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Production of Biosurfactant Lipopeptides Iturin A, Fengycin, and Surfactin A from *Bacillus subtilis* CMB32 for Control of *Colletotrichum gloeosporioides*

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A bacterial strain isolated from soil for its potential to control the anthracnose disease caused by *Colletotrichum gloeosporioides* was identified as a *Bacillus subtilis*. *Bacillus subtilis* CMB32 produced antifungal agents on M9 broth at 30°C. Biosurfactant lipopeptides produced by *Bacillus subtilis* CMB32 were precipitated by adjusting to pH 2 and extracting using chloroform/methanol, and then were purified using column chromatography and reverse-phase HPLC. The molecular masses of the lipopeptides were estimated by MALDI–TOF mass spectrometry as (a) 1,080, (b) 1,486, and (c) 1,044 Da, respectively. They had cyclic structures and amino acid compositions of (a) Pro, Asx, Ser, Tyr, Glx, (b) Glx, Tyr, Thr, Ala, Pro, Ile, and (c) Glx, Leu, Val, Asx, respectively. Further analysis revealed that *Bacillus subtilis* CMB32 produced three antifungal lipopeptides: (a) iturin A, (b) fengycin, and (c) surfactin A.

Keywords: *Bacillus subtilis* CMB32, *Colletotrichum gloeosporioides*, iturin A, fengycin, surfactin A, MALDI–TOF mass spectrometry

Anthracnose disease, which provokes heavy yield and quality losses in pepper, blueberry, mango, and other crops of commercial interest around the world, is caused by the fungal pathogen *Colletotrichum gloeosporioides*. To control various phytopathogenic fungi, including *C. gloeosporioides*, agrochemicals have been used for a long time. Widespread use of agrochemicals has certainly decreased the outbreak of fungal diseases, but at the same time has contributed to the development of resistant pathogens [3, 36]. Moreover, such chemicals can be lethal to beneficial microorganisms in the rhizosphere and useful soil insects, and they may also enter the food chain and accumulate in the human body as undesirable chemical residues [2].

To overcome the above problems, a non-hazardous alternative such as biological control has been extensively studied, and various microorganisms have been reported in the literature to suppress the phytopathogenic fungi [1, 6, 15, 30, 31, 48].

Antibiotics produced by some bacteria are responsible for disease suppression [35]. Gram-negative and Gram-positive bacteria have been extensively studied for production of a variety of antibacterial and antifungal antibiotics, such as zwittermicin-A [16], kanosamine [41], and lipopeptide biosurfactants [24, 42]. Surfactant [7, 18], bacillomycin [5], iturin A [51], mycosubtilin [34], plipastatin [32], halobacillin [44], lichenysins A/B/C/G [14, 20, 27, 49], and fengycin [21] are biosurfactant lipopeptides produced by *Bacillus* strains. These cyclic lipopeptides produced by *Bacillus* strains are also used as biocontrol agents for plant disease reduction [10, 21, 26, 29, 50, 51]. Among them, iturins and fengycins exhibit powerful antifungal activity and growth inhibition against a wide range of phytopathogens (Figs. 7A and 7B) [21, 22]. Surfactins are not toxic for fungal pathogens by themselves but sustain some synergistic effect on the antifungal activity of iturin A (Fig. 7C) [28]. They act on phospholipids and are able to form selective ionic pores in lipid bilayers of cytoplasmic membranes [40].

The objectives of this study were to screen antifungal bacteria from soil for use in the biological control of phytopathogenic fungi. Additionally, biosurfactant lipopeptides produced by an antagonist were purified, characterized, and analyzed for its structural properties.

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MATERIALS AND METHODS

Microbial Strains and Culture Conditions

Bacillus subtilis CMB32, which was selected for this study, was isolated from soil in Gwangju, Korea. Soil samples (1 g) were 10-fold serially diluted in phosphate-buffered saline (PBS, 0.05 M, pH 7.4) and soil suspensions were plated on Luria–Bertani (LB) agar. The plates were incubated at 37°C for 1 day and kept on LB broth at −80°C as 20% glycerol stocks. The phytopathogenic fungi, *Botrytis cinerea* KACC 40573, *Fusarium oxysporum* KACC 40037, *Rhizoctonia solani* AG-2-2 (IIIB) KACC 40151, *Phytophthora capsici* KACC 40157, and *Fusarium solani* KACC 40037, were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Korea. *Colletotrichum gloeosporioides* KCTC 6169 and *Fusarium solani* KCTC 6328 were kindly provided by the Korean Collection for Type Collection (KCTC), Daejeon, Korea. The pathogenic fungi were incubated at 25–28°C on potato dextrose agar (PDA) (Acumedia, Lansing, MI, U.S.A.).

