

# Production of Sophorolipids from Non-edible Jatropha Oil by *Stamerella bombicola* NBRC 10243 and Evaluation of their Interfacial Properties

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**Abstract:** To facilitate the development of bio-based chemicals from renewable and inexpensive natural resources, we sought to produce biosurfactants using non-edible jatropha oil. Twenty yeasts known to produce biosurfactants were tested in this study, and *Stamerella bombicola* NBRC 10243 was found to use jatropha oil efficiently to produce sophorolipids (SLs) as a mixture of lactone-form SL (L-SL) and acid-form SL (A-SL). Under culture conditions using rice bran as the source of organic nutrients, the yield of SLs reached 122.6 g/L in 5-L jar fermentors after 9 d in culture. HPLC analysis of the culture medium showed that the levels of phorbol esters (PEs), major toxic components of the oil, decreased markedly with an increase in culture time, suggesting that the yeast degrades PEs. Although the SLs obtained by solvent extraction of the culture medium contained a small amount of PEs, the sodium salt of A-SL (A-SL-Na) obtained by alkaline treatment (5N NaOH, 80°C) showed no PE peaks upon HPLC analysis. A-SL-Na had excellent surface activity with low CMC ( $9.0 \times 10^{-4}$  M) and  $\gamma_{\text{CMC}}$  (29.6 mN/m), which are lower than that of sodium dodecyl sulfate (SDS). The solubilizing ability of A-SL-Na toward for octanoic acid ([octanoic acid]/[A-SL-Na]) was found to be 2.0, which is half that of SDS. Our findings should help improve SL production from non-edible feedstock and broaden the use of promising bio-based surfactants.

**Key words:** biosurfactant, sophorolipid, jatropha oil, phorbol esters, *Stamerella bombicola*

## 1 INTRODUCTION

Biosurfactants (BSs) are surface-active compounds that are produced abundantly by a variety of microorganisms. BSs have received considerable attention because of their unique properties, which include higher biodegradability, lower toxicity, and more versatile biological functionality than established petroleum-based surfactants<sup>1-3</sup>. Because BSs are produced from renewable resources such as vegetable oils<sup>4-7</sup> and carbohydrates<sup>8,9</sup>, they are expected to be environmentally friendly as well as functional materials that contribute to the development of a sustainable society. However, producing BSs from edible vegetable oils is undesirable because that competes with the use of edible oils in the food supply chain and makes market prices of the oils

volatile.

Jatropha is a drought-resistant shrub or tree that is distributed widely in the wild and in semi-cultivated areas in India, South East Asia, Africa, and Central and South America<sup>10</sup>. Jatropha's seed kernels contain more than 60% oil with a fatty acid composition similar to that of common edible oils<sup>11,12</sup>, and jatropha oil has been proposed as a low-cost feedstock for biodiesel production. Nutritional uses of jatropha oil are limited because the oil contains the toxin curcin (a 28.2 kDa protein) as well as phorbol esters (PEs), which are known to promote tumors<sup>13</sup>. Although curcin becomes nontoxic when denatured by heating, PE persists in oil fractions. Therefore, techniques to remove or degrade PEs must be developed if jatropha oil is to be used

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widely.

Mannosylerythritol lipid (MEL)<sup>2, 14, 15</sup>, cellobiose lipid (CL)<sup>16</sup>, and sophorose lipid (SL)<sup>5-7</sup> are BSs that are well known for their productivity and versatile functions. SLs, in particular, are produced in large amounts from diverse carbon sources by yeasts such as *Stamerella bombicola*<sup>17</sup> and *Candida batistae*<sup>18</sup>. SLs are commonly a mixture of lactone-form SL (L-SL) and acid-form SL (A-SL) (Fig. 1) that can be obtained easily from culture media by precipitation<sup>19</sup>. Moreover, SLs and their derivatives have antimicrobial<sup>20</sup>, anticancer<sup>21</sup>, and antiviral<sup>22</sup> activities, and therefore the cosmetic, food, and detergent industries are increasingly interested in using SLs.

