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Purification and Structural Characterization of Glycolipid Biosurfactants from *Pseudomonas aeruginosa* YPJ-80

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Glycolipids produced by *Pseudomonas aeruginosa* YPJ-80 were characterized by chromatographic and spectroscopic techniques as a mixture of two rhamnolipids. For recovery of glycolipids from the culture broth, various isolation methods including ultrafiltration, adsorption and solvent extraction were compared. Ultrafiltration showed the best results in terms of glycolipids recovery. Further purification for spectroscopic analysis was carried out by adsorption chromatography and preparative thin layer chromatography. From the spectroscopic analysis, such as IR spectroscopy, FAB-MS, ^{1}H-NMR and ^{13}C-NMR and hydrolysis analysis, the glycolipids were identified as L-α-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate and 2-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate. Monorhamnolipid and dirhamnolipid lowered the surface tension of water to 28.1 mN/m and 29.3 mN/m, respectively.

Keywords: *Pseudomonas*, glycolipid, biosurfactant, emulsification,

INTRODUCTION

Various microorganisms, such as bacteria, yeasts and fungi, are known to grow on hydrophobic substrates as the only carbon source and to produce extracellular surface active agents during cultivation or they use cell wall-associated surfactants to facilitate the penetration of hydrocarbons to the periplasmic space. These substances are of commercial interest because of their biodegradability and low toxicity. Applications could be found in the food, pharmaceutical , cosmetics and specialty chemical industries, basically in any industry or processes where surface activity properties in multiphase systems are encountered [1]. These properties can be used in drug delivery, clean-up of oil spills, and enhanced oil recovery. Among these biosurfactants, glycolipids show various chemical structures including, rhamnose, trehalose, mannose and sophorose as hydrophilic moieties. In addition to surface activity, recently microbial glycolipids are shown to have biological activities to induce cell differentiation [2, 3], have antimicrobial activities for plant disease control [4, 5] and even some immunological activities [6].

Pseudomonas aeruginosa YPJ-80 was isolated and was found to produce several surface active agents when grown on glucose and hydrophobic substrates like n-alkanes and vegetable oils. The objective of the study is to optimize the recovery procedure from the culture broth and to confirm the structure of bioemulsifiers. This paper describes the isolationl and purification of the glycolipids produced from glycerol and the characterization of the two main glycolipids.

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MATERIALS AND METHODS

Microorganisms

Strain *Pseudomonas aeruginosa* YPJ-80 was isolated from soils as bioemulsifier producer in our laboratory. The strain was maintained on plate count agar plates (PCA, Difco, USA) at 4℃.

Media and Culture of Microorganisms

The culture medium contained carbon source (glucose, glycerol) 20 g/L, $NaNO_3$ 2.125 g/L, $(NH_4)_2SO_4$ 1.65 g/L, NH_4NO_3 1.0 g/L, $MgSO_4$-$7H_2O$ 0.5 g/L, K_2HPO_4 2.0 g/L, KH_2PO_4 2.0 g/L, yeast extract 1.0 g/L, trace metals NaCl 1.0 g/L, $CaCl_2$-$2H_2O$ 0.01 g/L, $MnSO_4$-$5H_2O$ 0.01 g/L, $FeSO_4$-$7H_2O$ 0.01 g/L. Carbon sources were sterilized separately.

The seed cultures were prepared by inoculating loopfuls of cells of strain YPJ-80 grown on PCA plates into 250 mL Erlenmeyer flask containing 50 mL of culture media, followed by incubation at 30℃ on

a rotary shaker with 250 rpm for 10 hrs. Fermentor experiments were performed in a 2.5 L stirred fermentor (Korea Fermentor Co., Korea) with the following culture conditions: Incubation temperature 32.5℃, agitation initially 300 rpm and then after foam formation 600 rpm; working volume 1.2 L broth; aeration rate 1 vvm. To obtain enough samples for purification procedures using adsorption chromatography on XAD-4, fermentation in 50 L fermentor (working volume 30 L; Korea Fermentor Co., Korea) was carried out at 32.5℃ under agitation at 300-400 rpm and aeration at 1vvm. The pH was automatically maintained at 8.0 with 1N NaOH. Cell growth was monitored by using Hewlett Packard 8452A spectrophotometer (USA) at 660nm.

