UTILIZATION OF *Spirulina maxima* TO ENHANCE YIELD AND CORDYCEPIN CONTENT IN *Cordyceps militaris* ARTIFICIAL CULTIVATION

Adisak Kaewkam[1] **Piyanast Sornchai**[2,4] **Sermsiri Chanprame**[1,3] **and Siriluck Iamtham**[1,2,4*]
[1] Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom 73140, Thailand and Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand
[2] Division of Genetics, Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Saen campus, Nakhon Pathom 73140, Thailand
[3] Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen campus, Nakhon Pathom 73140, Thailand
[4] Center for Advanced Studies in Tropical Natural Resource, NRU-KU, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.
*Corresponding author: faassli@ku.ac.th

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ABSTRACT

Cordyceps militaris has higher cordycepin content compared to other species of the genus *Cordyceps.* It can be cultured artificially but requires the development of appropriate medium formulation as some consumers are allergic to the larvae or pupae used as a supplementary protein in commercial cultivation media. Therefore, in the preliminary study, *C. militaris* was cultured in fruiting body induction medium (FIM) containing various sources of protein (*Spirulina maxima*, *Chorella vulgaris*, soybean, mung bean, rice bran and fresh chicken egg). The FIM containing *S. maxima* produced the highest fresh weight and dry weight (DW) of *C. militaris* and the highest amount of cordycepin. Therefore, a set of FIM containing various concentrations of dry and fresh *S. maxima* were further investigated to achieve high productivity and cordycepin content. *C. militaris* was cultivated in 11 different FIM culture media. In comparison with the commercial FIM using 50 g/L pupae, the powder of dry *S. maxima* was used at different rates: 1, 2.5, 5 and 10% w/v, or with fresh *S. maxima* at 10, 25, 50 and 100% (w/v). The best FIM was 1% fresh *S. maxima* which produced the highest DW of *C. militaris* fruiting bodies and the highest cordycepin content which is higher than the commercial product.

Key words: *Cordyceps,* culture medium, microalgae

INTRODUCTION

Cordycep militaris is an entomopathogenic fungi belonging to the order Hypocreales, family Cordycipitaceae (Kirk et al. 2001), which parasitize internally arthropod larvae or pupae of the Lepidoptera, Diptera or Heminoptera (Lee et al. 2005). The mycelium develops inside the body of the host, feeding on its nutrients and forms fruiting bodies in its insect host (Sung et al. 2007). *C. militaris* has long been used in traditional oriental medicines due to its pharmacological properties as it contains many interesting bioactive substances such as cordycepin, adenosine, polysaccharides and minerals (Gu et al. 2007; Nag and Wang 2005). Cordycepin is considered as one of the important biologically active substances, which has antitumor properties on cancer cells, antimicrobial, antioxidant, anti-influenza virus activities, improves sperm production, suppresses the expression of diabetes regulating genes, has fibrin binding activity and anti-inflammatory effect (Liu et al. 2015; Patel

and Ingalhalli 2013). The natural fruiting bodies of *C. militaris* are expensive and rare due to its host specificity, its growth being restricted to a specific area and its small size. Therefore, *C. militaris* growing in the wild is not sufficient to meet the demand for use as a folk medicine or nutritional supplementary foods. Since the industrial value of *C. militaris* has increased the demand for cordycepin, several methods of *C. militaris* cultivation have been reported to enhance cordycepin production including surface culture (Masuda et al. 2007), submerged culture (Mao et al. 2005) and even growing on silk worm (Li et al. 2002). The main disadvantage for surface cultivation is that the thick mycelia of the mushroom cover the surface of the medium throughout the culture and submerge the culture of mushrooms resulting in non-formation of mushroom nonmycelia; instead, a yeast-like substance is formed that is easily contaminated and productivity is generally low but with high associated costs (Suparmin et al, 2017; Kang et al. 2014). The present study used solid substrate fermentation to enrich the cordycepin content for *C. militaris* cultivation as this technique has many advantages since the mushroom is grown on readily available solid substrates at low cost and produces concentrated bioproducts using an environmental-friendly process (Thomas et al. 2013). The solid substrate comprises of grains of rice, wheat, rye, cereals or even arthropod larvae like silkworm. Thus, the cordycepin content depends on the nutrient composition of the solid substrate. However, allergic symptoms have been reported after consumption of silkworm larvae, which is mediated by both IgE and non- IgE mechanisms, and cross reacts with silkworm pupae (Choi et al. 2010). To avoid such allergic problems, other sources of protein should be used instead of insects. Such protein sources as soybean, mung bean, egg, rice bran and microalgae have been used to replace the use of silkworm pupae due to their bioactive components. Among the various solid substrates, soybean was the best choice for improving the cordycepin content (Lim et al. 2012). Microalgae are one of the most promising organisms that could be used as an alternative protein source with *Spirulina* and *Chlorella* being among pragmatic candidates due to their commercial readiness. *Chlorella* sp. is a rich source of protein (51%–58% DW) and valuable essential amino acid, vitamins, minerals, β-carotene, chlorophyll, and Chlorella growth factor (CGF) (Becker 2007). *Spirulin*a sp. also contains high amounts of essential nutrients including proteins (50-70% by weight), vitamins, fatty acids, minerals and pigments as well as having potential therapeutic benefits (Habib et al. 2008).

