NOTE

Screening of a Bacillus subtilis Strain Producing Multiple Types of Cyclic Lipopeptides and Evaluation of Their Surface-tension-lowering Activities

Hiroshi Habe¹,* , Toshiaki Taira² and Tomohiro Imura²

¹ Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, JAPAN
² Research Institute for Chemical Process Technology, National Institute of Advanced Industrial Science and Technology (AIST), Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN

Abstract: Sixty Bacillus subtilis strains were investigated for their ability to produce cyclic lipopeptides (CLPs). Among them, B. subtilis NBRC 109107 produced at least three types of CLPs by high-performance liquid chromatography (HPLC) analysis, and these CLPs were thought to be surfactin, iturin A, and fengycin by polymerase chain reaction amplification of respective CLP synthetase-encoding genes. However, after HPLC fractionation and purification, structural analysis of the CLPs revealed that these were surfactin homologues, iturin A, and unknown CLPs, whose surface-tension-lowering activities were 29.4, 56.7, and 48.6 mN/m, respectively. By contrast, fengycin was not detected.

Key words: cyclic lipopeptide, surfactin, iturin, Bacillus subtilis, biosurfactant

1 INTRODUCTION

Biosurfactants (BSs) are amphiphilic and surface-active compounds that can be produced by a variety of microorganisms from renewable resources and that have some advantages over chemically synthesized surfactants¹⁻³. Microbial cyclic lipopeptides (CLPs) are one of the five major classes of BSs, and they have received a lot attention from industries due to their excellent interfacial and biological activities, and their environmentally friendly characteristics⁴⁻⁵. In particular, surfactin is of interest because it is the most common CLP produced by the genus Bacillus⁶.

Surfactin can reduce the surface tension of an aqueous solution from 72 to 27 mN/m at concentrations to the order of 10⁻³ M⁶, indicating that it is one of the most powerful BSs so far. In addition, surfactin is reported to exhibit biological activities such as antiviral⁷, antifungal⁸, antitumor⁹, and hemolytic¹⁰ properties. Surfactin is known to act as an ionophore, wherein alkali metal ions can be trapped in the cyclic peptide¹¹⁻¹³. Recently, Taira et al. demonstrated the specific interaction between surfactin and cesium ion (Cs⁺) and succeeded in highly efficient removal of Cs⁺ from water using giant micelles as a natural sorbent¹⁴.

In addition to surfactins, the well-known families of CLPs are the iturins¹⁵⁻¹⁸ and the fengycins¹⁷,¹⁸. Other CLPs such as kurstarkin have also identified¹⁹. Both iturins and fengycins are known to have antifungal activities, and fengycin more strongly inhibits the growth of filamentous fungi²⁰; however, little is known about the interfacial properties of these CLPs, especially fengycins.

Singh et al. investigated the carbon-source-dependent surface tension reduction by Bacillus amylolafaciens strain AR2. The surface tension of culture media was in the range of 30–37 mN/m depending on the types of carbon sources²¹. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis revealed the presence of two prominent and separate peaks corresponding to the iturins and the fengycins when sucrose, dextrose, or glycerol was used as a carbon source²¹. However, the surface-tension-lowering activity of CLPs extracted and purified from culture media have not been determined.

*Correspondence to: Hiroshi Habe, Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, JAPAN
E-mail: hiroshi.habe@aist.go.jp
Accepted March 17, 2017 (received for review February 10, 2017)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs

785
In contrast to surfactin, which contains seven amino acid residues and a β-hydroxy fatty acid, iturin is a CLP with a β-amino fatty acid linked by amide bonds to the constituent amino acid residues, and fengycin is a CLP with 10 amino acid residues containing unusual amino acids such as ornithine and allo-threonyl. Considering these structural differences, the comparative studies of interfacial properties between surfactin and other CLPs will be important for expanding a variety of CLP surfactants.

The goal of this study was to investigate interfacial properties of CLPs other than the surfactins. First, we screened a *B. subtilis* strain, producing multiple types of CLPs including surfactin, and then we separated each CLP by high-performance liquid chromatography (HPLC). We also detected gene fragments for CLP synthesis in the selected strain by polymerase chain reaction (PCR).

2 EXPERIMENTAL

2.1 Materials

All reagents and solvents were of the highest purity commercially available. Authentic surfactin sodium salt was kindly supplied by Kaneka Corporation, Japan.

