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ORIGINAL ARTICLE

Production of biosurfactant from a new and promising strain of *Leucobacter komagatae* 183

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Abstract The *Leucobacter komagatae* 183 strain, isolated from mangrove sediment in Trang, southern Thailand, was evaluated as a potential biosurfactant producer. The biosurfactant production was carried out by using a mineral salt medium with commercial sugar and monosodium glutamate as the carbon and nitrogen source, respectively. The microbial growth was investigated and the best cultivation time for the biosurfactant was found to be 54 h. After the microbial cultivation at 30°C under optimized conditions, the biosurfactant produced was found to reduce the surface tension of pure water to 26.5 mN/m with the critical micelle concentration of about 9 mg/l. The stability of the biosurfactant at different salinities, pH and temperature and also its emulsifying activity has been investigated. It is an effective surfactant at very low concentrations over a wide range of temperatures, pHs and salt concentrations. The crude biosurfactant showed a broad spectrum of antimicrobial activity and also had the ability to emulsify oil and enhance PAHs solubility.

Keywords Biosurfactants · *Leucobacter komagatae* · Surface tension · Oil recovery · Polyaromatic hydrocarbon

Introduction

Surfactants are amphipathic molecules consisting both of hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases having different degrees of polarity and hydrogen bonding, e.g., oil and water or air and water interfaces (Banat et al. 2010). The surface and interfacial tension-reducing properties of surfactants confer excellent detergency, emulsifying, foaming and dispersing traits; these make them some of the most versatile process chemicals. With increasing environmental awareness and emphasis on a sustainable society in harmony with the global environment during recent years, natural surfactants of microbial origin, commonly referred to as biosurfactants, are getting much more attention compared to chemical surfactants owing to mild production conditions, lower toxicity, higher biodegradability and environmental compatibility (Mulligan 2009). All these qualities of biosurfactants have prompted their numerous applications in environmental protection as well as in the food, cosmetic, biopesticide and pharmaceutical industries (Singh and Cameotra 2004).

Based on the types of biosurfactant-producing microbial species and the nature of their chemical structures, biosurfactants can be categorized into four main groups: lipopeptides and lipoproteins, glycolipids, phospholipids, and polymeric surfactants (Fathabad 2011). Among these four groups, the most common biosurfactants that have been isolated and studied are the lipopeptides produced by *Bacillus subtilis* strains and the glycolipids produced by *Pseudomonas aeruginosa* strains (Pornsunthorntawee et al. 2008).

Biosurfactants have advantages over their chemical counterparts because they are bio-degradable, have low toxicity, are effective at extreme temperatures or pH values and show better environmental compatibility (Mulligan 2009). Nevertheless, from an economic standpoint, biosurfactants are not yet competitive with the synthetics. Biosurfactants can only replace synthetic surfactants if the cost of the raw material and the process is minimized

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(Nitschke and Coast 2007). *Leucobacter komagatae* 183 produces biosurfactants by using a mineral salt medium with commercial sugar and monosodium glutamate as the carbon and nitrogen source, respectively. So far, several renewable substrates from various sources, especially from industrial wastes, have been intensively studied for microorganism cultivation and surfactant production at an experimental scale (Banat et al. 2010). However, this is the first report for biosurfactant production using commercial sugar and monosodium glutamate. In addition, the characteristics of the biosurfactants obtained show versatile functions (antimicrobial activity, enhancing oil recovery, emulsifying several hydrophobic substrate and enhancing polyaromatic hydrocarbon solubility) compared to biosurfactants produced by other strains, as previously reported. Biosurfactants obtain from *L. komagatae* 183 also have good activity in extreme conditions (e.g., pH 5–12, temperature 4–121°C and NaCl up to 16%).

The objective of the present study was to examine the production and characterization of biosurfactants produced by bacteria isolated from mangrove sediments and evaluated for prospective applications including antimicrobial activity and microbially enhanced oil recovery. This is the first report describing biosurfactant production by *Leucobacter komagatae*.

Materials and methods

Biosurfactant producing strain

Leucobacter komagatae 183 (accession number AB542942) was isolated from mangrove sediment collected from Trang Province in the south of Thailand, during a screening study for biosurfactant-producing bacteria in mangrove sediment (Saimmai et al. 2010). The entire 16S rRNA gene sequence from *L. komagatae* 183 showed 99% homology to *Leucobacter komagatae* (accession number AB007419). *Leucobacter komagatae* 183 was maintained on NA plate 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 a liter 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 room temperature (30±2°C). This was used as inoculum at the 2% (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, 30.0 (Yin et al. 2005). pH of the medium was adjusted to 7.0±0.2. Carbon and nitrogen sources were added separately. Cultivation was performed in 250-ml flasks containing 50 ml medium at room temperature, and stirred 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% (w/v) of commercial sugar (CS; saccharose), glucose, molasses, used lubricating oil (ULO) and glycerol, a waste from biodiesel production, with $(NH_4)_2SO_4$ as nitrogen source. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, peptone and monosodium glutamate (MSG) 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 10 to 50 by keeping a constant nitrogen source concentration 1 g/l.

Recovery of biosurfactant

Four solvent systems; a mixture of chloroform:methanol (2:1) (Nitschke and Coast 2007), cold acetone (Das and Mukherjee 2007), dichloromethane (Joshi et al. 2008) and ethyl acetate (Maneerat and Phetrong 2007) were examined for biosurfactant extraction. The method showing the highest biosurfactant activity was used to recover biosurfactant from *L. komagatae* 183.

