

Production of Sophorolipid Biosurfactant by *Pichia anomala*

Jiraporn THANİYAVARN,^{1,†} Tanusta CHIANGUTHAI,¹ Polakit SANGVANICH,²
Niran ROONGSAWANG,³ Keji WASHIO,³ Masaaki MORIKAWA,³ and Suthep THANİYAVARN¹

¹Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

²Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

³Graduate School of Environmental Science, Hokkaido University, Sapporo 060-0810, Japan

Received March 17, 2008; Accepted May 7, 2008; Online Publication, August 7, 2008

[doi:10.1271/bbb.80166]

Biosurfactant production by *Pichia anomala* PY1, a thermotolerant strain isolated from fermented food, was examined as grown in media containing various carbon and nitrogen sources. The optimal conditions for biosurfactant production included 4% soybean oil as carbon source at pH 5.5 at 30 °C for 7 d. Under these conditions, the surface tension of the medium decreased to 28 mN/m with oil displacement measured at 69.43 cm². Comparative studies of biosurfactant production in media containing glucose or soybean oil were performed. The biosurfactants obtained were isolated and purified by chromatographic methods. The molecular weights of samples were further investigated by mass spectrometry. In medium containing glucose, biosurfactants of molecular weights of 675, 691, and 707 were obtained, while those isolated from medium containing soybean oil were of molecular weights of 658, 675, and 691. These results reveal that sophorolipid compounds containing fatty acids of C20 and C18:1 were produced from both media.

Key words: *Pichia anomala*; thermotolerance; soybean oil; biosurfactant; sophorolipid

Microbial processes are most commonly used in the production of biosurfactants, due to their economic efficiency. Most known biosurfactants are of bacterial origin, and only a few come from yeast or fungi.^{1–3} Several yeasts in the genus *Candida* are known to produce large amounts of glycolipid biosurfactants, such as mannosylerythritols and sophorolipids, from various substrates, such as carbohydrates, vegetable oils, animal fats, n-alkanes, and mixtures of these substrates.^{4,5}

The yeasts *C. apicola* and *C. bombicola* are known to produce extracellular sophorolipid biosurfactants.⁵ Their sophorolipids are produced as a mixture of acidic and lactonic forms. The hydrophilic portions of both forms consist of the dimeric sugar sophorose, while the lipophilic parts are composed of long-chain fatty acids with hydroxyl groups. The lengths of the fatty acids vary

depending on the medium content, 16–18 carbons being the usual composition.^{6,7} Hydroxyl fatty acids such as C20 and C20:1 were reported to be minor components by Davila *et al.* (1997).⁸ Unsaturated fatty acid chains and acetylated compounds are usually obtained as mixtures.^{7–9} Recently, Nunez *et al.* (2004)¹⁰ reported that the predominant form of sophorolipid produced by *Rhodotorula bogoriensis* is a C22 hydroxy carboxylic acid, although traces of C24 hydroxy carboxylic acid are also found. These compounds are used in the food, pharmaceutical, cosmetics, and specialized chemical industries, essentially in any industry or process in which surface activity properties figure.⁵

We isolated a yeast strain, PY1, from Khao Mhak, a Thai fermented food, which can produce biosurfactants within a temperature range of 20–40 °C. The aim of this study was to investigate the ability of PY1 to produce biosurfactants under optimum conditions. Growth and biosurfactant production were studied in media containing various carbon and nitrogen sources at various pH levels. Furthermore, comparative studies of biosurfactant production as between glucose and soybean oil media were performed, and the crude extracts obtained were used in structural analysis.

Materials and Methods

Microorganism. We isolated a yeast strain, PY1, from Thai fermented food from the central part of Thailand, and this was screened for biosurfactant producers on YM agar overlaid with crude oil medium, as previously described.¹¹ The yeast strain was identified by physiological activities and biochemical characteristics,^{12,13} and confirmed by the Internal Transcribed Spacer (ITS) and D1-D2 domains of large subunit (LSU) rDNA sequences analysis. Yeast strain PY1 was grown in 5 ml of YEPD cultivated with shaking for 48 h at room temperature. Chromosomal DNA was extracted using glass beads, as previously described.¹⁴ The ITS region of rDNA of PY1 was amplified and sequenced using

[†] To whom correspondence should be addressed. Tel: +662-2185070; Fax: +662-2530337; E-mail: jiraporn.Th@chula.ac.th

primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The amplification reaction of the D1-D2 region used NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') as primers. PCR was performed using Gene Amp PCR system 2400 (PerkinElmer, Massachusetts, USA). The PCR products were purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The nucleotide sequence was compared by BLAST homology search. The nucleotide sequences of the ITS and D1-D2 regions have been deposited in DDBJ databases, under accession nos. AB331898 and AB426548 respectively.

Growth and biosurfactant activity at various temperatures in YM medium. Growth at various temperatures (20–45 °C) was determined by cultivation of PY1 in YM broth by rotary shaking at 200 rpm. Cell growth was determined by measuring OD at 650 nm using appropriate dilutions. Biosurfactant activity was determined by cultivating PY1 on YM agar overlaid with crude oil medium at various temperatures for 5 d and observing the occurrence of halo zones surrounding the colonies that produced biosurfactants.

