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Molasses as a Whole Medium for Biosurfactants Production by *Bacillus* Strains and Their Application

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Abstract Two types of biosurfactant (BS)-producing bacteria, *Bacillus licheniformis* TR7 and *Bacillus subtilis* SA9, were isolated from mangrove sediment in the south of Thailand. The BS production was done by using only molasses as a whole medium for growth and production. Under optimized conditions, the yields of TR7 and SA9 BS were found to be 3.30 and 3.78 g/l, respectively. It could reduce the surface tension of pure water to 28.5 and 29.5 mN/m, with the critical micelle concentrations of about 10 and 30 mg/l, respectively. Good thermal, pH, and salt stability were exhibited. Both BSs could recover oil more effectively than the two synthetic surfactants. In addition, TR7 and SA9 BS could enhance the solubility of polyaromatic hydrocarbons (PAHs). Thus, these BSs have the potential for the removal of oil and PAHs from the combined contaminated environment and facilitate its bioremediation. These studies indicate that molasses, as a renewable, relatively inexpensive and easily available resource, can be used for important biotechnological processes.

Keywords Biosurfactant · Mangrove sediment · *Bacillus* spp. · Molasses · Oil recovery · Polyaromatic hydrocarbon

Introduction

Surfactants and emulsifiers are widely used in the petroleum, environmental remediation, pharmaceutical, cosmetic, and food industries. Most of these compounds are chemically synthesized, and it is only in the past few decades that surface-active molecules of biological origin have been described. Biosurfactants (BSs) are readily biodegradable, have lower toxicity and better environmental compatibility, and can be produced from renewable

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and cheaper substrates. Thus, they might be able to replace their chemically synthesized counterparts [9]. BSs are extracellular macromolecules produced by bacteria, yeast, and fungi and, in particular, by natural and recombinant bacteria when grown on different carbon sources. At present, BSs are unable to compete with the chemical surfactants due to their high production costs. Several low-cost or by-product carbon sources such as cassava wastewater, whey, waste motor lubricant oil, and hydrocarbon have been used to produce BS [29, 33, 45]. Specifically, the *Bacillus* species is well known for its ability to produce lipopeptide-type BS with potential surface-active properties when grown on different carbon substrates [10]. Lipopeptide BS produced by these species has great potential for industrial application and bioremediation [7]. In general, the lipopeptides were produced when crude oil, glucose, glycerol, or peptone was the substrate [20]. On the other hand, *Bacillus* spp. can use the various renewal resources, especially agro-industrial wastes, as the potential carbon sources [6]. One of the important points in the biotechnological process is to obtain maximum metabolite production with a low-cost substrate. This leads to the greater possibility for economical productions and reduced pollution caused by those wastes [23].

Molasses is a by-product of the sugar cane industry, which is low in price compared to other conventional sugar sources like sucrose or glucose. The principal reasons for the widespread use of molasses as substrate are its low price compared to other sources of carbon and the presence of several other compounds besides sucrose. These include minerals, organic compounds, and vitamins, which are valuable for the fermentation process. The present study was conducted with the following objectives: the screening of BS-producing bacteria from mangrove sediment by using only molasses as a whole medium for BS production, the selecting of two strains produced the highest of BS activity, examining for BS production at different molasses concentrations and inoculums size, and characterization of BS from selected strains. The effect of BS on oil mobilization and polyaromatic hydrocarbons (PAHs) solubilization was also investigated in this study.

Materials and Methods

Materials

Aliphatic hydrocarbons (cyclohexane, hexane, xylene, benzene, *n*-hexadecane, kerosene, and toluene) and PAHs (anthracene, phenanthrene, and pyrene) were purchased from Lab-Scan (Bangkok, Thailand). Synthetic surfactants, sodium dodecyl sulfate (SDS) and polyoxyethylene sorbitan monooleate (Tween 80), were obtained from Bio-Rad (Hercules, CA, USA) and Ajax Finechem Ltd. (Auckland, New Zealand), respectively. Used lubricating oil (ULO) was provided by the motor vehicle maintenance garage located at the Prince of Songkla University, Hat Yai, Songkhla, Thailand. Molasses was purchased from the agency in Hat Yai, Songkhla, Thailand. All other chemicals used were of analytical grade.

Substrate Preparation

Nutrient broth (NB) was purchased from Difco (Detroit, MI, USA). The mineral salts medium (MSM) used was composed of (gram per liter) K_2HPO_4, 0.8; KH_2PO_4, 0.2; $CaCl_2$, 0.05; $MgCl_2$, 0.5; $FeCl_2$, 0.01; $(NH_4)_2SO_4$, 1.0; and NaCl, 30.0 [48]. The pH was adjusted

to 7.0 with either hydrochloric acid (HCl) or sodium hydroxide (NaOH) solutions. Agar was added at concentration of 15 g/l. The molasses solution medium was prepared by diluted molasses with distilled water to required concentrations; pH was adjusted to 7.0. All mediums were sterilized by autoclaving at 121 °C for 15 min.

Isolation of BS-Producing Bacteria

Soil samples were collected at six sites from mangrove sediment in the south of Thailand. BS-producing bacteria were isolated by serially diluting samples in 0.85% sterile saline. Aliquots were spread on MSM agar plates containing ULO (1%, *w*/*v*) as the carbon source. The plates were then incubated at room temperature (30±2 °C) for 3–5 days. If emulsification circles formed by colonies were observed on MSM agar plates, these colonies were transferred to fresh MSM agar with 1% (*w*/*v*) of ULO several times until pure strains were obtained. To evaluate the production of BS, each single colony was cultivated firstly in NB and incubated at room temperature for 20 h. Cells grown in NB were initially used for BS production as the inoculums. BS productions by selected strains were carried out in 250 ml flask containing 50 ml of a molasses solution medium (2%, *w*/*v*). The culture solution was then incubated at 30 °C in a shaking incubator at 150 rpm for 24 h. After that, bacterial cells were removed by centrifugation, and the sample was then submitted for oil displacement test, surface tension measurement, and the emulsification index (E_{24}). The surface activity of all isolated strains was compared. Two bacterial strains with the highest activity towards the BS production were identified and maintained in a NB containing a glycerol solution at a concentration of 30% (*v*/*v*) at −20 °C.

16S rRNA Sequence Analysis

Selected strains were identified by 16S rRNA sequence analysis. Briefly, chromosomal DNA from an overnight liquid culture was isolated using a Roche kit (Roche Applied Science, Mannheim, Germany) following the manufacture's instruction. The 16S rRNA gene was amplified using the polymerase chain reaction (PCR) method with a 1 U *Taq* DNA polymerase (Bio-Lab Ltd., Auckland, New Zealand) and universal bacterial primers UFUL (GCCTAACACATGCAAGTCGA) and URUL (CGTATTACCGCGGC TGCTGG) [28]. These primers target two highly conserved regions known to be variable among bacterial species [34] and gave a PCR product of about 500 bp. The 16S rRNA gene was sequenced by using the ABI Prism BigDye terminator kit (Perkin-Elmer Applied Biosystems, Beverly, MA, USA), according to the manufacturer's protocol, with UFUL as primer. The 16S rRNA gene sequences obtained at about 500 bp were aligned along with the sequences of type strains obtained from the GenBank by using the program ClustalW [46]. Sequence homologies were examined using the BLAST version 2.2.12 of the National Center for Biotechnology Information, and a consensus neighbor-joining tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0 [43]. The 16S rRNA gene sequences were submitted to GenBank with an accession number.

Conditions of Cultivation

Growth studies and BS production were done in 250-ml flasks containing 50 ml molasses solution medium at 30 °C in a shaking incubator at 150 rpm for 48 h. The effects of molasses concentrations (2% to 10%, *w*/*v*) and inoculum concentrations (2% to 10%, *v*/*v*) on growth and BS production were studied.

Recovery of BS

Four solvent systems, a mixture of chloroform:methanol (2:1) [29], cold acetone [7], dichloromethane [15], and ethyl acetate [24], were examined for BS extraction. The method showing the highest BS activity was used to recover BS from selected strains.

