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Research article

Effects of preservation method on fruiting body formation and cordycepin production of *Cordyceps militaris* culture

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Abstract

Preservation was investigated of *Cordyceps militaris* mycelial cultures under eight different methods: freezing at -80°C; chilling at 5°C in 10% (volume per volume; v/v) glycerol; chilling at 5°C on rice grains that had been dried at 35°C, 45°C or 55°C; and three subculturings involving holding at 5°C. The cultivation of the original strain before the preservation tests was used as the control. The viability, purity and stability of the mycelia were tested after 4 mth storage. Fruiting body production and physico-chemical properties, together with the cordycepin production were determined. All preservation methods resulted in high viability and purity of the cultures. However, the stability of the preserved culture was significantly ($p < 0.05$) different among the preservation methods. Cultures chilled at 5°C on rice grains dried at 35°C, 45°C or 55°C and the three subculturing methods affected the size of the colony diameter ($p < 0.05$). Freezing at -80°C, chilling at 5°C in 10% (v/v) glycerol or keeping on rice grains dried at 35 or 45°C did not affect the number of fruiting bodies produced, fresh weight, firmness, total soluble solids or color ($p > 0.05$). Cultures frozen at -80°C, chilled at 5°C in 10% (v/v) glycerol, or kept on rice grains dried at 35°C compared to the control did not show any significant ($p > 0.05$) differences in cordycepin production, while subculturing negatively affected cordycepin production after 4 mth storage.

Introduction

Cordyceps militaris, a caterpillar-shaped, Chinese, traditional medicinal mushroom, is an entomopathogenic fungi in the class Ascomycetes and in the DongChong-XiaCao group of Chinese herbs (Buenz et al., 2005; Das et al., 2010). This medicinal mushroom has similar pharmacological activities to *Ophiocordyceps sinensis* (formerly *Cordyceps sinensis*) which has been used extensively as a crude drug and a folk tonic food in East Asia (Bhandari et al., 2010; Das et al., 2010). Various bioactive compounds have been found in *Cordyceps* spp. (Shashidhar et al. 2013) and Li et al. (2006) reported that the main bioactive constituent of *C. militaris* was cordycepin (3′-deoxyadenosine). Cordycepin was first extracted from *C. militaris* by Cunningham et al. (1950). It is a nucleoside analogue which exhibits a broad spectrum of biological activities including anti-virus, anti-cancer, anti-diabetic, anti-inflammatory, renoprotective and

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immunomodulatory activities (Sugar and Mccaffrey, 1988; Ahn et al., 2000; Zhou et al., 2002; Cho et al., 2003; Yun et al., 2003; Kim et al., 2006; Shin et al., 2009; Joung et al., 2014). *C. militaris* has become one of the most valuable substitutes for *C. sinensis* because it can be cultivated in various media and it is able to be maintained under laboratory conditions more readily than *C. sinensis* (Huang et al., 2009).

Currently, there is a growing interest in obtaining and studying the production of fruiting bodies and biologically active compounds from *C. militaris*, but there appear to have been be very few studies concerning the preservation method of this mushroom. The main objective of culture preservation is to store cultures in a viable and stable form for long periods without losing genotypic, phenotypic and physiological traits (Chang and Miles, 2004). Cultures of known fungi have been stored either as spores or as vegetative mycelia. The main preservation methods evaluated can be divided into "simple and cheap", such as subculturing, storage under oil, under water, in soil and in silica gel, or they may be "complex and expensive", such as lyophilization and cryopreserved in liquid nitrogen (Humber, 1997; Mata and Perez-Merlo, 2003; Nakasone et al., 2004). However, some of these methods are not compatible with all fungi due to the particular characteristics of each species (Elisashvili, 2012). Most *Cordyceps* mushroom growers in Thailand and elsewhere commonly preserve using repeated subculturing of mycelia that are stored at low temperature because this technique is simple and inexpensive (Smith and Onions 1994). Although storage of subcultures at low temperatures (4–7°C) can minimize the metabolism of isolates and prolong their life (Smith and Onions, 1994), this technique that may lead to an increased risk of accidental contamination, mutation and degenerative problems. Sung et al. (2006) reported fruiting bodies of *C. militaris* in vitro from subcultures were stable until the second–sixth subcultures, after which there was a sharp decrease, the colony color of subcultures changed, and less fruiting bodies formed. Therefore, the aims of this work were to evaluate the effects of preservation methods on the viability, purity and stability relevant to the macro-morphological characteristics of *C. militaris* mycelial cultures.

