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Research Article

CULTIVATION AND ANTIOXIDANT PROPERTIES OF WILD *SCHIZOPHYLLUM COMMUNE* PRODUCED ON DIFFERENT AGRO-INDUSTRIAL WASTES

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ABSTRACT

This study explores the cultivation and antioxidant properties of *Schizophyllum commune*, commonly known as the split-gill mushroom, as a sustainable natural food source. The growth performance of *S. commune* was evaluated on two lignocellulosic substrates, which are rubber wood and cocopeat. Key antioxidant activities include 2,2-diphenyl-1- (2,4,6-trinitrophenyl) hydrazyl stable free radical (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ferric reducing antioxidant (FRAP) assays were analyzed to assess the bioactive potential of the mushrooms. The highest inhibition in rubber wood samples was recorded in 100% sawdust (55%), with the lowest inhibition concentration (IC_{50}) value being 0.957 µg/ml in ABTS, while 75% inhibition in DPPH. Rubberwood substrates yielded superior growth and higher levels of antioxidant activity, suggesting that the substrate can optimize both biomass and bioactive compound production. This research highlights *S. commune* as a viable alternative food source with significant antioxidant potential, promoting sustainability and contributing to the search for natural supplements and antioxidant sources for food.

1.Introduction

Increment of global demand for natural food sources and bioactive compounds has driven interest in the sustainable cultivation of edible wild mushrooms. *Schizophyllum commune*, commonly known as split-gill mushroom, is a widely distributed fungus found in diverse ecosystems. It has gained attention for its potential as a sustainable natural food source due to its ease of cultivation and rich nutritional profile (Suwannarach *et al.,* 2022).

Schizophyllum commune is a good source of protein, fiber, and bioactive compounds, particularly antioxidants (Wunjuntuk *et al.,* 2021). Antioxidants can neutralize harmful free

radicals in the body, thus helping to prevent oxidative stress-related diseases (Roncero-Ramos & Delgado-Andrade, 2017).

Mushroom cultivation not only provides an eco-friendly food source but also utilizes agricultural by-products as substrates, reducing waste and promoting sustainability (Basso *et al.,* 2020). Cultivation of *S. commune* is relatively simple, making it an attractive option for low-cost food production, especially in regions where food security is a concern. However, optimizing the choice of substrate can significantly influence both the growth and bioactive compound production of cultivated fungi, which can affect their nutritional value.

To address this, the present study evaluates the growth performance and antioxidant properties of *S. commune* cultivated on two lignocellulosic substrates: rubber wood and cocopeat. By employing key antioxidant assays such as DPPH, ABTS, and FRAP, the study aims to determine the bioactive potential of the mushrooms across different growth substrates. The findings contribute to the optimization of cultivation practices, emphasizing the importance of substrate selection for enhancing biomass yield and antioxidant capacity. This research not only emphasizes the value of *S. commune* as a natural food source with high antioxidant potential but also aligns with the global movement towards sustainable and health-promoting dietary alternatives.

2. Materials and methods

2.1. Fungal sampling and isolation

Sporocarps of *S. commune* were sampled in Sultan Idris Shah Forest Education Centre (SISFEC), UPM, Selangor on 16 March 2022. The samples were kept in paper bags for identification and cultivation. Fruiting bodies of the wild *S. commune* were surface sterilized using 10% Clorox and rinsed using sterile distilled water. *Schizophyllum commune* tissue was thereafter aseptically broken with the aid of sterile forceps. A small piece of 2 × 2 mm of the fruiting body tissue was aseptically transferred onto plates containing PDA. The formed fungal mycelium was subsequently transferred onto fresh PDA plates and incubated at room temperature. The pure fungal culture is ready for spawn inoculation.

2.2. Fungal identification

Schizophyllum commune was identified based on morphological characteristics and Internal Transcribed Spacer (ITS) sequence analysis. Morphological characteristics include stipe, pileus, and lamella arrangement of sporocarps were observed.

For molecular identification, the samples were cut into small pieces and ground using liquid nitrogen, and proceeded to DNA extraction using the Ultra Clean® Microbial DNA isolation kit (MO-BIO, Carlsbad, CA, USA) by following the manufacturer's guidelines.

