Products Analysis and Molecular identification of Flavonoids-Produce Endophytic fungi in Ginkgo Biloba

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Abstract: Endophytic fungi (Y6, Y8, Y10, G8) of ginkgo were studied in this paper to clarify what species of them, what kind of flavonoids of their metabolite, and the bacteriostatic activity of fermentation liquid from endophytic fungi. Disc diffusion method was used to determine antibacterial properties that the target strains to dominant spoilage bacteria X15 (bacillus), T10 (proteus), X5 (aureus), N4 (Serratia marcescens) from low temperature meat products, the results showed that Y6, Y8 and Y10 had best strains antibacterial properties for X15, T10, N4, respectively, and G8 is the best strain in antibacterial performance. The metabolites of four endophytic fungi of ginkgo biloba were analysed by high performance liquid chromatography (HPLC), the results showed that quercetin and isorhamnetin were contained in the fermented liquid of Y6 strain, isorhamnetin and quercetin were contained in the fermented liquid of Y8 and Y10 respectively. Four strains were identified by molecular biology by extracting DNA, the result shows that Y6, Y8, Y10, G8 strains were Penicillium sp., Dothiorella gregaria., Phoma sp., Fusarium nematophilum.

1. Introduction

Ginkgo biloba L. is regarded as “living fossil” and “plant kingdom pandas”[1]. Many medical studies have shown that Ginkgo nut could produce advantage effect in cough asthma, clearing lung and phlegm, prevent cardiovascular and cerebrovascular diseases and expand capillaries[2, 3]. The metabolite of endophytic fungi of ginkgo biloba have antibacterial effects on some pathogenic fungi and bacteria because of its biological activity. Yu H.S, Deng Z.S, Liu X.L and others found that the endophytic fungus of ginkgo biloba has extensive bacteriostatic properties[4-6], which indicates that the endophytic fungus of ginkgo biloba can be used as a new natural bacteriostatic agent for further exploration. In order to provide a new solution for the preservative of low-temperature meat products, four strains of endophytic fungi of ginkgo, which produce the highest amounts of flavonoids, were selected to study their bacteriostasis performance against dominant spoilage bacteria X15, T10, X5 and N4 from low-
temperature meat products. Although a new material resource was obtained from the endophytic fungi with high yield flavonoid of ginkgo, the current research is still not comprehensive. It is not clear whether the flavonoids produced by endophytic fungi from ginkgo biloba is consistent with the flavonoids from host plants. Current studies shown that glycoside compounds are the main existing forms of ginkgo flavones, which mainly produce three kinds of flavonoids aglycones after hydrolysis: quercetin, kaempferol and isorhamnetin[7, 8]. Therefore, this study analyzed the metabolites of endophytic fungi of ginkgo biloba by high phase liquid chromatography, and studied whether the same substances existed in the fermentation products of strains and flavonoids of ginkgo biloba, explored the relationship between endophytic fungi of ginkgo biloba and ginkgo biloba plants preliminarily. Finally, this study identified four endophytic fungi of ginkgo biloba by molecular biology.

2. Materials and methods

2.1 Study on antimicrobial properties of metabolite from endophytic fungi

The paper diffusion method was used to determine the bacteriostasis performance of the target strain to the dominant putrefaction bacteria in low-temperature meat products[9, 10]. The experimental strains were Y6, Y8, Y10 and G8 which were selected from the endophytic fungi of ginkgo biloba, the indicated strains were X15 (bacillus), T10 (proteus), X5 (aureus), N4 (Serratia marcescens) which were the dominant putificient bacteria isolated from low-temperature meat products, both were come from laboratory 416, Food building, Beijing university of agricultural.

The target strain of high-yielding flavonoids preserved on the bevelled surface was inoculated into PDA medium. After culturing for 5-7 days under 28 conditions, mycelium blocks of 0.5 cm and 0.5 cm were selected by the inoculation needle and inoculated into conical flask (250 mL) containing 100 mL sterile PD medium to oscillate for 7 days at 28 ℃, 150 r/min.

