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Original Article

The effects of temperature, pH, and media on mycelium growth of *Isaria tenuipes* (Peck.) Samson (DL0099) from Lang Biang Mountain, Lam Vien Plateau, Vietnam

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Abstract

Isaria tenuipes (Peck.) Samson is an entomopathogenic fungus that has potential in pharmacology and biocontrol. The study aimed to determine the effects of temperature, pH, and media on mycelium growth of *Isaria tenuipes*(Peck.) Samson (DL0099) from Lang Biang Mountain, Lam Vien Plateau, Vietnam. In this study, we used the single-factor method to culture DL0099 mycelium on both surface liquid and agar media. The results indicated that the optimal temperature for the mycelium growth was 20~25 ºC. This mushroom mycelium can tolerate temperatures at 35 ºC for 8 days. The initial media pH range of 6~9 was found to be the most favorable to mycelia growth. Sabouraud's agar (SA) and Sabouraud's dextroseagar plus yeast (SDYA) media were the optimal agar media for the mycelium growth rate. Sabouraud broth (S) medium was the most suitable medium for mycelia biomass production in surface liquid culture. Yeast malt agar (YMA), malt agar (MEA), maltose agar (MA), and malt extract yeast agar (MYA) media were suitable for fruit body formation. Our results provided optimal temperature, pH, and media conditions for *Isaria tenuipes* (DL0099) mycelium growth on surface liquid and agar media. They lay the basis for effective propagation, biomass production, and fruit body formation of the Vietnam native *Isaria tenuipes* (Peck.) Samson.

Keywords: Entomopathogenic fungi, *Isaria tenuipes* Peck. (DL0099), Lang Biang Mountain, Mycelium growth

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Introduction

Insect parasitic fungi are fungi that cause disease in insects. They belong to 12 classes in 6 phyla of the fungal kingdom (Álvarez et al., 2019; Maina et al., 2018). Entomopathogenic fungi are very rich in genus and species. There have been recorded cases of 12 Oomycetes species, 238 Basidiomycota species, 339

Microsporidia species, 474 Entomophtoromycota species and 476 Ascomycota species (Araújo and Hughes, 2016). Among insect parasitic fungi, the genus *Isaria* is an important genus of fungi, comprising more than 100 different species, which play important roles in pharmacology and biological control (Elkhateeb et al., 2021; Karthi et al., 2020; Luangsa-Ard et al., 2005).

I. tenuipes is the most common species of the genus *Isaria*. This is an insect parasitic fungus that is considered traditional medicine in China, Japan, and Korea (Hong et al., 2007). It fruit body is used to create products with health benefits, contributing to hypoglycemia, anti-tumor, antibacterial, anti-depressant, antioxidant, lipid-lowering, and immunomodulatory effects (Prommaban et al., 2022; Shin et al., 2003; Zhang et al., 2019). The polysaccharide extracts from the mycelium of these fungi constitute the major bioactive agents and exhibit a variety of pharmacological activities, including anticancer, anti-inflammatory, immune-enhancing, hypoglycemic, protective neurons against free radical-induced cytotoxicity, steroidogenic, and antioxidant activities (Chhetri et al., 2020; Kenkhunthot and Labua, 2020; Sharma, 2015). Many secondary compounds have been found in *I. tenuipes* such as pseudo-dipeptide hanasanagin; isariotins A–D (1–4) from *I. tenuipes* BCC 7831 (Haritakun et al., 2007). Isariotins E and F, Spirocyclic and Bicyclic Hemiacetals from *I. tenuipes* BCC 12625 (Bunyapaiboonsri et al., 2009), Isariotins G-J from *I. tenuipes* BCC 15621, BCC 23112, BCC 21283 (Bunyapaiboonsri et al., 2011); cordycepin, tenuipesine, sterols, trichothecanes, cyclopeptides have been found from *I. tenuipes* (Isaka et al., 2007). Besides, they also have many insecticidal activities, especially against agricultural pests of the scale group (Elkhateeb et al., 2021; Maina et al., 2018).

