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Cultural characteristics and cordycepin production of some *Cordyceps militaris* strains under artificial cultivation conditions

LUYEN THI NGUYEN [1], VE VAN LE [2], BICH THUY THI NGUYEN [1], NGHIEN XUAN NGO [1], HUYEN TRANG THI NGUYEN [1], QUAN DINH NGUYEN [3], SIKANDAR MULLA [4]*

[1] Vietnam National University of Agriculture, Hanoi, Vietnam
[2] University of Science and Technology, Seoul, South Korea
[3] National Institutes of Medicinal Materials, Hanoi, Vietnam
[4] REVA University, Bangalore, India

Abstract

Cordyceps militaris, a precious medical mushroom, has attracted wide attention in industrial fields. Currently, the degeneration phenomenon of *C. militaris* commercial strains is amongst the major challenges for cultivation at the industrial scale. The screening for superior strains with high yield and medicinal value is considered a realistic approach to overcome degeneration problems. In the present study, the mycelial growth, primordia formation, yield performance, and cordycepin content of five strains (DT1, DT2, DT3, DT4, and DT5) under artificial cultivation conditions were investigated. All strains showed mycelial growth on SDAY and liquid medium. The strains were successfully cultivated in brown rice medium and required 18 (strain DT3) to 25 days (strain DT5) to form primordia. Additionally, morphological characteristics of fruiting bodies varied among the strains. Strains DT4 and DT3 exhibited the highest fruiting body length with 74.23 ± 5.13 mm and 72.63 ± 2.62 mm, respectively whereas the highest diameter was recorded for strains DT1 (4.05 ± 0.18 mm) and DT2 (3.63 ± 0.12 mm). Of note, among the investigated strains, strain DT3 exhibited the highest biological efficiency (8.95 ± 0.07%) and cordycepin content (1.68 mg/g). Therefore, strain DT3 could be selected as a potential strain for commercial cultivation.

Key words: *Cordyceps militaris*, mycelial, fruiting body, cordycepin

Introduction

As one of the most precious medicinal fungi, *Cordyceps militaris* belonging to the genus *Cordyceps*, family *Cordycipitaceae*, and class *Ascomycetes* has received considerable attention in several fields, including medicinal herbs, pharmaceutical products, tonic foods, and biocontrol agents against pests (Akata et al., 2016; Cui et al., 2015; Chiu et al., 2016; Zhang et al., 2019; Das et al., 2010; Yang et al., 2014). The fruiting body of *C. militaris* contains several bioactive metabolites such as cordycepin, adenosine, inosine, mannitol, sterols, polysaccharides, and pentostatin (Cui et al., 2015). Earlier studies reported positive health effects of these compounds to humans in the treatment of cancer, inflammatory diseases, and diabetes and for immunity improvement (Sun et al., 2018; Yin et al., 2017; Zhao et al., 2014; Yang et al., 2014). Among the bioactive metabolites, cordycepin – a nucleoside analog of adenosine – is a chemical marker used to distinguish genus *Cordyceps* from other fungi (Chamyuang et al., 2019).

Owing to strict growth requirements, *C. militaris* is scarce in nature (Yin et al., 2017). Thus, in order to improve the equality between the supply and demand chain, especially in Asian countries, artificial cultivation of *Cordyceps militaris* has been found to be a useful method (Yin et al., 2017). Unlike other species in genus

* Corresponding author: REVA University, Bangalore, India; e-mail: sikandar.mulla@gmail.com

Cordyceps, *C. militaris* can grow easily and form fruiting bodies on artificial media, and therefore, it is considered as the only species of this genus that could be cultivated for cordycepin production at the industrial scale (Zhang et al., 2019; Lin et al., 2017; Sun et al., 2018; Chamyuang et al., 2019). As described previously by Chiang et al. (2017), the cultivation of *C. militaris* could be classified into three periods based on growth characteristics (mycelium colonization, primordia formation, and fruiting body development stage). To improve biological efficiency, several parameters such as light conditions, temperature, humidity, pH, nutrition source, and minerals should be optimized (Chamyuang et al., 2019). To cultivate *C. militaris* in a large scale, cereal grains were widely used as the primary carbon source (Lin et al., 2017). Rice, cottonseed shells, wheat grain, corn grain, millet, sorghum, and corn cob particles have been used as main substrates and nutrient sources for the production of fruiting bodies and bioactive compounds by *C. militaris* (Lin et al., 2017; Shrestha et al., 2012b). Several studies have revealed that during the cultivation of *C. militaris*, light is the key environmental factor in determining pigment formation, stroma production, and cordycepin and carotenoid formation (Chiang et al., 2017; Yang et al., 2016; Shrestha et al., 2012b; Shrestha et al., 2006). The optimal light intensity for fruiting body induction was found to be 500 to 1000 lx (Hong et al., 2010). The suitable light condition for the growth of fruiting body is the light/dark cycle of 12 : 12 h at 25 °C (Chen et al., 2011). Similar to light conditions, temperature is known to be one of the most significant physical factors that noticeably affect stroma production by *C. militaris* (Shrestha et al., 2012b). The optimum temperature for mycelial growth and stroma development of *C. militaris* was determined to be 20–25 °C and 18–22 °C, respectively (Lee et al., 2010). The maintenance of high humidity of 70–90% has been recommended for the cultivation of *C. militaris* (Shrestha et al., 2012b). The optimal medium (yeast extract 10.33 g/l, sucrose 27.24 g/l, KH_2PO_4 5.60 g/l) for the mycelial growth of *C. militaris* was a submerged culture obtained by response surface methodology (Yang et al., 2014).

