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Conversion of Oil Palm By-Products into Value-Added Products through Oyster Mushroom (*Pleurotus ostreatus*) Cultivation

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Abstract: The oyster mushroom (*Pleurotus* species) is a popular and widely cultivated edible mushroom that can be found worldwide, including in Malaysia. However, its local production is unable to fulfil the market demand, partly due to the limited availability of rubber wood sawdust (RWS) as the conventional cultivation substrate. Furthermore, the palm oil industry in Malaysia generates large volumes of organic by-products that have caused environmental concerns. Therefore, the potential utilisation of oil palm waste-based substrates in order to develop a substitute RWS for *Pleurotus ostreatus* mushroom production is evaluated in this study, based on their agronomic performance and nutritional properties. Empty fruit bunches (EFBs), oil palm fronds (OPFs), and oil palm trunks (OPTs) were used to formulate the substrates. The control used was 100% RWS. Generally, 100% EFB showed a better agronomic performance, and mushroom growth was 1.9 times faster compared to the control, with a comparable mushroom yield. The crude protein and beta glucan content of mushrooms grown on oil palm by-product-formulated substrates were significantly higher than those grown using the control. Additionally, the number of fruiting bodies, crude protein, and beta glucan content of the mushrooms were positively correlated with potassium in the substrate. Therefore, 100% EFB could be used as a potential substitute for RWS for the cultivation and production of *P. ostreatus*.

Keywords: empty fruit bunch; oil palm frond; oil palm trunk; proximate analysis; mushroom substrates; physicochemical properties; correlation analysis; beta glucan

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1. Introduction

Mushroom consumption dates back to ancient times and mushrooms are believed to have a high nutritional value and medicinal properties. The oyster mushroom is the most cultivated and consumed mushroom variety in Malaysia [1]. The variety's essential amino acid content is highly appreciated [2]. In a ddition to being consumed as a food source, oyster mushrooms are also used in traditional medicines due to their high abundance of bioactive compounds that exhibit promising nutraceutical properties [3]. Numerous studies have proved that oyster mushrooms possess antitumor, anti-inflammatory, antioxidant, and antimicrobial potential [4–6]. The growing recognition of mushrooms' nutritional benefits has led to increasing market demand [1]. However, Malaysia has yet to achieve self-sufficiency, and the country relies heavily on mushroom imports from other countries (i.e., mainly from China and Japan), in both fresh and dried forms [1].

One of the rising concerns among local mushroom growers is the limited availability of substrate materials. The use of rubber wood sawdust as a substrate is the conventional practice for mushroom cultivation in Malaysia [7]. As the rubber industry has declined over the years, and rubber farmers have switched from rubber cultivation to oil palm

cultivation—as oil palm plantations are more profitable and less labour intensive [8]—this scenario has negatively affected the country's mushroom industry. Meanwhile, oil palm plantations generate 85.5% of their by-products in plantations and mills [9]. The poor disposal management of these by-products is causing growing concern with regard to environmental pollution [10]. Numerous, past studies on the utilisation of agricultural wastes for mushroom production have shown promising outcomes [11–14]. Thus, the utilisation of oil palm by-products as substrates for oyster mushroom cultivation could represent a solution to address both the problems related to the lack of substrates faced by mushroom growers and the overload of by-products left unattended by the palm oil industry.

Herein, the potential utilisation of oil palm by-products as substitute substrates to replace rubber wood sawdust for the production of *Pleurotus ostreatus* oyster mushrooms is evaluated. In the first instance, the physicochemical properties of the formulated substrates are determined. Furthermore, the growth and yield of *P. ostreatus* mushrooms cultivated on various formulations of oil palm empty fruit bunches (EFBs), oil palm fronds (OPFs), and oil palm trunks (OPTs), in comparison to rubber wood sawdust, are evaluated. Lastly, the nutritional composition of the *P. ostreatus* mushrooms grown on the formulated substrates is analysed. The correlation between the agronomic performance and nutritional properties of the mushrooms produced and the physicochemical properties of the substrates are also analysed.

2. Materials and Methods

2.1. Pure Culture Isolation

The pure culture isolation of *Pleurotus ostreatus* was performed aseptically under a laminar flow cabinet (AD03V, Advancelab, Kulaijaya, Malaysia). The basidiocarp of *P. ostreatus* was cut into halves, fresh tissue 5 cm × 5 cm in size was obtained from the centre of the basidiocarp, and was then transferred onto a potato dextrose agar (PDA) plate. It was incubated in dark conditions at a temperature of 25 ± 2 °C until the mycelia had grown across the entire plate. Then, it was subcultured onto a new, fresh PDA plate using a 5 cm cork borer to obtain a pure culture [15]. Working cultures were maintained on PDA plates, while stock cultures were kept in PDA slants at a temperature of 5–7 °C.

2.2. Spawn Preparation

The rice husk (1 kg) was rinsed under running tap water and soaked for 30 min, softened by boiling it for 15 min, and left on a strainer for an hour to remove excess water. The rice husk was packed into heat-resistant polypropylene bags 7.5 cm × 29 cm in size; each substrate bag weighed 500 g. The openings of the polypropylene bags were fitted with polyvinyl rings, plugged with cotton, and enclosed with a polyvinyl cap. The rice husk was sterilised at 121 °C for 20 min using an autoclave. After sterilisation, the ricehusk-filled bags were left to cool to room temperature before being inoculated aseptically with 10 pieces of 5 mm mycelial plugs of *P. ostreatus*. The inoculated rice husk-filled bags were incubated in dark conditions at a temperature of 25 ± 2 °C until the mycelia were completely spread throughout the spawn [15].

2.3. Substrate Preparation

Empty fruit bunches (EFBs), oil palm fronds (OPFs), and oil palm trunks (OPTs) are lignocellulosic residues generated in large quantities at oil palm plantations and palm oil mills. At the plantations, about 24% of the OPFs are obtained from the oil palm trees during pruning over a year, while 70% of the OPTs are obtained during replanting. The quantity of oil palm by-products generated at the mills depends on the quantity of fresh fruit bunches processed. For every tonne of fresh fruit bunch being processed, 0.18 tonnes of EFBs (wet basis) were generated [16]. Hence, EFBs, OPFs, and, OPTs were the oil palm by-products used as the basal substrates in this study. Rubber wood sawdust (RWS) served as the control. Prior to processing, EFBs were chopped into 5–7 cm sections, whereas RWS

was immersed in water for 24 h. Oil palm by-products and RWS were dried in a biomass drying facility, which is an enclosed room where sunlight is allowed to penetrate to dry the biomass at a temperature up to 50 °C. The basal substrates were supplemented with rice bran (10%) and calcium sulphate (1%), mixed thoroughly with water before packing into heat-resistant polypropylene bags sized 7.5 cm × 29 cm. The opening of the polypropylene bags was fitted with a polyvinyl ring, plugged with cotton, and enclosed with a polyvinyl cap. Each substrate bag weighed 500 g. The substrates were pasteurised at 80 °C for 8 h with a steam pasteuriser. After the substrates were cooled to room temperature, they were inoculated with 2% spawn dose (*w*/*w*) aseptically.