Lang Biang Mountain, Lam Vien Plateau in Lam Dong province is one of the four biodiversity centers of Vietnam, with a rich source of fungi, especially insect parasitic fungi (Lao et al., 2021). *I. tenuipes* (Peck.) Samson (DL0099) is one of 11 strains belonging to the species *I. tenuipes* (Peck.) Samson that have been collected in the wild at Lang Biang Mountain by Taynguyen Institute for Scientific Research, Vietnam Academy of Science and Technology (VAST) (Ly et al., 2015). *I. tenuipes* (Peck.) Samson has been cultivated in Japan, Korea, and China (Cho et al., 1999; Choi et al., 1999; Fukatsu et al., 1997; Kang et al., 2010; Lee et al., 2013; Nam et al., 1999; Xu et al., 2006). In Vietnam, studies related to mycelia growth, mycelia density, and fruiting body formation of Vietnam native *I. tenuipes* (Peck.) Samson is still limited. In general, the mushroom growth was affected by many factors, including intrinsic factors such as substrate composition, carbon source, nitrogen source, mineral source, pH, moisture, and levels of spawning, and extrinsic factors such as temperature, humidity, light, air composition, and encasement. They can act separately or interact with each other (Bellettini et al., 2019). Optimizing the conditions affecting fungal growth contributes to optimum efficiency in mushroom production, reducing losses, and reducing costs (Dulay et al., 2015; Zhang et al., 2012). *I. tenuipes* (Peck.) Samson has potential in pharmacology and biological control. Therefore, it is necessary to develop a method for mycelia growth with faster growth rate, dense mycelium density, and more fruiting body formation in *in vitro* conditions. In this study, we conducted a study to optimize some mycelia culture conditions such as temperature, pH, and media to the mycelium biomass and growth rate of *I. tenuipes* (Peck.) Samson (DL0099). The results in this present study were the basis for effective propagation, biomass production, and fruit body formation of the Vietnam native *I. tenuipes* (Peck.) Samson.

Material and Methods

Culture media

The culture agar used included water agar (WA), potato dextrose agar (PDA), Sabouraud's dextrose agar plus yeast extract (SDYA), yeast malt agar (YMA), hamada media (HMA), malt agar (MEA), maltose agar (MA), malt extract yeast agar (MYA), Sabouraud's agar (SA), Matin's peptone dextrose agar (MPDA), Czapek-dox agar (CDA) (Table-1).

Table-1. Composition of culture media

Nutritional regents	Medium (g/L)										
	WA	PDA	SDYA	YMA	HYA	CDA	MEA	MYA	SA	MA	MPDA
Potato		200									
Dextrose		20	20	10	20		20	4	20		10
Malt extract				3			20	10		20	
Peptone			5	5			1		10		5
Yeast extract			5	3	3			4			
HMA					3						
$MgSO_4.7H_2O$						0.5					0.5
KH_2PO_4						1					0.1
KCl						0.5					
Fe_7H_2O						0.01					
Sucrose						30					
$NaNO_3$						3					
Agar	20	20	15	20	20	20	20	15	20	20	20

Fungal culture

Isaria tenuipes Peck. (DL0099) was collected from Lang Biang Mountain, Lam Vien Plateau, Vietnam (N 11°02′19.8″, E102°46′04.7″, elevation 1650 m), in August 2011 and identified by Dr. Truong Binh Nguyen from Taynguyen Institute for Scientific Research, VAST (Ly et al., 2015) and were deposited at the Department of Microbiology, Taynguyen Institute for Scientific Research, Vietnam Academy of Science and Technology (VAST). They were maintained in potato dextrose agar medium (PDA) at 4 °C.

Inoculum preparation

The PDA medium (200 g/L of potato extract, 20 g/L of glucose, and 15 g/L of agar) was prepared by first being autoclaved for 15 minutes at 121 °C before being poured into sterile Petri dishes.

