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"Gene-coculture-activity" coupling-driven deep mining of terpenoids with high antifungal activity from pleurotus ostreatus

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Abstract. In our team's previous study, five highly active sesterterpenes against human pathogenic fungi were obtained from Pleurotus ostreatus via coculture with Trametes robiniophia. Genetic analysis indicated that these compounds feature structural diversity and are worthy of in-depth exploration. To obtain more novel terpenoid lead compounds for drug development, this study conducts deep mining of terpenoids with high antifungal activity from P. ostreatus under the drive of the "gene-coculture-activity" coupling strategy. Strategies including coculture, signal molecule supplementation and genetic engineering were attempted to activate silent genes in macrofungi, and the condition with the optimal effect was selected for in-depth excavation of novel terpenoids. Specifically, the coculture system was mainly constructed with P. ostreatus and the pathogenic fungus Candida albicans; signal molecule supplementation mainly involved Ca^{2+} and methyl jasmonate; the genetic engineering strategy mainly focused on transferring terpenoid-related biosynthetic gene clusters from P. ostreatus into Escherichia coli for heterologous expression. LC-MS was applied to analyze the effects of these different approaches on the biosynthesis of antifungal terpenoids in P. ostreatus. By comparing different conditions, coculture of P. ostreatus with the pathogenic fungus C. albicans was finally determined to achieve the best activation effect. Accordingly, fermentation culture was performed under this condition, and the crude extract was obtained via extraction and rotary evaporation. The target compounds were then separated using normal-phase column chromatography and High-Performance Liquid Chromatography (HPLC). At present, one target compound has been obtained. In the next step, structural identification and antifungal activity evaluation will be carried out, and other antifungal active substances will continue to be isolated and prepared. Coculture of Pleurotus ostreatus with the pathogenic fungus Candida albicans can effectively induce the production of antifungal active substances, laying a foundation for the screening and clinical application of novel anti-human pathogenic fungi drugs.

Keywords: edible and medicinal fungi, coculture, secondary metabolites, antifungal activity

1. Introduction

In recent years, diseases caused by human pathogenic fungal infections have seriously threatened human health. These fungi can invade human tissues such as skin and mucous membranes as well as certain organs,

causing systemic infections. The main pathogens causing systemic infections in humans include Candida albicans, Cryptococcus neoformans, Aspergillus, and Pneumocystis, among others [1]. At present, the main active pharmaceutical ingredients used to treat fungal infections include polyenes, azoles, pyrimidine analogs, echinocandins, and allylamines [2]. However, long-term use of these drugs causes damage to the body and leads to the development of drug resistance [3, 4]. Therefore, there is an urgent need for research and development of novel antifungal drugs.

Relevant studies have shown that edible and medicinal fungi can produce substances such as terpenoids, steroids, phenolic acids, and polysaccharides [5], which possess functions including antifungal, antiviral, antitumor, and immunity-enhancing effects. This points out the direction for solving the shortage of specific clinical antifungal drugs. In traditional laboratories, pure culture and liquid medium cultivation methods are mostly adopted. Most gene clusters related to macrofungi are in a silent or low-expression state, resulting in fewer types of secondary metabolites with low yields. To fully exploit these potential active substances, various strategies have been developed to activate silent metabolic pathways in fungi. Among them, commonly used methods include signal molecule supplementation, genetic engineering, and strain coculture [6]. For example, Zhang et al. conducted coculture of the basidiomycete Phellinus punctatus with another basidiomycete Inonotus obliquus, and found increased yields of some secondary metabolites such as phelligridin C, phelligridin H, methyl inoscavin A, inoscavin C, inoscavin B, davallialactone, 21,24-cyclopentalanosta-3β,21,25-triol-8-ene, methyl davallialactone, and foscoparianol D. These metabolites exhibit potential in scavenging free radicals and inhibiting the proliferation of HeLa 229 cells [7]. Treatment with a certain concentration of exogenous calcium can promote the synthesis of ganoderma triterpenoids in Ganoderma lucidum fruiting bodies, while the expression levels of HMGR, LS, and SQS are significantly reduced after the addition of cyclosporin A or chlorpromazine to inhibit Ca^{2+} influx [8]. Li Yan, Feng Jianju and colleagues constructed a heterologous expression system using Saccharomyces cerevisiae as the host, and successfully introduced and expressed the genes encoding Sesquiterpene Synthases (STSs) from the Armillaria mellea strain CPCC 400891, obtaining multiple sesquiterpene molecules [9]. These are all typical successful cases of obtaining active products from edible and medicinal fungi by improving cultivation methods.

