Chemical Characterization and Physicochemical Behavior of Biosurfactants

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Microbes have been isolated from soil and water samples after an enrichment culture process with kerosene and tested for biosurfactant production by measuring the surface and interfacial tensions and emulsification power of culture broths. The isolation and characterization of extracellular surface-active agents from the culture broth of *Pseudomonas aeruginosa* 44T1 strain have been made. Preliminary structure identification with specific TLC reagents of the $CHCl_3$:MeOH (2:1) extracts showed two spots with a glycolipidic structure and Rf values of 0.70 and 0.45, respectively, using the solvent system $CHCl_3$:MeOH:H_2O (65:25:4). Separation of surface active agents by chromatographic absorption column in Florisil or silica gel and further spectroscopic study (IR, ^{1}H-NMR, ^{13}C-NMR) and chemical degradation techniques (acid and alkaline hydrolysis) gave a structure of β[β(2-0-α-L-rhamnopyranosyloxy)decanoyl] decanoic acid and β[β (2-0-α-L-rhamnopyranosyl-α-L-rhamnopyranosyloxy) decanoyloxy] decanoic acid (glycolipid A and glycolipid B, respectively) for the two glycolipids detected. A new method of mass spectrometry, fast atom bombardment mass spectrometry (FABMS), was used to probe molecular structure. The mass spectra obtained contain molecular weight recognition and sequence information signals, and they are in agreement with the proposed structures. Physicochemical evaluations of the two isolated glycolipids were made. The minimum surface tension obtained was 25 mN/m in water solutions. At pH 7 the CMC value was 11 ppm. In both cases, at pH 3 the CMC was displaced to lower and at pH 9 it was displaced to higher concentration values. Glycolipid B showed a lower value of interfacial tension (0.2 mN/m) than glycolipid A (1.0 mN/m). Glycolipid A showed a lower CMC value at alkaline pH, whereas glycolipid B had a lower CMC at acidic pH than at alkaline pH.

Many microorganisms produce surface active compounds (extracellular or not) when grown in media with hydrocarbons as a carbon source. These biocompounds can modify the interfacial and surface conditions. The production of these compounds has been related mainly to the problem of substrate transport during hydrocarbon fermentation. Microbes require a large contact area with the lipophilic phase in order to assimilate such water-insoluble substrates.

The release of surface active compounds promotes an emulsification of the hydrocarbon phase, rendering such lipophilic molecules available to the metabolic pathways of microorganisms (1). These conclusions are supported by the fact that addition of surfactants to oil and water culture media often stimulates microbial growth (2).

Chemically, synthetic surfactant molecules usually are classified according to the nature of the polar group in cationic, anionic and nonionic types. Surface active agents obtained from microorganisms are classified according to the biochemical nature of the surfactant. Generally, these metabolites are amphiphilic compounds; the hydrophobic part is built from long-chain fatty acids, hydroxy fatty acids or α-alkyl-β-hydroxy-fatty acids. The hydrophilic part in the molecule can be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid, an alcohol, etc. (Table 1) (3).

TABLE 1

Biosurfactants Produced by Microorganisms

Glycolipids	Trehalose lipids	*Nocardia, Mycobacterium, Corynebacterium, Arthrobacter*
	Rhamnose lipids	*Pseudomonas aeruginosa*
	Sophorose lipids	*Torulopsis sps*
	Polysaccharide lipids	*Candida tropicalis, Acinetobacter calcoaceticus*
Amino acid lipids	Lipopeptides	*Bacillus, Streptomyces, Corynebacterium, Mycobacterium*
	Ornithine lipids	*Pseudomonas, Thiobacillus, Agrobacterium, Gluconobacter,*
Phospholipids		*Thiobacillus, Corynebacterium, Candida, Micrococcus*
Fatty acids	Neutral lipids	*Pseudomonas, Acinetobacter, Micrococcus, Candida*

Research on the biosurfactant producing microorganisms has increased in recent years (4). Biosurfactants have several advantages over synthetic surfactants, such as: Biosurfactants present surface-active properties differing in some cases from synthetic surfactants, providing new possibilities for industrial applications; microbial surfactants have been shown to be more effective and specific than many conventional synthetic surfactants in specific applications; and they are usually non-toxic and biodegradable. Several properties of these surface active agents have been described, including surfactants, emulsifiers, de-emulsifiers, flocculating agents, foam stabilizers, etc. (4).

