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Characterization of a Novel Lipopolysaccharide Biosurfactant from *Klebsiella oxitoca*

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Abstract The chemical, physical, and emulsifying properties of BSF-1, which is an extracellular lipopolysaccharide biosurfactant produced by *Klebsiella oxytoca* strain BSF-1, were studied. BSF-1 was found to be composed mainly of carbohydrate and fatty acids. The average molecular weight was 1,700~2,000 kDa. The polysaccharide fraction contained L-rhamnose, D-galactose, D-glucose, and D-glucuronic acid at a molar ratio of 3:1:1:1. The fatty acid content was 1.1% (w/w) and consisted mainly of palmitic acid (C16:0), 3-hydroxylauric acid (3-OH-C12:0), and lauric acid (C12:0). In terms of thermal properties, BSF-1 was revealed to have inter- and intra-molecular hydrogen bonds. The hydrodynamic volume (intrinsic viscosity) of BSF-1 was 22.8 dL/g. BSF-1 could be maintained as a stable emulsion for 48 h through a low-level reduction in surface tension. The optimal emulsification temperature was 30 °C. Emulsification by BSF-1 was efficient at both acidic and neutral pH values.

Keywords: biosurfactant, lipopolysaccharide, isolation, characterization, *Klebsiella oxytoca*

INTRODUCTION

An emulsion can be described as the dispersion of one liquid in another liquid. In order to prepare an emulsion from a mixture of immiscible solvents, a third component, surfactant, must be added. Surfactant molecules have both hydrophilic and hydrophobic moieties. Thus, it can solubilize both hydrophilic and hydrophobic solvents to form an emulsion [1].

The term 'biosurfactant' refers to any surfactant that is synthesized from biological sources. In general, biosurfactants are microbial metabolites with a typical amphiphilic structure. The hydrophobic moiety consists of a hydrocarbon chain of fatty acids. The hydrophilic moiety may be an ester, the alcohol function of neutral lipid, the carboxyl group of fatty acid, amino acids, phosphate-containing phospholipids, or the carbohydrate part of a glycolipid [2,3]. Biosurfactants can be categorized into four major groups: (i) glycolipid; (ii) phospholipid and fatty acid; (iii) lipopeptide/lipoprotein; and (iv) polymeric surfactant. Biosurfactants have many advantages over chemically synthesized surfactants in that they are highly specific, less toxic, and biodegradable. In addition, biosurfactants are effective at extremes of temperatures, pH, and salinity. They are also easy to produce from cheaper and renewable feedstock. The striking advantages of biosurfactants over chemically synthesized surface-active compounds include their broad range of novel structural characteristics and physical properties, and their capacity to be modified by genetic engineering, as well as by biological or biochemical techniques. Thus, they can be tailored to meet a specific application [4].

In spite of all these advantages, current industrial use of biosurfactants has been limited to applications in the petroleum industry. Much effort has been put into the application of biosurfactants to microbial enhanced oil recovery (MEOR). Much higher yields can be achieved using MEOR, whereby the biosurfactants decrease the surface tension and interfacial tension between water and oil within the well [5-10]. Biosurfactants could replace the relatively expensive petroleum sulfonates and lignosulfonates that are currently used. In 1987, the only commercial industrial biosurfactant on the market was emulsan, which has been patented by Gutnick *et al.* [11]. Among the various biosurfactants, emulsan is known to form a stable emulsion. Due to its polymeric nature, emulsan can maintain the emulsion once it is formed.

Because of the commercial potential of biosurfactants, it is desirable to screen and characterize new biosurfactants. In this study, we describe the characterization of a new lipopolysaccharide biosurfactant isolated from oil-contaminated soil.

