Isolation, screening and confirmative identification of high Hypocrellin A-Producing *Shiraia bambusicola* isolates

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ABSTRACT: The main objective of this study was to obtain high hypocrellin A (HA)-producing isolates of fungus *Shiraia bambusicola*. Fungal cultures were obtained from stromata of presumably *S. bambusicola*, found on Pleioblastus sp., *Brachystachyum* sp. and *Phyllostachys* sp. bamboos by isolation of single spore, multispores and stroma tissue. Appropriate HA sampling time and screening procedure were elucidated by selecting representative isolates to determine their mycelia biomass and HA content. Both morphological characteristics and ITS sequences were analyzed for confirmative identification of the isolates. Altogether 32 isolates of the fungus were obtained from Anhui and Zhejiang province of southern China in this study. All isolates were found to produce HA but its content in the single spore isolates were much lower than that of the isolates from multispore and stroma tissue during the sampling time at the 11th to 13th days of fermentation. The GZAAS2.0629 isolate was screened out as the highest HA yielding. It was confirmatively identified as *S. bambusicola* based on morphological and ITS sequence analysis.
Key words: fungal isolation, fungal identification, hypocrellin A, *Shiraia bambusicola*

Introduction

Hypocrellin A (HA) is one of naturally occurring perylenequinonoid pigments with excellent photosensitive property and has promising application in the photodynamic therapy (PDT) for anticancer treatment (Zhang et al., 1998; Yang et al., 2001; Deininger et al., 2002). HA is functional to kill tumour cells (Dong et al., 1987; Fu et al., 1988; Fu et al., 1989; Diwu, 1995; Fei et al., 2006; Yang and Huang, 2007), inhibit viruses and cure diabetic retinopathy (Chen et al., 2005b; Tong et al., 2004), and has been successfully employed in the clinical PDT treatment of certain skin diseases, such as white lesion of vulva, keloid, vitiligo, psoriasis, tinea capitis and lichen amyloidosis (Xu, 1982; Liang et al., 1982; Wang and Bao, 1985; Fu and Chu, 1989). With the presence of visible light and oxygen, hypocrellins has a striking antiviral activity against human immunodeficiency virus type 1 (HIV-1) (Hudson et al., 1994).

HA was originally isolated from stromata of *Shiraia bambusicola* and *Hypcrella bambusae*. *S. bambusicola* has been reported as parasitic fungus on branches of several genera of bamboo and found in part of south regions of China and Japan (Kishi et al.. 1991), The genus *Shiraia* was first established by Hennings (1900) and placed in family *Nectriaceae* of Ascomycetes, but Saccardo (1902) categorized it into the family Hypocreace based on its large fleshy ascocarp. Description of earlier mycologists (Hennings 1900; Teng,

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1934; Yamamoto and Okada, 1966) on asci of the fungus was unitunicate, but Amano (1980) found that the genus having bitunicate asci rather than unitunicate and suggested placement of the genus in the family *Pleosporaceae (Pleosporales)*. Kirk et al. (2001) placed the fungus as genus *Shiraia* with family *incertae sedis* in Dothideales (Dothideomycetes). Based on 18S rDNA and ITS-5.8S rDNA sequences analysis, Cheng et al. (2004) classified the genus into *Pleosporales* as Amano (1980) proposed but suggested it in the family *Phaeosphaeriaceae*. So far *S. bambusicola* is the only species in the genus. With the narrow distribution, wild sources of *S. bambusicola* are gradually decreasing. Moreover, *H. bambusae* has even narrower distribution existing only in the northwestern region of Yunnan province in China (Wan et al., 1981; Wu et al., 1989). Natural source of HA is far not enough to meet its extensively potential applications.

Fungi species in genus *Shiraia* are the important source of HA produced by fermentation. In order to find a way to produce HA industrially, some fungal isolates have been obtained from stromata or bamboo tissues to find hypocrellin-producing strains (Zhang et al., 2002; Li et al., 2003; Chen et al., 2005a; Chen et al., 2006; Liang et al., 2009). Among those reported HA-produce isolates, species concept of *Shiraia* was not elucidated clearly and fermentation times were different. Hence, isolation of *S. bambusicola* and establishment of screening procedure on high HA-producing isolate are of importance for industrial HA production.

