

Mycelial biomass and biological activities of Philippine mushroom *Pycnoporus sanguineus* in time-course submerged culture

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ABSTRACT

Pycnoporus sanguineus is a bright orange-red colored thin fan-like shaped bracket basidiomycetes. This paper highlights the effects of time-course submerged culture on the growth of mycelia and biological activities of *P. sanguineus*. Prolonged incubation of mycelia in submerged cultivation using coconut water up to 25 days significantly increased the mycelial biomass from 0.24 g to 0.51 g (dry weight), and correspondingly the biomass concentration from 4.8 g/L to 10.2 g/L. The characteristics of mycelia varied in every period of incubation. The radical scavenging activity of mycelial extracts increased in extending time of incubation, whereas the phenolic content peaked after 20 days of fermentation. Extracts of all ages of mycelia exhibited antibacterial activity against both bacteria, having a zone of inhibition diameter ranges of 8.00–9.30 mm in *Staphylococcus aureus* and 7.11–7.38 mm in *Escherichia coli*. Prolonged incubation caused the decrease in LC₅₀ values from 154.83 µg/mL to 16.02 µg/mL after 25 days, indicating an increasing toxicity of the mycelial extracts. The 20-day-old mycelia contain nine groups of fungal chemicals that include triterpenes, flavonoids, tannins, phenols, steroids, alkaloids, anthraquinones, anthrones, and fatty acids, which are responsible to the above-mentioned biological activities. Therefore, prolonged submerged culture of *P. sanguineus* mycelia up to 25 days enhances the production of mycelial biomass and improves the biological activities.

1. INTRODUCTION

Mushrooms have been utilized since historic time not only as simple food but also as a natural medicine to treat a wide range of diseases. As nutritious food, mushrooms are rich crude proteins, soluble polysaccharides, dietary fiber, vitamins, and minerals and accumulate a variety of active metabolites, including saponins, flavonoids, alkaloids, anthraquinones, anthrones, phenols, coumarins, fatty acids, and steroids [1,2]. These components of mushrooms are attributed to their biological activities, including hypoglycemic, antibacterial, antioxidant, and teratogenic activities [3-5]. Moreover, they are also responsible for immune system modulation, inflammation inhibition, hypertension and atherosclerosis prevention, antitumor, hypocholesterolemic, hypoglycemic, antithrombotic, and antimicrobial activities of mushrooms [6].

Pycnoporus sanguineus is a bright orange-red colored thin fan-like shaped bracket mushroom that is naturally growing on fallen hardwoods. This mushroom is usually ignored and not utilized by Filipinos. However, in Brazil, some indigenous people use this mushroom to stop hemorrhages [7]. It was also reported that cinnabarin

is one of the important secondary metabolites of *P. sanguineus* that has antibacterial activities and pharmacological properties [8]. However, Borderes *et al.* [9] revealed that cinnabarin was not the main compound produced by *P. sanguineus*. Recently, the mycelial growth performance in various solid culture media and the fruiting body production in rice straw-sawdust based substrate were studied (unpublished data) in our aim to establish techniques for its biomass production and popularize the utilization of this mushroom in the Philippines.

Submerged culture is an efficient, fast, and practical technique of producing mycelial biomass of mushroom, especially those that do not successfully produce fruiting bodies or with very low biomass yield under solid-fermentation conditions. Dulay *et al.* [10] optimized the submerged culture conditions for the production of mycelial biomass of *Ganoderma lucidum*, *Volvariella volvacea*, *Pleurotus cystidiosus*, and *Schizophyllum commune* as a potential source of functional lipids. Moreover, submerged cultivation was utilized for the production of mycelia and compounds with biological activities, including endopolysaccharides, exopolysaccharides, and ganoderic acids of *G. lucidum* [11]. Accordingly, this cultivation technique can be used to standardize the mycelial biomass production as well as the nutritive values and pharmacological properties of mushrooms.

In our interest to improve the mycelial biomass production and bioactivity and to address the low biological efficiency in solid-fermentation condition, this research had its main objective to

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determine the effect of time-course submerged culture on the mycelial production and functional properties (antioxidant, antibacterial, and cytotoxic) of *P. sanguineus*. The mycochemical constituents of the mycelia were also elucidated.

