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ORIGINAL PAPER

Isolation and characterization of a biosurfactant producing strain, *Brevibacilis brevis* HOB1

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Abstract Biosurfactant-producing bacteria were isolated from the production water of an oil field. Isolates were screened for biosurfactant production using surface tension test. The highest reduction of surface tension was achieved with a bacterial strain which was identified by 16S rRNA gene sequencing as *Brevibacilis brevis* HOB1. It has been investigated using different carbon and nitrogen sources. It showed that the strain was able to grow and reduce the surface tension of the broth to 29 mN/m on commercial sugar and maltose, and to 32 mN/m on glucose after 72 h of growth. The maximum amount of biosurfactant was obtained when nitrate ions were supplied as nitrogen source. Biosurfactant produced by *Brevibacilis brevis* HOB1 was confirmed as a lipopeptide class of biosurfactant using TLC test and mass spectra. Lipopeptide isoforms were isolated from cell-free supernatants by acid-precipitation followed by one step of chromatographic separation on solid-phase ODS C18 column. The separation was confirmed by HPLC and ESI Q-TOF MS spectroscopy. Comparing the mass data obtained and the mass numbers reported for the lipopeptide complexes from other strains, it can be concluded that the major lipopeptide product of *Brevibacilis brevis* HOB1 is the surfactin isoform. This lipopeptide showed strong antibacterial and antifungal activity. It is a candidate for the biocontrol of pathogens in agriculture and other industries.

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Introduction

Biosurfactants are biologically surface-active compounds produced mainly by microorganisms. A microbial surfactant generally consists of a hydrophilic moiety composed of amino acids or peptides, and a hydrophobic portion, which is often made up of hydroxylated fatty acid [1, 2]. Interest in biosurfactants has increased considerably in recent years since they are potential candidates for many applications in the petroleum, pharmaceutical, cosmetic, and food processing industries [3–6]. Several types of biosurfactant have been isolated, including lipopeptides, glycolipids, phospholipids, neutral lipids, fatty acids, peptidolipids, and lipopolysaccharides [7–9]. They are produced by bacteria, yeasts and fungi, and particularly in bacteria which are in a state of growth on a water-immiscible substrate, which is a source of food, for example crude oil spillage treated with selected microorganisms [10, 11]. By evolution, bacteria have adapted themselves to feeding on water-immiscible materials by manufacturing and using a surface active product that helps the bacteria, which are in the aqueous phase to adsorb, emulsify, wet, and disperse or solubilize the water-immiscible material.

Among many classes of biosurfactants, lipopeptides, a class including iturins, surfactins, and lichenysins, are particularly interesting because of their high surface activities and therapeutic potential [12, 13]. While surfactins and lichenysins are now reputed as the most powerful bacterial biosurfactants so far known [14], iturins are interesting in their remarkable efficacy against a broad variety of clinically important pathogenic yeasts and fungi [15]. However

surfactins are very efficient anti-*Mycoplasma,* antiviral, antitumoral agents as well as inhibitors of enzymes [16, 17]. Surfactin lipopeptides have been isolated from several strains of *Bacillus subtilis* and *Bacillus pumilis* [14, 18, 19]. Lichenysin B, lichenysin C, and biosurfactant BL86, have been isolated by different strains of *Bacillus licheniformis* [20, 21], while arthrofactin was produced by *Arthrobacter* sp. strain MIS38 [22], and viscosin was produced by *Pseudomonas fluorescens* [23]. Identification of newly-found biological entities, such as bacterium strains, subcellular localization of proteins [24] and their various biological attributes and structural features may provide very useful information for both basic research and drug discovery [25, 26]. As far as we know, production of lipopeptide biosurfactant as well as any surface active compound has never been reported for *Brevibacilis brevis* strains. We show here that a novel *Brevibacilis brevis* HOB1, screened from a deep oil field, can grow and produce a surfactant which substantially changes the surface tension of the culture medium. We reported the isolation, identification, and optimization of media and growth conditions. A tentative structure analysis of this surface-active compound has also been presented.

Materials and methods

Screening of microorganisms and surface activity measurement

Oil field production-water samples (5 ml) were diluted two times by phosphate-buffered saline (PBS, pH 7.5) and then incubated on LB agar at 30 °C for 2 days. Bacterial isolates were obtained, and then each isolate was incubated into a 500-ml Erlenmeyer flask containing 150 ml Luria broth. The flask was incubated on a rotary shaker at 120 *rev*/min for 48 h at 30 °C. Culture samples were centrifuged at 7500×*g* for 30 min for cell removal and the supernatant was submitted to surface activity measurements. Surface tension was determined with a DCA 315 series system (Thermo-Cahn DCA315 Instruments, Inc. USA) using the plate method.

