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Cryopreservation of *Cordyceps militaris* (L.) link using a vitrification method

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Abstract

Cordyceps militaris (L.) link has been traditionally consumed as an edible and medicinal fungus due to its pharmacological potential. *C. militaris* is widely cultivated on artificial mediums, but their growth and production are usually affected by excessive sub-culturing. The aim of this study was to investigate the effects of preservation conditions, including the concentrations of cryoprotectants, freezing temperatures, and storage times, on the colony diameters and the fruiting body production of *C. militaris*. The results indicated that at the freezing temperature of -196 ˚C, the fruiting bodies of *C. militaris* could be stored for 6 months. After 3 weeks of regrowth, the highest recovery percentage of cryopreserved fruiting bodies (100%) and mycelial discs (100%) were obtained after the samples had been exposed to 10% glycerol at -196 ˚C. Moreover, the development of fruiting bodies from the fungal preservation under the optimized conditions had been able to be completely induced. This study has established a simple and efficient preservation method for *C. militaris*.

Keywords: *Cordyceps militaris*, Cryoprotectants, Cryopreservation, Freezing temperature

1. Introduction

Cordyceps militaris (L.) Link is an entomopathogenic fungus. Many of its bioactive compounds, including cordycepin, adenosine, and polysaccharides, have multifunctional pharmacological effects. The chemical components are considered to promote human biological functions. Previous reports have revealed that the bioactive compounds of *C. militaris* exhibit strong antioxidant activity, and can prevent coronary heart disease, reduce blood cholesterol, and can decrease the risk of certain types of cancer [1-4]. In general, *C. militaris* has been consumed as a traditional Chinese medicinal fungus. Potentially, it is an economically efficient fungus with great possibilities for both the food and pharmaceutical industries. However, the ability and quality of the production of the fruiting bodies have normally been affected by repeated sub-culturing, degeneration, irregular mycelial growth, and the abnormal development of fruiting bodies [5,6]. The degeneration of fungal strains is a common phenomenon that occurs during the process of subculture and preservation. In order to solve this problem, the preservation of elite clones for commercial trade requires the development of a long-term storage protocol, which reduces the appearance of abnormalities in fruiting body production. Suitable research into long-term preservation enhances the underlying physical and biological factors that can affect the ability to produce fruiting bodies and that can allow for the establishment of stable sample stocks for commercial production. The successful cryopreservation of fungi has been gradually achieved through the use of this delicate method. In particular, many factors, including the types of samples, the concentrations of the cryoprotectants, and the preservation temperatures, need to be optimized. Cryopreservation is an ideal process for long-term storage under freezing temperatures. There are several advantages of cryopreservation, which include genetic stability, non-contamination, minimal space requirements, and less labor maintenance [7,8]. Cryoprotectants are known as the chemical substances that are used to protect the cell from ice formation. The formation of ice crystals during cryopreservation is harmful to the integrity of the cellular structure and causes physical damage to the fungal cells. The most popular cryoprotectants are glycerol, followed by dimethyl sulfoxide, glucose, and sucrose. Glycerol is an effective cryoprotectant in fungal cryopreservation that differs

from dimethyl sulfoxide, which is toxic to cells. Furthermore, glycerol has the property to decrease the solute concentrations in the fungal cells. Their protective effects are represented by colligative properties and the reduction of the freezing point temperature, which avoid ice formation and cryoinjury. Furthermore, freezing temperatures are also necessary factors, which can have a direct effect upon fungal cryopreservation [9-11]. However, the development of a rapid and efficient cryopreservation method for *C. militaris* is critical to the process of fungal conservation. In this study, the influence of preservation temperatures on fungal growth was estimated. The temperature range, which was normally used, was from -20 ˚C to -196 ˚C. The aim of this study was to establish a simple and reproducible long-term storage protocol for *C. militaris*. Therefore, the cryoprotectant concentrations, storage times, and freezing temperatures were all optimized.