2. MATERIALS AND METHODS

2.1. Mushroom Source and Inocula

P. sanguineus (BIL7137) culture was acquired from the Bioassay Laboratory, Department of Biological Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines. The cell line of this wild mushroom strain was rescued from Lingap Kalikasan Park in the same university. An agar block of mycelia was aseptically inoculated onto a sterilized potato dextrose agar (PDA) plate and incubated at 30°C for 7 days. A 10 mm-diameter cork borer was used to prepare mycelial disks as inoculant.

2.2. Evaluation of Mycelial Biomass Production

Coconut water from mature *Cocos nucifera* L. was used as a culture medium in the evaluation of mycelial biomass production in submerged culture. A 50 ml medium was dispensed into each 250 ml-capacity glass culture bottles with cotton plugged and sterilized at 15 psi, 121°C for 20 min. Each treatment was replicated 10 times. Bottled media were inoculated with mycelial disks and incubated at different periods at 30°C and static conditions in an incubation chamber. After incubation, the mycelia were harvested, air-dried, and weighed, and the culture spent volume was also recorded. The concentration of mycelial biomass was calculated by the ratio between mycelia (dry weight) and the volume of the medium used. The specific growth rate was also determined by computing for the natural log of the biomass (lnX) plotted against the fermentation time (t). For any given moment, the slope of the line gives the specific growth rate. Mycelia were pulverized using a food processor and extracted for the assays.

2.3. Ethanol Extraction

Three grams of pulverized mycelia from each incubation period were soaked in 300 ml of 80% ethanol for 48 h and filtered using No. 2 Whatman filter. Filtrates were concentrated to dryness in a rotary evaporator, which yielded 10.0–13.5% extract on a dry weight basis. The extracts were used for analyses and assays.

2.4. Scavenging Activity Assay and Phenolic Content Analysis

The stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the extracts was determined following the procedure described by Kolak *et al.* [12] with modifications. Catechin was used as the standard. Absorbance readings were determined at 517 nm using a UV VIS spectrophotometer (Spectrumlab 752S, Hinotek Instrument Co., LTD, China) and percentage radical scavenging activity was computed using this formula: %RSA = $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$. However, the Folin-Ciocalteu method of Sunita and Dhananjay [13] was followed with modifications to estimate the phenolic content of the extracts. The absorbance was read at 760 nm using the same UV VIS spectrophotometer and the phenolic content was expressed as mg gallic acid equivalent (GAE)/g of sample.

2.5. Antibacterial Activity Assay

Disk diffusion method was carried out to evaluate the antibacterial effects of the extracts. *Escherichia coli* and *Staphylococcus aureus* cultures were obtained from the National Institute of Molecular

Biology and Biotechnology, University of the Philippines-Los Banos, Los Banos, Laguna, Philippines. Nutrient broth bacterial suspensions were prepared and the turbidity was compared to 0.5 McFarland standard to equal to approximately 1.5×10^8 bacterial cells/mL. A 0.5 mL of bacterial suspension was inoculated and spread on Mueller-Hinton agar plate. Six mm-diameter paper disks impregnated with 20 μ L of mushroom extract and streptomycin were equidistantly placed on the inoculated medium and subsequently incubated at 37°C. Each treatment was replicated three times in triplicate tests. After 24 h of incubation, zones of inhibition were measured.

2.6. Brine Shrimp Toxicity Assay

Artemia salina lethality assay of Olowa and Nuñez [14] was carried out to assess the cytotoxic effects of the mushroom extracts. The brine shrimps were acquired from the Bureau of Fisheries and Aquatic Resources-National Freshwater Fisheries Technology Center in the Philippines. Extracts at different concentrations (μ g/mL) were prepared in triplicate vials. Ten nauplii were exposed into each vial for 24 h and the percentage mortality was recorded. The LC₅₀ values were determined using probit analysis to determine the cytotoxicity level based on the established Clarkson *et al.* [15] toxicity index.