In light of the above findings, we focused our attention on producing BSs from the non-edible jatropha oil. Recently, Wadekar et al. reported the production of SLs from jatropha oil by *S. bombicola*<sup>23</sup>, but the yield was low (6.0 g/L) and the amount of PEs in the culture medium or final products was not clarified. In this study, we examined several yeasts known to produce BSs for their ability to use jatropha oil, and then investigated the culture conditions (specially, the organic nutrient sources) that are optimal for *S. bombicola* NBRC 10243 to produce SLs. We also purified the SLs and evaluated the interfacial properties of L-SL, A-SL, and the sodium salt of A-SL (A-SL-Na). This is the first report on the efficient production of BSs from a non-edible feedstock.

## 2 EXPERIMENTAL

### 2.1 Microorganisms

Twenty yeasts known to produce CL, MEL, and SL were used in this study. *Pseudozyma parantarctica* JCM 11752, *P. aphidis* JCM 10318, *P. rugulosa* JCM 10323, *P. antarctica* JCM 10317, *P. tsukubaensis* JCM 16987, and *Cryptococcus humicola* JCM 1461 were obtained from RIKEN BioResource Center (Saitama, Japan). *P. tsukubaensis* NBRC 1940, *Starmellera bombicola* NBRC 10243, *Ustilago maydis* NBRC 5346, *U. cynodontis* NBRC 9758, and *U. scitaminea* NBRC 32730 were obtained from the

National Institute of Technology and Evaluation of Japan (Chiba, Japan). *P. hubeiensis* CBS 10070, *P. graminicola* CBS 10092, *P. crassa* CBS 9959, *P. shanxiensis* CBS 10075, *P. siamensis* CBS 9960, and *Candida batistae* CBS 8550 were obtained from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). *P. antarctica* T-34<sup>24</sup>, *P. hubeiensis* KM59<sup>25</sup>, and *P. churashimaensis* OK96<sup>26</sup> were from our laboratory stock.

### 2.2 Screening of BS-producing yeasts using jatropha oil

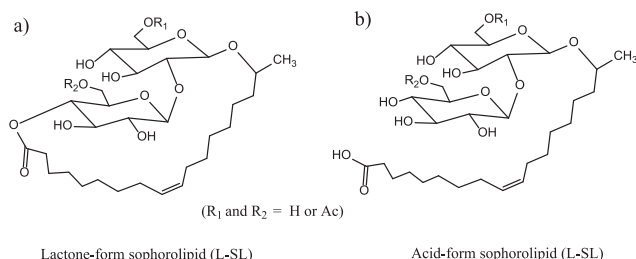
Seed cultures were prepared by inoculating cells grown on slants into test tubes containing YM medium [1% (w/v) glucose, 0.3% peptone, 0.3% yeast extract, 0.3% malt extract, and distilled water] and placing the test tubes on a reciprocal shaker at 200 strokes/min for 2 d at 25°C. Seed cultures (1 mL) were transferred to 200-mL Erlenmeyer flasks containing 20 mL of fermentation medium (1) [2% (w/v) Jatropha oil, 0.3% NaNO<sub>3</sub>, 0.03% MgSO<sub>4</sub>, 0.03% KH<sub>2</sub>PO<sub>4</sub>, and 0.1% yeast extract] or medium (2) [2% (w/v) Jatropha oil, 1% (w/v) glucose, 0.3% peptone, 0.3% yeast extract, and 0.3% malt extract], and then incubated at 25°C on a rotary-shaker (200 rpm) for 7 d. Commercial jatropha oil (produced from *Jatropha curcas* L.) was kindly supplied by Allied Carbon Solutions Co., Ltd (Tokyo, Japan).

The products synthesized by yeasts were extracted from the culture medium and analyzed by thin-layer chromatography (TLC) on silica plates (Silica gel 60F; Wako) with a solvent system consisting of chloroform/ethanol/7N ammonium hydroxide (65:15:2 by vol.). The compounds on the plates were located by charring at 110°C for 5 min after spraying an anthrone/sulfuric acid reagent as described<sup>27</sup>. The standards used were an SL mixture and an MEL mixture produced from olive oil by *S. bombicola* NBRC 10243<sup>28</sup> and *P. antarctica* T-34<sup>29</sup>, respectively, and a CL mixture produced from glucose by *C. humicola* JCM1416<sup>30</sup>.

