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Tuning surface-active properties of bio-surfactant sophorolipids by varying fatty-acid chain lengths

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Abstract−Sophorolipids are a kind of glycolipid-based bio-surfactant, and some known yeasts can produce them by utilizing plant oil at elevated glucose. In spite of a high production yield, sophorolipids have a limited industrial application, due to their narrow range of HLB (hydrophile - lipophile balance) value. These molecules have regained the attention of researchers and industrialists due to their surface properties and versatile applicability in herbal medicine and cosmetics. The bioactivity and surface properties of sophorolipids are mainly governed by chain length and type of the fatty acid. Therefore, the present study was designed to produce and characterize sophorolipids with varying fatty acid chain lengths. Surface-properties like critical micelle concentration of produced sophorolipids were varying from 43 to 62 (dyne/cm). Foamability, dispersion power, and detergence were found to be higher for short chained fatty acids than the longer ones. Cleaning ability of sophorolipid for FLUX coated PCB was found to be better than the chemical surfactants. Biodegradation rates of the sophorolipids were found to be higher than of the linear alkylbenzene sulfonate (LAS) at room temperature as well as 4 °C. These results showed that the properties of sophorolipids can be tuned by varying the chain's properties of fatty acids, and it may be possible to customize the properties of sophorolipids for specific industrial applications.

Keywords: Sophorolipids, *Candida bombicola*, Foamability, Dispersion Power, Detergency

INTRODUCTION

Sophorolipids are kind of bio-surfactant, which are mainly synthesized by yeasts, such as *C. apicola*, *C. batistae*, *Candida bombicola*, *Pichia anomala*, *Rhodotorula bogoriensis*, *Starmerella bombicola* and *Wickerhamiella domericqiae* [1]. Chemically, sophorolipids have two components, a hydroxy fatty acid and sophorose (a diglucose), which are condensed by glyosidic bond. The sophorose usually have an acetyle group at 6′ and/or 6″ positions [2]. Based on self-lactonification, sophorolipids are grouped into linear acidic (hydrophilic) and ringed lactonic (hydrophobic) forms [1,2]. The sophorolipids are well recognized for their surface-active properties and commonly known as green surfactant, offered as alternative to petrochemical based surfactants [2]. These molecules have been widely explored for their bioactivity. Researchers have identified the potential of the sophorolipids, as an alternative to laundry detergents [3], an emulsifier for the food-industry [1], and cosmetic constituent (antimicrobial and emulsifying properties) [1,2]. In spite of all potential, still major applications of sophorolipids are as household/laundry detergents [1,2]. Studies argue that the chain lengths of fatty acid are the major determinant of the bioactivity and surface like properties of sophorolipid [2,3]. Thus, the present study was designed to understand the relation between chain length and surface properties of sophorolipids. The methylated fatty acid of different chain length was used as a substrate for production of sophorolipid. Among known producers the *Candida bombicola* ATTC 2214, a well-studied sophorolipid producing organism, was therefore, used for this study [2,4]. However, most of the studies were mainly carried out with C18 or higher fatty acid; therefore, a comparative data on production and characteristics, based on fatty acid chain length, is being needed. In this study, we attempted to simplify the process for production of the C10 to C18 containing sophorolipid using different chain length fatty acids as a substrate. In addition, surface like properties and yield were also compared.

MATERIALS AND METHODS

The chemicals were procured from Sigma-Aldrich and the culture media and components from Difco and Merck. The oils were obtained from Shindongbang Co. (Korea).

1. Growth and Maintenance of Yeast

The cryopreserved (−70 °C) *C. bombicola* (ATCC 22214) was revived in YM broth (per liter, 3.0 g of yeast extract, 3.0 g of malt extract, 5.0 g of peptone, 10.0 g of glucose) media, incubated at 25 °C, at 250 rpm; a 24 hour grown culture was used as seed as well as stored for further application.