2.2 Apparatus

HPLC analysis of CLPs was performed by gradient elution with a Shimadzu LC-MS 2020 system (Shimadzu, Otsu, Japan) equipped with a SOURCE™ 15RPC (4.6 × 100 mm, GE Healthcare, Buckinghamshire, UK). The mobile phase, composed of solvent A (acetonitrile:water (80:20) containing 0.4% (v/v) acetic acid) and solvent B (acetonitrile: water (20:80) containing 0.4% (v/v) acetic acid), was used at a flow rate of 1.0 mL/min, and the column was kept at 30°C during analysis. The samples were detected and quantified at 210 nm using a UV detector.

MALDI-TOF-MS analysis was carried out with autoflex speed TOF/TOF (Bruker Daltonics, Inc.), and synthetic acid was used as the matrix. The instrument was operated in reflector positive-ion RP mode employing a mass range of 400–3,000 m/z. One μL of fractionated and concentrated CLP solutions was spotted on top of 1 μL of saturated matrix solution (acetonitrile:water = 1:1) over the MALDI plate using a droplet spotting method to ensure that mixing occurs within the drop.

The surface tension of the aqueous solution was determined by the Wilhelmy plate method at 25°C using a DY-500 surface tension meter (Kyowa Kaimen Kagaku Co., Niiza, Japan), and its accuracy was intermittently checked using ultrapure water.

2.3 Bacterial strains, media, and cultivation

Sixty *B. subtilis* strains were obtained from the National Institute of Technology and Evaluation (NITE) of Japan. The NBRC numbers are as follows: 3009, 3013, 3026, 3027, 3018, 3134, 3214, 3215, 3335, 3336, 3513, 3936, 12112, 12113, 12114, 12210, 13169, 13719, 13722, 14132, 14133, 14140, 14144, 14191, 14192, 14557, 14411, 14412, 14413, 14414, 14415, 14417, 14418, 14419, 14473, 14474, 16449, 100519, 100520, 100521, 104440, 104443, 104449, 104461, 104463, 104468, 109107, 101239, 101240, 102143, 101245, 101246, 101247, 101581, 101582, 101584, 101585, 101590, 101592. Stock cultures were grown using media and temperatures recommended by the NITE. All strains were successfully cultured on NITE medium number 702 (designated as 702 medium) containing 10 g/L of hipolypeptin (Wako Pure Chemical Industry), 2 g/L of yeast extract (Difco), and 1 g/L of MgSO₄·7H₂O (pH 7.0). For screening of CLP-producing strains, a single colony of each strain was added to 5 mL of 702 medium in a test tube and incubated aerobically at 37°C on a reciprocal shaker (200 rpm) for 24 h. When the growth of bacteria was observed, the seed cultures (0.5 mL) were transferred to 300-mL Erlenmeyer flasks containing 30 mL of 702 medium. The flasks were incubated for another 24 h at 37°C on a rotary shaker (200 rpm). After removing the cells by centrifugation, 1 mL of the respective supernatants was acidified with concentrated HCl and extracted with 1 mL of ethyl acetate. The ethyl acetate extracts were dried and then dissolved in an appropriate amount of methanol for HPLC.

2.4 PCR amplification of the synthetic genes for CLPs

Total DNA was isolated from *B. subtilis* NBRC 109107 grown on 702 medium according to standard protocols. Using total DNA from the strain as a template, the non-ribosomal peptide synthase-encoding genes were amplified by PCR with the forward and reverse primer sets SrfA-AF (5′-ACACAGATATCAGGCAAGC-3′) and SrfA-AR (5′-GTCCCATGTTCTTCA-3′) for srfA (surfactin, 908 bp PCR product), LocDF (5′-TCAGTTACACGATGAAACAATTGT-3′) and LocDR (5′-TTGTCATTACGATCAGTGTT-3′) for locD (locilinomycin, 812 bp PCR product), FenAF (5′-GCTTTGATGGTGCACTAGCA-3′) and FenAR (5′-CTG-GACCTGGTTGTCTTGGT-3′) for fenA (fengycin, 729 bp PCR product), ITUP1-F (5′-AGCTTAGGAACATATTGCTATGGGCTTCTC-3′) and ITUP1-R (5′-AAGAGGTTGATCAGCCGCGC-3′) for the intergenic region between ituA and ituB (iturin A, 2003 bp PCR product), LchAF (5′-ACTGAACGATTGCAAGTATT-3′) and LchAR (5′-TCTGCTCATATTGGTGCTTCT-3′) for lchA (lichenysin, 472 bp PCR product). PCR reactions were performed using *Premix Taq™* (Takara Shuzo, Kyoto, Japan) as recommended by the manufacturer, and the PCR conditions were as described previously.

