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Novel characteristics of sophorolipids, yeast glycolipid biosurfactants, as biodegradable low-foaming surfactants

Yoshihiko Hirata,* Mizuyuki Ryu, Yuka Oda, Keisuke Igarashi, Asami Nagatsuka, Taro Furuta, and Masaki Sugiura

Biochemical Laboratory, Saraya Co., Ltd., 24-12, Tamate-cho, Kashiwara, Osaka, 582-0028, Japan

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Sophorolipids (SLs) are a family of glycolipid type biosurfactants, which are largely produced by the non-pathogenic yeast, *Candida bombicola*. In order to investigate the possibility of SLs for industrial use, here we examined the interfacial activities, cytotoxicity and biodegradability of SLs, and compared these properties with those of two lipopeptide type biosurfactants (surfactin and arthrofactin), sodium laurate (soap, SP) and four kinds of chemically synthesized surfactants including two block-copolymer nonionic surfactants (BPs), polyoxyethylene lauryl ether (AE) and sodium dodecyl sulfate (SDS). It was indicated that SLs had extremely low-foaming properties and high detergency comparable with commercially available low-foaming BPs. These interfacial activities of SLs were maintained under 100 ppm water hardness. Cytotoxicity of SLs on human keratinocytes was the same as surfactin, which has already been commercialized as cosmetic material, but higher than BPs. Moreover, biodegradability of SLs using the OECD Guidelines for Testing of Chemicals (301C, Modified MITI Test) displayed that SLs can be classified as "readily" biodegradable chemicals, which are defined as chemicals that are degraded 60% within 28 days under specified test methods. We observed 61% degradation of SLs on the eighth day of cultivation. Our results indicate that SLs are low-foaming surfactants with high detergency, which also exhibit both low cytotoxicity and readily biodegradable properties.

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[**Key words:** Biosurfactants; Sophorolipids; Low-foaming ability; Biodegradability; Detergency; Cytotoxicity]

Surfactants are one of the most versatile products of the chemical industry and are characterized by an amphiphilic structure (1). Foaming ability and stability are key properties of surface active agents. In some industrial fields, such as spray cleaning, paper-making or textile dyeing processes, it is often useful to add surfactants that can show certain types of surface activity with low-foaming property. Several types of block-copolymer nonionic surfactants, which are petrochemical surface active agents containing propylene oxide (PO) and ethylene oxide (EO), are usually applied in these cases, but it is known that they have poor biodegradability due to their high degree of branching (2, 3). In order to improve their biodegradability, attempts to synthesize the block-copolymers with an altered degree of copolymerization of the PO and an alkylated terminal are currently in progress.

Biological surfactants, or 'biosurfactants', are biomolecules with both a hydrophilic and hydrophobic moiety. According to the structures of the hydrophilic moieties, they are classified as glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acid (4). Glycolipid biosurfactants, especially mannosylerythritol lipids (MEL), have been extensively studied to improve their productivity and to reveal their unique properties (5–7). Most biosurfactants are produced by bacteria, yeasts, and fungi during cultivation on various carbon sources (8). Therefore, they have clear advantages to the chemically synthesized surfactants, that include lower toxicity, higher biodegradability and better environmental compatibility. Nevertheless, the use of biosurfactants has been limited to a few specialized applications because they have been economically uncompetitive (4, 9). Regarding the practical application of biosurfactants, crude or impure biosurfactants, which are available at lower costs, can be used for environmental applications and oil recovery processes. Alternatively, highly purified biosurfactants can be utilized as high value added products, such as pharmaceuticals or cosmetics (4, 10). Furthermore, there are a few reports on the application of biosurfactants to biodetergents, such as spiculisporic acid (11), agaric acid (12) and corynomycolic acids (13); however, no commercial products have been developed yet.

SLs, which are produced by the non-pathogenic yeast *Candida bombicola*, are glycolipid biosurfactants first reported by Gorin et al. in 1961 (14). Their complete structures have been determined as a mixture of lactonic and acidic sophorosides of 17-L-hydroxydecanoic acid (Fig. 1) (15). Since then, in order to improve SLs productivity, extensive examinations on culture conditions have been carried out using various substrates, such as carbohydrates, vegetable oils, animal fat, whey, soy molasses and waste frying oil (16). Additionally, various new properties of SLs have been reported such as anti-HIV virulent activity, antibacterial activity, anticancer properties and apoptosis induction (16, 17). However, despite their high potential, the actual

* Corresponding author. Tel.: +81 729 77 8000; fax: +81 729 77-2224.
E-mail address: hirata@saraya.com (Y. Hirata).