DL0099 mycelium was taken out of the refrigerator and placed at room temperature for 24 hours, then the mycelium (0.8 cm diameter) was transferred to the medium dishes prepared above, incubated in dark for 10 days. The healthy, disease-free seed plates were used for next experiments.

Single factor experimental design

The single factor test was conducted with media, temperature, and pH value as the factors on liquid and agar culture. The optimum medium was selected through preliminary experiments as the base medium for each group of experiments. Each treatment was replicated thrice. The diameter of the mycelium expansion was measured nine days after the inoculation and the mycelium density was noted. Biomass was harvested after 20 days of culture. The dry weight of biomass was estimated by filtering the culture to separate fungal biomass, which was rinsed twice with distilled water and dried at 70 °C to constant weight. The mycelia dry weight per volume of culture media was measured. Compare mycelium growth rate, biomass, and density of strain DL0099 under different conditions.

Growth in agar media

Effect of various agar media

The DL0099 mycelia growth on agar media was evaluated by using eleven different kinds of culture media (Table-1). Before autoclaving, the media were adjusted to pH 7. In a Petri dish, 15 mL of every medium was poured aseptically after autoclaving (1.5 kg/cm2) for 15Mins at 121 °C**.** To obtain similar sized mycelia dishes, we used a sterile punch tool (8 mm in diameters) to cut the mycelium. The mycelium disc was placed in Petri dish containing culture medium (15 mL) under aseptic conditions and incubated at 25 °C in the darkness.

Effect of temperature

In order to determine the best temperatures for mycelia growth, DL0099 mycelium was cultivated at seven different temperatures (8; 13; 20; 25; 30; 35; and 37 °C). The evaluation of the effect of temperature on mycelium growth was based on the standard protocol by Kang et al., 2010. An 8 mm diameter agar plug of the inoculums was cut from 10 days old culture grown and placed in the middle of each agar plate in 15 mL of SDYA.

Temperature tolerance

DL0099 mycelium was grown on SDYA for 7-15 days in total dark condition at 35 and 37 °C then it was shifted to 25 °C in total dark condition for 7 days. Four Petri plates (replicates) every time were used for performing this experiment. The diameter of the mycelium expansion was measured nine days after the inoculation and the mycelium density was noted.

Effect of initial pH

Under aseptic circumstances, the mycelium discs (8 mm in diameter) were inoculated into the Petri dishes containing sterilized SDYA media (15 mL), and then they were incubated in the dark at seven different pH levels (4; 5; 6; 7; 8; 9; 10). The initial pH values were adjusted before sterilization using 0.1 M NaOH or HCl.

Growth in surface liquid culture

For the effects media on mycelium biomass: Using 11 kinds of culture medium as in the experiment effect of different agar media on mycelia growth. However, these experiments did not use agar. The media were adjusted to pH 7.0. 100 mL of each medium was put into 500 mL flasks containing and autoclaved for 15 minutes at 121 °C. The mycelium discs were used as inoculums (8 mm in diameter). Biomass was harvested after 20 days of culture.

For the effects temperature on mycelium biomass: 100 mL of the Sabouraud broth medium was distributed into various flasks to measure temperature. The flasks were autoclaved for 15 minutes at 121 °C before being inoculated with mycelium discs (8 mm in diameter) at 8; 13; 20; 25; 30; 35; and 37 °C, as stated in the growth media experiment, incubated and examined.

For the effects pH on mycelium biomass: The same Sabouraud broth medium was used for the pH experiment, but the medium was altered to pH 4; 5; 6; 7; 8; 9, and 10 by using 0.1 M NaOH or HCl. 100 mL of each treatment was put into a 500 mL flask and autoclaved before being inoculated, incubated, and then analysed as was done for the temperature experiment.

Statistical analysis

Data were analysed in the Statistical Package for Social Sciences (SPSS) version 16 using one-way analysis of variance (ANOVA) and Tukey test to analyse the significant treatment comparison at 5% level of significance. Each value is expressed as mean ± standard deviation. All the experiments were conducted in triplicates.