Materials and Methods

Source of culture, medium, inoculum preparation and fruiting body production

Cordyceps militaris mycelia were obtained from the Tang Chao Tongham Company Ltd., Thailand. The mycelia were cultivated on potato dextrose agar (PDA) medium in a Petri dish. The PDA medium contained: 200 g/L potato infusion, 20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 200 mg/L vitamin B1 and 15 g/L agar. The pH was initially adjusted to 6 and autoclaved at 121°C for 20 min. The culture was inoculated on the medium under static conditions at 22°C for 14 d. This culture was used as inoculum for the following experiment.

Potato dextrose broth (PDB) medium was prepared to increase the mycelial inoculum quantity. The ingredients of the PDB medium was the same as in the PDA medium, without the agar. The PDB medium in 250 mL flasks was autoclaved at 121°C for 20 min. Mycelial discs (5 mm diameter) were taken from the cultures grown on PDA plates (2 inoculum discs per flask), and the flasks were then incubated on a rotary shaker at 120 rpm at 22°C in the dark for 14 d. This culture was used as inoculum in the rice culture medium for the formation of the fruiting bodies of the mushroom.

The rice culture medium was made up of white rice (Sao-Hai rice) mixed with 50 mL PDB medium in a 30 mL bottle and autoclaved at 121°C for 20 min. The inoculum from the PDB (2 mL) was transferred to sterilized rice culture medium in a 30 mL bottle (3 replications, 3 bottles each). The inoculated medium was incubated at 22°C in the dark for 7–14 d. To induce fruiting bodies, this culture was kept at 18°C under 12 hr light and 12 hr dark and 60–70% relative humidity (RH) until the mycelia transformed into fruiting body primordia. Subsequently, the bottles were held at 22°C and 80–90% RH for 64 d to allow for the production of fruiting bodies.

Preservation methods and assessments of culture characteristics

Preservation under freezing at -80°C

The method was carried out as described by Smith (1991). The resultant inoculum discs were punched out from the margin of the mycelial colony on the PDA medium (5 mm in diameter) using a sterilized cork-borer under aseptic conditions and then the inoculum discs were transferred into 1.8 mL sterile cryotubes (1 inoculum disc per cryotube), each cryotube containing 1.5 mL of sterile cryoprotectant solution prepared with 10% glycerol (volume per volume; v/v) in water. The cultures were initially stored at -20°C for 2 hr and then kept at -80°C for 4 mth. The frozen cultures were thawed in water at 25°C for 30 min before inoculation onto PDA medium plates which were incubated in the dark at 22°C for 14 d for viability and purity assessments. Viability of the cultures was determined by measurement of plate numbers of mycelial growth and colony diameters. The period of mycelial growth recovery was also recorded. Plate numbers of the sample recovery and the contamination were calculated as the percentage of purity.

For stability assessments of the cultures, the mycelial colony on the PDA medium after viability and purity evaluation was induced for fruiting body production in the rice medium according to the above method in the fruiting body production session. Physico-chemical properties and cordycepin production were measured as the stability of the fruiting body by the following methods:

1. Physico-chemical of fruiting body evaluation

Fresh *C. militaris* fruiting bodies from each treatment were analyzed for measurement of quality as: number of fruiting bodies, fresh weight, firmness using a texture analyzer with 0.2 cm of pinhead (model QTS 25; Brookfield, USA) expressed in kilograms per square centimeter. The total soluble solids concentration (%TSS) was measured using a digital refractometer (PAL-1: Atago; Saitama 369-1246, Japan) after the sample had been extracted in 1 mL of distilled water for 5 min. The color of samples was measured using the CIE L*a*b* system with a color reader (CR-20: Konica Minolta; Bangkok, Thailand).