2. Fatty Acids Methylation

Fatty acids are toxic to yeast; therefore, the direct use of fatty acid as a substrate for production of sophorolipid was not possible. Thus, methylation was performed to diminish the toxicity [6]. In this study, different chain lengths of fatty acid were used as substrates; Capric acid (10:0), Lauric acid (12:0), Myristic acid (14:0), Palmitic acid (16:0) and Stearic acid (18:0) were methylated, prior to use as substrate. In brief, the methylation was carried in sealed containers,

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containing 300 ml methanol, 6 g of fatty acid and 4.9 ml sulfuric acid; this mixture was incubated at 65 °C for 4 hours. The produced fatty acid methyl ester was extracted in hexane. The methylated fatty acid was extracted by a phase separation, followed by evaporation using the rotary vacuum evaporator (EYELA, N-1000) [6].

3. Sophorolipid Production

A previously standardized media was used for the production of sophorolipid [4]; in brief, media contained, 50 g of glucose, 50 g of fatty acid (methylated), 5 g of yeast extract, 0.7 g Peptone, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 0.1 g of NaCl, mixed in one liter of water. The overnight grown culture was inoculated in above media. About 2.0% (V/V) of seed culture was inoculated in production media. The fermentation was in a 500 ml flask containing 200 ml of media, which was incubated at 25 °C with an agitation of 200 rpm for 20 days.

4. Residual Fatty Acid Determination and Extraction of Sophorolipid

To calculate the production yield, the residual fatty acid was extracted from the culture filtrate (after completion of fermentation) using n-Hexane [2,6]. The top non-aqueous layer, containing fatty acid, was separated through a separation funnel. The residual fatty acid was recovered by evaporation of n-Hexane using rotary vacuum evaporator, and weighed. The recovered aqueous phase from above was extracted three times with the same volume of ethyl acetate. The ethyl acetate layer was separated through separating funnel and by evaporation of the ethyl acetate using a rotary vacuum evaporator (Eyela, Japan), and the sophorolipid was recovered and weighed [6].

5. Measurements of Surface-active Properties of Sophorolipids: Surface Tension, Critical Micelle Concentration, Foaming Power, and Detergency

The surface tension of crude sophorolipid was determined by the ring method, using a du Nuoy tensiometer (Surface Tensiomat model 21, Fisher Scientific, Pittsburgh, PA, U.S.A.) [2,7]. The measured surface tension was used for calculation of the critical micelle concentration (CMC) at room temperature.

The dispersion power was determined by standard protocol [7]. In brief, 0.12 g of Fe_2O_3 was mixed in 40 ml of sophorolipid solution (ranging from 0.0-1.5%, w/v). This mixture was stirred vigorously and poured into a measuring cylinder (50 ml) and this mixture was allowed to rest for 15 min. 1 ml sample from top layer was diluted in 12.5 ml of distilled water, and the absorbance was measured at 660 nm (experiments performed in triplicate). The foaming power was measured by a slightly modified procedure from Ross and Miles method (as described ISO-696 at 50 °C) [8]. Emulsification capacity of the produced crude sophorolipids was determined by standard procedure [2]; briefly, 2.0 ml of 0.1% (w/v) solution of sophorolipid was mixed with 2.0 ml soybean oil in presence of 2.0 ml phosphate buffer (200 mM, pH 7.0). This mixture was vertexed for one minute. The emulsifying capability was monitored at every 10 min interval by measuring the absorbance at 540 nm.

6. Measurement of Detergency

Detergency was determined by previously standardized procedure [2,4]: The cotton fabric was soaked in oil; this contaminated fabric was washed by 100 ml of water, 0.2% solution of sophorolipid, and chemical surfactant (LAS), incubated for 30 min at 120 rpm and 25 °C [2,6,9]. Cleaning power was calculated by the following formula:

$$\text{Detergency } (C) = (W_0 - W_2)/(W_0 - W_1) \times 100$$

Where,
W_0=weight of cotton with oil
W_1=weight of cotton
W_2=weight of cotton after washing

In addition, the cleaning power of sophorolipid samples were also evaluated by cleaning of PCB electronic board.

7. Measurement of Biodegradability

Biodegradability was measured to ensure the eco-friendly nature of sophorolipids. The degradation of sophorolipids was monitored in Lake Water of Inha University. The degradability was monitored by decrease in surface tension. Experiments were conducted in 250 mL Erlenmeyer flask, containing 100 mL Lake water with sophorolipid and incubated at 25 °C for 8 days (180 rpm).

