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Full Length Research Paper

Chemical composition of *Hirsutella beakdumountainsis*, a potential substitute for *Cordyceps sinensis*

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A strain of Hirsutella beakdumountainsis parasitized on Antheraea pernyi pupa was isolated from the fruiting body. The objective of this work was to optimize the artificial cultivation condition for the fruiting bodies of H. beakdumountainsis and compare the compositions of natural fruiting bodies of Cordyceps sinensis (NFCS), mycelia from shake culture (SCHS) and the fruiting bodies from artificial cultivation (ACHS) of *H.* beakdumountainsis. The crude protein of NFCS accounted for 22.97%; obviously lower than those of SCHS (27.99%) and ACHS (31.89%). The contents of total and essential amino acids were in the following descending order: SCHS>ACHS>NFCS, respectively. The total content of five nucleosides in SCHS (16.82 mg/g) was significantly higher than those of NFCS (5.08 mg/g) and ACHS (4.45 mg/g). The content of D-mannitol in NFCS, SCHS and ACHS was 8.9, 11.5 and 10.2%, respectively. The above results suggest that *H.* beakdumountainsis can probably be used as a substitute for *C.* sinensis.

Key words: Hirsutella beakdumountainsis, Cordyceps sinensis, protein, amino acid, nucleoside, D-mannitol.

INTRODUCTION

Cordyceps sinensis (Bark) Sacc. Link (Claviceptaceae) (Ascomycetes), one of the well-known traditional Chinese medicines, is an insect parasitizing fungus. This fungus lives primarily on the head of the larva of one particular species of moth, Hepialus armoricanus, but is also occasionally found growing on other moth species (Zhu et al., 1998). It is commonly used in China to replenish the kidney and soothe the lung for the treatment of fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthemia after severe illness, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart disease, and liver disease (Pharmacopoeia of the People's Republic of China,2005). Modern pharmacological studies revealed that it has a broad medicinal effect, and its function of immunity regulation plays an important role in antitumor effects and organ transplantation (Ma et al., 2001; Shi, 2005). Furthermore, modern experimental methods in bio-chemistry have proved that *C. sinensis* consists of active constituents such as polysaccharides, mannitol, nucleosides, ergosterol, amino acids and trace elements (Cai et al., 2003; Kiho et al, 1999; Zhi et al., 1991).

However, its usage has been limited during the past decades due to the high price and the difficulty of its supply. Therefore, the isolation of anamorph strain from *C. sinensis* is a trend of many scientists to achieve a large-scale production of mycelia by fermentation. Nevertheless, the major problem here is that there are 22 hyphomyces from 13 genera that are associated with the anamorph of *C. sinensis*. A correct anamorphic isolate of it is thus of highest priority. On the basis of morphological and molecular evidence, *Hirsutella sinensis* (Liu et al., 2004) is currently considered as the correct anamorph of *C. sinensis* (Jiang and Yao, 2002). Unfortunately, fewer studies have been done on the fermentation by *H.* sinensis. In addition, the production of fruiting bodies of *C.* sinensis in artificial culture has proved to be extremely

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difficult and only feasible at laboratory scale. Successful cultivation for commercial purpose has not yet been achieved (Yin and Tang, 1995).

A strain of H. beakdumountainsis parasitized on Antheraea pernyi pupa was collected in Baekdu Mountain, China. It was isolated from the fruiting body. The strain was identified by Prof. Jiang X.L. (Ocean University of China). The optimization fermentation condition for the mycelia of *H.* beakdumountainsis has already been achieved in the previous study (Rong et al., 2010). After fermentation of the strain for four days, the mycelia biomass yield reached 10.06 g/L. Hence, the objective of the present research was to optimize the artificial cultivation condition for the fruiting bodies and analyze the chemical compositions of mycelia from shake culture (SCHS) and the fruiting body of artificial cultivation (ACHS), which were compared with that of the natural fruiting bodies of *C. sinensis* (NFCS) in order to find a new substitute of C. sinensis.

MATERIALS AND METHODS

Strain and samples

The mycelia of H. beakdumountainsis were produced by shake culture. The fruiting bodies of the strain were produced by artificial cultivation. Samples before experiments, except by specific indication, were subjected to a dry oven at 60°C for 24 h. The natural fruiting bodies of *C. sinensis* (NFCS) were purchased in Tibet and identified. The materials were milled in a mortar and dried prior to analysis.

