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Full Length Research Paper

Isolation and characterization of antifungal lipopeptides produced by endophytic *Bacillus amyloliquefaciens* TF28

Shu-Mei Zhang[1,2], Yu-Xia Wang[2], Li-Qiang Meng[2], Jing Li[2], Xiao-Yu Zhao[2], Xu Cao[2], Xiu-Ling Chen[1], Ao-Xue Wang[1]* and Jing-Fu Li[1]

[1]College of Horticulture, Northeast Agricultural University, 150030 Harbin, Heilongjiang Province, PR China.
[2]Institute of Microbiology, Heilongjiang Academy of Sciences, 150010 Harbin, Heilongjiang Province, PR China.

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***Bacillus amyloliquefaciens* TF28, an endophytic bacterium isolated from soybean root, showed strong antifungal activity *in vitro*. In this study, crude lipopeptides were extracted with methanol from the precipitate by adding concentrated HCl to culture filtrate. They exhibited highest antifungal activity against the rice bakanae fungus *Fusarium moniliforme*. Besides *F. moniliforme*, the crude lipopeptides also inhibited the growth of other phytopathogens such as *Botrytis cinerea*, *Fusarium oxysporum* etc. Microscopic analysis found that the crude lipopetides distorted hyphae and spore of *F. moniliforme*. The crude lipopetides were very stable to heat and insensitive to pH. They still retained strong antifungal activity after treatment at pH values ranging from 2 to 12 for 24 h or at 100°C for 30 min. Therefore, it is a candidate biocontrol agent for rice bakanae controlling. Biologically active fractions were isolated by high-performance liquid chromatography (HPLC). A component of a molecular weight of 1057 Da was identified as iturin A after electrospray ionization quadrupole time of flight tandem mass spectrometry analysis (ESI-Q-TOF-MS/MS).**

Key words: *Bacillus amyloliquefaciens*, lipopetides, iturin A, endophytic bacterium, fungal inhibition.

INTRODUCTION

Widely distributes in China, rice bakanae caused by *Fusarium moniliforme* caused 10 ~ 20% yield losses every year. Chemical fungicides have been used to control this pathogen. However, chemical fungicides cause serious environmental pollution and the efficiency is decreasing owing to the development of resistant pathogens (Chan et al., 2002). More effective and safer alternative control methods are needed but not available at present.

Biocontrol agents using antagonistic microorganisms or their active metabolites have received increasing attention as a promising supplement or alternative to chemical control (Haggag and Mohamed, 2007). Some species of the *Bacillus* have been reported to be effective against plant pathogens (Toure et al., 2004; Patel et al., 2004; Bottone and Peluso, 2003; Yoshida et al., 2001; Souto et al., 2004). They can antagonize pathogens by competing for niche and nutrients, by stimulating the defensive capacities of the host plant and by producing fungal toxic compounds (Timmusk et al., 2005; Bais et al., 2004; Ongena et al., 2005; Compant et al., 2005). Among antifungal compounds, lipopeptides play a major role in disease suppression (Romero et al., 2007). Several strains of *B. subtilis* and *B. amyloliquefaciens* are reported to produce lipopeptides. Some lipopeptides such as surfactin, iturin and fengycin families are isolated and identified. These lipopeptides display strong antifungal activity and have low toxicity, high biodegradability and are environmentally friendly. The role in biocontrol has been studied (Yu et al., 2002; Athukorala et al., 2009; Arrebola et al., 2010; Kim et al., 2010; Caldeira et al.,

*Corresponding author. E-mail: wangaoxueneau@gmail.com. Fax: 86-451-55191671.

2007; Preecha et al., 2008; Chen et al., 2008).

Endophytic bacteria are one of the most potential biocontrol agents in plant disease control. Many promising endophytic bacteria have been reported as biocontrol candidates against plant pathogens (He et al., 2009). Endophytic *B. amyloliquefaciens* strain TF 28 showed strong pathogen inhibition activity, especially effective against rice bakanae pathogen *F. moniliforme*. However, the composition of the antifungal compounds has not been elucidated. Identification of antifungal compounds may improve our understanding of biocontrol mechanism. The aim of this study was to isolate and identify the lipopeptides from strain TF28, and further evaluate the antifungal activity and stability to pH and heat.

