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แหล่งคาร์บอนและไนโตรเจนที่เหมาะสมในการเพาะถั่งเช่าหิมะเพื่อเพิ่มการผลิตสารออกฤทธิ์ทางชีวภาพ

Optimum Carbon and Nitrogen Sources for Enhancing Bioactive Compound Production of *Isaria tenuipes*

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งานวิจัยนี้เป็นการศึกษาแหล่งคาร์บอนและไนโตรเจนที่เหมาะสมต่อการสร้างดอกเห็ด และการสร้างสารสำคัญของถั่งเช่าหิมะ (*Isaria tenuipes*) โดยใช้แหล่งคาร์บอนที่ต่างกัน 5 ชนิดคือ ข้าวไรซ์เบอรี่ ข้าวสังข์หยด ข้าวหอมนิล ข้าวมันปู และข้าวเหนียวดำลืมผัว โดยแหล่งคาร์บอนที่ให้น้ำหนักแห้งของถั่งเช่าหิมะมากที่สุดคือ ข้าวไรซ์เบอรี่ (2.16 ± 0.11 ก./100 ก. ของสารตั้งต้น) ซึ่งสูงกว่าแหล่งคาร์บอนอื่น ๆ อย่างมีนัยสำคัญ แต่อย่างไรก็ตามพบว่าปริมาณของคอร์ไดเซปิน อะดีโนซีน และพอลิแซ็กคาไรด์ที่ได้จากข้าวเหนียวดำลืมผัวเป็นแหล่งคาร์บอนมีค่าสูงที่สุดคือ 1.03 ± 0.04, 7.5 ± 0.04 และ 0.23 ± 0.01 มล./ก. ตามลำดับ และเมื่อใช้แหล่งไนโตรเจนที่แตกต่างกัน 6 ชนิดคือ ยีสต์สกัด เปปโตน ไข่ไก่ กากถั่วเหลืองบด ทริปโตน และยีสต์สกัดผสมเปปโตน ผลการศึกษาพบว่าแหล่งไนโตรเจนจากไข่ไก่ให้น้ำหนักแห้งของดอกถั่งเช่าหิมะ 1.81 ± 0.03 ก./ 100 ก. ของสารตั้งต้น ปริมาณของคอร์ไดเซปิน อะดีโนซีน และพอลิแซ็กคาไรด์ให้ค่าสูงที่สุดคือ 1.39 ± 0.10, 14.86 ± 1.52 และ 0.24 ± 0.003 มล./ก. ตามลำดับ งานวิจัยนี้อาจเป็นประโยชน์โดยตรงต่อฟาร์มขนาดเล็กและระดับอุตสาหกรรม เพื่อผลิตถั่งเช่าหิมะที่ให้ผลผลิตและสารออกฤทธิ์ในปริมาณสูงสุด

คำสำคัญ : อะดีโนซีน ; คอร์ไดเซปิน ; ถั่งเช่าหิมะ

Abstract

The objective of this study was to find out the optimal carbon and nitrogen sources for enhancing bioactive compound production of *Isaria tenuipes,* Snow-flake Dongchunghacho. *Isaria tenuipes* was cultivated on five different carbon sources - Riceberry rice, Sungyod rice, Hom Nil rice, Mun-pu rice, and Luem Pua glutinous rice. The significantly highest dry weight ($p \leq 0.5$) of *Isaria tenuipes* itself was from Riceberry rice carbon source, at 2.16 ± 0.11 g/100 g of substrate. The highest yields of bioactive compounds were the following: cordycepin (1.03 ± 0.04 mg/g), adenosine (7.5 ± 0.04 mg/g) and polysaccharides (0.23 ± 0.01 mg/g) obtained from Luem Pua Glutinous rice carbon source. Riceberry rice carbon source also provided the highest yield of adenosine. In another experiment, six nitrogen sources were assessed—yeast extract, peptone, egg, ground soybean, tryptone, and yeast extract combined with peptone. The highest yields of *I. tenuipes* and the bioactive compounds were the following: dry weight of the fruiting body (1.81 ± 0.03 g/100 g of substrate), yield of cordycepin (1.39 ± 0.10 mg/g), yield of adenosine (14.86 ± 1.52 mg/g) and yield of polysaccharides (0.24 ± 0.003 mg/g), all of which were obtained from egg nitrogen source. These findings may directly benefit to small farms and industrial scales of *I. tenuipes* cultivations in their effort to harvest the highest amount of its bioactive compounds.