Isolation and Purification of Glycolipid Biosurfactants

Culture broths were centrifuged at 6,000 rpm for 30min at 4℃. The volume of culture broth, at pH 3, 7 and 8, was reduced to 20% with ultrafiltration using magnetically-stirred ultrafiltration cell 8400 (Amicon, USA) with YM10 membrane(molecular cut-off 10kDa). Retentate was acidified with 6N HCl to pH 2.0 and was extracted with an equal volume of chloroform:methanol mixture (2:1, v/v), which was repeated three times. The organic phase was carefully saponified off and after dehydration, the solvent was removed by vacuum evaporation in a rotary evaporator. A crude biosurfactant was obtained as a brown colored oil.

Also to compare with ultrafiltration methods, we used adsorption chromatography column (50×300 cm, 100 g) on Amberlite XAD-4 ion exchange resin (Rohm & Hass, USA) with cell free culture broth(25 mL/min). Adsorbed substances were eluted with a solvent mixture of chloroform:methanol (2:1, v/v).

Further purification of the biosurfactants was achieved by an adsorption chromatography (30×450 mm) on silica gel 60 (Merck Co., mesh 230-400). The 10 ml fractions were collected and assayed for surface activity. The fractions containing surface active agents were pooled and concentrated in vacuum. The residue was dissolved in a minimum volume of methanol and further purified by repeated preparative thin-layer chromatography (silica gel 60 F_{254}, 2 mm thick, Merck Co., Germany) with a solvent system (chloroform:methanol:water=65:25:4, v/v).

Surface Space Tension Measurement and Emulsification Activity

The surface tension of the culture broth and surfactant solution was measured at 25℃ with a ring tensiometer (K10ST; Kruss, Hamburg, Germany) by the De Nouy method. The measurement of emulsification activity was based on Rosenberg's emulsification test [7]. Hexadecane/2-methylnaphthalene mixture (1:1, v/v) was prepared. The hydrocarbon mixture (0.1 mL) was added to 10 mL of 20 mM citrate-phosphate buffer (pH 5.4) containing an appropriate volume of the culture broth in a 50 mL flask. After reciprocal shaking (150 strokes per min) for 1 hour at 25℃, the resulting emulsion was allowed to stand for 10 min. Its absorbance through 1 cm path length was then measured at 620 nm with Hewlett Packard UV-VIS spectrophotometer (HP8452A, USA). The emulsification activity was expressed as the absorbance.

Spectroscopic Analysis

Culture broth was extracted with chloroform: methanol mixture (2:1, v/v) and solvent phase was concentrated with vacuum evaporation. Thin layer chromatography was carried out on Silica gel plates (silica gel 60 F_{254}, 0.2 mm thick, Merck Co., Germany) was developed with solvents (chloroform:methanol: water=65:25:4, v/v) and visualized with different colorizing agents for chemical moieties.

Infrared spectroscopy was performed with a BOMEM FT-IR (USA) in KBr pellet. ^{1}H-NMR and ^{13}C-NMR spectra were acquired at 500 MHz in $CDCl_3$ solution on a Bruker AMX FT 500 MHz spectrometer (Germany). Fast atom bombardment -mass spectrometry (FAB-MS) was performed on a Kratos (Kratos Analytical Ltd., Manchester, UK). 3-Nitrobenzyl alcohol(NBA) and glycerol were used as matrices for glycolipid A and B, respectively.

Glycolipds Hydrolysis Analysis

Purified glycolipids were hydrolyzed in 1N HCl: dioxane (1:1, v/v) under reflux conditions (120℃, silicone oil bath) for 90 minutes. Hydrophilic and lipophilic moieties were separated by adding diethyl ether and analyzed by TLC and ^{1}H-NMR spectroscopy, respectively.