2. Materials and methods

2.1 Cryopreservation

2.1.1 Materials preparation and culture conditions

C. militaris was obtained from the Agricultural Science Program of Mahidol University in Kanchanaburi. The potato dextrose agar medium (PDA) was prepared for mycelial culture. *C. militaris* was cultured on petri dishes (90 mm x 25 mm) and incubated at 20 ± 2 ˚C under dark condition for 3 weeks.

2.1.2 Cryopreservation experiments

The mycelial discs and fruiting bodies of *C. militaris* were used for cryopreservation. Three pieces of mycelial discs (0.5 cm diameter) and fruiting bodies (1 cm length) were transferred to 1.8 mL cryotubes (Nunc, USA), which contained different concentrations of cryoprotectants (10% , 15% , and 20% glycerol). The cryotubes were stored at different freezing temperatures at -20 ˚C, -80 ˚C, and -196 ˚C (liquid nitrogen) for periods of 1, 3, and 6 months. After that, the cryotubes were thawed in a water bath at 40 ˚C for 2 min. The glycerol was removed immediately, and the samples were then washed twice with sterile distilled water under sterile conditions. For recovery, the samples were cultured on PDA media for 3 weeks under dark conditions. This step was followed by inducing the fruiting bodies on a modified potato medium (30 g rice, 10 g/L glucose, 10 g/L yeast extract, 1 g/L KH_2PO_4, 1 g/L $MgSO_4$, 1 g/L Vitamin B_1, and 1000 mL potato water) for 8 weeks with a photoperiod of 16 h light/ 8 h dark. After 3 weeks of culture, the recovery percentages and colony diameters were measured. In addition, the length of fruiting bodies (cm), the fresh weight (g/bottle), and the dry weight (g/bottle), as well as the cordycepin (mg/g) and adenosine contents (mg/g), were all recorded after 8 weeks of culture.

2.2 HPLC analysis

2.2.1 Sample preparation

One gram of dried *C. militaris* powder was extracted with 10 ml 50% (v/v) methanol in a 50 mL centrifuge tube. After that, the centrifuge tube was placed in an ultrasonic cleaner at 125 watts for 30 min. The supernatant was separated by centrifugation at 9,900 rpm for 10 min and was then filtered through a 0.45 µm membrane filter. The extractions of the fruiting bodies were assayed in order to determine the cordycepin and adenosine contents following Huang et al. (2009) [12].

2.2.2 Chromatograph conditions

To measure the cordycepin and adenosine contents, the HPLC system was equipped with a C_{18} analytical column (Inertsil ODS-4). The sample solutions were detected using a UV Visible Detector (Shimadzu LC-20A) at 254 nm. The mobile phase was performed by using 85: 15 (V/ V) deionized water and methanol. The separation was conducted at a flow rate of 1.0 mL/min. The injection volume was 20 µL. The cordycepin and adenosine standards were purchased from Sigma Chemical Corporation.

2.3 Statistical analysis

One-way analysis of variance (ANOVA) was conducted along with Duncan's multiple range tests to compare the mean significant differences ($P < 0.05$) among the treatments using the SPSS program version 22.0. All of the experiments were repeated three times with six replicates.

3. Results

3.1 Regrowth, recovery percentages, and the colony diameters of C. Militaris

The effects of fungal preservation with the cryoprotectants under different freezing temperatures were evaluated by assessing the ability for mycelial formation and for fruiting body production in *C. militaris*. To determine the maximum mycelial regrowth, the recovery percentages and colony diameters were investigated (Table 1-4). It was noted that those samples stored in the freezing temperature of -196 ˚C for 6 months had achieved the highest recovery percentage (100%) in both mycelial discs and fruiting bodies. The recovery percentage of the cryopreserved mycelial discs and fruiting bodies were found to be higher than the control. The results showed that the freezing temperature of -196 ˚C had exhibited the best colony diameter after regrowth. Additionally, the freezing temperature at -80 ˚C offered a challenge to the cryopreservation of *C. Militaris* since that temperature had resulted in cryoinjuries caused by ice formation. In the same way, cryopreservation at -20 ˚C had also been found to have decreased the recovery percentage.

Table 1 Effects of preservation condition on colony diameters of cryopreserved mycerial discs.