In this report we show the process of obtaining the fungus isolates, establishment of screening process for high HA-producing isolates, and confirmative identification of the selected isolates by morphological and ITS sequence analysis.

Materials and Methods

Isolation

Stromata of presumably *S. bambusicola* were collected from 3 species of host bamboos, *Pleioblastus* sp., *Brachystachyum* sp. and *Phyllostachys* sp. in Anhui and Zhejiang province of China. The fresh stromata were surface sterilized with 75% ethanol for 30 sec to 1 min, then washed with sterilized water and dried with sterilized filter paper. Subsequently, the stroma was cut to pick conidia or stromal tissue and transferred directly onto PDA plate for multispore or stromal tissue isolation. Single spore isolation was also performed using the capillary method as described by Liu et al. (2008). All the culture plates were incubated at 26°C and stored on PDA slants at 4°C.

Selection of representative isolates and HA sampling time

PDA cultures of the isolates were cut with 4 mm cork-borer, then transferred onto new PDA plates and incubated at 26°C. Growth rate of all isolates were determined everyday starting 4 days after the inoculation. Based on statistics of the growth rate, the isolates were divided into groups of fast and slow growing. A representative isolate was randomly selected out from each group to check mycelia biomass and HA content everyday after fermentation for 4 days in BPD broth (containing 0.5% bamboo powder, 20% potato and 2% dextrose) shaking at 120 rpm at 26°C. The fermented mycelia were collected and washed with distilled water then dried at 60°C to constant weight. Dry mycelia was made into powder and extracted in acetone. Absorbance of the extracted solution was detected by using spectrophotometer at 465 nm. Then HA contents was calculated based on regression equation y= 3 .2x +0.02 (Shi et al., 2004). Three HA sampling times were selected at one day

before, one day after and at the peak of HA production day of each representative isolate.

Screening of HA producing isolate

All isolates were cultured on BPD agar plates (10 ml/petri dish) at 26°C for 7 days to obtain uniform colonies. Inocula were prepared by using a 4 mm cork-borer to cut the colonies into same size pieces. The pieces of each isolate were inoculated into BPD broth and cultured on a rotary shaker at 120 rpm and kept at 26°C. The HA content of each isolate during the sampling times were determined by the same method as described previously.

Confirmative identification of the fungal isolate

Morphological description

Stomata collected above were observed using microscopy to describe relevant characteristics of taxonomy.

Genomic DNA extraction, PCR amplification and sequencing

The screened isolate and 4 single spore isolates from different host bamboos were selected as testing isolates. Total genomic DNA was extracted from actively growing mycelium scraped out from BPD agar plates. The ITS region was amplified using the universal primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR amplifications were performed with a thermal cycler (MyclerTM, Bio-Rad, Hercules, CA, USA) with following details: 2 min at 95°C for initial denaturation; 40 s at 95°C for denaturation, 30 s at 53°C for annealing, 30 s at 72°C for extension (for a total of 30 cycles of amplification), and final extension for 7 min at 72°C. PCR products were purified by using TIANGEL Mini Purification kit (TIANGEN Co. China). Purified samples were sequenced by SinoGenoMax Co., China.

Sequence alignment and analysis

BLAST (http://www.ncbi.nlm.nih.gov/BLAST) searches were conducted with sequence of the testing isolates to find homologous sequences. Some relevant sequence was downloaded in drawing the trees from GeneBank. Multiple sequence alignments were generated using Clustal W 2.0.10 (Larkin et al., 2007). ITS sequences were analyzed using maximum parsimony (MP) by PAUP* version 4.0 (Swofford, 2002). Trees were inferred using the heuristic search option with tree bisection-reconnection (TBR) branch swapping and 1000 random sequence additions. Clade stability of the trees resulting from the parsimony analyses were assessed by bootstrap analysis with 1000 replicates, each with 10 replicates of random stemwise addition of taxa. Trees were figured by Treeview.

