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Full Length Research Paper

Identification of antifungal compound produced by *Bacillus subtilis* LB5 with ability to control anthracnose disease caused by *Colletotrichum gloeosporioides*

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***Bacillus subtilis* strain LB5 showed inhibitory activity on conidial germination of *Colletotrichum gloesporioides* causal agent of mango and wax apple anthracnose disease. The antifungal metabolites produced by *B. subtilis* strain LB5 were highly stable to heat, pH ranging from 3 to 11, and ultraviolet (UV) light. Cell-free supernatant of LB5 culture broth was extracted by using *n*-butanol to give the *n*-butanol soluble fraction, which was purified by reversed phase high-performance liquid chromatography (RP-HPLC) to obtain the major antifungal compound. The structure of antifungal compound was identified as iturin A-2, a cyclic lipopeptide antibiotic, based on the analysis of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and fast atom bombardment mass spectrometry (FAB-MS) spectra.**

Key words: *Bacillus* subtilis LB5, *Colletotrichum gloeosporioides*, reversed phase high-performance liquid chromatography (RP-HPLC), nuclear magnetic resonance (NMR), fast atom bombardment mass spectrometry (FAB-MS), antifungal compound, iturin A-2.

INTRODUCTION

The filamentous fungus *Colletotrichum gloeosporioides* (Penzig) Penzig et Sacc. causes anthracnose disease on various temperate, subtropical and tropical fruits worldwide (Freeman et al., 1996). To control the disease, agricultural chemicals have been used for a long time (Leroux, 2003). However, the excessive use of chemical pesticides may cause the adverse effects on human health, environment, and induce the pathogen resistance to chemical. Due to these limitations of chemical fungicides, an additional control strategy is needed. The use of microorganisms or their secretions to prevent plant diseases is an attractive alternative or a supplement to pesticides and genetic resistance for the management of plant diseases without the negative impact of chemical control (Wang et al., 2002). The use of bacterial strains as biological control agents has received great attention because of the ability of such strains to suppress different plant diseases involving a blend of diverse modes of action (Cazorla et al., 2007) and the possibilities to be combined with other control methods. *Bacillus subtilis* species is a gram-positive and spore forming bacterium and shows antagonistic activities against several plant pathogens (Shih et al., 2009) because they have a well-developed secretory system producing diverse secondary metabolites with a wide spectrum of antibiotic activity.

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Therefore, they are widely used in biocontrol of plant diseases and become very valuable for medical and agricultural applications (Liu et al., 2007).

In previous study, *B. subtilis* strain LB5 was isolated from the wax apple orchard in the Pingtung County in Taiwan (ROC). The strain LB5 was successfully used as biological control agent on wax apple and mango (Jingkwang cultivar) anthracnose disease caused by *C. gloeosporiodes*. Application of LB5 can prevent early fruit infection in orchard, thereby reducing significantly anthracnose incidence in ripening fruits after harvesting. The aim of this study was to purify and identify the antifungal compounds produced by *B. subtilis* LB5.

MATERIALS AND METHODS

Microorganisms and cultures conditions

B. subtilis strain LB5 was cultured in potato sucrose agar medium (PSA; 200 g/l of potato, 20 g of sucrose, 18 g of agar) at 25°C for 2 days, then 1 ml of *B. subtilis* LB5 suspension at 10^8 cfu/ml was added in 1 L of potato sucrose broth (PSB) and cultured for 10 days under shacking at 100 rpm, at 25°C. *C. gloeosporioides* was grown on PSA at 25°C for 7 days for further experiment. To produce the conidia, *C. gloeosporioides* was cultured in oat meal agar (OMA; 72.5 g/l of oatmeal agar: Difco Laboratories, Augsburg, Germany) at 25°C for 7 days under continuous fluorescent light.

Antifungal activity *in vitro*

Inhibitory activity on mycelial growth

Mycelial growth inhibition activity was performed by using glass ring method. The PSA plates were prepared with glass rings by placing them as central wells. A one hundred microliters of PS culture broth of *B. subtilis* LB5 (cultured for 10 days) was added into the central well and new single agar inoculum disk (0.5 cm in diameter) of *C. gloeosporioides* was placed at the opposite edge of plates and incubated under 12 dark/12 light at 25°C for 5 days. Plates added with sterile distilled water were used as the control.