MATERIALS AND METHODS

Microorganisms and culture conditions

Endophytic *B. amyloliquefaciens* TF28 was isolated from soybean root in our lab. A loop of TF28 cell was incubated in a 100 ml Erlenmeyer flask with 10 ml seed medium (0.5% beef extract, 0.8% yeast extract and 1% glucose) for 18 h at 30°C on a rotary shaker (160 r/min), and then 2 ml of culture liquid was added to a 500 ml Erlenmeyer flask containing 100 ml fermentation medium (0.2% beef extract, 0.3% yeast extract, 0.8% glucose, 0.1% NaCl), The culture was incubated for 48 h at 30°C under shaking condition (200 r/min).

The phytopathogenic fungi listed in Table 1 were maintained on potato dextrose agar (PDA) at 4°C ready for use. Conidia of *F. moniliforme* were prepared from plate cultures on PDA for 7 days at 28°C. Conidia were collected by adding 5 ml sterile distilled water into the plate and rubbing the surface mycelia gently with a sample collection swab. The suspension was filtered by four layers of cheesecloth to remove mycelia. The conidia were collected by centrifugation and adjusted to the desired concentration using a hemocytometer.

Extraction of crude lipopeptides

After fermentation for 48 h at 30°C, one liter of the culture broth was centrifuged at 6,000 g for 20 min to remove the pellet. The cell-free culture broth was precipitated by adjusting pH to 2.0 with concentrated HCl and was stored overnight at 4ºC. The precipitates were collected by centrifugation at 1, 0000 g for 20 min and then extracted three times with methanol. The solution was evaporated under vacuum. The dried remains were dissolved in 0.5 ml methanol and filtered through a 0.45 µm pore size polytetrafluoroethylene membrane (Millipore, USA).

In vitro antifungal activity

The antifungal activity of crude lipopeptides was tested against twelve phytopathogenic fungi with mycelial growth inhibition method. 50 µl of lipopeptide (2.0 µg/µl) was spread on PDA plates. Sterile methanol was used to replace lipopeptides as a negative control. Fermentation liquid was used as a positive control. The mycelial plug of each fungus was deposited in the centre of plates. After incubation for 7 days at 28°C, the diameter of mycelium growth was measured. Growth inhibition effect was evaluated by comparing the percentage of reduction of mycelium expansion with control plates without lipopeptides. The experiment was performed twice.

Stability tests of crude lipopeptides

For pH stability test, crude lipopeptides were adjusted to pH 2, 4, 6, 8, 10, 12 and 14 using 1 N of HCl or NaOH. After maintaining for 24 h at room temperature, each sample was restored to pH 7. The antifungal activity was assayed as follows: PDA plates containing 100 µl *F. moniliforme* spores (1×10^5 spores/ml) were prepared. Sterile steel loops with a diameter of 6 mm were put into the plates. 10 µl of different samples was added into the loops. After incubating for 7 days at 28ºC, the diameter of inhibition zone was measured. The experiment was performed twice.

For thermostability test, crude lipopeptides were stored for 30 min at 20, 40, 60, 80 and 100°C and autoclaved for 15 min at 121°C. After the temperature of the heated samples lowered to 20°C, the antifungal activity was assayed with the method as above. The experiment was performed twice.

Mycelium growth inhibition assay

Dual culture was used to detect the inhibition of crude lipopeptides to *F. moniliforme* mycelium growth. The mycelium plug of *F. moniliforme* was deposited in the center of the plate containing PDA medium. After incubation for 3 days at 28°C, sterile filter paper disk (6 mm in diameter) was placed onto the plate at a distance of 5 mm away from the edge of mycelium colony. 10 µl of lipopetides (2.0 µg/µl) and sterile methanol were added to filter paper disk. The plates were incubated for 7 days at 28°C. Mycelium morphology was observed under light microscope (Olympus Japan). The experiment was performed three times.

Spore germination inhibition assay

The effect of crude lipopeptides against spore germination of *F. moniliforme* was carried out as followed: 50 µl of sterile PDA liquid medium, 10 µl of lipopeptides (2.0 µg/µl) and 10 µl of *F. moniliforme* conidial suspension (1×10^5 spore/ml) were mixed completely in an 1.5 ml tube and incubated in the dark for 48 h at 28°C. The sample was observed under the microscope at regular intervals. Sterile methanol was used to replace lipopeptides as control. The experiment was performed twice.

