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Optimization conditions, antibacterial and antioxidant activities of *Clitopilus chalybescens*

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Abstract

The aim of this work was to study the effect of media, temperature and pH on the mycelial growth of *Clitopilus chalybescens* strain MFLUCC 13–0809 collected from Lampang, Thailand. The antioxidant and antibacterial activities from the crude mycelial extracts were evaluated. The study indicated that the optimal medium, pH and temperature were observed on yeast extract agar (YEA) pH 5 to 7, and 20–29˚C, respectively. For antibacterial activities, crude extracts from mycelium slightly inhibited the growth of Gram positive bacteria (*Bacillus subtilis, Staphylococcus aureus* and *Micrococcus luteus*), but no activities were observed on Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). Antioxidant assay indicated that the crude extract had noticeable scavenging activity on 2,2-diphenyl-1-picrylhydrazyle (DPPH) radical. The results suggest that the crude extract from this mushroom mycelium deem to have a potential for further development on antibacterial and antioxidant applications.

Keywords : *Clitopilus,* optimal condition, antibacterial activity, antioxidant activity

1. Introduction

Many published data indicate that fruiting body of mushrooms has various biological functions such as antitumor, antioxidant, anti-aging, and immunological properties (1). Few reports are so far available in the antioxidant properties of mycelial extracts.

Clitopilus is a small genus of fungi in the family Entolomataceae (Basidiomycota, Agaicales). The distinct characteristic which could be easily recognized for this genus is farinaceous odor, pink or brownish pink spores and peculiarly spores shaped with longitudinal ribs (2, 3, 4). The cap is whitish to grayish when dry and then slimy

surface and sometime a wavy margin. The gill is decurrent attached with the stipe, spaced together rather than closely. The stipe is thick and white and ellipsoid in profile but rounded in polar view (5, 6, 7). This genus has a widespread distribution, especially in temperate areas or subtropics such as Spain (7), Taiwan (8), Thailand (9) and China (8, 10).

The basidiocarp of many *Clitopilus* sp. is reported to produce a biologically activity compound. This study aims to determine the culture condition for mycelium growth and evaluate the antibacterial and antioxidant activities of *C. chalybescens* strain MFLUCC 13–0809.

2. Materials and Methods

2.1 The sample collection and fungal isolation

Fruiting bodies of *C. chalybescens* strain MFLUCC 13–0809 were collected in wet season from Lampang, Thailand. The specimens were photographed in the field, characterized based on morphology, then the specimens were dried in electric drier at 40˚C for 24 hrs. Dried specimens were maintained in plastic bags with silica gel and were deposited at the herbarium collection of Mae Fah Luang University. The process to obtain an axenic culture of the mushroom is the first stage for mushroom cultivation. The starting mycelia culture was isolated from inside of mushroom fruiting bodies and transferring to potato dextrose agar (PDA), incubated at 25˚C for 10 days and re-subculture until obtain axenic cultures. The axenic cultures were maintained on PDA slants and deposited at the culture collection of Mae Fah Luang University (MFLUCC).

2.2 Optimization conditions for growing mycelium on solid media

2.2.1 Effect of culture media

A plug of mycelium of *C. chalybescens* strain MFLUCC 13–0809 with a 5 mm diameter was separately grown on five different culture media including potato dextrose agar (PDA), malt extract agar (MEA), corn meal agar (CMA), sabouraud dextrose agar (SDA) and yeast extract agar (YEA). The culture plates were incubated in darkness at 25˚C. Mycelial growth was evaluated by determination of the colony radial growth and mycelial dry weight in three replicates every 3 days for 12 days. The mycelial dry weight was processed by placed agar mycelial in a beaker, then the medium was melted and washed away with hot water. Mycelium was dried at 40˚C for 24 hrs and evaluated using analytical balance (Mettler Toledo ML204).

2.2.2. Effect of temperatures

The optimal media obtained from previous experiment (2.2.1) were used to determine the best temperature. The plug of mycelium was centrally placed on the media plates and incubated in the dark at different temperatures; 20, 25, 26, 27, 28, 29 and 30˚C for 12 days. Mycelial growth was determined by measuring the colony diameter and mycelial dry weight as previously describe in 2.2.1.