8. HPLC Analysis of Sophorolipids

The extracted sophorolipids (produced by different sized fatty acids) were dissolved in ethanol and subjected to HPLC analysis (Waters, Japan), using ACE-5 C18 4.6×250 mm column (ACE HPLC column). The acetonitrile/water was used as the mobile phase; a mobile phase gradient was varied between 40-60%; 20 μL of sample was injected for analysis; flow rate was maintained 1.0 mL/min; analysis window was for 30 min. Column temperature was maintained at 40 °C throughout the analysis and elute was monitored by UV detector using 195/210 nm wavelength.

9. Statistical Analysis

The experiments were carried out in triplicate. The results were presented as the average of data with respective standard deviation.

RESULTS AND DISCUSSION

Based on lactonization, two types of sophorolipids exist in nature. These two are usually synthesized simultaneously during fermentation [4]. Among most of the sophorolipid producing organisms, the *C. bombicola* have been placed at the highest rank, in terms of production yield. The synchronized, de-novo and bio-conversional production of sophorolipid by this organism is an additional advantage, which makes them first choice as a producer [4,6]. However, producing single kinds of sophorolipid with short chained fatty acid is still a challenge.

1. Production of Sophorolipid in Batch Culture

In this study sophorolipids were produced by *Candida bombicola* ATCC 22214 in a batch culture using methylated fatty acid as hydrocarbon feed. To achieve that, methylated fatty acids, namely capric acid (C10: 0), lauric acid (C12: 0), myristic acid (C14: 0), palmitic acid (C16: 0) and stearic acid (C18: 0), were used. From ten days old culture, the residual fatty acid, produced sophorolipid and dry mass was calculated and shown in Fig. 1(a), (b), (c). As revealed in Fig. 1(a) most of the supplemented fatty acid was consumed after 20 days of incubation. However, cell dry weight was not significantly increased, (Fig. 1(b)). It was also revealed that the production yield was chain length dependent, i.e., longer chain has