Glycolipids and Rhamnose Quantification

Cell-free culture broth was applied to Sep-Pak catridge (WAT05910, Waters, USA) and adsorbed glycolipids were eluted with methanol. After dehydration and evaporation, the residue was dissolved in acetone and was derivatized with 2,4'-dibromoacetophenone:triethylamine mixture (2:1, mol ratio). Separation of the derivatized glycolipids was performed by high performance liquid chromatography (HPLC) using a (μBondapak C18 column (3.9×300 mm, Waters, USA) and Waters UV detector at 265 nm. Flow rate was 1ml/min and the isocratic elution of the acetonitrile:water mixture (70:30, v/v) was used. Also glycolipids were assayed indirectly by rhamnose content [8] and standard emulsification dilution method(SED) [9].

RESULTS

Production of Biosurfactants by *Pseudomonas aeruginosa* YPJ-80

Pseudomonas aeruginosa YPJ-80 grows well on glucose, glycerol and vegetable oils as carbon sources and produces biosurfactants. When grown on glycerol, the surface tension of the broth was lowered to 30.1 mN/m. Fig. 1 shows the pattern of growth and biosurfactant production in a 2.5 L jar fermentor with glycerol as a carbon source. During exponential growth, there was a temporary decrease in pH, which again stabilized after 35 hrs to near the initial pH. Along with the decrease in pH, the color of cul-

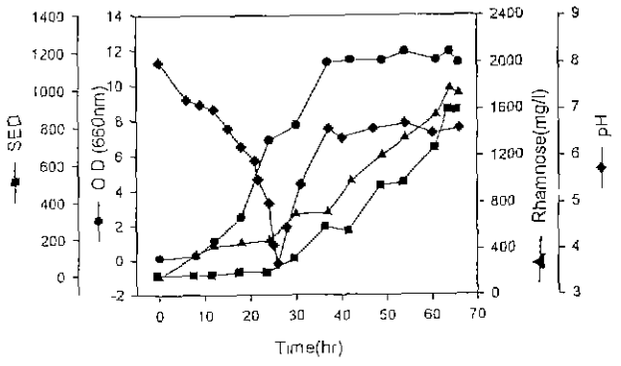


Fig. 1. Time course of glycolipid production in a 2.5 L jar fermentor by batch fermentation of *Pseudomonas aeruginosa* YPJ-80. Glycerol was used as a carbon source (2%, w/v) SED (Standard Emulsification Activity) was defined as the dilution factor where the emulsification activity was one [9].

ture broth became blue-green indicating the presence of a pigment [10, 11]. After stationary phase, *Pseudomonas aeruginosa* YPJ-80 was shown to still produce biosurfactants by monitoring emulsification activity and deoxysugar content assay.

Recovery and Purification of Glycolipids

For industrial applications of biosurfactants, the efficient recovery from the culture broth is desired. Table 1. shows the recovery of glycolipids from the culture broth by monitoring emulsification activity and rhamnose content assay. Ultrafiltration gave the highest yield at pH 3.0. Glycolipids were concentrated by ultrafiltration because they are able to form micelles at concentration above the critical micelle concentration, which allows these aggregates to be retained by relatively high molecular weight cut-off membranes. Low molecular weight impurities such as salts, free amino acids, peptides and small proteins could be easily removed. From the above results, the isolation and purification of glycolipids were summarized in Fig. 2. Further concentration could be achieved by solvent extraction (chloro

Table 1. Recovery of biosurfactants with various separation methods

Methods	Recovery conditions	Rhamnose recovery(%)	SED recovery(%)
Precipitation	Acidification pH 2.0	63.9	87.4
	Ammonium sulfate(2%) pH 2.0	57.5	59.5
	pH 8.0	78.8	78.8
Extraction	pH 2.0($CHCl_3$:MeOH=2:1,v/v)	89.0	97.9
	pH 8.0($CHCl_3$:MeOH=2:1,v/v)	52.0	19.2
	pH 2.0(ethyl acetate)	71.9	74.5
	pH 8.0(ethyl acetate)	49.2	71.1
XAD-4[a]	1st elution[b]	29.6	n.d.[c]
	6th elution	11.4	
	after regeneration[d]	24.1	
Ultrafiltration	pH 3.0	91.0	99.0
	pH 7.0	87.1	91.8
	pH 8.0	75.7	89.5

a: Rohm & Haas Amberlite XAD-4(100g, 5×30 cm) equilibrated with 50 mM citrate-phosphate buffer(pH5.0),
b: loaded at a rate of 25 mL/min.
c: not determined. d: regenerated with chloroform:methanol=2:1(v/v)