2.5 Production, separation, and characterization of CLPs

For large-scale production of CLPs for separation and subsequent characterization, *B. subtilis* NBRC 109107 was
A Bacillus subtilis strain producing multiple types of cyclic lipopeptides

3 RESULTS AND DISCUSSION

3.1 Screening of Bacillus subtilis strains producing multiple types of CLP

In the first screening, 60 B. subtilis strains were examined for their ability to produce CLPs using 702 medium. As the medium was not necessarily suitable for all strains, the amounts of CLPs analyzed by HPLC were only traceable or not detected in 39 strains.

The other strains yielded at least one CLP from the main peak regions, as shown in Fig. 1 (CLP-I, CLP-II, and CLP-III). Among the remaining 21 strains, 13 strains gave one peak region: four strains (NBRC numbers 14413, 14414, 14415, and 14418) in the CLP-I peak region, one strain (NBRC 101245) in the CLP-II peak region, and eight strains (NBRC numbers 14132, 14133, 14412, 16449, 104468, 101239, 101247, and 101584) in the CLP-III peak region. In addition, two peak regions of both CLP-II and CLP-III were confirmed in seven strains (NBRC numbers: 13719, 104440, 104443, 104449, 104463, 101246, and 101590). By contrast, all three peak regions were detected in strain NBRC 109107 (Fig. 1). Therefore, strain NBRC 109107 was used for further analysis.

The carbon source is known to be an important factor influencing microbial BSs production. Not only was the CLP yield affected, but the CLP types were also affected by media components including the carbon source\(^{21,27,28}\). Considering the finding that B. amyloliquefaciens AR2 produced both the iturins and the fengycins when sucrose, dextrose, or glycerol was used as a carbon source, but only produced the iturins when maltose, lactose, or sorbitol was used\(^{21}\), we expect that different CLP production results would be obtained if media other than the 702 medium were used in the first screening.

3.2 Detection of the presence of CLP synthetase-encoding genes in the B. subtilis NBRC 109107 genome

To predict the types of CLPs produced by strain NBRC

---

![HPLC profile of products extracted from Bacillus subtilis NBRC 109107 culture supernatant with ethyl acetate after acidification.](image)
109107, the presence of the first structural genes of the surfactin A synthetase operon (sfAA), the locillomycin synthetase operon (locD), the fengycin synthetase operon (fenA), the iturin A synthetase operon (itergenic region between ituA and ituB), and the lichenysin synthetase operon (lchAA) was investigated by PCR. The designed DNA regions within srfAA, fenA, and ituA-B were amplified, but locD and lchAA were not. There are many reports of B. subtilis strains producing surfactin, fengycin, and iturin together, so the detection of these three genes in one strain is understandable. Hence, these three peak regions were thought to be surfactin, iturin A, and fengycin by PCR analysis.

3.3 Separation and structure analysis of CLPs produced by B. subtilis NBRC 109107

For separation and subsequent characterization, enough CLP needed to be collected. The 5-L jar fermenter experiments were repeated 10 times, and the CLP-containing extracts from culture were mixed and concentrated. The resultant CLPs were separated and purified by HPLC (Fig. 1). After fractionation of CLP-I, II, and III, each fraction was concentrated, and their concentrations were measured by HPLC. The resultant concentrations for CLP-I, II, and III were 0.12, 0.08, and 1.84 g/L, respectively, by comparing their peak areas with that of 1 g/L authentic surfactin. Then, the CLPs were analyzed by MALDI-TOF-MS (Table 1).

A MALDI-TOF-MS spectral analysis showed that CLP-III contained several molecules having m/z 1053, 1039, 1035, 1025, 1021, and 1007 (Table 1). Since authentic surfactin showed its HPLC peaks in the CLP-III region, these spectra were putative surfactin homologues. The spectra of the major molecules[M]+ observed at m/z 1035, 1021, and 1007 corresponded to those of the authentic surfactin. The differences in mass of 14 Da suggested a series of homologues having C15, C14, and C13 β-hydroxy fatty acids. Microbial CLPs sometimes contain small amounts of their linear form, in which the lactone ring is cleaved to form a linear conformation. The results suggested that the surfactin homologues gained a mass of 18 Da by hydrolysis of the lactone ring; therefore, the linear molecules observed at m/z 1053, 1039, and 1025 were derived from surfactin.

