

Full Paper

Multiple biosurfactant production by *Aureobasidium pullulans* strain YTP6-14 in aqueous and heavy oil layers

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Aureobasidium pullulans YTP6-14 was demonstrated to be an excellent multiple biosurfactant producer utilizing cheap carbon sources available in Thailand, including glycerol and cassava flour hydrolysate. *A. pullulans* YTP6-14 maximally produced 1.81 g/l biosurfactant in an aqueous layer (BS-AQ) in a medium containing glycerol, and 7.37 or 6.37 g/l biosurfactant in a heavy oil layer (BS-HO) in cassava flour hydrolysate or a glucose containing medium, respectively. Each BS-AQ and BS-HO had critical micelle concentration values of 41.32 mg/l and 13.51 mg/l, and both biosurfactants formed a stable food oil emulsion and reduced the amount of biofilms formed by *Streptococcus sobrinus* and *Streptococcus mutans*. BS-AQ and BS-HO were mainly composed of liamocins or exophilins and massoia lactone, respectively.

Key Words: *Aureobasidium pullulans*; biosurfactant; exophilin; heavy oil; liamocin; massoia lactone

Introduction

Aureobasidium pullulans is a yeast like-fungus which is found in various environments; e.g., soil, leaf surfaces, walls, rock, hypersaline water, and even the North Pole (Deshpande et al., 1992; Gostin et al., 2014; Gunde-Cimerman et al., 2000; Urzi et al., 1999; Zalar et al., 2008). Its product polysaccharide, named “pullulan”, is widely used in the food industries as a thickener. *A. pullulans* also produces other useful bioactive compounds, such as beta-glucan, extracellular enzymes, fungicidal and antibacte-

rial compounds, heavy oil, and biosurfactants (Gostin et al., 2014; Luepongpatana et al., 2017). The heavy oil was composed of fatty acid esters of arabitol and mannitol, in which the fatty acids were 3,5-dihydroxydecanoic acid and 5-hydroxy-2-decanoic acid (Kurosawa et al., 1994).

A. pullulans produces biosurfactants in both an aqueous layer (BS-AQ) and a heavy oil layer (BS-HO) of the culture liquid. BS-HO was produced by *A. pullulans* strain CU43 at 6 g/l in 5% sucrose medium (Manitchotpisit et al., 2011). The BS-HO was then characterized by Price et al. (2013), and they found novel biosurfactants: liamocins. Liamocins consist of a single mannitol head group partially *O*-acylated with three or four 3,5-hydroxydecanoic ester groups, thus they were classified into four types (A1, A2, B1 and B2). The relationships between the types of liamocin produced and the carbon sources used for fungal growth are unclear. Different strains of *A. pullulans* and different culture media were reported to affect the cell growth, BS-HO production, and pigmentation (Leathers et al., 2015; Price et al., 2016). BS-HO was reported to have antibacterial properties against Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus* spp. In addition, BS-HO can inhibit the growth of mammalian breast and cervical cancer cell lines (Bischoff et al., 2015; Manitchotpisit et al., 2014).

A. pullulans also produces several biosurfactants in an aqueous layer, BS-AQ. For example, strain L3-GPY produces glycerol-liamocin, aureosurfactin, and 3-deoxyaureosurfactin (Kim et al., 2015, 2016). We previously reported massoia lactone (ML), a flavor biosurfactant, produced by *A. pullulans* YTP6-14 in a medium containing 2.5% each of glucose and glycerol (1.98% total carbon content) (Luepongpatana et al., 2017). ML is industrially produced by extraction from the bark of the tree *Cryptocarya massoia* (Rali et al., 2007; Rolli

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et al., 2016) or chemically synthesized (Vesonder et al., 1972). ML is widely used as an additive in food due to its aromas of coconut, cream, and butter (Mineeva, 2012).

Biosurfactants often reduced biofilm formation by several bacteria, including the producing strains (Banat et al., 2014). For instance, putisolvins produced by *Pseudomonas putida* PCL1445 decreased the formation of its own biofilms (Kuiper et al., 2004). *Streptococcus sobrinus* and *Streptococcus mutans* are the main causes of dental caries due to the production of robust biofilms on the surface of tooth and dental materials (Hahnel et al., 2012). Moreover, tooth surfaces are often damaged by the products of organic acids, such as lactic acid, by the fermentation of sugars such as sucrose, fructose and glucose present in foods and drinks (Choi et al., 2016).

This research examined the productivity of BS-AQ and BS-HO by *A. pullulans* YTP6-14 grown on four different carbon sources (glucose, sucrose, glycerol, and cassava flour hydrolysate). The components of BS-AQ and BS-HO were analyzed by high-performance liquid chromatography and gas chromatography/mass spectrometry. Biofilm reduction of *A. pullulans* YTP6-14, *S. sobrinus* ATCC 6715, and *S. mutans* ATCC 25175 by BS-AQ and BS-HO was also investigated.

Materials and Methods

Microorganisms and culture conditions. *A. pullulans* YTP6-14 was isolated from the coastal area of Koh Si Chang, Chonburi, Thailand (Thaniyavarn et al., 2013) and deposited in the Thailand Bioresource Research Center (TBRC) under accession number TBRC 5448. *A. pullulans* YTP6-14 was kept at 20°C and twice re-cultured on YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar, pH 4.5) at 30°C for 4 days.

Dental plaque-forming strains, *S. sobrinus* ATCC 6715 and *S. mutans* ATCC 25175 were obtained from Dr. Ratchapin Laovanitch Srisatjaluk, Department of Oral Microbiology, Faculty of Dentistry, Mahidol University. Stocks were kept in Brain Heart Infusion (BHI) containing 2% sucrose and 15% glycerol at 20°C. The stocks were grown on BHI agar at 37°C in anaerobic conditions for 24 h. After that, colonies were transferred into BHI broth and incubated at 37°C for 24 h.

