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RESEARCH PAPER

Isolation, Purification, and Characterization of Novel Fengycin S from *Bacillus amyloliquefaciens* LSC04 Degrading-Crude Oil

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Abstract In this study, a biosurfactant-producing bacterial strain was isolated from oil-contaminated soil on the basis of its ability to degrade crude oil and tributyrin ($C_{4:0}$). LSC04 was identified as *Bacillus amyloliquefaciens* LSC04 *via* 16S rRNA gene analysis and partial *gyrA* gene sequence analysis. The biosurfactants were purified and structural analysis results showed that *B. amyloliquefaciens* LSC04 generated a lipopeptide biosurfactant. Two main ions of 1,086.9 and 1,491.2 were measured *via* matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The m/z 1,491.2 was shown to correspond to the lipopeptide fengycin B, but the m/z 1,086.9 ion did not correspond to any known lipopeptide. As constituents of the peptides and the lipophilic portion of the m/z 1,491.2; 10 amino acids (Ile-Tyr-Gln-Pro-Val-Glu-Ser-Tyr-Orn-Glu); and β-hydroxy-C17 fatty acid were identified *via* ESI-MS/MS. Structurally, the lipopeptide of a molecular mass of 1,491.2 differed from fengycin B and fengycin A by a substitution of serine for the threonine residue in position 4, and the amino acid residue in position 6 was equal to that of fengycin A. The major compound, which had a molecular mass of 1,491.2 Da was designated "Fengycin S".

Keywords: biosurfactant, *Bacillus amyloliquefaciens*, fengycin, lipopeptide

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1. Introduction

Many microorganisms generate a variety of biosurfactants and can exist in a variety of environments, including soil, marine, desert, and aqueous environments. Biosurfactants are biologically surface-active agents that can be biosynthesized by diverse microorganisms, including bacteria, fungi, and yeasts. Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic residues. Due to this molecular structure, biosurfactants display many interesting properties, for example, detergency, emulsification, foaming, and dispersion [1,2]. Biosurfactants evidence a number of advantageous abilities compared with those of synthesized surfactants including, toxicity, biodegradability, and environmentally compatible specific activities at extreme temperatures, pH levels, and salinity [3-6]. Biosurfactants can be separated into low-molecular mass and high-molecular mass variants; the low-molecular mass biosurfactants include compounds such as glycolipids, lipopeptides, corynomycolic acid, and phospholipids. High-molecular mass biosurfactants include emulsans, alasan, liposan, polysaccharides, and protein complexes [7]. The lipopeptides of bacterially-produced low-molecular mass biosurfactants have been identified as members of the iturin families (iturin, mycosubtilins, and bacillomycin), fengycin, surfactin, arthrofactin, and putisolvin [8-10]. These are interesting groups of microbial biosurfactants due to their many attractive properties, which include bioremediation, oil-degradation, in addition to anti-fungal, anti-bacterial, and anti-viral properties.

Fengycin belongs to a group of lipopeptides generated by *Bacillus* species [9,11-14]. It is a well-known anti-fungal agent against phyto-pathogenic fungi [12,15]. Fengycin exists as a mixture of isoforms with variations in both the length and branching of the β-hydroxy fatty acid

moiety, as well as, in the amino-acid composition of its peptide ring [10]. For example, at position 6, D-alanine (as in fengycin A) can be replaced by D-valine (as in fengycin) [13]. The β-hydroxy fatty moiety of fengycin evidences a variety of peaks from C_{14} to C_{17}, as evidenced by MALDI-TOF mass spectra. Additionally, the mass spectra of fengycin groups evidence peaks at m/z values between 1,450 and 1,550.

In this study, we have isolated and identified *B. amyloliquefaciens* LSC04, which produces effective biosurfactants. Additionally, we purified and analyzed the structure of the biosurfactant. The isolated biosurfactant was designated as fengycin S, and it was compared with fengycin A and fengycin B.

2. Materials and Methods

2.1. Microorganism and culture conditions

B. amyloliquefaciens LSC04 was employed throughout this work, after being isolated from oil-contaminated soil samples. The strain was cultivated in Luria-Bertani (LB, Sigma-Aldrich Co., St. Louis, MO, USA) medium (10 g/L, casein peptone; 5 g/L, yeast; 5 g/L, extract; and 5 g/L, NaCl; pH 7.0) containing 2% (v/v) crude oil and tributyrin. The chemicals used for the LB medium were obtained from the Sigma-Aldrich Co. (St. Louis, MO, USA) and crude oil produced by the United Arab Emirates was obtained from the S-oil Refining Co. Ltd., Ulsan, Korea. Individual bacterial isolates were stored at −70°C in LB medium containing 20% glycerol.

