Genetics and Molecular Research

Isolation, identification, and optimal cultivation of a marine bacterium antagonistic to *Magnaporthe grisea*

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Genet. Mol. Res. 15 (2): gmr.15028646
Received March 23, 2016
Accepted April 11, 2016
Published May 13, 2016
DOI http://dx.doi.org/10.4238/gmr.15028646

ABSTRACT. In this paper, a plate confrontation method was used to isolate bacteria antagonistic to the rice blast fungus *Magnaporthe grisea* from samples collected from China's Dalian Bay. The antagonist strain LM-031 was obtained. We studied this strain's morphological, physiological and biochemical characteristics and analyzed its 16S rDNA sequence. We compared the effects of different culture conditions (type of media, carbon and nitrogen source, incubation temperature and time, and initial pH value) on the inhibitory effect against *M. grisea*. Strain LM-031 was preliminarily identified as *Bacillus pumilus* and was found to strongly inhibit *M. grisea*, especially when grown on BPY medium at an initial pH 7 for 72 h at 30°C. The optimum carbon and nitrogen sources for growth were lactose and peptone, respectively. The most suitable carbon and nitrogen sources for production of active substances were glucose and NH_4Cl, respectively. Our results show that development and

utilization of *B. pumilus* LM-031 has great potential for biological control of *M. grisea*.

Key words: Marine microorganisms; Rice blast fungus; Antagonistic bacteria; Identification; *Bacillus pumilus*

INTRODUCTION

The blast fungus or rice blast *Magnaporthe grisea* is a fungal disease that affects rice crops (Zhang, 2007). The disease can cause harm to rice throughout the year and can occur on various parts of the plant. Global annual rice yield losses caused by *M. grisea* range from 11 to 30% (Tang et al., 2013; Li et al., 2014; Zhu et al., 2014; Shanmugapackiam et al., 2015), and infection can even cause crop failure. Spores are mainly responsible for infection (Choi et al., 2013; Motallebi et al., 2013; Li et al., 2014; Nehls and Dietz, 2014; Babu et al., 2015).

Currently, methods for control of *M. grisea* involve the use of resistant varieties and chemical pesticides. Due to several reasons, including the fact that disease-resistant varieties are more singleness, resistant varieties do not easily meet production goals. In addition, the long-term use of chemical pesticides not only causes pollution, posing a serious threat to human health, but also drives pathogenesis in *M. grisea*, reducing the antifungal efficacy of resistant varieties. Therefore, there is a need for more efficient and environmentally friendly methods for controlling *M. grisea.* Biological control offers such an approach for the prevention and treatment of *M. grisea*.

Due to having evolved in a different environment, the metabolites of marine microbes are different from those of terrestrial microbes in their structure and nature. Hence, marine microbes offer a wide range of development and application. In a previous study, screening for marine strains antagonistic to *M. grisea* led to the identification of such a strain and the extraction of its extracellular metabolite protein substances (Guo, 2003). In this study, we separated and screened samples collected from China's Dalian Bay using Japonica *M. grisea* as the target species, and we obtained an antagonistic strain with antifungal activity. We conducted morphological, physiological, and biochemical assessments of the strain and sequenced the 16S rDNA for species identification.

MATERIAL AND METHODS

Strains and media

M. grisea strain 815; YYY; 3.3283 was purchased from the Institute of Microbiology, Chinese Academy of Sciences. The strain was cultured on PDA medium (200 g potato, 20 g sucrose, 20 g agar, and 1000 mL distilled water) for five days at 28°C. Other media used in this study were: modified PDA medium (same as PDA medium but with sea water replacing distilled water), beef extract peptone sea water medium (3 g beef extract, 10 g peptone, 5 g NaCl, 15-20 g agar, and 1000 mL sea water, pH 7.0-7.2), modified Zobell 2216E medium (5 g peptone, 1 g yeast extract, 0.1 g ferric phosphate, 15-20 g agar, and 1000 mL sea water, pH 7.0), and modified BPY medium (5 g glucose, 10 g peptone, 5 g beef extract, 5 g NaCl, 5 g yeast extract, 15 g agar, and 1000 mL sea water, pH 7.0). The above media without agar were also used as liquid media. Chemicals were obtained from Sangon, Ltd. (Shanghai, China).

