Appl Biochem Biotechnol (2010) 160:812–821
DOI 10.1007/s12010-009-8536-9

Structural Characterization of Lipopeptides from *Brevibacillus brevis* HOB1

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Received: 23 October 2008 / Accepted: 20 January 2009 /
Published online: 18 February 2009

Abstract *Brevibacillus brevis* HOB1 was isolated from the formation water of an oil field and found to produce lipopeptides. The separation of lipopeptides was successfully achieved by reversed-phase high-performance liquid chromatography (HPLC) leading to nine separated peaks. The chemical structures of these lipopeptides were studied by means of electrospray ionization mass spectrometry (ESI-MS), gas chromatography-mass spectrometry (GC/MS), HPLC and electrospray ionization tandem mass spectrometry (ESI-MS/MS). As the results, all the lipopeptides had peptide parts with the same amino acid composition of Asp, Glu, Val, and Leu in the molar ratio 1:1:1:4, while the lipid part was composed of C_{13}–C_{15} β-hydroxy fatty acids. As the sequence of fraction 1 was determined to be N-Glu-Leu-Leu-Val-Asp-Leu-Leu-C, the same as surfactin, they were proposed to be surfactin isoforms. Fraction 4 (C_{15} surfactin) exhibited a good surface activity of 26.8 mN/m with CMC of 9×10^{-6} M. Surfactin is a powerful biosurfactant possessing biological activities. As far as we know, *Br. brevis* is a new surfactin-producing species.

Keywords Biosurfactant · Lipopeptide · Structure · *Brevibacillus brevis* · Surfactin

Introduction

Surface active compounds which are derive from living organisms are classified as biosurfactants. Biosurfactants are mainly produced by microorganisms and fungi, such as the genus of *Pseudomonas*, *Bacillus*, *Candida*, *and Torulopsis* [1–4]. These compounds are of great interest with respect to their high biodegradability [5] and low toxicity which qualified them for potential applications in environmental protection [6–9], enhanced oil recovery [10, 11], food-processing industries, cosmetic [12], and in various fields of biomedicine. Among the many classes of biosurfactants including glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids, and neutral lipids [13],

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lipopeptides are particularly interesting due to their high surface activities and therapeutic potential. The biological activities of lipopeptides have been reported include antifungal, antibacterial, antiviral activity, cytolytic activity, inhibition of fibrin clot formation, and macrophage-stimulating activity [14–17].

Although there are a huge number of strains with diverse genus and species that can produce a biosurfactant, only a few have been identified to be lipopeptide producers. For instance, surfactin, fengycin, and iturin were isolated from *Bacillus subtilis* strains [17–19]. Lichenysin was produced only by *Bacillus licheniformis* [20, 21], and arthrofactin, produced by *Arthrobacter* sp. strain MIS38 [22]. In our previous studies, a new lipopeptide producer was isolated from oil field formation water and identified as *Brevibacillus brevis* HOB1 by analysis of 16 S rRNA gene [23]. Preliminary study showed that the biosurfactant produced by this strain had certain antimicrobial activity. An analysis by high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS) showed it was a mixture of lipopeptide homologues. In the present work, we report the separation and structural analysis of these lipopeptide homologues from *Br. brevis* HOB1.

Materials and Methods

Microorganisms and Culture Conditions

Br. brevis HOB1 was isolated from the oil-field formation water. The strain was grown aerobically in a liquid medium containing (g/l): sucrose, 10.0; yeast extract, 0.5; NH_4NO_3, 3.0; $MgSO_4 \cdot 7H_2O$, 0.5; $KH_2PO_4 \cdot H_2O$, 3.0; $K_2HPO_4 \cdot 3H_2O$, 7.0; $CaCl_2$, 0.01; $EDTA \cdot Na_2$, 0.01; and 1 ml/l of trace metals solution with the following composition (g/l): $FeSO_4 \cdot 7H_2O$, 0.116; H_3BO_3, 0.232; $CoCl_2 \cdot 6H_2O$, 0.41; $CuSO_4 \cdot 5H_2O$, 0.008; $MnSO_4 \cdot H_2O$, 0.008; $[NH_4]_6Mo_7O_{24}$, 0.022; and $ZnSO_4$, 0.174. For the production of lipopeptide, fermentations were performed using a 15-l fermentor with the working volume of 10 l. The cultures were grown at 30°C for 72 h stirred at 200 rpm, with an air supply of 1.0 vvm.

