Vietnam Journal of Agricultural Sciences

ISSN 2588-1299

VJAS 2018; 1(2): 117-126
https://doi.org/10.31817/vjas.2018.1.2.01

Identification of Optimal Culture Conditions for Mycelial Growth and Cultivation of Monkey Head Mushrooms *(Hericium erinaceus* (Bull.: fr.) Pers)

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Abstract

Monkey head mushrooms (*Hericium erinaceus* (Bull.: Fr.) Pers) have been broadly cultivated and widely consumed as traditional medicinal herbs as well as functional food in the Orient for several hundred years of history. The identification of optimal culture conditions for mycelium growth and fruiting body formation is one of the most important steps in cultivation of mushroom. The aim of this study was to investigate the optimal culture conditions including pH level, temperature, media and substrate mixtures for the mycelium growth and cultivation of *Hericium erinaceus* strain He-2. Results of the study revealed that the optimal conditions for mycelial growth were observed at 25 ± 1^{o}C and pH 8.0. *H. erinaceus* was cultured on five different types of culture media: Czapek, Raper, PGA (potato, glucose, agar), PGA supplemented with rice bran, and PGA supplemented with fresh mushrooms. PGA supplemented with fresh mushrooms was found to be the best medium for the growth of mycelia. A media containing 99% grain of rice + 1% $CaCO_3$ was considered as the best mother spawn media for mycelial growth. Among various culture media, the highest mycelium growth rate and biological efficiency of *H. erinaceus* were obtained when grown on a treatment of 87% sawdust + 4% corn bran + 8% rice bran + 1% $CaCO_3$.

Received: March 14, 2017
Accepted: September 7, 2018

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Keywords

Monkey head mushroom, mycelium, media, fruiting bodies

Introduction

Hericium erinaceus (Bull.: Fr.) Pers., commonly known as the monkey head mushroom, is considered as one of the best edible and medical mushrooms belonging to the family Hericiaceae, order

Russulales, and class Agaricomycetes (Kirk *et al.*, 2008). It has been widely consumed as traditional medicine and functional food in Asian countries for several hundred years. *H. erinaceus* fruiting bodies and mycelia are known to produce several extensive bioactive compounds, including health promoting substances like γ-aminobutyric acid (GABA), ergothioneine, and lovastatin (Cohen *et al.*, 2014) with different positive effects on the human body. As reported previously, various substances extracted from monkey head mushrooms have multiple pharmacological activities such as anti-microbial, anti-cancer (Gue *et al.*, 2006), and antioxidant activities (Chyi *et al.*, 2005), and can be used in the treatment of cancer, hepatic disorders, Alzheimer's and Parkinson's diseases, and wound healing (Sokół, 2015). In addition, *H. erinaceus* offers neuroprotective effects after ischemic brain injuries, peripheral nerve regenerative effects, and enhancement of sensory as well as functional recovery after nerve injury (Wong *et al.*, 2012; Lee *et al.*, 2014; Wong *et al.*, 2015; Wong *et al.*, 2016).

In order to obtain high quality mushroom spawn, the identification of optimal growth conditions is considered as one of the most critical steps. Therefore, the aims of this research were to evaluate various culture media, pH, temperature and substrate mixtures for the mycelia growth and fruiting body formation of *H. erinaceus.*

Materials and Methods

Mushroom strain

Monkey head mushroom *Hericium erinaceus* strain He-2 was obtained from the NN08 project. The culture was maintained on PGA (potato, glucose, agar) medium and stored in a refrigerator at 5-7^{o}C.

Media preparation

In order to prepare the culture media, potatoes were peeled, cut into small pieces, and boiled with distilled water for 30 m. The extract was filtered using steel mesh. Glucose and agar were added to the extract and dissolved. Water was added up to 1000 mL and then the media was poured into bottles. The media bottles were sterilized by autoclaving them at 121^{o}C for 60 min.

Paddy grains were prepared by washing and soaking them in water for 12 h to moisten them. The grains were boiled with an equal volume of fresh water until the grains became soft.

