J. Gen. Appl. Microbiol., **52**, 215–222 (2006)

Full Paper

Biosurfactant production by *Pseudomonas aeruginosa* A41 using palm oil as carbon source

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(Received June 29, 2005; Accepted July 28, 2006)

Biosurfactant production by *Pseudomonas aeruginosa* A41, a strain isolated from seawater in the gulf of Thailand, was examined when grown in defined medium containing 2% vegetable oil or fatty acid as a carbon source in the presence of vitamins, trace elements and 0.4% NH_4NO_3, at pH 7 and 30°C with 200 rpm-shaking for 7 days. The yield of biosurfactant steadily increased even after a stationary phase. Under such conditions the surface tension of the medium was lowered from 55–70 mN/m to 27.8–30 mN/m with every carbon source tested. However, types of carbon sources were found to affect biosurfactant yield. The yields of rhamnolipid biosurfactant were 6.58 g/L, 2.91 g/L and 2.93 g/L determined as rhamnose content when olive oil, palm oil and coconut oil, respectively, were used as a carbon source. Among them, biosurfactant obtained from palm oil was the best in lowering surface tension of the medium. Increase in biosurfactant activities in terms of oil displacement test and rhamnose content were observed to be higher with shorter chain fatty acids than that of the longer chains (C12>C14>C16). In addition, we found that C18:2, highly unsaturated fatty acid, showed higher oil displacement activity and rhamnose content than that of C18:1. The optimal oil displacement activity was found at pH 7–9 and in the presence of 0.5–3% NaCl. The oil displacement activity was stable to temperatures up to 100°C for 15 h. Surface tension reduction activity was relatively stable at pH 2–12 and 0–5% of NaCl. Emusification activity tested with various types of hydrocarbons and vegetable oils showed similarity of up to 60% stability. The partially purified biosurfactant via TLC and silica gel column chromatography gave three main peaks on HPLC with mass spectra of 527, 272, and 661 *m*/*z* respectively, corresponding to sodium-monorhamnodecanoate, hydroxyhexadecanoic acid and an unknown compound, respectively.

Key Words——biosurfactant; coconut oil; fatty acids; palm oil; *Pseudomonas aeruginosa*; rhamnolipids

Introduction

Biosurfactants are biological amphiphatic compounds consisting of hydrophilic and hydrophobic domains. Microorganisms have been reported to produce several classes of biosurfactants such as glycolipids,

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lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants (Cooper, 1986; Cooper and Zajic, 1980; Kosaric, 1993). These compounds are produced during the growth of microorganisms on water-soluble and water immiscible substrates (Jenny et al., 1993; Ron and Rosenberg, 2001; Sheppard and Mulligan, 1987).

Up to now, the most commonly isolated and best studied groups of biosurfactants are those of glycolipid compounds and phospholipids (Mata-Sandoval et al., 1999). Rhamnolipids are glycolipid compounds produced by *Pseudomonas* sp. which could reduce water surface tension and emulsify oil (Babu et al., 1996; Deziel et al., 1999; Lang and Wullbrandt, 1999; Mata-Sandoval et al., 1999; Patel and Desai, 1997; Rahman et al., 2002). These compounds are biodegradable and have potential industrial and environmental applications.

The rhamnolipids produced by *Pseudomonas aeruginosa* in liquid culture mainly contained one or two molecules of rhamnose linked to one or two β-hydroxy fatty acids as mono-rhamnolipid acid group (L-rhamonsyl-β-hydroxydecanoyl-β-hydroxydecanoate, Rha $C_{10}C_{10}$) and dirhamnolipid acid group (L-rhamonsyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, Rha Rha $C_{10}C_{10}$). Unsaturated mono- and di-rhamnolipids containing dehydrogenated lipid ($C_{10}C_{12:1}$ or $C_{10}C_{14:1}$) have also been reported (Deziel et al., 1999, 2000; Mata-Sandoval et al., 1999; Rendell et al., 1990). Although the potential for biosurfactant production is determined by the genetics of the microorganisms, other factors such as environmental conditions and the nature of the substrates also influence the level of expression. There are reports regarding the production of biosurfactants from different substrates such as some vegetable oil, whey, molasses, wastewater from olive oil processing and distillery wastes (Babu et al., 1996; Daniel et al., 1988; Patel and Desai, 1997; Rahman et al., 2002). However, there have been no reports regarding the production of biosurfactant from some substrates such as palm oil which is cheap and abundantly available in Southeast Asia and fatty acids by bacteria. Therefore, biosurfactant production using palm oil and fatty acids of various chain lengths as a carbon source will be described in this paper.

