



Development of an Edible Wild Strain of Thai Oyster Mushroom for Economic Mushroom Production

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ABSTRACT

The edible oyster mushroom genus *Pleurotus* are collected and cultivated in many countries. In 2011, fruiting body of *Pleurotus giganteus* was collected from northern Thailand and pure culture was isolated. The fungal identification based on morphology and molecular characteristics. In this paper we report the optimum *in vitro* culture conditions and the fruiting bodies production. Among ten culture media tested, potato dextrose agar was the best for the mycelia growth. The fungus was able to grow at temperatures ranging from 15-35°C, with an optimal growth temperature of 25°C. The optimum pH for mycelia growth was 7.0. In addition, the fruiting bodies induction conditions of this fungus were examined in pure culture. The primordia were formed under lowered temperature, high humidity and a 12 h light photoperiod conditions and developed to mature fruit-body. For the spawn production, sorghum grain was the suitable solid substrates for spawn production of *P. giganteus*. *Pleurotus giganteus* could grow on sawdust and produced the mature fruiting bodies as well as casing with soil layer induced the primordial production. This report provides valuable information for the first time concerning the possibility to cultivation *P. giganteus* in Thailand.

Keywords: agaricomycetous mushroom, cultivation, *Pleurotus giganteus*

1. INTRODUCTION

The cultivation of edible mushrooms has become an attractive economic alternative over past few years, mainly due to increase in its demand and market value [1,2]. Mushrooms are delicious food and are rich in proteins, vitamins, and minerals while containing little fat [3]. Several species of mushrooms are important because of their medicinal properties such as; they are active against human pathogen, cancer, diabetes, hypertension, hypercholesterolemia condition

and tumor [4, 5]. Cultivation methods for edible mushrooms varied considerably around the world. Depending on location of production, many specialty mushrooms can be grown using either indoor and outdoor method. In most countries, the consumers accept the cultivated mushroom such as *Agaricus bisporus*, *Auricularia* spp., *Lentinus edodes*, *Pleurotus* spp. and *Volvariella volvacea* for consumption [6]. In 2002, world production of cultivated mushroom

was estimated to be 12,250 thousand tons and China's mushroom production was over 14 million tons in 2006 which now China has become a leading mushroom producer and consumer in the world [1]. In Thailand, mushroom production in 2007 was estimated at 10,000 tons [7]. An edible mushroom such as, *A. auricula*, *Coprinus comatus*, *L. edodes*, *L. sarjov-coju*, *P. cystidiosus*, *P. ostreatus*, *Schizophyllum commune*, *Trimella fuciformis* and *V. volvacea* were grown on a commercial scale [8,9]. However, many types of wild mushrooms are also collected for sale and consumption.

The most well known species of *Pleurotus* are the largest group of cultivated in the world and they were found wide in temperate forests and some species in tropical forests [10,11]. In addition, morphological differences among members of this group are few; therefore, their taxonomic assignment has been in the past an issue of debate. Recently, various molecular analyses have been introduced for the verification of them, such as RFLP, RAPD, SSU rDNA and ITS sequence analyses [2,12,13]. Many wild species of this genus such as, *P. cornucopie*, *P. cystidiosis*, *P. eryngii*, *P. flabellatus*, *P. florida* and *P. ostreatus* were selected for cultivation in the commercial scale [18]. Oyster mushroom can be cultivated within a wide range of temperature on different natural resources and agricultural wastes [14]. However, the specific conditions of nutrition, humidity, carbon dioxide levels and temperature will affect mycelial growth and fruiting bodies production of mushroom [15-18]. This study focused on the identification of fungus base on morphology and phylogeny. In addition, the culture conditions for mycelial growth and fruiting bodies production in pure culture were studied.

Moreover, the evaluation of suitable solid media for the best spawn production was also investigated.