2. Determination of cordycepin production

For extraction of cordycepin from the fruiting bodies, the samples were dried in a hot-air oven at 55°C for 48 hr and then the dried samples were ground into a powder using a grinder (WF-04; Nonthaburi, Thailand). Approx. 1.0 g of powder was added to 10 mL methanol:water (1:1, v/v) solution in a 50 mL centrifuge tube. The tube was placed in an ultrasonic machine for 30 min and centrifuged at 9,900×g for 15 min. After centrifugation, the extraction procedure was repeated. The supernatants obtained from the two centrifugations were mixed and the volume was measured. The resultant sample was filtered through a 0.45 µm nylon filter prior to high performance liquid chromatography (HPLC) analysis. The concentrations of cordycepin were determined according to Huang et al. (2009) with some modifications. Standards of cordycepin were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). A mixture of standards was consecutively injected five times to prepare calibration curves into an HPLC. analyzer (Shimadzu; Kyoto 604-8511, Japan) using an Ultra IBD (150 mm × 4.6 mm, 5µm particle size) column (Restek; State College, PA, USA) and an ultraviolet-visible spectrum detector. The mobile phase was a mixture of water and methanol (90:10, v/v). The injection volume was 20 µL, the column temperature was 35°C, the flow rate was set at 1 mL/min and the eluent was monitored at 254 nm.

Preservation under chilling storage at 5°C in 10% (v/v) glycerol

This preservation method was conducted in the same manner as in the freezing at -80°C method, except the temperature for storage was at 5°C for 4 mth without thawing the sample before inoculation on the PDA medium plates. Viability, purity and stability of the cultures were evaluated sing methods described above.

Preservation under chilling storage at 5°C on dried rice grains

C. militaris mycelia were stored as cultures on rice grains (spawn) after the grain had been dried at three different temperatures (35°C, 45°C and 55°C). Thai white rice grains (Sao-Hai rice) were soaked in water overnight. Water was decanted off, 5 g rice grains were transferred to a 10 mL glass bottle and autoclaved at 121°C for 1 hr. The rice grains were dried in a hot-air oven at the different temperatures for 48 hr, and the moisture content of each dried rice grain sample was measured using a moisture analyzer (MA35; Sartorius AG; Göttingen, Germany). The inoculum discs from the PDA medium were transferred to each bottle containing the dried rice grains (1 inoculum disc per bottle) and stored at 5°C for 4 mth. After preservation, the viability and purity of the mycelial colony were evaluated according to the methods described above by placing a dried rice grain covered with the mycelia on a PDA medium plate (one grain per plate) and incubating in the dark at 22°C for 14 d. These cultures were transferred into the PDB medium and rice culture medium, respectively, for fruiting body production as described above. The fruiting bodies were evaluated for culture stability using the method mentioned in the freezing preservation session above.

Constant subculturing method

The original culture was subcultured three times by transferring the inoculum discs (5 mm diameter) and then punching out with a sterilized cork borer under aseptic conditions onto a fresh PDA medium plate and incubating in the dark at 22°C for 14 d. This culture was referred to as the first subculture stage. The first subculture was again inoculated onto a fresh PDA medium plate as the second subculture and incubated in the dark at 22°C for 14 d. The third subculture was also prepared from the second subculture in the same manner. These three subcultures were stored at 5°C for 4 mth. Then, each colony on the PDA plate was assessed for viability and purity using the same approach described in the freezing preservation at -80°C method above. The fruiting bodies of the cultures were produced and evaluated in the same manner as described above.

For the control experiment, the original strain of *C. militaris* without preservation was cultured in PDB and rice medium prepared as mentioned above for the different assessments of viability, purity and stability characteristics.

Statistical analysis of data

The results were expressed as mean ± SD. The data were analysed using the one-way ANOVA facilitated by SPSS version 15 (SPSS Inc.; Chicago, IL, USA). Significant differences between treatments were tested using Duncan's new multiple range test at $p < 0.05$.

Results

Effect of preservation methods on viability and purity

The first morphological characterization was carried out at the start of the storage treatments and used as a standard for evaluating the changes in viability, purity and macro- morphology. The results of the viability and purity are shown in Table 1. All cultures had 100& purity after 4 mth storage. Most cultures recovered in 1 d after being placed in culture medium, but following storage at -80°C, recovery took 2 d (Table 1).

The average diameter of the original mycelia (control) after incubation in the dark for 14 d and before preservation was 56.8 mm. After preservation for 4 mth, the average mycelial diameter was significantly different after incubation in the dark for 14 d, depending on the treatment (Table 1). The highest mycelial diameters were recorded after freezing at -80°C and in 10% (v/v) glycerol at 5°C which showed the lowest changes in mycelia diameter compared to the control. In the treatments based on rice grain storage techniques, the moisture content was 35.82%, 33.65% and 29.21% when dried at 35, 45 and 55°C, respectively. The values for mycelial development of cultures preserved under chilling at 5°C on rice grains dried at 35°C, 45°C and 55°C were not statistically different but were smaller than those from the glycerol and -80°C treatments (Table 1 and Figs. 1B–F). Within the subculture treatments, those from all cultures were the smallest overall with those from the third subculture having the lowest mycelia diameters and the highest change in diameter compared to the control (Table 1 and Figs. 1A, G–I). In all cultures tested, the recovered mycelia on PDA medium had a normal appearance with a white color and typical texture with no apparent alterations (Figs. 1A–I).

