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ORIGINAL ARTICLE

Using Corn Husk Powder as a Novel Substrate to Produce a Surface Active Compound from *Labrenzia aggregate* KP-5

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Abstract In this study, surface active compound (SAC)-producing bacterial isolates were evaluated for SAC production using corn husk powder (CHP) as a sole carbon source. From the 51 isolates screened, *Labrenzia aggregate* KP-5 produced the highest SAC activity. The highest SAC production (3.51 g L^{-1}) was obtained when the strain was cultivated in a minimal salt medium containing 40 g L^{-1} CHP and 1 g L^{-1} commercial monosodium glutamate at 30 °C and 150 rpm after 51 h of cultivation. The produced SAC had the ability to decrease the surface tension of water from 72.0 to 25.5 mN m^{-1}, with the critical micelle concentration of 9 mg L^{-1} (11.07 mM) and exhibited the highest emulsification activity (EA) of 81% against motor oil. The SAC showed stability at 4–121 °C and pH 4–10 against the surface and EA of vegetable oils and hydrocarbons, and showed tolerance at high salt concentrations (1–10% NaCl). The chemical structure of the SAC was confirmed as a rhamnolipid using Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectrometric analysis. The SAC did not exhibit inhibitory effects on various vegetables tested; however, strong inhibitory activity against Gram-positive and Gram-negative bacteria was observed. The application of SAC for microbial enhanced oil recovery by sand saturated with used lubricating oil resulted in above 89% of oil removal. The properties of the SAC we obtained from CHP have potential applications especially for microbial enhanced oil recovery and/or reducing the intensity of environmental contamination. In addition, the obtained SAC is a suitable alternative to antimicrobial agents.

Keywords Surface active compound · *Labrenzia aggregate* · Corn husks powder · Novel substrate · Biosurfactant

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Introduction

Surface active compounds (SAC) are amphiphilic molecules produced from a variety of microorganisms that contain hydrophobic and hydrophilic parts. These compounds are divided into low molecular weight, also termed biosurfactants, and high molecular weight or bioemulsifiers (Satpute, Banat, Dhakephalkar, Banpurkar, & Chopade, 2010; Sharma, Oberoi, Sharma, & Oberoi, 2017; Varjani & Upasani, 2017). The former generally consist of glycolipids and lipopeptides, which decrease the interfacial tensions of the liquid into which they are added. The latter include emulsan, alasan, biodispersan, and extracellular or cell membrane-bound bioemulsifiers (such as exopolysaccharides [EPS] usually more efficient in stabilizing oil-in-water emulsions; Santos, Rufino, Luna, Santos, & Sarubbo, 2016; Sharma, Oberoi, et al., 2017; Varjani & Upasani, 2017).

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A variety of microorganisms produce low-molecular-weight SAC (Ron & Rosenberg, 2001); the best investigated among them are surfactin from *Bacillus* sp. and rhamnolipid from *Pseudomonas* sp. (Liu et al., 2017; Santos et al., 2016; Varjani & Upasani, 2017). In the past few decades, SAC have been widely studied due to their practically functional properties such as low toxicity, environmentally friendly, wetting, foaming, solubilization, biodegradability, and ability to be produced from renewable, by-product, or low-cost substrates (Kourmentza, Freitas, Alves, & Reis, 2017; Santos et al., 2016; Satpute et al., 2016). Presently, SAC are applied in several industries including food, cosmetics, pharmaceutical formulations, agriculture, petroleum industry, special chemical substances, cleansers, and bioremediation of pollutants (Liu et al., 2017; Santos et al., 2016; Sharma, Oberoi, et al., 2017; Varjani & Upasani, 2017).

It is estimated that about 30–40% of the production cost of many industrial biologically SAC was caused by the cost of the growth substrate (Vijayaraghavan, Lazarus, & Vincent, 2014). Low-cost growth substrates used for the production of SAC are expected to greatly reduce the production costs. Exploration of locally available, inexpensive, substrates was important for production of valuable products by selecting novel microorganisms. Thailand is a major sweet-corn-growing country in the world having 4.8 million tons in year 2014 (Office of Agricultural Economics, 2015). Corn husks are the large-volume solid wastes that result from sweet-corn processing. The high cellulose content (382 g kg^{-1}) of corn husks enables them to be used in making paper and developing cellulose-rich fibers, or as substrates for antibiotics (Mahalaxmi, Sathish, Rao, & Prakasham, 2010), cellulase (Prakash, Jayalaksmi, Prakash, Rubul, & Sreeramulu, 2012), and citric acid production (Mahalaxmi et al., 2010). Corn husks are normally used for animal feed and fertilization. In this investigation, we report for the first time the utilization of corn husks as potential substrates for production of SAC. Because of their advantages such as vast availability, renewable nature, and easy release of carbon sources, the role of corn husks as the substrates for SAC production from isolated *Labrenzia aggregata* KP-5 was investigated in the present study.

Experimental

Materials and Methods

Substrates, Samples, and Isolation of Microorganisms

Corn husks obtained from a local farm in Songkhla Province, Thailand, were used as substrates. Corn husks were washed with tap water and cut into small pieces, dried at 60 °C for 24 h, and ground to 24 mesh size. Chemical composition of corn husk powder (CHP) was (g 100 g^{-1} dry weight): moisture 6.76, cellulose 42.58, hemicellulose 38.79, ash 1.50, lignin 9.25, crude protein 0.75, and fat 0.15.

SAC-producing bacteria were isolated from soils or water contaminated with palm oil from a palm oil refinery factory in southern Thailand. Samples were transported in sterile plastics bags and stored at 4 °C when not used immediately. For bacterial isolation, 1 g of the sediment or 1 mL of water was cultivated in a screening mineral salt medium (MSM): (g L^{-1}) K_2HPO_4, 0.8; KH_2PO_4, 0.2; $CaCl_2$, 0.05; $MgCl_2$, 0.5; $FeCl_2$, 0.01; and NaCl, 5.0 (Chooklin, Maneerat, & Saimmai, 2014; Chooklin, Petmeaun, Maneerat, & Saimmai, 2014) using CHP (2%, w/v) as a carbon source and then plates were incubated at 30 °C for 2–3 days. Morphologically distinct colonies on the plates were purified by transferring them onto new MSM plates and subsequently Gram-stained. Pure cultures were stored at −20 °C in the MSM mixed with sterile glycerol at a final concentration of 30%.

Screening of Potential SAC-Producing Strains

One hundred and seventy-five isolates were streaked on MSM agar containing 1% (w/v) of CHP for 24 h at 30 °C. Subsequently, one loop full of each isolate was transferred to test tubes containing 5 mL of nutrient broth (Hi-Media, Mumbai, India) and shaken (150 rpm) at 30 °C overnight. Two hundred microliters of the cell culture were transferred to 5 mL of the MSM medium supplemented with 1% (w/v) of CHP in a rotary shaker (Vision Scientific Co., Daejon, Korea) at 30 °C and 150 rpm for 2 days. The screening of SAC-producing isolates was performed using the drop-collapse test (DCT) and the emulsification activity (EA) of the culture supernatant was measured after centrifugation at 9000*g* at 4 °C for 10 min.

Identification and Characterization of the Isolate KP-5

The procedures outlined in Bergey's manual of systematic bacteriology were considered as standard to determine physiological characteristics (Sneath, 1986). The method described by Weisburg, Barns, Pelletier, and Lane (1991) was used as standard to amplify and sequence the 16S rRNA gene. The BlastN program was used to compare the 16S rRNA gene sequences with the public database. The Basic Local Alignment Search Tool (BLAST) searching program was used to obtain related sequences from the Genbank database (National Center for Biotechnology Information [NCBI], National Library of Medicine). The two-parameter and neighbor-joining method using BioEdit v7.0.0 (Hall, 1999; Ibis Therapeutics Carlsbad, CA) was chosen as standard to carry out phylogenetic analysis.

Medium Optimization

To study the effects of carbon concentration on the production of the SAC, *Labrenzia aggregata* KP-5 was cultivated on MSM supplemented with 4–20% (w/v) of CHP using 0.1% (w/v) of NH_4Cl as the N source. In the study of the effects of nitrogen sources, 4% (w/v) of CHP was used as the sole carbon source, and NH_4Cl in MSM was substituted with ammonium chloride, ammonium sulfate, ammonium molybdate, ammonium citrate, potassium nitrate, sodium nitrate, or urea, separately, at the same nitrogen concentration (0.1%, w/v). The ratio of carbon and nitrogen was varied (5–45) by keeping constant the nitrogen source (concentration, 1 g L^{-1}).

SAC Recovery

Centrifugation was used to remove bacterial cells from the culture broth (10,000*g* at 4 °C for 15 min) for SAC recovery as described by Chooklin et al. (2014). Briefly, we examined four solvent systems, namely, a mixture of chloroform:methanol (2:1), cold acetone, dichloromethane, and ethyl acetate, 150 mL of the cell-free culture supernatant was placed in a 500 mL separating funnel, and 150 mL of the solvent was added. The mixture of the cell-free culture supernatant and the solvent formed two phases, the upper phase was collected in a clean vial and the solvent was removed using a vacuum rotary evaporator. The solvent system showing the highest SAC activity was used to recover SAC from strain KP-5.

Chemical Analysis of the SAC

The protein content of the SAC was estimated using the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard. The carbohydrate was determined using the phenol–sulfuric acid method described by Chaplin and Kennedy (1986), with glucose as standard and the lipid content was estimated for free fatty acid using the method of Folch, Lees, and Stanly (1956) with cholesterol as standard. The chemical nature of the SAC obtained was determined using thin-layer chromatography as previously described by Saimmai, Onlamool, Sobhon, and Maneerat (2013).