Pseudomonas aeruginosa A41 was isolated from marine environment and was able to produce substances with surface properties upon growing in a defined medium (Chongchin, 1999). The present study elucidates I) production of biosurfactant by *Pseudomonas aeruginosa* A41 grown on various carbon sources; II) properties and structural analysis of biosurfactants produced from the culture grown in palm oil medium; and III) biosurfactant activity and stabilities in a wide range of pH, temperature and NaCl concentration.

Materials and Methods

Bacterial strain. Pseudomonas aeruginosa A41, an isolate from the gulf of Thailand (Chongchin, 1999; Thaniyavarn et al., 2001), was used throughout this work.

Biosurfactant production. Pseudomonas A41 was cultivated in 50 ml-defined medium (pH 7.0) containing 0.4% NH_4NO_3, 0.04% $MgSO_4 \cdot 7H_2O$, 0.02% KCl, 0.01% $CaCl_2 \cdot 2H_2O$, 0.14% $NaHPO_4 \cdot 12H_2O$, vitamins and trace elements (Jenny et al., 1993) and 2% (w/v) carbon source of different types which included coconut oil, palm oil, olive oil, lauric acid, myristic acid, palmitic acid , stearic acid, oleic acid and linoleic acid. Cultivation was at 30°C for 7 days with agitation speed of 200 rpm. The culture supernatant was used for biosurfactant determination.

Determination of growth and biosurfactant production. The surface tension of the culture supernatant was measured by using a ring tensiometer (K_6, Kruss, Germany). Oil displacement test was performed by determining the oil displacement area as described by Morikawa et al. (1993). Rhamnolipid production was determined as the amount of rhamnose by a specific colorimetric method (Dische and Shettles, 1948). The microbial growth was determined by cell dry weight.

Preparation of the biosurfactant crude extract. For biosurfactant production, *Pseudomonas aeruginosa* A41 was grown in 3 L-defined medium containing 2% palm oil at 30°C for 72 h with agitation speed of 200 rpm. Cells were removed by centrifugation at 8,000 rpm and the biosurfactant was recovered by acidifying the supernatant to pH 2.0 with 6 M HCl, and the precipitate was collected by centrifugation at 12,000 rpm for 20 min. It was dissolved in 0.1 M Tris-HCl (pH 8) and extracted three times with chloroform : ethanol (2 : 1). The organic solvent layer was evaporated under vacuum on a rotary evaporator to dryness, and used as the crude extract for further analysis.

Determination of fatty acids. Methylation of fatty

acid components in biosurfactants was performed according to Jenkin et al. (2001). Analysis of fatty acid compositions of the biosurfactants from every carbon source were analyzed by GC-MS using HP 5973B Agilent Technologies equipped with a DB-5 MS crosslinked 5% Ph Me Silicone column (25 m×0.25 cm I.D.).

Effect of pH, NaCl and temperature on biosurfactant activity. The crude extract (100 mg) was dissolved in 10 ml of 50 mM Tris-HCl and kept at pH 2–12 or in the presence of 0.5–5.0% NaCl or at 4–100°C. Then oil displacement activity and surface tension reduction activity were measured.

Effect of pH, NaCl and temperature on biosurfactant stability. Preparation of biosurfactant for stability test towards temperature, pH and NaCl was done by dissolving 100 mg of the crude extract in 10 ml of 50 mM Tris-HCl. For temperature stability, it was incubated at 100°C at pH 8.0 for 15 h or at 121°C for 20 min; for pH stability, it was tested at 2–12 for 6 months; for NaCl stability, it was tested at 0.5–5.0% NaCl at pH 8.0 for 6 months. After that, oil displacement activity and surface tension reduction activity were measured.