Due to their high effectiveness, biosurfactants have been tested for some applications such as enhanced oil recovery and transport of heavy crude oils. Therefore, many applications of biosurfactants may be found in other fields, such as pharmacology, biocosmetics, textiles and food (5).

The chemical structures of biosurfactants have not been studied extensively. They are very important in order to perform a rigorous physicochemical study and obtain better knowledge of metabolic pathways, thus affording the best production conditions and developing modifications in the structure of biosurfactants in order to improve surfactant properties.

The aim of this work has been to study the extracellular materials with surface activity obtained from microorgan-

isms isolated from soil and water samples. Isolation and separation of surface active agents from the *Pseudomonas aeruginosa* 44T1 strain and structural identification of biocompounds has been studied by: (a) Preliminary structure identification with specific TLC reagents; (b) Spectroscopic (IR, ^{1}H-NMR, ^{13}C-NMR) study of the isolated active compounds; (c) Chemical degradation techniques (acid and alkaline hydrolysis); (d) Spectrometric study (FABMS) without derivatization of bioactive agents; (e) Physicochemical evaluations (surface and interfacial tension) of both culture broth and isolated biosurfactants as a function of pH.

EXPERIMENTAL

Procedure for isolating biosurfactants from culture broth. Microbes have been isolated from soil and water samples after an enrichment culture process with kerosene. They have been tested for biosurfactant production by measuring surface and interfacial tensions and emulsification power of culture broth (6).

Isolation of surface active compounds from culture broth of the 44T1 strain has been made by conventional methods used in the isolation of glycolipidic structures (7).

Chemical detection of surface active compounds. Culture broth has been freeze-dried in a Telstar Liobeta apparatus; extraction of the sample at pH 2.0 with a solvent mixture of $CHCl_3$:MeOH (2:1) has been carried out. The chloroform used was dried over calcium chloride, distilled off and stored in brown bottles to avoid further production of phosgene. The organic phase was separated by column chromatography with silica gel 60 (Merck) or Florisil adsorbent (Fluka). The column was eluted using solvent gradient techniques starting with $CHCl_3$ followed by $CHCl_3$:MeOH (9:1). The components were purified by preparative thin layer chromatography on silica gel 60 G (Merck) in chloroform:methanol: distilled water (65:25:4). A vast number of solvents and detection methods for separating and identifying chemical compounds with spray reagents has been used.

The TLC reagents (8) used were ammonium molybdate-concentrated HCl for phosphate stain (Vaskovsky reagent); for free amino groups, ninhydrin; amide identification was made with o-tolidine; amine-deoxysugar was analyzed by Elson-Morgan assay; lipid stain was detected with iodine atmosphere and Rhodamine 6 G; free carboxylic acid was studied with bromocresol-green and α-Naftol-H_2SO_4 acid (Molish reagent) was used for sugar detection.

Structural characterization of the extracted components. Infrared spectra were recorded with a Perkin Elmer 399 B spectrophotometer. ^{1}H- and ^{13}C-NMR were recorded with a Bruker WP 80 SY spectrophotometer (80 MHz and 20 MHz, respectively) and sometimes with a Varian XL 200 (200 MHz) spectrophotometer. Chemical shifts are in δ scale reported with respect to internal tetramethylsilane.

Studies of fatty acids were done by reaction with diazomethane and final analysis by GLC-MS with a Hewlett-Packard 5890 instrument coupled with a Hewlett-Packard 5988 mass spectrometer.

Positive FABMS spectra were recorded on a MS-9 VG updated (VG Analytical J.K.). Glycolipidic samples ($\leq$ 0.1 mg) were dispersed in glycerol, thioglycerol or diethanolamine as probe matrix. We tried all the matrices described above, but only the best results are reported.

Alkaline hydrolysis of glycolipids was carried out by refluxing with 0.5 N sodium hydroxide solution for 12 hr at 40 C. Extraction of fatty acids with $CHCl_3$ and methylation with diazomethane afforded a lipidic sample for their ulterior GC-MS analysis.