The strains X15, T10, X5, N4 frozen at -80 ℃ was thawed at room temperature, inoculated into 50 mL nutrient broth medium, and activated for 12 h under 37 ℃ conditions. Then, the activated bacterial liquid was inoculated into 10 mL fresh nutrient broth medium at an amount of 2%, cultured for 10 h under 37 ℃ conditions for later use.

Filter paper sheets with a diameter of 6 mm were prepared with a hole punch and autoclaved at 121 ℃ for 20 min for later use.

The fermentation liquid of the target strain was treated with ultrasound for 30 min under 30 ℃, 45 kHz to destroy the fungal cells, so as to dissolve the intracellular substances fully and ensure the full extraction of flavonoids. Mycelium was removed from fermentation liquid which was treated by ultrasonic. The fermentation liquid were extracted three times by ethyl acetate and n-butanol sequentially in the proportion of 5:2. The extracted liquid was evaporated to dry under 50 ℃, then redissolved in 4 mL methanol, and stored in 4 ℃ after filtrating by 0.45 μm membrane.

Nutrient agar medium that high-pressure sterilization was cooled to 50-60 ℃, then added indicator bacteria at 1% of the dosage, poured into sterile petri dish for cooling and setting for later use.

The filter paper pieces were soaked in bacteriostatic agent for 5 min and then dried. They were placed on different indicator plates and wetted with a small amount of sterile water so that the bacteriostatic agent could be fully dispersed. The three filter paper pieces were placed on a flat plate, then observe its bacteriostatic effect after culture for 16-18 h in 37 ℃. The diameter of bacteriostatic circle was measured with a vernier caliper. The filter paper pieces soaked in methanol were used as the blank control.

2.2 Analysis of fermentation liquid by HPLC

The 4 mg/L standard solution of Kaempferol, Quercetin and isorhamninetin were prepared with methanol respectively, for later use. Preparation of HPLC samples is like 2.1. HPLC conditions is as follow:
Chromatographic column: Zorbax SB-C18 (250 mm×4.6 mm, 5 μm), detector: UV-detector, detection wavelength: λ=360 nm, column temperature: 30 ℃, sample size: 10 μL, flow velocity: 0.8 mL/min, mobile phase: A(0.3% aqueous phosphate):B(methanol)=40:60.

2.3 molecular biological identification

Fungal genomic DNA extraction kit from Beijing solarbio life science was used to extract DNA.

ITS (Internally Transcribed Spacer), length in 500-800 bp, is a small gene between 18 S, 5.8 S and 28 S rRNA encoding gene, it could be expanded easily by using rDNA universal primers, including conservative and variation sequences. At present, ITS is a common method to identify fungal species. In this paper, universal primers ITS1 and ITS4 were used to amplify the DNA sequences in 5.8 S, rDNA, ITS and ITS regions. The amplified gene sequence was sequenced by Beijing BGI.

ITS1: 5’-TCCG TAGG TGAA CCTG CGG-3’
ITS4: 5’-TCCT CCGC TTAT TGAT ATGC-3’

The PCR constituent of ITS-5.8S rDNA: Mix (25 μL), Primer ITS1 (1 μL), Primer ITS4 (1 μL), DNA template (2 μL), ddH2O (21 μL).

The PCR condition of ITS-5.8 S rDNA: Firstly, 94 ℃ for 2 min; secondly, 94 ℃ for 90 s, 55 ℃ for 30 s, 72 ℃ for 90 s, 34 cycles; then 72 ℃ for 10 min; finally, 4 ℃ constant forever.

In this paper, MEGA5.1 software was used to construct phylogenetic trees from the identified gene sequences, and to analyze the evolutionary relationship of high-yielding flavonoid fungi.