Study of BS Stability

The crude BS at critical micelle concentration (CMC) level from each strain in distilled water was prepared. To investigate the effects of pH, sodium chloride (NaCl) concentrations, and temperature on BS activity, the BS 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, the BS 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.

Preliminary Characterization of BS

Fourier transform infrared spectroscopy (FT-IR) of the TR7 and SA9 BS was done on a Nexus-870 FT-IR spectrometer (Thermo Electron Co., Yokohama, Japan) by the KBr pellet method. The dried BS samples (0.3–0.5 mg) were ground in about 80 mg of spectral grade KBr (Merck, Darmstadt, Germany) and pressed into pellets under about 6 t/cm^2 pressure with the help of a hydraulic press (Specac, Orpington, Kent, UK). A ^{1}H nuclear magnetic resonance (NMR) spectrum was recorded at 298 K on an AMX 300 NMR spectrometer (Bruker, 300 MHz). This was equipped with an Aspect 3000 computer (Bruker) locked to the deuterium resonance of solvent, $CDCl_3$, without spinning. Data were recorded at 32 K (the number of data points per parts per million of the plot).

The electrospray ionization mass spectrometry (ESI-MS) analysis of the BS was carried out in an LCQ™ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). This utilizes ESI. The electrospray source was operated at ionization source temperature 80 °C, electrolyte voltage 200 V, and spray inlet temperature 120 °C. The equipment was run in a positive ion mode.

Application of the BS in ULO Removal from Contaminated Sand

BS 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, Tween 80, TR7, and SA9 BS 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 5000 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 BS was gravimetrically determined as the amount of material extracted from the sand by hexane [42].

PAH Solubilization Assay

PAHs solubilization assay was done as described by Barkay et al. [2]. Briefly, any of the following 0.6 μg of anthracene or phenanthrene or pyrene (from 0.6 mg/ml stock in

acetone) was placed in a glass test tube (10×170 mm). This was 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 BS in increasing concentration (10–50 mg/ml) were obtained from respective bacterial strains used in this study. Assay buffer containing BS but no PAH was used as a blank. Tubes were capped with plastic closures and incubated overnight at 30 °C with shaking (200 rpm) in the 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 8,500 rpm for 10 min to separate the aqueous and hexane phases. A concentration of PAH was measured spectrophotometrically (Libra S22, Biochrom Ltd., Cambridge, England) at 250, 252, or 273 nm for anthracene, phenanthrene, or pyrene, respectively. From a calibration curve of individual PAH (in hexane), the concentration of each PAH was determined. Assay buffer with BS but without PAH was extracted with hexane identically and served as a blank.

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 8,500 rpm for 30 min. The biomass obtained was dried overnight at 105 °C and weighed.

Emulsification index (E_{24}) was performed accordingly to Cooper and Goldenberg [5]. 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 E_{24} was calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100.

The oil displacement test was carried out in a Petri dish (250 mm in diameter). Ten microliters of ULO was placed on the surface of distilled water (40 mL) in a Petri dish. Then, 10 μL of the culture supernatant was gently put on the center of the oil film. The diameter of the clear halo area seen under visible light was measured and calculated as described by Morikawa et al. [25].

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

The chemical characterization of BS was done by thin layer chromatography (TLC). The components of chloroform:methanol extract were separated on silica gel 60 plates (Merck, Darmstadt, Germany) using $CHCl_3$:CH_3OH:H_2O (65:15:1) as solvent system. Spots were revealed by spraying with (a) distilled water for detection of hydrophilic compounds and (b) ninhydrin 0.05% (*w*/*v*, in methanol/water, 1:1 *v*/*v*) for detection of compound with free amino groups. Treatments (a) and (b) were viewed after heating at 110 °C for 5 min. For detecting the presence of lipids, TLC plate was viewed under ultraviolet light after being sprayed with rhodamine B 0.25% (*w*/*v*, in absolute ethanol).

All experiments were carried out at least in triplicate. Two well-defined synthetic surfactants, i.e., Tween 80 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 Inc., Chicago, IL, USA).

Results and Discussion

Isolation and Identification of BS-Producing Bacteria

The following were among the 327 strains isolated from the samples taken from various sources of mangrove sediment in the south of Thailand. Trang Province samples (TR7, TR8, TR19, and TR21), Satun Province samples (SA3, SA4, SA9, and SA15), Songkhla Provinces samples (SO2, SO16, SO17, SO22, SO24, and SO25), and Nakhonsrithamarat Province samples (NA2, NA3, NA4, NA5, NA6, NA8, NA9, and NA12). Only 22 positive bacterial strains showed oil-displaced halo forming around the colonies. Figure 1 illustrates the activities of the BS produced by different isolated bacteria. These are shown in terms of the diameter of the clear zone by oil displacement test, E_{24} toward xylene, and the surface tension of the culture supernatant after 48 h of cultivation. Isolates TR7 and SA9 showed a comparable activity in terms of E_{24} and diameter of clear zone in oil displacement test (Fig. 2). In addition, they also reduced the surface tension considerably compared to other isolates, hence were selected for further study.

BS activity can be measured by changes in surface and interfacial tensions and emulsification/emulsion stabilization. According to Olivera et al. [32], a microorganism is considered promising for BS production if it is able to reduce the surface tension to values lower than 40 mN/m. Another approach for screening potential BS-producing microorganisms is estimation of the E_{24}. A criterion cited for emulsion-stabilizing capacity is the ability to maintain at least 50% of E_{24} [47]. Strain TR7 and SA9 produced extracellular BS and produced a strong BS capable of generating a stable emulsion form over several hours. As shown in Fig. 1, TR7 and SA9 reduced the surface tension of the culture medium from 55 to below 30 mN/m. This resulted in a surface tension reduction of about 40% and stabilized emulsion of xylene up to ≈65% of E_{24}. In addition, TR7 and SA9 also provided the largest clear zone diameter, indicating the high surface activities of the excreted BS.

The 16S rRNA sequences of selected strains were determined and deposited in the GeneBank database under accession numbers AB569640 and AB569641 for TR7 and SA9,

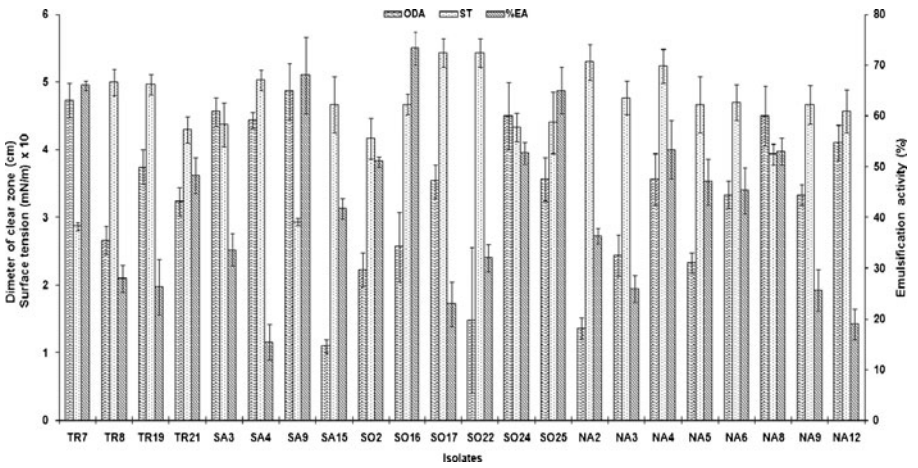

Fig. 1 Comparisons of surface activities of biosurfactant produced by the isolated strains. *Bars* represent the standard deviation from three determinations