Treatments	Glycerol concentration	Freezing temperature	Recovery percentage of mycerial discs (cm)		
			1 month	3 months	6 months
T1	control	-20 ˚C	66.67%	0.00%	0.00%
T2	10 %		100.00%	100.00%	0.00%
T3	15 %		0.00%	0.00%	0.00%
T4	20 %		0.00%	0.00%	0.00%
T5	control	-80 ˚C	0.00%	0.00%	0.00%
T6	10 %		100.00%	100.00%	83.33%
T7	15 %		100.00%	100.00%	0.00%
T8	20 %		100.00%	83.33%	83.33%
T9	control	-196 ˚C	0.00%	0.00%	0.00%
T10	10 %		100.00%	100.00%	100.00%
T11	15 %		100.00%	100.00%	100.00%
T12	20 %		100.00%	83.33%	100.00%

*****Each value is expressed as mean ± SD (n = 6). Means with different letters within column are significantly different ($P < 0.05$).

Table 2 Effects of preservation condition on colony diameters of cryopreserved fruiting bodies.

Treatments	Glycerol concentration	Freezing temperature	Recovery percentage of fruiting bodies (cm)		
			1 month	3 months	6 months
T1	control	-20 ˚C	50.00%	0.00%	0.00%
T2	10 %		83.33%	66.67%	83.33%
T3	15 %		100.00%	83.33%	0.00%
T4	20 %		100.00%	0.00%	16.67%
T5	control	-80 ˚C	0.00%	0.00%	0.00%
T6	10 %		100.00%	100.00%	66.67%
T7	15 %		66.67%	100.00%	66.67%
T8	20 %		100.00%	83.33%	100.00%
T9	control	-196 ˚C	0.00%	0.00%	0.00%
T10	10 %		83.33%	100.00%	100.00%
T11	15 %		100.00%	100.00%	100.00%
T12	20 %		66.67%	100.00%	100.00%

*****Each value is expressed as mean ± SD (n = 6). Means with different letters within column are significantly different ($P < 0.05$).

Table 3 Effects of preservation condition on colony diameters of cryopreserved mycerial discs.

Treatments	Glycerol concentration	Freezing temperature	Colony diameters of mycerial discs (cm)		
			1 month	3 months	6 months
T1	control	-20 ˚C	4.21±0.97^{c}	0.00±0.00^{e}	0.00±0.00^{c}
T2	10 %		5.57±0.39^{b}	5.57±0.97^{c}	0.00±0.00^{c}
T3	15 %		0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{c}
T4	20 %		0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{c}
T5	control	-80 ˚C	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{c}
T6	10 %		7.16±0.23^{a}	6.86±0.37^{ab}	5.61±0.90^{b}
T7	15 %		5.92±1.15^{b}	5.96±0.48^{bc}	0.00±0.00^{c}
T8	20 %		6.15±0.26^{b}	2.10±0.88^{d}	5.78±0.86^{b}
T9	control	-196 ˚C	0.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{c}
T10	10 %		7.27±0.16^{a}	7.25±0.55^{a}	7.84±0.33^{a}
T11	15 %		7.13±0.14^{a}	6.88±0.16^{ab}	7.53±0.31^{a}
T12	20 %		6.11±0.89^{b}	6.92±0.33^{ab}	7.60±0.17^{a}

*****Each value is expressed as mean ± SD (n = 6). Means with different letters within column are significantly different ($P < 0.05$).

Table 4 Effects of preservation condition on colony diameters of cryopreserved fruiting bodies.

