RESEARCH ARTICLE

A Novel Huperzine A-producing Endophytic Fungus NWUHS002 Isolated from Huperzia Serrata Trev.

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Abstract

Huperzine A is an important drug for Alzheimer's disease (AD) treatment, which is mainly extracted from the medicinal plant Huperzia serrata (Thunb.) Trev (*Huperzia serrata* Trev.). In present study, a new endophytic fungus (NWUHS002) producing Huperzine A was isolated from the *Huperzia serrata Trev.* with a yield of 5.20 μg/g. NWUHS002 was identified as Staphylotrichum by morphology and internal transcribed spacer (ITS) sequence. The discovery of NWUHS002 in *Huperzia serrata Trev.* can be used for the breeding of high-producing Huperzine A strains and the study of Huperzine A biosynthesis.

Keywords: huperzine A, alzheimer's disease, Huperzia serrata Trev., endophyte.

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1 Introduction

Alzheimer's disease (AD) is a dementia with progressive dementia, with a mortality rate of 71% within 5 to 12 years of onset, and has been recognised by the World Health Foundation (WHO) as a global public health priority [1]. With the demographic shift to older ages, the prevalence of dementia in older adults is expected to double every 20 years and reach 135 million by the year 2050 [2, 3].

Despite the fact that Alzheimer's disease has been clinically defined for about one hundred years, its molecular mechanisms and pathogenesis remain incompletely understood [4].At present, the most effective way to treat AD is to reduce acetylcholine hydrolysis, and acetylcholinesterase inhibitors (AChEIs) are the most efficacious mechanism in the clinical treatment of AD. [5].

Huperzine A (Huperzine A, Hup A) is a new type of effective monomer extracted from Chinese herbal Huperzia serrata [*Huperzia serrata* Trev.], which is a highly efficient, highly selective and reversible central Acetylcholinesterase (AChE) inhibitor [6]. In addition, Hup A can increase the activity of α-secretase, reduce the concentration of Aβ [7], and block the production of Aβ in vitro. These advantages mean that Hup A has a wide-ranging future in the field of AD therapeutics [8, 9]. However, Hup A concentrations in

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Huperzia serrata Trev. plants are unfortunately very low (about 0.007%), and the plant's growth cycle is very long about 8-10 years [10]. And the chemical synthesis method is relatively complex, high cost, and a lot of toxic by-products [11, 12].

2 Related Work

Since the microorganisms had previously been identified as substitutes alternatives for paclitaxel production , the usage of endophytic fungi for the production of secondary metabolites has already become a new study orientation, thus providing an environmentally friendly, relatively simple and inexpensive alternative to plants [13].Because fungi are more suitable for research than plants due to their simpler genetics and ease of manipulation [14]. So far, some endophytic fungi isolated with *Huperzia serrata* Trev. are known to generate Hup A.However, there are currently problems such as unstable metabolism and low efficiency, which cannot meet the requirements of industrial production. Therefore, it is critical to find more new efficient strains. In present study, we isolated and identified endophytic fungi capable of producing Hup A from *Huperzia serrata* Trev. plants, which can be used for the breeding of high-producing Hup A strains and the study of Hup A biosynthesis.

3 Experiments

3.1 The source of *Huperzia serrata* Trev. material

The *Huperzia serrata* Trev. plant in this experiment was obtained from Yan zi bian Town, Ning Qiang County, Han Zhong City, Shan Xi Province.(N:32°55′, E:105°55′, H:840m)

3.2 Isolation and Purification of Endophytic Fungi from *Huperzia serrata* Trev. Leaves

For *Huperzia serrata* Trev., the leaves were rinsed in tap water and surface sterilised according to the following procedure. Rinse with 75% ethanol solution for 1 min, sterile distilled water twice, and 0.1% mercuric chloride solution for 8 min. Finally, the leaves were rinsed 6 times in sterile distilled water and then cut into small pieces (0.5 - 1.0 cm^3) using a sterile scalpel.A small leaf from each section was placed in a potato glucose agar containing 200 g/L potato extract, 20 g/L glucose, 15 g/L agar, the antibiotics streptomycin (60 μg/mL) and penicillin (100 μg/mL).Subsequently, the petri dish was cultured in the dark at 25°C, and the cultured endophytic fungi were separated and purified to obtain a single colonies, which were used the following studies.