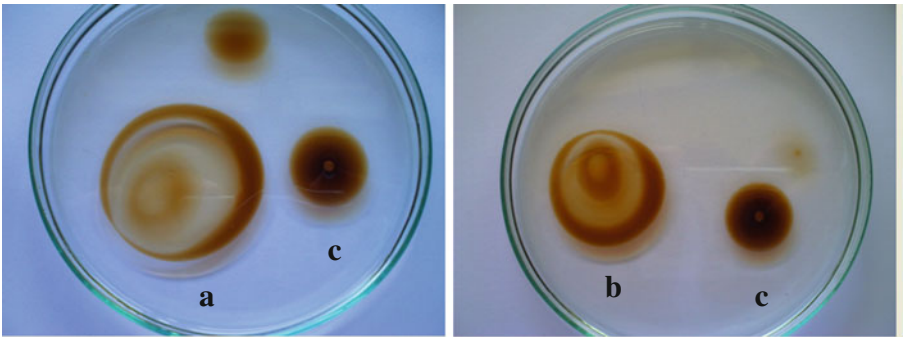

Fig. 2 Oil displacement test of the biosurfactant produced by *Bacillus licheniformis* TR7 (*a*), *Bacillus subtilis* SA9 (*b*), and mineral salts medium (*c*)

respectively. The phylogenetic analysis of strain TR7 and SA9 was undertaken using the 16S rRNA gene nucleotide sequences data. This showed that these strains had the highest homology (over 99.9%) with *Bacillus licheniformis* and *Bacillus subtilis*, respectively. The phylogenetic tree based on neighbor-joining analysis of the 16S rRNA gene nucleotide sequences is given in Fig. 3. Therefore, the isolates were named as *B. licheniformis* TR7 and *B. subtilis* SA9 by the phylogenetic analysis.

Mangroves are dominant inter-tidal wetlands along coastlines of tropical and subtropical regions. They have been considered as significant sinks for pollution from freshwater discharges as well as from contaminated tidal water [3]. Diverse groups of indigenous microorganisms capable of utilizing and degrading contaminants such as hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) might have been present in the contaminated sediment [16]. However, intrinsic biodegradation often takes a long time to complete because of the low water solubility of hydrocarbon [41]. The microbial cell itself is a BS and adheres to hydrocarbon [23]. Whereas there are many reports on BS in the last few decades [24, 38], reports on BS produced by mangrove sediment microorganisms have been limited so far. It is also noted that to our best knowledge, this work is one of the early attempts to isolate and characterize BS-producing bacteria from mangrove sediment.

Culture Media and Fermentation Conditions

Optimization of Molasses Concentration for BS Production

To find the optimum amount of molasses for the BS production, distillated water was used as diluents to reach the final concentration of molasses of 2% to 10% (*w*/*v*). Growth showed increase in dry cell weight, with the increased in concentration of molasses from 2% to 10%. The maximum productions of dry cell weight from both two strains were obtained when 10% (*w*/*v*) molasses was used (Table 1). The BS activity of both two strains increased with the increasing of molasses concentration, and maximum production occurred when 4% (*w*/*v*) of molasses was used. Further increase in the concentration of molasses did not affect BS production significantly ($p>0.05$). This result is in accordance with the findings of Joshi et al. [15] who reported that BS production increased with the increase in the concentration of molasses. The maximum production occurred when 5% (*v*/*v*) of molasses was used. Moreover, increases in molasses concentration of more than 7% (*v*/*v*) results in a decrease in rhamnolipid production by *Pseudomonas aeruginosa* [36].

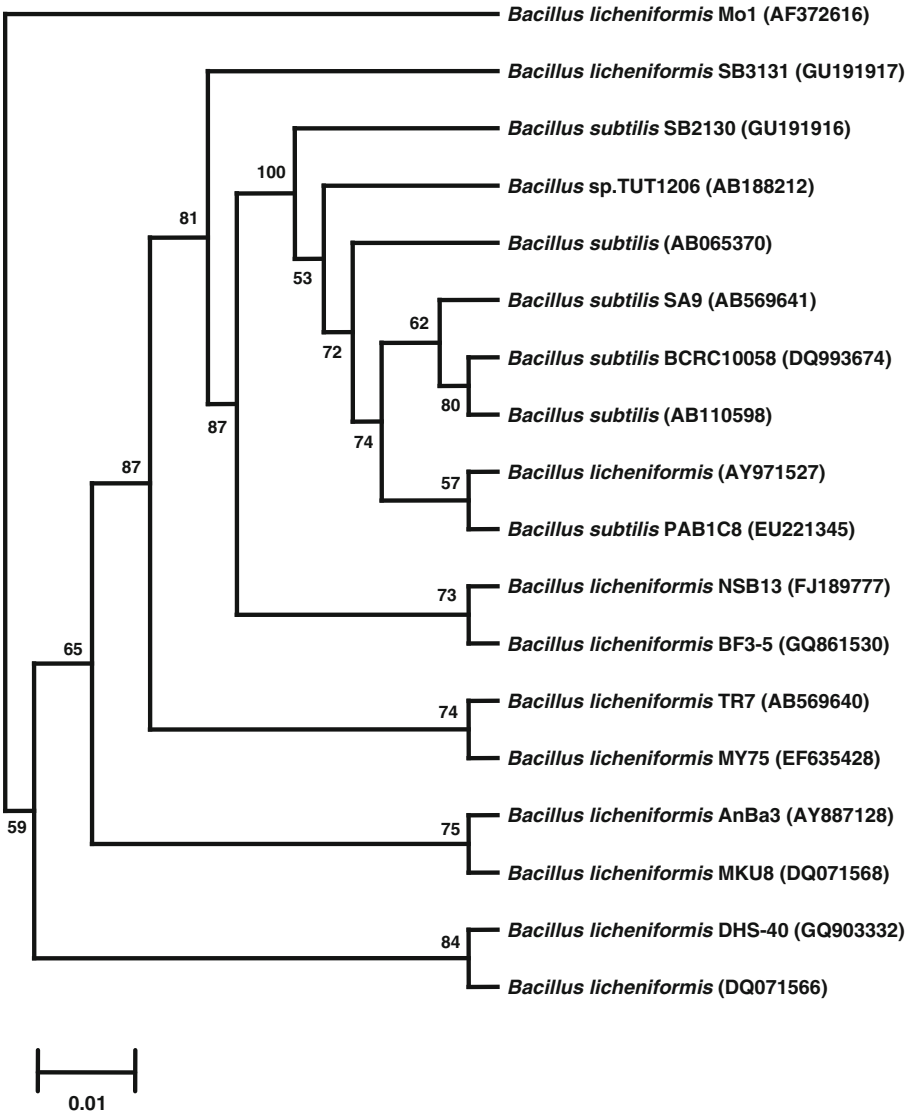

Fig. 3 Phylogenetic tree of the strain TR7 and SA9 and closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences (neighbor-joining tree method). The *scale bar* indicates 0.01 nucleotide substitutions per nucleotide position. The *numbers at the node* show the bootstrap values obtained with 1,000 re-sampling analyses. The GenBank accession numbers were reported in *parenthesis*

Isolates TR7 and SA9 had surface tension reduction in the range of 30 mN/m, and maximum yield was observed as 3.26 and 3.56 g/l, respectively. Therefore, molasses concentration 4% (*w*/*v*) was chosen as the appropriate culture medium for the next experiments of BS production by the two selected bacterial strains. Joshi et al. [15] used molasses and whey as the sole source of nutrition to produce BS from *Bacillus* spp. The BS production reached maximum when 7% (*w*/*v*) of molasses was used. Maximal surfactant production occurred after 72 h of incubation, when cells reached the stationary phase of

Table 1 Effect of the molasses concentration on growth and biosurfactant production by *Bacillus licheniformis* TR7 and *Bacillus subtilis* SA9, which were cultivated in 250-ml flask containing 50 ml molasses solution medium at 30 °C in a shaking incubator at 150 rpm for 48 h

