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Low toxicity and high surface activity of sophorolipids from *Starmerella bombicola* in aquatic species: A preliminary study

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Authors Info

W. Kumano, K. Namakoshi, M. Araki, Y. Oda, A. Ueda, and Y. Hirata*

Biochemical Laboratory,
Saraya Co. Ltd.,
Osaka- 582-0028, Japan

***Corresponding Author Email :** hirata@saraya.com

Edited by

Dr. V.K. Tiwari

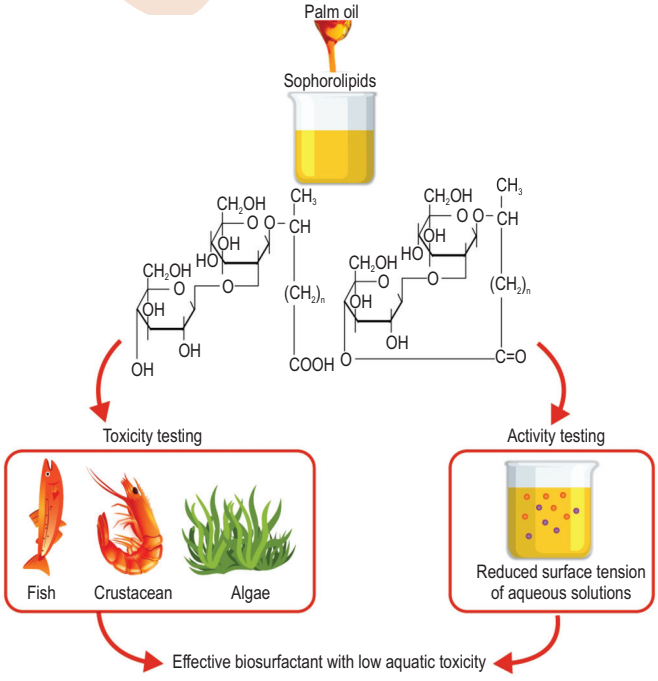
Reviewed by

Dr. S.K. Chakraborty
Dr. R.B. Raizada

Abstract

Aim : This study was carried out to evaluate aquatic toxicity and surface activity of sophorolipids extracted from yeast *Starmerella bombicola.*

Methodology : The half maximal effective concentration (EC_{50}) values of sophorolipids were determined in three aquatic species, and the surface activity of sophorolipids in aqueous solutions was examined at EC_{50} concentration.



Results : The EC_{50} of sophorolipid surfactant >473 mg l^{-1} was found in alga *Pseudokirchneriella subcapitata,* a median lethal concentration (LC_{50}) of 64.8 mg l^{-1} in *fish Oryzias latipes*, whereas a high EC_{50} of 48.2 mg l^{-1} was noted in crustacean *Daphnia magna,* respectively. Sophorolipids effectively reduced the aquatic surface tension to 39 mN m^{-1} in *O. latipes* and to 41 mN m^{-1} in *D. magna*.

Interpretation : These results show that sophorolipids from *S. bombicola* are a promising biosurfactant with an ideal balance of low aquatic toxicity and high surface activity.

Key words: Aquatic toxicity, Biosurfactant, Sophorolipid, Surfactant

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Introduction

Surface active agents or surfactants are amphiphilic compounds that lower surface and interfacial tensions, thereby making them useful as detergents or emulsifiers with a wide range of residential, commercial, medical laundering and sanitation (Scheibel, 2004), cosmetic (Falbe, 2012) and drug delivery (Torchilin, 2001) applications. However, given the ability of surfactant to reduce surface tension, they can also have significant toxicity towards aquatic life. Oya *et al.* (2007) reported that the surface tensions associated with half-maximal toxicity of several chemical surfactants ranged from 45 mN m^{-1} to 70 mN m^{-1}, depending on the surfactant examined. Biosurfactants are surface-active amphiphilic compounds produced by micro-organisms that contain both hydrophilic and hydrophobic regions that enable them to aggregate at the interfaces between fluids with different polarities, such as hydrocarbons and water, thereby decreasing the interfacial surface tension (Vijayakumar and Saravanan, 2015). Recently, there has been a growing interest in the identification and optimization of biosurfactants produced by yeast, bacteria and fungi from substrates such as sugar beet molasses (Onbasli and Aslim, 2008), oils (Kumar *et al.,* 2014), waste (Santos *et al.,* 2016) and other rhizospheric/phyloplanic sources (Tomar *et al.,* 2013).

