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Effects of Rubber Sawdust and Paper waste as Substrates for Cultivating American Oyster Mushrooms (*Pleurotus ostreatus*): Their Influence on Nutrient Composition, Bioactive Compound Levels and Antioxidant Capacity

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Abstract

Pleurotus ostreatus (American oyster) species are a type of extensively cultivated mushrooms worldwide and they can be grown on a variety of lignocellulosic substrates. As an agriculture-based country, substrates like sawdust and paddy straw are commonly used to cultivate *Pleurotus ostreatus* in Sri Lanka. However, there are many other potential waste products that can be used as substrates. Thus, the main objective of this study was to analyze the use of Rubber Sawdust (SD) and Paper Waste (PW) to grow *Pleurotus ostreatus* and to determine their influence on nutrient composition, bioactive compound levels and antioxidant capacity. Mushrooms were grown in 5 different substrate combinations (100% SD, 100% PW, 50:50 SD and PW, 75% SD and 25% PW, 75% PW and 25% SD) and the water holding capacity of each substrate was determined. Aqueous extracts were prepared from the matured fruiting bodies and the total protein and total carbohydrate concentrations were evaluated using Lowry and phenol-sulfuric assays respectively. Bioactive compounds were analyzed using qualitative tests and the total phenolic content was measured. Antioxidant capacity was determined using total antioxidant assay and DPPH assay. The use of 100% SD substrate was effective in increasing the mycelial growth, yield, and antioxidant activity. Highest cap diameter, stipe thickness and protein content were observed in 75% SD, 25% PW combination. 50:50 SD, PW displayed the highest carbohydrate and phenolic contents. Qualitative results indicated that all mushrooms contained saponins, polyphenols, terpenoids and steroids. Overall, it can be concluded that 100% SD is a better substrate in terms of incubation and harvesting period and mushroom yield. Furthermore, in terms of nutrient composition and antioxidant capacity, 100% SD and SD combined with paper waste (50:50, 75:25) can be used effectively.

Keywords: *Pleurotus ostreatus*, Sawdust, Paper waste, Nutritional composition, Antioxidant activity

1. Introduction

Mushrooms are succulent, spore-bearing fruiting structures belonging to the fungus kingdom. They are saprophytic multicellular organisms that conventionally thrive above ground or on their redundant food source. There are numerous species of mushrooms, few of which are edible while some fall under the poisonous category. Examples of edible mushrooms include *Cantharellus cibarius*, *Agaricus bisporus* and *Pleurotus ostreatus*.[1]

Pleurotus species (oyster mushrooms) which belong to the class Basidiomycetes, family Pleurotaceae and the order Agaricales, feature a distinctive shell or oyster-shaped basidiocarp that develops in a variety of colors.[2,3]

They comprise over 40 species, all of which naturally grow in a wide range of temperatures.[4] Few of these include *Pleurotus purpurea-olivaceus*, *Pleurotus giganteus* and *Pleurotus ostreatus*.[5,6]

Figure 1. *Pleurotus species: A) Pleurotus purpurea- olivaceus. B) Pleurotus djamor C) Pleurotus ostreatus D) Pleurotus citrinopileatus E) Pleurotus giganteus.*[7]

Pleurotus species are among the most extensively cultivated edible mushrooms worldwide, particularly in parts of Asia, North America, and Europe.[1,8] This is because these species of mushrooms grow at a faster rate when compared to other edible mushrooms. In addition to this, it grows on substrates that does not need to be pasteurized thereby reducing the overall cost for its cultivation.[9,10] The production of oyster mushrooms is thought to be a highly lucrative industry because a substantial fraction of the substrates used are transformed into fruiting bodies.[11] Furthermore, these species require few environmental conditions, and their fruiting bodies are less vulnerable to disease and pest attacks. Nevertheless, *Pleurotus* species is hence considered the ideal mushrooms to be cultivated when compared to the others.[4]

Generally, all mushroom species receive their aliment by decomposing their surroundings through the production of extracellular enzymes. They are frequently observed colonizing moist wood trunks of trees and decaying organic detritus abundant in lignin and phenol degrading enzymes. As a result, farmers have been employing agricultural wastes as substrates for the cultivation of mushrooms.[6] Examples of these agro-wastes include paddy straw, wheat straw, cereal straw, sawdust, and banana leaves. This innovative approach has not only benefitted farmers but has also had a positive impact on the environment itself by reducing the quantity of agro-wastes produced globally and by narrowing the nutritional gap that exists among the populations of China, India, and Africa.[1] These remaining substrates are occasionally repurposed as fertilizers, animal feeds and in the manufacture of biogas.[12]