2.4. Physicochemical Properties of Substrates

Substrate samples were prepared by oven drying at 70 °C until a constant weight was achieved. The wet bulk density of the substrate was determined according to Mihilall et al. [17], while the particle density and porosity of substrate were determined according to Almomany et al. [18]. The pH of the substrate was determined in distilled water at a ratio of 1:10 following the method of Owaid et al. [19]. Moisture content, volatile solids content, and ash content were determined according to Mihilall et al. [17].

Lignin, hemicellulose, and cellulose of the substrate were determined followed by the method of Van Soeat et al. [20]; neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) of the substrate were analysed before mushroom cultivation. Lignin content was determined by the percentage of ADL. Hemicellulose was determined by the difference between NDF and ADF, whilst cellulose was determined by the difference between ADF and ADL.

Total C and N contents were determined using a CHN analyser (CHN-600, LECO Corporation, St. Joseph, MI, USA) with a dry combustion method [21]. In addition, total K, P, Ca, Mg, Fe, and Zn were determined using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES Optima 5300 DV, Perkin Elmer, Whaltam, MA, USA). Substrate samples were extracted using the dry-ashing method according to Isaac et al. [22] with modifications, where the sample (1 g) was weighed into a porcelain crucible and ashed in a muffle furnace (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 300 °C for an hour. The temperature was increased to 520 °C for 4 h. The samples turned white after ashing. Once cooled down, a few drops of deionised water and 1 mL of concentrated HCl (36%) were added to the sample. The samples were evaporated to dryness on a hot plate in a fume hood. Then, 5 mL of 20% HNO_3 was added to the sample and placed in a water bath for an hour. The solution was filtered with a syringe filter (0.45 µm, PTFE) and made up to 50 mL with deionised water.

2.5. Cultivation of Mushroom

There was a total of 11 treatments in this study (Table 1), and each treatment consisted of 8 replicates. Experimental units were arranged horizontally in a completely randomised design and incubated in the dark at a 26–32 °C temperature and 70% relative humidity, until the mycelia fully colonised the substrate. A polyvinyl cap and cotton plug were removed from the opening of substrate bags to initiate primordia. The substrate bags were exposed to 12 h of dimmed sunlight and 12 h of darkness, the temperature in the cultivation area was maintained at 26–32 °C, and the relative humidity was maintained between 80–85% by spraying with water [23].

Table 1. Substrate formulation for each treatment.

Treatment	Substrate Formulation
Control	100% RWS
T1	100% EFB
T2	100% OPF
T3	100% OPT
T4	50% EFB + 50% OPF
T5	50% EFB + 50% OPT
T6	50% OPF + 50% OPT
T7	33.3% EFB + 33.3% OPF + 33.3% OPT
T8	66.6% EFB + 16.7% OPF + 16.7% OPT
T9	16.7% EFB + 66.6% OPF + 16.7% OPT
T10	16.7% EFB + 16.7% OPF + 66.6% OPT

Note: RWS = rubber wood sawdust, EFB = empty fruit bunch, OPF = oil palm frond, OPT = oil palm trunk.

2.6. Agronomic Performance of Mushroom

The days taken for mycelia to fully colonised substrate, days taken for primordia initiation from a fully colonised substrate, and days taken to complete first flush growth were recorded. The pileus diameter and stipe length were measured using a vernier calliper. Biological efficiency was calculated based on Equation (1) below [24]:

$$\text{Biological efficiency } (\%) = \frac{\text{Fresh weight of fruiting bodies}}{\text{Dry weight of substrate after harvest}} \times 100 \quad (1)$$

The yield performance was evaluated based on the fresh weight of fruiting bodies, number of total fruiting bodies, and number of well-developed and marketable fruiting bodies, which are also known as effective fruiting bodies.

2.7. Mushroom Sample Preparation

Harvested fruiting bodies were sliced into 1 cm x 1 cm sizes and frozen at −80 °C. The frozen mushroom samples were lyophilised (Labconco, freezone 2.5 L −84 °C benchtop freeze dryer, Kansas City, MO, USA) for 24 h and ground into powder form. The freeze-dried and ground mushroom samples were stored at −20 °C for proximate analysis and beta glucan content.

2.8. Proximate Analysis of Mushroom

2.8.1. Determination of Moisture Content

The fresh weight of the fruiting bodies (W1) and weight of freeze-dried fruiting bodies (W2) were recorded. The moisture content of fruiting bodies was calculated using Equation (2) below:

$$\text{Moisture content } (\%) = \frac{W1 - W2}{W1} \times 100 \quad (2)$$

W1 = fresh weight of fruiting bodies;
W2 = weight of freeze-dried fruiting bodies.

2.8.2. Determination of Ash Content

A porcelain crucible was oven-dried for 24 h and its weight was recorded (W3). Mushroom sample: 2 g was weighed in the porcelain crucible (W4). The porcelain crucible containing the mushroom sample was burnt at 550 °C for 4 h in a muffle furnace. The weights of the mushroom ash sample and porcelain crucible (W5) were recorded, and the ash content was calculated using Equation (3) [25]:

$$\text{Ash content } (\%) = \frac{W5 - W3}{W4 - W3} \times 100 \quad (3)$$

W3 = weight of empty porcelain crucible;
W4 = weight of dried, ground mushroom and porcelain crucible;
W5 = weights of mushroom ash sample and porcelain crucible.