2.2.3. Effect of initial pH

The optimal pH was evaluated in optimal media that was adjusted separately to pH 4, 5, 6, 7, 8 and 9 with 1N NaOH or 1N HCl using a digital pH meter before sterilization. The medium was poured into Petri dish. The media plates were inoculated with a mycelium plug. All

cultures were incubated at the optimal temperature. Mycelial growth rate was determined by measuring the colony diameter and mycelial dry weight as previously describe in 2.2.1.

2.3 Antibacterial and antioxidant activities

2.3.1 Preparation of crude extracts from mushroom mycelium

The mycelium of *C. chalybescens* was grown on yeast extract agar (YEA) at 28˚**C** for 30 days. The mycelium was macerated with 75 mL ethly acetate (EtOAc) and soak overnight at the room temperature for the first extaction, and eight hours each for the second and third extraction. The combined supernatants were concentrated and dried to afford crude extract. The samples were stored at 4˚C for further use.

2.3.2 Antibacterial activity assay

Antibacterial activity of the mushroom extracts was tested against Gram positive bacteria; *Bacillus subtilis* TISTR 008, *Micrococcus luteus* TISTR 884, *Staphylococcus aureus* TISTR 1466 and Gram negative bacteria; *Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781 by the disc diffusion method. Tested microorganisms in this study were obtained from the TISTR Culture Collection of the Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand. Bacterial suspensions were inoculated into nutrient broth (NB) at 37˚**C** to obtain approximately $3x10^8$ CFU/mL. The crude extracts were prepared at 50 mg/mL concentration in HPLC grade methanol. A 20 µL of each crude extract were placed in a 6 mm diameter sterilized paper discs prior bioassays. The Petri dish (90 mm diameter) containing Nutrient Agar (NA) was inoculated with 100 µL of bacterial suspension. The prepared extract discs were then placed on the bacterial loan using methanol as a control. Inoculated plates were incubated at 37˚**C** for 24 hrs. Three replicate experiments were carried out. Inhibition zone was recorded after 24 hrs incubation.

2.3.3 Antioxidant activity assay

The scavenging activity of the DPPH free radical was assayed according to the method of Brand-William et al. (11) with slight modification. The range of crude extract used included 5 to 50 mg/mL. The assay was performed in 96-well microtiter plates. The reaction mixture in each of the 96-wells included 30 µL of the crude extract and 220 µL of methanolic solution of DPPH. The mixture was incubated in the dark at room temperature and measured absorbance at 517 nm every 30 minutes for 2 hrs. All measurements were performed in triplicate. Standard antioxidants of Butylated hydroxytoluene (BHT) was used as positive control by varying the concentration to 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL. The DPPH radical scavenging activity percentage was calculated using the following formula:

Scavenging effect (%)

$$= ([A_{blank} - A_{sample}]/A_{blank}) \times 100$$

Where,

A_{blank} = Absorbance of the control solution, DPPH solution without the tested sample.

A_{sample} = Absorbance of the test extract, DPPH solution with the tested sample.

2.4 Statistical analysis

Experimental values were given as mean ± standard deviation (SD). The data of the experiments was analyzed by using one way analysis of variance (ANOVA) in SPSS version 11.5 program for windows and treatments mean compared using Duncan' test ($p \leq 0.05$) followed by post-hoc tests.

3. Results

3.1 Morphology of *C. chalybescens* strain MFLUCC 13-0809

The fresh specimens of *C. chalybescens* were collected from soil. Fresh fruiting body was shown with the umbillicate when young then infudibuliform (funnel-shaped) with white to yellowish white surface (Figure 1a). Basidiocarps occur in gregarious in soil with pileus in diameter in size 2.1–2.6 cm. The microscopic was observed under the microscope, its basidiospores with longitudinal ribs (Figure 1b). The internal tissues of *C. chalybescens* fruiting bodies were cut and placed on PDA plates and incubated at 25°C to get the pure mycelium culture (Figure 1c). The pure culture was maintained in PDA slant tubes at 4°C and in 15% glycerol at –20°C, and deposited at Mae Fah Luang University culture collection.