### 2.3 Production of SLs using jatropha oil

For large-scale production of SLs, *S. bombicola* NBRC 10243 was cultivated in a 5-L jar fermentor (MDL-N.5L, Marubishi, Tokyo, Japan) at 25°C, 500 rpm, and 2.5 vvm of aeration for 7 d using fermentation medium (3) [10% (w/v) Jatropha oil, 5% (w/v) glucose, 0.3% yeast extract, 0.1% urea, 0.05% MgSO<sub>4</sub>-7 H<sub>2</sub>O, 0.01% NaCl, and 0.1% KH<sub>2</sub>PO<sub>4</sub>], unless otherwise indicated. Rice and wheat bran were kindly supplied by Allied Carbon Solutions Co., Ltd. When rice or wheat bran was used instead of yeast extract, the jatropha oil was the only carbon source.

The mixture of SLs produced in the culture medium was extracted with an equal volume of ethyl acetate. The organic layer was separated and evaporated. The resulting yellow oil (50 g) was dissolved in chloroform, and loaded on a column (5 × 40 cm) and chromatographed with a close



**Fig. 1** Structures of lactone-form sophorolipid (L-SL) and acid-form sophorolipid (A-SL). (a) L-SL (b) A-SL.

gradient elution of chloroform-acetone (10:0 to 0:10).

#### 2.4 Alkaline hydrolysis of SLs

The SL mixture was subjected to alkaline hydrolysis to open the lactone ring and to obtain the sodium salt of A-SL. SLs (1.0 g) were dissolved in 0.5 mL of 5N NaOH aq. and placed in a test tube, which was sealed tightly with a screw cap before heating (80°C) in a water-bath for 1 h. After cooling, the reaction medium was acidified to pH 1.0 by adding aqueous HCl drop-wise, and then centrifuged (9000 rpm, 5 min). The upper layer was removed from the centrifuged sample, and the lower layer containing the A-SL was washed with water 3 times. The A-SL fraction was neutralized by adding 3N NaOH to generate the sodium salt (A-SL-Na), which was freeze-dried for 3 d.

#### 2.5 Analysis of PEs

PE amounts in the culture medium were monitored and quantified using high-performance liquid chromatography (HPLC)<sup>31)</sup>. Aliquots were loaded into an HPLC system equipped with the reversed-phase column LiChrospher 100 RP-18 (5 µm, 250 × 4 mm id, Merck) and separated at 25°C at a flow rate of 1.3 mL/min using an acetonitrile-water gradient elution buffer. Four PE peaks, detected by absorbance at 280 nm, appeared at retention times between 43.3 min and 46.5 min; phorbol-12-myristate 13-acetate (TPA; Sigma, St. Louis, MO), used as an external standard, appeared at 52.1 min. The areas of the 4 peaks were summed and converted to TPA equivalents based on the peak area and concentration of TPA.

#### 2.6 Coupled TLC and MALDI-TOF MS analysis<sup>32)</sup>

Coupled TLC and MALDI-TOF MS analysis was performed using aluminum-backed 50 × 75 mm TLC Silica gel 60 F<sub>254</sub> plates (200-µm layer thickness, Merck) mounted onto a TLC-MALDI adapter target (Bruker Daltonics). Matrix was added manually for TLC-MALDI imaging by using the manufacturer-recommended dip-coating protocol. A 200 mg/mL solution of 2, 5-dihydroxy-benzoic acid (DHB) in acetonitrile/water (9:1 by vol.) with 10 mM ammonium phosphate and 0.1% TFA was used.