2.2. Identification of biosurfactant-producing bacterium

Bacterial genomic DNA was isolated *via* the method of Cutting *et al.* [16] with some modifications. In brief, bacterial cells were cultured in 25 mL of LB medium at 37°C and 180 rpm until late-log phase (OD_{600}, 1.0~2.0) was reached, then harvested *via* 10 min of centrifugation at 8,000 rpm. The harvested cells were washed in 10 mL of lysis buffer (50 mM, EDTA; 0.1 M, NaCl; and 10 mM, Tris-HCl; (pH 8.0)) and centrifuged, and the pellet was resuspended in 4 mL of lysis buffer. Then, 10 mg of lysozyme was added to this suspension and incubated for 10 min without shaking at 37°C. A 0.3 mL of N-lauroylsarcosine (10% (w/v)) was then added and the mixture was incubated for an additional 5 min. The mixture was extracted with 4 mL of phenol, chloroform, and iso-amylalcohol (25:24:1) and subsequently re-extracted with 4 mL of chloroform and iso-amylalcohol (24:1). The DNA was precipitated with ethanol and 3 M sodium acetate (pH 5.2), and washed in chilled 70% ethanol, and finally resuspended in 10 mM Tris-HCl (pH 8.0). The extracted genomic DNA was utilized as a template for the polymerase chain reaction.

We conducted analyses of the partial 16S rRNA gene and the *gyrA* nucleotide sequences. The 16S rRNA gene and the *gyrA* fragment, corresponding to *Bacillus subtilis* 16S rRNA gene sequence positions were as follows: 96398-97941 (p16Sf, 5'-GAGTTTGATCCTGGCTCAG-3' p16Sr, 5'-AGAAAGGAGGTGATCCAGCC-3') and the *gyrA* were 43-1065 (pGyrAf, 5'-CAGTCAGGAAATGCGTACGTCCTT-3', pGyrAr, 5'-CAAGGTAATG CTCCAGGC ATTGCT-3'). The DNA was PCR amplified under the following conditions: 95°C for 3 min, 30 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 2 min and a final 10 min extension at 72°C. The PCR products were then extracted using a Gel Extraction Kit (NucleoGen Inc., Daejeon, Korea), and the DNAs were cloned into pGEM-T-easy vector (Promega Co., Madison, WI, USA) and transformed into *Escherichia coli* JM109 cells (Takara Bio. Inc., Shiga, Japan). The sequence was then compared to the rRNA gene and *gyrA* nucleotide sequences in the Genebank database ("http://www.ncbi.nlm.nih.gov/BLAST/"). Phylogenetic trees were constructed *via* the Neighbor-Joining method and Clustal-X software.

2.3. Surface tension of culture broth

The surface tension of the culture broth without cells was measured *via* the Ring Method [17] using a Du Nouy Tensiometer (Itoh Seisakusho, Ltd. Tokyo, Japan) every 6 h for 4 days.

2.4. Assay of emulsification activity and stability

The emulsification activities of the biosurfactants were assessed *via* a modified version of the method developed by Cirigliano and Carman [18,19]. The samples were diluted with distilled water to a final volume of 4 mL and the solution was mixed with 1 mL of a substrate (soybean oil, kerosene, crude oil, tributyrin, and hydrocarbons). Thereafter, the mixture was vigorously shaken for 2 min in a vortex mixer and then allowed to stand for 10 min before measuring turbidity at 540 nm. The absorbance was expressed as the emulsification activity.

The emulsion stability was analyzed on the basis of the emulsification activity [18,19]. The emulsified solutions were permitted to stand for 10 min at room temperature and absorbance readings were taken every 10 min for a period of 60 min. As such, the log of the absorbance was plotted versus time and the slope (decay constant, K_d) was calculated to express the emulsion stability.

2.5. Purification of biosurfactants of *B. amyloliquefaciens* LSC04

The bacterial cells were removed from the culture broth *via*

centrifugation at 13,000 rpm for 15 min at 4°C and the supernatant was acidified to pH 2.0 with 12 N HCl, following the formation of a precipitate at 4°C overnight. The precipitate was then collected *via* centrifugation (13,000 rpm, 4°C, and 15 min), washed 3 times in acidic water (pH 2.0 with 12 N HCl), and dried by vacuum. The dried surfactant was then extracted 3 times with methanol (100 %) for 3 h. The methanol was removed using an EYELA rotary evaporator (Tokyo Rikakikai Co. Ltd., Tokyo, Japan) under reduced pressure. The crude biosurfactant was obtained as a brown-colored material using an EYELA rotary evaporator, then purified by silica gel 60 chromatography (Merck Co. Inc., Damstadt, Germany; 2.5 ×30 cm, 230~400 mesh). The sample was purified further using a reverse-phase HPLC system and TLC.

2.6. Drop-collapsing assay of biosurfactant

The purified biosurfactant was evaluated for biosurfactant activity *via* a drop-collapsing assay [8]. Twenty micro liter of crude oil was placed on the surface of 20 mL of distilled water in petri dishes (90 mm in diameter). A thin oil film was immediately formed. Then, 10 μL of HPLC eluent was gently placed in the center of the oil film. A clear halo became visible under light and the diameter of the halo was measured.

2.7. Thin-layer chromatography

Thin-layer chromatography was conducted on pre-coated silica gel 60 F_{254} plates (Merck Co. Inc., Damstadt, Germany). The hydrolysis products were separated by thin-layer chromatography on silica gel using a mixture of chloroform:methanol:water (65:25:4 v/v) as the mobile phase. The plates were developed with sprayed water followed by incubating the plates for air dry.

2.8. High-pressure liquid chromatography

HPLC was conducted using a Waters 400 HPLC reverse-phase liquid chromatography system (Waters, USA) equipped with a BDS C18 column (Thermo Hypersil Ltd., Cheshire, UK; 5 μ, 4.6 × 250 mm) at room temperature. The system was operated at a flow rate of 2.0 mL/min with a solvent of 90% methanol as the mobile phase.