Screening and Identification of Antifungal Bacteria

Bacterial isolates were tested for their potential to inhibit the growth of *C. gloeosporioides* in petri dishes on PDA medium. Soil samples (1 g) were suspended in 9 ml of PBS and then diluted serially. The diluted samples were incubated on LB agar at 37°C for 1 day and the bacterial isolates were obtained. The bacterial culture broths were loaded onto paper disks on the edges of the plates, and mycelial plugs (5 mm) of the fungal pathogen were deposited in the center, 60 mm away from the bacterial colonies. Fungal pathogens with slow growth were inoculated 24 h before the bacterial isolates. Inhibition of mycelial growth was evaluated after further incubation for 3 days. Of the bacterial isolates, the strain CMB32 showing the strongest activity was selected. For the determination of 16S rDNA sequences, bacterial genomic DNA was extracted using phenol [37]. The 16S rRNA genes were amplified using polymerase chain reaction (PCR) with the universal primers fD1 and rP2. The sequences of fD1 and rP2 were 5'-AGAGTTTGATCCTCCCTCTCAG-3' and 5'-ACGGCTAC CTTGTTACGACTT-3', respectively [47]. The amplification protocol consisted of one cycle of 94°C for 3 min, 35 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 2 min, and one cycle of 72°C for 7 min. Following amplification, the PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, U.S.A.) and sequenced with an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Wellesley, MA, U.S.A.).

Extraction and Purification of the Lipopeptides

After cultivating the cells on M9 broth at 30°C, the bacterial cells were removed from the lipopeptide-containing medium by centrifugation at 6,000 ×*g* for 20 min followed by aseptic filtration (0.45 μm). Biosurfactant lipopeptides were precipitated from the supernatant by adding 3 N HCl to obtain a final pH of 2.0 and then stored at 4°C for 30 min. The acid precipitates were collected by centrifugation at 8,000 ×*g* for 1 h at 4°C and dissolved in a chloroform/methanol (2:1, v/v) solvent system. The solution was evaporated under a vacuum. The dried materials were dissolved in methanol and filtered through a 0.2-μm nonpyrogenic hydrophilic membrane (Sartorius AG, Goettingen, Germany). The filtrates were applied to a Sephadex LH-20 column (16 mm ID×70 cm L; Amersham Biosciences, Uppsala, Sweden) and fractionated by size-exclusion chromatography by using the methanol solvent for elution. The products were monitored by absorbance at 215 nm. Fractions were pooled and concentrated with a rotary evaporator, and then antifungal activity was tested against *C. gloeosporioides* KCTC 6169. The lipopeptide fraction showing antifungal activity was loaded on a Zorbax SB-C_{18} column (9.4 mm ID×25 cm L, 5-μm particle diameter; Agilent Technologies Co.) and separated by high-resolution reverse-phase high-performance liquid chromatography (HPLC) by using a Shimadzu HPLC system [PREP-ODS C_{18}, 20 mm ID×25 cm L, 15-μm particle diameter; Shimadzu, Columbia, MD, U.S.A.]. The mobile phase components were (A) 0.1% trifluoroacetic acid (TFA) in 10% acetonitrile and (B) 0.1% TFA in acetonitrile. The products were eluted at a flow rate of 4 ml/min with a linear gradient of solvent B, developed from 0 to 100%. The elution pattern was monitored by determining absorbance at 215 nm, and pooled fractions were collected and concentrated *in vacuo*, and detected by MALDI–TOF mass spectrometry.

MALDI–TOF Mass Spectrometry Analysis

MALDI–TOF mass spectrometric analysis was performed by a Voyager DE-STR MALDI–TOF instrument (Applied Biosystems, Foster City, CA, U.S.A.) equipped with pulsed extraction and a nitrogen laser operating at 337 nm. For mass spectrometric analysis, fractions (0.5 μl) of biosurfactant lipopeptides purified by gel filtration on a Sephadex LH-20 column and reverse-phase HPLC were mixed with an equal volume of 0.1% solution of α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile–water–TFA [50:50:0.1 (v/v/v)] used as the matrix. Mass spectra were accumulated over 100 individual laser shots and obtained in the reflector mode at an initial accelerating voltage of 20 kV. A molecular mass gate of 350 Da improved the measurement by filtering out most matrix ions.