Although *C. militaris* is known as a cultivable medicinal mushroom, the industry faces several huge challenges such as the high frequency of strain degeneration (Shrestha et al., 2012b; Sun et al., 2018; Yin et al., 2017). Such degenerated strains of *C. militaris* exhibit typical characteristics such as slow mycelium growth, reduced mycelial density, loss of the ability to form primordia, irregular shapes and sizes of fruiting bodies, excessive aerial hyphae, and poor development of stroma and fruiting bodies (Shrestha et al., 2012b; Sun et al., 2018; Wang et al., 2017). As reported by Yin et al. (2017), sub-culture is a major driving factor of the degeneration phenomenon of *C. militaris*. As observed, *C. militaris* started to degenerate at the third subcultured generation and therefore showed incomplete fruiting body development at the next generation. The mechanism of degeneration of *C. militaris* was directly related to genes responsible for toxin biosynthesis, DNA methylation, energy metabolism, and chromosome remodeling (Yin et al., 2017). To address the degeneration problem, the search for superior strains with high yield and medicinal value plays a crucial role in the cultivation of *C. militaris* at the industrial scale. In an attempt to look for potential strains, five strains -DT1, DT2, DT3, DT4 and DT5 were collected in Tam Dao mountain (21°31′0″N 105°33′0″E), Vietnam and deposited into Mushroom Research and Development Center, Vietnam National University of Agriculture. Taken together, the present study investigated 1) cultural characteristics of five *C. militaris* strains grown on solid-state and liquid media; 2) the potential of cultivation of *C. militaris* strains; and 3) the ability of *C. militaris* strains to produce cordycepin.

Materials and methods

Fungal strains

C. militaris strains DT1, DT2, DT3, DT4, and DT5 used in this study were obtained from Mushroom Research and Development Center, Vietnam. Pure mycelial cultures were isolated from the fresh fruiting bodies and maintained on PGA (potato, glucose, and agar) slants according to the procedure of Lin et al. (2017).

Culture conditions

To characterize the mycelium grown on solid-state medium, *C. militaris* strains were cultivated on SDAY medium (20 g/l dextrose, 5 g/l yeast extract, 5 g/l peptone, and 15 g/l agar) at 25 °C in dark. The diameter of the mycelium extension (mm) was measured at 5 to 25 days (5-day interval). The morphological characteristics of mycelia such as texture, density, and color were assessed by visual observations.

To characterize the mycelium grown in submerged cultures, 10 pieces (approximately 0.5 × 0.5 cm) of overgrown agar plugs were excised, transferred into 100 ml sterile water, and homogenized. Precultures were grown in a 500 ml flask containing 250 ml of the medium (20 g/l glucose, 1 g/l peptone, 1 g/l yeast extract, 1 g/l $MgSO_4 \cdot 7H_2O$, and 2 g/l KH_2PO_4) and kept on a rotary shaker incubator (120 rpm) at 22 °C. The density, size, and morphology of pellets were recorded for 1– 25 days. The dry weight of mycelium was determined following the protocol reported by Park et al. (2001). The mycelial pellets were washed with distilled water and dried overnight at 70 °C until a constant weight was achieved.

Substrate preparation and cultivation

C. militaris strains were cultivated in a brown rice medium (50 g of brown rice supplemented with 10 g of silkworm pupae powder) according to the method of Shrestha et al. (2012a). The liquid spawn was inoculated in the medium and incubated at 22 °C with relative humidity of 60–70% in dark at the spawn running stage after 15 days of incubation. After complete colonization of the medium, the strains were grown at 20 °C under 85–90% humidity with a dark/light cycle of 12 : 12 h for fruiting body formation and development.

Mycelial growth, period of substrate colonization, period of primordia formation, number of stromata, and length of stroma were measured as described previously by Ngo et al. (2019). The mycelial growth rate was calculated using the formula:

$$V = D / T,$$

where V is the mycelial growth rate (mm/day), D is the diameter growth (mm), and T is the incubation time (days).

The period of substrate colonization (days) was defined as the time required for the mycelium to completely colonize the full substrate. The period of primordia formation (days) was defined as the time required for the appearance of primordia after inoculation.

The primordia ratio was defined as the percentage of bottle exhibiting primordium. The average biological efficiency (BE) was determined according to the calculations by Nguyen et al. (2019):

$$\text{BE (\%)} = \frac{\text{Dry weight of mushrooms}}{\text{Dry weight of the substrate}} \times 100$$

Quantification of cordycepin content

To evaluate the medicinal value of five strains, the cordycepin content was quantified by HPLC. Powder of fruiting bodies (1 g) was precisely weighed, added to 10 ml of 80% ethanol (v/v) in a 50 ml corning polypropylene centrifuge tube, and sonicated for 30 minutes using an ultrasonic machine. The extracted solution was filtered using a 0.22 μm cellulose acetate membrane filter (Thermo Fisher Scientific). The standard for cordycepin was obtained from Sigma-Aldrich.

HPLC was performed on a Zorbax Eclipse XDB18 column (250 × 4.6 mm; 5 μm) with a flow rate of 0.8 ml/min and the injection amount of 20 μl. The mobile phase consisted of acetonitrile:water (8 : 92, vol/vol). The detection wavelength was set at 260 nm.

Statistical methods

Statistical evaluation was performed using *GraphPad Prism*, version 7.0 (GraphPad Software, Inc., San Diego, CA, USA). Each treatment was replicated three times. Significant differences were indicated with letters and assessed using one-way ANOVA followed by Tukey's multiple comparison test at $P < 0.05$.