Screening of antagonistic strains

Sea water, sea mud and sea creatures (seaweed, ostracean), 4 samples of each kind were collected from Dalian Bay. Pre-treated samples were separated via dilution series; single colonies with unique morphology, size, and color were chosen, streaked, and purified on beef peptone medium. Purified single colonies were transferred to the beef peptone slant medium and stored at 4°C for future use.

Initial screening was performed using the plate confrontation method. In cultured blast mycelia, a bacterial block with a diameter of 7 mm was cut and placed in the center of a PDA plate; isolated strains were inoculated approximately 2.5 cm from the center and incubated at 28°C for seven days to observe antifungal activity.

The strains with antifungal effects were cultured in liquid medium. The cylinder plate method was then used for further assessment of antifungal activity: after washing with sterile water, *M. grisea* was added to PDA medium, incubated at 45°C, and mixed and poured into plates. After plates had solidified, an oxford cup containing incubated broth without thalli was placed in the middle of the plate. After incubation, the size of the inhibition zone was measured.

Identification of antagonistic strains

Morphological identification

Isolate cultured for 48 h were used to observe the shape, size, edge, surface, protruding shape, transparency, and color of the colonies and to conduct Gram staining.

Physiological and biochemical identification

Preliminary identification of isolated strain was made based on morphological, physiological, and biochemical assessments. Tests for strain identification included Methyl Red, Voges-Proskauer (V-P), indole acid, nitrate reduction, casein hydrolysis, sugar fermentation, lecithin, oxidase, and catalase tests, as well as other physiological and biochemical tests, all of which were conducted according to *Bergey's Bacterial Identification Handbook* (Ninth Edition) (Zhou, 1986).

16S rDNA sequence analysis

Sequencing of 16S rDNA was conducted by China Takara (Engineering) Limited. A Blast analysis was performed for the 16S rDNA sequence using the GenBank database http://www.ncbi.nlm.nih.gov/. MEGA5.0 software was used for phylogenetic analysis and construction of a phylogenetic tree (Kim et al., 2007).

Optimization of cultivation

To determine the optimal growth medium, the activated strain was inoculated on modified Zobell 2216E liquid medium, beef extract peptone sea water liquid medium, modified PDA liquid medium, and modified BPY liquid medium for three days at 28°C and 160 rpm. The diameter of the inhibition zone was then measured (Zhou, 1986).

To determination the optimal carbon and nitrogen sources, other components of BPY medium were kept constant while glucose, lactose, maltose, starch, and fructose were individually used as sole carbon sources. In addition, NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, and peptone were tested individually as sole nitrogen sources. The strain was cultured for three days at 28°C and 160 rpm. The OD_{640} and the diameter of the inhibition zone were measured for each treatment.

For discovery of the optimal incubation time, the activated strain was inoculated at 2% in modified BPY medium and cultured at 28°C and 160 rpm for 24, 36, 48, 60, 72, 84, 96, 108, 120, or 132 h. The OD_{640} and the diameter of the inhibition zone were then measured for each treatment.

For assessment of the optimal temperature, the activated strain was inoculated at 2% on modified BPY medium for three days at 160 rpm. The culture temperature was set to 15°, 20°, 25°, 30°, 35° or 40°C, and the OD_{640} and the diameter of the inhibition zone were measured.

The optimal pH for antifungal activity was determined by inoculating the activated strain at 2% on modified BPY medium for three days at 28°C and 160 rpm. The pH value of the medium was adjusted to 5, 6, 7, 8, 9 or 10, and the OD_{640} and the diameter of the inhibition zone were measured.

