sec and then was allowed to stand for 5 min at room temperature prior to chromatography. GC-MSD was carried out on a Hewlett Packard 5890A Series II (column, SP-1; resolution, 600; EI, 70 eV; mass range, 30~600 m/e; temperature, 180~280°C; Detector, Hewlett Packard 5970 MSD) with a data system (Wiley 138).

Assay of Emulsion Stability

Emulsion stability was analyzed with emulsification activity [8]. The various synthetic surfactants and the biosurfactant from *Tsukamurella* sp. 26A were compared for stability of emulsification activities. The emulsified solutions were allowed to stand for 10 min at room temperature and then 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, and expressed as emulsion stability.

RESULTS AND DISCUSSION

Purification and Identification of Biosurfactant

The procedures including acid precipitation, ethylacetate extraction, and column chromatography were performed for the purification of the biosurfactant. Most of the surface activity was recovered in the acid precipitate. The ethylacetate solutions pooled by extraction of the samples three times were evaporated under reduced pressure, dissolved in small volumes of a mixture of chloroform and methanol (4:1), and then applied on a silica gel column. As a result, the eluent was separated into 4 fractions, but only one fraction showed surface activity. The active fraction was collected, dried, and dissolved in methanol to identify the purity. The sample was detected as one spot on the TLC with various solvent systems as described in Materials and Methods. The purified material also gave a single peak in analytical reverse phase HPLC (data not shown), indicating that the biosurfactant was purified to apparent homogeneity.

The purified biosurfactant was colorless and water soluble. The results of various color reactions suggested that the biosurfactant was a glycolipid composed of sugar and lipid (Table 1). According to the results of the infrared spectrum of the biosurfactant, the characteristic aliphatic chains (wave number 3,000 to 2,000, CH_2 and CH_3), the band corresponding to ester carbonyl group (wave number 1,737, CO), and hydroxyl group (wave number 3,406, OH) were observed (Fig. 1). These results

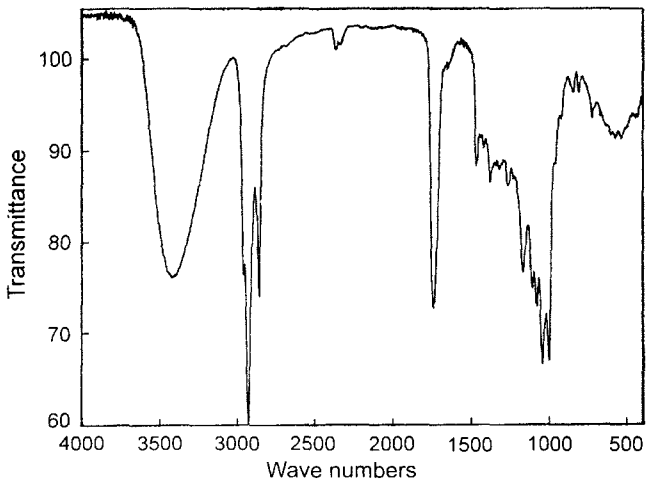

Fig. 1. The infrared spectrum of the biosurfactant.

indicated that this compound contained an esterified aliphatic hydrocarbon.

Sugar Moiety

The positive reaction of the purified biosurfactant with the Anthrone reagent [23] suggested that one of its components was a carbohydrate. The biosurfactant was therefore saponified and the water-soluble fraction from the saponified mixture was examined by various color reactions and HPLC. The sugar moiety of the biosurfactant had non-reducing power (Table 1) and the retention time corresponded to that of standard sugars (trehalose) in HPLC analysis (Fig. 2A). Furthermore, the mixture of trehalose and sugar moiety was sharply detected with a single peak (Fig. 2B). These results indicated that the sugar moiety of the surfactant should definitely be trehalose. To confirm this result, the water soluble fraction was further hydrolyzed with acid and analyzed with GC-MSD. Figure 3A gives three major peaks which had retention times of 3.355, 4.364, and 6.370 min. The first and second peaks were identified as α- and β-anomer of D-glucose, respectively, by data analysis and by comparision of their mass spectra with authentic glucose (Figs. 3B and 3C). The third peak (retention time, 6.37 min) was regarded as non-hydrolyzed trehalose. This finding confirmed that the hydrophilic moiety of the biosurfactant was trehalose. The hydrophobic component of the biosurfactant extracted by ether after saponification was methylated and then subjected to gas chromatography for the identification of its structure. It gave a single peak and seems to be composed of a single lipid with relatively short chains (data not shown), but the precise structure of the moiety is unknown.