2. MATERIALS AND METHODS

2.1 Fungal Strain

Fruiting body of *P. giganteus* CMU54-1 were collected from Medicinal Plant Garden (18°48'22"N, 98°54'52"E and altitudes 1,042 m), Doi Suthep-Pui National Park, Chiang Mai Province, Thailand in 15 May 2011 and kept at the Research Laboratory for Excellence in Sustainable Development of Biological Resources, Faculty of Science, Chiang Mai University. The mycelia were isolated from fruiting body by aseptically removing a small piece of mycelium from inside and transferring it to potato dextrose agar (PDA). The plates were incubated at 30°C in the dark. The mycelia emerging from tissue were picked and transferred to PDA. The pure cultures were kept on PDA slants for further use. Some cultures were kept in both sterile distilled water at 4°C and 20% glycerol at -20°C for long-term preservation.

2.2 Identification

The fruiting body was identified based on morphological characteristics [19,20] and a molecular analysis of the DNA of the fruit-body extracted through a modified CTAB method. Fruiting body and mycelia (1 g) were frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. The powder was placed in a 1.5 ml Eppendorf tube and 500 µl CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl, 1.2 M NaCl, 20 mM EDTA, pH 8.0) was added and mixed with the material. Samples were incubated at 65°C for 60 min in a water bath and occasionally mixed. An equal volume (500 µl) of chloroform: isoamyl alcohol (24:1) was

added to the samples and briefly mixed by vortexing. After centrifugation for 15 min at 13,000 rpm, the upper aqueous layer was transferred to a new sterile Eppendorf tube. The extraction was repeated until the interface was clear. The supernatant was removed to a new Eppendorf tube, containing 2 volumes of cold 100% ethanol. The DNA was precipitated at -20°C overnight and centrifuged 15 min at 14,000 rpm, 4°C. Then the pellet was washed with 70% ethanol and dried at room temperature. It was resuspended in 100 ml of 0.002% RNase (5 mg/ml) in TE buffer and incubated for one hour at 37°C. The suspension was stored at -20°C pending use for amplification.

2.3 Fungal ITS and LSU Regions Sequencing and Phylogenetic Analysis

The internal transcribed spacer (ITS) regions of nuclear rDNA were amplified by polymerase chain reaction (PCR) with primers ITS4 and ITS5 under the following thermal conditions: 94°C for 2 min, 35 revolution cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, and 72°C for 10 min. In addition, the 28S ribosomal RNA gene was also amplified with primer LROR and LRO5 under the following thermal conditions: 94°C for 2 min, 30 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 1 min, and 72°C for 10 min. The PCR products were checked on 1% agarose gels stained with ethidium bromide under UV light. PCR products were purified by using PCR clean up Gel extraction NucleoSpin^R Extract II purification Kit (Macherey-Nagel, Germany Catalog no. 740 609.50) following the manufacturer's protocol. The purified PCR products were directly sequenced. Sequencing reactions were preformed and the sequences were automatically determined in the genetic analyzer

(1ST Base, Malaysia) using PCR primers mentioned above. Sequences were used to query GenBank via BLAST (<http://blast.ddbj.nig.ac.jp/top-e.html>) and a multiple sequence alignment was carried out using the subroutines in Clustal X [21]. A phylogenetic tree was constructed using the PUAP beta 10 software version 4.0 [22].

2.4 Fungal Cultivation

Twenty five milliliters of culture media for each experiment were poured into Petri dishes (9.0 cm, in diam) after autoclaving at 121°C for 15 min. A sterilized cellophane disc (9.0 cm, in diam) was placed on the surfaces of the test media. Mycelial inocula were prepared by growing on PDA at 30°C in darkness for one week. Mycelial plugs (5 mm diam) from the periphery of the growing colony were then inoculated centrally of the media. All plates were sealed with parafilm after inoculation. Colony diameters at one week after inoculation were measured. The covered cellophane discs were removed, dried at 105°C overnight and maintained in desiccators for 20 min before weighing. Mycelium dry weights were calculated. Four replications were made for each treatment.

