The Influencing Factors of Bacillus megaterium Affect the Growth of the Antagonistic Strain and the Activities of Antibacterial Substances

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Abstract. 164 strains of Antagonistic bacteria were isolated from 131 mature-period soil samples from eight counties in Bijie City, Guizhou Province, China. In this study, the antagonistic bacteria that inhibited Alternaria alternata were screened and isolated. In addition, the antagonistic strain L2 was identified as Bacillus megaterium. Influencing factors also demonstrated that the suitable conditions for the production of antibacterial substances by strain L2 are as follows: liquid volume 50 mL/500 mL at pH 7.0 and 30°C under oscillation culture conditions, with beef extract as the carbon, nitrogen source.

1. Introduction

Alternaria brown spot is caused by the genera Alternaria [1-2]. Tobacco brown spot, is a major disease that attacks tobacco leaves at the late maturation stage. This disease is characterized by a short incubation period as well as rapid spreading, and it will directly impact tobacco quality and yield [3-5].

In previous decades, chemical-control methods have been the primary means of combating the brown spot. However, many of the chemical pesticides are detrimental to animal and human health alike. Accordingly, solutions have been sought to reduce the use of these chemicals while still effectively combatting the spread of pathogens. In 1978, Kloepper and Schrotth first reported that plant growth-promoting rhizobacteria (PGPR) can stimulate plant growth, increase yield, and reduce pathogen infections [6]. Since then, considerable research has been conducted concerning the capability of PGPR to provide resistance against plant pathogens as well as its role in promoting crop growth [7-12]. In this study, the antagonistic bacteria that inhibited Alternaria alternata were screened and the Influencing factors of antibacterial action was inspected, and in order to provide microbiological resources for the research of biocontrol and evaluate the values of L2 applications.
2. Materials and Methods

2.1. Materials

The antagonistic bacteria were isolated from 131 mature-period soil samples from eight counties in Bijie City, Guizhou Province, China. The antagonistic strain L2 was identified as *Bacillus megaterium*. The indicator fungi, *Alternaria alternata*, was isolated and preserved by The Institute of Fungi Resource, College of Life Science, Guizhou University.

2.2. Methods

2.2.1. Preparation of spore suspensions and seed solutions. *A. alternata* was cultured on potato dextrose agar (PDA)[13] plates at 29 °C for 15 d and then placed under an ultraviolet lamp for 3 d with continuous irradiation to induce spore production. Then, 10 mL of a 1% dextrose solution was added for 15 min of immersion. Next, spores and mycelia were scraped off with sterile cotton swabs, and the mycelia were removed through filtration with absorbent cotton. The spore concentration was adjusted to $2.0 \times 10^5$ CFU/mL using 1% dextrose.

The *B. megaterium* strain L2 was activated twice on nutrient agar (NA)[13] plates and then streak-inoculated for 24 h. Next, the strain was inoculated into the nutrient broth (NB)[13] culture solution and cultivated at 29 °C at 150 rpm/min for 12 h. Then, the seed bacterial liquid was prepared.

2.2.2. Bacteria-free filtrate preparation. 1 mL of the fermentation solution of L2 was collected and centrifuged at 5,500 rpm/min for 25 min. Then, the supernatant was collected and vacuum-filtered through 0.22-μm filter membranes to produce bacteria-free filtrates.

2.2.3. Influencing factors that affect the growth of the antagonistic strain and the activities of antibacterial substances. In preliminary experimentations, 30 °C, culture time 48 hours, liquid volume 50 mL, rotation speed 150 rpm/min and pH 7.0 has been found optimal for the growth of the antagonistic strain. Based on these results, the influencing factors of the growth of the antagonistic strain and the activities of antibacterial substances were investigated. The investigated influencing factors included temperature (20, 25, 30, 35, 40, 45, and 50 °C), pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0), liquid volume (five levels in 500-mL culture flasks: 50, 100, 150, 200, and 250 mL), and carbon, nitrogen (C, N) source (11 types, including yeast extract, beef extract, dextrose, sucrose, arginine, and cysteine). The bacterial growth was detected using the UV spectrophotometry [14], and the antibacterial activity was detected using the Oxford cup method [15].

2.2.4. Statistical analysis. Statistical analyses of quantification data were carried out using SPSS22 software, and were subject to one-way analysis of variance (ANOVA) analysis; means were analyzed using Dunnett’s multiple range tests. In the tables, the different superscript letters within the same column indicate statistically significant differences. For each experient, three replicates were included.

3. Results and Analysis

3.1. Influencing factors on bacterial growth and antibacterial substances

3.1.1. Effects of pH on bacterial growth and antibacterial substance activity. As is shown in Fig. 1, at pH 5.0-7.0, the increases were significant ($P = 5.12 \times 10^{-12} << 0.01$). The increment at pH 7.0 maximized at 1.2777, and then, as pH increased, it gradually declined. Furthermore, the activity levels of the antibacterial substances did not change greatly from pH 4.0 to 6.0, but the antibacterial substances produced at pH 6.0-7.0 had significantly greater activity levels ($P = 3.22 \times 10^{-5} << 0.01$). The activity was maximized at pH 7.0, and the diameter of the inhibition zone reached 1.47 cm. This result was consistent with the incremental changes, indicating that pH 7.0 was favorable for the
growth of strain L2 and the production of antibacterial substances. Moreover, the activity levels of the antibacterial substances gradually decreased as the pH increased further.