Results

The effects of different media on the mycelium growth

To evaluate the mycelium growth rate, we cultured the mycelium on 11 different agar media: PDA, YMA, SDYA, HMA, MEA, MY, SA, MA, CDA, MPDA, and WA. Simultaneously, to evaluate the effects media on mycelium biomass, we cultured mycelium on 11 media above without adding agar, surface liquid culture conditions, and room temperature. Table-2 and Figure-1 present the experimental findings.

Table-2. Effect of culture medium on the mycelium growth

Medium	Mycelium growth rate (mm/day)	Dry mycelium biomass (g/100mL)	Mycelium density
WA	2.80 ± 0.00^{d}	0.00 ± 0.00^{g}	+
PDA	5.01 ± 0.06^{b}	0.35 ± 0.03^{f}	++
SDAY	5.80 ± 0.10^{a}	0.98 ± 0.04^{b}	+++
YMA	5.13 ± 0.06^{b}	0.74 ± 0.04^{c}	+++
HMA	5.17 ± 0.06^{b}	0.75 ± 0.006^{c}	+++
CDA	4.87 ± 0.12^{c}	0.52 ± 0.07^{e}	+
MEA	5.10 ± 0.00^{b}	0.76 ± 0.04^{c}	+++
MYA	5.13 ± 0.06^{b}	0.77 ± 0.02^{c}	+++
SA	5.83 ± 0.06^{a}	1.3 ± 0.03^{a}	+++
MA	5.10 ± 0.10^{b}	0.74 ± 0.04^{c}	+++
MPDA	5.10 ± 0.10^{b}	0.67 ± 0.05^{d}	+++

Each value is the mean ± SD of three duplicate tests (n = 3). The difference between different characters (a, b, c, etc.) in the same column is significant at $p<0.05$.

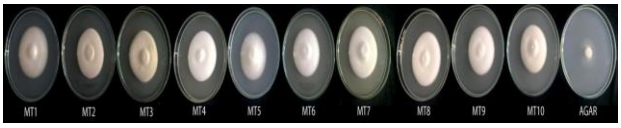

Figure-1. Growth of *I. tenuipes* (DL0099) mycelium on different agar media

MT1 = PDA: potato dextrose agar; *MT2=SDYA: Sabouraud's dextrose agar plus yeast extract; MT3=YMA: yeast malt agar; MT4=HMA: hamada media; MT5= CDA: Czapek-dox agar; MT6=MEA: malt agar; MT7=MY: malt extract yeast agar; MT8=SA: Sabouraud's agar; MT9=MA: maltose agar; MT10= MPDA: matin's peptone dextrose agar; Agar= WA: water agar.*

Experimental results showed that the mycelium can grow on 11 types of test media. The growth ability of the mycelium on the agar media was arranged follows: SDYA = SA > YMA = HMA = MYA = MEA = MA = MPDA > PDA > CDA > WA. The most suitable agar media for the mycelium growth were SA and SDAY medium, with an average growth rate was 5.8~5.83 mm/day, and Sabouraud broth (S) medium was the most suitable medium for mycelia biomass production with the dry mycelium biomass was 1.3 g/100 mL. Growth rate, as well as mycelium biomass on YMA, HMA, MYA, MEA, MA, and MPDA media were not significantly different. WA and CDA media should not be selected for mycelia culture because the mycelium is feeble, and the mycelium growth rate on WA and CDA was 2.8 mm/day and 4.87 mm/day, respectively.

Media containing organic nitrogen sources (SDYA, HMA, YMA, MEA, MY, SA, and MPDA) resulted in higher mycelium density, growth rate, and biomass compared with media containing inorganic nitrogen source (CDA and WA). Among the media containing organic nitrogen sources, YMA, MEA, MA and MY media promoted fruit body formation. However, the mycelium biomass in YM, ME, MA and MY media was lower than in SDY and S media. This experiment showed that the culture medium had a significant effect on mycelia density, growth rate, mycelium biomass, and fruiting body formation. Depending on the purpose of culture, we choose the appropriate medium. In these experimental conditions, the optimal agar medium for growth rate was SA and SDAY. The optimal medium for mycelium biomass was Sabouraud broth medium. YMA, MEA, MA and MYA media were suitable for the fruit body formation.