Keywords : adenosine ; cordycepin ; *Isaria tenuipes*

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Introduction

Isaria tenuipes is a fungus in the phylum Ascomycetes (Das *et al.,* 2010; Bunyapaiboonsri *et al.,* 2011). It is called "Snow-flake Dongchunghacho" in Korea (Che *et al.*, 2014). Its spores habituate as a parasite in the larvae of *Lepidoptera,* an order of insects that includes butterflies (Xu *et al.*, 2006). In autumn, the spores build fibers, and in summer, the fruiting body grows (Nam *et al.,* 2001).

Several important bioactive substances have been discovered in *Cordyceps*, fungal spores that grow in the body of *Lepidoptera* larvae. These are such as polysaccharides (Lu *et al.,* 2007), adenosine, and cordycepin or 3'-deoxyadenosine (Huang *et al.,* 2009). They help restore health, promote immune system (Chen *et al.,* 2008), possess anti-aging activities (Zhang *et al.,* 2019), reduce blood sugar (Park *et al.,* 2011), reduce blood fatty acid level in rats (Koh & Choi, 2003), inhibit cancer cell growth of various types such as leukemia (Park *et al.,* 2000; Shin *et al.,* 2003; Zhu *et al.,* 2016), and can be used as treatment for thrombosis, obesity, and diabetes (Kim *et al.,* 2011; Deng *et al.,* 2020).

Recently, there have been studied on the cultivation of *Cordyceps* by culturing them in silkworm pupae (Chaichana *et al.,* 2019). Those studies were supposed to guide commercial production. However, silkworms are expensive, and dry weight of fruiting body is low, so they are not suitable for commercial production. At the same time, many researchers have assessed agricultural products—such as barley, soybeans, and rice—as media for cultivation of *I. tenuipes*. Rice, especially, contains many nutrients for fungal growth. In this work, we studied rice, as the main carbon source for cultivation of *I. tenuipes*. It is cheap and readily available in Asian countries. We compared the growth rates and yields of *I. tenuipes*. We also determined the yields of its bioactive substances, i.e., cordycepin, adenosine, and polysaccharides. In addition, we investigated the optimal nitrogen sources for *I. tenuipes* cultivation.

Methods

<u>2.1 Preparation of inoculums of I. tenuipes</u>

Inoculum of *I. tenuipes* mycelium was prepared on a PDA medium in a 250 mL flask. The inoculums were cultured with 105 mL of supplementary PDB containing 200 g of potato, 50 g of baby corns, 20 g of glucose, 5 g of peptone, 5 g of yeast extract, and 30 g of ground silkworm pupae per 1 L distilled water. The PDB medium was autoclaved at 121 °C for 15 minutes. Then, four mycelial plugs were added into sterilized PDB medium, and incubated for 106 hours while being shaken at a speed of 178.9 rpm, in a dark room at 25°C.

<u>2.2 Carbon and Nitrogen Sources for Culturing I. tenuipes</u>

2.2.1 Optimization of carbon sources for the growth of I. tenuipes and the production of its bioactive substances

Five carbon sources—Riceberry rice, Sungyod rice, Hom Nil rice, Mun-pu rice, and Luem Pua glutinous rice—were investigated in this study. Forty grams of each kind of rice were placed in a glass bottle (7 x 14 cm), and 60 mL of PDB culture medium were added to the bottle. Next, the bottle was sterilized in an autoclave. The sterilized PDB medium with a tested carbon source in the bottle was added with 5 mL of the prepared inoculum of *I. tenuipes* described in

Section 2.1. After that, the bottle was left standing under controlled conditions: 25 °C temperature, 85-90 % humidity, and dark environment for 20 days then fluorescent light (300-400 lux) for 25 days to stimulate the growth of the fruiting body.