Pleurotus ostreatus species grow on a wide range of substrates. Some of these include wheat straw, banana leaves, sawdust, paper waste and cassava leaves.[8] Mushrooms require a high carbon source for their growth, and because paper waste and sawdust are high in carbon, they could be suitable substrates for harvesting them.[13,14] According to Tesfay *et al*., 2020, the combination of other substrates with paper waste appears to be a promising alternative to produce oyster mushrooms.[15]

In addition to having numerous positive effects on the environment and the economy, mushrooms have great nutritional value, and their cultivation is highly significant in the realm of medicine.[16] *Pleurotus* species have a high protein content which includes both essential and non-essential amino acids.[17] They are also considered as a great substitute for meat, fish, and vegetables due to their rich mineral content.[17] Additionally, they are rich in dietary fiber and vitamin C and B complexes.[18,19]

With regards to therapeutic benefits, mushrooms are highly recommended for diabetics and have been quite effective in the treatment of malignancies.[16] They are also known to contain many bioactive compounds. Bioactive compounds refer to the nutrients and non-nutrients available in the food matrix that exhibit physiological effects beyond their classical nutritional properties.[20] *Pleurotus* species contain a variety of bioactive compounds including terpenoids, phenols, steroids, and tannins.[21] Free radicals are highly reactive molecules that are

either formed in the body through normal metabolic processes or enter the body from the environment, such as pollution and other pollutants. The accumulation of these free radicals causes body harm. Antioxidants are nutrients that help the body protect itself from this damage.[22,23] The bioactive compounds found in Pleurotus species have been found to contain anticancer, antigenotoxic, antioxidant, antihypertensive, antiplatelet aggregating, antihyperglycemic, antibacterial, and antiviral properties.[6, 24, 25, 26]

Although mushroom cultivation has been increasing drastically in many parts of the world, its production has not reached to a very larger scale in Sri Lanka. This it principally due to challenges in farming and management, farmers lacking administrative and entrepreneurial abilities and pest and disease problems in mushrooms, which lead to severe losses in both its yield and profit.[27,28,29]

Therefore, it is necessary to study on better spawning techniques, effective substrates, innovative technologies, and management strategies for oyster mushroom production while avoiding significant challenges such as pest infestations. It is also crucial to find the optimal substrates that will produce mushrooms with the most yield and nutritional value. Moreover, it is essential that it remains accessible and affordable to purchase by all Sri Lankans while being profitable to farmers and economically beneficial to the country.[28]

Based on the background information, the following study was conducted to examine the efficacy of rubber Sawdust (SD) and Paper Waste (PW) as substrates for cultivating *Pleurotus ostreatus* (American oyster mushroom). The principal objective was to ascertain the impact of various substrate combinations on the nutrient composition, presence of bioactive compounds, and antioxidant capacity of these mushrooms. The ultimate aim of this study was to recommend the most suitable substrate combination to cultivate *Pleurotus ostreatus* efficiently and effectively.

2. Methodology

2.1. Preparation of substrate bags. The substrates, SD and PW were prepared by adding white rice bran, red rice bran, chemical mix, and adequate amountof water according to the ratios mentioned in Table 1.

Table 1. Preparation of substrate mix

Ingredient	**Amount (per 100 kg ofsubstrate**
White rice bran 8kg	8 kg
Red rice bran 2kg	2 kg
$CaCO_3$	2 kg
$MgSO_4$	200 g
Chlorinated/tap water	As required

The prepared substrate mixes were loaded into polypropylene bags. A total of 5 substrate combinations were prepared: 100% SD, 100% PW, 50:50 SD, PW, 75% SD and 25% PW, 75% PW and 25% SD. The substrate mixes were filled into bags which were all sealed using cotton wool. They were then autoclaved for 15 minutes at 121ºC.

2.2. Inoculation of substrate bags. The autoclaved bags were left to cool down for 24 hours at room temperature and were inoculated using *Pleurotus ostreatus* spawns under aseptic conditions. The bags were then transferred to the incubation room.

2.3. Harvesting. After the spawn run was complete, the bags were cut open and watered three times a day. Fully grown mushrooms were harvested, and the following parameters were recorded: no of days taken for spawn run to complete, no of days taken for first harvest from incubation, no of fruiting bodies per harvest and the parameters of the largest mushroom which included cap diameter, stipe length and stipe thickness.

