Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **45** Issue **4 Year 2024** Page **149-156***

Optimizing Culture Medium And Growth Conditions To Enhance The Biomass And Quality Of Medical Mushroom *Cordyceps Militaris* Under Controlled Conditions

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Abstract

Cordyceps militaris is widely used in many Asian countries as a traditional medicinal material for treating various ailments. This study aims to investigate how the growth of the medicinal mushroom *C. militaris* is influenced by the culture medium and culture conditions in a controlled laboratory setting. The results indicate that aromatic brown rice (BT7) significantly increases the fresh weight of all three collected materials of *C. militaris*. Shoot length and body weight were promoted by the glucose content, silkworm pupa powder and pH of the culture medium ranging from 6.0 to 7.0, with the optimal glucose content being 30.0g per bottle. The optimal temperature for growth is between 22°C and below 25°C, with a light intensity of 600-700 lux. For higher cordycepin content, the recommended harvesting time is 65 days, while for adenosine content should be 55 days after inoculation. These findings provide valuable information to improve both the biomass and quality of *C. militaris*, which is crucial for large-scale production in the pharmaceutical industry.

Keywords: Cordyceps militaris, medical fungus, biomass, culture medium, glucose

Introduction

Cordyceps is a large and diverse genus of fungi with over 627 species that belong to the family Cordycipitaceae (Hypocreales, Ascomycota). They are parasitic fungi that parasitize certain insects during their larva, adult, or pupa stages [1]. The fungus *C. militaris* is commonly found at altitudes between 2,000 and 3,000 meters above sea level. It primarily parasitizes worms and pupae of various Lepidoptera species. This particular species can be found in several countries worldwide, including China, Japan, Korea, Thailand, and Vietnam. However, the population density of *C. militaris* is quite low, and the stromata produced in the wild are small in size. Furthermore, the collection of wild specimens is insufficient to meet the growing demand of humans in many worldwide countries. Consequently, there has been a significant interest in cultivating its mycelia and stromata on a large scale [2]. There are two kinds of these species, namely C. sinensis and C.militaris have been used in food and medicine. However, obtaining C. sinensis from culture media is challenging, and its increasing

demand and limited natural resources have led to high prices. As a result, alternative sources have been sought. One potential solution is the use of a related species, *C. militaris* which can be cultivated and obtained *in vitro*. It is important to note that when *C. sinensis* mycelium is grown under *in vitro* culture conditions, it either cannot produce cordycepin or only small amounts. Moreover, the amino acids and D-mannitol content in the mycelium is lower than that in the fruiting bodies of C. sinensis found in natural habitats [3]. Currently, worldwide scientists have paid much interest to this fungus species and numerous biological effects are well documented including anti-cancer, anti-oxidant, anti-inflammatory, anti-aging, immunomodulatory, antimicrobials, immunosuppressive, hypolipidemic, hypoglycemic, neuroprotective, and fertility enhancer [2] Several bioactive compounds with nutraceutical potential, including cordycepin, adenosine, ergosterol, trehalose, mannitol, polysaccharides, nucleosides, and amino acids, have been identified in or extracted from *C. militaris*, in which cordyceps exhibits high levels of antioxidant activity and possesses anti-cancer properties. Recently, some reports showed that the anti-oxidation effect of cultured *C. militaris* was higher than the natural *C. sinensis* and had the ability to inhibit oxidation in liposomes. Moreover, *C. militaris* contains higher levels of polyphenolic and biologically active ingredients such as cordycepin and adenosine [3]. Therefore, *C. militaris* could be considered as a viable substitute for C. sinensis due to the similar qualitative and quantitative composition of bioactive compounds found in *in vitro*-cultivated *C. militaris* and C. sinensis fruiting bodies. [4]. Cordycepin shows promise as a possible option for the development of antiatherosclerotic medicines due to its ability to enhance vascular responses in smooth muscle cells [5]. Recent scientific reports elucidated the potential of cordycepin as an antiviral agent against SARS-CoV-2 in COVID-19 and HIV-1 protease [4, 6]. During the past decade, numerous studies have reported about potential and novel applications of *C. militaris* for human health. However, few studies have been conducted in Vietnam. Therefore, the objectives of this study were to examine the optimal culture medium and culture conditions for cultivating *C. militaris* mushrooms under laboratory conditions.