The TLC plates were used directly for MALDI imaging on an autoflex speed MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 1000 Hz smartbeam laser. The extraction voltage was 19 kV, and the gated matrix was suppressed up to m/z = 200 to prevent matrix ions from saturating the detector. All spectra were acquired in the reflector mode using delayed extraction. Data were collected automatically using FlexImaging 3.0 software (Bruker Daltonics). Briefly, a low-resolution optical image of the adapter target and the TLC plate were aligned using laser-focus positions. A rectangular acquisition region comprising the TLC plate was outlined on the optical image and a pixel raster of 650 µm × 650 µm spots was defined

within the boundaries of the acquisition region. For each pixel, a spectrum comprising 500 laser shots was acquired. Data were also visualized and analyzed using FlexImaging 3.0.

#### 2.7 Surface activities

Surface tensions were determined by the Wilhelmy plate method at 25°C using a DY-500 surface tension meter (Kyowa Kaimen kagaku Co., Saitama, Japan), which was calibrated with ultra-pure water before use. The Pt plate used was cleaned by flaming and glassware was rinsed sequentially with tap water and ultra-pure water.

L-SL, A-SL, and A-SL-Na stock solutions were prepared freshly and diluted to the desired concentrations for each measurement. Solutions were allowed to equilibrate overnight at 25°C, and surface tensions were measured thrice at each concentration. Solubilizing ability of A-SL-Na micelles was measured as described<sup>33)</sup> using octanoic acid as a typical solubilizate. A-SL-Na solutions containing various amount of octanoic acid were prepared and mixed vigorously overnight. The solutions became turbid when the amount of octanoic acid used was more than that which could be solubilized. The increase in turbidity was monitored by measuring absorbance at 650 nm.

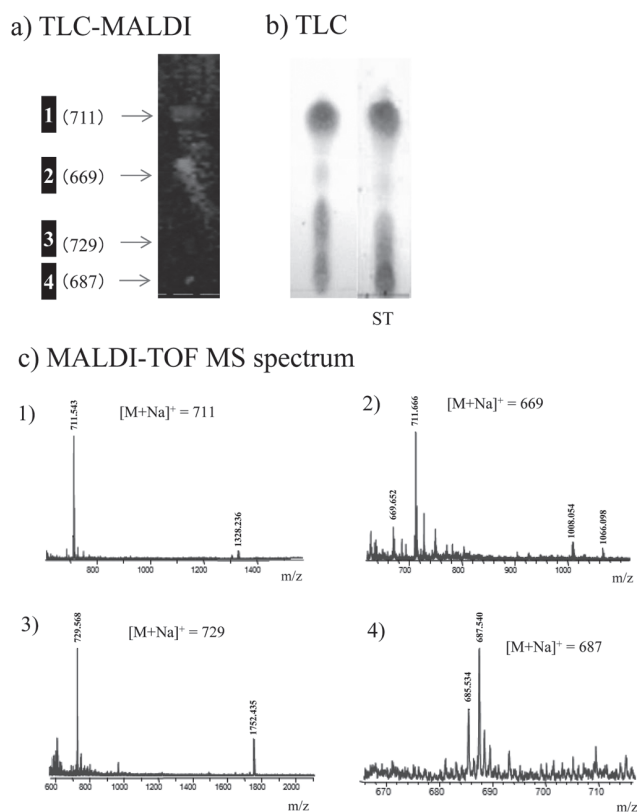
### 3 RESULTS AND DISCUSSION

#### 3.1 Screening of BS-producing yeasts using jatropha oil

We screened 20 yeasts known to produce CL, MEL, and SL for their ability to produce BSs from jatropha oil. The yeasts were grown in fermentation medium (1) or (2) containing 2% jatropha oil as the main source of carbon. After 7 d of cultivation, we extracted the culture media with equal volumes of ethyl acetate to examine for glycolipid products by using TLC. Of the yeasts tested, only the SL-producing strains—*S. bombicola* NBRC 10243 and *C. batistae* CBS 8550—synthesized glycolipid products, whereas the CL- and MEL-producers did not. This result suggests that bioactive compounds such as curcun and PEs contained in the oil inhibit the biosynthesis of MEL and CL but not SL. *S. bombicola* NBRC 10243 produced substantially more glycolipids than *C. batistae* CBS 8550, and therefore we focused on *S. bombicola*.