Sawdust without volatile oil and poisons can be used as a main substrate for the cultivation of *Hericium erinaceus*. Sawdust was mixed with a lime solution (4 kg of lime per 1000 L of water). The substrates were fermented for 5-7 days and then allowed to sit an extra 1-2 days until the substrates reached a 65% moisture level. The resulting substrate was poured into bottles. Each bottle contained 300 g and was autoclaved at 121^{o}C for 90 min.

Experiment design

Experiment 1: Effects of different initial pH levels on mycelial growth

The pH levels of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 were tested for the optimum mycelia growth of *H. erinaceus* (He-2). The medium was adjusted to the different pH levels with the addition of 1M NaOH or HCl.

Experiment 2: Effects of different temperature levels on mycelial growth

The petri dishes of PGA media were inoculated with *H. erinaceus* and incubated at four temperature levels (20˚C ± 1, 25˚C ± 1, 30˚C ± 1, and 35˚C ± 1) under darkness conditions. Mycelial growth was recorded daily (mm day^{-1}).

Experiment 3: Effects of different culture media on mycelial growth of pure spawn

The ingredients for the different culture media of pure spawn were as follows:

Treatment 1: Czapek (30 g Sucrose + 2 g $NaNO_3$ + 1 g KH_2PO_4 + 0.5 g $MgSO_4.7H_2O$ + 0.01 g $FeSO_4.7H_2O$ + 0.5 g KCl+ 20 g agar + 1000 mL distilled water)

Treatment 2: Raper (2 g yeast extract + 2 g peptone + 0.46 g KH_2PO_4 + 1 g K_2HPO_4 + 0.5 g $MgSO_4.7H_2O$ + 20 g glucose + 20 g agar + 1000 mL distilled water)

Treatment 3: PGA (20 g glucose + 250 g potatoes + 20 g agar + 1000 mL distilled water)

Treatment 4: PGA + 20 g rice bran

Treatment 5: PGA + 25 g fresh oyster mushrooms.

Experiment 4: Effects of different culture media on the mycelial growth of mother spawn

The ingredients for the different culture media used to grow the mother spawn were as follows:

Treatment A: 99% rice grain + 1% $CaCO_3$

Treatment B: 79% rice grain + 20% sawdust + 1% $CaCO_3$

Treatment C: 59% rice grain + 40% sawdust + 1% $CaCO_3$

Treatment D: 39% rice grain + 60% sawdust + 1% $CaCO_3$

Treatment E: 19% rice grain + 80% sawdust + 1% $CaCO_3$

The substrates were transferred into glass bottles and steam-sterilized for 90 min at 121°C. *H. erinaceus* was inoculated and grown on the culture media in glass bottles at 25°C under darkness conditions. The mycelial growth of *H. erinaceus* in the rice grain medium supplemented with sawdust was measured after several days of incubation.

Experiment 5: The growth and development of *H. erinaceus* cultivated on different substrates

For this experiment, *H. erinaceus* was cultivated on sawdust enriched by various types of supplements as follows:

Treatment I: 87% sawdust + 4% corn powder + 8% rice bran + 0% wheat bran + 1% $CaCO_3$

Treatment II: 87% sawdust + 4% corn powder + 6% rice bran + 2% wheat bran + 1% $CaCO_3$

Treatment III: 87% sawdust + 4% corn powder + 4% rice bran + 4% wheat bran + 1% $CaCO_3$

Treatment IV: 87% sawdust + 4% corn powder + 2% rice bran + 6% wheat bran + 1% $CaCO_3$

Treatment V: 87% sawdust + 4% corn powder + 0% rice bran + 8% wheat bran + 1% $CaCO_3$

Data collection

For the culture media, temperature, and pH experiments, data were recorded on the following parameters: mycelial growth rate (mm day^{-1}), characteristics of the mycelia, and diameter of the mycelia.

Mycelial growth was calculated using the following formula: V = D/T, where V is the mycelial growth rate (mm day^{-1}), D is the length of growth of the mycelia, and T is the duration of mycelial growth (days).

Data were also recorded on the period of surface colonization (days), the time required for mycelium to grow throughout the full media and establish total colonization on the bag surface, and the period of primordia formation (days), the time required for the formation of primordia.