Identification of isolates

Bacterial isolates that displayed high biosurfactant production were selected and identified by 16S rRNA sequencing. Bacterial small-subunit rRNA genes were amplified by PCR using primers corresponding to *Escherichia coli* positions 27F and 1492R (8f, 5′-AGA GTT TGA TYM TGG CTC AG-3′; 1492r, 5′-CGG TTA CCT TGT TAC GAC TT-3′) [27, 28]. PCR amplification was performed in a total volume of 100 µl. Each PCR mixture contained 1 µl template DNA, 10 µEx Taq reaction buffer, 100 µM of each dNTP, 2.5 U of Ex Taq DNA polymerase and 1 $\mu$of each primer.

The plasmid DNA was isolated from positive clones with an AxyPrep-96 Plasmid Kit (Axygen, USA). The rRNA gene inserts were sequenced on an automated ABI 377 sequencer (Dye-Terminator Cycle Sequencing Ready Reaction FS Kit; PE Applied Biosystems) using M13 universal sequencing primers. The resulting sequences (approximately 1,500 bp) were compared with sequences in the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST network service [29], to determine their approximate phylogenetic affiliations.

Cultivation conditions

Brevibacilis brevis HOB1 strain was streaked in a nutrient agar slant and incubated for 24 h at 30 °C. Two loops of culture were inoculated in 40 ml of nutrient broth (Difco) in a 100-ml Erlenmeyer flask and incubated in a rotary shaker, 150 *rev*/min at 30 °C for 8–12 h until cell numbers reached 10^8 cfu/mL. This was used as inoculum at the 5% (w/v) level. For biosurfactant synthesis, a mineral salt medium with the following composition was utilized: 2.5 g/l of $NaNO_3$, 0.1 g/l of KCl, 3.0 g/l of KH_2PO_4, 7.0 g/l of K_2HPO_4, 0.01 g/l of $CaCl_2$, 0.5 g/l of $MgSO_4{\cdot}7H_2O$, and 5 ml of a trace element solution. Trace element solution contained 0.116 g/l of $FeSO_4{\cdot}7H_2O$, 0.232 g/l of H_3BO_3, 0.41 g/l of $CoCl_2{\cdot}6H_2O$, 0.008 g/l of $CuSO_4{\cdot}5H_2O$, 0.008 g/l of $MnSO_4{\cdot}H_2O$, 0.022 g/l of $[NH_4]_6Mo_7O_{24}$ and 0.174 g/l of $ZnSO_4$. The respective carbohydrate (glucose, manitol, maltose, sucrose, and starch) was added to make a final concentration 2%. The concentration of ammonium sufate, soybean flour, ammonium nitrate and sodium nitrate was 0.2%. Cultivation studies have been done in 500-ml flasks containing 150 ml medium at 30 °C for 48 h. Experiments were conducted in three independent triplicates.

Purification of lipopeptide

The fermentation cultures were collected and the pH was adjusted to 8.0 by 20% NaOH. The bacterial cells were removed by centrifugation at 3500×*g* for 20 min. The cell-free biosurfactant was precipitated by adding 3 N HCl to a final pH of 2.0 and stored for 6 h at 4 °C. The pellets were obtained by centrifugation at 10,000×*g* for 20 min and washed by deionized water two times to remove the remaining HCl.

Crude lipopeptide was extracted three times with chloroform/methanol (2:1, v/v) solvent system. The solution was dried with a rotary vacuum evaporator R-124 (BÛCHI, Bern, Switzerland) and the resulting pellets was dissolved in methanol and filtered through a 0.2-µm nanopyrogenic

hydrophilic membrane. The crude sample was collected for further purification by the chromatographic procedure. About 3 ml of crude sample (0.1 mg crude lipopeptide/ml of methanol) was injected into an ODS C18 column with a size of (10 × 3.0 cm) at a flow rate of 3.5 ml/min at room temperature. The column was first washed with 100 ml of 70% methanol in water. Then lipopeptides were eluted with 80 ml of 100% methanol. Fifty tubes (25 ml/tube) were collected. Collection has been monitored using a spectrophotometer at a wavelength of 210 nm. The biosurfactants were detected and quantified by reversed-phase HPLC as follows.

