

Production and Characterization of a Biosurfactant from *Cyberlindnera samutprakarnensis* JP52[†]

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Cyberlindnera samutprakarnensis JP52[†], isolated from cosmetic industrial wastes in Thailand, was found to be an efficient biosurfactant-producing yeast when cultured in a medium containing (2% (w/v) glucose and 2% (v/v) palm oil at 30 °C, 200 rpm for 7 d. The crude biosurfactant had the ability to reduce the surface tension from 55.7 to 30.9 mN/m at 25 °C with a critical micelle concentration (CMC) of 0.046%. Physicochemical analysis of the crude biosurfactant revealed that it had wide ranges of optimum pH and pH stability at 6–9 and 3–10 respectively. It was also thermostable and retained 80% activity even after heat treatment, and it tolerated NaCl at 1.0–10%. Furthermore, it effectively emulsified various vegetable oils with an E24 value of over 80%. A partially purified biosurfactant fraction was analyzed for its structure by MALDI-TOF MS and NMR. This revealed that the biosurfactant mainly contained sophorolipids in C18 (MW 574) and C16-diacetylated (MW 662) forms.

Key words: *Cyberlindnera samutprakarnensis*; sophorolipids; biosurfactant properties; palm oil

Biosurfactants are surface-active compound, synthesized by microorganisms such as bacteria, yeasts, and filamentous fungi. They are amphiphilic compounds containing both polar and nonpolar moieties and so are soluble in both water and organic solvents. They are increasingly used in place of synthetic chemical surfactants, as they are biodegradable and have a lower toxicity and so are considered more environmentally friendly substances. In view of their diverse properties, new value-adding opportunities should result from the identification of new biosurfactants with different specific activities, especially in relation to their biological effects, due to which they have potential application as surface active agents, bioemulsifiers, solubilizers, cleansing agents, anti-adhesives, and antimicrobial agents.^{1,2)}

Sophorolipids are glycolipid-type biosurfactants, formerly known as sophorosides, that are excreted into the culture medium by *Candida* and related yeast genera.³⁾

Torulopsis magnolia, now known as *Candida apicola*, was the first reported sophorolipid-producing yeast⁴⁾ and the structure of its sophorolipid mixture was elucidated to be a partially acetylated 2-*O*- β -D-glucopyranosyl-D-glucopyranose unit (sophorose) attached β -glycosidically to 17-L-hydroxyoctadecanoic or 17-L-hydroxy- Δ 9-octadecanoic acid.^{5,6)} Later, *Candida bogoriensis* (now known as *Rhodotorula bogoriensis*) was found to be a sophorolipid-producing yeast, the product obtained being composed of a sophorose unit linked to 13-hydroxydocosanoic acid.⁷⁾ *Starmerella* (*Candida*) *bombicola*, reported by Spencer *et al.*⁸⁾ is the most commercially popular sophorolipid-producing yeast. *Wickerhamiella domericqiae*, isolated from oil-containing wastewater, was found to produce three main sophorolipids, one of which was 17-L(-oxy)-octadecanoic acid 1,4-lactone 6,6-diacetate, the major component of the sophorolipids of *C. apicola* and *S. bombicola*.⁹⁾ *Pichia anomala* PY1, isolated from fermented food, was found to produce sophorolipid compounds containing C20:0 and C18:1 fatty acids from media containing glucose and containing soybean oil.¹⁰⁾

Within the *Starmerella* yeast clade, *S. bombicola*, *C. apicola*, *Candida riodocensis*, *Candida stellata*, and *Candida* sp. NRRL Y-27208 have been reported to show significant sophorolipid productivity.¹¹⁾ Recently, novel sophorolipids from *Candida* sp. NRRL Y-27208 were reported to contain an ω -hydroxy-linked acyl group (typically 18-hydroxy- Δ 9-octadecenoate), and to occur predominantly in a non-lactone, anionic form. In addition, 17 dimeric and trimeric sophoroses were identified by MALDI-TOF MS.¹²⁾

Here we report that a new yeast species, *Cyberlindnera samutprakarnensis* JP52, produced significant amounts of sophorolipids in a medium containing 2% glucose and 2% palm oil. The biosurfactant also exhibited good performance as to ST reduction and had significant oil displacement activity. Additionally we focused on the purification and characterization of the most effective biosurfactant and certain included of its physical and chemical characteristics.

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Abbreviations: CD₃OD, deuterated methanol; EtOAc, ethyl acetate; OD, optical density; SDS, sodium dodecyl sulfate; ST, surface tension

Materials and Methods

Microorganism. *Cyberlindnera samutprakarnensis* strain JP52^T = BCC46825^T = CBS12528^T (Mycobank no. MB800879) was isolated from the wastewater of a cosmetics factory (Milott Laboratory) in Bangplee, Samutprakarn Province, Thailand.¹³ This strain was maintained in YM broth containing 10% (w/v) glycerol, and was stored at -20°C .

Growth studies and evaluation of the substrate used for biosurfactant production. For the preliminary studies, the production medium for biosurfactant producing yeast strain JP52 was a modified Hua's medium¹⁴ containing 0.4% (w/v) NaNO_3 , 0.02% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% (w/v) KH_2PO_4 , and 0.1% (w/v) yeast extracts supplemented with one of the various carbon sources indicated at initial pH 5.5. The culture was incubated at 30°C with shaking at 200 rpm for 7 d. Inoculums having OD 1.0 at wavelength 600 nm (10% v/v) were introduced to a production medium. Each experiment with three sets of flasks was checked at intervals for biomass and biosurfactant activities. Culture supernatants were tested in triplicate to analyze for biosurfactant activity. The production medium that gave maximum yields and its supernatant, having excellent biosurfactant producing capability, were selected for further studies.

