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*Full Length Research Paper*

# The antifungal mechanism of *Bacillus subtilis* against *Pestalotiopsis eugeniae* and its development for commercial applications against wax apple infection

**Hsiu Fen Lin[1], Tsang Hai Chen[2], and Shan Da Liu[3]***

[1]Graduate Institute of Bioresources, National Pingtung University of Science and Technology, Pingtung 912, Taiwan, Republic of China.
[2]Department of Plant Medicine, National Pingtung University of Science and Technology, Pingtung 912, Taiwan, Republic of China.
[3]Graduate Institute of Health and Biotechnology Industry, Meiho University, Pingtung 912, Taiwan, Republic of China.

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**The inhibition zones of three *Bacillus subtilis* isolates, BS-99, BS-23857 and BS-33608 against *Pestalotiopsis eugeniae* were, 13.5, 0, and 0 mm, respectively. BS-99 showed the strongest inhibitory activity, whereas, no inhibitory activity was reported for BS-23857 or BS-33608. The spore germination rates of *P. eugeniae* were not significantly inhibited by *n*-hexane, ethyl acetate (EtOAc), or methanol (MeOH) extracts of the fermentation broths of BS-99, BS-23857, and BS-33608. However, the hyphae of *P. eugeniae* became swollen and malformed after 12 h and stopped growing after 30 h of treatment with the MeOH extract of the BS-99 fermentation broth. Analysis by polymerase chain reaction (PCR), blood agar test plates, and high performance liquid chromatography (HPLC) indicated that, BS-99 produced the antibiotics, iturin A and surfactin, whereas, BS-23857 and BS-33608 only produced surfactin. These data suggest that, the antifungal activity of the *B. subtilis* BS-99 isolate against *P. eugeniae* is activated only when both iturin A and surfactin are present and not by surfactin alone. Unveiling the antifungal mechanism of *B. subtilis,* BS-99 could promote its commercial development and application as a biofungicide for controlling *P. eugeniae* infections of the wax apple, which is a highly valued fruit in Taiwan.**

**Key words:** *Bacillus subtilis*, iturin A, solvent extract, surfactin.

## INTRODUCTION

Wax apples are one of the most important tropical fruits in Taiwan, with a total planting area of 8700 ha and an annual production of 100 thousand metric tons. Due to many innovative growing techniques, the high quality wax apple of Taiwan is becoming a very competitive product in the international market. To harvest a good quality crop with high commercial value, fruit rot of the wax apple caused by *Pestalotiopsis eugeniae* has to be properly controlled. Biological control is becoming an important alternative to chemical control in managing plant diseases (Cubeta et al., 1985; El-Hassan and Gowen, 2006).

Several *Bacillus subtilis* isolates have been studied as biocontrol agents of plant pathogens because of their ability to produce various antibiotics (Sun et al., 2006). The potential of *B. subtilis* to produce antibiotics has been recognized for 50 years (Jamil et al., 2007). Cyclic lipopeptide antibiotics of the iturin, surfactin and fengycin families are important metabolites produced by *Bacillus* species (Hassan et al., 2010; Nihorimbere et al., 2010; Yu et al., 2002). Strong antibiotic activity of iturin A and surfactin is well known (Asaka and Shoda, 1996; Hassan et al., 2010). Iturin A contains seven amino acid residues (L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser) and one β amino acid residue (Yu et al., 2002; Hourdou et al., 1989). Surfactin contains a β-hydroxy fatty acid –with an ester peptide linkage to seven cyclic amino acid residues (L-Glu-L-Leu-DLeu-L-Val-L-Asp-D-Leu-L-Leu) (Kowall et al., 1998; Yakimov et al., 1995). The antifungal secondary metabolites of *B. subtilis* have been observed mainly

*Corresponding author. E-mail: mitsdliu@gmail.com. Tel: 88687799821 ext. 8101. Fax: 88687789837.

during microbial growth in media. The objectives of this study were to investigate the antifungal activities of various solvent extracts from fermented broth media of the *B. subtilis* isolates BS-99, BS-23857, and BS-3360; to analyze the antibiotic compounds in these media; and to evaluate the feasibility of using *B. subtilis* as a means of biological control in organic wax apple production.