Fourier Transform Infrared Spectroscopy

The chemical structure of SAC was elucidated using Fourier transform infrared spectroscopy (FT-IR) and its type of functional groups was identified. Translucent pellets were obtained by grinding 1 mg of purified SAC with 100 mg of KBr and pressing with 7500 kg for 30 s. A GX-FT-IR system (Perkin-Elmer, Norwalk, CT, USA) was used to record the infrared absorption spectra with a spectral resolution and a wave number accuracy of 4 and 0.01 cm^{-1}, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as the background reference.

Nuclear Magnetic Resonance Spectroscopy

The nuclear magnetic resonance (NMR) spectrum was recorded on a BrukerAvance-II spectrometer, Switzerland, at 500 MHz. The SAC was subjected to further analysis with NMR. All ^{1}H and ^{13}C NMR spectra were recorded on a BrukerAvance-II spectrometer at 500 MHz, managed with an Aspect 3000 computer (Bruker, Mumbai, India) set to the deuterium resonance of the solvent, $CDCl_3$, without spinning. Data were recorded at 32 K (the number of data points per ppm of the plot) (Saimmai, Onlamool, et al., 2013).

Mass Spectrometric Analysis of SAC

The chemical structure of the purified SAC was determined by electrospray ionization-mass spectrometric analysis (ESI-MS) in a LCQ™, a mass spectrometer system designed by Finnigan MAT, quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). The SAC was dissolved in MeOH/water (0.1% formic acid) and infused in the ESI source with a flow rate of 100 μL min^{-1}. In the ESI, nitrogen and auxiliary gas flows were maintained at 50 and 5 mL min^{-1}, respectively, and refer to arbitrary values set by the software. ESI-MS spectra in negative mode were acquired from *m/z* 50–1200 for 2.5 min. Performance conditions were as follows: accumulation time, 300 ms; temperature 325 °C; capillary voltage, 3500 V; nebulizer, 30 psi; and dry gas, dry gas flow rate 6 min L^{-1}. The trap drive values were close to 50, indicating an intermediate stability for our compound (Saimmai, Onlamool, et al., 2013).

Polycyclic Aromatic Hydrocarbon Solubilization Test

Procedures for sample equilibration and solubility tests were essentially the same as reported earlier (Saimmai, Onlamool, et al., 2013). Ten milliliter of SAC solutions were prepared at concentrations of 0.5 × critical micelle concentration (CMC), CMC, and 2 × CMC and placed into 25 mL Corex centrifuge tubes with Teflon cap liners. Ten milligrams of polycyclic aromatic hydrocarbons (PAH) including anthracene, fluoranthene, fluorine, naphthalene, phenanthrene, or pyrene was separately added to each tube. The sample solutions were then equilibrated on a rotary shaker at a speed of 250 rpm for 48 h at 25 °C. The samples were subsequently centrifuged for 30 min at a speed of 10,000*g* to separate the undissolved PAH. The supernatant

was filtered through 1.2 μm filters (Whatman, Springfield Mill, UK), and 2.0 mL of this filtrate was extracted with an equal volume of hexane. The aqueous and hexane phases were separated when the emulsion was centrifuged at 9693*g* for 10 min. The concentration of PAH was measured spectrophotometrically (Libra S22; Biochrom, Cambridge, UK) at the specific wavelength of each compound (Saimmai, Onlamool, et al., 2013). The calibration curve of individual PAH (in hexane) was used to determine the concentration of each PAH. Assay buffer with SAC but without PAH was used as a blank after being extracted identically with hexane.

Evaluation of the SAC in Oil Removal

The studies of submerged washing of oil removal using the SAC solution were performed according to Chooklin, Maneerat, et al. (2014). Briefly, used motor lubricating oil was added (20%, w/v) to the sand (50/80-mesh) and left at room temperature (30 ± 3 °C) for 7 days. Then, 20 g of the sand samples was added to 60 mL SAC solutions and put in a rotary shaker at 250 rpm over night at 30 °C and centrifuged at 9693*g* for 20 min for separation of the cleaning solution and the sand. The remaining oil was determined gravimetrically as the amount of material extracted from the sand by hexane.

Antimicrobial Activity of the SAC

SAC solution at concentrations of 0.5 × CMC, CMC, and 2 × CMC (50 μL) was tested for antimicrobial activity using the ager well diffusion method (Candan et al., 2003). The tested microorganisms were obtained from Songklanakarin Hospital, Prince of Songkla University, Thailand, and the cultures were collected from the Faculty of Agriculture Technology, Phuket Rajabhat University, including *Bacillus cereus*, *Botrytis cinerea*, *Candida albicans*, *Colletotrichum truncatum*, *Enterococcus faecium*, *Escherichia coli*, *Fusarium moniliforme*, *Gibberella zeae, Listeria monocytogenes*, *Penicillium candidum*, *Pseudomonas aeruginosa*, *Salmonella* sp., *Salmonella typhimurium*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Vibrio vulnificus.* The media used were Mueller-Hint on agar (Difco, Detroit, MI, USA) for bacteria and potato dextrose agar (Difco, Detroit, MI, USA) for fungi. The plates (triplicates) were incubated at 37 °C for 24 h in the case of bacteria and at 28 °C for 72 h in the case of fungi. The antimicrobial activities of the SAC were then determined by measuring the respective zones of inhibition in millimeter. The positive controls for antimicrobial activity were standard antibiotics including penicillin (Hi-Media, Mumbai, India), streptomycin (Hi-Media, Mumbai, India), and rhamnolipids (Sigma-Aldrich, St Louis, MO, USA).

Phytotoxicity Assessment of the SAC

The phytotoxicity of the SAC was evaluated in a static test based on the root growth and seed germination using the Tiquia, Tam, and Hodgkiss (1996) method with slight modifications. Six vegetable seeds namely *Brassica oleracea* L., *Lactuca sativa* L., *Raphanus sativus*, *Solanum gilo*, *Triticum aestivum*, and *Vigna radiate* were selected for our studies. Solutions of the isolated SAC were prepared with distilled water at concentrations of 0.5 × CMC, CMC, and 2 × CMC. Sterilized Petri dishes (1 cm × 10 cm) in which the toxicity was determined contained a Whatman No. 1 filter paper. Each Petri dish containing the pretreated seeds with sodium hypochlorite and 10 inoculated seeds was added and inoculated with 5 mL of the tested solution at 27 °C. Distilled water was used as the control. After 5 days of incubation in the dark, relative seed germination, relative root elongation, and the germination index (GI) were determined as follows:

$$\text{Relative seed germination}(\%) = \left(\frac{\text{Number of seeds germinated in the extract}}{\text{Number of seeds germinated in the control}}\right) \times 100$$

$$\text{Relative root length}(\%) = \left(\frac{\text{Mean root length in the extract}}{\text{Mean root length in the control}}\right) \times 100$$

$$\text{GI}(\%) = \frac{(\text{Relative seed germination}) \times (\text{Relative root elongation})}{100}$$

Analytical Methods

Biomass determination was carried out in terms of dry cell weight. Samples were mixed and centrifuged at 9693*g* for 30 min in preweighed tubes with chilled distilled water at different times of fermentation. Biomass obtained was dried overnight at 105 °C and weighed.

Drop-Collapse Test

The DCT was performed as described by Youssef et al. (2004). Briefly, each well of a 96-well microtiter plate lid was added with 2 μL of the used lubricating oil (ULO) (Biolog, Inc., Hayward, CA, USA) and these were left to equilibrate for 1 h at room temperature (30 ± 3 °C). Five microliters of the culture supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min with the aid of a magnifying glass. Bioemulsifier-producing cultures giving flat drops were scored as positive "+." Those cultures that gave rounded

drops were scored as negative "–," indicating no SAC production (Youssef et al., 2004).

EA Assay

The EA was measured as described by Plaza, Zjawiony, and Banat (2006). Briefly, equal volume of the cell-free supernatant (4 mL) was mixed with hydrocarbon or oil in a screw cap tube and vortexed at a high speed for 2 min. The emulsion stability was determined after 24 h. E24 was calculated by dividing the measured height of the emulsion layer by the total height of mixtures and multiplying by 100.

Surface Tension Measurement

Surface tension measurement was carried out as described by Saimmai, Udomsilp, and Maneerat (2013) using a Model 20 Tensiometer (Fisher Science Instrument Co., Pittsburgh, PA, USA) at 25 °C. CMC was determined by plotting the surface tension *versus* concentration of SAC in the solution.

Statistical Analysis

All experiments were carried out at least in triplicates. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 10.0, for Windows Inc., Chicago, IL, USA).

Results and Discussion

Screening of SAC-Producing Bacterial Isolates

SAC-producing bacterial strains from a palm oil refinery factory sample were isolated by a selective culture medium with CHP as a sole carbon source. The DCT and EA were used for the rapid detection of SAC-producing bacteria. From 22 soil and 14 water samples, 51 bacterial isolates were positive for the DCT and EA (7 Gram-positive bacilli, 9 Gram-positive cocci, 16 Gram-negative bacilli, and 19 Gram-negative cocci) (Table 1). Table 1 shows the Gram staining results, growth, EA, and surface activity of the selected bacterial isolates. The majority of the selected bacterial isolates were Gram-negative (35 from 51 isolates). This result was in accordance with the previously reported result that most bacteria isolated from sample sources were contaminated by oil or hydrocarbon are Gram-negative (Saimmai, Rukadee, Onlamool, Sobhon, & Maneerat, 2012; Saimmai, Tani, Sobhon, & Maneerat, 2012; Saisa-ard, Saimmai, & Maneerat, 2014). The explanation of these phenomena comes from the presence of outer membranes in Gram-negative bacteria composed of lipopolysaccharides, phospholipids, and lipoproteins, which act as SAC (Jan, 2017; Saisa-ard et al., 2014). In the present study, the highest SAC activity was identified for a Gram-negative bacterium (KP-5) isolated from a soil contaminated by a palm oil refinery in Krabi Province, with DCT and EA results of 3.5 mm 40.41%, respectively. Interestingly, this strain did not show the highest activity of EA, but it could produce SAC, which exhibited the highest surface tension activity by DCT. The nonproportionality between emulsification and surface tension activity of SAC maybe comes from the type of carbon source used for growth of SAC-producing bacteria. When a water-soluble carbon source was used, the system does not require SAC to make emulsion for an insoluble substrate (Saisa-ard et al., 2014).