Results and Discussion

Isolation

Thirty two isolates were obtained from the isolation including 10 single spore isolates, 11 stromatal tissue isolates and 11 multispore isolates (**Table 1**).

Selection of representative isolates and HA sampling time

All isolates were divided into two groups based on their growth rate (**Table 2**). Isolates GZAAS2.0703 and GZAAS2.0707 were selected as representatives of group A (1 mm/day mean growth rate) and group B (2 mm/day mean growth rate) respectively. Growth curve of the representative isolates (**Figure 1**) showed that the highest HA yields were found at the 12th day in both isolates. The biomass of mycelia gradually increased from the 5th day to the 13th day then kept in a steady stage. Therefore, the 11th to 13th day were selected as sampling time to screen for high HA-producing isolates.

Table 1 Isolation result of the bamboo fungus, presumably *Shiraia bambusicola* in China.

Isolate No. (GZAAS2. plus series number)	Host	Collection site	Source
0701	*Pleioblastus* sp.	Anhui province	Single spore
0702, 0703	*Phyllostachys* sp.	Linan, Zhejiang province	Single spore
0704, 0705, 0706, 0707, 0708	*Brachystachyum* sp.	Linan, Zhejiang province	Single spore
0709, 0710	*Pleioblastus* sp.	Linan, Zhejiang province	Single spore
0711	*Phyllostachys* sp.	Linan, Zhejiang province	Stromatal tissue0612
Pleioblastus sp.	Panan, Zhejiang province	Stromatal tissue	
0713	*Pleioblastus* sp.	Anhui province	Stromatal tissue
0714, 0715	*Phyllostachys* sp.	Linan, Zhejiang province	Stromatal tissue
0716, 0717	*Brachystachyum* sp.	Linan, Zhejiang province	Stromatal tissue
0718, 0719	*Pleioblastus* sp.	Linan, Zhejiang province	Stromatal tissue
0620, 0621	*Pleioblastus* sp.	Panan, Zhejiang province	Stromatal tissue
0722	*Pleioblastus* sp.	Anhui province	Multispore
0723, 0724	*Pleioblastus* sp.	Linan, Zhejiang province	Multispore
0725, 0726	*Phyllostachys* sp.	Linan, Zhejiang province	Multispore
0727, 0728	*Brachystachyum* sp.	Linan, Zhejiang province	Multispore
0629, 0630, 0631, 0632	*Pleioblastus* sp.	Panan, Zhejiang province	Multispore

Table 2 The presumably Shiraia bambusicola groups based on mean growth rate on PDA plate at 26°C.

Group	Mean growth rate (mm/day)	Isolate No.[1/]
A	1	02, 03, 12, 13, 15, 20, 21, 22, 29, 30, 31
B	2	01, 04, 05, 06, 07, 08, 09, 10, 11, 14, 16, 17, 18, 19, 23, 24, 25, 26, 27, 28, 32

[1/] Last two letters of the isolate number in Table 1.

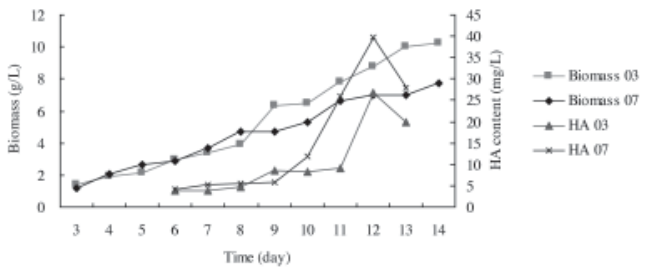

Figure 1 Biomass and HA producing curve of 2 representatives, presumably *Shiraia bambusicola* isolates, cultured in BPD broth and shaking at 120 rpm at 26°C.