Materials and Methods

Fungal materials

In this study, a total of 3 strains of *C. militaris* were used, namely Q1, Q2, and Q1, which were kindly provided by Agricultural Genetics Institute.

Methods

The *C. militaris* mushroom stock is stored and preserved at 40°C on PDA medium in the laboratory of the Department of Genetic Engineering, Institute of Agricultural Genetics. The liquid propagation medium consists of peptone (5 g), yeast extract (5 g), B1 (0.1 g), $MgSO_4$ (1 g), and KH_2PO_4 (0.5 g), with a pH of 6.5. This liquid was grown in a shaker at 120 rpm at a temperature of 25±10°C for 5 days.

For the initial experiment, BT7 fragrant rice was used as the medium to cultivate fruiting bodies, including white fragrant rice as CT1 treatment and brown fragrant rice (CT2). Each treatment was made up of rice (25g) and nutrient solution (50 ml), respectively. The nutrient solution is prepared by combining KH_2PO_4 (1 g/l), $MgSO_4$ (1 g/l), B1 (0.2 g/l), peptone (1 g/l), potatoes (50 g/l), and 10% coconut water, adjusting the pH to 7.0. The mixture is sterilized by placing it in a 500 ml cylinder, covering it with a plastic lid, and heating it at 121°C for 25 min. Each medium flask was then inoculated with 2 ml of the same liquid. The mycelial incubation stage took place in complete darkness at a temperature of 20-22°C for approximately 9-10 days. It is then transferred to room conditions with a temperature of 24±10°C, lighting intensity of 500-1.100 lux, a photoperiod of 12 light/12 night, and a humidity of 80-90% for around 55-65 days. In order to examine the effect of pH on the fruit bodies of *C. militaris*. The parameters and biomass of 3 strains of *C. militaris* which were affected by pH, temperatures and light, were averagely calculated and compared.

Development characteristics of fruiting body buds were observed 20 days after transplanting. The following parameters were measured: TW (total weight in grams per bottle), LS (fruiting body length), FW (fruiting body weight in grams per bottle), cordycepin content (in mg/g of dry matter), and adenosine content (in mg/g of dry matter). The contents of cordycepin and adenosine were identified and qualified by HPLC following the method of Quy et al. [7] with some minor revisions.

Statistical analysis

The values of control treatments and standards were expressed as means of standard deviations (SD). All data were statistically analyzed using Excel version 2017.

Results and Discussion

Effects of Substrate on Growth, Development, and Yield of *C. militaris*

The *C. militaris* fungus typically parasitizes insects in nature. To reduce costs and source raw materials for production, experiments have been conducted to cultivate *C. militaris* mushrooms in organic environments. Fortunately, cereals and other organic matter sources have been found to be suitable for cultivating *C. militaris* in artificial environments. Since 1941, Kobayashi has conducted experiments on growing *C. militaris* using rice as a substrate [6]. Rice has become the main substrate for cultivating *C. militaris* in artificial environments. However, not all types of rice are suitable for cultivating *C. militaris*. To determine the appropriate rice type, we conducted farming experiments using popular rice types in Vietnam - specifically, white fragrant and brown fragrant rice of BT7. After 65 days from inoculation, the mushrooms in the bottle were harvested, and the parameters were measured. The results are presented in Figure 1.

