Production, Isolation and Application of Biosurfactant/bioemulsifier by Oil Contaminated Soil Isolate *Enterobacter cloacae* LK5

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Abstract

The aims of this study were to optimize conditions for biosurfactant/bioemulsifier production by *E. cloacae* LK5 in fermentor, and to isolate and study the chemical characterization of the surface active component. The necessary biosurfactant/bioemulsifier properties for some applications were also determined. The isolate *E. cloacae* LK5 was screened for biosurfactant/bioemulsifier production in a medium with different carbon (cane sugar, soybean oil and molasses) and nitrogen (NH_4NO_3, glutamic acid and peptone) sources using an emulsification activity (EA_{24}) and an emulsification capacity (EC) tests. Scaling up and optimal growth condition studies were carried out in a 1.5-L bench scale fermentor using the optimal medium. Cane sugar (2%) and NH_4NO_3 (0.25%) produced the best biosurfactant activity in the fermentor using agitation and aeration rate at 250 rpm and 1.5 vvm, respectively, with the EC and EA_{24} values of 33.8% and 60.7%, respectively. The isolated biosurfactant (viscous yellowish product) corresponded to a yield of 7.88 g/L of medium or 1.34 g/g of bacterial biomass, and the bioemulsifier (white powder product) gave a yield of 11.06 g/L of medium or 1.88 g/g of bacterial biomass. The biosurfactant and bioemulsifier exhibited the critical micelle concentrations (CMCs) of 65 and 200 g/L, respectively, while the CMC of synthetic surfactant sodium dodecyl sulfate (SDS) was 2.6 g/L. At the CMC points, all biosurfactant, bioemulsifier and SDS could reduce the surface tension of water to about 38.0, 48.0 and 47.0 mN/m, respectively. The IR spectroscopy revealed that the biosurfactant produced contained aliphatic hydrocarbon as well as peptide and carbonyl moieties, and the bioemulsifier contained a mixture of several compounds. As a results of its action, 61.7-89.7% of the oil was recovered from the sand pack column when the different biosurfactant concentrations (1-30% v/v) was added, suggesting that the biosurfactant might be useful in oil removal, while the determination on foamability and stabilizing capacity of biosurfactant/bioemulsifier indicated that bioemulsifier with reducing surface tension property could prove to be a potential compound for emulsion application.

Keywords: Isolation; Biosurfactant; Bioemulsifier; *Enterobacter cloacae*

INTRODUCTION

Surface active compounds produced by microorganisms are divided into two main types. Those that reduce surface tension at air-water interface (biosurfactants) and those that reduce the interfacial tension between immiscible liquids, or at the solid-liquid interfaces (bioemulsifiers). Biosurfactants usually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce surface tension (Karanth et al., 1999). Biosurfactants and bioemulsifiers are a diverse group of surface active biomolecules, such as glycolipids, lipopeptides, polysaccharide-protein complexes, protein-like substances, lipopolysaccharides, phospholipids, fatty acids and neutral lipids (Rosenberg & Ron, 1999). Like chemical surfactants, these amphipathic compounds contain both hydrophilic and hydrophobic moieties and have ability to reduce surface and interfacial tension between difference fluid phases which confer excellent detergency, emulsifying, foaming, wetting and dispersing or solubilizing traits (Holmberg, 2001; Makkar & Cameotra, 2002). Nevertheless, in recent years, much attention has been directed towards biosurfactant according to their several advantages over the chemical surfactants, such as low toxicity, higher biodegradability, better environmental compatibility, high selectivity and specific activity at extreme temperature, pH, and salinity, and the ability to be synthesized from renewable feedstock (Banat et al., 2000; Desai & Banat, 1997). These favorable features make biosurfactants and bioemulsifiers potential alternatives of chemically synthesized surfactants in a variety of applications. The biosurfactants/ bioemulsifiers have potential for use in oil industry, such as cleaning oil sludge from storage tanks; enhancing oil recovery process; mobilizing heavy crude oil; transporting petroleum in pipeline; stabilizing fuel water-oil emulsions; and managing oil spill. The ability and stability of biosurfactants/bioemulsifier foams are also important and are to facilitate frothing as required in food, beverage, fermentation, pharmaceutical,

paper production, chemical industries and heavy metal-contaminated soil remediumtion (Das et al., 1998; Eisner et al., 2007; Makkar & Cameotra, 1997; Mulligan & Wang, 2006). Therefore, the reduction of surface tension at air-water interface or the reduction of interfacial tension between immiscible liquids, or at the solid-liquid interfaces, including solubilizing property, foamability, and stabilizing capacity of biosurfactant/bioemulsifier, are necessary for application.

Although biosurfactants and bioemulsifiers have many interesting properties, their high cost of production coupled with less production rate as compared to commercially available synthetic surfactants have been the major obstacle for industrial or commercial application. Recently, numerous efforts have been made on cost reduction of biosurfactants and to increase yields by focusing on strain screening and improvement, nutritional and environmental factors optimization, or fermentor operation and design as well as using cheaper and renewable substrates (Mulligan, 2005).

Despite some well known biosurfactants and bioemulsifiers, as for examples, surfactin produced by *Bacillus subtilis*, rhamnolipids produced by *Pseudomonas aeruginosa*, monosylerythritol lipids produced by *Candida antarctica* or succinol trehalose lipids produced by *Rhodococcus erythropolis* (Rodrigues et al., 2006), information on the biosurfactants and bioemulsifiers produced by *Enterobacter cloacae* strain have not been extensively studied.

The aim of the present study was to optimize conditions for biosurfactant/bioemulsifier production by *E. cloacae* LK5 in a fermentor. Isolation and chemical characterization of the surface active component from crude biosurfactant mixture were also discussed. The necessary biosurfactant/bioemulsifier properties for some applications were evaluated.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The biosurfactant producing strain LK5 was isolated from soil sediments of the Sirikitti oil field, Lankrabue, Kampangpet, Thailand, by the method described by Bodour (Bodour et al., 2003). It was identified as *E. cloacae* by morphological and biochemical tests and 16S rRNA gene sequencing. It was grown in nutrient broth (NB from Merck) and maintained in the NB containing 10% glycerol solution and stored below 0 ^{o}C until use. From frozen stock, bacteria were streaked on nutrient agar (NA from Merck) plates and incubated at 30 ^{o}C for further culturing. To prepare subcultures, the NA was inoculated with a colony from the plate and incubated overnight at 30 ^{o}C.