Molasses (%, *w*/*v*)	Dry cell weight[a] (g/l)	Surface tension[a] (mN/m)	Biosurfactant[a] (g/l)	Emulsification activity[a] (%)
B. licheniformis TR7				
2	6.17±0.15	34.5±1.32a[b]	3.20±0.02b	65.37±3.24
4	6.37±0.27	30.4±0.85b	3.26±0.04a	69.13±2.43
6	6.54±0.32	30.0±0.53b	3.26±0.18a	70.12±4.18
8	6.94±0.18	29.6±1.52b	3.24±0.23a	69.41±2.15
10	7.10±0.21	29.5±1.65b	3.25±0.43a	71.91±3.26
B. subtilis SA9				
2	6.54±0.06	32.96±2.11a	3.09±0.02b	67.45±2.11
4	6.67±0.10	29.75±1.28b	3.56±0.13a	70.32±2.95
6	6.77±0.27	30.43±1.17b	3.53±0.51a	71.28±1.63
8	6.98±0.33	30.05±0.52b	3.55±0.29a	70.42±1.93
10	7.34±0.67	29.63±0.63b	3.55±0.20a	69.72±2.32

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

[b] Different letters in the same column within the same species indicate significant differences ($p<0.05$)

growth. Rashedi et al. [36] studied BS production using medium A, with varying concentrations of molasses being used as the sole source of carbon. The BS production increased with the increase in the concentration of molasses, and maximum production occurred when 7% (*v*/*v*) of molasses was used. Both two authors reported that increasing the concentration of molasses did not affect surfactant production significantly. Our studies showed results which are in agreement with both studies reported in the literature. BS production using molasses or whey as a carbon source has already been reported [37]. Constituents such as peptone, nitrogen source, and some trace elements were supplemented in the medium for the growth and BS production. On the other hand, in the present study, only the molasses was diluted with distillated water and used as whole medium. This gave BS production without addition of a nitrogen source or metal supplements, implying that the process could be economical when used on a large scale.

Optimization of Inoculums for BS Production

After the optimization step of the culture medium, the concentration of the inoculum was studied. To optimize the amount of inoculum for BS production, the concentration of the inoculum was varied from 2% to 10% (*v*/*v*). The effect of the amount of inoculum on the BS production by *B. licheniformis* TR7 and *B. subtilis* SA9 after 48 h of cultivation is summarized in Table 2. BS production by both strains increased with an increase in inoculum size from 2% to 4% (*v*/*v*). Increasing the inoculum size more than 4% (*v*/*v*) did not affect impact in enhancing the BS yield. Increase in inoculum size beyond 4% (*v*/*v*) resulted in a steady decline in BS production by *B. subtilis* SA9. Although dry cell weight from two strains seemed to increase with an increase in the amount of inoculum, the excreted BS did not show superior surface activities. This result is in agreement with Das and Mukherjee [6], who reported that increased inoculum size above 10% results in a decreased BS production by *B. subtilis* DM-03. An inoculum size that is too large would

dilute the rate-limiting substrate concentration in the medium, resulting in a lower specific growth rate. On the other hand, a lower inoculum size would result in a long lag phase. Use of the appropriate inoculum size is critical for biological compound produced by microorganisms [19]. Thus, 4% inoculum was the optimum amount for the BS production by both *B. licheniformis* TR7 and *B. subtilis* SA9.

Times Course of BS Production

To maximize the BS production, both strains TR7 and SA9 were cultivated in a medium containing 4% molasses for 72 h. Figure 4 illustrates the growth profiles of both bacterial strains. It was observed that the TR7 strain started to excrete the BS after the lag phase, which was the first 9 h of the cultivation, as indicated by a decrease in the surface tension of the culture media (Fig. 4a). Under the study conditions, the log phase occurred and ranged from 12 to 36 h. In this period, the surface tension of the culture medium was markedly reduced and reached a minimum (30.0 mN/m). However, the highest of BS yield (3.30 g/l) was obtained as the cultivation time approached 48 h, which corresponded to the stationary phase of the microbial growth. From the obtained results, it can be seen that a cultivation time of 48 h gave the highest BS activity. Thus, it was selected as the optimum time for BS production by the strain TR7.

B. subtilis SA9 is also a significantly good BS producer since it possesses a short lag phase of 15 h and also provides a large reduction of surface tension, as shown in Fig. 4b. The log phase was subsequently observed and ranged from 15 to 51 h before the microbial system entered into the stationary phase. The surface tension of the culture media rapidly decreased in the early log phase and reached a minimum when the system approached the stationary phase. A minimum surface tension value (29.0 mN/m) and the highest of BS yield (3.78 g/l) were observed at a cultivation time of 51 h. Therefore, a cultivation time of

Table 2 Effect of the inoculums size on growth and biosurfactant production by *Bacillus licheniformis* TR7 and *Bacillus subtilis* SA9, which were cultivated in 250-ml flask containing 50 ml molasses solution medium at 30 °C in a shaking incubator at 150 rpm for 48 h

Inoculum (%, *v*/*v*)	Dry cell weight[a] (g/l)	Surface tension[a] (mN/m)	Biosurfactant[a] (g/l)	Emulsification activity[a] (%)
B. licheniformis TR7				
2	6.37±0.35	30.5±3.12a[b]	3.26±0.17c	69.13±4.25b
4	6.58±0.32	29.0±0.25b	3.30±1.21a	72.53±1.73a
6	6.92±0.61	29.0±2.31b	3.30±0.48a	72.56±3.17a
8	7.39±0.24	29.6±2.50b	3.29±1.31a	70.21±5.00ab
10	7.50±0.83	29.9±2.05b	3.28±1.16b	67.98±6.31c
B. subtilis SA9				
2	6.67±0.15	29.61±2.41a	3.56±0.12b	70.25±3.54b
4	6.89±0.52	28.75±2.10b	3.78±1.04a	72.82±4.85a
6	6.96±0.81	29.53±2.05a	3.78±0.21a	72.58±2.52a
8	7.05±0.27	30.04±1.53a	3.06±0.71c	64.32±2.67c
10	7.41±0.13	29.80±1.62a	2.99±0.81d	60.31±6.02d

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

[b] Different letters in the same column within the same species indicate significant differences ($p<0.05$)

