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# Optimization of medium composition and culture conditions for antifungal activity of a tomato endophytic bacterium

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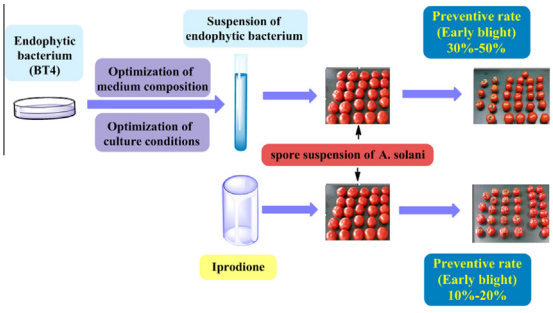
## HIGHLIGHTS

- Applications of antagonistic bacteria require optimization of culture conditions.
- The optimum medium composition and culture conditions for BT4 were determined.
- BT4 had antifungal effects that were better than common fungicides.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Early blight of tomato, which is mainly caused by *Alternaria solani*, is an important, globally distributed disease. We previously isolated an endophytic bacterium (BT4) from healthy tomatoes that has high antifungal activity against *A. solani*. Industrial applications of antagonistic bacteria require the optimization of culture conditions for growth and antifungal activity. Thus, in this study, the optimum medium composition and culture conditions for BT4 were determined using the "one factor at a time" method and an orthogonal design. Optimal cell growth and maximum antifungal activity were obtained at 30 °C, pH 7.0, with an inoculation proportion of 2% and incubation for 48 h in 12 g/L yeast extract, 10 g/L soluble starch and 7 g/L $CaCl_2$ in the culture medium. In addition, we compared the antifungal activity of BT4 with iprodione, a contact fungicide that is used on a variety of plants affected by fungal diseases. The results showed that BT4 had antifungal effects that were better than iprodione.



## 1. Introduction

The tomato *Lycopersicon esculentum* belongs to the family solanaceae and is one of the world's most widely grown fruits. It is a good source of vitamins and minerals, and its edible fruits can be consumed in either processed or fresh form. Tomato cultivation is popular and tomatoes are first among the processing crops. However, tomatoes are a perishable crop that is not available throughout the year. Postharvest decay of tomatoes causes substantial postharvest losses. In developing countries, postharvest losses of tomatoes are often severe because of inadequate transportation facilities and storage. Synthetic fungicides are used to control postharvest diseases of tomatoes. However, because of their environmental effects, use of synthetic fungicides is decreasing. Therefore, interest is high for finding safer alternatives for reducing tomato decay loss. The use of microbial antagonists such

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as fungi and yeasts is becoming popular and this biological approach is quite promising.

Microbial antagonists occur naturally inside or on the surface of vegetables and fruits. Isolated antagonists can be used to control of postharvest diseases (Janisiewicz and Korsten, 2002). Although the mechanisms by which microbial antagonists affect pathogens is not yet fully understood, several have been identified and introduced for use on a variety of harvested commodities for control of postharvest diseases. Several modes of action have been suggested to explain the activity of microbial antagonists. Antagonists usually compete with pathogens for nutrients and space (El Ghaouth et al., 2003; Filonow, 1998). In addition, microbial antagonists are thought to produce antibiotics, act through parasitism and induce fruits and vegetables to be resistant to postharvest pathogens (Castoria et al., 2001; El-Ghaouth et al., 1998, 2003; Filonow, 1998; Janisiewicz et al., 2000).

The optimization of culture conditions for the growth and antifungal activity of antagonistic bacteria is important for industrial applications. Evaluation of nutritional and environmental requirements of microorganism is crucial for bioprocess development (Abdel-Fattah and Olama, 2002). In addition, biochemical systems are multivariable processes (Rajendran and Thangavelu, 2007), and therefore production of biological control agents by antagonistic bacteria should also be performed by several regulatory mechanisms which respond to conditions in the culture environment. We previously isolated an endophytic bacterium (BT4) from healthy tomatoes. BT4 has high antifungal activity against *Alternaria solani*, a pathogenic microorganism that causes early blight of tomato. Based on 16S rDNA sequence, BT4 was identified as *Bacillus amyloliquefaciens*. In the present study, the optimal medium composition and culture conditions for BT4 were determined using the "one factor at a time" (OFAT) method and an orthogonal design. The antifungal activity of BT4 for tomato storage was also tested.

## 2. Materials and methods

### 2.1. Microorganisms and culture maintenance

Endophytic bacteria (BT4) were isolated in our previous work from healthy tomatoes grown in an area where postharvest early blight occurs in Hunan Province, China. *A. solani* was obtained from the Shanghai Institutes for Biological Sciences. BT4 was grown on Nutrient Broth or Nutrient Agar, while *A. solani* was grown on Potato Dextrose Agar.