2.2.2 Optimization of nitrogen sources for the growth of I. tenuipes and the production of its bioactive substances

After the optimal carbon source was obtained, it was used in all nitrogen source experiments. The nitrogen sources, i.e., yeast extract, peptone, eggs, ground soybean, tryptone, and yeast extract combined with peptone were investigated. Each nitrogen source was added to a PDB culture medium: PDB was added to 40 g of rice in a bottle, and the bottle was sterilized in an autoclave. Five milliliters of *I. tenuipes* inoculum was added to each bottle, and the inoculum was cultured under controlled conditions: 25 °C temperature, 85-90 % humidity, and dark environment for 20 days then fluorescent light (300-400 lux) for 25 days to stimulate the growth of fruiting body.

2.2.3 Extraction and analysis of bioactive compounds and analysis of the growth of I. tenuipes

Cordycepin and adenosine were extracted from fruiting body with ultra-pure water at a ratio 0.1:1 (mass per volume), following the extraction method reported by Wang *et al.* (2015). The amounts of cordycepin and adenosine in the extracted mixture were determined following an analysis method also reported by the same team of researchers. Namely, the method was a high-performance liquid chromatography (HPLC) assay. The HPLC setup was composed of a C18 column (4.6 mL x 250 mL, particle size 5 μm), at a column temperature of 40 °C, and a mobile phase of water: methanol at 85:15 V/V, flowing at 1 mL per minute. The sample volume was 1 μL. The absorbance of each fraction was measured at 260 nm. On the other hand, polysaccharides were extracted from samples with hot water (1 mg/20 mL) for 2 hours. Then, the supernatant was separated and precipitated with 95% ethanol (supernatant: ethanol was 1:4) at 4 °C for 24 hours, then the sediment was separated by centrifugation (10000×g, 20 minutes) (Zhu *et al.,* 2016). Nitrogen gas was used to blow away liquid from the sediment. Then, the sediment was incubated in a hot air oven at 60 °C for 30 minutes and weighed before an analysis. The total sugar analysis method was a widely used phenol-sulfuric acid method (Dubois *et al.,* 1956), while the reducing sugar was analyzed by a Dinitrosalicylic acid (DNS) method (Miller, 1959). The total sugar content minus reduced sugar content is the polysaccharide content (Zhu *et al.,* 2016).

To find out the yield of the fruiting body, samples of fruiting body were collected at 25 days after incubation. They were dried in a vacuum chamber at 40 °C for 24 hours, then the dried mass was weighed, and the dry weight was used to calculate the yield.

2.2.4 Statistical analysis

Significant differences at $p<0.05$ were analyzed with one-way ANOVA, and differences of the means were checked with Duncan's Multiple Range Test (DMRT) at $p<0.05$, using SPSS version 20.

Results

3.1 The optimal carbon sources for mycelial growth and bioactive compound production

Among the five tested carbon sources—Riceberry rice, Sungyod rice, Hom Nil rice, Mun-pu rice, and Luem Pua glutinous rice—Riceberry rice provided the highest yield of fruiting body (2.16 ± 0.11 g/100 g of substrate), significantly higher than the yields of all other sources (*p*<0.05). On the other hand, Luem Pua glutinous rice provided the highest yields of cordycepin (1.03 ± 0.04 mg/g), adenosine (7.54 ± 0.40 mg/g), and polysaccharides (0.23 ± 0.009 mg/g), significantly higher than the yields from all other sources (*p*<0.05). See Table 1 for all measurements based on dry mass weight.