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MATERIALS AND METHODS

Bacterial Culturing

Klebsiella oxytoca BSF-1 was used as the biosurfactant-producing strain [12]. This strain was isolated from

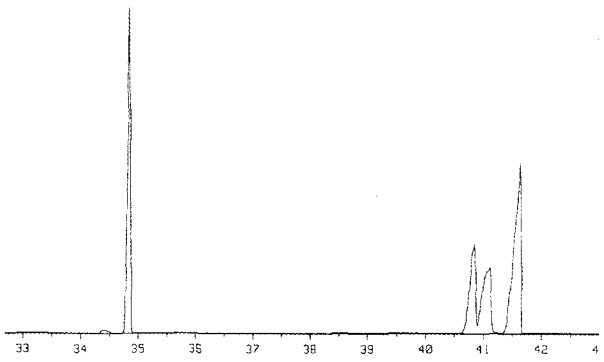


Fig. 1A. Gas chromatogram of BSF-1 monosaccharide. Analysis was performed after hydrolysis and acetylation, and BSF-1 carbohydrate composed of 4 kind of monosaccharides.

a bunker-C oil-contaminated soil in the Sangpyung Industrial Estate of Jinju, Korea [13]. The medium (pH 7.6) used for the production of biosurfactant contained 10 g/L glucose, 5 g/L KH_2PO_4, 1 g/L K_2HPO_4, 5 g/L tryptone, 2 g/L yeast extract, 200 mg/L $MgSO_4 \cdot 7H_2O$, 10 mg/L $MnSO_4 \cdot 7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4 \cdot 7H_2O$, and 10 mg/L $ZnSO_4 \cdot 7H_2O$. The cells were inoculated from the glycerol stock into 50 mL of the medium in a 250 mL Erlenmeyer flask, and incubated at 25°C with agitation at 250 rpm. After 12 h of cultivation, 5 mL of the cells were transferred to 500 mL of fresh medium in a Fernbach flask, and cultured for 72 h at 25°C with agitation at 250 rpm. The final culture supernatant was used for biosurfactant purification.

Purification of Biosurfactant BSF-1

After two rounds of centrifugation (6,000 rpm, 30 min, 4°C) to remove the cells, the supernatant was mixed with four volumes of ethanol, to induce precipitation. The precipitates were harvested by centrifugation (5,000 rpm, 15 min, 4°C), and then dissolved in 100 mL of distilled water (DW). The solution was dialyzed (MWCO of 12,000~14,000) for 24 h against cold water, and the sample was freeze-dried.

Chemical Analysis of BSF-1

The fatty acid composition and content were analyzed by gas chromatography and mass spectrometry using fatty acid methyl ester as the standard and following the method of Belsky *et al.* [14]. Purified BSF-1 (5 mg) was hydrolyzed for 16 h in 2 mL of 6 N HCl at 100°C under a nitrogen atmosphere. After cooling, the fatty acids were extracted three times with diethyl ether, and then dried over $MgSO_4$. Finally, the diethyl ether was evaporated, and the dried sample was analyzed by GLC-FID (DS 6200; Donam Instruments Inc., Seoul, Korea) with a capillary column (BP-1; Shimadzu, Kyoto, Japan). The temperature of the oven was raised from 100 to 280°C at a rate of 5°C/min. Benzoic acid (Sigma, St. Louis, MO, USA) was used as the internal standard. Each peak was assigned using GC-MS (JEOL JMS-SX 102A; JEOL, Akishima, Japan). The protein content was determined using a protein analysis kit (BSA; Sigma). The total carbohydrate content was determined by the phenol-sulfuric acid method with glucose as the standard [15].

The carbohydrate composition of BSF-1 was determined by both GC-MS and HPLC. BSF-1 was hydrolyzed using trifluoroacetate (TFA). Purified BSF-1 (5 mg) was reacted with 2 mL of 2 N TFA at 100°C for 2 h. After evaporation of the TFA, the reactant was mixed with 2 mL of DW and 5 mL chloroform. The solvent layer was discarded to remove the fatty acid contaminant. This product was used for HPLC analysis. Sugar-Pak column (Waters Corp., Milford, MA, USA) was used in the separation (DW elution at 0.5 mL/min, 90°C). The hydrolyzed monosaccharides were further reduced, and acetylized by conventional methods [16] for GC-MS analysis.