Identification of Selected Bacterial Strain

The colony morphology of the isolate KP-5 was slightly pink-colored, glistening, mucoid, smooth, and convex with an entire edge, which was rather circular with a diameter of 2–4 mm within 24 h growth on nutrient agar at 37 °C. It was Gram-negative rod-shaped and has uneven ends. Further identification was performed based on the 16S rRNA gene sequence analysis. The 16S rRNA sequence of strain KP-5 was deposited in the GeneBank database under accession number LC121774. The phylogenetic analysis of strain KP-5 was undertaken using the 16S rRNA gene nucleotide sequence data. This showed that this strain had the highest homology (100%) with *Labrenzia aggregate* IAM12614 (Fig. 1). We thus classified strain KP-5 as *Labrenzia aggregate* KP-5. It must also be noted that to the best of our knowledge, this work is the first to report and describe the SAC production from the genus *Labrenzia.* It has previously been reported that *Labrenzia* sp. was capable of degrading oil and hydrocarbon and also produced extracellular polymers (EPS) (Overholt et al., 2013; Priyanka, Arun, & Rekha, 2014). The microbial interaction and emulsification of various hydrophobic substrates are directly affected by EPS (Perfumo, Smyth, Marchant, & Banat, 2010; Satpute, Banpurkar, Dhakephalkar, Banat, & Chopade, 2010). The increase of the viscosity of solutions at a low pH value and the emulsification of several hydrocarbon compounds are also mainly dependent on EPS (Satpute, Banpurkar, et al., 2010). However, so far there has been no report found on the SAC production capability of this genus.

Effect of the CHP Concentration on Growth and SAC Production

The effect of the CHP concentration on growth and SAC production by *L. aggregate* KP-5 was investigated and the

Table 1 Source, Gram's staining, growth, and biosurfactant activity of the bacterial isolates

Sources	Isolate	Gram stain	Dry cell weight ($g\ L^{-1}$)[a]	DCT (cm)[a]	EA (%)[a]
Krabi Province	KP-1	Negative	1.3 ± 0.5	2.5 ± 0.2[b]	35.15 ± 4.20
	KP-2	Negative	1.8 ± 0.3	2.1 ± 0.5	32.25 ± 3.54
	KP-3	Positive	2.2 ± 0.2	2.2 ± 0.7	36.54 ± 2.78
	KP-4	Negative	1.5 ± 0.2	2.0 ± 0.5	35.21 ± 3.25
	KP-5	Negative	1.6 ± 0.3	3.5 ± 0.5	40.41 ± 5.20
	KP-6	Positive	2.1 ± 0.3	2.6 ± 0.4	25.20 ± 4.02
	KP-7	Negative	1.8 ± 0.8	2.3 ± 0.3	14.32 ± 2.45
	KP-8	Positive	2.0 ± 1.0	1.5 ± 0.2	30.13 ± 5.25
	KP-9	Negative	1.5 ± 0.2	1.8 ± 0.5	25.18 ± 5.58
	KP-10	Negative	1.8 ± 0.7	2.0 ± 1.0	22.50 ± 4.71
Satun Province	SP-1	Negative	1.7 ± 0.3	2.2 ± 0.6	27.19 ± 5.51
	SP-2	Negative	1.8 ± 0.3	2.5 ± 1.1	33.21 ± 5.85
	SP-3	Positive	2.0 ± 0.4	2.8 ± 0.5	18.25 ± 6.40
	SP-4	Negative	2.4 ± 0.8	2.0 ± 0.2	15.78 ± 2.75
	SP-5	Negative	2.5 ± 0.5	1.5 ± 0.6	28.70 ± 5.56
	SP-6	Positive	1.9 ± 0.2	1.3 ± 0.2	16.80 ± 3.57
	SP-7	Negative	2.0 ± 0.6	1.8 ± 0.0	28.78 ± 4.54
	SP-8	Negative	2.6 ± 0.2	2.7 ± 0.8	25.42 ± 3.05
	SP-9	Negative	2.1 ± 0.7	2.5 ± 0.3	29.50 ± 6.65
	SP-10	Positive	2.0 ± 0.2	1.7 ± 0.4	42.82 ± 2.50
	SP-11	Negative	1.8 ± 0.7	2.2 ± 0.6	25.50 ± 5.89
	SP-12	Positive	2.5 ± 0.5	1.5 ± 0.4	16.24 ± 4.18
	SP-13	Negative	2.0 ± 0.7	2.0 ± 0.1	18.55 ± 4.48
	SP-14	Negative	1.8 ± 0.4	2.7 ± 0.2	37.81 ± 3.05
	SP-15	Positive	2.7 ± 0.3	2.6 ± 0.4	36.79 ± 4.61
	SP-16	Negative	2.5 ± 0.2	3.0 ± 0.2	35.08 ± 3.74
	SP-17	Negative	1.5 ± 0.1	3.2 ± 0.2	35.12 ± 4.09
	SP-18	Positive	1.6 ± 0.2	2.5 ± 0.3	25.88 ± 3.54
Surat Thani Province	ST-1	Negative	1.7 ± 0.5	2.7 ± 0.4	41.70 ± 5.27
	ST-2	Negative	2.0 ± 0.1	1.7 ± 0.6	36.20 ± 6.05
	ST-3	Positive	1.5 ± 0.5	2.8 ± 0.5	18.15 ± 4.78
	ST-4	Negative	1.9 ± 0.3	1.7 ± 0.9	37.21 ± 2.62
	ST-5	Positive	1.7 ± 0.6	2.2 ± 0.7	29.62 ± 3.81
	ST-6	Negative	2.2 ± 0.3	2.0 ± 0.4	25.15 ± 4.25
	ST-7	Positive	2.0 ± 0.2	2.4 ± 0.3	33.58 ± 4.98
	ST-8	Negative	1.7 ± 0.5	2.2 ± 0.1	35.70 ± 3.91
	ST-9	Negative	2.2 ± 0.4	3.0 ± 0.5	38.08 ± 4.05
	ST-10	Negative	2.5 ± 0.7	2.8 ± 0.4	32.81 ± 5.51
Trang Province	TP-1	Positive	1.5 ± 0.1	2.8 ± 0.3	30.81 ± 5.18
	TP-2	Negative	2.0 ± 0.2	2.1 ± 0.3	31.47 ± 3.24
	TP-3	Negative	1.5 ± 0.4	2.8 ± 0.5	38.18 ± 6.81
	TP-4	Positive	1.7 ± 0.2	2.7 ± 0.9	30.28 ± 5.15
	TP-5	Negative	2.1 ± 0.2	3.0 ± 0.4	29.20 ± 7.50
	TP-6	Negative	1.6 ± 0.3	3.0 ± 0.4	25.25 ± 4.40
	TP-7	Negative	2.0 ± 0.5	2.7 ± 0.7	39.17 ± 3.18
	TP-8	Negative	2.6 ± 0.2	2.2 ± 0.1	35.71 ± 5.24
	TP-9	Positive	2.2 ± 0.1	2.7 ± 0.5	28.04 ± 5.10
	TP-10	Negative	1.2 ± 0.3	2.9 ± 0.3	32.18 ± 6.41
	TP-11	Positive	1.4 ± 0.2	2.0 ± 0.3	34.80 ± 3.48
	TP-12	Negative	1.6 ± 0.3	3.1 ± 0.6	31.18 ± 4.05
	TP-13	Negative	2.1 ± 0.5	2.3 ± 0.4	24.84 ± 3.47

[a] All values are mean ± SD from triplicate determinations.

[b] Diameter of negative control for the DCT was 1.0 cm.

results are shown in Table 2. As shown in Table 2, the highest activities toward SAC production were found when 8% (w/v) CHP was used in the cultivation of *L. aggregate* KP-5. Although the CHP concentration of 6% could not give the highest value of dry cell weight, the excreted SAC showed good surface activities in terms of surface tension and EA. Inconsistencies of cell growth and SAC activity observed in this study had been reported elsewhere (Pornsunthorntawee et al., 2008). Therefore, the culture medium supplemented with 8% CHP was chosen as the appropriate culture medium for the next experiments of SAC production by *L. aggregate* KP-5.

Effect of Nitrogen Sources on Growth and SAC Production

To determine the optimal conditions that yield the highest SAC production by *L. aggregate* KP-5, the effect of various nitrogen sources was evaluated (Table 2). *L. aggregate* KP-5 was able to grow and show SAC activity in all nitrogen sources tested with different activities and yields depending on the nitrogen source used. The highest biomass production of *L. aggregate* KP-5 was obtained using peptone as the sole nitrogen source (3.14 $g\ L^{-1}$). However, the lowest surface tension values (34.5), which corresponded to the highest EA (55.54%) and SAC yield (1.05 $g\ L^{-1}$), were obtained with commercial monosodium glutamate. This result was in accordance with Chooklin, Maneerat, et al. (2014), who reported that the highest biomass of *Halobacteriaceae archaeon* AS65 was obtained with peptone. However, the highest SAC production was obtained when using commercial monosodium glutamate as a nitrogen source. Other authors also reported the highest SAC production using commercial monosodium glutamate as a nitrogen source (Chooklin, Petmeaun, et al., 2014; Noparat, Maneerat, & Saimmai, 2014; Saimmai, Onlamool, et al., 2013; Saimmai, Udomsilp, et al., 2013).

