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# Antibiotic Spectrum and Stability of Crude Extract of Extracellular Metabolites from *Bacillus mageterium* LB01-17

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Crop pathogens including fungi and bacteria seriously affect the quality and yields of agricultural products. The purpose of this study was to detect the antibiotic spectrum of the crude extract of extracellular metabolites produced by *Bacillus megaterium* LB01-17 and its stability to heat, acid, alkali and ultraviolet light. The methods of mycelium growth rate and inhibitory zones were used to test the antimicrobial activity of the crude extract on 13 kinds of crop pathogenic fungi and 6 kinds of bacteria. The activity stability of the crude extract was determined under different pH values, acid-base environment and UV radiation time with *Colletotrichum gloeosporioides* in postharvest mango as the indicator strain. The crude extract displayed broad spectrum activity against all tested fungi with the inhibition rate on 5 kinds of fungi more than 83 %. Inhibitory effects of the crude extract on the growth of 6 kinds of bacteria were also observed and strongest inhibition to *Xanthomonas oryzae pv.oryzae* in rice was showed (inhibitory zone diameter 22.37 mm). By the detection of stability of the crude extract to acid, alkali, heat and UV, the results demonstrated that the inhibition rate of the crude extract against *Colletotrichum gloeosporioides* was stable at pH 2-8 and 77.13 % of inhibition rate was still kept after treated at 140 ºC for 20 min and the crude extract had a stable activity to UV, and the inhibition rate was almost unchanged after 6 h.

**Keywords: *Bacillus megaterium* LB01-17, Excellular metabolites, Antibiotic spectrum, Stability.**

## INTRODUCTION

Crop diseases caused by pathogenic microbial seriously affected the yields and quality of crops. Increasing use of chemical fungicides to control crop diseases has produced heavy soil pollution in recent years [1]. Biological control agents can act as a pathogen-specific, safe and pollution-free alternative to chemicals that have negative effects on the environment and animal and human health [2,3]. As an important biological agent, *Bacillus megaterium* is used in various fields because of its advantages, such as environmentally beneficial, non-pathogenic to humans and animals, small generation time, simple nutritional requirements, and strong stress resistance [4], especially in agriculture. *Bacillus megaterium* has been exten-sively studied as the biofungicide, biofertilizer, plant growth enhancer in the agriculture [5-7].

Recently, *Bacillus megaterium* LB01, which has strong antagonistic effect on *Colletotrichum gloeosporioides* of mango, was obtained from the rhizosphere soil of mango fruit trees in Karst area of Guangxi Province by the plant pathology laboratory of Baise University, P. R. China. The result of *in vitro* and *in vivo* tests showed that the strain LB01 had good control effect on *Colletotrichum gloeosporioides* in postharvest mango [8]. The preliminary study found that secretion of antifungal substance is one of the main mechanisms of the strain LB01 to control anthracnose in postharvest mangos [9].

At present, there are few studies about the crude extract of excellular metabolites produced by *Bacillus megaterium* in literature. In this article, the antibiotic spectrum and stability of the crude extract of excellular metabolites produced by *Bacillus megaterium* LB01-17 was studied in order to lay a foundation of further isolation, purification, and application of the crude extract in future.

## EXPERIMENTAL

The chemicals *viz*. ethyl acetate, sodium hydroxide and HCl (analytical purity) were purchased from Tianjin Concord Technology Co., Ltd.. The strain LB01-17 and all pathogenic microbial were provided by plant pathology laboratory of Baise

University, P.R. China. The GGC-X mintype tube overturning oscillation extractor and SE-1000 mintype rotary evaporator were purchased from Beijing Guohuan High Tech Automation Technology Research Institute for the preparation of the crude extract of excellar metabolites produced by strain LB01-17. The easydish round cell culture dish was used for *in vitro* antibiotic activities assays of the crude extract of excellar metabolites.

**Formulation of nutrient agar and potato sucrose agar:** The culture medium of nutrient agar and potato sucrose agar were prepared according to microbiology experimental technology [10].

**Preparation of crude extract of excellular metabolites produced from *Bacillus megaterium* LB01-17:** The strain LB01-17 preserved at 4 ºC was inoculated on nutrient agar culture medium and incubated at 32 ºC for 48 h. The mycelia were inoculated into nutrient agar culture broth again and incubated at 32 ºC for 6 d at 135 rpm. The fermentation broth of LB01-17 strain was centrifuged at 6000 rpm for 20 min, and the supernatant was filtered with microporous membrane (d = 0.22 μm). The cell-free culture filtrate (26 L) was obtained. The equal volume of ethyl acetate was added into the culture filtrate and shaken vigorously for 1 h. The ethyl acetate layer was separated after standing for 24 h. The water layer was also extracted with the equal volume of ethyl acetate. The obtained ethyl acetate layers were combined, dried with anhydrous $Na_2SO_4$ and concentrated on a rotary evaporator.