2.5 Effect of Media on Mycelial Growth

The following 10 different media were used in this experiment: corn meal agar (corn 20 g, glucose 10 g), Czapek Dox agar (NaNO₃ 2 g, KH₂PO₄ 1 g; MgSO₄·7H₂O 0.5 g, KCl 0.5 g, FeSO₄·7H₂O 0.01 g, sucrose 30 g), glucose yeast extract agar (glucose 10 g, yeast extract 2 g), malt extract agar (malt extract 20 g), oatmeal agar (oat 20 g, glucose 10 g), potato dextrose agar (potato 200 g, glucose 20 g), soybean meal agar (soybean 20 g, glucose 10 g), V8 medium (V8 juice 200 ml, CaCO₃ 2 g), wheat agar (wheat 20 g, glucose 10 g) and yeast extract

peptone glucose agar (yeast extract 10 g, peptone 20 g, glucose 20 g). All media were added with agar 20 g and adjusted volume to 1,000 ml with distilled water. Final pH was adjusted to 6.5 by using 1 N HCl and 1 N NaOH. After inoculated culture media were incubated in the darkness at 30°C for one week.

2.6 Effect of Temperature and pH on Mycelial Growth

In this experiment, cultures of *P. giganteus* CMU54-1 on PDA were incubated in darkness at 15, 20, 25, 30, 35, 40 and 45°C. The pH of the medium was adjusted to from 2.0-9.0 with 1N HCl or 1N NaOH before autoclaving. After inoculation, cultures were incubated at 25°C in the darkness.

2.7 Fruiting Bodies Induction

Potato dextrose agar was chosen for this test. The medium was autoclaved and transferred to Petri dishes. After inoculation with a 5 mm diam piece of mycelial disk cut from the growing edge of a 2 week-old culture, each Petri dish was sealed with Parafilm. The cultures were incubated at 25°C in the dark. After ten days of incubation, Parafilm was removed and the cultures were transferred to 20±2°C, 80±10% RH and illuminated for 12 h with fluorescent lamps at about 1,500 lux.

2.8 Determination of Suitable Spawn Production

Nine solid substrates such as, brown rice grain (*Oryza sativa*), corn grain (*Zea mays*), job's tear grain (*Coix lacryma-job*), black kidney bean (*Phaseolus vulgaris*), mung bean (*Phaseolus aureus*), rye grain (*Secale cereale*), sorghum grain (*Sorghum bicolor*), soy bean (*Glycine max*) and wheat grain (*Triticum aestivum*) were used in this

experiment. Each grain was prepared by boiling for 10-15 min. The grains were placed in 18×180 mm test tube to approximately 10 cm depth and autoclaved at 121°C for 30 min. After cooling, each tube was inoculated with a mycelial plug cut from the periphery of the growing colony on PDA and incubated at 25°C for two weeks in darkness with five replicates of each experiment. Linear growth of the mycelium was measured and growth rate was determined.

2.9 Cultivation for Fruiting Body Production

Sawdust of *Albiza saman* was used as main substrate for cultivation. Substrate was mixed with 6% rice bran, 2% CaCO₃, 0.2% Na₂SO₄ and 0.2% MgSO₄ on dry weight basis of substrate. The mixed substrate had reached a humidity of 70-75% with water and 300 g of mixed substrate was filled in plastic bag (12.5 cm wide and 18.5 cm long). The bags were sealed using cotton plugged polyvinyl chloride pipe ring and covered by piece of paper. The bags were autoclaved at 121°C for 30 min and the sterilized bags were cooled for 24 h. The bags were immediately inoculated with 5 g of master spawn (three week-old of *P. giganteus* CMU54-1 on sorghum grain) and bags were kept at room temperature (25±2°C). After 45 days, the mycelia covered the substrate and the bags were shift to 20±2°C, relative humidity above 75% and illuminated for 12 h. In addition, three different test casings including a control, scratched surface and added saturated soil with water were investigated the fruiting body production and the entire process was repeated twice with ten replicates. The number of primordia and fruiting body per bag was recorded.

2.10 Statistical Analysis

All data were analyzed using SPSS V16.0 for one-way analysis of variance (ANOVA) and Duncan's multiple range test was used for significant differences ($P < 0.05$) between treatments.