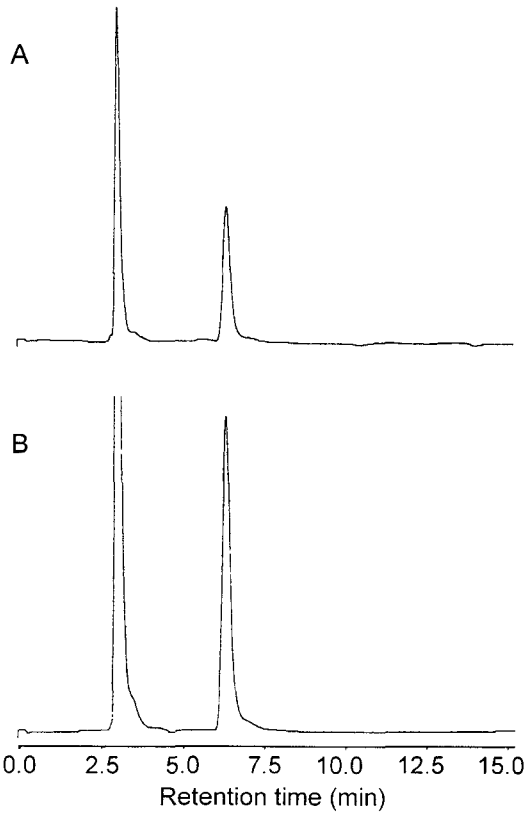

Fig. 2. HPLC chromatogram of aqueous layer after alkaline hydrolysis of biosurfactant.

Column, carbohydrate analysis; Flow rate, 1.0 ml/min; Detector, Waters 410 Differential Refractometer; Mobile phase, acetonitrile water (70:30); A, product of carbohydrate after alkaline hydrolysis; B, product A+ trehalose.

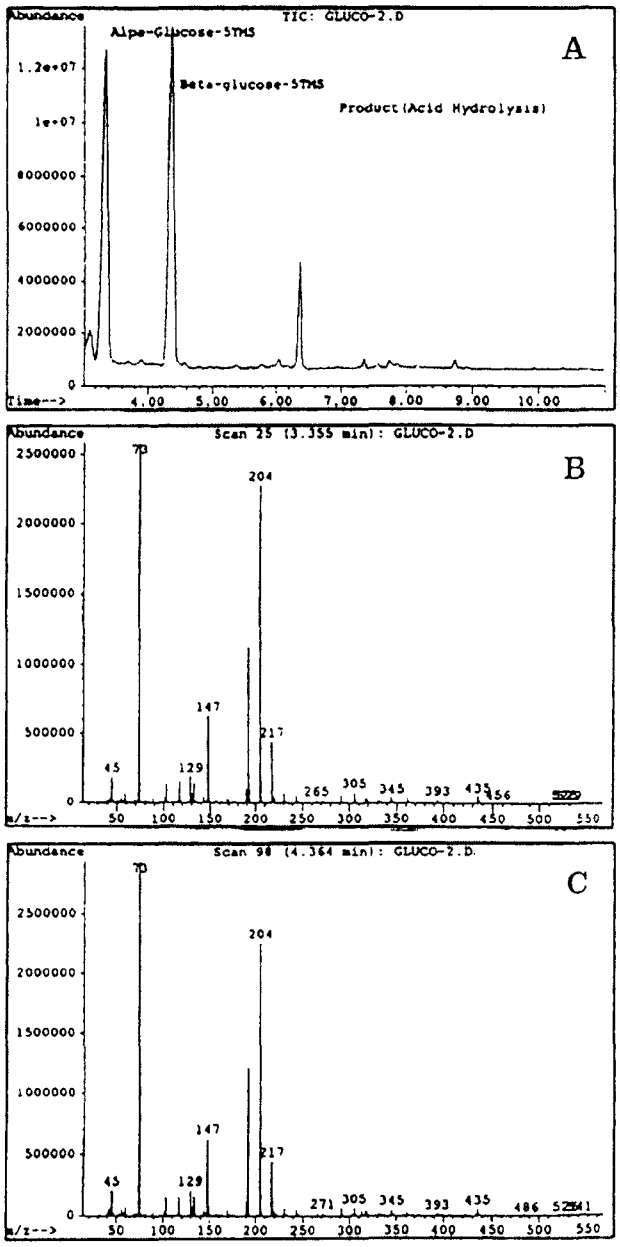

Fig. 3. Mass spectrum of trimethylsilylation (TMS) derivative of aqueous layer after acid hydrolysis of the biosurfactant.

