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RESEARCH ON ISOLATION, IDENTIFICATION, AND DETERMINATION OF SUITABLE CONDITIONS FOR THE CULTIVATION OF ALBINO *Cordyceps militaris* SHBTD FRUITING BODIES

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ABSTRACT

Cordyceps militaris is a valuable fungus that is well-known in many Asian countries. The fruiting bodies of *C. militaris* are commonly used in traditional medicine and the production of functional foods. Currently, *C. militaris* mushrooms are being cultivated on a large scale in Vietnam, yielding the characteristic yellow-orange fruiting bodies typical of *C. militaris*. In the mushroom cultivation industry, *C. militaris* is one of the most highly valued species. The yield and quality of the fruiting bodies are greatly dependant on the strain source. Therefore, the search for new strains of *C. militaris* with high productivity, quality, and uniqueness is always of interest to scientists. This study has isolated and identified the albino strain *C. militaris* SHBTD based on morphological characteristics and the rDNA ITS region gene sequence. The optimal conditions for fruiting body formation of the SHBTD strain are MT6 medium, a temperature of 22°C, a humidity of 90%, a light intensity of 500 lux (12 hours/day), and 65 days of cultivation. Additionally, this study determined that the albino strain *C. militaris* SHBDT carrying the single Mating-type *(MAT)* gene (*MAT1-1-1)*, could provide stable yield and quality of fruiting bodies over 5 consecutive cultivation generations. The obtained results demonstrate the potential applications of the *C. militaris* SHBTD in the industrial-scale production of unique and high-value mushroom products.

Keywords: Albino, cordycepin, *Cordyceps militaris*, mating-type (MAT) gene, fruiting body.

INTRODUCTION

The *Cordyceps* genus comprises over 400 species, many of which are considered important medicinal herbs used in traditional medicine in numerous Asian countries. Among them, *Cordyceps militaris* is widely cultivated on a large scale in countries such

as China, South Korea, Japan, Thailand and Vietnam, serving as both food and medicinal ingredients. Chemical analyses have revealed that *C. militaris* contains valuable compounds such as cordycepin, pentostatin, etc (Xia *et al*., 2017). *C. militaris* has been demonstrated to possess various biological activities such as cancer cell inhibition, antifungal, and antiviral properties (Vu *et al*., 2024; Verma, Aggarwal, 2021). Cordycepin - the main active compound in *C. militaris* is currently under research and has shown potential for developing a new drug for treating Covid-19. Cordycepin exhibits a high binding affinity to certain proteins that play crucial roles in the invasion and replication of SARS-CoV-2 in the host. Clinical research results indicated that cordycepin inhibited the replication ability of SARS-CoV-2 by 42-65% after 48 hours of treatment (Verma, Aggarwal, 2021). Therefore, the potential application of *C. militaris* in the future is substantial. The search for new strains of *C. militaris* with high yields, high levels of active ingredients, and unique characteristics is considered the key to successful cultivation of this fungus by commercial enterprises.

The fruiting bodies of *C. militaris* mushrooms typically exhibit yellow to orange coloration owing to the presence of numerous carotenoids. However, some studies have documented albino strains of *C. militaris*, characterized by white fruiting bodies with significantly higher cordycepin content than the original strain (Wang *et al*., 2018). These are promising new strains for research and development of functional foods for human health care. To date, there have been very few publications worldwide on albino strains of *C. militaris*. This may be attributed to the difficulty in collecting these strains, as albino mutant strains are rarely produced during conventional cultivation, making them challenging to obtain.