Study of biosurfactant stability

The crude biosurfactant at critical micelle concentration (CMC) level in distilled water was prepared. To investigate the effects of pH, sodium chloride (NaCl) concentrations and temperature on biosurfactant activity, the biosurfactant solution was adjusted with 1.0 N HCl or NaOH to obtain the pHs of 2.0–12.0. NaCl was added to the sample to obtain the final concentrations of 1.0–11.0% (w/v). For the thermal stability study, biosurfactant solution was incubated at 4–100°C for 1 h and at 121°C for 15 min and cooled to 25°C. The remaining activity was then determined.

Identification and characterization of the biosurfactant

The obtained biosurfactant was first identified and characterized by Fourier-transform infrared spectroscopy (FT-IR) with a Nexus-870 FT-IR spectrometer (Thermo Electron,

Yokohama, Japan) by the KBr pellet method (Das et al. 2008). Further characterization of the biosurfactant was carried out using ^1H nuclear magnetic resonance (NMR) using $CDCl_3$ with AMX 300 NMR spectrometer (Bruker, 500 MHz). Final characterization of the compound was performed by liquid chromatography-mass spectroscopy (LC-MS) with LCQ™ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) which utilizes electrospray ionization (Thavasi et al. 2007).

Application of the biosurfacant in ULO removal from contaminated sand

Biosurfactant suitability for enhance 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), and addition of 60.0 ml aqueous solutions of the SDS, Tween80, biosurfactant under the CMC, at the CMC and above the CMC of each compound. The samples were incubated on a rotary shaker (200 rpm) for 24 h at 30°C and centrifuged at 5,000 rpm 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). The experiment was carried out at 25, room temperature (30±2), 45 and 65°C to assess the influence of temperature on biosurfactant-induced oil recovery.

PAHs solubilization asssy

Polyaromatic hydrocarbons (PAHs) solubilization assay was done as described by Barkay et al. (1999). Briefly, any of the following 0.6 μg of PAHs (anthracene, fluoranthene, fluorine, naphthalene, phenanthrene or pyrene (from 0.6 mg/ml stock in acetone) was distributed into glass test tube (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) were 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 equal volume of hexane. This emulsion was centrifuged at 8,500 rpm for 10 min to separate the aqueous and hexane phases. Concentration of PAH was measured spectrophotometrically (Libra S22; Biochrom, Cambridge, UK) at specific wave length of each compounds (Barkay et al. 1999). From a calibration curve of individual PAH (in hexane), the concentration of each PAH was determined. Assay buffer with biosurfactant but without PAH was extracted with hexane identically and served as blank.

Antimicrobial activity of surfactive compound

The extracted compound was tested for antimicrobial activity using the agar well diffusion method and the area of the zone was measured (Candan et al. 2003). Extracted active compound was tested against pathogenic bacteria 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 weighed and dissolved in distilled water, 10 mg/ml, and the extract was filter-sterilized using a 0.2-μm membrane filter. Each tested microorganism was suspended in Brain Heart Infusion (BHI; Hi-Media Laboratories, Mumbai, India) and diluted to obtain 10^6 CFU/ml. They were “flood-inoculated” onto the surface of BHI agar. Agar plates were dried for 20 min at room temperature. The wells were cut from the agar and 50 μl of extract solution was added wells, incubated at 37°C for 24 h. After incubation, the clear zone was measured.

Analytical methods

Biomass determination was done in terms of dry cell weight. At different times of fermentation, samples were mixed in pre-weighted tubes with chilled distilled water and centrifuged at 8,500 rpm for 30 min. Biomass obtained was dried overnight at 105°C and weighed.

Emulsification index (E24) was performed accordingly to Cooper and Goldenberg (1987). 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 stability was determined after 24 h, and the E24 was calculated by dividing the measured height of emulsion layer by the mixtures total height and multiplying by 100.

Surface tension 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.

Chemical characterization of biosurfactant was done by thin layer chromatography (TLC). The components of chloroform:methanol extract were separated on silica gel 60 plates (Merck, Darmstadt, Germany) using as solvent

system $CHCl_3$:CH_3OH:H_2O (65:15:1). Spots were revealed by spraying with: (1) distilled water for detection of hydrophilic compounds, and (2) ninhydrin 0.05% (w/v, in methanol/water, 1:1 v/v) for detection of compound with free amino groups. Treatments (1) and (2) were visualized after heating at 110°C for 5 min. To detect the presence of lipids, TLC plate was visualized under ultraviolet light after sprayed with rhodamine B 0.25% (w/v, in absolute ethanol).

All experiments were carried out in triplicate for the calculation of the mean value. Two well-defined synthetic surfactants, Tween80 and SDS were used as positive controls, while distilled water and MSM medium were used as negative controls. All chemicals used were of analytical grade. Statistical analysis was performed using SPSS 10.0 for Windows (SPSS, Chicago, IL, USA).