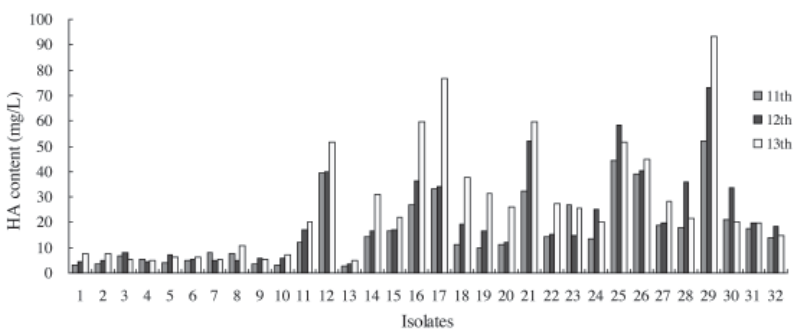

Figure 2 HA content during fermentation at the 11th to 13th day of presumably *Shiraia bambusicola* isolates, cultured in BPD broth and shaking at 120 rpm at 26°C.

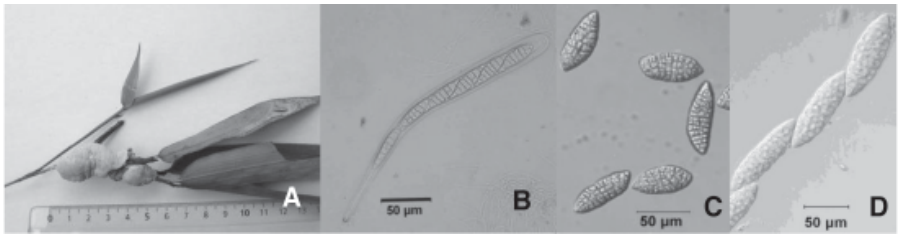

Figure 3 Morphology of the bamboo fungus, presumably *Shiraia bambusicola* used in the study. (A) Stroma; (B) Bitunicate ascus; (C) Conidia; (D) Ascospores.

Screening of HA producing isolates

All isolates in this study could produce HA during liquid fermentation (**Figure 2**). Most isolates produced the highest HA content at the 13th day. Among all the testing isolates, GZAAS2.0629 was found to produce the highest HA at the three sampling days. The single spore isolates, no. 1 to 10, showed uniformly lower HA contents. Thus the GZAAS2.0629 was screened out as a promising isolate in HA production and further identified.

Confirmative identification of *S. bambusicola*

As shown in **Figure 3**, the fungus used in this study formed pinkish, irregular tuberculate stroma covering the host bamboo branches. The sizes of stroma are varied depending on the host bamboo species. Bitunicate asci containing 6 ascospores were found in the stroma. Both conidia and ascospores were dictyospores with transverse and longitudinal septa. The conidia were 53~79 x 26~31 µm and the ascospores were 55~80 x 14~25 µm in size. These characteristics correspond with the descriptions of *S. bambusicola* as given by Cheng et al. (2004).

The cultures obtained in this study were difficult to identify by colony characteristics because of their similarity. The mycelia were initially white on PDA but became grey after 10 days. The back of colonies was black if looked from the underside. Sporulation was not found in in vitro cultures in all isolates. Thus identity of the isolates has to be confirmed by comparing their ITS sequences with that of the reference *S. bambusicola* in the Genebank (**Table 3**). The phylogenetic analysis of the fungal isolates under the study was as shown in **Figure 4.** It can be seen that GZAAS2.0629 and the 4 single spore isolates had very high sequence similarity with that of the reported isolates (AB105798, AY536372, AY536374, AY536373 and AY515304) of *S. bambusicola.* From the result it can be concluded that all these isolates are *S. bambusicola.* There are no inter-host differences among GZAAS2.0629 and the four single spore isolates from different species of bamboo, which is in accordant with the result of Cheng et al. (2004).

It is interesting to note that no sporulation was found when all the isolates were cultured under the laboratory conditions. This finding is the main distinction comparing with that of some reported species of *Shiraia* (Zhang et al., 2002; Chen et al., 2005b; Chen et al., 2006; Liang et al., 2009). So far *S. bambusicola* is the only species in the genus *Shiraia*, but some reported endophytes, *Shiraia*-like fungi, from bamboo closely related to *S. bambusicola* (Morakotkarn et al., 2008). Phylogenetic analysis in this study also found that isolates from stromata and bamboo tissues were divided into different group (**Figure 4**). Although no stroma of *S. bambusicola* were obtained from our anamorphic cultures, molecular analysis indicates that the genus *Shiraia* might contain more than one species.