PCR amplification of the ITS region was completed using a Professional Standard Thermocycler (Biometra Company, USA). A total of 50 µl reaction master mix containing 5× Green *GoTaq* Buffer, 2 mM dNTPs, 25 mM $MgCl_2$, 10 mM primer ITS1, 10 mM primer ITS4, Taq Polymerase, sterile distilled water (Promega, Madison, WI, USA). The quality of extracted gDNA was assessed by subjecting it to agarose gel electrophoresis. To amplify the ITS region of macrofungi, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') were applied (White *et al.,* 1990). PCR was run with the following cycle: initial denaturation at 95 ºC for 30 seconds, followed by 35 cycles of denaturation, annealing and extension at 95 ºC for 10 seconds, 59 ºC for 15 seconds and 72 ºC for 30 seconds, respectively. The final extension was running at 72 ºC for 5 minutes.

The PCR product undergoes the gel electrophoresis process by using 1% agarose gel consisting of 1× TBE buffer composed of 5.5 g of boric acid, 1 L of sterilized distilled water, 10.8 g of Tris-acetate and 0.93 g of Ethylenediaminetetraacetic acid disodium salt dihydrate. FloroSafe DNA was used to stain the amplified band. The produced band was observed under ultraviolet light using the Labnet Enduro TM Gel Documentation System (LABNET, USA). After obtaining a good DNA quality, it was processed for ITS sequencing by

the Nextgene Company using a Sanger dideoxy sequencer, 3730xl DNA Analyzer.

The sequences were aligned using BioEdit software. The aligned DNA sequences were blasted using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.gov) against sequences in GenBank to determine the closest matched sequence from the database. For depositing, the ITS sequences were prepared in FASTA format. Then, the document was submitted through the GenBank submission portal (https://submit.ncbi.nlm.nih.gov/subs/genbank/).

2.3. Cultivation of *S. commune*

2.3.1 Mushroom spawn preparation

Mushroom spawn was prepared with slight modifications to the volume and type of substrates following. Corn grains (500 g) were washed with running water to remove debris and soaked in water for 8 hours. Then, the grains were drained and left to air-dry overnight. The grains were mixed with 1% limestone (based on dry weight), and 250 g of the mixture was packed into a polypropylene spawn bag (13 x 6 cm). The bags were sealed with a plastic neck and cap before being autoclaved. The sterile corn grains were inoculated with a pure fungal culture. The inoculated spawn bags were incubated for 14 days.

2.3.2. *Mushroom substrate preparation and inoculation*

The mushroom substrate was prepared following the method of Hassan *et al.* (2022), with modifications based on the treatment sets. The ratios of rubber sawdust : cocopeat : cornbran : $CaCO_3$ were adjusted as follows: Treatment 1 (100:0:50:1), Treatment 2 (90:10:50:1), Treatment 3 (60:40:50:1), Treatment 4 (30:70:50:1), and Treatment 5 (0:100:50:1).

The corn grains that spawned with full mycelial growth were poured adequately, capped tightly in the inoculated substrate bag, and stored in an incubation room, 28 ± 2°C with an 8-hour photoperiod and 70-85% relative humidity. A total of five vertical notches were made on a bag of ripe mushrooms to allow the fruiting bodies to mature. Fungal growth was observed weekly until the bags were completely coated at 30-35 days after inoculation. After seven to 10 days of primordial creation, mushrooms were harvested. After 12-14 days from the first flush, a second flush was performed, and the same schedule was followed for subsequent flushes, or depending on the commencement of the mushroom primordia. Harvested fruiting bodies from this cultivation were used as mushroom samples in the antioxidant analysis. The biological efficiencies of mushroom specimens were calculated according to De Siquiera *et al.* (2011).

$$\text{Biological efficiency (\%)} = \frac{\text{Fresh fruiting body (g) x 100}}{\text{Dry substrate (g)}} \quad (1)$$