2.8.3. Determination of Crude Protein

The crude protein of fruiting bodies was determined using the Dumas method [26]. Mushroom sample: 100 mg was wrapped in a tin-foil cup and inserted into the CHN analyser (CHN-600, LECO Corporation, St. Joseph, MI, USA) to obtain the total N. Total N was multiplied by a conversion factor of 4.38 following the method of Rashidi and Yang [27] (Equation (4)):

$$\text{Crude protein } (\%) = \text{N} \times 4.38 \quad (4)$$

2.8.4. Determination of Crude Fibre

The crude fibre of the fruiting bodies was determined followed by the method of James [28]. Mushroom sample: 2 g was filled in a FibreBag and sealed (W6). The FibreBag was boiled in 1.25% H_2SO_4 for 30 min, removed from the solution, and rinsed with hot, distilled water 3 times. FibreBag was then boiled in 1.25% NaOH for 30 min, removed from the solution, and rinsed again with hot, distilled water 3 times. The FibreBag was oven-dried at 105 °C to constant weight (W7) and burnt in the muffle furnace at 550 °C for 3 h. The weight (W8) was recorded. The crude fibre was calculated using Equation (5):

$$\text{Crude fibre } (\%) = \frac{\text{W7} - \text{W8}}{\text{W6}} \times 100 \quad (5)$$

W6 = initial weight of sample;
W7 = weight of sample after oven drying;
W8 = weight of sample after burning.

2.8.5. Determination of Crude Lipid

The crude lipid of the fruiting bodies was determined using the solvent extraction gravimetric method [29] with modifications. An empty round-bottom flask was rinsed with acetone and oven-dried for 24 h before its weight was recorded (W9). Sample: 2 g of a freeze-dried and ground mushroom sample (W10) was wrapped with Whatman filter paper No. 2, and inserted into a thimble of a Soxhlet extractor (Standard Scientific Glass Industries, Mumbai, Maharashtra, India). A round-bottom flask was filled with two-thirds of petroleum ether and connected to the Soxhlet extractor. The Soxhlet was heated for 6 h for oil extraction before the extraction solvent petroleum ether was removed by a rotary evaporator. The flask containing oil extract was oven-dried at 80 °C for an hour, and its weight was recorded (W11). The crude lipid was calculated using Equation (6):

$$\text{Crude lipid } (\%) = \frac{\text{W11} - \text{W9}}{\text{W10}} \times 100 \quad (6)$$

W9 = weight of empty round-bottom flask;
W10 = weight of mushroom sample;
W11 = weights of round-bottom flask and oil extract.

2.8.6. Determination of Beta Glucan Content

The beta glucan of *Pleurotus ostreatus* was extracted using the method of Huang et al. [30]. The total and alpha glucan contents of the fruiting bodies were determined using Megazyme Yeast and a Mushroom Beta Glucan Assay Kit (K-YBGL 02/21, Wicklow, Ireland). The beta glucan content of the fruiting bodies was determined by the difference between total and alpha glucan contents of the fruiting bodies [31].

2.9. Statistical Analysis

The data were analysed using One-Way Analysis of Variance (ANOVA). Mean values were compared with Tukey's Honest Significance Test ($p \leq 0.05$), except for the proximate analysis of the mushrooms, where the mean values were compared with Duncan's Multiple Range Test ($p \leq 0.05$). Pearson's correlation was performed between the physicochemical properties of the substrate, agronomic performance, and nutritive properties of *Pleurotus ostreatus*. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 26.

3. Results and Discussion

3.1. Physicochemical Properties of Substrate

Table 2 shows that the wet bulk density, particle density, porosity, pH, and moisture content of substrate formulated with RWS and various oil palm by-products results in significant differences. The wet bulk density of the substrate in this study is comparable to Mihilall et al.'s study [17], which reported that substrate formulated with green grass (shredded to 5 cm in length) and pinewood shavings were 0.05 g cm^{-3} and 0.14 g cm^{-3}, respectively. Substrate formulated with 100% RWS had the heaviest weight because rubber wood sawdust holds more water content, has a smaller particle size, and thus creates more compaction in the substrate.

Table 2. Physical properties, pH, moisture content, ash content, and volatile solids of substrates.

Treatment	Wet Bulk Density (g cm^{-3})	Particle Density (g cm^{-3})	Porosity (%)	pH	Moisture Content (%)	Ash Content (%)	Volatile Solids Content (%)
Control	0.15 ^{a}	1.94 ^{a}	91.97 ^{a,b}	6.37 ^{g}	64.58 ^{a,b}	12.96 ^{a}	87.04 ^{a}
T1	0.10 ^{b,c,d}	0.08 ^{b}	87.11 ^{a,b,c}	7.66 ^{a}	58.16 ^{b}	13.94 ^{a}	86.06 ^{a}
T2	0.13 ^{a}	1.32a ^{b}	85.17 ^{a,b,c}	6.99 ^{c,d}	69.80 ^{a,b}	14.89 ^{a}	85.11 ^{a}
T3	0.12 ^{a,b,c}	0.76 ^{b}	83.83 ^{b,c}	6.43 ^{f,g}	63.64 ^{b}	10.55 ^{a}	89.45 ^{a}
T4	0.14 ^{a}	1.05 ^{a,b}	86.24 ^{a,b,c}	6.02 ^{h}	66.23 ^{a,b}	13.12 ^{a}	86.88 ^{a}
T5	0.13a ^{b}	0.96 ^{a,b}	86.5 ^{a,b,c}	5.95 ^{h}	65.33 ^{a,b}	14.44 ^{a}	85.56 ^{a}
T6	0.15 ^{a}	0.82 ^{b}	80.89 ^{c}	6.06 ^{h}	69.69 ^{a,b}	13.52 ^{a}	86.48 ^{a}
T7	0.14 ^{a}	0.83 ^{b}	82.89 ^{c}	6.84 ^{d,e}	76.70 ^{a}	14.19 ^{a}	85.81 ^{a}
T8	0.06 ^{e}	0.87 ^{b}	93.57 ^{a}	7.34 ^{b}	69.91 ^{a,b}	11.92 ^{a}	88.08 ^{a}
T9	0.07 ^{d,e}	0.87 ^{b}	91.5 ^{a,b}	6.65 ^{e,f}	42.98 ^{c}	14.51 ^{a}	85.49 ^{a}
T10	0.09 ^{c,d}	0.73 ^{b}	87.03 ^{a,b,c}	7.23 ^{b,c}	54.52 ^{a,b}	12.40 ^{a}	87.60 ^{a}

Means followed by different letters within column are significantly different at $p \leq 0.05$ by Tukey's Honest Significance Test. Note: control = 100% rubber wood sawdust (RWS), T1 = 100% empty fruit bunch (EFB), T2 = 100% oil palm frond (OPF), T3 = 100% oil palm trunk (OPT), T4 = 50% EFB + 50% OPF, T5 = 50% EFB + 50% OPT, T6 = 50% OPF + 50% OPT, T7 = 33.3% EFB + 33.3% OPF +33.3% OPT, T8 = 66.6% EFB + 16.7% OPF + 16.7% OPT, T9 = 16.7% EFB + 66.6% OPF + 16.7% OPT, T10 = 16.7% EFB + 16.7% OPF + 66.6% OPT.

