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Glycolipid Biosurfactants Produced by *Pseudomonas aeruginosa* D2D2 from Diesel-Contaminated Soil

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Abstract A biosurfactant-producing bacterial strain was selected from diesel-contaminated soil by measuring the oil-film collapsing activity and identified as *Pseudomonas aeruginosa* D2D2. When glucose and olive oil were used as carbon sources, 11.46 g/l of biosurfactant was obtained. Based on TLC analysis, the biosurfactant produced from *P. aeruginosa* D2D2 was identified as a glycolipid, consisting of two types of biosurfactants (Type I and Type II). The purified glycolipid reduced the surface tension of the culture from 72 dyne/cm to 27 dyne/cm. The hydrophilic and hydrophobic moiety of the biosurfactant were rhamnose and β-hydroxydecanoic acid, as determined by FAB-MS and NMR analyses, respectively.

Key words: Biosurfactant, glycolipid, *Pseudomonas aeruginosa* D2D2, rhamnolipid

Surfactants are amphiphilic molecules, consisting of hydrophilic and hydrophobic domains, which tend to partition preferentially at the interface between fluids with different degrees of polarity and hydrogen bonding. The formation of an ordered molecular layer at the interface lowers the interfacial tension, thereby providing the special surface properties of surfactants. Due to their unique interfacial behavior, surfactants are applied in various industrial processes involving emulsification, foaming, detergency, wetting, and phase dispersion or solubilization [15].

Many biological molecules exhibiting a particularly high surface activity are classified as biosurfactants. Microbial biosurfactants include a wide variety of chemical structures, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids. In terms of their physicochemical properties, such as their surface activity, pH, and heat stability, many biosurfactants are comparable to synthetic surfactants [2, 6, 13], however, biosurfactants possess certain advantages over synthetic surfactants such as low critical micelle concentration (CMC) and high biodegradability. Therefore, they are particularly well suited for environmental applications; for example, bioremediation and the dispersion of oil spills [7]. In addition, biosurfactants are also used in the petroleum industry to enhance oil recovery [10]. However, biosurfactants are not utilized extensively in industry due to their relatively high production and recovery costs. To reduce the cost of biosurfactant production, it is first necessary to select a microorganism capable of producing the required product in high concentrations.

To select bacteria that produce biosurfactants, they are traditionally grown individually in a liquid medium. After several days, the culture filtrates are then tested for their surface activity by measuring the surface tension or interfacial tension [8]. Yet this method is both labor-intensive and time-consuming.

Accordingly, in the current study, highly productive bacteria were screened using a sensitive and rapid method for measuring the oil-film collapsing activity. The selected strain was identified and its biosurfactant production patterns were investigated. The biosurfactant was then purified from a culture of the selected strain to apparent homogeneity. The structure of the compound was finally characterized using various spectroscopic techniques.

Materials and Methods

Medium and Culture Conditions

The medium used to isolate the biosurfactant-producing bacteria was a minimal salts medium (MSM: 1.5 g/l K_2HPO_4, 0.2 g/l NaH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l NaCl, 0.5 g/l

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$MnSO_4 \cdot 5H_2O$, and 0.05 g/l $CaCl_2 \cdot 2H_2O$). As the carbon source, glucose, *n*-hexadecane, and an olive oil were used at concentrations of 30 g/l in combination or individually. The incubation was carried out at 25°C for 3 days with 150 rpm rotary shaking. The agar plates were prepared with 1.8% Bacto agar (Difco).