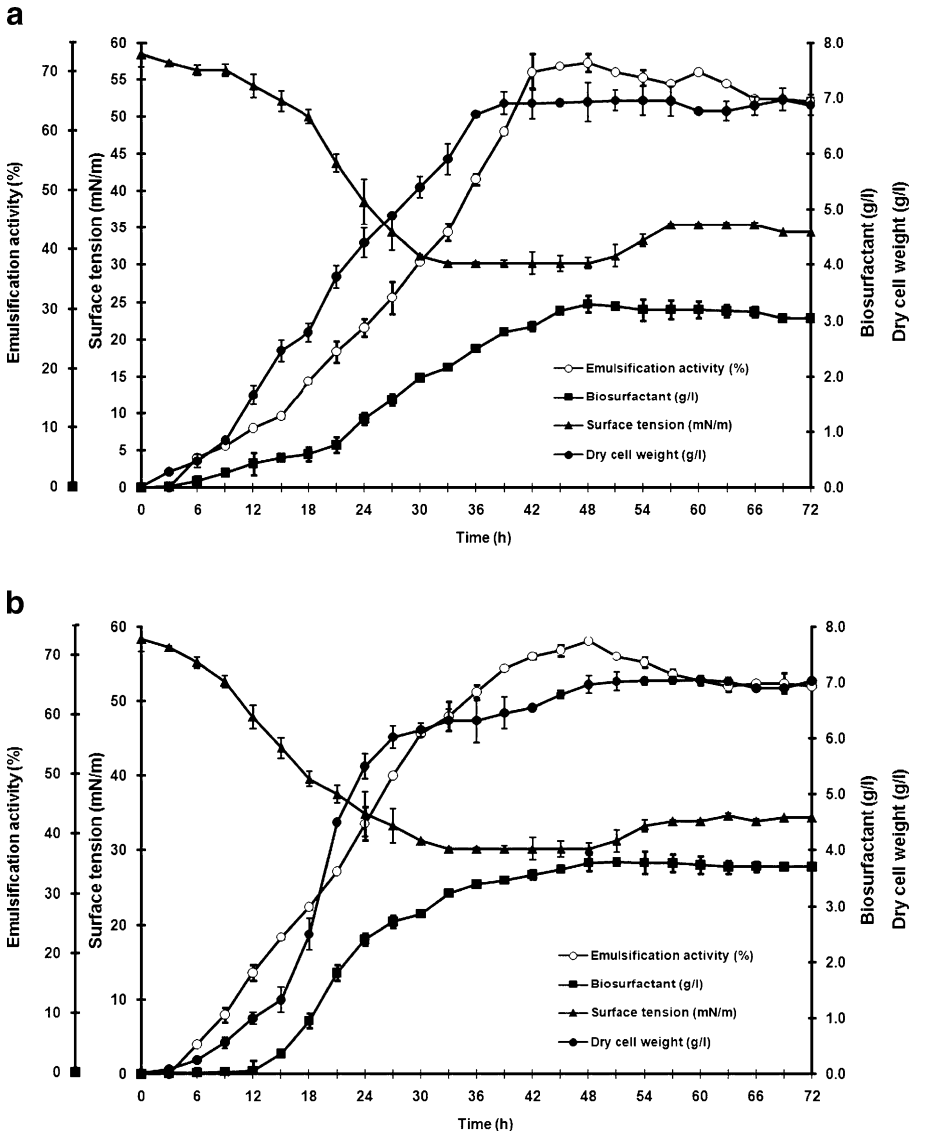

Fig. 4 Time course of biosurfactant production in molasses medium with 4% molasses by **a** *Bacillus licheniformis* TR7 and **b** *Bacillus subtilis* SA9. *Bars* represent the standard deviation from three determinations

51 h was selected for BS production by the strain SA9. In addition, the results showed that the BSs production by both two strains were growth associated. Most extracellularly secreted BSs are growth associated [12]. There was an almost parallel relationship between BS production, cell growth, and substrate utilization. The growth-associated production of emulsifiers has been reported from several other microorganisms [8, 11, 35]. The growth-associated production of BS has also been reported for *B. subtilis* [30], *P. aeruginosa* EMS1 [4], and *Rhodococcus* spp. [39].

Recovery of BS

Crude extract of the BS was recovered from the culture supernatant of *B. licheniformis* TR7 and *B. subtilis* SA9 by extraction with several organic solvents. Among four solvent systems, chloroform:methanol (2:1) was the most efficient in BS recovery from culture supernatant of two strains (data not shown). Recovery yields of 3.26 and 3.56 g/l were obtained from *B. licheniformis* TR7 and *B. subtilis* SA9, respectively. In addition, CMCs were 10 and 30 g/l for *B. licheniformis* TR7 and *B. subtilis* SA9, respectively. Mixtures of solvents were commonly used to facilitate adjustment of the polarity between the solvent as the extraction agent and the BS to be extracted [18]. The structure of BS which was composed of hydrophilic and hydrophobic moieties could easily be 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 the single solvent (ethyl acetate, Log P=0.73) used [40]. Extraction with chloroform:methanol (2:1) was chosen as the solvent system for BS recovery from both strains in this study.

Physicochemical Properties

The surface tension of the culture supernatants of both *B. licheniformis* TR7 and *B. subtilis* SA9 was measured as a function of concentration in order to compare the surface activities of the two excreted BS. Surface tensions versus BS concentrations were plotted as shown in Fig. 5. The surface tension of pure water rapidly decreased as the concentration of the TR7 BS increased, and a minimum surface tension of 28.5 mN/m was observed. From the break point of the plot of surface tension versus BS concentration, the CMC was about 10 mg/l (Fig. 5a). In the case of *B. subtilis* SA9, the excreted BS in the culture supernatant could decrease the surface tension of pure water from 72.0 to 29.5 mN/m, and the CMC was estimated to be 30 mg/l (Fig. 5b). The BS showed better properties in terms of higher surface tension reduction and a lower CMC. The obtained values of the minimum surface tension and the CMC of the TR7 and SA9 BS are consistent with the previously reported values.

In general, BS produced by *Bacillus* strain was found to reduce the surface tension of pure water from 72.0 to 27.0–37.0 mN/m with CMCs in the range of 0.02–20 mg/l, depending on their components [9, 26, 30]. BS isolated from *B. subtilis* 20B [14] showed similar reduction of the surface tension of water to 29.5 mN/m. The various isoforms of the longest known BS lichenysin and surfactin produced by different strains of *Bacillus* strain have been reported to lower the surface tension to 25.0–31.0 mN/m with CMC 10–160 mg/l [10, 27].

Stability of BS

Environmental factors such as pH, temperature, and salinity affected to TR7 and SA9 BS activity and stability were performed at several concentrations of each parameter (data not shown). The results obtained from thermal stability analysis of the TR7 and SA9 BS over a wide range of temperature (4–121 °C) revealed that the BSs from both strains are thermostable. Heating of the crude BS from two strains up to 100 °C (or its autoclaving at 121 °C) caused no effect on the surface tension reduction and its emulsion capacity. A 5% reduction in emulsification activity was observed on storage of crude BS at 4 °C. The surface tension reduction and E_{24} of crude BS from two strains were relatively stable at the temperatures used (surface tension≈31 mN/m and E_{24}≈68%).

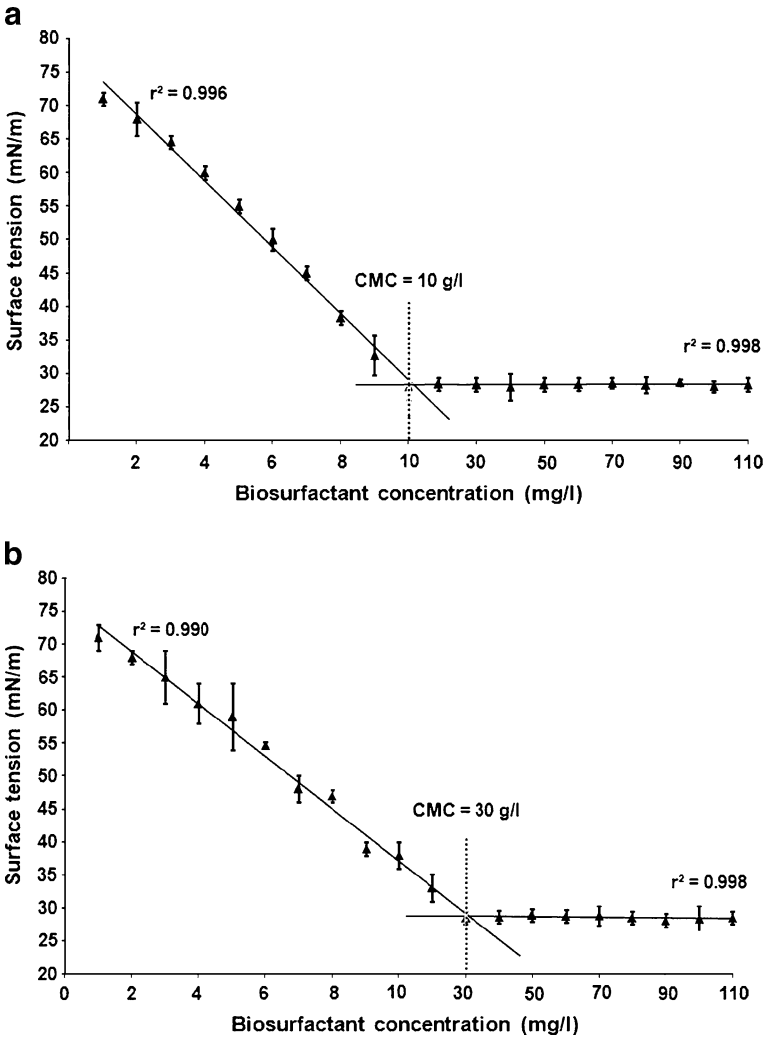

Fig. 5 Surface tension as a function of biosurfactant concentration produced by *Bacillus licheniformis* TR7 (**a**) and *Bacillus subtilis* SA9 (**b**). *Bars* represent the standard deviation from three determinations

The activity of TR7 BS decreased obviously with decreasing pH below 4.0. The surface tension of BS from two strains remained stable over a pH range from 6.0 to 12.0, although a marginal decrease at pH 12.0 was detected with SA9 BS. The surface tension was the lowest at pH 6.0, reaching values of 28.7 and 29.5 mN/m for TR7 and SA9 BS, respectively. On the other side, the E_{24} was in accordance with changes of surface tension. The E_{24} of both two strains decreased when pH varied from 6.0 to 12.0 and reached the highest at pH 6.0. However, when pH reached 4.0, the E_{24} from two strains declined and came to its lower point due to precipitation of BS [30]. Similar result had been reported for BS produced by *B. subtilis*, which remained stable in the range of pH 4.0 to 12.0 [22]. The crude BS produced by *B. subtilis* PTCC 1696 was also stable in a wide range of pH (4.0–11.0) [10].