2.9. Mass spectrometry analysis

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and Electro spray ionization mass spectrometry (ESI-MS/MS) were conducted on a Voyager-DE PRO instrument manufactured by Perkin-Elmer Cetus (Foster City, CA, USA), and a Waters/Micromass QTOF2 and Micromass (Milford, MA., USA). The samples were infused with a syringe. The electro-spray source was

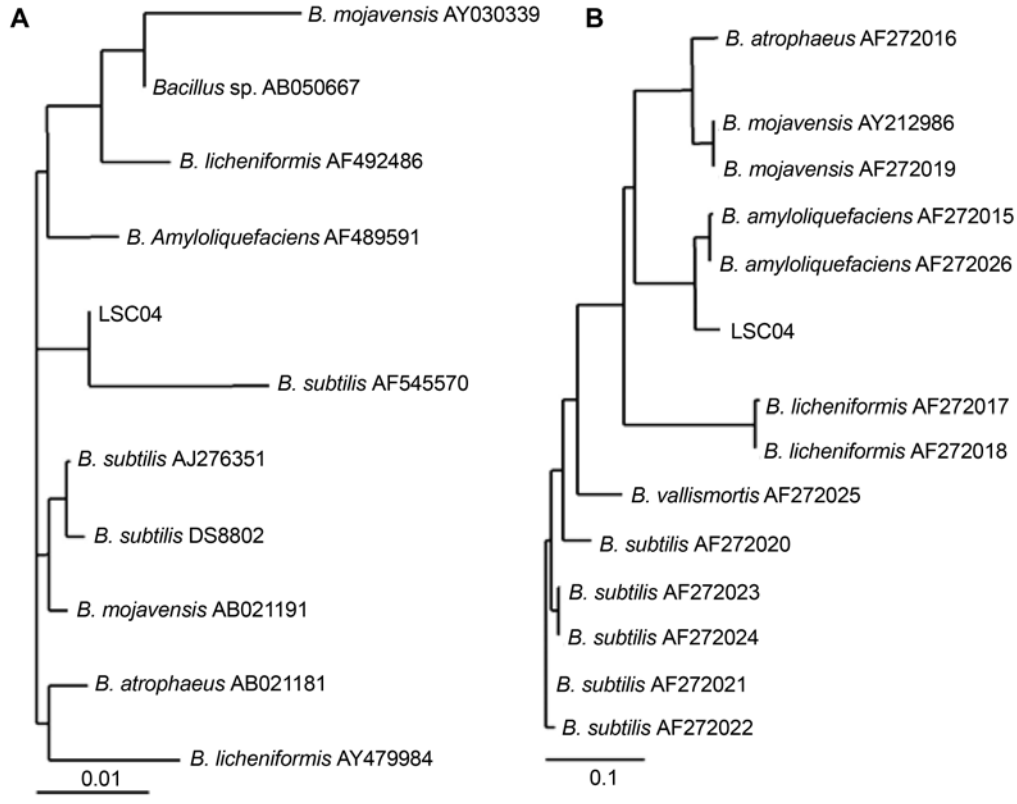

Fig. 1. Phylogenetic tree based on (A) partial 16S rRNA gene and (B) *gyrA* NT sequences.

operated at a capillary voltage of 32 V, a spray voltage of 5 kV, and a capillary temperature of 320°C.

3. Results and Discussion

3.1. Identification of biosurfactant-producing *B. amyloliquefaciens* LSC04

A biosurfactant-producing bacterial strain was selected from oil-contaminated soil owing to its ability to degrade crude oil and tributyrin ($C_{4:0}$). LSC04 was grown with crude oil or tributyrin as a sole source of carbon. Additionally, LSC04 evidenced marked crude oil and tributyrin-degrading activity, as visualized by the clear zone that developed around the colony after 12 h of incubation at 37°C. LSCO4 also evidenced an ability to reduce surface tension.

The isolate was identified as *B. amyloliquefaciens* LSC04 *via* an analysis of the partial 16S rRNA and partial *gyrA* nucleotide (NT) sequences. The phylogenetic trees based on a neighbor-joining analysis of the 16S rRNA and *gyrA* NT sequences are shown in Fig. 1. A 1,541 bp region of the LSC04 16S rRNA gene was amplified with p16Sf and p16Sr primers, and the sequence was compared to those in the 16S rRNA database. The closely related 16S rRNA genes in the database were from *Bacillus* species, which evidence an identity of more than 98% to *B. amyloliquefaciens* LSC04. Also, a 927 bp region of *B. amyloliquefaciens* LSC04 *gyrA* gene was amplified using pGryAf and pGryAr. The identities of *gyrA* NT sequences were in excess of 95% for *B. amyloliquefaciens* strains, but were under 83% for a variety of *Bacillus* species, including *B. mojavensis, B. subtilis, B. licheniformis, B. halodurans, B. anthracis*, and *B. cereus*. The *gyrA* NT sequences are a source of significantly higher genetic variation than the 16S rDNA sequences.