The highest particle density of substrate was observed for the control (1.94 g cm^{-3}) and the lowest particle density of substrate was observed for T1 (0.08 g cm^{-3}). The porosity of the substrate is related to the wet bulk and particle densities of the substrate [15]. The highest porosity of substrate was observed for T8 (93.57%) and the lowest porosity of substrate was observed for T6 (80.89%). In the current study, it was observed that T6 had the highest wet bulk density and the lowest porosity. This aligned with the statement reported by Mihilall et al. [17], where the wet bulk density of the substrate influences the porosity of the substrate. By increasing the moisture content of the substrate, the porosity of the substrate reduces [32].

The pH and moisture content of the substrates showed significant differences (Table 2). The pH value of all substrates ranged between 5.95–7.66. The pH of the substrate influenced the growth and development of the mushrooms [33]. Yadav [34] stated that a pH value of 5.0–8.0 is suitable for the cultivation of *P. ostreatus*. Therefore, the pH of substrates used in this study was in the appropriate range for the growth of *P. ostreatus*. Oyster mushrooms

require 50–75% of moisture content in the substrate to grow [32]. The moisture content of most substrates in the current study ranged between 54.52–76.70%, except for T9 (16.7% EFB + 66.6% OPF + 16.7% OPT), which only held a 42.98% moisture content.

No significant difference was observed for the ash and volatile solids contents of the substrates (Table 2). Ash content measures the inorganic material in a substrate; it is mainly composed of minerals. On the other hand, volatile solids content measures the carbon materials in the substrate, which quantifies the organic constituents that are converted into carbon dioxide [35]. In the current study, the ash content of the substrates ranged between 10.55–14.89%, while the volatile solids content of the substrates ranged between 85.11–89.45%. This is comparable with a study conducted by Sulaiman et al. [36], which reported that unwashed OPF and OPT contained 8.01% and 10.93% ash content, as well as 81.03% and 67.57% volatile solids content, respectively.

Lignin and hemicellulose contents of substrates showed significant differences among treatments, whilst the cellulose content (42.39–58.29%) showed no significant difference (Table 3). However, the cellulose content of the substrate in this study was higher than those reported by Kaniapan et al. [37], whilst the lignin and hemicellulose contents of EFB, OPF, and OPT in this study were lower than those reported by Kaniapan et al. [37]. Empty fruit bunches in this study were reported to have lower lignin and higher cellulose contents as compared to Kaniapan et al.'s results [37]. Empty fruit bunches are made up of spikelets and stalks. According to Md Yunos et al. [38], an EFB stalk has lower lignin and higher cellulose contents. The EFBs used in this study had more stalks, hence resulting in lower lignin and higher cellulose contents. The lignin content observed in the control (100% RWS) was similar to Hassan's and Abd-Aziz's result [39], who reported that the RWS substrate had the highest lignin content (29.3%).

Table 3. Fibre fractions of substrates before cultivation.

Treatment	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Control	24.63 ± 9.11 ^{a}	15.46 ± 2.45 ^{a,b}	53.42 ± 3.12 ^{a}
T1	8.87 ± 1.78 ^{b}	16.91 ± 1.57 ^{a,b}	50.73 ± 1.31 ^{a}
T2	8.10 ± 0.94 ^{b}	13.28 ± 3.2 ^{b}	43.06 ± 9.83 ^{a}
T3	9.68 ± 0.07 ^{b}	18.35 ± 2.26 ^{a,b}	50.63 ± 5.34 ^{a}
T4	7.33 ± 2.41 ^{b}	17.10 ± 5.23 ^{a,b}	52.92 ± 8.08 ^{a}
T5	8.93 ± 2.72 ^{b}	26.44 ± 2.89 ^{a}	47.84 ± 3.04 ^{a}
T6	15.31 ± 3.00 ^{a,b}	23.59 ± 4.93 ^{a,b}	45.74 ± 2.52 ^{a}
T7	5.07 ± 0.25 ^{b}	15.83 ± 7.4 ^{a,b}	58.29 ± 9.00 ^{a}
T8	6.99 ± 2.0 ^{b}	18.93 ± 0.44 ^{a,b}	53.38 ± 1.98 ^{a}
T9	12.59 ± 3.02 ^{b}	15.86 ± 1.47 ^{a,b}	48.49 ± 5.61 ^{a}
T10	12.57 ± 4.56 ^{b}	17.68 ± 7.48 ^{a,b}	42.39 ± 3.72 ^{a}

Means ± standard deviation followed by different superscripted letters within column are significantly different at $p \leq 0.05$ by Tukey's Honest Significance Test. Note: control = 100% rubber wood sawdust (RWS), T1 = 100% empty fruit bunch (EFB), T2 = 100% oil palm frond (OPF), T3 = 100% oil palm trunk (OPT), T4 = 50% EFB + 50% OPF, T5 = 50% EFB + 50% OPT, T6 = 50% OPF + 50% OPT, T7 = 33.3% EFB + 33.3% OPF +33.3% OPT, T8 = 66.6% EFB + 16.7% OPF + 16.7% OPT, T9 = 16.7% EFB + 66.6% OPF + 16.7% OPT, T10 = 16.7% EFB + 16.7% OPF + 66.6% OPT.

Total C and N contents and the C/N ratio showed significant differences (Table 4). The highest total C content of the substrate was determined for the control (44.8%), and the lowest total C content of the substrate was observed for T3 (38.27%). The highest total N content of the substrate was achieved by T2 and T6 (5.98%), and the lowest total N content of substrate was observed for T4 (5.57%). The highest C/N ratio of substrate was observed for T8 (8.00), and the lowest C/N ratio of substrate was observed for T3 (6.49). The N content of all substrates was higher compared to those reported by Ali et al. [40] and Cueve et al. [41], who stated that the total N content of substrates formulated with various agricultural wastes, such as oil palm frond, corn stubble, barley straw, lentil stubble, wheat straws, sugarcane bagasse, and soybean meal, did not exceed 1.5%. The C/N ratio of the substrate formulated with oil palm by-products was observed to be much lower compared

to those formulated with other agronomic wastes, such as viticulture waste, paddy straw, sesame straw, date palm fibre, and wheat straw [19,42].

Table 4. C, N, C/N, and mineral concentration of substrates.