Acid hydrolysis was done by refluxing the samples with 2 N HCl for 2 hr 30 min. The sugar moiety was analyzed by TLC with different solvent systems (ethyl methyl ketone/benzene/2-propanol/benzoic acid; ethyl acetate/pyridine/water and n-butanol/pyridine/O, 1 M HCl) and spectrophotometrically comparing with authentic samples of several monosaccharides such as L(+)-Rhamnose.

Physicochemical estimations. Surface and interfacial tensions of organic extract and of the identified components were measured with a Lauda Automatic Tensiometer TE-1 by the De Nouy ring method. Aqueous solutions of the compounds in this study were acidified, pH 3, with 1 N HCl, and made basic, pH 9, with 1N NaOH. The pH was measured using a Radiometer pH-meter 28.

RESULTS AND DISCUSSION

Isolation and separation of surface active agents. Approximately 10% of the microorganisms isolated after enrichment culturing showed surface activity against 4% of isolates without previous selection.

When samples were obtained from zones with hydrocarbon contamination, 22% of isolated microorganisms showed surface activity.

These results suggest a high correlation between hydrocarbon degradation and biosurfactant production from microorganisms.

When *Pseudomonas* 44T1 isolate grows on glucose as the carbon source, a lowering of surface and interfacial tension against kerosene to 26 mN/m and 5 mN/m, respectively, takes place. Emulsification power of the culture broth was 62.5% with a CMC^{-1} of 50.

Freeze-drying of the culture broth, followed by acidification and extraction with $CHCl_3$:MeOH (2:1), gave an organic extract with surface active properties. Two anionic agents were found to be responsible for the majority of the low surface and interfacial tension seen above. They were named Glycolipid A and Glycolipid B.

TABLE 2

Identification Methods by TLC Reagents

Solvent system	Rf a[a]	Rf b[a]	Rf c[a]	1-4[b]	5[b]	6[b]	7[b]	8[b]	9[b]
Dyestuff	0.59	0.62	0.93	–	–	+	+	+	–
Glycolipid A	0.05	0.34	0.70	–	+	–	+	+	+
Glycolipid B	0	0.18	0.45	–	+	–	+	+	+

[a]a, $CHCl_3$:MeOH (9:1); b, $CHCl_3$:MeOH (1:1); c, $CHCl_3$:MeOH:H_2O (65:25:4).

[b]1, Phospholipid test; 2, amine test; 3, amide test; 4, aminedeoxysugar; 5, lipid test; 6, UV; 7, I_2; 8, carboxylic acid test; 9, sugar test.

A preliminary structure of the organic phase was obtained. This structure was determined using the specific TLC reagents described above. The results obtained are shown in Table 2.

A yellow spot with an Rf value of 0.59, using the solvent system $CHCl_3$:MeOH (9:1), gave a positive detection under UV light, iodine atmosphere and bromocresol-green. The

two next spots, with an Rf value of 0.70 and 0.45, using the solvent system $CHCl_3$:MeOH:H_2O (65:25:4), were detected by Rhodamine 6G, iodine atmosphere, bromocresol-green and Molish reagent. The former results predict the existence of a highly conjugated system with some free carboxylic acid group in the first molecule. The remaining two compounds showed the presence of a glycolipidic structure with a free carboxylic group. With this preliminary study we can conclude the absence of structures such as amine compounds, amine-deoxysugars, peptides and phospholipid molecules.

Separation of the surface active agents was done by chromatographic absorption column in Florisil or silica gel. No change in the structure of compounds by either chromatographic method was observed.

Chemical identification of active compounds. The yellow spot was identified as phenazine-1-carboxylic acid (9) by spectroscopic methods. Characterization of biocompounds has been done by degradation methods using acid and alkaline hydrolysis. The acidic hydrolysis of the compound with Rf 0.70 (glycolipid A) gave one rhamnoseas sugar moiety identified by TLC with a standard sample and two units of β-hydroxydecanoic acid identified by ^{1}H-NMR, and after methylation reaction, the methyl ester was analyzed by GC-MS. Alkaline hydrolysis afforded a glycolipidic molecule, glycolipid A′, soluble in the water phase, and β-hydroxydecanoic acid, soluble in the organic phase. This means that one lipid moiety is linked by an ester bond. Acid hydrolysis of the remaining glycolipid A′ afforded one rhamnose sugar moiety and another unit of β-hydroxydecanoic acid as lipid component. These results involve a glycosidic bond between the sugar moiety and the second lipid molecule by the β-hydroxy group of the fatty acid.