## MATERIALS AND METHODS

### Bacterial isolate and culturing conditions

The bacterial antagonist, isolate BS-99, was isolated from soil and identified by PCR as *B. subtilis*. The *B. subtilis* isolates, BS-23857 and BS-33608 from the American Type Culture Collection (ATCC) were purchased from the Bioresources Collection and Research Center (Hsinchu, Taiwan, R.O.C). *B. subtilis* cells from a fresh slant culture were seeded into a 300 ml flask containing 50 ml nutrient broth (NB) (1% peptone, 0.3% beef extract, 0.5% NaCl, pH 7.0) and cultivated in a rotary shaker at 28°C for 18 h at 100 rpm. For production of inhibitory compounds, this seed culture was inoculated into a 2 L flask containing, 1000 ml NB medium and cultivated at the same conditions for 10 days.

### *In vitro* inhibitory plate trial by dual culture

The dual culture technique was used to test the inhibitory activity of the *B. subtilis* isolates, BS-99, BS-23857 and BS-33608 on the growth of plant pathogenic fungi *P. eugeniae*, *Botryodiplodia theobromae*, *Rhizotonia solani*, *Sclerotium rolfsii*, *Colletotrichum gloeosporioides*, *Phytophthora capsici* and *Fusarium oxysporum* f. sp. *cubense*. Pure cultures of these fungi were initially grown in Petri dishes containing standard PDA (20% potato extract, 2% dextrose and 1.5% agar) medium and incubated at 28°C for 5 days. After this period, 8 mm disks were cut from the edge of actively growing colonies of each fungus with the aid of a cork borer. Two plugs were placed at opposite edges of each dish. Each *B. subtilis* isolate was streaked on the center of the PDA plate at the time of fungi transplanting. After incubation for 2 to 5 days at room temperature, two perpendicular directions of radial growth of the fungal colony were measured using a vernier caliper (Leelasuphakul et al., 2008).

### Detection of the production of Iturin A and surfactin by molecular techniques

Genomic DNA from *B. subtilis* was isolated using QIAGEN QIAprep miniprep kits. Agarose gel electrophoretic analysis was performed according to the manufacturer's instructions. Surfactin was PCR-amplified using the oligonucleotide primer pair of *sfp*-f (5′-ATG AAG ATT TAC GGA ATT TA-3′) and *sfp*-r (5′-TTA TAA AAG CTC TTC GTA CG-3′). Iturin A was PCR-amplified, using the primer pairs of *ituD*-f (5′-ATG AAC AAT CTT GCC TTT TTA-3′) and *ituD*-r (5′-TTA TTT TAA AAT CCG CAA TT-3′), and *lpa-14*f (5′-ATG AAA ATT TAC GGA GTA TA-3′) and *lpa-14*r (5′-TTA TAA CAG CTC TTC ATA CG-3′). The amplification reaction of iturin A was performed with a DNA thermal cycler, using a step-cycle program set for denaturing at 94°C for 60 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s, for a total of 30 cycles (Hsieh et al., 2008). The amplification reaction of surfactin was performed using the same procedure, except annealing was set at 46°C for 30 s, extension at 72°C for 60 s, for a total of 25 cycles (Hsieh et al., 2004).

### Extraction of metabolites by solvents

The fermentation broths (1 l) of *B. subtilis* BS-99, BS-23857 and BS-33608 were extracted by maceration for three days with the following organic solvents of increasing polarity: n-hexane (2 l × 3) and ethyl acetate (2 l × 3). The residue of fermentation broth was centrifuged at 10,000 rpm for 20 min to remove the cells. The cell-free supernatants were adjusted to a pH of 2.0 with 12 N HCl and the precipitates were harvested by centrifugation at 12,000 rpm for 20 min. The pellet was extracted with methanol (MeOH). After each extraction, the solutions were filtered through 0.22 µm filter paper, and the solvents were evaporated in a rotatory evaporator at reduced pressure. The n-hexane extract contained 23 mg of BS-99, 17 mg of BS-23857, and 20 mg of BS-33608. The EtOAc extract contained 184 mg of BS-99, 100 mg of BS-23857, and 132 mg of BS-33608. The MeOH extract contained 78 mg of BS-99, 77 mg of BS-23857, and 74 mg of BS-33608.

