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Antifungal activity of the bioactive substance from *Bacillus atrophaeus* strain HAB-5 and its toxicity assessment on *Danio rerio*

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ABSTRACT

The prevalence of pathogen inhibitors bacteria has motivate the study for antimicrobial compounds. Bioactive fungicide have always received considerable attention. A bacterial isolated strain HAB-5 showed antifungal activity against plant fungi. Based on morphological, physiological, biochemical and 16SrDNA sequence analysis, the strain was identified to be a *Bacillus atrophaeus*. This strain possessed a broad spectrum antifungal activity against various plant pathogenic fungi. Extraction of antifungal substance was performed and the crude extract had potent antifungal ability and showed great potential for swelling and inhibiting spore germination. This antifungal displayed heat stability and active in a wide pH range 5.0–10.0. Moreover no reduction was found in its activity after enzyme treatment. The toxicity test was evaluated in *Danio rerio*. The acute toxicity test indicated that the 24, 48, 72, 96 h LC_{50} values of UMTLS to the zebrafish were 14.4, 13.8, 13.4, and 12.9%, respectively. Based on the results obtained in this study, antifungal substance was not toxic to zebra. Analyses of disease suppression showed that HAB-5 was effective to reduce the incidence of anthracnose symptoms on mango fruits, also prevent disease infection and protect tobacco seedling from *Phytophtora nicotianae*. The bioactive substance from *Bacillus atrophaeus* HAB-5 could be a candidate in the generation of new antifungal agents in crop.

1. Introduction

Pathogenic fungi that cause disease in other organisms are widespread in nature. That is the important reason for contamination of food crops, food raw materials and processed products. They can have an impact in many areas because of the broad host range, causing severe damage. Fungal contamination causes not only huge economic losses, but also food safety issues. They also lead to industrial contamination and some result in human and animal diseases [1].

Control of plant diseases is crucial to the reliable production of food, and provides significant reductions in agricultural use of land. Plants in both natural and cultivated populations carry inherent disease resistance, but there are numerous examples of devastating plant disease impacts, as well as recurrent severe plant diseases.

Prevention and control of fungal contamination is an important issue in the field of industry and agriculture. Chemical preservatives are generally used in food industry, to control the growth and reproduction of fungi. However, due to abuse of chemical preservatives, its residues and food safety issues become more and more serious. In addition, number of available drugs for treatment of fungal infections remains limited and they show different degrees of undesirable side effects [2].

Exploring and researching new antifungal agents from new resources have attracted major attention of research scientists. Controlling plant diseases through the overuse of chemical pesticides or fungicides is of concern to the environment and human health, with the increasing demand for ecologically safe biotechnological pesticides in the plant and crop industry. Thus, alternate solutions to replace chemicals in crop disease management are encouraged. Biological control through the application of antagonistic microbes of plant pathogens is one of the most promising options [3,4], which may be alternatives to chemical pesticides.

Various microbes can produce antimicrobial metabolites which can inhibit or extenuate other microorganism, has provided a new choice to prevent and control the fungal contamination of agricultural products. Many antagonistic microbes that produce inhibitory substances have been recently reported [5–7]. Bacterial genomes are one of the most important natural defenses which were against the invading of most fungal pathogens.

Bacillus spp. are known as producers of a number of antifungal compounds, have been explored for biocontrol agents for several years [8–13]. It has been fully demonstrated that those *Bacillus* species could inhibit some phytopathogenic fungi successfully and their study is of great significance. The genome sequence of *B. atrophaeus* HAB-5 may also

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provide insight into the evolutionary adaptation to its niche and the discovery of putative antimicrobial compounds. Here, we report an isolated *Bacillus atrophaeus* HAB-5, which exhibited a broad antifungal spectrum. Moreover stabilities and toxicity of antifungal substance generated by this bacterium were investigated.

2. Materials and methods

2.1. Bacteria strain

In this study, the bacterium was previously isolated from the soil in our laboratory according to the standard procedures and was designed as HAB-5 strain.

2.2. Growth conditions

The microorganism culture was carried out in various production media. The LB medium containing 5.0 g/L yeast extract, 10.0 g/L tryptone, and 10.0 g/L NaCl showed maximum zone of inhibition against and considered as the most suitable medium for metabolite production, and used as the production medium for further studies.

2.3. Identification of bacterial strain

Identification and characterization of the HAB-5 strain was carried out on the basis of the colony and cell morphology, growth characteristics, various staining and various biochemical tests, according Bergey's manual of systematic bacteriology instructions [14]. The results of the biochemical and biophysical tests were furthermore verified by molecular tests.