The experimental work already described was also carried out with glycolipid B (Rf 0.45, using the solvent system $CHCl_3$:MeOH:H_2O (65:25:4). In this case, the compound is composed of two molecules of rhamnose glycosidically linked with one unit of β-hydroxydecanoic acid, and another molecule of β-hydroxydecanoic acid linked by an ester bond to the former fatty acid.

Characterization of biocompounds was also done by rigorous study of the IR, ^{1}H-NMR and ^{13}C-NMR spectra, in agreement with the results described by Syldatk et al, (10).

Recently, FABMS has been introduced as a new mass spectrometric technique, useful for the analysis of a wide range of biomolecules such as glycoalkaloids, glycoproteins, polysaccharides and peptides (11). The results obtained in our work using this technique are in agreement with the proposed structures.

The mass spectra obtained by FABMS contains molecular weight recognition as well as sequence information. The abundance of alkali-metal cationized ions was dependent on the different amounts of inorganic salts present from isolation procedures. Sometimes, $(M + Na \text{ or } K)^+$, $(M + Na \text{ or } K + \text{matrix})^+$, $(M + H + \text{matrix})^+$, $(M + 2Na - H)^+$ and other peaks corresponding with the elimination of an H_2O molecule as $(X + nNa \text{ or } n'K - H_2O)^+$ were detected.

Figure 1 shows the positive FABMS spectrum of glycolipid A, β[β(2-0-α-L-rhamnopyranosyloxy)decanoyl]decanoic acid using thioglycerol as probe matrix. That is, signals at m/z 527, 549 and 1076 are in agreement with $(M + Na)^+$, $(M + 2Na - H)^+$, and $(2M + 3Na - H)^+$, respectively. The sequence signals shown in Figure 1 are assigned to the fragments indicated, pointing out the existence of two units of β-hydroxydecanoic acid and one rhamnose molecule.

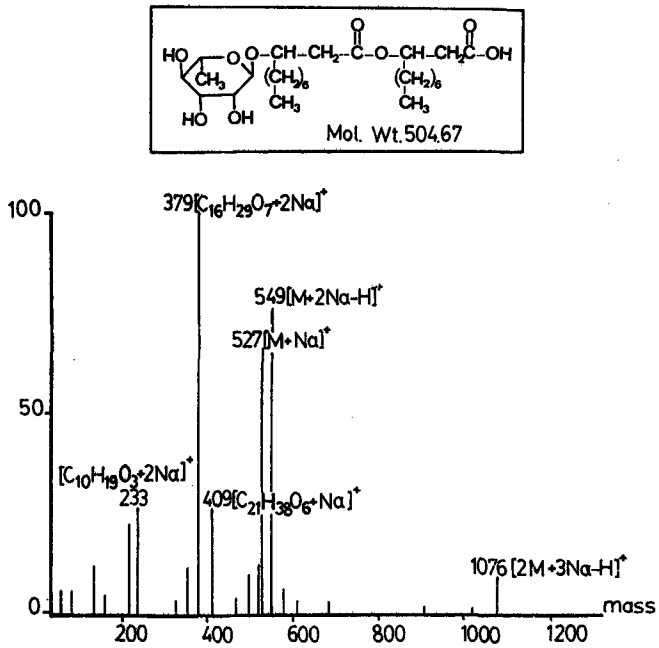

FIG. 1. FABMS spectra of glycolipid A.