### Preparation and detection of Iturin A and surfactin by HPLC and blood agar plates

The pellet collected from the MeOH extract was subsequently dissolved in 1 ml methanol, and then, the solution was filtered with a 0.22 µm PTFE membrane filter (Advantec, Tokyo, Japan). The 20 µl MeOH extracts of BS-99, BS-23857 and BS-33608 were injected into a reverse phase HPLC column (RP-18 column, 5 µm, 4 × 250 mm; Merck) for detecting Iturin A and surfactin. A mixture of acetonitrile and 10 mM/l ammonium acetate (2:3, v/v) was used as the mobile phase with a flow rate of 1.0 ml/min and monitored at 280 nm to detect Iturin A. The detection conditions for surfactin were a mobile phase of acetonitrile/3.8 mM trifluoroacetic acid (4/1, v/v) with a flow rate of 1.0 ml/min and detection at 210 nm. Pure Iturin A and surfactin from Sigma were used as references for identification. There are six isoforms for Iturin A (Kowall et al., 1998; Hsieh et al., 2004). The evaluation of biosurfactant activities of *B. subtilis* BS-99, BS-23857 and BS-33608 on blood agar plates was carried out using the method of Hsieh et al. (2004).

### Inhibition of conidia germination of *P. eugeniae* by solvent extracts

A 10 mg sample of the extracted substance was dissolved in 1 ml of dimethyl sulfoxide (DMSO). Two 10 µl solutions were placed separately into two round depressions of depression glass slides, then, two 10 µl samples of *P. eugeniae* conidial suspensions ($10^5$ conidia per ml) were placed separately into each depression. Two 10 µl samples of sterile distilled water and DMSO were placed into two depressions of another slide as a positive and a negative control. To increase conidial germination, 5 µl of potato dextrose broth (20% potato extract and 2% dextrose) was added to each depression. The slides were incubated in a moist chamber at room temperature for 12 h (Yoshida et al., 2001). Three microscopic fields of 100 conidia were selected and observed. The numbers of germinated conidia were identified by a doubling of the conidia diameter. The two depressions on each slide were considered subsamples. Treatments were replicated three times. The test was repeated twice.

## RESULTS AND DISCUSSION

Inhibition zones were measured and compared among 12 isolates of *B. subtilis* by the dual culture method. It has been proposed that substances or antibiotics, toxic to the

**Table 1.** *In vitro* inhibitory plate trial by the dual culture method.

| Pathogen | Inhibition zones (mm) | | |
|---|---|---|---|
| | BS-99 | BS-23857 | BS-33608 |
| *Pestalotiopsis eugeniae* | 13.5 | 0 | 0 |
| *Botryodiplodia theobromae* | 11.0 | 0 | 0 |
| *Rhizotonia solani* | 10.0 | 0 | 0 |
| *Sclerotium rolfsii* | 3.6 | 0 | 0 |
| *Colletotrichum gloeosporioides* | 6.8 | 0 | 0 |
| *Phytophthora capsici* | 7.0 | 0 | 0 |
| *Fusarium oxysporum* f. sp. *cubense* | 10.8 | 0 | 0 |