Isolation

Culture broth was centrifuged at 5,000×*g* for 30 min to remove the bacterial cells, and the cell-free supernatant was acidified to pH 2.0 with concentrated HCl, and allowing the precipitate to form at 4°C overnight. The pellet was collected by centrifugation (5,000×*g*, 20 min) and lyophilized overnight. The dried lipopeptide was extracted three times with methanol for 6 h. Solvent was removed with the aid of a rotary evaporator under reduced pressure. After evaporation, the lipopeptide-containing extracts were purified by a normal pressure liquid chromatography with an ODS C_{18} reversed phase column (50 μm, 10× 3.0 cm). The column was first washed with 200 ml of 70% (*v*/*v*) methanol and then washed by another 200 ml of 100% (*v*/*v*) methanol. Fractions (10 ml/tube) were monitored by determining the absorbance at 210 nm.

Thin-Layer Chromatography

The eluted fractions in each peak were pooled and condensed, then subjected to ninhydrin reaction after thin-layer chromatography (TLC) and acid hydrolysis in situ. The thin-layer

chromatography (TLC) was carried out with silica gel 60-precoated plates using $CHCl_3$/$CHOH/H_2O$ (65:25:4) as developing system. Acid hydrolysis was performed in a sealed vessel with plates and concentrated HCl both inside at 110°C for 1.5 h. Then plates were tested by spraying with 0.1% ninhydrin in ethyl acetate and heated at 110°C for 30 min. The peak exhibiting positive reaction was tentatively identified to be lipopeptide which would expose free amino groups after acid hydrolysis.

Prep Reverse Phase HPLC

The purified lipopeptide mixture was separated into single ones by preparative reverse phase HPLC with a JASCO HPLC System (JASCO, Japan) equipped with a HiQ sil C18 W column (KYA TECH, Japan; 5 μm, 250×21.2 mm). The system was operated at 30°C with a 85% to 100% linear gradient of acetonitrile with 0.05% (*v*/*v*) TFA as mobile phase. The peaks were eluted at a flow rate of 13 ml/min and detected by a UV monitor at 214 nm.

ESI-MS

Electrospray ionization (ESI) mass spectra were measured with a Micromass LCT TOF-MS instrument (Micromass, UK). The TOF-MS instrument was operated in the positive mode with capillary, sample cone, and extraction cone voltages of 3 kV, 100 V, and 6 V, respectively. Data were acquired with a MasslynxTM 3.5 data system.

Fatty Acid Analysis

Acid hydrolysis of lipopeptides were performed with 1 ml 6 M HCl at 90°C for 18 h in sealed tubes. The fatty acids were extracted with ether and esterified in a 1:9 mixture of 98% H_2SO_4 and methanol (*v*/*v*) at 55°C for 6 h. Fatty acid methyl esters were obtained and analyzed by GC/MS with a 6890 GC (Agilent, USA) equipped with a 5975 MSD (Agilent, USA) and a HP-5MS capillary column, 30 m×0.25 mm×0.25 μm (Agilent, USA). The helium was used as carrier gas at a flow rate of 1.0 ml/min. The column temperature was maintained at 120°C for 3 min and then gradually increased (8°C/min) to 260°C.

Amino Acid Analysis

After the lipid parts were extracted from the hydrolysate, the aqueous phase was dried under vacuum. The samples were derivatized with phenylisothiocyanate (PITC) and analyzed on a Hypersil ODS C_{18} column (5 μm, 250×4.6 mm) at 38°C, using gradient elution with detection at 254 nm. The detailed method was described by Yang et al. [24]. A mixture of L-aspartic acid, L-glutamic acid, L-valine, L-leucine, and L-isoleucine taken in the equimolar ratio served as a standard.