Nutritional optimization of biosurfactant production

A loopful of subculture strain was grown on NB with shaking (200 rpm) at 30 ^{o}C for 18 h. The seed culture at 10% (v/v) was used as an inoculum into 50 mL of mineral salt medium (MSM) containing 2% of glucose as the sole carbon and energy source (Bodour et al., 2003) in a 250-mL Erlenmeyer flask. The MSM was a mixture of solution A and solution B. Solution A contained (per liter) 2.5 g $NaNO_3$, 0.4 g $MgSO_4 \cdot 7H_2O$, 1.0 g NaCl, 1.0 g KCl, 0.05 g $CaCl_2 \cdot 2H_2O$, and 10 mL concentrated phosphoric acid (85%). This solution was adjusted to pH 7.2 with KOH. Solution B contained (per liter) 0.5 g $FeSO_4 \cdot 7H_2O$, 1.5 g $ZnSO_4 \cdot 7H_2O$, 1.5 g $MnSO_4 \cdot H_2O$, 0.3 g K_3BO_3, 0.15 g $CuSO_4 \cdot 5H_2O$, and 0.1 g $Na_2MoO_4 \cdot 2H_2O$. One milliliter of solution B was added to 1,000 mL of solution A to form the MSM. The broth culture was incubated with shaking (200 rpm) at 30 ^{o}C for 5 days. Instead of glucose, the various carbon sources (cane sugar, soybean oil and molasses) were added to make the final concentration of 1, 2 and 4% (w/v). The inorganic and organic nitrogen sources (NH_4NO_3, glutamic acid and peptone) were added to make the final concentration of 0.125, 0.25 and 0.5% (w/v) in substitution of $NaNO_3$. A fractional factorial design (FFD) involving lower, medium and upper level of each factor was statistically used in this experiment (Table 1). The biosurfactant activity was evaluated by emulsification activity (EA_{24}) and emulsification capacity (EC) tests of fermentation broth. The EA_{24} of fermentation broth was determined by adding 2 ml of kerosene oil to the same amount of fermentation broth in a test tube (1.5x15 cm), mixed at maximum speed for 2 min with a vortex mixer and allowed to stand for 24 h. The EA_{24} was defined as percentage of height of emulsified layer (cm) divided by the total height of the liquid column (cm) (Cooper & Glodenberg, 1987). To measure the EC, the weighed test tube (W) containing 1 mL of fermentation broth and 1 mL of 0.02 M Tris-HCl buffer pH 7.3 supplemented with 2 drops of lubricating oil (10W-40) was weighed (W1). The mixture was then vortexed for 30 s and allowed to stand for 5 min. After each addition of 2 drops of the lubricating oil, the mixture was again vortexed for 30 s and allowed to stand for 5 min. This procedure was repeated by adding the lubricating oil until the emulsifier layer collapsed and the test tube was finally weighed (W2) (Ghurye & Vipulanandan, 1994). The EC was defined as percentage of weight of W2-W1 (g) divided by the weight of W1-W (g). Statistical results from analytical methods of the EA_{24} and EC of fermentation broth were used to

evaluate and select the best carbon and nitrogen sources for biosurfactant production in the fermentor.

Environmental optimization of biosurfactant production in fermentor

The effect of environmental conditions was determined by adding 10% (v/v) of the bacterial inoculum into a 1.5-L fermentor containing 1000 ml of the optimum medium. A foam collector with a volume of 1.0 L was connected to the gas effluent of the fermentor. A peristaltic pump was designed to take up a portion of liquid culture back to the fermentor from the bottom of foam collector (Yeh et al., 2006). The overflowed foams were introduced to an acid tank. Batch cultures were incubated at 30 ^{o}C for 5 days with the agitation rate of 250, 300 and 350 rpm and the aeration (compress air) rate of 0.5, 1.0, and 1.5 vvm using FFD experimental design (Table 1). All parameters were statistical analyzed as mentioned in the shake-flask experiment to evaluate the best environmental conditions.

Biosurfactant/bioemulsifier production, isolation and partial purification

Growth characteristics and biosurfactant production on the optimum conditions in the fermentor were observed. After 5 days, the fermentation broth obtained was centrifuged at 10,000g for 15 min at 4 ^{o}C to remove the bacterial cells. The supernatant pH was adjusted to 2.0 with 6 N H_2SO_4, and an equal volume of $CHCl_3/CH_3OH$ (2:1) was added. The mixture was vigorously shaken for 5 min and allowed to set until phase separation occurred. For crude biosurfactant extraction, the bottom solvent phase was removed and the upper aqueous phase was re-extracted as before. For crude bioemulsifier extraction, the bottom solvent phase with the emulsifying layer was removed and the operation was also repeated again. Both products were concentrated from the pooled solvent phases using a rotary evaporator at 40 ^{o}C.

The critical micelle concentrations (CMCs) of the crude biosurfactants and the bioemulsifiers were estimated by quantifying the surface tension by the duNouy method using a ring tensiometer (Cole-Parmer). Biosurfactant/bioemulsifier dilutions were prepared in 0.02 M Tris-HCl buffer pH 7.3 and their surface tensions were measured. The CMCs were estimated graphically by plotting surface tension as a function of the logarithm of biosurfactant concentration and is found as the point at which the baseline of minimal surface tension intersects the slope where surface tension show a linear decline (Rodrigues et al., 2006).

Analysis of the components of the partially purified biosurfactant

The crude biosurfactants were separated by TLC using aluminum sheets silica gel 60 F_{254} plates (Merck) with various solvent systems. The components were observed under UV light (wavelength 254 and 360 nm) and visualized by staining with ninhydrin, rhodamine B, alkali potassium permanganate, and iodine vapor in the presence of amino acids, lipids, organic compounds/sugar and sugar/lipids, respectively (Prommachan, 2002). These samples were also analyzed for functional groups using a FTIR spectroscopy (Perkin Elmer, Spectrum GX) at the Department of Chemistry, Naresuan University.