Treatment	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Fe (mg kg^{-1})	Zn (mg kg^{-1})	C (%)	C/N Ratio
Control	5.73 ^{a,b,c}	27.62 ^{e}	33.38 ^{d}	61.79 ^{d}	13.61 ^{f}	1.83 ^{c}	0.33 ^{ab}	44.80 ^{a}	7.83 ^{a,b,c}
T1	5.87 ^{a,b}	38.32 ^{b,c,d,e}	222.37 ^{b}	74.45 ^{d}	25.68 ^{d,e}	6.64 ^{c}	0.51 ^{a}	44.16 ^{c,d}	7.54 ^{c,d,e}
T2	5.98 ^{a}	77.70 ^{a}	206.61 ^{b}	235.34 ^{a}	56.94 ^{a}	5.81 ^{c}	0.34 ^{a,b}	43.02 ^{g}	7.19 ^{f}
T3	5.90 ^{a,b}	46.11 ^{b,c,d}	200.14 ^{b}	189.27 ^{a,b}	29.17 ^{c,d}	33.44 ^{a}	0.48 ^{a,b}	38.27 ^{h}	6.49 ^{g}
T4	5.57 ^{c}	40.14 ^{b,c,d,e}	195.36 ^{b,c}	88.05 ^{d}	27.81 ^{c,d,e}	5.62 ^{c}	0.38 ^{a,b}	44.34 ^{b,c}	7.97 ^{a,b}
T5	5.77 ^{a,b,c}	59.69 ^{a,b}	306.43 ^{a}	142.77 ^{b,c}	38.26 ^{b,c}	18.31 ^{b}	0.46 ^{a,b}	43.62 ^{f}	7.56 ^{c,d,e}
T6	5.98 ^{a}	44.33 ^{b,c,d,e}	179.10 ^{b,c}	141.21 ^{b,c}	37.09 ^{b,c,d}	4.76 ^{c}	0.24 ^{a,b}	43.84 ^{d,e,f}	7.33 ^{d,e,f}
T7	5.76 ^{a,b,c}	51.04 ^{b,c}	227.05 ^{b}	145.30 ^{b,c}	36.07 ^{b,c,d}	5.99 ^{c}	0.39 ^{a,b}	44.02 ^{c,d,e}	7.64 ^{b,c,d}
T8	5.58 ^{c}	37.32 ^{c,d,e}	228.54 ^{b}	100.86 ^{c,d}	26.81 ^{c,d,e}	4.70 ^{c}	0.30 ^{a,b}	44.66 ^{a,b}	8.00 ^{a}
T9	5.95 ^{a}	49.91 ^{b,c}	204.85 ^{b}	212.42 ^{a}	47.11 ^{a,b}	3.20 ^{c}	0.23 ^{b}	43.00 ^{g}	7.23 ^{e,f}
T10	5.66 ^{b,c}	22.90 ^{e}	120.71 ^{c}	65.89 ^{d}	16.66 ^{e,f}	5.57 ^{c}	0.22 ^{b}	43.59 ^{f}	7.70 ^{a,b,c}

Means followed by different superscripted letters within column are significantly different at $p \leq 0.05$ by Tukey's Honest Significance Test. Note: control = 100% rubber wood sawdust (RWS), T1 = 100% empty fruit bunch (EFB), T2 = 100% oil palm frond (OPF), T3 = 100% oil palm trunk (OPT), T4 = 50% EFB + 50% OPF, T5 = 50% EFB + 50% OPT, T6 = 50% OPF + 50% OPT, T7 = 33.3% EFB + 33.3% OPF +33.3% OPT, T8 = 66.6% EFB + 16.7% OPF + 16.7% OPT, T9 = 16.7% EFB + 66.6% OPF + 16.7% OPT, T10 = 16.7% EFB + 16.7% OPF + 66.6% OPT.

K, P, Ca, Mg, Fe, and Zn concetrations of the substrates presented significant differences (Table 4). The substrate with the highest P level was T2 (77.7 mg kg^{-1}), and the lowest P level was presented by T10 (22.9 mg kg^{-1}). The substrate with the highest K level was T5 (306.43 mg kg^{-1}), and the lowest K level was observed for the control (33.38 mg kg^{-1}). The highest Ca level of substrate was observed for T2 (235.34 mg kg^{-1}) and T9 (212.42 mg kg^{-1}), while the lowest was for the control (61.79 mg kg^{-1}). The substrate with the highest Mg level was observed for T2 (56.94 mg kg^{-1}), and the lowest Mg level was observed for the control (13.61 mg kg^{-1}). The substrate with the highest Fe level was observed for T3 (33.44 mg kg^{-1}), and the lowest Fe level was observed for the control (1.83 mg kg^{-1}). The highest Zn level of substrate was for T1 (0.51 mg kg^{-1}), and the lowest Zn level was for T3 (6.49 mg kg^{-1}). Concentrations of N, P, K, Mg, and Ca for EFB, OPF, and OPT in the current study were lower compared to the previous studies conducted by Tas et al. [43] and Khalid et al. [44]. Palm age, nutrient element, and tissue type could influence the concentration of the nutrients [43]. According to Tas et al. [43], most nutrients from oil palm trunk and oil palm frond (petiole and leaflets) tend to appear at a higher concentration in immature palms, aged between 29–48 months.

3.2. Growth and Yield of Mushroom

Figure 1 presents the general growth stages of *Pleurotus ostreatus*. It started from the mycelia colonisation of the substrate, initiation of primordia, development of primordia, and development of fruiting bodies. All treatments successfully underwent these stages, except for T3 (100% OPT) and T6 (50% OPF + 50% OPT). T3 and T6 substrates were mainly formulated with OPT. The failure of mycelia growth and fruiting body development might have been caused by the presence of a serine/threonine protein kinase known as glycogen synthase kinase-3 (GSK-3) inhibitor in OPT. The GSK-3 inhibitor may directly or indirectly influence the growth of mushrooms as it plays an important role in cell differentiation. It was reported that the presence of lithium, which is a GSK-3 inhibitor, at 3 g L^{-1} LiCl and 2 g L^{-1} LiCl on PDA inhibited the mycelia growth of *C. cinerea* and fruiting body development of *P. djamor* [45]. Receptor-like serine/threonine protein kinase was observed in the core of OPT [46], which could have possibly disrupted the cell differentiation process of mycelia, and eventually inactivated fruiting body development in T3 and T6.