In addition to various classes of biosurfactants, such as glycolipids, lipoproteins, lipopeptides and phospholipids, produced by different micro-organisms, sophorolipids (Gorin *et al.,* 1961) have recently become a focus of attention. Sophorolipids, along with rhamnolipids and trehalolipids, are classified as glycolipids (Jarvis and Johnson, 1949) and are known to occur as a mixture of lactonic and acidic molecules (Tulloch *et al.,* 1968; Hu and Ju, 2001). Sophorolipids are low-foaming biosurfactants that are readily biodegradable and have low levels of cytotoxicity (Hirata *et al.,* 2009), thus making them potential candidates for laundering and bioremediation.

Production of sophorolipids by non-pathogenic yeast *Starmerella bombicola* has been the focus of many optimization efforts and has been examined using a variety of culture substrates, including carbohydrates, vegetable oils (such as sustainable palm oil), animal fat, whey, soy molasses and waste frying oil (Van Bogaert *et al.,* 2007). Although, a large number of studies have been conducted on the production and molecular characterization of sophorolipids produced by *S. bombicola* (Shah *et al.,* 2017; Van Renterghem *et al.,* 2018; Li *et al.,* 2016), however these compounds have not been extensively investigated as other glycolipids (De Graeve *et al.,* 2018). Only few studies have examined the toxicity of these sophorolipids to aquatic life in conjunction with evaluation of their surfactant properties, which is of ecological significance.

Given that there is a trade-off relationship between the surface activity of a surfactant and aquatic toxicity, the present study was carried out to evaluate toxicity and surface activity of sophorolipids extracted from *S. bombicola.* Toxicity of these sophorolipids were assessed against three aquatic species, *Pseudokirchneriella subcapitata,* an alga *Oryzias latipes*, a fish and *Daphnia magna, a* crustacean.

Materials and Methods

Preparation of sophorolipids from *S. bombicola:* Sophorolipids evaluated in this study were produced by *S. bombicola* ATCC22214 grown in a medium comprising 10% glucose, 8% palm oil [certified by the Roundtable on Sustainable Palm Oil (https://rspo.org/)], 0.8% KH_2PO_4, 0.2% $MgSO_4$, 0.1% urea and 0.2% yeast extract. Sophorolipids were purified as described by Cooper and Paddock (1984). Briefly, a 3 l batch was fermented in a 5 l jar fermenter. Fermentation was carried out for 12 days at 30°C and 600 rpm, with an air flow rate of 0.6 vvm. Sophorolipids were produced as a mixture of 1', 4''-lactone and free acid form in a ratio of approximately 7:3.

Preparation of hard water: Hard water for toxicity and surface tension tests was prepared in accordance with the AOAC International procedure for synthetic hard water (Beloian, 1995). Briefly, a 4 ml of Solution II (56.03 g $NaHCO_3$ made up to 1l with distilled water) was added to 1 ml of Solution I (67.71 g $MgCl_2.6H_2O$ and 73.99 g of $CaCl_2$ made up to 1 l with distilled water). The volume was then made up to 100 ml distilled water. This solution was prepared to have a hardness of 1000 ppm and was diluted with distilled water before use.

Toxicity tests

Algal growth inhibition test: *P. subcapitata* (ATCC No.: 22662) was maintained in media in accordance with the Organisation for Economic Co-operation and Development (OECD) guidelines for Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006). The algae were exposed to different concentrations of sophorolipids (0, 100, 200, 300, 400 and 500 mg l^{-1}) for 72 hr, and thereafter algal biomass was determined using a hemocytometer. The EC_{50} (mg l^{-1}) of *P. subcapitata* was compared with the established reference data for determining the acute toxicity and genotoxicity of five selected anionic and nonionic surfactants described by Liwarska-Bizukojc (2005).

