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Full Length Research Paper

Isolation and characterization of a potential biocontrol *Brevibacillus laterosporus*

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An antagonist bacterium ZQ2 was isolated from an apple rhizosphere at Mount Tai in China. The bacterium strongly inhibited the growth of numerous apple phytopathogens *in vitro*, such as *Rhizoctonia solani*, *Fusarium oxysporum*, *Fusarium solani*, and *Physalospora piricola*. The inhibition rates against the different fungi ranged from 55.26 to 88.17. The strain ZQ2 was identified as *Brevibacillus laterosporus* based on morphology, biochemical tests, and 16S rDNA sequence analysis. The antifungal metabolites produced by ZQ2 were thermally stable even after being maintained at 121°C for 30 min. Meanwhile, the activity against the growth of *R. solani* was almost unchanged when the culture filtrate was irradiated under ultraviolet (UV) or at pH ranging from 1 to 11, and was only reduced under pH conditions from 12 to 14. When observed under a light microscope, the mycelia of *R. solani* inhibited by the antifungal metabolites appeared abnormal in growth. The strong antifungal activity and relatively stable active substances of *B. laterosporus* ZQ2 showed great potential for controlling fungal diseases in apples.

Key words: *Brevibacillus laterosporus*, characterization, antifungal activity.

INTRODUCTION

Rhizoctonia solani, Fusarium oxysporum, Fusarium solani, and Physalospora piricola, common and damaging phytopathogenic fungi of apple trees, have a wide range of hosts and are major constraints to apple production. At present, chemical control remains the primary means of preventing the diseases caused by these fungi. Numerous synthetic chemicals comprise the major market share of fungicides (Imre et al., 2009; Jonssson et al., 2010). However, due to the increasing resistant mutants and environmental pollution, there is an increasing demand for more effective and safer fungicides with novel modes of action (Zhenzhen et al., 2010). Consequently, the selection of antagonistic microorganisms for biological control is considered as an alternative method (Li et al., 2009).

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In the last few years, various antifungal bacteria were investigated as potential biocontrol agents. Scientists have focused on the use of antagonistic bacteria and their active substances. Members of *Brevibacillus* clade, established as an independent genus from the reclassification of *Bacillus brevis* in 1996 (Shida et al., 1996), are well-known biocontrol microorganisms that produce structurally diverse secondary metabolites with broad antibiotic spectra. Some of these metabolites, such as chitinase and gramicidin oviet, have been extensively studied (Tatsushi and Kiyoshi, 2009). Numerous *Brevibacillus* species have potential as biocontrol agents in agricultural production, and some of these strains have become research hotspots.

In the present study, an antagonist *B. laterosporus* strain, ZQ2, was isolated from an apple rhizosphere via an *in vitro* screening technique and identified through morphologic observation, biochemical tests, and 16S rDNA sequence analysis. This strain produces stable metabolites that strongly inhibit numerous phytopathogenic

fungi of apple trees, including *R*. *solani*, *F*. *oxysporum*, *P*. *piricola*, and so on. The present research aims to screen novel and effective antagonistic bacterium and hopes to provide an alternative resource for the biocontrol of fungal diseases in apples.

MATERIALS AND METHODS

Isolation and cultivation of the rhizosphere bacteria

Soil samples were collected from an apple rhizosphere at Mount Tai in Shandong Province, China and stored at low temperature at the College of Forestry in Shandong Agricultural University. One gram of soil was suspended aseptically with 100 ml of physiologic saline, agitated in a rotary shaker at 200 rpm for 30 min, and centrifuged. Afterward, serial dilutions of the sample supernate were prepared up to 10^{-6} with sterile water. Then, 0.1 ml of each gradient was spread on potato dextrose agar plates (PDA: 200 g potato, 20 g glucose, 5 g beef extract, 5 g sodium chloride, and 20 g agar in 1 L distilled water) and incubated for approximately 36 h at 28°C. The visible single colony was purified from the proper gradient by streak-plate method. The isolates were cultured on PDA plates and stored at 4°C.

