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ORIGINAL ARTICLE

Production and characterization of biosurfactant from marine bacterium *Inquilinus limosus* KB3 grown on low-cost raw materials

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Abstract *Inquilinus limosus* strain KB3, isolated from marine sediment in the south of Thailand, was used to produce a biosurfactant from a mineral salts medium (MSM) with palm oil decanter cake (PODC) as a carbon source. It was found that cellular growth and biosurfactant production in MSM were greatly affected by the medium components. *I. limosus* KB3 was able to grow and to produce surfactant reducing the surface tension of medium to 28.2 mN/m and giving a crude surfactant concentration of 5.13 g/l after 54 h. The biosurfactant obtained was found to reduce the surface tension of pure water to 25.5 mN/m with the critical micelle concentration of 9 mg/l, and retained its properties during exposure to elevated temperatures (121 °C), high salinity (12 % NaCl), and a wide range of pH values. Chemical characterization by FT-IR, NMR, and ESI-MS revealed that the biosurfactant has a lipopeptide composition with molecular mass (m/z) of 1,032. The biosurfactant was capable of forming stable emulsions with various hydrocarbons and had the ability to enhance oil recovery, PAHs solubility, and antimicrobial activity.

Keywords *Inquilinus limosus* · Biosurfactant · Palm oil decanter cake · Oil recovery · Polyaromatic hydrocarbon

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Introduction

The interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications in the biomedical, pharmaceuticals, petroleum, and food processing industries (Banat et al. 2010). The biosurfactants have several advantages over chemical surfactants including high ionic strength tolerance, high temperature tolerance, higher biodegradability, lower toxicity, lower critical micelle concentration (CMC), and higher surface activity (Abdel-Mawgoud et al. 2009; Banat et al. 2010; Gudina et al. 2011). In spite of the advantages, fermentation must be cost competitive with chemical synthesis, and many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically. The choice of inexpensive raw materials is important to overall economy of the process because they account for 50 % of the final product cost and also reduce the expense of waste treatment (Makkar and Cameotra 2002). In recent years, much work has been carried out towards efficient utilization of agro-industrial residues such as cassava wastewater (Nitschke and Pastore 2006), ground-nut oil refinery residue and corn steep liquor (Sobrinho et al. 2008), molasses (Joshi et al. 2008; Saimmai et al. 2011), and potato peels (Das and Mukherjee 2007). Palm oil decanter cake, a by-product from the palm oil milling decantation process, is an abundant and low cost agricultural waste residue. It is easily available in large quantities in the south of Thailand. It accounts for about 3 % of the weight of the empty fruit bunch of palm oil and is rich in oil residues and various vitamins and mineral elements (Yahya et al. 2010). However, palm oil decanter cake has not found any significant commercial application until now and is generally disposed of in open areas, leading to

potentially serious environmental problems. It is thus necessary to explore its industrial reutilization.

This study was carried out to explore the feasibility of using palm oil decanter cake as the substrate for the production of biosurfactant by the marine bacterial *Inquilinus limosus* KB3 isolated from marine sediment and the potential application of biosurfactant obtained for enhanced solubilization of hydrophobic compounds.

Materials and methods

Biosurfactant-producing strain

Inquilinus limosus KB3 (accession number AB685266) was isolated from marine sediment collected from the southern part of Thailand, during a screening study for biosurfactant-producing bacteria in mangrove sediment (unpublished data). *I. limosus* KB3 was maintained on nutrient agar plates and transferred monthly.

Media and cultivation conditions

Nutrient broth was used for preparation of the inoculum. The composition of the nutrient broth used was as follows: beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g in 1 l of distilled water. To make nutrient agar, 15.0 g of agar was added to the nutrient broth. The culture was grown in this broth for 20–24 h at 30 °C. This was used as inoculum at the 3 % (v/v) level. For biosurfactant synthesis, a mineral salt medium (MSM) with the following composition (g/l) was utilized: K_2HPO_4, 0.8; KH_2PO_4, 0.2; $CaCl_2$, 0.05; $MgCl_2$, 0.5; $FeCl_2$, 0.01; NaCl, 10.0 (Saimmai et al. 2012a). pH of the medium was adjusted to 7.0. Carbon and nitrogen sources were added separately. Cultivation was performed in 250-ml flasks containing 50 ml medium at room temperature (30±3 °C), and shaking in a rotary shaker at 150 rpm for 48 h.

Medium optimization

The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed at a specific set of conditions. Three factors were chosen aiming to obtain higher productivity of the biosurfactant: carbon source (C), nitrogen source (N), and C:N ratio. The carbon sources used were 1 g/l of glucose, glycerol, molasses, oleic acid, palm oil decanter cake, soybean oil, stearic acid, used lubricating oil (ULO), and used vegetable oil (UVO), with $(NH_4)_2SO_4$ as a nitrogen source. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, beef extract, monosodium glutamate, $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, peptone, and yeast extract were employed at a concentration of 1 g/l with the optimum carbon source. The C:N ratio (with optimized carbon and nitrogen sources) was varied from 5 to 40 by keeping a constant nitrogen source concentration of 1 g/l.

Recovery of biosurfactant

Four solvent systems; a mixture of chloroform:methanol (2:1), cold acetone, dichloromethane, and ethyl acetate were examined for biosurfactant extraction (Saimmai et al. 2012b). The method showing the highest biosurfactant activity was used to recover biosurfactant from *I. limosus* KB3.

Chemical analysis of biosurfactant

The chemical nature of the biosurfactants obtained was determined with thin layer chromatography (TLC). The biosurfactant was spotted in triplicate on ready-made silica gel TLC plates (Merck, Darmstadt, Germany) using $CHCl_3$:CH_3OH:H_2O (65:15:1) as the solvent system. One of the plates was put into a jar saturated with iodine vapor to detect lipids (Das et al. 2009). Another plate was sprayed with anisaldehyde and ninhydrin reagent (0.2 % ninhydrin solution in acetone) and dried. It was then heated at 120 °C for 5 min for detection of sugars and peptides (Das et al. 2009), respectively.

Fourier transform infrared spectroscopy (FT-IR) of the biosurfactant obtained was done on a Nexus-870 FT-IR spectrometer (Thermo Electron, Yokohama, Japan) by the KBr pellet method. Further characterization of the biosurfactant was carried out using nuclear magnetic resonance (NMR) using $CDCl_3$ with an AMX 300 NMR spectrometer (500 MHz; Bruker). Final characterization of the compound was performed by liquid chromatography-mass spectroscopy (LC-MS) with an LCQ™ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) which utilizes electrospray ionization (ESI) (Thavasi et al. 2008).

Application of the biosurfactant in ULO removal from contaminated sand

Biosurfactant suitability for enhancing oil recovery was investigated using 800.0 g of acid-washed sand impregnated with 50.0 ml of ULO. Fractions of 20.0 g of the contaminated sand were transferred to 250-ml flasks which were submitted to the following treatments: addition of 60.0 ml distilled water (control), addition of 60.0 ml aqueous solutions of the SDS, Triton X-100 and biosurfactant at the CMC level of each compound. The samples were incubated on a rotary shaker (200 rpm) for 24 h at 30 °C and centrifuged at 3,354 *g* for 20 min for separation of the laundering

solution and the sand. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of material extracted from the sand by hexane (Sobrinho et al. 2008).

Laboratory experiment on biodegradation of ULO with biosurfactant

An experiment was conducted to study the impact of the biosurfactant isolated from *I. limosus* KB3 on biodegradation of ULO in natural seawater. Shake flask biodegradation experiments were carried out in 500-ml Erlenmeyer flasks with 100 ml of sterilized seawater. The experiment was conducted with four different sets: (1) bacterial cells alone; (2) with fertilizer and cells; (3) with cells and biosurfactants (0.1 %, w/v); and (4) with fertilizer and biosurfactant. Exactly 2.0 % (w/v) of ULO was added to the sterilized seawater, and inoculation was performed with 24-h-old culture at the rate of 1 % (v/v, 10^3–10^4 CFU/ml) concentration. Flasks were shaken at 150 rpm in dark at room temperature for a period of 168 h. The biodegradation of oil was estimated fluorimeterically as described in Intergovernmental Oceanographic Commission (IOC) Manuals and Guide No. 13 (1982). An uninoculated control was kept to assess the natural weathering of oil and degradation.