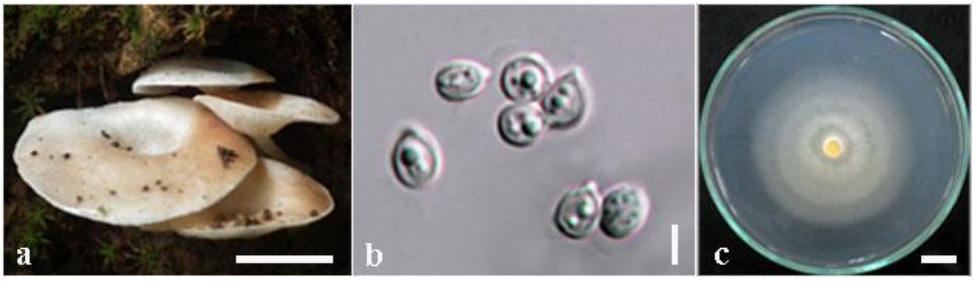

Figure 1 C. chalybescens strain MFLUCC 13-0809. a. Fruiting bodies on the field b. Basidiospores c. The pure culture mycelium. Scale bar: a,c =1 cm, b = 5 μm.

3.2 Optimization conditions for growing mycelium

3.2.1 Effect of culture media

Five different culture media were used to examine a suitable media for mycelial growth of *C. chalybescens* strain MFLUCC 13-0809. The result showed that after 12 days of incubation at 25°C, the highest dry weight of mycelial was occurred on MEA media followed by YEA media with an average of 69.97 mg and 62.27 mg, respectively (Table 1). The fastest growth rate covered on the agar plate with an average 8.63 cm of colony radial growth, the highest colony radial growth was observed in YEA media followed by CMA, MEA, SDA and PDA, respectively. While, (12),(13) reported a mycological agar was selected as a suitable agar medium for *C. passeckerianus* but this study YEA which promoted the fungal growth both in colony diameter and mycelial cell dry weight was selected for the further studies.

Table 1. Mycelial growth and mycelial density of *C. chalybescens* at different media inoculated for 12 days.

Culture media	Colony diameter (cm)	Mycelial dry weight (mg)
SDA	6.63±0.42^{c}	56.63±2.04^{b}
CMA	8.47±0.06^{ab}	54.23±7.62^{b}
PDA	2.27±0.50^{d}	14.03±2.30^{c}
MEA	7.90±0.36^{b}	69.97±2.78^{a}
YEA	8.63±0.21^{a}	62.27±6.48^{ab}

Mean with the same letter are not significantly different ($p \leq 0.05$) by the Duncan's test.

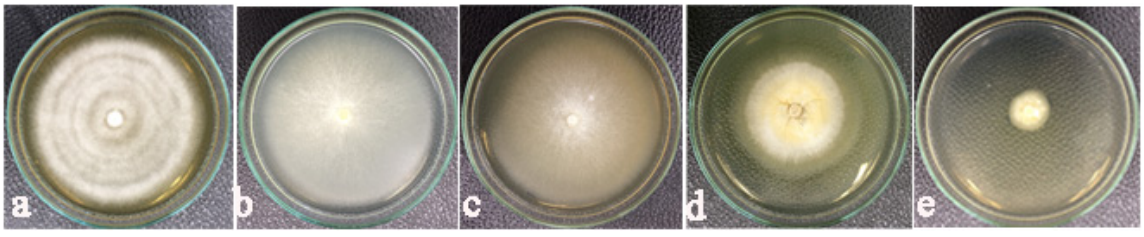

Figure 2. Mycelial growth on different solid media when inoculated at 28˚C for 12 days; a. yeast extract agar (YEA), b. corn meal agar (CMA), c. malt extract agar (MEA), d. sabouraud dextrose agar (SDA), e. potato dextrose agar (PDA)