Results

Mycelial growth of C. militaris strains on SDAY medium

As shown in Figure 1A and Figure 1B, the results indicate that all investigated strains exhibited the ability to grow on SDAY medium. In terms of texture, the five strains were similar to each other and showed only one type of texture (floccose) (Fig. 1A). The mycelial density of strains on the 25th day was found to be compact. Statistically significant differences ($P < 0.05$) were observed in the growth rate among the strains. The mycelium colony diameter of the five strains ranged from 78.07 ± 6.57 mm (DT5) to 90.00 mm (DT4) on the 25th day. Remarkably, compared to other strains, strains DT3 (90.00 mm) and DT4 (90.00 mm) showed a significantly higher growth rate on 15, 20, and 25 days of incubation (Fig. 1B). The colony color characteristics of all strains were observed to be white when grown in dark but yellow when exposed to white light.

Mycelial growth of C. militaris strains in submerged culture

Among the five strains used for this study, strains DT3 and DT4 adapted better to submerged culture con-

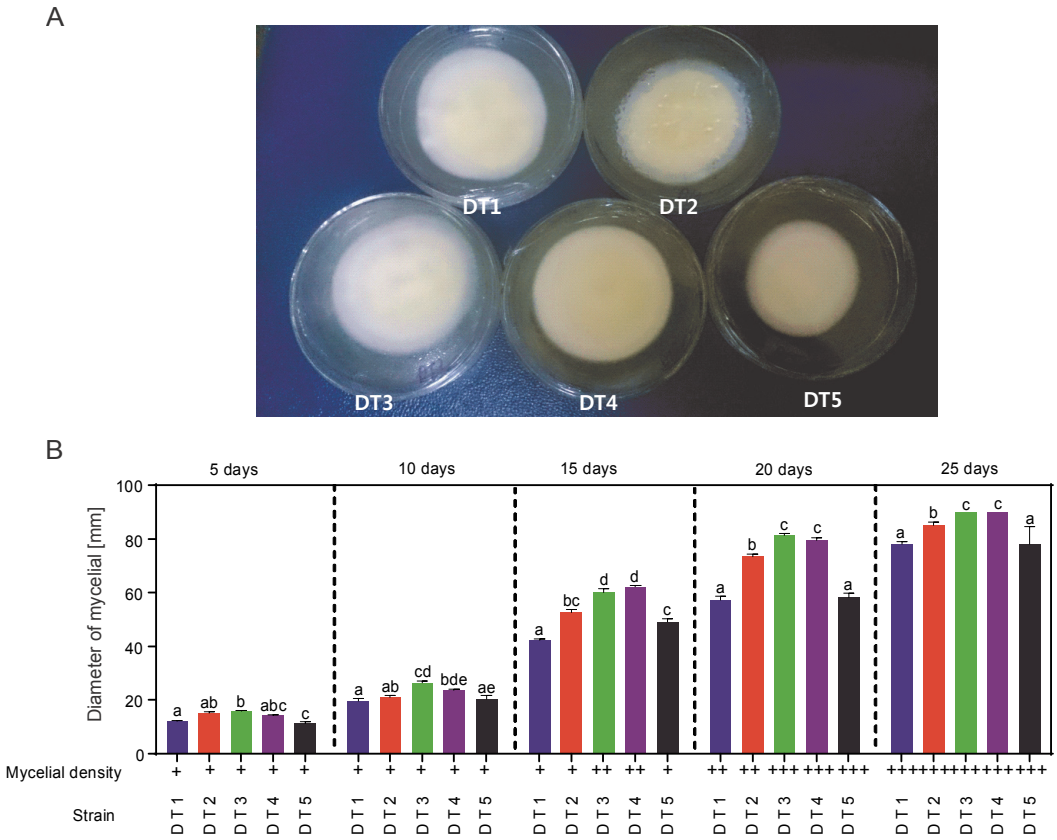

Fig. 1. A) Mycelial characteristics and B) growth of *Cordyceps militaris* strains on SDAY medium; bars in the same time period with different letters show significant differences at $P < 0.05$

ditions than other strains. Under submerged culture conditions, the highest biomass production was obtained for DT4 and DT3 at 6 days after inoculation with 11.08 ± 0.65 g/l and 11.04 ± 0.18 g/l, respectively (Fig. 2C). Remarkably, both filamentous and pellet were identified as growth forms of all strains during cultivation in submerged conditions (Fig. 2A and Fig. 2B). The pellet morphology of the five strains was found to be hairy, fluffy, and circular in shape (Fig. 2B). The pellet diameter and density of all strains started to increase gradually after 48 hours from inoculation. However, a remarkable difference in pellet size was observed. Compared to other strains, the pellet size of strain DT5 (1.11 mm/pellet) was smaller after 6 days from inoculation.