Amino Acid Analysis

Peptide bonds in the purified lipopeptides were hydrolyzed by boiling in 6 N HCl containing 1% phenol at 110°C for 22 h. Amino acid analysis was carried out according to the Waters Pico Tag method by pre-column derivatization with phenylisothiocyanate [9].

Determination of the Lactone Bond

To determine the lactone linkage, the lipopeptides were dissolved in 1 M KOH and allowed to react overnight at room temperature. The excess KOH was removed using ZipTip C_{18} (Millipore, Billerica, MA, U.S.A.). The samples were washed twice with 20 μl of 1% TFA, and then eluted from the cartridge with CHCA matrix solution. The reaction products were characterized by MALDI–TOF MS.

RESULTS

Screening and Identification of Antifungal Bacteria

For the selection of a potential antagonist to inhibit *C. gloeosporioides*, over 200 bacterial strains were isolated from soil. Among these isolates, strain CMB32 showed the strongest antagonistic effect (Fig. 1). Strain CMB32 also had antifungal activity against other fungal plant pathogens, including *Fusarium solani* KCTC 6328, *Botrytis cinerea* KACC 40573, *Fusarium oxysporum* KACC 40037, *Rhizoctonia solani* KACC 40151, and *Phytophthora capsici* KACC 40157 (data not shown). 16S rDNA sequence analysis

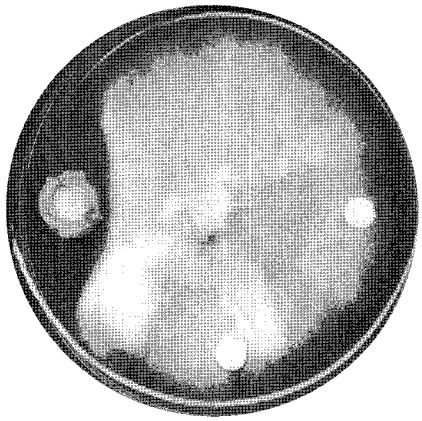

Fig. 1. Antifungal activity of *Bacillus subtilis* CMB32 against *Colletotrichum gloeosporioides*.
Growth inhibition of fungal mycelia was examined by pairing cultures on a PDA agar plate.

showed that strain CMB32 had high homology (≥95%) with *Bacillus subtilis* (data not shown).

Purification of Biosurfactant Lipopeptides

The biosurfactant lipopeptides from cell-free supernatant were purified by using Sephadex LH-20 column chromatography and the reverse-phase HPLC system (Fig. 2). After fractionation, active fractions were determined by monitoring antifungal activity against *C. gloeosporioides* (data not shown). In the fractions obtained, three lipopeptide classes were detected by MALDI–TOF mass spectrometry (Table 1). The table shows that there were well-resolved groups of peaks at *m/z* values between 1,066 and 1,110, between 1,450 and 1,544, and between 1,016 and 1,072, respectively. The groups of peaks could be attributed to

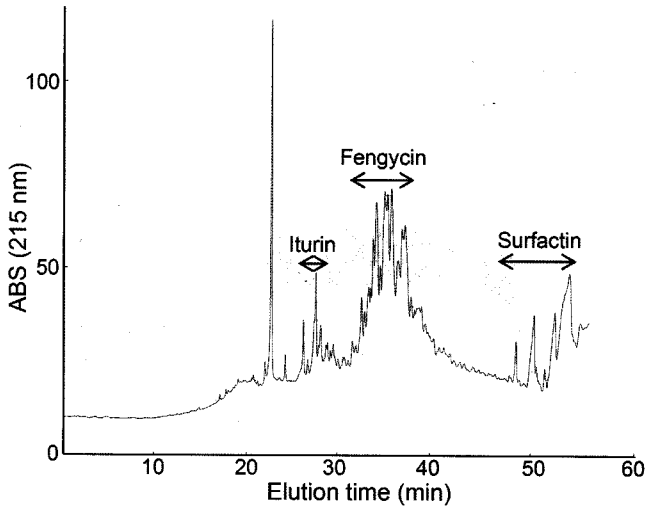

Fig. 2. HPLC chromatogram of compounds obtained from *Bacillus subtilis* CMB32 culture supernatant, after precipitation by adjusting the pH with 3 N HCl and extraction by a chloroform/methanol [2:1 (v/v)] solvent system.