3.2.2. Effect of temperature

Different mushrooms have different optimal temperature such as the growth rate of *C. passeckerianus* was optimal at 24˚C (13), *Pleurotus eryngii* and *Veriticillium fungicol* was at 25˚C (14, 15), *Pleurotus ostreatus* and *Pleurotus pulmonarius* was at 30˚C and *Volvariella volvaceae* was at 35˚C (14), therefore, study of effect of temperature on the mushroom growth should be investigated. In this study, *C. chalybescens* mycelia were grown under different temperatures (20–30˚C). The result showed that this mushroom was able to grow at the temperature range from 20–30˚C. Nevertheless, the statistical analysis indicated that the temperature 20–29˚C was the optimal temperature for the mycelial growth of this mushroom strain (Table 2). The optimum temperature is one of the most important factors in mushrooms cultivation for growth, production of metabolic products and sporulation of mushrooms (16). This result shown that optimal temperature was 25–30˚C.

Table 2. Mycelial growth and mycelial dry weight of C. chalybescens at different temperature inoculated on YEA media for 12 days.

Temperature (˚C)	Colony diameter (cm)	Mycelial dry weight (mg)
20	9.00±0.00[a]	48.70±18.71[ab]
25	9.00±0.00[a]	56.17±8.03[a]
26	9.00±0.00[a]	45.97±1.12[ab]
27	9.00±0.00[a]	42.23±3.69[ab]
28	9.00±0.00[a]	51.77±1.43[a]
29	8.73±0.25[a]	40.86±6.35[ab]
30	6.77±0.31[b]	25.83±4.37[b]

Mean with the same letter are not significantly different ($p \leq 0.05$) by the Duncan's test.

3.2.3. Effect of initial pH

The effect of initial pH on *C. chalybescens* mycelial biomass was studied under different initial pH (4–9). The optimal pH for mycelial biomass was found at pH 5 with a maximum growth rate of 9.00 cm of colony radial growth and mycelial dry weight of 61.8 mg in 12 days (Table 3).

Table 3. Mycelial growth and mycelial dry weight of C. chalybescens at different pH inoculated on YEA media for 12 days.

pH	**Colony diameter (cm)**	**Mycelial dry weight (mg)**
4	5.30±0.26[d]	15.33±8.02[d]
5	9.00±0.00[a]	61.80±5.66[a]
6	8.70±0.52[a]	45.47±3.55[bc]
7	8.73±0.25[a]	54.76±5.20[ab]
8	7.23±0.05[c]	41.07±7.46[c]
9	8.20±0.12[b]	37.03±10.08[c]

Mean with the same letter are not significantly different ($p \leq 0.05$) by the Duncan's test.

3.3 Antibacterial and antioxidant activities

3.3.1 Antibacterial activity

Antibacterial activities of *C. chalybescens* mycelial extract was studied using disc diffusion method. Crude extract was tested against five species of bacteria. The result was shown in Table 4.

Table 4. Antibacterial activities of crude mycelial extracts

Bacteria	**Inhibition zone (mm)**
B. subtilis	13.2 ± 0.58
S. aureus	19.0 ± 1.32
M. luteus	12.5 ± 1.32
P. aeruginosa	nd*
E. coli	nd*

nd* means cannot detect inhibition zone

Crude extract showed activities against *S. aureus*, *B. subtilis* and *M. luteus* respectively but did not inhibit the growth of *E.coli* and *P. aeruginosa* (Figure 3). The result is consistent with the previous study that antimicrobial activity against bacteria and yeast can be found from several Basidiomycetes (*Agrocybe perfecta, Hexagonia hydnoides, Irpex lacteus, Nothopanus hygrophanus, Pycnoporus sanguineus* and *Tyromyces duracinus*) (17). The extracts of *Agaricus bisporus* has been showed activity against *B. subtilis, S. aureus*, but not activity against *E. coli* (18). According to (19),(12) reported a biologically active compound; pleuromutilin produced by *Clitopilus passeckerianus* (*Pleurotus passeckerianus*), *Clitopilus scyphoides* (*Pleurotus mutilus*) and other species of the genus *Clitopilus* inhibited gram positive bacteria such as *S. aureus*, *Streptococcus haemolyticus*, and *B. subtilis* and had no effect on *E. coli* (21). Also, Yamac and Bilgili (22) reported that *Clavariadelphus truncates* had wider antibacterial properties.