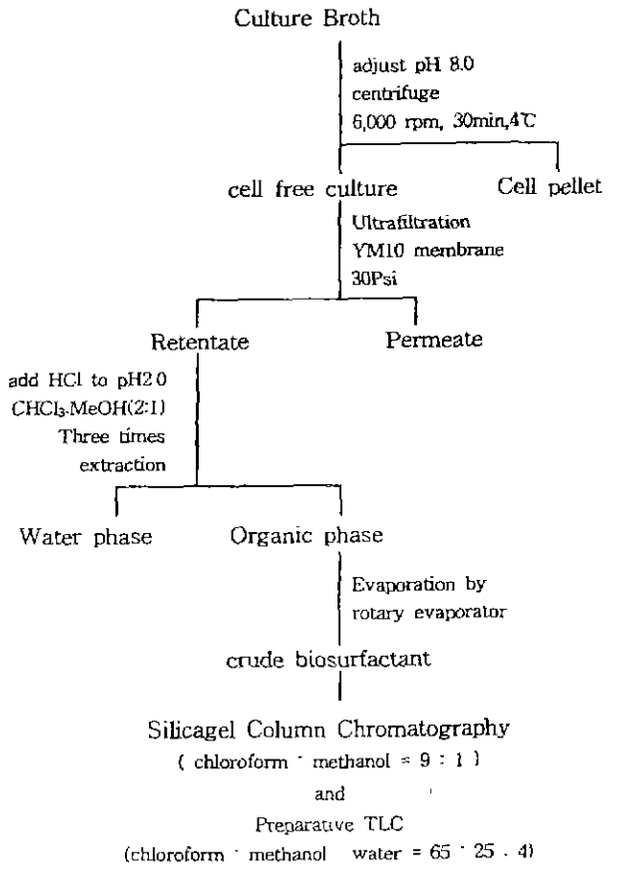


Fig. 2. Optimized separation of glycolipids from *Pseudomonas aeruginosa* YPJ-80.

form:methanol = 2:1, v/v). After evaporation, the crude biosurfactant was obtained as brownish material. Three major spots were detected on TLC, with two major types of glycolipids (R_f = 0.70 and 0.45), were present in the mixture, which were separated on a TLC plate (chloroform:methanol: water = 65:25:4, v/v). The component at R_f = 0.93 showed positive detection under UV light and iodine vapor. The next two components (R_f = 0.70 and 0.45) were detected using Rhodamine B, (α-naphthol and anthrone reagent, indicating the presence of sugar and free carboxylic acid. A further purification was performed on a silica gel column developed with a solvent mixture (chloroform:methanol = 9:1, v/v). The anthrone-positive compounds were scraped off from the repeated preparative TLC (2 mm thick). We named two compounds as glycolipid A and B (R_f = 0.70 and 0.45), respectively. When dissolved in 20 mM citrate-phosphate buffer (pH 5.0), glycolipid A and B lowered the surface tension to 28.1 and 29.3 mN/m, respectively.

Structural and Spectroscopic Analysis of Glycolpids

The sugar moiety of the glycolipids was shown to be a deoxysugar using sulfuric acid-thioglycolic acid assay [8].

The IR spectra of glycolipid (Fig. 3) showed the characteristic absorption bands at 3600 (hydroxyl), 3450 and 1720 (acid carbonyl), 2900 (long chain -CH_2-), 1180 (-CH_2- vibration), 1440 (-CH_3), suggest-