As a highly valuable alga, *Spirulina* gained popularity in various industries, including therapeutics and cosmetics. In medical fields, it has the potential to enhance brain function, boost the immune system, regulate cholesterol, stimulate antibodies and improve the white blood cell count (Khan et al. 2005). The major pigment, phycocyanin, which accounts for 20% of total algal protein can be extracted from *Spirulina* sp. and has been used mainly as a food pigment and in cosmetics. It shows anti-cancer and anti-inflammatory properties and is hepatoprotective (Romay et al. 2003) as well as having antioxidant properties (Estrada et al. 2001). In addition, *Spirulina* sp. does not produce any toxins and has a completely digestible cell wall including peptidoglycans and can be immediately consumed and digested with ease with no adverse effect (Eykelenburg 1977).

The composition of the medium for *C. militaris* artificial cultivation has important effects on the productivity of this mushroom. The present study focused on optimizing the ingredients in the medium by developing various formulae using different sources of proteins to enhance the growth of *C. militaris* and also to increase the cordycepin content in mushroom cultivation. The yield and the cordycepin content were evaluated and compared with those cultured in a standard medium.

MATERIALS AND METHODS

***C. militaris* stock culture.** The *C. militaris* isolate used in this study was provided by T.S. Twin Product Ltd. (Chachoengsao province, Thailand), maintained on potato dextrose agar and incubated in the dark for 14 days. Hyphal tips (1 cm^2) were cut and inoculated in 100 mL of potato dextrose broth (PDB) and incubated at 25°C in the dark with shaking at 200 rpm for 3 days, after which, the product was transferred to the fruiting body induction medium (FIM).

Algal stock culture. To prepare a stock culture, samples of *Spirulina maxima* (IFRPD 1183) and *Chlorella vulgaris* (IFRPD 1018) were obtained from the Institute of Food Research and Product Development, Kasetsart University, Bangkok, Thailand. *S. maxima* was transferred into 200 mL Erlenmeyer flasks containing 100 mL Zarrouk medium (Zarrouk 1996). *C. vulgaris* was cultured in N-8 medium at 30 °C under fluorescent lighting (12 Klux) for 16 h/day with 1-2% CO_2 until the optical density reached 1.0 (OD 560 nm). The algal samples were then filtered using a plankton net (30-50 µm) and washed with distilled water to remove the culture medium; this was used as the fresh algal source. For dry *S. maxima* powder, an algal sample was incubated in a hot-air oven at 60-70 °C for 6-8 h. The algal products were ground into a fine powder (85 mesh), which was suitable for further experiments.

Fruiting body induction medium preparation. The FIM for *C. militaris* cultivation is generally comprised of solid and liquid parts (1:1, w/v). In general, the solid part is raw white rice and the liquid part is modified PDB (200 g potato, 20 g glucose, 5 g yeast extract 5 g peptone and tested supplements in 1,000 mL of sterile water). The analytical and biochemical regents were all from Conda-Pronadisa, Spain and the food grade supplements were bought from a local supermarket in Nakhon Pathom, Thailand. The protein sources (soybean, mung bean, chicken egg and rice bran) were bought from a local supermarket in Nakhon Pathom, Thailand. The eri silk larvae or pupae were obtained from the Center for Excellence in Silk, Kasetsart University, Kamphaeng Saen campus, Thailand. To obtain the ready-to-use FIM, a 15 mL aliquot of liquid media was added to a 250 mL glass bottle containing 15 g white rice. Each bottle was sterilized at 121 °C for 15 min.

Preliminary study using FIM supplemented with various protein sources. Mycelia of *C. militaris* were cultured on FIM supplemented with 50 g/L of dry *S. maxima*, *C. vulgaris*, soybean, mung bean, rice bran and fresh chicken egg. The commercial FIM (liquid medium supplemented with 50 g/L stage 5 eri silk larvae or pupae) was used in the control treatments. An amount of 50 g/L larvae and pupae was blended in 100 mL sterile water, then filtered through cheesecloth and added into the liquid media.