Shake culture method

The stock culture was maintained on potato dextrose agar (PDA) slants, which were incubated at 24°C for 15 day and then stored at 4°C. Each 250 ml flask with 100 ml of liquid special media was inoculated with mycelia mat (ca. 10 cm^2) from a plate culture and incubated on a shaker at 180 rpm for seven days at 2°C. About 10 ml of the seeding culture was transferred to a 500 ml flask with 200 ml incubated on a shaker at 180 rpm for four days at 24°C. The culture medium contained sucrose (2.5%), yeast extract (0.5%), K_2HPO_4 (0.2%), and $MgSO_4$ (0.05%). The initial pH was adjusted to 5.5 before sterilization. The mycelia were collected by filtering mycelia through filter paper (Whatman No.1) and drying to a constant weight at 70°C overnight. All samples were stored at 4°C.

Artificial cultivation method

The process of producing *H. beakdumountainsis* fruiting bodies was the same as for other cultivated edible mushrooms. The method used was the cooked rice in bottle procedure, which was most widely adopted for commercial production (Zhang, 2005). The process can be divided into two major stages. The first step was preparing the seeding culture. The inoculation seeds were supported by the process of shake culture. Then, we prepared the rice medium as the second step. The rice medium included 30 g rice, the powder of *A. pernyi* pupa and 50 ml nutrient solution, which was composed of peptone (0.5%), sucrose (2%), K_2HPO_4 (0.15%), $MgSO_4$ (0.05%) and vitamin B1 (0.001%). The entire medium was added to the bottles, sterilized at 120°C for 2 h, and cooled to room temperature. After inoculation of 10 ml of the seeding culture, the rice was incubated at 25°C for approximately one week until mycelia appeared at the surface of the rice. Then the bottles were arranged under light (500xl) and the fruiting bodies maturated for about 45 days.

Analytical methods

Protein analysis

Protein was analyzed according to the Micro-Kjeldahl method (Micro-Kjeldahl apparatus 1030, Tecator Company, Sweden). Protein content was calculated using nitrogen factor (6.25).

Amino acid analysis

Amino acids were determined using an automatic amino acid analyzer (Beckman 6300, Beckman Instrument, Fullerton, CA) according to the method described by Moore and Stein (1963) and Danell and Eaker (1992). Hydrolysis of the samples was performed in the presence of 5.5 mol/L HCl at 120°C for 24 h under a nitrogen atmosphere. The hydrolysate was evaporated and the residue was redissolved in 1 ml 0.02 mol/L HCl. The sample was filtered through a 0.45 µm filter membrane prior to analysis.

Nucleoside analysis

Adenosine, guanosine, uridnine, cytidine and inosine used as reference substances, were purchased from Sigma. Accurate amounts of nucleosides were dissolved in mobile phase to give various concentrations for calibration. Samples were extracted by ultra-sonication with 70% aqueous EtOH for 30 min and filtered through a 0.45 µm filter membrane prior to analysis. Analysis was performed using high performance liquid chromatography (HPLC) Agilent 1100 with diode-array detection. A pre-packed RP column Waters Spherisorb ODS (4.6 × 250mm i.d., 5 µm) was used. Solvents that constituted the mobile phase were Water (A) and methanol (B). The elution conditions applied were: 0 to 15 min, linear gradient 5 to 20% B; 15 to 25 min, linear gradient 20 to 80% B; 25 to 30 min, linear gradient 80 to 5% B. The flow rate was 1 ml/min, and the injection volume was 10 µl. The analytes were monitored at 254 nm.

D-Mannitol analysis

The content of mannitol in each sample was determined using colorimetric method (Li et al., 1999). In brief, for the contents of mannitol, 1 ml of the solution containing 0.4 mg of extract and 1 ml of 0.015 mol/L periodate potassium were mixed. After 10 min, 2 ml of 5.5 mmol/L rhamnose and 4 ml of fresh Nash reagent (1000 ml of 2 mol/l ammonium acetate mixed with 2ml of acetic acid and 2 ml of acetyl acetone) were added to the mixture which was placed in a water bath at 53°C for 15 min. The absorbance was measured at a wavelength of 413 nm on a APL-752 spectrophotometer (Shanghai, China). A blank test was prepared by substituting distilled water for the extract solution. A standard curve was prepared using a mannitol standard. 1 ml of solution containing up to 50 mg/ml of mannitol was determined by the above method and the mannitol content of samples was calculated by the linear regression equation from the standard curve.