![Graph showing pH vs. OD and inhibition zone size](image1)

**Fig. 1** Strain L2 growth and antibacterial substances’ activity levels at varying pH values.

3.1.2. Effects of liquid volumes on L2 bacterial growth and antibacterial substances’ activity levels.

As is shown in Fig. 2, when 50 mL of liquid was added, the increment was maximized, and the OD_{620nm} was 1.4037. Furthermore, the activity levels of the antibacterial substances were also maximized, and the diameter of the inhibition zones was 1.46 cm. As the liquid volume increased, the increment dropped significantly (P = 1.39 × 10^{-5} << 0.01). Although the activity levels of the antibacterial substances were significantly decreased as the liquid volume increased (analysis of variance, P = 0.0025 < 0.01), the declining amplitude was very small compared with the change in the growth mass. This result indicated that, compared with the incremental change in the strain growth, the activity levels of the antibacterial substances were less affected by the liquid volume.

![Graph showing liquid volume vs. OD and inhibition zone size](image2)

**Fig. 2** Effects of liquid volumes on strain L2’s growth and the antibacterial substances’ activity levels.

3.1.3. Effects of temperature on bacterial growth and antibacterial substances’ activity levels.

As is shown in Fig. 3, at both 25 and 30°C, the growth change increments were both significantly positive (P = 4.75 × 10^{-6} << 0.01). The incremental change reached a maximum of 1.304 at 30°C. However, the incremental growth changes decreased as the temperature continued to rise, reaching a minimum (0.4677) at 50°C. The activity levels of the antibacterial substances produced at both 20 and 25°C were not significantly different, but the activity level at 30°C was significantly greater (P = 3.47 × 10^{-6} << 0.01), with an inhibition zone diameter of 1.30 cm, which was the maximum. The activity levels of the antibacterial substances at 35 and 30°C were not significantly different, but as the temperature rose, the activity levels of the antibacterial substances gradually decreased.
3.1.4. Effects of C, N sources on strain L2’s growth and the antibacterial substances’ activity levels.

As detailed in Table 1, the growth levels of strains that were inoculated into media that contained beef extract, yeast extract, sucrose, or dextrose increased greatly, with an OD$_{620nm}$ of 1.2837, and the activity levels of the antibacterial substances produced by the strain feeding on the beef extract were the greatest, producing an inhibition zone diameter of 1.51 cm. Although the strains could grow on sucrose or dextrose, no active antibacterial substances were detected, which indicated that these two C, N sources were suitable for strain growth but not for producing antibacterial substances.

| C, N source          | OD$_{620nm}$       | Inhibitory zone diameter(cm) |
|----------------------|-------------------|------------------------------|
| peptone (0.5%)       | 0.6937±0.0110c    | 0.8367±0.0404a               |
| beef extract(0.3%)   | 1.2837±0.0045d    | 1.5100±0.0351b               |
| yeast extract(0.1%)  | 1.0013±0.4225bcd  | 1.0766±0.0839ab              |
| sucrose(1%)          | 1.0970±0.0108b    | 0.7233±0.0115a               |
| glucose(1%)          | 1.1573±0.0021b    | 0.7067±0.0058a               |
| KNO$_3$(0.1%)        | 0.4110±0.0080a    | 0.7100±0.0100a               |
| inositol(0.2%)       | 0.1227±0.0025e    | 0.7067±0.0115a               |
| glycine(0.5%)        | 0.7443±0.0032c    | 0.7367±0.0351a               |
| glutamic(0.5%)       | 0.4177±0.0049a    | 0.8200±0.0436a               |
| aspartic(0.5%)       | 0.3240±0.0017f    | 0.7700±0.0173a               |
| tyrosine(0.5%)       | 0.2673±0.0032g    | 0.7300±0.0264a               |

4. Conclusion

Based on the above experimental results, we concluded that the most suitable conditions for the production of antibacterial substances by strain L2 were as follows: liquid volume of 50 mL, pH 7.0, 30 °C, oscillation culture conditions, and beef extract as the C, N source. In comparison to previous reports, this study highlighted the correlation between bacterial growth and the activity levels of the antibacterial substances. Generally, the incremental increases in the growth of the antagonistic strain L2 were positively correlated [R$^2$(pH) = 0.78, R$^2$(Liquid volumes) = 0.96, R$^2$(Temperature) = 0.45] with the activity levels of the antibacterial substances. However, while the strain grew in dextrose- and sucrose-based C, N culture solutions, the activity levels of the antibacterial substances were low. We, therefore, hypothesize that the growth of the antagonistic strain and the production of the antibacterial substances required different C, N sources. Moreover, the incremental growth changes in the antagonistic strain and the activity levels of the antibacterial substances could be detected throughout the entire tested pH range, the entire temperature gradient, and with all of the different C, N sources, indicating that the antagonistic strain possessed a strong environmental adaptability.

We then investigated its antibacterial mechanism. It was strongly inhibitory to *A. alternata* because the *A. alternata* mycelia were damaged and decomposed. Additionally, we found that the gross
metabolites from L2 inhibited A. alternata spore germination. In the future, we will systematically and specifically probe into the prevention and treatment of tobacco brown spot and evaluate the values of L2 applications.

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