Biosurfactant production by PY1 grown in various carbon sources. The biosurfactant was produced by cultivation of *Pichia anomala* PY1 in 50 ml, Hua medium¹⁵⁾ containing 0.2% NaNO₃, 0.02% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.1% yeast extract, and 8% of various carbon sources, such as glucose, cassava starch, soybean oil, palm oil, and coconut oil, in a 250-ml Erlenmeyer flask with shaking at 200 rpm for 7 d at 30 °C. Cell culture (6% v/v) was prepared as inoculums in YM broth at pH 4.5 and incubated at 30 °C with shaking at 200 rpm for 18 h. Aliquots of the culture were removed periodically for growth and biosurfactant activity analysis. To determine the optimum concentration of the carbon source, concentrations of 1–8% soybean oil were used in the analysis of biosurfactant production by PY1. Biosurfactant activity in the culture broth was determined by measuring the reduction in surface tension by the Du Nouy Ring method (Tensiometer, K6; Kruss, Hamburg, Germany) and the oil displacement area, as described by Morikawa *et al.*¹¹⁾ Growth was measured in terms of dry cell mass. Both oil displacement activity and Δ surface tension b-t were used as criteria to select the condition used.

Biosurfactant production by PY1 grown in various nitrogen sources. The biosurfactant was produced by cultivation of *Pichia anomala* PY1 in 50 ml-medium containing 0.02% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.1% yeast extract, 4% soybean oil, and 0.2% of various nitrogen sources, such as NH₄NO₃, (NH₄)₂SO₄, NH₄Cl, and NaNO₃, in a 250-ml Erlenmeyer flask with shaking at 200 rpm for 7 d at 30 °C. Aliquots of the culture were

periodically removed for growth, and supernatants were used in biosurfactant activity analysis. To determine the optimum concentration of the nitrogen source, concentrations of 0.1–0.5% NaNO₃ were used in the analysis of biosurfactant production by PY1.

Effect of pH on biosurfactant production. To determine the optimum pH of the production medium, cells were cultivated in media containing 4% soybean oil as a carbon source and 0.4% NaNO₃ as a nitrogen source at various pH levels, ranging from 3.5 to 6.5. The supernatants were collected and analyzed for biosurfactant activity every d for 7 d.

Production and isolation of biosurfactant. Biosurfactant production was analyzed in 3 liters of production medium with 4% glucose or soybean oil as the carbon source, pH 5.5, with shaking at 200 rpm for 7 d at 30 °C. After 7 d, cells were removed by centrifugation at 7,500 rpm for 15 min. Then the supernatants were kept at 4 °C overnight, after which clear separation of the two phases was observed. The aqueous phase was harvested and extracted 3 times with ethyl acetate. The solvent layer was evaporated to dryness in a vacuum at 40 °C. The crude extracts were obtained and analyzed for the structural characteristics of the biosurfactants present.

Purification of biosurfactants. Crude extracts, prepared as described above, were dissolved in ethyl acetate and analyzed by thin layer chromatography (TLC) on silica gel plates (G60, Merck, Darmstadt, Germany). The samples were then resolved in chloroform:methanol:acetic acid (65:25:4 v/v), and visualized with iodine vapor and molish reagent for lipid and carbohydrate detection respectively. All positive fractions were scraped and subjected to an oil displacement test. Positive fractions with high oil displacement activity were purified by high performance liquid chromatography (HPLC) using a cosmosil 5C18-AR column (4.6 × 150 mm, Waters, Massachusetts, USA) operating at 0.5 ml/min with a UV detector set at 210 nm. The mobile phase of these HPLC conditions was a linear gradient elution of acetonitrile (10–100%) in 0.1% trifluoroacetic acid. The gradient solvent elution profile used was as follows: 10% acetonitrile for 5 min to a obtain linear gradient, 10–100% acetonitrile for an additional 10 min, 100% acetonitrile for 30 min, and then 10% acetonitrile until the end (45 min). The purified biosurfactants were eluted at different retention times and tested for oil displacement activity. The effluents were connected to LC-MS and subjected to molecular mass analysis.

HPLC analysis of standard sophorolipid. Standard sophorolipid (provided by Saraya, Osaka, Japan) was dissolved in 50% acetonitrile at a final concentration of 1 mg/ml. This showed oil displacement activity of

about 5–6 cm in diameter. The sample (50 μ l) was then injected into a HPLC-UV detection system (LCQ, ThermoFinnigan, San Jose, CA).

LC-MS analysis. The purified samples were analysed with an electron-spray ionization mass spectrometer (LCQ, ThermoFinnigan, San Jose, CA) in positive mode at Hokkaido University and in the Department of Chemistry, Faculty of Science, of Chulalongkorn University.