The effect of addition of NaCl on the surface tension and E_{24} of TR7 and SA9 BS was tested. The results showed negligible changes occurred in the BS activity with an increase in the NaCl concentration up to 7.0%. Likewise, an increase in NaCl concentration up to 9.0% did not have effects on E_{24}. However, at the highest level of NaCl (11.0%), E_{24} was severely dropped to 20.0%, and surface activity was increased as well (around 40 mN/m). NaCl activated BS activity of many strains isolated from seawater or petroleum reservoirs [24]. Nevertheless, BS extracted from *Pseudomonas* sp. LP1 exhibited zero saline tolerance [31], whereas the BS from *Penicillium* sp. tolerated 10% NaCl and lost 5% of its activity in 20% NaCl [21]. Recently, BS produced by *B. subtilis* PTCC 1696 has shown good stability of emulsion and surface tension in the presence of salt [10].

Emulsification Properties of BS

BS isolated from *B. licheniformis* TR7 and *B. subtilis* SA9 showed a good E_{24} against several hydrophobic substrates (Fig. 6). The E_{24} of BS from two *Bacillus* strains were higher than that of the chemical surfactants since it more effectively emulsified aromatic and aliphatic hydrocarbons and several plant oils. TR7 BS emulsified more than 70% of all the hydrocarbons tested such as cyclohexane, hexane, xylene, benzene, *n*-hexadecane, kerosene, and toluene. It ranged from 60% to 80% for palm oil, soybean oil, and olive oil. In addition, SA9 BS was found to be able to form emulsion of more than 65% toward all hydrocarbons tested and ranging from 60% to 75% for vegetable oils. The ability of the BS produced by strain TR7 and SA9 to form stable emulsions with hydrocarbon and vegetable oils indicated that they have a good potential for applications in microbial-enhanced oil recovery. They can also be used as emulsifying agents in the food industry.

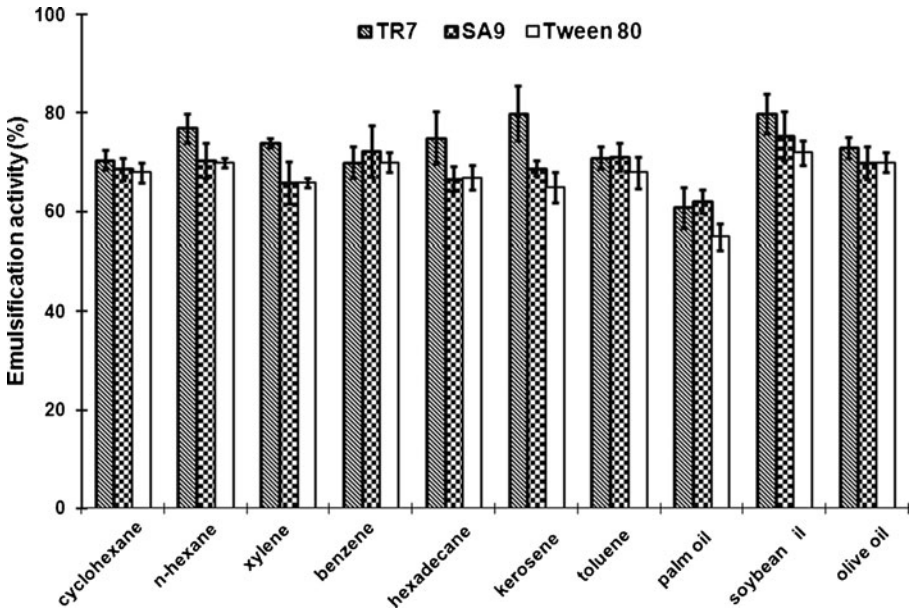

Fig. 6 Emulsification index (E_{24}) of the biosurfactant against different hydrocarbons and vegetable oils. *Bars* represent the standard deviation from three determinations

BS Characteristics

The chemical nature of the TR7 and SA9 BS was seen as a single spot on TLC. This fraction showed a positive reaction with ninhydrin reagent and iodine vapor, indicating the presence of peptide and lipid moieties in the molecule (data not shown). The lipopeptide nature of the BS was further confirmed by the IR spectra of the compound (Fig. 7a, b). The

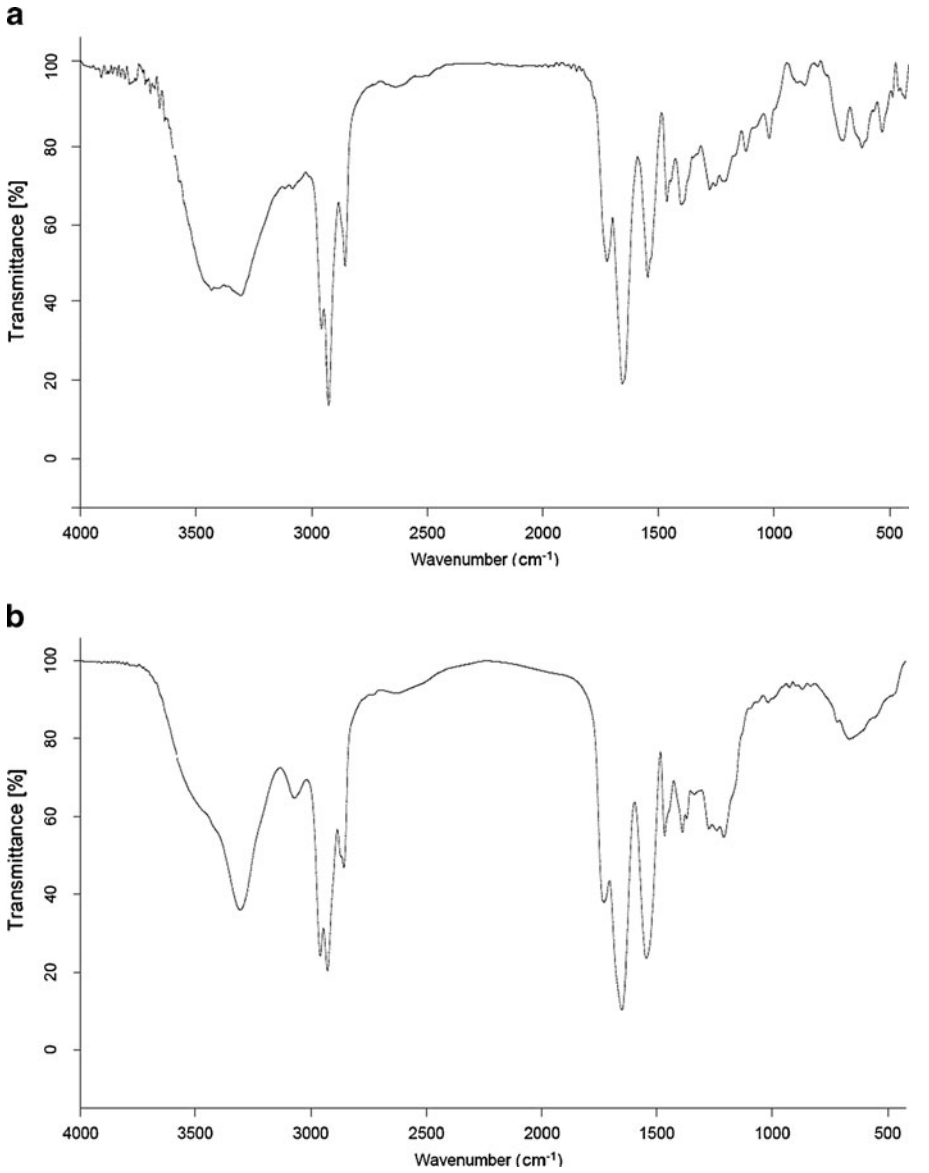

Fig. 7 Fourier transform infrared spectrum of the biosurfactant produced by *Bacillus licheniformis* TR7 (**a**) and *Bacillus subtilis* SA9 (**b**)