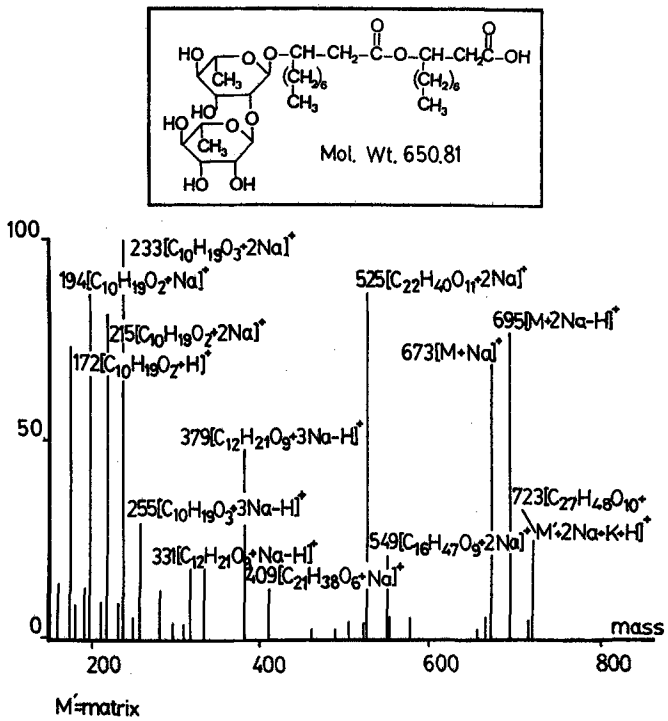

FIG. 2. FABMS spectra of glycolipid B.

Figure 2 shows the positive FAB spectrum of glycolipid B, β[β(2-0-α-L-rhamnopyranosyl-α-L-rhamnopyranosyloxy) decanoyloxy]decanoic acid, using diethanolamine as probe matrix. The more characteristic signals are at m/z 673 $(M + Na)^+$ and 695 $(M + 2Na - H)^+$. Also, the other sequence fragment ions as shown in Figure 2 could be assigned.

These signals are in agreement with the presence of two molecules of rhamnose and two units of β-hydroxydecanoic acid.

The studied structures are also supported by FABMS measurements.

FABMS in the positive ion mode is a straightforward, useful technique for direct analysis of small amounts of underivatized purified glycolipids. This technique allows rhamnolipid molecular weight determination.

Rhamnolipids produced by the 44T1 strain and isolated from culture broth are extracellular biosurfactants with improved surface properties and stimulated the growth of this strain in hydrocarbon sources. This strain also produces rhamnolipids when grown in glucose as carbon source.

Surface and interfacial values of culture broth and isolated biosurfactants as a function of pH. Figure 3 shows the dependence of the surface tension versus concentration in water solutions of the organic extract obtained after acidification of culture broth at the pH already described. At pH 3, the CMC value is lowered to 5.5 ppm; comparatively, at pH 7 the CMC value is 11 ppm and at pH 9 it is displaced to greater concentration values.

Dependence of the surface tension versus concentration in water solutions of glycolipid A and glycolipid B at pH 3, pH 7 and pH 9 are described in Figure 4. The minimum surface tension obtained was 25 mN/m. In both samples, the CMC value is lowered at pH 3 while at alkaline pH the CMC value is similar to that obtained at pH 7. On the other hand, the CMC value afforded at basic pH in the mixture (Fig. 3) is shifted as stated previously.

Figure 5 shows the dependence of the interfacial tension of the organic extract versus concentration in water solutions against kerosene. The minimum interfacial tension reached was 1.0 mN/m at pH 7 and pH 3.

In Figure 6 the dependence of interfacial tension of the two isolated rhamnolipids versus concentration in water solutions against kerosene is shown. Glycolipid A shows a lower CMC value at alkaline pH, as described by Syldatk et al. (10). Glycolipid B shows a lower interfacial tension with a minimum value of ≤ 1 mN/m (0.2 mN/m). The interfacial

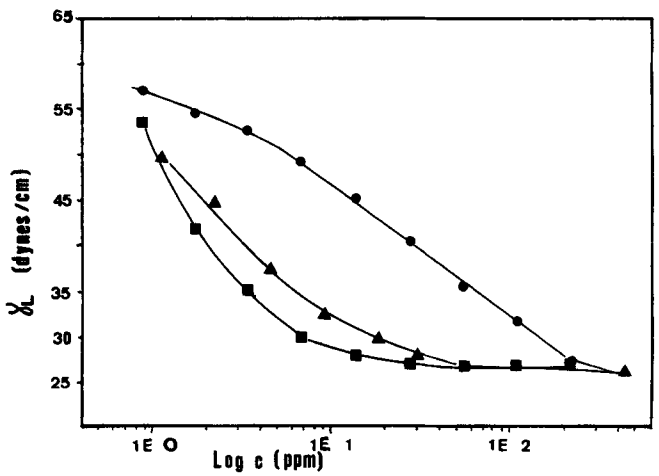

FIG. 3. Surface tension vs concentration of organic extract at different pH values. ▲, neutral pH; ■, acid pH; •, alkaline pH.