The effects of temperature on the mycelium growth

When examining the impact of temperature on DL0099's mycelium growth at levels: 8; 13; 20; 25; 30; 35, and 37 °C. After 9 days incubated, a favorable temperature condition for the mycelia growth of DL0099 was observed at 20~25 °C. DL0099 mycelium could grow at 13~30 °C. No growth occurred at or above 35 °C. The mycelium growth was very slow and thin at 13 °C. They did not expand at 8 °C after 9 days of incubation. At 20~25 °C temperature, the growth rate and mycelium biomass were highest. Table-3 and Figure-2 displays the test findings.

Table 3. Effect of temperature on mycelia growth of DL0099

Temperature (°C)	Mycelium growth rate (mm/day)	Dry mycelium biomass (g/100mL)
8	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}
13	3.27 ± 0.26^{b}	0.17 ± 0.03^{c}
20	5.30 ± 0.10^{a}	1.26 ± 0.04^{a}
25	5.20 ± 0.76^{a}	1.28 ± 0.11^{a}
30	3.17 ± 0.16^{b}	0.36 ± 0.04^{b}
35	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}
37	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}

Each value is the mean ± SD of three duplicate tests (n = 3). The difference between different characters (a, b, c, etc.) in the same column is significant at $p<0.05$

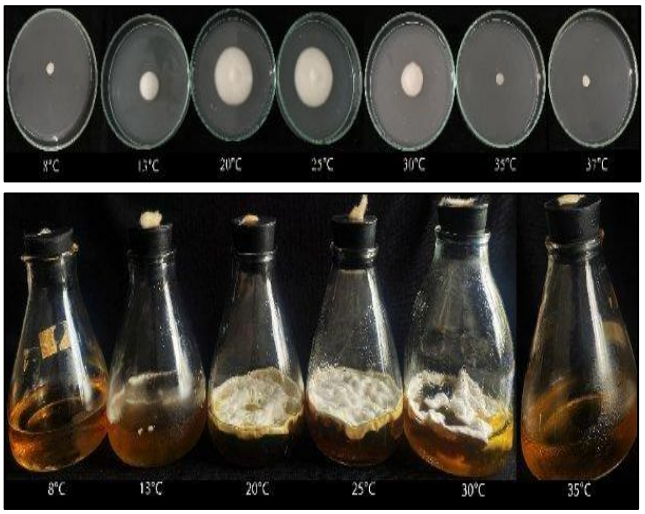

Figure-2. Effect of different temperatures on the mycelium growth of *I. tenuipes* (DL0099)

Table-4. Mycelium growth rate and dry biomass after 9 days incubated (mm) when transferred to 25 °C

Initial incubation Temperature (°C)	Initial incubation time (days)	Mycelium growth rate (mm/day)	Dry mycelium biomass (g/100mL)
35	7	5.31 ± 0.00^{a}	1.15 ± 0.15^{a}
	8	5.27 ± 0.06^{a}	1.02 ± 0.11^{a}
	9	-	-
	10	-	-
37	7	-	-
	10	-	-
	15	-	-

Each value is the mean ± SD of three duplicate tests (n = 3). The difference between different characters (a, b, c, etc.) in the same column is significant at $p<0.05$. (-) : No grow

Tolerance temperatures of DL0099

To evaluate tolerance temperature of *I. tenuipes* (DL0099), DL0099 mycelium was incubated at 35 and 37 °C for 7 to 15 days and then transferred to 25 °C. The findings demonstrated that after being initially cultured for 7 and 15 days at 35 °C and 37 °C, the fungus did not exhibit mycelia growth (Table-4). However, when transferred from 35 °C to 25 °C and continuing to culture, they were restored and developed normally with a 5.27 mm/day growth rate.