Effect of carbon sources on cell growth and biosurfactant production. *A. pullulans* YTP6-14 was precultured in 50 ml of Ymbro that 30°C with shaking at 200 rpm for 24 h. A preculture of 0.8–1.0 absorbance units (λ 600 nm) was inoculated at 10% (v/v) into 50 ml of production medium: 1.98% total carbon, 0.04% yeast extract, 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% KH_2PO_4 , 0.1% NaCl, and 0.03% peptone, pH 6.5 (Luepongpatana et al., 2017). The medium was supplemented, respectively, with different carbon sources: glucose, sucrose, glycerol, and cassava flour hydrolysate, at an amount of 1.98% total carbon. Each culture was incubated at 30°C with shaking at 200 rpm for 1, 3, 5, 7, 9, and 11 days, in triplicate.

Cassava flour hydrolysate was prepared by dissolving 15 g of cassava flour in 50 ml of deionized water, and adjusting the pH to 5.8 before the addition of 20 μl α -amylase (spezyme® alpha, Genencor, Danisco US Inc.,

USA) and then incubating at 85°C with shaking at 60 rpm for 3 h. After that, the solution was cooled in an ice bath and the pH was adjusted to 4.5 before the addition of 400 μl glucoamylase (GC 147, Genencor) and incubation at 60°C and 60 rpm for 30 min. A sugar solution was obtained after centrifugation separated out the remaining flour. The amount of reducing sugars in the solution was measured using 2,4-dinitrosalicylic acid (DNS) assay, according to the method of Miller (1959).

The cell growth (dry cell weight, DCW), the pH, the remaining carbon source(s), and BS-AQ and BS-HO, were assessed. BS-AQ and BS-HO were extracted and quantified by dry weight. Each carbon source remaining was analyzed as follows: DNS assay was used for measuring the reducing sugar, the method as developed by Kuhn et al. (2015) was used for glycerol detection, and high-performance liquid chromatography (HPLC) with a Sugar Sz5532 column (Shodex, Japan) was used for the sucrose measurement, respectively.

Biosurfactant extraction. The culture broth was centrifuged at $10,000 \times g$ for 40 min into three separated layers: an aqueous layer, cells, and heavy oil. BS-AQ was extracted three times from the aqueous layer by shaking with one volume of ethyl acetate in a separating funnel for 3 min and then collecting the ethylacetate layer. The ethylacetate fraction was rotary vacuum evaporated, transferred into 1.5 ml tubes and dried using a centrifuge evaporator (BUCHI, Switzerland). BS-AQ was collected and weighed. BS-HO was directly dissolved with ethyl acetate after removal of the layer of cells. The ethyl acetate was evaporated, and the remaining BS-HO was collected.

Oil displacement area (ODA) measurement. ODA was measured following the method of Morikawa et al. (2000). Briefly, 10 μl of BS-AQ or BS-HO at 1 mg/ml was dropped onto 20 μl of mineral oil film previously formed on 40 ml of deionized water in a 150-mm-diameter glass plate.

Critical micelle concentration (CMC) measurement. CMC is the concentration of the surfactant at which no further decrease in surface tension is detected. In this study, biosurfactants produced from a glucose medium were used for further studies. Each of BS-AQ and BS-HO was dissolved in deionized water at 0.01 to 1,000 mg/l and the surface tension (ST) was measured with 5 samples being repeated. The ST values were plotted against the log of the biosurfactant concentration for CMC determination.

Biosurfactant stability studies. The change of ST was studied over a wide range of NaCl concentrations, pH and temperature after 15 min of incubation. First, each of BS-AQ and BS-HO at twice the CMC was mixed equally with NaCl solution to a final concentration of 2–12% (w/v) NaCl. Second, each of BS-AQ and BS-HO was dissolved in deionized water (pH 2–12, adjusted with 1 M NaOH or HCl) and diluted to the CMC. Third, each of BS-AQ and BS-HO at the CMC was incubated at 4, 30, 60, 100°C, and in autoclave conditions (121°C for 15 min). ST values of each sample were measured in triplicate and are expressed as the mean ST value.

Emulsification activity (E24). Each of BS-AQ and BS-HO at 1 mg/ml (5 ml) were mixed equally with different