ESI-MS/MS

The lipopeptide was analyzed by tandem mass spectrometry (Q-Tof micro, Micromass, UK) in positive electrospray mode. Capillary voltage, sample cone voltage, ion energy and collision energy were 3 kV, 40 V, 1.5 V, and 65 V, respectively.

Surface Tension

Surface tension of the lipopeptide solution was measured at 25°C with a DCA 315 series system (Thermo-Cahn, USA), using the ring method. Samples were dissolved in 10 mM phosphate buffer (pH 8.0) and prepared in the 1–100 μM concentration range. The surface tension versus log concentration curve was used to determine critical micelle concentration (CMC).

Results and Discussion

Isolation and Purification

The isolation and purification of lipopeptides was mainly based on the procedure as described by Haddad et al. [25]. The process included acid precipitation, methanol extraction, ODS C_{18} column chromatography, and reversed phase (RP)-HPLC. In the ODS chromatography step, the target peak was identified by TLC, exhibiting a red spot in the plate. The lipopeptide-positive fraction was collected. In order to get a good separation of each lipopeptide, we have optimized isolation conditions by preparative RP-HPLC. The chromatographic profile showed nine different-sized peaks (Fig. 1).

Structural Characterization

The molecular weight of each peak was obtained by ESI-MS. The lipopeptide molecular appeared mainly as its sodium and potassium adducts in ESI-MS with a positive ion mode. The weight of the complete molecules was determined to be 1,007, 1,021, 1,021, 1,035, 1,035, 1,035, 1,035, 1,049, and 1,063, for fractions 1, 2, 3, 4, 5, 6, 7, 8, and 9, respectively. The result revealed that most fractions contained only one major molecular mass in ESI-MS.

Each fraction was hydrolyzed under acidic conditions for characterization of the hydrophilic moiety and hydrophobic moiety (fractions 6, 8, and 9 were not analyzed due to insufficient materials). The lipophilic fractions were methylated and analyzed by GC/MS. β-hydroxy fatty acid methyl esters were identified by the presence of base peak at m/z 103 ($[CH(OH)CH_2COOCH_3]^+$). A series of ions corresponding to M-1, M-18, M-50 were also detected in the mass spectrum, which can be used to define the chain lengths of the fatty acids. The gas chromatograms of fractions 1, 5, and 7 are shown in Fig. 2, the peaks identified to be β-hydroxy fatty acid methyl ester appeared as the main components in each fraction. So the lipid parts of fractions 1, 5, and 7 are corresponding to C_{13}, C_{14} and C_{15}

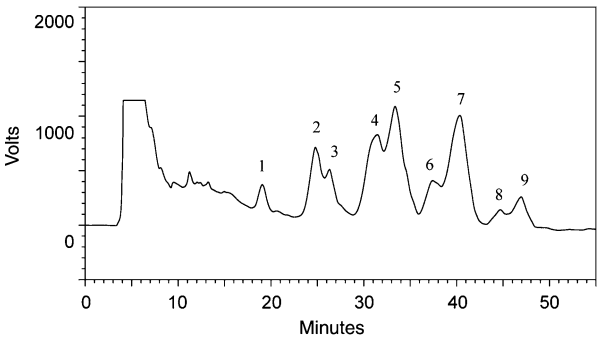

Fig. 1 Separation pattern of lipopeptides on preparative C_{18} reverse phase HPLC