Table-5. Effect of pH on mycelia growth (DL0099)

pH	Mycelium growth rate (mm/day)	Dry mycelium biomass (g/100mL)
4	2.67 ± 0.40^{c}	0.32 ± 0.05^{d}
5	4.83 ± 0.40^{b}	1.16 ± 0.09^{c}
6	5.2 ± 0.10^{a}	1.28 ± 0.01^{a}
7	5.27 ± 0.06^{a}	1.31 ± 0.05^{a}
8	5.2 ± 0.10^{a}	1.30 ± 0.07^{a}
9	5.23 ± 0.06^{a}	1.29 ± 0.01^{a}
10	5.07 ± 0.06^{ab}	1.20 ± 0.01^{b}

Each value is the mean ± SD of three duplicate tests (n = 3). The difference between different characters (a, b, c, etc.) in the same column is significant at $p<0.05$.

The effects of pH on the mycelium growth

The mushroom can tolerate a wide pH range, they can grow in initial media pH range of 4~10. There was not much difference ($p<0.05$) mycelium growth rate and dry mycelium biomass obtained at pH from 6 to 9 in Sabouraud broth medium. The mycelium growth rate and dry mycelium biomass were highest in initial media pH range of 6~9. At pH 5 and 10, the mycelium growth rate and dry mycelium biomass tend to decrease. The mycelia density was somewhat thin at pH 5 and 10. In this experimental condition, the initial media pH range 6~9 were favorable for mycelium growth.

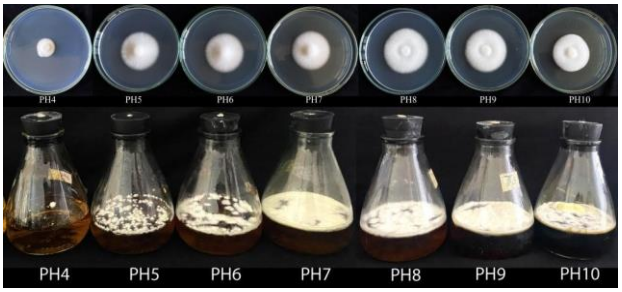

Figure-3. Effect of pH on mycelia growth of *I. tenuipes* (DL0099)

Discussion

In this paper, temperature, pH, and media were factors that have a significant impact on the growth of the *Isaria tenuipes* (DL0099). *I. tenuipes* (DL0099) mycelium can grow in temperature range of 13~30 °C. 20~25 °C was the ideal temperature for the mycelium growth rate, mycelia density, and biomass production. The mycelium growth was slow at 30 °C and 13 °C. The results of the effect temperature on *I. tenuipes* (DL0099) mycelium growth were quite similar to those reported previously. Kang et al. (2010) reported that the optimum temperature for the growth of *Paecilomyces tenuipes* PJ-1 was 25 °C in solid culture. The growth of the *P. tenuipes* PJ-1 mycelium rapidly decreased above 35 °C and below 15°C. Fukatsu et al. (1997) successfully isolated and cultured *P. tenuipes* on liquid and agar media at 20~25 °C. When evaluating the temperature tolerance of Vietnam *I. tenuipes* (DL0099) native, we found that the temperature tolerance of this strain is lower than the Korean P. *tenuipes* PJ-1. *P. tenuipes* PJ-1 can expand the mycelium slowly at 35 °C. However, DL0099 mycelium did not expand when incubated at 35 °C, they can survive at 35 °C for 8 days. At 37 °C, DL0099 mycelium stopped growing. This can be explained by their different geographical origin. The intraspecific variability was partially related to the microclimate of the fungal biotopes (Vidal et al., 1997). Vidal et al. (1997) showed the majority of *P. fumosoroseus* isolates from Europe (mild climates) grow best at temperatures between 8 and 30 °C, with ideal growth rates occurring between 20 and 25 °C. The temperature range was broader (8~35 °C) for *P. fumosoroseus* isolates from western Asia (wet tropical climates) and the southern United States (dry subtropical climates and humid climates), with growth optimal at 25~28 °C. *P. fumosoroseus* species that can tolerate high temperatures (between 32 and 35 °C) are those that are isolated in India's monsoon climate. Da Lat City of Lam Dong province, Vietnam has an average altitude of about 1500 m above sea level and its average temperature ranges 18~25 °C. The highest temperatures are less than 30 degrees. Experimental results shown in Table 3.1 showed that *I. tenuipes* (DL0099) mycelium grows most strongly at the temperature range of 20~25 °C. Therefore, *I. tenuipes* (DL0099) is quite suitable for cultivation in Da Lat City of Lam Dong province, Vietnam.