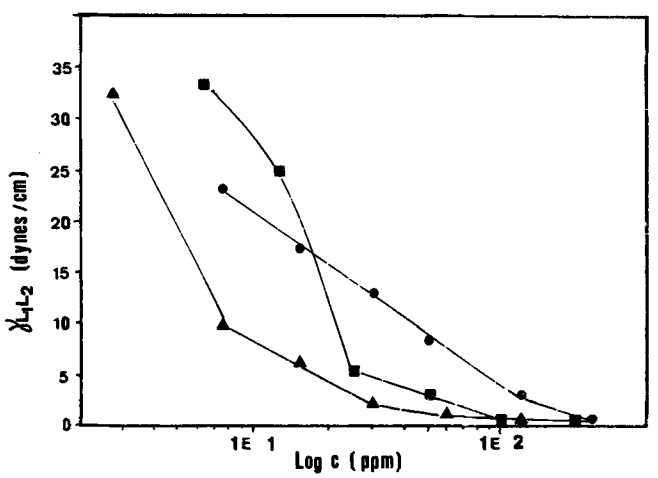

FIG. 5. Interfacial tension vs concentration of organic extract at different pH values. ▲, neutral pH; ■, acid pH; •, alkaline pH.

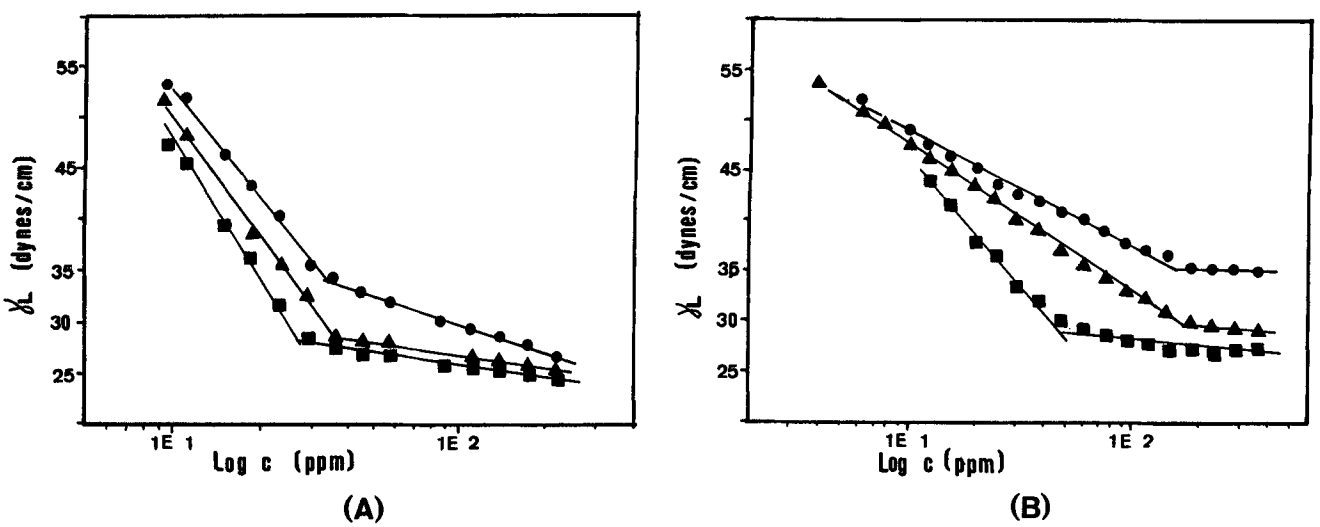

FIG. 4. Surface tension vs concentration of glycolipid A and B at different pH values. ▲, neutral pH; ■, acid pH; •, alkaline pH.