For molecular identification, 16SrDNA gene fragment was amplified by PCR. The genomic DNA was extracted from overnight bacterial culture, using PCR purification kit (Trans5α Chemical Competent Cell). For PCR amplification, the conditions for thermal cycling were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, annealing at 50 °C for 45 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. Universal primers 27 forward primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492 reverse primer (5′-GGYTACCTTGTTACGACTT-3′) were used. The PCR product was separated on 1% of agarose gel and recovered by the high pure PCR purification kit. Nucleotide sequencing analysis was performed. The sequence was reversed, aligned, and compared to similar database sequences by BLAST algorithm.

2.4. Screening for antifungal activity against fungal pathogens

22 fungal pathogens strain (Table 2) were selected and their pathogenicity was verified. The target fungi were cultured on potato dextrose agar (PDA) medium (200.0 g fresh potato, 20.0 g glucose, 15.0 g agar, 1.0 L distilled water, pH not adjusted). The antagonism of HAB-5 was checked with respect to the ability to suppress fungal growth. Antifungal bioassay was performed with the dual culture method. 6-mm mycelial disk of pathogenic fungus, collected from the edge of actively growing colonies, was placed into the center of plates containing fresh PDA. Bacterial isolates were grown around the target fungus with a distance of 3.0 cm. The plates were incubation at 28 °C, and checked every 5 days after inoculation. All treatments were tested in triplicate. The inhibition rate was calculated as follow: Inhibition rate (%) = (Diameter fungal colony on the control − Diameter fungal colony on the treatment with bacteria) / Diameter fungal colony on the control × 100%.

2.5. Analyses of antimicrobial and antagonistic substances

Based on antagonistic effects and the ability to suppress fungal pathogens, HAB-5 was carried for antimicrobial and antagonistic substances analysis. Chitin degrading capability, proteolytic activity, cellulase production, Phosphate solubilization, and siderophore production was measured using the modified instruction [15].

2.6. Fermentation and extraction of metabolites

Submerged fermentation experiments were carried out in 1-L fermentor with shaking 180 rpm at 28 °C, containing 500 mL of production medium. The supernatant was collected by centrifugation at 10.000 r.p.m. for 20 min at 4 °C followed by filtration of the supernatant through a 0.2 μm pore size cellulose acetate filter. The clear filtrates were tested for their activities against the test organism. All experiments were repeated at least three times in order to acquire high accuracy. This procedure gave consistent and reproducible results.

The antimicrobial substance was extracted from the mycelium-free supernatant by the solvent-liquid extraction method. The culture supernatant was first washed with petroleum ether which is added to the supernatant in the ratio of 1:1 (v/v) and shaken vigorously. The raffinate was extracted with methanol. The methanol extract was dried by a vacuum pump at 45 °C, 90 rpm. The crude extract was dissolved approximately in 2 mL of distilled water and lyophilized. The resulted powder was dissolved in distilled water. Control was extracted with the same procedure, by using medium nutrient in place of fermentation broth.

2.7. Determination of the antimicrobial activity of crude extract substance

The antimicrobial activity was determined by the disk diffusion method against the selected fungi pathogenic strain. The crude extract antifungal compounds were filtrated through a 0.22-μm membrane. The ability of the substance to produce antifungal substances was assessed as follow. Molten PDA precooled to 45 °C media was seeded with the indicative strains. Fresh phytopathogenic mycelium were seeded agar and then poured immediately into sterile Petri dishes. After the plates were cooled, 10 μL of the sample solution (1 mg/mL) was soaked into 6 mm-diameter filter paper, dried and placed on the plates. The plates were incubated at 28 °C for 4 days. Experiment was carried out with three replicates. The inhibition zone around the disk was measured with a millimeter scale and the results were tabulated and analyzed.

2.8. Thermal, pH and proteolytic enzymes stabilities on antifungal substances

To analyze heat stability, the antifungal substance was treated with different temperature 40 °C–100 °C and autoclaved at 121 °C for 20 min, and then placed at room temperature for 4 days. The residual inhibitory activity to fungi was then disk-diffusing assayed. The pH of substance was adjusted from 2 to 12 with NaOH or HCl and then placed 2 h at 37 °C. For resistance to proteolytic enzymes test, sample was treated at 28 °C for 1 h with 2 mg/mL final concentration of trypsin, pepsin, chymotrypsin, pronase E, amylase, and proteinase K; then incubated at 37 °C for 2 h. The enzyme activity was terminated by heating at 80 °C before the activity were confirmed. These treated samples were subjected for antifungal activity. The antifungal activity to fungi was then disk-diffusing assayed, and antimicrobial circle diameter was measured.

2.9. EC_{50} determination

To estimate the antifungal potency of the substance, the EC_{50} (concentration required to obtain a 50% antifungal effect) was determined. Different concentration of the substance was mixed PDA. A mycelial plug (6 mm diameter) obtained from fresh culture of *C. gloeosporioides* was inoculated in the center of each plates and incubated at 28 °C for 5 days. The mycelial growth were measured and analyzed.

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