The MALDI-TOF-MS spectra of CLP-I observed at m/z 1058, 1044, and 1030 were putative iturin homologues because the spectrum of the major [M]+ observed at m/z 1058 was the same as the spectrum reported for iturin A (C15)29. These spectra revealed a difference in mass of 14 Da, suggesting a series of homologues having C14 and C13 fatty acids. The amino acid compositions of CLP-I was analyzed by HPLC after PTC-derivatization of CLP-I hydrolysate. CLP-I contained Asx, Pro, Ser, Tyr, and Glx in a molar ratio of 3:1:1:1:1 (Fig. 2), which are the same sequences of iturin A in previous reports29, 31.

Table 1 Possible assignment of the structures and measurement of the surface-tension-lowering activities of cyclic lipopeptides (CLPs) in HPLC peak regions.

| Peak region | Main mass peak (m/z) | Possible assignment of structure | Concentration (M)a | Surface-tension-lowering activity (mN/m) |
|-------------|---------------------|---------------------------------|-------------------|----------------------------------------|
| CLP-I       | 1058, 1044, 1030    | Iturin A class with C15, C14, and C13 β-amino fatty acid | 3.1 × 10⁻³       | 56.7                                   |
| CLP-II      | 1043, 1073          | Unknown                         | 3.5 × 10⁻³       | 48.6                                   |
| CLP-III     | 1035, 1021, 1007    | Surfactin class with C15, C14, and C13 β-hydroxy fatty acid | 1.3 × 10⁻³       | 29.4                                   |
|             | 1053, 1039, 1025    | Surfactin class (linear form) with C15, C14, and C13 β-hydroxy fatty acid |                |                                        |

a The concentrations of CLPs in CLP-I to CLP-III regions used for evaluating surface-tension-lowering activity were measured by comparing their peak areas from HPLC with that of authentic surfactin.
A Bacillus subtilis strain producing multiple types of cyclic lipopeptides

J. Oleo Sci. 66, (7) 785-790 (2017)

By contrast, the spectra of CLP-II exhibited the major [M]+ at m/z 1073 and 1043. However, the spectra of CLP-II were different from the data that were previously reported for fengycin[11], and the compounds could not be identified based on the MALDI-TOF-MS analysis alone. According to the results of the PCR, strain NBRC 109107 should produce the fengycin homologues. This is probably due to unsuitable medium composition or deletion of subunit genes other than fengycin.

3.4 Surface-tension-lowering activities of the CLPs

After CLP-I, II, and III were further concentrated and neutralized with NaOH to form their sodium salt, the concentrations of each CLP sample were determined again by the Wilhelmy plate method, and the decrease in the surface tension was observed in respective groups (Table 1). The surface tension of respective CLPs in water was evaluated by the Wilhelmy plate method, and the decrease in the surface tension was observed in respective groups (Table 1).

The surface tension of CLP-III at 1.7 × 10⁻³ M (above the CMC) was 29.4 mN/m. This value was expected because CLP-III is a surfactin homolog, whose surface tension in an aqueous solution at a concentration on the order of 10⁻⁵ M is 27 mN/m[6].

By contrast, the surface tension of CLP-I at 3.1 × 10⁻³ M (above the CMC)[10] was 56.7 mN/m. This value was expected because the surface tension in an aqueous solution was reduced from 72 to around 52 mN/m by iturin A[10].

Although the compounds have not been identified, the surface tension of CLP-II at 3.5 × 10⁻³ M was 48.6 mN/m, which is superior to that of iturin A.

4 CONCLUSION

Among sixty tested strains, we found that Bacillus subtilis NBRC 109107 produced multiple types of CLPs in our screening medium. Although the exact amounts of each CLP in the batch culture is unknown, strain NBRC 109107 produced surfactin homologues, iturin A, and unknown compounds showing surface-tension-lowering activity. To clarify the structure of the unknown CLP and the reason that the strain did not produce fengycin, the draft genome analysis of B. subtilis NBRC 109107 is now underway.

ACKNOWLEDGMENT

This work was financially supported by Kaneka Corporation, Japan. The authors thank Susumu Kondo and Kiyomi Fujimata for technical assistance.