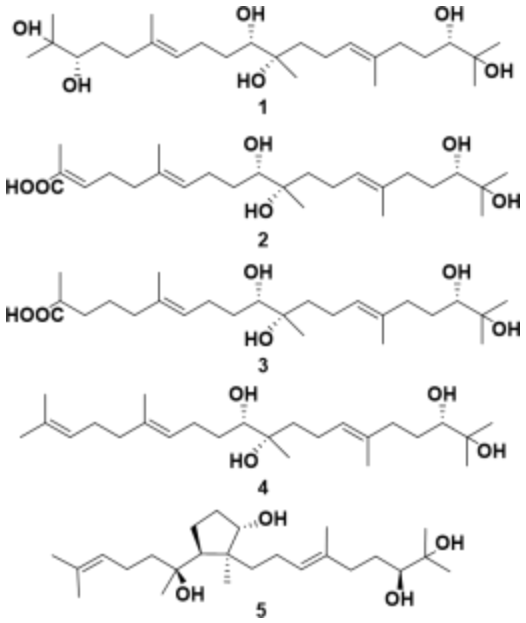

Figure 1. Five previously obtained sesterterpene compounds against human pathogenic fungi

In our team's previous study, five highly active sesterterpenes against human pathogenic fungi were obtained from Pleurotus ostreatus via coculture with Trametes robiniophia [10] (Figure 1). Genetic analysis indicated that the gene clusters responsible for synthesizing these compounds are rich in post-modification genes, with great biosynthetic potential and worthy of in-depth exploration (Figure 2). Therefore, to obtain more novel terpenoid lead compounds for drug development, this study performs deep mining of terpenoids with high antifungal activity from P. ostreatus under the drive of the "gene-coculture-activity" coupling strategy. This project attempts to activate silent genes in edible and medicinal fungi through strategies such as coculture, signal molecule supplementation, and genetic engineering, and selects the condition with the optimal effect for in-depth excavation of novel terpenoids, providing a basis for research on the synthesis of anti-human pathogenic fungi drugs using edible and medicinal fungi.