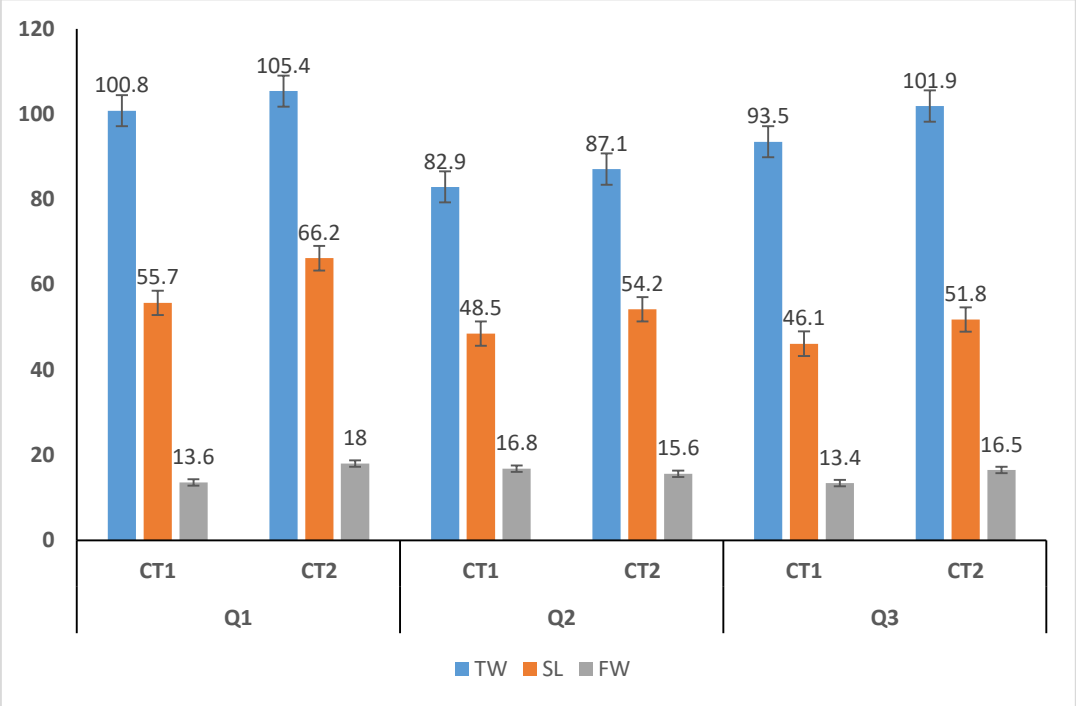

Figure 1. Effects of substrate on *C. militaris* fruiting bodies 60 days after inoculation. *TW: total weight (g/bottle); LS: fruiting body length (mm); FW: fruiting body weight (g/bottle)*

The results presented in Figure 1 show that all mushroom strains cultivated in brown fragrant rice (CT2) were higher than those of white fragrant rice (CT1), respectively. Specifically, the highest total weight (TW) of Q1 strain was 105.4 g, followed by Q3 (101.9g), and Q1 (100.8 g). Similarly, the length of the fruiting body (LS) Q1 was 66.2mm, Q2 (54.2) and Q3 was 51.8 mm. Also, the fruiting body weight of Q1 (g/bottle) was higher than both Q2 and Q3, respectively. Thus, it can be seen that using brown fragrant rice BT7 as a substrate yields a higher volume of fresh fruiting bodies than using white fragrant BT7 by 17.0-31.0%, depending on the mushroom strains.

Effect of glucose content on growth, development, and yield of *C. militaris*

In this experiment, except for the rice carbon source provided, we added glucose to the culture medium with gradually increasing levels from 0 to 50 g/l. Experimental results show that the sugar content in the culture medium had negligible effects on the formation time as well as the morphological and developmental characteristics of fruiting body length but does affect the number of fruiting bodies of *C. militaris* fungus (Figure 2).