Determination of the Physical Properties of BSF-1

The molecular weight of BSF-1 was determined using serial GFC columns (Ultrahydrogel 500 and 2000; Waters) with an RI detector (Shimadzu, Kyoto, Japan). The samples were eluted with DW at 0.8 mL/min at 30°C during the separation. Dextrans with various molecular weights (2,000 kDa, 500 kDa, 71 kDa, and 11.3 kDa; Sigma) were used as the polysaccharide standards. The thermal properties of BSF-1 were measured using the DSC 920 differential scanning calorimeter (Dupont, Wilmington, DE, USA). The surface tension of the BSF-1 solution was measured using the K10ST digital tensiometer (Kruss, Hamburg, Germany). The stabilization of an emulsion by BSF-1 over time at 30°C was determined using the DU650 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA). The emulsifying activities were measured according to the Zuckerberg method [17]. In 7.5 mL of TM buffer [20 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$], 0.1 mL of test oil (1:1 mixture of hexadecane and 2-methylnaphthalane) and 1 mg of polymeric biosurfactant sample were dissolved in a 125-mL Erlenmeyer flask. After reciprocal shaking (150 strokes/min, 30°C) for 1 h, the absorbance was measured and the emulsifying activity was calculated on the basis of one unit = one absorbance unit at 600 nm.

RESULTS AND DISCUSSION

Chemical Characterization of BSF-1

The chemical properties of the BSF-1 biosurfactant were characterized. The total carbohydrate content, fatty acid content, and protein content were 71.1%, 1.1%, and 3.2%, respectively, as assayed by the phenol-sulfuric acid method, GLC method, and bicinchoninic acid method, respectively. The hydrophobic moiety consisted mainly of palmitic acid (C16:0), 3-hydroxylauric acid (3-OH-C12:0), and lauric acid (C12:0). The protein content could be reduced by extraction with solvents, such as phenol. Therefore, BSF-1 is composed mainly of carbohydrate

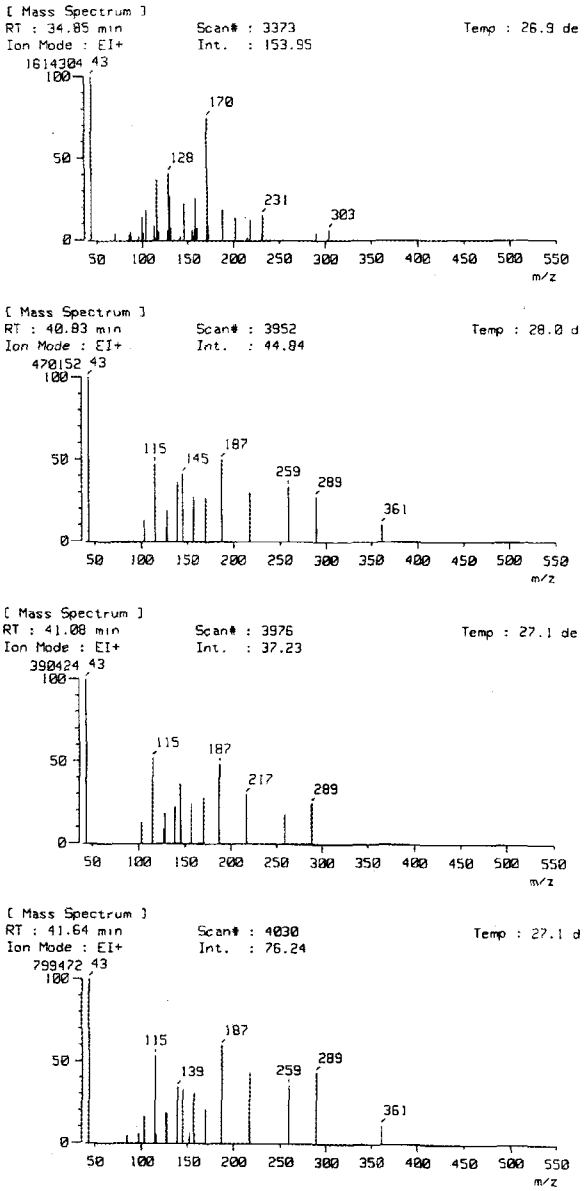


Fig. 1B. *Mass fragmentation of BSF-1 monosaccharide. Each peak was suggested as penta-o-acetyl-6-deoxy-L-manitol, hexa-o-acetyl-D-galacitol, hexa-o-acetyl-D-glucitol, and hexa-o-acetyl-D-iditol, respectively.*

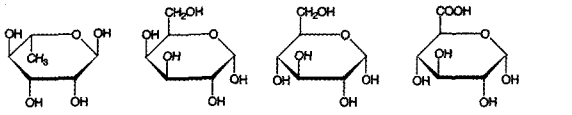
and fatty acids, and is contaminated by non-covalently bound protein.