3.3 Extracting of Hup A from Endophytic Fungi

Each selected strain of endophytic fungi (26 strains in total) was then inoculated into 200 ml/500 ml of PDB medium and grown on a rotary shaker at 25 degrees Celsius at 160 rpm/min for 6 d. The mycelium was obtained with a Buchner funnel, dried overnight at 40 °C, and then ground into powder with a grinding rod. 1 g powder was leached with 20 mL 0.5% hydrochloric acid overnight, and then sonicated for 15 minutes each time, and extracted 3 times.

The hydrochloric acid (60 mL in total) was collected with ammonia and the pH was adjusted to 9.5. The hydrochloric acid component is extracted with trichloromethane in a ratio of 1:1, the trichloromethane is evaporated to dryness using a spiral evaporator and the extract is solubilised in methanol and filtered through a 0.45 μm filter membrane.

3.4 Identification of Hup A by HPLC and LC-MS

The content of Hup A in the crude extract of the fungus by high performance liquid chromatography (HPLC) was determined.The HPLC was performed on Shimadzu LC-20AT, using a YWG C18 reversed-phase column (4.6 mm × 250 mm). The mobile phase solution and the analytical samples were filtered through a 0.45 μm filter before injection.The mobile phase is methanol-acetonitrile-0.08 M ammonium acetate, the ratio is 3:1:6, and the flow rate is 0.8 mL/min. Configure Hup A standards of 0.2 mg/mL, 0.15 mg/mL, 0.1 mg/mL, 0.05 mg/mL, 0.04 mg/mL, 0.02 mg/mL, 0.01 mg/mL, 0.005 mg/mL, 0.0025 mg/mL. After filtering the sample solution, the peak area is measured, and then the Hup A concentration is used as the abscissa and the peak area is the ordinate to draw a standard curve. Then the samples are in sequence, and the peak area of the sample is to be tested with the peak time of the Hup A standard product into the standard curve for content analysis. The Agilent Ultra High Performance Liquid Chromatography system (LC Agilent 1290) uses Agilent Eclipse Plus C18RRHD chromatographic column (2.1×50 mm, particle size 1.8μm), the injection volume is 2 μL, and the gradient elution is carried out with 0.1% formic acid and methanol. It is 0.3 mL/min. It is analyzed by Selected Ion Record (SIR) and Multiple Reaction Monitoring (MRM). Mass spectrometry is performed on an Agilent 6460 QQQ equipped with an electrospray ionization source (ESI). The above is performed in positive ion mode. The LC-MS data processing software is MassHunter.