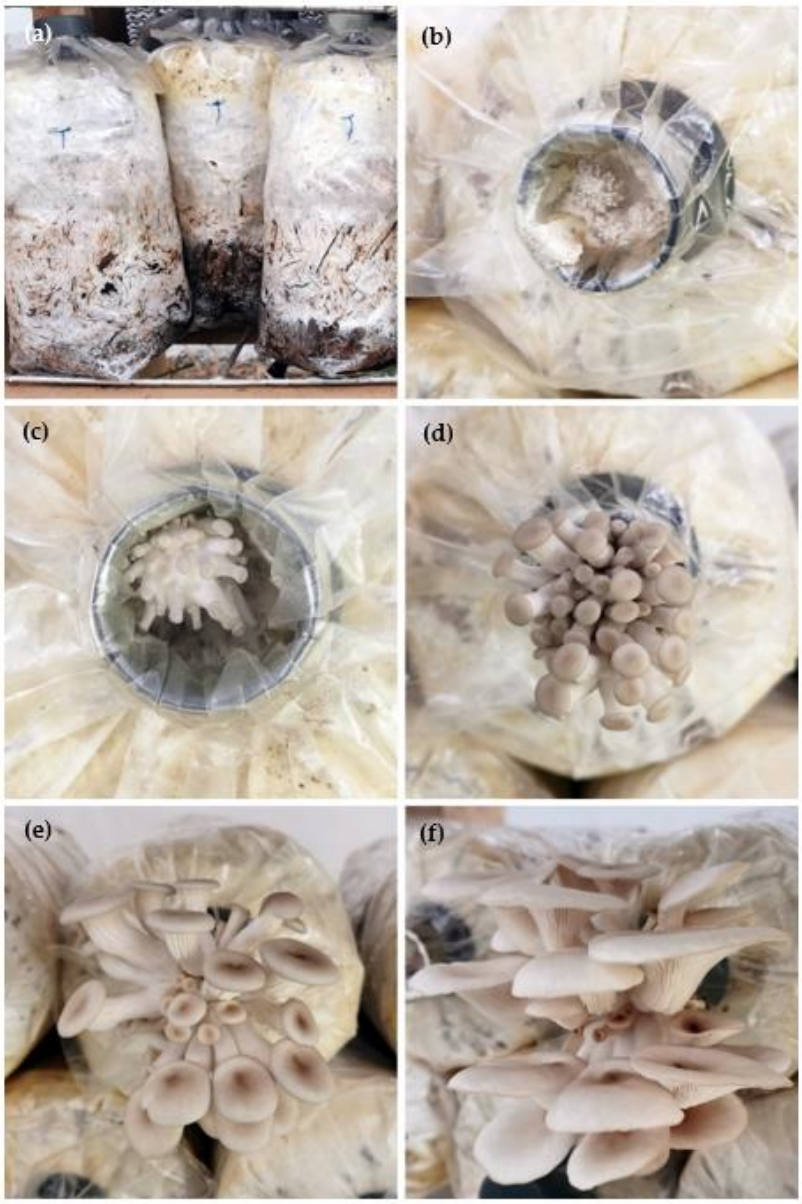

Figure 1. Growth stages of *Pleurotus ostreatus*. (**a**) Mycelia colonisation of substrate, (**b**) initiation of primordia, (**c**) early development of primordia, (**d**) later development of primordia, (**e**) early development of fruiting bodies, and (**f**) fruiting bodies ready to be harvested.

Tables 5 and 6 show the growth and yield for treatments that successfully produced fruiting bodies. Days taken for mycelia to fully colonise substrate, days taken for primordia initiation from fully colonise substrate, and days taken to complete first flush growth resulted in significant differences. T4 and T9 took 24 days for mycelia to fully colonise the substrate, which was the fastest among treatments. However, the same treatments (T4 and T9) took the longest time for primordia initiation, which were 14.17 and 20.83 days, respectively. Overall, T1 took the shortest time to complete first flush growth (40.25 days), while the control and T10 took the longest time to complete the first flush growth (77 days). It was 1.9 times slower than T1. Ali et al. [47] reported that *Pleurotus ostreatus* grown on rubber wood sawdust took 55 days to complete the first flush growth; this was 22 days faster compared to the current study.

Table 5. Growth of *Pleurotus ostreatus*.

Treatment	Days Taken for Mycelia to Fully Colonise Substrate	Days Taken for Primordia Initiation from Fully Colonised Substrate	Days Taken to Complete First Flush Growth	Stipe Length (mm)	Pileus Diameter (mm)
Control	37.00 ^{b}	36.33 ^{a,b}	77.00 ^{a}	44.21 ^{b,c}	94.41 ^{a}
T1	27.13 ^{c}	9.13 ^{c}	40.25 ^{c}	35.35 ^{c}	62.75 ^{a}
T2	25.00 ^{c}	46.00 ^{a}	75.67 ^{a}	61.44 ^{a}	73.62 ^{a}
T4	24.00 ^{c}	14.17 ^{c}	43.67 ^{b,c}	38.21 ^{b,c}	84.69 ^{a}
T5	48.67 ^{a}	7.00 ^{c}	56.67 ^{a,b,c}	34.44 ^{c}	78.87 ^{a}
T7	30.67 ^{b,c}	34.00 ^{a,b}	61.67 ^{a,b}	53.9 ^{a,b}	76.05 ^{a}
T8	26.25 ^{c}	14.75 ^{c}	45.00 ^{b,c}	36.95 ^{c}	59.30 ^{a}
T9	24.00 ^{c}	20.83 ^{b,c}	53.67 ^{b,c}	32.24 ^{c}	59.22 ^{a}
T10	31.33 ^{b,c}	41.00 ^{a}	77.00 ^{a}	44.88 ^{b,c}	73.03 ^{a}

Means followed by different letters within column are significantly different at $p \leq 0.05$ by Tukey's Honest Significance Test. Note: control = 100% rubber wood sawdust (RWS), T1 = 100% empty fruit bunch (EFB), T2 = 100% oil palm frond (OPF), T4 = 50% EFB + 50% OPF, T5 = 50% EFB + 50% OPT, T7 = 33.3% EFB + 33.3% OPF +33.3% OPT, T8 = 66.6% EFB + 16.7% OPF + 16.7% OPT, T9 = 16.7% EFB + 66.6% OPF + 16.7% OPT, T10 = 16.7% EFB + 16.7% OPF + 66.6% OPT.

Table 6. Yield of *Pleurotus ostreatus*.