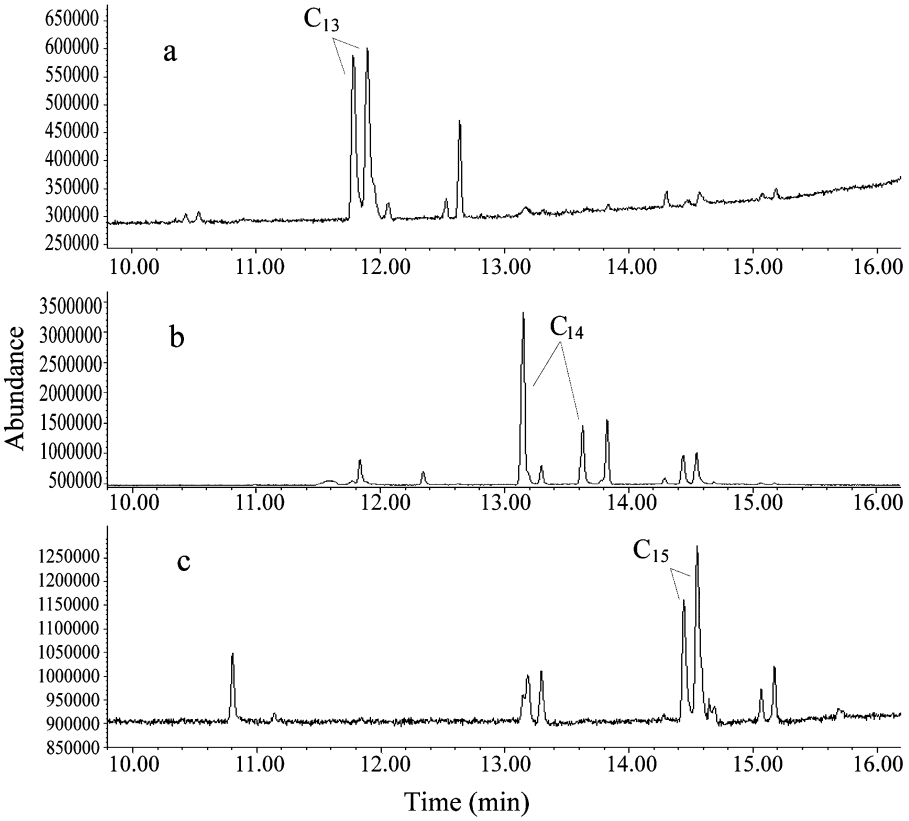

Fig. 2 Gas chromatograms of β-hydroxy fatty acid methyl esters of fractions 1 (**a**), 5 (**b**), and 7 (**c**). The peaks identified as β-hydroxy fatty acid methyl esters by MS were labeled aside

β-hydroxy fatty acids, respectively. In all the fractions which had been analyzed, totally six β-hydroxy fatty acids were found, two C_{13}, two C_{14}, and two C_{15}. β-hydroxy fatty acid methyl esters having the same chain length but differing in retention time were attributed to different chain type as *iso* or *n* or *anteiso* configuration. Here, a much more retention time shift of β-hydroxy fatty acids was observed in GC spectrum for neighboring chain length, than that with different chain types in the same length. As Hosono et al. has reported, there are linear relationships between retention times and carbon numbers with respective series of homologous fatty acid methyl esters [26]. Here, our result fits this rule well (see Fig. 3). Thus, these six β-hydroxy fatty acids were tentatively identified to be *iso*-C_{13}, *anteiso*-C_{13}, *iso*-C_{14}, *normal*-C_{14}, *iso*-C_{15} and *anteiso*-C_{15}, respectively. These results are in agreement with that reported in the literatures for lipopeptides [27–29], namely, only *iso* and *anteiso* types were reported for C_{13}, *iso* and *normal* types for C_{14}, *iso* and *anteiso* types for C_{15}. The fatty acid structural data of all fractions by GC/MS are summarized in Table 1.