Inhibitory activity on conidial germination

Conidia of *C. gloeosporioides* were harvested from 7-day-old culture growing on OMA by gentle scraping into sterile distilled water. The conidial suspension was filtered through two layers of cotton cloth to remove mycelia and the conidia were removed by centrifugation (Allegra™ X-22R Centrifuge, BECKMAN COULTER TM) at 5,000 rpm for 5 min. The conidia were resuspended in sterile distilled water and collected by centrifugation. The concentration of conidial suspension was adjusted to 10^5 conidia/ml. Ten microliters of conidial suspension were placed onto glass slide then mixed with 10 µl of antifungal compound or 10 days of cultivated broth. A conidial suspension mixed with sterile distilled water was used as a control. Slides were kept moist by placing on moistened paper towels in Petri dishes (9 cm in diameter) and sealed with parafilm. After incubation at 25°C for 12 h, conidial germination were recorded on 100 conidia per replication under compound light microscope and only those conidia with germ tubes longer than the conidia length were counted as germination. For positive and negative check, sterile distill water and azoxystrobin + difenconazole (1 ml/3000 ml) were used, respectively.

The stability of antifungal compound

Effect of heat on the inhibitory activity

The LB5 was cultured for 10 days in PSB medium, and then the culture broth were centrifuge at 8,000 rpm for 20 min at 4°C. The supernatant was heated by boiling at 100°C for 30 min or autoclaving at 121°C for 10 min. Heat treated supernatant was tested for inhibitory activity on conidial germination as previously described.

Effect of pH on the inhibitory activity

The LB5 culture broth of 10 days incubation was adjusted to pH range of 3, 4, 5, 6, 7, 8, 9, 10 or 11, using 0.5 N of NaOH or HCl and incubated for 14 h at 25°C. Each culture broth and control was readjusted to the original of pH 7 before tested for its inhibitory activity.

Effect of ultraviolet (UV) light on the inhibitory activity

One hundred microliters of bacterial culture filtrate was loaded into wells of PSA plate as previously described and exposed to UV light at 365 nm for 24 h at distance of 50 cm. Then the plates were seeded with the conidial mass of *C. gloeosporioides* by placing it at the edge of the plates at two opposite sides of the well and incubated at 25°C, under 12 dark/12 light for 5 days.

Extraction and purification of antifungal compound

The strain LB5 was cultured in 2 L-Erlenmeyer flask containing 1.5 L of PSB medium under shacking at 100 rpm at 25°C for 10 days. Figure 1 shows the extraction and isolation procedure of antifungal compound from cell-free supernatant of *B. subtilis* strain LB5 (12 L). The bacterial cells were removed by centrifugation at 8,000 rpm (Himac CR 21G, HITACHI), at 4°C for 20 min and cell-free supernatant of strain LB5 culture was concentrated *in vacuo*. The concentrate of cell-free supernatant (4 L) was partitioned with an equal volume of *n*-butanol (*n*-BuOH) to separate into two fractions, water (7 g) and *n*-BuOH (50 g) soluble fractions. Each fraction was tested for antifungal activity on conidia germination of *C. gloeosporioides* as previously described. The active fraction was passed through a reversed phase C-18 gel (RP-C18) column (7 × 60 cm) and eluted with H_2O:MeOH (2:8; 4:6; 5:5) to resolve into 20 subfractions, 1 to 20. Each fraction was tested for antifungal activity with those conidia. Fraction no.11 with antifungal activity was further chromatographed on a RP-C18 column (5 × 45 cm), eluted with H_2O:ACN (acetronitrile) (1:9) to obtained 20 fractions, 11A-11T. The most active fraction, no.11F, was purified by using a reversed phase high-performance liquid chromatography (RP-HPLC), eluted with H_2O-ACN (60:40; 45:55), 2 ml/min, to yield a pure compound (25 mg, t_R =31.5 min).