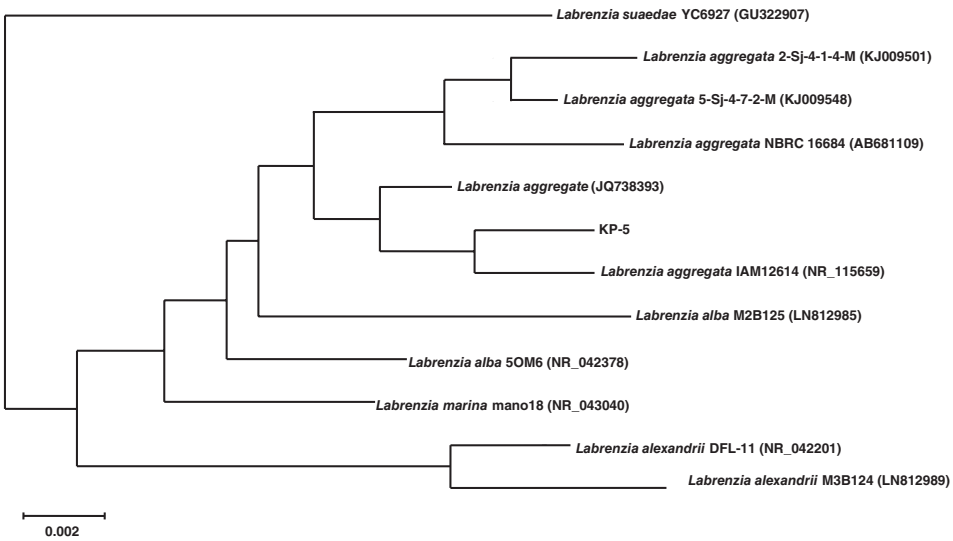

Fig. 1 Phylogenetic tree of isolate KP-5 and closest NCBI (BLASTn) strains based on 16S rRNA gene sequences (neighbor joining tree method). Scale bar 0.01 nucleotide substitutions per nucleotide position. Numbers at node. Bootstrap values obtained with 1000 resampling analyses. The GenBank accession numbers are reported in parentheses

Effect of the Carbon:Nitrogen (C:N) Ratio on Growth and SAC Production

The results showed that the C:N ratio was fundamental to improve SAC productivity by *L. aggregate* KP-5 (Table 2). Among nine different C:N ratios examined, with CHP and commercial monosodium glutamate as carbon and nitrogen sources, respectively, the maximum SAC activity in surface tension and EA of 25 mN m^{-1} and 65%, respectively, was achieved using the 30:1 ratio of C:N (Table 2). However, the highest SAC yield (3.50 g L^{-1}) was obtained when the C:N ratio of 40:1 was used. Increasing the C:N ratio to more than 40:1 does not significantly increase either bacterial cell growth or SAC yield. SAC is often produced under growth-limiting conditions of nitrogen (Franzetti et al., 2009; Santos et al., 2016). Nitrogen limitation caused an increase in lipopeptides from *H. archaeon* AS65 (Chooklin, Maneerat, et al., 2014), glycolipid production from *Ochrobactrum anthropi* 2/3 (Noparat et al., 2014) and *Candida* sp. SY16 (Kim et al., 2006), and rhamnolipids from *P. aeruginosa* LBI (Benincasa & Accorsini, 2008).

Kinetics of SAC Production

A time course study of SAC production by *L. aggregate* KP-5 was carried out using a production medium with 40 g L^{-1} CHP and 1 g L^{-1} commercial monosodium glutamate as a carbon and nitrogen source, respectively, at 30 °C, 150 rpm. SAC yields, surface tension, EA, and pH of the culture broth were dependent on the growth of the culture in the fermentation medium (Fig. 2). The kinetic growth curve of *L. aggregate* KP-5 and the SAC production curve with upward trends indicated a parallel relationship between bacterial growth and SAC production. As shown in Fig. 2, the highest dry cell weight of 4.62 g L^{-1} was observed after the cultivation time of 54 h, which is the exponential phase, before gradually entering the dead phase. The highest values of surface activity and EA were obtained after about 48 h of cultivation with 25.5 mN m^{-1} and 65%, respectively. However, the highest SAC yield (3.51 g L^{-1}) was obtained as the cultivation time approached 51 h, which also corresponded to the stationary phase of microbial growth. The results from the present study reveal that SAC production from *L. aggregate* KP-5 was growth associated. In the case of a growth-associated SAC production, there is a parallel relationship between the substrate consumption, growth, and SAC production (Rodrigues, Teixeira, Oliveira, & Mei, 2006; Uzoigwe, Burgess, Ennis, & Rahman, 2015). Growth-associated SAC production has been described for the production of lipopeptides from *H. archaeon* AS65 (Chooklin, Maneerat, et al., 2014), glycolipid from *O. anthropi* 2/3 (Noparat et al., 2014), and rhamnolipid from *P. aeruginosa* RS29 (Saikia, Deka, Deka, & Banat, 2012).

Recovery of the SAC

The ability of various solvent systems to harvest the SAC from the 51 h culture supernatant of *L. aggregate* KP-5 was investigated. Among the five solvent systems tested, the highest SAC yield, CMC, surface tension, and EA were obtained when using ethyl acetate as a solvent system (data not shown). The SAC obtained from ethyl acetate extraction can dissolve completely in distilled water. Recently, ethyl acetate has been successfully used as a solvent for

Table 2 Effects of the carbon concentration, nitrogen source, and carbon:nitrogen ratio on growth and biosurfactant production from *Labrenzia aggregate* KP-5, cultivated in a 250 mL flask containing 50 mL MSM at 30 °C in a shaking incubator at 150 rpm for 48 h

Parameters	Dry cell weight ($g\ L^{-1}$)[a]	Surface tension ($mN\ m^{-1}$)[a]	Biosurfactant ($g\ L^{-1}$)[a]	EA (%)[a]
Corn husks powder (g L^{-1}) (nitrogen sources, 1 g L^{-1} $(NH_4)_2SO_4$)				
2	1.60±0.30e	50.5±1.4c	0.10±0.01c	40.41±5.20d
4	1.82±0.13d	45.7±1.4b	0.12±0.02b	42.68±4.13cd
6	1.98±0.25c	43.2±1.4b	0.13±0.01b	44.24±3.95bc
8	2.03±0.15b	39.6±1.3a	0.25±0.03a	48.33±5.14a
10	2.14±0.04a*	39.0±2.2a	0.25±0.02a	48.40±6.20a
Nitrogen sources, 1 g L^{-1} (carbon source, 8 g L^{-1} CHP)				
Beef extract	2.95±0.12b	41.0±1.5de	0.15±0.01h	38.51±5.67f
commercial name of monosodium glutamate (CMSG)	2.71±0.35c	34.5±2.4g	1.05±0.12a	55.54±3.68e
Meat extract	2.91±0.14b	40.0±3.2de	0.14±0.14f	44.93±1.73d
$C_6H_{17}N_3O_7$	2.13±0.20e	45.5±0.9b	0.12±0.03e	45.53±2.83c
$(NH_4)_2SO_4$	2.03±0.15f	39.6±1.3e	0.25±0.03a	48.33±5.14a
$NaNO_3$	2.09±0.50f	36.0±3.1f	0.59±0.01b	51.51±1.47b
NH_4Cl	2.27±0.32d	37.5±1.8f	0.52±0.04b	50.54±3.38a
NH_4NO_3	2.39±0.13d	45.0±2.3b	0.13±0.05a	35.93±1.63a
Peptone	3.14±0.27a	42.5±1.5cd	0.15±0.06a	41.82±2.15a
Urea	3.05±0.23b	52.3±2.8a	0.11±0.15a	30.51±8.47a
Yeast extract	3.07±0.14b	47.5±3.5b	0.13±0.06a	35.54±6.08a
Carbon:nitrogen ratio				
5:1	2.56±0.87h	42.0±3.3a	0.95±0.04h	52.31±4.03e
8:1	2.71±0.35g	34.5±2.4b	1.05±0.12g	55.54±3.68de
15:1	3.05±0.56f	30.2±3.3c	1.38±0.15f	58.25±4.35cd
20:1	3.34±0.24e	27.0±2.4d	1.87±0.11e	60.37±5.27bc
25:1	3.56±0.57d	26.3±1.5e	2.14±0.08d	62.59±6.15ab
30:1	3.98±0.42c	25.0±2.0e	2.90±0.24c	65.41±3.03a
35:1	4.28±0.28b	25.2±1.4e	3.27±0.31b	65.28±4.31a
40:1	4.59±0.35a	25.5±2.7e	3.50±0.25a	65.63±5.04a
45:1	4.58±0.12a	25.3±4.0e	3.50±0.10a	65.87±4.13a

[a] All values are mean ± SD from triplicate determinations.

*Different superscript letters in the same column indicate significant differences ($P < 0.05$).

extraction of biosurfactants from *H. archaeon* AS65 (Chooklin, Maneerat, et al., 2014), *Inquilinus limosus* KB3 (Saimmai, Onlamool, et al., 2013), and *O. anthropi* 2/3 (Noparat et al., 2014). In general, the extraction has a drawback of using highly toxic chloro-organic compounds or the loss of biosurfactant activity and its expensive nature makes it practically less attractive (Chen, Chen, & Juang, 2008; Santos et al., 2016; Varjani & Upasani, 2017). Accordingly, it is desired to use ethyl acetate, a more economic and environmentally friendly, to improve the recovery yield and purity of the SAC.