A quantitative analysis of amino acids in water-soluble part of lipopeptide hydrolysates was carried out by a precolumn derivatization procedure using phenyl isothiocyanate (PITC). A standard and a negative control were determined to identify the target peaks and the background. Additionally, the relative concentration was measured by the area under these peaks. The molar ratio relative to Val is calculated for each peak by putting Val equal to 1. The result showed that the six fractions had almost the same amino acid composition

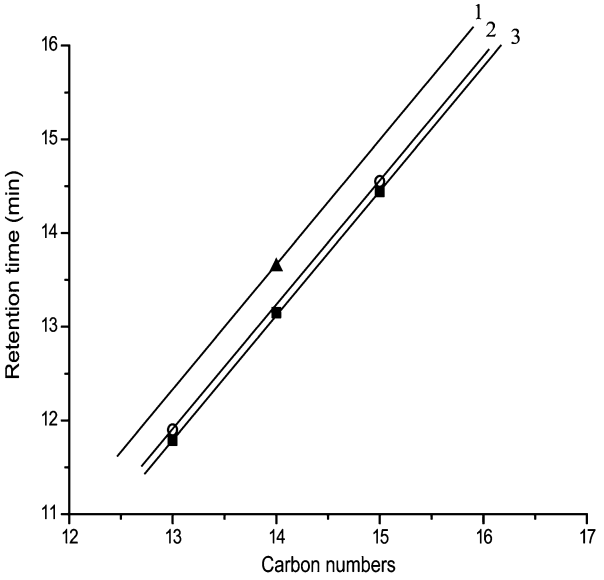

Fig. 3 Relationships between retention times and carbon numbers of β-hydroxy fatty acid methyl esters. *1 normal* fatty acid esters, *2 anteiso* fatty acid esters, *3 iso* fatty acid esters

of Asp, Glu, Val, and Leu in a molar ratio of approximately 1:1:1:4. Isoleucine was also detected, but the amount was very small, significantly below molar amounts.

To determine the amino acid sequence, fraction 1 has been subjected to tandem mass analysis with the sodium-ionized molecular ion at *m*/*z* 1,030 as precursor ion (Fig. 4). Under ESI processes, the degradation of cyclic lipopeptides was carried out through decyclization due to β-elimination of a C-terminal amino acid from a β-hydroxy fatty acid residue followed by rupture of the peptide chain [20, 29, 30]. Sequential losses of the C-terminal amino acid were observed. Fragment ions at *m*/*z* (1,030), 917, 804, 689, 590 suggested amino acid sequence of Val-Asp-Leu-Leu. Besides, the Mclafferty reagement with double hydrogen transfer (DHT) also occurs during electrospray ionization tandem

Table 1 Structural data for the isolated lipopeptides.

Fraction number on HPLC	MW of fatty acid methyl ester	Retention time on GC (min)	Lipid portion	MW of lipopeptides	Surfactin species
1	244	11.79	*iso* C_{13}	1,007	C_{13} surfactin
	244	11.90	*anteiso* C_{13}		
2	258	13.16	*iso* C_{14}	1,021	C_{14} surfactin
3	258	13.15	*iso* C_{14}	1,021	C_{14} surfactin
	258	13.64	*n* C_{14}		
4	272	14.44	*iso* C_{15}	1,035	C_{15} surfactin
	272	14.55	*anteiso* C_{15}		
5	258	13.14	*iso* C_{14}	1,035	C_{14} surfactin methyl ester
	258	13.63	*n* C_{14}		
6	–	–	–	1,035	–
7	272	14.44	*iso* C_{15}	1,035	C_{15} surfactin
	272	14.55	*anteiso* C_{15}		
8	–	–	–	1,049	–
9	–	–	–	1,063	–