Fish toxicity test: *O. latipes* were obtained from the National Institute of Environmental Research (Environmental Research Complex, Incheon, Korea). They were bred in accordance with OECD guidelines for Fish, Acute Toxicity Test (1992) at 23 ± 2°C under 16 hr light and 8 hr dark photoperiod, with illumination being provided by artificial lighting. Prior to the initiation of experimentation, fish without any visible gross abnormalities were selected and acclimated for 10 days. During the acclimation period, the water temperature and dissolved oxygen concentration were maintained at 21.6–23.2°C and 90.0%-105%, respectively. Following acclimation, 10 fish were randomly selected and placed in a test chamber containing 3 l of one of the six sophorolipid solutions in water (0, 100, 200, 300, 400 or 500 mg l^{-1}) at 23 ± 2°C, with a dissolved oxygen level of at least 60%, and pH 6.0–8.5. Temperature and oxygen levels were maintained

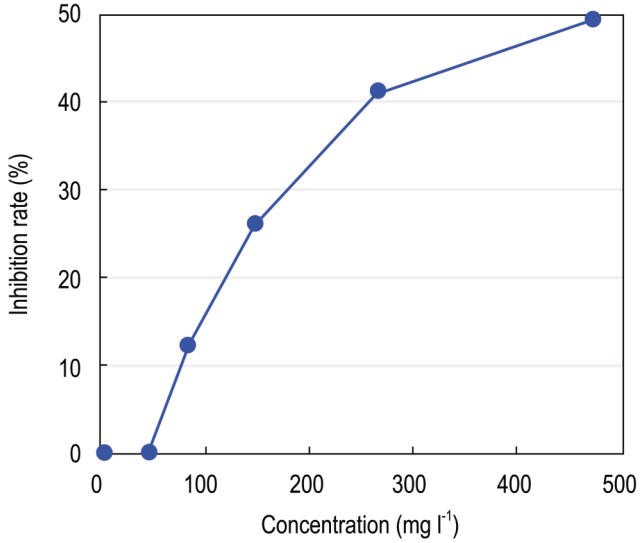

Fig. 1: Toxicity of sophorolipids on the growth of *Pseudokirchneriella subcapitata*: The algae were exposed to several different concentrations of sophorolipid and the resulting growth inhibition rates were calculated.

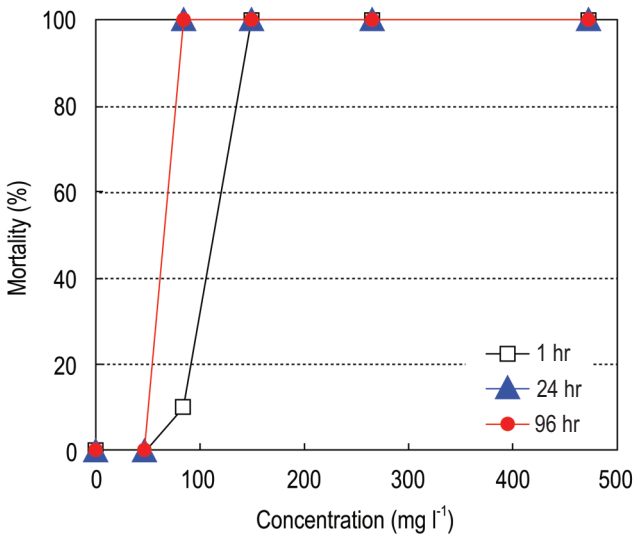

Fig. 2: Acute toxicity of sophorolipids towards *Oryzias latipes*. The fish were exposed to several different concentrations of sophorolipid and the resulting percentage mortalities were calculated.