Polyaromatic hydrocarbons (PAHs) solubilization assay

PAHs solubilization assay was done as described by Barkay et al. (1999). Briefly, 0.6 μg each of the following PAHs (anthracene, fluoranthene, fluorine, naphthalene, phenanthrene or pyrene; all from 0.6 mg/ml stock in acetone) were distributed into glass test tubes (10 mm × 170 mm) and kept open inside an operating chemical fume hood to remove the solvent. Subsequently, 3.0 ml of assay buffer (20 mM Tris-HCl, pH 7.0) and the biosurfactant at increasing concentrations (0–50 mg/ml) obtained from the bacterial strain used in this study. Assay buffer containing the biosurfactant, but no PAH, was used as blank. Tubes were capped with plastic closures and incubated overnight at 30 °C with shaking (200 rpm) in dark. Samples were 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. This emulsion was centrifuged at 9,693 *g* for 10 min to separate the aqueous and hexane phases. The concentration of the PAHs was measured spectrophotometrically (Libra S22; Biochrom, Cambridge, UK) at the specific wave lengths of each compound (Barkay et al. 1999). From a calibration curve of individual PAHs (in hexane), the concentration of each PAH was determined. Assay buffer with biosurfactant without PAH was identically extracted with hexane and served as blank.

Antimicrobial activity of surface active compound

The extracted compound was tested for antimicrobial activity using the agar well diffusion method and the inhibition zone was measured (Candan et al. 2003). Extracted active compound was tested against pathogenic microorganisms including *Bacillus cereus*, *Candida albicans*, *Enterococcus faecium*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella* sp., *Salmonella typhimurium*, *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio vulnificus*. All strains were obtained from Songklanagarind Hospital, Prince of Songkla University, Thailand. Briefly, the extract was dissolved in distilled water at a concentration of 10 mg/ml and filter-sterilized using a 0.2-μm membrane filter. Each tested microorganism was grown in brain heart infusion (BHI; Hi-Media Laboratories, Mumbai, India) and diluted to a concentration of 10^6 CFU/ml. They were overlaid onto the surface of BHI agar. The agar plates were dried for 20 min at room temperature. Microbial suspensions were plated on BHI agar plates, which were dried for 20 min at room temperature. The wells were cut from the agar and 100 μl of extract solution were added to the wells. The plates were incubated at 37 °C for 24 h; after incubation, the clear zone was measured.

Analytical methods

Biomass determination was done in terms of the dry cell weight. At different times of fermentation, samples were mixed in pre-weighted tubes with chilled distilled water and centrifuged at 9,693 *g* for 30 min. The biomass obtained was dried overnight at 105 °C and weighed.

Emulsification activity was performed according to Wu et al. (2008). Briefly, 4 ml of hydrocarbon or oil was added to 4 ml of aqueous solution of culture supernatant in a screw cap tube, and vortexed at high speed for 2 min. The emulsion activity (E24) was determined after 24 h. E24 was calculated by dividing the measured height of the emulsion layer by the total height of the mixtures and multiplying by 100.

The surface tension of culture supernatant was measured using a Model 20 Tensiometer (Fisher Science Instrument, PA, USA) at 25 °C. CMC was determined by plotting the surface tension versus concentration of biosurfactant in the solution.

All experiments were carried out at least in triplicate. Two well-defined synthetic surfactants, Triton X-100 and SDS were used as positive controls, while distilled water and MSM medium were used as negative controls. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 10.0 for Windows; Chicago, IL, USA).

Results and discussion

Effect of carbon source on growth and biosurfactant production

The literature has revealed that the type and concentration of carbon and nitrogen substrates markedly affected the production yield of biosurfactant (Wu et al. 2008). In light of this, this study started with the investigation of carbon and nitrogen sources on biosurfactant production. *I. limosus* KB3 was grown on each of 9 types of carbon sources. As seen in Table 1, the type of carbon source affected both the biosurfactant production and E24. Palm oil decanter cake differed from the others in relation to the biosurfactant concentration and E24, being the most appropriate carbon source; surface tension reduction reached 25.25 mN/m with 1.10 g/l and achieved an E24 of 19.25 % toward xylene. Table 1 also shows that there seems to be clear trend between biomass and biosurfactant yields which strongly depends on the carbon source used. Although vegetable oils or glucose have been frequently used as the carbon substrates for biosurfactant production (Banat et al. 2010), *I. limosus* KB3 attained a lower biosurfactant yield from soybean oil, UVO, and glucose than that from palm oil decanter cake, molasses, and glycerol (Table 1). Direct use of fatty acids (i.e., oleic acid and stearic acid) as the carbon source did not improve biosurfactant production, suggesting that hydrolysis of the oils was not the bottleneck step. Moreover, ULO and UVO were also inefficient in cell growth and biosurfactant production, resulting in a low biosurfactant yield of only 0.50 and 0.48 g/l, respectively, probably due to its poor biodegradability (Chayabutra et al. 2001) or toxicity toward bacterial cells (Li and Chen 2009).

Effect of nitrogen source on growth and biosurfactant production

After examining the most commonly used organic and inorganic nitrogen sources reported in the literature (Abdel-Mawgoud et al. 2008), it was found that the type of nitrogen source affected the growth and biosurfactant production of *I. limosus* KB3 (Table 2). The highest biomass was obtained when yeast extract was used. However, $NaNO_3$ exhibited the highest surface tension reduction and biosurfactant yield (35.49 mN/m and 1.79 g/l, respectively). This yield was nearly 2-fold that obtained from using $(NH_4)_2SO_4$ as the nitrogen source. Moreover, using $NaNO_3$ as the inorganic nitrogen source not only increased the biosurfactant yield but also improved the biomass and E24 at 4.12 g/l and 25.35 %, respectively.

Effect of C:N ratio on growth and biosurfactant production

The C:N ratio is also known as a vital factor influencing the performance of biosurfactant production (Santos et al. 2002). As indicated in Table 3, the best biosurfactant activity in surface tension reduction and yield (44.35 mN/m and 4.90 g/l, respectively) were obtained at a C:N ratio of 25; this yield was 2.6-fold that obtained from control (C:N ratio at 1:1). The productivity of biosurfactant tended to decrease as the C:N ratio increased from 30 to 40, especially for C:N ratio >35. Some reports mentioned that biosurfactant production is more efficient under nitrogen-limiting conditions (Benincasa et al. 2002). The results show that a possible inhibitory effect on the bacterial metabolism may occur due

Table 1 Effect of carbon source on biosurfactant production by *Inquilinus limosus* KB3, which were cultivated in 250-ml flasks containing 50 ml MSM medium at 30 °C in a shaking incubator at 150 rpm for 48 h (nitrogen source: 1 g/l of $(NH_4)_2SO_4$)

Carbon source (1 g/l)	Dry cell weight[a] (g/l)	Surface tension (mN/m)[a]			Biosurfactant[a] (g/l)	E24[a] (%)
		Initial	Final	Reduction		
No carbon source	0.06±0.01	72.01±1.27	70.01±0.04	1.51±0.04	0.05±0.01	0
Glucose	4.05±0.20	71.51±2.27	59.21±1.24	12.11±2.27	0.53±0.01	20.51±5.07
Glycerol	3.95±0.92	70.03±0.53	53.56±2.18	18.03±5.53	0.91±0.01	20.03±4.73
Molasses	4.54±1.62	65.08±2.57	44.64±1.03	20.08±2.57	1.08±0.08	20.08±2.67
Oleic acid	2.48±0.63	68.35±2.67	59.19±2.06	8.35±2.67	0.32±0.05	8.05±2.54
Palm oil decanter cake	3.72±0.63	69.25±1.58	45.21±4.18	25.25±1.58	1.10±0.02	19.25±4.50
Soybean oil	3.54±0.41	62.23±0.87	46.13±3.13	16.23±0.87	0.88±0.04	15.01±2.93
Stearic acid	1.04±0.12	70.23±0.36	60.25±0.15	8.23±0.36	0.32±0.02	10.03±4.57
Used lubricating oil	0.40±0.09	57.62±2.52	46.20±1.86	10.62±2.52	0.50±0.06	17.61±5.01
Used vegetable oil	0.61±0.02	65.61±2.05	56.25±4.04	9.61±2.05	0.48±0.04	12.12±4.07