#### 3.2 Coupled TLC and MALDI-TOF MS analysis

The ethyl-acetate extract obtained from the medium of *S. bombicola* NBRC 10243 was analyzed by coupled TLC and MALDI-TOF MS. Figure 2 shows the TLC-MALDI image (a), together with typical TLC staining by the anthrone/sulfuric acid reagent (b). The m/z values of the peaks used for image calculation are also given in the figure. MALDI-TOF mass spectra related to spots on TLC plates are shown in Fig. 2 (b).



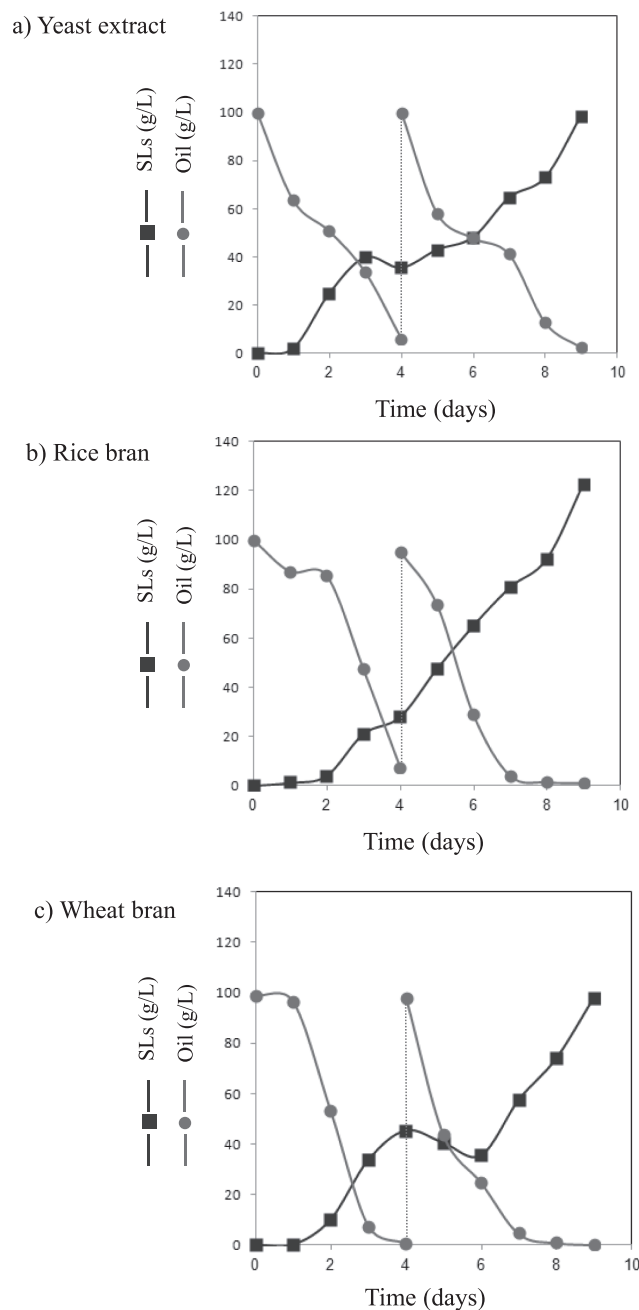
**Fig. 2** MALDI- TLC (a) and typical TLC (b) images of SL mixture produced by *S. bombicola* NBRC 10243, and MALDI-TOF MS spectra related to each spot (c).

Based on  $m/z$  values obtained, spots 1 and 2 were determined to be lactone-form structures: spot 1 and spot 2 corresponded to di-acetylated ( $m/z = 711$ ) and mono-acetylated ( $m/z = 669$ ) L-SL, respectively; and spots 3 and 4 were acid-form structures: spot 3 and spot 4 corresponded to di-acetylated ( $m/z = 729$ ) and mono-acetylated ( $m/z = 687$ ) A-SL, respectively. The detailed structures of the SLs produced by *S. bombicola* were assigned using <sup>1</sup>H NMR, LCMS, HPLC, and FTIR by Wadekar *et al.*<sup>23)</sup>. The observed tailing of spots is likely because of the high concentration of samples used for TLC imaging compared with that used for general TLC.