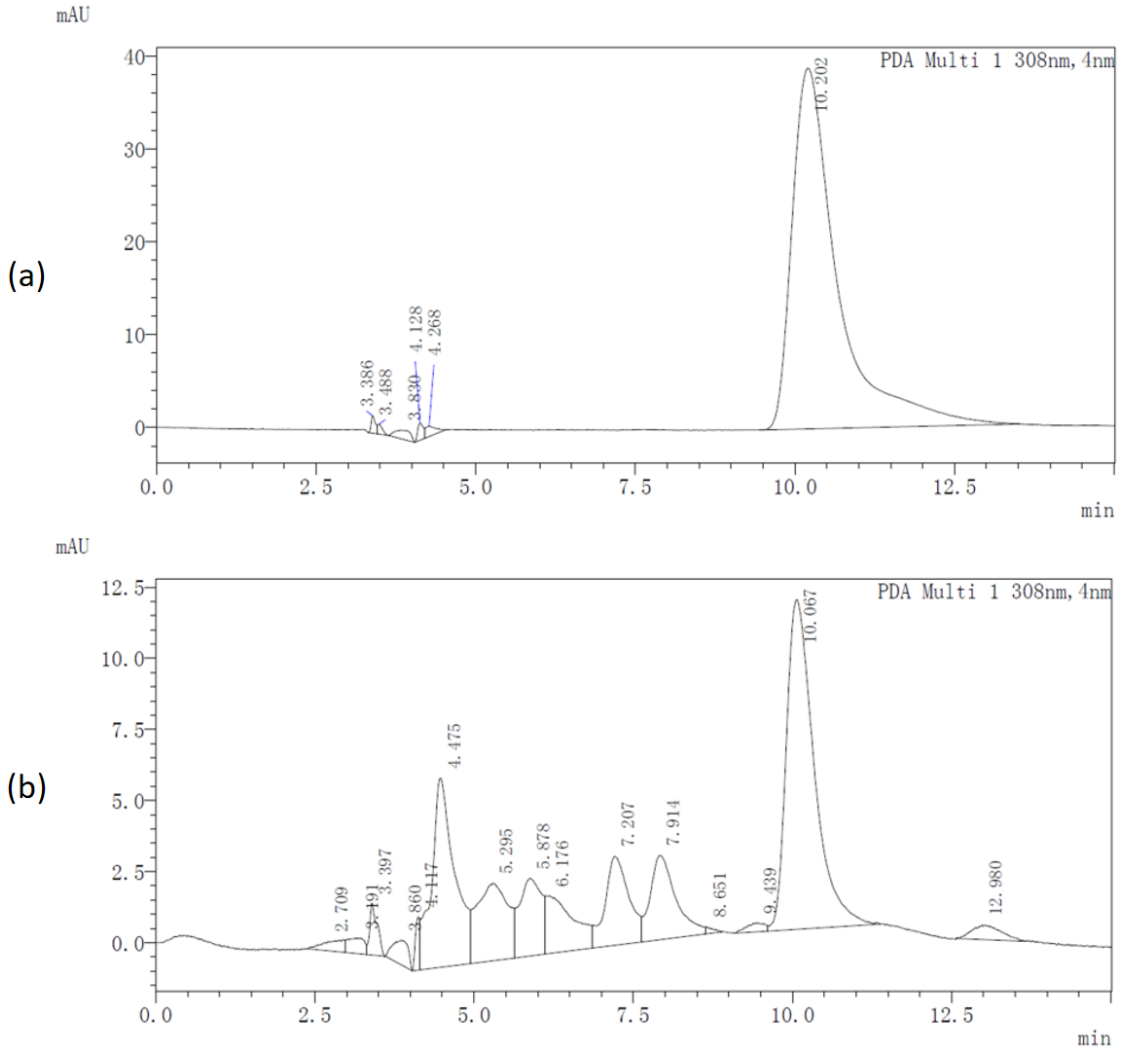

Figure 1. The HPLC chromatogram of Hup A (a) Hup A Standard (b) produced by strain NWUHS002.

3.5 Determination of Inhibitory Activity of AchE in Vitro

According to published methods slightly changed [15]. 300 μL of 0.1 mol/L PBS buffer with pH 7.2, 10 μL of samples of different concentrations, 10 μL of 1 U/mL AChE, 30 μL of 5 mmol/L Acetylthiocholine(ATCh), set a control, and mix well Then, react at 37°C for 20 minutes, and then add 10 μL of 6% HClO4 to stop the reaction. Take 100 μL of the mixed sample and add it to the microplate well and add 100 μL of 5 mmol/L DTNB(5,5′-Dithiobis(2-nitrobenzoic acid)) for color development for 5 minutes. Use a microplate reader to detect at 410 nm. The above procedure was performed three times, and the suppression rate was measured by the following formula: (Control absorbance-Sample absorbance/Control absorbance) • 100.

3.6 Identification of Hup A producing Endophytic Fungi

The Hup-A-containing strains were incubated in PDA medium at 25 °Cfor 6 days., and preliminary identification was carried out based on the colony morphology. Use Leica DM500 optical microscope to observe the structure of mycelium conidia and so on by immersing oil under 100 times microscope. Methods of molecular identification that are based on internal transcribed segregation (ITS) sequence analysis and PCR amplification are used to identify genera or species of fungi. The mycelium was crushed by liquid nitrogen and endophytic fungal genomic DNA was extracted by a modified CTAB method. The rDNA fragments containing ITS1-5, 8S-ITS2 were amplified by PCR using primers ITS(15′-TCCCTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATGATATATGC-3′).The amplified product (25 μL) consists of 2 μL genomic DNA, 2.5 μL 10×Eaq buffer, 2 μL dNTP, 0.125 μL ExTaq, 0.5 μL primer and 17.375 μL ddH2O. The PCR reaction procedure was as follows: pre-denaturation at 95 °C for 3 minutes, heat cycle of 95 °C for 30 seconds, 55 °C for 15 seconds, 72°C for 1 minute for a total of 34