2.7. Mycochemical Screening

The mycochemicals present in the ethanol extract of mycelia from the cultures incubated for 25 days were screened following the protocol of Guevara [16]. The different mycochemicals were detected as spots in thin layer chromatography (TLC) through the use of UV light, hot plate, and several reagents used for a typical visualization of the secondary metabolites. Vanillin-sulfuric acid was used to determine the presence of phenols, sterols, fatty acids, triterpenes, and essential oil. Methanolic potassium hydroxide was used to visualize anthraquinones, coumarins, and anthrones, while potassium ferricyanide-ferric chloride was used to test phenolic compounds and tannins. Alkaloids and flavonoids were detected using Dragendorff's reagent and antimony (III) chloride, respectively.

2.8. Statistical Analysis

Analysis of variance (ANOVA) and Tukey's honestly significant difference at 5% level of significance in the SAS System Version 9.0 was used to analyze and compare the data.

3. RESULTS AND DISCUSSION

3.1. Mycelial Biomass Production

Mycelium is the vegetative structure of mushroom that could also be a source of biomass and bioactive compounds. Hence, evaluation of the biomass production of mushroom is indeed necessary. The production of mycelial biomass of *P. sanguineus* using coconut water as a culture medium at different periods of incubation was evaluated in this study. The fresh and dry weight of mycelia as affected by different incubation periods were determined (Table 1). The 25-day-old culture produced the highest yield (6.08 g fresh and 0.51 g dry), while the 10-day-old culture had the lowest. Statistically, the different periods of incubation significantly influenced the mycelial biomass production. It can be noticed that the mycelial biomass increased as the incubation period prolonged up to 25 days. The biomass concentration was computed and revealed that *P. sanguineus* incubated for 10 days produced 4.8 g/L of mycelial biomass and significantly increased up to 10.2 g/L as the incubation time extended up to 25 days with a specific growth rate of 0.640 g/L/day. These

results strongly suggest that prolonged incubation can be a useful technique for mass production the mycelia, which could mask out the need for lengthy fruiting body production of mushrooms. Studies have found out the efficiency of prolonged incubation time to obtain higher mycelial yield in submerged culture condition. For instance, the wood ear mushroom, *Auricularia polytricha*, produced more cell mass as a time of fermentation extended to 20 days [17]. The same was also observed in the production mycelial biomass of a novel edible mushroom species, *Lentinus tuberregium* [18]. Contrastingly, the biomass production of three *Auricularia* species (*A. delicata*, *A. polytricha*, and *A. auricula*) in submerged fermentation in a longer incubation period of 30–40 days resulted to a decrease in the biomass yield [19]. They also added that the optimum incubation period for mycelial biomass production of the three *Auricularia* was 20–25 days. Therefore, it is too early to mention in the present study that 25 days was the optimum incubation period. Further investigation on the biomass production in the lengthier period of incubation is imperative to establish the maximum period of incubation.

The morphology of mycelia as affected by the different periods of incubation was also observed (Figure 1). A 10-day-old culture had semi-transparent, very thin mycelia with white powder-like structures scattered on the top of mycelia (Figure 1a). These powder-like structures turned into light orange and the mycelia became thicker when incubated further up to 15 days (Figure 1b). After 20 days of incubation, the light orange mycelia became bright orange to red-orange with very thick mycelia (Figure 1c). Finally, at 25th day, the very thick mycelia produced pinkish to light orange massive primordia (Figure 1d). The high biomass yield in 20- and 25-day-old cultures is most likely attributed to the very thick mycelia and formation of primordia. The same cultural characteristics of *P. sanguineus* were also observed by Tellez-Tellez *et al.* [20]. They observed that the presence of salmon-red color or vermilion pigment becomes more intense as the mycelia aged, and they also believed that this characteristic greatly participates in bioactivities of the mycelia. Pigment characterization and biosynthesis revealed that phenoxazine-3-one type structure is the one accountable for the red color pigment of several *Pycnoporus* strains and for their bioactivities [20,21].

