Isolation and identification of *Morchella* pathogenic bacteria and determination of antibacterial activity of 22 essential oils

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**Abstract.** To extend the storage period and shelf life of *Morchella* and reduce environmental pollution problems, in this experiment, pathogenic fungi were isolated and identified from the naturally-occurring *Morchella*, and the antibacterial activity of 22 kinds of plant essential oils was determined by the plate dilution antibacterial method. The results showed that a strong pathogenic fungus strain YDJ-P was isolated and identified from the naturally-occurring *Morchella*, and it was identified as *Rhizopus stolonifer* based on morphology and molecular biology. The antibacterial activity of 22 essential oils showed that the essential oils of Clove, Styrax and Spearmint had obvious inhibitory effect on the strain YDJ-P, and the inhibitory rate was 100% at a concentration of 1000 μl/L. The bacteriostatic rate of Patchouli, Inula, Oregano, Neroli and Myrrh essential oils against strain YDJ-P were significantly different from that of completely bacteriostatic essential oils (*P*<0.01), all >50%.

1. Introduction

*Morchella importuna* belongs to Morvellaceae *Morchella* [1]. It is rich in a variety of biologically active ingredients and is a precious edible and medicinal fungus, which has great value in medicine and health food [2-3]. However, *Morchella* has a strong physiological metabolism and high water content. It is susceptible to mechanical damage and microbial infestation during storage and transportation, resulting in decay and deterioration, resulting in loss of edible value and commodity value [4]. Among them, the infection of pathogenic microorganisms is the main cause of postharvest rot, and fungi are also the main rot-causing pathogens of fruits and vegetables after harvest. Therefore, inhibiting the rot caused by the infection of *Morchella* pathogenic fungi and prolonging the storage period and shelf life of *Morchella* are urgent problems to be solved.

Chemical preservation is one of the commonly used preservation methods for edible fungi. At present, the commonly used chemical reagents mainly include sodium sulfite and citric acid [5]. Because most traditional chemical reagents are teratogenic and carcinogenic to the human body, and harmful to the environment. With the enhancement of people's health awareness, the green physical preservation technology without residue is more and more popular.

Plant essential oils, as natural plant extracts, have a significant inhibitory effect on pathogenic bacteria after harvesting fruits and vegetables [6]. Cinnamon essential oil had the best antibacterial effect on rot-causing mushrooms, followed by Thyme essential oil [7]. Lavender essential oil and Fennel oil can significantly inhibit the growth of edible fungi pathogenic fungi [8]. Embedded Cinnamon essential
oil and Garlic essential oil in microcapsules, which had a better preservation effect on mushrooms [9]. Plant essential oils have a good preservation effect on edible fungi after harvest, but there are few reports on the application of plant essential oils to the antibacterial research of pathogenic fungi of Morchella.

In this test, the pathogen was isolated and purified from the naturally-occurring Morchella, and its pathogenicity was determined according to Koch's law, and the pathogenic bacteria were identified by 18S rDNA sequence analysis. The antibacterial effect of 22 kinds of plant essential oils studied by previous researchers on the main pathogenic bacteria of morels after harvest was tested, and the essential oils with better antibacterial effects were screened to provide green prevention and control technology for morels storage and preservation for reference, reduce environmental pollution caused by chemical reagents.

2. Materials and Method

2.1. Experimental materials

2.1.1. Experimental essential oil.
Clove essential oil, Styrax essential oil, Spearmint essential oil, Patchouli essential oil, Inula essential oil, Oregano essential oil, Neroli essential oil, Myrrh essential oil, Geranium essential oil, Sandalwood oil, Cypress essential oil, Agarwood essential oil, Nepeta essential oil, Bupleurum essential oil, Asarum essential oil, Peppermint essential oil, Chuanxiong Essential oil, Fennel essential oil, Amomum essential oil, Cedarwood Essential oil, Cinnamon essential oil, Catnip essential oil, a total of 22 plant essential oils were purchased from Jiangxi Ji'an Shengda Spice Oil Company.

2.1.2. Experimental medium.
Weigh 46.0 g of potato dextrose agar finished medium, dissolve it in 1000 ml of distilled water, autoclave at 115 °C for 20 minutes, and place it in a refrigerator at 4 °C for storage after cooling. Potato Dextrose Medium (PDB) is the liquid state of Potato Dextrose Agar Medium.