Structure identification of antifungal compound

Optical rotations were measured using an optical activity AA-10R automatic spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded in dimethyl sulfoxide (DMSO) at room temperature on a Varian Mercury plus 400 NMR spectrometer, and the solvent resonance was used as internal shift reference (TMS as standard). Fast atom bombardment-mass spectrophotometer (FAB-MS) was recorded on a Finnigan/Thermo Quest MAT 95XL spectrometer. TLC was performed by using silica gel 60 F_{254} plates (Merck). RP-C18 silica gel (Merck, 230–400 mesh) was used for

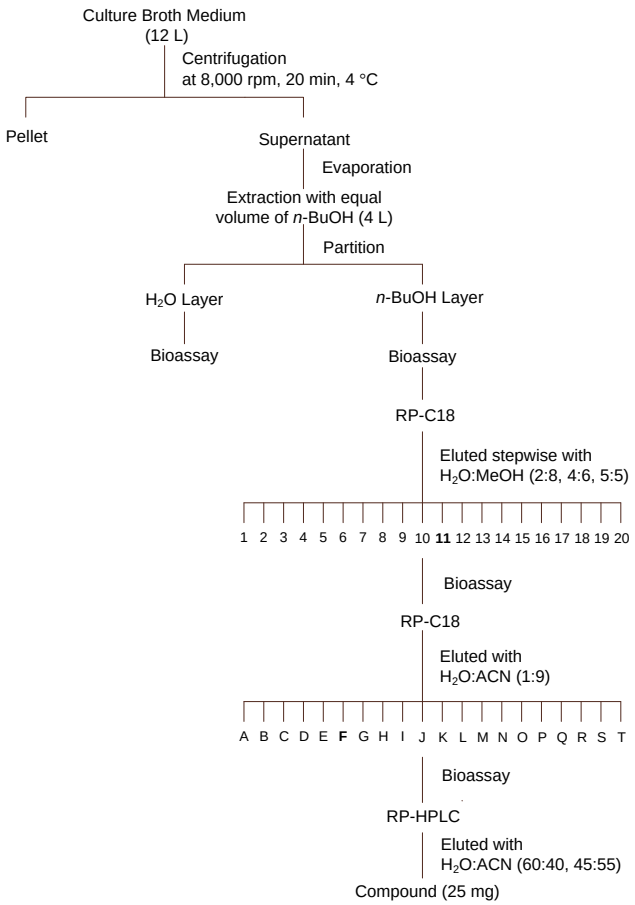

Figure 1. Extraction and purification procedure of antifungal compound from free-cell supernatant of *B. subtilis* strainLB5.

column chromatography. HPLC was performed on a Hitachi L-7000 chromatograph with a Thermo Betasil C-18 column (5 µm, 250 x 10 mm).

RESULTS

Inhibitory activity

B. subtilis strain LB5 showed antagonistic activity by inhibiting mycelial growth and conidial germination of *C. gloeosporioides*. For the mycelial growth, normal growth was observed from control (Figure 2A) and inhibition zones were obtained from culture broth medium of strain LB5 after 5 days of incubation (Figure 2B). The phenomenon of conidial germination inhibition showed that most conidia could not germinate (Figure 2D) and some conidia produced swollen germ tube immediately after germination (Figure 2E) when incubated with culture broth of LB5 for 12 h. However, in the control, conidia germinated by forming germ tube and produced appressoria (Figure 2C) after incubated with sterile distilled water for 12 h.

The effect of thermal condition, pH and UV light

The stability of antifungal compound in the culture broth was examined. Inhibitory activity of antifungal compound from LB5 still showed strong activity after being subjected to different pH, UV light and thermal condition (Table 1). Conidia germination of *C. gloeosporioides* was completely inhibited by culture broth after treated at pH 3 through 11. Besides, antifungal activity in culture broth was not affected under the UV light at 365 nm for 24 h or after autoclaving for 15 min at 121°C or heating for 30 min at 100°C.

Extraction, purification and identification of antifungal compound

In order to study the antifungal compounds produced by *B. subtilis* LB5, the active compound is presented in the culture broth by showing inhibitory activity on conidial germination of *C. gloeosporioides*. The supernatant (12 L) of culture broth was concentrated to reduce the volume to 4 L and then partitioned with *n*-BuOH to give *n*-BuOH and water soluble layers. The *n*-BuOH soluble layer (50 g) showed inhibitory activity on conidial germination of *C. gloeosporioides* at the concentration of 0.01 g/ml. However, water layer (17 g) at 0.01 g/ml did not show inhibitory activity.

The crude extract of *n*-butanol layer was chromatographed on RP-C18 column to obtain 20 fractions (Table 2), five of which showed antifungal activity on conidial germination at the concentration 5 mg/ml. However, only 1 fraction, fraction no. 11, has highest amount (7.4 g). This fraction was further purified by RP-HPLC. Out of 20 fractions from RP-HPLC, only 2 fractions, fractions no.11F and 11G, (Table 3) showed 100% of inhibition on the conidial germination at the concentration 5 mg/ml.

