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Molecular identification and optimization of liquid culture conditions of *Trametes sanguinea* isolate from Mt. Arayat Protected Landscape, the Philippines

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Abstract

In this present report, the *Trametes sanguinea* isolate from the Mt. Arayat Protected Landscape, Philippines, was molecularly identified to confirm its identity and optimized for mycelial growth through submerged cultivation. Its genomic DNA was extracted, and ITS1F/ITS4R primers were used for ITS gene amplification and sequencing, followed by BLASTn nucleotide sequence homology analysis, which was confirmed by phylogenetic analysis. The results revealed the mushroom sample was identified as *Trametes sanguinea* with 99.1% identity with NCBI Accession Code (PV776394). Meanwhile, the optimal nutritional and physical liquid culture conditions for *Trametes sanguinea* revealed that coconut water yielded the highest, 311.67 mg/100 mL, at an optimal pH of 6.0. Biomass production of *Trametes sanguinea* was satisfactorily observed at a temperature of 30°C under agitated conditions. This achieved result suggests that *Trametes sanguinea*'s high mycelial biomass productivity can be further analyzed for commercial cultivation and a potential source of nutraceuticals for the pharmaceutical industry.

Keywords – BLASTN – mycelial growth – nutraceuticals – phylogenetic analysis

Introduction

Mushrooms are fungi with a unique appearance of the fruiting body that are widely collected, cultivated, and consumed by people. Typically, they are categorized as edible, medicinal, and poisonous (Lopez et al. 2016). Mushrooms are commonly found in woodlands and pastures and are used as food, a source of traditional medicine, and at times for the maintenance, prevention, and treatment of various health conditions (Valverde et al. 2015, Aguilar et al. 2016). Technological advancement and discoveries, however, have elucidated mushrooms as factories of nutritional contents (fibers, proteins, and low-fat) and medicinal values (anticancer, antioxidants, anti-glycemic), which are beneficial to human health (Samsudin & Abdullah 2019).

In the Philippines, the popularity of mushrooms has emerged due to their biological and industrial significance, thus diversity assessment and cultivation of various strains become highly evident. Because of the known promising nutraceutical properties, mushrooms have been mass-produced now in both traditional and advanced techniques to fully exploit their potential as food and additives to pharmaceutical and therapeutic drugs. Likewise, in the Philippines, mushrooms have been commercially cultivated using different formulations, including indigenous materials.

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The use of agro-industrial waste has been utilized as substrates like rice bran, rice straws, sawdust, and as alternatives for efficient mushroom fructification (Garcia et al. 2020).

At present, numerous studies reported the distribution and cultivation of wild mushrooms in the Philippines due to its favorable tropical status, which is attributed to its rich mycological resources (Bustillos et al. 2024). Several reports on rely specifically on diversity and identification on morphological identification including those that are found in Puncan, Carranglan, Nueva Ecija (Sibounavong et al. 2008), Bungkalot tribal community, Nueva Viscaya (Torres et al. 2020), Mt. Makiling, Laguna (Nacua et al. 2018), Mt. Maculot, Provine of Batangas (Arenas et al. 2018), Mt. Isarog National Park (Paguirigan et al. 2020), Quezon Protected Landscape (Tadiosa 2022), Aeta communities Bataan (Tantengco & Ragragio 2018), Mt. Arayat Protected Landscape (Bustillos et al. 2024a,2025b), and selected areas in Nueva Ecija Province (Bustillos et al. 2025). Despite these comprehensive diversity reports, the identification and classification of these documented macrofungal species is morphological and traditional. Morphological characterization as a method of identification is sometimes confusing, which led to misidentification owing to the fact that mushroom diversity is complex (Undan et al. 2016, Reyes et al. 2016, Guzman et al. 2018). Thus, taxonomic classification must be combined with modern molecular techniques to resolve the issues and inconsistencies of the traditional system. As they are underutilized and understudied, only a few species have been cultivated.

This study confirmed the identity of *Trametes sanguinea*, a wild mushroom collected from the Mt. Arayat Protected Landscape, using a molecular approach, and established efficient submerged culture conditions for mycelial mass production of this basidiomycete.

