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Purification and Characterization of Bioemulsifier Produced by *Acinetobacter* sp. BE-254

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The *Acinetobacter* sp. BE-254 isolated from soil sources produced a bioemulsifier in the medium supplemented with n-hexadecane. This bioemulsifier was purified by the procedures of fractionation (ammonium sulfate and chilled acetone), extraction by hexane, and column chromatography on silica gel 60. The results from various color reactions indicated that the bioemulsifier was a glycolipid. The purified emulsifier was very stable at pHs ranging from 4 to 10 and under heat treatment at 100°C for 30 min. Emulsification activity was also hardly influenced by pH. The critical micelle concentration (CMC) and surface tension at the point (γ_{cmc}) of the bioemulsifier were approximately 35 mg/l and 30 mN/m, respectively. The bioemulsifier showed a fairly good emulsification activity and stability in comparison with other commercial emulsifiers in the basic formula composed of emulsifier, oil, and water.

Surface-activity, i.e. the ability to adsorb at interfaces, is a consequence of both hydrophilic and lipophilic moieties being present simultaneously in the molecules or particles (6, 11). The interactions of different surfactants and different interfaces (liquid/liquid, solid/liquid, liquid/gas, etc.) can give rise to a variety of commercially important surfactant functions (emulsification, de-emulsification, foaming, defoaming, detergency, solubilization, wetting, and spreading, etc.). Therefore, surfactants can be applied in industries as diverse as medical pharmaceuticals, food and beverages, cosmetics, agriculture, petroleum and petrochemical production, textiles, etc (6, 8, 10, 11).

Commercially available, synthetic surfactants, however, have several problems as some of them are toxic and not readily biodegradable (13, 19). Also, as most of them are produced from hydrocarbons, and through a series of processing steps where harmful side-products (sulfurous acid, carbon monoxide, carbon dioxide, etc.) are discharged, a number of costly purification steps are necessary (12, 18). Furthermore a single surfactant is not appropriate for all required applications. This in turn has led to the idea that surfactants of biological origin will expand the range of available agent and may in some cases displace the synthetics, either because of safety for humans or for economical and environmental reasons (8, 10, 13). Additionally, emulsifiers produced by naturally occuring microbes have another advantage, they can be produced on a large scale by relatively simple and inexpensive procedures (8, 10, 13).

For this reason we tried to develop a microbial bioemulsifier which can substituted for synthetic emulsifiers in a variety of circumstances. We first isolated a microbe, named *Acinetobacter* sp. BE-254 from soil sources (9) and purified the bioemulsifier produced by it. We also characterized several chemical and physical properties of the bioemulsifier and compared their emulsification ability and stability with those of commercial emulsifier and stabilizer.

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MATERIALS AND METHODS

Microorganism and Culture Conditions

The strain, *Acinetobacter* sp. BE-254, used throughout this work, was isolated from soil sources. Stock cultures were maintained on agar slant (2% glucose, 0.1% beef extract, 1.0% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% NaCl, and 1.6% agar, pH 7.0), and transferred once a month. The optimum culture conditions for the production of bioemulsifier are shown in Table 1.

Detection of Bioemulsifier

The bioemulsifier was identified by TLC on silicagel 60 F_{254} plate (Merck, Germany). The three solvent systems used for the TLC were as follows: (1) chloroform/methanol/H_2O (6:2:0.2, v/v/v); (2) ethylacetate/butanol/methanol/H_2O (16:3:2:1, v/v/v/v); (3) ethylacetate/acetate/methanol/H_2O (18:5:2:1, v/v/v/v). The compounds were visualized by spraying the plates with 50% H_2SO_4 and charring at 120°C. Alternatively, the plates

were sprayed with α-naphthol solution, and then with concentrated H_2SO_4 and anisaldehyde-concentrated H_2SO_4-CH_3COOH (1:2:100, v/v/v) to investigate the presence of glycolipids (4, 5, 14).

Emulsification Activity

For measuring the emulsification activity of an extracelullar emulsifier, a modification of the method developed by Rosenberg *et al.* (15, 16, 22) was employed. The sample (culture broth or purified material) was diluted with 0.05 M Tris-HCl buffer (pH 8.0) supplemented with 0.01 M $MgSO_4 \cdot 7H_2O$ to a final volume of 7.5 ml and placed in a test tube (18×150 mm); 0.1 ml of n-hexadecane and 2-methylnaphthalene mixture was added, and was voltexed vigorously for 2 min at 25°C. The resulting uniform emulsion was allowed to sit for 10 min, after which its mixture was measured at 540 nm. Reference experiments were conducted with samples excluding either emulsifier solution or substrates.