Chemical Characterization of the SAC

Compositional analysis of the obtained SAC revealed that the SAC produced from *L. aggregate* KP-5 was a glycolipid, consisting of 68% lipids and 30% carbohydrates. A minor fraction of protein (1–2%) was found from the obtained SAC possibly arising from the existence of the residual cell debris in broth coprecipitated with SAC during its extraction process (Lotfabad et al., 2009). The agar double diffusion test revealed the appearance of precipitation lines between the SAC produced from *L. aggregate* KP-5 and the cationic compound used (barium chloride). However, no lines were formed between the SAC and the anionic compound (sodium dodecyl sulfate [SDS]). Under the experimental conditions of the present study, this simple test demonstrated the anionic character of the SAC produced.

The molecular composition of the SAC produced from *L. aggregate* KP-5 was evaluated using FT-IR and the result is shown in Fig. 3. The important adsorption bands

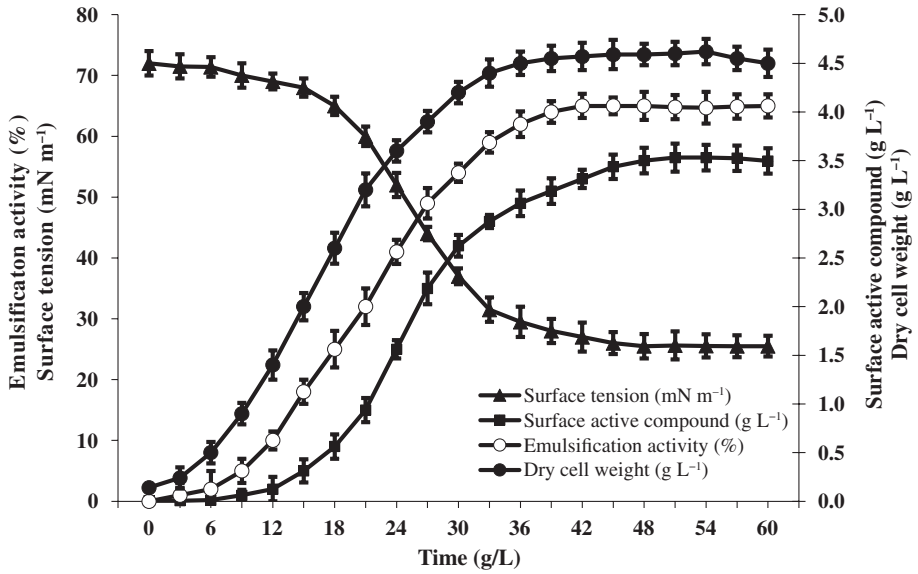

Fig. 2 Time course of growth and SAC production from *Labrenzia aggregate* KP-5, cultivated in a 250 mL flask containing 50 mL MSM at 30 °C in a shaking incubator at 150 rpm

involving strong adsorption peaks at 3424 cm^{-1} indicate the presence of O—H stretching vibrations. Absorption around 2856–3002 cm^{-1} was assigned to the symmetric stretch (CH) of CH_2 and CH_3 groups of aliphatic chains since lactones and esters have two strong absorption bands arising at 1747 and 1648 cm^{-1}, respectively. The stretch of the C—O band of C(—O)—O—C in lactones exists at 1240 cm^{-1}, while that from the acetyl esters was found to be at 1370–1457 cm^{-1}. Moreover, sugar C—O stretch of C—O—H groups was found to be at 1037 cm^{-1} and the band at 1648 cm^{-1} corresponded to the C—O—H in-plane bending of carboxylic acid (—COOH) in the structure of the product. The above information from the respective wave numbers confirmed the rhamnolipid nature of the biosurfactant (Abdel-Mawgoud, Lépine, & Déziel, 2010; Noparat et al., 2014).

The ^1H NMR spectrum of the obtained SAC (Fig. 4a) had a number of signals corresponding to different sugar moieties and protein content. The chemical shift (δ) values between 1.60 and 1.25 ppm and those between 5.4

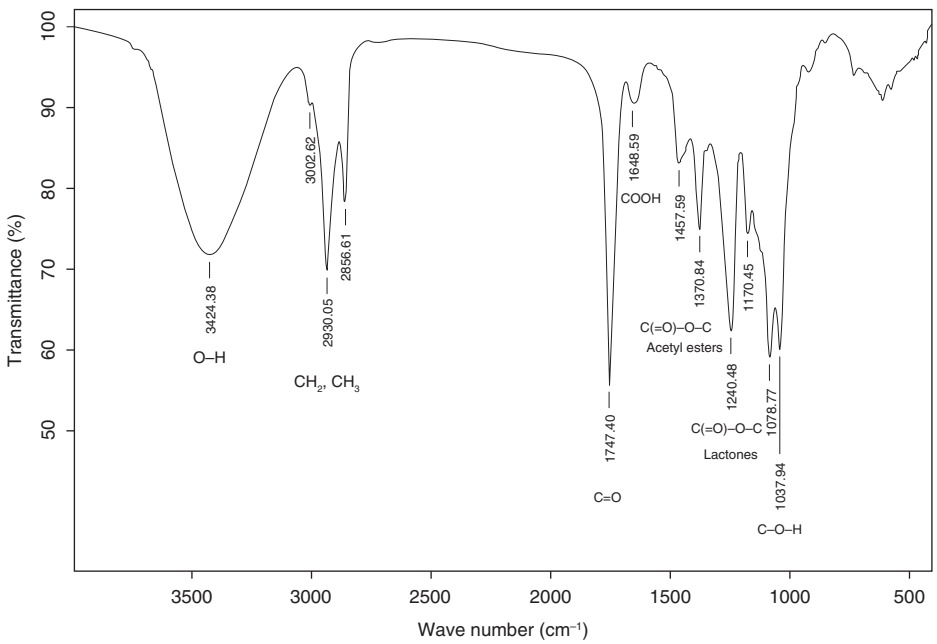

Fig. 3 FT-IR of the SAC produced from *Labrenzia aggregate* KP-5

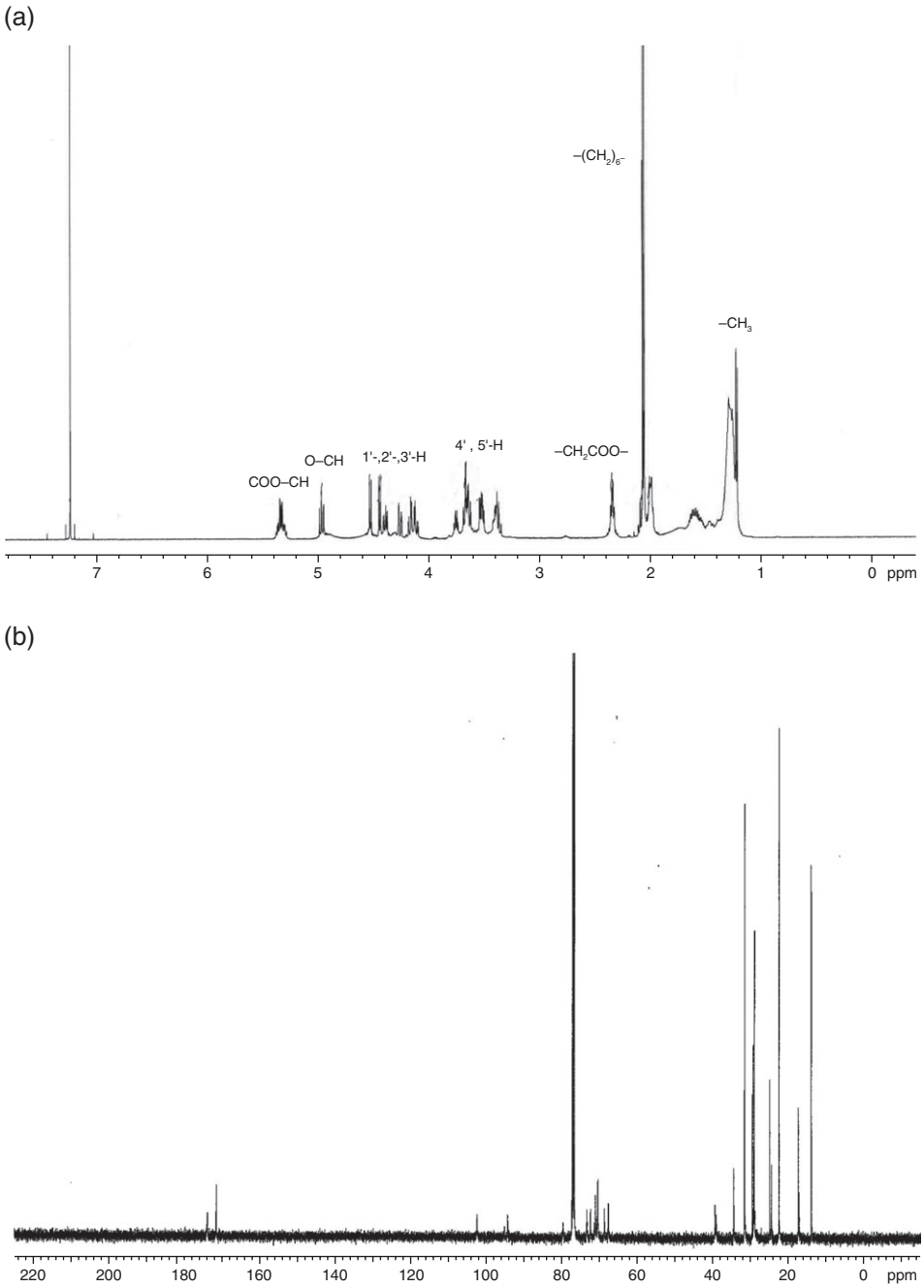

Fig. 4 ^{1}H NMR spectrum (**a**) and ^{13}C NMR (**b**) of the SAC produced form *Labrenzia aggregate* KP-5

and 5.0 ppm were attributed to aliphatic nature of the sugar moiety and olefinic groups, respectively. The signal at 4.60–3.40 was assigned to the protons on β-anomeric carbon of sugar moieties. ^{1}H NMR exhibited well resolved peaks of anomeric protons in the region from 5.40–4.05 to 5.74 ppm. These chemical shifts indicated most probably the α-glucogalacto configuration of glycosidic linkages (Nikonenko, Buslov, Sushko, & Zhbankov, 2000). Figure 4b is the ^{13}C NMR spectrum of the obtained SAC, which shows the presence of several —CH_3— and —CH_2— groups in the fatty chain moiety that were also resonated at 40–12 ppm. The peak signal in the range of (δ) 80–64 ppm confirmed the presence of the acetyl group in the SAC. To further elucidate the structure of the SAC produced in the study, liquid chromatography (LC)–MS analysis was performed. The mass spectrum of the compound indicates that the highest signal was observed at *m*/*z* 813 (Fig. 5). This corresponds to the di-rhamno-di-lipidic congeners reported by Abdel-Mawgoud et al. (2010). There are numerous reports on