A time-course study of the biosurfactant production monitored the following parameters: dry cell weight (DCW), pH change, biosurfactant activity from surface tension (ST) values (mN/m), oil displacement area (ODA), and substrate consumption during cultivation. Modified Hua's medium supplemented with 2% (w/v) glucose and 2% (v/v) palm oil was selected for this study, as it showed excellent growth and biosurfactant activity (see "Results and Discussion" below). Biomass as cell dry weight was determined by centrifugation of the culture at $10,160 \times g$, at 4°C for 20 min. The pellet was washed twice with distilled water, placed on a pre-weighed foil plate, and dried at 105°C to achieve a constant weight. The supernatant was examined for biosurfactant activity by oil displacement test and ST value measurement. For substrate analysis, the reducing sugar was measured from the supernatant without oil by the dinitrosalicylic acid (DNS) method.¹⁵ The residual vegetable oil was measured from oils extracted by hexane from the cell pellet combined with oils from the supernatant, as reported by Kawashima *et al.*¹⁶ The nitrate concentration was determined colorimetrically by the brucine sulfate method.¹⁷ All data are reported as mean values of triplicate samples.

Biosurfactant productivity test. After centrifugation of the culture broth, the supernatant was harvested, rough filtration to remove oil. We did an oil displacement test and determined ST value for ability to reduce the surface tension. The oil displacement test was done by measuring the diameter of the clear zone that formed on the surface of an oil-water phase and measuring the displacement area, as previously reported.¹⁸ The ST value was measured by the Du Nouy Ring method (Tensiometer, K6; Kruss, Hamburg, Germany). The measurement was repeated 3 times and an average value was obtained. The ΔST value was derived from the ST of the supernatant minus the ST of the initial medium. The ΔST value, defined as the ST reducing value, was used as the criterion for selecting the optimum carbon source in the culture medium by selecting the highest ΔST and the highest biosurfactant activity of the producing yeast strain.

Isolation of crude biosurfactant. Strain JP52 was grown in modified Hua's medium supplemented with various carbon sources at 30°C with shaking at 200 rpm for 7 d. The supernatant was harvested by centrifugation ($10,160 \times g$, 4°C , 20 min), and then extracted with 1:3 (v/v) hexane: supernatant. The aqueous phase was harvested and extracted 3 times with 1:1 (v/v) ethyl acetate: aqueous phase. The ethyl acetate extracts were pooled, evaporated to dryness in a vacuum at 40°C , and weighed to determine the yield.

Measurement of the critical micelle concentration (CMC) of the biosurfactant. The crude extracted biosurfactant from a medium supplemented with glucose and palm oil (GP) dissolved in 50 mM Tris-HCl pH 8.0 and serially diluted to achieve concentrations at 10^{-2} – 10^5 and 5×10^5 mg/L prior to measurement of surface tension. The CMC was obtained from a plot of the surface tension as a function of the

biosurfactant concentration. The concentration at which micelles began to form was taken to be the CMC. Above this concentration, no increment was detected in the reduction of surface tension.

Measurement of emulsification activity. The emulsification activity of the crude extracts from GP medium towards various vegetable oils and hydrocarbons was studied. A mixture of 6 mL of vegetable oils or hydrocarbons and 4 mL of 1 mg/mL of the crude biosurfactant solution was vortexed at high speed for 2 min. The emulsion activity was determined after 24 h, and the emulsification index (E24) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying that by 100.¹⁹ Those emulsions were left standing in order to re-examine them at days 7, 28, and 60 for emulsion stabilization.

Effects of pH, salt, and temperature on biosurfactant activity and stability. These studies were carried out using 1 mg/mL of the extracted biosurfactant from GP medium suspended in 50 mM Tris-HCl pH 8.0, and we investigated the effects of several environmental parameters (pH, salt, and temperature variation) on the surface activity of the biosurfactant. For the effects of pH, the biosurfactant solution was adjusted with 0.1 M NaOH or HCl in a pH range of 2–10, and for the salt tolerance test was prepared at a concentration of 0–10% w/v NaCl. All the culture samples were analyzed for activity of surface tension reduction, and we measured ST value when left standing for 3 h (d 1) and 72 h (d 3). For the heat stability of the crude biosurfactant solution, we incubated samples at various temperatures (4, 30, 60, or 100°C) or by the sterilization process *via* autoclave and then monitored biosurfactant activity at interval incubation time durations up to 9 h, or sterilization 5 times.

Purification and chemical structure analysis of the biosurfactant. Crude extracts of the biosurfactant from GP medium were dissolved in 10% (v/v) acetonitrile to 20 mg/mL, and 10 μL aliquots were used to measure oil displacement activity. The crude extracts were resolved on silica gel thin layer chromatography (TLC) plates (G60, Merck, Darmstadt, Germany) with a chloroform: methanol: water mixture (65:25:4 v/v) as mobile phase. Positive areas of glycolipids on the TLC plate were visualized by iodine vapor for detection of fatty acid, seen as dark yellowish spots. The relative mobility (Rf) values of the positive fractions were recorded, and then the fractions were harvested, dissolved in ethyl acetate, and assayed for carbohydrate components by the Molish test, and oil displacement activity, as previously reported.¹⁰ Positive fractions with high oil displacement activity were further analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) to identify the glycolipid type in terms of a sophorolipid, mannosyl erythritol lipid, or rhamnolipid, as reported previously.^{9,20,21}

MALDI-TOF-MS analysis was performed at the Equipment Management Center, Creative Research Institution, Hokkaido University, as previously described^{11,21} on Voyager-DE STR-H operating in linear mode with positive ion detection (Bruker Daltonics, Billerica, MA). Samples were suspended in acetonitrile to 100 $\mu\text{g}/\mu\text{L}$, and then 0.5 μL was mixed with 5 μL of the matrix (2,5-dihydroxybenzoic acid at 10 mg/mL in 50% acetonitrile) and allowed to dry in 100-well plates at room temperature prior to analysis. An acceleration voltage of 20 kV and time delay of 110 ns was applied, and a total of 100 laser shots was summed for each spectrum.