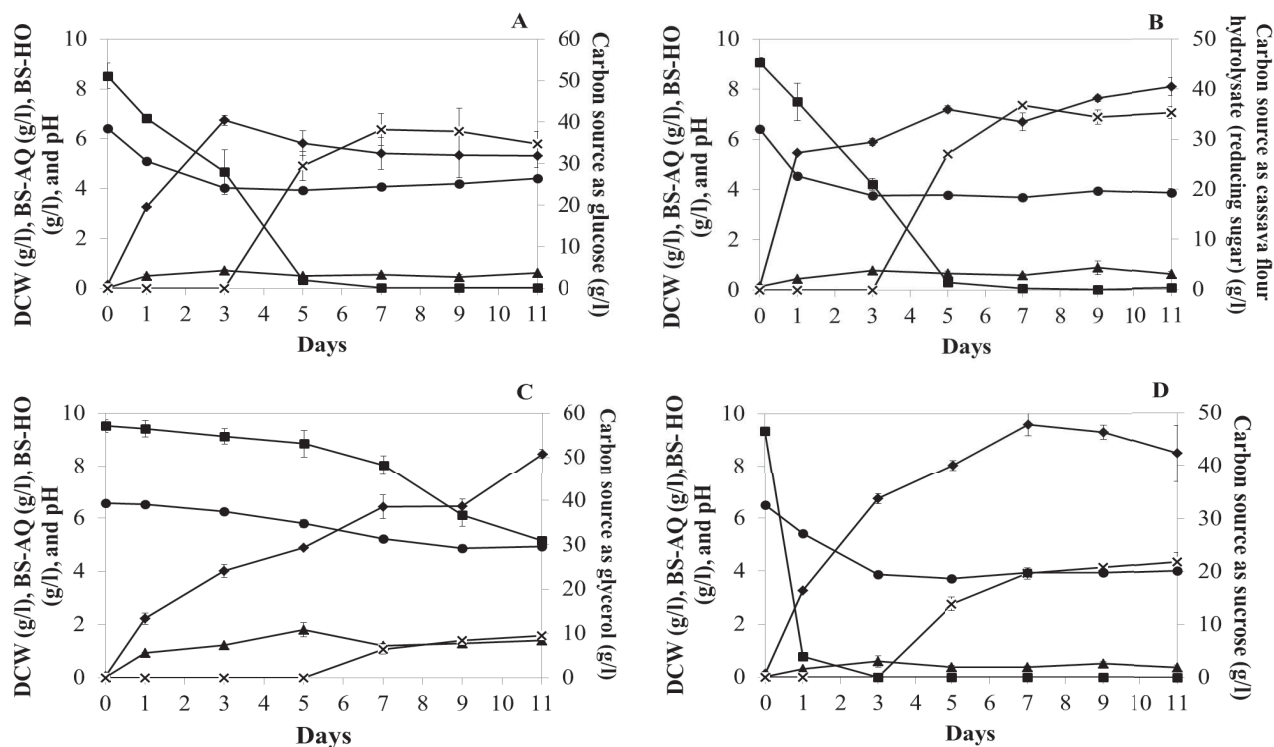


Fig. 1. Time course profiles of cell growth, pH, remaining carbon source, and amount of crude BS-AQ and BS-HO produced by *Aureobasidium pullulans* YTP6-14 in production medium containing different carbon sources.

A: glucose, B: cassava flour hydrolysate, C: glycerol, D: sucrose. Symbols: cell growth (dry cell weight, diamond), pH (circle), remaining carbon source (square), amount of BS-AQ (triangle), and amount of BS-HO (cross). Data points are average of 3 samples. The bars indicate standard deviations.

oils (olive oil, palm oil, soy bean oil, coconut oil, rice bran oil, sunflower seed oil, and sesame oil) by vortex for 2 min. The emulsion was left at room temperature for 24 h (emulsification index, E24) and then the stability was observed for 60 days (samples stored at room temperature). The emulsion was measured and quantified as the percentage of emulsion height by solution height.

Biofilm inhibition. Biofilm inhibition assays with BS-AQ and BS-HO were performed with three microorganisms (*A. pullulans* YTP6-14, *S. sobrinus* ATCC 6715 and *S. mutans* ATCC 25175). The biofilm of *A. pullulans* was grown in a yeast nitrogen base (YNB; Difco, USA) medium (0.67% YNB and 0.5% glucose) at 30°C with shaking at 200 rpm for 24 h. BHI with 2% sucrose was used for *S. sobrinus* and *S. mutans*, which were grown in an aerobic conditions at 37°C for 8 and 10 h, respectively. Each of BS-AQ and BS-HO was dissolved in solvent (2.5% of 1:1 DMSO:butanone) at 2500 µg/ml and passed through a 0.45-µm filter. Biofilm inhibition assays were performed in “pre-coating”, “co-incubation”, and “mid post-formation” conditions.

In pre-coating, flat-bottomed polystyrene 96-well plates were filled with BS-AQ in columns 3–7 and BS-HO in columns 8–12. Two-fold dilution was carried out vertically in rows 1–7 for a final volume of 100 µl and final BS concentrations of 1250, 625, 312, 156, 78, 39, and 20 µg/ml. The positive control (no cells and no biosurfactant) was in column 1, and the negative control (no

biosurfactant) was in row 8. The plates were incubated at 4°C for 24 h and then emptied and washed with sterile water. After that, 25 µl of 4× culture medium, 25 µl of cell aliquot (OD = 0.4 at λ600 nm), and 50 µl of sterile normal saline were added into the test wells. The plates were incubated under different conditions according to the type of microorganism.

In the co-incubation experiments, BS-AQ and BS-HO were prepared in a final volume of 50 µl and final concentrations of 1250, 625, 312, 156, 78, 39, and 20 µg/ml. Then, 25 µl of 4× culture medium and 25 µl of cell aliquot (OD = 0.4 at λ600 nm) were added into the test wells. The plates were incubated under different conditions according to the type of microorganism.

In the mid post-formation experiments, 25 µl of 4× culture medium and 25 µl of cell aliquot (OD = 0.4 at λ600 nm) were added into 50 µl of 0.85% NaCl. The plate was incubated for half the time that the experiment was scheduled to run (“half time”). Then, 100 µl of BS-AQ or BS-HO stock were added to final concentrations of 1250, 625, 312, 156, 78, 39 and 20 µg/ml. Incubation was then continued to the end of the experiment (“full time”; *A. pullulans* 24 h, *S. mutans* 8 h, *S. sobrinus* 10 h).

In all biofilm assays, each concentration was repeated 5 times and the highest and lowest values were excluded.