Treatment	Fresh Weight of Fruiting Bodies (g)	Number of Total Fruiting Bodies	Number of Effective Fruiting Bodies	Biological Efficiency (%)
Control	59.64 ^{a}	2.33 ^{a}	2.00 ^{a}	41.74 ^{a}
T1	44.78 ^{a,b}	9.50 ^{a}	5.00 ^{a}	47.96 ^{a}
T2	34.55 ^{a,b}	6.00 ^{a}	3.33 ^{a}	37.80 ^{a}
T4	24.9 ^{b}	3.33 ^{a}	1.67 ^{a}	48.97 ^{a}
T5	32.77 ^{a,b}	6.00 ^{a}	4.50 ^{a}	35.07 ^{a}
T7	40.47 ^{a,b}	5.33 ^{a}	3.33 ^{a}	43.92 ^{a}
T8	40.75 ^{a,b}	5.00 ^{a}	2.75 ^{a}	47.81 ^{a}
T9	24.81 ^{b}	5.83 ^{a}	3.67 ^{a}	34.88 ^{a}
T10	21.72 ^{b}	2.33 ^{a}	1.67 ^{a}	18.36 ^{a}

Means followed by different letters within column are significantly different at $p \leq 0.05$ by Tukey's Honest Significance Test. Note: control = 100% rubber wood sawdust (RWS), T1 = 100% empty fruit bunch (EFB), T2 = 100% oil palm frond (OPF), T4 = 50% EFB + 50% OPF, T5 = 50% EFB + 50% OPT, T7 = 33.3% EFB + 33.3% OPF +33.3% OPT, T8 = 66.6% EFB + 16.7% OPF + 16.7% OPT, T9 = 16.7% EFB + 66.6% OPF + 16.7% OPT, T10 = 16.7% EFB + 16.7% OPF + 66.6% OPT.

The low lignin content in EFB could have contributed to the rapid growth rate of *P. ostreatus*. Lignin acts as a barrier and needs to be broken down so that *P. ostreatus* has access to carbon and energy sources in the forms of hemicellulose and cellulose to support their growth [48]. The results of Alsanad et al. [49] reported that substrate formulated with a high lignin content resulted in the slowest growth of *P. ostreatus*. T3, T4, and T7 substrates were also formulated with low lignin contents. However, the possible presence of GSK-3 inhibitor in OPT might have slowed down the growth rate of the mycelia and fruiting bodies.

Apart from that, the water holding capacity of EFB also could contribute to the growth of *P. ostreatus*. EFB could maintain adequate moisture in the substrate to ease nutrient transport. This study found that 58.16% is the optimal moisture content of substrate for the growth of *P. ostreatus*. When too much moisture is trapped in a substrate, it suffocates *P. ostreatus* as gas exchange, particularly oxygen transfer, is reduced, which could lead to the growth inhibition of mycelia and the development of fruiting bodies [32]. In addition, there was less compaction in the EFB substrate due to its larger particle size compared to RWS. Thus, the mycelia network could easily spread throughout the substrate and assist in nutrient uptake [17].

As for the size of the fruiting bodies, the shortest stipe length was for T9 (32.24 mm) and the longest stipe length was for T2 (61.44 mm), and there was no significant difference in pileus diameter. For the yield performance, the highest fresh weight was observed for the control (59.64 g) followed by T1 (44.78 g), and the lowest fresh weight was observed for T10 (21.72 g). No significant difference was observed for the number of total fruiting bodies, number of effective fruiting bodies, and biological efficiency.

3.3. Nutritional Properties of Mushrooms

3.3.1. Proximate Composition of Fruiting Bodies

The moisture and crude lipid contents of the fruiting bodies were not significantly affected by substrate formulation. Table 7 presents the ash content, crude protein, and crude fibre of fruiting bodies. The highest ash content was observed for the control (4.60%), and the lowest ash content was observed for T10 (0.37%). Substrates formulated with oil palm by-products resulted in significantly higher crude protein content, as compared to the control, by more than three-fold. This is particularly helpful in the development of protein-enriched vegetarian foods as vegetarians face challenges to fulfil their nutritional needs, and plant-based foods generally have lower protein quality as compared with animal-based foods [43]. In general, the protein content of *Pleurotus* ranges from 17–42% [50]. The high crude protein found in this study could have been influenced by the drying method used on the harvested fruiting bodies. Different drying temperatures and methods, such as drying, solar drying, convection drying, and low-heat air blow, affected the nutritional properties of *Pleurotus* mushrooms [51,52]. Convection drying is the most commonly used drying method in the food industry [51]. However, freeze-drying was used in this study. Thus, it could have contributed to the highly preserved crude protein content in *Pleurotus ostreatus*. The highest crude fibre content (54.27%) was observed in fruiting bodies grown on T10.

Table 7. Proximate composition of *Pleurotus ostreatus*.

Treatment	Moisture Content (%)	Ash Content (%)	Crude Protein (%)	Crude Fibre (%)	Crude Lipid (%)
Control	80.00 ^{a}	4.60 ^{a}	11.05 ^{f}	46.02 ^{a,b}	3.53 ^{a}
T1	80.98 ^{a}	1.99 ^{b,c}	44.49 ^{d,e}	42.30 ^{b,c}	3.27 ^{a}
T2	82.84 ^{a}	2.69 ^{b}	52.09 ^{a}	31.58 ^{c,d,e}	3.19 ^{a}
T4	78.20 ^{a}	0.83 ^{d,e}	45.94 ^{c,d}	24.86 ^{e}	4.03 ^{a}
T5	80.77 ^{a}	1.50 ^{c,d}	43.33 ^{e}	29.16 ^{d,e}	3.83 ^{a}
T7	82.21 ^{a}	2.01 ^{b,c}	49.02 ^{b}	39.02 ^{b,c,d}	7.00 ^{a}
T8	72.47 ^{a}	0.63 ^{d,e}	44.67 ^{d,e}	25.96 ^{e}	10.41 ^{a}
T9	77.41 ^{a}	0.55 ^{d,e}	45.25 ^{d,e}	30.31 ^{d,e}	9.17 ^{a}
T10	78.73 ^{a}	0.37 ^{e}	47.73 ^{b,c}	54.27 ^{a}	1.51 ^{a}

Means followed with different letters within column are significantly different at $p \leq 0.05$ by Duncan's Multiple Range Test. Note: All samples are analysed based on a dry basis. Control = 100% rubber wood sawdust (RWS), T1 = 100% empty fruit bunch (EFB), T2 = 100% oil palm frond (OPF), T4 = 50% EFB + 50% OPF, T5 = 50% EFB + 50% OPT, T7 = 33.3% EFB + 33.3% OPF +33.3% OPT, T8 = 66.6% EFB + 16.7% OPF + 16.7% OPT, T9 = 16.7% EFB + 66.6% OPF + 16.7% OPT, T10 = 16.7% EFB + 16.7% OPF + 66.6% OPT.