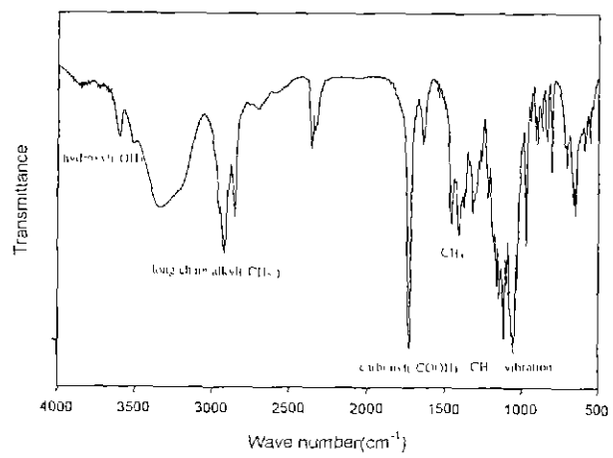
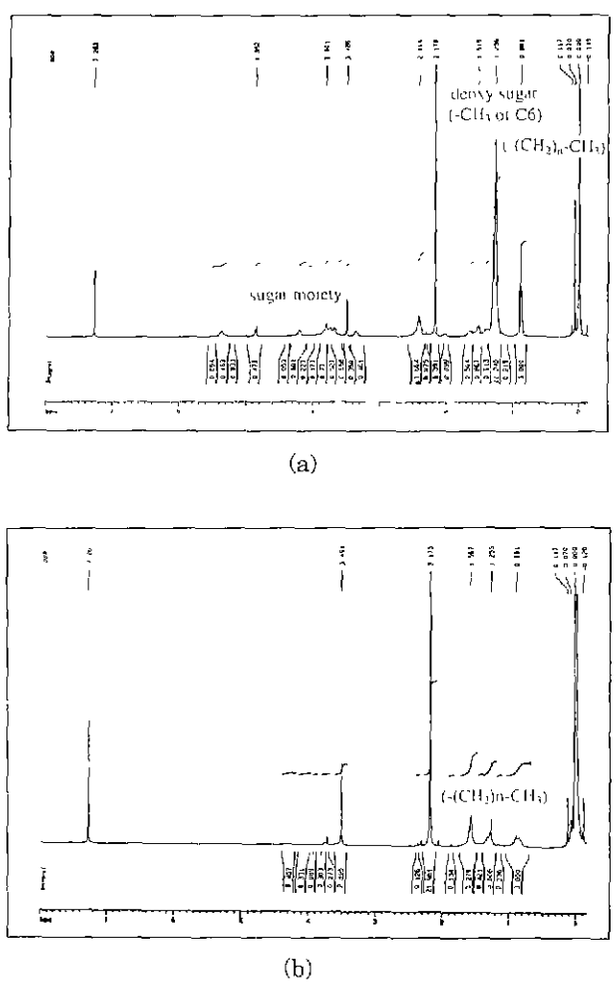


Fig. 3. FT-IR spectroscopy of glycolipids isolated from *Pseudomonas aeruginosa* YPJ-80.



(a)

(b)

Fig. 4. ^{1}H-NMR spectra of (a) glycolipid A (b) ether-soluble lipophilic moiety.

ing the presence of aliphatic fatty acid and sugar moiety.

The glycolipids were hydrolyzed under acidic conditions and the water soluble component showed the same R_f value by TLC with a standard rhamnose sample. Ether-soluble part was analyzed on ^{1}H-NMR and showed similar spectra to a hydroxy fatty acid (Fig. 4-b).

NMR Spectroscopy

After preparative TLC, the glycolpids were identified using ^{1}H-NMR, ^{13}C-NMR and mass spectrometry.

The proton spectra of glycolipid A is shown in Fig. 4-a. When glycolipid A was hydrolyzed under acidic conditions, the signals at δ 4.85, 3.80, 3.49, and 3.65-3.75 disappeared. And the intensity of a signal at δ 1.25 was reduced remarkably. This is because the signal corresponds to the overlap of deoxysugar (-CH_3, C6') and aliphatic chain of lipid part, indicating that the sugar moiety is likely to be rhamnose. The signals at δ 0.88, 1.15-1.65 and 1.25 are characteristic of long chain aliphatic fatty acids. The proton spectra of glycolipid B showed the similar spectra to glycolipid A, suggesting they are build up with the related chemical moieties(data not shown).