A weight of 0.881 mg concentrated crude extract of the extracellular metabolites from strain LB01-17 was then dissolved in 15 mL of sterile distilled water for further use in the *in vitro* antibiotic activity assay.

**Effect of crude extract of excellular metabolites produced from *Bacillus megaterium* LB01-17 on crops pathogenic fungi growth:** Mycelial growth rate method [11] was used to test the effect of crude extract of the extracellular metabolites from strain LB01-17 on pathogenic fungi of crops. The pathogenic fungi was activated on PSA culture medium. A punch (d = 5 mm) was used to cut the edge when the colony of fungi grew more than half of the culture dish. 1 mL of the sample was mixed with 9 mL of PSA culture medium. After the mixture was cooled, the fungus cake was inoculated in opposite direction and incubated at 28 ºC. Once the mycelia of the control covered the edge of the culture dish, the mycelia growth diameter of treatment was measured. Each pathogenic fungus was tested three times in parallel and 1 mL of water instead of the sample was used as control. The inhibition rate of mycelial growth was calculated according to the formula below [12]:

$$\text{Inhibition rate of mycelial growth (\%)} = \frac{\text{Mycelia growth diameter of the control} - \text{Mycelia growth diameter of the treatment}}{\text{Mycelia growth diameter of the control} - 5\text{ mm})} \times 100$$

**Effect of crude extract of excellular metabolites produced from *Bacillus megaterium* LB01-17 on crops pathogenic bacteria growth:** Inhibition zone method [13] was used to test the effect of the sample on pathogenic bacteria of crop. A 15 mL aqueous agar (agar content 3%, v:v) was poured into the culture dish to form a thin layer. After the thin layer was solidified, 25 mL nutrient agar medium (t = 50-60 ºC) containing $1.0 \times 10^7$ CFU / mL of pathogenic bacteria was added to the thin layer. When the nutrient agar medium was coagulated, 800 μL of sample was added to each hole drilled with a sterile puncher (d = 7 mm), incubated at 28 ºC for 48 h, and the equal volume of water was added as the control. Each treatment was repeated for 3 times and the diameter of inhibition zone was measured by cross method.

**Stability of crude extract of excellular metabolites produced from *Bacillus megaterium* LB01-17:** The stability of antagonistic activity of the sample was determined under different conditions such as temprature, pH and UV light. *C. gloeosporioides* in mangos was used as indicator strain. A sample at room temperature, pH 7.0 and UV light was served as the control.

The pH of the sample was regulated from 1.0 to 14.0 by 1 mol/L HCl or 1 mol/L NaOH. After keeping at 4 ºC for 12 h, the pH levels of the samples were regulated to 7.0 and antifungal assays were performed to investigate the activity. The effect of temperature was also evaluated by keeping the sample at room temperature, 60, 100 and 140 ºC for 30 min. In the UV test, 560 μL of the sample was poured into a 7.5 cm wide culture dish, which was placed 30 cm under a 30 W UV lamp. Then, 56 μL sample was taken every hour for 6 h. The variations in the remaining activities were detected. All assays were conducted in triplicate.

**Statistical analysis:** The data were processed by SPSS 19.0 software (SPSS Inc., Chicago, USA). The measurement data was given in the form of mean ± standard deviation and categorical data were expressed as a percentage. Duncan multiple comparison method was adopted to analyze significant difference ($P < 0.05$) and extremely significant difference ($P < 0.01$).

## RESULTS AND DISCUSSION

**Inhibitory effect of crude extract of excellular metabolites produced from the strain LB01-17 on crops pathogenic fungi growth:** In recent years, there have been many reports about the fermentation broth from *B. megaterium* inhibiting crops pathogenic fungi. Zhou *et al.* [14] reported that *Bacillus megaterium* 196 had good biocontrol effect on *Rhizoctonia solani* in rice. Chen *et al.* [15] demonstrated that *B. megaterium* Y-30 strongly inhibited the growth of *B. cinerea* in tomato, while Kong *et al.* [16] confirmed that marine *B. megaterium* had inhibitory effect on *Aspergillus flavus* in peanut. Ji *et al.* [17] showed that the culture filtrate of *B. megaterium* LB01 strongly inhibited the mycelial growth of *Colletotrichum gloeosporioides* in postharvest mango.