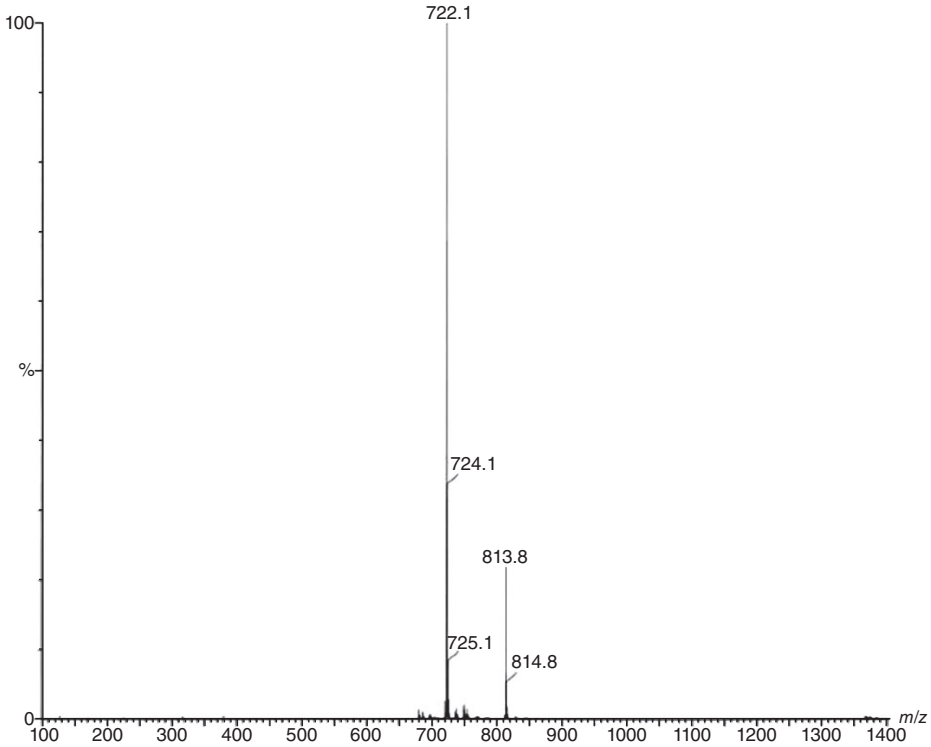

Fig. 5 Mass spectrum of SAC concentration produced from *Labrenzia aggregate* KP-5

the isolation and rhamnolipid biosurfactant production of different species of the genus *Acinetobacter*, *Bacillus*, *Candida*, and *Seratia* (Kim et al., 2006; Liu et al., 2017; Pornsunthorntawee et al., 2008; Saimmai, Onlamool, et al., 2013; Sriram et al., 2011; Varjani & Upasani, 2017; Vaz, Gudina, Alameda, Teixeira, & Rodrigues, 2012). However, in the case of *Labrenzia*, this is the first report for the production of rhamnolipid.

Physicochemical Properties of the SAC

The solubility of surfactants within the aqueous phase is defined by the CMC and is used as a measurement for the efficiency of a SAC. The lower the value of the CMC, the lower the amount of SAC required to reduce the surface tension to the minimum level and hence indicating a higher efficiency (Santos et al., 2016; Sriram et al., 2011; Varjani & Upasani, 2017). The CMC of the SAC produced from *L. aggregate* KP-5 was 9 mg L^{-1} (11.07 mM) at a surface tension value of 25.5 mN m^{-1} as shown in Fig. 6. The result indicated that the SAC produced from *L. aggregate* KP-5 using CHP as a substrate showed a lower minimum surface tension and a CMC value than the SAC produced from *H. archaeon* AS65 using banana peel as the carbon source (25.5 mN m^{-1}, 10 mg L^{-1}) (Chooklin, Maneerat, et al., 2014), *Bacillus subtilis* using crude oil as the carbon source (29.0 mN m^{-1}, 40 mg L^{-1}) (Vaz et al., 2012), *Bacillus tequilensis* ZSB10 using a mixture of hemicellulosic and cellulosic hydrolyzate as the carbon source (38.60 mN m^{-1}, 177.14 mg L^{-1}) (Cortes-Camargoa, Pérez-Rodríguezb, Souza Oliveirad, Barragán Huertaa, & Domínguez, 2016), *P. aeruginosa* MR01 using residues from the soybean oil industry (27.0 mN m^{-1}, 24 mg L^{-1}) (Lotfabad, Ebadipour, Roostaazad, Partovi, & Bahmaei, 2017), *Pseudomonas* sp. BUP6 using groundnut oil as the carbon source (34.0 mN m^{-1}, 48 mg L^{-1}) (Silva et al., 2017), and *Pseudomonas* sp. using molasses and corn steep liquor as substrates (27.0 mN m^{-1}, 24 mg L^{-1}) (Silva et al., 2017).

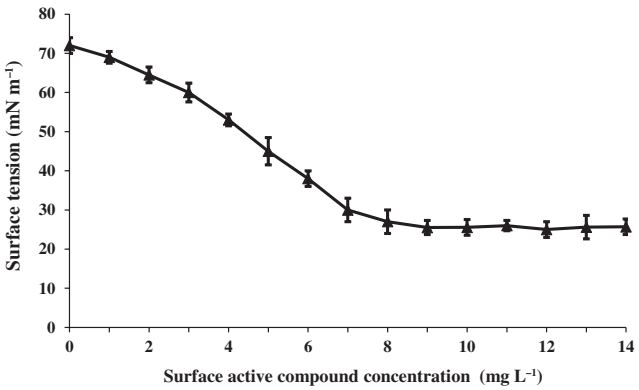

Fig. 6 Surface tension as a function of SAC concentration produced from *Labrenzia aggregate* KP-5. Bars represent the SD from three determinations

Emulsification Specificity of SAC

The emulsification property of a SAC depends on the organic solvent system used, *i.e.*, oil, aliphatic, or aromatic hydrocarbon. In the present study, the specificity of emulsion formation was highly variable, depending on the hydrophobic substrate (Fig. 7a). Formation of stable emulsion was observed with benzene, dichloromethane, hexane, motor oil, *n*-hexadecane, paraffin, and petroleum ether at the CMC, which was reflected by the high emulsification indices in the range from 70% to 82% up to 72 h. Diesel oil, olive oil, palm oil, soybean oil, and sunflower oil also formed stable emulsions in the range of 50–68%. Decane, kerosene, toluene, ULO, and xylene resulted in poor emulsification with EA and E24 less than 50%, probably due to the inability of the SAC to stabilize the microscopic droplets of these compounds. Chooklin, Maneerat, et al. (2014) and Chooklin, Petmeaun, et al. 2014) reported a similar result, for which the SAC showed activity against various hydrocarbons or oil, with the maximum toward aromatics and the least with ULO and xylene. The ability of SAC from *L. aggregate* KP-5 to emulsify various hydrophobic substrates indicates that it has good potential for application in microbial enhanced oil recovery and can also be used as an emulsifying agent in the food industry.

Effects of Temperature, pH, and Salinity SAC Stability

Two important properties of the SAC are to reduce the surface tension of solution and stabilize the emulsion between two phases (Perfumo et al., 2010; Santos et al., 2016).