The carbohydrate components were determined by GLC after hydrolysis and acetylation. Using gas chromatography, the carbohydrate was elucidated as being composed of four components (Fig. 1A). The peaks were certified by GC-MS (Fig. 1B) as being penta-*o*-acetyl-6-deoxy-L-mannitol, hexa-*o*-acetyl-D-galacitol, hexa-*o*-acetyl-D-glucitol, and hexa-*o*-acetyl-D-iditol, respectively. Thus, the carbohydrate components of BSF-1 are likely to be 6-deoxy-L-mannose (L-rhamnose), D-galactose, D-glucose, and D-idose. Three of these four components were identified using standards for rhamnose, galactose, and glucose. Due to modification before analysis by GC, carboxy- or amine-group-containing monosaccharides may have been misidentified. To verify these potential errors, the monosaccharides were double-checked by HPLC-RID without modification. In this case, three peaks were noted and exposed to glucuronic acid, galactose, and glu-

Table 1. Summary of chemical characteristics of BSF-1

Chemical composition	
Carbohydrate	71.1 ± 0.7%
Fatty acid	1.1 ± 0.1%
Protein	3.2 ± 0.5%

Carbohydrate components



L-Rhamnose(3) D-Galactose(1) D-Glucose(1) D-Glucuronic acid(1)

Rhamnose:Galactose:Glucose:Glucuronic acid ≈ 3:1:1:1

Fatty acid components

C10:0 : 0.02%; C12:0 : 0.11%; 3OH-C12 : 0.28%; C14:0 : 0.09% C16:1 : trace; C16:0 : 0.46%; C18:0 : 0.05%

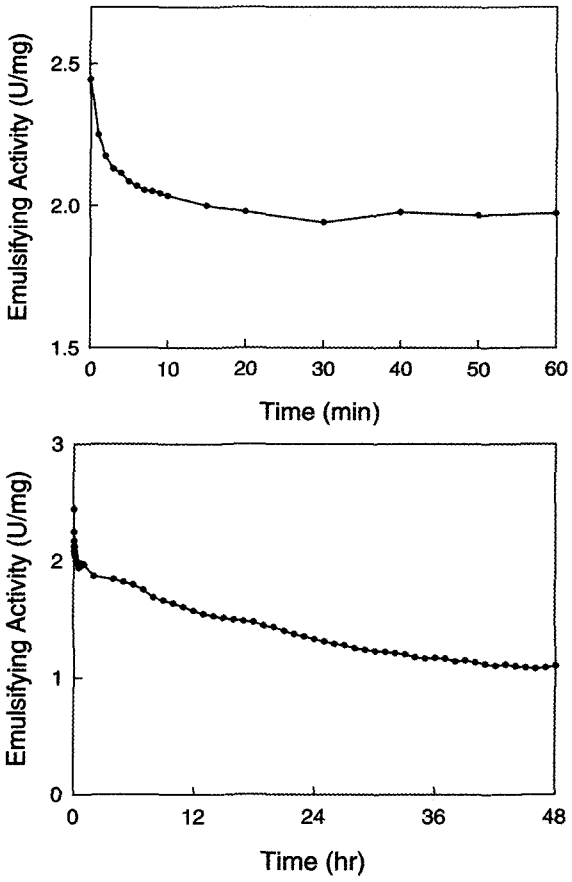


Fig. 2. Emulsion stability by BSF-1.

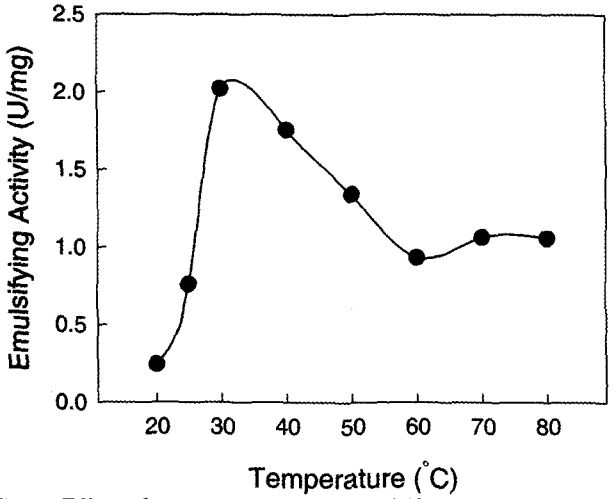


Fig. 3. Effect of temperature on the emulsification of BSF-1.