RESULTS

Isolation of antagonistic strains

We isolated 106 bacterial strains from 12 samples collected from China's Dalian Bay. Through inhibition experiments, we found that strain LM-031 exhibited a strong inhibitory effect on *M. grisea*, with the diameter of the inhibition zone reaching 22 mm (Figure 1). When placed in the middle of LM-031, *M. grisea* is unable to grow (Figure 1A), and LM-031 inhibits *M. grisea* growth (Figure 1B).

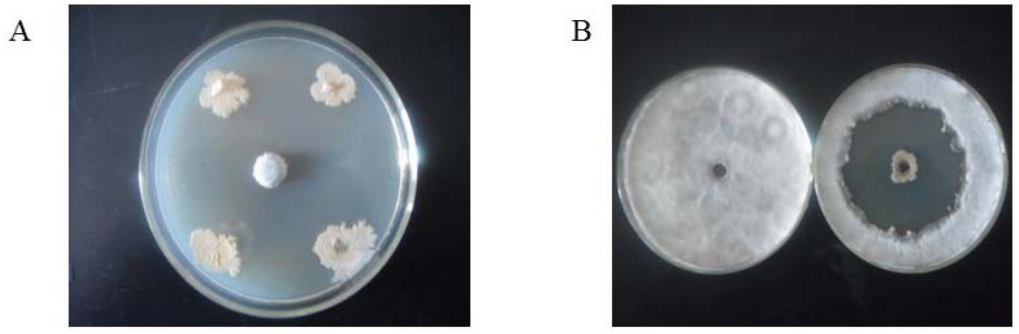

Figure 1. Screening and inhibition of *M. grisea* by strain LM-031. **A.** *M. grisea* is unable to grow in the midst of LM-031. **B.** LM-031 inhibited the growth of *M. grisea*, with the diameter of the inhibition zone reaching 22 mm.

Identification results

LM-031 colonies grew well in beef extract peptone sea water medium and were rod-shaped under electron microscope (Figure 2). Colonies were Gram-positive, round, jagged, white, shiny, and opaque.

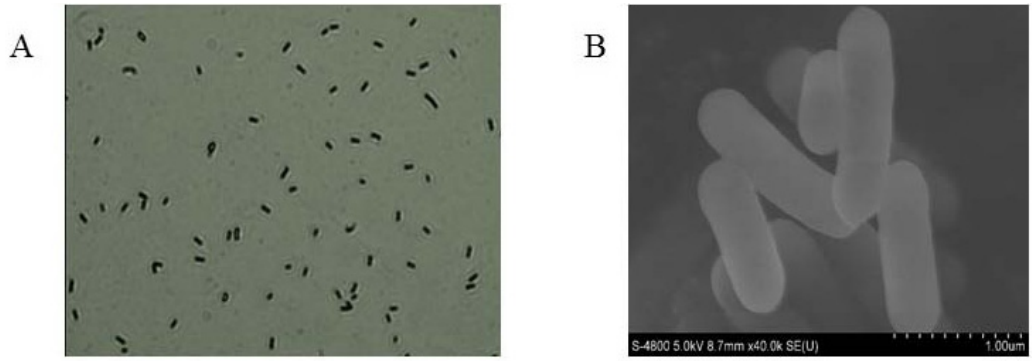

Figure 2. Cell morphology of strain LM-031. **A.** Gram staining of LM-031. **B.** LM-031 cells were rod-shaped under the scanning electron microscope.

Physiological and biochemical tests

The results of the physiological and biochemical tests are shown in Table 1. The Methyl red test was negative, indicating that LM-031 does not perform mixed acids fermentation when supplied glucose. The V-P test was positive, indicating that the strain produces 2, 3-butanediol from glucose as a fermentation product. The indole acid test was negative, indicating that the strain cannot convert tryptophan into an indole. The nitrate reduction test was positive, indicating that the strain reduces nitrate. The casein test was positive, indicating that the strain decomposes casein. The sugar fermentation test was positive, indicating that the strain decomposes sugar. A test for lecithinase was negative, indicating that the strain decomposes lecithinase, while oxidase and catalase tests were positive, indicating that the strain contains cytochrome C and produces the enzyme catalase.