[a] Values are given as means ± SD from triplicate determinations

Table 2 Effect of nitrogen source on biosurfactant production by *Inquilinus limosus* KB3, which were cultivated in 250-ml flasks containing 50 ml MSM medium at 30 °C in a shaking incubator at 150 rpm for 48 h (carbon source: 1 g/l of palm oil decanter cake)

Nitrogen source (1 g/l)	Dry cell weight[a] (g/l)	Surface tension (mN/m)[a]			Biosurfactant[a] (g/l)	E24[a] (%)
		Initial	Final	Reduction		
No nitrogen source	0.21±0.03	70.01±1.27	60.10±0.14	9.20±0.56	0.45±0.04	4.52±0.47
Beef extract	5.59±1.50	70.51±2.27	59.21±1.24	15.75±2.56	0.89±0.05	27.72±3.07
Monosodium glutamate	5.02±1.00	68.03±0.53	43.56±2.18	25.49±2.08	1.19±0.13	20.74±2.78
$NaNO_3$	4.12±0.58	71.08±2.57	44.64±1.03	30.23±3.50	1.91±0.04	25.35±5.4
$(NH_4)_2SO_4$	3.72±0.63	69.25±1.58	45.21±4.18	25.25±1.58	1.10±0.02	19.25±4.50
NH_4Cl	3.61±0.82	68.20±1.58	46.21±4.18	22.70±2.82	1.04±0.06	20.51±5.00
NH_4NO_3	3.05±0.81	69.23±0.87	58.13±3.13	10.41±3.67	0.81±0.04	25.51±5.60
Peptone	6.84±1.01	70.23±0.36	39.25±0.15	20.05±2.35	0.98±0.02	21.68±3.73
Yeast extract	6.91±0.71	69.62±2.52	46.20±1.86	22.50±2.02	1.35±0.03	24.01±0.90

[a] Values are given as means ± SD from triplicate determinations

to a likely nutrient transport deficiency. That is, nitrate first undergoes dissimilatory nitrate reduction to ammonium and then is assimilated by glutamine–glutamate metabolism. It is likely that assimilation of nitrate as the nitrogen source is so low, leading to a simulated nitrogen-limiting condition (Barber and Stuckey 2000).

Time course of growth and biosurfactant production

The results in Fig. 1a show that biosurfactant production started early in the exponential phase and the production kinetics paralleled the biomass kinetics up to 2 days of incubation. On the basis of these facts, it can be concluded that biosurfactant production is growth-associated. It was found that the maximum level of cell biomass was obtained after 48 h of incubation. However, maximum biosurfactant concentration was obtained 6 h later (5.13 g/l), i.e., after 54 h of incubation. After those periods, a sharp reduction in either biomass or biosurfactant production levels was observed. Growth-associated production of biosurfactant has been reported for *Aeromonas* sp. (Ilori et al. 2005), *Bacillus subtilis* (Abdel-Mawgoud et al. 2008), *Leucobacter komagatae* 183 (Saimmai et al. 2012b), and *Pseudomonas* sp. (Obayori et al. 2009). Tabatabaee et al. (2005) also documented that a biosurfactant synthesized by a strain of *Bacillus* sp. was a primary metabolite produced during cellular biomass formation. From the obtained result, it can be seen that a cultivation time of 54 h gave the highest biosurfactant yield.

Recovery of biosurfactant

The ability of various solvent systems to recover surface-active components from the culture supernatant of *I. limosus* KB3 after 54 h of cultivation was examined. The use of ethyl acetate resulted in greater activity of crude extract against systems based on mixtures of chloroform and

Table 3 Effect of C:N ratio on biosurfactant production by *Inquilinus limosus* KB3, which were cultivated in 250-ml flasks containing 50 ml MSM medium at 30 °C in a shaking incubator at 150 rpm for 48 h

C:N ratio	Dry cell weight[a] (g/l)	Surface tension (mN/m)[a]			Biosurfactant[a] (g/l)	E24[a] (%)
		Initial	Final	Reduction		
Control	4.12±0.58	71.08±2.57	40.64±1.03	30.23±3.50	1.91±0.04	35.35±5.41
5	4.26±0.92	71.51±1.27	36.21±1.24	35.30±4.00	2.73±0.36	40.41±6.20
10	4.48±0.23	71.03±0.53	32.56±2.18	39.09±2.28	3.92±0.39	46.64±4.38
15	4.61±0.92	71.08±0.57	31.64±1.03	40.03±2.22	4.22±0.53	50.70±3.60
20	4.85±0.52	71.25±1.58	28.21±4.18	43.11±2.70	4.81±0.41	55.64±2.07
25	5.00±0.64	72.20±2.08	28.21±4.18	44.35±2.56	4.90±0.51	59.62±5.20
30	5.18±0.43	72.23±0.87	32.13±3.13	40.59±3.28	4.85±0.05	55.50±4.70
35	5.36±0.14	72.23±0.87	40.13±3.13	32.59±3.28	4.01±0.05	50.50±2.10
40	5.49±0.55	72.23±0.36	41.25±0.15	31.15±5.25	3.52±0.13	48.58±3.83

[a] Values are given as means ± SD from triplicate determinations

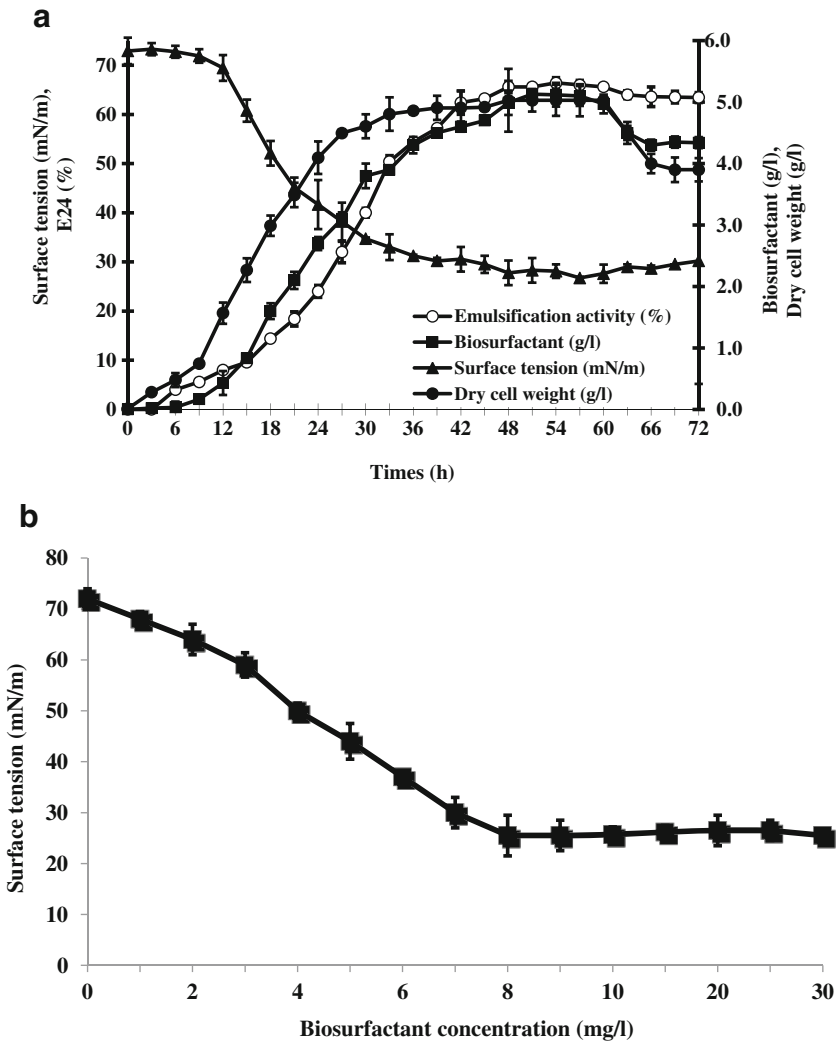


Fig. 1 Time course of growth and biosurfactant production by *Inquilinus limosus* KB3 in optimal medium at 150 rpm and 30 °C (**a**) and surface tension versus biosurfactant concentration produced by *Inquilinus limosus* KB3 (**b**). *Bars* indicate the standard deviation from triplicate determinations

methanol, cold acetone or dichloromethane (Table 4). It has also been reported previously that the extraction of bioproducts with considerably high polarity by ethyl acetate solvent is rather efficient (Chen and Juang 2008). Because the recovery and concentration of biosurfactants from fermentation broth largely determines the production cost, ethyl acetate is a better choice than the highly toxic chloro-organic compounds.