Determination of emulsification index. Emulsification index was determined by adding 6 ml of hydrocarbons or vegetable oils which included cyclohexane, hexane, toluene, benzene, palm oil, soybean oil and olive oil to 10 mg crude extract dissolved in 4 ml of 50 mM Tris-HCl pH 8. The mixture was vortexed for 5 min and left standing for 24 h. The emulsification index was determined as the percentage of the height of the emulsified layer divided by the total height of liquid column as described by Patel and Desai (1997). The stability of the emulsions was also assayed for up to 1 month.

Purification of biosurfactant by chromatography. Three-liter-culture supernatant from the palm oil medium was used for biosurfactant purification. The crude extract prepared as described above was dissolved in chloroform and analyzed by analytical and preparative thin layer chromatography (TLC) on silica gel plates (G60: Merck, Germany), which were developed with chloroform : methanol : acetic acid (65 : 25 : 4 v/v) and visualized with TLC reagents, i.e. iodine vapors for lipid staining and α-naphthol-H_2SO_4 (molish reagent) for sugar detection. All fractions were then subjected to oil displacement test. The positive fractions from preparative TLC were further purified by silica gel column chromatography eluted stepwisely with 100–40% chloroform in methanol (containing 0.5% acetic acid) with 10% decrements for each step. Each fraction was evaporated to dryness and subjected to oil displacement test. The active fractions were further purified by HPLC using Cosmosil 5 C18-AR column (4.6×150 mm, Nacalai Tesque, Kyoto, Japan) and a HP1100 system (Hewlett-Packard, Fort Collins, CO, USA) operating at 0.6 ml/min with a UV detector set at 220 nm. Elution was with a linear gradient of acetonitrile (10–100%) in 0.1% trifluoroacetic acid (TFA). The active fractions were subjected to LC-MS analysis.

LC-MS analysis. The purified samples were analyzed by an electron-spray ionization mass spectrometer (LCQ, ThermoFinnigan, San Jose, CA, USA) under the conditions recommended by the manufacturer.

Results

Biosurfactant production and culture conditions

Biosurfactant production by *Pseudomonas aeruginosa* A41 was examined under different carbon sources in defined medium containing 2% oil or fatty acid and 0.4% NH_4NO_3 cultivated at 30°C with agitation speed of 200 rpm for 7 days (pH 7). The yield of biosurfactant steadily increased even after the bacterial culture reached the stationary phase. The surface tension of the medium was lowered from 55–70 mN/m to 27.8–30 mN/m with every carbon source tested. However the biosurfactant yield was affected by the type of carbon source used (Table 1). The amounts of rhamnolipid produced were found to be 6.58 g/L, 2.93 g/L and 2.91 g/L as rhamnose content when grown on olive oil, palm oil and coconut oil, respectively. Under such conditions, the Δ surface tension of the palm oil culture showed the highest value, from 70 mN/m to 30 mN/m (Fig. 1). Increased biosurfactant activities in terms of oil displacement test and rhamnose content were observed to be higher in the culture with shorter chain fatty acids than those of longer chains (C12>C14>C16). Furthermore we found that with highly unsaturated fatty acid, linoleic acid (C18:2), the biosurfactant possessed higher oil displacement activity and rhamnose content than those with C18:1.

Analysis of fatty acid(s) component in biosurfactants obtained from culture grown on various carbon sources

GC-MS analysis showed the predominance of 3-hydroxydecanoic acid in biosurfactants produced from all

Table 1. Comparative study of growth and biosurfactant production in a culture medium containing difference carbon sources.