In this study, we have collected some bacterial isolates from the soil samples. For the selection biosurfactant-producing strains, we assessed crude oil and tributyrin degradation in the agar plates, as well as, surface tension in the culture broth. LSC04 isolated strain was selected for production of biosurfactant. The biosurfactant-producing LSC04 was similar to *B. subtilis* in terms of its size, colony type, and physical properties. Accordingly, to identify LSC04, we analyzed the nucleotide (NT) sequences of the partial 16S rRNA gene and partial *gyrA* sequences, and identified the organism as *B. amyloliquefaciens*. Many studies have been conducted recently regarding bacterial diversity in soil, marine, desert, and aqueous environments, using extensive 16S rRNA sequence evaluations [20-24]. However, as the partial 16S rRNA gene sequences of a variety of *Bacillus* species are very similar to *B. subtilis* (over 99%), the partial 16S rRNA sequences provide insufficient resolution to distinguish various subspecies and close relatives of *Bacillus* species [25]. The partial *gyrA* sequences were then compared with those of other *Bacillus* species, an approach that has previously proven useful for distinguishing between *Bacillus* species [26].

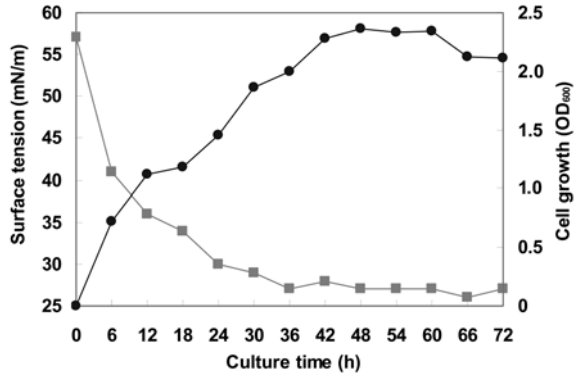

Fig. 2. Cell growth and surface tension decreases in culture broth by the LP04.

3.2. Surface tension of biosurfactant-producing bacteria

The surface tension of the culture filtrates of *B. subtilis* A8-8, *Bacillus atrophaeus* DYL130, and LSC04 decreased from 58 to 26 mN/m, 27 mN/m, and 26 mN/m, respectively (Fig. 2). Generally, the surface tension of various biosurfactants has been reported to range from approximately 27 to 35 mN/m [27-29]. A reduction in the surface tension of media as a consequence of biosurfactant production, and accumulation during the period between the exponential growth and stationary phases has already been reported for several other microorganisms [27,29,30]. Biosurfactants generated by microorganisms are of 2 main types: those that reduce surface tension at the air-water interface and those that reduce the interfacial tension between immiscible liquids or at the solid-liquid interface (bioemulsifiers). Biosurfactants generally evidence emulsifying ability, but bioemulsifiers do not necessarily reduce surface tension [31].

3.3. Emulsifying activity and stability of biosurfactant from bacteria

The emulsification activity of the biosurfactants from *B. subtilis* A8-8, *B. atrophaeus* DYL130, and LSC04 was measured with a variety of water-immiscible substrates (Table 1). The stabilization ability (Table 2) of the biosurfactants from isolates was described by the decay content, K_d (the slope of the emulsion decay plot). Additionally, emulsion decay plots were constructed for a variety of emulsifying substrates in the presence of the biosurfactants from isolates, after which the respective K_d values were

Table 1. Emulsification activity of biosurfactant from *B. subtilis* A8-8, *B. atropaeus* DYL130, and *B. amyloliquefaciens* LSC04

Substrate	Emulsification activity ($OD_{540\,nm}$)[a]		
	A8-8	DYL130	LSC04
Soybean oil	2.75	2.10	2.79
Trybutyrin ($C_{4:0}$)	1.94	1.99	1.54
Kerosene	1.46	2.88	1.75
Crude oil	1.08	1.16	1.18
Hexadecane (C_{16})	1.06	1.59	0.79
Tetradecane (C_{14})	0.55	1.40	1.29
Dodecane (C_{12})	0.71	1.39	0.39
Decane (C_{10})	0.60	1.30	0.30

[a]The emulsification assay was performed in the presence of the biosurfactant as described in the text. After an initial 10 min holding period, absorbance readings were taken every 10 min for 60 min.

Table 2. Emulsification stability of biosurfactant from *B. subtilis* A8-8, *B. atropaeus* DYL130, and *B. amyloliquefaciens* LSC04

Substrate	Decay constant (K_d, 10^{-3})[a]		
	A8-8	DYL130	LSC04
Soybean oil	−0.00	−0.00	−0.13
Trybutyrin ($C_{4:0}$)	−1.88	−9.56	−2.22
Kerosene	−2.32	−11.32	−0.54
Crude oil	−5.89	−2.21	−5.25
Hexadecane (C_{16})	−2.54	−3.18	−3.26
Tetradecane (C_{14})	−4.48	−5.08	−3.08
Dodecane (C_{12})	−6.41	−3.65	−3.65
Decane (C_{10})	−6.11	−5.43	−7.53

[a]The log of the absorbance was plotted versus time and the slop (decay constant, K_d) was calculated.