Determination of antifungal activities

The antifungal activities of all bacterial isolates were determined in Petri dishes containing PDA medium using *R. solani* as the indicating fungus. The isolated bacteria were inoculated into Luria–Bertani medium (10 g peptone, 5 g yeast extract, and 10 g NaCl in 1 L distilled water) and incubated for 12 h at 30°C. After centrifugation, the filtrate was diluted with potato dextrose broth medium (PDB: 200 g potato, 20 g glucose, 5 g beef extract, and 5 g sodium chloride in 1 L distilled water) at 1:25 (v/v) and poured into Petri dishes. Six-millimeter disks of the tested fungi were placed at the center of the mixed PDA plate. Inhibition of fungal growth was evaluated as the percentage reduction of mycelial growth contrasted with that of the control plates without bacterial cell-free filtrate in the medium. The antifungal activities were calculated using the following equation: Growth inhibition (%) = [(mycelia length in the control plate – mycelia length in the treated plate)/ mycelia length in the control plate × 100] (Dake et al., 2007).

Identification of strain ZQ2

The bacterial isolate ZQ2, which exhibits high antifungal activity against *R. solani*, was screened for strain identification. Cell morphology was observed via a light microscope (Nikon Eclipse E200, Japan), including Gram-staining and microexamination (shape and size of the cell, spore formation, and so on). Physiological and biochemical tests were performed according to literature (Xiuzhu and Miaoying, 2001), such as growth in pH 5.5 and 9.0, at temperature 15 and 50°C, and in NaCl concentration at 2 and 5%. Molecular technology was employed for exact identification of the strain. The bacterial genomic DNA was extracted and was purified using TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit (Takara, Japan). The 16S rDNA of the bacterium was amplified via polymerase chain reaction (PCR) and sequenced. The primers used for the amplification were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR mixtures contained 5 µL 10 × buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), 4 µl of 2.5 mmol/L deoxynucleoside triphosphates, 1 µl of each 10 µmol/L primer, 2 µl 2 U/µl TaKaRa Ex Taq, and 20 ng template DNA. The thermal cycling conditions were as follows: 2 min denaturation at 94°C, then 25 cycles of denaturation for 0.5 min at 94°C, annealing for 1 min at 50°C, extension for 1 min at 72°C, followed by a final 10 min extension at 72°C. The products were examined and recovered by electrophoresis in a 1% (w/v) agarose gel and then sequenced.

Correlation between culture time and antifungal activity

A loop of single ZQ2 colony was inoculated from the PDA plate into a 250 ml Erlenmeyer flask with 50 ml of potato dextrose broth medium (PDB: 200 g potato, 20 g glucose, 5 g beef extract, and 5 g sodium chloride in 1 L distilled water) and incubated for 18 h at 28°C in a rotary shaker at 200 rpm to prepare the liquid seed. One milliliter of the seed culture was then transferred aseptically into a 250 ml flask containing 50 ml and fermented 200 rpm for 72 h at 30°C. Samples were taken every 6 h to test the antifungal activity against *R. solani* with the aforementioned technique.

Stability of the antifungal metabolites produced by strain ZQ2

The pH of the culture filtrate of strain ZQ2 was adjusted from 1.0 to 14.0 using 1 M HCl or 1 M NaOH, respectively. After maintaining at 4°C for 12 h, the pH levels of the samples were adjusted to 7.0 and antifungal bioassays were conducted to detect the strain activities. The effect of temperature was also assessed by maintaining the culture filtrate at 60, 80, 100, and 120°C for 30 min. The remaining activities against *R. solani* were tested after cooling to room temperature. A sample at room temperature and pH 7.0 was assayed as the control.