Properties of biosurfactant/bioemulsifier for potential application

Solubilizing properties of biosurfactant

The application of the biosurfactant in enhancing oil removal was evaluated using the sand pack technique described by Makkar & Cameotra (1997). A burette column was packed with 50 g of acid-washed sand ranged in sizes from 250-500 μm. The column was then saturated with 10 mL of kerosene. The activity of the biosurfactant in oil recovery was estimated by pouring 10 mL of the extracted biosurfactant (10% w/v) prepared in 0.02 M Tris-HCl buffer pH 7.3 onto the column. The treatment was compared to those performed by a 1% (w/v) solution of the synthetic surfactant sodium dodecyl sulfate (SDS, Bio Basic Inc.) in 0.02 M Tris-HCl buffer pH 7.3 and the cell-free fermentation broth. The amount of oil released was measured and calculated for percentage of oil removal.

Table 1. Levels of the variables tested in the fractional factorial design

Variables	Levels		
	Low (-1)	Medium (0)	Upper (+1)
Carbon sources (Cane sugar, Molasses, Soybean oil)			
Carbon concentration (% w/v)	1	2	4
Nitrogen sources (NH_4NO_3, Peptone, Glutamic acid)			
Nitrogen concentration (% w/v)	0.125	0.25	0.50
Agitation rate (rpm)	250	300	350
Aeration rate (vvm)	0.5	1.0	1.5

Foamability of biosurfactant/bioemulsifier

Foamability or foam growth was measured in terms of foam volume (FV) per minute (Das et al., 1998). Air at a constant flow rate of 100 mL/min was passed through a nozzle placed at the bottom of a 40 mm dia., 250 mL graduated tall measuring glass cylinder containing 20 mL of the crude biosurfactant/bioemulsifier (10% w/v) prepared in 0.02 M Tris-HCl buffer pH 7.3. The maximum foam height at which the foam reached to the top scale of cylinder (250 mL) was measured against the time. The FV was estimated as the difference between the volume occupied by the liquid-plus-foam and the volume of the liquid at rest.

Stabilizing capacity of biosurfactant/bioemulsifier

The procedure used to evaluate the relative emulsion volume (EV,%) and stability (ES,%) were similar to the method used to evaluate the EA_{24} described before but it was measured in the intervals of 0 h up to 72 h using the following equations (Das et al., 1998):

$$\%EV = \frac{\text{emulsion height (cm) x cross-section area (cm}^2\text{)}}{\text{total liquid volume (cm}^3\text{)}}$$

$$\%ES = \frac{\%EV \text{ at time } t \text{ h x } 100}{\%EV \text{ at time 0 h}} \quad .$$

It should be noted that when using the formula for relative emulsion above, values can be greater than 100% when the emulsion has a greater volume than that of the original liquid mixture (which was 4 cm^3) because of entrained air. The emulsions formed by the *E. cloacae* LK5 were compared to those formed by a 1% (w/v) solution of the SDS in 0.02 M Tris-HCl buffer pH 7.3.

Statistical analysis

All screening and biosurfactant characterization studies were performed at least twice with three to five replicates of each test in all experiments. Means and standard errors of means (SEM) were calculated for pooled results. Data in percentages were arcsine transformed before further analysis. Analysis of variance (ANOVA) and post hoc comparison were performed using SPSS software. A p-value of less than 0.05 was considered statistically significant using Tukey HSD test.

RESULTS

Effect of nutrients on biosurfactant production in the shake-flask

The *E. cloacae* LK5 could grow in the medium containing all carbon sources tested with significant difference ($p < 0.05$). The maximum EC occurred when 2% of cane sugar and 0.25% of NH_4NO_3 were used as the nutrient source and concentration (Table 2), but the EA_{24} could be obtained when 1% of cane sugar and 0.125% of peptone were applied (Table 3). Since biosurfactants usually exhibited emulsifying capacity with their properties on the emulsion activity, therefore, the optimal nutritional sources for biosurfactant production were selected by statistically evaluating the EC of the fermentation broth rather than the EA_{24}. Moreover, production of exopolymers by *E. cloacae* LK5 also appeared when the peptone was used as the organic nitrogen source leading to a decrease of the EC property. Thus, 2% of cane sugar and 0.25% of NH_4NO_3, the best and cheap substrates for the biosurfactant formation with the EC and EA_{24} values of 16.0% and 45.1%, respectively, were used as the nutrient sources for further scaling up studies in the fermentor.

Effect of environmental condition on biosurfactant production in the fermentor

Scaling up studies were carried out in the 1.5-L bench scale formentor using the optimal medium. The results showed that the efficient biosurfactant was obtained from the fermentor cultivation conditions using agitation and aeration rate at 250 rpm and 1.5 vvm with the EC and EA_{24} values of 33.8% and 60.7%, respectively (Table 4).

Growth characteristics and biosurfactant production on optimum conditions in the fermentor

Since biosurfactants reduced the surface tension of the medium in which they grew, the biosurfactant production was monitored by measuring the reduction in surface tension of cell-free broth. Figure 1 illustrates the biosurfactant production and growth characteristics of the *E. cloacae* LK5 on 2% cane sugar and 0.25% NH_4NO_3 at 30 ^oC in the 1.5-L fermentor. The EA_{24} of biosurfactant reached at the maximum in 24 h of the fermentation. At this point the biosurfactant exhibited a low surface tension of 57.6 mN/m, decreased the surface tension of the medium (72.3 mN/m), and retained its surface activity (54.3-58.8 mN/m) even after the fermentation for 120 h while the EC values slowly increased at 6-120 h. The substrate, indicated as the reduced sugar, was highly utilized by the strain within 24 h of growth and still had left enough through out the fermentation period. The strain reached the maximum of growth at 72 h of cultivation with the log CFU of 10.1 and the medium pH of the whole fermentation time was within the pH range of 6.4-7.8.