Table 1. Physiological and biochemical characteristics of strain LM-031.

Characteristics	LM-031
Methyl red test	-
V-P test	+
Indole acid test	-
Nitrate reduction test	+
Casein test	+
Sugar fermentation test	+
Lecithinase test	-
Oxidase test	+
Catalase test	+

(+) Positive; (-) negative.

Analysis of 16S rDNA sequence

According to the sequencing results, the length of the 16S rDNA of LM-031 is 607 bp. The nucleotide sequence was uploaded to NCBI with accession number KJ933349.1. BLAST was used to find related sequences in GenBank and to determine the similarity between the LM-031 sequence and resulting hits. The similarity between LM-031 and *Bacillus pumilus* was 99%.

Figure 3 illustrates the phylogenetic relationship between LM-031 and related strains. LM-031 and *B. pumilus* are placed in the same group and exhibit the closest relationship. In accordance with morphological characteristics and sequence and phylogenetic analysis of 16S rDNA, therefore, strain LM-031 was preliminarily identified as *B. pumilus*.

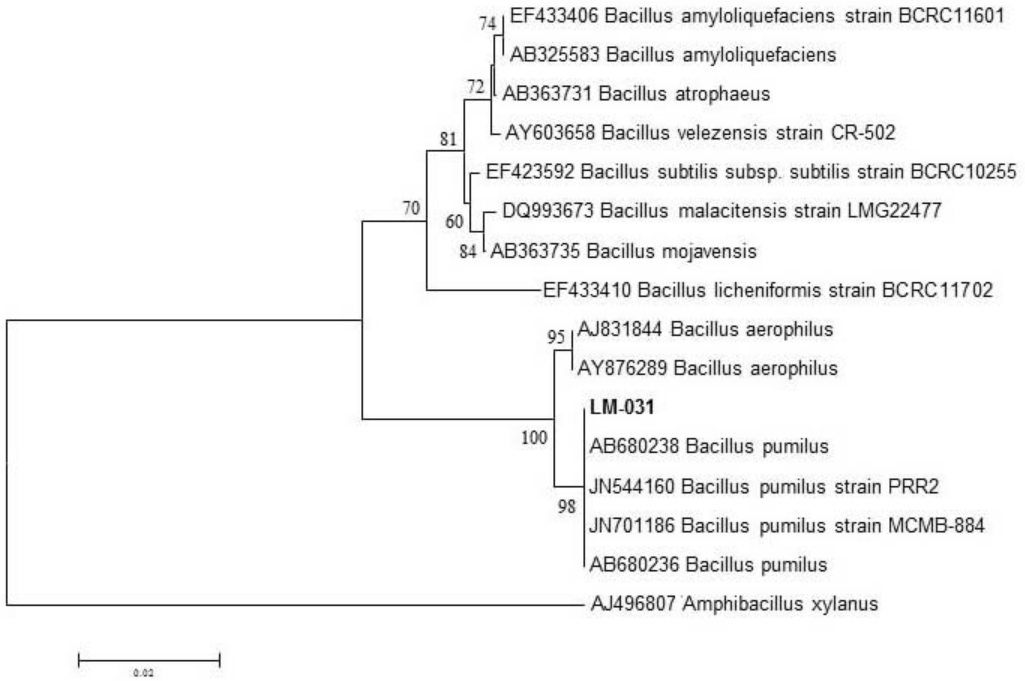

Figure 3. Phylogenetic tree of LM-031 and related strains based on 16S rDNA sequences. The MEGA5.0 software was used for phylogenetic analysis and tree construction.

Optimization of cultivation

Effect of growth medium

Differences in the compositions of the tested media resulted in varying antifungal activities. LM-031 exhibited high antifungal activity when grown in Zobell 2216E medium, modified BPY medium, and modified PDA medium (Figure 4). The strain showed the lowest antifungal activity in beef extract peptone medium. In additional experiments, modified BPY medium was used as incubation medium.