Biofilms were quantified by the method of Stepanović et al. (2000). Briefly, a biofilm was incubated with 100 µl of 99% methanol for 15 min. The methanol was removed

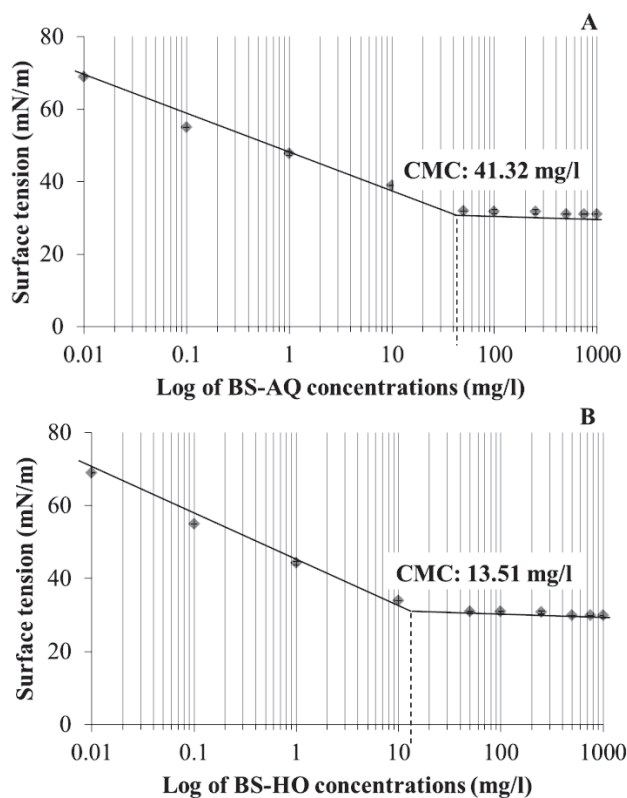


Fig. 2. The relationship between the log value of biosurfactant (BS) concentration and surface tension.

CMC indicates the critical micelle concentration. Panels show BS-AQ (A) and BS-HO (B). Data points are average of 3 samples.

and the biofilm was left to dry before staining for 15 min with 120 μ l of 0.02% crystal violet. The excess dye was removed and the well washed with 150 μ l of water. The remaining dye was dissolved with 200 μ l of 33% acetic acid and the absorbance measured at λ 540 nm.

Molecular analyses. Each of the BS-AQ and BS-HO was dissolved in 100% acetonitrile and passed through a 0.22- μ m filter. The filtrate was analyzed by HPLC (Prostar, Varian, USA) using a 5C18-AR Cosmosil reverse phase column, 4.6 \times 150 mm (Nacalai Tesque Inc., Kyoto, Japan) with gradient elution by solvent A (0.1% trifluoroacetic acid [TFA] in 10% acetonitrile) and solvent B (0.1% TFA in 100% acetonitrile) at a flow rate of 0.5 ml/min, monitored at 200 nm using a UV detector. Fractions obtained from HPLC were detected for ODA. High activity fractions were analyzed by gas chromatography/mass spectrometry (GC/MS) and matrix-assisted laser desorption/ionization-time of flight MS (MALDI-TOF/MS) at the Instrumental Analysis Center, Hokkaido University, Japan. Crude BS-HO was also analyzed by MALDI-TOF/MS. GC/MS was performed using a JMS-T100GCV mass spectrometer (JEOL, Tokyo).

Statistical analysis. Statistically significant differences were examined using one-way analysis of variance, tested in Duncan's multiple comparison at a significance level of $P \leq 0.05$. Statistical analysis was carried out using IBM SPSS software, version 22 (IBM Corp., USA).

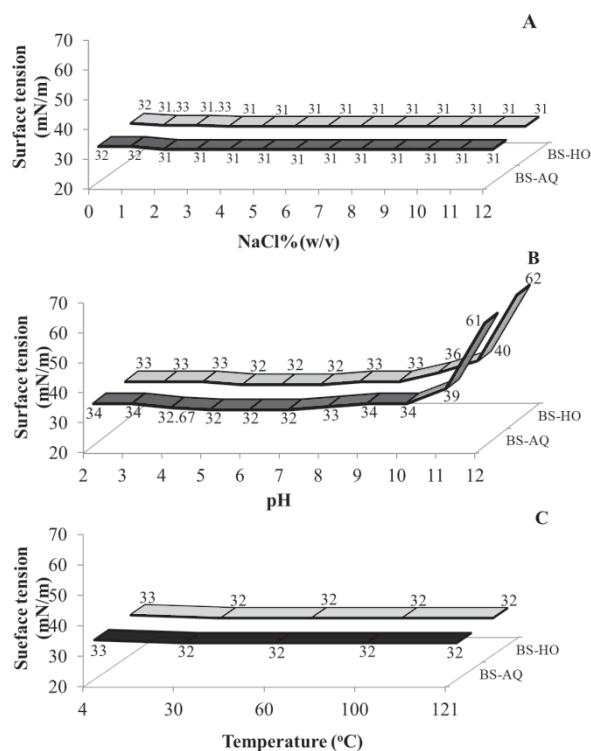


Fig. 3. Effect of salinity, pH, and temperature on surface tension of BS-AQ and BS-HO.

A: NaCl, B: pH, C: temperature. Black graph, BS-AQ; grey, BS-HO. The values shown are average of 3 samples.

Results

Growth of *A. pullulans* YTP6-14 and production of BS-AQ and BS-HO

Making wide use of abundant agricultural products and wastes is of great economic significance in Thailand. To examine the growth and productivity of BS-AQ and BS-HO, *A. pullulans* YTP6-14 was grown in a production medium supplemented, respectively, with four different carbon sources (glucose, sucrose, glycerol, and cassava flour hydrolysate) for 11 days. BS-AQ was produced maximally (1.81 ± 0.27 g/l) in a glycerol medium on Day 5. BS-HO was produced maximally in cassava flour hydrolysate (7.37 ± 0.16 g/l on Day 7), followed by the glucose medium (6.37 ± 0.64 g/l on Day 7). Growth of *A. pullulans* YTP6-14 was highest in the sucrose medium (DCW of 9.59 ± 0.43 g/l on day 7). Sucrose was consumed by Day 3, glucose and cassava flour hydrolysate by Day 5, while glycerol still remained on Day 11. Carbon source consumption correlated with the culture pH value, as shown in Fig. 1. The relationship between the growth of *A. pullulans* YTP6-14, and the production of BS-AQ and BS-HO, in different carbon sources is shown in Fig. 1.