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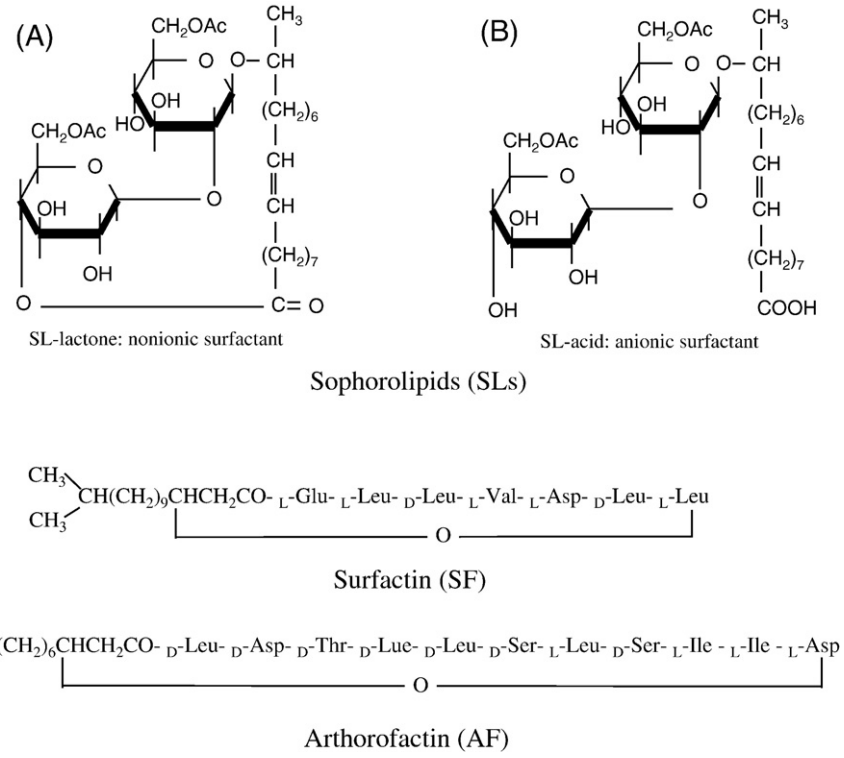

FIG. 1. Structures of biosurfactants prepared in this study. Sophorolipids (SLs) are glycolipid biosurfactants containing (A) SL-lactone (nonionic surfactant) and (B) SL-acid (anionic surfactant). Surfactin (SF) and arthrofactin (AF) are cyclic lipopeptide biosurfactants, which are containing anionic amino acid residues (glutamic acid and aspartic acid).

use of SLs has not yet to be reported. In view of the possibility of SLs for industrial application, this study was carried out to examine several important properties of SLs, focusing on their interfacial activities, cytotoxicity and biodegradability.

MATERIALS AND METHODS

Biosurfactants, soap and chemically synthesized surfactants The sophorolipids (SLs) (Fig. 1) tested in this report were produced by *Candida bombicola* ATCC22214 using a jar-fermenter (18) with a medium 10% glucose, 10% soybean oil, 1% KH_2PO_4, 0.5% $MgSO_4$, 0.1% NaCl, 0.1% urea and 0.25% yeast extract, and purified as described by Cooper et al. (19). SLs were produced the mixtures with 1′, 4″-lactone (A) and free acid form (B) in about a 7 to 3 ratio. Surfactin (SF) (Fig. 1), sodium laurate ($C_{11}H_{23}COO^-Na^+$, SP), sodium dodecyl sulfate (SDS), and polyoxyethylene lauryl ether ($C_{12}H_{25}(OC_2H_4)_{23}OH$, AE) were purchased from Wako chemicals. Pluronic L31 (BPL31) and L64 (BPL64) were purchased from BASF Japan. They are block-copolymers of EO and PO with an average structure H–$(EO)_2$–$(PO)_{16}$–$(EO)_2$–OH (average MW = 1100) and H–$(EO)_{13}$–$(PO)_{30}$–$(EO)_{13}$–OH (average MW = 2900), respectively. Arthrofactin (AF) was a cyclic lipopeptide biosurfactant (Fig. 1) first reported by Morikawa et al. and closely studied on the structure–function relationship (20, 21). AF was kindly gifted by Profs. Tadayuki Imanaka (Kyoto University, Japan) and Masaaki Morikawa (Hokkaido University, Japan). Basic surface activities of surfactants and their abbreviations were shown in Table 1.