Carbon source	Rhamnose (g/L)	Surface tension (mN/m)	Δ Surface tension **b-t** (mN/m)	Oil displacement test (cm^2) Dilution 10^{-1}
Olive oil	6.58	30	30.8	70.63
Palm oil	2.91	30	40	66.85
Coconut oil	2.93	30	31	50.24
Lauric acid (C12)	3.39	29.5	33.9	60.79
Myristic acid (C14)	1.89	27.8	34.5	41.30
Plamitic acid (C16)	0.26	29.7	38	0.05
Stearic acid (C18)	2.26	30	35	56.72
Oleic acid (C18:1)	1.58	29.9	34	52.78
Linoleic acid (C18:2)	4.99	29.9	33.5	71.25

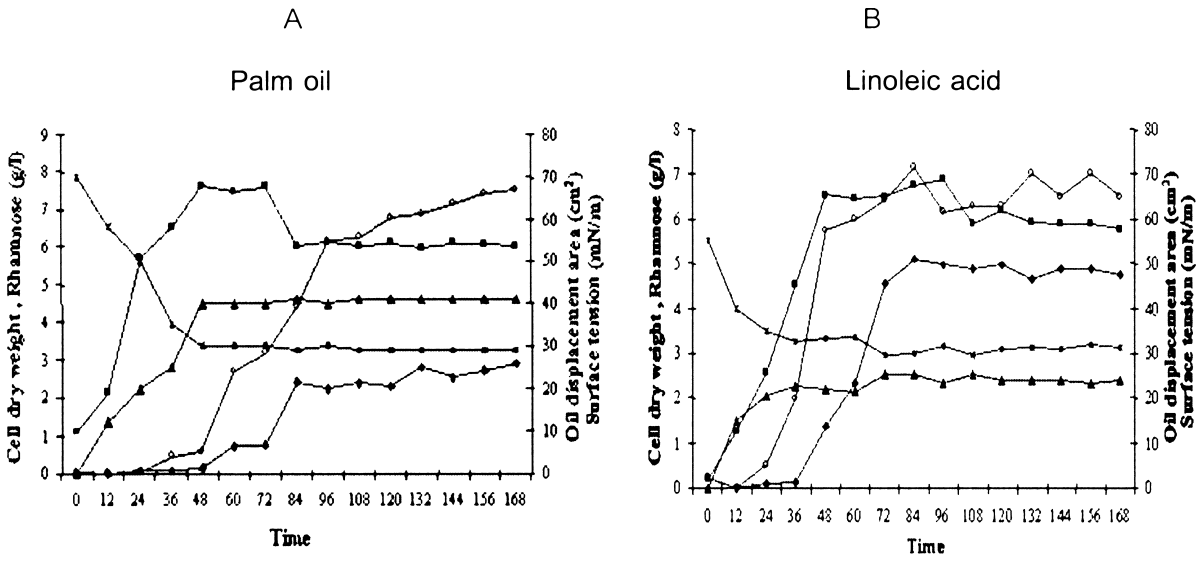

Fig. 1. The representative profiles of growth, rhamnose content, oil displacement and surface tension of supernatant from *Pseudomonas* sp. A41 when cultivated in a defined medium with two types of carbon source.

A) Palm oil; B) Linoleic acid. (■) dry weight (g/L); (◆) rhamnose content (g/L); (×) surface tension (mN/m); (▲) surface tension **b-t**; (○) area (cm^2) dilution 10^{-1}. Δ surface tension **b-t** is defined as the surface tension of culture medium at the beginning minus at the interval time.

types of carbon sources within 36 h of cultivation except from palmitic acid (Fig. 2, Table 2), indicating that the biosurfactants were glycolipid species with C10 as the main component. Unsaturated fatty acid-carbon sources gave faster production and higher yield than those of other fatty acids.

In the early results we showed that olive oil yielded the highest biosurfactant production compared to palm oil and coconut oil. However, it is the most expensive substrate. Palm oil is cheap and abundantly available in Southeast Asia and gave considerably high yield of biosurfactant with highest value of Δ surface tension from 70 mN/m to 30 mN/m. Therefore, we chose palm oil as a carbon source for further study on biosurfactant production, properties and structural analysis.