In Vietnam, most research on *C. militaris* currently focuses on studying diversity and cultivating fruiting bodies. A few studies have delved deeper into genetic characteristics and gene expression in *C. militaris* (Tran Van Tuan, Vu Xuan Tao, 2020). In production facilities, *C. militaris* is commonly cultivated on various scales. However, the quality of the fruiting body products is not consistently high. Furthermore, all cultivation facilities only cultivate typical strains of *C. militaris* with orange-yellow fruiting bodies, leading to a lack of differentiation and uniqueness in the products among these facilities. Overall, there are not many studies addressing albino mutant strains of *C. militaris* in Vietnam. Therefore, the search for *C. militaris* strains not only ensures high yields and active ingredients but also focuses on uniqueness and novelty, which is a matter of research concern. Hence, this study aims to isolate, identify, and determine suitable conditions for cultivating albino mutant strains of *C. militaris* to effectively exploit the genetic resources of albino mutant strains of *C. militaris* in Vietnam.

MATERIALS AND METHODS

Materials

A fresh fruiting body box of *C. militaris* strain TND (with some white-colored fruiting bodies present) was collected from a mushroom production unit in Thai Nguyen. The mushroom sample was preserved in clean nylon bags. After collection, the mushroom sample was promptly transported to the laboratory for isolation. All chemicals used in this study were purchased from

Sigma-Aldrich, Merck, Biobasic. The chemicals were used according to the manufacturer's instructions.

Isolation and determination of morphological characteristics of the albino strain SHBTD

The white-colored mushroom fruiting body sample was separated from the fruiting body box of *C. militaris* strain TND (the separation process ensured that the white-colored samples did not come into contact with the yellow-colored fruiting bodies nearby). After separation, the white mushroom fruiting body sample was cut into small slices and placed on the surface of the Potato Dextrose Agar (PDA) medium supplemented with the antibiotic chloramphenicol to inhibit bacterial growth. Petri dishes containing the white fruiting body samples were then incubated at 22 - 25°C in the dark for 3 days. White fungal mycelia appearing from the sliced fruiting bodies were transferred to PDA medium plates. The isolated mushroom strain was named SHBTD. The SHBTD mushroom strain was directly cultured on sterile microscope slides containing PDA medium for observing the morphology of the fungal mycelium and spore under a light microscope (Tran Van Tuan, Vu Xuan Tao, 2020).

Identification of the albino strain SHBTD based on the rDNA ITS region sequence

The albino strain SHBTD mushroom was cultured in PDB medium (shaken at 150 rpm at 25°C in the dark), and after 4 days, fungal mycelium was harvested for DNA extraction (Tran Van Tuan, Vu Xuan Tao, 2020). Total DNA samples were used as templates for amplifying the ITS region using the primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). The PCR products were electrophoresed and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified products were sequenced by the 1st BASE company. The ITS sequences were compared to data in the GenBank database using the BLAST program. A phylogenetic tree based on the ITS sequences was constructed using the MEGA6 software.

Determination of some optimal conditions for cultivating fruiting bodies of the albino strain SHBTD

The study aimed to determine the influence of factors including nutrient medium, temperature, humidity, light, cultivation time, etc on fruiting body yield. The impact of these factors was investigated independently by varying the factors under study. The appropriate experimental results would be applied to subsequent experiments. The factors under investigation included: *Nutrient medium*: The study evaluated different types of media in a 650 ml cylindrical box containing 30 g of brown rice and 60 ml of nutrient solution. The composition of the nutrient solution in different media was as follows (components per liter of nutrient solution): MT1: The extract solution of 200 g of potatoes, 30 g of glucose, 1 g of $MgSO_4.7H_2O$, 1 g of KH_2PO_4, 1 g of peptone, 100 g of silkworm pupae; MT2: The extract solution of 200 g of potatoes, 30 g of sucrose, 1 g of $MgSO_4.7H_2O$, 1 g of KH_2PO_4, 1 g of peptone, and 100 g of silkworm pupae; MT3: The extract solution of 200 g of potatoes, 30 g of glucose, 1 g of $MgSO_4.7H_2O$, 1 g of KH_2PO_4, 1 g of yeast