CMC (critical micelle concentration)

Expensive manufacturing cost is often an obstacle when using a biosurfactant for practical use. Therefore, in this research, in order to save the cost required for purification, various properties of the crude sample were examined rather than pure compounds. CMC is an important

Table 1. Emulsification Index (E24) and emulsion stability of BS-AQ and BS-HO in vegetable oils.

Biosurfactant and oil	Emulsification index (E24)	Emulsion stability (%) after shelf life (days)				
		1	7	15	30	60
BS-AQ + Coconut oil	52.22 ± 0.93 ^c	100	98.81	71.04	00.00	00.00
BS-AQ + Palm oil	54.31 ± 1.36 ^d	100	90.84	90.84	90.84	90.07
BS-AQ + Soy bean oil	52.14 ± 0.57 ^c	100	92.86	68.43	17.88	00.00
BS-AQ + Rice bran oil	49.12 ± 1.63 ^b	100	90.57	34.62	00.00	00.00
BS-AQ + Olive oil	55.62 ± 0.77 ^d	100	96.07	96.07	96.07	96.08
BS-AQ + Sunflower seed oil	49.25 ± 0.67 ^b	100	94.44	68.84	24.90	00.00
BS-AQ + Sesame oil	14.12 ± 0.67 ^a	100	70.24	70.24	70.24	66.07
BS-HO + Coconut oil	54.77 ± 1.16 ^{cd}	100	92.02	77.22	00.00	00.00
BS-HO + Palm oil	55.01 ± 1.99 ^{cd}	100	93.61	93.61	93.61	92.43
BS-HO + Soy bean oil	53.45 ± 2.35 ^c	100	80.30	42.38	28.58	11.49
BS-HO + Rice bran oil	53.19 ± 1.80 ^c	100	94.78	35.61	15.87	04.74
BS-HO + Olive oil	57.00 ± 1.42 ^d	100	97.39	97.39	97.39	97.39
BS-HO + Sunflower seed oil	49.24 ± 0.84 ^b	100	96.25	74.69	31.27	04.98
BS-HO + Sesame oil	14.60 ± 1.36 ^a	100	76.20	76.20	76.20	69.05

The E24 values and the stability values shown are an average of 5 samples. The statistically significant ($P \leq 0.05$) values are designated by different superscript letters comparison between oils in each groups of BS-AQ and BS-HO.

parameter to examine the efficiency of the biosurfactant. Crude BS-AQ and BS-HO produced from a glucose medium showed an excellent reduction of water surface tension. CMC values of BS-AQ and BS-HO were 41.32 and 13.51 mg/l, and minimal surface tensions were 32.88 and 31.68 mN/m, respectively (Fig. 2).

Effect of NaCl, pH, and temperature on ST of BS-AQ and BS-HO

The stability of biosurfactants under various conditions, such as NaCl, pH, and temperature is one of the factors which indicates the usefulness of industrial applications. The STs (surface tensions) of BS-AQ and BS-HO at CMC concentrations were remained around 31 mN/m in a wide range of NaCl concentration (Fig. 3A). Alkaline pH (10–12) affected the surface tension of BS-AQ and BS-HO; the STs increased from 34 to 62 mN/m (Fig. 3B). The STs remained stable at 4, 30, 60, 100°C, and in autoclave conditions (121°C for 15 min) (Fig. 3C).

Emulsifying activity

Biosurfactants with a high emulsifying activity are beneficial in the food industry. BS-AQ and BS-HO emulsified well, with high E24 values, with olive oil, palm oil, coconut oil, soybean oil, rice bran oil, and sunflower seed oil, but not sesame oil (Table 1). The emulsion of BS-AQ and BS-HO with olive oil showed the highest stability at 96.08% and 97.39% at 60 days, respectively, followed by palm oil.

Effects of BS-AQ and BS-HO on biofilm formation

One of the expected useful functions of biosurfactants is the reduction of biofilm formation by cariogenic bacteria. To examine the activities of BS-AQ and BS-HO on biofilm formation, three biofilm-forming microorganisms (*A. pullulans* YTP6-14, *S. mutans* ATCC 25175 and *S. sobrinus* ATCC 6715) were tested by three methods: pre-coating, co-incubation and mid post-formation. BS-AQ and BS-HO did not decrease biofilm formation by *A. pullulans*, while the amount of *S. mutans* and *S. sobrinus*

Table 2. ODA of BS-AQ and BS-HO fractions from HPLC.

Fraction (8 mg/ml, 10 µl)	HPLC retention time (min)	ODA (cm ²)
BSAQ-1	3.645	0 ± 0.00
BSAQ-2	4.744	0.13 ± 0.00
BSAQ-3	6.732	0.13 ± 0.04
BSAQ-4	11.154	10.07 ± 0.05
BSHO-1	7.427	1.87 ± 0.01
BSHO-2	11.161	35.98 ± 0.20

biofilms were reduced differently by the surfactants (Fig. 4). BS-HO generally showed stronger effects than BS-AQ. In co-incubation experiments, *S. sobrinus* biofilm was reduced 24.73% by 312 µg/ml BS-AQ, and 31.08% by 156 µg/ml BS-HO. *S. mutans* biofilm was reduced 38.00% by 156 µg/ml BS-AQ and 79.76% by 78 µg/ml BS-HO. In pre-coating experiments, 1250 µg/ml of BS-AQ or BS-HO reduced the biofilm amounts of *S. mutans* by 14.28% and 71.63%, respectively. In mid post-formation experiments, the amount of *S. mutans* biofilm was decreased by 16.69% with 312 µg/ml BS-AQ and 21.49% with 156 µg/ml BS-HO.