Table 2. Effect of nutrients on the emulsification capacity (EC) of biosurfactants produced in the shake-flask

Nitrogen sources		Carbon sources								
		Cane sugar			Molasses			Soybean oil		
		1 %*	2 %*	4 %*	1 %*	2 %	4 %	1 %	2 %	4 %*
NH_4NO_3 (%)	0.125	15.0±8.9^{a}	11.2±2.9^{ab}	6.8±0.1^{bcd}	1.4±0.0^{ab}	1.4±0.1	1.4±0.1	1.5±0.1	1.5±0.1	1.4±0.0^{b}
	0.25	11.3±2.3^{ab}	16.0±1.6^{a}	15.5±0.1^{a}	1.5±0.1^{ab}	1.4±0.1	1.5±0.1	1.5±0.3	1.5±0.2	1.5±0.1^{ab}
	0.5	12.4±1.9^{ab}	13.8±1.1^{ab}	9.2±0.8^{b}	1.4±0.0^{ab}	1.4±0.1	1.4±0.1	1.4±0.5	1.5±0.1	1.5±0.0^{b}
Peptone (%)	0.125	6.1±0.3^{bc}	3.4±0.9^{de}	4.2±0.1b^{cd}	1.3±0.2^{b}	1.4±0.0	1.4±0.1	1.5±0.1	1.4±0.1	1.4±0.1^{b}
	0.25	3.1±0.5^{c}	3.4±0.9^{de}	3.8±0.7^{cd}	1.3±0.3^{b}	1.7±0.8	1.4±0.1	1.4±0.1	1.5±0.1	1.7±0.3^{a}
	0.5	9.0±3.4^{abc}	8.8±0.6^{bcd}	2.7±0.0^{d}	1.5±0.1^{ab}	1.3±0.0	1.4±0.2	1.5±0.1	1.5±0.2	1.5±0.1^{ab}
Glutamic acid (%)	0.125	3.4±0.7^{c}	2.7±1.3^{e}	3.0±0.0^{cd}	1.6±0.1^{a}	1.4±0.1	1.4±0.1	1.5±0.1	1.4±0.2	1.5±0.1^{ab}
	0.25	2.9±0.1^{c}	6.0±2.4^{cde}	5.2±0.2^{bcd}	1.6±0.1^{a}	1.5±0.1	1.4±0.1	1.5±0.1	1.4±0.1	1.4±0.2^{ab}
	0.5	5.1±1.4^{bc}	9.1±7.6^{ab}	7.5±0.7^{bc}	1.5±0.0^{ab}	1.5±0.1	1.5±0.3	1.4±0.1	1.5±0.1	1.4±0.1^{ab}

*Values in the same column with the same superscripted letters do not differ significantly ($p \geq 0.05$) as determined by Tukey HSD test.

Table 3. Effect of nutrients on the emulsification activity (EA_{24}) of biosurfactants produced in the shake-flask

Nitrogen sources		Carbon sources								
		Cane sugar			Molasses			Soybean oil		
		1 %*	2 %*	4 %*	1 %*	2 %*	4 %*	1 %*	2 %	4 %*
NH_4NO_3 (%)	0.125	54.0±3.3^{bc}	58.9±9.3^{b}	42.2±2.66^{c}	26.6±8.9^{bc}	38.2±5.5^{bcd}	20.7±11.5^{bc}	0±0^{c}	0±0^{b}	0±0^{c}
	0.25	39.9±17.2^{c}	45.1±18.7^{b}	60.2±0.85^{bc}	27.7±12.5^{bc}	51.8±9.5^{ab}	24.6±6.4^{b}	0±0^{c}	0±0^{b}	0±0^{c}
	0.5	53.4±10.4^{bc}	55.1±9.1^{b}	60.3±8.50^{bc}	47.0±6.6^{ab}	27.8±14.1^{cd}	15.0±10.4^{bc}	0±0^{c}	8.7±9.5^{ab}	0±0^{c}
Peptone (%)	0.125	97.2±1.0^{a}	93.2±1.5^{a}	91.3±4.47^{a}	24.9±15.2^{c}	43.2±4.7^{bc}	6.9±5.6^{c}	13.0±14.6b^{c}	15.9±14.8^{ab}	0±0^{c}
	0.25	47.8±5.1^{bc}	96.0±1.8^{a}	73.5±9.96^{ab}	29.1±10.3^{bc}	27.1±12.0^{cd}	10.4±4.4^{c}	0±0^{c}	26.0±29.2^{a}	0±0^{c}
	0.5	59.1±1.6^{bc}	42.6±14.1^{b}	58.0±6.25^{bc}	25.5±18.4^{c}	22.4±13.9^{d}	10.6±7.0^{c}	0±0^{c}	18.8±20.7^{ab}	5.4±3.4^{c}
Glutamic acid (%)	0.125	90.9±8.8^{a}	39.2±31.2^{b}	27.0±1.30^{cd}	43.1±4.2^{abc}	53.3±2.8^{ab}	52.2±2.3^{a}	43.6±9.0^{a}	6.0±4.3^{ab}	39.4±5.3^{ab}
	0.25	91.5±9.3^{a}	35.5±10.4^{b}	3.3±1.15^{d}	50.4±13.5^{a}	53.5±3.2^{ab}	53.7±8.2^{a}	19.7±21.7^{b}	17.3±8.4^{ab}	32.6±2.8^{b}
	0.5	65.2±4.0^{b}	42.3±34.2^{b}	2.0±0^{d}	62.1±0.7^{a}	61.8±1.6^{a}	55.0±3.2^{a}	55.2±2.2^{a}	26.1±12.9^{ab}	46.6±10.5^{a}

*Values in the same column with the same superscripted letters do not differ significantly ($p \geq 0.05$) as determined by Tukey HSD test.

Table 4. Effect of environmental conditions on the emulsification capacity (EC) and the emulsification activity (EA_{24}) of biosurfactant produced in the fermentor

Environmental conditions		EC (%)*	EA_{24} (%)*
Aeration (vvm)	Agitation (rpm)		
0.5	250	1.4 ± 0.0^{f}	27.1 ± 2.5^{d}
	300	1.5 ± 0.1^{f}	26.7 ± 6.3^{d}
	350	3.1 ± 0.2^{f}	39.1 ± 1.8^{c}
1.0	250	28.9 ± 0.6^{bc}	56.5 ± 2.3^{b}
	300	31.0 ± 1.1^{b}	62.4 ± 0.3^{b}
	350	20.5 ± 0.8^{d}	70.4 ± 4.2^{a}
1.5	250	33.8 ± 1.8^{a}	60.7 ± 1.9^{b}
	300	27.1 ± 0.7^{c}	61.1 ± 2.3^{b}
	350	9.1 ± 0.3^{e}	62.1 ± 1.3^{b}

*Values in the same column with the same superscripted letters do not differ significantly ($p = 0.05$) as determined by Tukey HSD test.