extract, 100 g of silkworm pupae; MT4: The extract solution of 200 g of potatoes, 30 g of sucrose, 1 g of $MgSO_4.7H_2O$, 1 g of KH_2PO_4, 1 g of yeast extract, 100 g of silkworm pupae; MT5: The extract solution of 200 g of potatoes, 30 g of glucose, 1 g of $MgSO_4.7H_2O$, 1 g of KH_2PO_4, 1 g of peptone, 1 g of yeast extract, 100 g of silkworm pupae; MT6: The extract solution of 200 g of potatoes, 30 g of sucrose, 1 g of $MgSO_4.7H_2O$, 1 g of KH2PO4, 1 g of peptone, 1 g of yeast extract, 100 g of silkworm pupae; MT7: The extract solution of 200 g of potatoes, 15 g of glucose, 15 g of sucrose, 1 g of $MgSO_4.7H_2O$, 1 g of KH_2PO_4, 1 g of peptone, 1 g of yeast extract, 100 g of silkworm pupae. *Temperature*: After a 7-day mycelial incubation period in the dark at 23 - 25°C, the cultivation boxes were transferred to the fruiting stage at the following temperatures: 20, 22, 25°C. *Humidity*: The fruiting stage was conducted under the following humidity conditions: 70, 80, and 90%. *Light*: The fruiting stage was conducted under a 12-hour/day light condition using white LED lights with intensities of 250, 500, 750, and 1000 lux. *Cultivation time*: Mushroom fruiting bodies were harvested for yield assessment after 55, 65, 75, and 85 days of cultivation (counted from the inoculation time). The monitored parameter was biological efficiency (BE). BE (%) = (dry weight of fruiting bodies/substrate weight) x 100 (Shrestha *et al*., 2012).

Identification of the albino strain SHBTD *MAT1-1-1* and *MAT1-2-1* genes

The DNA of the SHBTD strain was used for PCR amplification of the MAT1-1-1 and MAT1-2-1 genes with specific primer pairs, which were MAT1-1-1-F (5'-ATGGAACACAGATCGAGCGACAC-3')/MAT1-1-1-R (5'-ATATACCTTCGCGATCATTGCCCAG-3') and MAT1-2-1-F (5'-TGTTTTGTCGCGATGGTTCTGG-3')/MAT1-2-1-R (5'-CCTCTGGAGGTTCTGCATTCCA-3') (Kang *et al*., 2017). Identification of the *MAT1-1-1* and *MAT1-2-1* genes was based on the electrophoresis results of the PCR products on a 0.7% agarose gel.

Evaluation of fruiting body yield stability and the content of cordycepin in the fruiting bodies of the albino strain SHBTD

The process of assessing the fruiting body formation ability of the SHBTD strain over 5 consecutive generations was conducted according to a previous study (Vu *et al*., 2023). The fruiting body cultivation procedure was carried out based on the results determining the optimal conditions for cultivating the SHBTD strain identified in the previous experiment. The fruiting bodies obtained in each generation were evaluated for biological efficiency (BE) and cordycepin content. The cordycepin content was determined using high-performance liquid chromatography (HPLC) (Vu *et al*., 2024).

RESULTS AND DISCUSSION

Isolation and identification of the albino strain SHBTD

The white fruiting body sample observed during the cultivation of the *C. militaris* TND strain (Figure 1A) was used for the isolation of the SHBTD albino strain. The sample formed white mycelial colonies after 3 days of cultivation on PDA medium at 25°C (Figure 1B). The fungal mycelia were

further re-incubated on PDA plates for pure culture isolation (Figure 1C). Observation of the morphological characteristics of the SHBTD strain revealed that it developed white mycelial colonies on the PDA medium (Figure 2A). The preliminary results of this study show that the SHBTD strain grows more slowly on PDA medium compared to the TND strain. This may be due to the growth vigor of the SHBTD strain being affected after mutating into an albino strain. The growth and development of the albino strain have been reported to be different from the original strain by Wang *et al*., 2018 (Wang *et al*., 2018). Observed under a microscope, the SHBTD strain's mycelia developed elongatedly to form spore-producing structures, the spores formed were ovoid (Figure 2B). The morphology of the collected fruiting body samples as well as the characteristics of the mycelia and spores, indicate that these exhibit the typical morphological characteristics of *C. militaris* (Zheng *et al*., 2011).