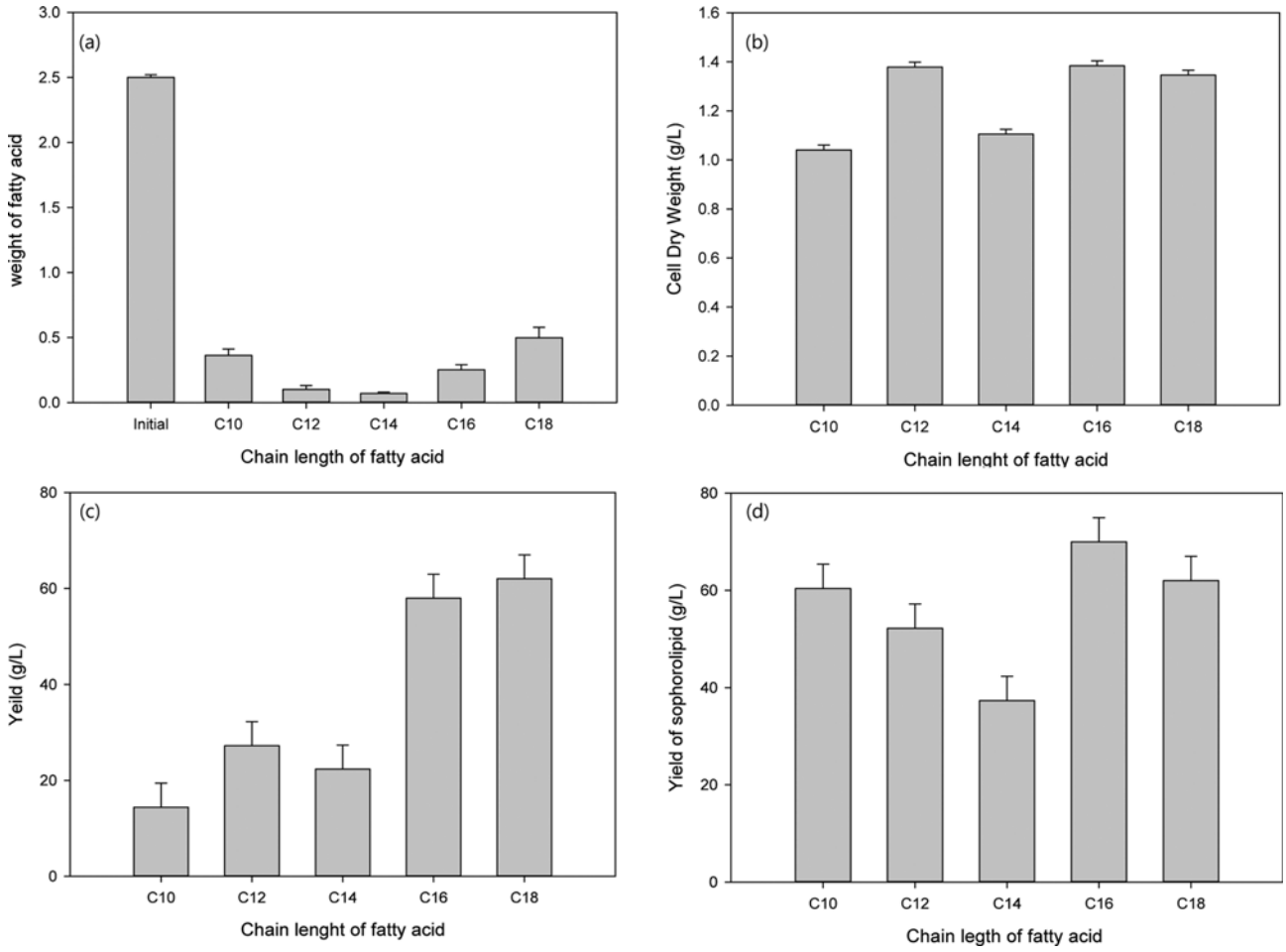

Fig. 1. Measurement of cell dry weight (CDW) (a), residual fatty acid (b), sophorolipid lipid production yield (c), yield of sophorolipid in a fed batch culture (feeding after 10 days) (d) after 20 days of fermentation.

better productivity. However, the C14 fatty acid was found as exception, thus not following the general trend (reason not known) (Fig. 1(c). The reason behind lower production yield, using short chained fatty acid as substrate, ie probably the metabolization of fatty acid (as reported in literature) [5], and the result shown in Fig. 1(c) showed a similar pattern as mentioned in previous reports [2,5]. However, fermentation carried out with additional feeding of fatty acid after 10 days, in a 20 days of incubation period was found to be significantly higher productivity as shown in Fig. 1(d). However, the medium chained fatty acid C14 showed a lowered yield, whereas the C16 and C18 retained their production yield (Fig. 1(c), (d)).

2. Surface Active Properties

Produced sophorolipids were compared for their surface properties against control (linear alkylbenzene sulfonate). Surface properties and bioactivities of sophorolipid are dependent on both the hydrophilic as well as hydrophobic regions of molecules [2]. Therefore, any structural anomaly in sophorolipid may cause significant change in surface properties like CMC, Min. S.T., and emulsification ability, etc. [5,7,9]. The calculated surface active properties of the sophorolipids derived from different fatty acid are discussed below.

3. Minimum Surface Tension and CMC

Sophorolipids produced with different fatty acid were examined to evaluate their minimum surface tension and critical micelle concentration (CMC) (Fig. 2). These values were compared with commercial detergent, linear alkylbenzene sulfonate, which CMC values were reported between 90-100 ppm. Surface tension was found to be decreased with increasing chain length of fatty acid in sophorolipid. Data presented in Table 1 and results shown in Fig. 2, reveals that C10 and C12 fatty acid containing sophorolipid had higher surface tension than the LAS. In general, the CMC value was highly influenced by chain length, whereas the C14, C16 and C18 showed almost similar CMC value and was calculated 40. The results are consistent with augmentation presents by Shin et al. [6]. However, the C14, C16 and C18 containing sophorolipids showed lowered S.T. than the LAS (Fig. 2, Table 1). Similar observations were reported by previous researchers [2,6,10]. There are many factors which also contribute to surface properties, but the chain length is the major one [2,4,6,10].