Table 3. Comparison of emulsification activity and stabilization by biosurfactant and commercial surfactants (soybean oil as substrate)

Surfactant	Emulsification activity ($OD_{540\,nm}$)[a]	Decay constant (K_d, 10^{-3})[b]
LSC04	2.79	−0.13
B. subtilis A8-8	2.75	−0.00
Tween 20	2.71	−0.14
Tween 40	2.63	−0.12
Tween 80	2.60	−0.14
Span 40	1.80	−0.43
Span 85	0.88	−5.68
Triton X-100	2.36	−0.18
SDS	1.51	−16.05

[a]The emulsification assay was performed in the presence of the biosurfactant as described in the text. After an initial 10 min holding period, absorbance readings were taken every 10 min for 60 min.
[b]The log of the absorbance was plotted versus time and the slop (decay constant, K_d) was calculated.

calculated (Table 2). Biosurfactants of *B. subtilis* A8-8 and LSC04 were showed highest emulsification activity with soybean oil as a substrate, whereas *B. atrophaeus* DYL130 showed highest emulsification activity with Kerosene (Table 1). The stability of biosurfactants was found to be higher in substrates with high emulsification activity (Table 2), which is comparable to the results observed with the biosurfactant from *Nocordia* sp. L-417 [28]. In our previous studies, we isolated and characterized the biosurfactants generated by *B. atrophaeus* DYL-130 [32] and *B. subtilis* A8-8 [33]. Therefore, these characteristics of the LSC04 biosurfactant indicate that it might potentially function as a substitute for chemically synthesized surfactants.

3.4. Comparison of biosurfactant with commercial surfactants

Several commercial surfactants have been investigated to compare their emulsification activities and emulsion stabilities with those of the biosurfactants from *B. subtilis* A8-8, *B. atrophaeus* DYL130, and LSC04 against the substrates with the highest emulsification activity (Table 3). The results showed that the emulsification activities of the biosurfactants from *B. subtilis* A8-8, *B. atrophaeus* DYL130, and LSC04 were superior to those of several commercially available surfactants, with the notable exception of the Tween detergents (20, 40, and 80); however, its emulsification stability was far superior to that of all the commercial surfactants tested, thereby indicating that the biosurfactants from *B. subtilis* A8-8, *B. atrophaeus* DYL130, and LSC04 might prove useful as emulsifying and emulsion-stabilizing agents.

3.5. Purification of biosurfactant from *B. amyloliquefaciens* LSC04

The biosurfactant was purified from the culture filtrate of *B. amyloliquefaciens* LSC04 grown in an LB-medium, as described in the Materials and Methods section. The purification procedure consisted of 4 steps: HCl fractionation, methanol treatments, silica gel column chromatography, and HPLC. The brown-colored precipitate containing the biosurfactant was acquired *via* treatment with 12 N HCl and precipitation at 4°C overnight. The crude biosurfactant was then extracted 3 times with methanol and concentrated in a rotary evaporator. It was subsequently identified as several spots on a TLC plate developed using solvent system chloroform: methanol:water (65:25:4 v/v). Thereafter, the crude biosurfactant was concentrated in a rotary evaporator, dissolved in butanol (100%), and loaded onto a silica gel 60 column equilibrated with butanol (100%). The active fraction was then collected, concentrated, and dissolved in methanol in order to estimate its purity *via* reverse-phase HPLC. The HPLC chromatogram showed one major peak and evidenced a retention time of 10.183

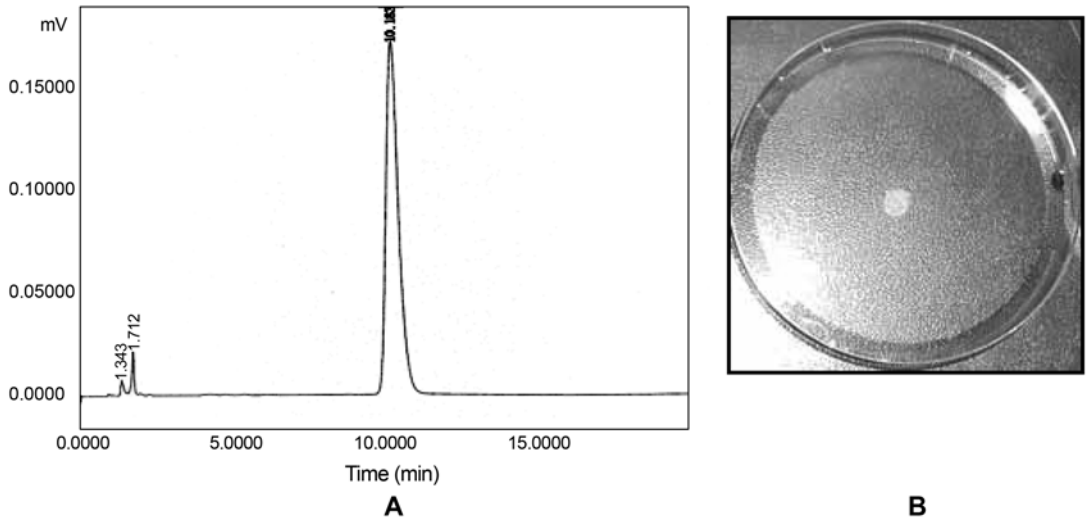

Fig. 3. (A) HPLC chromatogram and (B) oil film-collapsing of purified biosurfactant.