2.3.3. Mushroom extraction for antioxidant bioassay

Mushroom samples were prepared for antioxidant tests according to Tsai (2009). The moisture content of fresh fruiting bodies was calculated by weighing them. Mushrooms were sliced and lyophilized for 3 days (0,045 mBar, -51°C, Labconco, Missouri) before being milled into a fine powder using a blender. By subtracting the fresh and lyophilized weights of mushroom fruiting bodies, the moisture content was estimated. Mushroom powder (10 g) was extracted with 100 mL of distilled water and filtered through Whatman No. 1 filter paper using a water bath shaker (3.25 hours, 42.5°C, 160 rpm). The mixture was centrifuged for 10 min at 4000 rpm. The supernatant was collected and passed through Whatman No. 1 filter paper supernatant was collected and passed through Whatman No.1filter paper. All aqueous extracts were then freeze-dried and diluted for further analysis.

2.4. Scavenging activity towards 2,2-diphenyl-1- (2,4,6-trinitrophenyl) hydrazyl (DPPH) radical

With slight modification on a series of concentrations, the DPPH assay was carried out

according to Bloise's technique (Kebaili *et al.,* 2021). Fresh DPPH solution was made by dissolving 5 mg DPPH crystals in 2 ml ethanol, sealing it in aluminum foil, and keeping it at 4°C. In 96-well microtiter plates, extracts were diluted to generate a series of concentrations (0.13-1.00 µg/ml, volume 100 µL). Each well was filled with a 5 µL DPPH solution and left to react in the dark for 30 minutes. A microplate reader set to 515 nm was used to measure absorbance (MultiskanSky 1530-80079, Sweden) 8. All test analyses were averaged after at least three replicates. As standard antioxidants, ascorbic acid (Sigma) was utilised. To determine the blank absorbance, methanol was utilised instead of the mushroom extract. The extracts' antioxidant potential was determined using the equation below (Mwangi et al., 2022):

$$\text{Scavenging activity (\%)} = \frac{(\Delta A_{\text{blank}} - \Delta A_{\text{extract}})\text{x } 100\%}{\Delta A_{blank}} \quad (2)$$

Where:

$\Delta \boldsymbol{A_{blank}}$ = Average of blank absorbance (t=30 min)

$\Delta \boldsymbol{A_{sample}}$= Average of extract absorbance (t=30 min)

2.5. Ferric reducing antioxidant (FRAP) assay

With few adjustments, the FRAP test of mushroom extracts were adapted from Oyaizu (1986). Phosphate buffer (1 ml, 0.2 M, pH 6.6) and CNFeKz were combined with different quantities of extract (0.125 µl, 1 µ g/L) (1 L,1 percent). The mixture was incubated for 20 minutes at 50°C before being cooled on ice. $CHC_{13}O_2$ (1 ml, 10%) aliquots were added to the mixture and centrifuged (6000 rpm) for 10 minutes for the upper layer of the solution (2 ml) to react with distilled water (2 ml) and $FeCl_3$ solution (1 ml, 0.1 percent). The solution's absorbance was measured at 593 nm according to Madhanraj *et al.* (2017).

2.6. Determination of total phenolic content (TPC)

The total phenolic contents (TPC) of *S. commune* were determined by the Folin–Ciocalteau method developed by Singleton and Rossi (1965). The extract with distilled water (1200 ml), NaCO; (450 L) and Folin-Ciocalteu reagent (450 µl). The mixture was agitated and allowed to stand for 90 min. Absorbance of the blue coloration mixture were determined at 760 nm by using a UV visible spectrophotometer. Results were expressed as mg/g gallic acid equivalent (GAE) as followed, based on the calibration curve y=0.0007x+0.1886, RP=0.9865 (Liu *et al.,* 2017).

Total phenolic content = (mg TAE/g) – CXV x df/M (3)

Where,

C-concentration of tannic acid established from calibration curve (mg/ml)

V = volume of extraction solvent (ml)

df=dilution factor

M=weight of plant material (g)

2.7. Determination of 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

ABTS was formed by creating a mixture of 7 mM ABTS stock solution combined with 2.45 mM potassium persulfate (1/1, v/v) (Wongaem *et al.,* 2020). The mixture was permitted to stand at room temperature in complete darkness for 12 h prior to use. The mixture was allowed to stand for 4–16 h to ensure that the reaction has been completed, and a state of stable absorbance achieved. Distilled water was used to dilute the ABTS solution such that the level of absorbance measured 0.7 ± 0.02 at 734 nm. 750 µL sample of the ABTS solution was collected for the photometric assay with test samples of 25 µL which underwent vortexing for 5s before the absorbance was recorded at A 734 nm after being allowed to stand for 10 min. The reference compound chosen for this assay was ascorbic acid.