4. Foaming Power

Foamability is one of the major criteria for selection of a biosurfactant as detergent or emulsifier. Therefore, foaming properties of produced sophorolipids were compared with typical non-

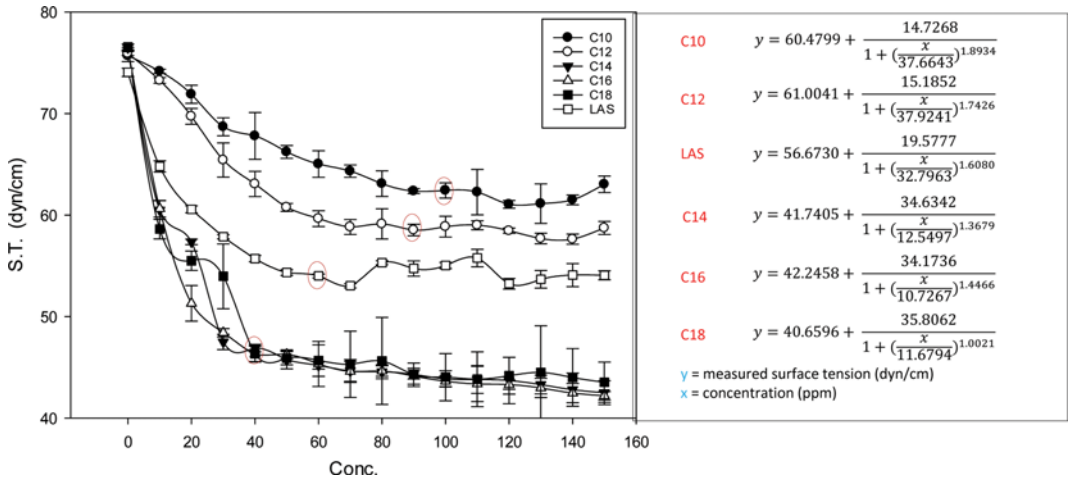

Fig. 2. Measurements of the surface tension of different sized fatty acid containing sophorolipids and calculation method for CMC values, using mentioned equation.

Table 1. Calculated CMC values for different fatty acids in compare to LAS

2nd Substrate	CMC (ppm)	Surface tension (dyne/cm)
LAS	100	46.2
C-10 oil	90	61.05
C-12 oil	60	57.52
C-14 oil	40	42.82
C-16 oil	40	41.64
C-18 oil	40	43.70

Table 2. The foamability of the sophorolipids compared to LAS and SA

Time (min)	The height of the bubble (cm)						
	LAS	SA	C-18	C-16	C-14	C-12	C-10
0	1.7	1.3	0.49	0.21	0.28	0.19	0.11
1	1.6	1.3	0.38	0.09	0.11	0	0
2	1.5	1.3	0.26	0.02	0.02	0	0
3	1.5	1.3	0.21	0.02	0.02	0	0
4	1.5	1.3	0.17	0.02	0.02	0	0
5	1.5	1.3	0.13	0	0	0	0

ionic chemical surfactants, i.e. linear alkylbenzene sulfonate and sugar ester, as shown in Table 2. The foamability of sophorolipid was found to be lower than the LAS and SA. Initial foaming heights, and their stability, both were found be xtremely lower (measured by the foaming heights after 5 min). The foaming properties of sophorolipids are reciprocal to the chain length [6]. The results from present study revealed that the C18 sophorolipid had highest foaming capacity among all sophorolipids (Table 2). The fraction of lactonic and acidic form of sophorolipid significantly affected the foamability; however, it requires further study.

5. Emulsifying Ability and Cleaning Power

Emulsification capacity is one of the major constraints while selecting a bio-surfactant as an emulsifier for the cosmetics or food industry [2]. Emulsification capacity of sophorolipids was measured against soybean oil (Fig. 3). The emulsification property of surface active molecule (surfactant) cannot determined by the surface tension alone; however, other factors like direct interaction of the hydrophobic moiety of the molecule with hydrophobic substrate/s also matter [1,2,10]. Results shown in Fig. 3(a)-(b), sug-