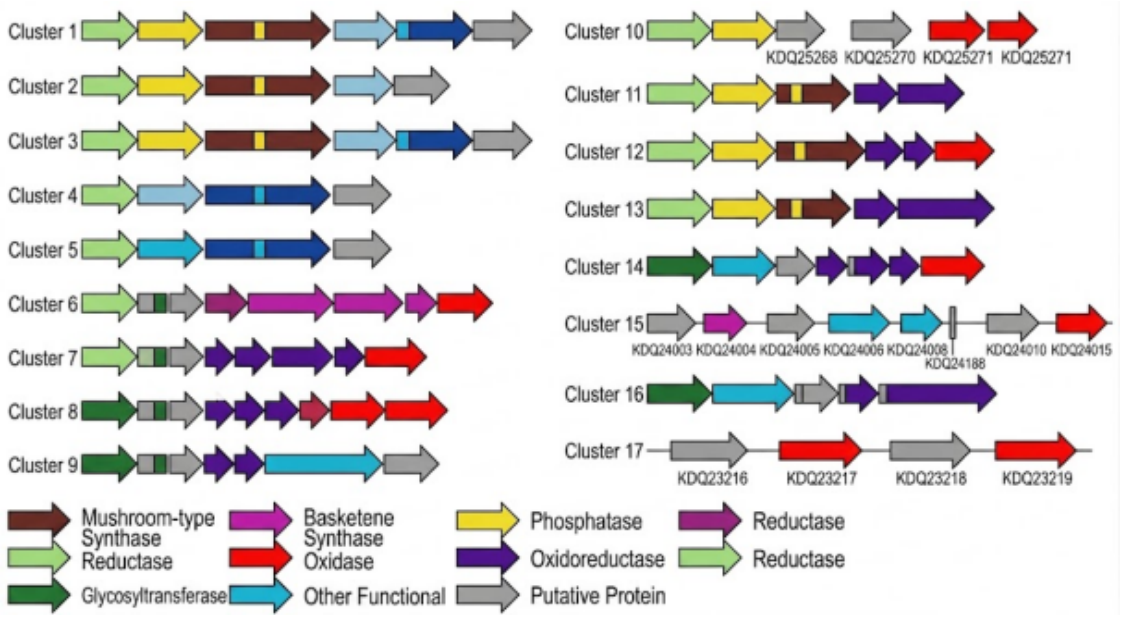

Figure 2. Seventeen putative gene clusters for terpenoid biosynthesis

2. Materials and methods

2.1. Test strains

Cryptococcus neoformans (ATCC 90012) and Candida albicans (ATCC 10231) are strains preserved in the Microbiology Laboratory of our university; Trametes robiniophia (SY636) and Pleurotus ostreatus (SY10) are strains preserved in the Edible Fungi Laboratory of our university.

2.2. Main reagents and instruments

Glucose (analytical grade) was purchased from Tianjin Dingshengxin Chemical Co., Ltd.; peptone (analytical grade) was purchased from Beijing Shuangxuan Microbial Medium Products Factory; Milli-Q ultrapure water was purchased from Millipore, France. The BHC-1300 II A clean bench was purchased from Suzhou Purification Equipment Co., Ltd.; the QYC-200 air constant temperature shaker was purchased from Shanghai Fuma Experimental Equipment Co., Ltd.; the autoclave was purchased from the Medical Equipment Factory of Shanghai Boxun Industrial Co., Ltd.; the BS 124 S precision electronic analytical balance was purchased

from Ohaus Instruments (Changzhou) Co., Ltd.; the AllegraX-12R high-speed centrifuge was purchased from Beckman Coulter Trading (China) Co., Ltd.

2.3. Medium preparation

Fungal basal medium: glucose 10.0 g/L, peptone 2.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KH_2PO_4 1.0 g/L. Sterilized at 115 ℃ for 30 min.

Fungal optimized medium: glucose 45.5 g/L, sucrose 14.5 g/L, peptone 6.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KH_2PO_4 1.0 g/L. Sterilized at 115 ℃ for 30 min.

YPD liquid medium: 10 g yeast extract, 20 g peptone, 20 g glucose, add water to 1000 mL and heat to dissolve. Sterilized at 115 ℃ for 30 min.

YPD solid medium: 10 g yeast extract, 20 g peptone, 20 g glucose, 20 g agar, add water to 1000 mL and heat to dissolve. Sterilized at 115 ℃ for 30 min.

PDA medium: 200 g potato, 20 g glucose, 20 g agar, 1000 mL ultrapure water. Sterilized at 115 ℃ for 30 min.

2.4. Strain activation

2.4.1. Pleurotus ostreatus SY10

The SY10 strain stored in a 4 ℃ refrigerator was used. A piece of mycelium with a diameter of 5 mm was transferred to the center of PDA solid medium and cultured in a 28 ℃ incubator for 7 days for later use.

2.4.2. Pathogen activation

3 mL of YPD medium was added to each test tube. A small amount of pathogenic fungi was drawn with a 10 μL pipette tip and inoculated into the test tube containing YPD medium. The tube was sealed and placed in a shaker, and cultured at 30 ℃ and 220 rpm until the OD_{600} value reached approximately 0.8–1.0.