Results

Microorganism and identification

The morphological and physiological characteristics of the yeast strain PY1 were identified, and described in the literature.¹³⁾ The results of the assimilation test and the growth characteristics suggest that it belongs to the genus *Pichia*. Comparative sequence analysis of strain PY1 suggested its similarity to *Pichia anomala* YS117 (100%) according to its D1-D2 domain of 26S rDNA, and to *P. anomala* MTCC462 (AY231607) (98%) by ITS sequence analysis.

Growth and biosurfactant activity at various temperatures in YM medium

The optimum temperature for growth was 20–30 °C, with low growth 40 °C and no growth at 45 °C (Fig. 1). Biosurfactant activity was recognized by the occurrence of halo zones surrounding the colonies on YM medium after cultivation at 20–37 °C, slightly at 40 °C, but was absent at 45 °C. In some reports, thermophilic yeasts are defined as yeasts with a minimal temperature for growth of 20 °C but with no maximal temperature limit. By this definition, we concluded that the yeast PY1 strain that grows well at 20 °C, and that up to high temperatures, such as 37 °C, it is thermotolerant.^{16,17)}

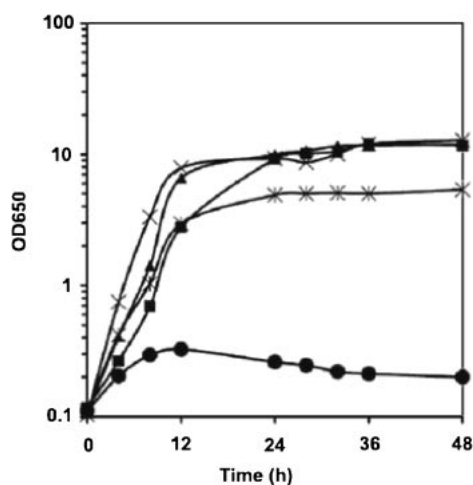


Fig. 1. Growth Profile of Yeast PY1 in YM Media at Different Temperatures.

■— 20 °C; ▲— 25 °C; ×— 30 °C; *— 37 °C; ●— 40 °C.

Table 1. Effects of Carbon Sources on Biosurfactant Production by *P. anomala* PY1 at 7 d

Carbon source (8% w/v)	Dry weight (g/l)	Initial surface tension (mN/m) ^a	Surface tension (mN/m) ^a	Δ Surface tension _{b-t} ^b (mN/m)	Oil displacement test (cm ²)
Glucose	15.21	47	43	4	7.07
Starch	10.78	66	61	5	< 1
Soybean oil	14.94	52	33	19	34.23
Palm oil	13.48	43.5	33	10.5	28.29
Coconut oil	9.55	49	46	3	< 1

^aMilli Newton/meters

^b Δ Surface tension_{b-t} was defined as the initial surface tension of the culture medium minus that of day 7.

All of the values in the table are averages of three measurements.

Table 2. Effects of Various Concentrations of Soybean Oil on Biosurfactant Production at 7 d

Soybean oil conc.	Dry weight (g/l)	Initial surface tension (mN/m) ^a	Surface tension (mN/m) ^a	Δ Surface tension _{b-t} ^b (mN/m)	Oil displacement test (cm ²)
0%	0.57	65	59	6	11.35
1%	8.31	53	35	18	19.64
2%	7.01	52	34	18	28.29
4%	11.52	52	31	21	35.50
8%	10.01	52	34	18	30.20

^aMilli Newton/meters

^b Δ Surface tension_{b-t} was defined as the initial surface tension of the culture medium minus that of day 7.

All of the values in the table are averages of three measurements.

Biosurfactant production in various carbon sources

Five different carbon sources were used: glucose, starch, soybean oil, palm oil, and coconut oil. These sources were chosen according to previously reported results^{7,15,18)} and commercial availability in Southeast Asia. In this study, biosurfactant production was determined by biosurfactant activities, both in terms of surface-tension reduction and by oil displacement test. Measurements of reductions in surface tension and oil displacement activity indicated that the biosurfactant was produced throughout the 7 d of yeast cultivation.

Between the two hydrophilic substrates, glucose supported growth and biosurfactant production better than starch. As for the hydrophobic substrate, soybean and palm oil supported growth and biosurfactant production, while only trace amounts of biosurfactants were produced in coconut oil (Table 1). Based on Δ surface tension b-t and oil displacement activity, we found that 4% soybean oil was the most effective carbon source for biosurfactant production by *Pichia anomala* PY1 (Table 2).

Biosurfactant production in various nitrogen sources

The nitrogen source and its concentration affected biosurfactant production. We found that NaNO₃ supported growth and biosurfactant production, and that other nitrogen sources did not (Table 3a). The highest

Table 3. Effects of Nitrogen Sources and Concentrations on Biosurfactant Production at 7 d

a Effects of nitrogen sources on biosurfactant production at 7 d

Nitrogen source (0.2% w/v)	Dry weight (g/l)	Initial surface tension (mN/m) ^a	Surface tension (mN/m) ^a	ΔSurface tension _{b-t} ^b (mN/m)	Oil displacement test (cm ²)
NH ₄ NO ₃	3.71	46	45	1	< 1
(NH ₄) ₂ SO ₄	2.62	52	47	5	< 1
NH ₄ Cl	3.37	50	46	5	< 1
NaNO ₃	10.08	53	33	20	28.29

^aMilli Newton/meters^bΔSurface tension_{b-t} was defined as the initial surface tension of the culture medium minus that of day 7.