Results and discussion

Effect of carbon source

The literature revealed that the type and concentration of carbon and nitrogen substrates markedly affected the production yield of biosurfactant (Jeong et al. 2004; Wu et al. 2008). In light of this, this study started with the investigation of carbon and nitrogen sources on biosurfactant production. *L. komagatae* 183 was grown on each of 9 carbon sources. After cultivation for 48 h, the culture with CS and glucose produced 5.31 and 5.25 g/l of biomass, respectively. Meanwhile, the maximum biosurfactant production was 0.70 and 0.61 mg/l for CS and glucose, respectively. The resulting biosurfactant-containing supernatant had a surface tension reduction of 35.8 mN/m and achieved an E24 of over 70% for both kerosene and xylene, when CS was used as carbon source. Table 1 also shows that there seems to be a clear trend between biomass yield and biosurfactant yield, strongly dependent on the carbon source used. Although vegetable oils have been frequently used as the carbon substrates for biosurfactant production (Rahman et al. 2002), *L. komagatae* 183 attained a lower biosurfactant yield from olive oil and soybean oil than that from glucose and CS (Table 1). The biosurfactant production was 0.52 and 0.43 g/l for palm oil and soybean oil, respectively. The biosurfactant productivity obtained from using palm oil and soybean oil was also much lower than that obtained from using CS and glucose. 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 bottle-neck step. Moreover, ULO was also inefficient in cell growth and biosurfactant production, resulting in a low biosurfactant yield of only 0.25 g/l, probably due to its poor biodegradability (Chayabutra et al. 2001).

Effect of nitrogen source

With CS as a carbon source, the choice of nitrogen source affects the biosurfactant production is depicted in Table 2. After examining the most commonly used organic and inorganic nitrogen sources reported in the literature (Wei et al. 2005), it was found that MSG was the most efficient nitrogen source for *L. komagatae* 183 to produce biosurfactant, giving a high biosurfactant yield of 4.02 g/l. This yield is nearly 6-fold of that obtained from using $(NH_4)_2SO_4$ as the nitrogen source. Moreover, using MSG as the organic nitrogen source not only increased in biosurfactant yield but also improved in biomass and

Table 1 Effect of carbon source on biosurfactant production by *Leucobacter komagatae* 183, cultivated in a 250-ml flask containing 50 ml MSM medium at 30°C in a shaking incubator at 150 rpm for 48 h (nitrogen source: 1% $(NH_4)_2SO_4$)

Carbon source	(%, w/v)	Dry cell weight[a] (g/l)	Surface tension reduction[a] (mN/m)	Biosurfactant[a] (g/l)	Emulsification activity[a] (%)	
					Kerosene	Xylene
No carbon source	(%, w/v)	0.16±0.03	0	0	0	0
Commercial sugar	(%, w/v)	5.31±0.52	35.81±1.45	0.70±0.31	70.37±3.24	72.08±3.8
Glucose	(%, w/v)	5.25±1.01	30.51±2.27	0.61±0.24	66.33±4.20	51.81±5.45
Glycerol	(%, w/v)	4.84±0.92	6.0±0.73	0.36±0.08	40.22±2.88	38.41±5.57
Molasses	(%, w/v)	4.54±1.62	3.6±0.62	0.24±0.03	69.41±2.15	5.51±0.75
Used lubricating oil	(%, w/v)	3.10±0.21	2.6±0.22	0.25±0.03	5.31±2.01	4.41±0.30
Palm oil	(%, w/v)	2.42±0.50	9.25±1.58	0.52±0.28	4.02±1.05	10.52±2.25
Soybean oil	(%, w/v)	2.74±0.21	6.23±0.87	0.43±0.30	8.38±2.03	11.48±1.83
Oleic acid	(%, w/v)	0.98±0.23	2.05±0.27	0.52±0.26	6.32±1.76	13.44±1.68
Stearic acid	(%, w/v)	1.04±0.12	3.23±0.36	0.55±0.15	9.78±2.5	14.60±2.42

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

Table 2 Effect of nitrogen source on biosurfactant production by *Leucobacter komagatae* 183, cultivated in a 250-ml flask containing 50 ml MSM medium at 30°C in a shaking incubator at 150 rpm for 48 h (carbon source: 1% CS)

Nitrogen source	(%, w/v)	Dry cell weight[a] (g/l)	Surface tension reduction[a] (mN/m)	Biosurfactant[a] (g/l)	Emulsification activity[a] (%)	
					Kerosene	Xylene
No nitrogen source	(%, w/v)	0.41±0.13	0	0	0	0
$(NH_4)_2SO_4$	(%, w/v)	5.31±0.52	35.81±1.45	0.70±0.31	70.37±3.24	72.08±3.8
$NaNO_3$	(%, w/v)	4.32±0.38	30.23±2.00	0.40±0.31	65.00±5.42	70.05±5.0
NH_4Cl	(%, w/v)	3.71±0.92	22.70±2.82	0.74±0.13	59.71±3.15	50.61±4.05
NH_4NO_3	(%, w/v)	4.75±1.81	30.31±3.87	1.04±0.44	56.35±2.20	50.41±5.65
Beef extract	(%, w/v)	5.49±1.00	19.65±2.56	0.85±0.80	44.06±4.05	40.62±3.27
Monosodium glutamate	(%, w/v)	6.68±1.03	45.09±2.28	4.02±0.19	70.52±4.76	70.84±6.38
Peptone	(%, w/v)	5.94±1.01	36.03±0.35	1.13±1.80	58.68±3.03	61.58±6.83
Yeast extract	(%, w/v)	6.12±0.81	22.70±5.02	0.95±0.12	45.61±2.00	54.01±0.20

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

surface tension reduction as 6.68 g/l and 45.09 mN/m, respectively.