Structure elucidation of the glycolipids was also performed at the Equipment Management Center by nuclear magnetic resonance (NMR) analysis at GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University. The ^1H and ^{13}C NMR spectra of the partially purified glycolipids were recorded in deuterated methanol (CD_3OD) on AMX500 a Bruker Avance spectrometer operating at 500.11 MHz (Bruker BioSpin Corp., Billerica, MA) with a standard 5 mm z-gradient BBI probe at 27°C . Two-dimensional NMR spectra, ^1H - ^1H -correlation spectroscopy (COSY), ^1H - ^1H -total correlation spectroscopy (TOCSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) were performed at 600 MHz by standard procedures. Chemical shifts were expressed in parts per million (ppm) downfield of an internal standard tetramethylsilane. Standard sophorolipid (kindly gifted by Saraya, Osaka, Japan) was also analyzed as mentioned above.

Table 1. Effects of Carbon Source in the Medium on the Cell Growth and Biosurfactant Production Levels of *C. samutprakarnensis* JP52^T

Carbon sources	Cell dry weight ^a (g/L)	Surface tension ^b (mN/m)	Δ Surface tension ^b (mN/m)	Crude BS weight ^c (g/L)	ODA of crude BS ^d (cm ²)
<i>Single carbon source at 2% (w/v) of:</i>					
Glucose	7.01 ± 0.01	34.5 ± 2.8	25.8 ± 2.8	0.030 ± 0.01	67.2 ± 0.06
Glycerol	8.44 ± 0.01	35.8 ± 0.7	17.5 ± 0.7	0.013 ± 0.00	44.5 ± 0.06
Palm oil	0.48 ± 0.00	38.5 ± 1.8	2.50 ± 1.8	ND	ND
Soybean oil	0.43 ± 0.00	39.9 ± 0.7	6.80 ± 0.7	ND	ND
<i>Mixed carbon source of 2% (w/v) glucose plus 2% (v/v) of:</i>					
Glycerol	8.68 ± 0.01	37.8 ± 0.7	12.4 ± 0.7	0.234 ± 0.02	10.7 ± 0.08
Soybean oil	6.74 ± 0.00	41.8 ± 2.5	12.9 ± 2.5	0.63 ± 0.05	10.8 ± 0.10
Palm oil	7.40 ± 0.01	33.3 ± 2.3	20.0 ± 2.3	1.89 ± 0.32	123.9 ± 0.21

Data are presented as mean ± 1SD. All experiments were conducted in triplicate.

^aCell dry weight is at d3 of culture (maximal growth).

^bThe surface tension value is at d7 of culture (minimum surface tension).

^cWeight of crude biosurfactant (BS) was analyzed at d7 of culture (maximal biosurfactant activity).

^dODA, The oil displacement area was evaluated with 20 mg/mL of biosurfactant solution.

ND, not determined

Results and Discussion

Effects of carbon sources on biosurfactant production by Cyberlindnera samutprakarnensis strain JP52

A yeast species *C. samutprakarnensis* JP52 was used in this study, as it showed the highest biosurfactant producing ability in our laboratory yeast strain collection, as determined by surface tension reduction.¹⁰⁾ We examined biosurfactant productivity in various 2% carbon sources, including glucose, glycerol, palm oil, and soybean oil, or a mixture of glucose and glycerol, palm oil, or soybean oil (Table 1). Strain JP52 showed maximum cell growth on day 3 of all the tested media (not shown). Strain JP52 showed good growth in glucose and on glycerol medium, but biosurfactant production (as crude extracted BS) was not very high. However, in a mixed medium of glucose and glycerol, biosurfactant production increased 7.8-fold and 18-fold compared with that of glucose alone and glycerol alone respectively. The result that glycerol supported good cell growth but was an inefficient carbon source for sophorolipid production is consistent with a report on *C. bombicola*.²²⁾ As Table 1 indicates, palm oil and soybean oil were inefficient growth carbon sources, but they had a significant effect on crude biosurfactant production when mixed with glucose. The crude biosurfactant level for the glucose-palm oil (GP) medium was by far the highest, at 1.89 g/L, some 63-fold higher than that obtained at 0.03 g/L for 2% (w/v) glucose as sole carbon source.

The highest ΔST value was obtained with 2% (w/v) glucose as sole carbon source (25.8 mN/m), followed by GP medium (20.0 mN/m). In addition, tested at a concentration of 20 mg/mL, it showed the highest biosurfactant activity on the oil displacement test, at 123.9 cm², some 1.84-fold higher than that 2% (w/v) glucose as sole carbon source. There have been many reports on biosurfactant production for combination substrates of glucose and vegetable oil, *e.g.* glucose and corn oil utilized by *C. bombicola*,²³⁾ glucose plus cotton seed oil by *C. glabrata*,²⁴⁾ and glucose and canola oil by *C. lipolytica*,²⁵⁾ but there are only few reports on biosurfactants production from palm oil, such as sophorolipid production by *C. bombicola* using palm

oil and a methyl ester of palm oil.²⁶⁾ Palm oil is a popular domestic plant and used as raw material for biodiesel production and so on. It is abundantly available in Southeast Asia and cheaper than many other vegetable oils. Hence the production of biosurfactants from palm oil and the used palm oil in combination with carbohydrates is a low cost strategy for the industry, and might also reduce the generation of waste. Thus *Cyberlindnera samutprakarnensis* JP52 has high potential to produce biosurfactants economically in Southeast Asia.