RP-HPLC chromatogram of fraction no. 11F monitored by UV detector at wavelength 256 nm revealed it in pure form and the structure of purified compound was characterized by the spectral techniques. The FAB-MS spectrum of fraction no. 11F showed a sodinated molecular ion peak $[M+Na]^+$ at *m/z* 1,065 (Figure 3), indicating the molecular weight is 1,042. The ^{1}H-NMR spectrum of this compound showed signals for N-binding protons at δ 8.70-6.86 and α-protons at δ 4.48-4.00 of peptide, one set of A_2B_2 coupling pattern protons of benzene ring at δ 7.01 and 6.64 (each *d*, *J*=8.4 Hz, 2H), methylene protons of long aliphatic chain at δ 1.30-1.06 and one terminal methyl protons at δ 0.83 (t, *J*=6.4 Hz, 3H). The ^{13}C-NMR and DEPT spectra revealed signals for 12 carbonyl groups at δ 174.3-170.4, one para-substitued benzene at δ 155.9 (1C), 129.9 (2C), 128.0 (1C) and 115.2 (2C), oxymethylene carbon at δ 61.5, and one methyl carbon at δ 14.1. Based on these results, this major antifungal compound produced by *B. subtilis* LB5 was identified as iturin A-2 and its structure was

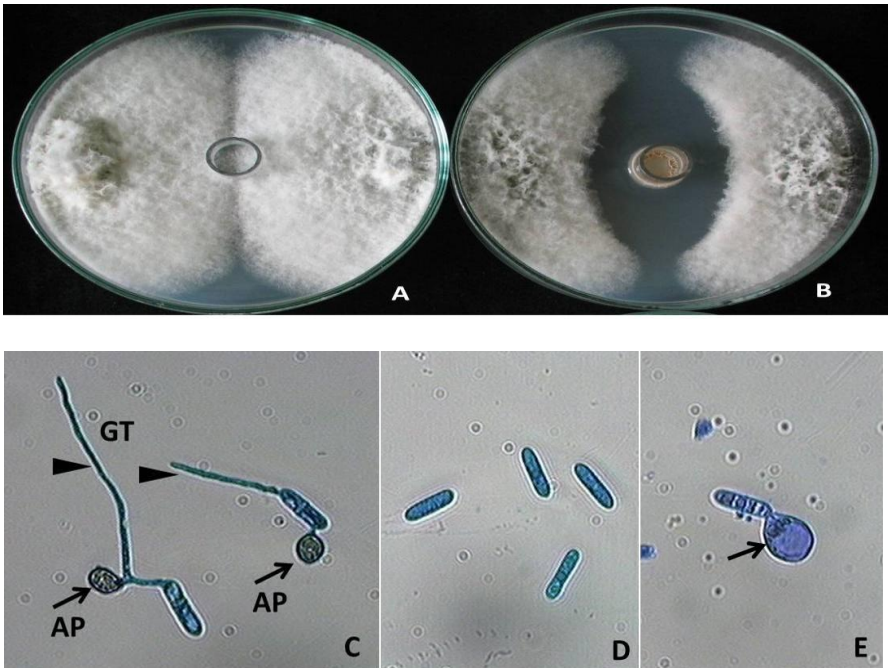

Figure 2. Antagonistic activity on mycelial growth and conidial germination of *C. gloeosporioides* by *B. subtilis* strain LB5. A, Normal mycelial growth; B, inhibited mycelial growth by culture broth of strain LB5 after 5 days incubation on PSA medium; C, normal conidial germination exhibiting germ tube (GT) (arrowheads) and appressoria (AP) (arrows); D, nongerminated conidia; E, conidium showed swollen germ tube (arrow) after 12 h of incubated with culture broth of strain LB5.

Table 1. Effect of pH, UV light, and thermal condition on the antifungal activities of *B. subtilis* strain LB5 on conidia germination of *C. gloeosporioides.*

Condition	Antifungal activity
After treating at pH 3 to 11	+*
UV light at 365 nm	+
121°C for 5 min	+
100°C for 30 min	+

*Positive effect: showed antifungal activity.

showed in Figure 4.