^{13}C NMR spectra data of glycolipid B is given in Table 2. The signals from δ 67 to 79.6, along with two signals at δ 94.5 and 102.4 indicates two carbohydrate molecules are present. The downfield shift of signals for C2' by 9 ppm from that of C2 is indicative of the 1,2 linkage between the two carbohydrate molecules [12]. The number and intensity of the signals of ^{13}C NMR spectra confirmed the presence of two rhamnose moieties and two hydroxy fatty acids in glycolipid B.

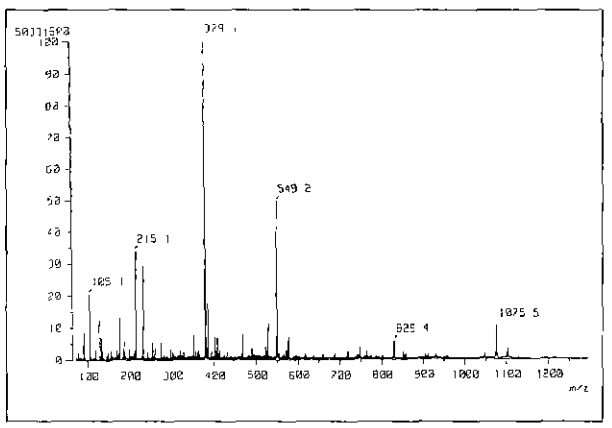
Table 2. ^{13}C-NMR spectra data of glycolipid B (500MHz, in $CDCl_3$)

Carbon(sugar moiety)	δ (ppm)	Carbon(aglycon moiety)	δ (ppm)
C1, C1'	94.5, 102.4	1, 1'	173.6, 171.4
C2, C2'	79.6, 70.6[a]	2, 2'	39.5, 39.2
C3, C3'	71.1[a], 70.6[a]	3, 3'	71.1[a], 70.6[a]
C4, C4'	73.4, 72.4	4, 4'	34.2, 34.2
C5, C5'	68.8, 67.9	5, 5'	25.0, 24.7
C6, C6'	17.4, 17.4	6, 6'	29.1 - 29.4
		7, 7'	29.1 - 29.4
		8, 8'	31.7, 31.7
		9, 9'	22.6, 22.6
		10, 10'	14.0, 14.0

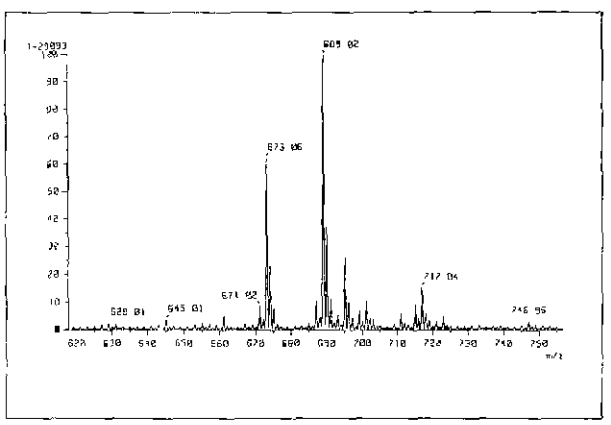
a; assignments may be interchangeable

FAB-MS Analysis

The purity of purified glycolipid A and B was established by HPLC after derivatization with p-dibromoacetophenone to form phenacyl ester of glycolipids. Their retention time were 11.8 and 9.1 min, respectively. The molecular weight of glycolipids A and B was determined by fast atom bombardment ionization mass spectroscopy (Fig. 5-a and 5-b). FAB-MS spectra of glycolipid A exhibited peaks at m/z 379, 549 and 1076. The fragment ion at m/z 379 is due to the loss of one terminal lipid (hydroxydecanoic acid, $CH_3(CH_2)_6CHOHCH_2COOH$), i.e. corresponds to $\{M\text{-}C_{10}H_{18}O_2\text{+}2Na\text{-}H\}^+$. Peaks at 549 and 1076 could be assigned to $\{M + 2Na\text{- }H\}^+$ and $\{2M + 3Na\text{-}2H\}^+$. The FAB-MS spectra of glycolipid B gave signals at m/z 673, 689 and 717 in agreement with $\{M + Na\}^+$, $\{M + K\}^+$ and $\{M + 3Na\text{- }2H\}^+$,