3. RESULTS AND DISCUSSION

3.1 Identification

The morphological identification indicated that the fungus isolate CMU54-1 was *Pleurotus giganteus* (Berk.) Karunarathna & Hyde (sym. *Lentinus giganteus* Berk. and *Panus giganteus* (Berk.) Corner) [23] which a popular edible mushroom in Sri Lanka [24]. Pileus was 40-118 mm broad, plano-convex becoming centrally depressed at maturity, grayish brown to dark brown, margin wavy to lobed, inrolled when young, incurved at maturity, surface smooth and small flattened scales in age. Lamellae was deeply decurrent, anastomosing ridges on the stipe, subdistant to crowded, 3.5-5.0 mm broad, cream to white. Stipe was 162-175 mm long and 5-12 mm thick, central, roughly, tapering to narrowed

base, attachment laterally, solid, the same color as the pileus (Figure. 1A, 1B). Spore print white. Basidiospore 4.0-9.0×7.0-10.0 μm , $Q = 2.1$, elongate to cylindrical, smooth, hyaline, inamyloid, thin-walled (Figure. 1C). Basidia 8.0-10.5×20.5-40.0 μm , clavate, bearing 4 spores (Figure. 1D). Sterigma 5.8-11.2 μm long, Pleurocystidia absent. Cheilocystidia 6.0-12.2×12.0-44.0 μm , narrowly clavate, some with a tapering apex, 0.3-0.5 μm diam, hyaline, thin-walled (Figure. 1E). Pileipellis 4.0-6.0 μm diam, with hyphae arranged parallel to the pileus but somewhat irregularly entangled and clamped. Hyphal was system dimitic. Hyphae of context 3.8-4.5 μm diam, branched, with clamped. Hyphae of stipe 5.0-6.5 μm diam, scantily branched, with clamped. This study reported that the new collection site of *P. giganteus* from Medicinal Plant Garden, Doi Suthep-Pui National Park, Thailand. However, Karunarathna et al [31] reported the three collection site of this mushroom in Thailand such as, Mushroom Research Centre (Ban Pha Deng, Mae Taeng District),

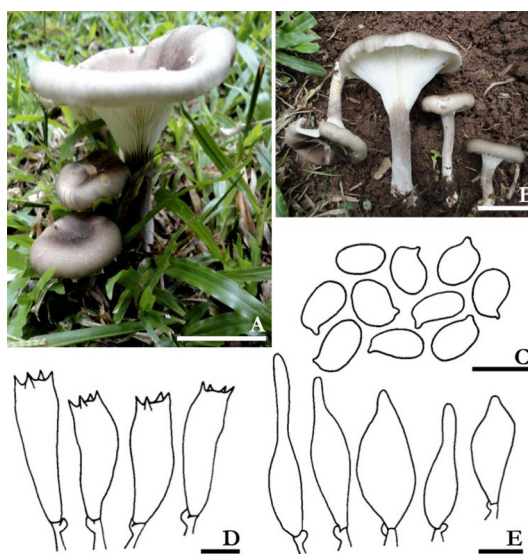


Figure 1. *Pleurotus giganteus*. A-B, Fruiting bodies at natural habitat, C, Basidiospore, D, Basidia and E, Cheilocystidia. Bar A-B = 5.0 cm; C-E = 10.0 μm .

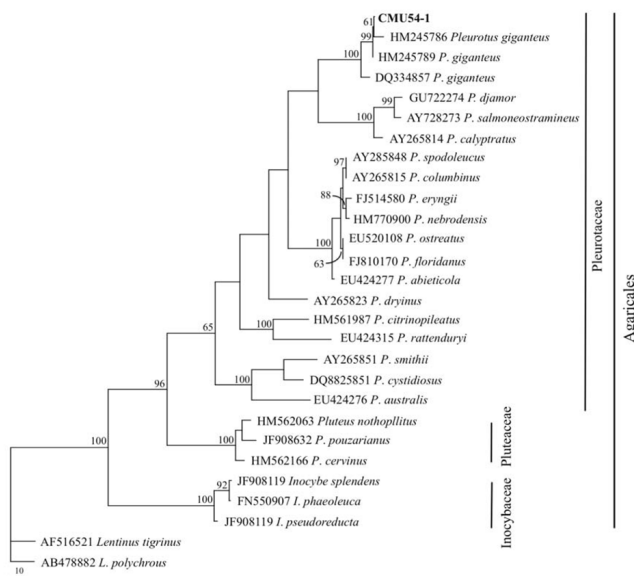


Figure 2. Maximum-parsimonious trees inferred from a heuristic search of the internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 sequence alignments of 28 isolates. *Lentinus tigrinus* and *L. polychrous* were used to root the tree. Branches with bootstrap values $\geq 50\%$ are shown at each branch and bar represents 10 showed substitutions per nucleotide position.