Effect of carbon to nitrogen (C/N) ratio on biosurfactant production

The C/N ratio was also known as a vital factor influencing the performance of biosurfactant production (Santos et al. 2002). The effect of C/N ratio on biosurfactant production was thus investigated by keeping a constant nitrogen source (1 g/l of MSG) using. The lowest C:N ratio used (C/N=10) was limited in terms of carbon, since cell growth was about 80% lower compared with that using the highest C:N ratio (data not shown). The best biosurfactant yield (4.51 g/l) was obtained at a C/N ratio of 30, whereas the productivity tended to decrease as the C/N ratio increased from 35 to 50, especially for C/N ratio >40. Some reports mentioned that biosurfactant production is more efficient under nitrogen-limiting conditions (Benincasa et al. 2002; Kim et al. 2006). The results showed that a possible inhibitory effect on the bacterial metabolism may occur due to a likely nutrient transport deficiency. In addition, the present study also shows that a proper amount of nitrogen source is a prerequisite for efficient biosurfactant production with *L. komagatae* 183.

Effect of inoculum size on biosurfactant production

To optimize the amount of inoculum for biosurfactant production, the concentration of the inoculum was varied from 2 to 8% (v/v). The result showed that the highest percentage of the biosurfactant yield (4.51 g/l) was obtained at 2% inoculum for this bacterial strain (data not shown). Although dry cell weight seemed to increase with an increase in the amount of inoculum, the excreted biosurfactants did not show superior surface activities and yield.

Time course of growth and biosurfactant production

Time course studies were conducted on growth and biosurfactant production by *L. komagatae* 183 in MSM (pH 7.0) using 3% CS and 1% MSG as the carbon and nitrogen source, respectively, for 72 h at room temperature (Fig. 1). It was observed that this strain started to excrete biosurfactant after the lag phase, which was after 12 h of cultivation, as indicated by a decrease in the surface tension of the culture media. Under the studied conditions, the log phase occurred and ranged from 24 to 48 h. In this period, the surface tension of the culture medium was markedly reduced and reached a minimum (27.0 mN/m). However, the highest of biosurfactant yield (4.52 g/l) was obtained as the cultivation time approached 54 h, which corresponded to the stationary phase of the microbial growth. Therefore, it can be concluded that the biosurfactant produced by *L. komagatae* 183 is a primary metabolite. Growth-associated production of biosurfactant has been reported for *Brevibacillus brevis* (Haddad et al. 2008), *Pseudomonas* sp. (Obayori et al. 2009) and *Aeromonas* sp. (Ilori et al. 2005). 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 results obtained, it can be seen that a cultivation time of 54 h gave the highest biosurfactant activity.

Recovery of biosurfactant

Crude extract of the biosurfactant was recovered from the culture supernatant of *L. komagatae* 183 by extraction with

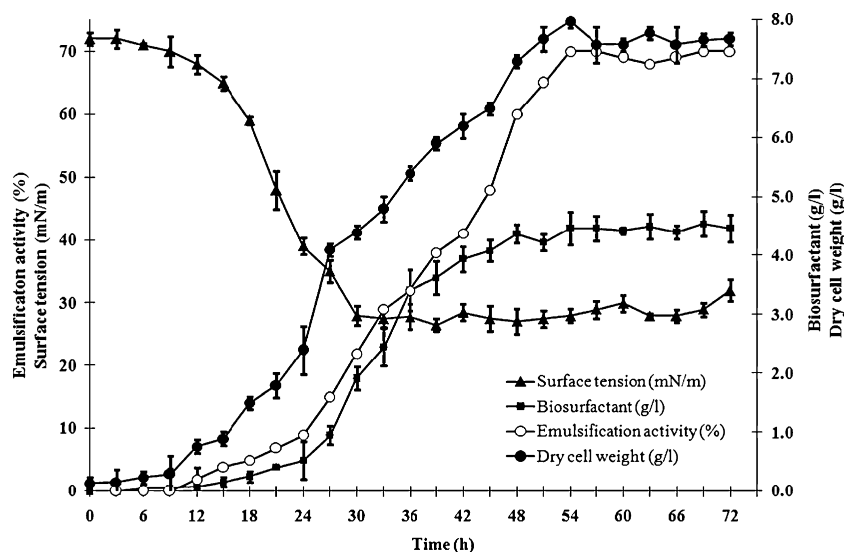

Fig. 1 Time course of growth and biosurfactant production by *Leucobacter komagatae* 183 in optimal medium (3% commercial sugar, 1% monosodium glutamate and 2% inoculation concentration) at 150 rpm and 30° C). *Bars* indicate standard deviations from triplicate determinations

several organic solvents. Among four solvent systems, chloroform:methanol (2:1) was the most efficient in biosurfactant recovery from culture supernatant of this strain (data not shown). Recovery yield of 3.03 g/l was obtained from *L. komagatae* 183. Mixtures of solvents were commonly used to facilitate adjustment of the polarity between the solvent as the extraction agent and the biosurfactant to be extracted (Kuyukina et al. 2005). The biosurfactant which was composed by hydrophilic and hydrophobic moieties, could be easily extracted by this solvent system because the solvent system contained both non-polar (chloroform, Log P=1.97) and quite polar (methanol, Log P=−0.74) solvent. Thus, it is better than single solvent (ethyl acetate, Log P=0.73) used (Sangster 1989). Extraction with chloroform:methanol (2:1) was chosen as solvent system for biosurfactant recovery from *L. komagatae* 183.