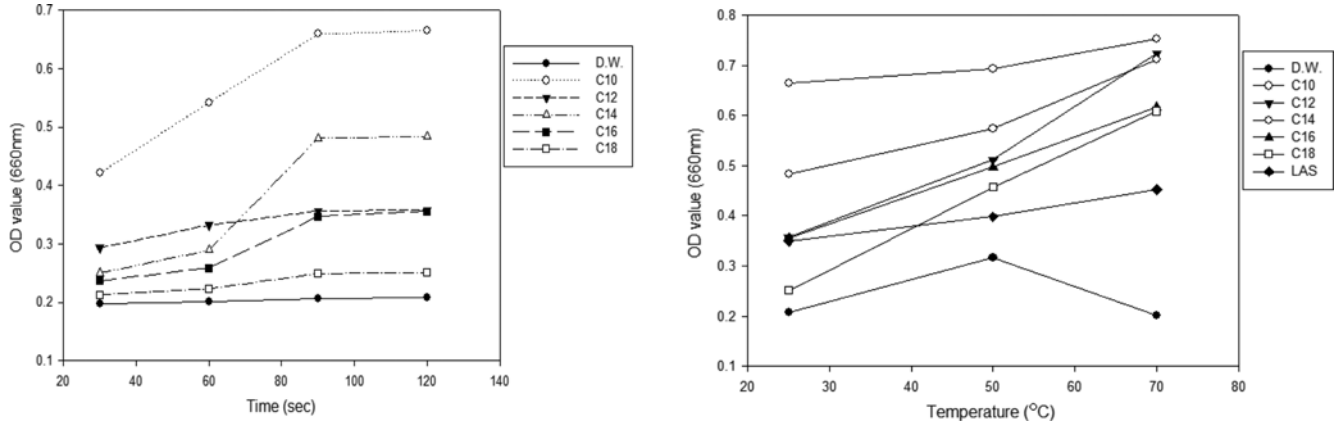

Fig. 3. Showing emulsifying ability of the sophorolipids in compare LAS (a) time dependent stability (b) effect of temperature.

gesting, a short chain fatty acid containing sophorolipid had better ability to emulsify. As shown in Fig. 3(a), the C10 sophorolipid had better emulsifying properties than the other sophorolipids. Thus, emulsification capacity of sophorolipid is a chain length dependent property and reciprocal to chain length. Suspension temperature was also studied, and results shown in Fig. 3(b); the elevation in temperature significantly increased the emulsifying ability of the sophorolipids (Fig. 3(b)). Temperature is always considered as an important factor for enhancement of the emulsification (however, not applicable to C10 sophorolipid). Probably, the C10 sophorolipid had attained maximum emulsifying ability at room temperature. It is anticipated these properties of C10 and C12 sophorolipid may be helpful to fix the petroleum-caused environmental problems.

The surface properties of any surfactant are important for selection of them for a specific application. The sophorolipids are nonionic and biodegradable surfactants; therefore, they may be good candidate for cleaning of semiconductor devices. In the present study, the detergency of sophorolipids was compared with that of linear alkylbenzene sulfonate and sugar ester, and results provided in Table 3. The cleaning ability (experimented with dirty cloth/fabric) of studied sophorolipids was recorded around 82%, i.e., no relation has been observed between chain length and detergency (Table 3). However, the cleaning ability was found to be chain length dependent with PCB electronic board washing. The C10 sophorolipid was found to be better than the LAS and SA in case of PCB electronic board.

6. Biodegradability

Biodegradability is one of the major advantages of bio-surfactant over chemical detergents. Therefore, the biodegradability pattern of sophorolipids with different chain length was examined at room temperature (25 °C) and 4 °C. The sophorolipid were incubated with Lake Water (Inha University) at respective temperature. Fig. 4(a) and (b) show the degradation pattern of the sophorolipid. To achieve complete degradation at room temperature ten days of incubation was sufficient (Fig. 4(a)). Whereas, at very low temperature the degradation was found to be very slow as well as not completed, even up to 25 days (Fig. 4(b)) and 50% of degradation was achieved within 15 days at 4 °C. Due to lowered microbial activity at low temperature, the degradation was extremely slow. These results showed that the biodegradation rate of sophorolipid is very significant at room temperature.