Study of FIM supplemented with different forms and concentrations of *S. maxima*. To find suitable forms and concentrations of *S. maxima* for *C. militaris* growth and cordycepin production, we added various forms (fresh and dry) and concentrations (w/v of liquid medium) of *S. maxima* to the liquid basal media of FIM. The treatments are summarized in Table 1.

Table 1. FIM medium containing different forms and concentrations of *S. maxima* and controls.

Treatment	Component
1	Control (only 15 g rice medium and 15 mL basal liquid medium)
2	Silkworm pupae 5% (external control)
3	*Spirulina* powder 1% (1 g/100 mL medium)
4	*Spirulina* powder 2.5% (2.5 g/100 mL medium)
5	*Spirulina* powder 5% (5 g/100 mL medium)
6	*Spirulina* powder 10% (10 g/100 mL medium)
7	Fresh *Spirulina* 1% (1 g/100 mL medium)
8	Fresh *Spirulina* 10% (10 g/100 mL medium)
9	Fresh *Spirulina* 25% (25 g/100 mL medium)
10	Fresh *Spirulina* 50% (50 g/100 mL medium)
11	Internal control (medium containing only 15 g of rice medium supplemented with 100% fresh *Spirulina*, without the 15 mL of basal liquid medium).

***C. militaris* inoculation and cultivation.** Aliquots of 5 mL stock of *C. militaris* in PDB were inoculated into each bottle and incubated at 18-20 °C in the dark for 4 weeks for mycelial growth. For fruiting body induction, the culture room was illuminated (55 µmol /m^2/s) for 16 h at 20 °C for 2 months (Fig.1). The fresh weights of fruiting bodies were determined immediately on harvest and dry weights were determined after drying at 60°C for 24 h.

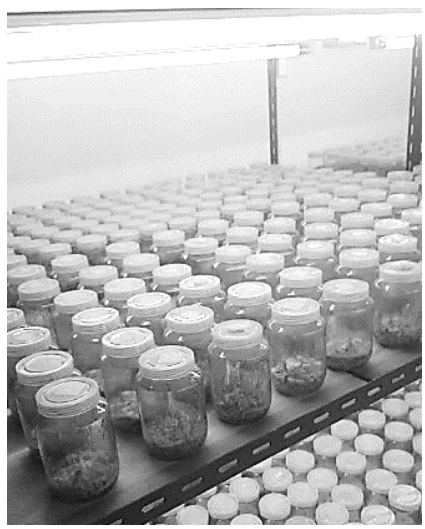


Fig. 1. *Cordyceps militaris* culture with mushrooms cultivated in 250 mL bottles containing fruiting body induction medium under illumination (55 µmol/m^2/s) for 16 h at 20 °C for 2 months.

Cordycepin determination. The cordycepin concentration was determined in dried fruiting bodies of *C. militaris* using high performance liquid chromatography (HPLC; Shimadzu Corp., Kyoto, Japan). A ground sample of 0.5 g was placed in a 100 mL flask containing 80 mL of sterilized distilled water and subjected to ultrasonic extraction at 60° C for 6 h, after which 1 mL was centrifuged at 4,000 rpm. The supernatant fluid was filtered through a 0.45 µm microporous membrane and the filtrate was used for HPLC analysis. The samples were separated and analyzed using a Waters Symmetry C18 column (250 mm, 4.6 mm, 5 µm) at 35° C. The mobile phase consisted of 10% solvent A (methanol) and 90% solvent B (water) at a flow rate of1.0 mL/min. The detecting wavelength was set between 190 and 380 nm, and the chromatographic peaks were measured at a wavelength of 260 nm.

Statistical analysis. This experiment used a completely randomized design with three replicates for each treatment, each replicate had 5 culture bottles. The fresh and dry weights of *C. militaris* and the cordycepin content were tested using analysis of variance at a significance of $p<0.05$.

RESULTS AND DISCUSSION

***C. militaris* yield in media formulated using different protein sources.** In nature, *C. militaris* has a slow growth rate and specific culture conditions, which are main problems resulting in inadequate supply to meet industry demand, especially for cordycepin and bioactive compounds with wide therapeutic activities that are produced by this mushroom. Since there is an increasing demand for large amounts of cordycepin, there have been attempts to artificially cultivate *C. militaris* using different culture medium to achieve higher productivity and a greater cordycepin content in the mushroom. The present study produced significantly different fresh and dry weights of the mushroom from the different protein sources. Among the various protein sources used in this study, *Spirulina* produced the

significantly highest fresh and dry weights (18.28 and 5.78 g, respectively), followed by *Chlorella* (16.07 and 3.69 g, respectively) and pupae (15.95 and 3.60 g, respectively) (Fig. 2).