A, acid hydrolysis product; B, standard (glucose).

Biosynthesis of glycolipids from a variety of bacteria has been reported [19, 26]. Trehalose lipid has been detected in some microorganisms such as *Mycobacteria, Brevibacterium, Arthrobacter, Corynebacterium, Nocardia, Rhodococcus* [11, 22, 31] and their structure was deduced, but the lipid moiety of most glycolipids is not clear. Among trehalose lipids previously reported, a biosurfactant from *Rhodococcus erythropolis* DSH 43215 consisted predominently of saturated long-chain α-branched β-hydroxy fatty acids (mycolic acids) ranging from $C_{32}H_{64}O_3$ to $C_{38}H_{76}O_3$, of which $C_{34}H_{68}O_3$ and $C_{35}H_{70}O_3$ predominate [19]. Ioneda *et al.* [13] have also isolated trehalose dimycolates with saturated and mono-unsaturated fatty acids ranging from $C_{38}H_{76}O_3$ to $C_{46}H_{90}O_3$ from *Nocardia* and *Rhodococcus* grown on glycerol. The trehalose lipid extracted from *N. asteroides* contained saturated and mono-unsaturated mycolic acids ranging from $C_{32}H_{66}O_3$ to $C_{36}H_{72}O_3$, while the trehalose dimycolate from *C. diphtheriae* contained the saturated and mono-unsaturated mycolic acids of C_{32} [14]. Consequently, their lipid moieties consisted mostly of mycolic acids with a long chain. In contrast, the biosurfactant from *Tsukamurella* sp. 26A seems to consist of one type of lipid with relatively short chains according to the retention time of GC analysis.

Surface and Interfacial Activity

The purified biosurfactant was dissolved in distilled water and the surface tension was measured (Fig. 4). The biosurfactant reduced the surface tension of water from 72 mN/m to 30 mN/m at a concentration of 250 mg/l, while the minimum interfacial tension against *n*-hexadecane was lowered to 1.5 mN/m at a concentration of 40 mg/l, suggesting that the biosurfactant from *Tsukamurella* sp. 26A is a good surfactant, especially for interfacial activity rather than surface activity. The biosurfactant produced by *Tsukamurella* sp. 26A exhibited a strong surface activity and interfacial activity compared with trehalose lipids produced by other microorganisms [19, 23].

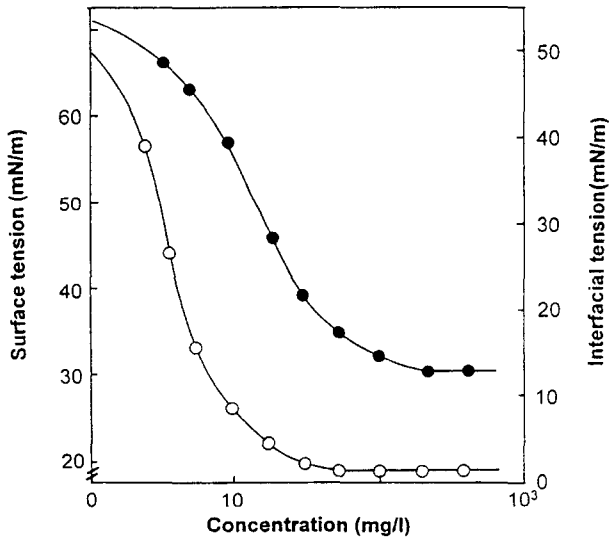

Fig. 4. Surface and interfacial tension versus concentration of the purified biosurfactant.
—●—, surface tension; —○—, interfacial tension.

Temperature and pH Stability

The effects of temperature and pH on the surface activity were investigated. The surface tension values of the biosurfactant were measured at the pH range 2~12 and at 100°C over 3 h with 10 min intervals. The CMC value means the point where surface-active compounds begin to aggregate to form micelles.

The biosurfactant remained stable after exposure to a high temperature of 100°C for 3 h. Surface tension activity was uniformly maintained at pHs ranging from 2 to 12 at room temperature, indicating that the pH variation has no appreciable effect on surface tension (data not shown). Most known biosurfactants are less stable over an extreme pH range [1, 7, 21, 34].