Screening by Oil Film-Collapsing Assay

Oil-polluted soil samples were taken from nearby oil reservoirs. The soil samples were enriched in 10% (v/v) diesel containing MSM at 25°C for 2 months. Water suspensions of the enriched soil samples were spread on nutrient agar plates, then the oil degradation ability of the colonies on the agar plates was tested using DCPIP (2,6-Dichlorphenolindophenol, from Sigma Co., St. Louis, U.S.A.) as follows: Each bacterial colony in 200 µl of MSM containing 10% (v/v) diesel was incubated with 5 µl of DCPIP (3 g/l) at 25°C for a week. The color of the reaction tubes containing oil-utilizing bacterial colonies then changed from blue to clear [1]. The isolated colonies were cultured in an isolation liquid medium containing glucose, *n*-hexadecane, and olive oil as the carbon source, then the biosurfactant producers were selected by measuring the oil-film collapsing activity of the culture broth. An oil-film collapsing assay is based on the fact that surfactants change the contact angle at the oil/water interface. The surface pressure of the surfactant is pushed over the oil film, thereby displacing the oil molecules. Twenty µl of crude oil was placed on the surface of 5 ml of distilled water in petri dishes (50 mm in diameter). A thin oil film formed immediately. Then, 5 µl of culture broth was gently placed in the center of the oil film. A clear halo became visible under light, and the diameter of this circle was measured.

Identification of Bacterial Strain

The morphological, physiological, and biochemical properties of the selected strain were investigated using the methods of Cowan and Steel [3] and MacFaddin [15], and then 16S rDNA sequencing was performed using modified version of the methods developed by Rochelle *et al.* [16], Lane [11], and Felsenstein [4].

Analyses

The cell concentrations were determined by measuring absorbance of the samples at 660 nm (OD_{660}). The surface tension of the culture broth and surfactant solution was determined at 25°C using a ring tensiometer (K10ST; Krüss, Hamburg, Germany).

TLC (Thin Layer Chromatography) was carried out on F_{254} plates of silica gel (Merck Co., No. 5715, Germany) and the compound were detected using 30% H_2SO_4, ninhydrin, DPH (1,6-diphynyl-2,3,5-hexatriene, Sigma), and thymol-sulfuric acid (0.5% thymol and 5% sulfuric acid in ethanol).

To determine the protein concentration of the culture broth, the cells were lysed by adding 20 µl of 1N NaOH, and the protein content of the lysed cells was then determined using the Bio-Rad protein dye-binding reagent (Bio-Rad, Mississauga, Canada). Bovine serum albumin (Sigma Chemical Co., U.S.A.) served as the standard [13].

Production of Biosurfactant

The effect of various carbon sources on the biosurfactant production was determined by measuring the cell concentration, protein assay, surface tension, and biosurfactant yield. MSM was used as the production medium along with ethanol, canola oil, corn oil, soybean oil, olive oil, glucose, glycerol, sucrose, hexadecane, and dodecane as the carbon sources at a concentration of 3 g/l. The cultivation was carried out at 30°C in a shaking incubator for 7 and 14 days.

To identify the optimum nitrogen sources, NH_4NO_3, $(NH_4)_2SO_4$, and $NaNO_3$ were used as inorganic nitrogen sources, and beef extract, soytone, peptone, tryptone, yeast extract, and malt extract as organic nitrogen sources. The concentrations of nitrogen sources were 2 g/l and the cultivation was carried out in a shaking incubator at 30°C for 3 days.

Purification of Biosurfactant

The biosurfactant was extracted from the cultures using a modified version of the method of Hisatsuka *et al.* [5]. The bacterial cells were removed from the culture broth by centrifugation (12,000 ×g, 4°C, 10 min), then the crude biosurfactant was extracted from the cell free supernatant three times with ethyl acetate. Na_2SO_4 was used to remove any residual water, then the crude biosurfactant was obtained as a brown-colored oil type by removing the solvent under reduced pressure using a rotary evaporator.

Further purification of the biosurfactant was done using an adsorption chromatography column (3×60 cm) on silica gel 60 (Merck Co., mesh 230–400). Six g of the crude biosurfactant and a 60 g of the heat-activated silica gel were mixed with chloroform to make a liquid slurry [19]. To remove any residual oil, the slurry was filtered through a Buchner funnel and rinsed with 1 liter of chloroform while stirring. The biosurfactant was applied to a chromatography column on the heat-activated silica gel and eluted with chloroform and methanol: Chloroform and methanol (chloroform:methanol=95:5 (v/v), 90:10 (v/v), 80:20 (v/v)) were used in order. Finally, the eluted samples were dried under vacuum at 40°C using a rotary evaporator. The homogeneity of the purified biosurfactant was determined by thin-layer chromatography.