Growth kinetics studies and biosurfactant production

The growth of *C. samutprakarnensis* JP52 and the production of biosurfactant illustrating biosurfactant activity are shown in Fig. 1. Maximum biomass as cell dry weight of yeast strain JP52 in GP medium was achieved for cultivation at d3 at 0.85 log₁₀ dry cell weight (g/L), and the level of cell growth decreased slightly until it became constant during the stationary phase, as shown in Fig. 1a. The pH increased from 5.0 to 7.9 (data not shown) in the exponential phase and remained unchanged until the end of cultivation. The biosurfactant produced showed an ability to reduce surface tension from the initial ST 52 to 43 mN/m and to increase the displaced oil area during exponential growth (d3). Maximum production of the biosurfactant was observed after the late exponential growth phase which gave the minimum ST (from initial ST 52 to 33 mN/m), and the largest oil displacement area occurred at day 7. Biosurfactant production rose gradually in the exponential phase, and increased significantly, while the yeast grew in the stationary phase, indicating that biosurfactant production is not growth associated. Similar observations have been reported for another biosurfactant-producing yeast, *C. lipolytica* UCP0988 with 10% glucose plus 10% canola oil.²⁵⁾

The kinetic behavior of *C. samutprakarnensis* JP52^T growth and biosurfactant production in GP medium were investigated. Cell biomass, biosurfactant activity as determined by the oil displacement test and surface tension reduction, substrate consumption determined as relative amounts of total reducing sugar, palm oil, and nitrate compound in culture broth are shown in Fig. 1b.

At the beginning of exponential growth on the first day, glucose was almost exhausted with a rapid increase in cell biomass to maximum, and then the remaining hydrophilic carbon compound in the culture was completely depleted, after d3 of cultivation. However, palm oil, which was more hydrophobic than the other carbon sources in the medium, was slowly utilized by yeast strain JP52, for which only a 20% consumption rate was observed at the end of the cultivation period as compared to that of the un-cultured broth. When the total amounts of nitrate supplied from sodium nitrate were determined, it was found that the amounts of nitrate decreased to about 50% within 24 h, and decreased significantly when yeast cell biomass reached maximum, and they were completely exhausted at d7. This finding is in agreement with other previous reports, as on sophorolipid production by *C. bombicola*.^{27,28)}

Critical micelle concentration (CMC) of the biosurfactant

The CMC value is defined as the minimum concentration of surfactant necessary to initiate micelle structure. A crude biosurfactant from *C. samutprakarnensis* JP52 showed high efficiency in reducing the surface tension of Tris buffer, used as control, from 55.7 to 30.9 mN/m at a CMC of 457.1 mg/L (0.046%), as shown in Fig. 2. In spite of the increasing biosurfactant concentration, no further reduction in surface tension was observed once the CMC reached this point. Compared to biosurfactants from other sources, the crude (partially purified) biosurfactant from *C. samutprakarnensis* JP52 had a minimum surface tension (γ ST) value less than those from *C. bombicola* (39–43.5 mN/m),²³⁾ from *C. antarctica* (35 mN/m),²⁹⁾ and from *Y. lipolytica* (50 mN/m).³⁰⁾ Furthermore, it also had a lower CMC value than those of other biosurfactants from other yeasts previously reported, including 0.6% for a biosurfactant from *C. antractica*,²⁹⁾ and 2.5% for a biosurfactant from *C. lipolytica*.²⁵⁾ Moreover, it had a greater CMC value than SDS, a chemical surfactant, at 1280 mg/L.³¹⁾

Measurement of emulsification activity

Emulsification activity and its stability are important physicochemical characteristics of biosurfactants in practical applications. The formation of an emulsion usually results from dispersion of the liquid phase as microscopic droplets in another liquid continuous

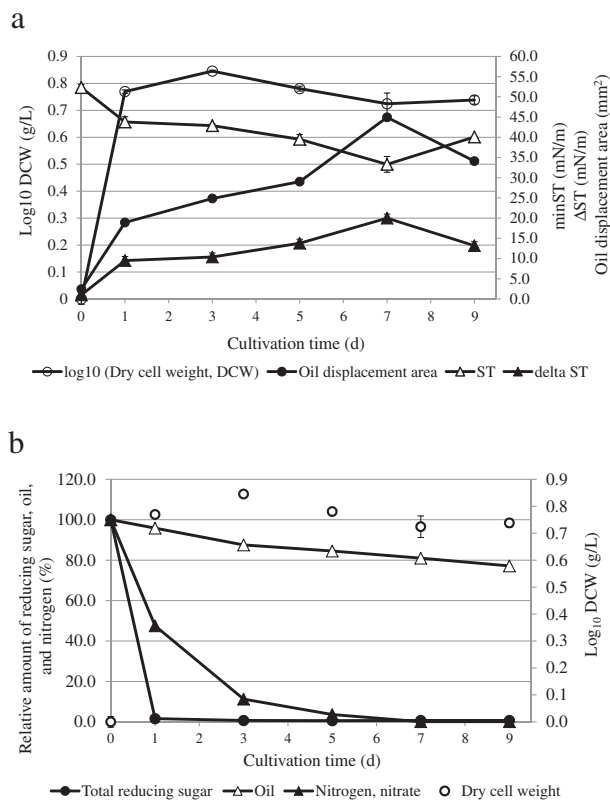


Fig. 1. Profiles of Cell Growth, pH, and Biosurfactant Activity (a). Kinetic studies of cell growth, substrate consumption (b) determined as relative amounts of total reducing sugars, oil and nitrate of *C. samutprakarnensis* JP52^T cultured in GP medium.

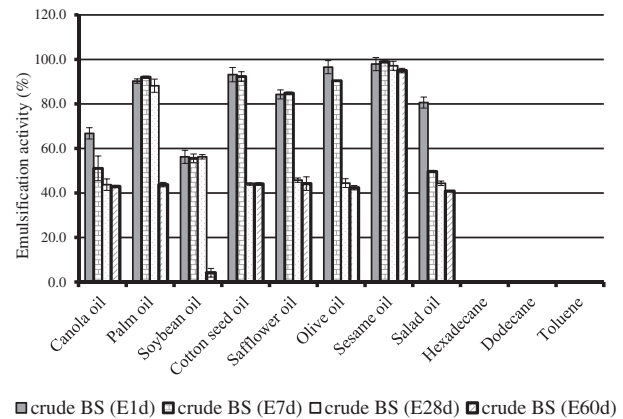


Fig. 3. Emulsification Stability at 1, 7, 28, and 60 d (E1d or E24 index, E7d, E28d, E60d) of Crude Biosurfactant from *C. samutprakarnensis* JP52^T Cultured in GP Medium.