Table 1. Main mass peaks of the lipopeptides produced from *Bacillus subtilis* CMB32 by MALDI–TOF mass spectrometry.

Fraction(s)	Main mass peak (*m/z*)	
28	1,066, 1,082	
29	1,066, 1,080, 1,082	Putative inturin class
30	1,080, 1,096	
31	1,080, 1,094, 1,096, 1,110	
32	1,094, 1,108, 1,110	
33	1,094, 1,108, 1,436	
34	1,450, 1,472, 1,488	
35	1,472, 1,486, 1,500, 1,502, 1,516	
36	1,486, 1,500	
37	1,478, 1,500, 1,516	Putative fengycin class
38	1,500, 1,514, 1,530	
39	1,514, 1,528	
40	1,514, 1,528, 1,544	
41	1,506, 1,528, 1,542, 1,544	
42	1,060, 1,520, 1,542	
43–45	1,060, 1,074	
46–48	1,074, 1,088	
49–50	1,016	
51–52	1,030, 1,046	
53	1,044, 1,060	Putative surfactin class
54	1,044, 1,058	
55	1,044, 1,058, 1,074	
56–58	1,058, 1,072	

the biosurfactant families, such as surfactins, iturins, and fengycins, produced by *B. subtilis* strains [11, 25, 46].

Structure Analysis of Biosurfactant Lipopeptides

To detect and identify the biosurfactant lipopeptides produced by strain CMB32, active fractions obtained by a Sephadex LH-20 column and reverse-phase HPLC were investigated by performing MALDI–TOF mass spectrometry. A MALDI–TOF mass spectral analysis revealed a cluster containing several molecules observed at *m/z* 1,066, 1,080, 1,094 (putative iturin class) and 1,472, 1,486, 1,500, 1,514, 1,528, 1,542 (putative fengycin class), and 1,016, 1,030, 1,044, 1,060, 1,074 (putative surfactin class) (Table 1). These peaks revealed differences of 14 Da, suggesting a series of homologous molecules having different lengths of fatty acid chains (*i.e.*, CH_2=14 Da). The MALDI–TOF MS/MS spectrum of the major protonated molecule $[M+H]^+$, observed at *m/z* 1,058 in the putative iturin class, was exactly the same as those of the $[M+H]^+$ and $[M+Na]^+$ of iturin A (Fig. 3, Table 1). The major protonated molecules $[M+H]^+$, observed at *m/z* 1,464 and 1,022 in the putative fengycin and surfactin classes, respectively, were also detected as those of the $[M+Na]^+$ (Table 1). The structure of the linear derivative obtained after alkaline treatment of the lactone cyclic lipopeptide is described in Fig. 4. The putative fengycin and surfactin classes with major protonated