Surface tension and critical micelle concentration (CMC)

The relationship between surface tension and concentration of the crude extracted biosurfactant solution was investigated (Fig. 1b). The biosurfactant produced exhibited excellent surface tension reducing activity. The surface tension of water of 72 mN/m decreased to 25.5 mN/m by increasing the solution concentration up to 9 mg/l. Further increases in

Table 4 Effect extraction methods on yield, critical micelle concentration (CMC) and activity of biosurfactant produced by *Inquilinus limosus* KB3

Recovery method	Yield (g/l)[a]	CMC (mg/l)	Surface tension (mN/m)[a]
Acetone precipitation	6.65±0.85	16	61.5±2.2
Acid precipitation	7.34±1.24	30	50.0±3.4
Ammonium sulfate precipitation	3.58±0.23	25	31.5±0.5
Chloroform:methanol extraction	4.21±0.55	12	32.5±0.4
Dichloromethane extraction	5.00±0.15	15	29.0±0.5
Ethanol precipitation	3.27±0.27	24	33.5±0.5
Ethyl acetate extraction	5.13±0.31	9	28.2±0.7

[a]Values are given as means ± SD from triplicate determinations

the concentration of the biosurfactant solution did not reduce the surface tension of the water, indicating that the CMC was reached at this concentration. The biosurfactant from *I. limosus* KB3 showed a minimum surface tension and CMC value compared with the biosurfactant from *B. subtilis* (26.7 mN/m, 10 mg/l, respectively) (Ghojavand et al. 2008), from *Lactobacillus paracasei* (41.8 mN/m, 250 mg/l) (Gudina et al. 2011), and from *Pseudomonas aeruginosa* Bs 20 (29.5 mN/m, 13.4 mg/l) (Abdel-Mawgoud et al. 2009).

Study of biosurfactant stability

The results obtained from thermal stability analysis of biosurfactant over a wide range of temperature (4–121 °C) revealed that the biosurfactant from *I. limosus* KB3 was thermostable (Fig. 2a). Heating of the biosurfactant solution up to 100 °C (or its autoclaving at 121 °C) caused little effect on the biosurfactant performance and its emulsification ability. The surface tension activity and E24 were relatively stable at the temperatures used (ST≈30 mN/m, E24≈57 %), respectively. The activity of the biosurfactant and its emulsification ability were also affected by the pH. When the pH was acidic and set to 2, 3, and 4, the biosurfactant activities were 50, 47, and 40 mN/m, respectively (Fig. 2b). Correspondingly, the emulsification ability was limited to the acid to neutral pH and emulsification activity up to 36, 40, and 45 %, respectively, was obtained. The optimum pH for both parameters, namely biosurfactant activity (ST=25.5 mN/m) and emulsification activity (E24=60 %) was determined to be 7–10. However, the emulsification activity was relatively stable between pH 8 and 12. Figure 2c demonstrates the effect of the addition of sodium chloride on the surface tension and E24 of the biosurfactant obtained. As is shown, negligible changes occurred in surface tension activity with an increase in the NaCl concentration up to 12 %. Likewise, an increase in NaCl concentration up to 15 % did not have a significant effect on E24. However, at the highest level of NaCl (21 %), E24 was severely decreased to 42 % and surface tension was also increased (48 mN/m).

Emulsification properties of biosurfactants

Biosurfactant isolated from *I. limosus* KB3 showed a good emulsification against several hydrophobic substrates (Fig. 3a). The E24 of biosurfactant from *I. limosus* KB3 was higher than that of the chemical surfactants, since it more effectively emulsified aromatic and aliphatic hydrocarbons and several plant oils. Olive oil, soybean oil, and toluene were good substrates for E24 by the KB3 biosurfactant, showing no significant differences. Benzene, hexadecane, hexane, kerosene, and motor oil also formed stable emulsions. Xylene and ULO differed from the others, resulting in poor emulsification, probably due to the inability of

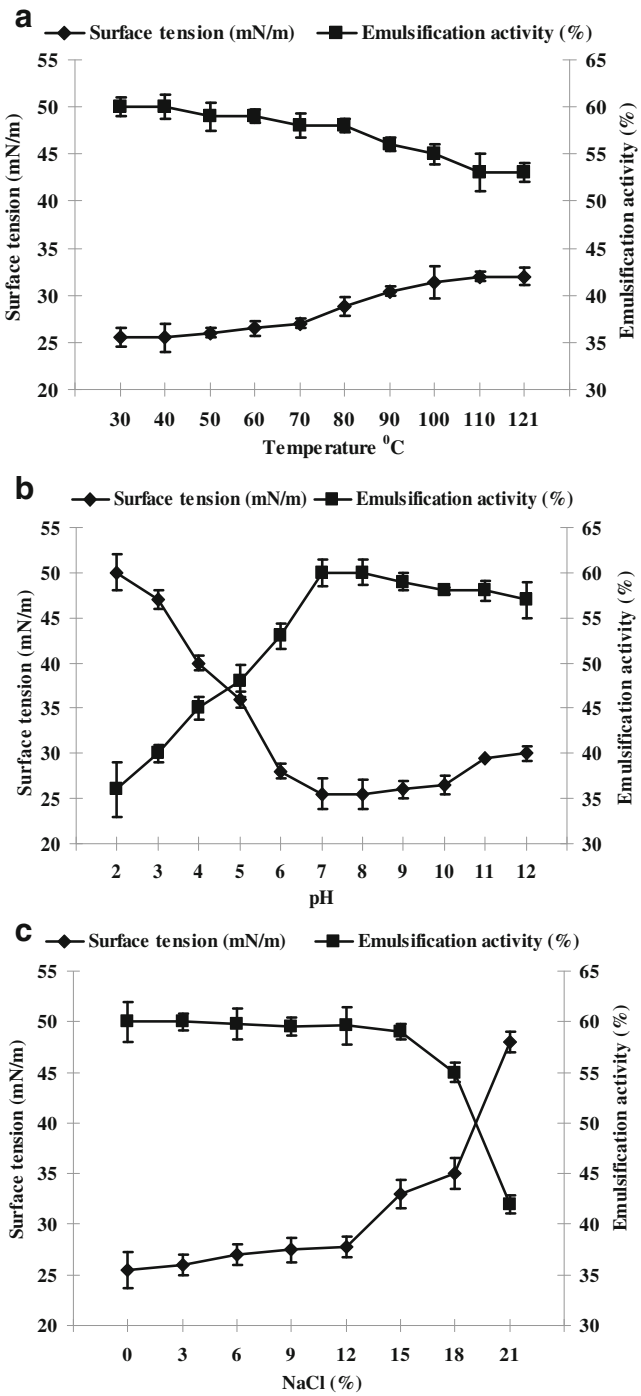


Fig. 2 Effect of temperature (**a**), pH (**b**), and NaCl (**c**) on activity of crude biosurfactant produced by *Inquilinus limosus* KB3. *Bars* indicate the standard deviation from triplicate determinations

the biosurfactant to stabilize the microscopic droplets of these compounds. The explanation of these results could come from the structure of these compounds, consisting of a mixture of paraffin, naphthalene, and aromatic hydrocarbon, which was difficult to emulsify by crude biosurfactant (Muthusamy et al. 2008). The ability of the biosurfactant produced by *I. limosus* KB3 to emulsify various

hydrophobic substrates indicates that it has a good potential for applications in microbial-enhanced oil recovery, as a cleaning and emulsifying agent in food industry, and also for bioremediation.