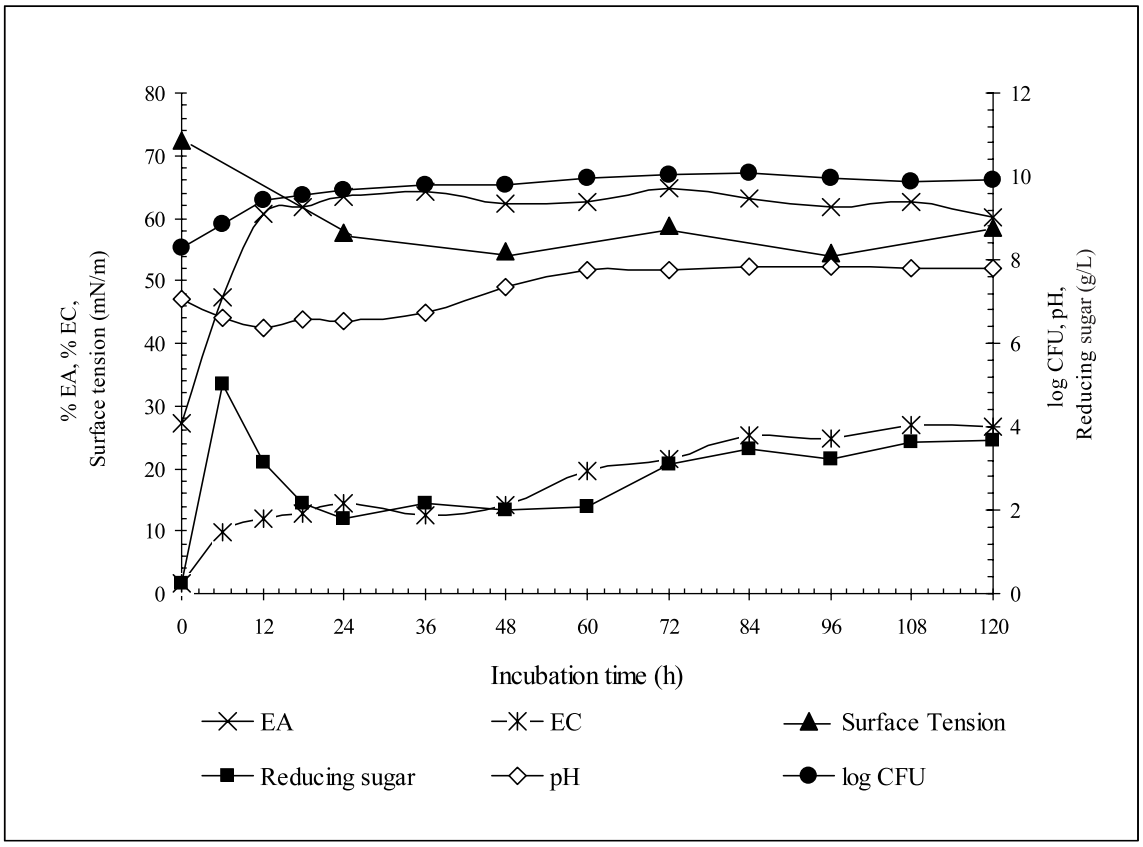

Figure 1. Growth characteristics and biosurfactant production by *Enterobacter cloacae* LK 5 in the fermentor (the MSM medium contained 2% cane sugar and 0.25% NH_4NO_3, 30 °C).

Biosurfactant production, isolation and partial purification

The viscous yellowish product and the white powder product obtained from the extraction and purification processes were defined as a crude biosurfactant and a crude bioemulsifier, respectively. The isolated biosurfactant corresponded to a yield of 7.88 g/L of medium or 1.34 g/g of bacterial biomass, and the bioemulsifier gave a yield of 11.06 g/L of medium or 1.88 g/g of bacterial biomass.

A plot of surface tension versus the biosurfactant concentration was used to estimate a CMC (Figure 2). The biosurfactant and bioemulsifier exhibited the CMCs of 65 and 200 g/L, respectively, which were high compared to the CMC of the SDS control (2.6 g/L). All biosurfactant, bioemulsifier and SDS could reduce the surface tension of water to about 38.0, 48.0 and 47.0 mN/m, respectively, at the CMC points.