References

1) Flechter, A. Biosurfactant: moving towards industrial application. Trends Biotechnol. 10, 208-217 (1992).
2) Kitamoto, D.; Isoda, H.; Nakahara, T. Functions and potential applications of glycolipid biosurfactants: from energy-saving materials to gene delivery carriers. J. Biosci. Bioeng. 94, 187-201 (2002).
3) Mulligan, C.N. Environmental applications for biosurfactants. Environ. Pollut. 133, 183-198 (2005).
4) Ongena, M.; Jacques, P. Bacillus lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. 16, 115-125 (2007).
5) Mnif, I.; Ghribi, D. Lipopeptides biosurfactants: mean classes and new insights for industrial, biomedical, and environmental applications. Biopolymers 104, 129-147 (2015).
6) Peypoux, F.; Bonmatin, J.M.; Wallach, J. Recent trends in the biochemistry of surfactin. Appl. Microbiol. Biotechnol. 51, 553-563 (1999).
7) Vollenbroich, D.; Ozel, M.; Vater, J.; Kamp, R.M.; Pauli, G. Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from Bacillus subtilis. Biologicales 25, 289-297 (1997).
8) Tendulkar, S.R.; Saikumari, Y.K.; Patel, V.; Raghotama, S.; Munshi, T.K.; Balamur, P.; Chattou, B.B. Isolation, purification and characterization of an antifungal molecule produced by Bacillus licheniformis BC98, and its effect on phytophathogen Magnaporthe grisea. J. Appl. Microbiol. 103, 2331-2339 (2007).
9) Kamed, Y.; Kato, H.; Sagai, H.; Yamada, T.; Matsui, K. Antitumor activity of Bacillus natto. 3. Isolation and characterization of a cytolytic substance on Ehrlich ascites carcinoma cells in culture medium of Bacillus natto-KMD 1126. Chem. Pharm. Bull. 20, 1551-1553 (1974).
10) Kracht, M.; Rokos, H.; Ozel, M.; Kowall, M.; Pauli, G.; Vater, J. Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. J. Antibiot. 52, 613-619 (1999).
11) Arutchelvi, J.; Sangeetha, J.; Philip, J.; Doble, M. Self-assembly of surfactin in aqueous solution: role of divalent counterions. Colloids Surf. B 116, 396-402 (2014).
12) Déjugnat, C.; Diat, O.; Zemb, T. Surfactin self-assembles into direct and reverse aggregates in equilibrium and performs selective metal cation extraction. ChemPhysChem 12, 2138-2144 (2011).
13) Li, Y.; Zou, A.-H.; Ye, R.-Q.; Mu, B.-Z. Counterion-induced changes to the micellization of surfactin-C₁₆ aqueous solution. J. Phys. Chem. B 113, 15272-15277 (2009).
14) Taira, T.; Yanagisawa, S.; Nagano, T.; Zhu, Y.; Kuroiwa, T.; Koumura, N.; Kitamoto, D.; Imura, T. Selective encapsulation of cesium ions using the cyclic peptide

J. Oleo Sci. 66, (7) 785-790 (2017)
moiety of surfactin: highly efficient removal based on an aqueous giant micellar system. Colloids Surf. B 134, 59-64 (2015).

15) Hiradate, S.; Yoshida, S.; Sugie, H.; Yada, H.; Fujii, Y. Mulberry anthracnose antagonists (iturins) produced by Bacillus amyloliquefaciens RC-2. Phytochemistry 61, 693-698 (2002).

16) Yu, G.Y.; Sinclair, J.B.; Hartman, G.L.; Bertagnolli, B.L. Production of iturin A by Bacillus amyloliquefaciens suppressing Rhizoctonia solani. Soil Biol. Biochem. 34, 955-963 (2002).

17) Vanittanakom, N.; Loeffler, W.; Koch, U.; Jung, G. Fengycin—a novel antifungal lipopeptide antibiotic produced by Bacillus subtilis F-29-3. J. Antibiot. 39, 888-901 (1986).

18) Villegas-Escarob, V.; Ceballos, I.; Mira, J.J.; Argel, L.E.; Peralta, S.O.; Romero-Tabarez, M. Fengycin C produced by Bacillus subtilis EA-CEB0015. J. Nat. Prod. 76, 503-509 (2013).