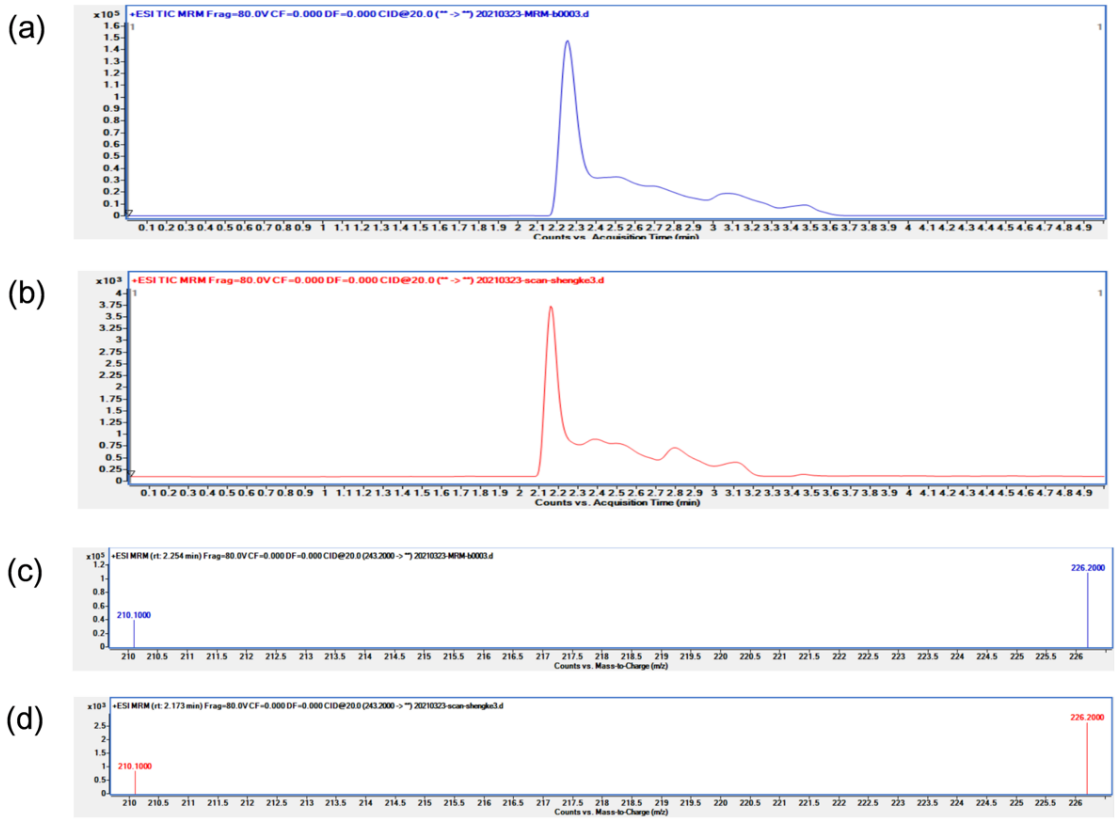

Figure 2. The chromatograms of LC-MS/MS for Hup A. (a) standard Hup A product ion scan (b) Hup A obtained from the fungus product ion scan (c) standard product molecular ion of Hup A at 226.2 and 210.1 (d) fungus product molecular ion of Hup A at 226.2 and 210.1.

cycles, and finally extension for 5 minutes at 72°C. PCR products were electrophoresed with 1% agarose gel at 1×TAE buffer and then were purified with EZ-10 Spin Column DNA Gel Extraction Kit. Afterwards, all PCR samples were subjected to Sangon Bitech sequencing.

3.7 Phylogenetic Analysis of Hup A-producing Endophytic Fungi

Performing BLAST on the measured ITS sequence from NCBI, finding the homologous sequence of the bacteria in the database,and selecting the reference sequence with the highest homology for constructing phylogenetic trees.The evolution history was deduced from the neighbor joining method.The bootstrap consensus tree deduced from one hundred years of repetition indicates the evolutionary history of the analysed taxa.Evolutionary distances were calculated in base substitutions per site by Jukes-Cantor method [16–18]. Evolutionary analyses were performed in MEGA7 [19].

3.8 Statistical Analysis

All results were expressed in mean ± standard deviation and correlation analyses were performed using SPSS 25.0 and Origin 2018 software.

4 Results

4.1 Isolation of Endophytic fungi and Hup A detection

Wild *Huperzia serrata* Trev. is a rich source of endophytic fungi.We fermented 26 species of endophytic fungi isolated from *Huperzia serrata* Trev. leaves. The Hup A in endophytic fungi was extracted and identification by HPLC and LC-MS.