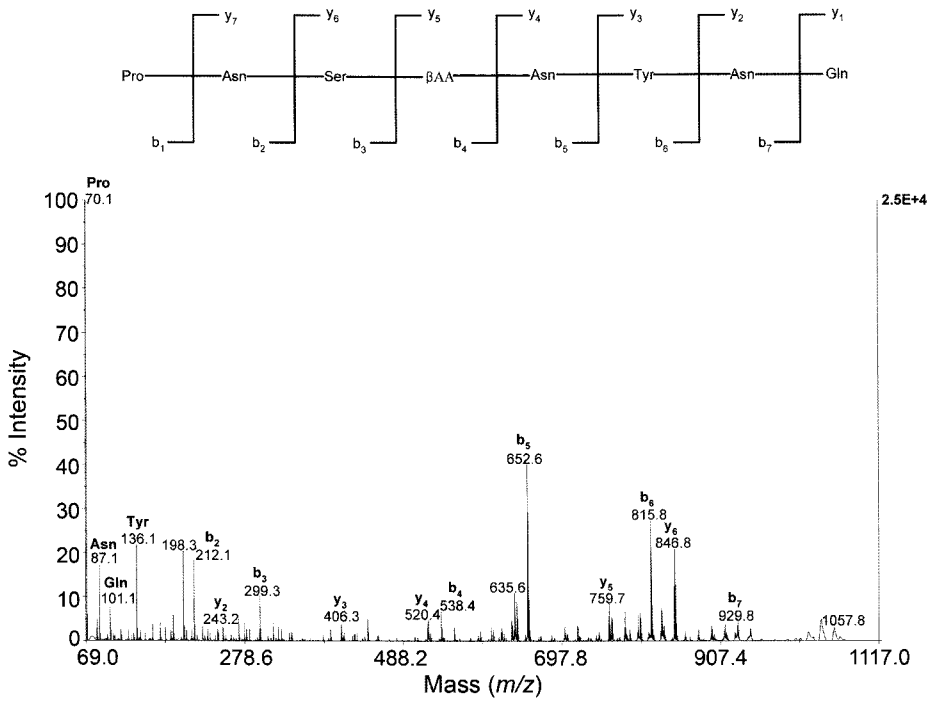

Fig. 3. MALDI-TOF MS/MS spectra obtained from the protonated linear derivatives of the $[M+H]^+$ of iturin A at *m/z* 1,058.

molecular masses of 1,464 and 1,022 Da were subjected to mild alkaline hydrolysis. Each molecule yielded a new product, with masses of 1,482 Da (protonated mass of putative fengycin) and 1,062 Da ($[M+Na]^+$ mass of putative surfactin), respectively. From the above results, it was suggested that each product gained a mass of 18 Da by hydrolysis of the lactone ring. The linear molecules observed at *m/z* 1,482 and 1,062 were determined by MALDI-TOF MS/MS analysis as fengycin and surfactin A, respectively (Figs. 5 and 6).

Amino Acid Analysis

The amino acid compositions of each biosurfactant lipopeptide follow. Putative iturin is Pro, Asx, Ser, Tyr, Glx in a molar ratio of 1:3:1:1:1, putative fengycin is Glx, Tyr, Ala, Pro, Ile in a molar ratio of 3:2:1:1:1:1, and putative surfactin is Glx, Leu, Val, Asx in a molar ratio of 1:4:1:1, which are the same sequences as the lipopeptides reported previously [46].

DISCUSSION

We isolated eight antagonists to suppress the growth of a fungal pathogen, *C. gloeosporioides*, causing anthracnose disease in a variety of crops. Among them, *Bacillus subtilis* CMB32 showed the strongest activity against many fungal plant pathogens described in the Materials and Methods and Results sections. The production level of biosurfactant lipopeptides increased proportionally with the number of bacterial cells cultured in M9 broth medium (data not shown).

The *Bacillus* strains have been reported to produce biosurfactant lipopeptides that suppress growth of phytopathogenic fungi [8, 17, 21, 43, 51]. In this study, *B. subtilis* CMB32 secreted three lipopeptides, which play important roles in inhibition of the growth of fungal pathogens, when cultured in M9 broth. The production of biosurfactant lipopeptides was also investigated by adding various metal ions (Fe^{2+}, Fe^{3+}, Co^{2+}, Cu^{2+}, Cs^{+}, Ni^{+}, Zn^{2+}, Mn^{2+}) into M9 broth. As the result, the lipopeptide production

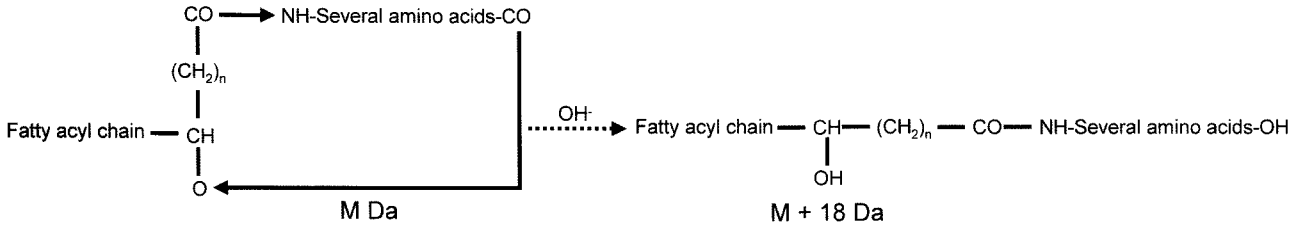

Fig. 4. Structure of the linear derivative obtained after hydrolysis of lactone cyclic lipopeptide by 1 M KOH.