On silica-gel column chromatography, the fractions corresponding to spot 1 and 2 were corrected as L-SL, and the fractions to spot 3 and 4 were corrected as A-SL, and used in subsequent experiments.

### 3.3 Time course of the SL production from jatropha oil

Because using yeast extract makes BS production expensive, and because *S. bombicola* NBRC10243 converted jatropha oil efficiently into SLs, we attempted to optimize culture conditions for this yeast, especially the source of organic nutrients used. Figure 3 shows the typical time



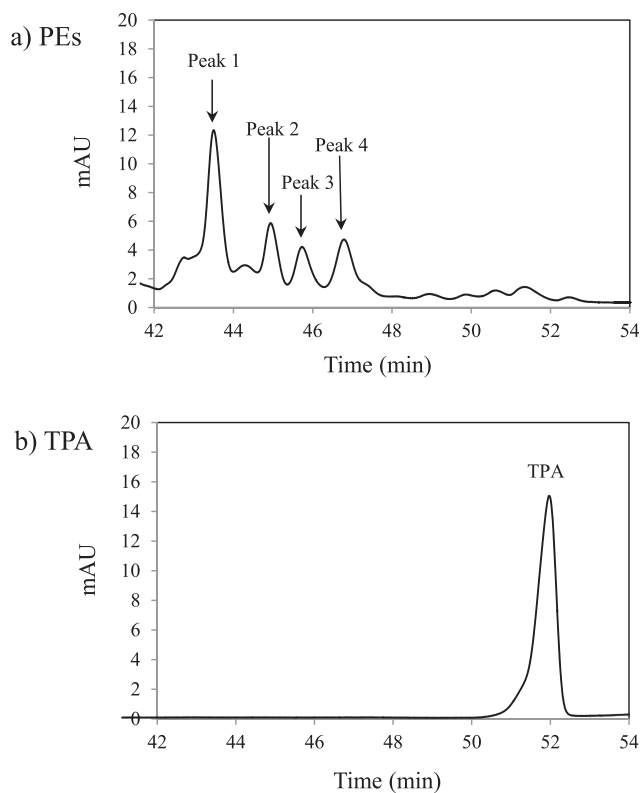
**Fig. 3** Time course of the production of SLs by *S. bombicola* NBRC 10243 from jatropha oil in a 5L-jar fermentor.

courses of SL production by the yeast cultured with jatropha oil in 5-L jar fermentors, using 3 organic-nutrient sources for cultivation: 0.3% (w/v) yeast extract (a), 1% (w/v) rice bran (b), and 1% (w/v) wheat bran (c). After 4 d, 10% (w/v) jatropha oil was added again because by that time the oil was consumed almost completely. Adding jatropha oil dilutes the culture media, and a change in the composition of media can affect the growth of microorganisms. As Fig. 3 shows, adding jatropha oil affected SLs pro-

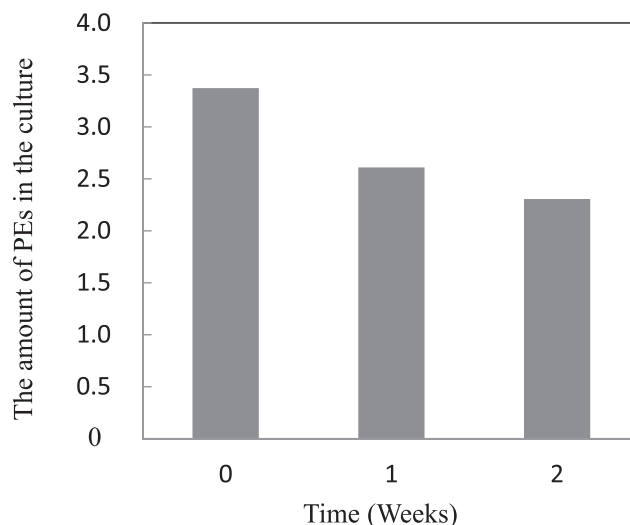
duced with rice bran less than that with yeast extract or wheat bran. SLs were produced successfully by the yeasts even though inexpensive rice and wheat bran were used instead of yeast extract. Moreover, rice bran yielded the most SLs, 122.6 g/L after 9 d cultivation, which is more than 20-times higher than the amount of SLs produced from jatropha oil in the work of Wadekar et al. Thus, this is the first report of high-yield SL production from non-edible jatropha oil. Rice bran contains 15%-25% oleic acid<sup>34</sup>, and this lipid fraction, being an excellent carbon source, could have improved the SL yield: yeasts can produce SLs efficiently from oleic acid<sup>35</sup>.