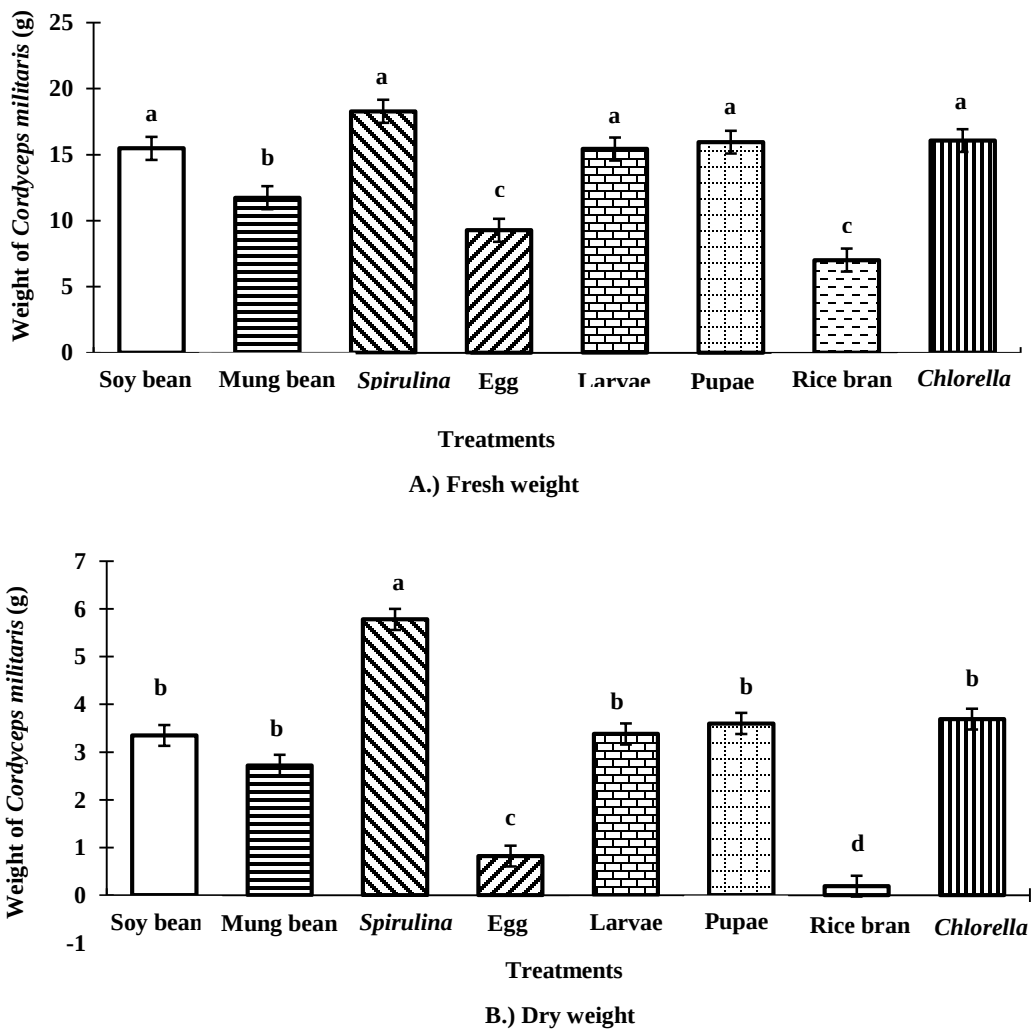


Fig. 2. Average fresh (A) and dry (B) weights of *C. militaris* in basal medium supplement with different protein sources (soybean, mung bean, *Spirulina*, egg, larva, pupa, rice bran and *Chlorella*), where columns with different superscripts are significantly different at $p<0.05$ and error bars indicate standard deviation of data.

A comparison of various solid substrates (brown rice, millet, sorghum, corn, wheat and glutinous rice) revealed the highest DW yield of 1.03 g/ in fruiting bodies of *C. militaris* resulted from using brown rice used as the substrate (Wen et al. 2014). In this respect, *Spirulina* sp. is the best source of nutrients and is particularly rich in proteins. *Spirulina* sp. contains 56-77% protein of its dry weight which is higher than other protein sources such as eggs, milk, meat, fish, soybeans and other grains (Ciferri 1983; Richmond 1980, 1986). This alga lacks a cellulose cell wall, allows the organism to absorb and then supply an abundance of nutrients (Shimamatsu 2004). Among all protein sources, *Spirulina* sp. is the richest source of proteins which contain about 60-70% DW (Ravindran et al. 2016), compared to 51%–58%, 35%, 20.97–31.32%, and 18–22% for the protein content in *Chlorella* sp., chicken egg, mung beans, and soy beans respectively (Becker 2007; Anwar et al. 2007; Xu et al. 2015). Moreover protein content of *Spirulina* sp. is about several fold higher than in rice bran and eri silklarva, with a lower storage protein content (10-15% and 16%) (Fabian et al. 2011: Longvah et al. 2011). *Spirulina* sp. also contains numerous essential minerals and carotenoid (a powerful antioxidant) as well as being especially rich in vitamin B (B1, B2, B3, B6, B12) and in particular a high concentration of vitamin B1 (0.5g/100g) which was reported to be an important substances for *C. militaris* growth. (Gershwin and Belay 2008). Cultivation of *C. militaris* in medium supplemented with vitamin B1 produced the maximum mycelium yield compared with other tested vitamins, being even higher than the control medium containing all vitamins and glucose (Dang et al. 2018).