7. HPLC Analysis of Sophorolipid

The results from HPLC data of different chain sized sophorolipid produced in this study are shown in Fig. 5. For detection, a UV detector (195/210 nm) was used. Analysis was performed with peaks obtained from 210 nm wavelength. This wavelength is lipid specific, and based on "end absorption" due to carboxyl, carbonyl and acyl groups the sophorolipids were analyzed [11]. Commercially purchased acid and lactone form of sophorolipid (procured from Bioland, Korea) were used as control (Fig. 5(f), (g)). The sophorolipids produced by fermentation using various methylated fatty acid (Capric acid (10:0), Lauric acid (12:0), Myristic acid (14:0), Palmitic acid (16:0), Stearic acid (18:0)), were compared. Analysis of the sophorolipid by HPLC and data was similar as mentioned in previous reports, i.e., peaks between 4 and 7 min for the acidic sophorolipid, while 10 to 20 min as lactonic sophorolipid [12-14]. The peaks were compared with control as well as the data from the literature [2,4,6,12-14]. The lactonic form was observed in C16 and C18 fatty acid chained sophorolipid. However,

Table 3. Cleaning ability of sophorolipids on dirty Cloth and PCB electronic board

	Water	LAS	SA	C-18	C-16	C-14	C-12	C-10
					With fabric			
C (%)	76.66	85.88	82.92	82.17	82.41	82.30	82.22	82.94
					PCB electronic board			
C (%)	14.06	36.99	34.78	55.26	58.62	68.85	56.25	79.41

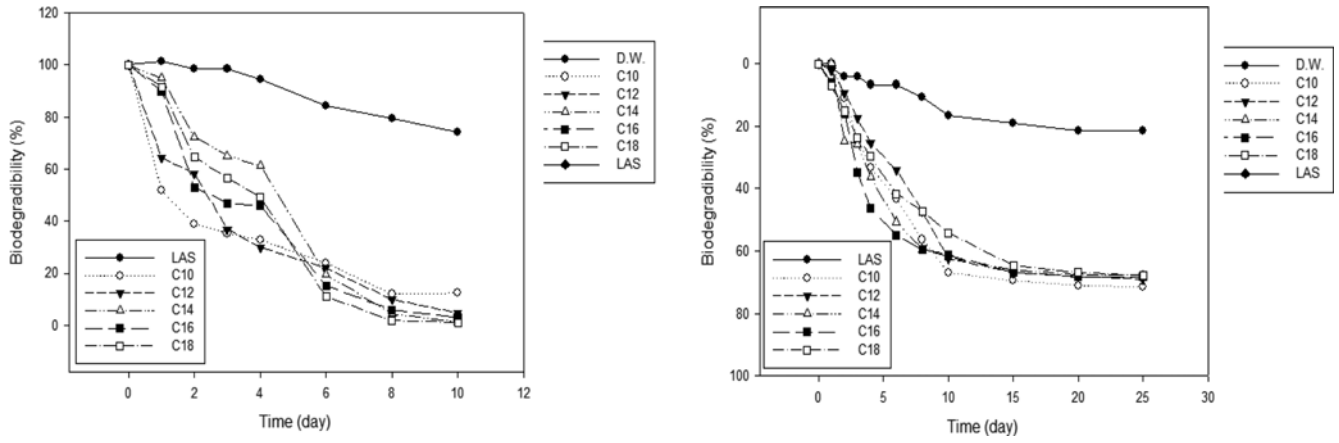

Fig. 4. Biodegradability of sophorolipid at (a) room temperature (25 °C) and (b) 4 °C.

Table 4. HPLC analysis of sophorolipid produced by different sized fatty acid

Sample (chain length)	Number of peak	Ret. time (min)	Area (mAU*sec)	Height	Area %
C10	1	12.618	632393	39609	100.00
C12	1	6.686	3272454	59232	96.14
	2	27.411	131289	3899	3.86
C14	1	0.727	3958	327	0.12
	2	0.917	2409	383	0.17
	3	1.070	8046	742	0.36
	4	1.897	897877	35379	53.23
C16	1	17.582	65509	2480	39.51
	2	19.133	72161	2563	43.52
	3	23.526	28117	779	16.90
C18	1	23.513	25125	800	100.00
Control (acid) (1.0 mg/ml)	1	10.224	43764	2602	100
Control (lactone) (1.0 mg/ml)	1	8.837	57136	1127	6.340
	2	10.076	3463	173	0.384
	3	12.629	12576	453	1.396
	4	14.184	17498	580	1.942