Surface tension and critical micelle concentration (CMC)

The CMC is a widely used index to evaluate surface activity. By definition, the CMC is the surfactant concentration at which an abrupt increase in surface tension is observed. Regardless of the surfactant concentration, a further decrease in the surface tension will not be observed once the CMC has been reached. The relationship between surface tension and concentration of the isolated biosurfactant solution was determined by a du Nouy'a ring tensiometer (data not shown). The biosurfactant produced exhibited excellent surface tension reducing activity. The surface tension of water of 72 mN/m decreased to 26.5 mN/m by increasing the solution concentration up to 9 mg/l. Further increase in the concentration of the biosurfactant solution did not reduce the surface tension of water, indicating that the CMC was reached at this concentration. The biosurfactant from *L. komagatae* 183 showed a lower minimum surface tension and CMC value than that of the biosurfactant from *Bacillus subtilis* (26.7 mN/m, 10 mg/l) (Ghojavand et al. 2008), from *Pseudomonas fluorescens* (31.5 mN/m, 72 mg/l) (Janek et al. 2010), from *Lactobacillus paracasei* (41.8 mN/m, 2.5 mg/ml) (Gudina et al. 2010), and from *Pseudomonas aeruginosa* (33.9 mN/m, 50 mg/l) (Yin et al. 2009).

Effect of temperature, pH and salinity on biosurfactant stability

Heating of the biosurfactant solution up to 100°C (or its autoclaving at 121°C) caused no effect on the biosurfactant performance and its emulsification capacity (data not shown). The surface tension reduction and emulsification activity were relatively stable at the temperatures used, indicating the usefulness of this biosurfactant in the food, pharmaceutical and cosmetics industries, where heat treatment may be used to achieve sterility. The activity of biosurfactant solution and its emulsification activity were also affected by the pH (data not shown). When the pH was acidic and set to 2.0, 3.0 and 4.0, the biosurfactant activities were 70, 67 and 66 mN/m, respectively. Correspondingly, the emulsification ability of biosurfactant was limited to the acid to neutral pH and E24 up to 15, 49 and 59%, respectively, was obtained. The result showed that negligible changes were observed in the biosurfactant activity with an increase in the NaCl concentration up to 18%. Likewise, an increase in NaCl concentration up to 16% did not cause a significant effect on E24 (data not shown). These findings suggested that the robust characteristics of the crude biosurfactant are very beneficial for applications under

extreme conditions of temperature, pH and NaCl, such as in oil recovery and in the bioremediation of a polluted marine environment.

Chemical characterization of the biosurfactant

Chemical nature of the biosurfactant from *L. komagatae* 183 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 indicated the existence of lipopeptide biosurfactant. Infrared analysis of the *L. komagatae* 183 biosurfactant revealed a pattern similar to that of surfactin, indicating the presence of lipopeptide component, as can be seen in Fig. 2. Based on the band characteristics information, the lipopeptidic nature of these compounds was confirmed: wave number 3,456 cm^{-1} showing stretching mode of the amine (N–H) and wave number 1,708 cm^{-1} showing stretching mode of the CO–N bond; both are indicative of the presence of peptides. Characteristic absorption bond for the presence of aliphatic chains was shown by wave number 2,925 and 1,567 cm^{-1} indicating C−H stretching modes, suggests the presence of an aliphatic chain. These results were strong evidences that the product contains aliphatic and peptide-like moieties (lipopeptide compound). The overall FT-IR spectrum of biosurfactant from *L. komagatae* 183 was very similar with cyclic lipopeptides produced by bacilli-like surfactin (produced by *B. subtilis*) and lichenysin (produced by *B. licheniformis*) are the most effective biosurfactant so far discovered (Joshi et al. 2008).

To further confirm the results of this study, a ^{1}H-NMR analysis was performed (Fig. 3). Results obtained from ^{1}H-NMR indicated that the molecule is a lipopeptide. Almost all the back bone amide NH groups are in the region from 7.5 to 8.5 ppm downfield from tetramethylsilane. Alpha hydrogens of the amino acids come into resonance from 3.98 to 5 ppm. A doublet at δ=0.854 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 result of the sidechain protons of the amino acids. Remaining spectra clearly confirmed the presence of β-hydroxy fatty acid. Therefore, biosurfactant produced by *L. komagatae* 183 could be a lipopeptide, possibly surfactin.

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 and 1,032, respectively (Fig. 4). 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 Roongsawang et al. (2002) who reported surfactin was a lipopeptide-type biosurfactant with a molecular mass in the range of 1,007–1,035 Da. To our knowledge, this is the first report of the production of lipopeptide by *Leucobacter komagatae*.

Application of the biosurfactant in motor oil removal from contaminated sand

For practical application of biosurfactant on oil removal from contaminated sand, it is of great interest to compare the performance of the biosurfactant obtained with that of two commonly used synthetic surfactants (Tween80 and SDS). Biosurfactant of *L. komagatae* 183 and Tween80 could recover 25–28% of motor oil from contaminated sand