Table 1 Viability and purity assessment of *C. militaris* preserved under different methods for 4 mth

Preservation method	Viability			Purity (%)
	Plate number of mycelia growth (n1/n2)	Mycelial growth recovery (d)	Mycelial colony diameter (mean ± SD) (mm)	
Before preservation (control)	5/5	1	56.8 ± 0.8[a]	100
Freezing at –80°C	5/5	2	56.6 ± 1.6[a]	100
Chilling storage at 5°C				
10% (v/v) glycerol	5/5	1	55.4 ± 1.1[a]	100
Rice grains dried at 35°C	5/5	1	51.0 ± 1.5[b]	100
Rice grains dried at 45°C	5/5	1	49.8 ± 0.8[b]	100
Rice grains dried at 55°C	5/5	1	50.8 ± 0.8[b]	100
1st subculture	5/5	1	47.0 ± 1.2[c]	100
2nd subculture	5/5	1	40.0 ± 1.5[d]	100
3rd subculture	5/5	1	34.6 ± 2.0[e]	100

n1= plate number of replications; n2 = plate number of mycelial growth.
Mean is taken for the total recovery of 5 samples.
Mean values with different superscript letters within each column are significantly ($p < 0.05$) different between groups.

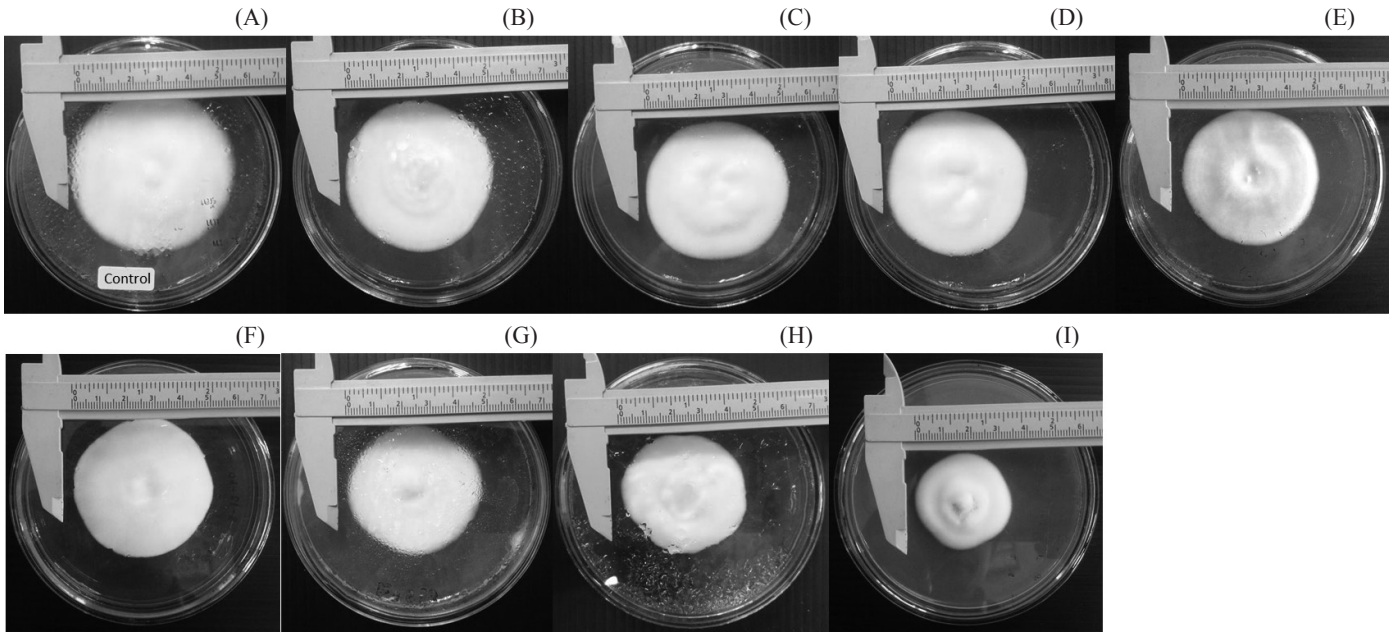

Fig. 1 Mycelial colony diameter from different preservation methods of mycelia of *C. militaris* after incubation on potato dextrose agar medium for 14 d: (A) before preservation (control); (B) freezing at -80°C; (C) chilling at 5°C in 10% (v/v) glycerol; (D) chilling at 5°C on rice grains dried at 35°C; (E) chilling at 5°C on rice grains dried at 45°C; (F) chilling at 5°C on rice grains dried at 55°C; (G) chilling at 5°C in the 1st subculture; (H) chilling at 5°C in the 2nd subculture; (I) chilling at 5°C in the 3rd subculture

Effect of preservation methods on stability

Fruiting body production

Cultures from all treatments produced profuse fruiting bodies (stromata) when they were cultivated in rice culture medium. Different preservation methods had significant effects on the number of fruiting bodies and on fresh weight (Table 2). Cultures preserved under freezing at -80°C, chilling at 5°C in 10% (v/v) glycerol, or held on rice grains dried at either 35°C or 45°C for 4 mth were not significantly different and furthermore, were not different to the control. In addition, the fruiting bodies in these