IR spectrum in KBr showed bands characteristic of peptides at 3,305 cm^{-1} (NH stretching mode) and at 1,650 cm^{-1} (stretching mode of the CO–N bond). The bands at 3,000–2,800, 2855, and at 1,464 cm^{-1} reflect aliphatic chains (–CH3, –CH2) of the fraction, while a band observed at 1,730 cm^{-1} is due to a carbonyl group. These patterns were identical to those of surfactin. ^{1}H NMR was performed for further confirmation of the results of this study. The results are shown in Fig. 8a, b. Results obtained with ^{1}H NMR indicated that those two molecules are lipopeptides. Almost all of the back bone–amide–NH groups are in the region

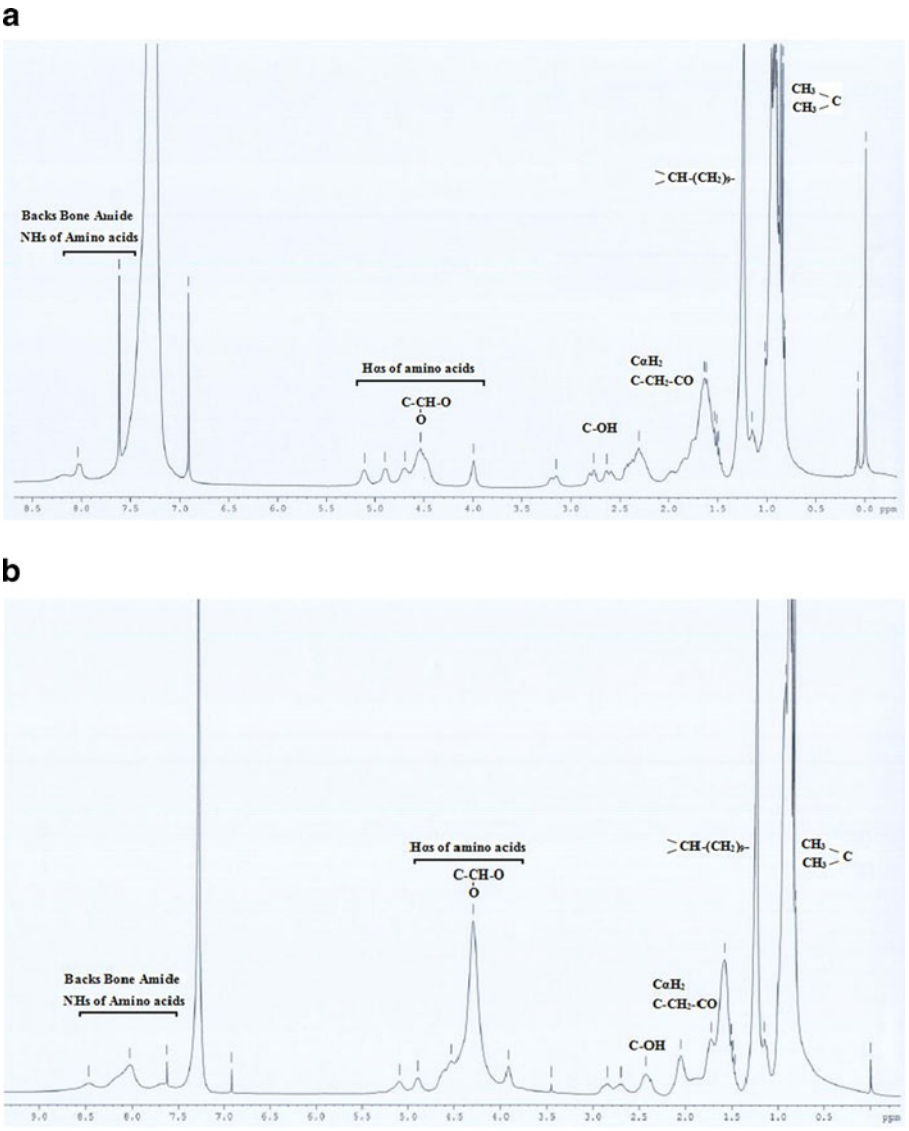

Fig. 8 ^{1}H nuclear magnetic resonance spectrum of the biosurfactant produced by *Bacillus licheniformis* TR7 (**a**) and *Bacillus subtilis* SA9 (**b**)

from 7.2 to 8 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. Remaining spectra clearly confirmed the presence of β-hydroxy fatty acid.

The structure of BS from boat strains was fully supported by its mass spectrometric analysis. Analysis of the intact molecules with LCQ-MS revealed molecular ion peaks of *m*/*z* 1,332.6 and 1,004.0, 1,017.9, and 1,031.9 for TR7 and SA9 BS, respectively (Fig. 9a, b). 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 a previous study that showed BSs produced by *Bacillus* spp. had molecular mass ranging in *m*/*z* 992–1,088 [20]. The chemical nature of the BS thus varies with both species and strains within the genus *Bacillus* [10, 44]. According to these results, the obtained TR7 and SA9 BS were the lipopeptide type.

Application of the BS in Motor Oil Removal from Contaminated Sand

Petroleum hydrocarbon compounds bind to soil components and are difficult to remove and degrade [42]. BS can emulsify hydrocarbons by enhancing their water solubility, decreasing surface tension, and increasing the displacement of oil substances from soil particles [1]. The ability of TR7 and SA9 BS to enhance motor oil removal from contaminated sand was examined in comparison with those of synthetic surfactants such as a nonionic surfactant Tween 80 and anionic surfactants SDS. The efficiency of oil removal from contaminated sand was shown to be dependent on the surfactant dosage (Table 3). The results obtained demonstrated that the BS from both strain at under the CMC removed the spiked oil around 45–49%. The distilled water (control) removed only 35% of the contaminated oil. At CMC level, the removal efficiency of the oil adsorbed in the sand by BS and synthetic surfactants was not significantly different ($p>0.05$).

An increase in the BS concentration above CMC (two times) resulted in an increase of oil removal more than 78% and 73% for TR7 and SA9 BS, respectively. At these concentrations, the removal efficiency of oil adsorbed in sand by BS was higher than those of synthetic surfactants ($p<0.05$). This characteristic was actually not predicted by the conventional theory because the surfactant at the concentration below its CMC generally showed no enhancement on sorbed hydrocarbon [18]. These results have implications on the potential use of a TR7 or SA9 BS to enhance sorbed motor oil from the environment.