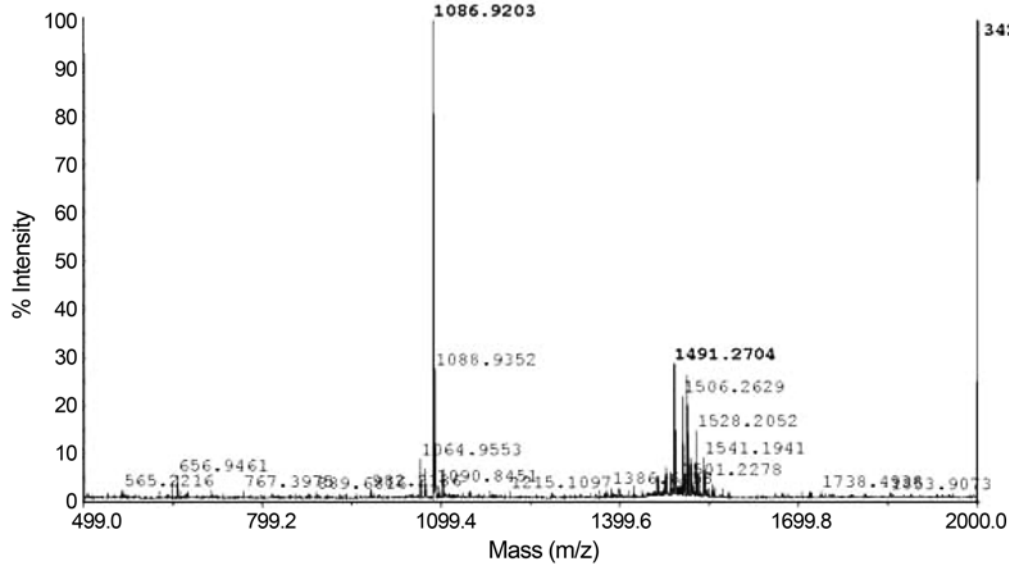

Fig. 4. MALDI-TOF mass spectrum of the purified biosurfactant by *B. amyloliquefaciens* LSC04.

min (Fig. 3A). The HPLC eluent evidenced oil film-collapsing activity and the diameter of the halo zone was 74 mm (Fig. 3B). The purified biosurfactant was identified as one white-colored spot (R_f value, 0.69) on a TLC plate sprayed with water (data not shown).

3.6. Structural characterization of the purified biosurfactant

The molecular mass of the purified compound was measured *via* MALDI-TOF mass spectrometry, providing 2 main signals at m/z 1,086.9 and 1,491.2 as (M + H) (Fig. 4). The m/z 1,491.2 corresponded to the lipopeptide fengycin B, but the m/z 1,086.9 did not correspond to any known lipopeptide. Each of the 2 ions was utilized as a precursor ion for further ESI-MS/MS analysis. Fig. 5 shows the product patterns determined by ESI-MS mass spectrometry for m/z 1,491.2, but m/z 1,086.9 was not analyzed. As constituents of the peptide and lipophilic part of the m/z 1,491.2, 10 amino acids (Ile-Tyr-Gln-Pro-Val-Glu-Ser-Tyr-Orn-Glu) and β-hydroxy-C17 fatty acid were identified *via* ESI-MS/MS. The product ions of m/z 980.8 and 1,094.9 in Fig. 3 were identified as Ile < -Tyr < -Gln < -Pro < -Val < -Glu < -Tyr and Ile < -Tyr < -Gln < -Pro < -Val < -Glu < -Tyr < -Orn (increased 114). The residue of m/z 1,941.2 was 397.25 (fatty acid (C_{17})-Glu). Structurally, the novel 1,491.2 Da lipopeptide was similar to fengycin B, but a component of the amino acid was different in that the threonine residue in position 4 was replaced by serine. The

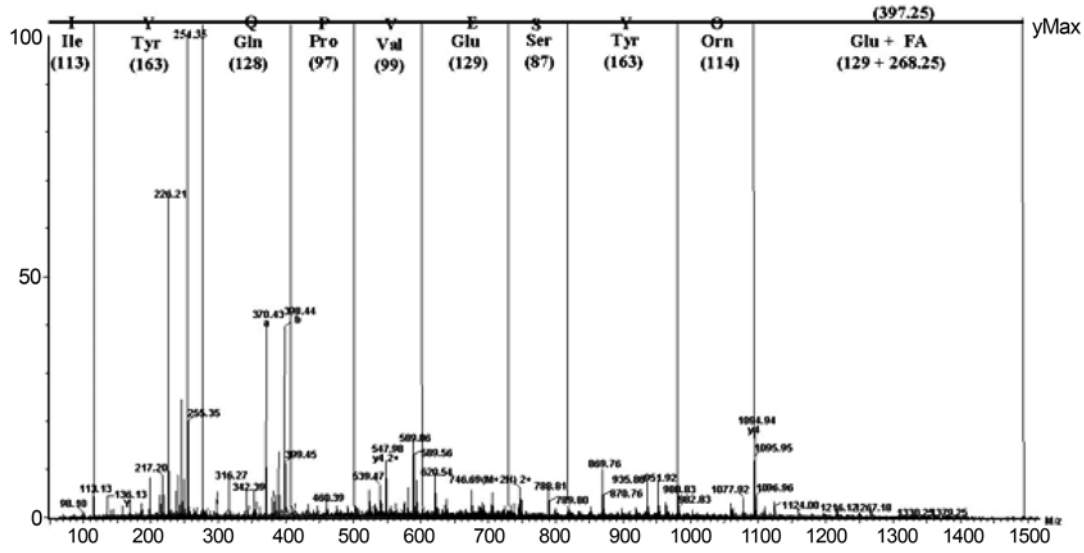

Fig. 5. ESI-MS/MS spectrum of the precursor ion m/z 1,941.