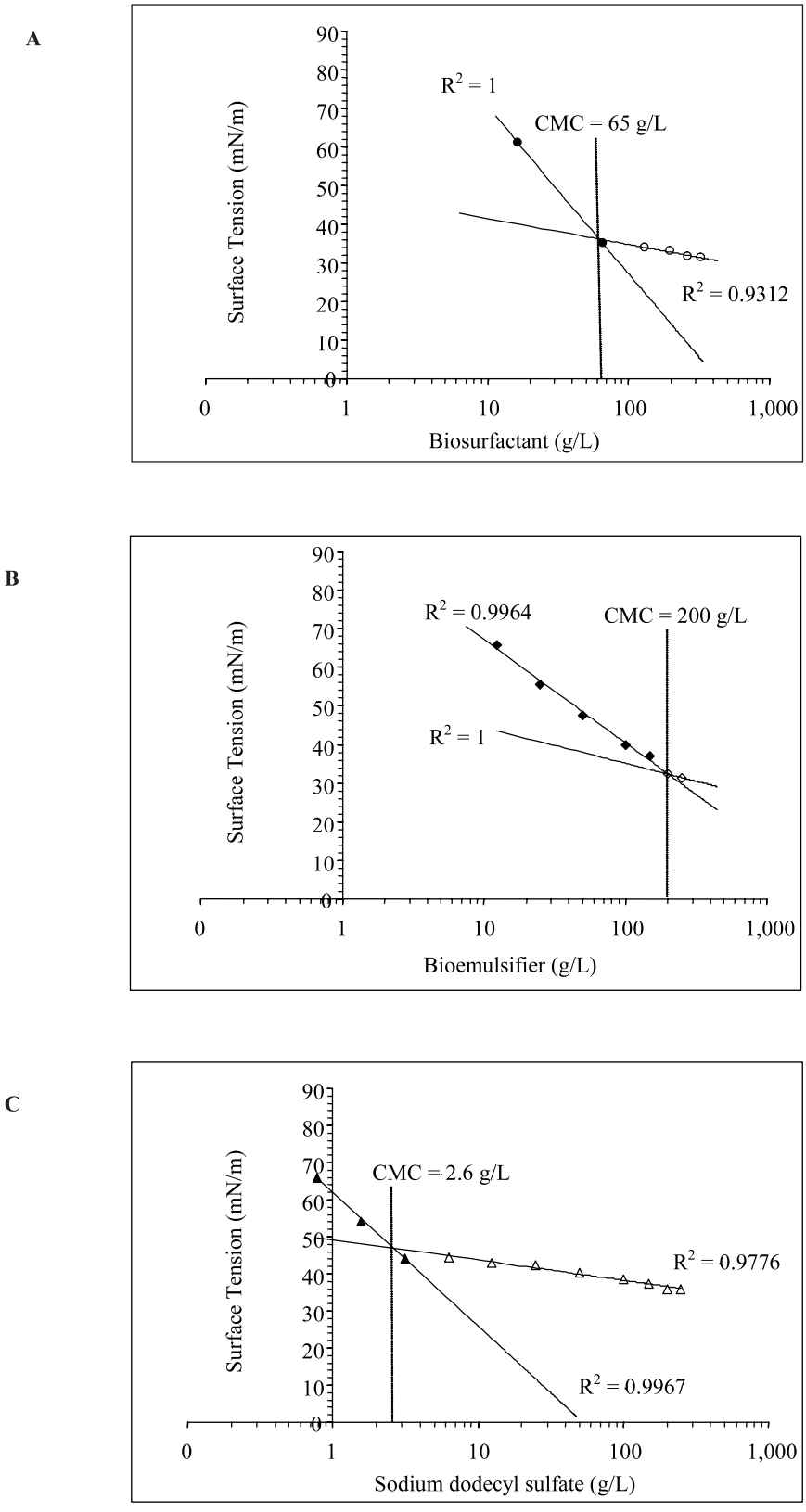

Figure 2. Surface tension vs. concentrations of the crude biosurfactant (A), bioemulsifier (B) obtained from *Enterobacter cloacae* LK5 and the SDS control (C).

Analysis of the components of the partially purified biosurfactant/bioemusifier

For detection of partially purified biosurfactant by TLC study, the solvent system used was toluene/acetone (1:1). The TLC plate showed the presence of amino acids and lipids when the components were observed under the UV light and sprayed with developing agents.

The IR spectrum of the crude biosurfactant was different (overlapping) to the broad spectrum of the crude bioemulsifier, indicating a mixture of several compounds in the crude extracts (Figure 3). However, the IR spectroscopy revealed that the biosurfactant showed absorption bands, indicating the presence of a peptide component at 3440 cm^{-1} resulting from the N-H stretching. At 1119-1360 cm^{-1}, the mode of C-N stretch was observed. The presence of an aliphatic chain was indicated by the C-H stretching mode at 2937-2983 cm^{-1} and 1417-1454 cm^{-1}. The band at 1715 cm^{-1} was due to carbonyl absorption. These results infer that the biosurfactant produced contains aliphatic hydrocarbon as well as peptide and carbonyl moieties.

Properties of biosurfactant/bioemulsifier for potential application

The sand pack column was washed with Tris-HCl buffer, only 50.7% of the kerosene oil was removed, while compared with the 1% SDS and the cell-free fermentation broth, 91.0% and 71.7% could be recovered, respectively. However, 61.7-89.7% of the oil was recovered from the sand pack column when the different crude biosurfactant concentrations was added (Table 5), suggesting that the biosurfactant might be useful in oil removal.

Results on the foamability or foam growth with the air at a constant flow rate of 100 mL/min showed unappreciable emulsion forming capacity of both the 10% of biosurfactant and bioemusifier when compared to the 1% SDS control. However, when the air at a constant flow rate of 700 mL/min was applied, the bioemusifier foamability rate could reach up to 295 mL/min. The bubbles appearance in foams were found to be equal and small in size, coalesce relatively fast and stable closely to the SDS test (Table 6), while the biosurfactant bubbles were large and unstable.

The results on the stabilizing capacity of biosurfactant/bioemulsifier test showed that stable and compact emulsions with emulsification activity of about 95% of kerosene oil were detected by application of the crude bioemulsifier. Moreover, the emulsification capacity remained practically unaltered after 2 h which were high efficiency and stability compared to the 1% SDS control, the fermentation broth and the cell-free fermentation broth whereas the biosurfactant exhibited only 10% of the emulsification activity and the emulsions reduced and collapsed in 2 h (Figure 4).

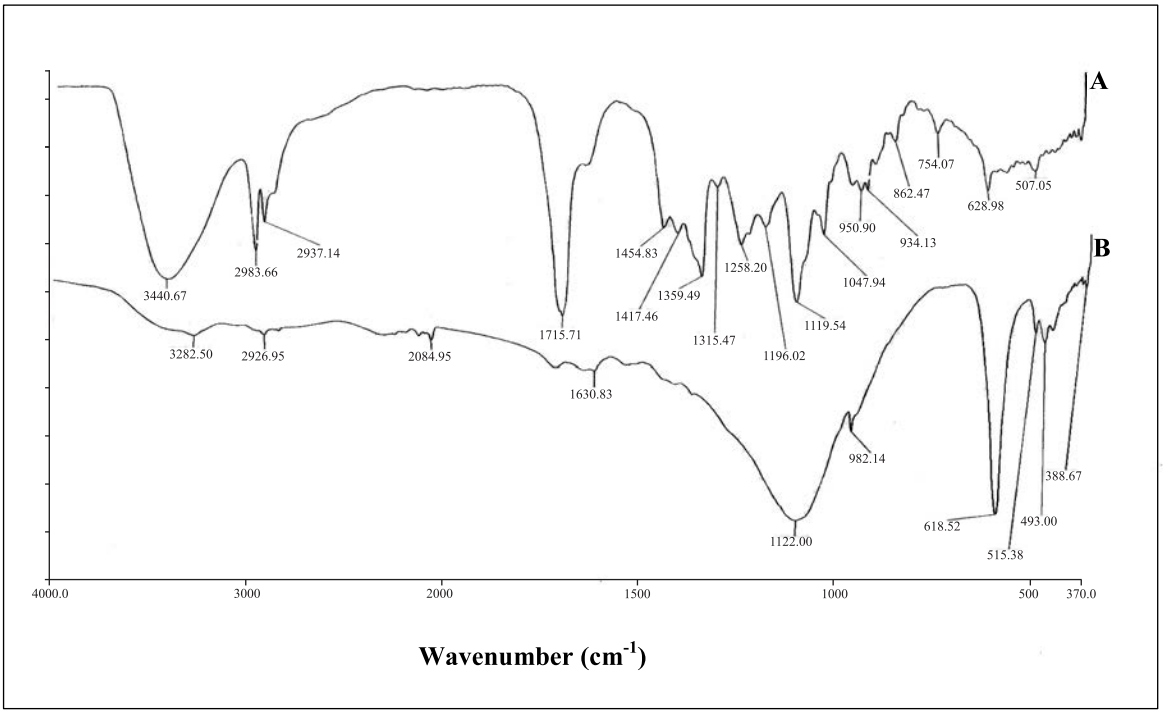

Figure 3. A comparison of IR spectrum of the crude biosurfactant (A) and bioemulsifier (B) produced by *Enterbacter cloacae* LK5 in fermentor.