Biological efficiency (BE) (%) was calculated with the following formula:

$$\frac{Weight\ of\ mushrooms}{Weight\ of\ substrates} \times 100$$

Statistical analysis

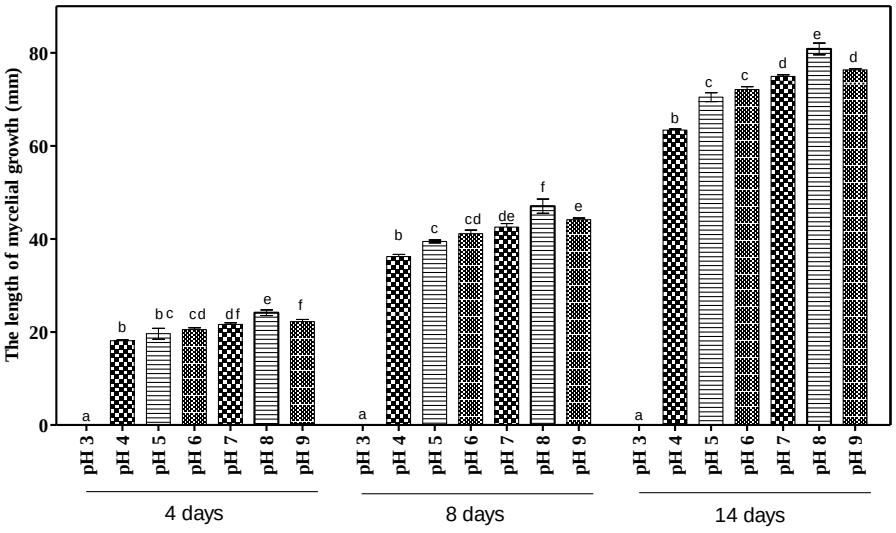
The data of experiment were statistically analyzed using IRRISTAT version 5.0 and GraphPad Prism version 5.0. Each treatment was replicated three times. Differences among the means of groups were assessed using the one-way or two-way analysis of variance (ANOVA) followed by a multiple-comparison test (Bonferroni post test).

Results and Discussion

Effects of pH on the mycelial growth of *H. erinaceus*

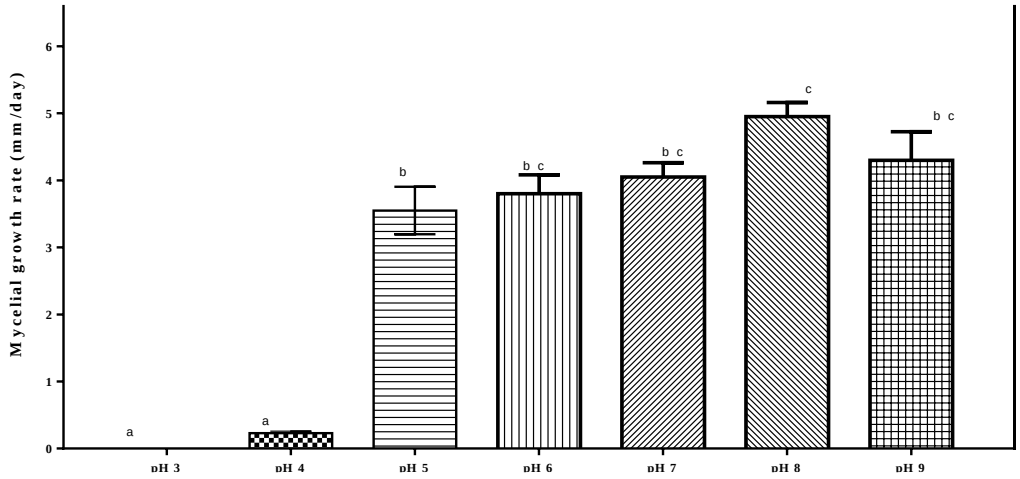
pH is generally considered to be one of the most important chemical factors that can affect cell membrane function, uptake of various nutrients, cell morphology and structure, solubility of salts, ionic state of substrates, enzyme activity, and product biosynthesis (Elisashvili, 2012). Most mushrooms grow and