Spectroscopic Analyses

A fast atom bombardment-mass spectroscopy (FAB-MS) analysis was performed on a Concept-1S instrument

(Kratos Analytical Ltd., Manchester, U.K.). Methanol and glycerol were used as the matrices. The mass spectra were obtained from 400–800 AMU and positive ions were detected. The ^{1}H-NMR was acquired at 500 MHz in $CDCl_3$ solution using AMX FT spectrometer (Bruker Co., Germany).

RESULTS AND DISCUSSIONS

Selection of Biosurfactant-Producing Bactera

One hundred and twenty eight bacterial strains using diesel as the sole carbon source were isolated from the soil samples. The isolated colonies were cultured in the isolation liquid medium in test tubes containing glucose, n-hexadecane, and olive oil as the carbon sources, and then four strains exhibiting more than 2 cm-diameter halo were isolated (D2D2, D2D3, D4G1, and I2G1) and further tested by measuring the oil-film collapsing activity of the culture broth. Among the four strains, strain D2D2 showed the lowest surface tension (27 dyne/cm) in the culture broth. Therefore, it was finally selected as a potential biosurfactant producer for the current study.

Table 1. Characteristics of selected strain D2D2.

Characteristics	Selected strain D2D2	*Pseudomonas aeruginosa*
Morphological		
Gram stain	–	–
Shape	rod	rod
Motility	+	+
Cultural		
Growth		
MacConkey	+	+
Anaerobic	+	+
pH 3.6	–	–
Physiological		
Catalase	+	+
Oxidase	+	+
O/F (Oxidation/Fermentation)	O	O
Nitrate reduction	–	d*
Hydrolysis of		
Starch	–	–
Gelatin	–	–
Casein	–	–
Tween 80	–	–
Urease	–	–
Acid production from		
Glucose	+	+
Fructose	–	+
Lactose	–	–
Maltose	–	d
Mannitol	–	d
Salicin	–	–
Sucrose	–	–
Xylose	+	+
Methanol	–	d
Ethanol	–	d

*Different reactions in different strains.

Identification of Strain D2D2

The morphological, physiological, and biochemical characteristics of strain D2D2 are shown in Table 1. Strain D2D2 was found to be a Gram-negative, motile, and rod-shaped bacterium. It showed a negative reaction to nitrate reduction, and did not hydrolyze starch, gelatin, or casein. The morphological, physiological, and taxonomical characteristics of D2D2 were very similar to those of *Peudomonas aeruginosa*, except for production of acid from fructose. The 16S rDNA sequencing confirmed that the strain was *Pseudomonas aeruginosa* D2D2. Many *Pseudomonas* sp. have already been investigated as glycolipid-producing bacteria [9, 19, 20]

Production of Biosurfactant by *P. aeruginosa* D2D2

The growth of *P. aeruginosa* D2D2 and biosurfactant production were examined in a 250-ml Erlenmeyer flask using MSM containing ethanol, canola oil, corn oil, soybean oil, olive oil, glucose, glycerol, hexadecane, and dodecane as carbon sources for 3 days. As shown in Table 2, *P. aeruginosa* D2D2 reduced the surface tension of the culture broth from 72 to 27.0 dyne/cm in MSM containing olive oil. The strain also showed high emulsifying activity (data not shown). Olive oil promoted the biosurfactant production ranging from 9.99 g/l to 11.48 g/l after 7 and 14 days, respectively. Olive oil was usually used as the carbon source in the large-scale production of biosurfactants [14].

When beef extract was used as an organic nitrogen source without any inorganic nitrogen sources added to the MSM, this produced the lowest surface tension of the culture broth at 31.5 dyne/cm and the highest growth (Table 3). When $(NH_4)_2SO_4$ and beef extract were added to the batch, this also produced low surface tension. When other nitrogen sources were added, they all showed different surface tension values and growth rates. These results demonstrated that *P. aeruginosa* D2D2 was dependent on the type of nitrogen source.