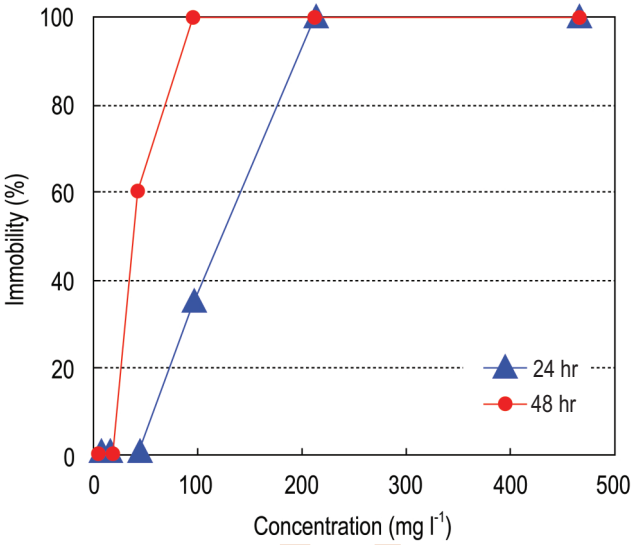

Fig. 3: Acute toxicity of sophorolipids towards *Daphnia magna*. The crustaceans were exposed to several different concentrations of sophorolipid and the resulting percentage mortalities were calculated.

at a constant level throughout the course of the exposure period. The room was artificially illuminated with fluorescent lighting on 16 hr light and 8 hr dark cycle. Fish were observed for abnormalities at 1, 3, 6, 24, 48, 72 and 96 hr after the initiation of exposure. Upon discovery, any dead fish were removed from the chamber and mortalities were recorded.

Crustacean immobilization test: *D. magna* were obtained from the National Institute of Agricultural Science & Technology (Gyeonggi-do, Korea) and treated in accordance with OECD guidelines for *Daphnia* sp. Acute Immobilisation Test (2004). Young daphnids, less than 24 hr old, were obtained from mature (>14-days-old) third-generation daphnids and were placed in a culture beaker containing Elendt M4 medium, in which they were maintained for 28 days. The culture beaker was maintained at 20 ± 1°C in a water bath under 16 hr light and 8 hr dark cycle. After preparation, the pH and hardness of water were 7.69–7.86 and 250 mg $CaCO_3$ l^{-1}, respectively.

During culture period, *Daphnia* were observed once daily for abnormal behavior, antennal movement, appearance, neonate production and ephippia. In addition, five daphnids were placed in 100 ml of diluted sophorolipid solution (0, 100, 200, 300, 400 or 500 mg l^{-1}) contained in test dishes in a water bath (20 ± 1°C), at a dissolved oxygen level of not less than 3 mg l^{-1} and pH 6–9, and exposed for 48-hr under a continuous 16 hr light and 8 hr dark cycle. During the exposure period, the daphnids remained fed, and at 24 and 48 hr were observed for general appearance, behavior, swimming or immobilization and abnormalities. Immobilization was considered as no movement or movement of antennae (posterior abdomen), however, failure to float or swim for approximately 15 sec after dishes had been gently shaken. Daphnids lying on their sides were considered to be immobile.

Surface tension measurements for determination of surface activity: Surface tension was measured as described by Hirata *et al.* (2009). Briefly, surface tension was measured at 20°C using a CBVP-Z Tensiometer (Kyowa Interface Science, Niiza City,

Saitama, Japan) according to the method described by Wilhelmy (1863) using solutions of pH 7.0 and the indicated water hardness and concentration of sophorolipids. This study was reviewed and approved by the IACUC of Biotoxtech Co., Ltd. based on the Animal Protection Act (Enactment May 31, 1991, No. 4379; Revision February 29, 2008, No. 8852) (Approval No.: 09765).

Statistical Analyses: One-way analysis of variance (ANOVA) was employed to analyze homogeneous data. If ANOVA results were significant, they were assessed using Dunnett's test for multiple comparisons. The trimmed Spearman–Karber method was used for estimating median lethal concentrations in toxicity bioassays, and the moving average-angle method was used to calculate LD_{50} values. Statistical analyses were performed using SAS software (version 9.1.3; SAS Institute Inc., Cary, NC, U.S.A.).