### 3.4 Analysis of PEs

Because PEs are tumor-promoters<sup>13</sup>, we investigated the amount of PEs present in culture media using reversed-phase HPLC together with UV-Vis spectroscopy. The ethyl-acetate extract from fermentation medium (3) (0-d cultivation) was analyzed first using HPLC (Fig. 4 (a)). Four peaks were detected, at 43.3 min (peak 1), 44.7 min (peak 2), 45.7 min (peak 3), and 46.5 min (peak 4). The wavelength of maximum absorbance ( $\lambda_{\max}$ ) of the peaks were 282.6 nm (peak 1), 280.4 nm (peak 2), 275.5 nm (peak 3), and 303.5 nm (peak 4), all of which correspond closely to the reported PEs in jatropha oil<sup>36</sup>. Using TPA as a standard ( $R_t = 52.1$  min, Fig. 4 (b)), we quantified the total amount of these 4



**Fig. 4** HPLC analysis of phorbol esters (PEs) in the culture medium. a) PEs b) TPA.



**Fig. 5** Relationship between the amount of PEs in the culture microbe and cultivation time.

compounds to be 3.4 mg/mL, which is within the range of the amount of PEs in jatropha oil (2-8 mg/mL).

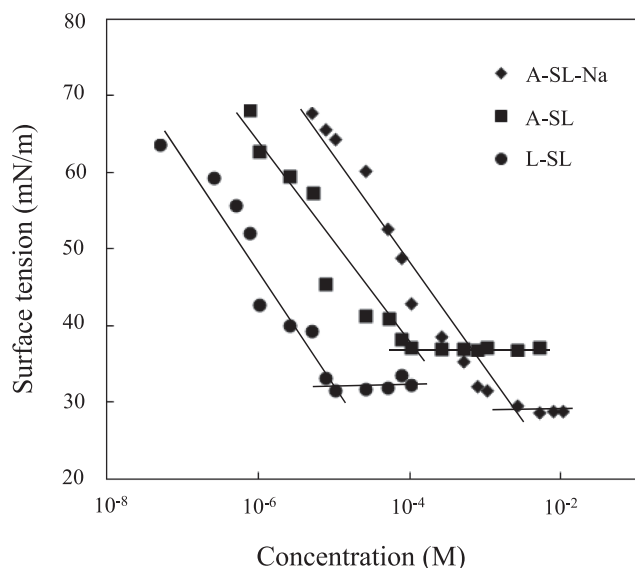
Interestingly, the amount of PEs in medium (3) decreased markedly with an increase in culture time (Fig. 5). Conversely, the amount of PEs in the medium showed no change in the absence of the yeast cells. Therefore, *S. bombicola* NBRC 10243 degrades the PEs in jatropha oil while assimilating the oil. The degradation rates of PEs after 1 and 2 wk of cultivation were estimated to be approximately 22.6% and 31.6%, respectively. The fraction of SLs extracted from the culture medium contained a small amount of PEs. Interestingly, however, the fraction of A-SL-Na obtained after alkaline treatment showed no peaks of PEs during HPLC analysis, which could be due to the hydrolytic degradation of PEs by the alkaline treatment.

### 3.5 Surface activity of L-SL, A-SL, and A-SL-Na

Surface tensions were measured for L-SL, A-SL, and A-SL-Na in water at 25°C (Fig. 6). CMC was estimated from the intersection of the straight-line fitting to the surface-tension data; values of CMC and the surface tension at CMC ( $\gamma_{\text{CMC}}$ ) are listed in Table 1.