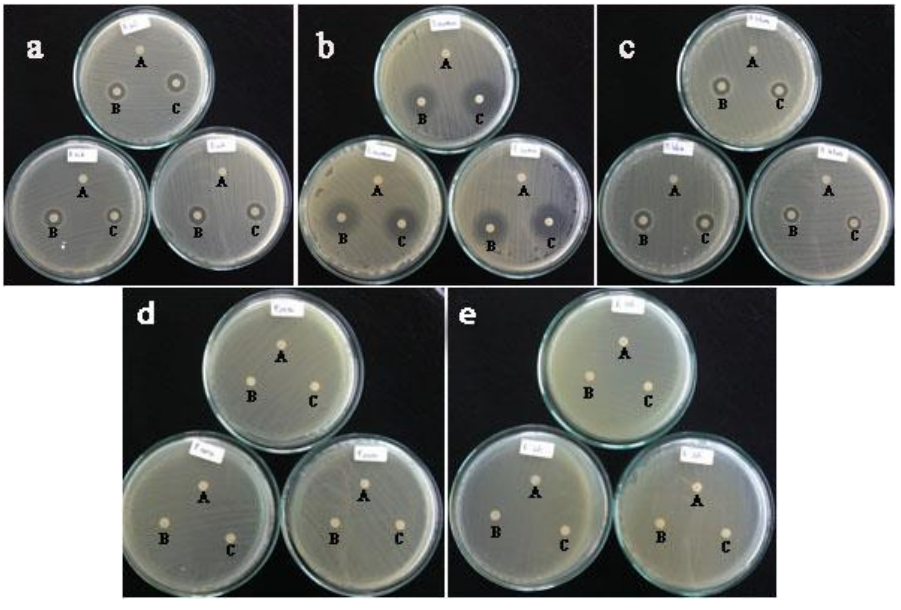

Figure 3. Antibacterial activities of C. chalybescens on different bacteria a) B. subtilis, b) S. aureus, c) M. luteus, d) P. aeruginosa and e) E. coli with A. control, B-C. Sample

3.3.2 Antioxidant activity

The ethanolic mycelial extract was subjected to evaluate antioxidant activities. The DPPH free radical scavenging method was use for the analysis. It is widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. DPPH exhibits a deep purple color with absorption maximum at 517 nm. Therefore, the antioxidant activity of a substance can be evaluated as its ability on scavenging the DPPH free radical.

Scavenging effects of *C. chalybescens* mycelial extract at different concentrations; 5, 10, 20, 30, 40, 50 mg/mL were shown in Figure 4. The scavenging effect of this mushroom on the DPPH radical increased

concentration dependently and were 21.41% and 26.45% at 40 and 50 mg/mL, respectively. According to the results of this study, it was not clearly indicated that the methanolic extract from mycelial of this mushroom species have high antioxidant activity, however, the antioxidant may exhibit at the higher concentration of crude extract and thus worth for further study.

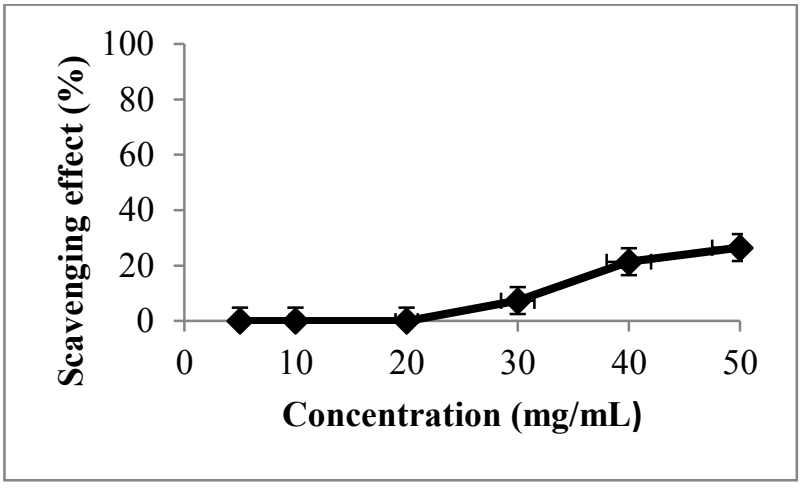

Figure 4. Scavenging effect of C. chalybescens mycelial extraction on DPPH radicals. The results were representative of three separated experiments.