The eluted samples were characterized on a JASCO LC2000 HPLC system (Hypersil ODS C18, particle size 5 µm, Φ 4.6 mm × 250 mm) at a flow rate of 1 ml/min. Lipopeptides were detected at 214 nm with an UV 2075 detector. The mobile phase was (A) 0.05% trifluoroacetic acid (TFA) in water and (B) acetonitrile (ACN). The gradient (A/B) was maintained at 50% in the first 10 min, at 50–75% in the next 15 min, at 75–100% in the subsequent 10 min, and then finally at 100% for the following 10 min (from the 35th to 45th minute).

Biomass determination

At different times of fermentation, 50 mm samples were withdrawn and centrifuged at 13,000×*g* for 30 min. Biomass obtained was dried overnight at 105 °C and weighted.

Thin-layer chromatography

Each fraction obtained was concentrated and spotted on silica gel plates, which were developed with chloroform/methanol/double distilled water (65:25:4, by vol). Before hydrolization (110 °C, 1.5 h), the plates were immerged into the 0.1% (v/v) ninhydrin in ethyl acetate [30]. The lipopeptides were determined by comparing the two results between hydrolization and nonhydrolization. The red spot which appeared only on the plate after the acid hydrolization step was recognized to contain amino acid from hydrolysis of lipopeptide.

Antimicrobial activity

Escherichia coli, Bacillus subtilis and *Staphylococcus aureus* bacterial strains were grown in beef extract and peptone medium and yeast strain *Pichia pastoris* was grown in yeast extract peptone dextrose (YEPD) broth medium till stationary phase at 37 °C. Serial dilutions of the microbial cultures were made in sterile phosphate buffered saline, pH 7.0 and plated in nutrient agar and YEPD agar plates for bacterial and yeast strains, respectively. Various concentrations of purified lipopeptides produced by *Brevibacilis brevis* HOB1, dissolved in sterile 0.025 M PBS, pH 8.6 were applied on the center of the plates and incubated for 24 h at 37 °C along with control as sterile 0.025 M PBS, pH 8.6. The diameter of the clear zone (if any) around the point of application of purified lipopeptide was measured.

Results and discussion

Screening and identification of strain

Three biosurfactant-producing microorganisms were isolated from the oil field production water based on surface tension test. The highest reduction of surface tension was achieved with *Brevibacilis brevis* HOB1 (data not shown), and was selected for further studies. The 16S rRNA sequences of strain *Brevibacilis brevis* HOB1 were determined and deposited in the GeneBank database under accession number EU327889. The 16S rRNA sequence analysis revealed that *Brevibacilis brevis* HOB1 had 99.2% homology with *Brevibacilis brevis*AY887081 (Fig. 1).

Effect of carbon source and nitrogen source on biosurfactant production

Brevibacilis brevis HOB1 was grown on different carbon sources. Table 1 presents the average values of cell growth and reduction in surface tension produced by *Brevibacilis brevis* HOB1 using different carbon sources. The biosurfactant production was monitored by measuring the reduction in surface tension of the cell-free broth. The surface tension values differed concerning the substrate used, with the best results obtained with commercial sugar and maltose, followed by glucose, mannitol and starch.

Great variation in final concentration of biomass cells has been achieved in the cultivation of *Brevibacilis brevis* HOB1 on different carbon sources. The maximum biomass values reached for glucose and starch were 3.8 and 2.9 g/l, respectively, whereas the bacterial growth on mannitol resulted in only 1.5 g/l (Table 1). The greatest reductions in surface tension 29, 29, and 32 mN/m, were achieved, when commercial sugar, maltose and glucose were used as carbon sources in a bacterial growth of 2.2, 2.4 and 3.8 g/l, respectively. These results revealed that there was no relationship between cell growth and biosurfactant production, similar to that reported by other investigators [31].