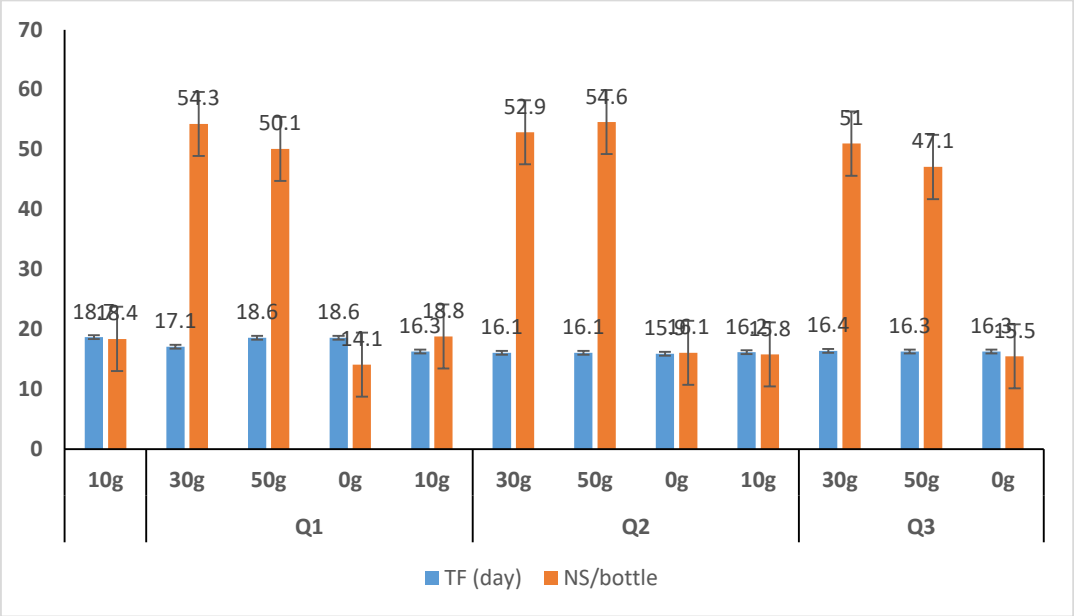

Figure 2. Effect of glucose content on the formation and development of fruiting bodies after 25 days of inoculation.*; TF: time of fruiting (day); TF: number of shoot/bottle.*

The results shown in Figure 2 show that the number of shoots of all three strains remarkably increased and ranged from 47,1 shoots to 54.6 shoots, while the control was 15.5 shoots, which was equal to 67.1% to 72.3% compared with the control treatments. However, it notes that the number of fruiting bodies in the two formulas without added sugar and with 10 g of sugar did not have a significant difference. Adding 20 g of sugar, the number of fruiting bodies increased significantly. When the amount of added sugar increased to 30 g, the number of fruiting bodies increased more than 2 times compared to the control treatment (0g), respectively. It notes that by increasing glucose content up to 50g, the number of shoots of Q1 and Q3 fungal strains decreased. However, the number of shoots in the Q2 strain was still slightly enhanced but was lower than 30g of glucose application (Figure 2). The most appropriate amount of glucose added to the culture medium is 30 g.

Effects of pupa powder content on growth, development, and yield of *C. militaris* mushroom

We have further examined the effects of silkworm pupa powder contents on the growth of *C. militaris* fungus after 60 days of inoculation. In nature, *C. militaris* fungus is parasitic on insects, so its need for natural protein is also quite high. Therefore, we added silkworm pupa powder to optimize the culture environment. Silkworm pupa powder was added to the medium at levels of 1, 2, and 3 g/bottle (Figure 3).

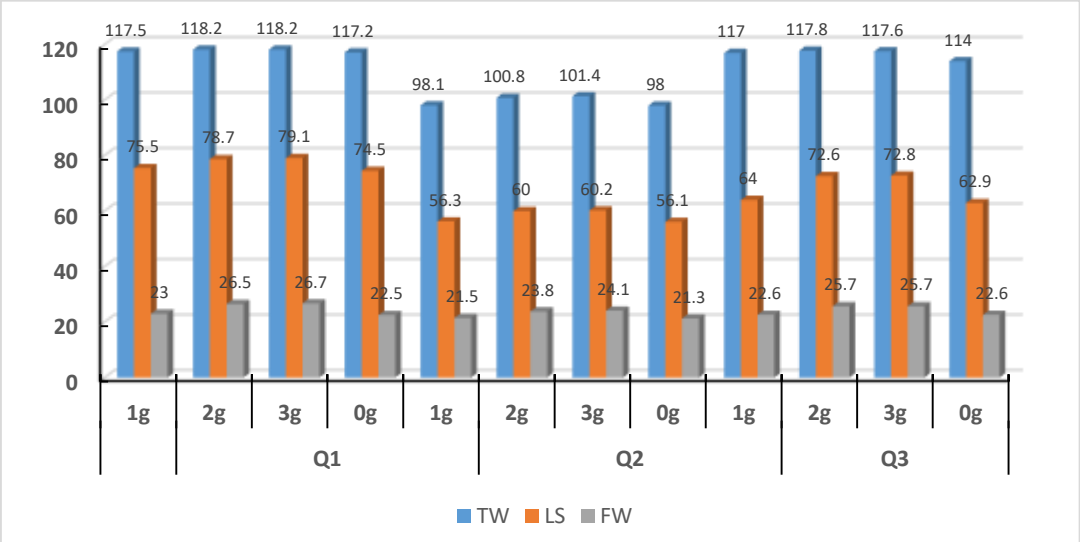

Figure 3. Effects of silkworm pupa powder content on growth of *C. militaris* after 60 days of inoculation. *TW: total weight (g/bottle); LS: fruiting body length (mm); FW: fruiting body weight (g/bottle).*