Effect of carbon and nitrogen source

As shown in Figure 5, LM-031 is able to use glucose, lactose, maltose, starch, fructose, or other carbon sources. Growth was best when lactose was used as the sole carbon source. LM-031 exhibited the highest antifungal activity when glucose and maltose were provided

as carbon sources. Due to cost and other issues, glucose was used as a sole carbon source in additional experiments. LM-031 is able to use NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, peptone, and other nitrogen sources. The strain exhibited the most vigorous growth and lowest inhibitory effect when using peptone. The antifungal activity was highest when NH_4Cl was used as the sole nitrogen source, and growth of the strain was satisfactory under this nitrogen source as well. Therefore, NH_4Cl was chosen as the sole nitrogen source for additional experiments.

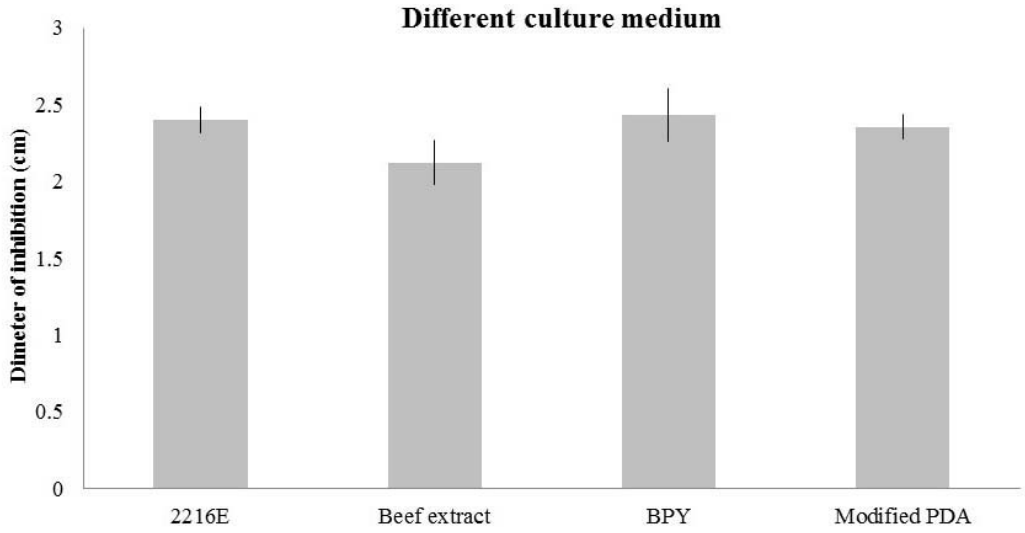

Figure 4. Effect of growth medium on antifungal activity of LM-031 (The error bars indicate standard error).

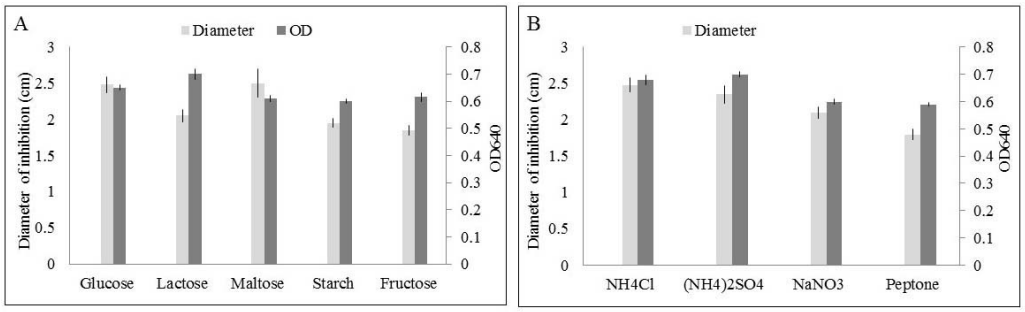

Figure 5. Effect of carbon source (**A**) and nitrogen source (**B**) on the growth and antifungal activity of LM-031.