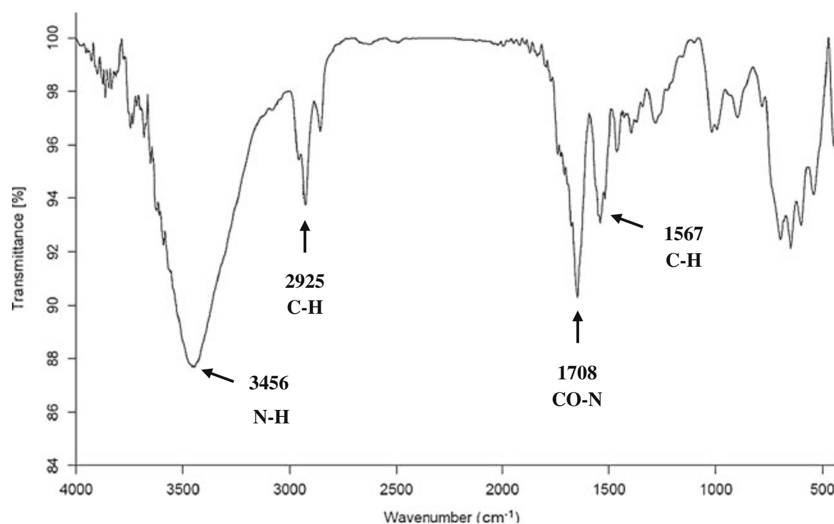

Fig. 2 Fourier transform infrared spectrum of the biosurfactant produced by *Leucobacter komagatae* 183

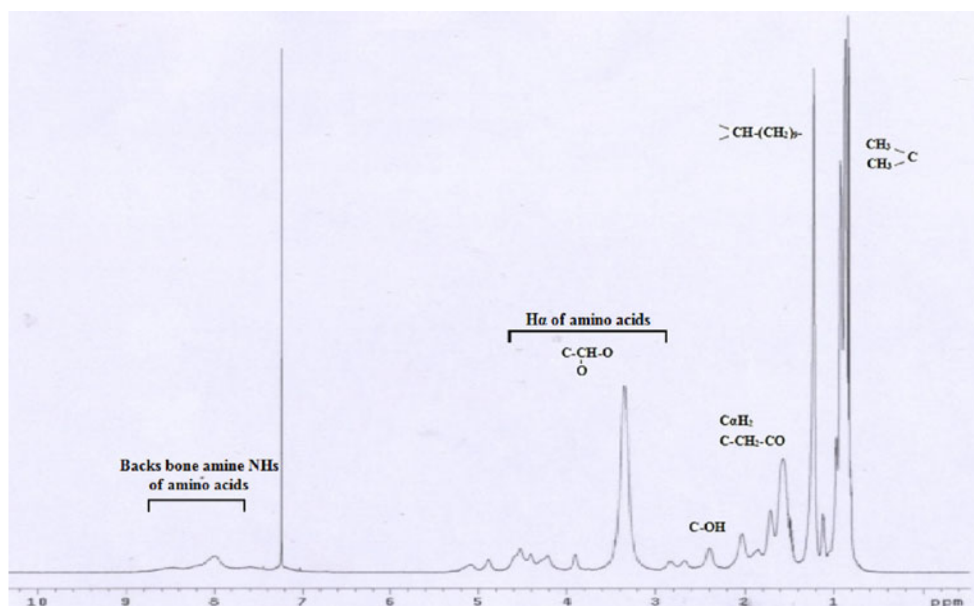

Fig. 3 ^{1}H nuclear magnetic resonance spectrum of the biosurfactant produced by *Leucobacter komagatae* 183

at 25°C, 43% at room temperature, 68% at 45°C and 80% at 60°C. The synthetic surfactant SDS was found to be less efficient. In the case of control (distilled water), very little recovery (5–20%) could be obtained in the temperature range (Fig. 5). These results indicated the superior performance of the biosurfactant over synthetic surfactants SDS in terms of mobilization of oil pollutants from the contaminated soil, and thus the biosurfactant examined in this work has the potential to be used as a biostimulation agent for bioremediation of oil-polluted soils.

Effect of biosurfactant on PAH solubilization

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 (5–30 mg/ml) is depicted in Table 3. In general, the biosurfactant enhanced the apparent

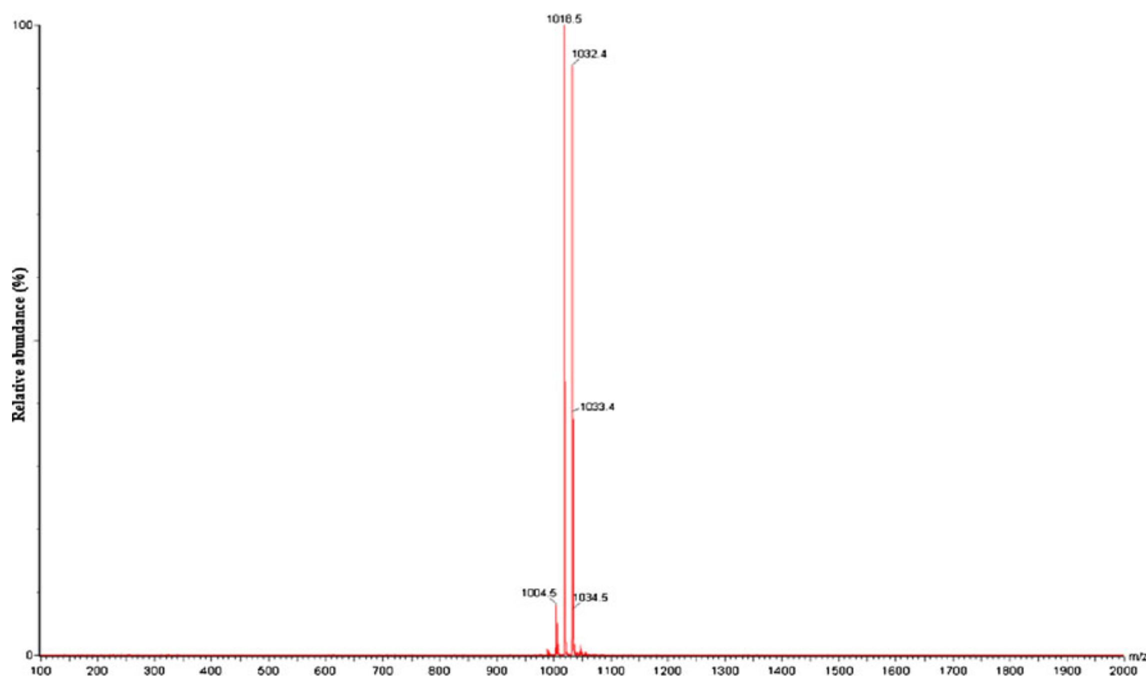

Fig. 4 Mass spectrum of the biosurfactant produced by *Leucobacter komagatae* 183