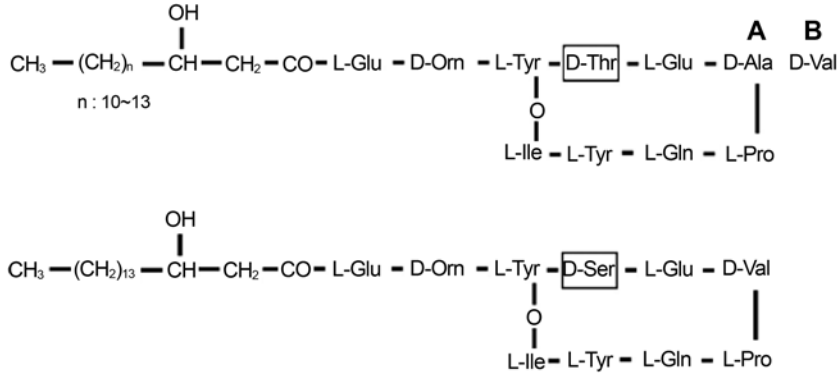

Fig. 6. Structure of fengycin A, B, and novel fengycin S of lipopeptide biosurfactants.

structure of fengycin A, B, and fengycin S are displayed in Fig. 6. Therefore, we designated the compound with a molecular mass of 1,491.2 Da as fengycin S.

Bacillus species are well-known to produce lipopeptides, such as bacillomycin, fengycin (or plipastatin), iturin, lichenysin, mycosubtilin, and surfactin [10]. The fengycin of lipopeptides is a well-known anti-fungal substance [11]. Moreover, it evidences strong properties as a lipopeptide biosurfactant [14]. Prominent groups of mass peaks in lipopeptides were observed in a mass range between m/z 1,000 and 1,530 by MALDI-TOF mass spectra [13]. The m/z 1,491 in the fengycin cluster was identified as C_{16}-Fengycin (M + H) [14,15]. Structurally, fengycin is composed of a cyclic chain of 10 amino acid residues containing a β-hydroxy fatty acid in the side chain [14]. In our analysis of ESI-MS/MS, fengycin S differs from fengycin A by 2 amino acid residues (threonine versus serine and alanine versus valine), and fengycin B by only a single amino acid residue (threonine versus serine). Fengycin exists as a variety of isoforms [14,15] because it changes from a carbon number in the fatty acid moiety of C_{14} to C_{17} and from amino acid moieties of 4 to 6.

4. Conclusion

In this study, the isolated and purified biosurfactant was designated as fengycin S, and was compared with fengycin A and fengycin B. *B. amyloliquefaciens* LSC04 was identified as a potential producer. It may potentially be used, either directly on oil spills in contaminated environments, or for the biotechnological production of biosurfactants.