In the UV test, 10 ml of culture filtrate was poured into a 7.5 cm wide Petri dish, which was placed 30 cm under a 30 W UV lamp. Then, 1 ml sample was taken every hour for 6 h. The variations in the remaining activities were studied. All experiments were performed in triplicate, and the mean value was analyzed with SAS 9.0 software.

In vitro antifungal activities of the antifungal metabolites

Eight pathogenic fungi, namely, *F. oxysporum*, *F. solani*, *P. piricola*, *Aspergillus fumigatus*, *Alternaria alternata*, *Valsa sordida*, *Colletotrichum gloeosporioides*, and *Botrytis cinerea*, were kindly provided by the Plant Protection College of the Shandong Agriculture University (Taian, China) and maintained on PDA plates at 4°C prior to use. The inhibitory abilities of the antifungal substances produced by strain ZQ2 against the pathogens were determined as the percentage reduction in mycelial growth. As a control, the target fungi were grown on PDA plates without culture filtrate. The results were recorded after 72 h of incubation at 30°C. The morphology of the inhibited *R. solani* mycelia, along the edges, was then examined under a light microscope (Nikon Eclipse E200, Japan).

RESULTS

Isolation of antifungal strains

A total of 127 bacterial strains were obtained from the soil sample and bioassayed. Among the 12 strains that showed antifungal activity (data not shown), strain ZQ2 most strongly inhibited *R. solani*. It exhibited high activity in the *in vitro* test, with an inhibition rate of up to 80.17%.

Table 1. Physiological and biochemical characterization of *B. laterosporus* ZQ2 and standard *B. laterosporus*

Characteristic	ZQ2	Standard *B. laterosporus*
Shape	Rod	Rod
Size	0.83 × 3.6 µm	0.7–0.9 µm × 3–5 µm
Endospore forming	-	-
Gram stain	-	-
Motility	-	-
Oxidase	-	-
Voges-Praskauer (V-P) test	-	-
Starch hydrolysis	-	-
Glucose fermentation	-	-
Growth in pH 5.5	-	-
Growth in pH 9.0	-	-
Growth at 15°C	-	-
Growth at 50°C	-	-
Growth in 2% NaCl	-	-
Growth in 5% NaCl	-	-
Anaerobic growth	-	-

Identification of strain ZQ2

Strain identification results suggest that ZQ2 should belong to the *Brevibacillus* genus. The strain is Gram-positive, rod-like (0.8 to 1.5 µm × 1.2 to 2.0 µm), motile, facultative anaerobic bacterium with endospores formed in the cell. The strain can hydrolyze starch and gelatin, and produces catalase and oxidase. However, it did not grow at temperatures higher than 55°C or lower than 15°C. Detailed morphologic and biochemical characteristics are summarized and compared with the standard *B. laterosporus* (Xiuzhu and Miaoying, 2001) in Table 1. The 16S rDNA of this strain was then amplified and sequenced, consisting of 1444 nucleotides. The phylogenetic tree, established via the SAS 9.0 software using the data obtained from NCBI Genbank (Figure 1), also demonstrated that the strain is most likely to be *B. laterosporus*. Therefore, ZQ2 was designated as *B. laterosporus* strain ZQ2, based on characterization and clustering similarity, and its 16S rDNA sequence was submitted to NCBI Genbank (Accession no. EU471747).

Correlation between culture time and antifungal metabolite production

The antifungal activity of strain ZQ2 was not detected until 12 h after inoculation (Figure 2), and then the active metabolites were continuously secreted along with the cell growth. The strongest activity against *R. solani* was observed at 60 h, after which the activity continuously declined until 72 h. This behavior demonstrates that the optimal time to harvest the antifungal metabolites produced by strain ZQ2 is 60 h after inoculation.

Stability of the antifungal metabolites

Figure 3a illustrates the thermal stability of the antifungal substances. The activity remained at more than 88% even when the culture filtrate was maintained at 120°C for 30 min. The same result was observed in the UV radiation test. The antifungal activity against *R. solani* was almost unchanged during the sampling period (Figure 3b), suggesting that the antifungal metabolites produced by ZQ2 are stable in open environments.