Table 1: Mycelial biomass of *P. sanguineus* at different periods of incubation

Incubation period (day)	Mycelial biomass (g)		Biomass concentration (g/L)	Specific growth rate (g/L/day)
	Fresh	Dry		
10	4.48±0.24 ^b	0.24±0.01 ^d	4.8	0.256
15	4.50±0.07 ^b	0.35±0.00 ^c	7.0	0.384
20	5.42±0.50 ^a	0.43±0.01 ^b	8.6	0.512
25	6.08±0.86 ^a	0.51±0.04 ^a	10.2	0.640

Values are expressed as mean±SEM (n=10). Means with the same letters of superscript are not significantly different from each other at 5% level of significance

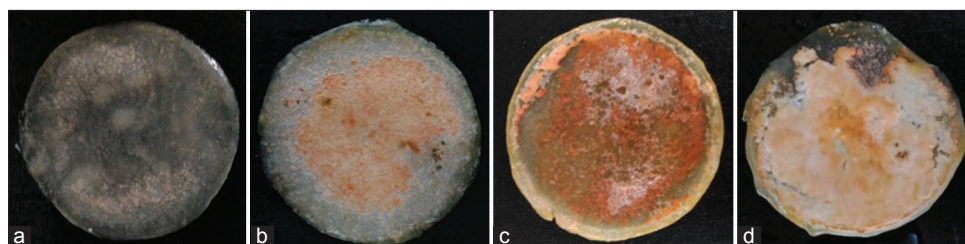


Figure 1: Mycelia of *P. sanguineus* grown in coconut water at different periods of incubation: (a) 10days, (b) 15 days, (c) 20 days, and (d) 25 days

3.2. Radical Scavenging Activity and Phenolic Content

The scavenging DPPH free radical assay can be utilized to analyze the antioxidant properties of compounds or extracts in a short time [22]. This work determined the radical scavenging activities of *P. sanguineus* mycelial extracts (Table 2). Notably, the extract of 25th-day mycelia recorded the highest scavenging activity of 37.25%, followed by 20th-day mycelial extract, which showed no statistical difference. In contrast, the lowest scavenging activity was noted in the extracts of early-harvested mycelia. These results suggest that the radical scavenging activity of *P. sanguineus* increased in extending time of incubation. Similarly, Borderes *et al.* [9] also investigated the effect of different time course in the radical scavenging activity of *P. sanguineus* mycelia harvested from 5th to 30th day (5 days interval) of incubation and they found out that as a time of incubation increased, the radical scavenging activity of the mycelia increased.

Phenolics represent the main antioxidant component in mushroom [23,24]. Phenolic antioxidants transfer hydrogen atom to peroxy radicals and then converting them to hydroperoxides [25]. Thus, it was also quantified in this work (Table 2). Noticeably, extract of 20-day-old mycelia contained the highest of phenolic content (86.29 mg GAE/g sample), whereas 10-day-old mycelial extract registered the lowest (78.89 mg GAE/g sample). Our results conform with the study of Sanchez [26], who reported the presence of phenolic antioxidant compounds in *P. sanguineus*. Moreover, the phenolic contents obtained in the present work are higher than the phenolic contents of other edible mushrooms, including *Ganoderma applanatum*, *Ganoderma lucidum*, *Schizophyllum commune*, *Auricularia auricula-judae*, *Lentinus tigrinus*, *Pleurotus ostreatus*, and *Pleurotus sajor-caju* [27].

It is noteworthy to mention that 20-day-old mycelia, which had the highest phenolic content, was not the optimum mycelial age for radical scavenging activity. The high scavenging activity of 25-day-old mycelia, despite the lower phenolic content, could be attributed to non-phenolic antioxidants such as triterpene, anthrones, steroids, and other fatty acids, which are found present in mycochemical screening of the mycelia of *P. sanguineus*. Huang *et al.* [28] disclosed that the total phenolic content and the radical scavenging activity of the extracts are sometimes indirectly correlated. Based on the results, it is safe to conclude that *P. sanguineus* mycelia cultured for 20 days can be a sweet spot in obtaining phenolic-rich components.