Figure 3 presents the effects of silkworm pupa powder (SPP) contents on the growth of *C. militaris*, including total weight (g), the length of the fruiting body (LS) and the weight of the fruiting body (FW) after 60 days of inoculation. The results revealed that silkworm pupa powder (1g) caused not much difference compared to the controlled treatment. However, the biomass of 3 strains showed significant change at 2g SPP of application. All parameters of *C.militaris* were also influenced by adding 3g of SPP but were lower than the application of 2g of SPP, respectively. The most effective to enhance the biomass of *C.militaris* was at 2 g of SSP application (Figure 3).

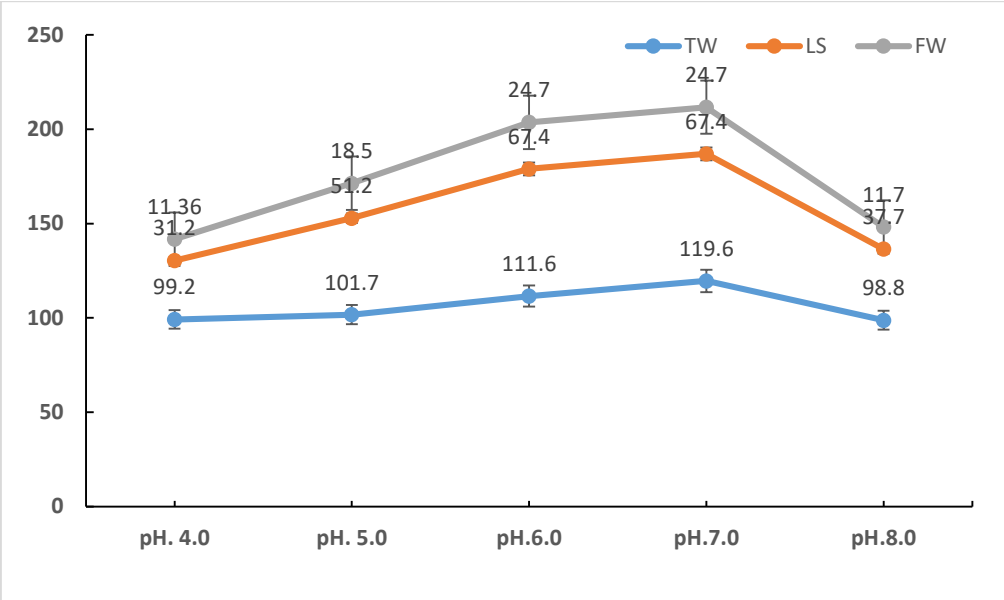

Figure 4. Effects of pH on fruiting bodies of *C. militaris* after 60 days of seeding. *TW: total weight (g/bottle); LS: fruiting body length (mm); FW: fruiting body weight (g/bottle)*

Effect of pH on the growth, development, and productivity of *C. militaris*

The pH is one of the important environmental factors related to the growth and development of fungi because it affects the ability to absorb nutrients. as well as the activity of enzyme interaction in mushrooms. To find the most suitable environmental pH for 3 strains of *C. militaris* fungus, we conducted experiments with environmental formulas with pH from 4.0 to 8.0 as shown in Figure 4. Our results showed that all strains can be grown and developed on the pH of media 4.0 and 8.0. However, the highest total average of biomass of 3 strains (TW, LS, and FW) was at pH 7.0 and followed by 6.0, for instance, the FW value was the highest (24.7g), followed by LS (67.4 mm) and TW (119.6g). However, pH was increased up to 8.0 causing significantly reduced all parameters as shown in Figure 4. Our results were in line with the previous studies, which reported that the great growth of *C.militaris* was at pH range from 6.0 to 7.0 [1]