Results and Discussion

Toxicity tests

Toxicity of sophorolipids on the growth *P. subcapitata*: Unicellular alga, *P. subcapitata* is routinely used in chemical toxicity studies (Brezovšek *et al.,* 2014; de Melo *et al.,* 2013). To assess the toxicity of sophorolipids towards this species, the algae were exposed to several different concentrations of these glycolipids and the resulting growth inhibition rate was calculated. No observable effects on algal growth were detected at or below a sophorolipid concentration of 47.3 mg l^{-1} (Fig. 1) whereas exposure to a concentration of 83.7 mg l^{-1} resulted in a small degree (12.4%) of growth inhibition. Increasing the concentration to 149 mg l^{-1} and 266 mg l^{-1} increased the growth inhibition to 26.1% and 41.2%, respectively; however, even after 72 hr of exposure to the highest concentration of sophorolipids tested (473 mg l^{-1}) inhibition of average specific growth rate remained below 50%. This half-maximal effective concentration (EC_{50}) of >473 mg l^{-1} is considerably higher than those reported by Liwarska-Bizukojc *et al.* (2005) for *P. subcapitata* inhibition by common chemical surfactants (Table 1). Indeed, the EC_{50} of sophorolipid was more than an order of magnitude higher than that of sodium dodecyl sulfate and alcohol ethoxylate, and more than four times higher than that of linear alkylbenzene sulfonate. These results indicate that compared to common chemical surfactants sophorolipids are biosurfactants with very low toxicity towards aquatic algae. The results of the current study are comparable with those previously reported for glycolipids, which were found to have high EC_{50} values for various algal species (Poremba *et al.,* 1991; Invally *et al.,* 2017; De Oliveira *et al.,* 2017). Hence, it can be concluded that if sophorolipids exhibit low toxicity towards other organisms and result in effective surface tension at or below their EC_{50} concentrations, they would be an ideal biosurfactant for development and commercialization.

Acute toxicity of sophorolipids towards *O. latipes*: To further investigate the potential applicability of sophorolipids as biosurfactants, the concentration-dependent effects of sophorolipids on the viability of Japanese rice fish, *O. latipes* were examined. Similar to *P. subcapitata* growth inhibition results, it was observed that sophorolipid concentrations up to 47.3 mg l^{-1} had no measurable effect on the viability of *O. latipes,* even after 96 hr exposure (Fig. 2). The effects of sophorolipids on *O. latipes* viability were partially dependent on exposure time, increasing noticeably from 1 - 24 hr whereas exposure for 24 - 96 hr had no measurable effect on the viability of these fish. Plotting mortality rates after 96 hr revealed that the median lethal concentration (LC_{50}) of sophorolipids towards this species was 64.8 mg l^{-1} (95% confidence limits: 59.6–72.4 mg l^{-1}). These results are broadly consistent with the findings of previous studies that had evaluated the toxicity of biosurfactants against various marine fish and recorded survival rates between 70% and 95% (Santos *et al.,* 2017; Saeki *et al.,* 2009).

Acute toxicity of sophorolipids towards *D. magna*: When toxicity of sophorolipids towards *D. magna,* a planktonic crustacean commonly used in ecotoxicology studies, was examined, no observable effects on swimming ability were detected at concentrations below 20.3 mg l^{-1}. This result is more favorable compared with that obtained for rhamnolipid, the LC_{50} of which was found to be 13.8 mg l^{-1} (Santa Anna *et al.,* 2002), and for sophorolipids derived from *Candida bombicola,* the LC_{50} of which was 15 mg l^{-1} (Otto *et al.,* 1999). At concentrations greater than

Table 1: Comparison of the toxicities of sophorolipds from *Starmerella bombicola* and anionic/nonionic surfactants toward *Pseudokirchneriella subcapitata*

Surfactant	EC_{50} (mgl^{-1})	Water Hardness (ppm)
Sophorolipids	>473 (72 hr)	24
Sodium dodecyl sulfate	37 (72 hr)[1]	24[1]
Linear alkylbenzene sulfonate	112 (72 hr)[1]	24[1]
Alcohol ethoxylates	7 (72 hr)[1]	24[1]