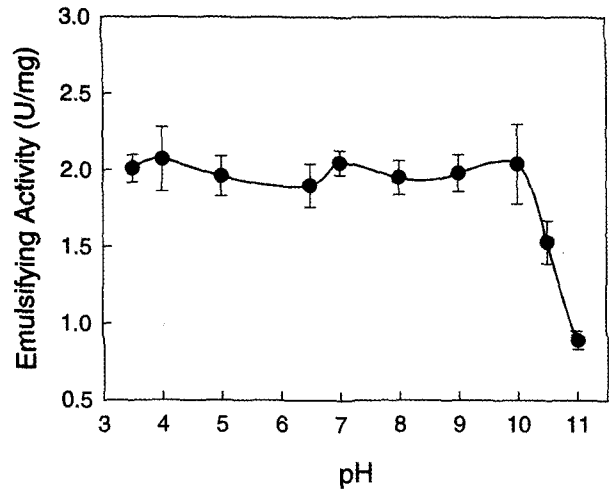


Fig. 4. Effect of pH on the emulsification of BSF-1.

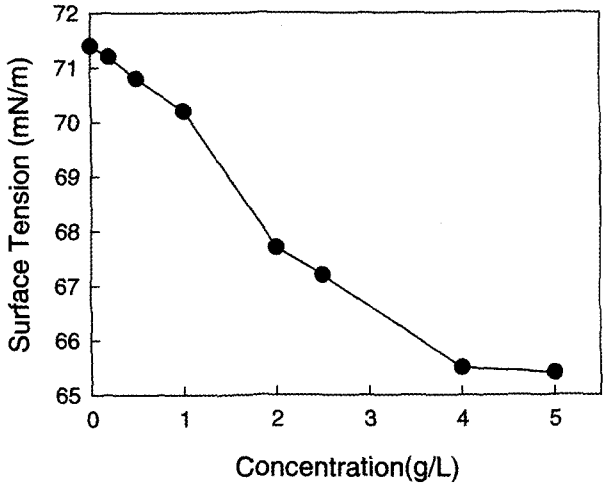


Fig. 5. Effect of BSF-1 concentration on tension reduction. The surface tension was measured based on the BSF-1 in D.W against air.

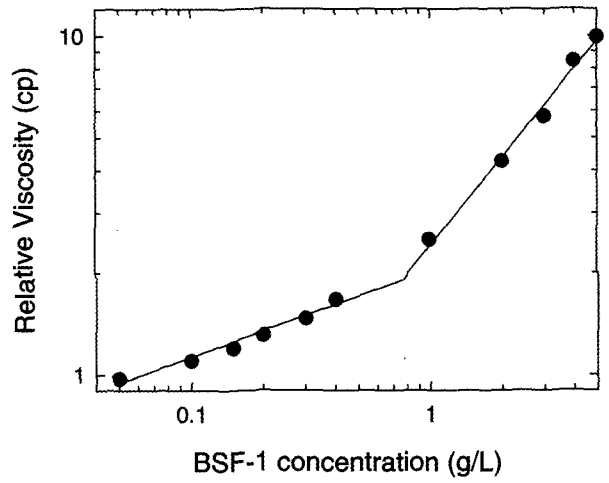


Fig. 6. Relative viscosity of BSF-1. Viscosity was measured at 25°C.

cose plus rhamnose, respectively. Each peak was confirmed by comparison with the respective standard compound. The glucose and rhamnose peaks did not separate, and showed the same retention time. Glucuronic acid was exposed to the fourth component in the BSF-1 carbohydrate instead of idose, and the result was confirmed by gas chromatography. Therefore, the carbohydrate portion of BSF-1 is composed of L-rhamnose, D-galactose, D-glucose, and D-glucuronic acid with a molar ratio of 3:1:1:1. The overall chemical properties are summarized in Table 1.

Emulsifying Properties of BSF-1

The emulsifying activity of BSF-1 was measured over time (Fig. 2). BSF-1 formed a stable emulsion within 10 min, and the emulsion was maintained for 48 h. Since BSF-1 is a polymer, it is considered to form more stable emulsions than other low-molecular-weight biosurfactants.