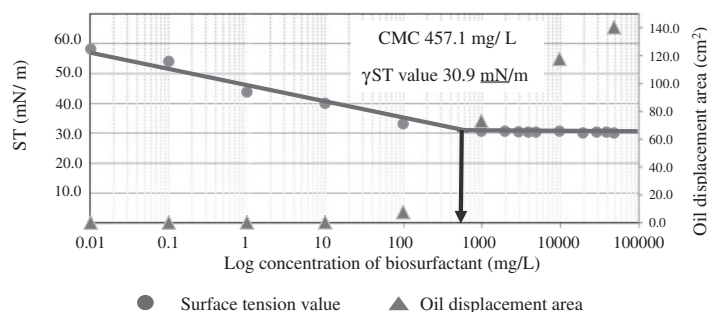


Fig. 2. Relationship of the Biosurfactant Capacity of Surface Tension Reduction and Oil Displacement Area Enhancement of Crude Biosurfactant from *C. samutprakarnensis* JP52^T Cultured in GP Medium. The γ ST value reached 30.9 mN/m at CMC 457.1 mg/L (0.046%).

phase.³²⁾ The present study determined the emulsification index (E24) and stability of an emulsion of the crude biosurfactant at 1 mg/mL from *C. samutprakarnensis* JP52 tested against various vegetable oils and several hydrocarbon compounds. As shown in Fig. 3, emulsion with five vegetable oils, olive oil, sesame oil, palm oil, cotton seed oil, and safflower oil, gave E24 values higher than 80%, and the emulsions were stable over a period of 7 d. On stability test, it was found that the emulsions formed with olive oil, cotton seed oil, and safflower oil collapsed from 90 to 50% after 1 month, but this was not so palm oil or sesame oil. In contrast, the biosurfactant failed to emulsify any of the hydrocarbons tested, hexadecane, dodecane, and toluene. A sophorolipid produced by *Torulopsis bombicola* ATCC 22214 did not stabilize emulsions containing water and hydrocarbons or vegetable oils.³³⁾ Sophorolipids from *T. bombicola* have been found to reduce surface and interfacial tension, but were not good as emulsifiers.³⁴⁾ As for the biosurfactant from *Candida glabrata* UCP1002, it was efficient in emulsification cotton seed

oil, and no significant difference in the emulsification index was observed for it as compared to *n*-hexadecane,²⁴⁾ but the present study found that a crude biosurfactant produced by *C. samutprakarnensis* JP52 grown on glucose and palm oil had better emulsification activity and stability than papers reported by others in the literature.^{24,33)} This suggests that the biosurfactant produced by *C. samutprakarnensis* JP52 has high potential in diverse industrial applications associated with emulsion.

Effects of pH, salt, and temperature on biosurfactant activity and stability

A crude biosurfactant from *C. samutprakarnensis* JP52 was tested as to pH range, salt concentrations, and temperature. For pH, the samples of crude extract of biosurfactant at 1 mg/mL dissolved in 50 mM Tris-HCl at various pHs from 5 to 9 showed high efficiency in reducing surface tension and retained strong activity for 3 d (Fig. 4a). It was found that its optimal pHs activity was in the neutral pH range, but it was still active under

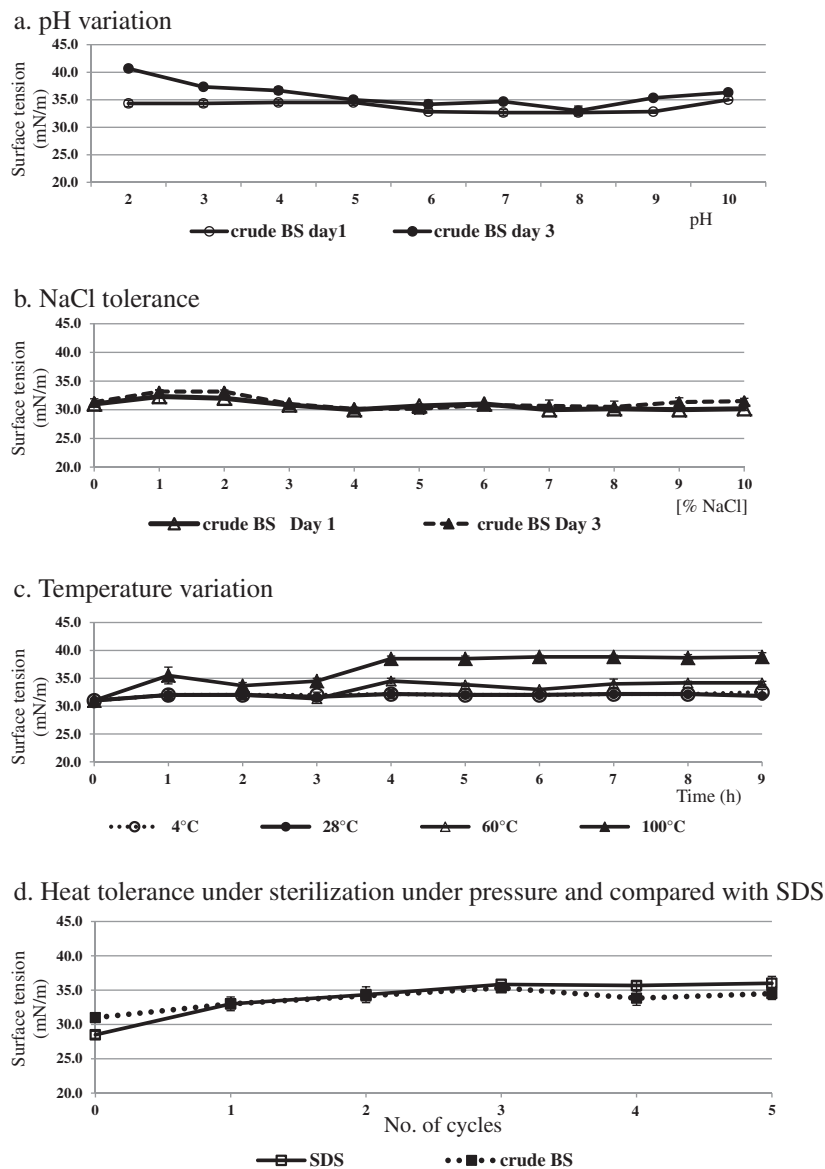


Fig. 4. Effects of pH (a), NaCl Concentration (b), Temperature (c), and sterilization (d) for Tensio-active Capacity of Crude Biosurfactant from *C. samutprakarnensis* JP52^T Cultured in GP Medium.