Sangasabhasri Lane to Huai Kok Ma village (Doi Suthep-Pui National Park) and highway No.110 to Mae Sai, Doi Tung (Chaing Rai).

The ITS and LSU gene regions were deposited in GenBank as JQ724360 and JQ724361, respectively. The maximum-parsimony (MP) analysis of the ITS gene of 28 sequences resulted in 759 characters, of which 316 characters were constant, 78 variable characters were parsimony uninformative, and 365 characters were parsimony informative. Heuristic searches resulted in a tree length = 1,136 steps, CI = 0.6391, RI = 0.7892, RC = 0.5044 and HI = 0.3909. While, The MP analysis of LSU data of 25 sequences results in 938 characters of which 728 characters were constant, 48 variable characters were parsimony uninformative, and 162 characters were parsimony informative. Heuristic searches resulted in a tree

length = 359 steps, CI = 0.6825, RI = 0.8331, RC = 0.5685 and HI = 0.3175. The phylogenetic studied of ITS and LSU gene regions of CMU54-1 were shown in Figure. 3 and 4, receptively. Both phylogenetic analyses indicated that *Inocybaceae*, *Pluteaceae* and *Pleurotaceae* are the family level of order *Agaricales* with 100% bootstrap support. The analysis confirmed that the ITS and LSU sequence of CMU54-1 were placed in the family *Pleurotaceae*, order *Agaricales* which related to the *Pleurotus* species. The result was supported that *P. giganteus* was transferred from *L. giganteus* to *Pleurotus* which the new combination mushroom [23].

3.2 Effect of Media on Mycelial Growth

Mycelial growth of *P. giganteus* on ten different growth media was presented in Table 1. After one week of incubation, the fungus grew well on malt extract agar,

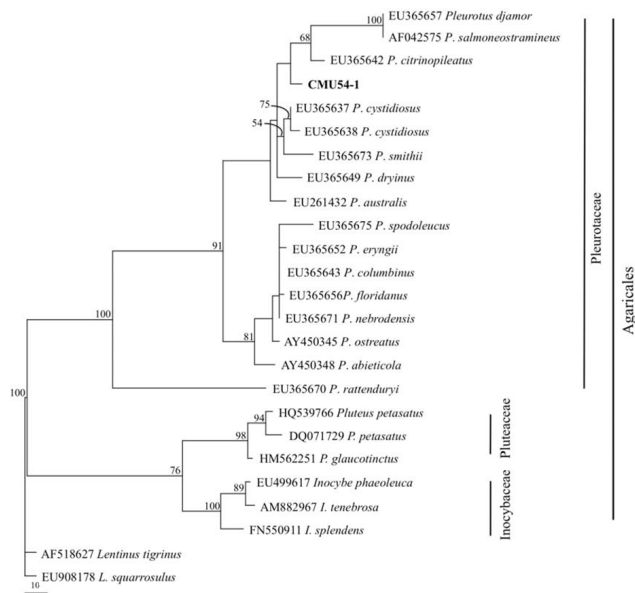


Figure 3. Maximum-parsimonious trees inferred from a heuristic search of the 28S ribosomal RNA gene alignments of 25 isolates. *Lentinus tigrinus* and *L. squarrosulus* were used to root the tree. Branches with bootstrap values $\geq 50\%$ are shown at each branch and bar represents 10 showed substitutions per nucleotide position.