Properties of biosurfactant

pH. The crude extract of biosurfactant dissolved in 50 mM Tris-HCl at pH 7–9 exhibited the best oil displacement activity whereas the surface tensions at pH between 2–12 were not different at about 30–29 mN/m. It could retain the oil displacement activities at

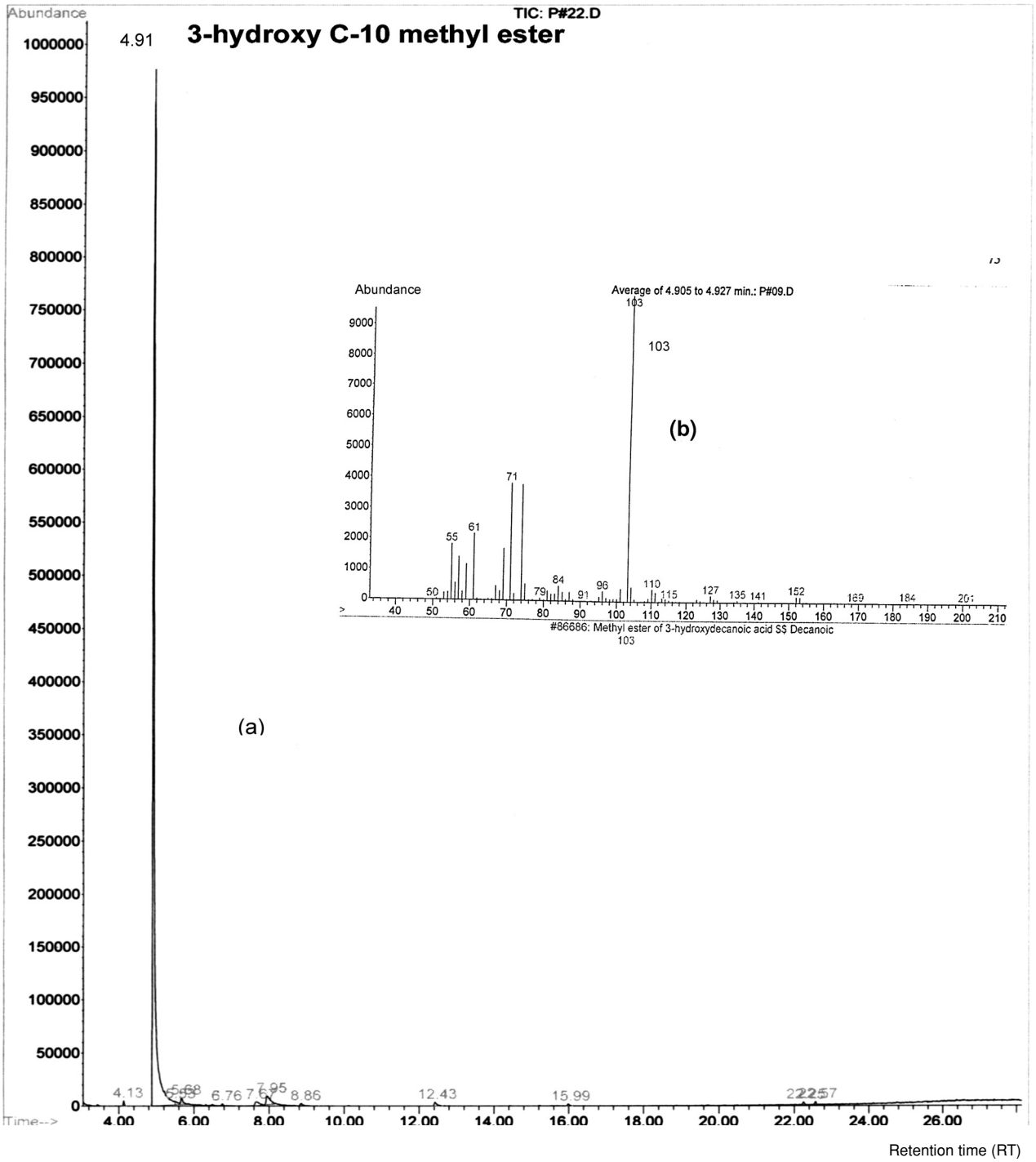

Fig. 2. (a) GC chromatogram of fatty acids composed in a biosurfactant produced from various carbon sources at 168 h of cultivation. (b) GC-MS chromatogram of methyl ester of 3-hydroxydecanoic acid at retention time 4.9.

pH 7–12 and surface tension activities at various pH for 6 months before bringing back to the optimum pH of 8 at 25°C.