Chemical characterization of the biosurfactant

The chemical nature of the biosurfactant from *I. limosus* KB3 was seen as a single spot on TLC. This fraction showed positive reaction with ninhydrin reagent and rhodamine B reagent indicating the presence of peptide and lipid moieties in the molecule (data not shown). These results indicate the existence of lipopeptide biosurfactant. The FT-IR spectrum of the biosurfactant from *I. limosus* KB3 showed strong absorption bands, indicating the presence of peptides at 3,360 cm^{-1} resulting from N-H stretching mode (Fig. 3b). At 1,660 cm^{-1}, the stretching mode of a CO-N bond was observed, and at 1,540 cm^{-1}, the deformation mode of the NH bond combined with N-H stretching mode occurred. The presence of an aliphatic chain was indicated by the C-H stretching modes at 2,920–2,854 cm^{-1} and 1,460–1,057 cm^{-1}. These results strongly indicate that the biosurfactant contains aliphatic and peptide-like moieties. The overall FT-IR spectrum of the biosurfactant from *I. limosus* KB3 was very similar to cyclic lipopeptides produced by bacilli-like surfactin (produced by *B. subtilis*) and lichenysin (produced by *B. licheniformis*) which are the most effective biosurfactants so far discovered (Roongsawang et al. 2002; Joshi et al. 2008).

To further confirm the results of this study, a NMR analysis was performed (Fig. 4a, b). Results obtained from ^{1}H-NMR indicated that the molecule is a lipopeptide. Almost all the backbone amide NH groups are in the region from 8.2 to 7.3 ppm downfield from tetramethylsilane. Alpha hydrogens of the amino acids come into resonance from 5.1 to 3.9 ppm. A doublet at δ=0.90 ppm for the $(CH_3)_2$-CH group indicated terminal branching in the fatty acid component. Owing to the presence of CH at 1.2 ppm, the ratio of the methylene and terminal groups could not be resolved. Other multiplets in the upfield region arise as a

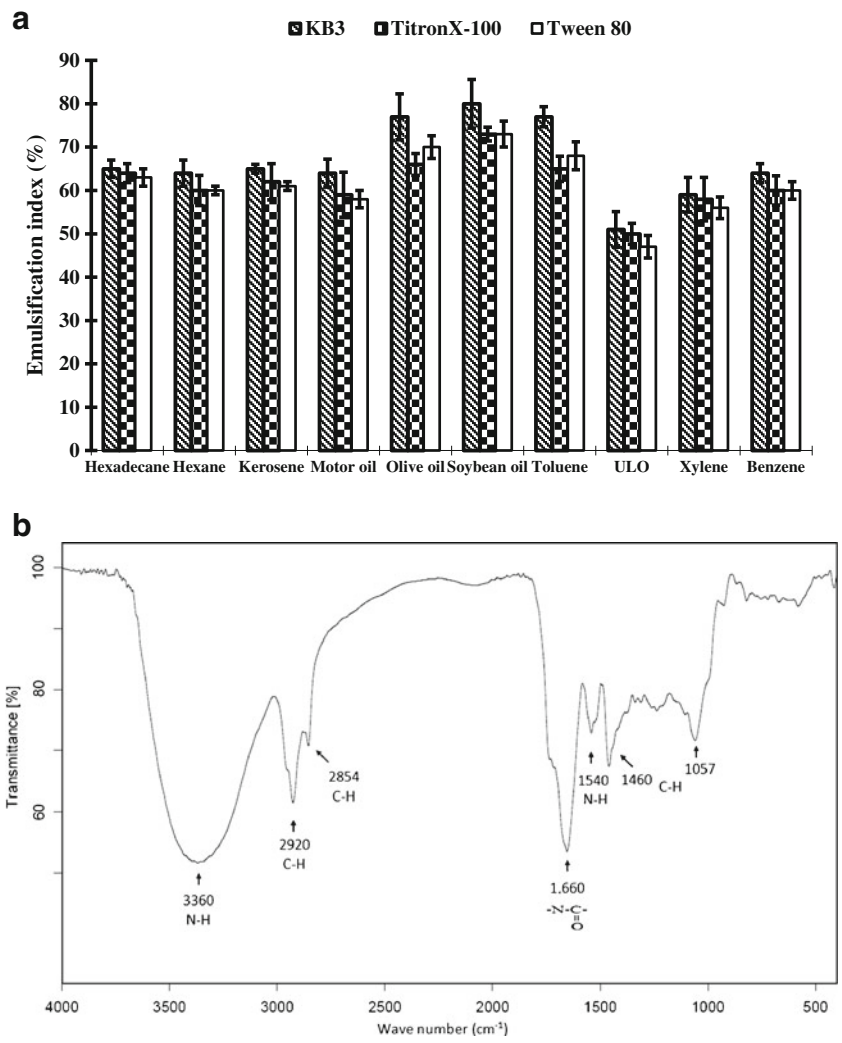


Fig. 3 Emulsification activity of the biosurfactant produced by *Inquilinus limosus* KB3 (**a**) and Fourier transform infrared spectrum (**b**) of the biosurfactant produced by *Inquilinus limosus* KB3. *Bars* indicate the standard deviation from triplicate determinations

result of the sidechain protons of the amino acids. Remaining spectra clearly confirmed the presence of β-hydroxy fatty acid. The ^{13}C-NMR spectrum showed strong signals at 14.0, 22.98–37.68, 171.4, and 174.2 ppm from methyl, methlene, ester, and the carboxyl group, respectively (Fig. 4b). Therefore, the biosurfactant produced by *I. limosus* KB3 could be a lipopeptide, possibly an isoform of surfactin (Tang et al. 2007).