2.2. Experimental method

2.2.1. Isolation and purification of pathogenic fungi of Morchella.
Select naturally susceptible Morchella, cut out 1 cm×1 cm disease-healthy junction tissue, sterilize the surface with 0.1 % mercury for 30 seconds, rinse with sterile water for 3 times, then transfer to PDA medium for culture. Used the plate streak separation method, single colonies with different colony morphologies were picked for separation and purification, and the pathogenic bacteria were obtained at least three times. The isolated and purified strains were observed and recorded in morphology and inoculated on the slant medium, and stored at 4°C.

2.2.2. Pathogenicity test.
According to Koch's law, the pathogenicity of the isolated and purified pathogenic fungi was determined. Used an inoculating loop to hook the pathogenic fungal hyphae and inoculate them in PDB medium, and culture them with shaking at 26 °C for 2 days. Centrifuge in a high-speed refrigerated centrifuge for 5 minutes at a speed of 8000 r/min, remove the supernatant, and add an equal volume of normal saline to prepare a spore suspension. 20 μl of spore suspension was inoculated on Morchella and treated with sterile water as a control. Put the inoculated morel into a plastic box, and inoculate two groups of 4 morel for each pathogen. The inoculated Morchella was placed in an incubator at 26 °C for 5 days, and the incidence and disease symptoms were recorded [10].

2.2.3. Molecular biological identification of pathogenic bacteria.
The mycelium of the strain with the highest incidence rate was inoculated into PDB medium and cultured with shaking at 26 °C for 48h to prepare the pathogenic fungus fermentation broth. The cultured
fermentation broth was sent to the Chengdu branch of Beijing Kinco Biotechnology Co., Ltd. for sequencing. The sequencing results were analyzed by BLAST in the NCBI database to search for known sequences with higher homology; the MEGA X software was used to construct a phylogenetic tree to clarify the classification status and kinship of pathogens.

2.2.4. Preparation of bacteria-containing plates and essential oil plates.

(1) Used an inoculating loop to pick the hyphae from the plate and inoculate it in 50 ml PDB medium, culture in a constant temperature shaker at 26 °C for 48h, draw 1 ml of fermentation broth and spread it on the PDA plate, put it in a plastic bag and place it in a 26 °C biochemical culture Incubate for 48 hours to make a plate containing pathogenic fungi. (2) Dissolve 22 essential oil crude oil in 5% Tween-80 to fully emulsify the final concentration to 20000 μl/L. Under aseptic conditions, draw 2 ml of 20000 μl/L essential oil in a centrifuge tube, added 38 ml of fungal culture medium, shake well, pour them into 2 sterile petri dishes, and prepare 1000 μl/L essential oil plates. Did 2 sets of repetitions for essential oils.

2.2.5. Determination of the antibacterial rate of essential oils.

Used a sterile puncher to take a 6 mm diameter colony on the plate containing bacteria that has been cultured for 2 days, transfer it to the center of the essential oil plate, and place it in a 26°C biochemical incubator for cultivation. After 72h, the diameter of the pathogenic bacteria plaque was measured, and the inhibition rate was calculated.

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\text{Inhibition rate} = \frac{C - T}{C} \times 100\%
\]

C: control plaque diameter (mm); T: different plant essential oil treatment plaque diameter (mm).

2.3. Statistical Analysis.

The DPS system was used for statistical analysis of the test data, and the Tukey method was used for analysis of variance.

3. Results and Discussion

3.1 Isolation and purification of pathogenic bacteria and determination of pathogenicity

In this experiment, three pathogenic fungi were isolated and purified from the naturally-occurring Morchella. The pathogenicity test was carried out according to Koch's rule. One of the pathogenic fungi was the most pathogenic, showing 100% pathogenicity rate within 2 days after inoculation, label it as YDJ-P. The plate colony morphology and pathogenicity determination of Morchella morchella was shown in Figure 1.

![Plate colony morphology](image1)

![Spore hypha morphology](image2)

![Pathogenic bacteria on Morchella](image3)

Figure 1. Morphology of the pathogenic bacteria YDJ-P colony and determination of pathogenicity

3.2 Molecular biological identification of pathogenic bacteria

Combined the 18S rDNA sequence analysis results and the phylogenetic tree analysis, the results were
shown in Figure 2. Strain YDJ-P and *Rhizopus stolonifer* are grouped together and have the highest homology. This shows that the strain YDJ-P has the closest genetic relationship with *Rhizopus stolonifer*. Combined with the observation of morphological characteristics, the strain YDJ-P was identified as *Rhizopus stolonifer*.