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References

1. Kretschner, A., H. Bock, and F. Wagnee (1982) Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erythropolis* grown on *n*-alkane. *Appl. Environ. Microbiol.* 44: 864-870.
2. Cho, W. S., E. H. Lee, E. H. Shim, J. S. Kim, H. W. Ryu, and K. S. Cho (2005) Bacterial communities of biofilms sampled from seepage groundwater contaminated with petroleum oil. *J. Microbiol. Biotechnol.* 15: 952-964.
3. Baek, K. H., H. S. Kim, S. H. Moon, I. S. Lee, H. M. Oh, and B. D. Yoon (2004) Effects of soil types on the biodegradation of crude oil by *Nocardia* sp. H17-1. *J. Microbiol. Biotechnol.* 15: 901-905.
4. Banat, I. M (1995) Characterization of biosurfactants and their use in pollution removal-state of the art. *Acta Biotechnol.* 15: 251-267.
5. Desai, J. D. and I. M. Banat (1997) Microbial production of surfactants and their commercial potential. *Micrbiol. Mol. Biol. Rev.* 61: 47-64.
6. Zajic, J. E., H. Guignard, and D. F. Gerson (1977) Properties and biodegradation of a bioemulsifier from *Corynebacterium hydrocarboclatus*. *Biotechnol. Bioeng.* 19: 1303-1302.
7. Rosenberg, E. and E. Z. Ron (1999) High- and low-molecular-mass microbial surfactants. *Appl. Microbiol. Biotechnol.* 52: 154-162.
8. Kuiper, I., E. L. Lagendijk, R. Pickford, J. P. Derrick, G. E. Lamers, J. E. T. Oates, B. J. Lugtenberg, and G. V. Bloemberg (2004) Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. *Mol. Microbiol.* 51: 97-113.
9. Roongsawang, N., J. Thaniyavarn, S. Thaniyavarn, T. Kameyama, M. Haruki, T. Imanaka, M. Moriawa, and S. Kanaya (2002) Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. *Extremophiles* 6: 499-506.
10. Steller, S. and J. Vater (2000) Purification of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213. *J. Chromatogr. B: Biomed. Sci. Appl.* 737: 267-275.
11. Hou, X., S. M. Boyetchko, M. Brkic, D. Olson, A. R. S. Ross, and D. D. Hegedus (2006) Characterization of the anti-fungal activity of a *Bacillus* spp. associated with sclerotia from *Sclerotinia scelerotiorum*. *Appl. Microbiol. Biotechnol.* 72: 644-653.
12. Ongena, M., P. Jacques, Y. Toure, J. Destain, A. Jabrane, and P. Thonart (2005) Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* 69: 29-38.
13. Steller, S., D. Vollenbroich, F. Leenders, T. Stein, B. Conrad, J. Hofemeister, P. Jacques, and J. Vater (1999) Structural and functional organization of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213 and A1/3. *Chem. Biol.* 6: 31-41.
14. Vater, J., B. Kablitz, C. Wilde, P. Franke, N. Mehta, and S. S. Cameotra (2002) Matrix-assisted laser desertion ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. *Appl. Environ. Microbiol.* 68: 6210-6219.
15. Wang, J., J. Liu, X. Wang, J. Yao, and Z. Yu (2004) Application of electrospray ionization mass spectrometry in rapid typing of fengycin homologues produced by *Bacillus subtilis*. *Lett. Appl. Microbiol.* 39: 98-102.
16. Cutting, S. M. and H. P. B. Vander (1990) Genetic analysis. pp. 27-74. In: C. R. Harwood and S. M. Cutting (eds). *Molecular Biological Methods for Bacillus*. John Wiley & Sons, Ltd., NJ, USA.
17. Magaritis, A., K. Kennedy, J. E. Zajic, and D. F. Gerson (1979) Biosurfactant production by *Nocardia erythropolis*. *Dev. Ind. Microbiol.* 20: 623-630.
18. Calvo, C., C. F. Martinez, A. Mota, V. Bejar, and E. Quesada (1998) Effect of cations, pH and sulfate content on the viscosity and emusifying activity on the *Halomonas eurithalina*. *J. Ind. Microbiol. Biotechnol.* 20: 205-209.
19. Cirigliano, M. C. and G. M. Carman (1985) Purification and characterization of liposan, a bioemusifier from *Candida lipolytica*. *Appl. Environ. Microbiol.* 50: 846-850.
20. Eppard, M. W., E. Krrumbein, C. Kock, E. Rhiel, J. T. Sraley, and E. Stackebrandt (1996) Morphological, physiological, and molecular characterization of actinomycetes isolated from dry soil, rocks, and monuments surfaces. *Arch. Microbiol.* 166: 12-22.
21. Gray, J. P. and R. P. Herwig (1996) Phylogenetic analysis of the bacterial of the bacterial communities in marine sediments. *Appl. Environ. Microbiol.* 62: 4049-4059.
22. Kuske, C. R., S. M. Bams, and J. D. Busch (1997) Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. *Appl. Environ. Microbiol.* 63: 3614-3621.
23. Siefert, J. L., S. M. Larios, L. K. Nakamura, R. A. Slepecky, J. H. Paul, E. R. Moore, G. E. Fox, and P. J. Jurtshuk (2000) Phylogeny of marine *Bacillus* isolates from the Gulf of Mexico. *Curr. Microbiol.* 41: 84-88.
24. Wise, W. G, J. V. McArthur, and L. J. Skimkets (1997) Bacterial diversity of a Carolima bay as determined by 16S rRNA gene analysis: confirmation of novel taxa. *Appl. Environ. Micrbiol.* 63: 1505-1514.
25. Reva, O. N., C. Dixelius, J. Meijer, and F. G. Priest (2004) Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *FEMS Ecol. Microbiol.* 48: 249-259.
26. Chun, J. and K. S. Bae (2000) Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequence. *Antonie van Leewenhoek* 78: 123-127.
27. Lee, S. C., Y. J. Jung, J. S. Yoo, Y. S. Cho, I. H. Cha, and Y. L. Choi (2002) Characteristic of biosurfactants produced by *Bacillus* sp. LSC11. *Kor. J. Life Sci.* 12: 745-751.
28. Kim, S. H., E. J. Lim, S. O. Lee, J. D. Lee, and T. H. Lee (2000) Purification and characterization of biosurfactants from *Nocardia* sp. L-417. *Biotechnol. Appl. Biochem.* 31: 249-253.
29. Suk, W. S., H. J. Son, G. Lee, and S. J. Lee (1999) Purification and characterization of biosurfactants produced by *Pseudomonas* sp. SW 1. *J. Microbiol. Biotechnol.* 9: 56-61.
30. Yakimov, M. M., K. N. Timmis, V. Wray, and H. L. Fredrickson (1995) Characterisation of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl. Environ. Microbiol.* 61: 1706-1713.
31. Batista, S. B., A. H. Mounteer, F. R. Amorim, and M. R. Totola (2005) Isolation and characterization of biosurfactant/bioemusifier producing bacteria from petroleum contaminated sites. *Bioresour. Technol.* 97: 868-875.
32. Kim, S. H., S. C. Lee, I. H. Park, J. S. Yoo, W. H. Joo, C. W. Hwang, and Y. L. Choi (2005) Isolation and characterization of biosurfactant from *Bacillus atrophaeus* DYL-130. *Kor. J. Life Sci.* 15: 679-684.
33. Lee, S. C., J. S. Yoo, S. H. Kim, S. Y. Chung, C. W. Hwang, W. H. Joo, and Y. L. Choi (2006) Production and characterization of lipopeptide biosurfactant from *Bacillus subtilis* A8-8. *J. Microbiol. Biotechnol.* 16: 716-723.