Molecular structure analysis data from NMR of Iturin A-2

[α] 24D 3.1° (*c* 0.5, MeOH). ^{1}H NMR (400 MHz, DMSO-d6) δ_H: 8.70 (d, *J* = 6.8 Hz, 2H, Tyr and Asn-6 NH), 8.05 (d, *J* = 7.6 Hz, 1H, Asn-3 NH), 7.71 (d, *J* = 6.4 Hz, 1H, Asn-1 NH), 7.39 (s, 1H, Asn-6 NH_2), 7.34 (s, 1H, Asn-1 NH_2), 7.33 (d, 1H, Ser NH, overlapped by δ 7.34), 7.24 (s, 1H, Asn-3 NH_2), 7.17 (s, 1H, Gln NH_2), 7.13 (d, *J* = 9.6 Hz, 1H, β-amino acid NH), 7.01 (d, *J* = 8.4 Hz, 2H, Tyr δ), 6.98 (d, 1H, Gln NH_2, overlapped by δ 7.01), 6.92 (s, 1H, Asn-1 NH_2), 6.88 (s, 1H, Asn-3 NH_2), 6.86 (s, 2H, Asn-6 and Gln NH_2), 6.64 (d, *J* = 8.4 Hz, 2H, Tyr ε), 4.89 (br t, *J* = 6.0 Hz, 1H, Ser OH), 4.48 (m, 1H, Gln α), 4.43 (m, 3H, Asn-3 α, Asn-1 and Asn-6 α), 4.15 (m, 2H, Pro and Ser α), 4.00 (m, 1H, Tyr α), 3.98 (m, 1H, β-amino acid C_3H), 3.75 (m, 2H, Pro δ), 3.64 (m, 2H, Ser β), 2.95 (br d, *J* =14.6 Hz, 1H, Tyr β), 2.73 (br d, *J* = 14.2 Hz, 1H, Tyr β), 2.71 (m, 1H, Asn-6 β), 2.57 (dd, *J* = 9.2, 15.4 Hz, 1H, Asn-3 β), 2.47 (m, 2H, Asn-3 β and Asn-6 β), 2.32 (m, 2H, β-amino acid C_2H_2), 2.26 (m, 1H, Asn-1 β), 2.16 (m, 2H, Asn-1 β and Pro β), 2.08 (m, 3H, Gln β and Gln γ), 1.99 (m, 1H, Pro γ), 1.86 (m, 1H, Pro γ), 1.74 (m, 2H, Gln β and Pro β), 1.39 (m, 2H, β-amino acid C_4H_2), 1.30–1.06 (m, β-amino acid aliphatic CH_2), 0.83 (t, *J* = 6.4 Hz, 3H, β-amino acid $C_{14}H_3$); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 174.3 (C=O), 173.4 (C=O), 172.9 (C=O), 172.0 (C=O), 171.6 (C=O), 171.5 (C=O), 171.4 (C=O), 171.2 (C=O), 171.0 (C=O), 170.9 (C=O), 170.8 (C=O), 170.4 (C=O), 155.9 (Tyr, ζ), 129.9 (2C, Tyr, δ), 128.0 (Tyr, γ), 115.2 (2C, Tyr, ε), 61.5 (Ser, β), 60.9 (Pro, α), 56.5 (Tyr, α), 56.3 (Ser, α), 50.9 (2C, Asn-6, α and Asn-1, α), 49.7 (2C, Asn-3, α and Gln, α), 47.4 (Pro, δ), 45.6 (β-amino acid, C_3), 41.9 (β-amino acid, C_2), 36.4 (Asn-1, β), 36.1 (Asn-3, β), 35.3 (Asn-6, β), 35.0 (Tyr, β), 34.7 (β-amino acid, C_4), 31.4 (β-amino acid, C_{12}), 30.7 (Gln, γ), 29.3-29.2 (5C, β-amino acid and Pro, β), 28.9 (β-amino acid), 28.7 (β-amino acid), 26.6 (Gln, β), 25.5 (β-amino acid, C_5), 24.8 (Pro, γ), 22.2, (β-amino acid, C_{13}), 14.1 (β-amino acid,

Table 2. Antifungal activity of the first eluted fractions collected from RP-C18 column on conidial germination of *C. gloeosporioides*.