Isolation of crude lipopeptides

Preparative HPLC was carried out on Waters DeltaPrep instrument with a 5 µm Waters Sunfire C18 column (250 × 4.6 mm). The mobile phase consisted of acetonitrile and 10 mM ammonium acetate (40:60, v/v) with flow-rate of 1 ml/min. The detector monitored absorption at 214 nm. 500 µl of sample was injected. Peaks were collected and the antagonistic activity against *F. moniliforme* was assayed. The eluted fractions with antifungal activity were analyzed by ESI–Q-TOF –MS/MS.

ES1- Q-TOF-MS analysis

Electrospray ionization quadrupole time of flight tandem mass spectrometry analysis was performed on Q-TOF2 instrument (Waters Micromass) to determine the molecular weight and structure of the purified lipopeptide. The electrospray source was operated at a capillary voltage of 32 V, a spray voltage of 5 kV and a capillary temperature of 320°C Positive ionization mode was used.

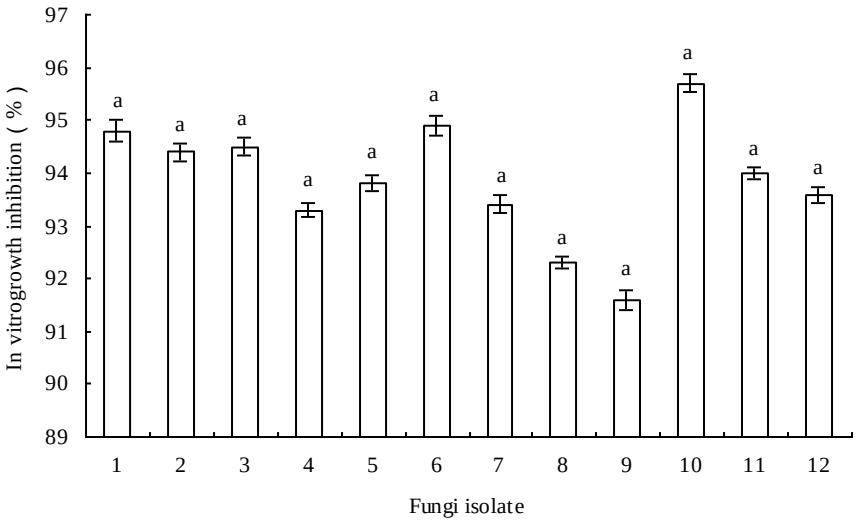

Figure 1. Inhibition of crude lipopeptides against pathogenic fungi on plates.
1. *F. oxysporum f.sp. vedolens*; 2. *R. solani f.sp. vedolens*; 3. *Phythium sp.*; 4. *F. oxysporum f. sp cucumerinum*; 5. *F. oxysporum f.sp. melonis*; 6. *F. oxysporum f.sp.niveum*; 7. *B. cinerea*; 8. *A. solani*; 9. *S. sclerotiorum*; 10. *F. moniliforme*; 11. *F. oxysporum f. sp. lycopersici*; 12. *R. solani f. sp cucumerinum*. Capped bar represents standard errors of the means, and letter a indicates no significant difference between different pathogens.

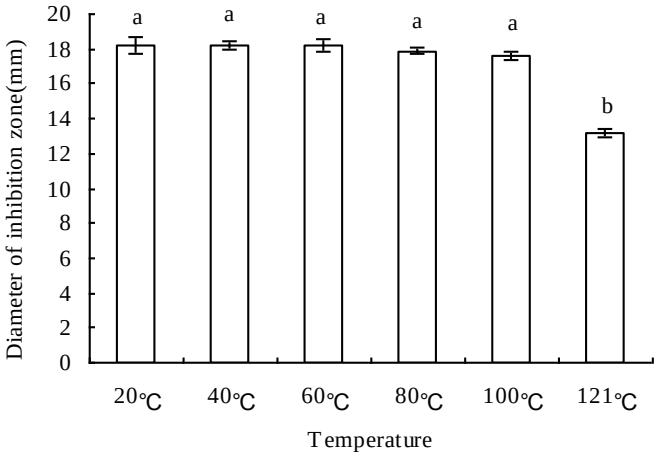

Figure 2A. Effect of different thermal treatments on antifungal activity of crude lipopeptides. Capped bar represents standard errors of the means, and different letter indicates significant difference between different treatments.