Table 1 Effects of carbon sources on the growth and bioactive compound production of *I. tenuipes*

Carbon source	Fruiting body (g dry weight/100 g of substrate)	Cordycepin (mg/ g)	Adenosine (mg/ g)	Polysaccharides (mg/ g)
Riceberry rice	2.16±0.11^{a}	0.87±0.01^{b}	7.52±0.19^{a}	0.13±0.007^{b}
Sungyod rice	1.94±0.05^{b}	0.71±0.04^{d}	6.18±0.62^{b}	0.14±0.006^{b}
Hom Nil rice	1.92±0.08^{b}	0.75±0.04$^{c d}$	6.42±0.28^{b}	0.14±0.014^{b}
Mun-pu rice	1.38±0.05^{d}	0.79±0.04^{c}	6.47±0.46^{b}	0.13±0.017^{b}
Luem Pua glutinous rice	1.75±0.05^{c}	1.03±0.04^{a}	7.54±0.40^{a}	0.23±0.009^{a}

Notes : Different letters in the same column imply significant differences (p<0.05), while the same letters imply insignificant differences.

3.2 The optimal nitrogen sources for mycelial growth and bioactive compound production

Eggs, as a nitrogen source, provided the highest yield of fruiting body at 1.81±0.03 g/100 g of substrate. All results are shown in Table 2. All measurements were based on dry mass. Eggs also provided the highest yields of cordycepin (1.39±0.10 mg/g), adenosine (14.86±1.52 mg/g) and polysaccharides (0.24±0.003 mg/g). Nevertheless, yeast extract provided slightly lower, but insignificant, yields of these compounds.

Table 2 Effects of nitrogen sources on the growth and bioactive compound production of *I. tenuipes*

Nitrogen source	Fruiting body (g dry weight/100 g of substrate)	Cordycepin (mg/ g)	Adenosine (mg/ g)	Polysaccharides (mg/ g)
Yeast extract combined with peptone	1.61±0.02^{b}	0.89±0.08^{c}	10.50±0.11^{c}	0.18±0.008^{b}
Yeast extract	1.78±0.06^{a}	1.36±0.07$^{a b}$	14.23±0.19^{a}	0.23±0.009^{a}
Peptone	1.37±0.06^{c}	0.69±0.10^{d}	12.31±0.24$^{b c}$	0.18±0.003^{b}
Eggs	1.81±0.03^{a}	1.39±0.10^{a}	14.86±1.52^{a}	0.24±0.003^{a}
Ground soybean	1.41±0.03^{c}	1.21±0.12^{b}	11.92±1.36^{b}	0.15±0.016^{b}
Tryptone	1.35±0.04^{c}	0.76±0.01$^{c d}$	8.11±0.56^{d}	0.15±0.007^{b}

Notes : Different letters in the same column imply significant differences (p<0.05), while the same letters imply insignificant differences.

Discussion

4.1 The optimal carbon sources for mycelial growth and bioactive compound production

Carbohydrates are an important component of the fruiting body structure. They are essential nutrients for growth and development of highly developed fungi. Even for the same species, different carbon sources may affect the growth and production of bioactive compounds differently. Most important for a sustainable culturing of *I. tenuipes* is the cost of the carbon source. Nutrients from agricultural products or by-products are inexpensive and hence recommended (Kim & Yun, 2005; Chioza & Ohga, 2013).

In general, cordycepin content determines the commercial price of *Cordyceps* (Tapingkae, 2016). Therefore, it is the most influential factor in carbon source selection. Although Riceberry rice provided the highest fruiting body yield, Luem Pua glutinous rice provided the highest cordycepin yield, and it was much less expensive than Riceberry rice. Therefore, Luem Pua glutinous rice was selected as the primary carbon source for the subsequent nitrogen source experiment.

4.2 The optimal nitrogen sources for mycelial growth and bioactive compound production

From table 1 and 2, when the nitrogen source was added to Leum Pua glutinous rice, mycelial growth and bioactive compound production increased. Especially, adenosine increased significantly. Therefore nitrogen source affects the bioactive compound. Nitrogen promotes faster mycelium growth (Kang *et al.,* 2014). Both the tested egg and yeast extract as nitrogen sources promote *I. tenuipes* growth well, but eggs are much cheaper. Chandra and Shoji (2007) investigated different nitrogen sources that affected mycelium and polysaccharide production of *Lyophyllum decastes* and found that yeast extract provided a mycelium yield of 7 g/L and a polysaccharide yield of 325 mg/g of dry mycelium. In addition, Das *et al.* (2010) found that culturing *Cordyceps militaris* with yeast extract provided a mycelium yield of 94 g/L of substrate and a cordycepin yield of up to 6.8 g/L of substrate. Higher fungus can utilize organic nitrogen compounds better than inorganic ones (Fang & Zhong, 2002).