Effect of culture duration

The diameter of the inhibition zone was greater than 2 cm after 48 h of incubation, indicating that the strain had already produced antifungal compounds (Figure 6). The diameter of the inhibition zone reached a maximum at 72 h. As culture duration continued past 84 h, the antifungal activity did not increase further. Therefore, the best duration for fermentation was found to be 72 h.

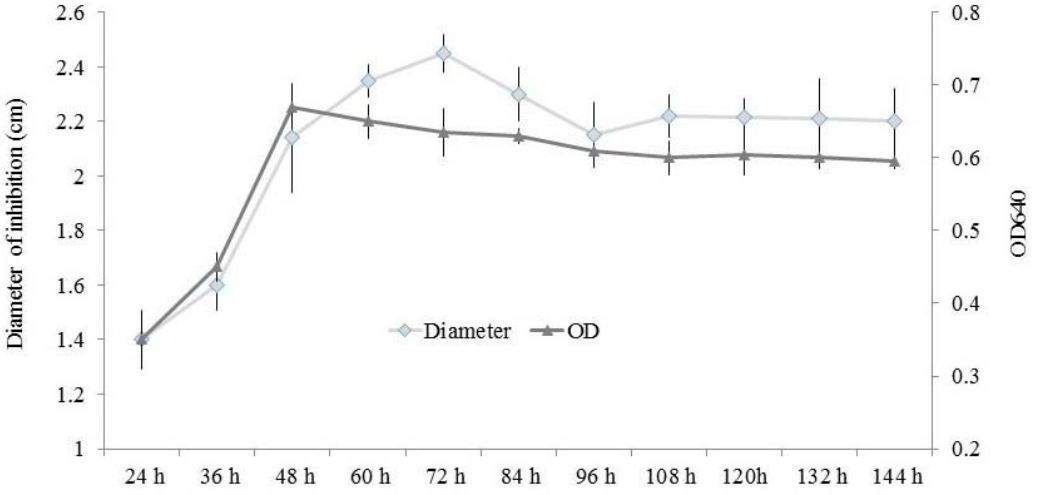

Figure 6. Effect of culture duration on growth and antifungal activity of LM-031.

Effect of temperature

The growth and antifungal activity of LM-031 varied under different temperatures (Figure 7). The strain produced more antifungal compounds at 25°C than 15°C, 20°C, reaching a maximum at 30°C. As the temperature increased further, the antifungal activity decreased significantly. When the temperature reached 40°C, LM-031 produced few inhibitory compounds.

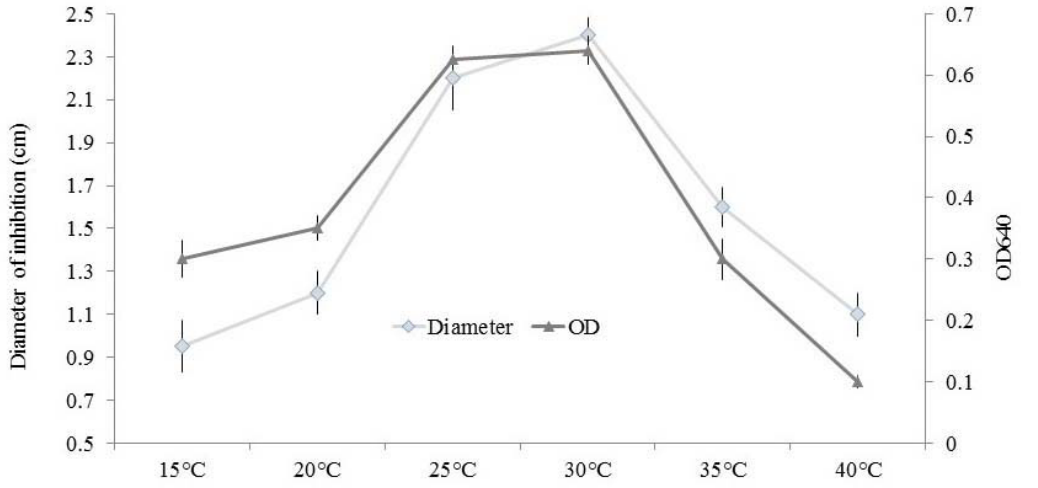

Figure 7. Effect of culture temperature on antifungal activity of LM-031.