TABLE 1. Surfactants tested in this study.

Surfactants	Abbreviations	CMC (mg/L)	Min. ST (mN/m)
Sophorolipids	SLs	43.0	37.2
Surfactin	SF	35.6	28.3
Arthrofactin	AF	9.0	27.0
Sodium laurate	SP	2200[a]	21.5[a]
Pluronic L31	BPL31	–[b]	45.0[c]
Pluronic L64	BPL64	–[b]	41.3[c]
Sodium dodecyl sulfate	SDS	270	33.7
Polyoxyethylene lauryl ether	AE	42.0	42.2

[a] Data were obtained using the solution diluted by distilled water.
[b] Clear CMC was not detected in this method.
[c] The value was obtained at 1000 mg/L of surfactant.

Foaming tests Foaming properties of surfactants were determined according to Ross–Miles method (22). In brief, using special pour test apparatus, 200 ml of test solutions in concentration of 10–10,000 mg/L were poured onto surface of the same liquid at 20 °C from 900 mm height taking 30 s. All test solvents were adjusted to 0 or 100 ppm water hardness calculated as ppm $CaCO_3$ and a buffer (pH 8.94) containing 5 mM Na_2CO_3 and 90 mM $NaHCO_3$. A hundred ppm hard water was prepared as described in the Association of Official Analytical Chemists (23), and contained about 26.7 mg/L Ca^{2+} and 8.1 mg/L Mg^{2+}. The foam heights were measured at immediately (0 time) and five minutes later (5 min) after all of the liquid has run out.

Washing tests Washing tests were carried out using standard soiled swatches as specified by the Association of Washing Chemistry Foundation, Tokyo. The same solvent system as described in foaming tests was used in these tests. Five swatches were washed in a Terg-O-Tometer (Daiei Kagaku Seiki, Japan) with 1000 ml of the 500 mg/L test solution for 10 min at 20 °C. Reflectance of the soiled swatches before and after the washing was measured by a colorimeter CR-300 (Minolta), and detergency (W) of the test solutions was calculated from the following formula as a washing rate.

The detergency (W) was defined as:

$$W = [(Y - C)/(A - C)] \cdot 100\%$$

Where Y and C denote the average degree of whiteness of the fabric after and before washing, respectively, and A is the average degree of whiteness of unsoiled fabric.

Cytotoxicity Cytotoxicity of SLs was determined by MTT method with normal human epidermal keratinocytes (Kurabo). The medium used in these tests were HuMedia-KG2 (Kurabo). Keratinocytes suspended in 150 μL HuMedia–KG2 medium were seeded into 96-well plates at 2×10^4 cells/well and incubated at 37 °C, 5% CO_2 for 72 h. After the incubation, surfactant dissolved in 150 μL of DMEM medium was added and the plates were incubated for 48 h. The viability of keratinocytes was then determined by an MTT assay in which the absorbance at 570 nm was measured with microplate reader (Spectra Thermo, Tecan Austria). Viability was calculated according to the following equation:

$$\text{Viability} \quad (\%) = (\text{As}/\text{Ac}) \cdot 100$$

As = absorbance of the extract when surfactant was applied to keratinocytes
Ac = absorbance of the extract when only medium was applied to keratinocytes

CMC and surface tension measurement Surface tension was measured with Tensiometer CBVP-Z (Kyowa Interfacial Science) according to the Wilhelmy method by using a series of various concentrations of surfactant solutions at 20 °C with the above buffer system (pH 8.94 and water hardness 0 ppm). The solutions were aged at 20 °C before each measurement until equilibrium was reached between adsorption and desorption of the amphiphilic molecules at the surface. Measurements were repeated

TABLE 2. Foaming properties and surface activities of surfactants.

Surfactants (1000 mg/L)	Foam height (mm)			
	0 ppm water hardness		100 ppm water hardness	
	Initial	After 5 min	Initial	After 5 min
SLs	24	20	23	9
SF	145	135	117	95
AF	134	118	94	86
SP	237	201	10	8
BPL31	18	1	10	0
BPL64	27	8	15	0
SDS	202	195	227	202
AE	69	60	65	56

three times, and the mean value was taken. Critical micelle concentration (CMC) and minimum surface tension (min. ST) were determined from the curve.