Materials & Methods

Source of mushrooms

Samples of *T. sanguinea* were collected from the Mt. Arayat Protected Landscape, Arayat, Pampanga, Philippines. The collected fruiting bodies were covered with brown paper, labeled, and revived in the laboratory. Since MAPL is a protected area, the collection was conducted with the permission of the Department of Environment and Natural Resources, San Fernando, Pampanga, The Philippines, with Permit No. III-2023-008.

Genomic DNA extraction

The freshly ramified mycelia of *T. sanguinea* were carefully scraped on the media surface through the use of a flat tweezer and were transferred into a mortar with 1000 µL volume of pre-warmed 2× CTAB buffer and 100 µL of 20% sodium dodecyl sulfate (SDS). A micropestele was used to crush the mcyelia. After homogenization, samples were transferred to microtubes, by vortexed, and incubated at 55°C for 50 min in a digital dry bath (Labnet Accublock™ Digital Dry Bath, USA). Then 600 µL of chloroform-isoamyl alcohol (1:20, isoamyl alcohol:20 chloroform) was added, the tubes were mixed using a vortex (DLAB MX-S, USA), and centrifuged (HETTICH MIKRO 185, Germany) at 12,000 rpm for 10 min. The aqueous phase, or the upper layer, was then transferred to a 1.5 mL Eppendorf tube. Then, 400 µL of ice-cold isopropanol was added, and the samples were incubated at -20°C overnight. After incubation, the samples were spun at centrifuged at 12,000 rpm for 10 minutes, and the supernatant was decanted. Using 700 µL of 70% ethanol, the pellet was washed and centrifuged at 12,000 rpm for 10 minutes. Ethanol was decanted, and tubes were carefully inverted on a sterile paper towel inside the biobase chamber for about 5-10 min. The DNA pellet was redissolved in a buffer solution containing 30 µL 1× TE buffer (Milli-Q water) and incubated at room temperature.

PCR amplification

The primer pair or gene marker ITS1 F (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 R (5’-TCCTCCGCTTATTGATATGC-3’) of the Internal Transcribed Spacer (ITS) Region of genomic rDNA was utilized for Polymerase Chain Reaction (PCR) amplification (White et al.

1990, Gardes & Bruns 1993). The genomic DNA was diluted in a 1:100 ratio by molecular biology-grade water, pH 7.5. Each PCR tubes with 25 µL PCR reaction mixture contained the following: 1 µL of fungal genomic DNA as DNA template, 1 µL of ITS 1 and 1 µL ITS 4 primer, 1 µL of deoxynucleoside triphosphates (dNTPs) mix, 2.5 µL of 10× PCR buffer, 1.5 µL of $MgCl_2$, 0.1 µL of KAPA Taq DNA polymerase, and 16.9 µL of nuclease-free water. The PCR conditions were set using the Applied Biosystem® 2720 with the following profile: a 3-minute initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 51°C for 30 seconds, and extension at 72°C for 60 seconds. The final cycle will consist of an extension at 72°C for 10 minutes. The quality of the PCR products was evaluated using gel electrophoresis. Amplified samples were sent to Apical Scientific Sequencing SdnBhd Laboratory in Malaysia for sequencing. The sequenced samples of the ITS regions were assembled, aligned, trimmed, and analyzed using BioEdit. Nucleotide sequence homology was determined using BLASTn and compared against the GenBank database (NCBI). The phylogenetic analysis was built using the MEGA 7.0 program. The neighbor-joining technique was used to infer evolutionary relationships at 1000 bootstrap repetitions.