Cultivation characteristics of C. militaris strains

As the first step to evaluate the cultivation potential of the five strains used in this study, their ability to produce primordia was analyzed. As shown in Figure 4, Figure 5, and Figure 6, all the five strains could form and develop primordia to the maturity stage. All the investigated strains required 18 days (DT3) to 25 days (DT5) to form primordia (Fig. 3). The highest length of the fruiting body was found for strains DT4 and DT3 with 74.23 ± 5.13 mm and 72.63 ± 2.62 mm, respectively (Fig. 5A). The highest diameter was observed for strains DT1 and DT2 (4.05 ± 0.18 mm and 3.63 ± 0.12, respectively) (Fig. 5B). As shown in Figure 5C, the number of fruiting bodies varied significantly among the strains and ranged from 8.53 ± 0.16 (DT1) to 120.07 ± 2.46 (DT3). The morphological characteristics of fruiting bodies of the investigated strains is shown in Figure 6A. Compared to other strains, the primordia ratio of strain DT1 showed a lower percentage of 68 ± 6%. The BE of the investigated strains varied significantly. Notably, the highest BE of 8.95 ± 0.07% was observed for strain DT3 (Fig. 6B). Therefore, strain DT3 was found to be superior over other strains in terms of BE and should be used for commercial cultivation.

Cordycepin content of C. militaris strains

A calibration curve of cordycepin was constructed by plotting peak areas of the standard sample measured from 12.7 µg/ml to 203 µg/ml. Cordycepin isolated from

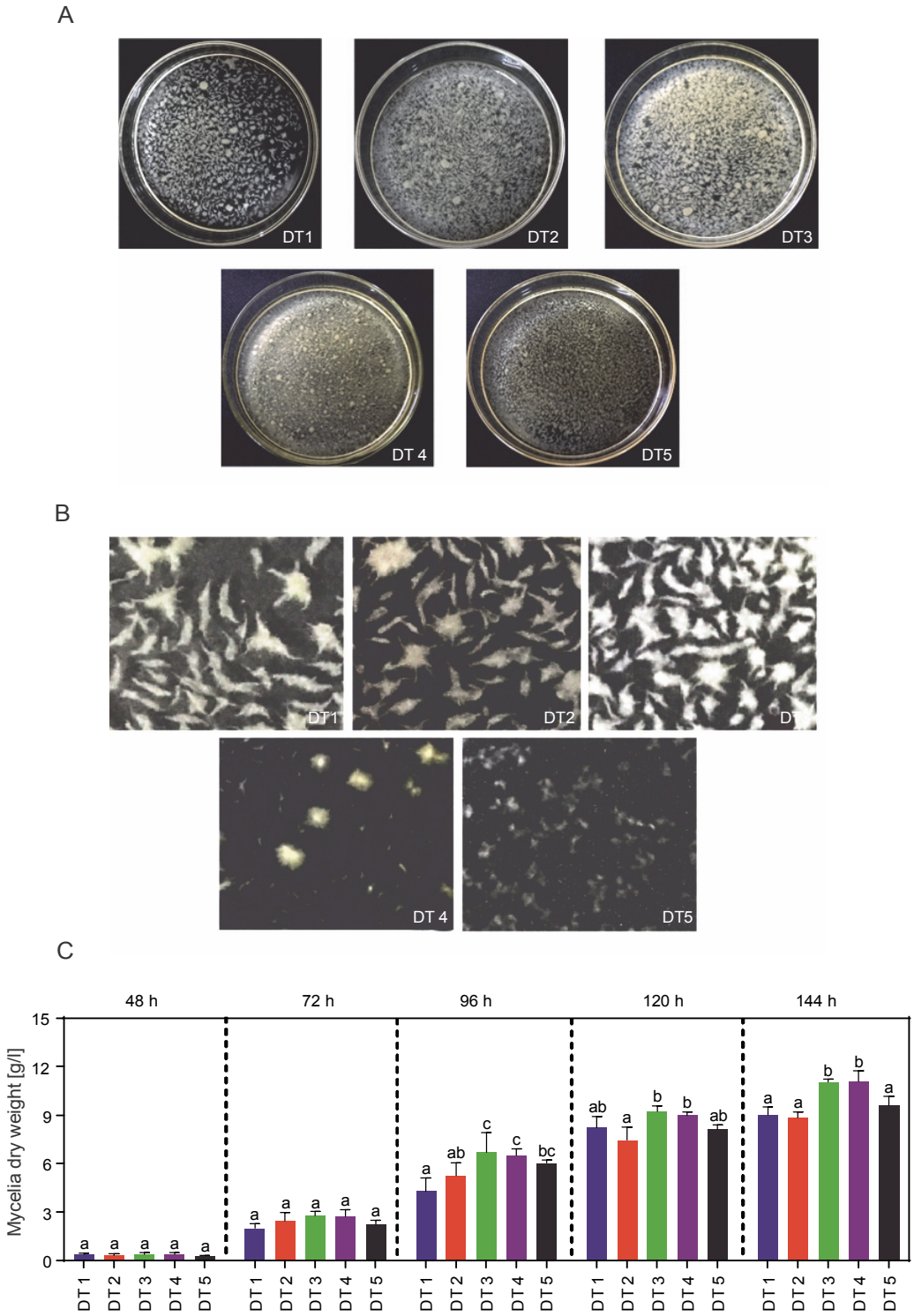

Fig. 2. A) Pellet density, B) morphology, and C) mycelia dry weight of *Cordyceps militaris* cultivated in submerged culture; bars in the same time period with different letters show significant differences at $P < 0.05$

five strains was detected based on the retention time of samples and a standard (Fig. 7A). All strains exhibited the ability to produce cordycepin. However, in a detailed comparison, strain DT3 was found to produce cordycepin with the highest concentration (1.68 mg/g). In contrast, the lowest cordycepin content was observed for strain DT5 (0.24 mg/g) (Fig. 7B).