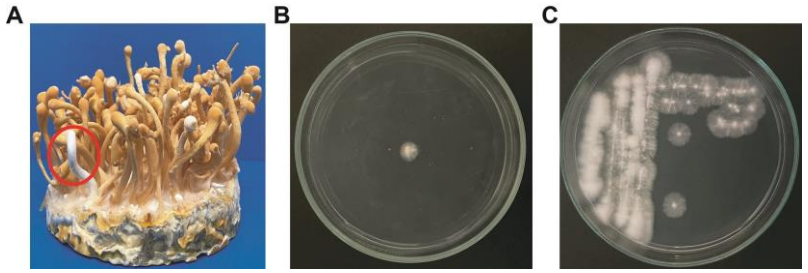

Figure 1. Isolation of the albino strain SHBTD from fruiting body samples of the *C. militaris* TND strain collected in Thai Nguyen. (A) Fruiting body of C. militaris TND collected; (B) Isolation of the SHBTD strain from the white fruiting body sample; (C) SHBTD strain colonies on PDA medium after 7 days. The red circle indicates the portion of the white mushroom specimen used for isolating the SHBTD strain.

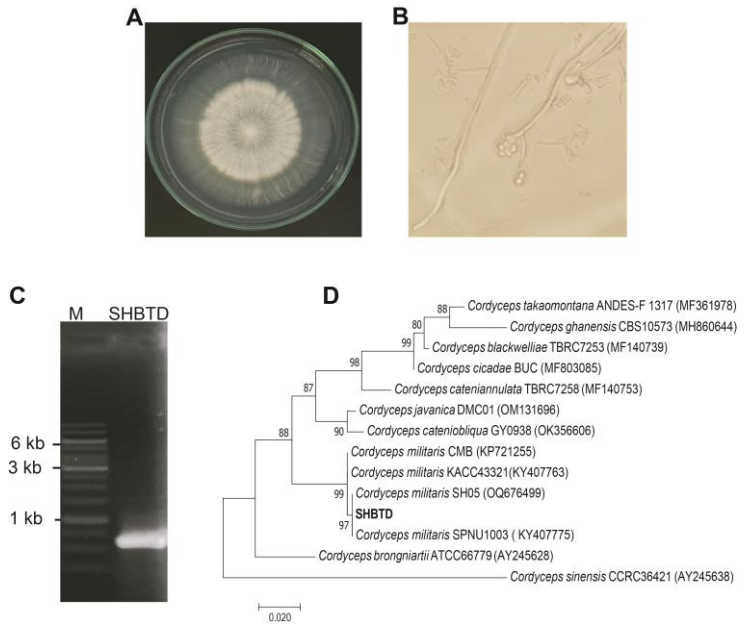

Figure 2. Identification of the SHBTD strain based on morphological characteristics and the rDNA ITS region sequence. (A) Mycelial morphology of the SHBTD strain on PDA medium after 15 days; (B) Mycelial structure and spore-producing structure under a microscope (40x); (C) Analysis of the

PCR products of the ITS on a 0.7% agarose gel; (D) The phylogenetic tree was constructed with MEGA6 using the neighbor-joining method and 1000 bootstrap replications; M: DNA marker 1 kb (Thermo Fisher Scientific, USA).