Molecular analyses of BS-AQ and BS-HO

A. pullulans YTP6-14 produced complex biosurfactants, BS-AQ and BS-HO, in aqueous and heavy oil layers, respectively. The molecular compositions of BS-AQ and BS-HO produced from the glucose medium were characterized by HPLC fractionation. Fractions with retention times of 11.154 min (BSAQ-4) and 11.161 min (BSHO-2) showed a high ODA activity (Table 2). BSAQ-4 and BSHO-2 were further analyzed by GC/MS and MALDI-TOF/MS (Table 3). The GC/MS results were compared with the reports of Vesonder et al. (1972) and Luepongpatana et al. (2017), which revealed that BS-AQ and BS-HO contained *m/z* peaks of 168.15 (corresponding to massoia lactone, ML), 187.16 (3,5-dihydroxydecanoic acid delta lactone), 115.06 (the lactone ring of the 187.16 peak), and 97.04 (the lactone ring

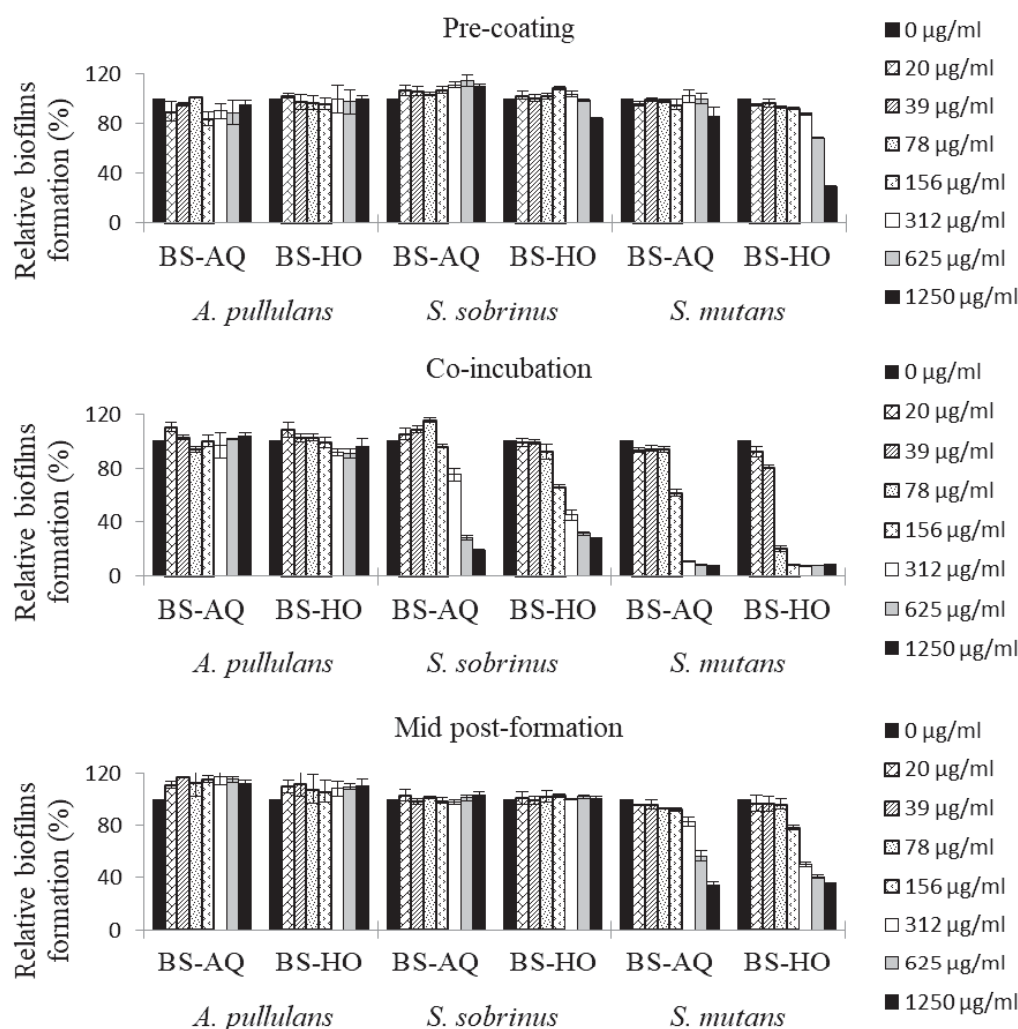


Fig. 4. The effect of BS-AQ and BS-HO on biofilms formation of *A. pullulans* YTP6-14, *S. sobrinus* ATCC 6715 and *S. mutans* ATCC 25175.

The values shown are average of 3 samples. The lines indicate standard deviations.

Table 3. Peaks (m/z) and possible compounds contained in fractions BSAQ-4 and BSHO-2 analyzed by GC/MS and MALDI-TOF/MS.