Statistical analysis

The data was statistically analyzed according to Minitab 15.0 for

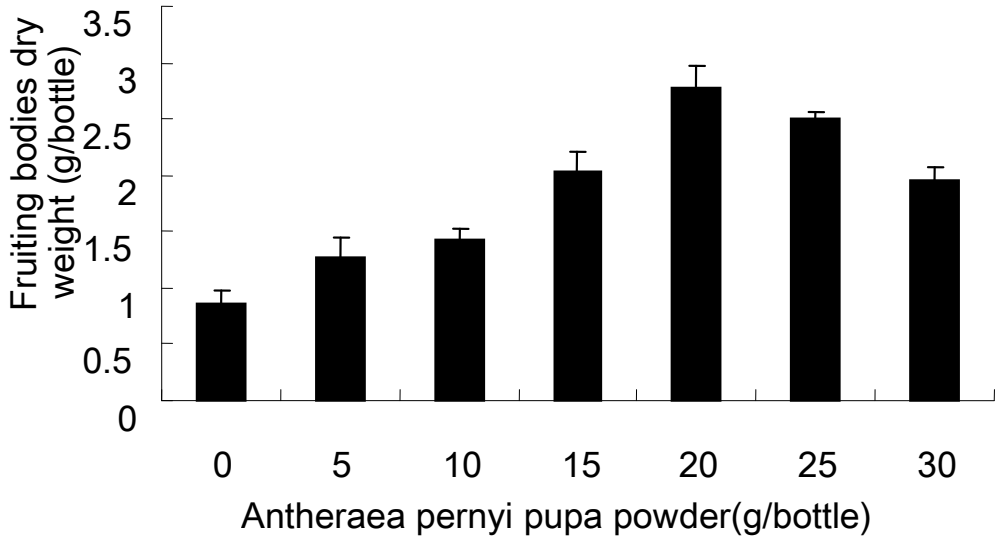

Figure 1. Effect of *A pernyi* pupa powder on the fruiting bodies growth. All points represent the mean ± SD of triplicates.

windows (Minitab Inc., USA). Significant differences between any two means were determined at the 0.05 level.

RESULTS AND DISCUSSION

Optimization of artificial cultivation condition

It was reported that the content of *A. pernyi* pupa powder was the important factor for fruiting bodies production in *C.* militaris (Wang and Yang, 2006). As illustrated in Figure 1, the maximum yield of fruiting bodies was 2.78 g/bottle, when we added 20 g *A. pernyi* pupa powder in the medium. Nevertheless, the yield of fruiting bodies decreased to 1.96 g/bottle, when the medium included 30 g *A. pernyi* pupa powder. After 45 days cultured on the optimized condition, the fruiting bodies were matured, which were the same as the natural ones from the original sample in the morphology (Figures 2 and 3).

The composition analysis

Crude protein

Crude protein contents in NFCS were significantly different from those of SCHS and ACHS. They were in the following descending order: ACHS> SCHS>NFCS. Crude protein contents of ACHS and SCHS were 31.89 and 27.99%, respectively, higher than those of NFCS (22.97%). Previous papers reported that the content of crude protein in *C. sinensis* was in the range of 20.06 to 33.00% (Hsu et al., 2002; Ji et al., 1999). The protein content is mainly due to different geographical origins.

Amino acids

Over 20% of amino acids can be found in Cordyceps, which should be responsible for the tonic and immuno-potentiating activity of Cordyceps (Li et al., 2006). Amino acid content and composition in NFCS, ACHS and SCHS are presented in Table 1. There are statistically significant differences in the contents of total amino acids between NFCS and SCHS. The contents of the total and essential amino acids in SCHS were 28.18 g/100 g and 7.50 g/100 g, respectively; highest among the three samples. As illustrated in the Figure 4, the compounds percentage of the total amino acids was similar in NFCS, SCHS and ACHS. The four principal amino acids were glutamic acid, arginine, aspartic acid and valine. However, their levels were different in NFCS, SCHS and ACHS as showed in Table 1; glutamic acid: 1.39, 5.85, 5.23(g/100 g); arginine: 1.35, 4.35, 2.00(g/100 g), aspartic acid: 1.43, 2.40, 2.13(g/100 g); valine: 1.05, 2.19, 1.66(g/100 g), respectively. Glutamine acid is the most abundant compound in ACHS and SCHS, representing 24 and 21% of total compounds (Figure 4). In addition, the level of total amino acids in the NFCS was about 12.99(g/100 g) in the study; lower than 16.4 to 18.1(g/100 g) as reported (Hsu et al., 2002). It is found that the content of amino acids in *C. sinensis* after hydrolysis is mostly 20 to 25%; the lowest being 5.53%, and the highest being 39.22% (Ji et al., 1999).

Nucleosides

Nucleosides are one of the major components in *Cordyceps.* Adenosine, inosine or cordycepin are used as

Figure 2. The matured fruiting bodies after artificial cultivation for 45 days.