The antifungal activity was significantly reduced when the compounds were exposed to basic conditions from pH 12 to 14 (Figure 3c), but the active substances remained stable even after exposure to pH ranging from 1 to 11 (>80%).

In vitro antifungal test

The antifungal substances produced by ZQ2 displayed strong *in vitro* inhibition against *F. oxysporum*, *F. solani*, *P. piricola*, *A. fumigatus*, *A. alternata*, *V. sordida*, *C. gloeosporioides*, and *B. cinerea* after 3 days of incubation (Table 2). The maximum inhibition rate was observed against *V. sordida* (88.17%), followed by *A. alternata* (79.02%), and *A. fumigatus* (77.13%). When observed under a light microscope, the mycelia of *R. solani* along the edges of interaction zone appeared thick and opaque, their cytoplasmic contents became cumulate, and parts of the mycelia ruptured. There were no spores in the field of vision (Figure 4a). However, the fungal mycelia on the control plate showed normal thin and clear radial growth with visible spores (Figure 4b).

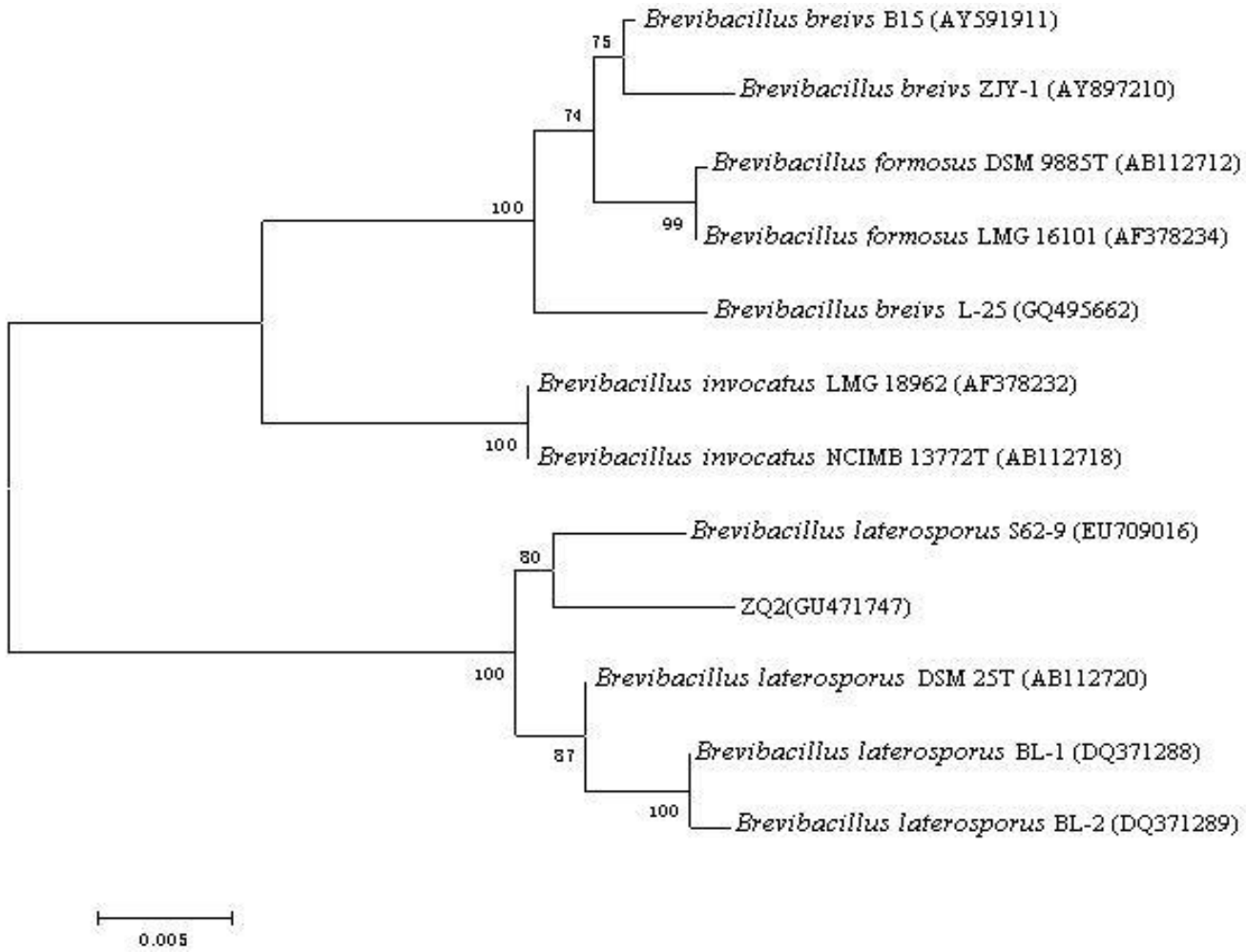

Figure 1. Phylogenetic tree of *B. laterosporus* ZQ2 based on 16S rDNA sequence analysis using the neighbor-joining method.

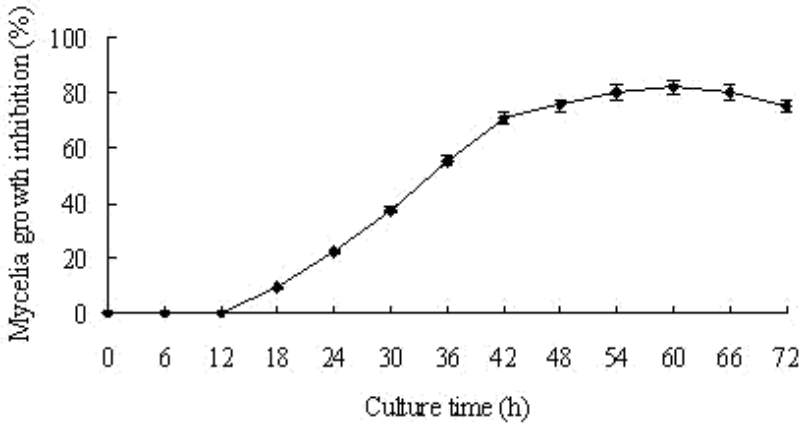

Figure 2. Correlation between culture time and antifungal activity.

DISCUSSION

Combined technologies were used in the present study to identify the bacterium strain ZQ2. According to its morphology, the bacterium is rod-shaped, Gram-positive, motile, and spore-forming (0.8 to 1.5 µm × 1.2 to 2.0 µm).