Other assays were carried out with several components as nitrogen sources for growth and biosurfactant production. The results are shown in Table 2. Five components, ammonium nitrate, ammonium sulfate, sodium nitrate, potassium nitrate and soybean flour were used as nitrogen substrate. The greatest reduction in surface tension was obtained with sodium nitrate. It is observed that *Brevibacilis*

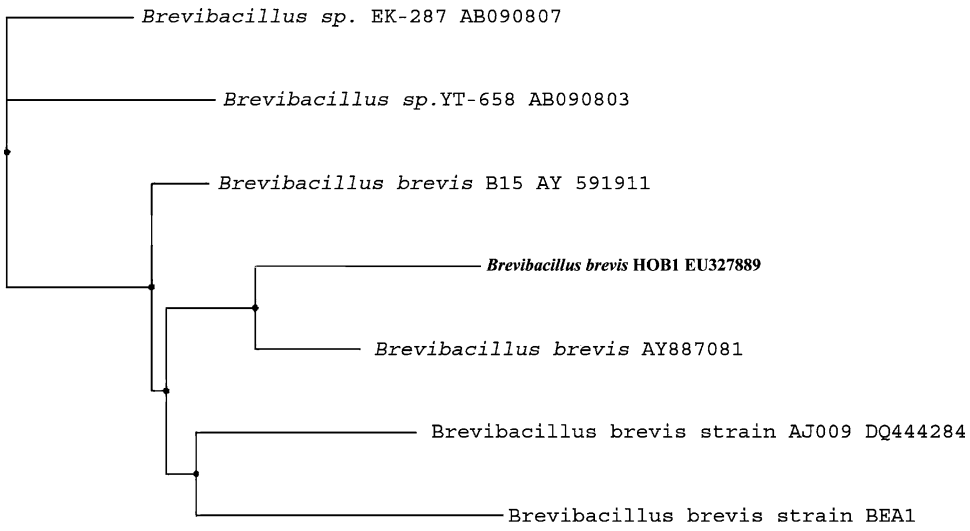

Fig. 1 Phylogenetic tree of the *Brevibacilis brevis* HOB1. 16S rRNA gene phylotypes and closely related sequences from EMBL database. The topology shown was obtained with the Neighbor-joining method

Table 1 The effect of different substrates on growth and biosurfactant production by *Brevibacilis brevis* HOB1

Carbon source	Surface tension (mN/m) at 30 °C	Biomass (g/l)
Blank	65	–
Glucose	32	3.8
Starch	37	2.9
Manitol	34	1.5
Maltose	29	2.4
Commercial sugar	29	2.2

Initial conditions: 2% substrate concentration; batch fermentation conditions: 150 ml of mineral salt medium with the following composition: 2.5 g/l $NaNO_3$, 0.1 g/l KCl, 3.0 g/l KH_2PO_4, 7.0 g/l K_2HPO_4, 0.01 g/l $CaCl_2$, 0.5 g/l $MgSO_4{\cdot}7H_2O$, and 5 ml of a trace element solution. Trace element solution contains 0.116 g/l of $FeSO_4{\cdot}7H_2O$, 0.232 g/l of H_3BO_3, 0.41 g/l of $CoCl_2{\cdot}\,6H_2O$, 0.008 g/l of $CuSO_4{\cdot}5H_2O$, 0.008 g/l of $MnSO_4{\cdot}H_2O$, 0.022 g/l of $[NH_4]_6Mo_7O_{24}$ and 0.174 g/l of $ZnSO_4$. pH 7.0, 150 *rev*/min, 48 h

Table 2 The effect of different nitrogen sources on the biosurfactant production by *Brevibacilis brevis* HOB1

Nitrogen source	Surface tension (mN/m) at 30 °C
Ammonium nitrate	31.6
Ammonium sulfate	34.5
Sodium nitrate	28.0
Potassium nitrate	30.8
Soybean flour	45.8

Initial conditions: 2% commercial sugar as substrate; the concentration of nitrogen source was 0.2%; batch fermentation conditions: 150 ml of mineral salt medium with the following composition: 0.1 g/l of KCl, 3.0 g/l of KH_2PO_4, 7.0 g/l of K_2HPO_4, 0.01 g/l of $CaCl_2$, 0.5 g/l of $MgSO_4{\cdot}7H_2O$, and 5 ml of a trace element solution. Trace element solution contains 0.116 g/l of $FeSO_4{\cdot}7H_2O$, 0.232 g/l of H_3BO_3, 0.41 g/l of $CoCl_2{\cdot}6H_2O$, 0.008 g/l of $CuSO_4{\cdot}5H_2O$, 0.008 g/l of $MnSO_4{\cdot}H_2O$, 0.022 g/l of $[NH_4]_6Mo_7O_{24}$ and 0.174 g/l of $ZnSO_4$. pH 7.0, 150 *rev*/min, 48 h

brevis HOB1 did not prefer ammonium ions, but exhibited a preference for nitrate ions. Ammonium ions inhibited the utilization of nitrate ions for biosurfactant production as observed by less reduction in surface tension when ammonium nitrate was provided as nitrogen source (Table 2). These results are in agreement with the findings of previous reports [32, 33]. Thus under the optimized experimental conditions used, *Brevibacilis brevis* HOB1 gave a maximum yield of biosurfactant at about 210 mg/l.