Figure 3. The original sample collected in Baekdu Mountain, China.

Table 1. Amino acid composition of NFCS, SCHS and ACHS (g/100 g).

Amino acid	NFCS	SCHS	ACHS
Aspartic acid	1.43[b]	2.40[a]	2.13[a]
Threonine	0.75[b]	1.42[a]	1.16[a]
Serine	0.75[b]	1.48[a]	1.43[a]
Glutamic acid	1.39[b]	5.85[a]	5.23[a]
Glycine	0.65[b]	1.50[a]	1.20[a]
Proline	0.45[b]	0.86[a]	0.70[a]
Alanine	0.81[b]	1.39[a]	1.43[a]
Valine	1.05[c]	2.19[a]	1.66[b]
Methionine	0.41[a]	0.26[b]	0.21[b]
Isoleucine	0.46[c]	0.81[a]	0.62[b]
Leucine	0.83[c]	1.28[a]	1.00[b]
Tyrosine	0.36[b]	0.59[a]	0.73[a]
Phenylalanine	0.58[a]	0.61[a]	0.54[b]
Lysine	0.94[a]	0.93[a]	0.97[a]
Histidine	0.78[b]	2.26[a]	0.80[b]
Argnine	1.35[c]	4.35[a]	2.00[b]
Total amino acid	12.99[b]	28.18[a]	21.81[a]
Total essential amino acid	5.20[c]	7.50[a]	6.16[b]

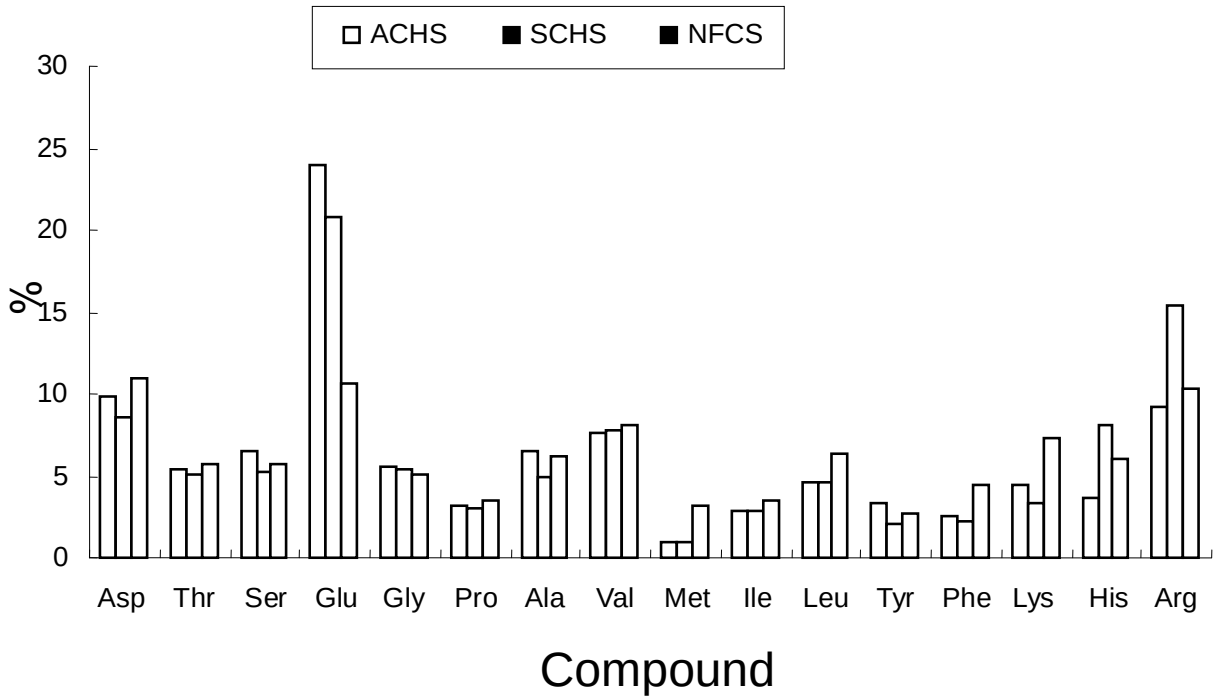

Figure 4. Amino acid profiles of NFCS, SCHS and ACHS. Asp, aspartic acid; Thr, Threonine; Ser, Serine; Glu, Glutamic acid; Gly, Glycine; Pro, Proline; Ala, Alanine; Val, Valine; Met, Methionine; Ile, Isoleucine; Leu, Leucine; Tyr, Tyrosine; Phe, Phenylalanine; Lys, Lysine; His, Histidine; Arg, Argnine.

indexing ingredients for quality control, which differentiate *Cordyceps* from different species and the counterfeit (Gong et al., 2004; Huang et al., 2003; Li et al., 2004). Indeed, nucleosides are involved in the regulation and modulation of various physiological processes in the central nervous system (CNS). Adenosine is known to

Table 2. Composition of the five nucleosides in NFCS, SCHS and ACHS (mg/g).