Following the sequencing, a phylogenetic analysis was constructed, supported by a bootstrap value. The phylogenetic tree was generated from the aligned sequences of the T. sanguinea species of macrofungi obtained from the NCBI GenBank. Maximum Likelihood phylogenetic tree of T. sanguinea isolates constructed in MEGA 7. Bootstrap values were derived from 1000 replicates. The scale bar indicates the number of substitutions per site. [o

Influence of nutritional factors

Following the revival of pure culture, evaluation of four indigenous culture conditions, namely: potato sucrose broth, rice bran broth, corn grit broth, and coconut water, followed. Preparation of culture media was employed using Dulay et al. (2015a) with slight modifications. Culture bottles were dispensed with 100 ml of media and sterilized for 30 minutes (21 psi). A mycelial disc (10 mm) from 7-day-old *T. sanguinea* mycelia was inoculated into each bottle containing 100 ml of medium, and the bottles were incubated at room temperature. Each setup was triplicated. After mycelial harvest, they are air-dried and weighed. The media with the best result were evaluated for pH levels (4.0 pH to 8.0 pH) with 1 M HCl or 1 M NaOH, and sterilized at 15 psi and 121°C for 30 minutes. The bottles containing the liquid culture were incubated for 10 days and harvested, air-dried, and weighed.

Influence of physical factors

The most suitable pH culture medium was used to assess the optimal physical factors (temperature and agitation) in *T. sanguinea*. The most suitable temperature was determined using three conditions: refrigerated (0°C), air-conditioned (20°C), and room temperature (30°C). The liquid cultures were incubated separately in these conditions. Based on the temperature results, culture bottles were evaluated for agitation. The two conditions used were static (0 rpm) and agitated (100 rpm). Mycelial dry weight was determined 10 days after incubation. Both temperature and agitation setups were replicated three times.

Statistical analysis

A complete randomized design (CRD) and analysis of variance (ANOVA) were used to analyze the data. A Duncan Multiple Range Test (DMRT) was also used to determine differences between means at the 5% level of significance.

Results

Molecular Identification of Mushroom

The amplified ITS sequences at 514 bp (Table 1). The sequence was subjected to phylogenetic analysis. The isolate was analyzed through BLASTn and shown in Table 1, which summarizes different samples using nuclear transcribed sequences.

Table 1 The collected sample, site of collection, and BLASTn analysis with maximum identity, and GenBank accession.

Sample Code	Identified name of Samples	BP Length	BLASTn Identity	Query Coverage	GenBank Accession Number	Elevation and Coordinates of collection site			Location
						Elevation	Latitude	Longitude	
Species	*Trametes sanguinea*	514	99.61%	100%	PV776394	750 masl	15°12'16"N	120°44'12"E	North Peak

Sequence and analyzed genomic DNA of *T. sanguinea* was confirmed by BLASTn and presented in Table 1. The sample was identified down to species level with an identity of 99.61% (*Trametes sanguineus*- PV776394) similarity with their GenBank accessions.