2.5. Construction of liquid coculture system

2.5.1. Construction of coculture system of pleurotus ostreatus SY10 and pathogenic fungi

Three pieces of P. ostreatus SY10 mycelium with a diameter of 5 mm each were inoculated into a 500 mL Erlenmeyer flask containing 200 mL of optimized medium. After pure culture in a shaker at 28 ℃ and 180 rpm for 7 days, 2 mL of pathogenic fungus suspension was added respectively, and the culture was continued for another 7 days (Figure 3).

Figure 3. Coculture of Pleurotus ostreatus (SY10) and Candida albicans (liquid medium)

2.6. Extraction and analysis of coculture fermentation products

The mycelia and fermentation broth were separated. The mycelia were extracted with methanol, and the fermentation broth was extracted with ethyl acetate. The extract of secondary metabolites produced by fermentation was obtained through ultrasonic extraction, rotary evaporation, and centrifugation. Liquid Chromatography-Mass Spectrometry (LC-MS) and High-Performance Liquid Chromatography (HPLC) were used to analyze, compare, and observe the absorption peaks of the produced secondary metabolites.

2.7. Isolation of active compounds

Semi-preparative HPLC analysis and triple quadrupole LC-MS analysis were performed to analyze active fractions, determine the preparation and separation scheme, and further isolate and obtain target active products.

3. Experimental results

3.1. Analysis of results under various culture conditions

According to the experimental design, this experiment aimed to activate terpenoid gene clusters by constructing a coculture system of P. ostreatus SY10 with pathogenic fungi, supplementing signal molecules (Ca^{2+}, methyl jasmonate) based on the coculture system of P. ostreatus SY10 and T. robiniophia SY636, or performing heterologous expression of P. ostreatus genes. Preliminary exploration showed that heterologous gene expression did not achieve the expected effect. The yield of target compounds in the signal molecule supplementation groups (Ca^{2+}, methyl jasmonate) increased to a certain extent, but the effect was more significant in the coculture of P. ostreatus SY10 with the pathogenic fungus (C. albicans), with the content of active sesterterpene compounds increased by 3–10 times, which was more in line with experimental expectations. Therefore, this strategy was adopted for further experiments (Figure 4).

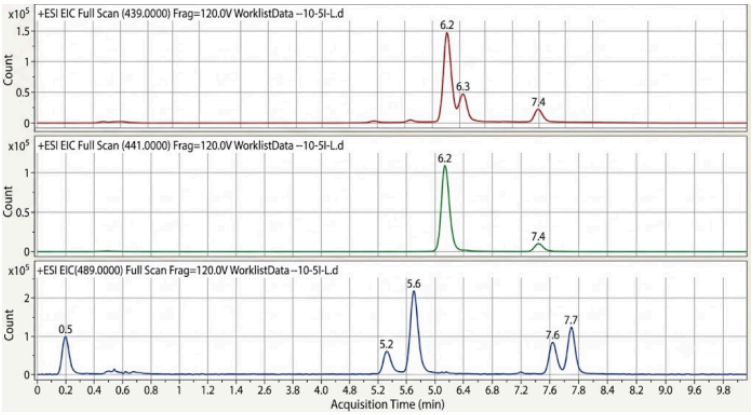

Figure 4. Partial LC-MS detection results of coculture of Pleurotus ostreatus SY10 and Candida albicans

3.2. Fermentation of pleurotus ostreatus SY10 and Candida albicans, and extraction and preliminary analysis of obtained samples

By constructing the coculture system of P. ostreatus SY10 and C. albicans, fermentation was carried out in a shaker for 7 days. The total extract of fermentation products was extracted with ethyl acetate, concentrated with a rotary evaporator, dissolved with methanol, transferred to penicillin vials, dried and weighed to obtain

17.5 g of total extract. The secondary metabolites were preliminarily analyzed by HPLC, and the results are shown below (Figures 5 and 6).