PAH Solubilization Effect of BS

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 [50]. The effect of BS on the apparent aqueous solubility of PAHs was determined by test tube solubilization assays in the presence of increasing concentrations of BS (10 to 50 mg/ml). This is depicted in Table 4. In general, the TR7 and SA9 BS enhanced the apparent solubility of PAHs in a dose-dependent manner. However, solubilization of phenanthrene by BS from two bacterial strains (about 1.7–1.9 times higher apparent solubility compared to control) was significantly lower ($p<0.05$) when compared with anthracene or pyrene solubilization affected by BS (five to six times higher compared to control). In addition, the TR7 BS displayed higher pyrene solubilization effect compared to SA9 BS produced. The differences in PAH solubilization effects of BS from different

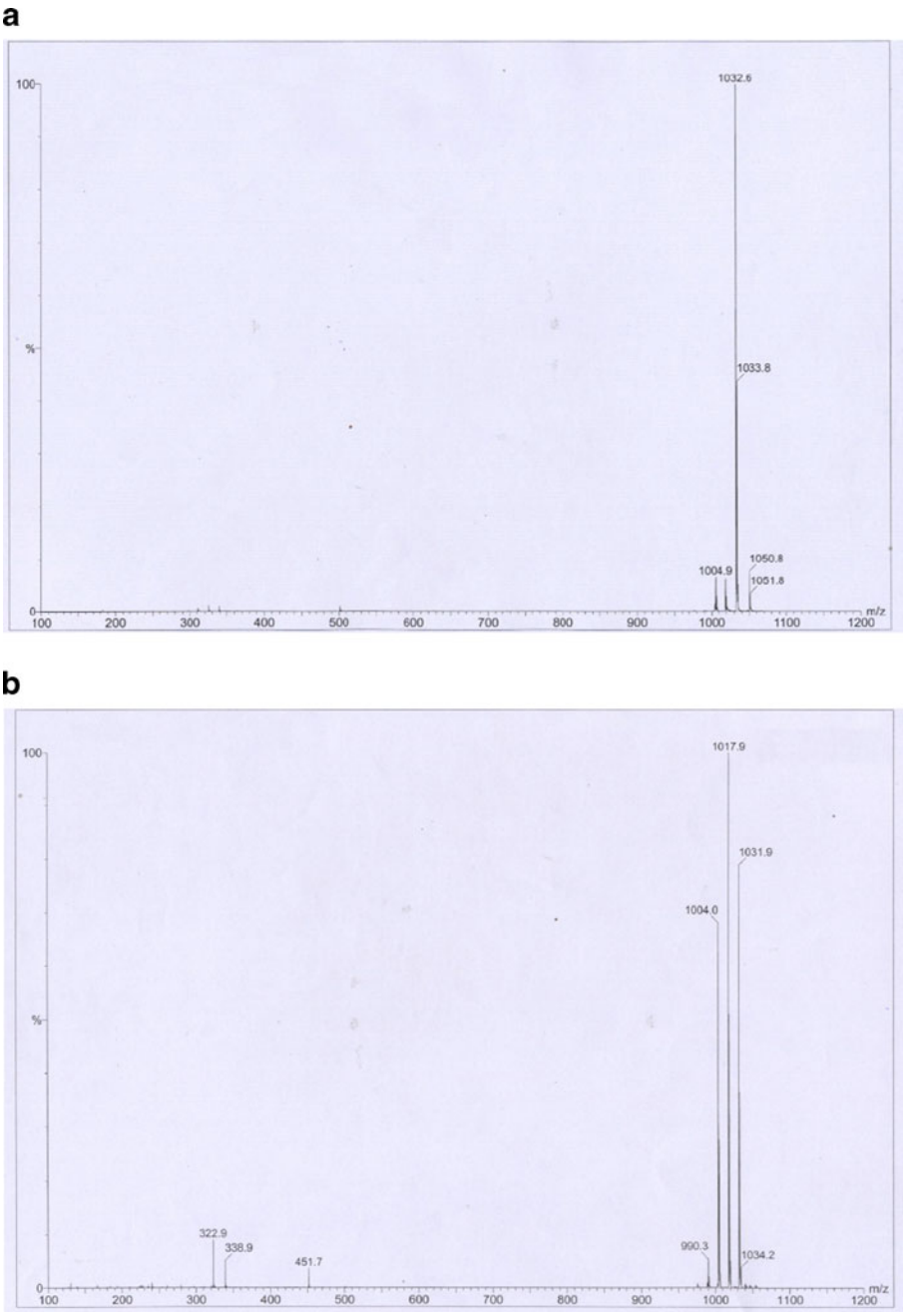

Fig. 9 Mass spectrum of the biosurfactant produced by *Bacillus licheniformis* TR7 (**a**) and *Bacillus subtilis* SA9 (**b**)

bacterial strains in this study could be related to the chemical nature as well as a minor variation in BS isoforms between these two strains [10, 20]. These might result in a large variation in the emulsification property and the PAH solubilization effect of BS [26]. In the

Table 3 Dose-dependence of motor oil removal from contaminated sand by crude biosurfactants isolated from *Bacillus licheniformis* TR7, *Bacillus subtilis* SA9, and synthetics surfactant Tween 80 and SDS

Fold CMCs of surfactant[b]	Oil removal[a] (%)			
	TR7	SA9	Tween 80	SDS
0	35.0±1.3A[c]f[d]	33.5±3.7Ad	34.2±2.1Ac	35.7±2.8Ac
0.5	49.3±4.1Ae	45.4±5.1ABc	40.3±6.0Bb	39.5±6.8Bc
1.0	71.5±6.7Ad	70.4±3.7Ab	68.7±4.8Aa	68.3±4.5Ab
2.0	78.8±1.9Abc	73.3±2.2Bb	69.8±1.3Ba	70.8±2.7Bb
4.0	85.3±9.4Aab	82.3±6.3Aa	72.6±7.5Ba	76.6±4.5ABa
8.0	87.1±11.7Aa	83.1±12.4Aa	73.1±5.9Ca	78.1±2.9Ba

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

[b] Fold CMCs of each chemical (10, 30, 2, and 18 mg/ml for TR7, SA9, Tween 80, and SDS, respectively)

[c] Different capital letters in the same row indicate significant differences ($p<0.05$)

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

present study, the crude BS also showed the ability to solubilize PAHs in the aqueous phase, indicating its possible role in increasing the bioavailability of non-soluble organic compounds for bacterial metabolism.

The application of surfactants to soil environments contaminated with PAHs has become a possible means to increase the bioavailability of these hydrophobic compounds and to facilitate their biodegradation. BS-enhanced biodegradation was observed with anthracene [13], phenanthrene [49], and pyrene [17]. BSs enhance overall biodegradation by facilitating interphase mass transfer of PAHs at the soil solution interface. Solubilization and lowering of the surface and interfacial tension are thought to be main reasons for

Table 4 Dose-dependent solubilization of PAHs by crude biosurfactants isolated from *Bacillus licheniformis* TR7 and *Bacillus subtilis* SA9

Source and concentration of crude biosurfactant (mg/ml)	Solubility of PAHs[a] (ng/ml)		
	Anthracene	Phenanthrene	Pyrene
B. licheniformis TR7			
0	153.5±12.5f[b]	480.0±21.4f	30.0±5.7f
10	220.4±21.7e	529.3±14.2e	59.3±8.1e
20	389.3±13.2d	731.5±36.7d	133.7±5.8d
30	542.6±12.6c	842.8±21.5c	153.8±4.2c
40	942.3±36.3b	893.3±39.4b	162.6±4.5b
50	1053.1±12.7a	933.1±13.2a	184.1±5.7a
B. subtilis SA9			
0	153.0±15.3f	481.0±25.5f	30.9±2.5f
10	196.3±10.2e	502.5±24.5e	54.1±4.2e
20	331.5±23.5d	643.2±16.8d	117.7±12.0d
30	493.8±20.5c	674.3±11.3c	129.5±5.5c
40	832.3±18.0b	742.4±29.6b	149.4±3.1b
50	1013.1±12.4a	812.1±12.7a	163.1±7.8a

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

[b] Different letters in the same column within the same species indicate significant differences ($p<0.05$)

facilitating the transport of pollutants adsorbed on the solid phase to the surfactant containing aqueous phases [49]. Data presented here suggest that interactions with hydrophobic regions in BS are the most plausible explanation for the mechanism by which BS solubilizes compounds with limited aqueous solubility.

Conclusions

B. licheniformis TR7 and *B. subtilis* SA9 isolated from mangrove sediment produce release extracellular BS into the culture medium which will simplify the recovery procedures. BS production is supported by low-cost renewable substrates that will contribute to the reduction of process costs. Overall results indicated that the two BSs produced by *B. licheniformis* TR7 and *B. subtilis* SA9 have good potential for use in the petroleum industry and in environmental applications. These included enhanced oil recovery, cleaning of oil reservoirs, reducing oil viscosity for crude oil transportation, and decomposition of spilled oils in soil or marine environments.

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