RESULTS

In vitro antifungal activity of crude lipopeptides

The antifungal activity of crude lipopeptides was tested by mycelial growth inhibition method. Strong activity was detected with all twelve tested fungal phytopathogens. The antifungal activity is not significantly different among different fungi. The inhibition rate against all tested fungi was over 90% (Figure 1). Among them, *F. moniliforme* was the most sensitive species with 95.7% relative inhibition of mycelial growth.

Stability of crude lipopeptides to heat and pH

Crude lipopeptides were insensitive to heat and pH. Thermal stability results showed that different thermal treatments below 100°C did not affect the antifungal activity of the crude lipopeptides. No activity loss was detected after the incubation of crude lipopeptides at 20, 40 and 60°C for 30 min.

The antifungal activity was a little bit lower when it was incubated at 80 and 100°C for 30 min. However, the activity was reduced by 27.5% when crude lipopeptides were autoclaved at 121°C for 15 min (Figure 2A). pH stability results indicated that the crude lipopeptides were stable in wide pH condition. The activity was not affected at pH 2 to 10, and reduced moderately at pH 12. However, no activity was detected at pH 14 (Figure 2B). Therefore, the crude lipopeptides were stable enough for routine laboratory study and agricultural application.

Effect of crude lipopeptides on mycelium growth and spore germination

Dual culture showed that the mycelia close to lipopeptides could not extend as normal. They were distorted and granulated (Figure 3Bb), whereas the mycelia close to the control sample were normal and

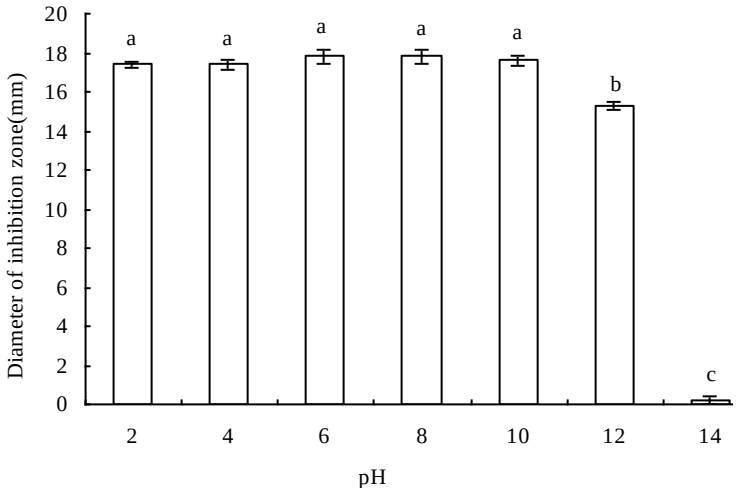

Figure 2B. Effect of different pH treatments on antifungal activity of crude lipopeptides.Capped bar represents standard errors of the means, and different letter indicates significant difference between different treatments.

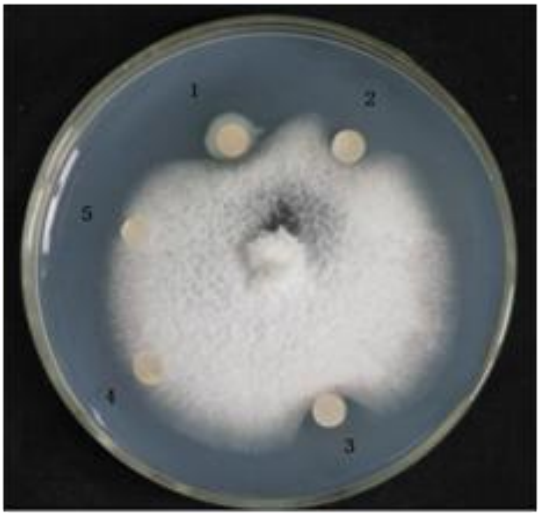

Figure 3A. Antifungal activity of crude lipopeptides against *F. monoliforme*.1. Fermentation liquid; 2, 3: Crude lipopeptides; 4, 5: Methanol。

healthy (Figure 3Ba). A crescent inhibition line was formed around the disk containing lipopeptide sample (Figure 3A 2, 3), whereas the mycelia close to the control sample covered the disk (Figures 3A 4, 5). When Lipopeptides and spores were incubated together for 8 h, no spore germinated. When incubation was conducted for about 24 h, 98% abnormal spores were observed. They appeared bulbous and swollen (Figure 4b). However, percentage of spore germination of the control increased from 14% at 8 h to 95% at 24 h. The hyphae grew strongly after spore germ-tube emergence, growth and elongation (Figure 4a). The results indicated that lipopeptides strongly inhibited *F. moniliforme* mycelium growth and spore germination.