All of the values in the table are averages of three measurements.

b Effects of various concentrations of NaNO₃ on biosurfactant production at 7 d

NaNO ₃	Dry weight (g/l)	Initial surface tension (mN/m) ^a	Surface tension (mN/m) ^a	ΔSurface tension _{b-t} ^b (mN/m)	Oil displacement test (cm ²)
0%	9.09	55	36	19	7.07
0.1%	15.08	53	34	19	19.63
0.2%	16.01	53	32	21	19.63
0.3%	12.97	52	31	21	36.33
0.4%	14.15	51	29	22	45.48
0.5%	11.94	51	30	21	34.22

^aMilli Newton/meters^bΔSurface tension_{b-t} was defined as the initial surface tension of the culture medium minus that of day 7.

All of the values in the table are averages of three measurements.

yield was obtained with NaNO₃ at a concentration of 0.4% (Table 3b). Acidic nitrogen sources such as (NH₄)₂SO₄ and NH₄NO₃ gave low pHs, of 3 and 4 (from an initial pH of 5.5), as well as low biosurfactant production.

Effect of pH on biosurfactant production

The influence of the initial pH of the culture medium on biosurfactant production was studied in medium containing 4% soybean oil as the carbon source, and 0.4% NaNO₃ as the nitrogen source. This was incubated at 30 °C with shaking at 200 rpm for 7 d. As Table 4 shows, optimal performance was obtained when fermentation was carried out at an initial pH of 5.5. At the optimal pH, the products reduced surface tension from 50 mN/m to 28 mN/m, and oil displacement reached 63.64 cm².

Production and structural analysis of biosurfactant

After cultivation of the yeast strain for 7 d in production media that contained 4% glucose or soybean oil as carbon source and 0.4% NaNO₃ as nitrogen source at an initial pH of 5.5 at 30 °C with shaking at 200 rpm, the surface tension of the culture broth containing glucose fell from 50 mN/m to 42 mN/m, and the oil displacement test yielded a measurement of 3.14 cm². The surface tension of culture medium

Table 4. Effects of pH on Biosurfactant Production When Cultivating Cells in 4% Soybean Oil and 0.4% NaNO₃ Medium at 7 d

pH	Dry weight (g/l)	Initial surface tension (mN/m) ^a	Surface tension (mN/m) ^a	ΔSurface tension _{b-t} ^b (mN/m)	Oil displacement test (cm ²)	Final pH
3.5	13.45	50	31	19	30.20	6.87
4.5	10.02	50	29	21	32.18	7.11
5.0	11.34	50	29	21	55.44	7.37
5.5	10.39	50	28	22	63.64	7.44
6.5	12.83	49	29	20	52.83	8.04

^aMilli Newton/meters^bΔSurface tension_{b-t} was defined as the initial surface tension of the culture medium minus that of day 7.

All of the values in the table are averages of three measurements.

containing soybean oil fell from 47 mN/m to 28 mN/m, and the measurement on the oil displacement test was 69.43 cm² (Fig. 2). Crude extracts of biosurfactants were prepared from the culture media, as described in "Materials and Methods." The dry crude extract was obtained at 0.06 g/l from glucose medium and at 0.2 g/l from soy bean oil medium. These samples were then analyzed by TLC and visualized with iodine vapor and molish reagent. The positive spots obtained from the iodine and molish reagent tests for crude extract from the glucose medium are shown in Fig. 3a and b, while the six spots obtained for crude extract from the soybean oil medium are shown in Fig. 4a and b. All fractions were collected and extracted with ethyl acetate for determination of their oil displacement activities. It was found that the biosurfactant activity from soybean oil culture was higher than that from the glucose culture. This might have been due to the presence of biosurfactants and fatty acids released from the soybean oil by lipase activity.

The F2 and F2g fractions, which were found to have the highest oil displacement ability, were collected for further purification by preparative TLC and linear gradient HPLC by the UV detector system. The most active fraction eluted from the sample from the glucose medium was obtained at a retention time of 9.269 min. The main active fraction eluted from the sample obtained from the soybean oil medium by HPLC was obtained at a retention time of 9.779 min. Subsequently, the active fractions were subjected to LC-MS analysis. From the results, a peak at RT of 9.646 min for the sophorolipid standard, *m/z* of 648.760 and 650.816 were found. *M/z* of 648.760 corresponded to a sodium adduct of C18 sophorolipid which is added 23 Da of the sodium molecule, whereas the *m/z* of 650.816 corresponded to C20:1 sophorolipid.