3.3. Antibacterial Property

Mushrooms have their own fighting mechanism for their competitor (e.g., bacteria). One of these mechanisms is by secreting compounds that contribute to the antimicrobial potential. The antibacterial activity of *P. sanguineus* mycelia ethanolic extract against *E. coli* and *S. aureus* was also studied (Table 3). All extracts of mycelia exhibited antibacterial activity against both bacteria. *S. aureus* was found to be more sensitive on the extract having a range of 8.00–9.30 mm diameter

Table 2: Radical scavenging activity and phenolic content of ethanolic extracts of *P. sanguineus* mycelia grown at different periods of incubation

Extract of mycelia grown after	Radical scavenging activity (%)	Phenolic content (mg GAE/g sample)
10 days	31.69 ^c	73.89 ^d
15 days	31.44 ^c	78.17 ^c
20 days	36.62 ^b	86.29 ^a
25 days	37.25 ^b	81.50 ^b
Catechin	72.10 ^a	---

Values are expressed as mean ($n=3$). Means with the same letters of superscript are not significantly different from each other at 5% level of significance

Table 3: Diameter of zone of inhibition of ethanolic extract of mycelia of *P. sanguineus* against *S. aureus* and *E. coli*

Extract of mycelia grown after	Diameter of zone of inhibition (mm)	
	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>
10 days	9.30±0.58 ^b	7.38±0.08 ^b
15 days	8.00±0.44 ^c	7.11±0.28 ^b
20 days	9.05±0.49 ^b	7.25±0.22 ^b
25 days	8.12±0.16 ^c	7.34±0.06 ^b
Streptomycin	28.27±0.98 ^a	27.57±0.40 ^a
Ethanol	6.00±0.00 ^d	6.00±0.00 ^c

Values are expressed as mean±SEM ($n=3$). Means with the same letters of superscript are not significantly different from each other at 5% level of significance

zone of inhibition. However, *E. coli* showed sensitivity ranging from 7.11 to 7.38 mm diameter zone of inhibition. The zones of inhibition exhibited by the extract of 25-day-old mycelia against the two bacteria are shown in Figure 2. Although extract from 10-day-old mycelia showed the widest diameter zone of inhibition in both bacteria, the difference of all mycelial extracts was statistically not significant. Therefore, we cannot presume that the antibacterial agents are most likely developed and produced at the younger stage of mycelia. However, the presence of the zone of inhibition strongly suggests the promising antibacterial potential of mycelial extract of *P. sanguineus*.

In a similar study, a significant increase in antibacterial activity of *Polyporus tricholoma* against *S. aureus* was found in the extract of later harvest mycelia [29]. *P. sanguineus* has been reported to possess an excellent antibacterial property, particularly cinnabarin, which is usually extracted by ethyl acetate [30]. Moreover, the antibacterial property of *P. sanguineus* could be accounted to the present mycochemicals such as triterpenes, tannins, steroids, alkaloids, anthraquinones, and anthrones which are known to exhibit antibacterial properties.

3.4. Cytotoxic Effect

A brine shrimp lethality assay was used as an indicator for broad toxicity. This is one of the important tools for the isolation of bioactive compounds [14]. Our paper is the first one to report the cytotoxic activity of *P. sanguineus* mycelia using this assay. Results of the assay showed that the mortality of brine shrimp nauplii increased as the concentrations of all mycelial extracts increased. Table 4 presents the computed LC₅₀ values of the four extracts. Noticeably, the LC₅₀ values of mycelial extracts decreased as the time of incubation in submerged culture prolonged, indicating that prolonging the incubation period could contribute to the toxicity of the mycelia. Interestingly, the obtained LC₅₀ value of the 25-day-old mycelial extract is lower

Table 4: LC₅₀ values and toxicity levels of ethanolic extracts of mycelia of *P. sanguineus* on brine shrimp nauplii after 24 h of exposure

Extract of mycelia grown after	LC ₅₀ (µg/mL)	Toxicity Level ^a
10 days	154.83	Medium toxic
15 days	90.45	Highly toxic
20 days	33.50	Highly toxic
25 days	16.02	Highly toxic

^aBased on the cytotoxicity level established by Clarkson *et al.* [15]