Isolation of crude lipopeptides

The HPLC analysis appeared four major peaks (Figure 5A). Samples from each peak were condensed and assayed for antifungal activity against *F. monoliforme*. Only fraction 1 showed antifungal activity. The antifungal activity couldn't be detected in other three peaks (Figure 5B).

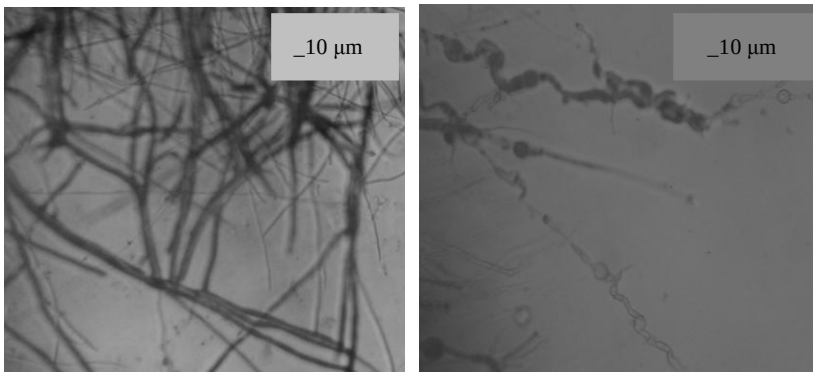

Figure 3B. Inhibition of crude lipopeptides to *F. monoliforme* mycelial growth (a) Normal mycelia morphology close to the control sample; (b) Abmormal mycelia morphology close to lipopeptides.

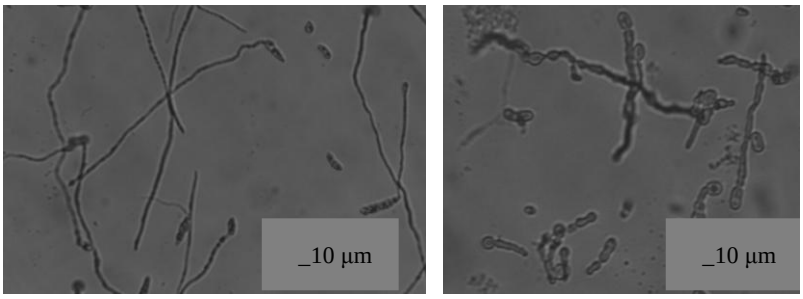

Figure 4. Inhibition of crude lipopeptides to *F. monoliforme* spore germination. (a) Normal germinated spores of *F. monoliforme*; (b) Abnormal germinated spores of *F. monoliforme* appeared bulbous and swollen.

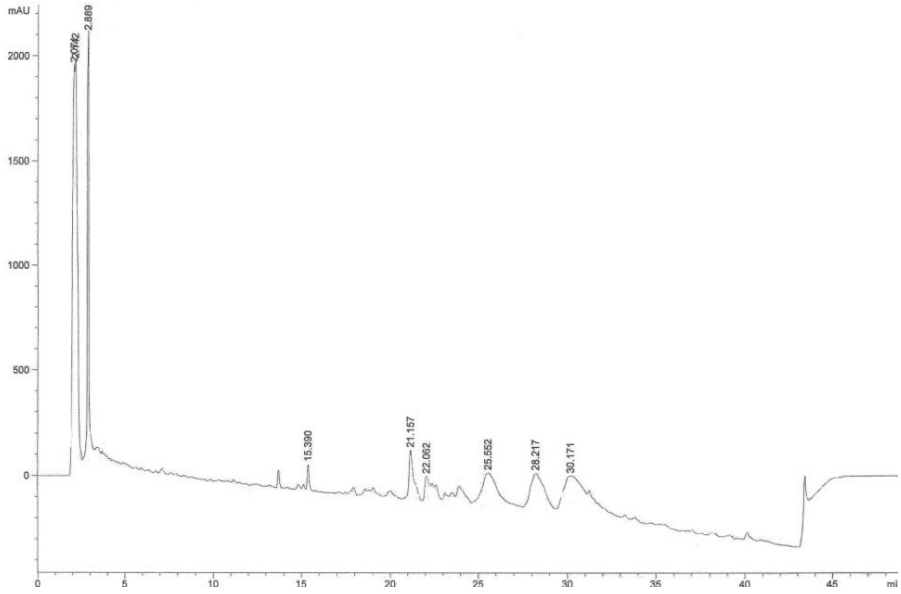

Figure 5A. HPLC chromatogram of crude lipopeptides produced by *Bacillus amyloliquefaciens* TF28.