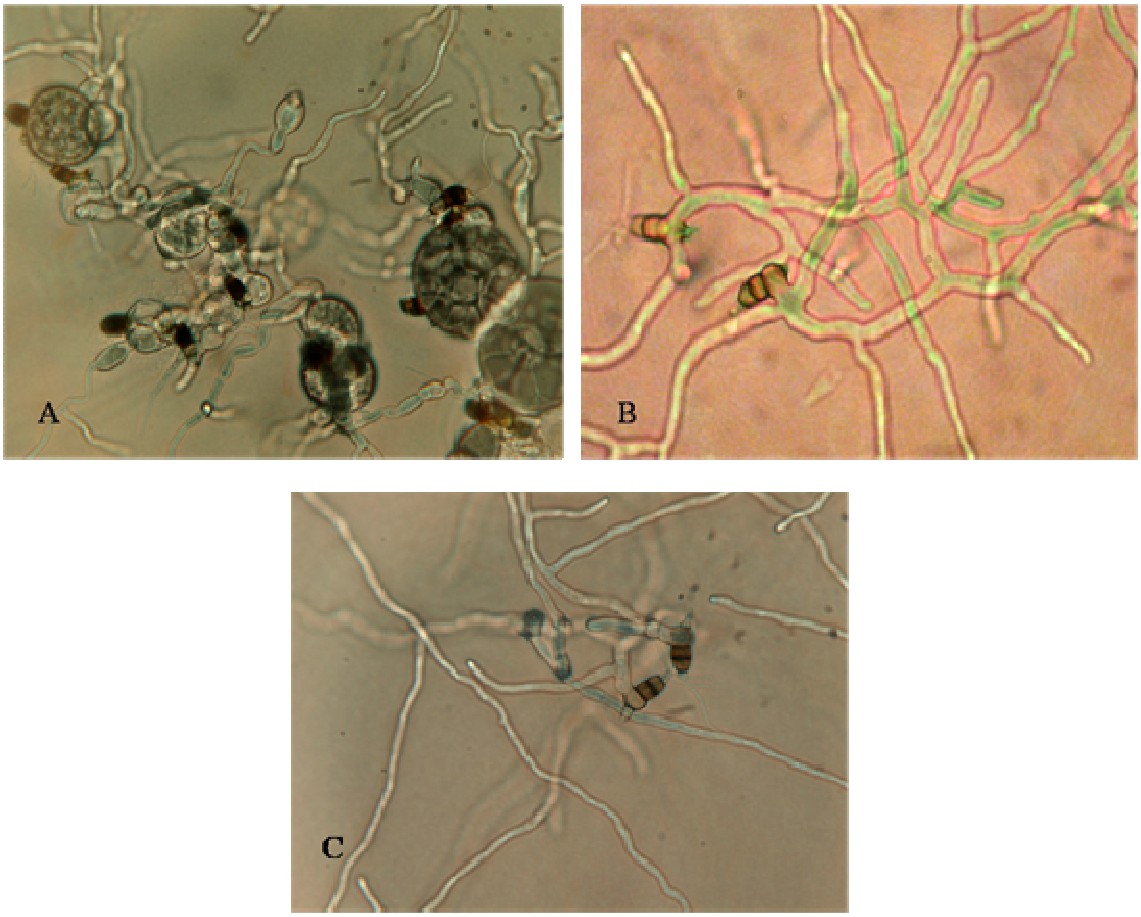


**Figure 1.** Hyphae growth during spore germination of *P. eugeniae* 12 h after treatment with MeOH extracts from fermentation broths of BS-99, BS-23857, and BS-33608; (A) swollen and deformed hyphae growth after treatment with the BS-99 extract; (B) normal growth after treatment with the BS-23857 extract; (C) normal growth after treatment with the BS-33608 extract.

potential pathogens, are secreted into the growth medium (Leelasuphakul et al., 2006). The inhibitory activities of *B. subtilis* isolates BS-99, BS-23857, and BS-33608 were significantly different (Table 1). The highest activity was exhibited by BS-99, with an inhibition zone of 13.5 mm against *P. eugeniae* and 11.0 mm against *Botryodiplodia theobromae*. Antifungal activity against the seven selected pathogens was not observed for BS-23857 or BS-33608.