Treatments	Glycerol concentration	Freezing temperature	Colony diameters of fruiting bodies (cm)		
			1 month	3 months	6 months
T1	control	-20 ˚C	1.49±0.70^{b}	0.00±0.00^{d}	0.00±0.00^{e}
T2	10 %		4.73±0.43^{a}	5.23±0.10^{ab}	4.70±0.83^{cd}
T3	15 %		3.80±0.62^{a}	3.25±0.65^{c}	0.00±0.00^{e}
T4	20 %		4.51±0.44^{a}	0.00±0.00^{d}	0.44±0.89^{e}
T5	control	-80 ˚C	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{e}
T6	10 %		4.90±0.89^{a}	4.83±0.49^{b}	5.85±0.92^{b}
T7	15 %		4.95±0.17^{a}	5.33±0.23^{ab}	5.82±0.50^{b}
T8	20 %		4.43±0.77^{a}	6.04±0.34^{a}	4.06±0.43^{d}
T9	control	-196 ˚C	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{e}
T10	10 %		5.22±0.19^{a}	6.17±0.40^{a}	7.29±0.44^{a}
T11	15 %		4.55±0.57^{a}	4.48±0.98^{b}	6.99±0.19^{a}
T12	20 %		4.98±0.39^{a}	6.04±0.41^{a}	5.18±0.37^{bc}

*****Each value is expressed as mean ± SD (n = 6). Means with different letters within column are significantly different ($P < 0.05$).

In order to optimize the favorable conditions for preservation, *C. militaris* was stored at different freezing temperatures with various concentrations of glycerol. The optimal temperature for long-term storage was determined to be -196 ˚C, while the appropriate concentration of cryoprotectants was found after the samples had been stored in 10% glycerol. The effects of the preservation conditions on the colony diameters are shown in Figure 1. The colony diameters of the cryopreserved mycelial discs at -196 ˚C were better than those, which had been stored at the freezing temperature of -20 ˚C. The results demonstrated that a low concentration of glycerol had enhanced the development of colony diameters. In comparison with other treatments, treatment T10 stored with 10% glycerol at -196 ˚C was found to be the best preservation condition. The average colony diameters had slightly increased and ranged from 7.27±0.16 cm to 7.84±0.33 cm, which indicated a significant difference when compared to the other treatments.

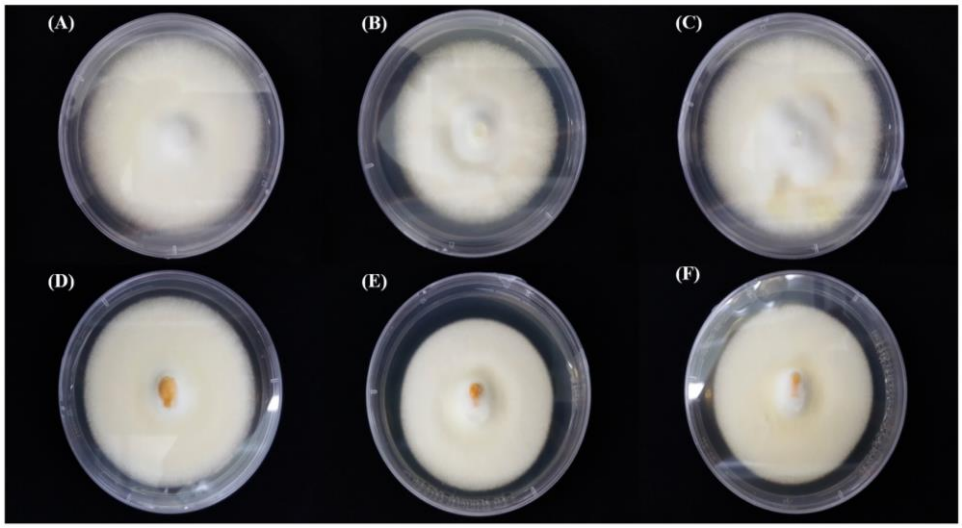

Figure 1 The mycelial growth after preservation. (A) cryopreserved mycerial discs for 1 month, (B) cryopreserved mycerial discs for 3 months, (C) cryopreserved mycerial discs for 6 months, (D) cryopreserved fruiting bodies for 1 month, (E) cryopreserved fruiting bodies for 3 months, (F) cryopreserved fruiting bodies for 6 months. Scale bar = 3 cm.