(a)



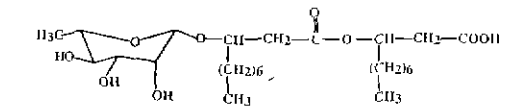
(b)

Fig. 5. FAB-MS spectra of (a) glycolipid A (b) glycolipid B

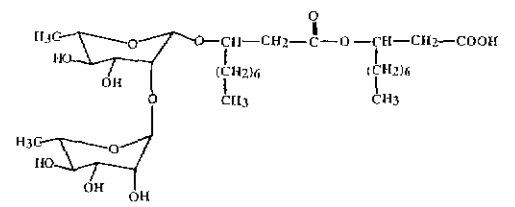
respectively. Through FAB-MS analysis of two glycolipids, it was confirmed that molecular weight of glycolipid A is 504 and that of glycolipid B is 650.

DISCUSSION

Downstream processes of biosurfactants from the fermentation broth could account for a large fraction of the total production cost. However, for most applications crude preparations could meet the requirements of users as long as the preparations maintain the desired properties. The methods employed will depend on the carbon sources used and fermentation operations [13]. Several techniques were used for large scale or continuos isolation of biosurfactants; solvent extraction, acid precipitation, adsorption and tangential flow filtration [14]. Continuos removal of biosurfactants during fermentation could eliminate product inhibition. Also foam fractionation was used for continuous removal of lipopeptide surfactin after acidification and solvent extraction with dichloromethane [15]. Preparations of crude rhamnolipids could be carried out by ultrafiltration without pH adjustment. Rhamnolipid preparations at pH 7.0 exhibits above 90% of emulsification activity. The recovery of rhamnolipids by ultrafiltration from the culture broth would be very effective as large volume could be processed rapidly and at low cost [6, 16, 17].



L α-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (monorhamnolipid)



2-*O*-α L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (dirhamnolipid)

Fig. 6. Structures of rhamnolipids produced by *Pseudomonas aeruginosa* YPJ-80.

The pigment compound (R_f = 0.93) could be used as a biodye and pH indicator because the aqueous solution shows color changes depending on pH. *Pseudomonas aeruginosa* strains were known to produce phenazine dyes along with glycolipids [10, 18].

^{1}H-NMR spectra of glycolipid B showed the broadening of signals as observed by Syldak *et al.* [19]. They attributed this to the presence of free carboxylic groups in the molecule and the possible formation of micelles. From spectroscopic analysis and comparison with literature data, the structure of rhamnolipids produced by *Pseudomonas aeruginosa* YPJ-80 on glycerol as a carbon source is shown in Fig. 6. The first report of rhamnolipids from *Pseudomonas aeruginosa* was in 1949. They also reported the compound had bacteriocidal activity against *Mycobacterium tuberculosis* [20]. Syldak *et al.* reported rhamnolipids containing only one β-hydroxydecanoic acid were produced with resting cells [21]. But they seemed to represent degradation products derived from partial hydrolysis of rhamnolipids with two β-hydroxydecanoic acids. Rendell *et al.* observed a number of related rhamnolipids with different fatty acids homologues from a clinical isolate *Pseudomonas aeruginosa* [22]. Ishigami *et al.* used rhamnolipids modified at C2 position with decenoyl moiety from *Pseudomonas aeroginosa* BOP 100 cultivated on 0.1% hexadecane as the sole carbon source [23]. However, the core structure of all the rhamnolipids reported in the literature is composed of rhamnose and hydroxyfatty acids [24]. Rhamnolipids could be utilized as biodegradable surfactants and antimicrobial agents [5] for their amphiphilic nature and also as a source of rhamnose which is used as a fine chemical for the synthesis of a widely used flavour 2,5-dimethyl-4-hydroxy-2, 3-dihydrofran-3-one (Furaneol®) [25, 26]. The production of rhamnolipids could be improved by media optimization and use of waste materials. Recently biorenewable waste was used as a carbon source for production of lipopeptides and glycolipids [27, 28]. The study on the structure-function relationship of rhamnolipids will be also used for the development for "tailor-made" biosurfactants [29].

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