Biodegradability Biochemical oxygen demand (BOD) was determined with a BOD Track (Hach USA) by the oxygen consumption method according to the OECD Guidelines for Testing of Chemicals (301C, Modified MITI Test) at 20 °C. The concentration of the test compounds in the incubation media was 25 mg/L. Actual total oxygen demand was determined with TOD analyzer Model 1548 (Yuasa Ionic).

RESULTS

Foaming properties of SLs Foaming properties of SLs solutions (1000 mg/L) determined with the Ross–Miles method at 20 °C, pH 8.94 are shown in Table 2. We observed that SLs have extremely low-foaming properties compared to SF, AF, SP and SDS. In fact, their properties were comparable to BPL31 and BPL64, which are block-copolymer nonionic surfactants already commercialized as low-foaming detergents with the product names of Pluronic L31 and Pluronic L64, respectively. Interestingly, the foaming properties, especially the foaming ability of SLs in 100 ppm hardness water, were almost equal to that in DW (0 ppm hardness). In contrast, the foaming properties of SF, AF and SP decreased with the increase of water hardness. The foaming ability of SP decreased dramatically to about 1/20 at 100 ppm water hardness compared to the level observed at 0 ppm hardness.

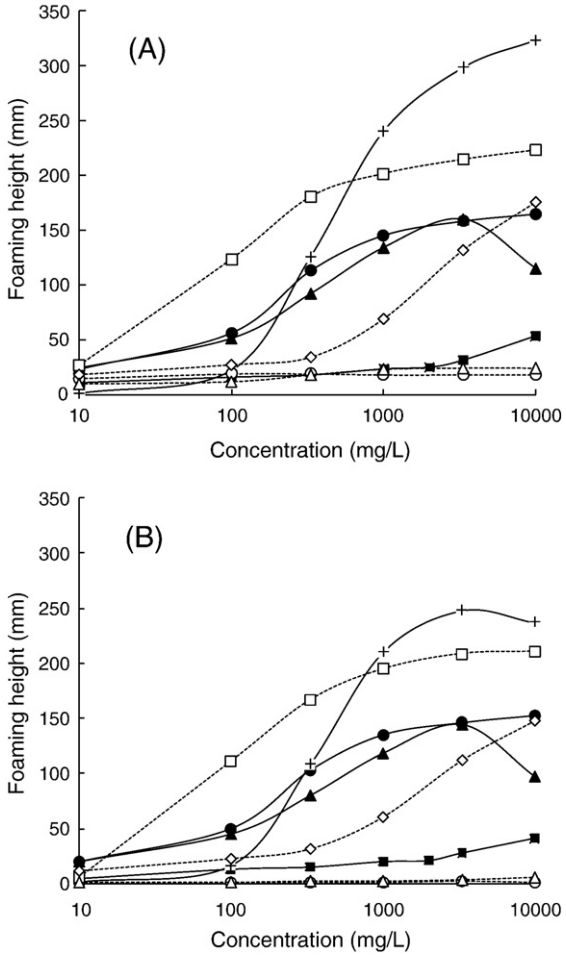

FIG. 2. Effect of surfactant concentration on the foaming ability (A) and stability (B). Symbols: filled squares, SLs; filled circles, SF; filled triangles, AF; crosses, SP; open circles, BPL31; open triangles, BPL64; open squares, SDS; open diamonds, AE. Foam height was measured immediately (a) and 5 min after (b) all of the liquid has run out. The experiment was repeated three times and the average value was shown.

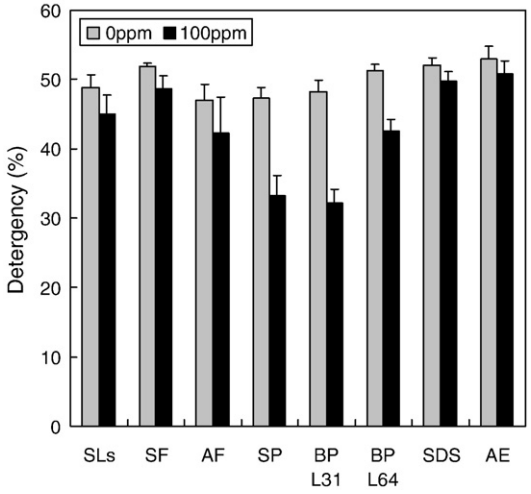

FIG. 3. Detergency of surfactants. Surfactant concentration was 500 mg/L, at 20 °C, pH 8.94. Water hardness was 0 ppm or 100 ppm. Error bars refer to standard deviation, SD ($n=5$).