Figure 5. Shaker fermentation of SY10 + Candida albicans coculture

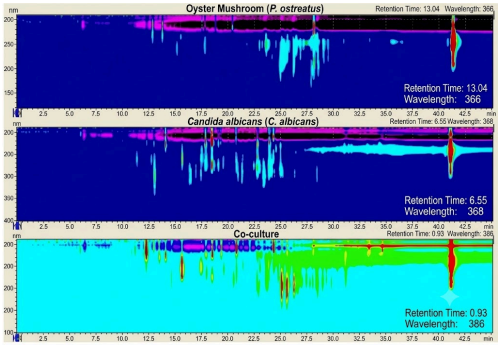

Figure 6. HPLC analysis results of SY10 pure culture and SY10 + C. albicans coculture

3.3. Tracking of target compounds based on molecular networking technology

MS/MS spectra were obtained by high-resolution tandem mass spectrometry detection of the crude extract of secondary metabolites, and molecular networking analysis was performed. It can be seen that the active sesterterpenes identified in previous studies [10] (Figure 1) and their analogs each form a cluster, based on which further excavation of analogs can be carried out (Figures 7–10).

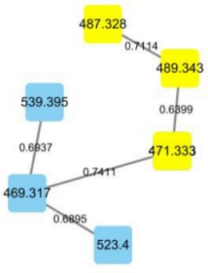

Figure 7. Molecular network diagram of target compound 1

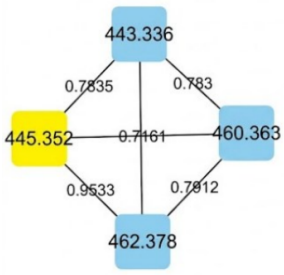

Figure 8. Molecular network result of analogs A

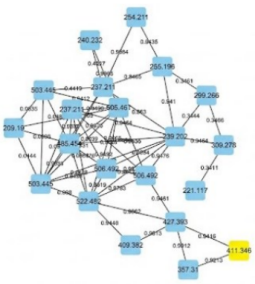

Figure 9. Molecular network result of analogs B

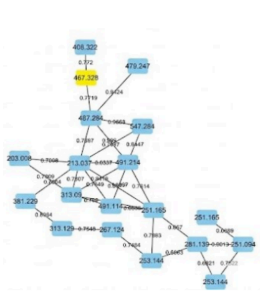

Figure 10. Molecular network result of analogs C

3.4. Verification by combined LC-MS and molecular networking analysis

The total extract was preliminarily separated into different fractions (1–7) by column chromatography. After LC-MS detection, target compounds with molecular weights consistent with those from molecular networking analysis were extracted from fraction 3. Under the combined guidance of the two analytical methods, we isolated compounds with target ion peaks at m/z 471, 487, 489, etc. (Figure 11).

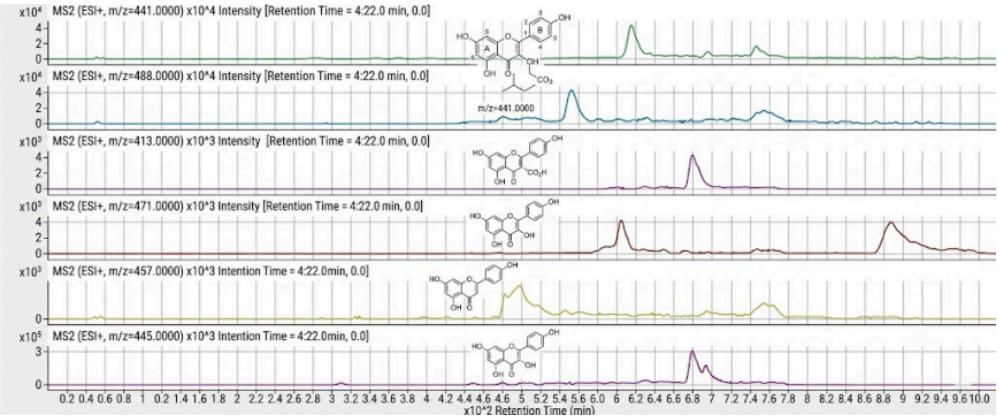

Figure 11. Different target products located by LC-MS analysis in fraction 3 (only partial results shown)