[1]These are the results of toxicity bioassays for *P. subcapitatae* xposed to selected anionic and nonionic surface-active agents reported by Liwarska-Bizukojc *et al.* (2005) and performed in accordance with the OECD Guidelines for Testing of Chemicals, Sect. 2: Effects on Biotic Systems, No. 201 "Alga, Growth Inhibition Test." Adopted June 1984. Paris: OECD (1981). A revised version of the same OECD guidelines (2006) was followed in the present study for the algal toxicity test

Table 2: Interfacial activity of sophorolipids at $EC(LC)_{50}$

		Toxicity test	Surface tension test
Species	**Water hardness**	**Measured EC $(LC)_{50}$ of sophorolipids (mgl^{-1})**	**γ_{tox}**
Oryzias latipes	61 ppm	65	39
Daphnia magna	250 ppm	48	41

The surface tension of aqueous solutions containing the indicated $EC(LC)_{50}$ concentration of sophorolipids was measured using a tensiometer as described in the Materials and Methods section. The surface tension values presented in this table are averages of six independent measurements. $EC(LC)_{50}$ = half-maximal (median lethal) concentration. γtox = surface tension at $EC(LC)_{50}$

44.5 mg l^{-1}, the effects of sophorolipids on *D. magna* swimming ability were found to be dependent on exposure time, increasing from 24 to 48 hr of exposure (Fig. 3). Quantification and graphical plotting of inhibition of *D. magna* swimming in response to a range of sophorolipid concentrations revealed that after exposure to sophorolipids for 48 hr, the EC_{50} was 48.2 mg l^{-1} (95% confidence limits: 40.6–57.2 mg l^{-1}), which was similar to the LC_{50} observed after 24 and 96 hr in *O. latipes* mortality test (Fig. 2). Taken together, the results of algal growth, crustacean mortality and fish immobility bioassays enabled identification of a range of concentrations at which sophorolipids exhibit low toxicity towards several aquatic species. Nevertheless, higher sophorolipid toxicity was detected in the crustacean mortality test compared with the algal growth test, and there was a small additional increase in toxicity in fish immobility test. These findings are probably related, at least in part, to the species used and nature of each toxicity test.

Surface activities of sophorolipids at $EC(LC)_{50}$: In the present study, we determined the surface tension of aqueous solutions of sophorolipids at median toxicity values (toxic surface tension, or γ_{tox}). As water hardness is known to affect both interfacial activity and toxicity of surfactants, a surface tension test was performed using the same water hardness conditions that were used for the toxicity tests, the results of which are summarized in Table 2. At LC_{50} towards *O. latipes* (65 mg l^{-1}), sophorolipids reduced the surface tension of aqueous solution to 39 mN m^{-1}. Similarly, at EC_{50} towards *D. magna* (48 mg l^{-1}), sophorolipids reduced the surface tension to 41 mN m^{-1}. Although the aquatic species, water hardness and median toxicity concentration of sophorolipids differed between these two toxicity tests, the γ_{tox} remained unchanged. Moreover, these γ_{tox} values for sophorolipids were lower than any of the γ_{tox} values reported for chemical surfactants in *O. latipes* and *D. magna* toxicity studies conducted by Oya *et al.* (2007). Furthermore, the results obtained in the present study are more favorable compared with the previously reported surface tensions of 32.1–34.2mN m^{-1} for sophorolipids (Develter *et al.,* 2010) and 37 mN m^{-1} for rhamnolipids (Cheng *et al.,* 2017).

The purpose of this research work was to gain insights into the efficacy of sophorolipids which have potential utility as environmental friendly low-toxicity biosurfactants. The data obtained in the current study indicate that sophorolipids have low aquatic toxicity and good interfacial activity, thus satisfying two important criteria for an ideal surfactant. Herein, it is demonstrated that sophorolipid biosurfactant produced using sustainable palm oil as a carbon source is an effective surfactant at its $EC(LC)_{50}$, possibly even more so than the commonly used chemical surfactants. Hence, the current findings have significant implications for global laundering, sanitization and environmental remediation applications. This study sets a precedent for the evaluation of other biosurfactants that may possess a similarly desirable profile of efficacy versus toxicity.

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