To accurately identify the species, the total DNA of the SHBTD strain was used for PCR amplification of the ITS region using the specific primer pair ITS1/ITS4. The PCR product analysis showed the appearance of a DNA band approximately 550 bp in size (Figure 2C). Analysis and comparison of the ITS sequence of the SHBTD strain using the BLAST program revealed that the ITS sequence obtained exhibited 99.6 - 100% similarity with the ITS sequences of various *C. militaris* strains in the GenBank database. Additionally, the phylogenetic tree constructed using MEGA6 software based on the ITS sequence confirmed that the SHBTD strain belongs to the species *C. militaris* (Figure 2D).

Some suitable conditions for cultivating the fruiting bodies of the albino strain *C. militaris* SHBTD

Mutant fungal strains often have different growth rates compared to the original strain. Therefore, to use the albino strain in fruiting body production, it is necessary to determine the appropriate conditions for the formation of fruiting bodies for this strain. Factors such as nutrient medium, temperature, humidity, and light intensity are important in the process of cultivating the fruiting bodies of *C. militaris* (Feng *et al.*, 2018). Research results have shown that the MT6 medium, a temperature of 22°C, a humidity of 90%, a light intensity of 500 lux (12 hours/day), and a cultivation period of 65 days are suitable conditions for cultivating the fruiting bodies of the albino strain *C. militaris* SHBTD (Figure 3). These are also suitable conditions for cultivating the fruiting bodies of the TND strain, but the cultivation time for the TND strain is shorter, specifically 55 days. Overall, these conditions are also commonly used for cultivating the fruiting bodies of *C. militaris* strains (Vu *et al*., 2023; Vu *et al*., 2024). Under these optimal conditions, the SHBTD strain yielded approximately 22 g of fresh fruiting bodies per 650 ml cultivation box, corresponding to a biological efficiency of 8.9%. The biological efficiency of the *C. militaris* SHBTD strain is lower than that of the TND strain (9.5%), but it is comparable to some reported strains such as *C. militaris* SH05 and DL01. (Vu *et al*., 2024). The study by Wang et al. (2018) showed that the albino *C. militaris* 505 strain could form fruiting bodies, but the study did not address the fruiting body yield of the 505 strain (Wang *et al*., 2018). Thus, this study has provided some suitable conditions for cultivating the fruiting bodies of the albino *C. militaris* strain. These parameters are crucial for developing a cultivation process for the SHBTD strain and integrating it into practical production.