perform well at a pH near to neutral or slightly basic (Khan *et al.*, 2013). According to Imtiaj *et al.* (2008), the pH values most suitable for the favorable growth of *H. erinaceus* were observed in the range of 5.0 ~ 9.0 and the best was pH 6.0. The other pH values also showed good mycelial growth, and pH 9.0 was better than pH 5.0 for the growth of the different strains of *H. erinaceus.* Grigansky *et al.* (1999) reported that for the growth of *H. erinaceus*, the optimum pH-level was between 5.8 and 6.2. To determine the optimum initial pH for mycelial growth, PGA media was inoculated with *H. erinaceus* at various initial pH values (3.0-9.0). The results presented in Figure 1 showed that the mycelial growth of *H. erinaceus* was affected by the initial pH. *H. erinaceus* was able to grow at the pH range of 4.0 to 9.0 (optimally at pH 8.0). Although *H. erinaceus* could grow over a wide range of pH values between 4.0 and 9.0, lower pH levels showed growth inhibition. A remarkable difference in terms of mycelial morphology was observed between acidic media (pH 4.0-6.0) and alkaline media (pH 7.0-9.0).



Note: pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 are values before being autoclaved. Bars in the same time period with different letters differ significantly at P<0.05.

Figure 1. Effects of different pH values on mycelial growth of *H. erinaceus*



Note: Mycelial growth rate of H. erinaceus on different pH levels. Bars with different letters differ significantly at P<0.05.

Figure 2. Effects of pH on the mycelial growth rate

The mycelia of *H. erinaceus* cultivated at the initial pH values of 4.0, 5.0, and 6.0 had a white color for the first 8 days, but changed to brown at the center of the plate after 20 days of incubation. In contrast, mycelia had a lighter white color for the first 10 days and remained white after 30 days of incubation in the alkaline media. The fastest spawn running time of the mycelia in pure culture was observed at pH 8.0 level. Therefore, we recommend this pH for the best mycelial growth of *H. erinaceus* in PGA media.

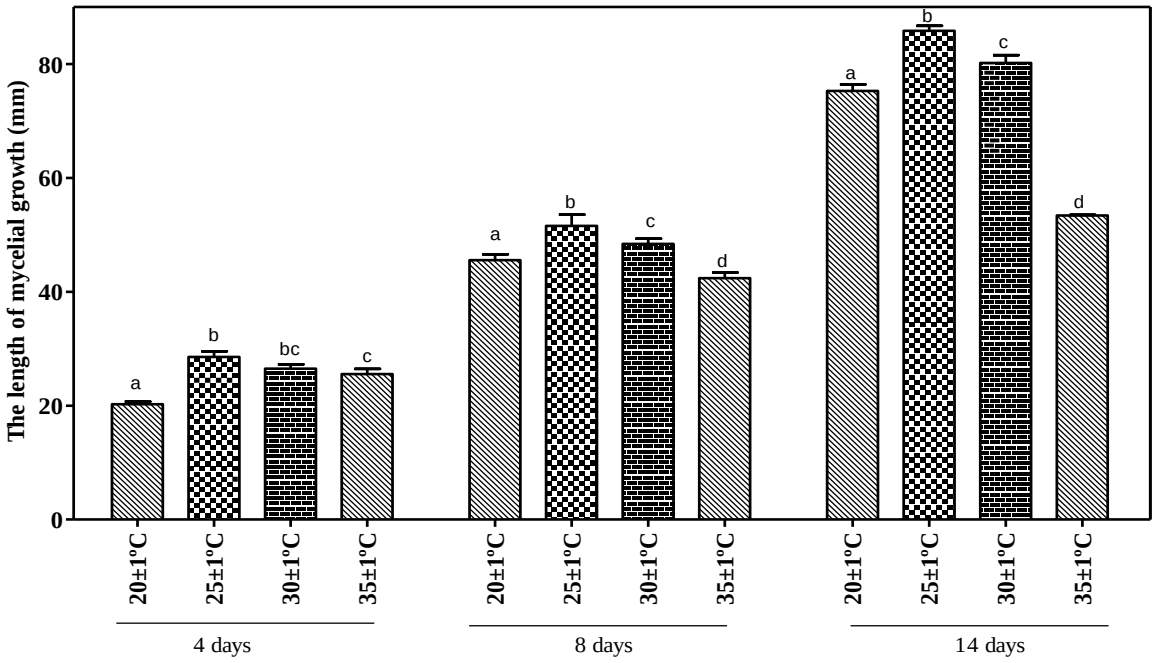
Effects of temperature on the mycelial growth of *H. erinaceus*