The scavenging effect of BTH at different concentration (3.125, 6.25, 12.5, 25, 50, 100 and 200) is shown in Figure 5. The scavenging effect of BHT was higher than *C. chalybescens* mycelial extracts at the same concentration, this might due to the degree of purity of used sample.

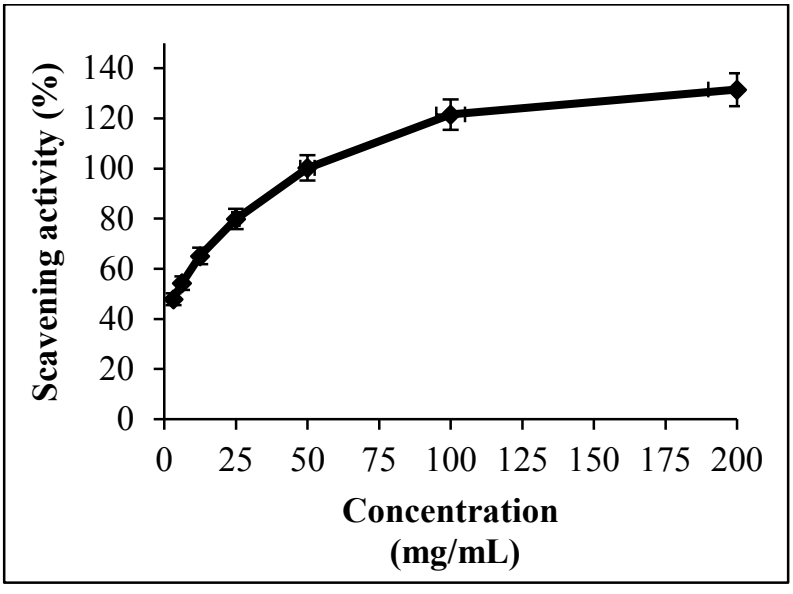

Figure 5. Scavenging effect of BHT on DPPH radicals. The results were representative of three separated experiments.

4. Conclusions

The data on optimal condition for mushroom growth is important for a possibility to grow the mushroom *in vitro*. In this study YEA was optimal medium for mycelial growth comparing to PDA which is a general medium and widely used for fungal cultivation. This mushroom can grow in a wide range of temperature and pH which was range from 20–29˚C and pH 5–7, respectively.

Even though we do not focus to obtain fruiting bodies of *C. chalybescens in vitro*, the data on optimal condition in this study are expected to possibly achieve this aim. The assay of the antibacterial and antioxidant activities *in vitro* demonstrated that crude mycelial extract had a potential of antibacterial activities against Gram positive bacteria and slightly showed scavenging activity of DPPH radical.

5. Acknowledgements

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6. References

(1) Zhong W, Liu N, Xie Y, Zhao Y, Song X, Zhong W. Antioxidant and anti-aging activities of mycelial polysaccharides from *Lepista sordida*. Int. J. Biol. Macromol. 2013;60:355-359.doi:10.1016/j.ijbiomac.2013.06.018.

(2) Singer R. The Agaricales in Modern Taxonomy. 4th Ed., Koeltz Scientific, Koenigstein; 1986.

(3) Noordelood ME. Funga Nordica. Nordsvamp, Copenhagen; 2008.

(4) Vyas D, Chaubey A, Dehariya P. Biodiversity of mushrooms in Patharia forest of Sagar (M.P.)-III. Int J Biodivers Conserv. 2014;6(8):600-607.doi:10.5897/IJBC2014.0681.