The MeOH extract from the fermentation broth of BS-99 effectively inhibited spore germination at a rate of 25.3%. The hyphae of *P. eugeniae* became swollen and malformed at 12 h and stopped growing, 30 h after treatment (Figure 1). Extracts from BS-23857 and

**Table 2.** Inhibition of spore germination of *Pestalotiopsis eugeniae* by *B. subtilis* extracts using different solvents.

| Extract layer | Inhibition of spore germination rate (%) | | |
|---|---|---|---|
| | BS-99 | BS-23857 | BS-33608 |
| *n*-hexane | 0 | 0 | 0 |
| EtOAc | 0 | 0 | 0 |
| MeOH | 25.3 [1] | 0 | 0 |

[1] The hyphae became swollen and malformed and stopped growing 30 h after treatment.

BS-33608 showed no inhibition against spore germination of *P. eugeniae* (Table 2). The growth and shapes of hyphae of *P. eugeniae* treated with extracts of BS-23857 and BS-33608 were normal (Figure 1). Although, *B. subtilis* BS-99 was found to be active against pathogenic fungi by the dual culture plate method, extracts by *n*-hexane or EtOAc did not have antifungal activities against *P. eugeniae*. These results indicated that extraction of the antibiotic substances from the fermentation broth of *B. subtilis* BS-99 using *n*-hexane or EtOAc as solvent was not effective.

Specific primers to *itu*D, *lpa*-14 and *sfp* were used for PCR amplification of the antibiotic-encoding genes, following the procedures of Hsieh et al. (2004, 2008) (Figures 2 and 3). *B. subtilis* BS-99 DNA contained the *itu*D, *lpa*-14, and *sfp* genes. Both 1203 and 675-bp fragments were amplified with the *itu*D and *lpa*-14 primers from *B. subtilis* DNA, which indicated the presence of iturin A. A 675-bp fragment was amplified with the *sfp* primer from three *B. subtilis* isolates; this technique could be used as an approach to identify *Bacillus* species that produce surfactin. Hassan et al. (2010) reported the use of the *sfp* gene as a molecular marker to confirm the production of surfactin by *B. subtilis* isolates. A similar result was also obtained by HPLC analysis, which showed the production of Iturin A and surfactin antibiotics by *B. subtilis* BS-99 (Table 3). However, the production of iturin by *B. subtilis* BS-23857 or BS-33608 was not detected by PCR or HPLC analysis (Figure 3 and Table 3).

Surfactin is known to inhibit fibrin clotting and, to lyse erythrocytes (Borchert et al., 1994). The blood agar plate method was used to detect surfactin. The hemolytic clear zones around each colony on blood agar plates with *B. subtilis* BS-99, BS-23857, and BS-33608 indicated surfactin was produced by the three isolates. Our results demonstrated the production of both cyclic lipopeptide antibiotics, iturin A and surfactin, by *B. subtilis* BS-99 and the inhibition of *P. eugeniae* by this isolate. We also found that the *B. subtilis* isolates BS-23857 and BS-33608, which did not produce surfactin, were not inhibitory to *P. eugeniae.* Iturin A has very strong antibiotic activity, while surfactin has weak antibiotic activity and could be used as a potent surfactant (Asaka and Shoda, 1996). Ohno et al. (1995) showed that the cytolytic activity of surfactin weakens cell membranes and enables the easy attack of iturin A. Several studies have demonstrated that iturin A