L-SL, with a lactonic structure, was more surface active than A-SL. Compared to A-SL, L-SL had a lower  $\gamma_{\text{CMC}}$ , and the CMC of L-SL was more than 10-fold lower than that of A-SL; this agrees broadly with previous data<sup>37</sup>. Moreover, the CMC and  $\gamma_{\text{CMC}}$  of A-SL-Na were lower than that of sodium dodecyl sulfate (SDS)<sup>38</sup>.

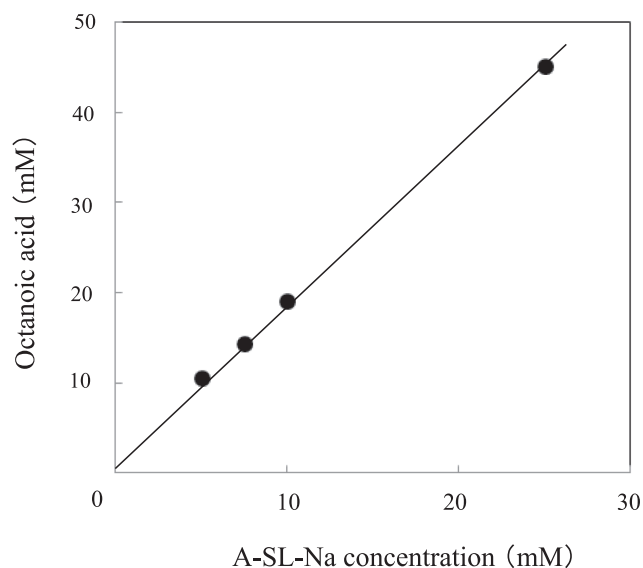
The solubilizing ability of A-SL-Na micelles toward octanoic acid was evaluated next because A-SL-Na was reported to form giant micelles above its CMC<sup>39</sup>. The relationship between the maximum amount of octanoic acid solubilized and A-SL-Na concentration is shown in Fig. 7. The slope of



**Fig. 6** Relationship between surface tension and surfactant concentration. ● L-SL ■ A-SL ◆ A-SL-Na.

**Table 1** Interfacial parameters of L-SL, A-SL, and A-SL-Na.

	CMC (M)	$\gamma_{\text{CMC}}$ (mN/m)
L-SL	$1.4 \times 10^{-5}$	32.3
A-SL	$1.2 \times 10^{-4}$	37.1
A-SL-Na	$9.0 \times 10^{-4}$	29.6
SDS	$8.2 \times 10^{-3}$	39.5



**Fig. 7** Relationship between maximum solubilizing amount of octanoic acid and A-SL-Na concentration.

the plots ( $[\text{octanoic acid}]/[\text{A-SL-Na}]$ ), which indicates the “solubilizing ability” of A-SL-Na micelle for octanoic acid, was estimated to be 2.0; this is lower than that of a representative solubilizing agent, SDS ( $[\text{octanoic acid}]/[\text{SDS}] = 4$ )<sup>33</sup>.

#### 4 CONCLUSION

Twenty BS-producing yeasts were examined for their ability to convert non-edible jatropha oil into BS. Of the yeasts tested, only *S. bombicola* NBRC 10243 was found to produce SLs efficiently. Under the culture conditions using rice bran as an inexpensive source of organic nutrients, the yield of SLs reached 122.6 g/L in 5-L jar fermentors after cultivation for 9 d; this yield of SLs from jatropha oil is 20-times higher than that reported previously.

PE levels in the culture medium fell considerably with an increase in culture time. More interestingly, although the SLs extracted from the medium contained a small amount of PEs, the A-SL-Na obtained after alkaline treatment showed no peaks of PEs upon HPLC analysis.

The A-SL-Na produced in this study from jatropha oil showed excellent surface activity and was able to solubilize octanoic acid. These results should help improve SL production from non-edible feedstock substantially and broaden the application of these promising bio-based surfactants.

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