Mass analysis	HPLC fraction	m/z	Possible compound
GC/MS	BSAQ-4 (11.154)	97.04	Ring of Massoia lactone
		115.06	Ring of 3,5-dihydroxydecanoic acid delta lactone
		168.14	Massoia lactone
		187.16	3,5-dihydroxydecanoic acid delta lactone
	BSHO-2 (11.161)	97.04	Ring of Massoia lactone
		115.06	Ring of 3,5-dihydroxydecanoic acid delta lactone
		168.15	Massoia lactone
		187.16	3,5-dihydroxydecanoic acid delta lactone
MALDI-TOF/MS	BSAQ-4 (11.154)	733.9	Liamocin Ara-A1 [M+Na] ⁺
		763.9	Liamocin A1 [M+Na] ⁺
		806.0	Liamocin A2 [M+Na] ⁺
	BSHO-2 (11.161)	641.1	Exophilin A2 [M+Na] ⁺

of ML), as well as other peaks at $m/z = 43.06, 68.04, 73.08, 108.11$ and 198.11 . Moreover, fractions BSAQ-4 and BSHO-2 were analyzed in the positive ion mode in MALDI-TOF/MS to give rise to pseudomolecular

[M+Na]⁺ adduct ions. Most of the peaks were matched to the structural characterization of heavy oil by Price et al. (2013). BSAQ-4 contained m/z peaks at 733.9, 763.9 and 806.0, which corresponded to liamocin Ara-A1, liamocin

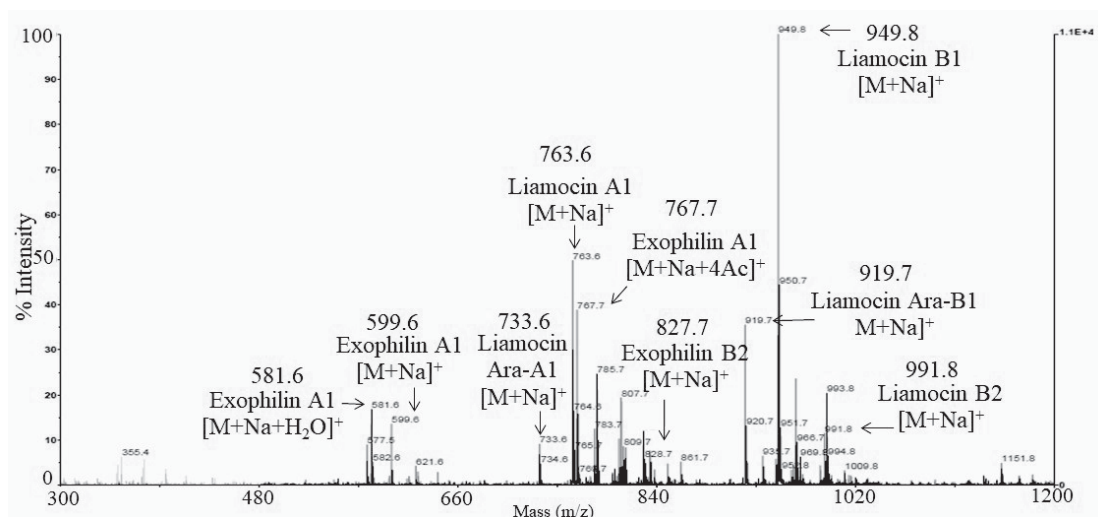


Fig. 5. MALDI-TOF/MS analysis of crude BS-HO.

A1 and liamocin A2, respectively. The dominant ion $[M+Na]^+$ of BSHO-2 was at $m/z = 641.1$, which corresponded to exophilin A2 (Table 3). In addition, crude BS-HO was also analyzed by MALDI-TOF/MS. The spectra corresponded to the heavy oil compositions reported by Price et al. (2013) and Price et al. (2016). The $[M+Na]^+$ ions were at $m/z = 581.6, 599.6, 733.43, 763.45, 767.49, 827.51, 919.56, 949.57$ and 991.58 , corresponding to exophilin A1-H₂O, exophilin A1, liamocin Ara-A, liamocin A, exophilin A1+4Ac, exophilin B, liamocin Ara-B, liamocin B1 and liamocin B2, respectively (Fig. 5).

Discussion

Production of BS-AQ and BS-HO

BS-AQ was produced in correlation with the growth of *A. pullulans* YTP6-14, while BS-HO started to be produced as a typical secondary metabolite after entering a stationary growth phase. BS-AQ was effectively produced in the glycerol medium. Glycerol is one of the abundant by products from biodiesel manufacturing factories in Thailand. In a glycerol medium, the growth and glycerol consumption were slow. Saito and Posas (2012) reported that glycerol permeability through the lipid bilayer is low in fungal species. A specific channel protein St11 (glycerol proton symporter) is used for glycerol uptake from the environment, whose gene expression is strongly induced by Hog1 upon saline, osmotic, temperature, and oxidative stress. On the other hand, Turk and Gostinčar (2018) reported that *A. pullulans* EXF-150 contains multiple genes involved in glycerol metabolism, including an NAD⁺-dependent glycerol-3-phosphate dehydrogenase, glycerol-3-phosphate phosphatase, mitochondrial glycerol-3-phosphate dehydrogenase, two copies of a glycerol kinase, and more than 10 copies of major facilitator superfamily transporters similar to glycerol proton symporters. The transcription of these genes also responded to hypersaline (5% and 10% w/v NaCl) and low temperature stress. Our observation of slow glycerol consumption by *A. pullulans* YTP6-14 in a medium with low NaCl [0.1% (w/v)] and moderate temperature is not inconsistent with the above reports.

BS-HO was produced at the highest level in cassava flour hydrolysate (7.37 g/l), followed by glucose (6.37 g/l) media. Cassava is one of the main agricultural products in Thailand, which accounts for 12% of the world production in 2009 (Howeler, 2012). A two-step enzymatic hydrolysis of cassava starch gives reducing sugars composed of glucose, maltose, and maltotriose in the ratio 25:5:1 (Wei et al., 2009). In our study, only reducing sugar was measured in the hydrolysate, so this resulted in a higher initial total carbon content (compared with the glucose, sucrose and glycerol media), which could be a reason why the use of cassava flour hydrolysate resulted in the maximum observed BS-HO production. Palasak (2014) reported that cassava flour hydrolysate contains nitrogen at 183 mg/kg and other elements, such as copper, calcium, zinc, manganese, potassium, phosphorus, and magnesium.