Table 5. Potential use of the crude biosurfactant in oil removal by the sand pack test

Treatments	Oil removal (%)*
0.02 M Tris-buffer (control)	50.7 ± 1.2^{c}
1% SDS (control)	91.1 ± 1.0^{a}
Cell-free fermentation broth	71.7 ± 2.1^{b}
Crude biosurfactant (v/v)	
1%	61.7 ± 2.9^{c}
5%	71.7 ± 2.9^{b}
10%	73.7 ± 2.9^{b}
30%	89.7 ± 1.5^{a}

*Values with the same superscripted letters do not differ significantly ($p \geq 0.05$) as determined by Tukey HSD test.

Table 6. Foamability rate and bubbles characteristics in foam

Treatments	Foamability rate (ml/min)	Bubbles characteristics
Tris-buffer (control)	0	No bubbles
1% SDS (control)	309.8 ± 18.6	Equal and small bubbles in size, stable
10% Biosurfactant	27.8 ± 1.27	Unequal and large bubbles in size, stable
10% Bioemulsifier	No detection*	Equal and small in size, stable
10% Bioemulsifier (with 700 mL/min air flow rate)	295.0 ± 20.5	Equal and small in size, stable

*Foam did not reach to the top scale of 250-mL cylinder.

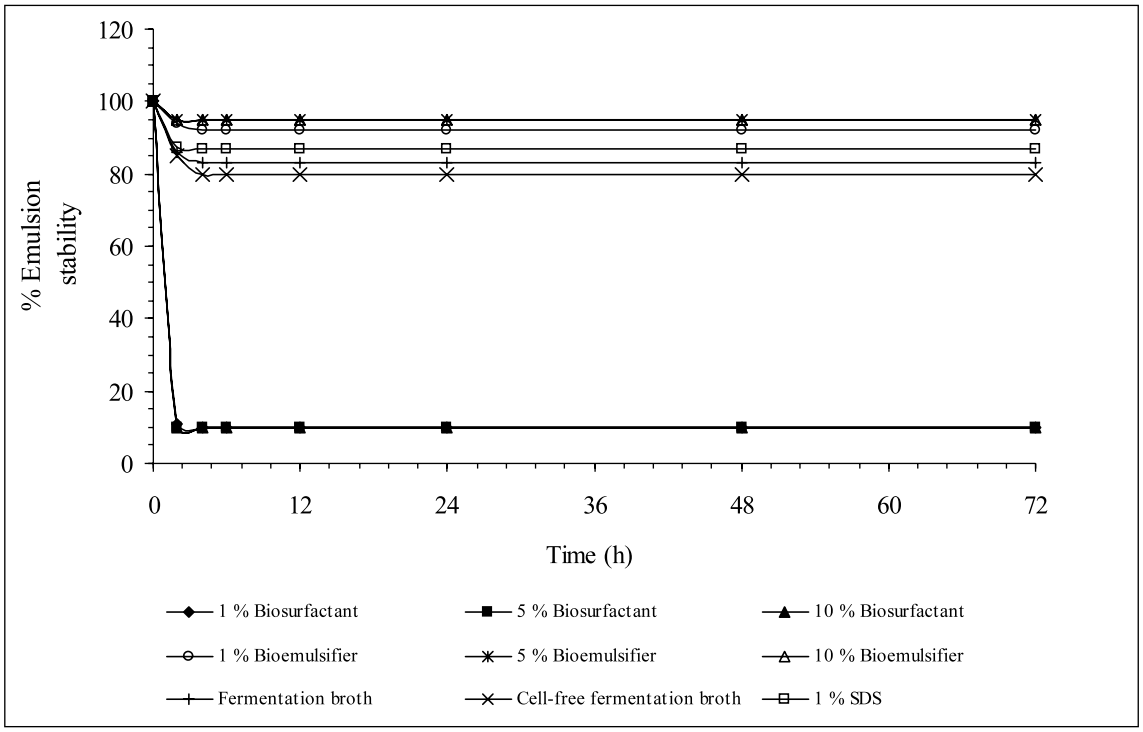

Figure 4. Stability of emulsions formed with equal volume of kerosene and fermentation broth or various concentrations of biosurfactant/bioemulsifier or SDS control.

DISCUSSION AND CONCLUSIONS

The finding that the MSM medium contained 2% of cane sugar and 0.25% of NH_4NO_3 were the best substrates for biosurfactant formation by the *E. cloacae* LK5 with the EC and EA_{24} values of 16.0% and 45.1%, respectively. The result was in agreement with the experimental results of Makkar & Cameotra (1997) and Yakimov et al. (1995). Although higher EA_{24} of the biosurfactant was obtained with the medium 1, 2 and 4% cane sugar content supplemented with peptone, resembling what was observed for molasses and soybean oil medium. A better compromise among good EC, EA_{24} and low-cost was achievable with the medium to which NH_4NO_3 was added. Once, the sucrose in the cane sugar is hydrolyzed into glucose and fructose, they will become an essential source of water-soluble carbon and energy for bacteria growth and biosurfactant production. Ammonium and nitrate are available to bacteria as essential nutrients that can be used for assimilation into cellular material due to easily forms of inorganic nitrogen. Generally a 'preference' is shown (Cheng et al.,1999; Flynn et al., 1997) for the nitrogen source with the assimilatory pathway that costs least in terms of energy required to assimilate the same quantity of inorganic nitrogen into amino acids. Ammonium, although requiring transportation into the cell, is already at the appropriate redox state for assimilation into amino acids, and hence requires the least energy. Thus, cane sugar and NH_4NO_3 can be used as an optimum and alternative economical medium for biosurfactant production by *E. cloacae* LK5.