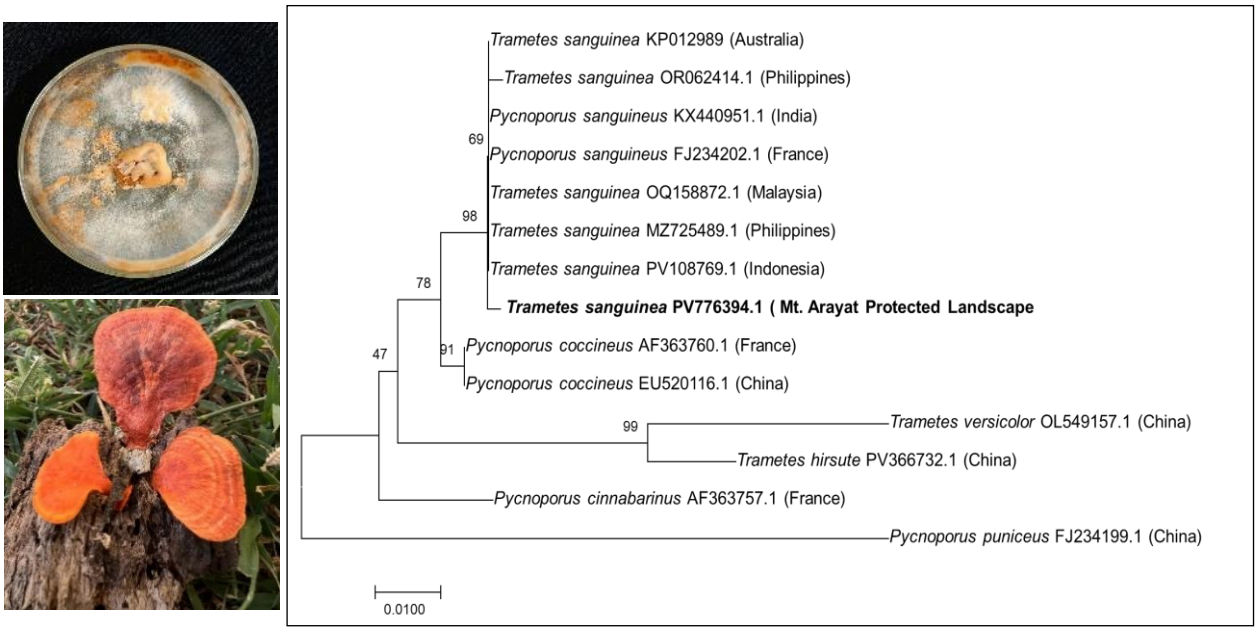

Fig. 1 – Constructed phylogeny tree illustrating the relationship of *T. sanguinea* with the assigned related species. Bootstrap values were derived from 1000 replicates, and *T. sanguinea* is represented as a taxon group. Meanwhile, the substitutions per site is indicated by the scale bar.

Fig. 1 shows that the constructed phylogenetic tree presented that the sample is closely related to *T. sanguinea* taxa with 98% bootstrap support.

Influence of liquid culture media

Liquid substrate is a very important factor in the mycelial ramification of mushrooms. Biomass production of *T. sanguinea* in various broth media was investigated 10 days after incubation. Among the four media evaluated, coconut water (Table 2) was statistically comparable to the other media, with the highest mycelial biomass of 311.67 mg. Lowest mycelial growth, however, was recorded in potato sucrose broth and coconut grit broth with 63.33 mg and 43.33 mg, respectively.

Table 2 The mycelial biomass yield of *T. sanguinea* using different indigenous broth media, 10 days after incubation.

	Biomass yield (mg/100mL)
Liquid culture media	*T. sanguinea*
Potato sucrose broth	63.33 ± 11.54^{c}

Table 2 Continued

	Biomass yield (mg/100mL)
Liquid culture media	*T. sanguinea*
Rice bran broth	263.33.00 ± 15.28[b]
Corn grit broth	43.33.00 ± 5.77[c]
Coconut water	311.67 ± 23.63[a]

*Similar superscript are not significantly different from each other at 5% level of significance.

Influence of pH

The most successful medium from the previous test was used to evaluate the optimal pH of the culture medium. The pH of a substrate is a very important factor in mycelial growth. The mean dry mycelial weight varied significantly across treatments. The mycelia grow optimally at pH 4.0-6.0, with the highest mycelial biomass recorded at 6.0 pH (311.0 mg), while 7.0 and 8.0 pH yielded the lowest mycelial biomass (180.67 mg and 157.67 mg, respectively).

Table 3 Mycelial biomass yield of *T. sanguinea* grown in coconut liquid culture media at varying pH conditions after 10 days of incubation.

	Biomass yield (mg/100 ml)
pH	*T. sanguinea*
pH 4.0	263.33 ± 20.31[bc]
pH 5.0	296.33 ± 12.74[b]
pH 6.0	311.00 ± 9.64[a]
pH 7.0	180.67 ± 41.04[c]
pH 8.0	157.67 ± 11.72[c]

*Similar superscript are not significantly different from each other at 5% level of significance.

Influence of temperature and agitation

Generally, temperature is a significant factor that affects fungal growth and sporulation. In this study, *T. sanguinea* grew rapidly between 20°C and 30°C (Table 3). The obtained mycelial weight was highest at 30°C with 348.00 mg. Comparison of treatments revealed a difference in the room-temperature preference of *T. sanguinea*. As gleaned (Table 3, Fig.2), no mycelial growth was observed in refrigerated. This suggests *T. sanguinea* grows preferably at room temperature.