19) Hathout, Y.; Ho, Y.P.; Ryzhov, V.; Demirev, P.; Fenselau, C. Kurstakins: a new class of lipopeptides isolated from Bacillus thuringiensis. J. Nat. Prod. 63, 1492-1496 (2000).

20) Liu, J.; Zhou, T.; He, D.; Li, X.-Z.; Wu, H.; Liu, W.; Gao, X. Functions of lipopeptides Bacillomycin D and fengycin in antagonism of Bacillus amyloliquefaciens C06 towards Monilia flaccum. J. Mol. Microbiol. Biotechnol. 20, 43-52 (2011).

21) Singh, A.K.; Rautela, R.; Cameotra, S.S. Substrate dependent in vitro antifungal activity of Bacillus sp strain AR2. Microb. Cell Fact. 13, 67 (2014).

22) Sambrook, J.; Russell, D.W. Molecular cloning: A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. (2001).

23) Luo, C.; Liu, X.; Zhou, H.; Wang, X.; Chen, Z. Nonribosomal peptide synthase gene cluster for lipopeptide biosynthesis in Bacillus subtilis 916 and their phenotypic functions. Appl. Environ. Microbiol. 81, 422-431 (2015).

24) Tsuge, K.; Inoue, S.; Ano, T.; Itaya, M.; Shoda, M. Horizontal transfer of iturin A operon, itu, to Bacillus subtilis 168 and conversion into an iturin A producer. Antimicrob. Agents Chemother. 49, 4641-4648 (2005).

25) Madslien, E.H.; Rønning, H.T.; Lindbæk, T.; Hassel, B.; Andersson, M.A.; Granum, P.E. Lichenysin is produced by most Bacillus licheniformis strains. J. Appl. Microbiol. 115, 1068-1080 (2013).

26) Nomura, T.; Habe, H.; Widad, J.; Chung, J.S.; Yoshida, T.; Nojiri, H.; Omori, T. Genetic characterization of the dibenzofuran-degrading Actinobacteria carrying the dbfA1A2 gene homologues isolated from activated sludge. FEMS Microbiol. Lett. 296, 233-240 (2004).

27) Akpa, E.; Jacques, P.; Wathelet, B.; Paquot, M.; Fuchs, R.; Budzikiewicz, H.; Thonart, P. Influence of culture conditions on lipopeptide production by Bacillus subtilis. Appl. Biochem. Biotechnol. 91, 511-561 (2001).

28) Li, J.-F.; Yang, J.; Yang, S.-Z.; Ye, R.-Q.; Mu, B.-Z. Effects of different amino acids in culture media on surfactin variants produced by Bacillus subtilis TD7. Appl. Biochem. Biotechnol. 166, 2091-2100 (2012).

29) Kim, I.-J.; Ryu, J.; Kim, H.Y.; Chl, Y.-T. Production of biosurfactant lipopeptides iturin A, fengycin, and surfactin A from Bacillus subtilis CMB32 for control of Colletorichum gloeosporioides. J. Microbiol. Biotechnol. 20, 138-145 (2010).

30) Taira, T.; Ikeda, S.; Kawamura, D.; Sakai, H.; Abe, M.; Kitamoto, D.; Imura, T. Monolayer behavior of cyclic and linear forms of surfactants: thermodynamic analysis of Langmuir monolayers and AFM study of Langmuir–Blodgett monolayers. J. Oleo Sci. 63, 407-412 (2014).

31) Vater, J.; Kablitz, B.; Wilde, C.; Franke, P.; Mehta, N.; Cameotra, S.S. Matrix-assisted laser desorption 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 (2004).

32) Yang, H.; Li, X.; Li, X.; Yu, H.; Shen, Z. Identification of lipopeptide isoforms by MALDI-TOF-MS/MS based on the simultaneous purification of iturin, fengycin, and surfactin by RP-HPLC. Anal. Bioanal. Chem. 407, 2529-2542 (2015).

33) Maget-Dana, R.; Thimon, L.; Peypoux, F.; Ptak, M. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on biological properties of iturin A. Biochimie 74, 1047-1051 (1992).

34) Razafindralambo, H.; Popineau, Y.; Deleu, M.; Hbib, C.; Jacques, P.; Thonart, P.; Paquot, M. Surface-active properties of surfactin/iturin A mixtures produced by Bacillus subtilis. Langmuir 13, 6026-6031 (1997).