In this article, based on previous study, the effect of crude extract of the excellular metabolites produced from the fermentation broth of *B. megaterium* LB01-17 on pathogenic fungi of crops was studied. The results showed that the crude extract of excellular metabolites from strain LB01-17 had broad-spectrum antifungal activity, as shown in Table-1. The crude extract of excellular metabolites has inhibitory activity against 13 kinds of crops pathogenic fungi, among which, the inhibitory

TABLE-1
INHIBITORY EFFECT OF CRUDE EXTRACT OF THE METABOLITES FROM THE STRAIN LB01-17 ON CROPS PATHOGENIC FUNGI GROWTH

| Pathogenic fungi in crop | Inhibition rate on mycelial growth of fungi (%) |
|---|---|
| *Rhizoctonia cerealis* in wheat | 84.13 ± 0.83[b] |
| *Fusarium oxysporum Schlecht* in barley | 64.25 ± 0.45[d] |
| *Drechslera avenacea* in oat | 74.26 ± 0.32[c] |
| *Pyricularia oryzae* in rice | 94.10 ± 1.61[a] |
| *Bipolaris maydis* in maize | 96.52 ± 1.52[a] |
| *Rhizoctonia solani* in sugar beet | 71.26 ± 0.43[c] |
| *Colletotrichum gloeosporioides* in avocado | 86.28 ± 0.79[b] |
| *Botrytis cinerea* in blueberry | 83.27 ± 0.73[b] |
| *Colletotrichum gloeosporioides* in cherry | 44.27 ± 0.13[e] |
| *Botrytis cinerea* in grape | 74.26 ± 0.43[c] |
| *Phytopythium helicoides* in kiwifruit | 33.15 ± 0.11[f] |
| *Colletotrichum gloeosporioides* in apple | 64.26 ± 0.23[d] |
| *Curvularia lunata* in banana | 73.35 ± 0.48[c] |

Average values in the same column with different letters (a-f) are significantly different ($p < 0.05$).

effect on *Rhizoctoniacerealis*in wheat, *Pyricularia oryzae* in rice, *Bipolaris maydis* in maize, *Colletotrichum gloeosporioides* in avocado and *Botrytis cinerea* in blueberry is better with the inhibition rates more than 83% in average. However, the inhibition effect on *Colletotrichum gloeosporioides* in cherry was poor and the effect on *Phytopythium helicoides* in kiwifruit was the lowest.

**Inhibitory effect of crude extract of excellular metabolites produced from the strain LB01-17 on crops pathogenic bacteria growth:** Recently, *Bacillus megaterium* had also been reported to inhibit crops pathogenic bacteria. Ji *et al*. [17] reported that the fermentation broth of *Bacillus megaterium* L2 had inhibitory effect on *Ralstonia solanacearum*. Zhao *et al*. [18] further studied and found that the fermentation broth of *Bacillus megaterium* L2 exhibited the antibacterial activity against *Erwinia carotovora* subsp. Carotovora.

In this study, the inhibition spectrum of the crude extract of excellular metabolites from strain LB01-17 against 6 kinds of pathogenic bacteria was investigated. The results showed that the diameter of inhibition zone against *Erwinia carotovora* subsp. *Carotovora* in Konjac, *Xanthomonas pruni* in plum and *Xanthomonas oryzae* pv. *Oryzicola* in rice and *Xanthomonas oryzae* pv. *oryzae* in rice was more than 19 mm and the diameter of inhibition zone to *Xanthomonas oryzae* pv.*oryzae* in rice was the largest, reaching 22.37 mm. However, the activity against *Pectobacterium carotovora* pv. *Crotovora* in cabbage and *Ralatonia solanacearum* in tomato was low and the inhibition zone diameter was 4.35 and 5.25 mm, respectively (Table-2).

**Stability of crude extract of excellular metabolites produced from strain LB01-17:** Several research showed that the fermentation broth of *Bacillus subtilis* SN-02 and other strains generally had higher activity against a variety of plant pathogenic fungi and bacteria [19-21]. The activity was stable in acidic and neutral environment, but unstable in strong alkaline environment. In addition, the activity was also stable at high temperature and ultraviolet light. All these results were similar to the results reported in present work.

TABLE-2
INHIBITORY EFFECT OF CRUDE EXTRACT OF THE METABOLITES FROM THE STRAIN LB01-17 ON CROPS PATHOGENIC BACTERIA GROWTH

| Pathogenic bacteria in crop | Diameter of bacteriostatic zone (mm) |
|---|---|
| *Erwinia carotovora* subsp. *Carotovora* in Konjac | 21.35 ± 0.50[a] |
| *Pectobacterium carotovora* pv. *Crotovora* in cabbage | 4.35 ± 0.43[b] |
| *Ralatonia solanacearum* in tomato | 5.25 ± 0.23[b] |
| *Xanthomonas pruni* in plum | 20.35 ± 0.17[a] |
| *Xanthomonas oryzae* pv. *Oryzicola* in rice | 19.24 ± 0.73[a] |
| *Xanthomonas oryzae* pv.*oryzae* in rice | 22.37 ± 0.53[a] |

Average values in the same column with different letters (a-b) are extremely different ($p < 0.01$).