Effects of temperature on growth, development, and yield of *C. militaris*

Temperature is considered as an important factor in stimulating fruit body formation as well as fruit body shape, length, and yield of *C. militaris*. To determine the influence of room temperature on the formation and development of fruiting bodies, we established 5 different mushroom culture temperatures ranging from 15 to 35°C. As presented in Figure 5, at all examined temperatures of 15°C, 20°C, 25°C and 30°C, TF was a negligible difference which ranged from 18.0 days to 18.7 days, respectively. However, it strikingly noted that the number of shoots on average was remarkably increased at 20°C and 25°C (43.6 and 48.9 shoots/bottle) after 25 days of inoculation, respectively. The optimal temperature should be applied below 20°C to below 25°C for good growth and development of *C. militaris* (Figure 5).

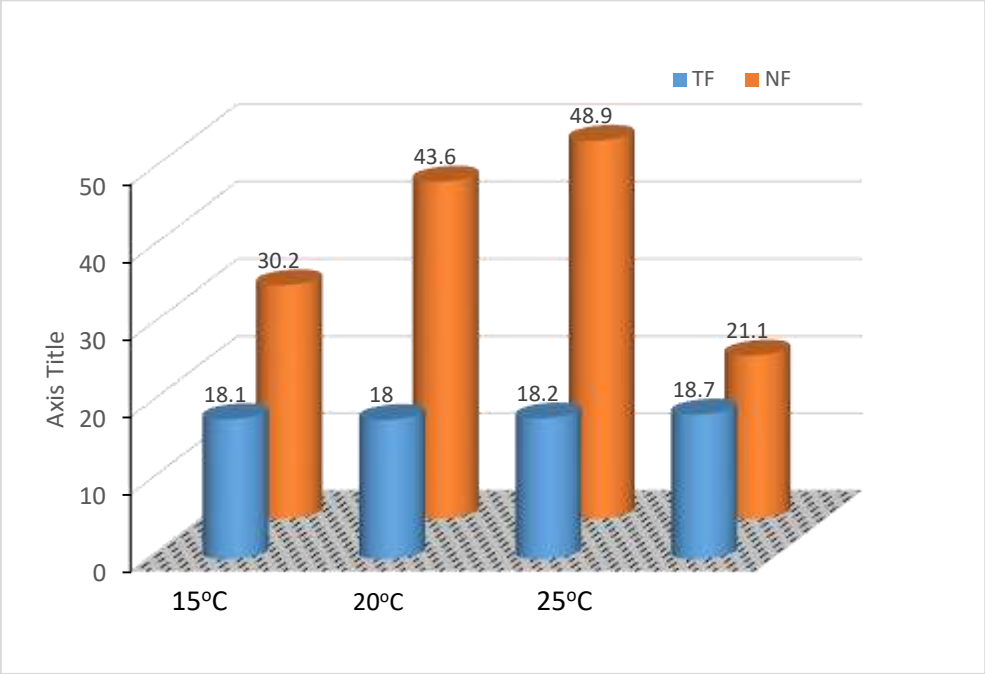

Figure 5. Effect of environmental temperature on the formation and development of the fruiting body of *C. militaris* after 25 days of inoculation. *TF: time of fruiting formation (day); TF: number of shoot/bottle*