treatments were thick and long while those in the third subculture treatment were thick and short (Figs. 2A–I). Cultures preserved under chilling at 5°C on rice grains dried at 55°C, or in the second and the third subcultureing methods were different to the control ($p < 0.05$). Culture preserved in the third subcultures at 5°C had the lowest number of fruiting bodies and fresh weight (Table 2).

Table 2 Average number of fruiting bodies produced, fresh weight, firmness, total soluble solid (TSS), color and cordycepin concentration for *C. militaris* preserved under different methods for 4 mth

Preservation method	Number of fruiting bodies (fruiting bodies/ bottle)	Fresh weight (g/bottle)	Firmness (kg/cm^2)	TSS (%)	Color			Cordycepin (mg/g)
					L*	a*	b*	
Before preservation (control)	114.33±1.52^a	22.33±0.41^a	0.29±0.0.2^b	13.33±0.11^a	42.53±0.83^c	24.40±0.45^a	52.73±0.40^a	3.57±0.03^a
Freezing at -80°C	112.66±1.52^a	22.43±0.80^a	0.26±0.02^b	13.30±0.10^a	42.70±0.44^c	24.32±0.26^a	52.32±1.26^a	3.51±0.07^a
Chilling storage at 5°C								
10% (v/v) glycerol	111.33±2.08^a	21.76±0.85^a	0.27±0.01^b	13.23±0.05^a	42.56±0.21^c	24.49±0.55^a	52.56±1.02^a	3.45±0.04^a
Rice grains dried at 35°C	110.33±1.52^a	21.86±0.55^a	0.25±0.04^b	13.30±0.10^a	42.52±0.59^c	24.51±0.52^a	52.91±0.48^a	3.35±0.25^a
Rice grains dried at 45°C	109.66±1.52^a	21.80±0.20^a	0.27±0.02^b	13.20±0.10^a	42.26±0.27^c	24.29±0.23^{ab}	52.70±0.53^a	2.34±0.10^b
Rice grains dried at 55°C	103.00±4.58^b	19.66±0.90^b	0.29±0.01^b	13.23±0.05^a	41.99±0.13^c	23.99±0.13^{ab}	51.14±0.81^{ab}	1.63±0.20^c
1st subculture	99.33±2.30^{bc}	17.70±0.96^{bc}	0.28±0.15^b	12.96±0.15^b	55.03±1.04^b	23.78±0.38^{ab}	51.42±0.96^a	1.43±0.09^c
2nd subculture	97.00±3.60^c	17.13±1.02^c	0.27±0.04^b	12.80±0.10^b	57.00±0.39^a	23.60±0.45^b	49.49±0.75^b	1.07±0.11^d
3rd subculture	66.66±2.51^d	14.66±0.35^d	0.51±0.06^a	12.20±0.10^c	57.84±0.41^a	22.84±0.41^c	44.16±1.76^c	0.54±0.04^e

TSS = total soluble solids.

^{a,b,c,d} Values (mean ± SD) with different superscript letters within each column denote significant differences ($p < 0.05$) between groups.