Emulsion Stability

The stability of oil-in-water emulsion formed by the bioemulsifier was tested as a modification of the method developed by Cirigliano *et al.* (2, 3). A mixture of n-hexadecane and 2-methylnaphthalene (1:1, v/v) was used as an emulsifying substance. The absorbance (A_{540}) of the emulsion formed by the bioemulsifier was determined over a 50-min time period. After the initial 10-min holding period, absorbance readings were taken every 10 min for 50 min. The log of the absorbance versus time was then plotted, and the slope of the line was calculated. The slope of the emulsion decay plot was used as decay constant (K_d) to present the stabilization ability of the bioemulsifier.

Surface Tension and CMC

Measurement of surface tension was performed with a Rigosha autotensiometer at room temperature. The concentration at which micelle began to form was represented as the critical micelle concentration (CMC). At CMC, sudden changes in some properties such as surface tension, electrical conductivity, and detergency were observed (17).

Table 1. The optimum culture condition for the production of bioemulsifier.

Medium (g/l)	n-Hexadecane	40 ml
	$NaNO_3$	2.0
	KH_2PO_4	0.5
	$MgSO_4 \cdot 7H_2O$	0.5
	$CaCl_2$	0.1
	Yeast Extract	0.1
	pH	6.8 - 7.0
Other conditions	Temperature	30°C
	Culture time	4 days
	Agitation	120 rev.×6 cm stroke
	100 ml of medium per 500 ml flask	

The CMC was determined by plotting surface tension as a function of bioemulsifier concentration, and surface tension at this point was designated as γ_{cmc} (17, 18).

RESULTS AND DISCUSSION

Purification of Bioemulsifier

The purification procedure consists of three steps as shown in Fig. 1; ammonium sulfate precipitation, chilled acetone and hexane treatments, and column chromatography on silica gel 60.

As most of the proteins and acetone-insoluble materials were removed by chilled acetone treatment, this step was critical in the purification procedures. After evaporating the solvent, the residue was separated by column chromatography on silica gel 60. The fractions showing emulsification activity were collected, and used for further characterization of the bioemulsifier properties. According to the elution profile of column chromatography on silica gel 60, the carbohydrate peak from O.D. at 490 nm by the phenol-sulfuric acid reaction (1) and the peak of the emulsification activity coincided, indicating that the bioemulsifier contains carbohydrate moieties(data not shown).

Fig. 2 shows a TLC chromatogram of the purified bioemulsifier conducted on a silicagel 60 F_{254} plate. Only one spot was detected on the plates with various solvent systems, indicating that the bioemulsifier was purified to apparent homogeniety. Sprayed with α-naphthol solution,

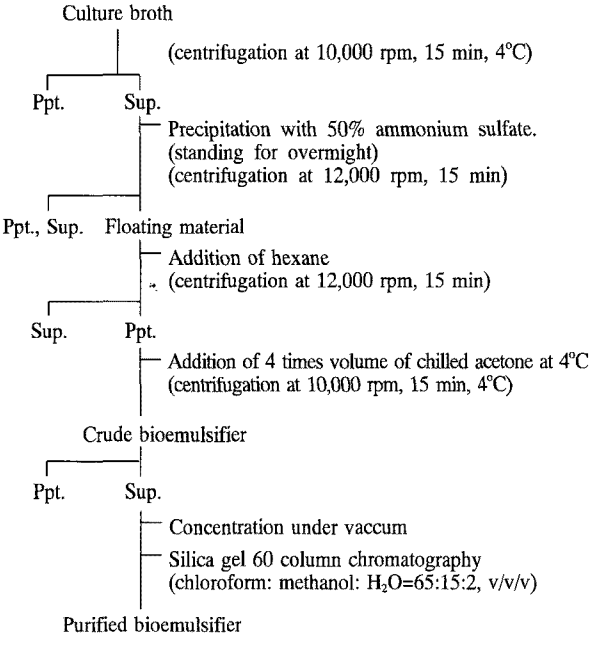

Fig. 1. Purification scheme of the bioemulsifier produced by *Acinetobacter* sp. BE-254.

Fig. 2. Thin layer chromatogram of the purified bioemulsifier.
The purified bioemulsifier was developed on silicagel 60 F_{254} plate with various solvent systems. A, chloroform/methanol/acetate (6:2:0.2); B, ethylacetate/butanol/H_2O (16:3:2:1); C, ethylacetate/acetate/methanol/H_2O (18:5:2:1).

followed by concentrated H_2SO_4, the spot on the plate generated a red-brown color, whereas it was green with anisaldehyde-concentrated H_2SO_4-CH_3COOH (1:2:100, v/v/v), indicating that the bioemulsifier was a glycolipid. Consistent with our observation, most of extracellular emulsifiers from microbial origins seemed to be either glycolipids or lipoproteins in nature (6, 12, 22).