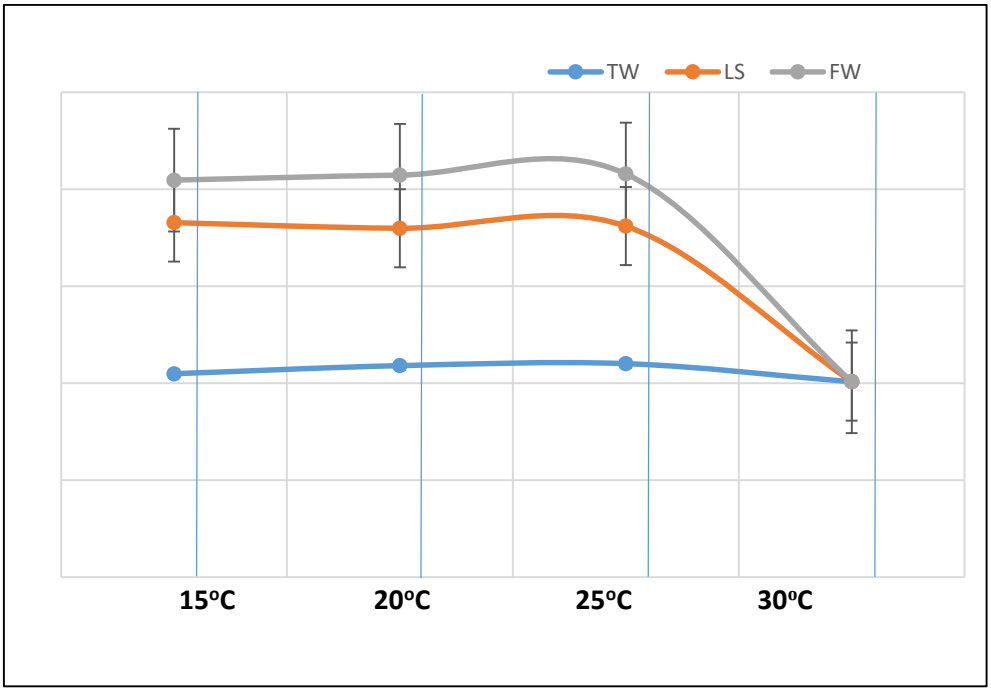

Figure 6. Effects of culture temperature on fruiting bodies *C. militaris* after 60 days of inoculation. *TW: total weight (g/bottle); LS: fruiting body length (mm); FW: fruiting body weight (g/bottle).*

As presented in Figure 6, after 60 days of inoculation, at 15°C, there was a slightly difference in total weight. However, the total average of parameters (TW, LS, and FW) also increased the greatest from 20°C to 25°C. In particular, TW was the highest (110.3 g/bottle), LS (70.9 mm), and FW (26.9g/bottle). Therefore, the most suitable culture temperature for *C. militaris* should be 20°C to below 25°C, respectively.

Effects of light intensity on growth, development, and yield of *C. militaris*

Light is one of the key factors affecting the formation and development of *C. militaris* mushroom fruiting bodies. To determine the influence of lighting intensity on this process, we set up five different lighting formulas with different intensities: 300, 500, 700, 900, and 1,100 lux. (Figure 7).

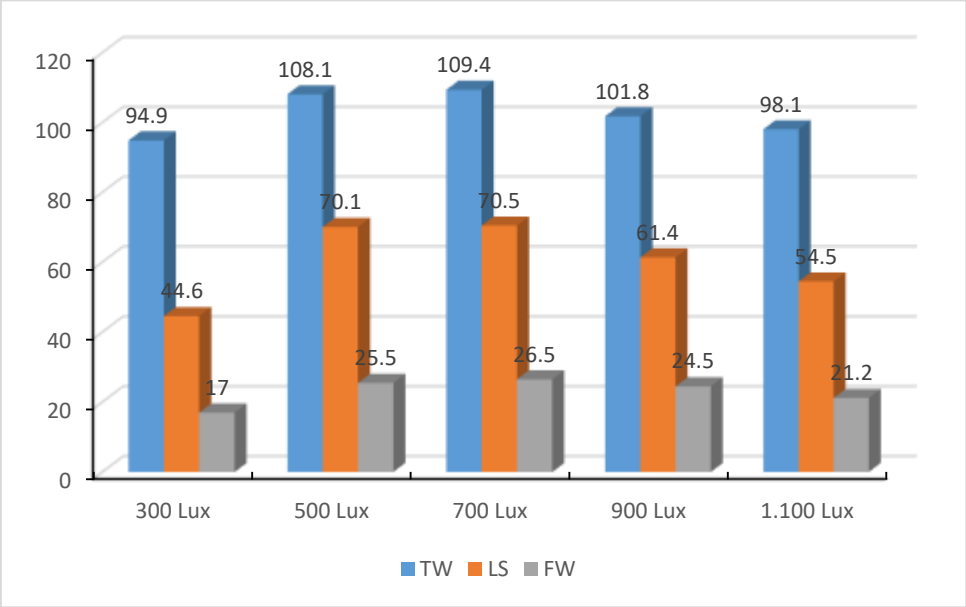

Figure 7. Effects of light intensity on fruiting bodies *C. militaris* fungus after 60 days of inoculation. *TW: total weight (g/bottle); LS: fruiting body length (mm); FW: fruiting body weight (g/bottle).*