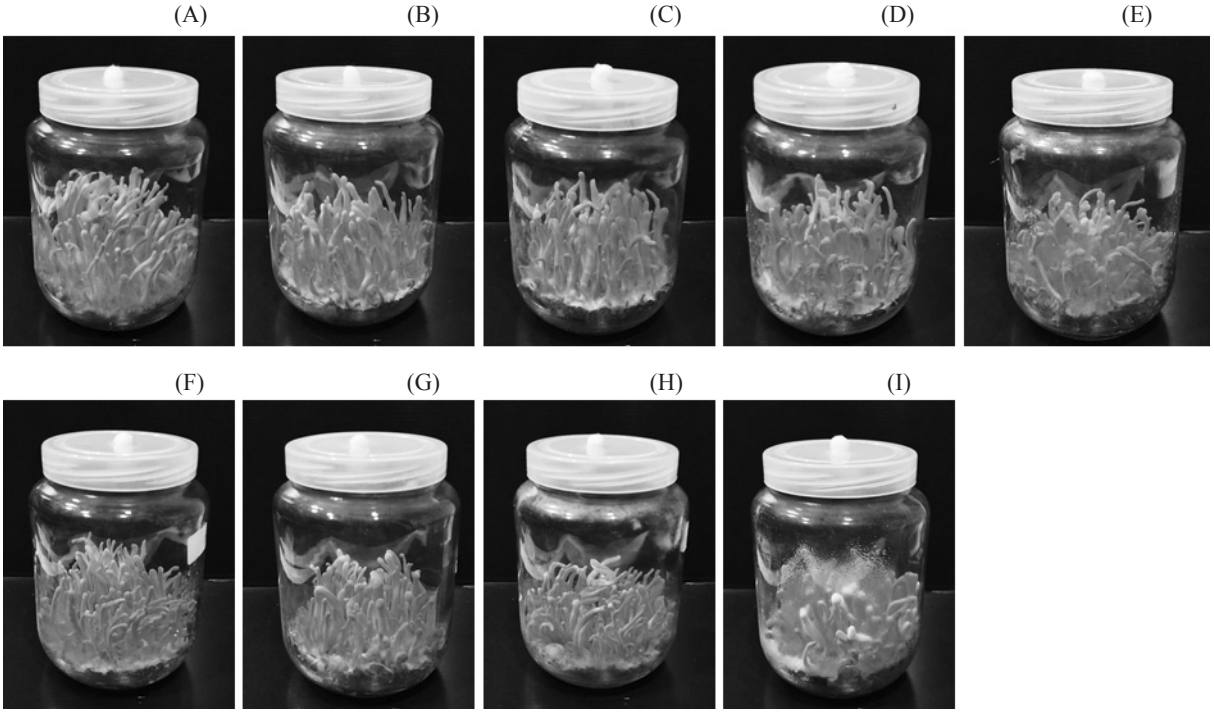

Fig. 2 Fruiting body formation and characteristics of *C. militaris* using different preservation methods: (A) before preservation (control); (B) freezing at -80°C; (C) chilling at 5°C in 10% volume per volume glycerol; (D) chilling at 5°C on rice grains dried at 35°C; (E) chilling at 5°C on rice grains dried at 45°C; (F) chilling at 5°C on rice grains dried at 55°C; (G) chilling at 5°C in the 1st subculture; (H) chilling at 5°C in the 2nd subculture; (I) chilling at 5°C in the 3rd subculture

Physico-chemical properties of fruiting bodies

Firmness

Cultures preserved under freezing at -80°C, chilling at 5°C in 10% (v/v) glycerol, or on rice grains dried at 35°C, 45°C and 55°C, or in either the first and second subcultures methods were not significantly different among treatments and not different from the control (Table 2). Highest firmness of fruiting bodies was recorded in the third subculture method and was significantly different compared to the control.

Total soluble solids

Cultures preserved under chilling at 5°C in the three subculturing methods had their total soluble solids (TSS) concentration of fruiting bodies affected while cultures preserved under freezing at -80°C, chilling at 5°C in 10% (v/v) glycerol, or on rice grains dried at 35°C, 45°C and 55°C methods were not affected after 4 mth storage. Culture preserved under chilling at 5°C in the third subcultures method had the significantly lowest TSS concentration of fruiting bodies (Table 2). The average TSS concentrations of fruiting bodies from the cultures that had been preserved under freezing at -80°C, chilling at 5°C in 10% (v/v) glycerol, or cultured on rice grains dried at 35°C, 45°C or 55°C were not significantly different among these treatments and were not different to the control (Table 2).