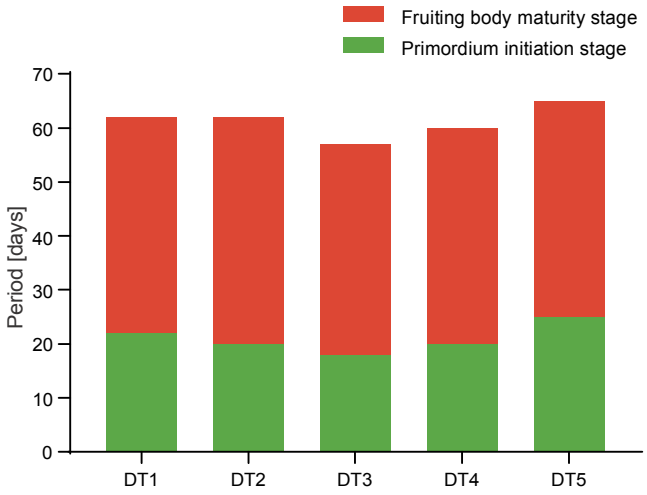

Fig. 3. Growth period of *Cordyceps militaris* strains in artificial cultivation condition

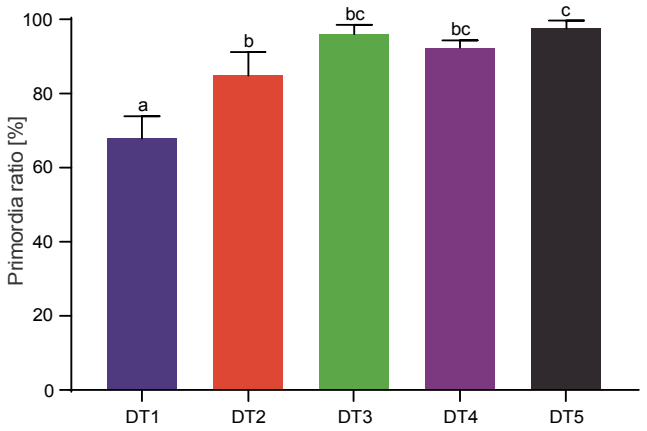

Fig. 4. Primordia ratio of *Cordyceps militaris* strains in artificial cultivation condition; bars with different letters show significant differences at $P < 0.05$

Discussion

Nutrients are well known to be the main factor that noticeably affect the growth of mushrooms (Nguyen et al., 2019). To meet all the nutrient requirements for the vegetative growth of *C. militaris*, various types of media were developed for its isolation, identification, and preservation (Shrestha et al., 2006). For the vegetative growth of *C. militaris*, sucrose, beef extract, zinc chloride, and folic acid were found to be optimal carbon, nitrogen, mineral, and vitamin sources, respectively (Pathania et al., 2015). As highlighted by Shrestha et al. (2006), SDAY, SMAY (maltose 40 g/l, peptone 10 g/l, yeast extract 10 g/l, agar 15 g/l), and CZYA (sucrose 30 g/l, yeast extract 5 g/l, $NaNO_3$ 3 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, KCl 0.5 g/l, KH_2PO_4 0.01 g/l, K_2HPO_4 1 g/l, and agar 20 g/l) were the optimal media for the vegetative growth of *C. militaris*. Therefore, SDAY was used as the medium to investigate the growth characteristics of *C. militaris* in the present study. As described previously by Shrestha et al. (2006) regarding mycelial texture, *C. militaris* showed cottony-type mycelial texture from the initial stage in the dark condition. The mycelial morphology of *C. militaris* growth on the solid-state medium was identified as wooly in texture, creamish white in color at the initial stage, which later changed to light brown at the mature stage (Pathania et al., 2015). In the present study, the five investigated strains showed floccose in terms of texture. Because of different culture periods, genotypes (Nopparat et al., 2018), and growth medium, the characteristics of the mycelium were considerably different for each of the *C. militaris* strains.

Unlike solid culture, submerged cultivation of fungi has several distinct advantages such as time-saving, enhanced bioactive compound content, and efficient biomass production. Therefore, submerged cultivation is becoming increasingly popular as a useful method for artificial field production (Jin et al., 2017). However, many studies emphasized that not all species of the higher fungi are capable of growing well as mycelial cultures in bioreactors (Turlo, 2014). During the submerged cultivation, the growth forms of the mycelium are directly linked to several factors such as culture medium, initial pH, inoculation size, aeration rate, and agitation speed. Generally, under fermentation conditions, filamentous and/or pelleted forms could be observed as growth forms (Park et al., 2002). In the present study, all the five strains were able to grow and form both filamentous and pelleted forms in submerged culture conditions. Therefore, it was concluded that submerged cultivation could be used as a spawn production technology for *C. militaris* cultivation at the industrial scale. As reported by Kim et al. (2003), during submerged cultivation of *C. militaris*, the cells formed mainly pellets at the initial stage and then maintained this growth form almost constant during the entire fermentation period. Several parameters such as diameter, circularity, roughness, and compactness are normally estimated to characterize pellet morphology (Park et al., 2002). Among these characteristics, pellet size is found to be one of the most important parameters. As reported previously, the pellet size greatly affects the transportation of oxygen and nutrients into the core region of pel-