Effects of surfactant concentrations on foaming properties We examined the effect of SL concentration on foaming properties and compared them to those of lipopeptide biosurfactants (SF and AF), SP, block-copolymer nonionic surfactants (BPL31 and 64), SDS and AE (Fig. 2). We found that SL had low-foaming properties comparable to those of BPL31 and 64 at concentrations below 3000 mg/L. The foaming ability (Fig. 2A) of SLs gradually increased at concentrations of

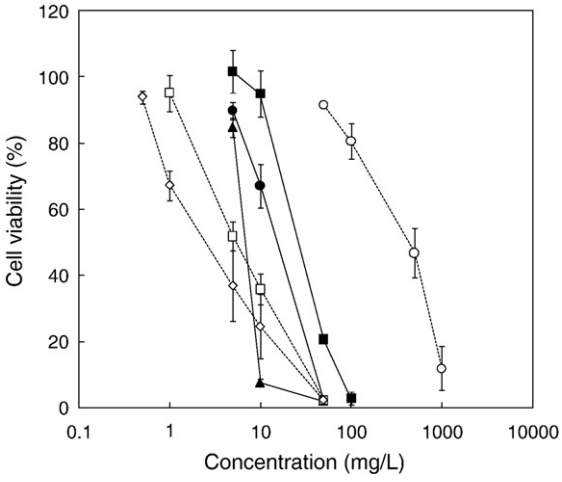

FIG. 4. Effects of surfactant concentrations on cell viability. Symbols: filled squares, SLs; filled circles, SF; filled triangles, AF; open circles, BPL31; open squares, SDS; open diamonds, AE. Error bars refer to standard deviation, SD ($n=3$).

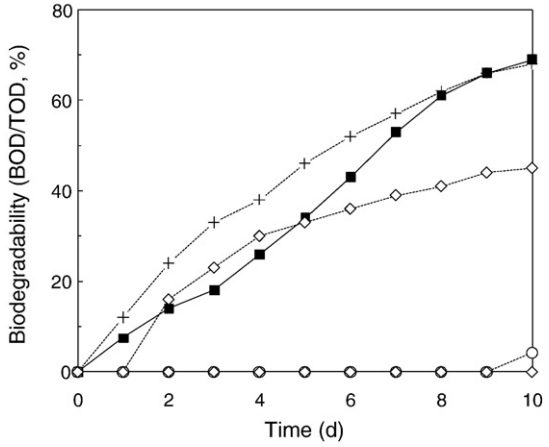

FIG. 5. Biodegradability of SLs and others surfactants. Symbols: filled squares, SLs; crosses, SP; open circles, BPL31; open diamonds, LAS; open diamonds, AE.

approximately 3000 mg/L and its maximum foaming height was 160 mm, which was similar to that of SF and AF at 10,000 mg/L (data not shown).

Detergency of SLs The detergency of SLs was investigated at 500 mg/L under the same conditions described above using Terg-O-Tometer, and was compared with those of other surfactants (Fig. 3). As a result, it was found that SLs had a high detergency level similar to AF, SP and BPL31, but was slightly lower than SF, BPL64, SDS and AE in 0 ppm water hardness. Meanwhile, in 100 ppm water hardness, the detergency of SLs was higher than SP and BPL31, and was very similar to those of AF and BPL64.

Cytotoxicity and biodegradability of SLs We examined the cytotoxicity of SLs using the MTT method with keratinocytes and compared it with the cytotoxicities SF, AF, BPL31, SDS and AE (Fig. 4). BPL31 showed the lowest cytotoxicity among the surfactants tested. SLs also exhibited relatively lower cytotoxicity than SF, AF, SDS and AE.

Fig. 5 shows the biodegradability of SLs and other surfactants tested in this study according to OECD guidelines. Biodegradability of SLs was observed immediately after cultivation was started and displayed 61% (BOD/TOD) biodegradability after 8 days of cultivation. This biodegradation pattern was similar to that of SP, which is well known as a good biodegradable natural surfactant. The chemicals tested in this method can be defined as "readily" biodegradable, when biodegradability (%) exceeds 60% within 28 days. According to this definition, SLs can clearly be regarded as "readily" biodegradable chemicals. In contrast, the other surfactants tested in this study do not fall into this category. In particular, the low-foaming block-copolymer nonionic surfactant, BPL31, and anionic surfactant, LAS, showed little biodegradability.