The HPLC of the biosurfactant from the glucose medium showed a peak at RT of 9.269 min, and consisted of three *m/z*, 675.687, 691.880, and 708.062. The *m/z* of 675.687 and 691.880 can be assigned as sodium adduct of C20 sophorolipid and the oxidized form of C20 sophorolipid respectively. The *m/z* of

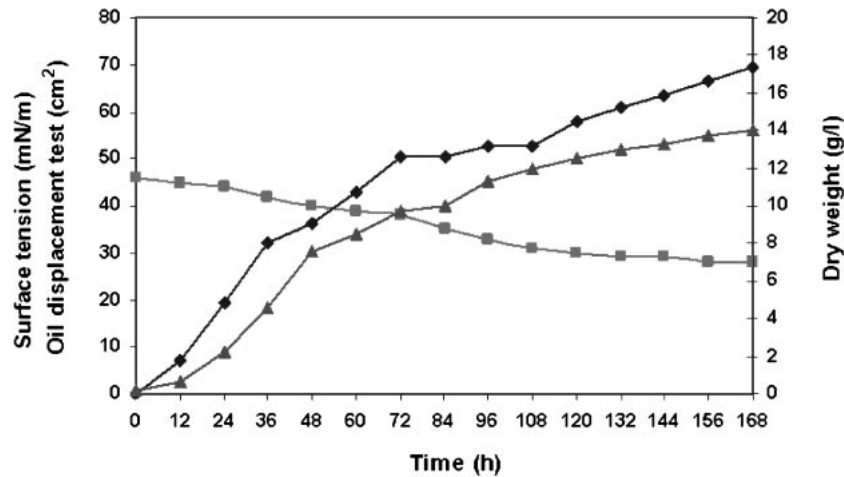


Fig. 2. Growth and Biosurfactant Production of *P. anomala* PY1 under Optimal Conditions: 4% Soybean Oil Medium Containing 0.4% NaNO₃ as Nitrogen Source with Shaking at 200rpm for 7 d at pH 5.5 with Incubation at 30 °C.
 -■- Surface tension (mN/m); -◆- Oil displacement test (cm²); -▲- Dry weight g/l.

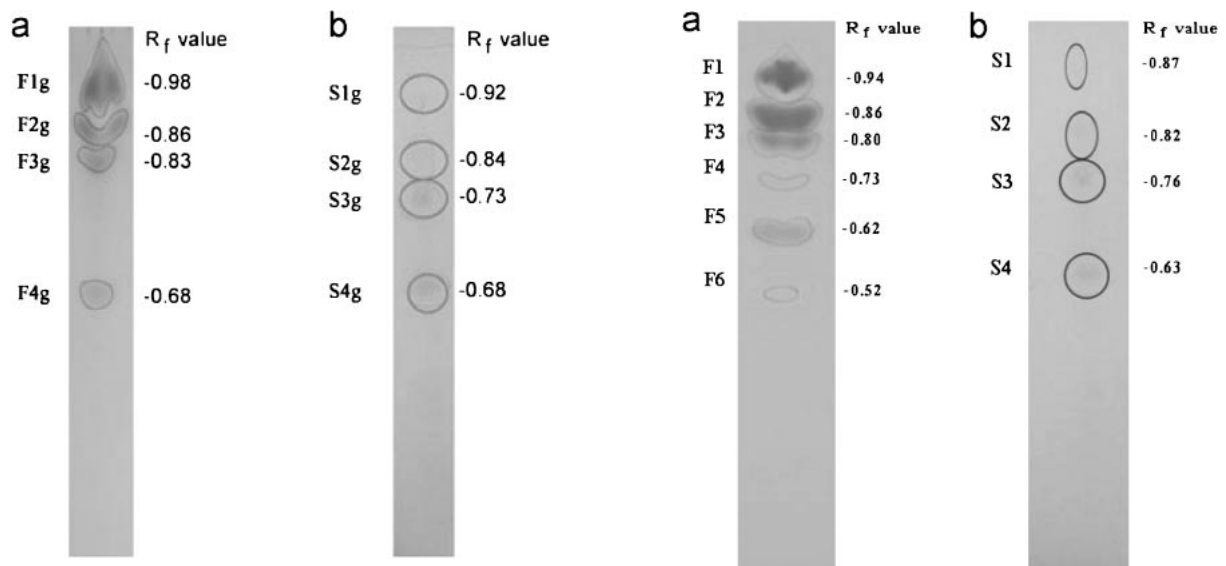


Fig. 3. TLC Analysis of Biosurfactants Produced by the PY1 Strain When Cultivated in Glucose Medium.

a, F1g-F4g, fractions that gave positive reactions with iodine vapor; b, S1g-S4g, fractions that gave positive reactions with molish reagent.

Fig. 4. TLC Analysis of Biosurfactants Produced by the PY1 Strain When Cultivated in Soybean Oil Medium.

a, F1-F6, fractions that gave positive reactions with iodine vapor; b, S1-S4, fractions that gave positive reactions with molish reagent.

708.062 has been deduced to be the two acetylated group to C18:1 sophorolipid.