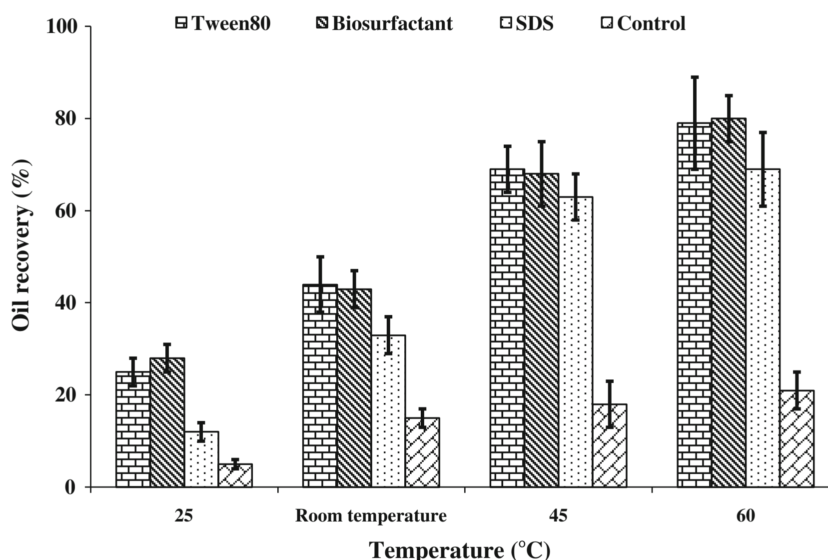

Fig. 5 Microbial enhanced oil recovery by the biosurfactant produced by *Leucobacter komagatae* 183 under different temperatures

solubility of PAHs in a dose-dependent manner. However, solubilization of fluorine, naphthalene or phenantrene by biosurfactant from this strain (about 2–3 times higher apparent solubility compared to control) was significantly lower ($p<0.05$) when compared with anthracene, fluoranthene or pyrene affect by the biosurfactant (18–20 times higher compared to control). In the present study, the crude biosurfactant showed an ability to solubilize PAHs in aqueous phase indicating its possible role in increasing the bioavailability of non-soluble organic compounds for bacterial metabolism.

Antimicrobial activity of biosurfactant

The crude biosurfactant of *L. komagatae* 183 was found to be an antimicrobial agent depending on the microorganism (Fig. 6). It was found that the biosurfactant exhibited a high antimicrobial activity against *P. aeruginosa, C. albicans, S. aureus* and *B. cereus* at tested concentrations. In addition, it was observed that the biosurfactant showed no antimicrobial activity against *V. vulnificus* and *V. cholerae* and low activity against *Enterococcus 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 of lipopeptide biosurfactants have shown various biological activities reflecting their structures (Seydlova and Svobodova 2008). The effect of the biosurfactant on bacteria appears more markedly 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 against the biosurfactant, because they contain an outer peptidoglycan layer, which is an infective permeability barrier (Negi et al. 2005). Moreover,

Table 3 Dose-dependent solubilization of PAHs by crude biosurfactant isolated from *Leucobacter komagatae* 183

Concentration of crude biosurfactant (mg/ml)	Solubility of PAHs[a] (mg/l)					
	Anthracene	Fluoranthene	Fluorene	Naphtalene	Phenantrene	Pyrene
0	0.07±0.01 g	0.25±0.03 f	1.98±0.21 g	30.31±4.0 e	1.39±0.17 g	0.15±0.02 e
5	0.22±0.02 f	0.93±0.08 e	2.18±0.09 f	39.3±5.8 d	1.67±0.11 f	0.91±0.08 d
10	0.47±.01 e	1.72±0.11 d	2.97±0.21 e	44.3±4.7 d	1.93±0.25 e	1.23±0.27 c
15	0.73±0.03 d	2.75±0.28 c	3.73±0.18 d	49.3±3.1 c	2.35±0.21 d	1.57±0.48 c
20	0.97±0.05 c	3.25±0.08 b	4.36±0.24 c	55.3±1.9 bc	2.79±0.15 c	1.95±0.44 bc
25	1.22±0.04 b	4.63±0.65 a	5.15±0.46 b	62.6±7.6 ab	3.26±1.3 b	2.43±0.57 ab
30	1.43±0.07 a	4.73±0.31 a	5.94±0.51 a	69.3±8.0 a	3.63±0.26 a	2.81±0.64 a