Growth curve was obtained for the *Brevibacilis brevis* HOB1 in order to establish the relationship between cell growth and surface activity of the biosurfactant in time at 30 °C, as can be seen in Fig. 2. Biosurfactant production occurred mainly after 36 h of fermentation, whereas cell growth occurred almost in mid-exponential phase. However, maximum yield of biosurfactant was obtained at 72 h of fermentation while at this point biomass was at the onset of the stationary phase and surface tension was at minimum. As shown in Fig. 2, the biosynthesis of the surface-active compound took place at the mid-exponential phase, achieving its maximum values at the beginning of the stationary phase. Therefore, it can be concluded that the biosurfactant produced by *Brevibacilis brevis* HOB1 is a primary metabolite, due to the production of growth-associated biosurfactant.

Isolation and purification of biosurfactant

Biosurfactants obtained by acid precipitation of the cell-free supernatants from *Brevibacilis brevis* HOB1 was confirmed as lipopetide class of biosurfactants by TLC test, with a surface tension of 29 mN/m. Crude lipopetide was subjected to ODS C18 column as shown on Fig. 3, the chromatographic elution profile of the separation. The

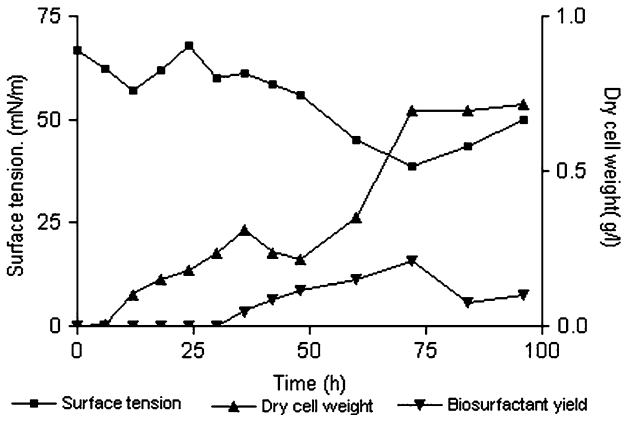

Fig. 2 Kinetics of growth and biosurfactant production of *Brevibacilis brevis* HOB1

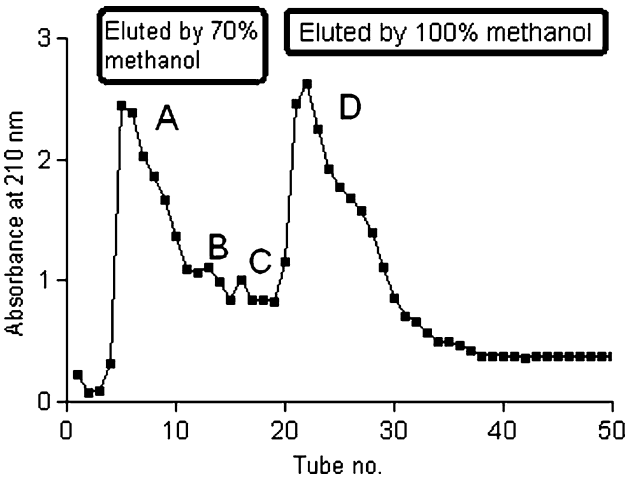

Fig. 3 Separation and purification profile of lipopeptides produced by *Brevibacilis brevis* HOB1 using ODS C18 column. Peaks *A*, *B* and *C* were eluted by 70% of methanol, while peak *D* was eluted by 100% of methanol