Cordycepin content of *C. militaris* in media formulated using different protein sources. Cordycepin, a nucleoside analogue, can convert into 5' monophosphate, diphosphate and triphosphate inside the cell, which can hinder purine or DNA/RNA biosynthesis or both, causing anti-metastasis anti-tumor and anti-microbial results and it can also modulate a wide range of signaling pathways involved in a variety of mechanisms including apoptosis, proliferation, metastasis, angiogenesis and inflammation (Tuli et al. 2013). From the current experimental results, the *C. militaris* culture using various protein sources instead of silkworm pupae produced significantly different cordycepin contents. The basal medium supplement with *Spirulina* produced the highest cordycepin content (1,100 mg/100 g), followed by the formula that used pupae and larvae (780 and 740 mg/100 g, respectively) as shown in Fig. 3.

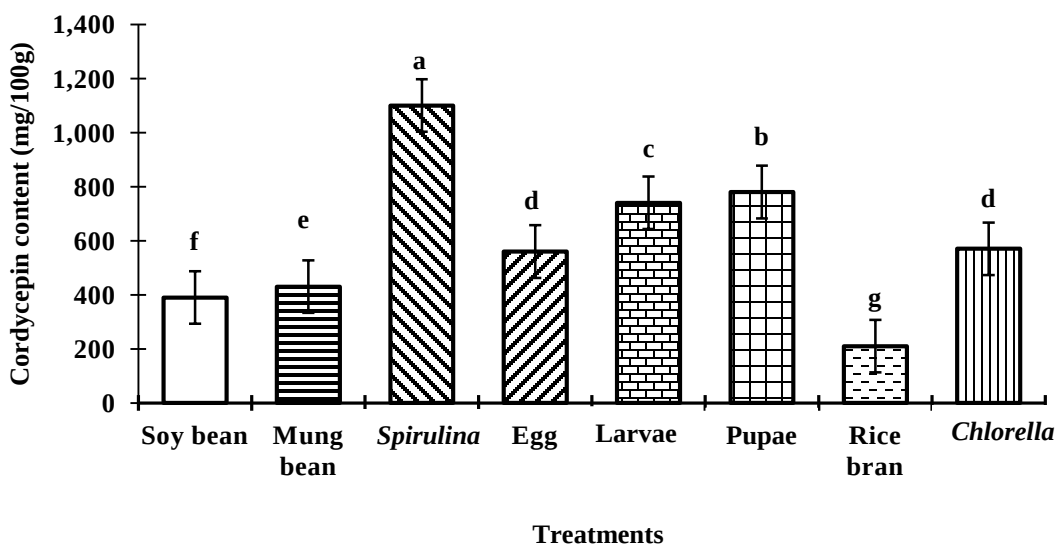
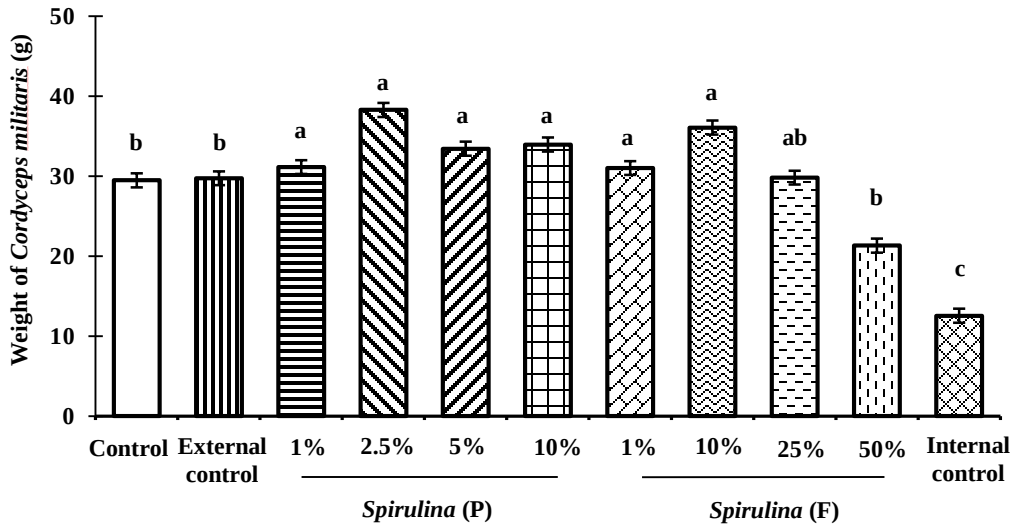


Fig. 3. Cordycepin content of *C. militaris* obtained from different protein sources (soybean, mung bean, *Spirulina*, egg, larva, pupa, rice bran and *Chlorella*), where columns with different superscripts are significantly different at $p<0.05$ and error bars indicate standard deviation of data.