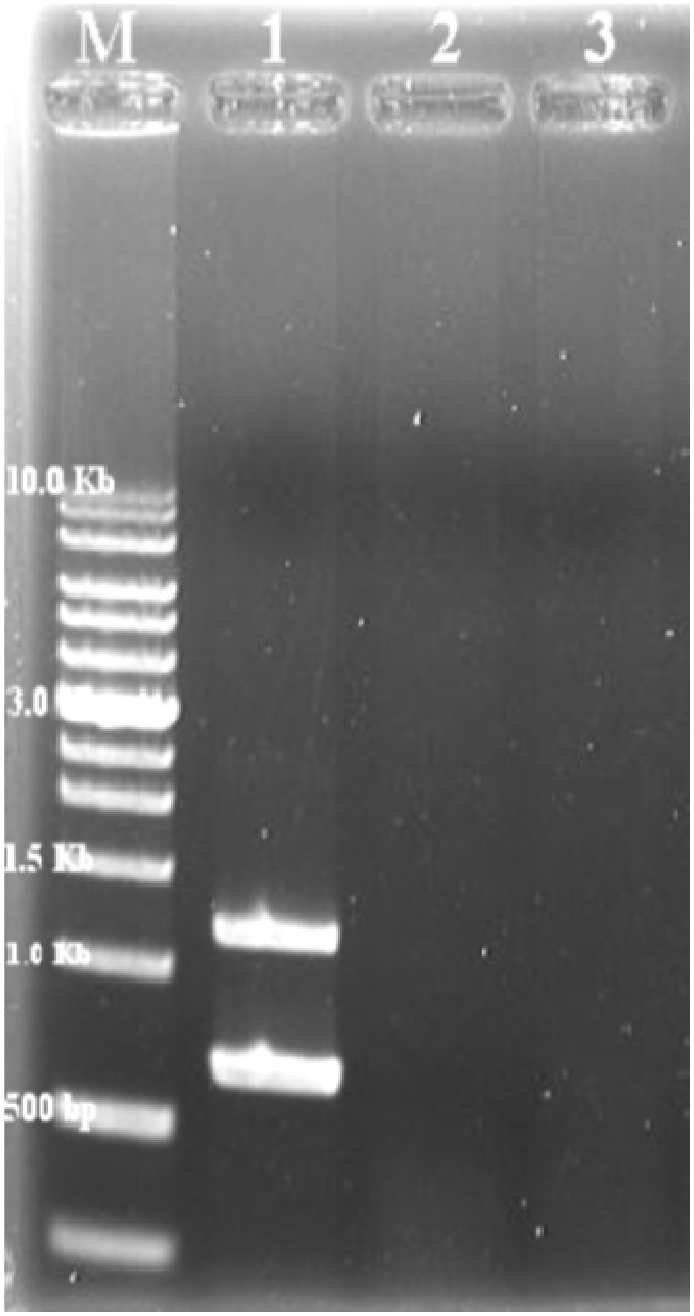


**Figure 2.** Detection of iturin A from the 3 isolates of *B. subtilis* by PCR with *itu*D- and *lpa*-14-specific primer pairs.M: Marker (1 Kb ladder); Lane 1: BS-99; Lane 2: BS-23857; Lane 3: BS-33608.