Fig. 2 – Mycelial biomass production in varying temperature conditions of *T. sanguinea.*

Table 4 Biomass growth yield of *T. sanguineus* in liquid media as influenced by temperature and agitating conditions 10 days after incubation.

	Biomass yield (mg/100 ml)
Temperature	***T. sanguineus***
0°C	0.00 ± 0.00[c]
20°C	175.00 ± 26.96[b]
30°C	348.00 ± 46.13[a]
Agitation	
Agitated (100 rpm)	659.67 ± 100.55[a]
Static (0 rpm)	350.33 ± 14.98[b]

*Means with the same superscript are not significantly different from each other at 5% level of significance.

Agitation is another important factor in mushroom biomass production. The effects of shaking and static conditions on the biomass yield of *T. sanguinea* were also investigated. As shown in Table 4, the biomass production is statistically comparable between the agitated and static conditions. The agitated condition significantly produced a higher mycelial weight of 659.67 mg and a lower one at the static state with 350.33 mg.

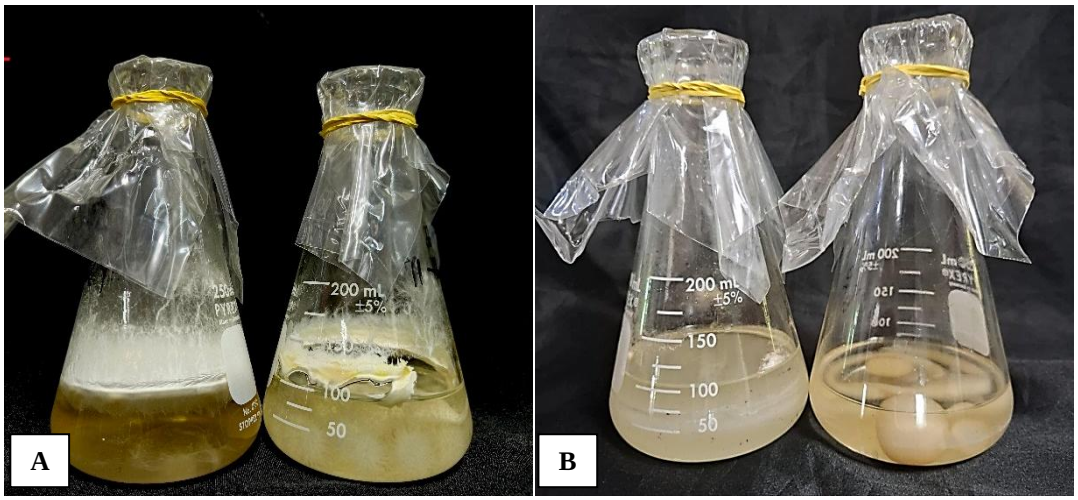

Fig. 3 – Mycelial biomass production in an agitated condition of *T. sanguinea* (A) temperature, and (B) agitated conditions.

Discussion

The Mt. Arayat Protected Landscape is a protected area that is recognized for its massive forest diversity. This condition favored the aggressive proliferation and fructification of macrofungal entities. In our previous report in Bustillos et al. (2024), we collected 108 species of wild macrofungi and identified one of them, *T. sanguinea*, through morphological means. The mycelia and DNA of this mushroom were successfully extracted, amplified, and analyzed. It was then subjected to nucleotide sequencing to determine its accurate identity and to reduce the challenges of inconspicuous and inconsistent morphological identities, a common problem in fungal diversity and taxonomy (Blackwell et al. 2006).

According to Raja et al. (2017), ≥97% to 100% sequence similarity and ≥80% query coverage is a reasonable result in assigning species names in accordance with the BLASTn and GenBank database. Thus, the result of the ITS analysis of *T. sanguinea* was found within these values, obliging its molecular identities. In congruence, Parnmen et al. (2016) investigated the of poisonous and non-edible mushroom molecular identity using the nuclear ITS region and peptide toxins in Thailand and found similarities ranging from 88% to 100%. Additionally, 91%-100% of five macrofungi, including a 99% similarity of *Ganoderma lucidum*, were isolated and identified from

Bangkay, Cuyapo, Nueva Ecija, Philippines (Undan et al. 2016). Likewise, seven *Lentinus* isolates from Luzon Island, Philippines, showed 97.90%- 100% confirmed similarity (Dulay et al. 2022).