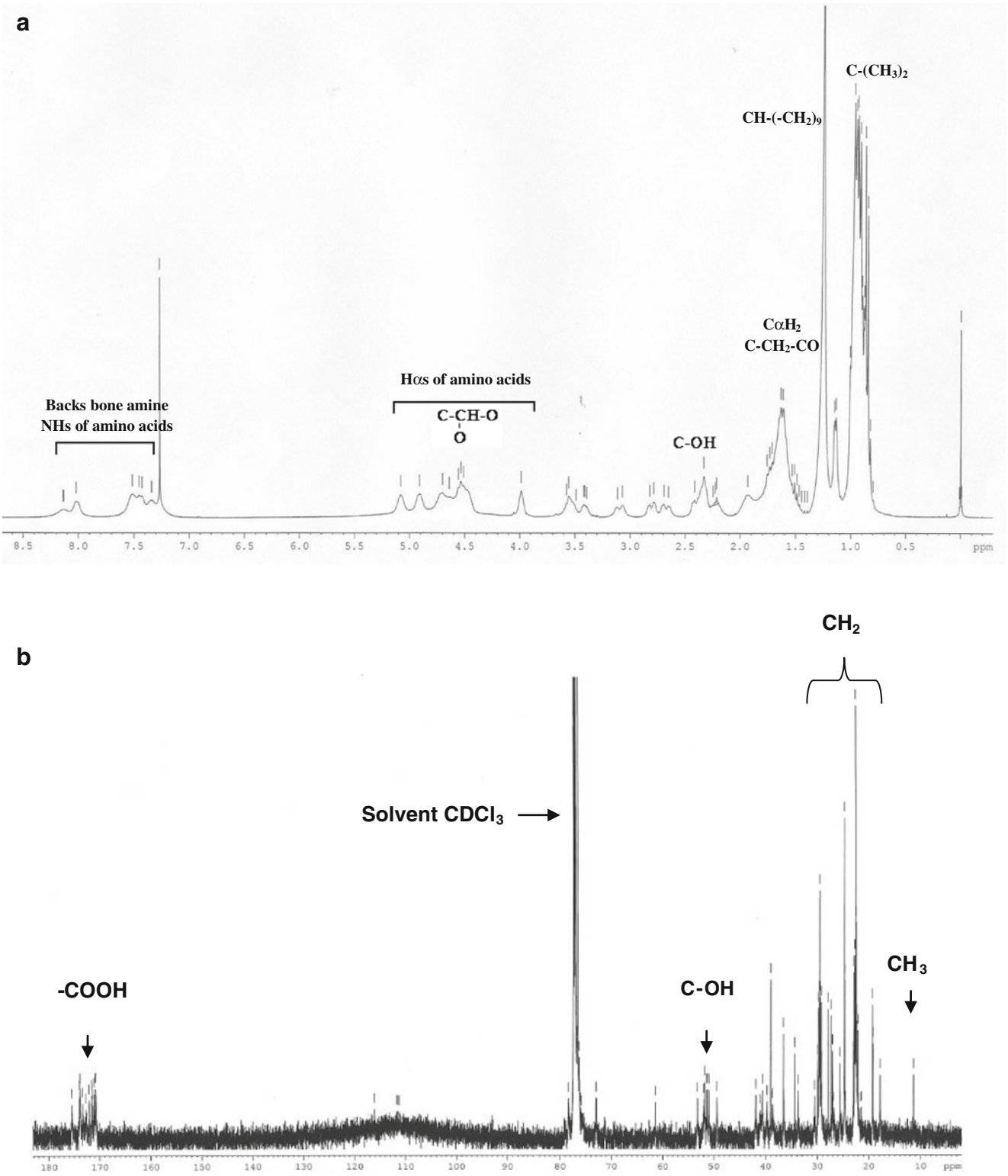


Fig. 4 ^{13}C (**a**) and ^{1}H nuclear magnetic resonance spectrum (**b**) of the biosurfactant produced by *Inquilinus limosus* KB3

Table 5 Dose-dependent solubilization of polyaromatic hydrocarbons by crude biosurfactant isolated from *Inquilinus limosus* KB3

Concentration of crude biosurfactant (mg/l)	Solubility of PAHs[a] (mg/l)					
	Anthracene	Fluoranthene	Fluorene	Naphtalene	Phenantrene	Pyrene
0	0.07±0.01 g	0.25±0.01 f	1.98±0.21 g	30.31±4.02 e	1.39±0.17 g	0.15±0.02 e
5	0.19±0.03 f	1.12±0.09 e	2.41±0.14 f	49.21±4.65 d	2.42±0.32 f	0.82±0.06 d
10	0.45±.009 e	1.81±0.23 d	3.27±0.35 e	58.21±5.10 d	2.41±0.31 e	1.68±0.31 c
15	0.79±0.04 d	2.91±0.34 c	3.82±0.11 d	64.16±4.03 c	2.58±0.42 d	2.01±0.36 c
20	1.07±0.11 c	3.31±0.09 b	4.51±0.41 c	76.45±4.63 bc	2.92±0.30 c	2.57±0.57 bc
25	1.32±0.05 b	4.81±0.31 a	5.61±0.25 b	84.34±5.38 ab	3.81±3.12 b	2.82±0.48 ab
30	1.51±0.09 a	4.98±0.20 a	6.41±0.62 a	91.47±9.23 a	3.98±0.58 a	3.05±0.32 a

Different letters in the same column indicate significant differences ($p<0.05$)

[a] Values are given as means ± SD from triplicate determinations

The above structure of the biosurfactant obtained was fully supported by its mass spectrometric analysis. Analysis of the intact molecules with LCQ-MS revealed four molecular ion peaks with molecular masses $[M+H]^+$ of 1,004, 1,018, 1,032, and 1,046, respectively (Fig. 5a). The spectra clearly indicate the presence of higher and lower homologs of surfactants for the difference between prominent M^+, peaks being around 14, corresponding to a difference in the number of methylene groups (CH_2). This finding was in accordance with Tang et al. (2007) who reported that surfactin was a lipopeptide-type biosurfactant with a molecular mass in the range of 1,007–1,072 Da. To our knowledge, this is the first report of the production of lipopeptide from the genus *Inquilinus*.

Application of the biosurfactant in ULO removal from contaminated sand

Petroleum hydrocarbon compounds bind to soil components and are difficult to remove and degrade (Sobrinho et al. 2008). Biosurfactants can emulsify hydrocarbons, enhancing their water solubility, decreasing surface tension, and increasing the displacement of oil substances from soil particles (Banat et al. 2010). The ability of the biosurfactant from *I. limosus* KB3 to enhance ULO removal from contaminated sand was examined in comparison with those of synthetic surfactants, i.e. a nonionic surfactant Triton X-100 and anionic surfactants SDS. The biosurfactant of *I. limosus* KB3 and Triton X-100 could recover 25–30 % of ULO from contaminated sand at 25 °C; 50 % at room temperature (30± 2 °C); 65 % at 45 °C; and 85 % at 60 °C. The synthetic surfactant SDS was found to be less efficient. In the case of control (distillated water), very little recovery (5–18 %) could be obtained in the temperature range. These results have implications on the potential use of a biosurfactant produced by *I. limosus* KB3 to enhance sorbed oil from the environment. However, it is important not to rule out that the removal efficiency could deviate depending on the characteristic of the contaminants and site characteristics.

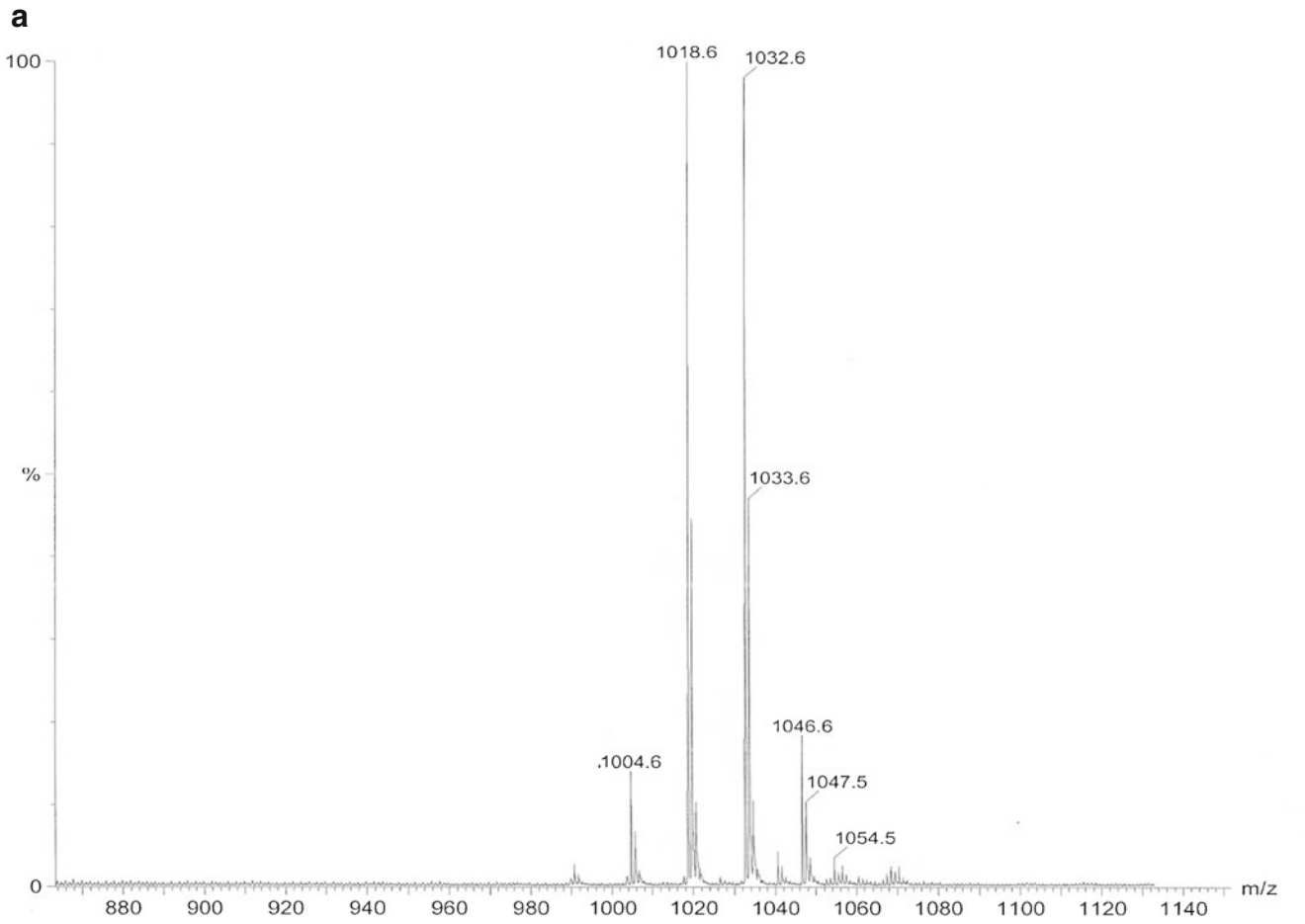
Laboratory experiment on biodegradation of ULO with biosurfactant