The effect of temperature on BSF-1-mediated emulsification was studied (Fig. 3). Maximal emulsifying activity was obtained at 30°C, while activity decreased dramatically at temperatures below 30°C. On the other hand, emulsifying activity decreased slightly up to 60°C, and reached a plateau above 60°C. The pH level also affected BSF-1-mediated emulsification (Fig. 4). Similar levels of emulsifying activity were maintained at acidic and neutral pH levels. At pH 10, the emulsifying activity began to decrease. Therefore, optimal emulsification by BSF-1 occurs under the conditions of 30°C and pH 4~10.

In the experiment to determine the critical micelle concentration (CMC), the surface tension decreased as more BSF-1 was added (Fig. 5). At 5.0 g/L BSF-1, the surface tension approached 65.4 mN/m. However, the CMC could not be determined because BSF-1 did not dissolve at concentrations >5 g/L. Similar to other polymeric biosurfactants, BSF-1 reduces the surface tension only

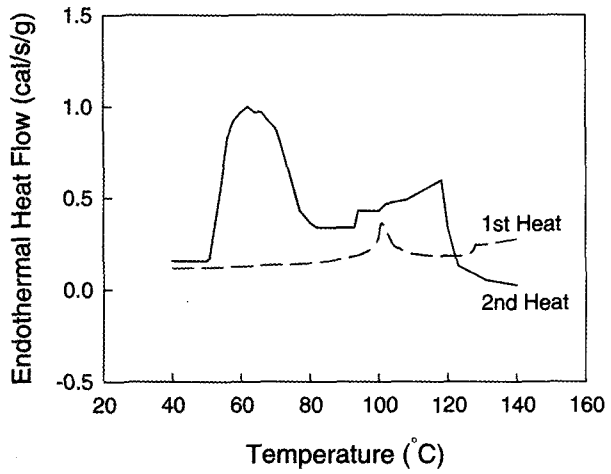


Fig. 7. Thermal flow graph of BSF-1.

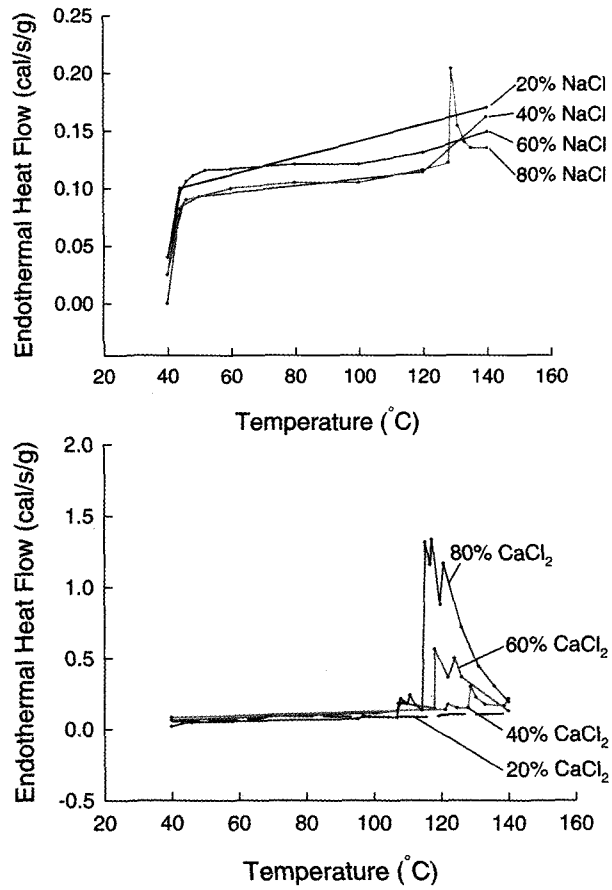


Fig. 8. Thermal flow graphs of BSF-1 with NaCl and $CaCl_2$.

slightly. Therefore, BSF-1 has value as a good emulsion stabilizer but not as a tension reducer.

Physical Characterization of BSF-1

The molecular weight of BSF-1 was determined by gel-filtration chromatography (GFC). Dextran (2,000 kDa, 500 kDa, 71 kDa, and 11.3 kDa; Sigma) and sucrose were used as the standards. By interpolation, the average molecular weight of BSF-1 was calculated as 1,700-2,000 kDa. Therefore, BSF-1 is considered to be a lipopolysaccharide biosurfactant.