Figure 1 shows the biosurfactant production pattern in the culture medium, containing olive oil as the carbon source, in a 250-ml Erlenmeyer flask. The surface tension, cell concentration, and surfactant production patterns were examined. The cell concentrations were determined by weighing the dry cells. The culture reached the stationary phase in 12 h. The biosurfactant production started after 48 h to overproduce. The similar result that biosurfactant production increased after the culture reached the stationary phase was also reported [19, 20]. The minimum surface tension of 32 dyne/cm was obtained during the late exponential growth phase in 12 h, while the maximum

Table 2. Surfactant production by *Pseudomonas aeruginosa* D2D2 with different carbon sources.

Carbon source (3%)	Cell concentration (OD_{660nm})		Protein assay (g/l)		Surfactant production (g/l)		Minimum surface tension (dyne/cm)	
	7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days
Ethanol	1.078	1.022	0.085	0.135	0.912	1.981	27.6	27.5
Canola oil	3.978	4.624	1.560	1.985	8.520	10.628	28.2	28.0
Corn oil	3.966	5.230	1.761	2.250	6.505	6.950	28.2	28.3
Soybean oil	4.240	3.327	2.105	1.985	6.380	9.320	28.8	29.0
Olive oil	4.369	4.667	2.095	2.113	9.996	11.480	27.0	27.3
Glucose	3.223	0.686	0.023	0.038	3.200	3.820	28.5	28.5
Glycerol	1.273	0.790	0.089	0.153	0.810	0.566	29.0	30.0
Sucrose	0.075	0.080	0.011	0.007	0.031	0.056	50.5	30.5
Hexadecane	0.037	0.709	0.020	0.053	0.593	0.651	50.0	32.8
Dodecane	0.181	0.098	0.017	0.010	1.250	1.255	46.2	35.7

surfactant concentration was 11.46 g/l in a 95 h culture. This production yield was much higher than previously reported results (7.65 g/l) [14]. The maximum biomass was 2.4 g/l in 25 h.

Purification of Biosurfactant

The biosurfactant from *P. aeruginosa* D2D2 was identified using TLC. After removing the cells by centrifugation, the supernatant was acidified and then the biosurfactants were extracted with ethyl acetate. The crude biosurfactant was dissolved in chloroform and then placed on a column of silica gel. Two major spots were detected on the TLC plate [Type I=0.65 R_f (retardation factor), Type II=0.4 R_f]. The 0.4-R_f component showed positive under UV light, by the presence of thymol-sulfuric acid, 30% H_2SO_4, DPH, and ninhydrin (data not shown). To further identify the sugar components of this glycolipid, the ethyl acetate extract was reacted with a thymol sulfuric acid coloring reagent. The results of this TLC analysis suggested that the biosurfactant was a glycolipid composed of sugars and lipids. The purified biosurfactant of *P. aeruginosa* D2D2 showed the same R_f values as rhamnolipids.

Table 3. Surfactant production by *Pseudomonas aeruginosa* D2D2 with different nitrogen sources.

Inorganic-N (2 g/l)	Organic-N (2 g/l)	OD_{660}	Surface tension[1] (dyne/cm)
NH_4NO_3	Beef extract	0.574	49.5
$(NH_4)_2SO_4$	Beef extract	3.312	31.6
NaNO3	Beef extract	0.345	40.9
NH_4NO_3	Soytone	0.754	38.1
NH_4NO_3	Peptone	1.824	47.6
NH_4NO_3	Tryptone	1.021	48.3
NH_4NO_3	Yeast extract	2.819	33.7
NH_4NO_3	Malt extract	0.901	48.7
NH_4NO_3	None	0.695	52.3
None	Beef extract	3.549	31.5

[1]Diluted culture broth (6 times).

Spectroscopic Analyses

The molecular weight of the glycolipid type I in the FAB-MS analysis exhibited peaks at m/z (mass to charge) 359 and 549 (Fig. 2). The fragment ion at m/z 359 was due to the loss of a terminal lipid (hydroxydecanoic acid, $CH_3(CH_2)_6CHOCHCH_2COOH$), which corresponded to $\{M\text{-}C_{10}H_{20}O_3\text{+}2Na\text{-}H\}^+$. The peaks at m/z 549 were assigned to $\{M\text{+}2Na\text{-}H\}^+$. The FAB-MS spectra of the glycolipid type II showed signals at m/z 673 in agreement with $\{M\text{+}Na^+\}$.