Figure 2. Phylogenetic tree constructed based on 18S rDNA sequence

3.3 Antibacterial effect of 22 essential oils on strain YDJ-P

Table 1 shown the antibacterial effects of 22 essential oils on strain YDJ-P at a concentration of 1000 μl/L. Compared with the control, the antibacterial rate of Clove, Styrax and Spearmint essential oil against strain YDJ-P was 100%, and the antibacterial effect was the most significant. The essential oils of Patchouli, Inula, Oregano, Neroli and Myrrh had general antibacterial effects on strain YDJ-P, with an antibacterial rate greater than 50%, and the difference was very significant compared with the complete antibacterial essential oils (*P*<0.01). The essential oils of Sandalwood, Cypress, Agarwood, Nepeta and Bupleurum had poor bacteriostatic effect on the strain YDJ-P, with a bacteriostatic rate of less than 50%. Compared with the completely bacteriostatic essential oils, the difference is extremely significant (*P*<0.01). Patchouli, Inula, Oregano, Neroli and Myrrh essential oils with general bacterial effect were significantly different (*P*<0.05). The essential oils of Asarum, Peppermint, Chuanxiong, Fennel, Amomum, Cedarwood, Cinnamon and Catnip had no antibacterial effect on the strain YDJ-P, and the antibacterial rate is 0, which was extremely significant different from the essential oils with antibacterial rate greater than 50% (*P*<0.01). The essential oils of Agarwood, Nepeta and Bupleurum had poorer effect on strain YDJ-P, but were not significantly different from essential oils such as Asarum and other essential oils with a bacteriostatic rate of 0 (*P*<0.05).

Table 1. 1000 μl/L 22 essential oils on the inhibition of strain YDJ-P.

| Essential oil type      | Inhibition rate | Essential oil type      | Inhibition rate |
|-------------------------|----------------|-------------------------|----------------|
| Clove essential oil     | 100.00^aA      | Agarwood essential oil  | 22.5^bcDEF     |
| Styrax essential oil    | 100.00^aA      | Nepeta essential oil    | 13.06^cEF      |
| Spearmint essential oil | 100.00^aA      | Bupleurum essential oil | 6.39^cEF       |
| Patchouli essential oil | 70.83^bB       | Asarum essential oil    | 0^FF           |
| Inula essential oil     | 68.89^bB       | Peppermint essential oil| 0^FF           |
| Oregano essential oil   | 65.56^bcB      | Chuanxiong Essential oil| 0^FF           |
| Neroli essential oil    | 56.39^bcB      | Fennel essential oil    | 0^FF           |
Myrrh essential oil 53.06bc B Amomum essential oil 0f F
Geranium essential oil 49.72bc BC Cedarwood Essential oil 0f F
Sandalwood oil 46.11dc BCD Cinnamon essential oil 0f F
Cypress essential oil 26.11de CDE Catnip essential oil 0f F

Note: Different lowercase letters indicate significant differences at the $P < 0.05$ level, different uppercase letters indicate significant differences at the $P < 0.01$ level.

4. Conclusions
In this experiment, a more pathogenic fungus was isolated and purified from *Morchella* naturally-occurring in storage, labeled as YDJ-P, and the relationship between the strain YDJ-P and *Rhizopus stolonifera* was found through 18S rDNA sequence comparison recently, combined with morphological characteristics, it was identified as *Rhizopus stolonifera*. The bacteriostatic test results of essential oils showed that among the 22 tested essential oils, Clove, Styrax and Spearmint essential oils had 100% bacteriostatic rate against strain YDJ-P, and the bacteriostatic effect was the most significant. The essential oils of Asarum, Peppermint, Chuanxiong, Fennel, Amomum, Cedarwood, Cinnamon and Catnip had no antibacterial effect on the strain YDJ-P, and the antibacterial rate was 0. In order to inhibit the diseases caused by *Morchella* pathogenic fungi, prepare natural, environmentally friendly and safe biological agents, thereby reducing the use of chemical agents in the modern agricultural system, laying a theoretical foundation.

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