The last HPLC was a sample from the biosurfactant from the soybean oil medium. It showed a peak at RT of 9.779 min. M/z of 659.499, 675.627, and 691.830 were found. According to the calculation, all three compounds were related to C20 sophorolipid. The m/z of 659.499 corresponded to a sodium adduct of [C20]_{Lactone}. M/z of 675.687 and 691.880, which were also found in the biosurfactant from the glucose medium, were identified as the same compounds. All the results and proposed compounds are summarized in Table 5, and the mass spectra for [M + H]⁺ are shown in Table 5 and Fig. 5a and b.

Discussion

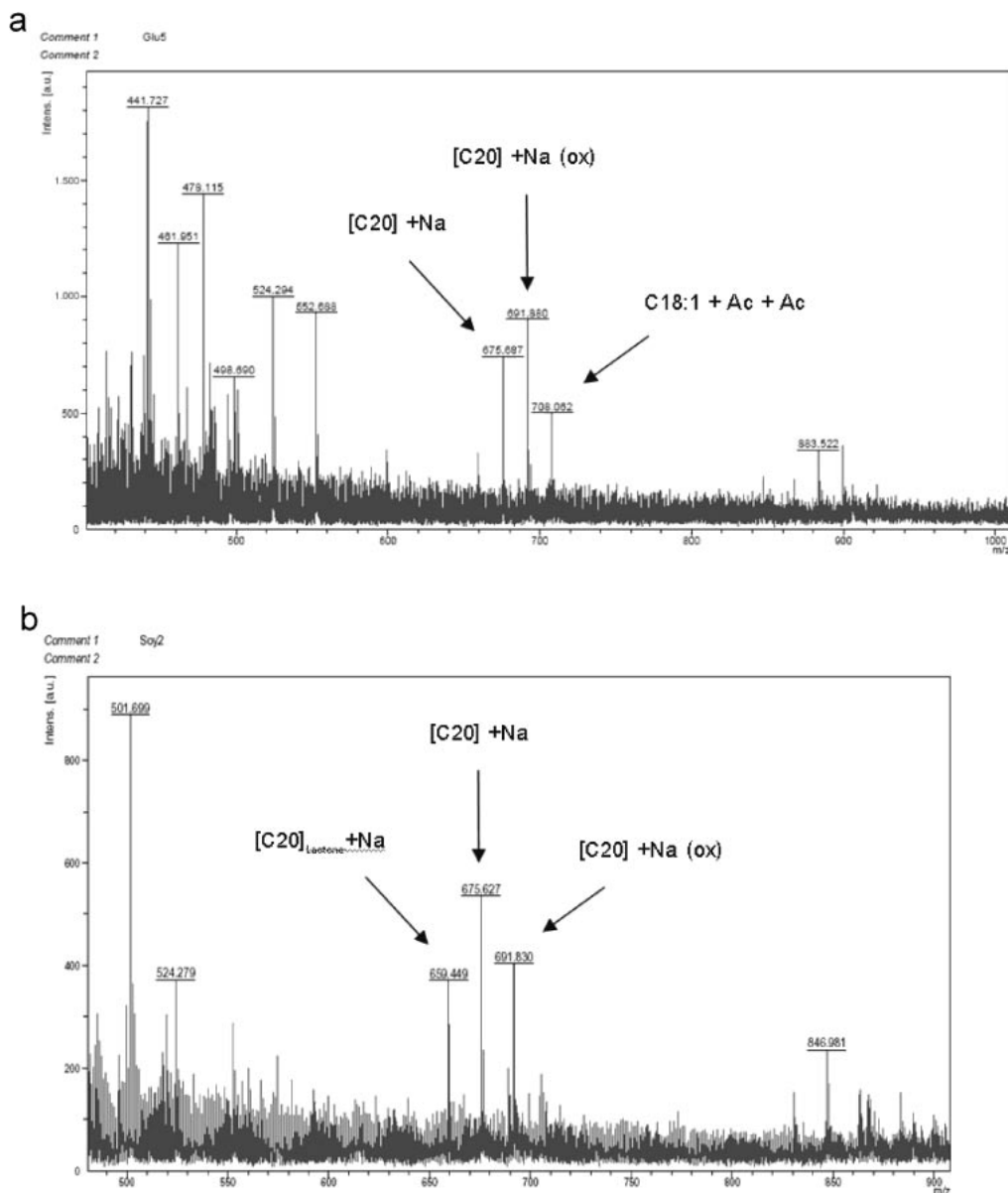
Here we report a study that examined biosurfactant production by *P. anomala* PY1 in culture media. The influence of carbon and nitrogen concentrations in the culture media at various pH levels was studied. When *P. anomala* PY1 was grown on glucose and soybean oil media, the biomass concentration reached 10–15 g/l after 7 d. Surfactant was produced at low levels during the early exponential phase, and the levels increased in the late exponential phase. We found that for the hydrophobic substrate, 4% soybean oil was the best carbon source and 0.4% NaNO₃ was the best inorganic nitrogen source for *P. anomala*. Additionally, we found