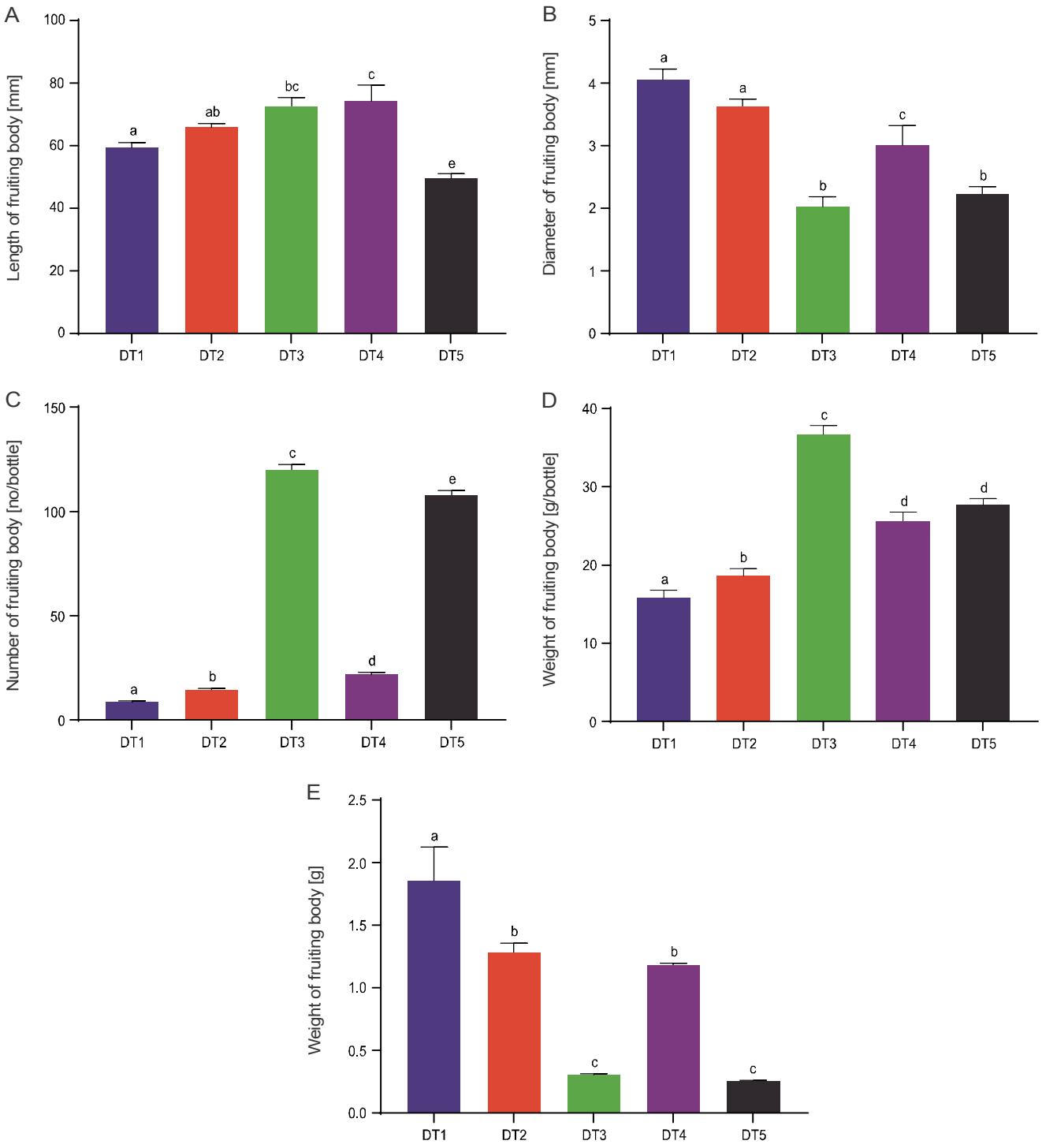

Fig. 5. Characteristics of the fruiting body of *Cordyceps militaris* strains; bars with different letters show significant differences at $P < 0.05$

let cells. The lack of oxygen and nutrients in the center leads to the death of large pellet cells. Thus, pellets with a small size are advantageous (Turlo, 2014). The size of pellet cells during incubation is strictly related to several factors such as agitation course, inoculum size, and carbon concentration in the medium (Turlo, 2014).

For the artificial cultivation of *C. militaris,* cottonseed shells, cob particles (Long, 2016), brown rice, German millet, and millet (Kim et al., 2010) could be used as the basal substrate and nutritional sources. Compared to other substrates, brown rice, German millet, and millet exhibitedhigher stromata lengths of approximately 50 mm

