Optimization of culture conditions for mycelial growth of the wild edible mushroom *Phlebopus portentosus* PPBR01

Santhaya Boonrung*, Teerayut Kanjak, Junjira Sombatwong, Pornpimon Vichuma and Nuchjaree Salee

Biology Program, Faculty of Science, Buriram Rajabhat University, Buriram Province, 31000, Thailand

* Corresponding author. E-mail address: Santhaya.br@bru.ac.th

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Abstract

The objective of the present study was to optimize the optimal media and culture conditions for the mycelial growth of the wild edible mushroom, *Phlebopus portentosus* PPBR01 on agar medium. The mycelium of the strain was isolated from the fruiting bodies and cultured on commercial potato dextrose agar (commercial PDA) medium. To optimize the culture conditions for the mycelium growth rates of the mushrooms, various solid media, carbon and nitrogen sources, temperatures and initial pH were determined with single-factor experiments. Five local crops consisting of potato, pumpkin, taro, corn and cassava were used as the nutrient source in agar media while commercial PDA was used as the control. Among the six culture media tested, potato agar and pumpkin agar were the best for mycelia growth. Starch was the best carbon source and $(NH_4)_2SO_4$ was the best nitrogen source for promoting fungal growth in terms of mycelial density. The mushrooms were found to grow best at temperatures ranging from 29-30°C. In addition, the mushroom was able to grow at a pH ranging from 5-9, with an optimal pH of 5. The present findings provide valuable information concerning optimal growth conditions for the *in vitro* culture of *P. portentosus*.

Keywords: mycelial cultivation, wild edible mushroom, *Phlebopus portentosus*

Introduction

Phlebopus portentosus (Berk. and Broome) Boedijn belongs to the family of Boletinellaceae and the order of Boletales. It is a popular edible mushroom in northern and northeastern Thailand. It is also known locally as the "Hed Har", "Hed Phueng Tham Kam" or "Hed Tub Tao Dam" (Kumla, Danell, Bussaban, & Lumyong, 2011; Sanmee, Dell, Lumyong, & Lumyong, 2010). However, it is still unclear whether *P. portentosus* is a saprotrophic, parasitic or ectomycorrhizal (ECM) fungus. *P. portentosus* can form ECM-like structures in greenhouse experiments (Kumla, Hobbie, Suwannarach, & Lumyong, 2016) while it can successfully produce sporocarps in the culture without a host plant (Ji et al., 2011; Sanmee et al., 2010; Zhang et al., 2017). This mushroom usually has a symbiotic association with soil mealy bugs by forming a special insect gall (crust) on the plant roots. (Sanmee et al., 2010; Zhang et al., 2015).

P. portentosus has a large fruiting body and delicious flavor. It is also rich in nutrients, including proteins, amino acids and minerals (Ji et al., 2011; Zhang et al., 2017), making it one of the most popular edible mushrooms in Thailand, Vietnam and China (Kumla et al., 2016; Zhang et al., 2017), and is sold at a relatively high price compared to some other wild edible mushroom species (Nhi et al., 2017). However, this edible mushroom produces fruiting bodies in natural habitats for a limited period from May to July of each year but is difficult to cultivate *in vitro* to produce fruiting bodies. Thus, there is a need to investigate optimal culture conditions for mycelial growth of *P. portentosus* before applying it to mass production in cereal media. Pure

cultures of the mushroom can be used as inoculum for the development of basidiocarp both *in vitro* and in pot culture experiments.

In this study, we researched the culture conditions for the mycelial growth of wild edible mushroom *P. portentosus* PPBR01 on an agar medium. The mycelial growth of this mushroom on different culture media was examined. The composition of the culture media from carbon and nitrogen sources best for the mycelial growth was then studied. In addition, temperature and initial pH were the growth conditions that were determined.

Methods and Materials

Isolation of mushroom

Fruiting bodies of *P. portentosus* were collected from a roadside market and other locations in Buriram Province, Thailand. The morphological characteristics of the strain were recorded from fresh fruiting bodies. Basidiospore and clamp connection of mushroom were also studied under a light microscope (Nikon Eclipse E100, Tokyo, Japan) Mycelia were isolated from the fresh fruiting bodies and cultured on potato dextrose agar (PDA; HiMedia Laboratories Pvt. Ltd., India) plates and tetracycline (200 µg/mL), pH 7. The plates were incubated at room temperature (29±2°C). The pure cultures were then kept on PDA slants for further study.