Effect of pH on Emulsification Activity

The emulsification activity for n-hexadecane and 2-methylnaphthalene mixture as oil phase was measured at various pHs ranging from 3 to 12 at room temperature. The emulsification activity of the bioemulsifier produced by *Acinetobacter* sp. BE-254 was little influenced in the pH 3 to 9 range. (Fig. 3), whereas many other microbial emulsifiers have been described to have emulsifying activity at low pH values (2, 16, 20, 21).

Effect of pH and Temperature on the Stability of Bioemulsifier

To investigate the effect of pH on the stability of the bioemulsifier, the bioemulsifier-containing solution was adjusted to different pHs ranging from 3 to 12 and stood for 48 h at room temperature. When the remaining emulsification activity was measured at pH 7.0, the

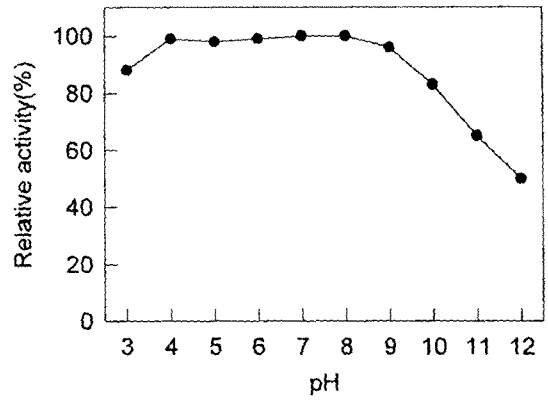

Fig. 3. Effect of pH on the emulsification activity.
The emulsification activity was measured at pHs ranging from 3 to 12 at room temperature.

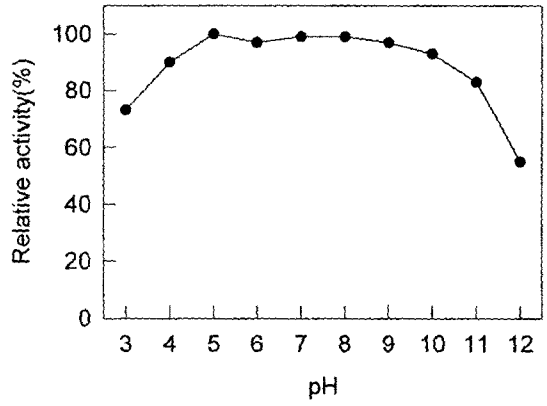

Fig. 4. Effect of pH on the stability of bioemulsifier.
After the bioemulsifier solution (0.02%, w/v) was adjusted to various pHs ranging from 3 to 12 at room temperature and stood for 48 h, the remaining emulsification activity was measured.

activity of the bioemulsifier was unchanged, therefore it was stable in the pH 4.0 to 10.0 range (Fig. 4).

To evaluate the stability against temperature, the bioemulsifier was incubated for 30 min at various temperatures ranging from 20 to 100°C at pH 7. As the bioemulsifier still showed a high emulsification activity at all ranges of temperature, it is believed that it was highly stable under heat treatment (Fig. 5).

Therefore, unlike many other microbial emulsifiers (eg. emulsan of *Arthrobacter* RAG-1 and liposan of *Candida lipolytica*) (3, 16), our bioemulsifier is very stable at a relatively broad range of pH and under heat treatment at 100°C for 30 min.

Emulsification Test

Several commercial emulsifiers and stabilizers were compared with the bioemulsifier produced by *Acinetobacter* sp. BE-254 for their emulsification activity and stabilization properties, using n-hexadecane and 2-methyl-

naphthalene mixture as a water-immiscible oil. As a results, our bioemulsifier had fairly good emulsification activity as compared with those of other commercial emulsifiers and stabilizers. It is similar to Triton X-100, and is three times higher than that of LAS which is a well-known synthetic detergent as shown in Table 2.

The stabilization effect of emulsions by the purified bioemulsifier and some commercial emulsifiers were measured as described in Materials and Methods. As shown in Fig. 6, this bioemulsifier has good emulsification stability as compared with several commercial emulsifiers. Since the methods of estimation on emulsification activity and emulsion stability are different from each other, an absolute comparison with the emulsifying agents produced by other microbes is currently not possible.