Biodegradation of crude oil in the laboratory-scale experiment inferred that maximum biodegradation was found with the biosurfactant (72.3 %), followed by the biosurfactant- and fertilizer-added set (65.2 %), fertilizer (52.4 %), and in a normal setup (40.7 %). The improved biodegradation levels obtained with the biosurfactant indicated that it represents the most efficient accelerators for hydrocarbon biodegradation through increasing oil bioavailability (Maneerat 2009). The use of the biosurfactant in combination with fertilizer could reduce the actual amount of fertilizer to be added to polluted sites. In some studies, water-soluble fertilizers encountered problems such as being washed away and rapid dilution in aquatic environments. Thavasi et al. (2001) reported that fertilizers only stimulate the early stage degradation rate of the oil and that the final degradation efficiencies with fertilizers were not significantly different from those where no fertilizers were used. It is important, however, to keep in mind that nutrient or fertilizer use may be essential in some environments with insufficient nutrient levels.

Effect of biosurfactant on PAHs solubilization

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental polluants. Excessive inputs from anthropogenic activities have caused serious contamination and adversely affect the health of aquatic and human through

Fig. 5 Mass spectrum (**a**) and microbial enhanced oil recovery (**b**) of the biosurfactant produced by *Inquilinus limosus* KB3 under different temperatures. *Bars* indicate the standard deviation from triplicate determinations


a
100
%
0
1018.6
1032.6
1033.6
1046.6
1004.6
1047.5
1054.5
880
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920
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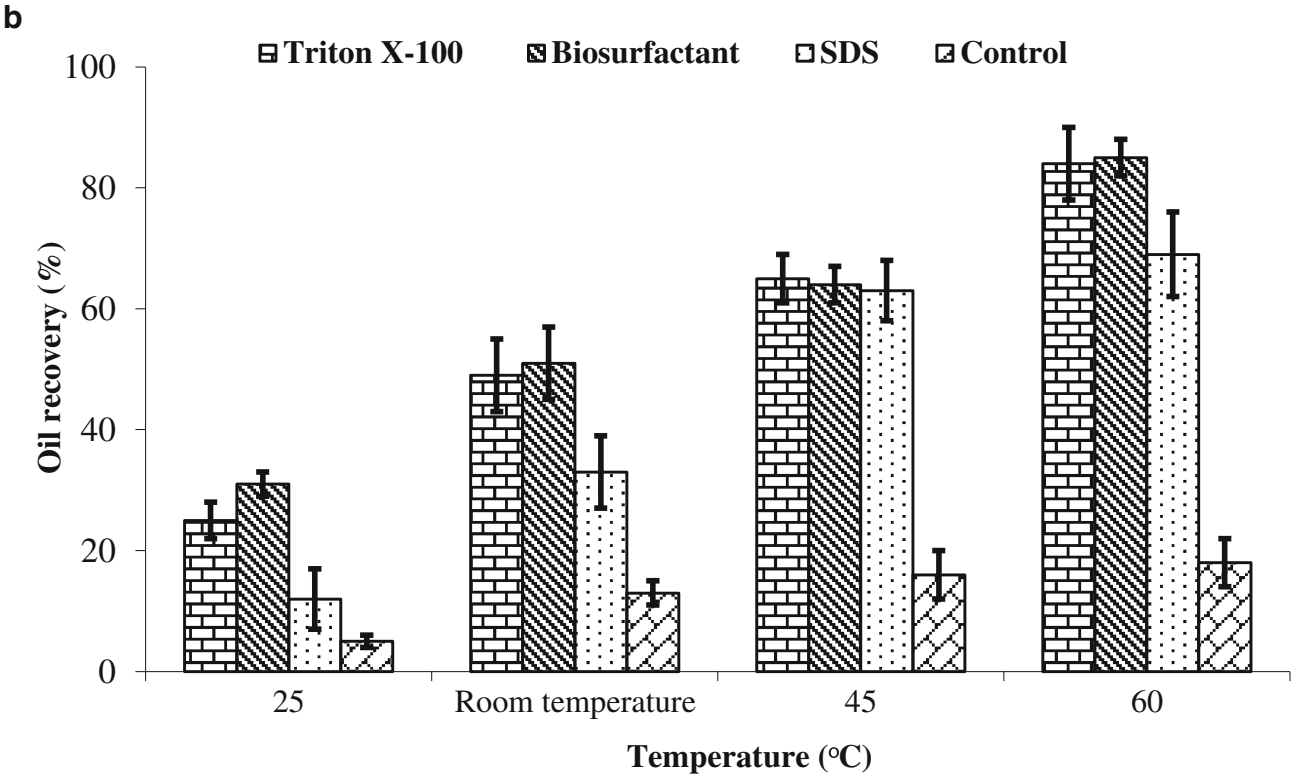
960
980
1000
1020
1040
1060
1080
1100
1120
1140
m/z


b
Triton X-100
Biosurfactant
SDS
Control
Oil recovery (%)
100
80
60
40
20
0
25
Room temperature
45
60
Temperature (°C)

bioaccumulation. PAHs are hydrophobic and readily adsorbed onto particulate matter; therefore, coastal and marine sediments become the ultimate sinks, and elevated concentrations have been recorded (Aniszewski et al. 2010). Solubilization of PAHs depends on the type and dose of the surfactant, the hydrophobicity, the surfactant–soil interactions and the time that the contaminant has been in contact with the soil (Zhou and Rhue 2000). The effect of the biosurfactant on the apparent aqueous solubility of PAHs was determined by test tube solubilization assays in the presence of increasing concentrations of biosurfactant (0 to 50 mg/l) and is depicted in Table 5. In general, the biosurfactant obtained enhanced the apparent solubility of PAHs in a dose-dependent manner. However, solubilization of fluorene, naphthalene, or phenantrene by the biosurfactant from this strain (about 3–5 times higher apparent solubility compared to control) was significantly lower ($p<0.05$) when compared with anthracene, fluoranthene, or pyrene affected by biosurfactant (15–20 times higher compared to control). In the present study, the crude biosurfactant showed ability to solubilize PAHs in aqueous phase indicating its possible role in increasing the bioavailability of non-soluble organic compounds for bacterial metabolism. These characteristics indicate the potential to use the biosurfactant obtained in environmental remediation.

Antimicrobial activity of biosurfactant

The crude biosurfactant of *I. limosus* KB3 was found to be an antimicrobial agent, depending on the microorganism (data not shown). It was found that the biosurfactant obtained exhibited a high antimicrobial activity against *B. cereus*, *C. albicans*, *P. aeruginosa*, and *S. aureus* at tested concentrations. In addition, it was observed that the biosurfactant obtained showed no antimicrobial activity against *V. vulnificus* and *V. cholerae* and low activity against *E. faecium*, *E. coli*, *L. monocytogenes*, *Salmonella* sp. and *S. typhimurium*.

Generally, surfactants having high surface-active properties show certain antimicrobial activities to some extent. Indeed, many lipopeptide biosurfactants show various biological activities reflecting their structures (Banat et al. 2010). The effect of the biosurfactant on bacteria appears more marked with Gram-positive bacteria than with Gram-negative bacteria because of the different cell wall structures. This is attributed to the presence of lipopolysaccharides in the outer membrane of Gram-negative bacteria, making them naturally resistant to certain antibacterial agents (Anderson and Yu 2005. On the other hand, Gram-positive bacteria showed higher sensitivity to the biosurfactant, because they contain an outer peptidoglycan layer, which is an effective permeability barrier (Negi et al. 2005). Moreover, some lipopeptide biosurfactants inhibit growth not only of microorganisms but also of viruses and cancer cells (Singh and Cameotra 2004).