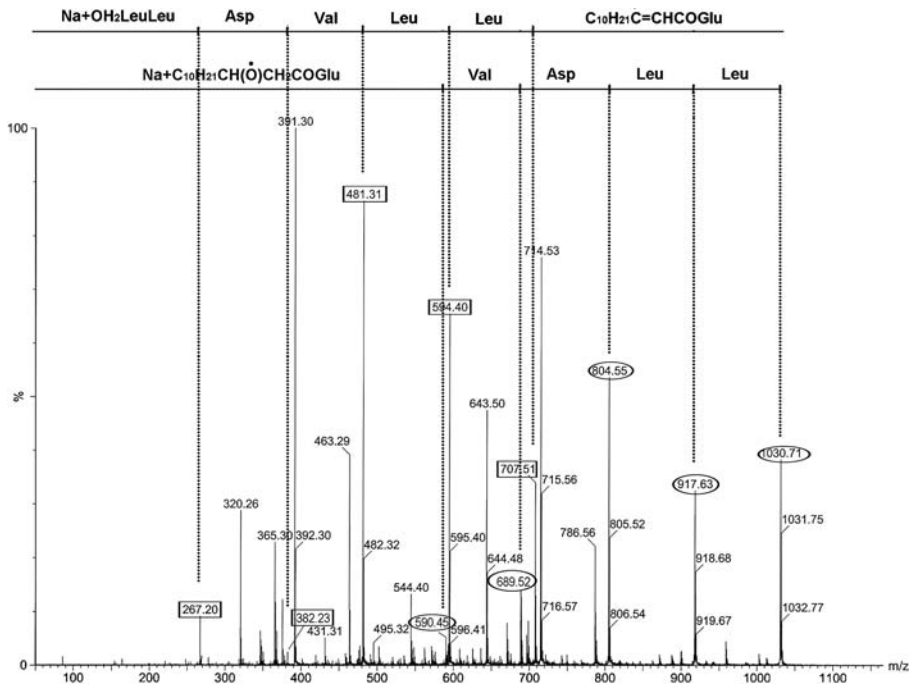

Fig. 4 Tandem mass spectrum of fraction 1. Proposed cleavage sites and fragments by the DHT mechanism and resulting from simple cleavage of the C-terminal amino acid residues are shown, respectively

mass spectrometry (ESI-MS/MS) [31]. As a result, the OH_2 would be added to C-terminal part, which allowed identification of C-terminal in a peptide and also indicated the existence of lactone ring connecting the hydroxyl group in fatty acid part and C-terminal of peptide part. The set of peaks 707, 594, 481, 382, 267 resulting from DHT implied the sequence of Leu-Leu-Val-Asp-C. Other intense peaks of fragments at *m*/*z* 320, 391, 463, and 643 were attributed to Na + LeuLeuVal–CO, Na + LeuLeuVal + CO + NH, Na + LeuLeuValAsp or Na + LeuValAspLeu or Na + ValAspLeuLeu and Na + M–(AspLeuLeu + CO + OH_2), which also support the above sequences. This method was well described by Yang et al. [31] and effectively used for determination of amino acid sequence in a cyclic lipopeptide without hydrolysis in our lab [32, 33].

From the ESI-MS/MS, no individual signals were shown about Glu residue. Considering the molecular weight, fatty acid part and amino acid composition already obtained, Glu was most likely located in the N-terminal of the peptide, since C-terminal has already been affirmed, and there is no other binding site in the fatty acid part besides carboxyl group. Finally, the amino acid sequence of fraction 1 was assigned to be N-Glu-Leu-Leu-Val-Asp-Leu-Leu-C.

As the data shown above, fraction 1 is clearly assigned to C_{13} surfactin [34, 35]. On the basis of the amino acid composition, lipophilic moiety, and total molecular weight, these lipopeptides are considered to be surfactin isoforms with high possibility. However, fraction 5, with a C_{14} β-hydroxy fatty acid, has a more molecular mass than C_{14} surfactin by 14 Da which most probably represents a methylene group. Because the amino acid composition was measured after hydrolysis under drastic acidic conditions, Glu-γ-methyl ester and Asp-β-methyl ester would be hydrolyzed and detected as glutamic acid and aspartic acid, respectively. So, the additional 14 Da in molecular mass of fraction 5 may come from the methylation of a free carboxyl group of Glu or Asp residue. Moreover, fraction 5 was eluted