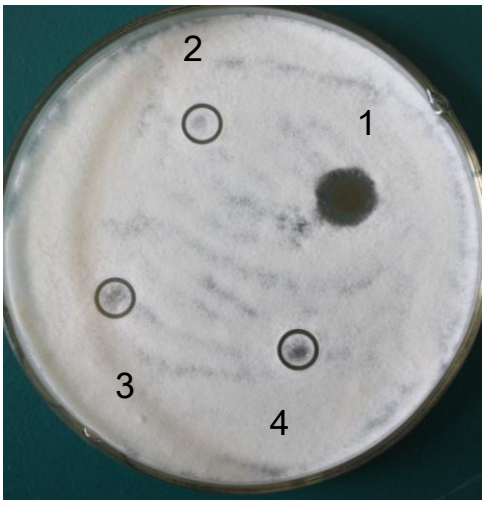

Figure 5B. Antifungal activity of different peaks by HPLC isolation.
1.Peak 1; 2. Peak 2; 3. Peak 3; 4. Peak 4.

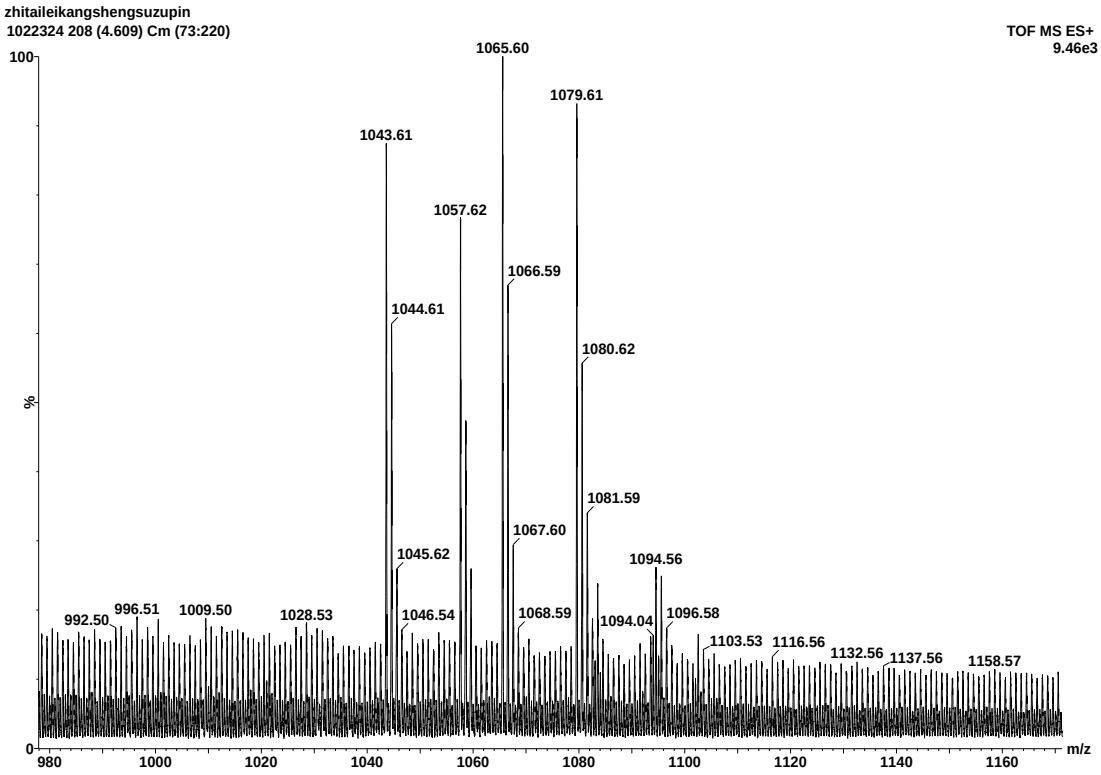

Figure 6. ESI-MS spectrum of the peak 1 compound.