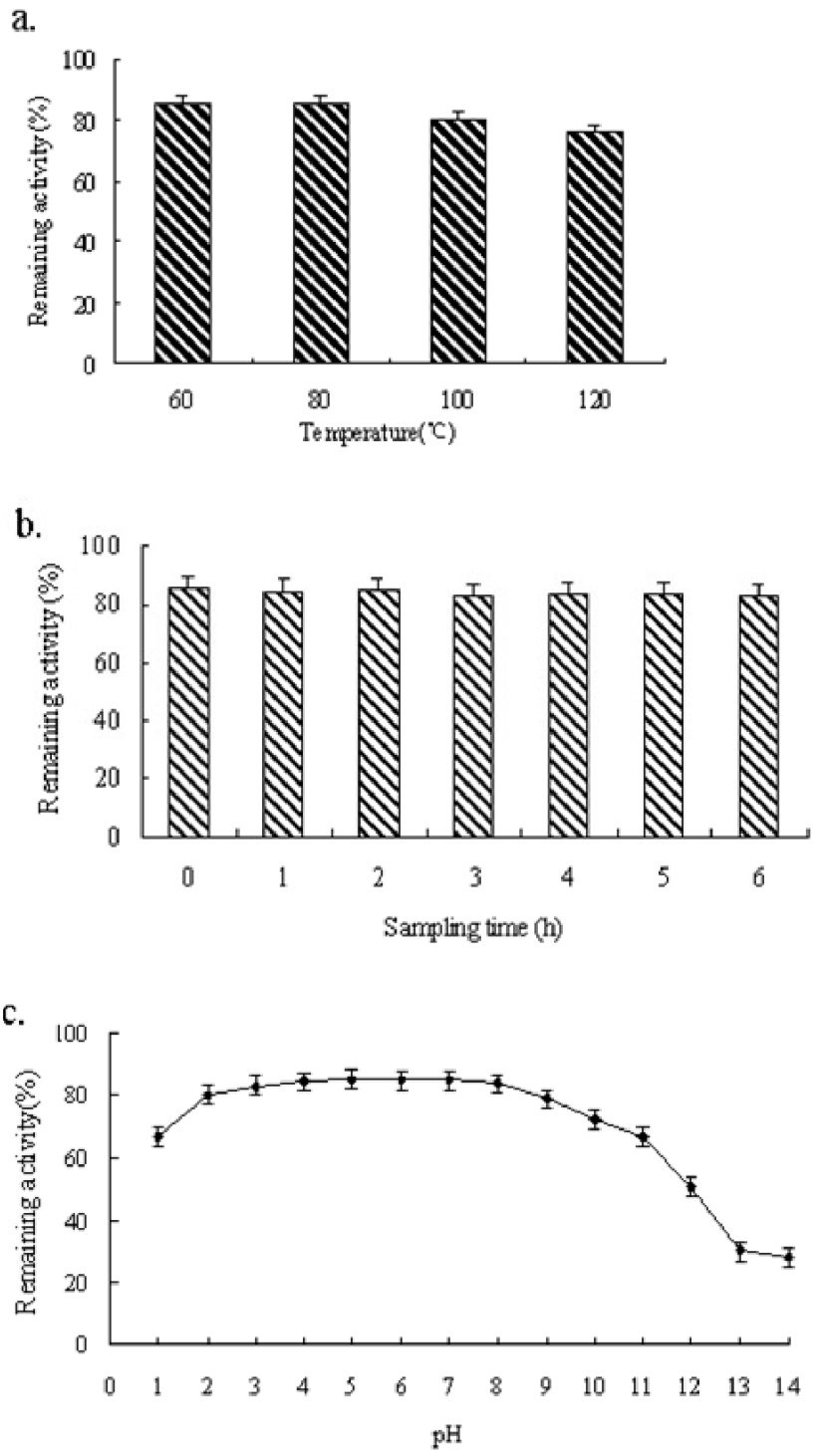

Figure 3. Effect of temperature (a), UV radiation (b), and pH conditions (c) on the stability of the antifungal substances from *B. laterosporus* ZQ2.

Table 2. Inhibition of phytopathogens using *B. laterosporus* strain ZQ2 antifungal metabolites.

Pathogenic fungi	*F. oxysporum*	*F. solani*	*P. piricola*	*A. fumigatus*	*A. alternata*	*V. sordida*	*C. gloeosporioides*	*B. cinerea*
Inhibition rates (%)	64.70	66.41	55.26	77.13	79.02	88.17	66.83	61.95