3.5. HPLC separation results

At present, one target compound 1 has been isolated and purified by HPLC preparation, with an estimated molecular weight of 487, and its structure is being elucidated. In the next step, its structure will be elucidated using Nuclear Magnetic Resonance (NMR) and mass spectrometry, and other antifungal active terpenoids will continue to be isolated and prepared, followed by antifungal activity evaluation (Figure 12).

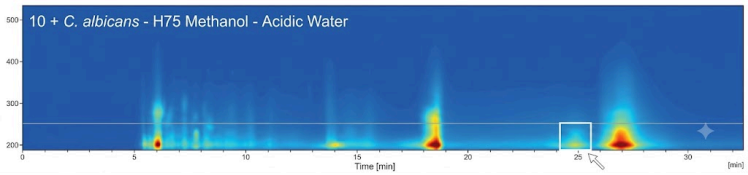

Figure 12. HPLC chromatogram of isolated and prepared target compound 1 (the white-framed part is target compound 1)

4. Discussion and prospects

Through preliminary condition exploration, this project finally selected the coculture system of P. ostreatus and C. albicans for in-depth excavation. Under this condition, the content and diversity of active sesterterpenoids increased significantly. After fermentation and enrichment, further isolation was carried out using methods such as column chromatography, LC-MS analysis, and HPLC preparation under the guidance of molecular networking technology. At present, one target compound has been purified. In the next step, its structure will be further elucidated through qualitative NMR analysis and LC-MS analysis, antifungal activity evaluation will be performed, and other antifungal active terpenoids will continue to be isolated and prepared.

Natural active substances are an important treasure trove for drugs. Obtaining chemical components with novel structures, clear activities, and low toxicity to humans through the cultivation of edible and medicinal fungi provides abundant resources and possibilities for the research and development of antifungal drugs. With the spread of viruses such as AIDS and novel coronavirus, as well as the increase in major surgical procedures, the situation of invasive fungal infections has become increasingly severe. The emergence of multidrug-

resistant "super fungi" with high mortality rates has brought greater challenges to the treatment of fungal infections [11]. Searching for more novel antifungal drugs concerns the health and well-being of thousands of households.

This experiment was carried out based on this background. Activating silent genes for expression through different culture methods to obtain more novel active compounds improves the possibility of discovering anti-human pathogenic fungi drugs, and also provides theoretical support for research on coculture of edible and medicinal fungi. However, this project still requires further in-depth research to obtain more active lead compounds. It is believed that the research on coculture of edible and medicinal fungi in this project will contribute to the R&D and screening of antifungal drugs as well as their later clinical application.

5. Conclusion

In summary, under the strategy of "gene-coculture-activity" coupling drive, this study successfully carried out deep mining of terpenoids with high antifungal activity from Pleurotus ostreatus. By comparing various fungal silent gene activation strategies including coculture, signal molecule supplementation, and genetic engineering, the study finally determined that coculture of P. ostreatus with the pathogenic fungus C. albicans achieved the optimal activation effect. Under this coculture system, the yield of active sesterterpene compounds was significantly increased, with their content elevated by 3 to 10 times. Based on this optimal condition for fermentation and enrichment, the research team, guided by molecular networking technology, performed isolation using physicochemical methods such as column chromatography, LC-MS analysis, and HPLC preparation. At present, one target compound with an estimated molecular weight of 487 has been successfully purified. This study not only verifies the high efficiency of coculture of edible and medicinal fungi with pathogenic fungi in inducing the production of antifungal active substances, but also provides high-quality lead compound resources and solid theoretical support for the screening and development of novel anti-human pathogenic fungi drugs to address the increasingly severe clinical challenges of invasive fungal infections and multidrug resistance.

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