collection has been monitored using spectrophotometer at wave length of 210 nm, and lipopeptide activity of the collected fractions was evaluated by TLC on silica gel as described above. To remove the contaminants compounds, peaks A, B and C, 70% methanol was used, while the lipopeptide fractions, peak D, were collected using 100% methanol (Fig. 3). The collected lipopeptide fractions (peak D fractions) were separated by analytical HPLC according to the retention behavior of lipopeptides [34, 35]. They were resolved into five (B1–B5) peaks, with the retention time of 16.58, 18.39, 21.05, 23.97 and 27.01 min, respectively. The HPLC profile of lipopeptide mixtures is described in Fig. 4a, Mass spectral analysis was chosen initially to determine the molecular masses of the mixtures and to identify the number of the molecular ion species in the collection, as shown in Fig. 4b. The results revealed that there are three main components with a molecular mass of 1,035, 1,049 and 1,063 with minor components of the isoforms with a molecular mass of 1,007 and 1,021. Comparing the mass data obtained and the mass numbers reported for the lipopeptide complexes from *Bacillus subtillis* strains [36, 37], the major lipopeptide products of *Brevibacilis brevis* HOB1 could be identified as surfactin. The mass spectra of these lipopeptides had peaks, which could be attributed to the protonated forms, as well as to the sodium adducts. Further investigations for detailed structures and new isoforms of lipopeptides from novel biosurfactant producer *Brevibacilis brevis* HOB1 are undergoing.

Antimicrobial activity

Purified lipopeptide biosurfactant from *Brevibacilis brevis* HOB1 showed significant antagonistic activity against *Escherichia coli* and *Bacillus licheniformis*, while the inhibition was shown to be less against *Pichia pastoris*. However, lipopeptides produced by *Brevibacilis brevis* HOB1 could not inhibit the *Escherichia coli* up to a does of 100 µg. Table 3 shows the antimicrobial activity of lipopeptide biosurfactant produced by *Brevibacilis brevis* HOB1 against *Escherichia coli, Bacillus licheniformis*, *Staphylococcus aureus,* and *Pichia pastoris* using different doses, 50, 100, 200 and 300 µg.

Microbial molecules that exhibit high surface activity are classified as biosurfactants. These molecules reduce surface or interfacial tension in both aqueous solution and hydrocarbon mixture making them potential agents for bioremediation [38]. Microbial candidates for biosurfactant production are expected to decrease surface tension to approximately 35 mN/m [10]. In our work, we achieved a reduction of 29 mN/m in surface tension with optimized culture components. *Bacillus* species is one of the most studied industrial microorganisms. It has been reported that *B. cereus* isolated from oil lowered the water surface tension to 28 mN/m in sucrose containing medium. Among the major types of biosurfactants produced by microorganisms, surfactin is one of the most well-known products with commercial application. Only *B. subtilis* and *B. pumilus* have been reported as surfactin producers [38]. We found a *Brevibacilis brevis* HOB1 that decreases surface tension to 29 mN/m and produces a surfactin family of biosurfactant. To our knowledge, this is the first report on biosurfactant production by *Brevibacilis brevis* strain.

Lipopeptide surfactants including the cyclic lipopeptides have been confirmed as potent antibiotics [39, 40]. The antibiotic potency of the lipopeptides from *Brevibacilis brevis* HOB1 strain may differ from others, which is reasonable to assume that this antibiotic specificity of lipopeptides may have a natural role in enhancing the growth of the producing bacteria, and to defense against the competitors to the producer in given environment. The high antimicrobial