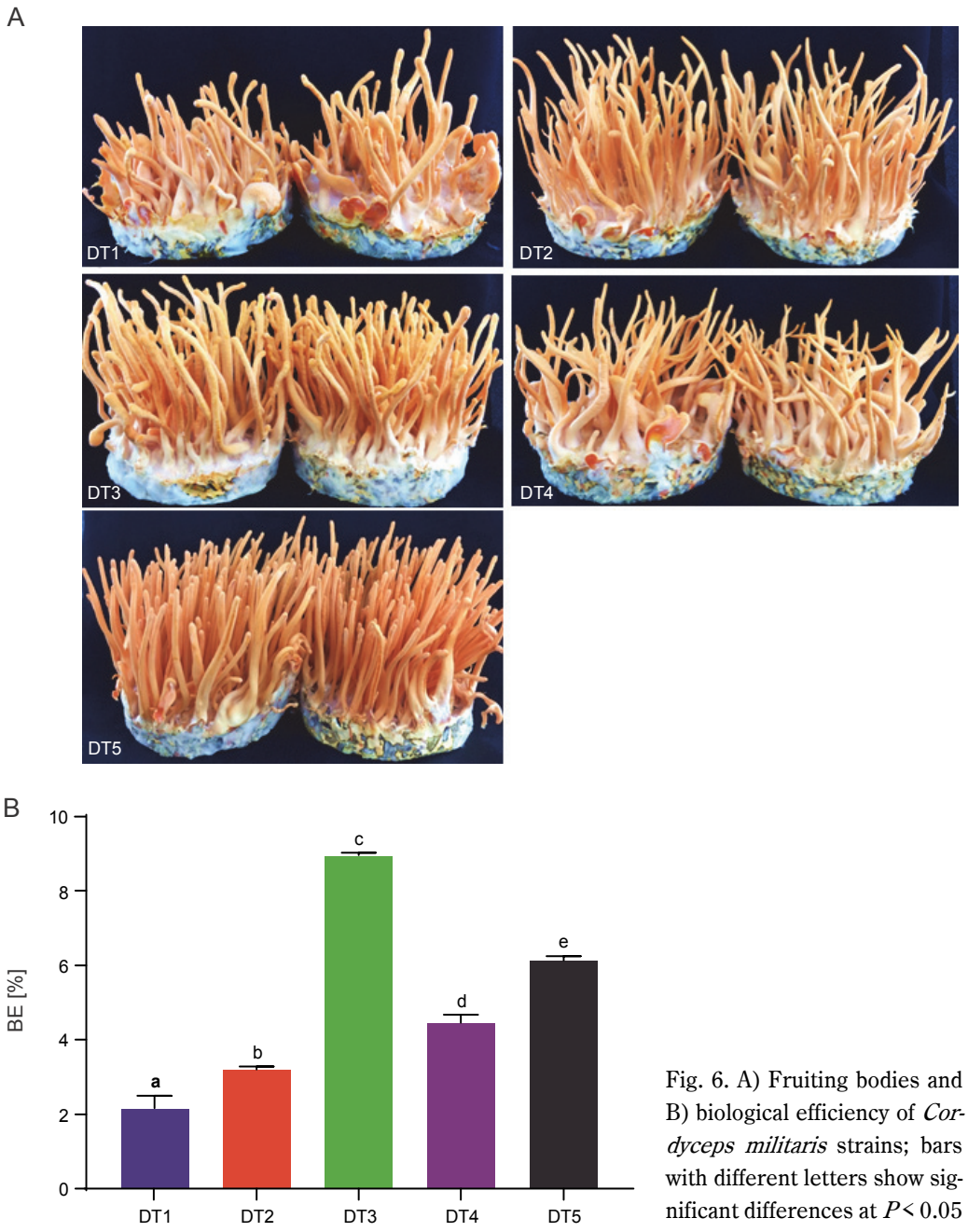

Fig. 6. A) Fruiting bodies and B) biological efficiency of *Cordyceps militaris* strains; bars with different letters show significant differences at $P < 0.05$

(Kim et al., 2010). Brown rice showed the highest yield of fruiting bodies with 6–7 g of fresh weight per bottle (Kim et al., 2010). Thus, brown rice supplemented with silkworm pupae powder as the main substrate was used in the present study. All five strains investigated in this study showed the ability to form and develop primordia to the maturity stage.

According to Lee et al. (2015), the fruiting bodies of strain Dowonhongcho and strain Yedang 3 were formed on 15 days after inoculation. Under favorable conditions, the period for substrate colonization, primordia formation, and harvest of *C. militaris* were 5–6 days, 12 days, and 45–50 days after inoculation, respectively (Du et al., 2010). Among all strains used for this study, the earliest primordia formation was recorded for strain DT3 with 18 days. This may be due to the different genotype and culture conditions. The length of the fruiting body is one of the most important morphological characteristics that affect the commercial value of *C. militaris*. As reported by Kim et al. (2010), higher lengths with fewer fruiting