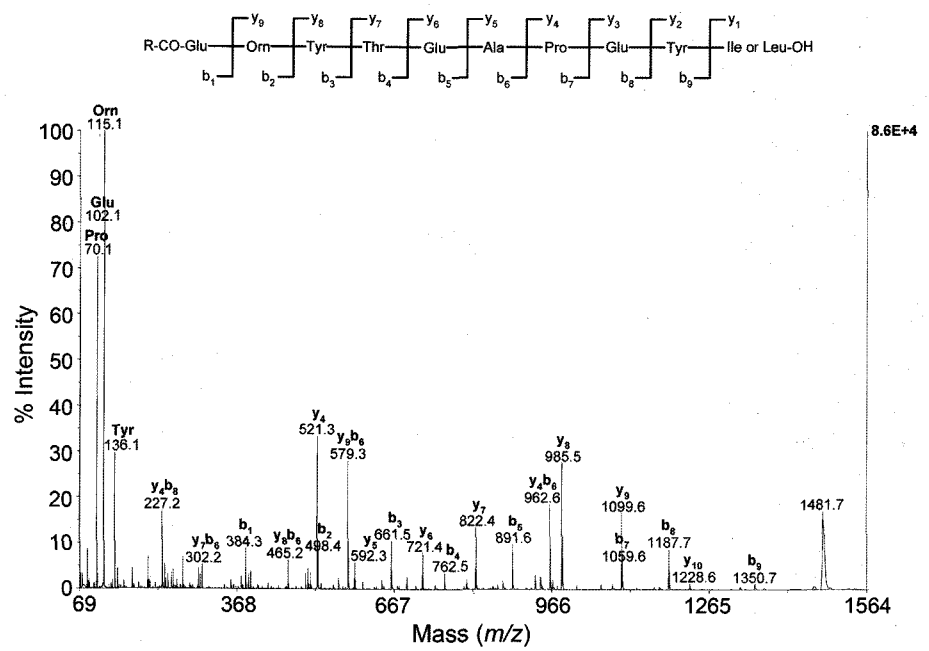

Fig. 5. MALDI–TOF MS/MS spectra obtained from the protonated linear derivatives of the $[M+H]^+$ of fengycin at *m/z* 1,482.

was enhanced 2.6-fold by the addition of 10^{-6} M Mn^{2+} ion (data not shown). The biosurfactant lipopeptides were purified by Sephadex LH-20 column chromatography and reversed-phase HPLC, and structural properties were determined using MALDI–TOF mass spectrometry.

Iturins are a member of an antifungal lipopeptide group that contains iturins A–E, bacillomycins D, F, and L, and mycosubtilin [4, 5, 33]. Both A- and B-types of fengycins, varying by the replacement of an alanine residue with valine, were detected. Many microorganisms produced fengycin A with various lengths of the acyl side-chain from C_{14} to C_{20}. Moreover, surfactins (C_{12} to C_{16}) were coproduced to increase the antifungal activity of iturin A. Compared with the mass data summarized in Table 1 and