Table 5. Retention Times and $[M + H]^+$ of Mass Spectrometry for Peaks from Standard Sophorolipid, Glucose Medium, and Soybean Oil Medium Samples

Sample	RT from HPLC	$[M + H]^+$	MW	Hydroxy carboxylic acid of sophorolipid
Sophorolipid	9.646	648.760 650.816	647 650	C18 + Na C20:1
Biosurfactant from glucose medium	9.269	675.687 691.880 708.062	675 691 707	C20 + Na C20 + Na (OX) ^a C18:1 + Ac + Ac
Biosurfactant from soybean oil medium	9.779	659.499 675.627 691.830	658 675 691	$[C20]_{Lactone} + Na$ C20 + Na C20 + Na (OX)

^aOX, oxidized form of sophorolipid

that pH 5.5 was the optimum pH for biosurfactant production of *P. anomala*. This finding contradicts previous reports state that pH levels of 2.5–3.5 are optimal for the biosynthesis of surfactants by *C. bombicola*.^{8,9,19)} The optimum production condition was in medium containing 0.4% NaNO₃, 0.02% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.1% yeast extract, and 4% soybean oil at pH 5.5, with incubation at 30 °C and shaking at 200 rpm for 7 d. Under such conditions, the surface tension of the medium fell from 47 mN/m to 28 mN/m, and an oil displacement test yielded 69.43 cm². Comparative studies of biosurfactant production and their compositions in glucose- and soybean oil-containing media were performed. We obtained biosurfactants at concentrations of 0.06 g/l and 0.2 g/l from glucose and soybean oil medium respectively. In agreement with

**Fig. 5.** LC-MS Chromatogram of Biosurfactants. a, from glucose medium; b, from soybean oil medium.

previously published reports, only small amounts of sophorolipid were produced by *C. bombicola* when glucose and soybean oil were used as the carbon source in the production medium.^{5,20} It is well established that sophorolipid production is significantly enhanced when two types of carbon sources, *i.e.*, hydrophilic and hydrophobic ones, are simultaneously provided.^{18,21–25} Enhancement of biosurfactant production using hydrophilic and hydrophobic sources as co-substrates will be examined in further studies.

Molecular mass determination by LC-MS showed that the molecular weights of the biosurfactants obtained from the glucose medium were 675, 691, and 707 Da, while those of the biosurfactants obtained from the soybean oil medium were 658, 675, and 691 Da. These values suggest that the biosurfactants produced were sophorolipid compounds containing C20 and C18:1 fatty acids in acidic, lactonic, and diacetylated forms. The structures were similar to the sophorolipids of *C. apicola* and *C. bombicola* in their hydroxy fatty acid moieties, in that the sophorose unit was linked to C18-20 hydroxy fatty acids and a hydrophilic carbohydrate head, the sophorose unit being partially acetylated. Moreover, Nunez *et al.* (2004)¹⁰ suggest the possibility of sophorolipid dimer formation by lipase lactonization.

Pichia anomala has attracted much interest, since it is regarded as a source of proteins and vitamins.²⁶ It has been reported to be a food flavoring agent, and has application in producing food bioemulsifiers.²⁷ Additionally, thermotolerant yeasts have certain advantages over mesophiles in industrial processes. It is possible that the *Pichia anomala* PY1 strain will prove an attractive organism for both physiological study and industrial application.

Acknowledgments

This study was supported by the JSPS-NRCT Core University Program on Microbial Resources and the Integration Project: Innovations for the Improvement of Food Safety and Food Quality for New World Economy, The Government Research Budget of Thailand, and Chulalongkorn University.