3. Results and discussions

3.1. Identification of *S. commune*

Schizophyllum commune is a distinctive basidiomycete fungus known for its unique fan-shaped fruit bodies, which are small in the range 3-5 cm with a pubescent, hairy upper surface (Figure 1B). The fruit bodies are typically grayish to brown. It has intercalcated lamellae and the stipe sessile (Figure 1A). *Schizophyllum commune* can be found on dead wood in a cluster form (Figure 1C). A hallmark feature of *S. commune* is its split-gills which are bifurcated.

Figure 1. *Schizophyllum commune* that found in Ayer Hitam Reserve Forest Selangor. (A) intercalcated lamellae; (B) hairy top surface; (C) attach to dead wood. (D) on PDA culture plate. Scale bar: 1 cm

Table 1. Moisture content and total extraction yield, quantity and quality fruiting bodies of *Schizophyllum commune* from rubber tree substrate

*Treatment	Moisture content (%)	Dry weight (g)	Number of fruiting bodies	Length of cap (mm)	Width of cap (mm)
T1	58.55 ± 0.007	40.16 ± 0.023	413	15-65	3-45
T2	53.96 ± 0.156	36.82 ± 0.157	399	15-41	10-52
T3	60.69 ± 0.018	35.80 ± 0.062	104	5-50	7-56
T4	67.47 ± 0.004	28.88 ± 0.027	94	15-40	5-40
T5	63.27 ± 0.004	25.28 ± 0.042	62	10-36	4-35

*Composition treatments: the ratios of rubber sawdust : cocopeat : cornbran : $CaCO_3$ were adjusted as follows: T1 (100:0:50:1), T2 (90:10:50:1), T3 (60:40:50:1), T4 (30:70:50:1), and T5 (0:100:50:1).

To verify the identification of the fungus species utilized in this investigation, the ITS region was sequenced as it is commonly recognized as the universal DNA barcode for fungi (White *et al.,* 1990). Following sequencing, species identification was verified by comparing the ITS sequences with reference sequences in GenBank using the BLAST program. A strong resemblance (99.84%) with *S. commune* was found in the analysis, supporting the samples' classification as a species. The obtained sequence was deposited in GenBank with the accession number OR178483

3.2. Growth and yield of *S. commune*

The moisture content of *S. commune* varied less significantly across treatments, though the highest content (67.47%) was observed in T4, while T2 exhibited the lowest (53.96%) (Table 1). The increased moisture in T4 suggests that the substrate or treatment applied enhanced the water-retention capacity. Moisture levels in

mushrooms are crucial for their physiological processes, as higher water content supports growth, development, and amplification (Dawadi *et al.,* 2022).

The moderate moisture content observed in the T1 group (58.55%) aligns with findings that untreated substrates typically provide a baseline for water availability in fungal cultivation (Muswati et al., 2021). The differences in moisture content across treatments imply that the physical and chemical properties of the rubber sawdust substrates were altered by treatment applications, affecting water retention and absorption efficiency. Substrate composition and environmental modifications are known to influence the microenvironment, thus impacting temperatures, and oxygen availability (Shakir *et al.,* 2023).

The T1 produces the highest number of fruiting bodies (413), suggesting that the untreated substrate provides optimal conditions for mushroom growth (Figure 2).

As treatments are introduced, particularly from T3 to T5, there is a significant decrease in the number of fruiting bodies, with T5 showing only 62 fruiting bodies. The size of the fruiting bodies, indicated by cap length and width, also decreases with more intense treatments. However, T2 exhibits the largest cap sizes, ranging from 15-41 mm in length and 10-52 mm in width, while T5 shows the smallest cap sizes, ranging from 10-36 mm in length and 4-35 mm in width.