Effect of initial pH value

The initial pH value had a greater impact on antifungal activity of LM-031 than other factors tested (Figure 8). The antimicrobial activity was greatest when the medium was at neutral pH. In acidic or alkaline media, the strain exhibited drastic changes in the amount of antifungal compounds produced. While LM-031 is able to grow at pH 5, no inhibitory compounds are produced. Likewise, when the pH value of the medium was higher than 9, the strain produced few inhibitory compounds.

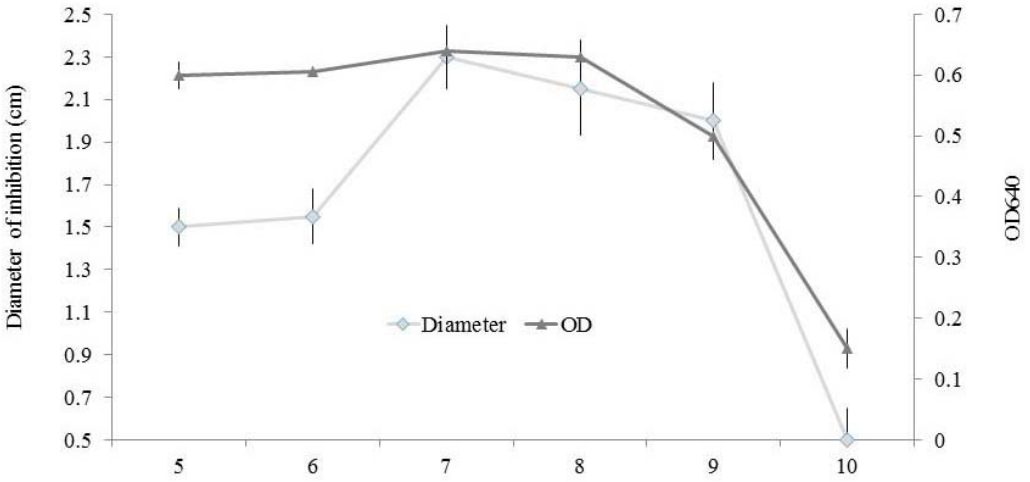

Figure 8. Effect of initial pH value on the antifungal activity of LM-031.

DISCUSSION

After isolation and purification of samples collected from China's Dalian Bay, we obtained a total of 106 bacterial strains, one of which, strain LM-031 (isolated from mud samples), exhibited highly antagonistic effects against the rice blast fungus *M. grisea*. In *M. grisea* inhibition experiments, the diameter of the inhibition zone reached 22 mm. Based on initial physiological and biochemical characteristics and 16s rDNA analysis, we preliminarily identified this strain as *B. pumilus*. Through further optimization experiments, we found that in order to achieve the greatest antifungal activity, strain LM-031 should be grown in BPY medium, with glucose as the carbon source and NH_4Cl as the nitrogen source at 30°C and an initial pH of 7 for 72 h.

M. grisea is known to affect more than fifty members of the Gramineae including rice, wheat, and finger millet (*Eleusine coracana*). The advantages of *B. pumilus* include high propagation speed, hardiness, and antifungal activity. It is therefore suitable for industrial production. Strain LM-031 was isolated from the ocean. Compared with terrestrial environments, the marine environment is characterized by high salt, high pressure, low temperature, and dilute nutrients, and therefore the metabolites produced by marine microorganisms are distinct from those produced by terrestrial microorganisms. In future experiments, the mechanism of

the antagonistic effect of LM-031 on *M. grisea* should be studied, as well as other production considerations for industrial use.

ACKNOWLEDGMENTS

Research supported by the Startup Foundation for Doctors of Dalian Nationalities University, the Natural Science Foundation of China (#31170168), the National Spark Program Project (#2013GA651006), the National Torch Plan Project (#2012GH531899), and the Dalian Science and Technology Project (#2014B11NC078).

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