References

- 1) Kosaric, N., Biosurfactant: production, property, applications. In "Surfactant Science Series" Vol. 48, Marcel Dekker, New York, p. 483 (1993).
- 2) Desai, J. D., and Banat, I., Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.*, **61**, 47–64 (1997).
- 3) Banat, I. M., Makkar, R. S., and Cmetra, S. S., Potential commercial application of microbial surfactants. *Appl. Microbiol. Biotechnol.*, **53**, 495–508 (2000).
- 4) Kitamoto, D., Isoda, H., and Nakahara, T., Functions and potential applications of glycolipid biosurfactants from energy-saving materials to gene delivery carriers. *J. Biosci. Bioeng.*, **94**, 187–201 (2002).
- 5) Van Bogert, I. N. A., Saerens, K., Muynck, C. D., Develter, D., Soetaert, W., and Vandamme, E. J., Microbial production and application of sophorolipids. *Appl. Microbiol. Biotechnol.*, **76**, 23–34 (2007).
- 6) De Koster, C. G., Heerma, W., Pepermans, H. A. M., Groenewegen, A., Peters, H., and Haverkamp, J., Tandem mass spectrometry and nuclear magnetic resonance spectroscopy studies of *Candida bombicola* sophorolipids and product formed on hydrolysis by cutinase. *Anal. Biochem.*, **230**, 135–148 (1995).
- 7) Hu, Y., and Ju, L. K., Sophorolipid production from different lipid precursors observed with LC-MS. *Enzyme Microbiol. Technol.*, **29**, 593–601 (2001).
- 8) Davila, A. M., Marchal, R., and Vandecasteele, J. P., Sophorose lipid fermentation with differentiated substrate supply for growth and production phases. *Appl. Microbiol. Biotechnol.*, **47**, 496–501 (1997).
- 9) Rau, U., Manzke, C., and Wagner, F., Influence of substrate supply on the production of sophorose lipids by *Candida bombicola* ATCC 22214. *Biotechnol. Lett.*, **18**, 149–154 (1996).
- 10) Nunez, A., Ashby, R., Foglia, T. A., and Solaiman, D. M. Y., LC/MS analysis and lipase modification of the sophorolipids produced by *Rhodotorula bogoriensis*. *Biotechnol. Lett.*, **26**, 1087–1093 (2004).
- 11) Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y., and Imanaka, T., A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. *J. Bacteriol.*, **175**, 6459–6466 (1993).
- 12) Barnett, J. A., Payne, R. W., and Yarrow, D., "Yeast: Characteristics and Identification" 3rd ed., Cambridge University Press, Cambridge, p. 1139 (2000).
- 13) Yarrow, D., Methods for isolation, maintenance and identification of yeasts. In "The Yeasts, a Taxonomic Study" 4th ed., eds. Kurtzman, C. P., and Fell, J. W., Elsevier, Amsterdam, pp. 77–100 (1998).
- 14) Bruke, D., Dawson, D., and Sterns, T., "Methods in Yeast Genetics: Cold Spring Harbor Laboratory," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 111–112 (2000).
- 15) Hua, Z., Chen, Y., Du, G., and Chen, J., Effects of biosurfactants produced by *Candida antractica* on the biodegradation of petroleum compounds. *World J. Microbiol. Biotechnol.*, **20**, 25–29 (2003).
- 16) Limtong, S., Srisuk, N., Yongmanitchai, W., Kawasaki, H., Yurimoto, H., Nakase, T., and Kato, N., Three new thermotolerant methylotrophic yeasts, *Candida krabiensis* sp. nov., *Candida sithepensis* sp. nov., and *Pichia siamensis* sp. nov., isolated in Thailand. *J. Gen. Appl. Microbiol.*, **50**, 119–127 (2004).
- 17) Limtong, S., Srisuk, N., Yongmanitchai, W., Yurimoto, H., Nakase, T., and Kato, N., *Pichia thermomethanolica* sp. nov., a novel thermotolerant methylotrophic yeast isolated in Thailand. *Int. J. Sys. Evol. Microbiol.*, **55**, 2225–2229 (2005).
- 18) Kim, S. Y., Oh, D. K., Lee, K. H., and Kim, J. H., Effect of soybean oil and glucose on sophorolipid fermentation by *Torulopsis bombicola* in continuous culture. *Appl. Microbiol. Biotechnol.*, **48**, 23–26 (1997).
- 19) McCaffrey, W. C., and Cooper, D. G., Sophorolipids production by *Candida bombicola* using self-cycling fermentation. *J. Ferment. Bioeng.*, **79**, 146–151 (1995).
- 20) Cooper, D. G., and Paddock, D. A., Production of a

- biosurfactant from *Torulopsis bombicola*. *Appl. Environ. Microbiol.*, **47**, 173–176 (1984).
- 21) Casas, J. A., and Garcia-Ochoa, F., Sophorolipid production by *Candida bombicola*: medium composition and culture methods. *J. Biosci. Bioeng.*, **88**, 488–494 (1999).
- 22) Daniel, H. J., Otto, R. T., Reuss, M., and Syldatk, C., Sophorolipid production with high yields on whey concentrate and rapeseed oil without consumption of lactose. *Biotechnol. Lett.*, **20**, 805–807 (1998).
- 23) David, A., Cavalero, D. A., and Cooper, D. G., The effect of medium composition on the structure and physical state of sophorolipids produced by *Candida bombicola* ATCC 22214. *J. Biotechnol.*, **103**, 31–41 (2003).
- 24) Gumienna, M., Czarnecka, M., and Czarnecki, Z., Effect of selected lipid substrates on the process of biosynthesis of surface-active compounds by the *Candida bombicola* yeasts. *EJPAU*, **8**(2), #12 (2005). Available online at <http://www.ejpau.media.pl/volume8/issue2/abs-12.html>
- 25) Zhou, Q., and Kosaric, N., Utilization of canola oil and lactose to produce biosurfactant with *Candida bombicola*. *J. Am. Oil Chem. Soc.*, **72**, 67–71 (1995).
- 26) Litchfield, J. N., Single-cell protein. *Science*, **219**, 740–746 (1983).
- 27) Shepherd, R., Rocky, J., Sutherland, J. W., and Roller, S., Novel bioemulsifiers from microorganisms for use in foods. *J. Biotechnol.*, **40**, 207–217 (1995).