Regarding extraction yield, the T1 group exhibited the highest yield (40.16%), indicating that untreated mushrooms were more efficient in providing extractable bioactive compounds. This observation supports previous studies that suggest excessive substrate modifications may reduce the availability of target metabolites, pH of substrate block, and available minerals that affect mycelial density (Khoo *et al.,* 2021). As more intensive treatments are applied, the extraction yield decreases, with T5 showing the lowest yield (25.28%).

Figure 2. Formation of fruiting bodies of *Schizophyllum commune* based on different substrate composition with the rations of rubber sawdust : cocopeat : cornbran : CaCO3 . A) T1 (100:0:50:1), B) T2 (90:10:50:1), C) T3 (60:40:50:1), D) T4 (30:70:50:1), and E) T5 (0:100:50:1).

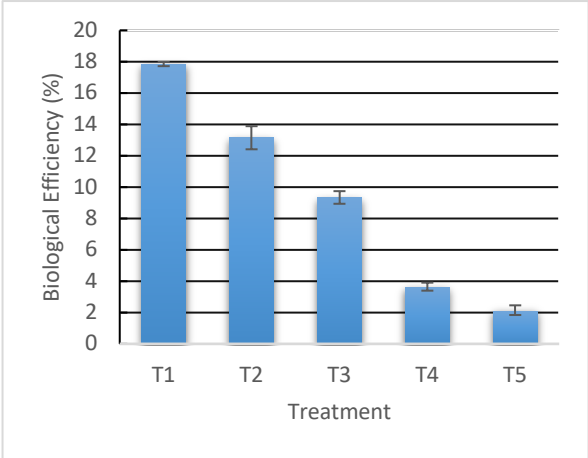

Figure 3. Biological efficiency of substrates on rubber dead wood at different treatments. Composition treatments: the ratios of rubber sawdust : cocopeat : cornbran : $CaCO_3$ were adjusted as follows: T1 (100:0:50:1), T2 (90:10:50:1), T3 (60:40:50:1), T4 (30:70:50:1), and T5 (0:100:50:1).

3.3. Biological Efficiency Ratio (BER) of *S. commune*

The Biological Efficiency Ratio (BER) reflects how efficiently a substrate is converted into mushroom biomass. The high BE in the T1 group, when compared to all treatments, raises the possibility that either the rubber wood substrate is more biologically efficient in its original state or that the treatments are preventing growth. The slow drop in BE from T2 to T5 may indicate that the modifications made to these treatments gradually impacted biological efficiency, perhaps by changing substrate structure, pH, or nutrient availability in ways that were detrimental to biological growth. BE in mushrooms can be impacted by changing the substrate's composition (Hoa *et al.,* 2015). Nitrogen and carbon supplementation are essential for mycelial growth and fruiting body formation (Singh *et al.,* 2021) is one example of how improper nutrition levels can hinder growth and decrease efficiency.

3.4. Antioxidant Properties of *S. commune*

Phenolic compounds contribute significantly to the antioxidant properties of mushrooms. Mushrooms grown on rubber dead trunk substrates show higher total phenolic content (TPC), particularly in the T2 (10% cocopeat) treatment, which achieves a TPC of 1924.57 GAE/g (Table 2). This high TPC value suggests that the rubber dead trunk substrate, especially when mixed with cocopeat, is effective in promoting the accumulation of phenolic compounds in the mushrooms. T2 treatment had the highest TPC value, suggesting specialized treatments can increase fungi's phenolic content, potentially influencing the formation of phenolic compounds linked to secondary metabolism or stress reactions (Ao & Deb, 2019).