Effect of culture media

To optimize the mycelial growth of *P. portentosus*, suitable culture media were first investigated. Six different culture media were used in this study: potato agar, pumpkin agar, taro agar, corn agar and cassava agar. The method for media preparation involved boiling 200 g of each crop in distilled water for 30 mins and subsequently filtering the broth through cheesecloth. Glucose (20 g) and agar (16 g) were then added to the filtrate. All media were adjusted to one liter with distilled water. A commercial PDA was used as the control. The pH of the medium was adjusted to 7.0 before sterilization (121°C, 15 min). All tested media were inoculated with an 8 mm diameter plug of a 7-day-old mycelial culture of the strain. The inoculated plates were incubated at room temperature for 9 days. After incubation, the mycelial growth was estimated by measuring the colony diameters. The experiment was done in triplicate. The suitable culture media from this experiment were added to different carbon and nitrogen sources.

Effect of different carbon and nitrogen sources

Potato agar was selected for this experiment. The medium was supplemented separately with 2% (w/v) carbon sources: glucose, lactose, sucrose, carboxymethyl cellulose (CMC) and starch. For the different nitrogen sources, potato agar was supplemented separately with 2% (w/v) nitrogen sources: peptone, yeast extract, NH_4Cl, $(NH_4)_2SO_4$ and NH_4NO_3. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated plates were incubated at room temperature for 9 days. Colony diameters after incubation were measured. The experiment was done in triplicate.

Effect of temperature and pH

Cultures of *P. portentosus* PPBR01 on potato agar, pH 7.0 were incubated at room temperature (29±2°C), 30°C, 35°C and 37°C. For the initial pH of the culture medium, *P. portentosus* PPBR01 was inoculated on potato agar with initial pH values ranging from 5.0-9.0 and incubated at 30°C. Colony diameters at 9 days after inoculation were measured. The experiment was done in triplicate.

Statistical analysis

The data were analyzed using the SPSS program for one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used for significant differences ($p<0.05$) between treatments.

Results and Discussion

Isolation of mushroom

Mushroom samples were collected from a roadside market in Buriram Province, Thailand. Three samples were named PPBR01, PPBR02 and PPBR03. The isolate PPBR01 grew faster than other isolates, and it was selected for further study. Morphological observation showed that pileus was mostly olive brown to dark olive brown, plano-convex to convex, and 5-9 cm in diameter. The context was thick and soft, pale yellow and turned blue when injured. Gills were crowded and yellowish brown. Stalk was 7-9 x 2-4 cm, clavate shaped, bulbous at the base, solid with a dry surface and yellowish brown to brown (Figure 1A). The basal mycelium was brownish-yellow (Figure 1B). For the microscopic morphology, basidiospores were ellipsoid, smooth surface and basidia were clavate shaped (Figure 1C, 1D). This mushroom grew on an agar medium with abundant clamp connections (Figure 1E). These data were similar to those reported in other literature (Ji et al., 2011; Thongklang, Hyde, Bussaban, & Lumyong, 2010).