Nucleoside	NFCS	SCHS	ACHS
Adenosine	0.98[c]	5.78[a]	1.68[b]
Guanosine	1.63[b]	5.30[a]	0.48[c]
Uridnine	1.85[b]	4.95[a]	2.30[b]
Inosine	0.63[a]	0.45[a]	ND
Thymine	ND	0.34	ND
Total	5.08[b]	16.82[a]	4.45[b]

ND= non-detectable. Means (n=3) with different letters in the same row are significantly different ($P<0.05$).

depress the excitability of CNS neurons and to inhibit release of various neurotransmitters presynaptically (Schmidt et al., 1995). Moreover, inosine, the major biochemical metabolite of adenosine due to oxidative deamination, can stimulate axon growth *in vitro* and in the adult central nerve system (Benowitz et al., 2002). However, there is great variation of nucleoside content in different sources of *C. sinensis* and the content of nucleosides from fresh NFCS is too low to be detected (Li et al., 2001). Adenosine has been detected in most natural and cultivated *Cordyceps* species in a content range of 0 to 3.2 mg/g depending on species, geographical location (for natural species) and culture conditions (for fungal mycelia) (Li et al., 2004).

In this study, the contents of five nucleosides (adenosine, guanosine, uridnine, inosine and thymine) in the NFCS, SCHS and ACHS are shown in Table 2. The content of the total five nucleosides in the SCHS was 16.82 mg/g, which was significantly higher than those of NFCS (5.08 mg/g) and ACHS (4.45 mg/g). For SCHS, adenosine, guanosine, uridnine were the main nucleosides. The results show that uridine was the most abundant nucleoside in NFCS and ACHS. The adenosine concentration, consisting of 5.78 mg/g in SCHS, was approximately six-fold higher than that in the NFCS. Even though, the content of inosine in NFCS (0.63 mg/g) was higher than that of SCHS (0.45 mg/g), there was no statistical difference. In addition, the results indicate that the shake culture method of *H. beakdumountainsis* was better than the artificial cultivation method for producing nucleosides.

D-Mannitol

D-Mannitol, also called cordycepic acid, was isolated from *C. sinensis* in 1957. It was one of the main active medicinal components. It has been shown to have diuretic, antitussive and anti-free radical activities (Xu, 2006). It was used not only in injections as raw material, but also as a supplement in other medicines (Zhan and Song, 2003). The content of mannitol in NFCS, SCHS and ACHS was 8.9, 11.5 and 10.2%, respectively. There were no statistical differences. It was reported that the content of mannitol in *C. sinensis* was 7 to 29%, differing in the various growing stages of the fruiting bodies (Jiang, 1987).

Conclusion

The demand for natural fruiting bodies of *C. sinensis* is continuously increasing, while the wild resource is decreasing rapidly due to non- sustainable collection. It is urgent to find new substitutes of *C*. sinensis, which can be applied successfully on a large scale for further development. *H.* beakdumountainsis parasitized on *A. pernyi* pupa was isolated from the fruiting body. After fermentation of the strain for four days, the mycelia biomass yield reached 10.06 g/L. Furthermore, the yield of fruiting bodies was 2.78 g/bottle after 45 days artificial cultivation.

The composition analysis of the NFCS, SCHS and ACHS is very important to determine the application of the strain. The content of crude protein in NFCS was lower than those of ACHS and SCHS. The total and essential amino acid contents were quantified in the following descending order: SCHS>ACHS>NFCS. Each of the five nucleosides contents in SCHS was significantly higher than those of NFCS and ACHS. ACHS contains less nucleoside than NFCS, especially inosine, which cannot be detected in ACHS. The content of mannitol in NFCS, SCHS and ACHS was 8.9, 11.5 and 10.2%, respectively but there were no statistical differences. Our findings suggest that *H. beakdumountainsis* can be regarded as a potential substitute of *C. sinensis*. Moreover, the method of shake culture of the strain seemed better than that of artificial culture based on the content of the active compounds. In addition, more attention will be placed on other active constituents such as polysaccharides in further research, and the identification of the strain is still in process.

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