Conclusions

An investigation into optimum medium for *I. tenuipes* indicated that the optimum culture medium for *I. tenuipes*, in terms of fruiting body yield, consisted of Riceberry rice carbon and egg nitrogen sources. Nevertheless, Luem Pua glutinous rice carbon source led to the best yields of cordycepin and polysaccharides, while yeast extract nitrogen source provided only slightly lower, but not significantly lower, yields. These findings may directly benefit small farm growers and industrial growers in their effort to obtain the best yield from each harvest. More improvements to the suggested culture medium are expected from further studies on the optimum mineral source and environmental conditions for growing *I. tenuipes*.

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References

Bunyapaiboonsri, T., Yoiprommarat, S., Srisanoh, U., Choowong, W., Tasanathai, K., Hywel-Jones, NL., Luangsa-Ard, JJ., & Isaka, M. (2011) Isariotins G-J from cultures of the Lepidoptera pathogenic fungus *Isaria tenuipes*. *Phytochemistry Letters*, *4*(3), 283-286.

Chandra, PP., & Shoji, O. (2007). Submerged culture conditions for mycelial yield and polysaccharides production by *Lyophyllum decastes*. *Food Chemistry*, *105*(2), 641-646.

Che, JH., Yun, JW., Che, EY., Kim, SH., Kim, YS., Kim, WH., Park, JH., Son, WC., Kim, MK., & Kang, BC. (2014). Toxicologic assessment of *Paecilomyces tenuipes* in rats: Renal toxicity and mutagenic potential. *Regulatory Toxicology and Pharmacology*, *70*(2), 527-534.

Chen, XM., Lu, JX., Zhang, YD., He, JT., Guo, XZ., Tian, GY., & Jin, LQ. (2008). Studies of macrophage immuno-modulating activity of polysaccharides isolated from *Paecilomyces tenuipes*, *International Journal of Biological Macromolecules*, *43*(3), 252-256.

Chaichana, T., Kulsarin, J., Suttiprapan, P., Buranapanichpan, S., & Tapingkae, T. (2019). Strain improvement of *Cordyceps tenuipes* (Peck) for Snowflake Cordyceps production on silkworm pupae. *Journal of Agriculture, 35*(3), 355-364. (in Thai)

Chioza, A., & Ohga, S. (2013). Mycelial Growth of *Paecilomyces hepiali* in Various Agar Media and Yield of Fruit Bodies in Rice Based Media. *Advances in Microbiology*, *3*, 529-536.

Das, SK., Masuda, M., Sakurai, A., & Sakukabara, M. (2010). Medicinal uses of the mushroom *Codyceps militaris*: Current state and prospects. *Fitoterapia*, *81*(8), 961-968.

Deng, Y., Kim, BY., Park, MJ., Ok, M., Lee, KS., & Jin, BR. (2020). *Paecilomyces tenuipes* extracts affect lipid and glucose metabolic parameters similarly to *Cordyceps militaris* extracts. *Journal of Asia-Pacific Entomology*, *23*(3), 746-755.

Dubois, M., Gilles, KA., Hamilton, JK., Rebers, PA., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350-356.

Fang, QH. & Zhong, JJ. (2002). Submerged fermentation of higher fungus *Ganoderma lucidum* for production of valuable bioactive metabolites—ganoderic acid and polysaccharide. *Biochemical Engineering Journal*, *10*(1), 61-65.

Huang, L., Li, QZ., Chen, YY., Wang, XF., & Zhou XW. (2009). Determination and analysis of cordycepin and adenosine in the products of *Cordyceps* spp. *African Journal of Microbiology Research*, *3*, 957-961.