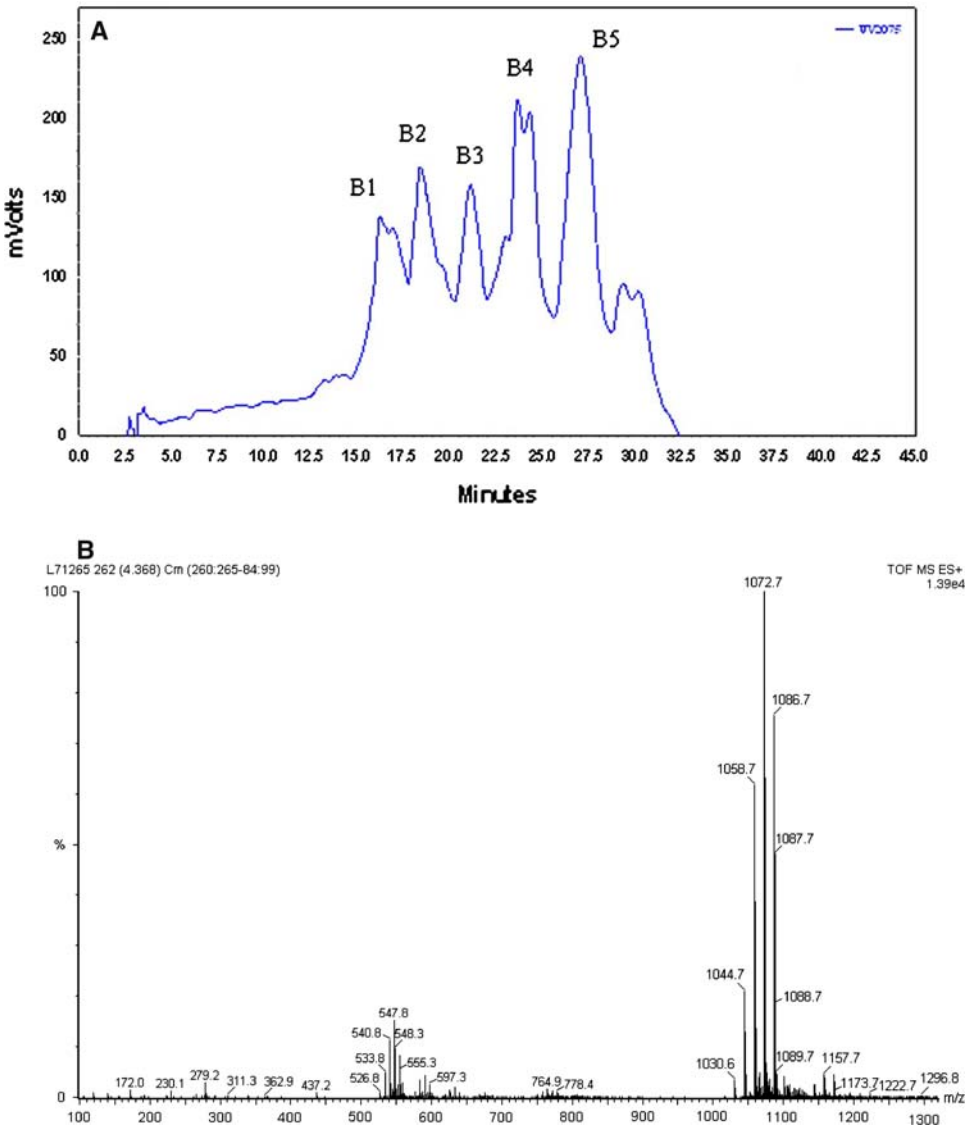

Fig. 4 **a** HPLC analysis profile for the lipopeptide produced from *Brevibacilis brevis* HOB1, and after the purification by ODS C18 column. Solution system used in **a** and **b** can be seen from "Materials and methods". **b** ESI Q-TOF MS of purified lipopeptides produced by *Brevibacilis brevis* HOB1

Table 3 Antimicrobial properties of purified cyclic lipopeptides fractions from *Brevibacilis brevis* HOB1

Microorganisms	Dose of lipopeptide from *Brevibacilis brevis* HOB1			
	50 μg	100 μg	200 μg	300 μg
Control	–	–	–	–
Escherichia coli	–	+	++	+++
Bacillus licheniformis	+	++	+++	++++
Staphylococcus aureus	–	–	–	–
Pichia pastoris	–	+	+	++

activity of the lipopeptides produced by *Brevibacilis brevis* HOB1 strain against *Bacillus licheniformis*, which were co-isolated along with *Brevibacilis brevis* HOB1 strain from oil-field production water, supported our hypothesis. It described the phenomena that appeared to the mixture of strains sample, to loose the surface activity or even the growth characteristic in the second step of cultivation, from seed culture to the production medium. Lipopeptides produced by *Brevibacilis brevis* HOB1 showed strong antibacterial and antifungal activity. Therefore, it is a candidate for the biocontrol of pathogens in agriculture.

Chemically-synthesized surfactants have been used in the oil industry to aid the clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds

are not biodegradable and can be toxic to the environment. Biosurfactants, however, have been shown in many cases to have equivalent emulsification properties and are biodegradable. Thus, there is an increasing interest in the possible use of biosurfactants in mobilizing heavy crude oil, transporting petroleum in pipelines, managing oil spills, oil pollution control, cleaning oil sludge from oil storage facilities, oil/sand bioremediation and microbially enhancing oil recovery (MEOR)[41]. Biosurfactants are used in food industry, cosmetics and special chemical industries; however, we expect that this biosurfactant production strain may be useful in agriculture and various industries, as a biosurfactant as well as a biocontrol agent, although further study is needed.

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