I. tenuipes (DL0099) can grow on surface liquid and agar media with pH between 4 and 10. The initial

media pH range of 6~9 was found to be the most favorable to mycelia growth. This is similar to previous studies on other insect parasitic fungi (Lee et al., 2013; Nam et al., 1999). It is observed that when the pH is too low, the metabolic activity of the cell decreases and inhibits the cell growth while at higher pH the growth time is shorter. The decrease in growth rate may reflect the disruption of metabolic processes or the death of cells. A mild osmotic acid can cause cytoplasm acidification by being added to the medium, where it will pass through the plasma membrane as a free and ionized acid and transform into its anion and proton inside the cell. The cell may be inhibited by this acidity. Their metabolic reactions, rates of development, and nutrient consumption needs for their growth and cordycepin contents can vary depending on pH changes (Leung and Wu, 2007).

By testing the mycelium culture on 11 different media types, we have determined that the optimal agar media for the mycelium growth were SA and SDYA media with an average growth rate was 5.8~5.83 mm/day. The optimal medium for mycelium biomass was Sabouraud broth medium with the dry mycelium biomass was 1.3 g/100 mL. With these results, we found that the media were more optimal than the report by Nam et al., 1999. Media containing organic nitrogen sources (SDYA, HMA, YMA, MEA, MY, SA, and MPDA) resulted in higher mycelium density, growth rate, and biomass compared with media containing inorganic nitrogen source (CDA and WA), as reported in other insect parasitic fungi species (Sung et al., 2010, 2011). Beside, in this study, we found YMA, MEA, MA and MYA media were suitable for the fruit body formation. This will be beneficial to mushroom growers in the cultivation to form *I. tenuipes* (DL0099) fruit bodies.

Conclusion

In conclusion, this study demonstrated the effects of temperature, pH, and media on the mycelium growth of *Isaria tenuipes* (Peck.) Samson (DL0099) from Lang Biang Mountain, Lam Vien Plateau, Vietnam. Maximum mycelium growth of *I. tenuipes* (Peck.) Samson (DL0099) was achieved by cultivating them at 20~25 °C and a pH range of 6~9. The mycelium can tolerate temperatures at 35 ºC for 8 days. The optimal agar and broth media for the mycelium growth of *Isaria tenuipes* (Peck.) Samson (DL0099) were Sabouraud's agar (SA), Sabouraud's dextrose agar plus yeast (SDYA), and Sabouraud broth (S), respectively. The fruit body formation was promoted on yeast malt agar (YMA), malt agar (MEA), maltose agar (MA), and malt extract yeast agar (MYA) media. The obtained data can be used as a guideline for mushroom growers. Further studies on strain selection, nutrition values, and antimicrobial activity can be investigated for applications in the pharmacology and biological control of this fungus.

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Contribution of Authors

Hoa PN: Conceived idea and designed the experimental study, performed the experiments, wrotemanuscript and approved the final draft for submission.
Hoa ND: Performed the experiments, data analysis.
Diep TK & Huyen PV: Literature review, edited and formatted the manuscript draft.
Nguyen TB: Contributed in write up and manuscript critiquing.