Conclusion

In the present study, the production of the biosurfactant from *I. limosus* KB3 which was isolated from marine sediment is reported. The growth characteristics were obtained and studies on the properties of the biosurfactant low-cost fermentative medium indicate the possibility of its industrial application. The spectra obtained from FT-IR spectroscopy, NMR, and ESI-MS confirmed the presence of lipopeptide in the sample. The properties of the biosurfactant obtained have potential application especially for microbial-enhanced oil recovery and/or reducing the intensity of environmental contamination. Finally, biosurfactants are a suitable alternative to synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents.

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References

Abdel-Mawgoud AM, Aboulwafa AM, Hassouna NAH (2008) Optimization of surfactin production by *Bacillus subtilis* isolate BS5. Appl Biochem Biotechnol 150:305–325

Abdel-Mawgoud AM, Aboulwafa MM, Hassouna NAH (2009) Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* Isolate Bs20. Appl Biochem Biotechnol 157:329–345

Anderson RC, Yu PK (2005) Factors affecting the antimicrobial activity of ovine-dervied cathelicidins aginst *Escherichia coli* O157: H7. Int J Antimicrob Ag 25:205–210

Aniszewski E, Peixoto SR, Mota FF, Leite SGF, Rosado SA (2010) Bioemulsifier production by *Microbacterium* sp. strains isolated from mangrove and their application to remove cadmium and zinc from hazardous industrial residue. Braz J Microbiol 41:235–245

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R (2010) Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol 87:427–444

Barber WP, Stuckey DC (2000) Nitrogen removal in a modified anaerobic baffled reactor (ABR): 1, denitrification. Water Res 10:2413–2422

Barkay T, Navon-Venezia S, Ron EZ, Rosenberg E (1999) Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl Environ Microbiol 65:2697–2702

Benincasa M, Contiero J, Manresa MA, Moraes IO (2002) Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source. J Food Eng 54:283–288

Candan F, Unlu M, Tepe B, Daferera D, Polissiou M, Sokmen A, Akpulat HA (2003) Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae). J Ethnopharmacol 87:215–220

Chayabutra C, Wu J, Ju LK (2001) Rhamnolipid production by *Pseudomonas aeruginosa* under denitrification: effects of limiting nutrients and carbon substrates. Biotechnol Bioeng 72:25–33

Chen HL, Juang RS (2008) Recovery and separation of surfactin from pretreated fermentation broths by physical and chemical extraction. Biochem Eng J 38:39–46

Das K, Mukherjee AK (2007) Comparison of lipopeptide biosurfactants production by *Bacillus subtilis* strains in submerged and solid state fermentation systems using a cheap carbon source: some industrial applications of biosurfactants. Process Biochem 42:1191–1199

Das P, Mukherjee S, Sen R (2009) Substrate dependent production of extracellular biosurfactant by a marine bacterium. Bioresource Technol 100:1015–1019

Ghojavand H, Vahabzadeh F, Roayaei E, Khodabandeh A (2008) Production and properties of a biosurfactant obtained from a member of the *Bacillus subtilis* group (PTCC 1696). J Colloid Interf Sci 324:172–176

Gudina EJ, Teixeira JA, Rodrigues LR (2011) Isolation and functional characterization of a biosurfactant produced by *Lactobacillus paracasei*. Colloid Surf B 76:298–304

Ilori MO, Amobi CJ, Odocha AC (2005) Factors affecting biosurfactant production by oil degrading *Aeromonas* sp., isolated from a tropical environment. Chemosphere 61:985–992

Intergovernmental oceanographic commission manuals and guide No.13 (1982) Manual for monitoring oil and dissolved/dispersed petroleum hydrocarbons in marine waters and beaches. UNESCO

Joshi S, Bharucha C, Jha S, Yadav S, Nerurkar A, Desai AJ (2008) Biosurfactant production using molasses and whey under thermophilic conditions. Bioresource Technol 99:195–199

Li JL, Chen BH (2009) Surfactant-mediated biodegradation of polycyclic aromatic hydrocarbons. Materials 2:76–94

Makkar R, Cameotra SS (2002) An update on the use of unconventional substrates for biosurfactant production and their new applications. Appl Microbiol Biotechnol 58:428–434

Maneerat S (2009) Biosurfactants from marine microorganisms. Songklanakarin J Sci Technol 27:1263–1272

Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P (2008) Biosurfactants: properties, commercial production and application. Curr Microbiol 94:736–747

Negi PS, Chauhan AS, Sadia GA, Rohinishree YS, Ramteke RS (2005) Antioxidant and antimicrobial activities of various seabuckthorn (*Hippophae rhamnoides* L.) seed extracts. Food Chem 92:119–124

Nitschke M, Pastore G (2006) Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Bioresource Technol 97:336–341

Obayori O, Ilori M, Adebusoye S, Oyetibo G, Omotayo A, Amund O (2009) Degradation of hydrocarbons and biosurfactant production by *Pseudomonas* sp. strain LP1. World J Microb Biot 25:1615–1623

Roongsawang N, Thaniyavarn J, Thaniyavarn S, Kameyama T, Haruki M, Imanaka T, Morikawa M, Kanaya S (2002) Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. Extremophiles 6:499–506

Saimmai A, Sobhon V, Maneerat S (2011) Molasses a whole medium for bosurfactants production by *Bacillus* strains and their application. Appl Biochem Biotech 165:315–335

Saimmai A, Sobhon V, Maneerat S (2012a) Mangrove sediment, a new source of potential biosurfactant producing bacteria. Ann Microbiol. doi:10.1007/s13213-012-0424-9

Saimmai A, Sobhon V, Maneerat S (2012b) Production of biosurfactant from a new and promising strain of *Leucobacter komagatae* 183. Ann Microbiol 62:391–402

Santos AS, Sampaio AP, Vasquez GS, Anna LMS Jr, Pereira N, Freire DM (2002) Evaluation of different carbon and nitrogen sources in the production of rhamnolipids by a strain of *Pseudomonas*. Appl Biochem Biotechnol 98:1025–1035

Singh P, Cameotra SS (2004) Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol 22:142–146

Sobrinho HBS, Rufino RD, Luna JM, Salgueiro AA, Campos-Takaki GM, Leite LFC, Sarubbo LA (2008) Utilization of two agroindustrial by-products for the production of a surfactant by *Candida sphaerica* UCP0995. Process Biochem 43:912–917

Tabatabaee A, Assadi MM, Noohi AA, Sajadian VA (2005) Isolation of biosurfactant producing bacteria from oil reservoirs. J Environ Health Sci Eng 2:6–12

Tang JS, Gao H, Hong K, Yu Y, Jiang MM, Lin HP, Ye WC, Yao XS (2007) Complete assignments of ^{1}H- and ^{13}C-NMR spectral data of nine surfactin isomers. Magn Reson Chem 45:792–796

Thavasi R, Jayalakshmi S, Banat IM (2001) Effect of biosurfactant and fertilizer on biodegradation of crude oil by marine isolates of *Bacillus megaterium*, *Corynebacterium kutscheri* and *Pseudomonas aeruginosa*. Bioresource Technol 102:772–778

Thavasi R, Jayalakshmi S, Balasubramaniam T, Banat IM (2008) Production and characterization of a glycolipid biosurfactant from *Bacillus megaterium* using economically cheaper sources. World J Microb Biot 24:917–925

Wu JY, Yeh KL, Lu WB, Lin CL, Chang JS (2008) Rhamnolipid production with indigenous *Pseudomonas aeruginosa* EM1 isolated from oil-contaminated site. Bioresource Technol 99:1157–1164

Yahya A, Sye CP, Ishola TA, Suryanto H (2010) Effect of adding palm oil mill decanter cake slurry with regular turning operation on the composting process and quality of compost from oil palm empty fruit bunches. Bioresource Technol 101:8736–8741

Zhou M, Rhue RD (2000) Screening commercial surfactants suitable for remeditating DNAPL source zones by solubility. Environ Sci Technol 34:1985–1990