Fraction No. (5 mg/ml)	Conidial germination (%)*
1	94
2	90
3	98.3
4	96
5	95
6	96.7
7	0
8	0
9	0
10	0
11	0
12	96.7
13	94
14	93
15	93.3
16	95.7
17	92.7
18	93.7
19	95.3
20	96.7
Azoxystrobin + difenconazole (1 ml/3000 ml)	0
dH_2O	90

*Value is means of three replications of each fraction.

C_{14}). FAB-MS *m/z* 1065 $[M+Na]^+$.

DISCUSSION

The antifungal compound was produced by *B. subtilis* LB5 cultivated in PSB medium. This antifungal compound could inhibit conidial germination and mycelial growth of *C. gloeosporioides*, the causal agent of anthracnose disease and successfully used as biological control agent on mango anthracnose in growing seasons. In this study, compound light micrograph demonstrated that the germ tube of conidia of *C. gloeosporioides* was swollen or the germination was completely inhibited when treated with culture broth of LB5. Besides, strain LB5 also had antifungal activity against conidial germination of other wax apple fungal pathogens, including *Lasildiplodia theobromae* and *Pestalotiopsis euginae* (unpublished data). Moreover, this antifungal compound was remarkably heat stable, active over a wide range of pH value (3 to 11), and resistant to UV light at 365 nm. The results were consistent with Yu et al. (2002) that reported the antifungal compound in culture filtrate from *Bacillus amyloliquefaciens* strain B94 was highly stable to heat, pH 3-11 and UV light and used as biocontrol agent to suppress *Rhizoctonia solani*. Therefore, these results were supported by the successful use of *B. subtilis* strain LB5 as a biological control agent on mango in the fields and suggested that the suppressive effect is due to inhibition of conidial germination on the fruits. Through the extraction and isolation procedures, the major anti-fungal compound produced by LB5 was purified in pure form and the correctness of the compound was confirmed by comparing their spectral data of NMR and Mass with those of iturin A-2 described in the literature (Hiradate et al., 2002; Peypoux et al., 1978; Isogai et al., 1982).

Iturins are a family of lipopeptides extracted from the culture media of various strains of *B. subtilis* (Regine and Peypoux, 1994). The lipopeptide iturin family consists of iturins A-E, bacillomycin D, F and L, and mycosubulin. They are amphiphilic cyclic peptides with seven α-amino acids (A1-A7) and one unique β-amino acid. Iturin A, in nature, is produced as a mixture of up to eight isomers that are iturins A1-A8 (Isogai et al., 1982). Besides, iturin A has low toxicity and low allergic effect on human and animal and was found to be very active against phytopathogenic fungi (Regine and Peypoux, 1994) but only limited antibacterial and no antiviral activities

Table 3. Antifungal activity of the second eluted fractions collected from fraction no.11.

Fraction No. (5 mg/ml)	Conidial germination (%)*
11A	88.7
11B	90.7
11C	91.3
11D	92.3
11E	88.7
11F	0
11G	0
11H	18
11I	69.7
11J	74.7
11K	87.7
11L	90.7
11M	88.7
11N	88.7
11O	91
11P	88.7
11Q	88
11R	88.3
11S	91.3
11T	91.7
Azoxystrobin + difenconazole (1 ml/3000 ml)	0
dH_2O	89

*Value is means of three replications of each fraction.