During the microbial production of biosurfactant in the present of aeration and agitation, excessive foam is produced in the fermentor because the biosurfactant lowers the surface tension. Therefore, the process of recovering the biosurfantant using a foam collector is also applied. During aerobic and viscous fermentation, oxygen supply is very important because an insufficient oxygen supply can lead to sub-optimal productivity rates (Shu & Yang, 1990), as well as products of low quality. Increasing the agitation and aeration was used to enhance the gas-liquid oxygen transport in this study. When cultivation of *E. cloacae* LK 5 was attempted in the fermentor with an increase in agitation and aeration rates, the results showed a significant improvement that the efficient biosurfactant was obtained from the fermentor cultivation conditions using agitation and aeration rate at 250 rpm and 1.5 vvm with the EC and EA_{24} values of 33.8% and 60.7%, respectively.

The surface tension of the fermentation broth was between 54.3 and 58.8 mN/m, whereas the control showed the value of 72.3 mN/m. This was evident due to the production of the extracellular biosurfactants by *E. cloacae* LK5 during the fermentation utilizing the specified carbon source, sucrose, which was consistent with the observation of Persson and Molin (1987) and Das et al. (1998). The increased biosurfactant production was observed by mean of the increased EC values mainly after bacterial growth had reached stationary phase suggesting the accumulation of biosurfactant as secondary metabolites (Raza et al., 2007).

The biosurfactant and bioemulsifier yields from the MSM medium containing 2% of cane sugar and 0.25% of NH_4NO_3 were 7.88 and 11.06 g/L of medium or 1.34 and 1.88 g/g of bacterial biomass. This yield was relatively high. The culture supernatant of *E. cloacae* LK5 reduced the surface tension to 54.3 mN/m, while the partially purified biosurfactant reduced the surface tension to a minimum 31.5 mN/m. This indicated strong activity comparable to that of other biosurfactants (Bodour & Maier, 2002). A plot of the surface tension versus the log of biosurfactant/bioemulsifier concentration was used to estimate the CMC of 65 and 200 g/L, respectively. This was quite high compared to the SDS control and to those of other lipoprotein biosurfactant, for example, lichenysin CMC values ranged from 0.01 to 0.03 g/L (Jenny et al., 1991; Lin et al., 1994; Yakimov et al., 1995), and for surfactin, iturin and fengycin, reported CMC values were 0.01, 0.02, and 0.01 g/L, respectively (Deleu et al., 1999). However, the CMC presumption could be improved with pH optimization because the applicability of the biosurfactant could be conditioned by its stability to the pH changes. Considering that the surface-active properties of the biosurfactant, the EC values obtained from the fermentation broth were high (Figure 1), but the crude biosurfactant and bioemulsifer were similar, giving very low solubilization ratios (about 2-3%, data not showed). It can be supposed that some components in the fermentation broth and the acidity of the crude biosurfactant/bioemulsifer solution (final pH of 2.3) influenced the characteristics of the biosurfactants (Prommachan, 2002; Sarubbo et al., 2006). However, it is important for several applications of the biosurfactants to establish their CMC, as above this concentration no further effect is expected in the surface activity.

As a result of analyzing the components of biosurfactant from *E. cloacae* LK5 suggested that it was lipopeptide surfactant, while thebioemulsifier was presumptively contained a polysaccharide moiety attached to lipid and/or protein. These results were supported by those of biosurfactant produced by *Bacillus* species, which usually produced lipopeptide or lipoprotein surfactant (Das & Mukherjee, 2007; Joshi et al., 2007; Makkar & Cameotra, 1997) and the bioemulsifier produced by a marine carbohydrate polymer-producing strain, *Enterobacter*

cloceae (Iyer et al., 2006).

In the sand pack test, compared with the Tris-buffer control, the biosurfactant (30%, v/v) could recover the oil of about 1.6 fold as similar to that achieved by 1% SDS, while the cell-free fermentation broth gave the potential of oil removal equal to the 5% biosurfactant solution. This may be presumed that after 120 h of incubation time, there was about 5% of bisurfactant contained in the fermentation broth. The result showed that the biosurfactant was effective in the removal of oil from the sand pack column, indicating the potential use of the biosurfactant in microbiologically enhanced oil recovery (MEOR), similar to those experiments done by Makkar & Cameotra (1997), and Prommachan (2002).

The biosurfactant/bioemulsifier was added to aqueous liquids to reduce their surface tension leading to the formation of small bubbles in the foam. When the diameter of bubbles was small on the result of bioemusifier test, the bubbles are nearly spherical in shape and usually encountered; it has been suggested that the surface tension is reduced and gas volume fraction is low. Therefore, it may be applied in food such as ice cream, which has about 50% air and typically with a mean bubble diameter between 20 and 60 μm (Berger et al., 1972; Chang & Hartel, 2002). Since the bubbles in foam are large on the result of biosurfactant test, the surface tension is low and the volume fraction of gas is high, bubbles deform to polyhedral bubbles with thin liquid films. Examples include beverage of beer foam (Bhandola et al., 1989; May et al., 1996), which have typically bubbles with diameter and gas volume larger than 500 μm and 75%, respectively. However, the foaming properties of biosurfactant depends on their chemical structures, concentrations in liquid and at the interface between liquid and bubble, ionic strength, temperature and the method of foam formation.

In Figure 3, it is apparent that a culture filtrate giving an initial higher %EV might not necessarily mean that the emulsion produced would be of higher stability (%ES) as well. The results have confirmed that the ability of biosurfactant to form a stable emulsion is not always associated with surface tension lowering (Plaza et al., 2005; Willumsen & Karlson, 1997). Moreover, the ability of the bioemulsifier to form stable emulsions may be attributed to the concentration and nature of the present proteins. The proteinic nature coupled with its good viscosity (even at acidic pH) promotes its application in food products containing citric acid and ascorbic acid when gelation is not required (Iyer et al., 2006). However, the results on the foamability and the stabilizing capacity of the biosurfactant/bioemulsifier suggest that the bioemulsifier with reducing surface tension property is appreciable for emulsion application.

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