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

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

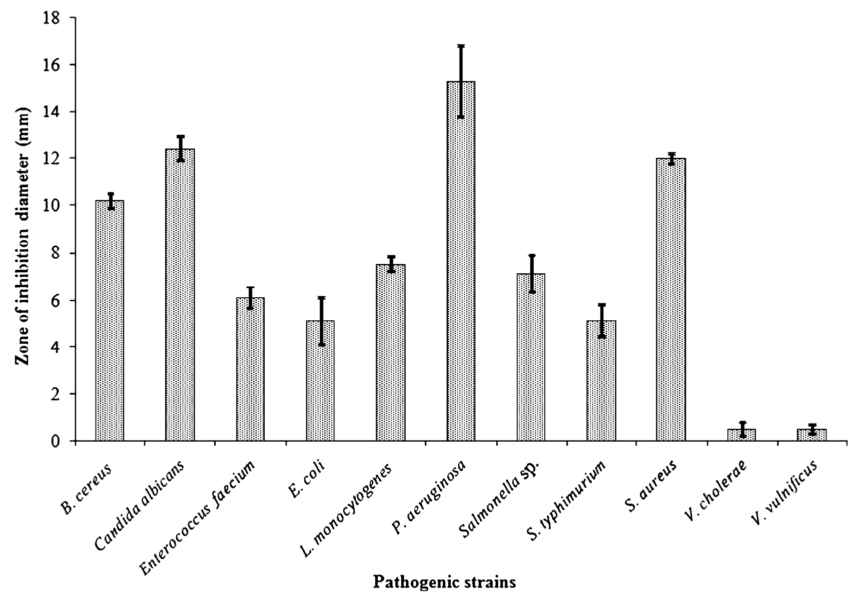

Fig. 6 Antimicrobial activity of biosurfactant produced by *Leucobacter komagatae* 183

some of lipopeptide biosurfactants inhibit not only growth of microorganisms but also that of viruses and tumor cells (Singh and Cameotra 2004). Surfactin, a lipopeptide-type biosurfactant produced by *Bacillus* spp. exhibits a wide range of interactions with target cell membranes and possesses potential for various medical applications. Besides its antifungal and antibacterial effect, surfactin can also inhibit several viruses including Semliki Forest virus, herpes simplex virus (HSV), suid herpes virus, vesicular stomatitis virus, simian immunodeficiency virus, feline calicivirus and murine encephalomyocarditis virus (Seydlova and Svobodova 2008). Surfactin has also been reported to have an antitumor activity against Ehrlich's ascite carcinoma cells (Rodrigues et al. 2006). A recent study on the effect of surfactin on the proliferation of a human colon carcinoma cell line showed that surfactin strongly blocked the cell proliferation (Kim et al. 2007).

Emulsification properties of biosurfactant

To determine hydrocarbon specificity for emulsification, a wide range of pure and mixed substrates were investigated. According to Fig. 7, emulsions can be formed with a wide range of hydrophobic compounds, an important property for environmental applications. Hexadecane, kerosene, toluene and xylene were good substrates for emulsification by the obtained biosurfactant, showing no significant differences. Hexane, motor oil and ULO also formed stable emulsions. Soybean oil, palm oil and olive oil differed from the others, resulting in poor emulsification, probably due to the inability of the biosurfactant to stabilize the microscopic droplets of these compounds. Dastgheib et al. (2008) reported a similar result, for which bioemulsifier showed activity against various hydrocarbons, with the maximum with aromatics and the least with olive oil.

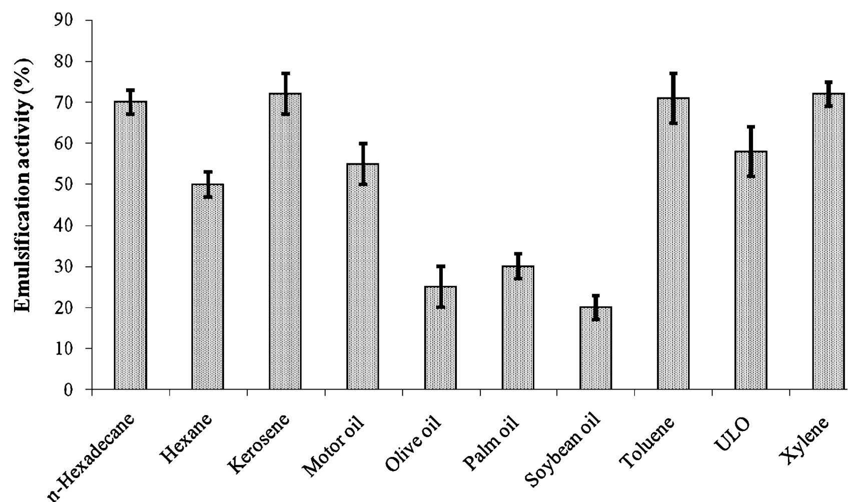

Fig. 7 Emulsification index (E24) of the biosurfactant against different hydrocarbons and vegetable oils

Conclusion

In the present study, the production of the biosurfactant from *L. komagatae* 183 which was isolated from mangrove sediment is reported. The growth characteristics were obtained and studies on the properties of the biosurfactant indicate the possibility of its industrial application. The spectra obtained from FT-IR spectroscopy, NMR, and LC-MS confirmed the presence of lipopeptide in the sample. The potential of this biosurfactant for industrial uses was shown by studying its physical properties, i.e., the surface tension, critical micelle concentration and emulsification activity, and its stability to environmental stresses such as salinity, pH and temperature. The surface tension of an aqueous solution of this biosurfactant at a CMC value of 9 mg/l reached 26.5 mN/m. These values are very low compared with other surfactants. The properties of the biosurfactant we obtained have potential applications 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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