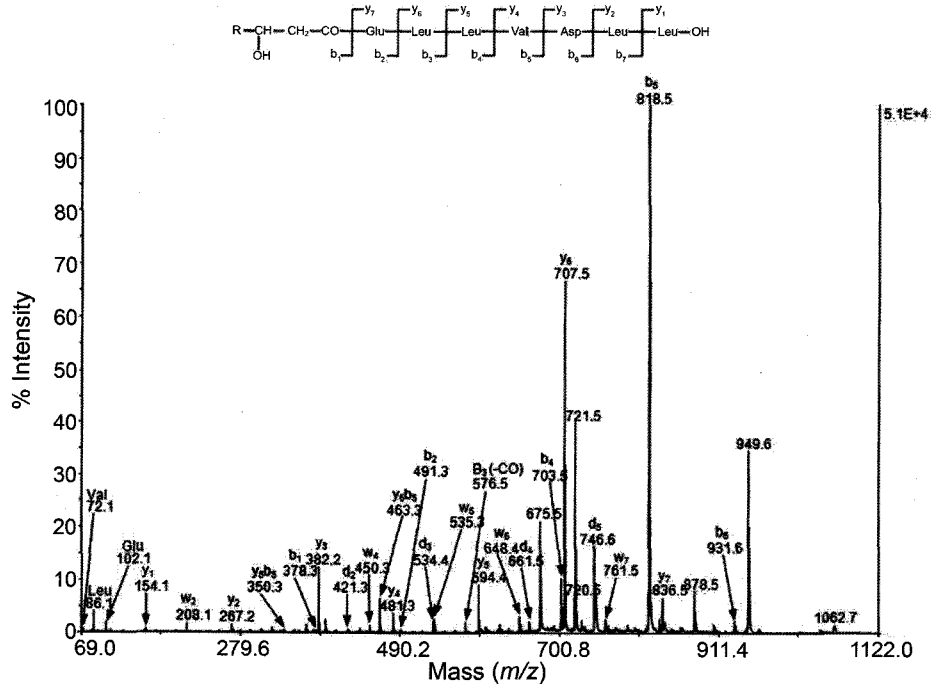

Fig. 6. MALDI–TOF MS/MS spectra obtained from the protonated linear derivatives of the $[M+H]^+$ of surfactin A at *m/z* 1,062.

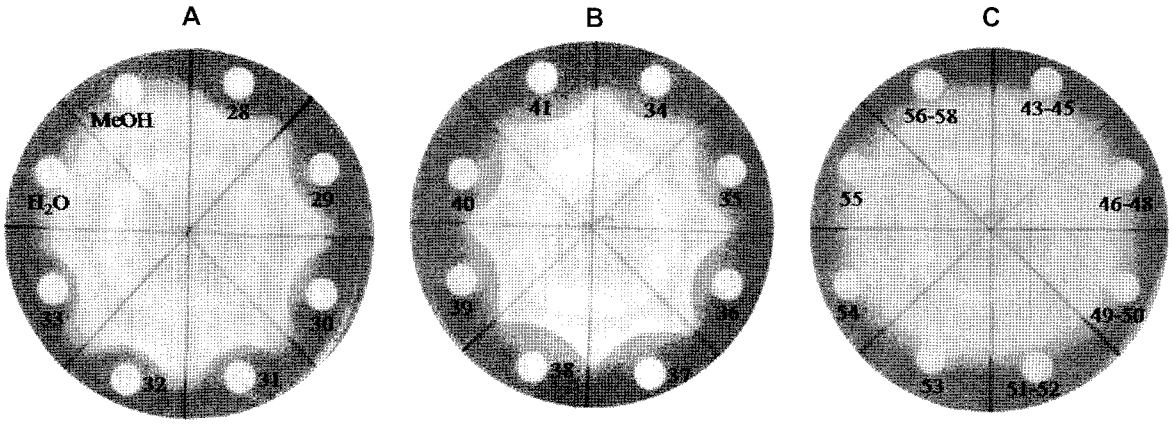

Fig. 7. Antifungal activities of iturin A, fengycin, and surfactin A obtained from HPLC fractions.
A. Iturin A; **B**. Fengycin; **C**. Surfactin A. The numbering means lipopeptide fraction(s) presented in Table 1. Water and methanol were used as negative controls.

Figs. 3, 5, and 6 with the mass numbers reported previously for the lipopeptide families from other *Bacillus* strains [19, 23, 25, 33, 38, 39], and by analyzing amino acid sequences, the antifungal substances produced by *B. subtilis* CMB32 were found to be similar to the lipopeptides iturin A, fengycin, and surfactin A. Among the three lipopeptides, iturin A and fengycin separately exhibited antifungal activity, whereas surfactin A retained the antifungal effect of iturin A as a synergistic factor (Fig. 7). The lipopeptides are less toxic and produce better reduction and control of phytopathogens than agrochemicals. From these results, we suggest that *Bacillus subtilis* CMB32 would seem to be a good biocontrol candidate and a successful antagonist, although the question remains as to how effective this antagonistic bacterium would be under field conditions.

Furthermore, the biosurfactant lipopeptides are used in various industrial fields, including the food industry, clinics, cosmetics and specialty chemical industries, and for cleaning oil spills by bioremediation [12, 13]. Therefore, we expect that these lipopeptides may be useful in agriculture and various industries as biocontrol agents and biosurfactants, respectively, although further study is needed.

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