The ST value of 50 mM Tris-HCl without biosurfactants was 55.7 mN/m.

acidic (pH < 5) and alkaline (pH < 8) conditions. The salt tolerance of the biosurfactant was analyzed in the presence of 0–10% (w/v) NaCl. As shown in Fig. 4b, the biosurfactant was active under all the NaCl conditions tested. For temperature stability, it was treated at 4, 28, 60, and 100 °C for up to 9 h, and then the samples were left standing at ambient temperature and the ST values were determined. The samples treated at 4 to 60 °C retained almost full activity throughout the treatment periods, whereas the sample treated at 100 °C retained more than 80% activity up to 3 h of treatment, and retained moderate activity to 9 h (Fig. 4c). We further investigated its heat tolerance by heating it at 121 °C under pressure *via* a sterilization process up to 5 times. Surprisingly it remained at 86% activity and was more thermostable than the chemical surfactant SDS (72% activity) (Fig. 4d). In view of the stability properties of the biosurfactant from *C. samutprakarnensis* JP52^T as to pH, salinity, and heat, it is a good candidate for use in various industries even under extreme conditions.

Determination of the structure of the biosurfactant

Crude extracts of biosurfactants were prepared from GP and 2% (w/v) glucose-supplemented media. The extract from the GP medium was an oily and yellowish, whereas that from the 2% (w/v) glucose medium was a dark brown. These samples were analyzed by preparative TLC and visualized with iodine vapor (Fig. 5). The resolved biosurfactant fraction obtained from the GP medium was separated into three major bands, F1GP, F2GP, and F3GP, with Rf values of 0.89, 0.77, and 0.69 respectively (Fig. 5a). The presence of three major bands from the 2% (w/v) glucose medium, F1G, F2G, and F3G, was also observed, but they had slightly different Rf values, of 0.90, 0.73, and 0.65 respectively (Fig. 5b). On the Molish test, F2GP and F3GP showed

obvious positive results after only a few minutes, as did F1G and F3G, suggesting that these biosurfactants were glycolipids. However, in agreement with the slightly different Rf values, the oil displacement activity (as area) was not similar between the extracted samples from the corresponding spots of the GP and 2% (w/v) glucose media. The oil displacement area was measured at a sample concentration of 20 mg/mL in Tris buffer, pH 8. These fractions of TLC and their oil displacement areas (cm²) gave results of F1GP/18.3 vs. F1G/41.9, F2GP/18.3 vs. F2G/11.6, and F3GP/71.3 vs. F3G/9.1 cm². The highest oil displacement activity was seen for F3GP (some 7.8-fold higher than that for F3G), followed by that of F1G (2.3-fold higher than that for F1GP). Based on the efficiency of biosurfactant production by *C. samutprakarnensis* JP52^T when cultured in GP medium and its biosurfactant activities, the F3GP fraction was selected for analysis of structure by MALDI-TOF-MS and NMR.

Possible structure of F3GP based on MALDI-TOF-MS analysis

The F3GP glycolipid extract was analyzed by MALDI-TOF-MS (Fig. 6), which revealed that the major spectra for (M + H)⁺ were *m/z* 574.57 and 663.99. These values are similar to the molecular weights of the compound and molecule adduct ions for the lactone and free acid forms of sophorolipids, according to the nominal mass calculation. Sophorolipids are typically produced as complex mixtures in fermentation broths, depending on the carbon source.^{28,35} When the yeast *S. bombicola* ATCC 22214 was cultured with glucose plus soybean oil as carbon source, the sophorolipid contained both lactonic and acidic forms with saturated and unsaturated C16 and C18 fatty acid moieties.²⁸ In the case of F3GP, molecular weights of 574 and 662 suggested that they are non-acetylated lactonic sophorolipids (SL-C18) and diacetylated lactonic sophorolipids (SL-Ac-Ac-C16) respectively.

Structure elucidation of F3GP by NMR analysis

NMR analysis of glycolipid F3GP was performed on CD₃OD. Although the sample was not pure, both the ¹H and ¹³C NMR spectra could be assigned to a typical sophorolipid-type structure. Compared to the NMR spectra of standard sophorolipid and previously known NMR assignments, F3GP presented NMR signals of ¹H and ¹³C nuclei derived from a sophorose sugar (2-*O*-glucopyranosyl-D-glucopyranose), as indicated by the H1'/C1' peak at 4.36 ppm/101.1 ppm and the H1''/C1'' peak at 4.57 ppm/106.3 ppm. These were similar to the signals of Glc1 and Glc2 for the sophorolipids of *S. bombicola*¹² (H1'/C1' peak at 4.46 ppm/102.2 ppm, and H1''/C1'' peak at 4.63 ppm/104.0 ppm) and *W. domericqiae*⁹ (H1'/C1' peak at 4.46 ppm/102.6 ppm and H1''/C1'' peak at 4.56 ppm/104.0 ppm).