Color

The lightness, redness and yellowness saturation indices of the cultures were expressed as L*, a* and b* values, respectively. Both the second and third subculture preservation methods affected the color stability of the fruiting bodies after 4 mth storage. The L*values of fruiting bodies from the cultures that had been preserved using either the second and third subculture methods increased and had the highest value compared to the other treatments (Table 2). The a* and b* values of fruiting bodies from the cultures that had been preserved in either the second or third subculture methods were significantly decreased from the other treatments (Table 2). The a* and b* values of the fruiting bodies from the cultures that had been preserved using freezing at –80°C, chilling at 5°C in 10% (v/v) glycerol, or on rice grains dried at 35°C, 45°C, and 55°C, or in the first subculture methods were not significantly different among these treatments and not different from the control (Table 2).

Cordycepin production

Cultures preserved under freezing at -80°C, chilling at 5°C in 10% (v/v) glycerol, or cultured on rice grains dried at 35°C were not significantly different and not different from the control (Table 2). The stability of cordycepin production in fruiting bodies of cultures preserved under chilling at 5°C on rice grains dried at 45°C or 55°C, and those in all subcultures were affected. Culture preserved under chilling at 5°C in the third subculture method had the significantly lowest concentration of cordycepin after 4 mth storage (Table 2).

Discussion

Methods of fungi preservation involve maintenance of cell viability and the metabolic properties of these organisms for long periods without losing genotypic, phenotypic and physiological characteristics (Chang and Miles, 2004). Suitable culture preservation is an integral part of the successful mushroom cultivation on a commercial scale. However, the cost and convenience of available methods must also be considered. Regarding *Cordyceps* mushrooms, mycelial preservation has never been reported. The fungus is heterothallic and cordycepin is produced in stroma making it impossible to be stored as ascospores. A cheap and reliable methods for culture maintenance could benefit small business operations, especially retaining the ability of the fungus to produce the valuable bioactive compound.

Viability, micro-morphological and macro morphological properties were the primary characteristics that are evaluated in microorganisms during and after preservation. The results obtained from this experiment demonstrated that all preservation methods tested produced no visible morphological changes, or contamination by bacteria or other fungi after 4 mth storage. Although viability was maintained for all samples tested, preservation of *C. militaris* mycelia under different techniques resulted in differences in stability.

The production of fruiting bodies and physico-chemical properties as firmness, color and TSS concentration, together with bioactive compounds production are the major quality traits of *C. militaris* cultivation because these largely determine the cost and consumer acceptance. Firmness is an indicator of fruiting body maturity, while the TSS concentration is an indicator of mushroom sweetness. The physico-chemical properties data of *C. militaris* fruiting bodies has not been previously reported. In this experiment, cultures frozen at -80°C, chilled at 5°C in 10% (v/v) glycerol or cultured on rice grains dried at 35°C or 45°C were not different from the control regarding the number of fruiting bodies and fresh weight. These results suggested that the tested preservation methods had no effect on fruiting body production after 4 mth storage. The highest firmness of fruiting bodies in this experiment was recorded in the third subculture method because the shape of the fruiting bodies in this treatment was thicker and shorter than other treatments. Most methods of preservation in this experiment produced no differences in the TSS concentration of fruiting bodies compared to the control. This result suggested that these preservation methods did not affect mushroom sweetness after 4 mth storage. The color of fruiting bodies in all treatments was orange, except in the third subculture where some fruiting bodies were white. The white color of some fruiting bodies was believed to have been due to a random mutant (Shrestha et al. 2005). The culture frozen at -80°C had the lowest change in the cordycepin concentration which decreased by 1.68% compared to the control. Although the cordycepin concentration in fruiting bodies from the frozen culture at -80°C were higher than those the other storage methods, the cordycepin concentrations from all storage methods were acceptable for commercial species (>20 mg/10g). On the other hand, the third subculture produced a cordycepin concentration of less than 20 mg/10g. Within the rice grain storage methods, the cordycepin concentration declined with an increase in the grain drying temperature (that is, a decrease in the grain moisture content). Similarly, the cordycepin concentration decreased as the number of subcultures increased. Huang et al. (2009) reported that the content of

cordycepin in fruiting bodies from artificial culture of *C. militaris* was 26.51 mg/10g, which was lower than in the current research where mycelial cultures of *C. militaris* were preserved by freezing at -80°C, or chilling at 5°C in 10% (v/v) glycerol, or by culturing on rice grains dried at 35°C for 4 mth.