In the same way, it was found that the colony diameters of the cryopreserved fruiting bodies, which had been stored in liquid nitrogen at -196 ˚C, also grew better than those, which had been stored at -20 ˚C. The average colony diameters had shown a considerably increased range from 5.22±0.19 cm to 7.29±0.44 cm. The results indicated that the highest colony diameter (7.29±0.44 cm) had been achieved at -196 ˚C. In contrast, the cryopreserved fruiting bodies stored at -20 ˚C had suffered from contamination. In conclusion, results from treatment T10, which had been stored with 10% glycerol and kept at -196 ˚C, were found to be significantly better than the other treatments.

3.2 Fruiting body induction after preservation

Based on the results mentioned above, treatment T10 stored with 10% glycerol at -196 ˚C from 1, 3 and 6 months was selected as a suitable treatment for further study. To confirm the morphology of *C. militaris* after preservation, the fruiting bodies were induced on the artificial medium for 8 weeks as shown in Figure 2. The growth parameters and bioactive components of the fruiting bodies were recorded (Table 5,6). The results revealed that after cryopreserving the mycelial discs, the fruiting body production had shown the longest fruiting bodies (5.31±0.07 cm), the highest fresh weight (12.54±0.06 g/bottle), and the highest dry weight (3.21±0.13 g/bottle) after 1 month of storage. Investigating the bioactive components was also of interest. Therefore, the cordycepin and adenosine contents were also measured. It was found that adenosine contents had been significantly enhanced by increasing the storage time to 6 months. The maximum adenosine contents (0.3003±0.0081 mg/g) and cordycepin contents (2.3136±0.0276 mg/g) had been obtained after a storage time of 1 month. Conversely, after cryopreserving the fruiting bodies, the fruiting body production showed the longest fruiting bodies (5.88±0.03 cm), the highest fresh weight (11.24±0.26 g/bottle), and the highest dry weight (2.79±0.04 g/bottle) after storage for 6 months. As shown in Table 6, the maximum adenosine (0.2483±0.0213 mg/g) and cordycepin contents (2.7902±0.0595 mg/g) had been found after 1 month of storage. After 6 months of preservation, the cryopreserved *C. militaris* with the cryoprotectant solution had produced well-developed fruiting bodies, while the fungus preserved without protective agents had shown a lower abundance and undeveloped fruiting bodies.

Table 5 Fruiting body production after cryopreserved mycerial discs.

	length of fruiting bodies (cm)	fresh weight (g/bottle)	dry weight (g/bottle)	adenosine contents (mg/g)	cordycepin contents (mg/g)
1 month	5.31±0.07^{a}	12.54±0.06^{a}	3.21±0.13^{a}	0.2520±0.0157^{b}	2.3136±0.0276^{a}
3 months	4.92±0.13^{b}	12.01±0.15^{b}	2.98±0.11^{a}	0.2306±0.0229^{b}	2.1202±0.1160^{b}
6 months	4.82±0.15^{b}	11.20±0.17^{c}	3.15±0.12^{a}	0.3003±0.0081^{a}	2.2907±0.0937^{ab}

*****Each value is expressed as mean ± SD (n = 6). Means with different letters within column are significantly different (P < 0.05).

Table 6 Fruiting body production after cryopreserved fruiting bodies.

	length of fruiting bodies (cm)	fresh weight (g/bottle)	dry weight (g/bottle)	adenosine contents (mg/g)	cordycepin contents (mg/g)
1 month	4.29±0.08[b]	10.74±0.06[b]	1.86±0.05[c]	0.2483±0.0213[a]	2.7902±0.0595[a]
3 months	4.37±0.04[b]	10.48±0.04[b]	2.41±0.04[b]	0.2321±0.0173[a]	2.6653±0.0332[a]
6 months	5.88±0.03[a]	11.24±0.26[a]	2.79±0.04[a]	0.2191±0.0158[a]	2.7048±0.1350[a]

*****Each value is expressed as mean ± SD (n = 6). Means with different letters within column are significantly different (P < 0.05).