Fig. 1 displays the thermal stability of crude extract of the excellular metabolites. The inhibition rate was maintained at more than 77% even when the crude extract was kept at 140 ºC for 20 min. The similar result was observed in the UV analysis. The inhibition rate against *C. gloeosporioides* in postharvest mango was almost constant during the period of UV light radiation (Fig. 2), indicating that the crude extract of the excellular metabolites were stable under UV radiation.

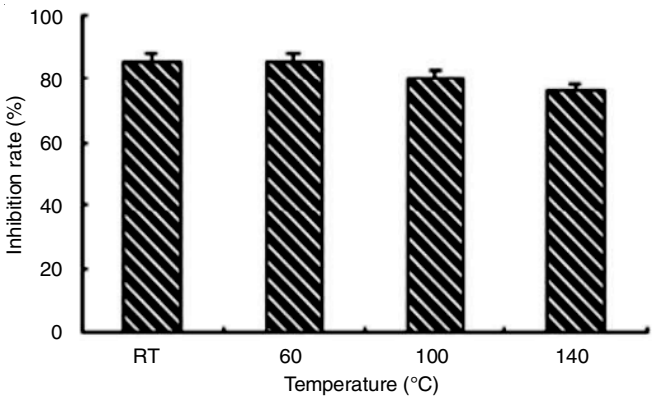


Fig. 1. Stability of the crude extract of extracellular metabolites produced by *B. megaterium* LB01-17 at different temperature. The sample at room temperature (rt) was served as the control. Error bars indicate standard deviations of the average value, which is is the same as the error bars in Figs. 2 and 3

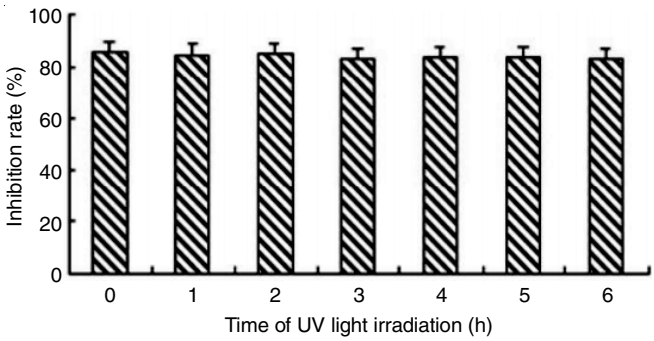


Fig. 2. Stability of the crude extract of extracellular metabolites produced by *B. megaterium* LB01-17 at different time of UV light radiation. The sample at 0 h of UV light radiation was served as the control

The antifungal activity was significantly reduced when the crude extract was exposed to basic conditions from pH 12

to 14 (Fig. 3), but the crude extract remained stable even after exposed to pH ranging from 2 to 8 (> 80%).

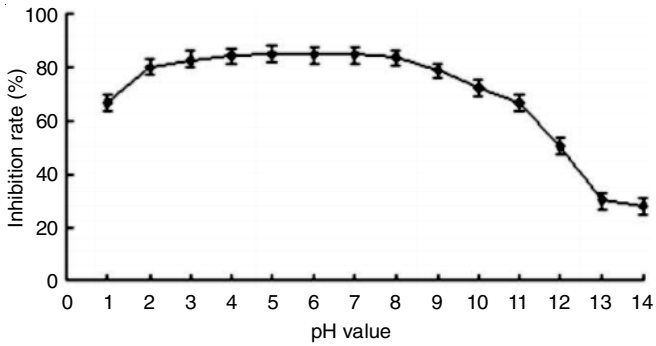


Fig. 3. Stability of the crude extract of extracellular metabolites produced by *B. megaterium* LB01-17 at different pH value. The sample at pH 7.0 was served as the control

## Conclusion

In summary, the crude extract of extracellular metabolites produced by *Bacillus megaterium* LB01-17 was obtained by the extraction of the culture filtrate of strain LB01-17 with ethyl acetate and the crude extract had strong inhibitory activity against 13 kinds of pathogenic fungi and 6 kinds of bacteria. Moreover, the activity of the crude extract had strong stability against heat, acidic conditions and ultraviolet radiation. The optimization of fermentation conditions of strain LB01-17, separation and purification of active components in crude extract and structural identification of active components are in progress. In this work, only *in vitro* assays were conducted to determine the antibiotic spectrum of the crude extract. Whether the crude extract has a good biocontrol effect on plant diseases still needs to be verified by a large number of i*n vivo* and field trials.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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