Mycelial cultures of *C. militaris* preserved by freezing at -80°C in cryoprotectants with 10% (v/v) glycerol maintained the best overall quality, most likely because the glycerol prevented the formation of ice crystals during freezing, which thus reduced the cellular damage caused by their formation (Mata and Pérez-Merlo, 2003). However, in this storage treatment, there was a subsequent delay in growth of mycelia until 2 d of incubation. Another study has similarly shown that the recovery of frozen mycelium of various mushroom varieties was delayed compared to a control group which was not frozen (Mata and Pérez-Merlo, 2003). Freezing methods have been described in literature that were able to maintain the viability of fungal cultures for periods of more than 5 yr (Nakasone et al., 2004). In general, vigorously growing and sporulating cultures survive the freezing process better than less vigorous strains. Many laboratories store cultures in ultracold mechanical freezers. However, the mushrooms strains subjected to such preservation must be able to withstand the extreme physical conditions. The disadvantages of this method are that expensive equipment is required, freezer temperatures favor ice crystal formation in the cultures and cultures may be subject to losses during power failures (Humber, 1997).

In all treatments where preservation was carried out by chilling at 5°C, in cultures immersed in 10% (v/v) glycerol or when stored with rice grains that had been dried at 35°C or 45°C, the cultures maintained good quality in fruiting body production and physico-chemical properties similar to those stored at -80°C or the control. Storage of mycelia in 10% (v/v) glycerol in distilled water suppressed morphological changes in *C. militaris* mycelia (Nakasone et al., 2004), and including storage at low temperatures (4–7°C) can minimize their metabolism and prolong their life (Smith and Onions, 1994). In the case of preservation using the rice grains method, samples that were preserved in rice grains that had been dried at either 35°C or 45°C maintained better fruiting body production and cordycepin content than when cultured on rice grains that had been dried at 55°C, likely because the moisture content of rice grains dried at 55°C was insufficient to support growth and allow the survival of the mycelia. This suggested that a moisture content in the range 33.6%–35.8% in the dried rice grains is required for preservation of *C. militaris* mycelia cultures during 4 mth storage. Tariq et al. (2015) showed that preservation of phytopathogenic fungi using cereal grains and storage at -20°C was able to hold fungi for up to 2 yr.

Mycelia in all the repeated subcultures remained viable. This preservation method showed the overall quality of the cultures decreased when the frequency of subcultures increased. With a high number of subcultures, the fruiting bodies were firmer and the shape of the fruiting bodies was thicker and shorter. Similar results were observed by Sung et al. (2006) who reported that the production of fruiting bodies of *C. militaris* in vitro from subcultures was stable until the sixth subculture, after which it decreased sharply, the colony color of subcultures changed and fewer fruiting bodies were formed. Roy et al. (2014) reported that preservation of pathogenic fungi using a continuous growth method caused a loss of viability in the long term. It has been suggested that this method causes changes in morphology and genetics and a loss in the ability to produce secondary substances (Nakasone et al., 2004).

For all the preservation methods evaluated, viability, purity and stability as morphological characteristics, fruiting body production and retention of physico-chemical properties, together with concentrations of a selected bioactive compound in the cultures, were achieved by freezing at -80°C overall, or by preservation with chilling at 5°C in 10% (v/v) glycerol or by culturing on rice grains that had been dried at either 35°C or 45°C. In contrast, subculturing of the preserved cultures resulted in a loss in fruiting body production and a loss of certain valuable culturing characteristics during 4 mth storage. Therefore, the preservation of this mushroom under chilling at 5°C in 10% (v/v) glycerol, or on rice grains that have been dried at 35°C and 45°C could be an alternative means for *C. militaris* production because of the resultant cheap cost, convenient routine practices, viability, purity and stability. It is possible to use these preservation methods as an appropriate choice for mycelia maintenance of *C. militaris* in short term preservation at small or large commercial-scale production. However, there is a need for further studies with longer preservation times and a comparison with other methods such as lyophilization and cryopreservation in liquid nitrogen, using a greater number of samples in order to establish the best preservation criteria for this particular species.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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