Table 3 List and details of the data used in ITS sequences analysis from GeneBank of presumably Shiraia bambusicola isolates.

Taxa	Strain	Accession number in GenBank	Host/source	Country
S. bambusicola	JCM 1879	AB105798	Bamboo	Japan
S. bambusicola	TFC Pn2003	AY536372	Bamboo/Stroma (Cheng et al., 2004)	China
S. bambusicola	TFC Bd2003	AY536374	Bamboo/Stroma (Cheng et al., 2004)	China
S. bambusicola	TFC Pa2003	AY536373	Bamboo/Stroma (Cheng et al., 2004)	China
S. bambusicola	TFC Ppp2003	AY515304	Bamboo/Stroma (Cheng et al., 2004)	China
Shiraia sp.	SUPER-H168	EU267793	Bamboo (Liang et al., 2009)	China
Shiraia sp.	A8	FJ560594	*Artemisia annua*	China
Shiraia sp.	JP232	AB255303	Bamboo (Morakotkarn, 2007)	Japan
Shiraia sp.	ML-2004	AY425966	Bamboo	China
Shiraia sp.	BFM-L31	AB369481	*Triticum aestivum*	China
Leptosphaeria dryadis	CBS 743	AF 439461	Dryas octopetala	Switzerland

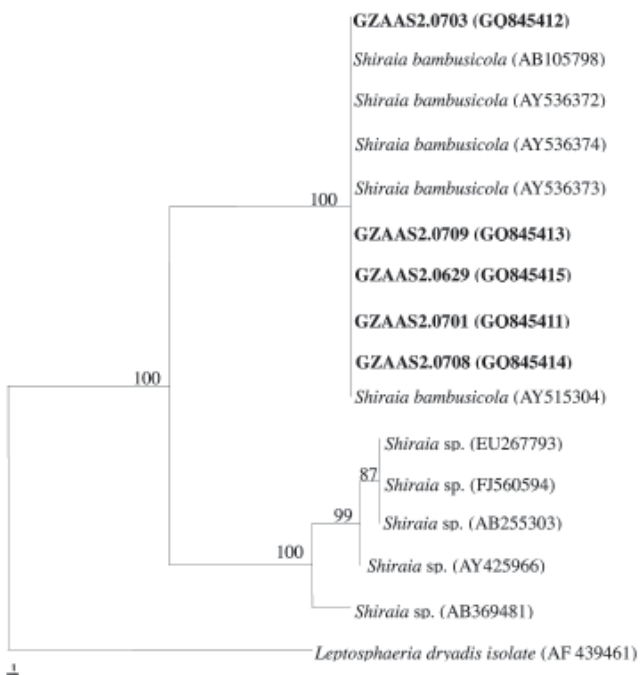

Figure 4 Maximum parsimony phylograms of the isolate GZAAS2.0629 inferred from I*TS1-5.8S-ITS2* showing phylogenetic relationships among isolates from Bamboo. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap (equal or above 50%). The tree is rooted with *Leptosphaeria dryadis*. The scale bar indicates the number of steps.

Conclusions

Thirty two isolates of bamboo fungus, presumably *Shiraia bambusicola* were isolated from 3 genera of bamboos from southern part of China. Most of the isolate produced hypocrellin A (HA) at 12th day of liquid fermentation. Among them GZAAS2.0629 yielded the highest HA content. The isolate was confirmatively identified as *S. bambusicola* based on its morphological and ITS sequence analysis.

Acknowledgement

This work was financially supported by Guizhou Academy of Agricultural Sciences in China (No. QianNongKeHe-Rencai 08005). This paper represents a portion of the dissertation submitted by Yongxiang Liu to Suranaree University of Technology in Thailand for requirement of a Ph D. degree.

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