Emulsification and Stabilization Properties

Emulsification activity of the biosurfactant was measured with various water-immiscible substrates (Table 2). The stabilization ability of the biosurfactant was described with the decay constant, K_d (the slope of the emulsion decay plot. $dOD_{540}/dt = K_dOD_{540}$). The greater the stability, the smaller the K_d value. These results showed that the biosurfactant produced by the *Tsukamurella* sp. 26A had high emulsification activities against soybean oil, *n*-hexadecane, and crude oil, but it had a rather lower efficiency to corn oil, peanut oil, and caster oil. The emulsification activity was lower to the mixtures of *n*-hexadecane and 2-methylnaphthalene than that of 2-methylnaphthalene alone. These findings are different

Table 2. Emulsification activity and stabilization of substrates by the biosurfactant.[a]

Substrate	Emulsification activity (OD_{540})	Decay constant (K_d, 10^{-3})
Castor oil	1.58	1.0
Corn oil	2.35	9.5
Crude oil	2.96	0.6
n-Hexadecane	2.15	1.0
n-Hexadecane/ 2-Methylnaphthalene (1:1)	2.30	1.1
2-Methylnaphthalene	2.54	1.1
Olive oil	2.09	3.0
Peanut oil	0.99	6.5
Soybean oil	2.31	1.0

[a]The emulsification assay was performed in the presence of biosurfactant (0.1%) as described in the text except that indicated oils were substituted for *n*-hexadecane.
Decay constant (K_d) was calculated as described in Materials and Methods ($dOD_{540}/dt = K_dOD_{540}$).

from that of Kaplan and Rosenberg [15] who reported that a mixture of an aliphatic hydrocarbon (*n*-hexadecane) and an aromatic hydrocarbon (2-methylnaphthalene) was required for maximum emulsion formation. Several other commercially available emulsifier-stabilizers were examined for their emulsification and stabilization properties using the *n*-hexadecane as a water-immiscible oil and compared with the purified biosurfactant (Table 3, Fig. 5). The result showed that both emulsification activity and stabilization properties of the biosurfactant from *Tsukamurella* sp. 26A were better, compared to those of synthetic commercial surfactants.

It still remains to conduct more chemical and structural studies about the biosurfactant from *Tsukamurella* sp. 26A. For application to industry, we also are considering studies for mass production of the biosurfactant and for safety evaluation.

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

Surfactant	Emulsification activity (OD_{540})	Decay constant (K_d, 10^{-3})
Biosurfactant	2.52	0.43
Emulex 600Di-IS	2.66	0.36
Emulex GWIS-115	1.19	0.20
Emulex NP-12	1.27	6.30
Span 80	1.74	3.04
Span 85	1.73	13.70
Tween 80	1.12	2.00
Tween 85	2.73	1.70
SDS	0.71	6.50
Triton X-100	2.12	1.70

*The indicated commercial surfactants (0.2%) was analyzed for emulsification activity by using *n*-hexadecane as described in the text. Decay constant (K_d) was calculated as described in Materials and Methods ($dOD_{540}/dt = K_d OD_{540}$).

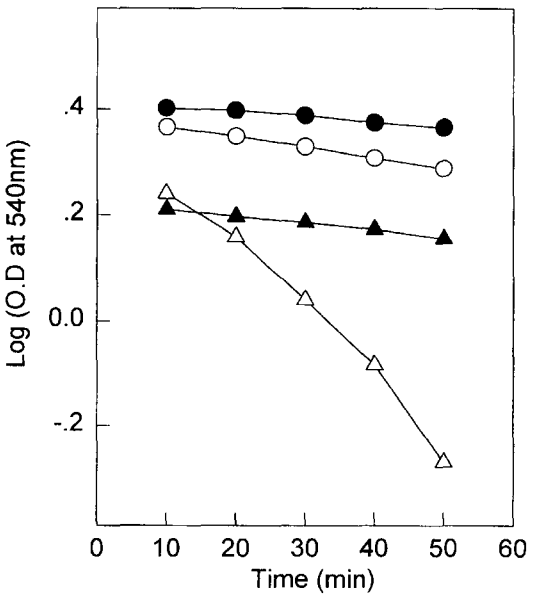

Fig. 5. Stabilization of emulsion by the purified biosurfactant.

Emulsifying substrate was *n*-hexadecane. The absorbance (A_{540}) of the emulsion was determined at the indicated times. After the initial 10 min holding period, absorbance readings were taken every 10 min. The log of the absorbance was then plotted versus time, and the slope (decay constant, K_d) of the line was calculated. —●—, biosurfactant; ○—, Triton X-100; —▲—, Tween 60; —■—, Span 85.

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