3. Result and analysis

Figure1. The anti-bacteria effect of the target strains

\[d \geq 20 \text{ mm is extremely sensitive, } 15 \text{ mm} < d < 20 \text{ mm is highly sensitive, } 10 \text{ mm} \leq d \leq 15 \text{ mm is moderately sensitive, } d < 10 \text{ mm is hyposensitive, } d = 0 \text{ is invalid.}\]

The disk diffusion method was used to determine the bacteriostatic performance of metabolites from four endophytic fungi against dominant spoilage bacteria from low-temperature meat products. The results showed that it had significant bacteriostatic effects against X15, T10, X5 and N4. Y6, Y8 and Y10 had the strongest bacteriostatic effect on X15, T10, N4 respectively, with the bacteriostatic zone diameter of 19.55±0.78 mm, 18.29±0.06 mm, 19.3±1.20 mm respectively, and the weaker bacteriostatic effect on N4, X5, X15 respectively, with the bacteriostatic zone diameter of 12.50±0.49 mm, 12.9±0.35 mm, 16.5±0.35 mm respectively. G8 has the best bacteriostatic effect on four putrefactive bacteria, which has the best bacteriostatic performance among four target strains. In the bacteriostasis test, the diameter of bacteriostasis circle of the four strains of fungi was all higher than 10 mm, which provided a new research direction for the preservative and preservation of low-temperature meat products.
Figure 2. The HPLC chromatogram of three standard substances

A: Y6 N-butanol extract from fermentation broth; B: Y6 Ethyl acetate extract from fermentation broth; C: Y8 N-butanol extract from fermentation broth; D: Y10 N-butanol extract from fermentation broth;

Figure 3. The HPLC chromatogram of extracts of strains

The peak retention time of quercetin, kaempferol and isorhamnetin in HPLC was 7.380 min, 10.983 min and 11.966 min, respectively. By comparing the peak retention time of the samples with the peak retention time of the standard samples, we can find that the corresponding peaks of quercetin and isorhamnatin were found in the chromatogram of ethyl acetate extraction from Y6, the peak retention time was 7.390 min and 11.982 min, respectively, which showed that the fermentation liquid from Y6 contains quercetin and isorhamnatin; the peak corresponding to the standard substance in the chromatogram of n-butanol extraction from fermentation liquid of Y8 is isorhamnetin; the peaks of n-butanol extraction from Y10 fermentation liquid corresponded with quercetin, the peak retention time was 7.367 min, which illustrated there is quercetin in Y10 fermentation liquid. According to the results of HPLC, there are three strains (Y6, Y8 and Y10) with the highest flavonoid production, their fermentation liquid contained the same chemicals as ginkgo flavones which are quercetin and isorhamnatin, of which the strains produced quercetin were Y6 and Y10, and the strains produced isorhamnetin were Y6 and Y8.
Figure 4. The Phylogenetic tree of strain Y6, Y8, Y10, G8

The Y6, Y8, Y10, G8 strain was identified as *Penicillium* sp., *Dothiorella gregaria* strain, *Phoma* sp., *Fusarium nematophilum* by means of the phylogenetic tree constructed by Neighbor-joining method according to gene sequence provided by Beijing BGI gene through comparison with close population and calculation of similarity of 1000 times Strain.

4. Conclusion

The results illustrated that four strains of fungal metabolites have obvious effect on X15, T10, X5 and N4, Y6, Y8 and Y10 strains showed strongest effect on bacillus proteus Mr Charest, respectively. The G8 strain has a good antibacterial effect on the four dominant spoilage bacteria, and is the best strain in the target strain. The Y6, Y8 and Y10 strains all detected the same chemical substances as gingko flavonoids, which were quercetin and isorhamnetin, and the strains producing quercetin were Y6 and Y10, Y6 and Y8 strains produce isorhamnetin. Molecular biological identification showed that Y6, Y8, Y10 and G8 strains were *Penicillium* sp., *Dothiorella gregaria* strain, *Phoma* sp., and *Fusarium nematophilum* strain.

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