For DPPH inhibition, T1 (100% sawdust) and T3 (40% cocopeat) show the best results, with inhibition percentages of 75% and 78%, respectively, and high IC_{50} values of 0.654 and 0.741 µg/ml, respectively. These values indicate that these treatments produce mushrooms with strong antioxidant properties, capable of effectively neutralizing free radicals. The ABTS assay, another method for measuring antioxidant activity, also shows moderate inhibition values for mushrooms grown on rubber dead trunk substrates. Treatment 1 (100% sawdust) again performs best with 55% inhibition and an IC_{50} of 0.957 µg/ml. This consistency across different antioxidant assays reinforces the conclusion that rubber dead trunk substrates support the production of mushrooms with strong antioxidant properties. The consistent performance of T1 across both assays suggests that 100% sawdust is particularly effective at supporting the production of compounds such as gallic acid, p-hydroxybenzoic acid, and protocatechuic that can neutralize free radicals (Abdelshafy *et al.,* 2021). The FRAP assay measures the antioxidant power in terms of the ability to reduce ferric ions. Interestingly, the T4 (30:70:50:1) treatment shows the highest FRAP value at 4217.90 GAE/g.

Table 2. Antioxidant properties of *Schizophyllum commune* in different treatments

Treatment	TPC (GAE/g)	DDPH inhibition activity		ABTS inhibition activity		FRAP (GAE/g)
		Inhibition (%) at 1 g/ml	IC_{50} (ug/ml)	Inhibition (%) at 1 ug/ml	IC_{50} (ug/ml)	
T1	1795.05	75	0.654	55	0.957	2521.40
T2	1924.57	74	0.686	49	1.032	2562.03
T3	1556.32	78	0.741	43	1.011	2577.27
T4	1439.49	64	0.827	37	1.047	4217.90
T5	1670.60	66	0.814	33	1.095	3864.89
Ascorbic acid	-	63.68	0.991	66	0.891	-

*Composition treatments: the ratios of rubber sawdust:cocopeat:cornbran:CaCO3 were adjusted as follows: T1 (100:0:50:1), T2 (90:10:50:1), T3 (60:40:50:1), T4 (30:70:50:1), and T5 (0:100:50:1).

Schizophyllum commune produces a polysaccharide known as schizophyllan which has various properties and applications, such as stimulating the immune system (Zhang *et al.,* 2013). It also possesses specialized enzymes, such as lignin peroxidases and laccases, that can break down lignin. The degradation of lignin can lead to the release of phenolic compounds, which are known for their antioxidant properties. Therefore, while lignin poses a challenge, it also offers an opportunity to produce valuable bioactive compounds. The lignocellulosic composition of rubber wood, which includes a moderate amount of lignin, creates an optimal environment for mushroom growth (Kumla *et al.,* 2020). The balance between cellulose, hemicellulose, and lignin in rubber wood ensures that the substrate is not only nutritious but also capable of supporting the complex metabolic processes required for the synthesis of bioactive compounds. The higher phenolic content in mushrooms grown on rubber wood is linked to increased antioxidant activity, as demonstrated by tests such as DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assays (Boonthatui *et al.,* 2021).

Fungal enzymes, such as cellulases, break down cellulose into glucose, which is then utilized by the fungus for energy and growth (Kumla *et al.,* 2020). The high cellulose content in rubber wood makes it an ideal substrate for mushrooms like *S. commune,* as it provides a steady and sufficient supply of carbon. An optimal C:N ratio is crucial for maintaining a balance between fungal growth and the production of bioactive compounds. If the ratio is too high (excess carbon), the fungus may grow rapidly but produce fewer bioactive compounds. Conversely, if the ratio is too low (excess nitrogen), the growth of the fungus may be stunted, leading to a lower yield. Rubber wood, with its high cellulose content, provides a rich carbon source, while the addition of nitrogen supplements can help achieve the desired C:N ratio. This balance ensures that the fungus not only grows well but also produces a high concentration of bioactive compounds, making rubber wood an ideal substrate for mushroom cultivation.

4. Conclusions

This study demonstrates the potential of *S. commune* as an alternative natural food source with promising antioxidant properties. The results highlight that the choice of substrate significantly impacts both the growth and antioxidant activity of the mushrooms. Rubberwood substrates support optimal mycelial development and higher antioxidant activity, as indicated by superior DPPH, ABTS, and FRAP assay results. The use of sustainable cultivation practices for *S. commune* could provide a cost-effective, environmentally friendly food source with functional health benefits, contributing to food security and the development of natural antioxidants.

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