Physicochemical properties of BS-AQ and BS-HO

BS-AQ and BS-HO produced from a glucose medium had CMC values of 41.32 and 13.51 mg/l, respectively, which were lower than the CMCs of crude rhamnolipid from *Pseudomonas aeruginosa* LBI, surfactin from *Bacillus subtilis* ATCC 21332, and sophorolipid from *Wickerhamiella domercqiae* var. *sophorolipid* CGMCC 1576 (Ma et al., 2012; Nitschke et al., 2010; Whang et al., 2008). BS-AQ and BS-HO were resistant to a wide range of NaCl concentrations, temperature, and pH (2–10). These properties are required for industrial applications (Mulligan, 2005). For example, salt-tolerant biosurfactants are beneficial for oil drilling because this often involves high-salt conditions (Shavandi et al., 2011). Nevertheless, the ST changed from 34 to 62 mN/m at high pH (10–12). The ST activity of these biosurfactants is affected by high pH because they contain ML and liamocins which have a single head sugar connected by an ester to a fatty acid. The structures of lactones and esters are easily broken down by base-catalyzed hydrolysis at an acyl-oxygen group. For example, two linear esters, four β -lactones, two γ -lactones, and one δ -lactone were hydrolyzed by base catalysis via a two-step pathway (B_{AC}2) (Gómez-Bombarelli et al., 2013).

BS-AQ and BS-HO emulsified well with many vegeta-

ble oils. The emulsion with olive oil was the best and retained >90% stability after 60 days of storage. Although high molecular weight biosurfactants such as emulsan, liposan and alasanare, are generally better emulsifiers than low molecular weight biosurfactants (Dams-Kozłowska et al., 2008; Nitschke and Pastore, 2006), the low molecular weight BS-AQ and BS-HO had good emulsification activity, in agreement with the report of Nitschke and Pastore (2006) that low molecular weight biosurfactants, such as lipopeptides produced by *B. subtilis* were good emulsifiers.

Effects of BS-AQ and BS-HO on biofilm formation

It is natural to consider that surfactants reduce interfacial tension resulting in interfering with cell-solid surface adhesion. The biosurfactant, putisolvin, produced by *Pseudomonas putida* PCL1445, decreased biofilm formation by that bacterium itself (Kuiper et al., 2004). However, BS-AQ and BS-HO did not decrease biofilm formation by *A. pullulans* YTP6-14. On the contrary, a slight biofilm promotive effect was observed specifically in the “mid post-formation” experiment (Fig. 5). This observation is not inconsistent with previous reports that the biosurfactant is important in higher-order column structure formation of the mature biofilm (Branda et al., 2001; Roongsawang et al., 2003).

Next, we tested BS-AQ and BS-HO against dental plaque-forming *S. mutans* ATCC 25175 and *S. sobrinus* ATCC 6715, because their biofilms are known to be virulence factors for tooth decay. Both biosurfactants showed significant but different biofilm inhibitory effects against the strains. In co-incubation experiments, the *S. mutans* biofilm was reduced by 88.87% by 312 µg/ml BS-AQ, while the *S. sobrinus* biofilm was reduced by only 24.73%. This showed dose differences between *S. mutans* and *S. sobrinus*. Moreover, we also observed a biofilm promotive effect of BS-AQ against *S. sobrinus* in the “pre-coating” experiments. Both *S. mutans* and *S. sobrinus* are classified among *Mutans Streptococci*. However, the cell wall of *S. mutans* contains rhamnose and glucose, while *S. sobrinus* contains rhamnose, glucose, and galactose. The peptide chain in *S. mutans* peptidoglycan is Lys-Ala_{2,3}, while that of *S. sobrinus* is Lys-Thr-Ala (Whiley and Beighton, 1998). It is worth noting that Bischoff et al. (2015) reported that liamocin of 78 µg/ml completely inhibited the colony formation of *S. mutans*. On the other hand, it did not inhibit the colony formation of *S. sobrinus*, at least up to 1,250 µg/ml.

Gudiña et al. (2010) reported that a biosurfactant from *Lactobacillus paracasei* ssp. *Paracasei* A20 decreased biofilms of *S. mutans* HG 985 and *S. mutans* NS by merely 31.4% and 38.6%, respectively, even when present at 50 mg/ml. In our mid post-formation experiments, the addition of BS-AQ or BS-HO after half time did not decrease the amount of *S. sobrinus* biofilm, but decreased *S. mutans* biofilms by 16.69% at 312 µg/ml BS-AQ and 21.49% at 156 µg/ml BS-HO.

Molecular compositions of BS-AQ and BS-HO

Price et al. (2013) found ML in the methanol-soluble fraction from methanolysis of liamocin oil. ML is easily

synthesized by dehydration of 3,5-dihydroxydecanoic acid delta lactone (Vesonder et al., 1972). We directly found ML ($m/z = 168.14$) and 3,5-dihydroxydecanoic acid delta lactone (187.16) in both BSAQ-4 and BSHO-2 by GC analysis. These findings indicate that ML is produced naturally by *A. pullulans* YTP6-14 as a precursor of liamocins, or that it may be a natural breakdown product of the liamocins. In conclusion *A. pullulans* YTP6-14 produced multiple biosurfactants in both BS-AQ and BS-HO, including a flavor biosurfactant ML, liamocins, and exophilins.

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