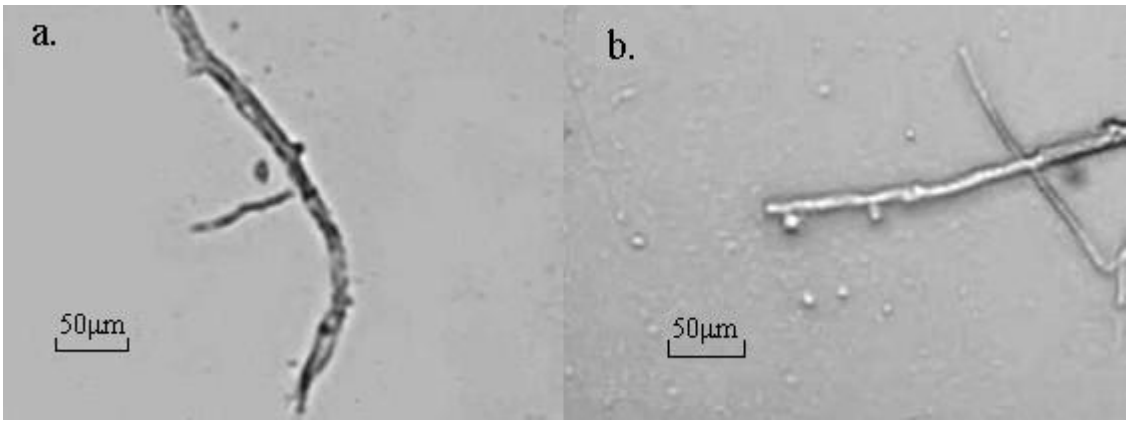

Figure 4. Effects of *B. laterosporus* ZQ2 active metabolites on the growth of *R. solani*: (a) mycelia inhibited by the antifungal substances and (b) healthy mycelia on the control plate.

Biochemical indices showed positive oxidase production and glucose fermentation but negative in starch hydrolysis and V-P tests. Strain ZQ2 showed almost identical properties to the members of *B. laterosporus* (Saikia, et al., 2010), based on 16S rDNA sequence analysis. Homology also revealed the same result.

B. laterosporus bacteria are potent biocontrol agents against numerous insects belonging to orders lepidoptera and coleoptera (Oliveira et al., 2004) and can be used for nematode control due to their production of extracellular neutral protease (Baoyu et al., 2006). However, there are few reports on the use of *B. laterosporus* as a biocontrol agent for pathogenic fungi. To the best of our knowledge, this is the first report of the isolation of *B. laterosporus* with broad-spectrum antifungal activity from an apple rhizosphere.

Brevibacillus spp. can produce a wide variety of metabolites with antifungal activity, which can control plant diseases as biocontrol agents (Sunita et al., 2010). A number of the active metabolites are fungicidal or fungistatic peptides that are non-ribosomally synthesized by multi-enzyme–catalyzed systems. Most of these peptides are very stable because of their low molecular weight and specific structure. Some non-ribosomal peptides have already been purified from *B. laterosporus*. Kelsey et al. (2007) isolated and elucidated the structure of the lipopeptide tauramamide from *B. laterosporus* Ren et al. (2007) purified an antimicrobial peptide, R-1, with a small molecular weight and composed of 9 kinds of amino acids from marine *B. laterosporus*. In the present study, the active substances produced by *B. laterosporus* ZQ2 were relatively stable under different conditions and have broad and strong antifungal activity. These features show great potential in the application of strain ZQ2 and its metabolites in controlling pathogenic apple fungi.

The mechanisms of action of many antifungal peptides are still undetermined although investigations were conducted during the last few decades. Daniel et al. (2010) found that many short cationic peptides accumulate on the cell membrane of fungal hyphae and disturb sterol-rich membrane domains. Thicker hyphae and depolarized cells were observed in micrographs, and most spores did not germinate after antifungal peptide treatment. The phenomenon is very similar to the results in the present study, signifying that *B. laterosporus* strain ZQ2 likely secretes peptides with the same inhibitory mechanism on fungi.

The antifungal substances produced by ZQ2 are being purified from the PDB medium of ZQ2, and elucidation of their structures is in progress. In further research, the bioactive products will be characterized and the medium for production will be optimized. We hope to provide an alternative resource for controlling fungal diseases in apples using *B. laterosporus* ZQ2 as a novel biocontrol agent.

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