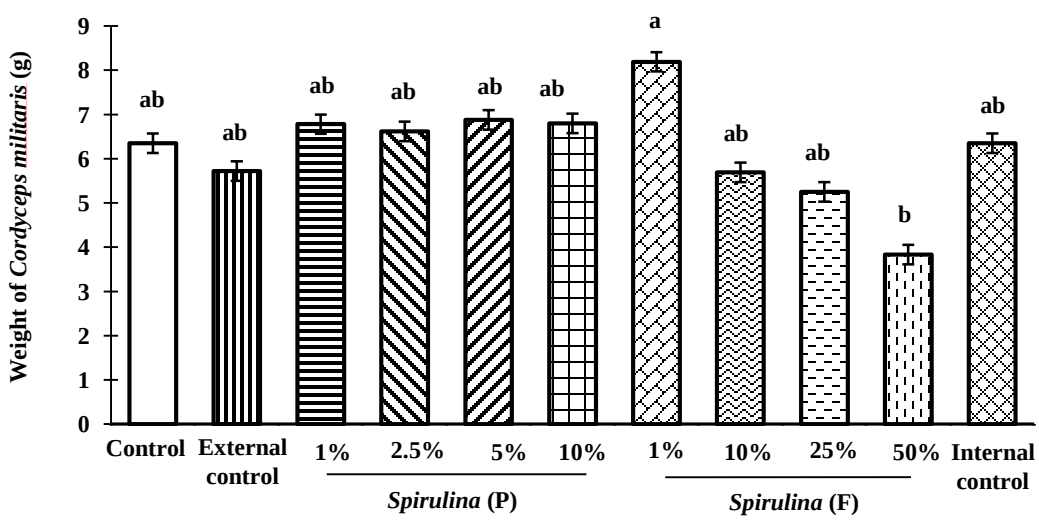
This may have been successful because *Spirulina* sp. is nutrient-rich and is a good source of protein (50-70%), carbohydrate (15-25%) and carotenoid content (6 mg/g) (Liestianty et al. 2019) and essential minerals, which help to balance and boost the immune system in the human body compared to using silk larva pupae which contained lower amounts of essential substances including protein (12-16%), carbohydrate (1.2-1.8%) (Mishra et al. 2003), carotenoid content (3-35 ug/g pupa) (Chieco et al. 2019). The yield of cordycepin in the present study was higher than yields of cordycepin of *C. militaris* following cultivation on a solid state medium containing spent brewery grain that produced 1,042 mg/100 g cordycepin concentration (Gregori 2014). *C. militaris* cultivated in a basal medium supplemented with different culture substrates (brown rice, millet, sorghum, corn, wheat and glutinous rice) produced the highest cordycepin content (562 mg/100g) when brown rice was used as the substrate (Wen et al. 2014). The optimum culture conditions to produce a high level of cordycepin (728.18 mg/100g) used soybean as the solid substrate (Lim et al. 2012). Therefore, in the current study, using *Spirulina* as the protein source provided highest amounts of cordycepin (1,100 mg/100 g) compared to other reports using solid state fermentation for the production of *C. militaris* on an industrial scale (Lim et al. 2012). The highest production of cordycepin was achieved, when *Spirulina* was used as the protein source perhaps because of the various types of amino acids and high dietary pyrimidine nucleoside content of *Spirulina* (Berthold et al.1995) which act as precursors of cordycepin synthesis (Chassy and Suhadolnik 1969). Nucleosides and amino acids are the major substances related to the purine biosynthetic pathway which could increase the cordycepin content by providing backbone precursors for cordycepin (Masuda et al. 2007). Among the 20 amino acids used as supplements in the basal medium, lysine, histidine and glycine greatly enhanced cordycepin production of *C. militaris* (7.18mg/g, 6.95mg/g, 7.08 mg/g, respectively) compared to the control without amino acid (3.99 mg/g) (Wen et al. 2016).