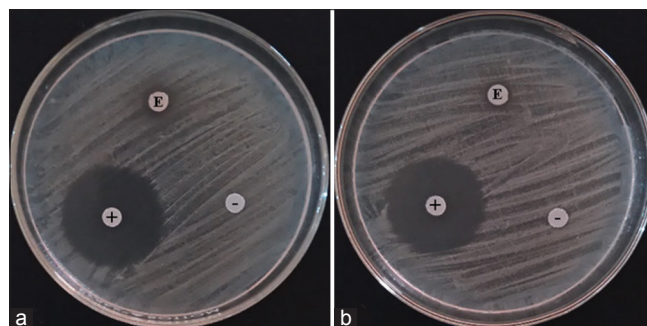


Figure 2: Antibacterial assay plates showing the zones of inhibition exhibited by the mycelial extract of 25-day old culture of *P. sanguineus* against (a) *S. aureus* and (b) *E. coli*. (E) mycelial extract, (+) streptomycin and (-) ethanol

than the reported LC₅₀ value (16.30 µg/mL) of cyclophosphamide, a commercially available anticancer drug [31]. This clearly suggests that *P. sanguineus* mycelia could be a valuable source of toxic compounds, which could be a potential anticancer drug.

3.5. Mycochemical Constituents

In the interest of knowing the different mycochemicals that contributed to the different bioactivities of *P. sanguineus* mycelial extract, a thin-layer chromatography (TLC) spot test was carried out. The term mycochemical refers to a classification system of different mycological chemicals which are present or detected. These are chemicals that fungi, particularly mushrooms, produce to perform metabolic functions and also to protect themselves [4]. Due to the notable performance of 25-day-old mycelia of *P. sanguineus*, this was considered in the mycochemical screening. Nine mycochemicals were detected including triterpenes, flavonoids, tannins, phenols, steroids, alkaloids, anthraquinones, anthrones, and fatty acids. Several studies reported the biological activities of the above-mentioned mycochemicals.

Triterpenes have antibacterial, antifungal, antiviral, anti-inflammatory, anti-oxidative, and anticancer properties [32]. Zuco *et al.* [33] stated that among the bioactive compounds, triterpenes showed low activity in normal cells but cytotoxic against tumor cells. Flavonoids are known for their antioxidant property, anticarcinogenesis potential, antiproliferative effects against cancer cell lines, and also chemopreventive agent [34]. Tannins are astringent, bitter polyphenols that are reported to have a major impact on animal nutrition due to their ability to bind to protein and other macromolecules and also has antioxidant properties [35]. Phenols are under an important class of antioxidants that reduce the oxidative degradation of organic materials [36]. Steroids are known to be important for their cardiotoxic activities and also possess insecticidal, antioxidant activity, and antimicrobial properties [37].

Alkaloids are found to have significant antibacterial and antifungal activities, have allelopathically active chemicals, and a possibility to have a cytotoxic effect [38]. Anthraquinones are known to be laxative compounds for constipation. They have cathartic and diuretic effects, anti-inflammatory, anticancer, antimicrobial, vasorelaxant activities and DNA binding ability. Recently anthraquinones are discovered to have therapeutic potential on autoimmune diabetes [39]. Anthrones possess antibacterial, antifungal, antioxidant, and anti-inflammatory properties [40]. Fatty acids, especially polyunsaturated ones, the omega-3 and omega-6 series, are essential in the human health for preventing physiologically-related diseases and autoimmune disorders. Fatty acids are also much needed in one's diet [41].

4. CONCLUSION

Collectively, this paper demonstrated the time-course submerged cultivation of mycelial biomass and its effects on the biological activities of *P. sanguineus*. A prolonged incubation up to 25 days enhances the mycelial production and improves antioxidant activity and toxicity. However, the antibacterial property is not affected by the periods of incubation. The 25-day-old mycelia contain a variety of mycochemicals, which are responsible to the antioxidant and antibacterial activities, and toxic effect exhibited in the present study, and most likely to other biological activities of *P. sanguineus* mycelia, which we need to further elucidate.

5. CONFLICTS OF INTEREST

Authors declared that they do not have any conflicts of interest.

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None.

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