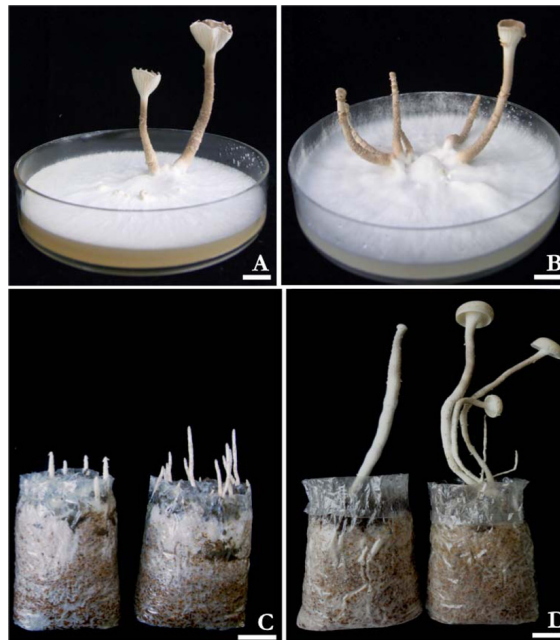


Figure 4. A-B, Fruiting bodies formation of *Pleurotus giganteus* in induction conditions, C-D, Cultivation of *Pleurotus giganteus*, C, Primordia formation, D, Fruiting bodies formation. Bar A-B = 1.0 mm; C-D = 5.0 cm.

Table 1. Growth of *Pleurotus giganteus* on different media.

Growth Media	Growth rate (mm/day)	Dry weight (mg/plate)
Corn meal agar	14.15±0.44c	23.68±0.91f
Czapek Dox agar	12.30±0.42d	18.93±0.62g
Glucose yeast extract agar	13.80±0.37c	38.95±4.67e
Malt extract agar	17.60±0.49a	57.65±3.54c
Oatmeal agar	11.35±0.82e	22.05±1.50fg
Potato dextrose agar	17.85±0.30a	82.65±3.63a
Soybean meal agar	12.30±0.77d	22.40±2.01fg
V8 medium	17.75±0.50a	38.43±1.50e
Wheat agar	13.65±0.90c	43.93±3.77d
Yeast extract peptone glucose agar	16.70±0.53b	69.55±3.82b

The results are mean and standard deviation of four replicated. Data with different letters with in the same column indicated the significant difference at $P < 0.05$ according to Duncan's multiple range test.

potato dextrose agar and V8 medium. The highest mycelial growth rate (17.85±0.30 mm/day) and biomass yield (82.65±3.63 mg/plate) were observed on potato dextrose agar. The smallest of the mycelial growth rate and biomass yield were found on oatmeal agar and V8 medium, respectively. These results agreed with the result of Gibriel et al [14] and Rawte and Diwan [16], who reported that the maximum biomass yield of *P. columbinus*, *P. florida* and *P. flabellatus* were observed on potato dextrose liquid and solid media. However, malt extract agar was the best media for mycelial growth of *P. eryngii* [18], *P. ostreatus* [25] and *P. pulmonarius* [26]. Furthermore, Khandakar et al. [27] reported that corn extract agar was a suitable medium for the mycelial growth of *P. citrinopileatus*.

3.3 Effect of Temperature and pH on Mycelial Growth

The mycelial growth and biomass production were responded on the different temperature treatment. *P. giganteus* grew at temperatures ranging from 15-35°C

(Table 2). The statistical analysis of the data showed that 25°C was the best temperature for mycelial growth and biomass production of this fungus. The results were according to the previous studies that the optimum temperature for mycelial growth of oyster mushrooms was range from 25-30°C [17,27]. After one week of incubation at 40 and 45°C were stunted and failed to resume growth after additional one week incubation at 25°C.

The effects of pH variations on *P. giganteus* mycelia growth are shown in Table 3. The fungus had the ability to grow at pH 4.0-9.0. The pH 7.0 was the optimum pH value which produced the highest mycelial growth rate (18.00 mm/day) and biomass yield (163.10±4.40 mg/plate). This result showed different growth respond to pH when compared with other *Pleurotus* spp. For examples, *P. citrinopileatus* was preferred alkaline pH values which pH 8.0 was the optimum pH value [27]. While, *P. eous*, *P. eryngii*, *P. flabellatus* and *P. florida* showed the maximum mycelial growth in acid condition [16, 28].

Table 2. Growth of *Pleurotus giganteus* on potato dextrose agar at different temperature.

Temperature (°C)	Growth rate (mm/day)	Dry weight (mg/plate)
15	6.85±1.39c	20.63±5.00d
20	10.80±0.49b	42.13±5.18c
25	17.90±0.20a	116.15±9.55a
30	17.75±0.50a	89.20±7.15b
35	2.75±0.70d	14.30±1.02d

The results are mean and standard deviation of four replicated. Data with different letters with in the same column indicated the significant difference at $P < 0.05$ according to Duncan's multiple range test.