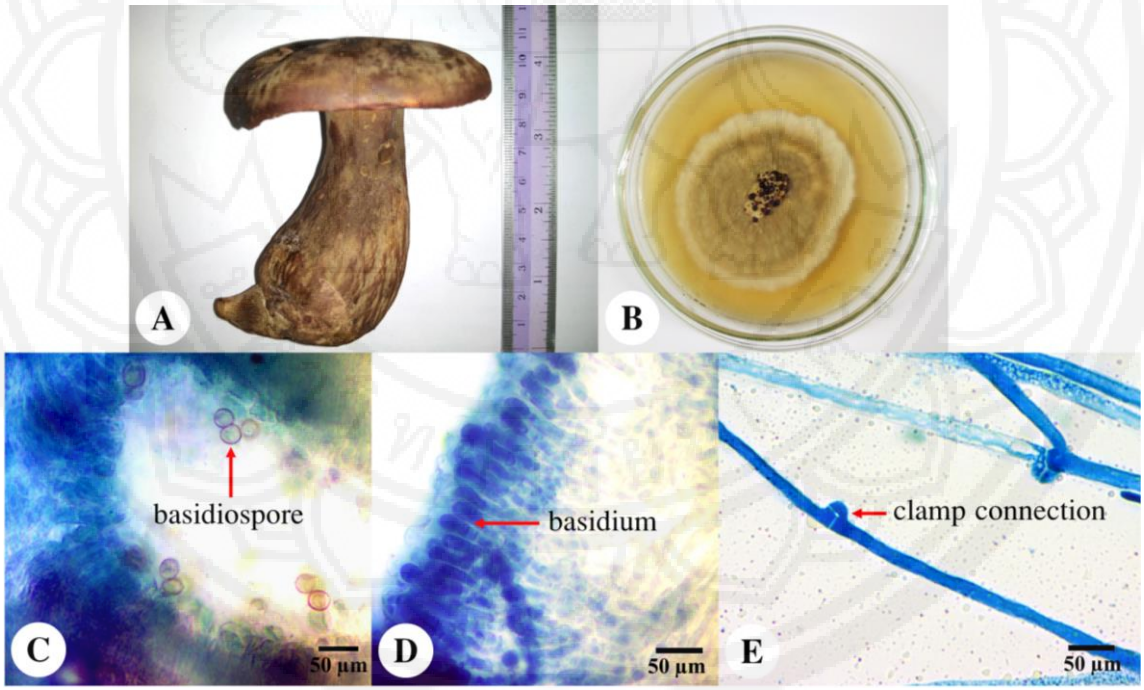

Figure 1 *Phlebopus portentosus* PPBR01. A, fruiting bodies in natural habitat; B, mycelium growth on PDA at 17 days; C, basidiospores; D, basidium; E, clamp connection; C-E are stained with lactophenol cotton blue

Effect of culture media

Mycelial growth of *P. portentosus* PPBR01 on six different culture media is summarized in Table 1. The results indicated that the mycelial growth of this mushroom was significantly affected by the culture media. Potato agar and pumpkin agar were the best media for the mycelial growth of *P. portentosus* PPBR01. Therefore, potato agar was selected as a suitable culture media for further study. The mycelium diameter of *P. portentosus*

PPBR01 on potato agar (34.7±0.23 mm) and pumpkin agar (31.3±0.38 mm) were significantly larger than those on the taro agar (25.7±0.21 mm), corn agar (11.7±0.29 mm), and commercial PDA (13.7±0.24 mm) after 9 days of incubation at room temperature. However, this edible mushroom did not grow on cassava agar. These data were in contrast to that reported by Thongjiem (2002), who found that cassava agar was a suitable medium for the mycelial growth of *Boletus colossus*. Our results were similar to that reported by Inyod et al. (2021), who found that potato glucose agar and potato carrot agar were the best media for the growth of *P. portentosus*. The observations of Thongklang et al. (2010) showed that *P. portentosus* CMUHH121-005 grew well on a variety agar media such as Murashige & Skoog agar, malt extract agar, glucose-peptone-yeast extract agar, oatmeal agar, potato sucrose agar and potato dextrose agar. These data indicated that mycelial growth is dependent on the types of plant and fungus species (Fang et al., 2020; Hoa & Wang, 2015). According to Ajdari et al. (2011), most fungi require several specific elements for growth and reproduction.

Table 1 Effect of different culture media on the mycelial growth of *P. portentosus* PPBR01 cultured at room temperature for 9 days

Culture media	Mycelium colony diameter (mm)
Potato agar	34.7±0.23[a]
Pumpkin agar	31.3±0.38[a]
Taro agar	25.7±0.21[b]
Corn agar	11.7±0.29[cd]
Cassava agar	8.0±0.00[d]
Commercial PDA	13.7±0.24[c]

Data are means ± standard deviation (SD). Data with different letters within the same column indicate a significant difference at $p<0.05$ according to Duncan's multiple range test (DMRT)

Effect of different carbon and nitrogen sources

The effects of different carbon and nitrogen sources on the mycelial growth of *P. portentosus* PPBR01 are summarized in Table 2. Among the various carbon sources tested, the largest colony diameter of *P. portentosus* PPBR01 was observed on the medium containing starch with a colony diameter of 36.7±0.15 mm. The smallest colony diameter of the mushroom was found on media supplemented with lactose (27.7±0.25 mm) and CMC (25.0±0.00 mm). These data were similar to that reported by Thongklang et al. (2010), who found that *P. portentosus* CMUHH121-005 grew well on media containing malt extract and starch, and that the lowest growth was found on media supplemented with glucose, sucrose and fructose. According to Nhi et al. (2017), *P. spongiosus* BC-F0075 grew well on the MS media containing saccharose, glucose, fructose and maltose, but did not grow well with lactose. In addition, Lazarević, Stojićić, and Keća (2016) reported that the growth of *Suillus collinitus* was faster on sucrose, dextrin and starch.