Kang, C., Wen, TC., Kang, JC., Meng, ZB., Li, GR., & Hyde, KD. (2014). Optimization of large-scale culture conditions for the production of cordycepin with *Cordyceps militaris* by liquid static culture. *The Scientific World Journal*, *4*, 1-15.

Kim, HC., Choi, BS., Sapkota, K., Kim, S., Lee, HJ., Yoo, JC., & Kim, SJ. (2011). Purification and characterization of a novel, highly potent fibrinolytic enzyme from *Paecilomyces tenuipes*. *Process Biochemistry*, *46*(8), 1545-1553.

Kim, HO., & Yun, JW. (2005). A comparative study on the production of exopolysaccharides between two entomopathogenic fungi *Cordyceps militaris* and *Cordyceps sinensis* in submerged mycelial cultures. *Journal of Applied Microbiology*, *99*(4), 728-738.

Koh, JB., & Choi, MA. (2003). Effect of *Paecilomyces japonica* on lipid metabolism in rats fed high fat diet. *Journal of the Korean Society of Food Science and Nutrition*, *32*(2), 238–243.

Lu, R., Miyakoshi, T., Tian, GY., & Yoshida, T. (2007). Structural studies of *Paecilomyces tenuipes* Samson polysaccharide-part-2. *Carbohydrate Polymers*, *67*(3), 343-346.

Miller, G. (1959). Use of dinitrisalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry*, *31*, 426-429.

Nam, KS., Jo, YS., Kim, YH., Hyun, JW., & Kim, HW. (2001). Cytotoxic activities of acetoxyscirpenediol and ergosterol peroxide from *Paecilomyces tenuipes*. *Life Sciences*, *69*, 229-237.

Park, JH., Park, NS., Lee, SM., & Park, E. (2011). Effect of Dongchunghacho rice on blood glucose level, lipid profile, and antioxidant metabolism in Streptozotocin induced Diabetic rats. *Food Science and Biotechnology*, *20*(4), 933-940.

Park, YH., Moon, EK., Shin, YK., Bae, MA., Kim, JG., & Kim, YH. (2000). Antitumor activity of *Paecilomyces japonica* is mediated by apoptotic cell-death. *Journal of Microbiology and Biotechnology*, *10*, 16-20.

Shin, KH., Lim, SS., Lee, S., Lee, YS., Jung, SH., & Cho, SY. (2003). Anti-tumour and immuno-stimulating activities of the fruiting bodies of *Paecilomyces japonica*, a new type of *Cordyceps* spp. *Phytotherapy Research*, *17*(7), 830-833.

Tapingkae, T. (2016). *Commercial Cordyceps mushroom cultivation*. (2^{nd} Ed.). Bangkok: Fram-Up Design LTD. Part. (in Thai)

Wang, JQ., Kan, LJ., Nie, SP., Chen, HH., Cui, SW., Phillips, AO., Phillips, GO., Li, YJ., an& Xia, MY. (2015). A comparison of chemical composition, bioactive components and antioxidant activity of natural and cultured *Cordyceps sinensis*. *LWT - Food Science and Technology*, *63*(1), 2-7.

Xu, CP., Sinha, J., Bae, JT., Kim, SW., & Yun, JW. (2006). Optimization of physical parameters for exo-biopolymer production in submerged mycelial cultures of two entomopathogenic fungi *Paecilomyces japonica* and *Paecilomyces tenuipes*. *Letters in Applied Microbiology*, *42*, 501-506.

Zhang, JX., Wen, CT., Duan, YG., Zhang, HH., & Ma, HL. (2019). Advance in *Cordyceps militaris* (Linn) polysaccharide: Isolation, structure, and bioactivities: A review. *International Journal of Biological Macromolecules*, *132*, 906-914.

Zhu ZY, Liu XC, Tang YL, Dong FY, Sun HQ, Chen L, Zhang YM. (2016). Effects of cultural medium on the formation and antitumor activity of polysaccharides by *Cordyceps gunnii*. *Journal of Bioscience and Bioengineering*, *122*, 494-498.