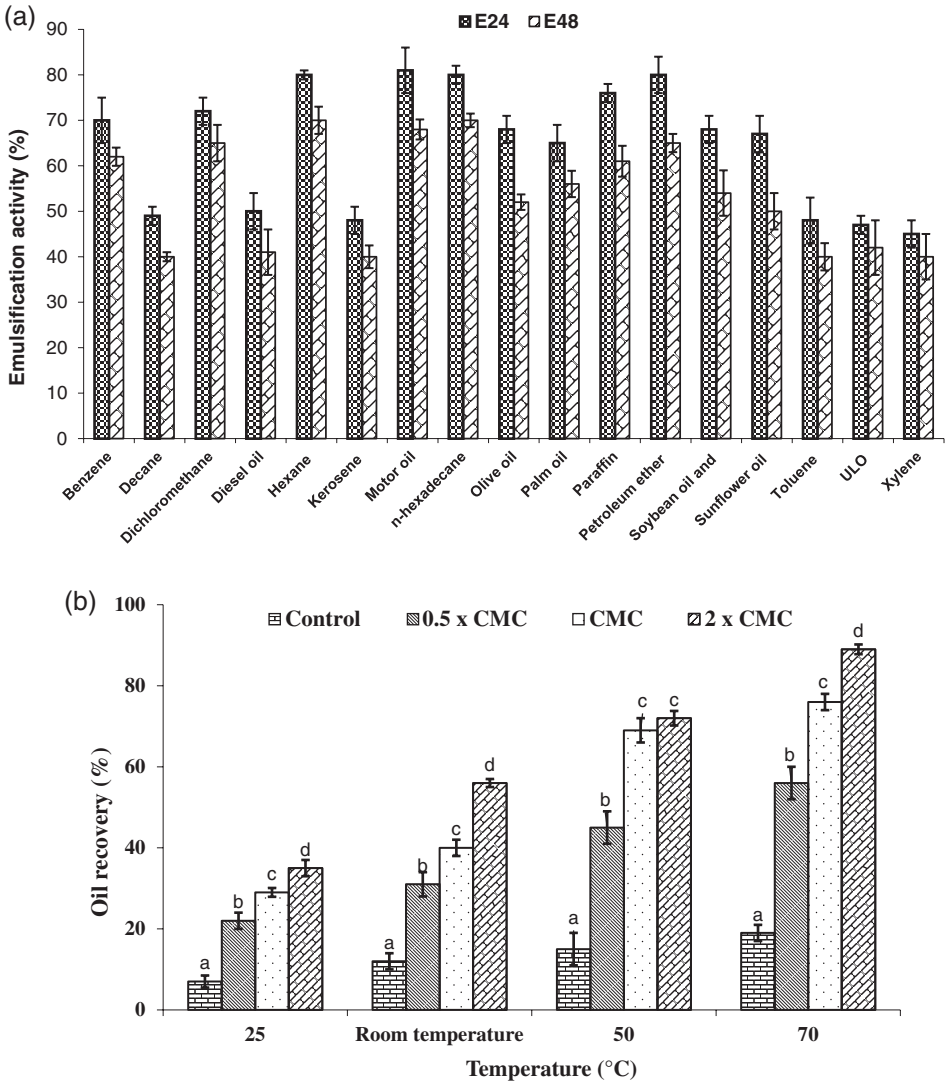

Fig. 7 EA and the emulsification index (E24) against different hydrocarbons and vegetable oils (**a**) and microbial enhanced oil recovery (**b**) of the SAC produced from *Labrenzia aggregate* KP-5. Bars represent the SD from three determinations

Stability of the SAC from *L. aggregate* KP-5 was tested over a wide range of temperatures and the results showed that the obtained SAC was thermostable (Table 3). The surface tension and EA of the SAC at CMC were stable from 4 to 100 °C with minimal variation (3.0 mN m^{-1}, 7%, and 6% for surface tension, EA, and E24, respectively) and about 20% reduction in surface tension, E24, and E48 was observed after autoclaving at 121 °C for 20 min. This property will be very useful in cosmetics and food industries, pharmaceutical industry, and also where heating to achieve sterility is of paramount importance. Tests performed with SAC solution demonstrated no significant changes in surface tension, EA, and E24 at different pH values. However, a slight increase in surface tension was found at pH 2–4 and 10–12. Silva, Rufino, Luna, Santos, and Sarubbo (2014) analyzed a biosurfactant produced from *Pseudomonas cepacia* CCT6659 and found a little change in surface activity in acidic pH 2–4 and alkali pH 12, but no significant changes in the other values tested (pH 6–10). The effect of NaCl addition on the stability of SAC produced from *L. aggregate* KP-5 was studied (Table 3). A negligible change was observed in surface tension, EA, and E24 with an increase in the NaCl concentration up to 10.0%. However, at 12% of NaCl, surface tension increased sharply (35.5 mN m^{-1}), and EA and E24 dropped as well (30% and 26%, respectively). This finding was in agreement with that observed in a relevant study reported by Chooklin, Maneerat, et al. (2014), who found that the activity of the biosurfactant from *H. archaeon* AS65 remained unaltered when subjected to concentrations of NaCl up to 10%.

Table 3 Influence of temperature, pH, and salt concentration on surface tension and EA of the biosurfactant produced from *Labrenzia aggregate* KP-5

Parameters	Surface tension (mN m^{-1})[a]	EA (%)[a]	
		EA	E24
Temperature (°C)			
4	25.5±2.0	65.0±3.8	56.5±3.0
20	25.5±1.5	65.8±5.3	56.8±4.3
40	26.5±0.8	65.5±5.0	56.5±5.0
80	28.2±3.0	65.8±5.4	52.8±5.2
100	28.5±1.8	58.4±4.0	50.4±8.1
110	30.5±2.1	51.4±6.0	48.4±4.0
121	32.0±2.4	50.0±5.7	45.0±5.4
pH			
2.0	56.4±1.5	56.8±2.0	41.5±3.0
3.0	38.3±0.4	60.5±1.5	43.8±4.3
4.0	38.3±1.2	61.5±2.5	48.8±2.3
5.0	27.3±2.8	63.5±2.4	54.8±3.3
6.0	25.9±1.5	65.7±2.7	56.5±1.0
7.0	25.5±2.8	65.5±3.5	56.8±2.2
8.0	26.0±3.4	64.3±2.0	56.8±3.7
9.0	26.3±0.8	63.5±2.5	54.8±2.3
10.0	27.1±2.9	60.8±1.0	50.4±1.0
11.0	38.3±2.8	54.5±3.5	45.8±2.2
12.0	49.7±1.6	50.0±2.4	40.0±4.0
NaCl (%, w/v)			
0	25.5±1.8	65.5±3.0	56.8±2.9
2	25.8±0.7	65.0±1.5	56.9±5.3
4	25.9±2.7	64.2±2.5	55.0±4.8
6	26.2±2.5	63.5±1.7	54.0±5.7
8	27.5±1.0	60.5±2.5	52.2±2.3
10	28.5±0.8	58.9±3.3	41.8±6.0
12	35.5±1.5	45.0±5.5	35.7±3.3

[a] All values are mean ± SD from triplicate determinations.

Application of the SAC in the Oil Removal

Hydrophobic substances such as oil or hydrocarbon bind to soil components and are difficult to remove and degrade. SAC can decrease surface tension, increase water solubility, and enhance the displacement of hydrophobic substances from soil particles (Liu et al., 2017; Santos et al., 2016; Satpute, Banpurkar, et al., 2010; Varjani & Upasani, 2017). The capacity of aqueous SAC solutions to remove crude oil from contaminated sand was investigated (Fig. 7b). The results obtained demonstrated that the SAC solution at 0.5 × CMC was capable of removing 22%, 31%, 45%, and 56% of the oil adsorbed in the sand at 25, room temperature (30 ± 3 °C), 50 °C and 70 °C, respectively (Fig. 7b). Increasing the SAC concentration from 0.5 × CMC to 2.0 × CMC resulted in 33% increase in oil recovery (from 56% to 89%) at 70 °C. The negative control (distilled water) removed only 19% of the contaminated oil at 70 °C. The biosurfactant from *H. archaeon* AS65 showed a high surface activity in the oil rinsing test from sand samples. After rinsing of the sand with solutions of biosurfactants at 60 °C, over 72% of oil was recovered (Chooklin, Maneerat, et al., 2014), while the biosurfactant solution at 10 × CMC of *Deinococcus caeni* PO5 had removed 92% of the motor oil adsorbed in sand samples (Chooklin, Petmeaun, et al., 2014). Based on the analysis of the results obtained in this study, the SAC produced from *L. aggregate* KP-5 showed good properties for use in enhanced oil recovery.

Two mechanisms are proposed for the removal of hydrophobic substrates from the matrix: mobilization and solubilization. The mobilization mechanism causing the reduction of surface tension between air/water and soil/water occurs at concentrations below the biosurfactant CMC. Also, the contact of biosurfactants with the soil/oil system raises the contact angle and reduces the capillary force holding oil and soil together because of the reduction of the interfacial force. Solubilization occurs when the

SAC concentration exceeds the CMC, as the apparent solubility of hydrophobic increases significantly because of the aggregation of SAC micelles. The tail or hydrophobic moieties of SAC cluster together inside the micellar structure with the head or the hydrophilic part exposed to exterior leaving the whole structure to remain in solution (Liu et al., 2017; Santos et al., 2016; Urum & Pekdemir, 2004). According to the observed data, solubilization is the main mechanism associated with the oil removal by the SAC obtained from *L. aggregate* KP-5, because increasing the SAC concentration enhanced the removal of oil significantly, because of the mixing of these molecules into micelles. The results herein obtained are in accordance with the previous report (Saimmai, Onlamool, et al., 2013) describing the solubilization mechanism of rhamnolipid from *Selenomonas ruminantium* CT2 in the removal of ULO.

PAH Solubilization by the SAC

PAH represent a diverse class of organic compounds having a broad range of molecular weight, water solubility, volatility, sorption coefficients, *etc.* They are generally described as molecules, which comprise three or more fused aromatic rings in various structural configurations (Perfumo et al., 2010; Santos et al., 2016; Satpute et al., 2016). The low bioavailability makes PAH recalcitrant to degrade by bacteria or fungi. The rate of microbial degradation of PAH increases when the water solubility of PAH increases (Abdel-Mawgoud et al., 2010; Satpute, Banpurkar, et al., 2010; Varjani & Upasani, 2017). The effect of the SAC concentration on the water solubility of PAH was determined by the PAH solubilization test and the result is shown in Table 4. All of the PAH solubility tested was increased when increasing the SAC concentration from 0.5 × CMC to 2.0 × CMC (Table 2). Chrysene and phenanthrene showing the least increase (1.6- to 1.7-fold), fluorine, naphthalene, naphthylamine, and pyrene being intermediate (2.4- to 3.7-fold), and acenaphthylene, acenaphthene, anthracene, and fluoranthene giving the highest increase (4.1- to 5.1-fold).