The HPLC data showed that the retention time (Rt:10.210 min) of one of the 26 endophytic strains was similar to that of the standard (Rt:10.067 min) (Figure 1), which suggested this strain may be produce Hup A. For future research, we named this strain as NWUHS002. The evidence that NWUHS002 can produce Hup A was further confirmed by LC-MS. Basal peaks were chosen and further identified by LC-MS/MS by production ion mode. The fungal extract produced the same electrospray mass spectrum as the genuine Hup A, and its main molecular ion was (M+H+) = 243. The molecular ion 243 was selected for MRM analysis, and it was found that the Hup A standard product and the NWUHS002 sample produced the same ion pair (226.2 and 210.1) (Figure 2). Therefore, the NWUHS002 can produce

the secondary metabolite Hup A. Then, the Hup A production capacity of NWUHS002 was measured. The results suggested that the the production of Hup A is about 5.20 μg/g.

4.2 Detection of AChE Inhibitory Activity

The in vitro AChE inhibitory activity of NWUHS002 extract was compared with that of standard Hup A. With the increase of Hup A concentration, the inhibition rate of AChE also gradually increased. The inhibitory rate of the NWUHS002 extract reached 42.2% at approximately 20 μg/mL Hup A, while the standard Hup A reached 69.5% at 20 μg/mL, thus confirming that the strain extracted metabolites have AChE inhibitory activity.

4.3 Identification of Hup A–producing Strains

The morphological Characteristics of NWUHS002 was cultured on PDA medium. As displayed in Figure 3, the strain grew rapidly, reaching a coverage of 5 cm after about 5 d and expanding to the whole PDA medium after 8 d at 25°C. The hyphae are loose, with light-colored hairs on the edges, and light black cells are formed in the middle of the medium (Figure 3). Microscopically, there are septa and round spores in the hyphae. Accordingly, according to the classification of fungi by Ainsworth , the NWUHS002 was initially identified as Staphylotrichum.

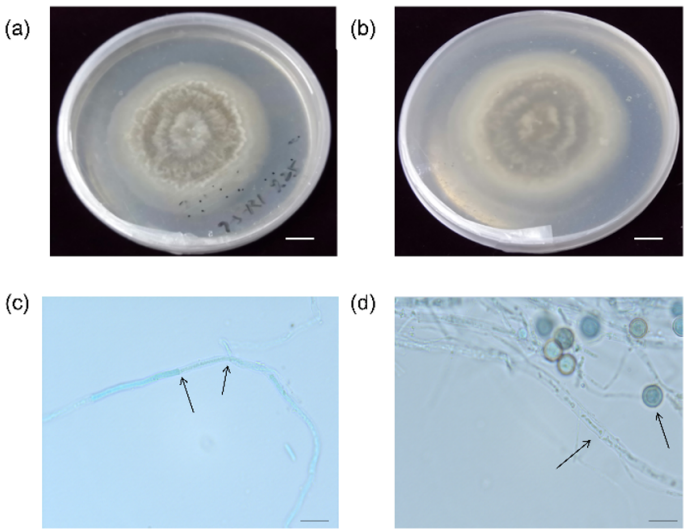

Figure 3. Morphological characteristics of NWUHS002. (a and b): Observation of colony morphology after 6 d of culture. (b and c): The morphology of mycelium under the microscope(10 μm).

In order to further confirm the species of this strain, we analyzed its ITS sequence.

Using both ITS1 and ITS4 as primers, we successfully amplified the genomic DNA of NWUHS002, which was detected by agarose gel electrophoresis. As the result, the amplified band size of NWUHS002 is 500 - 600 bp (Figure 4).

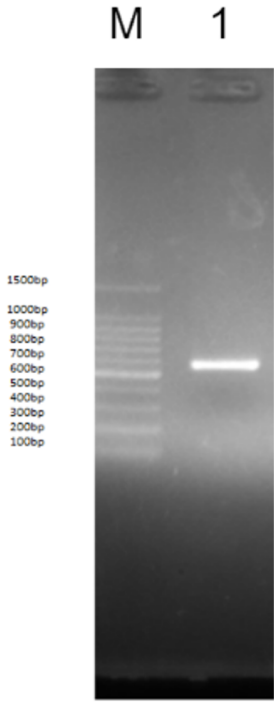

Figure 4. Analysis of the ITS sequence of NWUHS002 and reported Huperzine A-producing endophytic fungi.

The recovered DNA from agarose gel electrophoresis was subjected to ITS-rDNA sequence analysis. BLAST on NCBI further compared these sequences. The phylogenetic tree was constructed and shown in the Figure 5. The ITS-rDNA sequence of NWUHS002 showed 91% similarity to Staphylotrichum boninense strain: JCM 17908 (accession number AB625580.1) and Staphylotrichum coccosporum isolate: FKI-5577 (accession number AB625583.1), indicating the strain NWUHS002 has a close evolutionary distance to Staphylotrichum in the phylogenetic tree. Therefore, ITS sequence analysis combined with of morphological analysis identified NWUHS002 as Staphylotrichum.