3.3.2. Beta Glucan Content of Fruiting Bodies

Substrates formulated with oil palm by-products resulted in a significantly higher beta glucan content in fruiting bodies as compared to the control (100% RWS). The highest beta glucan content was observed in T8 (51.54%) and T1 (49.44%), while the lowest beta glucan content was observed in the control (30.87%) (Table 8). The results are higher than those of the previous studies conducted by Sari et al. [30], which reported that the beta glucan content of oyster mushrooms, namely, *P. ostreatus*, *P. eryngii*, *P. citrinopileatus*, *P. pulmonarius*, and *P. djamor*, ranged between 15.3–24.2%. Beta glucans are polysaccharides, a major component that makes up fungal cell walls [40]. For beta glucan isolated from the fruiting bodies of *Pleurotus ostreatus*, they are better known as pleuran. It has been used as a

dietary supplement to support the immune system and helps to overcome fatigue [53]. Beta glucans are reported to have antitumour and antiinfective activities; they are considered potent immunomodulators [54].

Table 8. Beta glucan content of *Pleurotus ostreatus*.

Treatment	* Beta Glucan Content (%)
Control	30.87 ^{d}
T1	49.44 ^{a}
T2	37.65 ^{c,d}
T4	46.13 ^{a,b}
T5	46.93 ^{a,b}
T7	38.57 ^{b,c,d}
T8	51.54 ^{a}
T9	46.19 ^{a,b}
T10	40.95 ^{b,c}

Means followed with different letters within column are significantly different at $p \leq 0.05$ by Tukey's Honest Significance Test. * Beta glucan content is determined based on water extract of the dried sample. Note: control = 100% rubber wood sawdust (RWS), T1 = 100% empty fruit bunch (EFB), T2 = 100% oil palm frond (OPF), T4 = 50% EFB + 50% OPF, T5 = 50% EFB + 50% OPT, T7 = 33.3% EFB + 33.3% OPF +33.3% OPT, T8 = 66.6% EFB + 16.7% OPF + 16.7% OPT, T9 = 16.7% EFB + 66.6% OPF + 16.7% OPT, T10 = 16.7% EFB + 16.7% OPF + 66.6% OPT.

3.3.3. Correlation between Physicochemical Properties of Substrate and Agronomic Performance of *Pleurotus ostreatus*

Supplementary Table S1 shows the correlation between the physicochemical properties of the cultivation substrate and the agronomic performance of the *P. ostreatus* mushroom. It was observed that faster colonisation of mycelia on the cultivation substrate was directly proportional to the total Fe (r = 0.731) and hemicellulose (r = 0.761) in the substrate. This aligns with the results of the study by Budzynska et al. [55], where the mycelium of *Pholiota nameko* grew slower with the addition of iron to the media. Meanwhile, hemicellulose releases xylose, glucuronic acid, and galacturonic acid where these sugars are converted into carbon sources, which is beneficial for *P. ostreatus* cultivation [56]. Moreover, the wet bulk (r = 0.844) and particle (r = 0.710) densities of the substrate were positively correlated with the size of the mushroom (pileus diameter). Higher wet bulk and particle densities created less compaction on the substrate, thus increasing the nutrient uptake by the mycelia and fruiting bodies, eventually resulting in a larger pileus size. Inversely, the substrate pH was negatively correlated to the pileus diameter (r = −0.749). *Pleurotus ostreatus* preferred a slightly basic substrate, as reported by Khan et al. [57], where a pH of 7.8 produced the most number of fruiting bodies; however, there was a decline in the number of fruiting bodies as the pH level of the substrate increased to 8.2 and 8.7. Additionally, the potassium concentration and cellulose in the substrate were positively correlated to the number of effective fruiting bodies (r = 0.675) and biological efficiency (r = 0.741), respectively. Potassium has a role in synthesising amino acids and proteins; this provides support for mushroom growth [58], thus resulting in more effective fruiting bodies being developed. Xie et al. [59] reported that there was a positive relationship between the biological efficiency of *P. eryngii* and lignocellulosic degradation.

3.3.4. Correlation between Physicochemical Properties of Substrate and Nutritional Properties of *Pleurotus ostreatus*

Supplementary Table S2 shows the correlation between the physicochemical properties of the cultivation substrate and nutritive properties of *P. ostreatus* mushroom crude protein content of fruiting bodies were higher when grown on a substrate with less lignin content (r = −0.901) and beta glucan (r = 0.701). Lignin forms a barrier, blocking the access between mycelia and its nutrient source in hemicellulose and cellulose, resulting in the lower crude protein content of fruiting bodies. In addition, it was observed that a higher concentration of potassium in the substrate produced fruiting bodies with a higher crude protein content

(r = 0.706), possibly due to the role of potassium in the synthesis of amino acids and protein [60].

4. Conclusions

It is important to consider the agronomic performance and nutritive composition of mushrooms when formulating an alternative substrate. This is to ensure that the nutrients of the mushroom grown are not compromised. This study concluded that substrate formulation T1 (100% EFB) has the potential to be used for the commercial production of *P. ostreatus*. Compared to the conventional substrate (100% RWS), T1 (100% EFB) showed the fastest growing rate of *P. ostreatus*. The growth cycle of *P. ostreatus* grown on 100% EFB was significantly shorter by 1.9 times due to the physicochemical properties of the cultivation substrate, including the pH, fibre composition, and concentration of K. The crude protein and beta glucan contents of *P. ostreatus* grown on 100% EFB were significantly higher than those grown on 100% RWS. In addition, it was observed that the physicochemical properties of the substrate affected the agronomic performance and nutritional properties of a mushroom based on the Pearson's correlation analysis; the potassium concentration in the substrate was especially beneficial for the development of fruiting bodies and the enhancement of protein and beta glucan contents in mushrooms.

The results obtained in this study suggest more oil palm plantations to be involved in the implementation of the waste-to-wealth concept by reducing the burden of oil palm industries in managing their by-products, especially EFB, and turning it into a value-added product through oyster mushroom cultivation. In addition, this study provided information on the nutritional composition of oyster mushrooms grown solely on empty fruit bunch biomass, which is not only capable of improvng the protein quality, but is potentially utilised in the development of vegetarian foods.

Supplementary Materials: The following supporting information can be downloaded at: https://doi.org/10.5281/zenodo.7106544, Table S1: Pearson's correlation coefficient (r) between physicochemical properties of cultivation substrate and agronomic performance of *Pleurotus ostreatus* mushroom; Table S2: Pearson's correlation coefficient (r) between physicochemical properties of cultivation substrate and nutritional properties of *Pleurotus ostreatus* mushroom.

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