In the ^{1}H-NMR analysis results (Fig. 3), the signals corresponded to an overlap of the deoxysugar (-CH_3, C6) and aliphatic chain of the lipid part, indicating that the sugar moiety was most likely rhamnose. The signals at δ 0.82, 1.19-1.51, and 1.22 were characteristic of long chain aliphatic fatty acids. The proton spectra of the glycolipid type II had similar spectra to those of the glycolipid type I. These results suggested that the type I and II were composed of related chemical moieties. It was

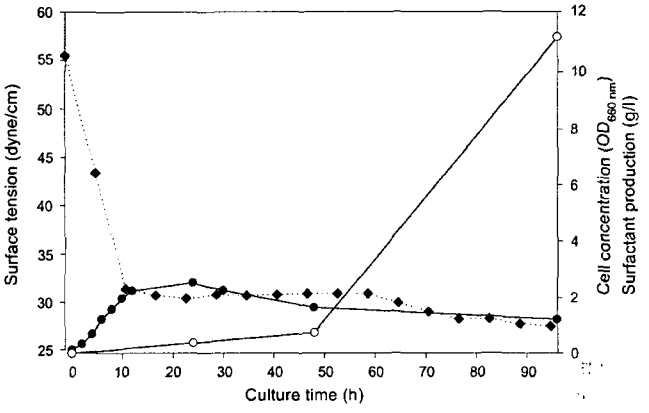

Fig. 1. Patterns of biosurfactant production by *Pseudomonas aeruginosa* D2D2.

The cultivation was performed in a basal medium containing olive oil (30 g/l) as the sole carbon source at 25°C. ◆: surface tension, ●: cell concentration, ○: rhamnolipid production.

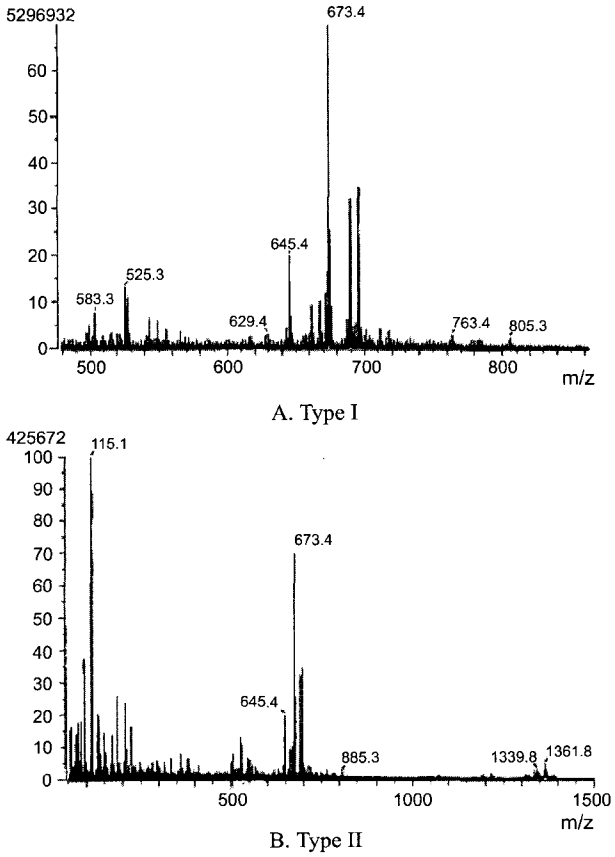

Fig. 2. FAB-MS spectrum of D2D2 surfactants.