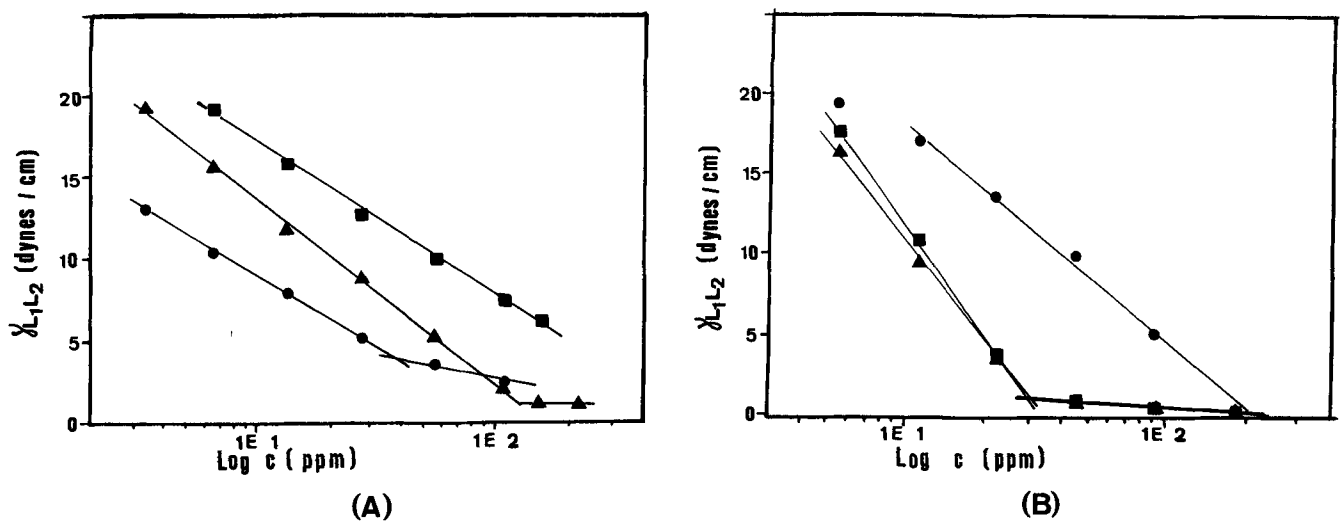

FIG. 6. Interfacial tension vs concentration of glycolipid A and B at different pH values. ▲, neutral pH; ■, acid pH; •, alkaline pH.

behavior of glycolipid B, with two rhamnose molecules, is slightly modified with the change of pH at acidic values, affording a lower CMC at acidic pH than alkaline pH.

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REFERENCES

1. Zajic, J.E., and W. Seffens, *Crit. Rev. Biotech 1:*87 (1984).
2. Hisatsuka, K., T. Nakahara, N. Sono and K. Yamada, *Agric. Biol. Chem. 35:*686 (1971).
3. Cooper, D.G., and J.E. Zajic, *Adv. Appl. Microbiol. 26:*229 (1980).
4. Kosaric, N., W.L. Cairns and N.C.C. Gray, *Biosurfactants and Biotechnology, Surfactant Science Series, Vol. 25,* Marcel Dekker, Inc., 1987.
5. Rosemberg, E., *Crit. Rev. Biotech. 3:*109 (1986).
6. Bosch, M.P., M. Robert, M.J. Espuny, M.E. Mercadé, J.L. Parra and J. Guinea, *Tenside 4:*208 (1988).
7. *Carbohydrate Analysis: A Practical Approach,* edited by M.F. Chaplin and J.F. Kennedy JRL Press, Oxford, 1986.
8. *Techniques of Lipidology: Isolation, Analysis and Identification of Lipids,* edited by M. Kates, Elsevier Amsterdam, 1972.
9. Wendenbaum, S., P. Demange, A. Dell, J.M. Meyer and M.A. Abdallah, *Tetrahedron Lett. 24:* 4877, (1983).
10. Syldatk, C., S. Lang, F. Wagner, V. Wray and L. Witte, *Naturforsch. 40:*51 (1985).
11. Barber, M., R.S. Bordoli, R.D. Sedgwick and A.N. Tyler, *J. Chem. Soc. Chem. Commun.* 325 (1980).

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