NaCl. Optimum oil displacement activity of the crude extract was found in the presence of NaCl at the concentrations between 0.5–3.0% in the range of 10.18–11.57 cm^2 but the activity decreased at higher NaCl concentration to the minimum activity of 0.5 cm^2 at 5.0% NaCl. No difference in terms of surface tension was observed at 0.5–5.0% NaCl (29.0 mN/m). Oil displacement activity was stable after it was incubated in the presence of 0.5–3% NaCl for 6 months whereas

Table 2. Incidence of 3-hydroxydecanoic acid of biosurfactants produced from *P. aeruginosa* A41 when grown on different carbon sources by GC analysis.

Time (h)	Carbon sources								
	Coconut oil	Palm oil	Olive oil	C12	C14	C16	C18	C18:1	C18:2
0	−	−	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−	−	−
24	−	−	−	trace	trace	−	trace	+	+
36	trace	trace	trace	+	+	−	+	++	++
48	trace	trace	trace	++	++	trace	++	+++	+++
60	trace	++	++	++	++	+	++	+++	+++
72	+	++	++	+++	+++	++	+++	+++	+++
84	++	+++	+++	+++	+++	+++	+++	+++	+++
96	+++	+++	+++	+++	+++	+++	+++	+++	+++
108	+++	+++	+++	+++	+++	++	+++	+++	+++
120/168	+++	+++	+++	+++	+++	+/−	+++	+++	+++

+, small amount; ++, medium amount; +++, large amount; −, negative test.

the surface tension activity was stable to NaCl at the concentration ranging from 0.5–5%.

Temperature. Oil displacement activity of the crude extract was not affected by the gradient of temperature when determined at 4–100°C for 10 min. However for surface tension activity, we could not determine at different temperatures as the suitable determination condition recommended by the manufacturer is 25°C. For temperature stability after incubation at 121°C for 20 min or 100°C for 15 h, oil displacement activity and surface tension activity remained.

Emulsification index and stability. Emulsion of hydrocarbons and vegetable oils with the crude extract showed the same values which were up to 60% in 24 h. The stability of the emulsion was relatively good; however, the emulsion formed with vegetable oil collapsed from 60 to 38% after 1 month.

Purification of biosurfactants and their molecular weights determination

After cultivation for 72 h in the palm oil medium, the crude extract of biosurfactant (13.35 g/L) was prepared from the culture supernatant as described in MATERIALS AND METHODS. It was analyzed by TLC and visualized with specific reagents showing positive spots at R_f 0.33, 0.61, 0.68, 0.75, 0.83 and 0.95 (F1–F6), respectively (Fig. 3). All fractions (F1–F6) were subjected to oil displacement test. Detection of F5 with α-naphthol–H_2SO_4 (R_f 0.83) indicated it is a glycolipid which is common with *Pseudomonas* which usually produces

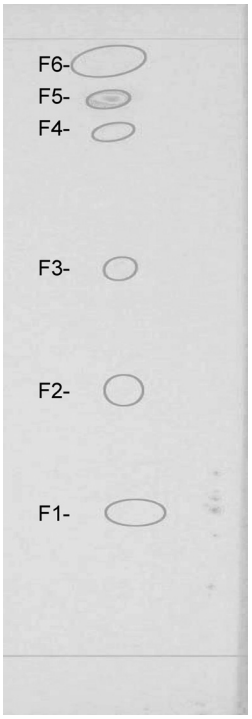

Fig. 3. TLC profile of the crude extract of biosurfactant from *Pseudomonas* sp. A41 when cultivated in palm oil medium.