Sophorolipid structures are usually characterized as *O*-(1→2)-glycoside linkages between two glucose units (Glc1, C2'-*O*-Glc2, C1''). In the case of F3GP, assigned H-1'' at 4.57 ppm, there was a HMBC long-range coupling across the 1'-anomeric oxygen to the C-2' carbon (C2' at 82.5 ppm) on the other glucosyl ring. In addition, the chemical shifts at H2'/C2' (3.55 ppm/

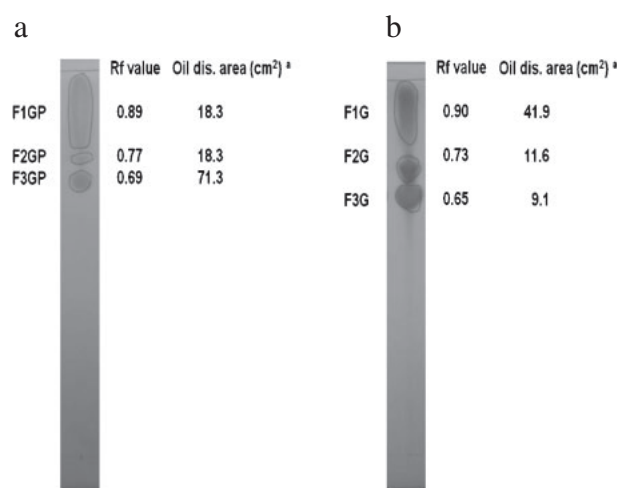


Fig. 5. TLC Analysis of the Biosurfactant Produced by *C. samutprakarnensis* JP52^T Cultured in Media Supplemented with (a) 2% (w/v) Glucose Plus 2% (v/v) Palm Oil (GP medium) and (b) 2% (w/v) Glucose, and the Rf and Oil Displacement Activity Values of Each Fraction.

Spots were visualized by iodine vapor. The Oil displacement area was measured at a sample concentration of 20 mg/mL. The TLC plates shown and associated values given are representative of those seen in three independent samples. (Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site)

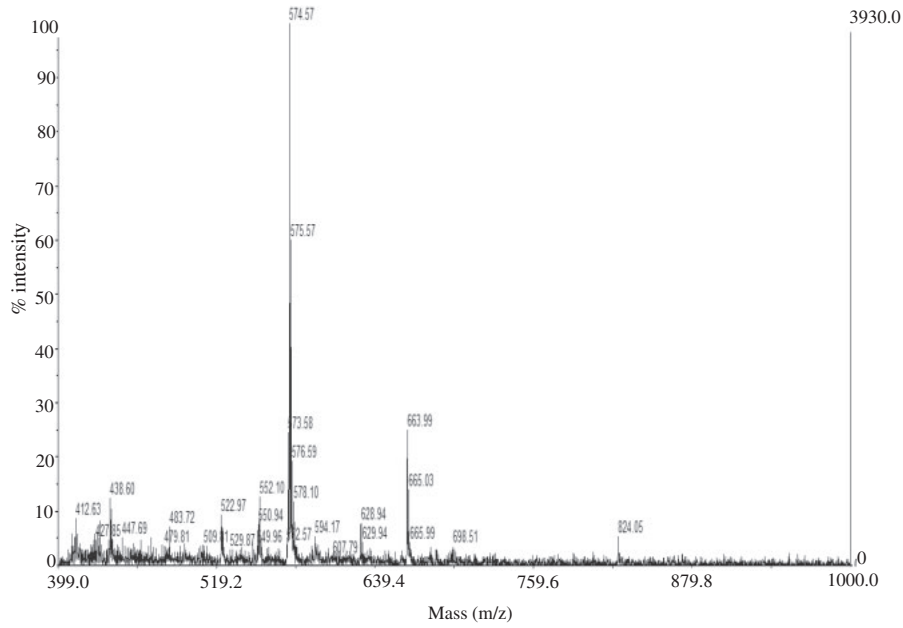


Fig. 6. MALDI-TOF-MS Spectra of Glycolipid F3GP from *C. samutprakarnensis* JP52^T. Spectra are shown representative of those seen in two independent samples.

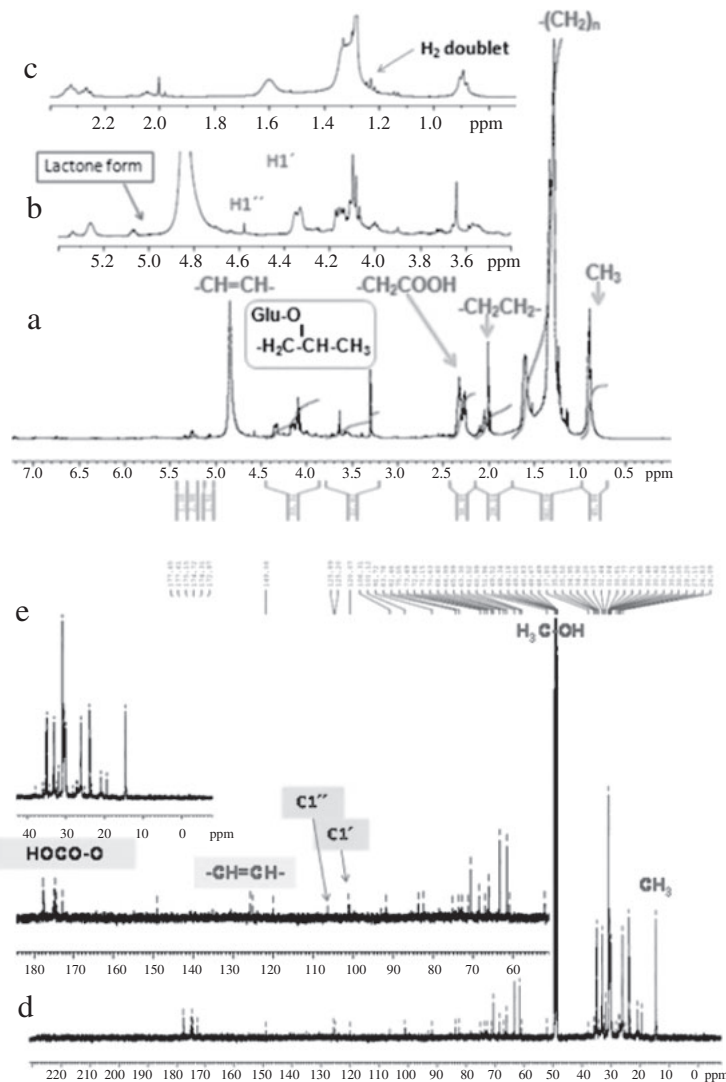


Fig. 7. NMR Data for F3GP Sophorolipids from *C. samutprakarnensis* JP52^T. Shown are the ¹H NMR spectra (a–c), and the ¹³C NMR spectra (d & e). Solvents CD₃OH and CH₃OD are indicated by * and ** respectively and the spectra shown are representative of those seen in two independent samples. (Supplemental Fig. 2; see *Biosci. Biotechnol. Biochem.* Web site)