Like pH and other external factors, temperature is a significant physical factor that affects the growth of mycelium as well as fruiting body formation. Enzymatic activity and vitamin synthesis of fungi are also affected by temperature (Miles and Chang, 1997; Colauto *et al.*, 2008). For assaying the effect of temperature, mycelial growth of *H. erinaceus* was recorded at four different temperature levels, including 20°C ± 1, 25°C ± 1, 30°C ± 1, and 35°C ± 1. The average values of three replications in each treatment were calculated and used as quantitative measures for comparing growth. According to Ahmed *et al.* (2008), the incubation temperature most suitable for the mycelial growth of *H. erinaceus*is was found to be 25^{o}C. The optimum temperature for vegetative growth was observed to be 26^{o}C (Grigansky *et al.*, 1999).

For this experiment, we used PGA as the medium for inoculating the *H. erinaceus* strain He-2. The results of the observations are shown in Figure 3 and Table 1. The results indicated that *H. erinaceus* can grow at all the temperatures tested between 20-35^{o}C. However, the maximum growth was achieved with the temperature 25°C ± 1 (85.84 mm) followed by 30°C ± 1 (80.20 mm), and 20°C ± 1 (75.26 mm) after 14 days of incubation. Therefore, these results suggest that the best temperature for maximum mycelial growth of *H. erinaceus* is 25 ± 1°C. Under this condition, the density of the mycelia was thick with a white color and corroborates with the results of Ahmed *et al.* (2008).

Effects of different culture media on the mycelial growth of pure spawn

There are many types of culture media with



Note: Bars in the same time period with different letters differ significantly at *P*<0.05.

Figure 3. The mycelial growth of *H. erinaceus* on different temperature levels

Table 1. Effects of different temperature levels on mycelial growth of *H. erinaceus*

Factors / Temp. level	Mycelium run rate (mm day^{-1})	Mycelial density	Mycelial characteristics
20 ± 1°C	3.16	++	Mycelia density was thick with a light white color. Media were colonized incompletely. Fruiting body formation occurred.
25°C ± 1	4.78	+++	Mycelial density was thick with a white color. Media were colonized completely.
30°C ± 1	4.30	++	Mycelia density was thin with a light white color. Media were colonized completely.
35°C ± 1	2.82	+	Mycelia density was very thin. Media were colonized incompletely.
CV%	1.00		
$LSD_{(0.05)}$	0.16		

Note: +++: High; ++: Regular; +: Low.

different nutrient compositions that can be used for the vegetative growth of mushrooms. In this experiment, five different culture media were screened to determine the optimal media for mycelial growth of *H. erinaceus*. As shownin Table 2, *H. erinaceus* was able to grow on all five types of media tested. However, comparatively, the most suitable medium for mycelial growth was PGA supplemented with fresh mushroom extracts, corresponding to the mycelial growth rate of 3.73 mm day^{-1}. In addition, in terms of mycelial characteristics, the color of the mycelium was white in three type of media (PGA supplemented with fresh oyster mushrooms, PGA supplemented with rice bran, and Raper media).

Effects of different culture media on the mycelial growth of mother spawn

Following the results of the pure spawn media experiment, pure cultured spawn was inoculated into new medium to produce mother spawn. Cereal, rice bran, and sawdust are considered as the basic ingredients of culture media for the growth of mother spawn. For this study, we selected paddy rice and sawdust supplemented with $CaCO_3$ as the main components of the experiment treatment. Treatment B and C were the common culture media used for mushroom cultivation with a ratio of 79% rice grain + 20% sawdust + 1% $CaCO_3$, and 59% rice grain + 40% sawdust + 1% $CaCO_3$, respectively, and showed the best mycelial growth. The mycelium extension rate was fast with a poor density in treatment 5. By contrast, because rice grain media was rich in nutrition, allowed for mycelium respiration, and allowed the mycelium to easily grow into the substrate, the mycelium run rate was fast with a high density in treatment 1. These results suggest that rice grain should be used as a nutrient source in the culture medium for the development of mother spawn.