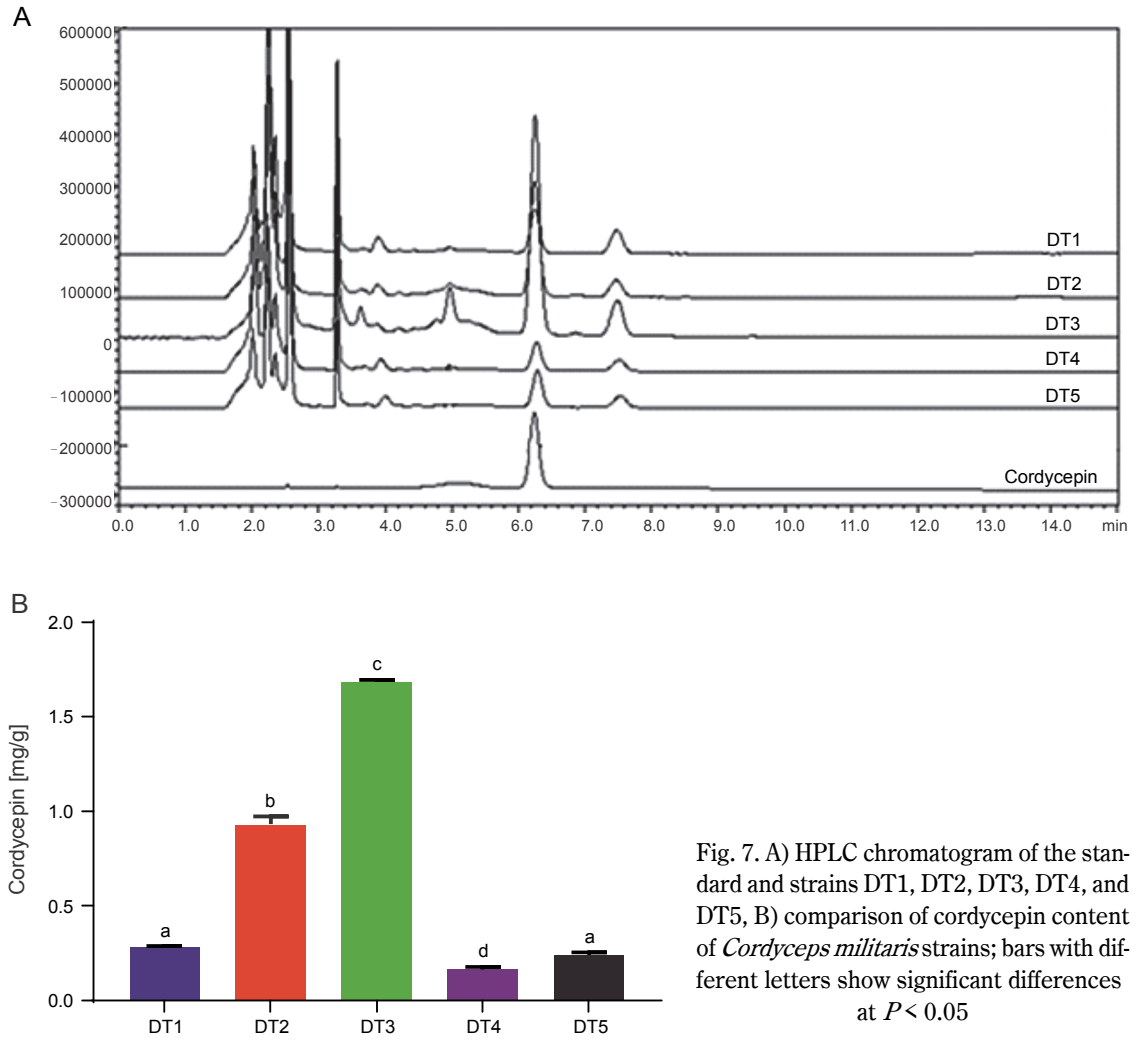

Fig. 7. A) HPLC chromatogram of the standard and strains DT1, DT2, DT3, DT4, and DT5, B) comparison of cordycepin content of *Cordyceps militaris* strains; bars with different letters show significant differences at $P < 0.05$

bodies often lead to reduced biomass and thus affect commercial value. However, higher biomass of fruiting bodies is related to the appearance of abnormally shaped fruiting bodies, thereby resulting in decline in commercial value (Kim et al., 2010). The size of the fruiting body ranged from 4.2 to 7.8 cm (Pathania et al., 2015). In our present study, strains DT4 and DT3 exhibited the highest length of the fruiting body with 74.23 ± 5.13 mm and 72.63 ± 2.62 mm, respectively.

Cordycepin, a well-known biomarker of genus *Cordyceps* (Ghatnur et al., 2015; Guo et al. 2016), has been widely used as a bioactive compound in immunological, hepatic, renal, and cardiovascular disorders and as an anticancer agent (Jin et al., 2018). The content of cordycepin in *C. militaris* is related to several factors such as culture condition, growth stage, and ingredients of medium. Thus, to enhance the production yield of cordycepin in *C. militaris* culture, numerous studies have been conducted to optimize culture conditions. Through optimal culture conditions (10 g/l glucose, 10 g/l peptone, 1.0 g/l $MgSO_4 \cdot 7H_2O$, 1.0 g/l K_2HPO_4, and 1.0 mg/l α-naphthylacetic acid), the average content of cordycepin isolated from fruiting bodies reached 9.17 ± 0.09 mg/g (Wen et al., 2014). *C. militaris* strains cultivated in silkworm exhibited the highest cordycepin concentration (4.17 ± 1.66 mg/g) followed by brown rice medium (2.98 ± 1.41 mg/g), while *C. militaris* cultivated in PDB showed the least content of cordycepin with 1.08 ± 0.73 mg/g (Kang et al., 2017). This may be because the silkworm pupae medium contains a high concentration of proteins and low carbohydrate content (Lee et al., 2017; Guo et al., 2016). Strain KSP8 with a high content of cordycepin (6.63 mg/g) was developed by mating (Kang et al., 2017). *C. militaris* cultivated on different

media could produce cordycepin in amounts ranging from 1 to 14 g (Cho et al., 2010). The contents of cordycepin, niacin, and potassium isolated from the fruiting bodies of *C. militaris* cultivated on soy power media were higher than those obtained from the strain cultivated on other media (Cho et al., 2010). The cordycepin content of strain Haizhou 1 was 24.98 mg/g DW (Du et al., 2010). In the present study, the cordycepin content of the five strains cultivated in rice medium ranged from 0.24 mg/g (DT5) to 1.68 mg/g (DT3). Therefore, further studies are needed to establish optimal culture conditions for cordycepin production.

Conclusions

All the tested fungal strains in the present study exhibited the ability to grow mycelium on both SDAY (dextrose 20 g/l, yeast extract 5 g/l, peptone 5 g/l, and agar 15 g/l) and liquid (peptone 1 g/l, yeast extract 1 g/l, $MgSO_4 \cdot 7H_2O$ 1 g/l, KH_2PO_4 2 g/l) media. Compared with other strains, DT3 exhibited the highest biological growth efficiency ($8.95 \pm 0.07\%$) and cordycepin content (1.68 mg/g). Therefore, strain DT3 could be considered as a potential strain for commercial cultivation.

Acknowledgments

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