ESI-TOF tandem mass spectrometry analysis of lipopeptides

ESI-TOF-MS/MS was chosen to measure the mass of peak 1. The results showed that the predominant ion mass peaks in the positive ion mode were that of m/z 1043.61, 1057.62, 1065.60 and 1079.61 (Figure 6). M/z 1043.61 and 1057.62 were $(M+H)^+$ ion peaks. M/z 1065.60 and 1079.61 were $(M+Na)^+$ ion peaks. Ions at m/z 1043.61 and 1057.62 were two homologues with a difference of 14 Da ($-CH_2-$), which had similar m/z values to the antifungal lipopeptide iturin A by comparison with

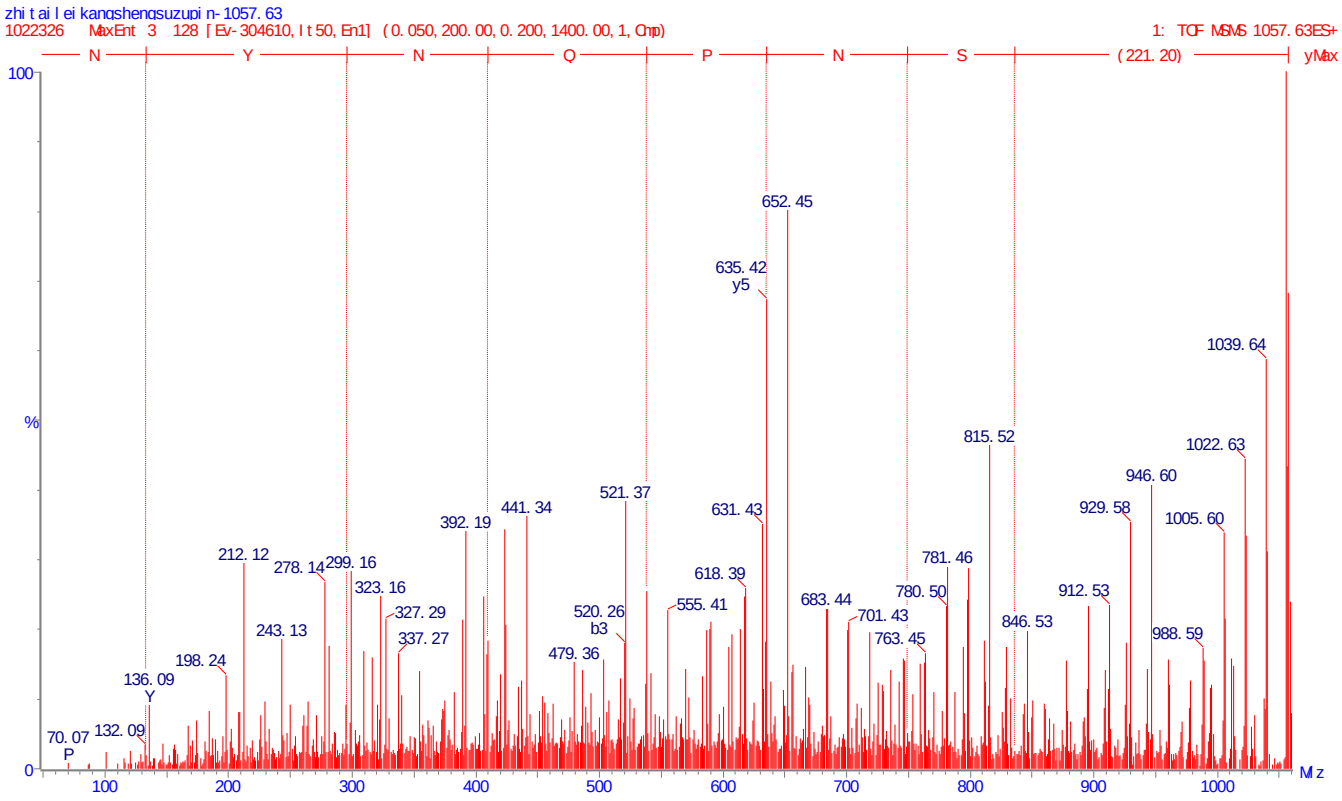

Figure 7A. ESI-TOF-MS /MS spectrum at m / z 1057.62 and its amino acid sequences.