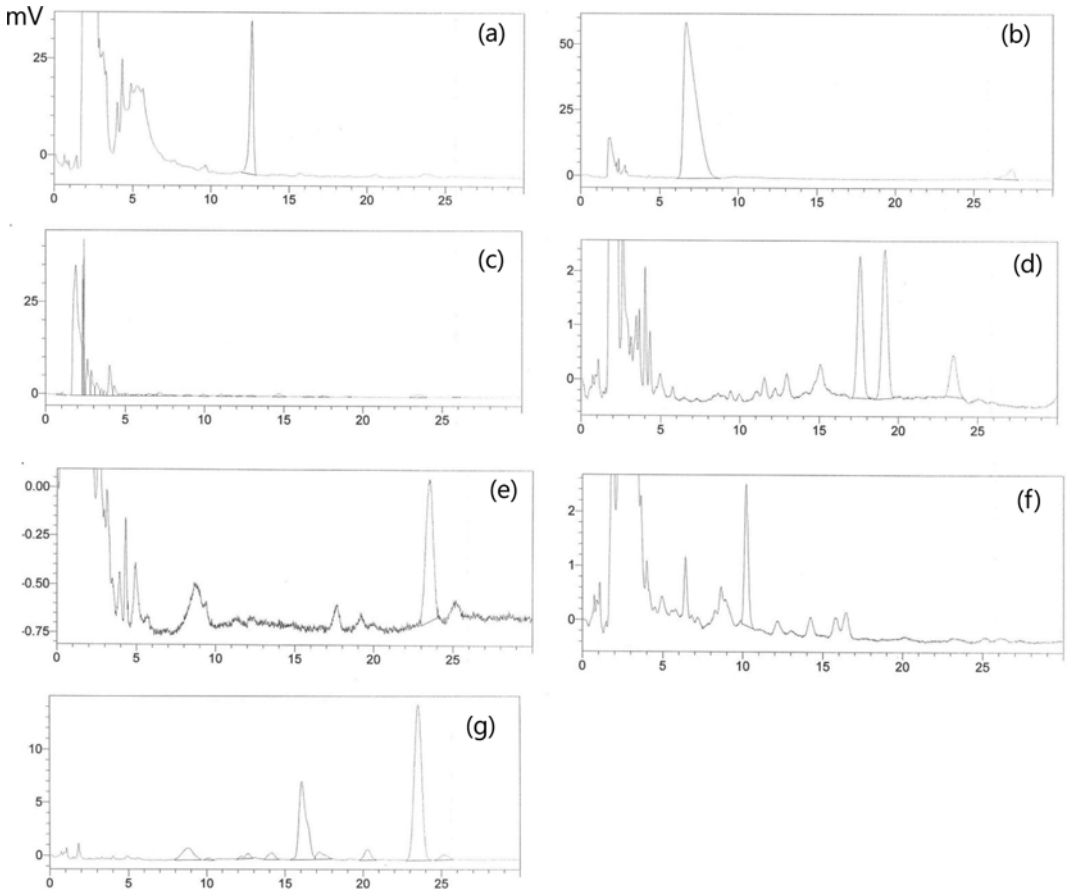

Fig. 5. HPLC analysis of produced sophorolipid using different sized fatty acid (a) C10, (b) C12, (c) C14, (d) C16 and (e) C18; control (commercial) (f) acid form sophorolipid (g) Lactone form Sophorolipid.

the sophorolipid with short chain length fatty acid had only acidic form; no lactonic form was observed (Fig. 5). Probably, the chain length from C10 to C14 fails to form a lactonic ring, or least stable structure; thus, only acidic form was recorded.

CONCLUSION

Production and characterization of different chain sized fatty acid derived sophorolipid in a batch culture, with a significant yield (especially low molecular weight fatty acid is a huge challenge) demonstrated that a single type of sophorolipid production is possible with C10-C14. This study was mainly focused on chain length variation to tune surface properties of sophorolipid, and production of short chained sophorolipid. The surface active properties of the sophorolipid synthesized from C10, C12, and C14, provide a better alternative to chemical or petroleum based surfactants. The production yield seems to be lower, 54 g/L; however, in comparison to secondary alcohol used for the production of short chain sophorolipid, the cost of this process is very effective. This method also allows a selective production of acid form of sophorolipid with short chained fatty acid, which can be applied in several industrial applications, especially for cleaning of PCB-electronic boards and cosmetic emulsifiers.

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