DISCUSSION

Biosurfactants are natural surfactants with both high biodegradability and low toxicity, and thus have recently received increased attention (1, 4). Much effort has been taken to increase their productivity through fermentation. However, few studies have reported on their interfacial properties in view of their application in industry. In this report, we have found novel interfacial characteristics of SLs. SLs exhibited strikingly low-foaming properties in a wide concentration range, which were comparable with commercially available block-copolymers (Table 2 and Fig. 2). Surfactants generally produce foam because they can be easily adsorbed at the liquid–gas interface. Therefore, low-foaming surfactants need to form specific molecular structures which retain their surface activity while producing an unstable foam (24). Thus, the low-foaming characteristics of SLs could be due to their rapid diffusion and/or a large occupied area per molecule at the liquid–air interface.

Interestingly, this property was maintained at concentrations well above (~100-fold) the CMC. Foam height generally increases with the increase in surfactant concentration until it nears the CMC, where foam height reaches a maximum value or increases slowly to a maximum value somewhat above the CMC (24). It is likely that this is due to the increase of the bulk viscosity and/or the alternation of the molecular assembly of SLs in their solutions. In view of industrial application, such as washing machines, these low-foaming surfactants are generally formulated at the ratio below approximately 10% (100 g/L) in most of these special detergents, which are actually used at the concentration below 1%. Accordingly, the practical concentrations of low-foaming surfactants will be estimated below 1000 mg/L. Thus, SLs are significantly expected for practical use as low-foaming detergents, since they have the low-foaming property at 10 times higher concentration than estimated ones (Fig. 2).

Another property that should be noted was that water hardness had almost no effect on the foaming property and the detergency of SLs. Interfacial activities of surfactants containing carboxyl groups generally decrease in hard water due to their binding to calcium and magnesium ions (25). This resistance to hard water may be due to the bola type structure of the SL-acid form, which not only contains a carboxylic group but also a sophorose (Fig. 1) in the hydrophilic moiety. In addition, SLs are composed of mixtures of anionic acid type and nonionic lactone type molecules. In general, it is well known that mixtures of two surfactants can generate a synergistic effect (26). These specific mixtures of SLs might be responsible for the resistance to hard water.

As for the cytotoxicity of SLs, it has been reported that SLs displayed low cytotoxicity towards human fibroblasts (27) and human keratinocytes cell line HPK II (28). In this report, we examined their effects on normal human epidermal keratinocytes (Fig. 4). As a result, SLs displayed relatively lower cytotoxicity than SF, which is a lipopeptide BS already commercialized as cosmetic material with extremely weak skin irritation (29). In contrast, regarding the relationship between cytotoxicity and efficiency of surfactants, the half-maximal inhibition concentrations (IC_{50}) of SDS and AE calculated from Fig. 4 were 5.3 mg/L and 2.3 mg/L, respectively. These values were about 1/50 and 1/20 of their respective CMC values (Table 1). On the other hand, IC_{50} of SLs are 24 mg/L, and are thus highly similar to their CMC (43 mg/L) presented in Table 1. This characteristic of SLs is also observed for other tested BSs, SF and AF which display 14 mg/L and 6.8 mg/L IC_{50} values, respectively. This property implies that biosurfactants not only exhibit high efficiency as a surfactant at low concentration, but also display low cytotoxicity at their effective concentrations.

Extremely low-foaming SLs displayed not only high detergency and low toxicity but also rapid biodegradability (Fig. 5). BPL31 and 61 tested in this study are block-copolymer nonionic surfactants containing PO–EO and have already been applied in a wide range of industrial areas. These surfactants harbor low-foaming ability and remarkably low toxicity (Fig. 4), and have been utilized as low-foaming detergents for washing machines such as laundry machines, automated dish washers and washer–disinfectors for cleaning medical devices. However, these surfactants have poor biodegradability (Fig. 5), as they are nonionic surfactants containing PO blocks which have a high degree of branching (2, 3). Biosurfactants are commonly said to be biodegradable. Indeed, examining the biodegradation of SLs according to the OECD 301C method revealed that SLs are readily biodegradable chemicals (Fig. 5s Renkin, using OECD 301F as an alternative biodegradable test, has reported that the biodegradation of SLs was sufficient to be classified as readily biodegradable chemicals (30). This report also supports that SLs are highly biodegradable surfactants.

Accordingly, these results indicate that SLs are biodegradable low-foaming surfactants with both excellent detergency and low cytotoxicity. Furthermore, the interfacial properties of SLs were not

affected by water hardness. All of these properties are favorable for the industrial use of SLs.

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