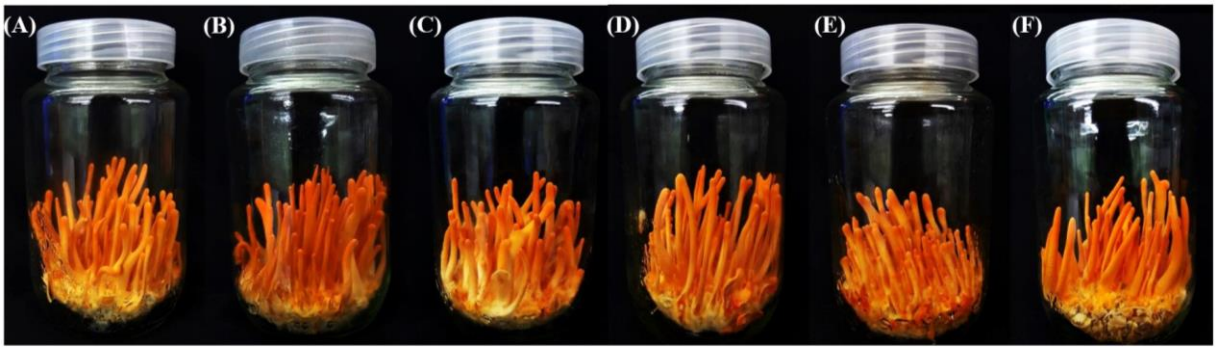

Figure 2 Fruiting body production of *C. militaris* after cryoprevation. (A) cryopreserved mycerial discs for 1 month, (B) cryopreserved mycerial discs for 3 months, (C) cryopreserved mycerial discs for 6 months, (D) cryopreserved fruiting bodies for 1 month, (E) cryopreserved fruiting bodies for 3 months, (F) cryopreserved fruiting bodies for 6 months. Scale bar = 2 cm.

4. Discussion

Cryopreservation, which is the most effective method for long-term preservation at ultra-low temperatures, provides great value for stable storage and prevents the unfavorable effects of culture periods, which include the risk of contamination, the loss of developing proficiency, and genetic modifications [13]. In storage conditions, there is no risk of new contamination by fungi or by bacteria, and cryogenically stored material has been reported to retain its genetic stability. For successful preservation, it is necessary to avoid the lethal intracellular freezing that occurs during the rapid cooling in liquid nitrogen. Most fungal cells have high quantities of water and are extremely sensitive to freezing temperatures. Therefore, their cells should be dehydrated with cryoprotectants to avoid the formation of ice crystals. However, due to the high concentration of internal solutes and protein denaturation, extreme desiccation also produces damage to the cell membrane and is harmful to the cell compartments [14]. The preservation technique can be categorized procedures, which require only a small area, are non-laborious, and which provide a pathogen-free germplasm. Additionally, this technique is an efficient method for long-time conservation. Normally, the freezing temperatures, ranging from -20 ˚C to -196 ˚C, were investigated with different concentrations of cryoprotectants and different cryopreservation times [15, 16]. In this study, the results revealed that those samples, which were stored at -196 ˚C, had shown that -196 ˚C was the most suitable temperature for cryopreservation. This obtained result was supported by findings from a study by Miguel et al. (2017), who noted that cryopreservation at -196 ˚C had been proposed as the best method to preserve entomopathogenic fungi (EPF) species, including fungi of the genera *Beauveria, Metarhizium, Nomuraea, Paecilomyces,* and *Verticillium* [17].

Cryoprotectants are known as the chemical substances that are used to protect cells from undergoing ice formation. Cryoprotectants mainly dehydrate the water potential gradient between the inside and the outside of the fungal cells. Cryoprotectants can help to avoid the phase change from liquid to crystalline and can prevent freezing injury. During cryopreservation, the formation of ice crystals is harmful to cellular structure and causes physical damage to the cells. Therefore, air-drying, dehydration, and the addition of cryoprotectants are processed as cryogenic strategies. Cryoprotectants are utilized to reduce cryoinjuries during freezing and cryopreservation. Different cryoprotectants are utilized in cryopreservation. Glycerol is one of the most effective cryoprotectants in fungal cryopreservation. The concentration of 5-15% glycerol showed greater mycelial viability. Furthermore, the mycelial viability was verified at greater than 75% after preservation [18]. The results of our study differed from the work of Tanaka et al. (2013), who reported that performing