Table 2. Summary of physical characteristics of BSF-1

	Physical properties
Molecular Weight (in average)	1.7~2.0×10^6 *Da*
Intrinsic Viscosity (in D.W.)	22.78 *dl/g*
Emulsifying Activity	1.2~1.9 *U/mg*
Thermal Property (by DSC)	Hydrogen bond and ionic bond are present in inter/intra-molecular reaction
Critical Micelle Concentration	ND
UV absorption	no peak

ND: not determined

The hydrodynamic volume was evaluated using a capillary viscometer. By extrapolation with Fouss's equation, the intrinsic viscosity was determined to be 22.8 dL/g, with r^2=0.9884. The relative viscosity was observed at 25°C (Fig. 6). In 5 g/L BSF-1, the viscosity was 10-fold higher than that of water (viscosity of water at 25°C: 0.89395 cp). By adding BSF-1, the viscosity of the solution as well as the degree of emulsification could be controlled.

The thermal properties of BSF-1 were determined. Fig. 7 shows the differential thermal gram of BSF-1 alone. In the first heat, the evaporation of free water was raised 100°C. The second heat showed a large peak at around 60~80°C, which indicates that the hydrogen bond involves inter-/intra-molecular conformational force. The temperature range of glassiness is around 110~120°C. The effects of ion additions were also examined (Fig. 8). In the case of monovalent ion addition to BSF-1, the thermal gram did not show any deviation. However, in the case of $CaCl_2$ addition, the heat flow increased, depending on the divalent ion concentration. These results indicate that an ionic bridge is also involved in the inter-/intra-molecular interactions. Due to the glucuronic acid in the polysaccharide backbone, BSF-1 carries a net negative charge. These negative charges may interact with divalent calcium ions. The overall physical properties of BSF-1 are summarized in Table 2.

In summary, BSF-1 has similar properties to emulsan, which is a well-known lipopolysaccharide biosurfactant used for MEOR. Both compounds have lipopolysaccharide qualities and are good emulsifying stabilizers; both can stabilize emulsions for at least two days. The polysaccharide backbones of BSF-1 and emulsan are negatively charged (glucuronic acid in BSF-1, galatosaminuronic acid in emulsan). The average molecular weight of BSF-1 (1,700 kDa) is about 2-fold higher than that of emulsan (1,000 kDa), while the hydrodynamic volume of BSF-1 is

10-fold larger (22.8 dL/g for BSF-1 versus 2.13 dL/g for emulsan). Although the fatty acid content of BSF-1 is lower than that of emulsan (1.1% for BSF-1 versus 5-11% for emulsan), their emulsifying activities are similar (both approximately 2.0 U/mg) [18]. We have reported that the emulsifying activity of a lipopolysaccharide biosurfactant is not affected by the fatty acid content or fatty acid composition, and that emulsifying stability is affected by the polysaccharide backbone structure, especially by the hydrodynamic volume [19,20]. Therefore, BSF-1 may represent a superior emulsion stabilizer, owing to its larger hydrodynamic volume and higher molecular weight.

CONCLUSION

BSF-1, which is an extracellular biosurfactant of *K. oxytoca* strain BSF-1, isolated from oil-contaminated soil, was characterized. This strain produces a 1,700~2,000 kDa extracellular lipopolysaccharide biosurfactant. BSF-1 is composed mainly of palmitic acid, 3-hydroxylauric acid, and lauric acid. The polysaccharide backbone is composed of rhamnose, galactose, glucose, and glucuronic acid with a molar ratio of 3:1:1:1. Although it contains only 1.1% fatty acids, BSF-1 can form very stable emulsions for up to two days. From our studies of the physical properties of BSF-1, we conclude that this compound has potential applications in the stabilization of emulsions and viscosity control.