Table 4 Effects of the SAC isolated from *Labrenzia aggregate* KP-5 on the aqueous solubility of PAH

	Solubility of PAH (mg L^{-1})[a]		
	0.5 × CMC (4.5 mg L^{-1})	CMC (9 mg L^{-1})	2 × CMC (18 mg L^{-1})
Acenaphthylene	12.58 ± 1.25c*	31.87 ± 5.57b	52.63 ± 6.14a
Acenaphthene	13.25 ± 2.87c	40.64 ± 4.32b	68.25 ± 2.87a
Anthracene	0.52 ± 0.12c	1.98 ± 0.81b	2.57 ± 0.54a
Chrysene	0.48 ± 0.03c	1.57 ± 0.28b	2.06 ± 0.89a
Fluoranthene	1.25 ± 0.24c	3.58 ± 1.14b	5.81 ± 1.25a
Fluorine	2.87 ± 0.35c	4.65 ± 1.56b	7.89 ± 3.21a
Naphthalene	22.96 ± 5.25c	57.25 ± 6.87b	85.21 ± 5.24a
Naphthylamine	14.58 ± 2.57c	35.10 ± 5.76b	52.74 ± 6.25a
Phenanthrene	2.14 ± 0.17c	3.98 ± 0.24b	3.57 ± 0.21a
Pyrene	1.59 ± 0.09c	2.75 ± 0.57b	3.89 ± 1.25a

[a] All values are mean ± SD from triplicate determinations.

*Different letters in the same row indicate significant differences ($P < 0.05$).

Antimicrobial Activity of the SAC

The SAC isolated from *L. aggregate* KP-5 showed a wide range of activity against the pathogenic microorganisms tested (Table 5). The antimicrobial activity increased with increasing concentration of the SAC. Table 5 shows that all tested microorganisms were sensitive to the SAC except some Gram-negative bacterium (*Klebsiella pneumonia*). The explanation of this result could come from the biofilm formation of *K. pneumonia.* It helps the strain resistant to antibiotic substances such as ampicillin and ciprofloxacin (Anderl, Franklin, & Stewart, 2000). These antimicrobial

Table 5 Antimicrobial activities of the SAC isolated from *Labrenzia aggregate* KP-5 at different concentrations using the agar diffusion method

Microorganisms	Zone of inhibition diameter (mm)[a]		
	0.5 × CMC (5.54 mM)	CMC (11.07 mM)	2 × CMC (22.14 mM)
Bacteria			
Bacillus cereus	6.51 ± 1.89	8.21 ± 2.71	9.57 ± 1.75
Enterococcus faecium	8.35 ± 2.23	10.67 ± 3.80	11.82 ± 3.17
Escherichia coli	7.24 ± 3.84	9.85 ± 2.12	12.55 ± 2.51
Klebsiella pneumonia	3.58 ± 2.96	5.23 ± 2.89	7.54 ± 2.51
Listeria monocytogenes	7.69 ± 2.18	11.22 ± 3.51	13.57 ± 1.50
Pseudomonas aeruginosa	8.07 ± 1.98	9.05 ± 3.56	11.08 ± 3.95
Salmonella sp.	5.86 ± 2.51	7.91 ± 2.64	9.59 ± 2.36
Salmonella typhimurium	6.51 ± 2.89	8.35 ± 1.64	10.06 ± 5.64
Staphylococcus aureus	9.31 ± 3.61	12.76 ± 2.28	15.25 ± 2.42
Streptococcus pneumonia	10.17 ± 1.84	12.54 ± 3.78	16.42 ± 2.42
Vibrio cholera	5.81 ± 2.74	7.03 ± 3.82	10.04 ± 3.78
Vibrio vulnificus	5.35 ± 3.62	7.51 ± 2.19	10.73 ± 2.31
Fungi			
Botrytis cinerea	7.54 ± 3.82	9.94 ± 2.67	11.92 ± 4.67
Candida albicans	8.89 ± 2.15	10.02 ± 2.91	12.04 ± 2.89
Colletotrichum truncatum	9.31 ± 2.96	11.23 ± 3.70	13.22 ± 3.07
Fusarium moniliforme	8.63 ± 2.53	10.78 ± 4.33	12.73 ± 1.03
Gibberella zeae	7.64 ± 3.51	10.02 ± 3.95	13.01 ± 3.12
Penicillium candidum	6.57 ± 1.85	8.57 ± 2.71	10.21 ± 2.87

[a] All values are mean ± SD from triplicate determinations.

Table 6 Phytotoxicity of the biosurfactant isolated from *Labrenzia aggregate* KP-5 grown in a MSM supplemented with 40 g L^{-1} CHP and 1 g L^{-1} commercial monosodium glutamate as a carbon and nitrogen source, respectively, at 30 °C, 150 rpm for 51 h

Tested plant	SAC concentration	Seed germination (%)[a]	Root elongation (%)[a]	GI (%)[a]
Brassica oleracea L.	0.5 × CMC (5.54 mM)	100 ± 2.51	102 ± 2.50	98 ± 2.51
	CMC (11.07 mM)	100 ± 3.12	101 ± 3.17	99 ± 1.78
	2 × CMC (22.14 mM)	99 ± 2.87	98 ± 1.69	101 ± 2.94
Lactuca sativa L.	0.5 × CMC (5.54 mM)	102 ± 3.98	99 ± 1.87	103 ± 3.74
	CMC (11.07 mM)	98 ± 2.51	106 ± 2.64	92 ± 4.31
	2 × CMC (22.14 mM)	100 ± 1.50	98 ± 4.32	102 ± 1.98
Raphanus sativus	0.5 × CMC (5.54 mM)	100 ± 3.95	97 ± 2.57	103 ± 2.41
	CMC (11.07 mM)	105 ± 2.36	107 ± 1.84	98 ± 3.25
	2 × CMC (22.14 mM)	100 ± 5.64	90 ± 3.62	111 ± 1.97
Solanum gilo	0.5 × CMC (5.54 mM)	97 ± 2.42	106 ± 1.32	92 ± 2.31
	CMC (11.07 mM)	100 ± 3.78	96 ± 2.87	104 ± 3.89
	2 × CMC (22.14 mM)	107 ± 2.31	90 ± 1.15	119 ± 4.54
Triticum aestivum	0.5 × CMC (5.54 mM)	100 ± 5.05	98 ± 3.98	102 ± 2.41
	CMC (11.07 mM)	99 ± 4.67	96 ± 4.75	103 ± 4.23
	2 × CMC (22.14 mM)	100 ± 2.89	92 ± 2.32	109 ± 2.75
Vigna radiate	0.5 × CMC (5.54 mM)	112 ± 3.07	106 ± 1.23	106 ± 3.36
	CMC (11.07 mM)	107 ± 1.03	104 ± 1.24	103 ± 2.69
	2 × CMC (22.14 mM)	98 ± 2.82	91 ± 3.97	108 ± 1.47

[a] All values are mean ± SD from triplicate determinations.

results were similar to the results obtained from Gomaa (Gomaa, 2013) where the biosurfactant produced from *Bacillus licheniformis* M104 grown on whey had a good surface activity and antimicrobial activity against several tested pathogenic microorganisms except *K. pneumonia.* The antimicrobial properties of rhamnolipid have often been reported (Gomaa, 2013; Liu et al., 2017; Santos et al., 2016; Varjani & Upasani, 2017). Very recently, defined rhamnolipids have shown good inhibition behavior against either photogenic bacteria (*e.g.*, *K. pneumonia*, *L. monocytogenes*, *P. aeruginosa*, and *S. aureus*) (de Araujo et al., 2016; Haba et al., 2014) or phytopathogenic fungal species (*e.g.*, *C. albicans*, *Mucor miehei, Neurospora crassa*, and *Yarrowia lipolytica*) (Sharma, Kalita, & Duarah, 2017; Silva et al., 2017).

Phytotoxicity Determination of the SAC

The phytotoxicity of the SAC was measured by the germination test due to its low execution cost. Seed germination and root elongation of selected vegetable species were used to test the phytotoxicity of the SAC. A series of experiments were conducted to assess the level of toxicity of the SAC against *B. oleracea* L., *L. sativa* L., *R. sativus*, *S. gilo*, *T. aestivum*, and *V. radiate.* All the solutions tested exhibited no inhibitory effect on both seed germination and root elongation (Table 6). SAC solutions also exhibited no inhibitory effect on the GI, *V. radiate* (103–108) had the highest GI followed by *S. gilo* (92–119), *T. aestivum* (102–109), *R. sativus* (98–111), *L. sativa* L. (92–103), and *B. oleracea* L. (98–101), consecutively. A GI of 80% has been used as a bioindicator to confirm the absence of phytotoxicity of the biosurfactant (Tiquia et al., 1996). The results obtained in the present study indicate that the SAC from *L. aggregate* KP-5 does not pose any inhibitory effect on seed germination and root elongation. Interestingly, leaves and secondary root growth were observed in almost all tested vegetable species. Similar results were observed by Luna, Rufino, Sarubbo, and Campos-Takaki (2013) for the biosurfactants produced from *Candida sphaerica* UCP0995 and *Bacillus methylotrophicus* USTBa, respectively.

Conclusions

Use of cost-free materials is an attractive alternative for the production of an efficient SAC, as the sources used in the present study are readily available and inexpensive. To the best of our knowledge, this report is the first one that describes the production of SAC using CHP as a carbon substrate. The SAC produced from *L. aggregate* KP-5 shows a stable surface activity and ability to emulsify different hydrophobic substrates. The stability of the SAC under varying pH, temperature, and

salinity is also another attractive characteristic for its applications in a diversity of environments. This complies with the recent need for the use of SAC in their crude form for the purpose of cost reduction. More importantly, the excellent biological activity, capacity to remove hydrophobic contaminants, and no toxicity of the SAC indicated its potential application in agricultural and environmental fields.

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