cryopreservation at -70 ˚C and using 5% glycerol had been effective for preserving *Agaricus blazei*, while cryopreservation at -20 ˚C had been ineffective in preserving fungal viability [19]. In the same way, the slow freezing process from 8 to -80 ˚C in 10% glycerol with *A. blazei* was found to be effective, whereas the fast freezing, from 8 to -196 ˚C had been ineffective [20]. Previous reports revealed that the preservation periods had been based on the freezing temperatures and the protective agents. The mycelial formation percentage and the development of fruiting bodies from the fungal storage were optimized in order to determine the suitable preservation conditions. Previous reports indicated that storage at 4 ˚C had maintained the fungal vitality and the fruiting body production [21], the results of which stand in contrast to our experiments. Our results indicated that storage at -196 ˚C had been suitable for long-term preservation. In this study, the preservation period of at least 6 months for *C. militaris* had been the most productive. Our findings were also supported by previous studies. Freitas et al. (2014) reported that *Metarhizium anisopliae* and *Nomuraea rileyi* had retained their growth stability for 3 and 6 months [22]. Moreover, *Hirsutella citriformis* was found to have also maintained the growth ability and sporulation after 6 months of preservation [23]. The different fungi had demonstrated different optimal preservation periods under their special conditions [24]. However, the simple, reliable, and cost-effective strategy of fungal preservation is a basic requirement for germplasm conservation. The effective method for cryopreservation would allow for more widespread use for fungal cryopreservation. Several preservation techniques have been developed to minimize the damaging effects of desiccation and freezing in order that a high recovery of cryopreserved materials could be ensured. The effective types and concentrations of cryoprotectants depend upon the types and species of the samples. The successful cryopreservation of fungi has been gradually increasing with the use of cryoprotectants and more delicate methods. Having a suitable long-term preservation method will allow for the establishment of stable fungal stocks for commercial production.

5. Conclusions

The mycelial discs and fruiting bodies of *C. militaris* were used as the samples in this study. The samples were transferred to cryotubes that contained cryoprotectant solutions with different concentrations. Then, cryotubes were stored at different freezing temperatures at -20 ˚C, -80 ˚C, and -196 ˚C (liquid nitrogen) for 1, 3, and 6 months, depending on the period of storage time. After preservation, the samples were cultured on PDA medium for 3 weeks under dark conditions in order that the recovery percentages and colony diameters could be measured. It was determined that freezing in liquid nitrogen (-196 ˚C) had been the best preservation condition. This determination was based on the highest recovery percentage (100 %) and the largest colony diameters for both the cryopreserved mycelial discs and the cryopreserved fruiting bodies after the samples had been stored with 10% glycerol as a cryoprotectant for 6 months. Furthermore, the results also showed that the cryopreserved mycelial discs and cryopreserved fruiting bodies had been able to produce vigorous fruiting bodies, which had a rich accumulation of bioactive components.

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7. References

[1] Shrestha B, Zhang W, Zhang Y, Liu X. The medicinal fungus *Cordyceps militaris*: research and development. Mycol Prog. 2012;11:599-614.
[2] Cui J. Biotechnological production and applications of *Cordyceps militaris*, a valued traditional Chinese medicine. Crit Rev Biotechnol. 2015;35(4):475-484.
[3] Luo X, Duan Y, Zhang W, Li C, Zhang J. Structural elucidation and immunostimulatory activity of polysaccharide isolated by subcritical water extraction from *Cordyceps militaris*. Carbohydr Polym. 2017;157:794-802.
[4] Wang B, Lee C, Chen Z, Yu H, Duh P. Comparison of the hepatoprotective activity between cultured *Cordyceps militaris* and natural *Cordyceps sinensis*. J Funct Foods. 2012;4:489-495.
[5] Sun S, Deng C, Zhang L, Hu K. Molecular analysis and biochemical characteristics of degenerated strains of *Cordyceps militaris*. Arch of Microbiol. 2017;199:939-944.
[6] Yin J, Xin X, Weng Y,Gui Z. Transcriptome-wide analysis reveals the progress of *Cordyceps militaris* subculture degeneration. PLoS One. 2017;12(10):1-14.