Table 3. Growth of *Pleurotus giganteus* on potato dextrose agar at different pH.

pH value	Growth rate (mm/day)	Dry weight (mg/plate)
4	9.45±1.05d	91.20±7.53e
5	15.55±1.06c	108.10±6.51cd
6	17.95±0.20a	112.90±5.92c
7	18.00±0.00a	163.10±4.40a
8	17.75±0.30ab	141.90±2.69b
9	16.90±0.53b	103.88±4.89d

The results are mean and standard deviation of four replicated. Data with different letters with in the same column indicated the significant difference at $P < 0.05$ according to Duncan's multiple range test.

3.4 Fruiting Bodies Induction

The white primordia of *P. giganteus* were found after two weeks of incubation in the low temperature, high humidity and illuminated conditions. The primordial were developed to mature fruit-bodies within 10-15 days. The mature fruiting bodies were collected and descriptions of these fruit-bodies were recorded. Pileus was 14.0-17.2 mm broad and stipe was 62.0-75.3 mm long and 5-8 mm thick. The other macroscopic and microscopic characteristics were similar the natural fruiting body (Figure 4A, 4B). The results similar the previous studies that lowering the temperature and light during incubation was necessary to induce the pure culture primordia formation and mature fruiting body development of *Pleurotus* spp. [29,30].

3.5 Determination of Suitable Spawn Production

The mycelium growth rates of *P. giganteus* in the test tube culture on various substrates were investigated and showed in Table 4. After two weeks of incubation, sorghum grain and corn grain had the thickest mycelial growth in comparison with other media by visually assess. The fungus significantly grew faster on sorghum grain (9.86±0.22 mm/day), followed by corn grain (9.12±0.19 mm/day) and jobs tear grain (9.28±0.29 mm/day). The results showed that sorghum grain was a suitable substrate of spawn production of *P. giganteus* based on the low cost. This agreed with Thulasi et al [28] who reported that that sorghum grain was the best substrate for spawn production of *P. eous* and *P. florida*. In addition, the previous studies

Table 4. Growth rate of *Pleurotus giganteus* on solid media after two weeks incubated in test tube.

Solid medium	Growth rate (mm/day)
Brown rice grain	2.70±0.47e
Corn grain	9.12±0.19b
Job's tear grain	9.28±0.29b
Black kidney bean	6.68±0.24d
Mung bean	2.58±0.33e
Rye grain	8.92±0.32b
Sorghum grain	9.86±0.22a
Soy bean	6.86±0.24d
Wheat grain	7.74±0.44c

The results are mean and standard deviation of five replicated. Data with different letters with in the same column indicated the significant difference at $P < 0.05$ according to Duncan's multiple range test.

reported that sorghum grain was worldwide used for spawn production of oyster mushroom [31].

3.6 Cultivation for Fruiting Body Production

After 30-40 days in the lower temperature, high humidity and illuminated conditions, white rhizomorphs and primordia were formed on top of soil treatment only (Figure. 4C) which gave 11.40±5.46 primordia/bag. In addition, soil treatments showed the highest of the primordia production. The mature fruiting bodies were developed in 10-15 days (Figure. 4D) which 2.40±1.02 fruiting body/bag. Pileus was 30.2-50.5 mm broad and stipe was 120.0-150.4 mm long and 6.0-12.3 mm thick. This results showed that *P. giganteus* was grown on sawdust as a substrate for fruiting bodies production it agreed to the previous studies that oyster mushroom can be cultivated within a wide

range of different natural resources and agricultural wastes such as, log, sawdust, straw dust and weed [14, 31]. Furthermore, previous reports indicated that the casing overlay with soil could improve the oyster mushroom production [32, 33].

4. CONCLUSIONS

Pleurotus giganteus could grow best on PDA and sorghum grain which proved to be suitable medium for mycelial growth and solid substrate for spawn production, respectively. The optimum temperature and pH values for the mycelial growth were 25°C and 7, respectively. Interestingly, the fungus was able to form the mature fruiting bodies in terms of mushroom productivity which provided valuable for cultivation. Future studies will attempt the selection of suitable techniques for a large scale commercial production of this mushroom in Thailand.

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