Surface Tension and CMC

One of the important characteristic properties of a potent surface active agent is the ability to reduce the surface tension of aqueous solutions, and the possession of a low critical micelle concentration (CMC) (13, 19). The CMC is a characteristic property of a surfactant, and can be found from a semilogarithmic plot of surface tension

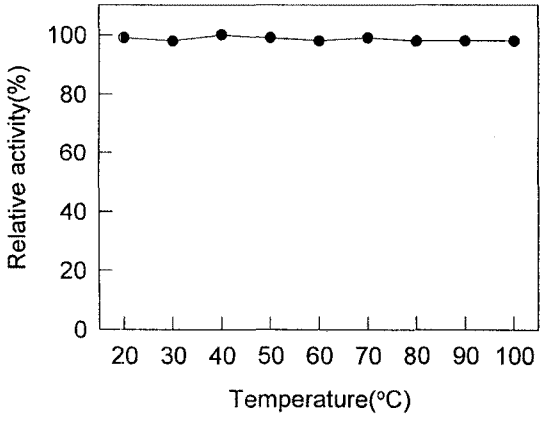

Fig. 5. Effect of temperature on the bioemulsifier stability.
After the bioemulsifier solution (0.02%, w/v) was adjusted to different temperature ranging from 20 to 100°C at pH 7 and stood for 30 min, the remaining emulsification activity was measured.

Table 2. Comparison of emulsifying and stabilizing properties of the purified bioemulsifier with other commercial emulsifiers and stabilizer.

Emulsifiers-stabilizers	Emulsification Activity (A_{540})	Emulsion Stability (K_d, 10^{-3})
Purified bioemulsifier	2.43	-2.05
Triton N-42	1.07	-7.27
Triton X-45	2.62	-4.33
Triton X-100	2.69	-1.75
Tween 20	0.83	-5.43
Tween 40	1.75	-2.06
Tween 80	1.18	-2.0
Span 40	0.60	-8.75
Span 80	0.80	-0.54
SDS	0.31	-7.0
LAS	0.74	-5.20
Gelatin	0.15	-10.32
Casein	1.18	-7.31

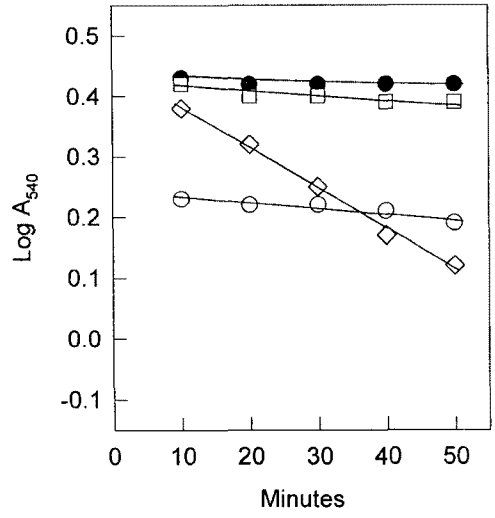

Fig. 6. Stabilization of the emulsions by purified bioemulsifier and commercial emulsifier.
A mixture of n-hexadecane and 2-methylnaphthalene (1:1, v/v)was used as an emulsifying substrate. The absorbance (A_{540}) of the emulsion was determined at the indicated times. After the initial 10 min holding period, absorbance readings were taken every 10 min. The log of the absorbance was then plotted versus time. —●—, purified bioemulsifier; —□—, Triton X-100; —◇—, Triton N-42; —○—, Tween 40.

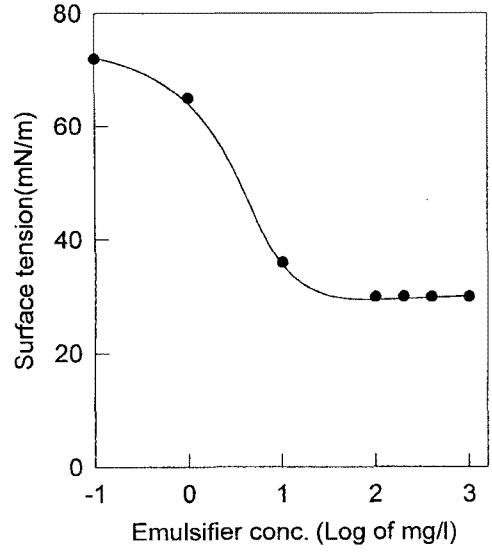

Fig. 7. Semilogarithmic plot of surface tension of the solution against the bioemulsifier concentration.
Measurement of surface tension was made with Rigosha autotensiometer at room temperature.

of the solution against the surfactant concentration (22). To measure CMC of the bioemulsifier, it was redissolved in distilled water and surface tension was measured at various concentrations. As seen in Fig. 7, CMC of the bioemulsifier produced by *Acinetobacter* sp. BE-254 was about 35 mg/l, and surface tension (γ_{cmc}) at this point was 33 mN/m. In particular, the bioemulsifier effectively reduced the surface tension of distilled water to near 30 mN/m, which was as effective as commercial, biodegradable surfactants, such as EP-680 and WS-1000 (7). These results indicate that the bioemulsifier was a good surface active agent in both aspects of efficiency and effectiveness.

The bioemulsifier produced by *Acinetobacter* sp. BE-254 needs further chemical, structural, and physiological studies. Before finding applications in various industries, we also have to solve some problems for mass production of the emulsifier and conducting safety evaluations, etc.

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