Effects of different substrates on fruiting body formation and biological efficiency of *H. erinaceus*

Sawdust was selected as the most preference basal ingredient in the substrate mixtures for *H. erinaceus* cultivation. In order to determine the best combination, correlation analyses were carried out. Sawdust, however, is well known as a substrate poor in nutrients. Therefore, to reduce the cultivation time and promote economic efficiency, the cultivation media was supplemented with essential nutrients for mycelial growth. In this experiment, we used corn bran, rice bran, and wheat bran in different percentages to determine the best formula for the growth and development of the mycelium.

BE is an important factor in mushroom cultivation and is the major purpose of this set of experiments. Sawdust was tested with three kinds of brans as supplements. Treatment I and treatment II had rice bran and corn bran which are high in vitamins and suitable for fruiting body development stage. As such, the highest

Table 2. Effects of different culture media on the mycelial growth of *H. erinaceus* pure spawn

Factors / Media	Mycelium run rate *(mm day^{-1})*	Mycelial density	Mycelial characteristics
Czapek	2.58	+	Mycelia density was very thin with a white color. Media were colonized incompletely.
Raper	3.42	++	Mycelia density was thick with a white color and colonized incompletely. Fruiting body formation did not occur.
PGA	3.38	++	Mycelia density was thick with a white color. Media were not colonized completely and stopped growing after 22 days of incubation.
PGA supplemented with rice bran	3.56	+++	Mycelia density was thick with a white color. Media were fully colonized after 24 days of incubation. Fruiting body formation did not occurred.
PGA supplemented with fresh mushrooms	3.73	+++	Mycelia density was thick with white color. Media were fully colonized after 21 days of incubation.
CV%	1.12		
$LSD_{0.05}$	0.65		

Note: +++: High; ++: Regular; +: Low.

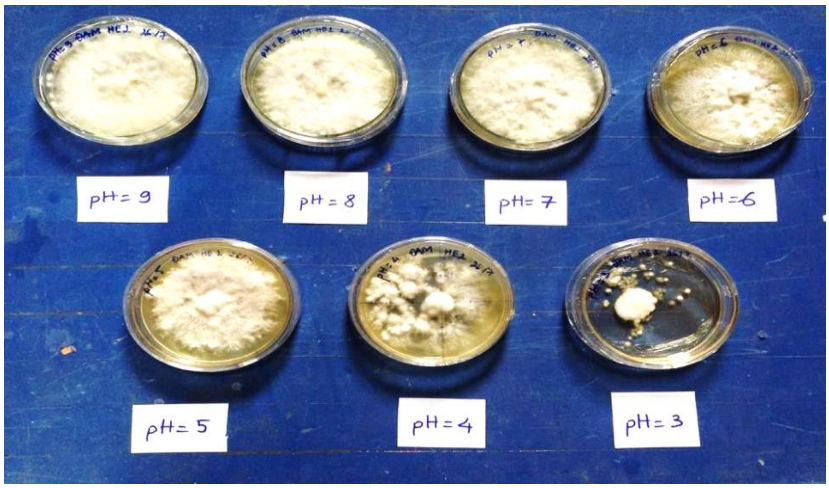


Figure 4. Mycelial growth on plates with different pH levels 20 days after inoculation