### 2.2. Antifungal activity assays

Bacterial isolates were screened for their ability to inhibit *A. solani* on PDA plates using a plate stand-opposite culture method. A thin layer of PDA (5 ml) was made in a petridish. An empty Oxford cup was put on the surface of the solid medium and another 10 ml of PDA mixed with 0.5 ml *A. solani* ($1 \times 10^8$ spore/ml) suspension was poured to make a second layer. The Oxford cup was removed and 100 μl endophytic bacteria suspension was added to the hole. Three replicates of each isolate were subjected to analysis of variance. Plates were incubated at 28 °C for 72 h to measure inhibitory zone diameter (IZD).

### 2.3. Effect of carbon and nitrogen source and metal ions on cell growth and antifungal activity

Different C-sources such as soluble starch, lotus root starch, glucose, lactose, trehalose, and sucrose were screened based on cell growth and antagonistic activity at 2% (w/v) level in medium with NaCl 5 g/L, beef extract 3 g/L, and peptone 5 g/L. Beef extract-peptone, peptone, beef extract, yeast extract, $NH_4Cl$, $NH_4NO_3$ and $(NH_4)_2SO_4$ were screened as sole N-source at 0.8% (w/v) level in medium with NaCl 5 g/L and glucose 20 g/L. Seven different metal ions [$KH_2PO_4$ ($K^+$), $FeSO_4$ ($Fe^{2+}$), $ZnSO_4$ ($Zn^{2+}$), NaCl ($Na^+$), $CuSO_4$ ($Cu^{2+}$), $MgSO_4$ ($Mg^{2+}$), and $CaCl_2$ ($Ca^{2+}$)] were screened at 0.5% (w/v) level in medium with beef extract 3 g/L, peptone 5 g/L and glucose 20 g/L. Cell growth at different temperatures was measured at 600 nm using a BioSpec-mini. Antagonistic activity was measured using a plate stand-opposite culture method. All incubations were done at 37 °C for 48 h.

### 2.4. Medium optimization

An OFAT method was used to determine the main factors affecting growth and antifungal activity. An orthogonal design ($L9[3^3]$) was applied to evaluate the effects of the following factors: soluble starch (A), yeast extract (B) and $CaCl_2$ (C). Nine experiments were performed to evaluate the best conditions for antifungal activity of BT4. Factors and test levels are in Table 1. Data were analyzed using SPSS statistical software (SPSS for windows, version 18.0; SPSS Inc.).

### 2.5. Optimal conditions for antifungal activity

To determine the optimal time for antifungal activity, an inoculation proportion of 1% was used with pH 7.0, 30 °C and five different times: 24, 36, 48, 60, and 72 h. To determine the optimal temperature, an inoculation proportion of 1% was used with pH 7.0 and 48 h and five different temperatures: 20, 25, 30, 35, and 40 °C. To determine the optimal pH, an inoculation proportion of 1% was used with 30 °C and 48 h and five different pH conditions: 6.0, 6.5, 7, 7.5, and 8. To determine the optimal inoculation proportion, pH 7.0, 30 °C and 48 h incubation were used with five different inoculation proportions: 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. BT4 was inoculated in a medium optimized by the orthogonal design and the described conditions. Cell growth at different temperatures was measured at 600 nm using a BioSpec-mini. Antifungal activity was measured using a plate stand-opposite culture method.

### 2.6. Tomato storage test

Tomatoes were obtained from a commercial greenhouse near Hunan Agricultural University. Fruit was harvested at mature-red stage and selected to have uniform color and size, without bruises or signs of infection. Upon arrival at the laboratory, fruits were washed properly and dried in open air. Fruits were then divided randomly into 3 groups (30 fruits in each group) and spayed with *A .solani*, BT4 or iprodione according to Table 2. After dried in open air, fruits were packaged and stored at room temperature for 15 or 30 days.

To prepare antagonistic bacterial suspensions, BT4 was inoculated in optimal medium with optimal conditions based on the results of tests described above to push BT4 to reach top form. Then cultures were centrifuged at 4500 g/min for 10 min. Pellets

**Table 1**
Factors and levels of the orthogonal design.

| Influence factors | Level | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Soluble starch (A) (g/L) | 10 | 15 | 20 |
| Yeast extract (B) (g/L) | 4 | 8 | 12 |
| $CaCl_2$ (C) (g/L) | 3 | 5 | 7 |