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REFERENCES

[1] Fiechter, A. (1992) Biosurfactants: moving towards industrial application. *Trends Biotechnol.* 10: 208-217.
[2] Kim, Y. J., T. W. Kim, H. Chung, C. I. Kwon, and S. Y. Jeong (2001) Counterion effects on transfection activity of cationic lipid emulsion. *Biotechnol. Bioprocess Eng.* 6: 279-283.
[3] Li, J., S. Y. B. Hu, and J. M. Wiencek (2001) Development of a supported emulsion liquid membrane system for propionic acid separation in a microgravity environment. *Biotechnol. Bioprocess Eng.* 6: 426-432.
[4] Desai, J. D. and I. M. Banat (1997) Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* 61: 47-64.
[5] Wei, Q. F., R. R. Mather, and A. F. Fotheringham (2005) Oil removal from used sorbents using a biosurfactant. *Bioresour. Technol.* 96: 331-334.
[6] Menezes Bento, F., F. A. de Oliveira Camargo, B. C. Okeke, and W. T. Frankenberger, Jr. (2005) Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. *Microbiol. Res.* 160: 249-255.
[7] Lu, X. X., X. Zhang, G. H. Li, and W. H. Zhang (2003) Production of biosurfactant and its role in the biodegradation of oil hydrocarbons. *J. Environ. Sci. Health. A Tox. Hazard Subst. Environ. Eng.* 38: 483-492.
[8] Miller, R. M. (1995) Biosurfactant-facilitated remediation of metal-contaminated soils. *Environ. Health Perspect.* 103 Suppl 1: 59-62.
[9] Garcia-Junco, M., C. Gomez-Lahoz, J. L. Niqui-Arroyo, and J. J. Ortega-Calvo (2003) Biosurfactant- and biodegradation-enhanced partitioning of polycyclic aromatic hydrocarbons from nonaqueous-phase liquids. *Environ. Sci. Technol.* 37: 2988-2996.
[10] Choi, D. B., K. A. Cho, W. S. Cha, and S. R. Rhy (2004) Effect of Triton X-100 on compactin production from *Penicillium citrinum*. *Biotechnol. Bioprocess Eng.* 9: 171-178.
[11] Gutnick, D. L., E. Rosenberg, I. Belsky, and Z. Zinaida (1983) alpha-Emulsan. *US Patent* 4,395,354.
[12] Kim, P. (1998) Structure-Emulsifying Activity Relationship of a Biosurfactant Emulsan and Characterization of a Novel Lipopolysaccharide Biosurfactant. in Ph.D. thesis. KAIST: Daejeon. Korea.
[13] Kim, J. H., W. H. Jeong, T. B. Karegoudar, and C. K. Kim (2002) Stable degradation of benzoate by *Klebsiella oxytoca* C302 immobilized in alginate and polyurethane. *Biotechnol. Bioprocess Eng.* 7: 347-351.
[14] Belsky, I., D. L. Gutnick, and E. Rosenberg (1979) Emulsifier of *Arthrobacter* RAG-1: determination of emulsifier-bound fatty acids. *FEBS Lett.* 101: 175-178.
[15] Lin, F. M. and Y. Pomeranz (1968) Effect of borate on colorimetric determinations of carbohydrates by the phenol-sulfuric acid method. *Anal. Biochem.* 24: 128-131.
[16] Stellner, K., H. Saito, and S. I. Hakomori (1973) Determination of aminosugar linkages in glycolipids by methylation. Aminosugar linkages of ceramide pentasaccharides of rabbit erythrocytes and of Forssman antigen. *Arch. Biochem. Biophys.* 155: 464-472.
[17] Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick, and E. Rosenberg (1979) Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. *Appl. Environ. Microbiol.* 37: 414-420.
[18] Kim, P., D. K. Oh, J. K. Lee, S. Y. Kim, and J. H. Kim (2000) Biological modification of the fatty acid group in an emulsan by supplementing fatty acids under conditions inhibiting fatty acid biosynthesis. *J. Biosci. Bioeng.* 90: 308-312.
[19] Kim, P., D. K. Oh, S. Y. Kim, and J. H. Kim (1997) Relationship between emulsifying activity and carbohydrate backbone structure of emulsan from *Acinetobacter calcoaceticus* RAG-1. *Biotechnol. Lett.* 19: 457-459.
[20] Kim, P., S. W. Kim, S. Y. Kim, and J. H. Kim (1998) Effects of hydrodynamic volume of anionic lipopolysaccharide, emulsan, on emulsifying activity. *Biosci. Biotechnol. Biochem.* 62: 603-604.

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