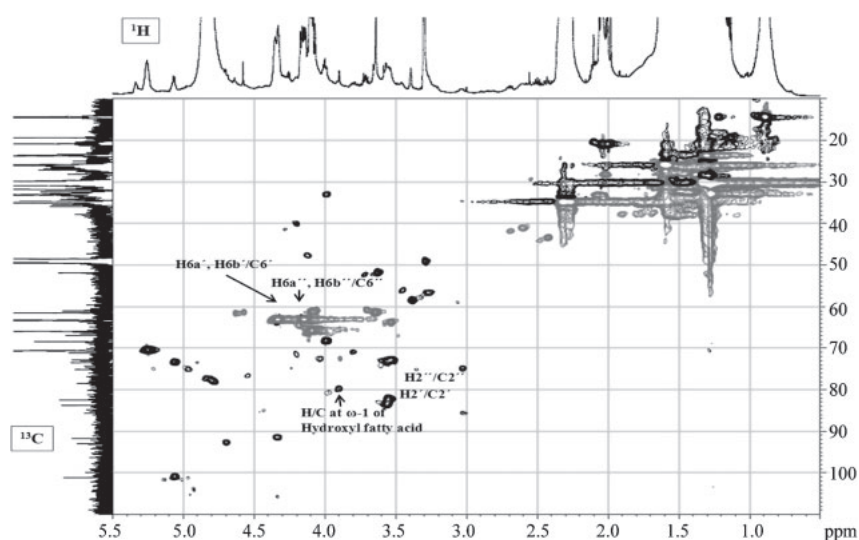


Fig. 8. Hetero-Nuclear Single Quantum Correlation (HSQC) Spectra of ^1H and ^{13}C NMR Spectra Data for F3GP Sophorolipids from *C. samutprakarnensis* JP52^T. (Supplemental Fig. 2; see *Biosci. Biotechnol. Biochem.* Web site)

82.5 ppm) and $\text{H}2''/\text{C}2''$ (3.31 ppm/75.1 ppm) confirmed that $\text{C}2'$ resonated at higher values than $\text{C}2''$ due to the de-shielding effect of the higher electro-negativity of $\text{C}2'-\text{O}$ in Glc1 (Fig. 7).

With respect to the $\text{C}6'$ and $\text{C}6''$ positions in glucose, normally located with an $-\text{O}(\text{CH}_2)$ -group, they differed in having $-\text{CH}-$, as revealed by HSQC analysis (Fig. 8). The two proton signals were shifted with $\text{C}6'$ at 4.40 and 4.25 ppm, and with $\text{C}6''$ at 4.25 and 4.10 ppm. The R group ($-\text{OH}$ or acetate) revealed two large chemical shifts in the ^{13}C -NMR spectrum, at 174.7 and 174.3 ppm, derived from the carbonyl groups ($-\text{CO}-$), indicating that these carbonyl groups were linked to $\text{C}6'$ and $\text{C}6''$ of glucose. This assignment was confirmed by HMBC analysis, with peaks at 4.2–4.40 ppm (data not shown). Together, these data suggest that the major component of the glycolipid F3GP was an acetyl group containing sophorose. The lactonic sophorolipid was formed by the esterification of the end carboxylic acid of the fatty acid linked to the $\text{C}4''$ of glucose, as was evident by the $\text{H}4''$ signals at 4.92 (Fig. 7b). The terminal fatty acid was assigned by the H/C NMR signal at 1.17 ppm/20.05 ppm as an acyl methyl group. The terminal proton showed doublet spectra at 1.17 ppm (Fig. 7c) that were influenced by the coupling with a nucleus proton at the $\omega-1$ position of the hydroxyl fatty acid.

With respect to the fatty acid moiety, carbonyl groups at the terminal C1 carbon were found to show a large chemical shift, of 177.6 ppm, in the ^{13}C -NMR. The $-\text{CH}=\text{CH}-$ group was represented by ^1H at 5.1–5.3 ppm and by ^{13}C of two carbons in a range of 120.0–125.9 ppm. Multiple signals of protons and ^{13}C in the long fatty acid chain were found at 1.2–2.25 ppm and 20.1–33.1 ppm respectively (Fig. 7d and e). HSQC analysis revealed that the NMR signals at the $\omega-1$ position of the hydroxyl fatty acid to be at chemical shift ^{13}C 79.1 ppm, and were coupled to the adjacent ^1H proton at 3.87 ppm (Fig. 8). This proton was shifted remarkably by the attached $\omega-1$ position of the hydroxyl fatty acid, linked with the glucose ring.

Conclusions

A yeast species, *C. samutprakarnensis* JP52^T, was found to produce significant amounts of glycolipid biosurfactants when cultured in a medium supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil as carbon source. Of the three main components found in the biosurfactant by TLC, F3GP gave the highest oil displacement activity and was confirmed to be a glycolipid by the Molish test. Chemical characterization by MALDI-TOF-MS and NMR analysis revealed that the major components of the F3GP biosurfactant fraction had molecular weights of 574 and 662, which corresponds to non-acetylated lactonic sophorolipids (SL-C18) and diacetylated lactonic sophorolipids (SL-Ac-Ac-C16) respectively. Thus *C. samutprakarnensis* JP52^T is a newly discovered of sophorolipid biosurfactant-producing yeast. Moreover, this biosurfactant was found to be a good emulsifier, and it had excellent tensio-active properties that might be useful in food and petroleum industrial applications.

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