Protein quality depends on the essential amino acid content. In the present study, *Spirulina* proteins are complete, since all the essential amino acids are present, forming 47% of total protein weight (Bujard et al. 1970), while total essential amino acid contents profile (45.3%), with the exception of sulphur amino acids is found in *Chlorella vulgaris* (Morris et al. 2009). The total essential amino acid contribution was comparatively high and complete in *Spirulina* sp. compared to that found in eri silk larva, mung beans, chicken egg, rice bran and soy beans which was 44%, 43.51%, 40.9-41.3%, 35%, 12.83 -19.02%, respectively (Longvah et al. 2011; Yi-Shen et al. 2018; Adeyeye et al. 2011; Zaky et al. 2020; Carrera et al. 2011). However, optimization of the appropriate amount of *Spirulina* could produce a larger amount of this important substance.

***C. militaris* yield in basal medium supplemented with different forms and concentrations of *Spirulina* sp.** The protein source affects the yield of cordycepin, making it essential to optimize the medium composition. Therefore, we varied the forms and concentrations of *Spirulina* in the supplement. In this study, the fresh and dry weights of the mushroom differed significantly among the basal media according to the various forms and concentrations of *Spirulina* sp. These results suggested that different forms and concentrations of *Spirulina* could affect the productivity of the mushroom. In the present study, 2.5% algal powder in the medium produced the greatest fresh weight, followed by 10% fresh *Spirulina* and 10% algal powder, respectively, with the three treatments having average weights of 38.28, 36.07 and 33.95 g, respectively (Fig. 4). However, 1% fresh alga produced the highest average dry weight, followed by 5% and 10% algal powder with average dry weights of 8.19, 6.88 and 6.80 g, respectively (Fig. 4), compared with the commercial formula that used silkworm pupae and produced average fresh and dry weights of 29.74 and 5.72 g, respectively.



A.) Fresh weight



B.) Dry weight

Fig. 4. Average fresh (A) and dried (B) weights of *C. militaris* in basal medium supplemented with different forms and concentrations of *Spirulina*, where columns with different superscripts are significantly different at $p < 0.05$ and error bars indicate standard deviation of data. *Spirulina* (F) = *Spirulina* fresh; *Spirulina* (P) = *Spirulina* powder.

Carbon and nitrogen sources were essential for cell proliferation and metabolic biosynthesis for mycelial growth and metabolite formation of *C. militaris* and if used in appropriate quantities or concentrations, those sources promoted growth and mycelial formation (Zhang et al. 2016). On the other hand, if the mushroom intake has insufficient amounts of these nutrients, there will an adverse impact on growth and secondary metabolite production (Yue 2012). Previously, *C. militaris* was cultured in different carbon (glucose, sucrose, amidulin, lactose, maltose, mannose) and nitrogen sources (wheat bran, soybean oil meal, beef extract, peptone, yeast extract, silkworm pupa) and the results revealed that glucose was the best carbon source producing the highest fruiting body dry weight (1.36 g), while peptone was the best nitrogen source for *C. militaris* growth in terms of dry weight (1.75 g) (Wen et al. 2014). Peptone was the best nitrogen source and the cordycepin yield was enhanced to 843.63 mg/L when sucrose was used as the carbon source (Kang et al. 2014).

Cordycepin content of *C. militaris* in basal medium supplemented with different forms and concentrations of *Spirulina* sp. From the experimental results, it was found that 1% fresh algal supplement in the fruiting body induction medium produced the highest amount of cordycepin (1,599 mg/100 g), followed by 10% and 5% powdered algal supplement in the medium which each contained cordycepin of 1,062 and 857 mg/100 g, respectively (Fig. 5).