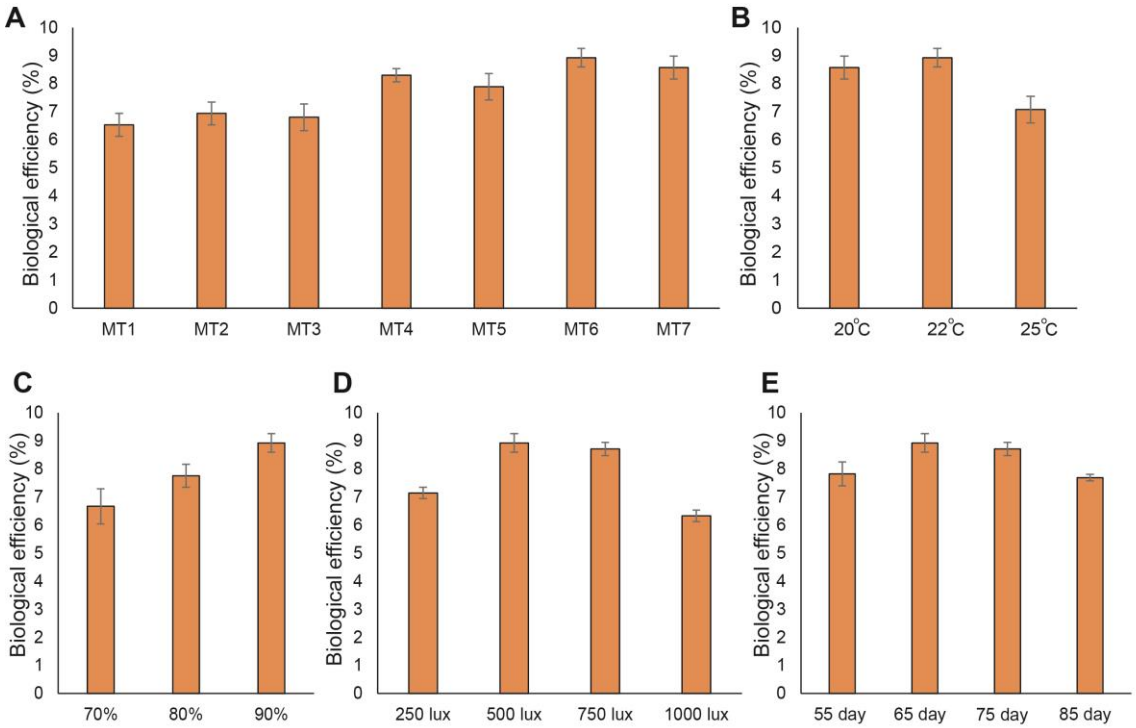

Figure 3. Influence of some factors on the yield of fruiting bodies of the albino strain *C. militaris* SHBTD. (A) Nutrient medium; (B) Temperature; (C) Humidity; (D) Light intensity; (E) Cultivation time.

Determination of the mating-type gene, evaluating the stability of fruiting body yield, and content of the cordycepin in the fruiting bodies of the SHBTD strain

The cultivation of *C. militaris* is currently facing rapid degeneration of strains, manifested by a significant decrease in fruiting body yield and cordycepin content over successive generations, posing significant challenges to the mushroom farming industry. The two *MAT1-1-1* and *MAT1-2-1* genes have been identified to play crucial roles in fruiting body formation and degeneration in *C. militaris* (Vu *et al*., 2023). However, there has been no study addressing these two genes in albino strains of *C. militaris*.

The PCR results for identifying the *MAT1-1-1* and *MAT1-2-1* genes in the albino strain *C. militaris* SHBTD showed that the SHBDT strain only carries the MAT1-1-1 gene (Figure 4). According to a previous study, *C. militaris* strains carrying only the *MAT1-1-1* gene have a better ability to form stable fruiting bodies compared to strains carrying both the *MAT1-1-1* and *MAT1-2-1* genes (Vu *et al*., 2023). However, to assess the SHBTD strain specifically and accurately, this study evaluated the fruiting body formation capability and the cordycepin content in the fruiting bodies of the SHBTD strain over 5 consecutive generations. The research results indicated that the fruiting body formation capability and the cordycepin content in the fruiting bodies of the albino strain *C. militaris* SHBTD did not vary significantly over the 5 generations (Figure 5). Specifically, the fruiting body yield in the first generation was

8.9%, and after 5 generations of cultivation, the fruiting body yield was 8.02%. Similarly, the cordycepin content in the fruiting bodies was 5.88 mg/g dry fruiting body in the first generation and 5.1 mg/g dry fruiting body after 5 generations of cultivation. Studies on degeneration in *C. militaris* indicated that the fungus began to degenerate when transferred to the third generation, severe degeneration appeared in the fourth generation, and by the fifth generation, the strain might lose its ability to produce fruiting bodies (Vu *et al*., 2023). Meanwhile, the yield and cordycepin content in the fruiting bodies of the albino strain *C. militaris* SHBTD remained stable with little variation over 5 generations. This is the first study on the stability of yield and fruiting body quality of the albino strain of *C. militaris*. Thus, the albino strain *C. militaris* SHBTD holds high potential for industrial-scale production.