from the reverse phase column later than fraction 2, fraction 3 (C_{14} surfactin), and fraction 4 (C_{15} surfactin), revealed that it has much higher hydrophobicity than the corresponding nonmodified surfactin. For whether this compound is a native product or a derivative produced during the procedure, it remains a question. However, no C_{13} surfactin methyl ester was found in the supposed eluted range. In this case, a native C_{14} surfactin methyl ester seemed to be the most probable. Fraction 7 was also identified to be C_{15} surfactin, the same as fraction 4, but eluted much later. A similar phenomenon was met by Kowall et al. [29]; this may attribute to the difference in advanced structure or micelle formation of lipopeptides during HPLC separation [36, 37]. The HPLC seems unable to separate surfactin isomers, having lipid portions of the same chain length but different chain types. Generally, the lipopeptides were eluted out successively with the increasing molecular weight. The primary structures of these fractions were shown in Table 1.

Surface Activity

Figure 5 shows the variation of surface tension versus log concentration for fraction 4. The critical micelle concentration (CMC) value was estimated to be around 9×10^{-6} M, while the minimum surface tension value was 26.8 mN/m, which was similar to that identified for surfactin [38, 39], providing further evidence to validate that these lipopeptides were surfactin isoforms.

Surfactin, a cyclic lipopeptide, demonstrated to have exceptional surface-active properties and is regarded as one of the most powerful biosurfactants. In addition to its strong surface activity, surfactin also exhibits various other properties such as antimicrobial, antiviral, antitumoral, and hypocholesterolemic activities. Natural surfactin is a mixture of isoforms consisting of a heptapeptide and a β-hydroxy fatty acid with chain length of 13–15 carbon atoms. Biosynthesis of surfactin is known to a property of members of the genus *Bacillus* (almost *Bacillus subtilis*) in past decades. *Br. brevis* has not been reported as a surfactin producer before. However, some of these species can produce a cyclic decapeptide antibiotic known as gramicidin S [40]. Both gramicidin S and surfactin are synthesized non-ribosomally by the thiotemplate mechanism with large multifunctional enzyme complexes.

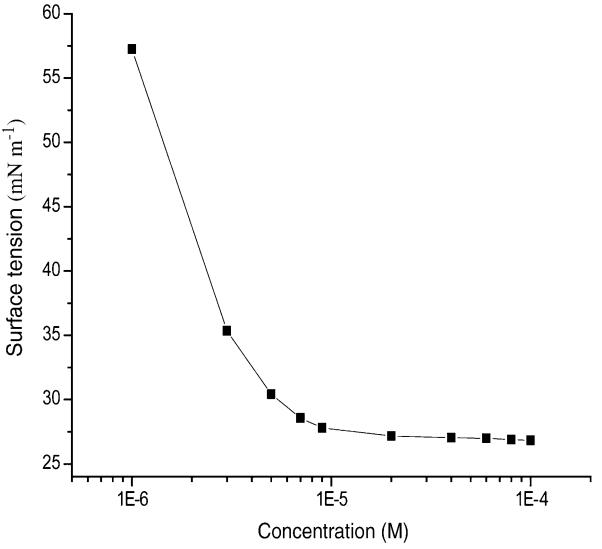

Fig. 5 Surface tension–concentration plots of fraction 4 (C_{15} surfactin)

Genetic evidence has shown that two putative components of the surfactin-synthesizing enzyme complex from the *B. subtilis* share homology with gramicidin S synthetase [41]. These findings leads to the conclusion that surfactin synthetic gene may have a common origin with gramicidin S in evolution. Moreover, our study also revealed that *Br. brevis* which named *B. brevis* previously and *B. subtilis* are two closely related genera. However, all the previous studies on the genetics of surfactin production were performed in *B. subtilis*. We propose a further investigation of surfactin synthetic gene in *Br. brevis*, which would help to provide more information about surfactin synthetic mechanism.

Acknowledgement This work was supported by the National Natural Science Foundation of China (No.50744016), the grant from the Ministry of Science and Technology of China (2007CB707801), and Shanghai Municipal Science and Technology Commission (071607014).

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