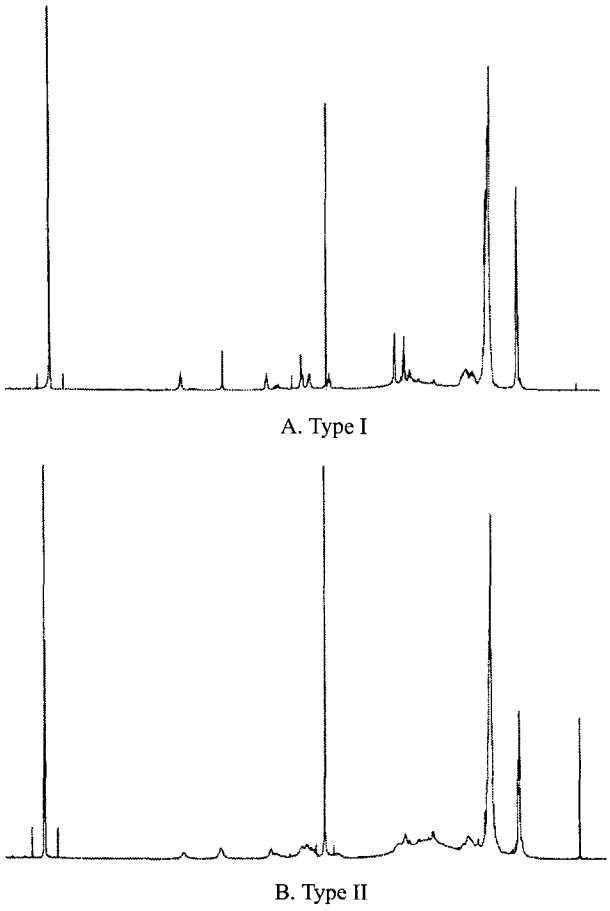

Fig. 3. ^{1}H-NMR spectrum of D2D2 surfactants.

confirmed through this analysis of the two glycolipids that the molecular weight of the rhamnolipid type I was m/z 505, while that of the rhamnolipid type II was m/z 650.

The structures of the two types of glycolipids were identified as rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and 2 rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, respectively (Fig. 4).

CONCLUSION

Pseudomonas aeruginosa D2D2 was selected by using a DCPIP test and by measuring the oil-film collapsing activity from diesel-contaminated soil. This bacterium produced a biosurfactant in a medium containing glucose and olive oil as carbon sources. The biosurfactant emulsified hydrocarbons, vegetable oils, diesel, and crude oils, and lowered the surface tension of the culture broth from 72 dyne/cm to 27 dyne/cm. The optimum carbon and nitrogen sources were olive oil (3 g/l) and beef extract (2 g/l), respectively. The results of TLC analysis confirmed that the biosurfactant produced by *P. aeruginosa* D2D2 consisted of two types of glycolipid (Types I and II). Using a FAB mass spectroscopy and NMR analysis, the hydrophilic and hydrophobic

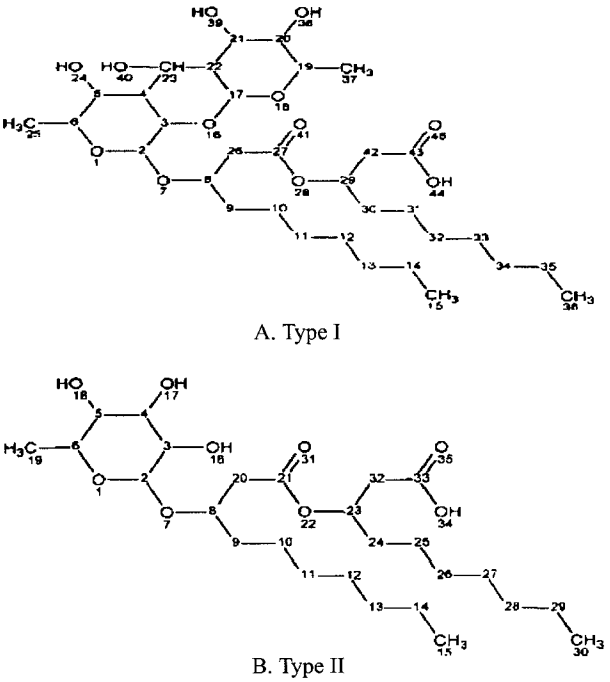

Fig. 4. Proposed structure of D2D2 surfactants.

moiety of the biosurfactant were determined to be rhamnose and β-hydroxydecanoic acid, respectively.