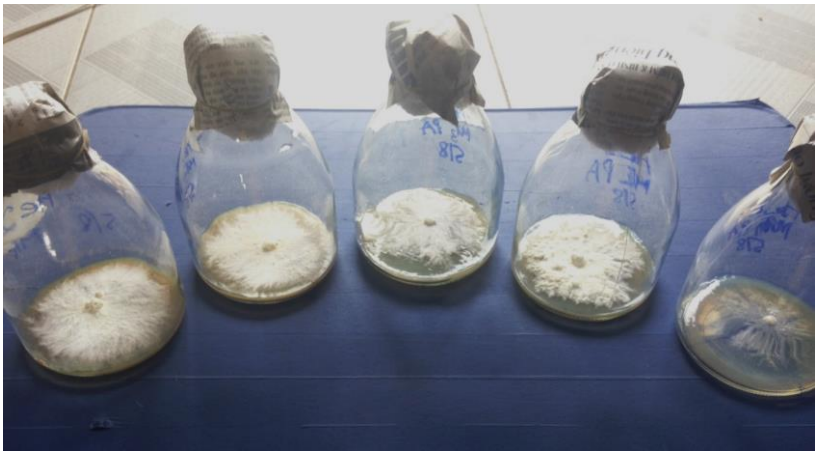
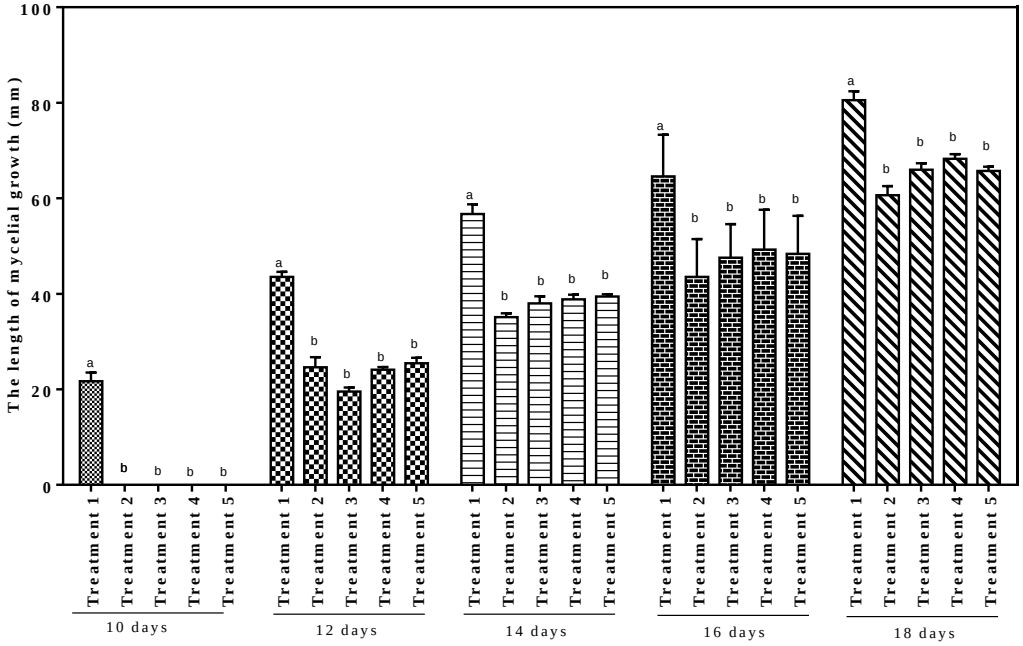


Figure 5. Mycelial growth on different media 20 days after inoculation *(right-to-left: media 1 to 5)*



Note: Bars in the same time period with different letters differ significantly at P<0.05.

Figure 6. Effects of different grade 1 culture media on the mycelial growth of *H. erinaceus*