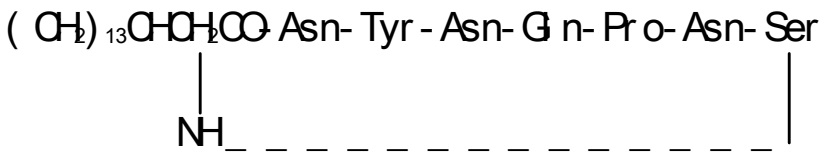

Figure 7B. The cyclic structure mode of iturin A.

reference data (Chen et al., 2008; Pueyo et al., 2009). For further identification, the amino acid sequences of m/z 1057.62 were analyzed by ESI-Q-TOF-MS/MS. The amino acid sequences were confirmed according to debris ions mass of y type: m/z 836.43, 749.40, 635.35, 538.30, 410.24, 296.20 and 133.14. The amino acid sequences were Ser, Asn, Pro, Gln, Asn, Tyr and Asn. In the sequences, mass difference of fatty acid was 221.20Da (Figure 7A). The sequences indicated that Ser and Asn were connected with fatty acid, respectively, which was deduced as cyclic lipopeptide according to tandem mass spectrometry regulation. The cyclic structure of the lipopeptide was analyzed as iturin A (Figure 7B).

DISCUSSION

Bacillus spp. is the important biocontrol agents that have shown strong antifungal activity against a number of plant pathogens. The antibiotics produced from them were generally assumed to be responsible for the control activities (Kim et al., 2010; Wang et al., 2010; Nagórska et al., 2007; Asaka and Shoda, 1996). *B. amyloliquefaciens* strain TF 28 is a particular endophytic biocontrol strain. It showed an effective inhibitory effect on *F. moniliforme*, suggesting that antibiosis may be involved in the disease control. In this study, it is evident that TF28 produced lipoppetides. To our knowledge, few reports were found to use *B. amyloliquefaciens* to control rice bakanae disease. Therefore, the strain is potential for agricultural application.

In this study, we extracted crude lipopeptides from cell-free culure broth of strain TF28 using HCl precipitation. Crude lipopeptides exhibited a wide-spectrum of antifungal activity. They strongly inhibited the growth of all tested fungal pathogens, especially significantly inhibited the growth and spore germination of *F.*

moniliforme. The crude lipopeptides are very stable to heat and pH, which is higher than previous studies (Gong et al., 2006; Qian et al., 2009). Therefore, the crude lipopeptides may be a potent candidate in controlling rice bakanae disease. However, the yield of crude lipopeptides is very low. Further research is needed to improve the yield of crude lipopeptides through changing medium components and culture condition. Chromatographic analysis showed that crude lipopeptides mainly included 4 compounds. Only one compound (peak 1) has antifungal activity. Its content in crude lipopeptides is about 14%. This compound was highly potent in its antagonistic activity as it could inhibit the growth of *F. moniliforme* at a low concentration. The compound was identified as iturin A based peptide ring sequence and the molecular weight by ESI-Q-TOF-MS/MS analytical techniques. It is known that some *B. amyloliquefaciens* strains could produce surfactin, iturin and fengycin production (Yoshida et al., 2001; Yu et al., 2002; Athukorala et al., 2009; Arrebola et al., 2010). Iturins are a group of antifungal, cyclic lipopeptodes, consisting of iturin A-E, bacillomycin D, F and L, and mycosubtilin. There are isomers for each of iturins because their fatty acid chain can be in the n-, iso-, or anteiso-form. Iturin A has a cyclic structure consisting of β-amino fatty acid integrated into a peptide moiety [Asn-Tyr-Asn-Gln-Pro-Asn-Ser]. In nature, iturin A is produced as a mixture of up to eight isomers. Iturin A may play an important role in disease suppression (Hiradate et al., 2002). Some of *B. subtilis* and *B. amyloliquefaciens* strain could co-produce surfactin, iturin A and fengycin. They showed synergistic effects on antifungal activity. Their anifungal activity is higher than only iturin A production (Athukorala et al., 2009; Arrebola et al., 2010; Chen et al., 2008). However, the co-production of lipopeptides makes purification difficult. *B. amyloliquefaciens* strain TF 28 only produces iturin A, which makes the purification easier than co-production of lipopeptides. The easy purification reinforces the potential application of bioproducts from this strain as a biocontrol agent.

The traditional method to determine the sequence of amino acid residues in lipopeptides is known as the Edman degradation, in which the amino acid sequence in a cyclic lipopeptides is determined based on several separated fragments after partially hydrolyzed. In this study, we used an ESI-TOF-MS/MS method to directly determine the amino acid sequence in a cyclic lipopeptide without hydrolysis.

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