[7] Homolka L. Preservation of live cultures of basidiomycetes - recent methods. Fungal Biol. 2014;118:107-125.
[8] Homolka L, Lisa L, Eichlerova I, Valaskova V, Baldrian P. Effect of long-term preservation of basidiomycetes on perlite in liquid nitrogen on their growth, morphological, enzymatic and genetic characteristics. Fungal Biol. 2010;114:929-935.
[9] Ifeanyi N, Okorie N, Marshall E. Germplasm preservation and propagation; the foundation of agricultural development-a review. J Pharm Biol Sci. 2016;11:70-73.
[10] Nam S, Li C, Li Z, Fan M, Kang S, Lee K, et al. Long-term preservation, regeneration, and cultivation of *Paecilomyces tenuipes* (Peck) Samson (Ascomycetes), an entomopathogenic gungus inoculated into the silkworm larva of *Bombyx mori*. Int J Med Mushrooms. 2011;13:83-91.
[11] Sung J, Park Y, Lee P, Han S, Lee W, Choi S, et al. Effect of preservation periods and subcultures on fruiting body formation of *Cordyceps militaris in vitro*. Mycobiology. 2006;34(4):196-199.
[12] Huang L, Li Q, Chen Y, Wang X, Zhou X. Determination and analysis of cordycepin and adenosine in the products of *Cordyceps* spp. Afr J Microbiol Res. 2009;3(12):957-961.
[13] Elliott G, Wang S, Fuller B. Cryoprotectants: a review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures. Cryobiology. 2017;76:74-91.
[14] Jang T, Park S, Yang J, Kim J, Jae H, Park U, et al. Cryopreservation and its clinical applications. Integr Med Res. 2017;6:12-18.
[15] Leibo S, Pool T. The principal variables of cryopreservation: solutions, temperatures, and rate changes. Fertil Steril. 2011;96(2):269-276.
[16] Linde G, Luciani A, Lopes A, Valle J, Colauto N. Long-term cryopreservation of basidiomycetes. Braz J Microbiol. 2018;49:220-231.
[17] Miguel A, Ayala ZM, Gallou A. Berlanga PA, Andrade MG, Rodriguez RJ, et al. Viability, purity, and genetic stability of entomopathogenic fungi species using different preservation methods. Fungal Biol. 2017;121:920-928.
[18] Singh S, Upadhyay R, Kamal S, Tiwari M. Mushroom cryopreservation and its effect on survival, yield and genetic stability. Cryo Letters. 2004;25:23-32.
[19] Tanaka H, Mantovani T, Santos M, Linde G, Colauto N. Cereal grains and glycerol in *Agaricus blazei* cryopreservation. J Biosci. 2013;29:627-633.
[20] Colauto N, Eira A, Linde G. Cryopreservation at -80 ˚C of *Agaricus blazei* on rice grains. World J Microbiol Biotechnol. 2011;27:3015-3018.
[21] Sun H, Hu T, Guo Y, Liang Y. Preservation affects the vegetative growth and fruiting body production of *Cordyceps militaris*. World J Microbiol Biotechnol. 2018;34(11):1-9.
[22] Freitas A, Araújo M, Hendges E, Azevedo A, Lima D, Cruz I, et al. Viability of *Metarhizium anisopliae* conidia (metsch.) sorok preserved in packages containing silica gel. BMC Proc. 2014;8(Suppl 4):1-8.
[23] Ayala ZM, Gallou A, Berlanga PA, Serna DM, Arredondo BH, Montesinos MR. Characterization of entomopathogenic fungi used in the biological control program of *Diaphorina citri* in Mexico. Bio control Sci Technol. 2015;25:1192-1207.
[24] Crahay C, Declerck S, Colpaert J, Pigeon M, Munaut F. Viability of ectomycorrhizal fungi following cryopreservation. Fungal Biol. 2013;117:103-111.