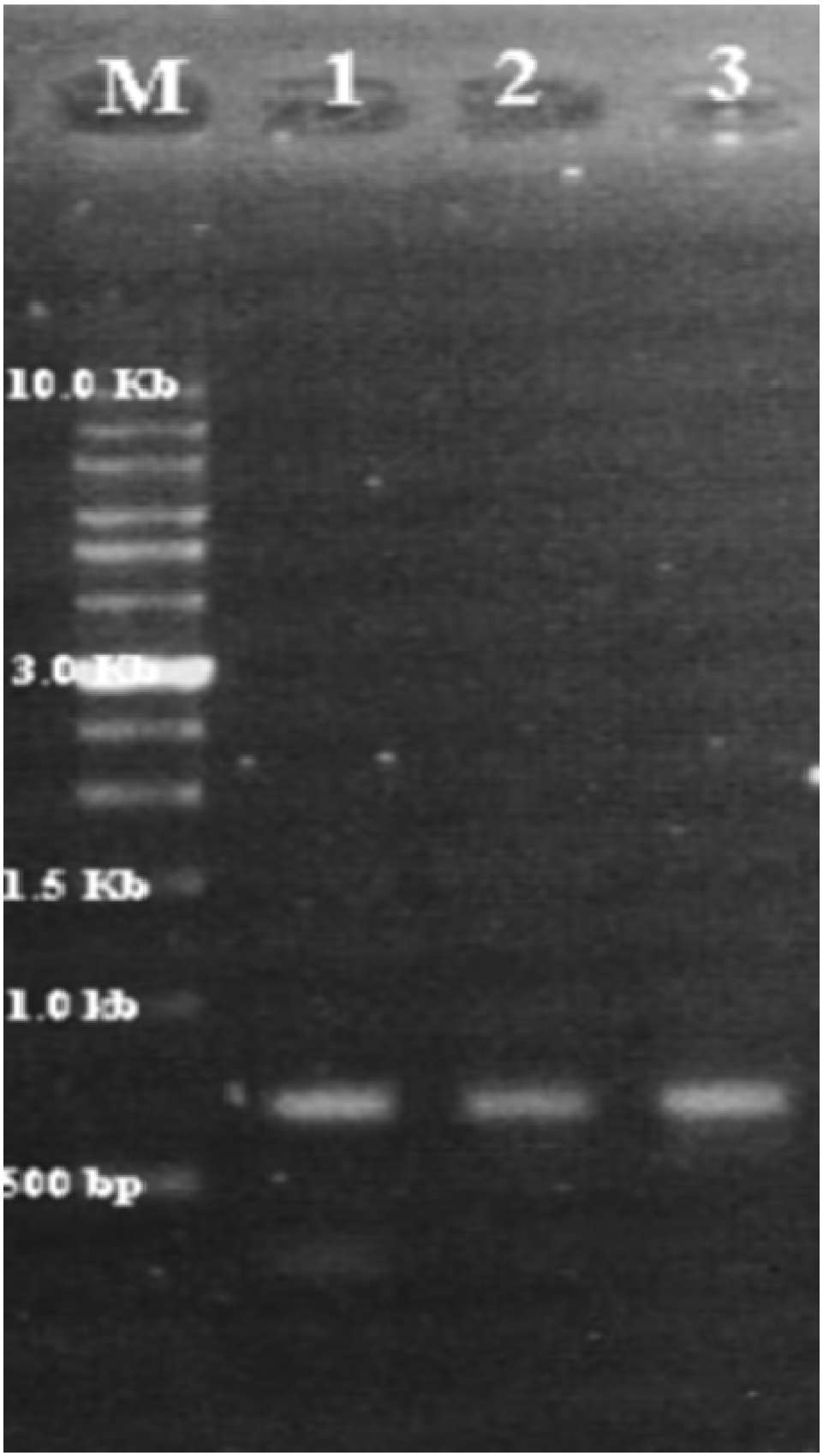


**Figure 3.** Detection of surfactin from the 3 isolates of *B. subtilis* by PCR with *sfp*-specific primer pairs. M: Marker (1 Kb ladder); Lane 1: BS-99; Lane 2: BS-23857; Lane 3: BS-33608.

**Table 3.** Antibody detection in different solvent extracts by HPLC.

| Extract layer | Quantitative Iturin A[1] (mg/L) | | | Quantitative Surfactin[1] (mg/L) | | |
|---|---|---|---|---|---|---|
| | BS-99 | BS-23857 | BS-33608 | BS-99 | BS-23857 | BS-33608 |
| EtOAc | -[2] | - | - | - | - | - |
| n-hexane | - | - | - | - | - | - |
| MeOH | +[3](180.3) | - | - | + (112.4) | + (94.0) | + (171.6) |

[1] Two standard antibiotics from Sigma were used as references. [2] Not detected. [3] Detected.

and surfactin from *B. subtilis* are potent biocontrol agents of fungi, acting synergistically through the degradation of the fungal cell walls (Ohno et al., 1995; Phae and Shoda, 1991; Sandrin et al., 1990). This study confirms that the two compounds exhibit a synergistic mechanism of antifungal activity against *P. eugeniae*. Our results suggest that the *B. subtilis* isolate BS-99 is a potential source for an effective commercial biofungicide. The antifungal activity of *B. subtilis* BS-99 demonstrated in this study provides very valuable information in promoting the commercialization of biofungicide for the management of wax apple disease in Taiwan.

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