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REFERENCES

1. Charlotte, S., H. Margrethe, S. Christensen, and S. Martin. 1998. Characterization of a cellobiose dehydrogenase from Humic insolens. *Biochem. J.* **330:** 565–571.
2. Choi, K. S., S. H. Kim, and T. H. Lee. 1999. Purification and characterization of biosurfactant from *Tsukamurella* sp. 26A. *J. Microbiol. Biotechnol.* **9:** 32–38.
3. Cowan, N. R. and K. J. Steel. 1974. *Manual for the Identification of Medical Bacteria*, 2nd ed. Cambridge University, London, U.K.
4. Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle, U.S.A.
5. Hisatsuka, K., T. Nakahara, N. Sano, and K. Yamada. 1971. Formation of rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. *Agric. Biol. Chem.* **35:** 686–692.
6. Hwang, K. A., J. R. Lee, S. J. Kim, Y. S. Kim, and H. J. Ahn. 1999. Surface-activity and environmental characteristics of biosurfactant produced by *Pseudomonas aeruginosa* JRT-4. *Korean J. Appl. Microbiol. Biotechnol.* **2:** 159–165.
7. Ishigami, Y. 1993. Biosurfactants face increasing interest. *Inform.* **4:** 1156–1165.
8. Jain, D. K., D. L. Collins-Thomapson, H. Lee, and J. T. Trevors. 1991. A drop-collapsing test for screening surfactant-producing microorganisms. *J. Microbiol. Methods* **13:** 271–279.
9. James, P. P., C. P. Everett, and B. H. Iglewski. 1997. Roles of *Pseudomonas aeruginosa* las and Quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol.* **179:** 5756–5767.
10. Khire, J. M. and M. I. Khan. 1994. Microbially enhanced oil recovery (MEOR). Part I. Importance and mechanisms of MEOR. *Enzyme Microb. Technol.* **16:** 170–172.
11. Lane, D. J. 1991. 16S/23S 16S rRNA sequencing, pp. 115–175. *In* E. Stackebrahdt and M. Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics.* John Wiley and Sons: New York, U.S.A.
12. Lin, S. C., Y. C. Chen, and Y. M. Lin. 1998. General approach for the development of high-performance liquid chromatography methods for biosurfactant analysis and purification. *J. Chrom.* **825:** 149–159.
13. MacElwee, C. G., H. Lee, and J. T. Trevors. 1990. Production of extracellular emulsifying agent by *Pseudomonas aeruginosa* UG1. *J. Microbiol.* **5:** 25–32.
14. Robert, M., M. E. Mercade, M. P. Bosch, J. L. Parra, M. J. Espuny, M. A. Manresa, and J. Guinea 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. *Biotech. Lett.* **11:** 871–874.
15. MacFaddin, J. F. 1984. *Biochemical Tests for Identification for Medical Bacteria*, 2nd ed. Williams and Wilkins Co., Baltimore, U.S.A.
16. Rochelle, P. A., J. C. Fry, R. J. Parkes, and A. J. Weightman 1992. DNA extraction for 16S rRNA gene analysis to determine genetic diversity in deep sediment communities. *FEMS Microbiol. Lett.* **100:** 59–66.
17. Rosen, M. J. 1989. *Surfactants and Interfacial Phenomena.* John Wiley and Sons, Inc., New York, U.S.A.
18. Sim, L., O. P. Ward, and Z. Y. Li. 1997. Production and characterization of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1. *J. Ind. Microbiol. Biotechnol.* **19:** 232–238.
19. Suk, W. S., H. J. Son, G. Lee, and S. J. Lee. 1999. Purification and characterization of biosurfactants produced by *Pseudomonas* sp. SW1. *J. Microbiol. Biotechnol.* **9:** 56–61.
20. Zhang, Y. and R. M. Miller. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant. *Appl. Env. Microbiol.* **58:** 3276–3282.