F1–F6 are positive spots with iodine vapor and F5 is positive spot with α-naphthol reagent.

rhamnolipid (Arino et al., 1996; Lang and Wullbrandt, 1999). The active fractions F1–F6 were further purified by preparative TLC following by silica gel column chromatography and HPLC as described in MATERIALS AND METHODS. The main active fractions eluted from HPLC

Table 3. Characteristics of samples A, B and C, respectively.

Sample	RT from LC (min)	$M+H^+$	Structure
A	7.28	272.3	Hydroxyhexadecanoic acid
	24.02	295.3	Sodiumhydroxyhexadecanoic acid
B	23.15	527.4	Rha $C_{10}C_{10}Na^+$
C	28.36	661.6	Unknown compound

were obtained at the retention times of 13.66 min (for F4), 15.99 min (for F5) and 19.76 min (for F5), respectively. These 3 fractions were tentatively named A, B and C, respectively. They were subjected to LC-MS analysis and revealed the mass spectra of $M+H^+$ at *m*/*z* 272.3 and 295.3 for A; at *m*/*z* 527.4 for B; and at *m*/*z* 661.6 for C which corresponded to hydroxylhexadeconoic acid and its sodium salt, sodium monorhamnodecanoate, and a rhamnolipid with unidentified fatty acids compound, respectively (Table 3).

Discussion

The present work demonstrated that rhamnolipid biosurfactant of 6.58, 2.91 and 2.93 g/L were produced by *P. aeruginosa* A41 when grown on 2% olive oil, palm oil and coconut oil, respectively. These were higher than that of *P. aeruginosa* (2.7 g/L) grown on 4% mixed waste frying olive and sunflower oils (Haba et al., 2000) and comparable to that of *P. aeruginosa* 44 T1 (7.65–10 g/L) grown on 2% olive oil (Manresa et al.,1991; Robert et al.,1989). However, there have been reports on about 100 g/L of rhamnolipid produced from 16% of soybean oil (Lang and Wullbrant, 1999) and about 4.3 g/L from 0.6% of soybean oil (Rahman et al., 2002). The surface tension reduction ability of the biosurfactant was found to be 27.8–30 mN/m which was in the same range as those of other rhamnolipids reported from *P. aeruginosa* DSM.2874 (28 mN/m) (Syldatk et al., 1985), *P. aeruginosa* 44 T1 (25 mN/m) and *P. aeruginosa* BP100 (28 mN/m) (Lang and Wullbrant, 1999). Although the biosurfactant yield with palm oil as a carbon source was lower than that of olive oil, it showed quite good properties in both surface tension reduction and oil displacement activity (Table 1). Moreover, this is the first report on using palm oil as a carbon source for biosurfactant production. This indicated that low cost-large scale production of the biosurfactant from this organism is possible as palm oil is cheap and abundantly available.

Furthermore, we found that biosurfactants produced from fatty acids with shorter chain length gave higher oil displacement activity and the rhamnose content than that with longer chain length (C12>C14>C16). In addition, using highly unsaturated fatty acid also gave higher oil displacement activity as well as the rhamnose content (C18:2>C18:1). This agreed with Ferraz et al. (2002) who studied the influence of vegetable oils on biosurfactant production by *Serratia marcescens* and suggested that linoleic acid which was about 60% composed in sunflower oil might be responsible for the increase in biosurfactant production. However, our work is the first in reporting using free fatty acids as a carbon source for biosurfactant production by bacteria.

For the characteristics of biosurfactants, mostly they were reported under moderate conditions (Lang and Wullbrant, 1999; Ron and Rosenberg, 2001) but very little information has been reported from extreme environments (Yakimov et al., 1995). The present work showed that the rhamnolipid biosurfactant produced by *P. aeruginosa* A41 from palm oil medium had good activity and stability in wide ranges of pH, temperature and NaCl concentration, indicating that it will have high potential for application in various environments.

Acknowledgments

The present study was partially supported by a research grant from National Research Council of Thailand through JSPS-NRCT Large scale Co-operative Programme in Biotechnology and Ratchadaphisek Somphot Endowment Fund from Chulalongkorn University.

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