Different nitrogen sources used in this study were peptone, yeast extract, NH_4Cl, $(NH_4)_2SO_4$ and NH_4NO_3. Among the various nitrogen sources tested, $(NH_4)_2SO_4$ was the best nitrogen source for the growth of *P. portentosus* PPBR01 with a colony diameter of 36.3±0.11 mm. These results were in agree with previous studies that report the best mycelial growth was found in the medium containing inorganic nitrogen. For instance, Kumla et al. (2011) reported that $(NH_4)H_2PO_4$ was the best inorganic nitrogen source for the growth of *P. portentosus*. Also, *P. spongiosus* BC-F0075 grew well on media containing $(NH_4)H_2PO_4$ and $(NH_4)_2SO_4$ (Nhi et al., 2017). Hatakeyama and Ohmasa (2004) reported that ammonium tartrate was the best nitrogen source

for the mycelial growth of *Boletinus* sp. In addition, Rangel-Castro, Danell and Taylor (2002) reported that the edible ectomycorrhizal mushroom *Cantharellus cibarius* SNGT2-A grew well on media containing ammonium. On the other hand, Thongklang et al. (2010) reported that yeast extract and peptone were the most effective nitrogen source for the mycelial growth of *P. portentosus* CMUHH121-005.

The statistical analysis in this study indicated that the fungal mycelium growth on the medium containing starch or $(NH_4)_2SO_4$ was not significantly different from that on media without carbon or nitrogen sources. However, the mycelia on media supplemented with starch and $(NH_4)_2SO_4$ were denser and thicker than those on the control media (data not shown).

Table 2 Effect of various carbon and nitrogen sources on the mycelial growth of *P. portentosus* PPBR01 cultured at room temperature for 9 days

Sources	Mycelium colony diameter (mm)
Carbon sources	
Glucose	32.3 ± 0.25^{b}
Lactose	27.7 ± 0.25^{c}
Sucrose	32.0 ± 0.10^{b}
CMC	25.0 ± 0.00^{c}
Starch	36.7 ± 0.15^{a}
Control	38.7 ± 0.23^{a}
Nitrogen sources	
Peptone	23.3 ± 0.15^{cd}
Yeast extract	25.0 ± 0.17^{c}
NH_4Cl	21.0 ± 0.10^{d}
$(NH_4)_2SO_4$	36.3 ± 0.11^{a}
NH_4NO_3	30.3 ± 0.05^{b}
Control	37.3 ± 0.15^{a}

Data are means ± standard deviation (SD). Data with different letters within the same column indicate a significant difference at $p<0.05$ according to Duncan's multiple range test (DMRT)

Effect of temperature

The effect of temperature on the mycelial growth of *P. portentosus* PPBR01 was studied (Table 3). The statistical analysis of the data revealed that the optimum temperature for the mycelial growth of the mushroom was ranged from 29°C-30°C. However, this mushroom did not grow at 35°C and 37°C. These results agreed with the observations of Sanmee et al. (2010), who found that the optimum temperature for the mycelial growth of *P. portentosus* WPPH2 was 30°C. According to Thongklang et al. (2010), *P. portentosus* CMUHH 121-005 grew well at 30°C, but did not grow at 40°C or 45°C. Kumla et al. (2011) reported that 30°C was the best temperature for the mycelial growth of *P. portentosus*. Also, the optimum temperature for the mycelial growth of *P. spongiosus* was 30°C (Kumla, Suwannarach, & Lumyong, 2020; Nhi et al., 2017). Wanwaen and Youpensuk (2019) reported that the optimum temperature for the mycelial growth of *Amanita princeps* was 28°C. Furthermore, the optimum growth temperature of *Amanita caesarea* was 24°C-28°C (Daza et al., 2006).

Effect of pH

The effect of pH on the mycelial growth of *P. portentosus* PPBR01 is summarized in Table 4. The results from this study showed that *P. portentosus* could be grown in all the tested media at a pH range of 5-9 (Figure

2). Significantly, the largest colony diameter (60.1±0.23 mm) was observed on potato agar at pH 5. However, the statistical analysis indicated that the mycelial growth on culture media in a range of 6-9 was not significantly different. These results agreed with the observations of Thongklang et al. (2010), who found that the optimum pH for the mycelial growth of *P. portentosus* CMUHH 121-005 was 4-5.5. According to Kumla et al. (2011), *P. portentosus* grew at a pH range of 3-9 and showed maximum mycelial growth at pH 4. Sanmee et al. (2010) reported that a strain of *P. portentosus* grew best at pH 4. The optimum pH for the mycelial growth of *P. spongiosus* was 4-5 (Kumla et al., 2020; Nhi et al., 2017). The observations of Lazarević et al. (2016) showed that ectomycorrhizal mushrooms *Lactarius deliciosus* and *Russula sanguinaria* grew well at pH between 5.8 and 6.5. Also, Wanwaen and Youpensuk (2019) reported that mycelia of *Amanita princeps* grew well at pH 6. In addition, the largest radial growth of *Amanita caesarea* was obtained at pH 6-7 (Daza et al., 2006). Previous studies reported that many ectomycorrhizal mushrooms grow at acidic pH (Ohta, 1998; Palmer & Hacskaylo, 1970).