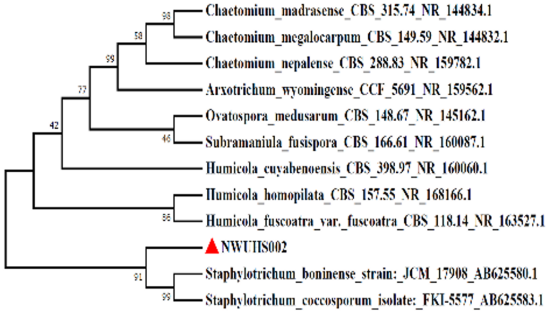

Figure 5. ITS sequence analysis of NWUHS002.

5 Discussion

Huperzia serrata Trev. produces Huperzine A, an important pharmaceutical ingredient in the treatment of Alzheimer's disease. And the price of Staphylococcus aureus is high in the market. In order to improve the process of industrial production of Huperzine A and to reduce the cost of production of Huperzine A, our isolation of strains upstream of industrial preparation and screening of suitable endophytes provide a reliable basic research for the preparation of engineered bacteria.

A variety of endophytic fungi were separated from *Huperzia serrata* Trev., and some them were identified to produce Hup A. Including *Acremonium sp.2F09P03B, Blastomyces sp. HA15, Botrytis sp. HA23, Cladosporium cladosporioides LF70, Alternaria sp. YD-01, Shiraia sp. Slf14, Trichoderma harzianum L44, Colletotrichum gloeosporioides ESO26, Paecilomyces tenuis YS-13, Fusarium sp. Rsp5.2, Penicillium polonicum hy4, Colletotrichum gloeosporioides Cg01, Fusarium C17, Mucor racemosus NSH-D, Fusarium verticillioides NSH-5, Mucor fragilis NSY-1, Trichoderma harzianum NSW-V, Fusarium oxysporum NSG-1* [20–31]. Among them, the genus *Colletotrichum* and *Fusarium* are relatively more. These endophytic Hup A-producing fungi provide a new source of Hup A. In addition, they are also capable of producing Hup A in a variety of ways.However, these are many problems for these huperzine A-producing endophytic fungus in production such as low metabolic content, instability and low biomass. Therefore, it is still very important to find more efficient endophytic fungi for Hup A production. We picked wild *Huperzia serrata* Trev. from Hanzhong City, Shaanxi Province, China, and isolated more than 100 endophytic fungi from leaves. After preliminary morphological screening, we selected 26 endophytic fungi for liquid fermentation and one of these endophytic fungi producing Hup A was obtained by LC- MS, which was named NWUHS002. Then we identified that NWUHS002 belongs to Staphylotrichum by morphology and molecular biology (ITS sequence). This is also the first report of Staphylotrichum in *Huperzia serrata* Trev..In present study, a new endophytic fungus from the *Huperzia serrata* Trev. was found and its species was identified, which can be used for the breeding of high-producing Hup A strains and the study of Hup A biosynthesis.

6 Conclusion

In this study, we successfully isolated and identified several endophytic fungi from *Huperzia serrata* Trev., including the novel strain Staphylotrichum NWUHS002, which is reported for the first time as a producer of Huperzine A (Hup A). These findings highlight the potential of various endophytes, particularly those from the genera Colletotrichum and Fusarium, as promising sources for Hup A biosynthesis. However, challenges such as low yield, metabolic instability, and limited biomass in existing Hup A-producing strains underscore the necessity for more efficient fungal strains. The identification of Staphylotrichum NWUHS002 not only provides a new avenue for Hup A production but also facilitates the development of engineered strains aimed at enhancing Hup A biosynthesis, which could lead to reduced production costs in industrial settings. Furthermore, this research contributes foundational insights for the artificial domestication of *Huperzia serrata* Trev. and deepens our understanding of its symbiotic relationship with endophytes, a factor that complicates tissue culture efforts. Overall, this study establishes a crucial basis for optimizing Hup A production through endophytic fungi and suggests sustainable approaches for the exploitation of *Huperzia serrata* Trev. in pharmaceutical applications.

Conflicts of Interest

The authors declare no conflicts of interest.

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