As shown in Figure 7, fruiting bodies were formed at all 5 light intensities. The results showed that light intensity from 500 to 700 lux was the most suitable for the growth and development of *C. militaris* fungus. For example, TW was 109.4g/bottle, while at 500 lux TW was 108.1 g/bottle. Similarly, LS was 70.5 mm at 700 lux, and found 70.1 mm at 500 lux, respectively. It notes that at lighting intensities of 300 lux and 1.100 lux, the parameters were significantly reduced when compared with the 500-700 lux application. Thus, the optimal light intensity in *C. militaris* mushroom culture conditions is 500-700 lux. Fruit bodies harvested on time led to good quality and high active ingredient contents. If harvested prematurely, the fruit bodies have not yet fully developed, and the active ingredient content in the fruit bodies has not accumulated, leading to both yield and quality not being high. If harvested too old, the mushroom fruiting bodies will lose water, and become rough, the resulting mushroom mycelium will not be beautiful, and fruiting body productivity will also decrease.

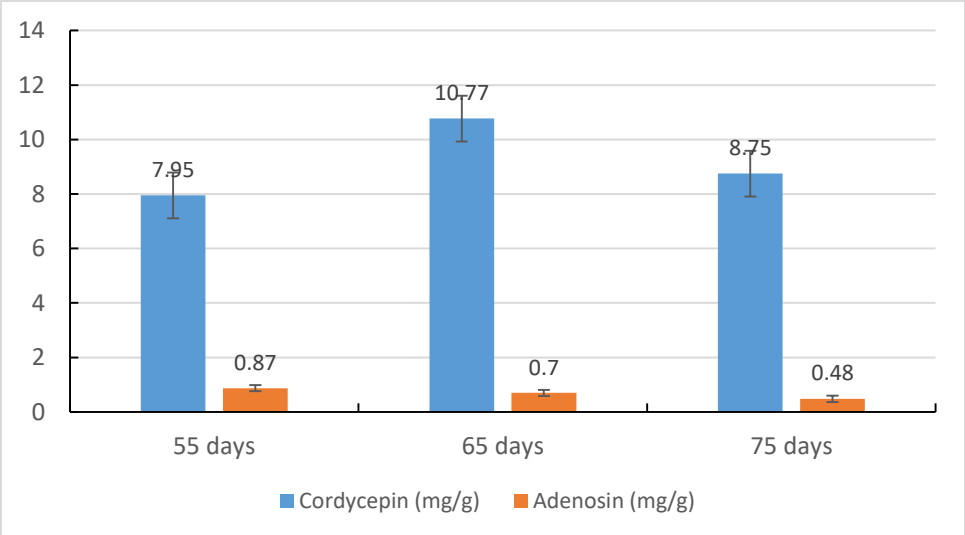

Figure 8. Effects of time harvest on the contents of cordycepin and adenosine

The influence of harvest time on yield and active ingredient content in fruit bodies is shown in Figure 8. The results show that the cordycepin content on average in fruit bodies was the highest when the harvest time was 65 days (10.7 mg/) while at 55 days was 7.95 mg/g and 75 days (8.75mg). Unlike cordycepin, adenosine content in mushroom fruiting bodies gradually decreases with harvest time. The longer the mushroom harvest time, the lower the adenosine content in the fruiting bodies.

Conclusions

In this study, we have identified the optimal culture medium and growth conditions that enhance the biomass and quality of *C. militaris*. Based on our findings, we recommend using brown fragrant rice as a substrate. Additionally, it is beneficial to add glucose (30g) and silkworm pupa powder (2.0 g) to the medium. We found that maintaining a pH of 7.0 and a temperature between 20°C and 25°C, with a lighting intensity of 500-700 lux, promotes better growth. For harvesting, we recommend waiting for 65 days to obtain a higher cordycepin content and 55 days for higher adenosine content.

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