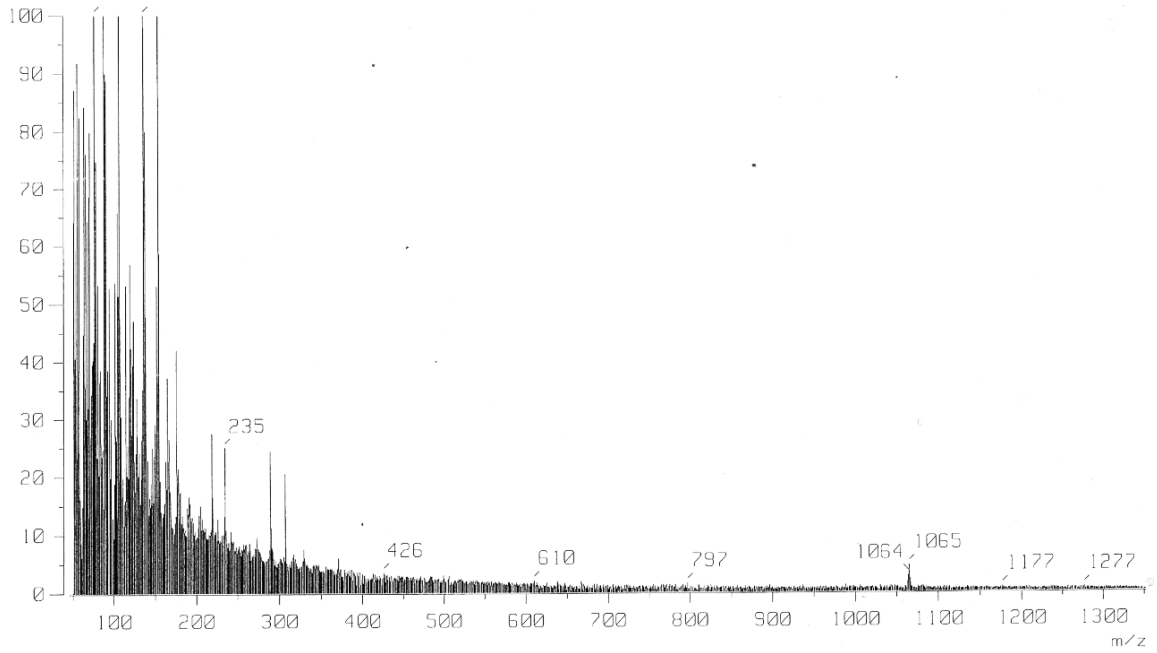

Figure 3. FAB-MS spectrum of fraction no. 11F.

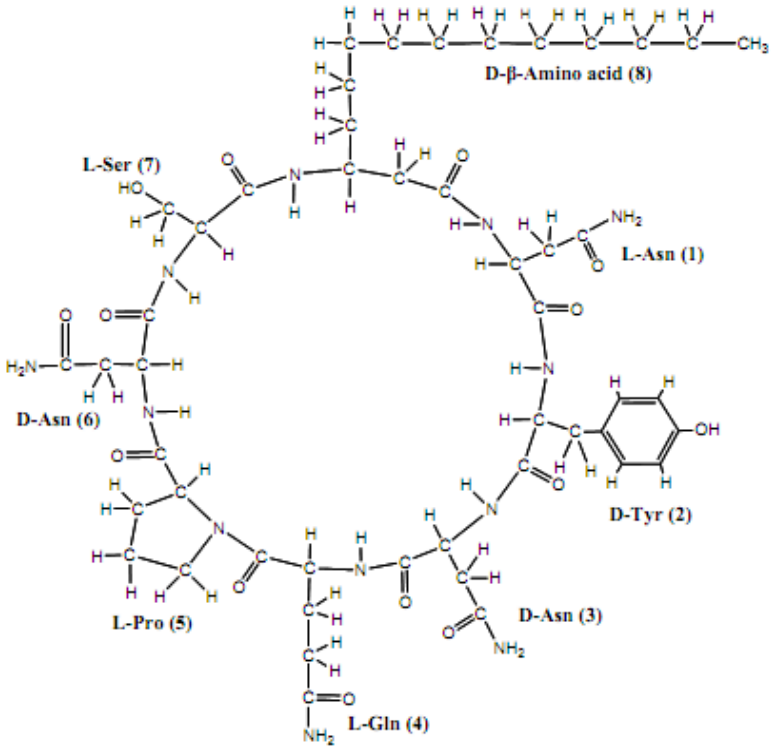

Figure 4. The structure of the antifungal compound isolated from *B. subtilis* strain LB5 and assigned as iturin-A2.

(Ongena and Jacques, 2007) and appeared as a good candidate to minimize the use of chemical fungicides (Regine and Peypoux, 1994). Iturins have been used for the biological control of a variety of phytopathogen such as *B. amyloliquefaciens* strain B94 used to suppress *R. solani* causing pre- and pot-emergence damping-off of soybean (Yu et al., 2002) and *B. amyloliquefaciens* strain RC-2 produced antifungal compounds iturins A-2 to A-7 which inhibited the development of mulberry anthracnose caused by *C. dematium*.

In conclusion, our results showed that *B. subtilis* strain LB5 produced lipopeptide antibiotic iturin A-2 which was the major antifungal compound that inhibited conidial germination of *Colletotrichum gloeosporioides* causal agent of anthracnose disease of mango and wax apple.

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