Based on the optimal mycelial production, the coconut water was found favorable culture both media for *T. sanguinea*. This significant result can be attributed to the mushroom's nutritional composition, which favors efficient mycelial proliferation. Coconut water is highly nutritious primarily due to the presence of sugars, vitamins, amino acids, minerals, and phytochromes (Yong et al. 2009). Additionally, 100 g of coconut water may contain 3.71g of carbohydrates, 2.61 g of sugars, 1.1 g of fiber, 0.72 g of protein, and 0.2 g of fat. Its mineral contents also include 250 mg potassium, 105 mg sodium, 25 mg magnesium, 24 mg calcium, 20 mg phosphorus, 0.29 mg iron, 0.14 manganese, 0.1 mg zinc, 0.04 mg copper, and vitamins such as ascorbic acid, thiamine, niacin, panthothenic acid, pyridoxine, cobalamin, folate, and choline (Food Data Central, USDA 2019). These naturally occurring compositions of coconut water favor the growth of several mushroom isolates of Lentinus species from Luzon Island, Philippines. In six *Lentinus* isolates, 5 of them recorded significantly highest biomass yield in coconut water medium (Dulay et al. 2021). In the study of Bustillos et al. (2014), the same culture medium exhibits significant mycelial growth (5.9 g) for *P. cyanescens,* which is statistically comparable to corn grit broth (3.8). Also, a very thick mycelium of *Fomitopsis feii* notably produced a wider mycelial diameter in coconut water-solid media 12 days after incubation (De Leon et al. 2020). This abundance of mycelia is often associated with the presence of sugar where it is consumed as a major carbon source for growth of mycelia (Bindu & Charya 2017). Furthermore, the mycelial growth results of 10 various mushrooms studied were found to be significantly higher in coconut water medium (Kalaw et al. 2016). This is further confirmed in the previous reports that *L. tigrinus* (Dulay et al. 2012) and *L. squarossulus* & *P. grammocephalus* (De Leon et al. 2013) ideally grow on coconut gulaman. Concurring this result, Magday et al. (2014) stated that coconut culture medium is proven to be the most appropriate substrate for the growth performance and development of species like *G. lucidum*.

Contrary to this result of Aguilar et al. (2023), rice bran broth was recorded with the most favorable condition on the growth of *P. sanguineus* (100.13 mg/30ml). The optimal growth of *P. sanguineus* in rice bran was attributed to mineral composition of the broth, including proteins, fats, fibers, and micronutrients like phytosterol and tocopherols (Nagendra et al. 2011). Similarly, mushrooms grown in rice husk medium also obtained excellent mycelial density. This favorably allowed substantial proliferation due to high nitrogen and carbon contents, permitting efficient biomass production (Marim et al. 2016, Narh et al. 2018). Several species of mushrooms, such as *T. elegans* and *L. tigrinus* were also recorded to be favorably growing in rice bran-associated culture media (Dulay et al. 2021b, Dulay et al. 2015b)