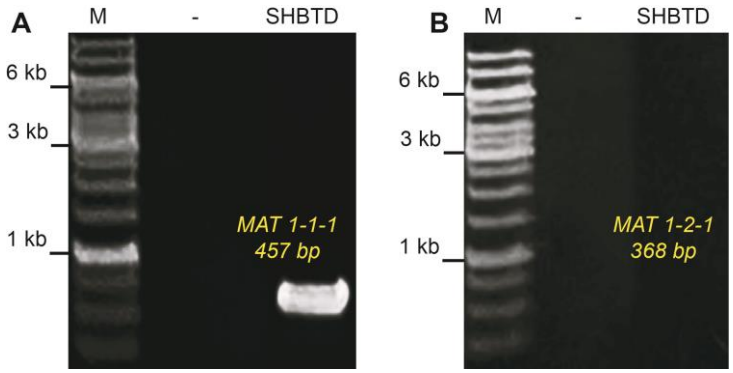

Figure 4. Identification of *MAT1-1-1* and *MAT1-2-1* genes of the albino strain *C. militaris* SHBTD**.** (A) Analysis of the PCR products of the *MAT1-1-1* on a 0.7% agarose gel; (B) Analysis of the PCR products of the *MAT1-2-1* on a 0.7% agarose gel; M: DNA marker 1 kb (Thermo Fisher Scientific, USA).

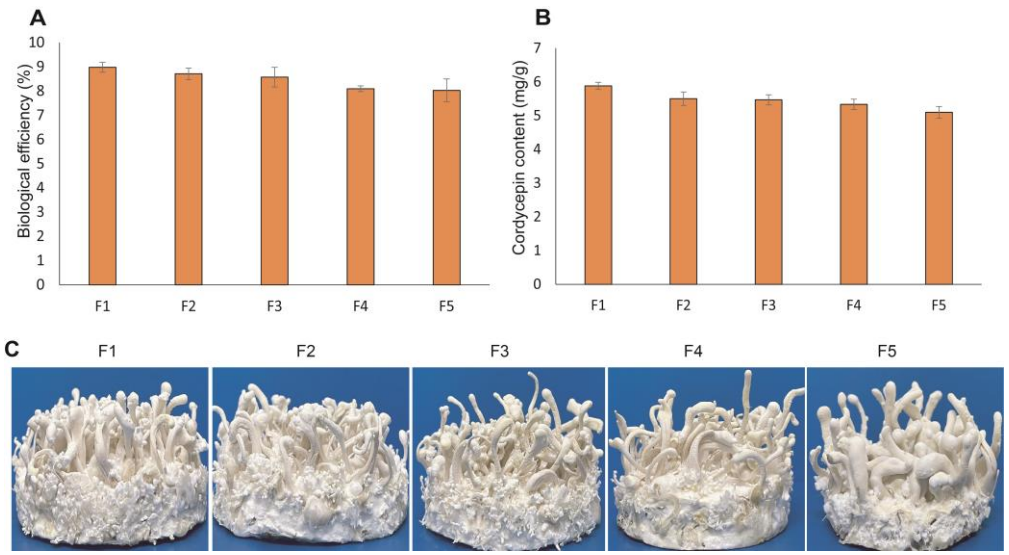

Figure 5. Fruiting body yield and cordycepin content in the fruiting bodies of the albino strain *C. militaris* SHBTD over 5 generations. (A) Biological efficiency; (B) Cordycepin content in the fruiting bodies; (C) Fruiting bodies over 5 consecutive generations.

CONCLUSION

This study successfully isolated and identified the albino strain *C. militaris* SHBTD based on morphological characteristics and the rDNA ITS region gene sequence. The optimal conditions for fruiting body formation of the *C. militaris* SHBTD strain are MT6 medium, a temperature of 22°C, a humidity of 90%, a light intensity of 500 lux (12 hours/day), and a cultivation period of 65 days. Additionally, this study determined that the albino strain *C. militaris* SHBDT, carrying the single mating-type gene (*MAT1-1-1*), could provide stable yield and quality of fruiting bodies over 5 consecutive cultivation generations. These findings suggest that the *C. militaris* SHBTD strain has the potential for industrial-scale production.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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