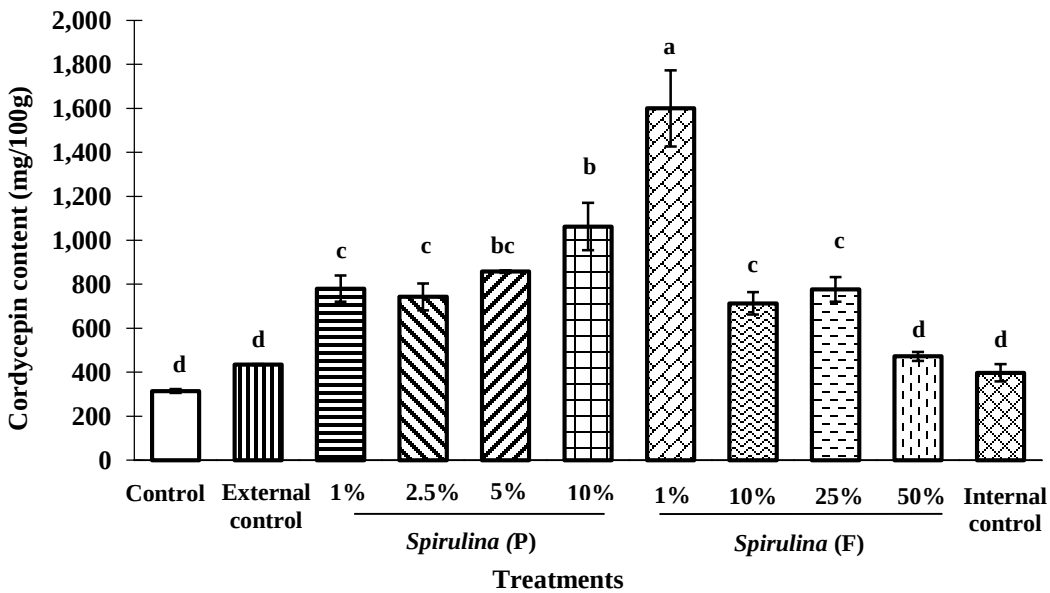


Fig. 5. Cordycepin content of *C. militaris* obtained from basal media supplemented with various forms and concentrations of *Spirulina*, where columns with different superscripts are significantly different at $p<0.05$ and error bars indicate standard deviation of data. *Spirulina* (F) = *Spirulina* fresh; *Spirulina* (P) = *Spirulina* powder

In this study, a novel supplement of 1% fresh *Spirulina* to the fruiting body medium formula produced the greatest dry weight of mushroom and enhanced cordycepin production. This study also found significant differences in the cordycepin content between 1% fresh and 1% powder of *Spirulina* added to the fruiting body medium, (1,599 mg/100 g and 780 mg/100 g, respectively), possibly due to the algal powder losing some nutritional value in the drying process, which resulted in a lower cordycepin content compared to using the medium with 1% fresh algal supplement. In addition, 100% fresh algal supplement in the media without yeast extract and peptone resulted in the lowest productivity and cordycepin content. This may be due to the very high amounts of protein sources but had less

nitrogen and carbon sources compared to other medium which contained peptone and yeast as nitrogen and carbon sources.

The carbon and nitrogen sources stimulate the growth of mycelia or the formation of polysaccharides and the production of secondary substances if used in appropriate quantities or concentrations (Zhang et al. 2016). In the medium containing 100% fresh alga, the medium was packed very tightly after autoclaving, which interfered with the development of mushroom mycelia and inhibited the production of secondary substances. Similarly, the volume of the medium could affect the increase in cordycepin production (Kang et al. 2014). A higher volume of medium did not necessarily help to increase the production of substances due to the large volume of the medium restricting the amounts of oxygen that could dissolve to the surface of the medium, which would limit the production of cordycepin. Therefore, using the appropriate forms and amounts of *Spirulina* in culturing the mushroom could produce higher levels of production and cordycepin content. This was consistent with the nutrient requirements of the *C. militaris* in nature, which can be divided into two groups. First, macronutrients which include carbon, hydrogen, oxygen and nitrogen, are obtained from the decomposition of organic and inorganic substances in the environment. Second, only small amounts of some micronutrients are required including zinc, manganese, copper, molybdenum and vitamins, which are components of co-enzymes in the metabolic processes. The lack of micronutrients, such as the lack of biotin, causes abnormal cell development. In nature, mushrooms obtain nutrients by releasing exoenzymes to digest the larger molecules. *C. militaris* cultivation in aromatic black rice medium and Mole cricket with supplementary nutrient produced the greatest amounts of cordycepin (Sornprasert et al. 2016). A new strain of *C. militaris* was developed that produced a higher cordycepin content than parent strains through mating-based sexual reproduction (Kang et al. 2017). Another study reported 225mg/100g cordycepin in commercial *C. sinensis* products obtained through solid-state cultivation and 65 mg/100g cordycepin in wild-collected *C. sinensis* stromata (Holliday et al. 2004). Spent *C. militaris* cultivating substrates were reported to contain 10-100 mg/g cordycepin content (Ni et al. 2009). A cordycepin content of 917 mg/100g was achieved in optimized solid-state composition for *C. militaris* cultivation (Wen et al. 2014). However, the cordycepin contents in these reports were somewhat lower than those obtained in our present study.

CONCLUSIONS

Spirulina is recommended for large-scale industrial applications for fruiting body and cordycepin production based on cost of culture medium. This study highlighted an aspect of artificial cultivation to make *C. militaris* a more affordable material for commercial trade. We developed the medium formula using 1% fresh alga as the protein source (instead of using silk larva pupae) that was added to basal media which could promote the growth and cordycepin content of *C. militaris*. The medium formula developed in the current study provides an effective way for improving the productivity and cordycepin production of this mushroom on a large scale. However, further research is necessary to optimize cultivation factors such as light, incubation time, aeration and temperature.

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