Microbiological Research **164** (2009) 9—17

Available online at www.sciencedirect.com

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Microbiological Research

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Isolation and identification of a new hypocrellin A-producing strain *Shiraia* sp. SUPER-H168

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Received 26 June 2008; received in revised form 7 August 2008; accepted 8 August 2008

KEYWORDS
Hypocrellin A;
Production;
Identification;
Shiraia sp.;
18S rDNA

Summary

A new hypocrellin A-producing strain, *Shiraia* sp. SUPER-H168, was isolated from tissues of bamboo, *Brachystachyum densiflorum*. The morphology of this strain was characterized with a light microscope and a scanning electronic microscope. The mycelia, conidia, pycnidia of fungus were observed. Small pycnidia (10—20 μm in length) full of small conidia appeared on the mycelia, which were first reported in this study. The 18S rDNA region of this strain was amplified and sequenced. Then a neighbor-joining tree of 18S rDNA was constructed. According to the result of analysis, the strain SUPER-H168 was proved to belong to the genus *Shiraia*. Hypocrellin A was produced by solid-state fermentation, extracted by acetone, isolated by preparative RP-HPLC, and identified by RP-HPLC, ESI-MS and ultraviolet—visible absorbing scanning with diode array detection. The HA production could reach 2.02 mg/g dry solid substrate.

Introduction

Naturally occurring perylenequinonoid pigments (PQP), mainly including hypocrellin A (HA), hypocrellin B, cercosporin, elsinochrome A and hypericin, have long been known as excellent photosensitizers and gained much attention in recent years owing to their light-induced antitumor, antifungal and antiviral activities (Wan and Chen, 1981; Ma et al., 1989, 2003; Daub and Ehrenshaft, 2000; Meille et al., 1989; Schinazi et al., 1990). Hypocrellins were isolated from stromata of the parasitic fungi *Hypocrella bambusae* and *Shiraia bambusicola* (Wan and Chen, 1981; Wu et al., 1989; Kishi et al., 1991). Among them, HA is the main component and possesses several

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doi:10.1016/j.micres.2008.08.004

advantages over the currently used photodynamic therapy (PDT) agent photofrin II with easy preparation and purification, high yields of reactive oxygen species (ROS), low toxicity and rapid metabolism *in vivo*, etc. (Miller et al., 1997). Thus, HA has great potential to be developed as new generation of PDT medicines. In clinical trials, HA has produced promising results in the treatment of various skin diseases such as white lesions of vulva and keloid cases (Wan and Chen, 1981). Damage to human erythrocyte membranes was also found when the cells were irradiated together with HA (Cheng and Wang, 1985). HA can kill tumor cells efficiently and accumulate selectively in cancer cells (Zhang et al., 1998). For example, Hela cells and S-180 solid tumor cells can be killed photodynamically in the presence of HA (Fu and Chu, 1989). In addition, HA has an antiviral activity against human immunodeficiency virus type 1 (Hudson et al., 1994). It has been proved that HA possesses promising potentials for PDT to tumors, viruses and some special vascular diseases, such as port wine stains and age-related macular degeneration.

HA has a wide application prospect but this useful PQP is found to exist only in the stromata of *H. bambusae* and *S. bambusicola* (Wan and Chen, 1981; Kishi et al., 1991), which are of low yield in the nature. It is necessary to find a strain that can produce HA for fermentation production. In this study, an HA-producing strain SUPER-H168 was successfully isolated and identified to belong to the genus *Shiraia*.

Materials and methods

Materials

HA standard reagent used in the study was obtained from Sigma, USA, other chemicals used were of analytical grade and obtained locally. The bamboo *Brachystachyum densiflorum* was collected from Ma Mountain, Wuxi, Jiangsu Province of China.

Isolation of HA-producing fungus

Fresh plant tissues such as nodes, leaves and internodes were surface sterilized by 0.1% $HgCl_2$ for 3 min, then cut into small pieces with knives, and these pieces were immersed in 3% sodium hypochlorite for 3–5 min, then rinsed with 70% ethanol for 30 s. After that, the tissues were washed with sterile distilled water. The pieces were dried and placed on PDA medium (containing 20% potato and 2% glucose) at the temperature of 26 °C for isolation. HA is red under acidic condition, becomes green under alkaline condition and turns dark purple when $FeCl_3$ is added (Wan and Chen, 1981). So color reaction was conducted to identify HA preliminarily.

Observation of morphology and structure

Mycelia, conidia and pycnidia of the fungus were observed with a light microscope (LEICA DMLB, LEICA Microsystems AG, Germany) and a scanning electron microscope (SEM, QUANTA-200, FEI, Netherlands).

Extraction of mycelia genomic DNA

Samples used for DNA extraction were collected from the fermentation broth culture in the shake flask, with the medium composition: glucose 20 g/L, potato extract 20% (w/v) at pH 6.0. Strain SUPER-H168 was inoculated in a 250 mL Erlenmeyer flask containing 50 mL of the medium, and cultivated at 26 °C on a reciprocal shaker at 200 rpm for 48 h. The mycelia were precipitated by centrifugation at 10,000*g*, 4 °C for 10 min. Genomic DNA was isolated as described by Yli-Mattila and Hyvönen (1996) and Paavanen-Huhtala et al. (1999). The integrity of the obtained genomic DNA was detected by electrophoresis in 1% agarose gel stained with ethidium bromide.

PCR amplification and sequencing

The 18S rDNA was amplified using the universal primer pairs of NS1 (5′-GTAGT CATAT GCTTG TCTC-3′)/NS8 (5′-TCCGC AGGTT CACCT ACGGA-3′) (White et al., 1990). The reaction mixture for PCR amplification was prepared in a total volume of 50 μL with 1 × PCR buffer (Mg^{2+} free), 2.5 m mol/L $MgCl_2$, 200 μ mol/L of each deoxynucleotide triphosphate, 2.5 U *Taq* DNA polymerase (Shanghai Shengon, China) and 200 n mol/L of each NS1/NS8 primer. The amplifications were performed in a ThermoHybaid PCR Sprint Thermal Cycler (Thermo Electron, USA). The PCR reaction details were as follows: 4 min at 94 °C for initial denaturation, 45 s at 94 °C for denaturation, 1 min at 50 °C for annealing of 18S rDNA, 1 min 45 s at 72 °C for extension with total 35 cycles of amplification and 10 min of final extension. The 18S rDNA (EU267792) were purified by UltraClean PCR Clean-up kit (Mo Bio, USA) and sequenced by Shanghai Shengon, China.