The culture medium pH is an intrinsic factor that influences the physiological functions of mushrooms. The use of an appropriate pH in culture media supports the luxuriant mycelial growth required for efficient mushroom biomass production. In this study, coconut water was adjusted to different pH. The biomass yields under varying pH conditions are shown in Table 2. According to Lee et al. (2008), *Ganoderma* species grow optimally in pH 5.5-6.0. This is nearly identical to the result of this present study. Apparently, *T. sanguinea* preference is slightly acidic pH condition. This finding is in congruence with those of the *Xylaria nigripes*, *Pleurotus flabellatus*, *Volvariella volvacea,* and *Ganoderma lucidum* (Chen et al. 2014, Mohamad et al. 2015, Dulay et al. 2015, Agudelo-Escobar et al. 2017). Similarly, the Philippine strain of *Chlorophyllum molybdites* exhibit highest mycelial growth between pH range 5-6 (Garcia et al. 2020). Consummately, the aggressive growth of mushrooms can be affected by a wide range of pH. Although most mushrooms were observed to be maximally growing at slightly acidic pH, some species can also efficiently produce thick mycelia in neutral or alkaline substrates. This important parameter triggers the utilization of various nutritional requirements for assembling their biological characteristics. Reports revealed that acidic pH usually provides a more suitable condition for metabolite production and mycelial growth of ascomycetes and basidiomycetes (Shah & Modi 2018, Shih et al. 2007). This evidence implies mushrooms have pH preferences depending on the species, in which mycelial shutdown occurs when exposed beyond the optimum pH (Basu et al. 2015).

The optimal growth of *T. sanguinea* occurred at room temperature. Temperature ranges were used to determine optimal growth and colonization of *Trametes sanguineus*. This study utilized 0°C, 20°C and 30°C to determine which phase mycelia spread throughout the substrate (Dulay et al. 2012). These are benchmarks are critical biological limits and optimal phase of fungal life cycle. The 0°C was used to determine cold tolerance, 20°C is the standard temperature for fruiting body production that can tolerate colder temperature, and 30°C is mainly for tropical mushrooms which can withstand higher temperatures. This also conforms to the result that this temperature requirement is suitable in the incubation of *G. lucidum*, *P. cystidiosus*, *V. volvaceae* (Dulay et al. 2015a, Dulay et al. 2015b) *Pycnoporus sanguineus* and *Pycnoporus cinnabarinus* (Sharma & Jaitly 2017), *Chlorophyllum molybdites* (Garcia et al., 2020). In contrast, growth was suppressed at 0°C. This drastic drop in temperature caused inactivation and denaturation of essential enzymes, which contribute to the metabolic activities of the two mushroom species. In conformity, Lee et al. (2004) observed that species of *P. sanguineus*, *O. canarii*, and *G. frondosa* have optimal growth below 25°C. Consequently, this result suggests that *Trametes sanguineus* can be incubated at 20–30°C to achieve mycelial growth. This is similar to the studies of Li et al. (2026), in which *Trametes sanguinea* showed optimal mycelial growth at the same temperature, while *Volvariella volvacea* has the highest mycelial biomass yield at 30°C (Asasira et al. 2025).

The positive effect of agitation is allowing mycelia to efficiently absorb available nutrients, including a considerable amount of oxygen as a result of shaking (Peng et al. 2000). In agitation, oxygen is evenly distributed, and nutrients are sufficiently absorbed (Dulay et al. 2015a). High biomass yields in both test samples yielded results similar to those of Aguilar et al. (2023) for P. sanguineus, where agitated submerged culture with 413.3 mg was significantly higher than 134.50 mg in static conditions. Although some mushrooms produce no significant effect in terms of agitating condition (Dulay et al. 2015a), others yield more at higher agitation intensities. Moreover, other mushrooms such as *G. lucidum* (Dulay et al. 2015a), and *P. pulmonarius* (Abdullah et al. 2013) produce high yield with the same agitation preference. Another positive indication of agitating at a higher speed is the production of bioactive metabolites. For instance, *G. lucidum* and *Tricholoma matsutake* agitation significantly produced higher exopolysaccharides at 200 rpm and 300 rpm, respectively (Kim et al. 2010). Consistently, the production of mushroom metabolites can be enhanced by agitation. In conclusion, the mushroom isolate from Mt. Arayat Protected Landscape, the Philippines, was identified as *Trametes sanguinea* using the ITS region of rDNA. The mycelial growth production of *T. sanguinea* optimally grows on coconut water medium at a slightly acidic pH range of 4.0-6.0, at a room temperature of 30°C, in an agitated condition of 100 rpm. This successful identification and nutritional and physical requirements for its optimum growth were elucidated. This significant result is a prelude to the mass cultivation of this mushroom to establish its biological activity as a potent pharmaceutical drug, which is an ongoing study in our laboratory.

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