(5) Bi ZS, Li TH, Zhang WM, Song B. A preliminary agaric flora of Hainan Province. Guangdong, China press; 1997.

(6) Yang ZL. *Clitopilus amygdaliformis*, a new species from tropical China. Mycotaxon. 2007;100:241-246.

(7) Vizzini A, Musumeci E, Ercole E, Contu M. *Clitopilus chrischonensis* sp. nov. (Agaricales, Entolomataceae), a striking new fungal species from Switzerland. Nova Hedwigia. 2011;92:425-434. doi:10.1127/0029-5035/2011/0092-0425.

(8) Qiu DW, Hui LT, Heng SY. A new variety of crepidotoid *Clitopilus* from Taiwan, China. Mycosystema. 2010;29(6):924-926.

(9) Baroni TJ, Desjardin DE, Hywel-Jones N. *Clitopilus chalybescens*, a new species from Thailand. Fungal Divers. 2001; 6:13-17.

(10) Deng WQ, Li TH, Shen YH. A new species of *Clitopilus* from southwestern China. Mycotaxon. 2012;122:443-447.

(11) Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. LWT. Food Sci Technol. 1995;28(1):25-30.

(12) Irene A. Papa IA, Zulaybar TO, Raymundo AK. Increasing Pleuromutilin Activity of *Clitopilus passeckerianus* ByChemical Mutagenesis and Improvement of Production Medium. 2006;89(1): 20-33.

(13) Vicente MA, Raymundo AK, Quimio TH. Optimization of growth conditions of the wild-type and mutant Strains of the Pleuromutilin-producing *Clitopilus passeckerianus* (PILAT) SING. NRRL 3100. NRCP Res J. 2006.8/9:20-27.

(14) Zervakis G, Philippoussis A, Ioannidou S, Diamantopoulou, P. Mycelium Growth Kinetics and Optimal Temperature Conditions for the Cultivation of Edible Mushroom Species on Lignocellulosic Substrates. Folia Microbiol. 2001;46(3):231-234.

(15) Marek Siwulski M, Sobieralski K, Górski R, Lisiecka J, Sas-Golak I. Temperature and pH impact on the Mycelium growth of *Mycogone perniciosa* and *Verticillium funggicola* isolates derived from polish and foreign mushroom growing houses. J Plant Prot Res. 2011;51(3):268-272.

(16) Chang ST, Miles PG. Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact. 2nd ed., CRC Press, Boca Raton; 2004.

(17) Rosa LH, Machado KMG, Jacob CC, Capelari M, Rosa CA, Zani CL. Screening of Brazilian Basidiomycetes for antimicrobial activity. Mem Inst Oswaldo Cruz. 2003;98:967-974.

(18) Akyuz M, Kirbag S. Antimicrobial activity of *Pleurotus eryngii* var. ferulae grown on various agro-wastes. Eurasia J Biosci. 2009;3:58-63.doi:10.5053/ ejobios.2009.3.0.8.

(19) Kavanagh F, Hervey A, and Robbins WJ. Antibiotic substances from basidiomycetes: IX. *Drosophila subatrata* (Batsch Ex Fr.) quel. Proc Natl Acad Sci USA. 1952;38(7):555-560.

(20) Hartley AJ, de Mattos-Shipley K, Collins CM, Kilaru S, Foster GD, Bailey AM. Investigating pleuromutilin producing basidiomycetes and related *Clitopilus* species. FEMS Microbiol Lett. 2009;297:24-30. doi:10.1111/j.1574-6968.2009.01656.x.

(21) Kavanagh F, Hervey A, Robbins WJ. Antibiotic substances from basidiomycetes. VIII. *Pleurotus multilus* (Fr.) Sacc. and *Pleurotus passeckerianus* Pilat Proc Natl Acad Sci USA. 1951; 37(9):570-574.

(22) Yamac M, Bilgili F. Antimicrobial activities of fruit bodies and/or mycelial cultures of some mushroom isolates. Pharmaceutic Biol. 2006; 44: 660–667.