Table 3 Effect of incubation temperature on the mycelial growth of *P. portentosus* PPBR01 cultured for 9 days

Temperature (°C)	Mycelium colony diameter (mm)
room temperature (29±2)	34.0±0.00[a]
30	37.3±0.50[a]
35	8.0±0.00[b]
37	8.0±0.00[b]

Data are means ± standard deviation (SD). Data with different letters within the same column indicate a significant difference at $p<0.05$ according to Duncan's multiple range test (DMRT).

Table 4 Effect of initial pH of the medium on the mycelial growth of *P. portentosus* PPBR01 cultured at 30°C for 9 days

initial pH	Mycelium colony diameter (mm)
5	60.1±0.23[a]
6	45.3±0.24[b]
7	47.3±0.17[b]
8	43.6±0.09[b]
9	45.2±0.27[b]

Data are means ± standard deviation (SD). Data with different letters within the same column indicate a significant difference at $p<0.05$ according to Duncan's multiple range test (DMRT).

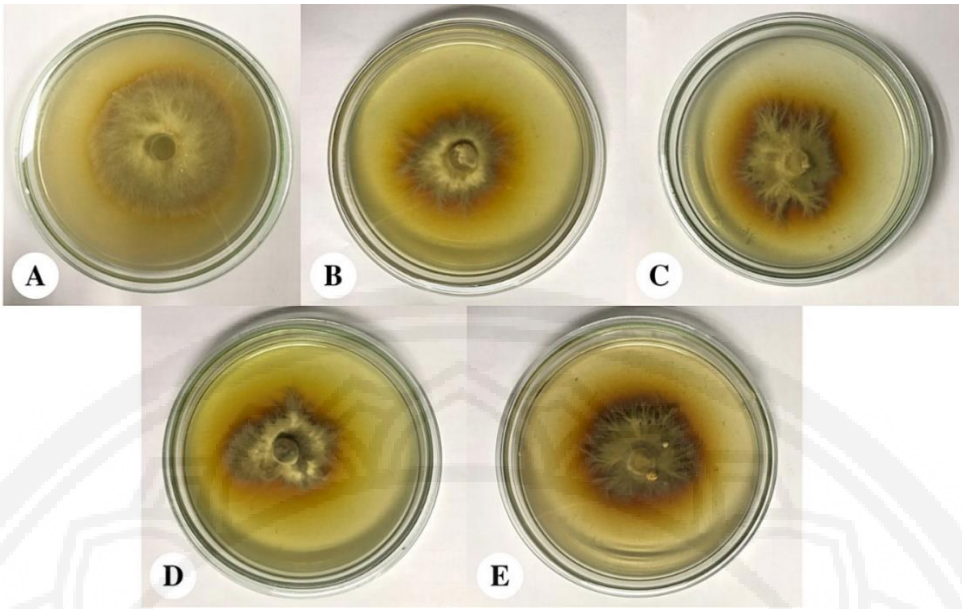

Figure 2 Mycelial growth of *P. portentosus* PPBR01 on potato agar with different pH. A, pH 5; B, pH 6; C, pH 7; D, pH 8; E, pH 9

Conclusion and Suggestions

The culture conditions for the mycelial growth of wild edible mushroom *P. portentosus* PPBR01 on agar medium were studied. Culture media, namely potato agar and pumpkin agar were found suitable for the mycelial growth of *P. portentosus* PPBR01. Starch was a suitable carbon and $(NH_4)_2SO_4$ was a suitable nitrogen source to promote fungal growth in terms of mycelial density. Temperatures of 29°C and 30°C, and pH 5, were the optimal values for the mycelial growth of this wild edible mushroom. Our results indicated that potato agar and pumpkin agar can replace commercial PDA for the cultivation of mycelia of *P. portentosus* PPBR01. Mycelial cultivation of the mushrooms using crop agar media not only enhanced the mycelial growth, but also reduced the cost of culture media. The knowledge of some *in vitro* growth requirements of *P. portentosus* PPBR01 is a first step towards inoculum production for the development of basidiocarp both *in vitro* and in pot culture experiments. However, future studies are needed to confirm the strain of *P. portentosus* PPBR01 using a molecular approach. As well, measurement of biomass yield on different culture media and the ectomycorrhizal status of *P. portentosus* PPBR01 need further study.

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