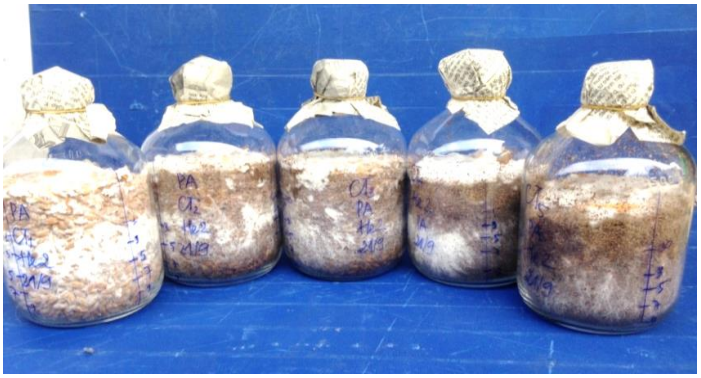


Figure 7. Mycelial growth on different substrates 20 days after inoculation

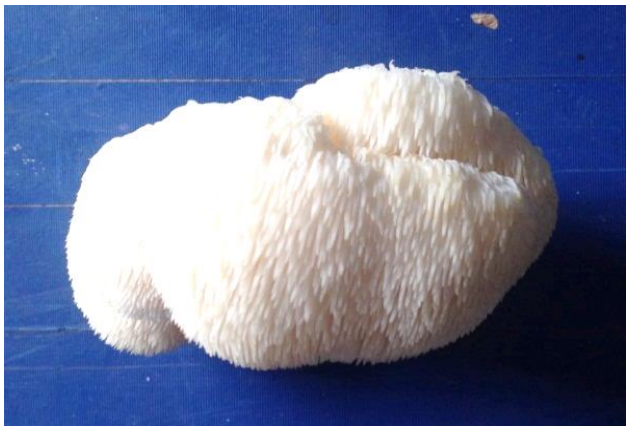
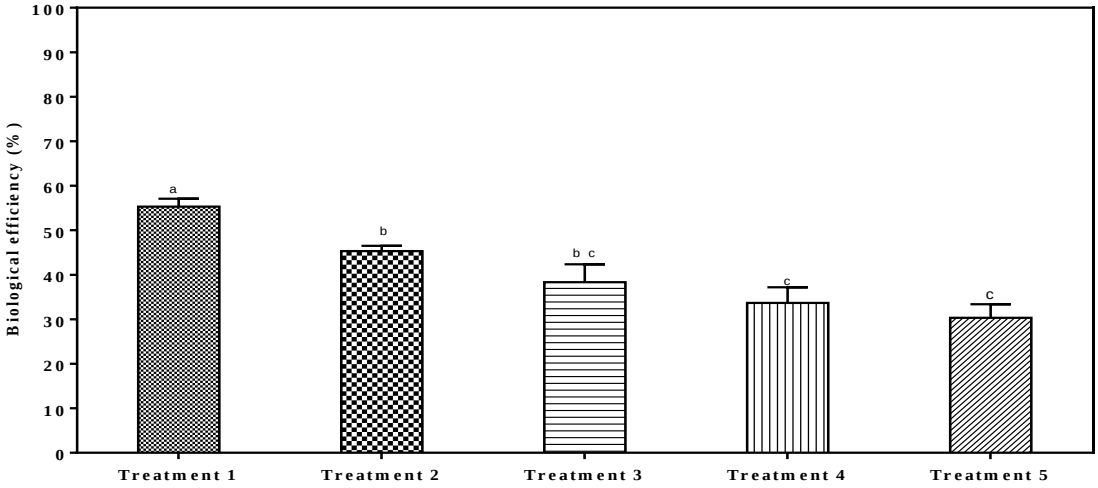


Figure 8. Fruiting body of *H. erinaceus* cultivated on formula



Note: Bars with different letters differ significantly at P<0.05.

Figure 9. Biological efficiencies of *H. erinaceus* grown on different substrates

BE value of 56.54% was obtained using treatment 1, and was followed by treatment 2 (45.44%). Treatments 4 and 5 had low BE values (33.02% and 30.56%, respectively) due to the lower percentage of rice bran than treatment 1 and 2. For treatment 3, the period of primordia formation required the longest time, and the biological efficiency had a higher value than treatments 4 and 5. The findings of the present study are in agreement with those obtained by Gyu *et al.* (2005) and Swiulski and Sobieralski (2005).

Conclusions

The pH value of 8.0 was determined to be the optimum pH for mycelial growth of *H. erinaceus* with a maximum growth diameter of 81.0 mm after 14 days of incubation. The ideal temperature for mycelial growth was determined to be 25°C. PGA enriched with fresh oyster mushrooms was the most suitable for mycelial growth of *H. erinaceus*, which showed a maximum growth of 3.73 mm day^{-1}. The treatment of 99% rice grain + 1% $CaCO_3$ was selected as the most favorable mother spawn media for the fastest mycelial growth rate and high mycelial density. With the biological productivity of 56.54%, the treatment containing 87% sawdust + 4% corn powder + 8% rice bran + 1% $CaCO_3$ was considered to be the most suitable substrate to cultivate *H. erinaceus.*

Acknowledgements

We thank the NN08 project for providing *H. erinaceus* strain He-2.

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