2.4. Water holding capacity. 50 g of sample was prepared for each combination and mixed with 100 ml of water in a beaker. For each

substrate combination, 3 beakers were prepared. The beakers were covered using aluminum foil and were left to sit for 24 hours at room temperature. After 24 hours, the contents of each beaker were filtered, and the volume of water eluted was recorded.[30]

2.5. Preparation of mushroom extracts. 5 g of dried mushroom powder was mixed with 50 ml distilled water in a falcon tube. The tubes were left in a rollermixer for 48 hours. After 48 hours the contents of each tube were filtered using Whatman No 1 filter paper to obtain the mushroom extract.[31]

2.6. Total protein concentration analysis using Lowry assay. A standard series of 200 µg/ml – 1000 µg/ml was prepared using Bovine Serum Albumin (BSA). Lowry A, B and C chemical mixes were prepared. 1 ml of each standard, 1 ml of distilled water (blank) and 1 ml from each sample extract (20 times diluted) were taken in duplicates. 5 ml of Lowry AB mix was added to all the tubes and were left to incubate for 10 minutes at room temperature. After this, 0.5 ml of Lowry C was added to all the tubes and left to incubate for 30 minutes at room temperature. The absorbance was measured at 660 nm using a UV-Vis spectrophotometer. Protein concentrations of the mushroom extracts were calculated using the BSA standard curve.[32]

2.7. Total carbohydrate content analysis using phenol sulfuric assay. A standard curve was prepared with Dextrose in the concentrations of 200-1000 µg/ml. Then 0.25 ml of each diluted mushroom extract was added to a test tube in duplicates. To each tube, 0.25 ml of concentrated sulfuric acid and 0.25 ml of phenol was added immediately. The tubes were all heated in a water bath at 100ºC for 5 minutes and then cooled at room temperature. The samples of each tube were measured for its absorbance using a UV-Vis spectrophotometer at 490 nm along with the blanks.[33,34]

2.8. Bioactive compound analysis using qualitative test.

Table 2. Qualitative test methods.[35, 36]

Bioactive compound	Method
Saponins	To 0.5ml of each sample, 0.5ml distilled water was added and shaken vigorously.
Flavonoids	To 1ml of each sample, 2ml of 2% Sodium Hydroxide was added along with 2 drops of diluted hydrochloric acid.
Polyphenols	To 1ml of each sample, a few drops of diluted Iodine was added.
Tannins	To 0.5ml of each sample, 5% Ferric Chloride solution was added.
Terpenoids	0.5ml of each sample was mixed with 2ml of Chloroform and 2ml of concentrated Sulfuric acid.
Anthraquinones	2ml of 10% Ammonium solution was mixed with 0.5ml of each sample.
Steroids	0.5ml of each sample was mixed with 0.5ml Chloroform followed by 1 drop of concentrated Sulfuric acid.

2.9. Total phenolic content analysis. To 0.3 ml of each mushroom extract (diluted 20 times), 1.2 ml of 10% Folin-Ciocalteu phenol reagent and 1.5 ml of 7.5% saturated Na2CO3 solution was added. The sample tubes were incubated for 1 hour at room temperature and the absorbance was measured at 765 nm. Gallic acid was used at concentrations of 20-100 µg/ml to plot a standard curve to determine the total phenolic content.[37]

2.10. DPPH assay for antioxidant activity. A series of mushroom extracts were prepared in test tubes (1-5 mg/ml) to which 2 ml of DPPH solution was added. The tubes were incubated at room temperature for 30 minutes in the dark. After using methanol as a blank, the absorbance of each sample was measured using a UV-Vis

Spectrophotometer at 517 nm. The antioxidant activity was calculated using the equation below and IC_{50} (Inhibitory Concentration) values were determined.[38]

2.11. Statistical analysis. Microsoft Excel 365, Version 2302 was used to analyze the data produced. Results are expressed as mean values ± Standard Error. One-way Analysis of Variance (ANOVA) was used to determine the statistical differences. Where $p < 0.05$, the values were deemed statistically significant.

3. Results

3.1. Mushroom Harvest

Table 3. Images of Harvest Produced by Bag 1 and 2 in each Substrate Combinations

Substrate Combination	Bag 1	Bag 2
100% SD		
100% PW		
50:50 PW, SD		
75% SD 25% PW		
75% PW 25% SD		

Key: SD = Sawdust, PW = Paper Waste

3.2. Water Holding Capacity (WHC) of Substrates All the substrate combinations did not elute any amount of water upon filtration. Hence, all of them contained 100% WHC.

3.3 Spawn run

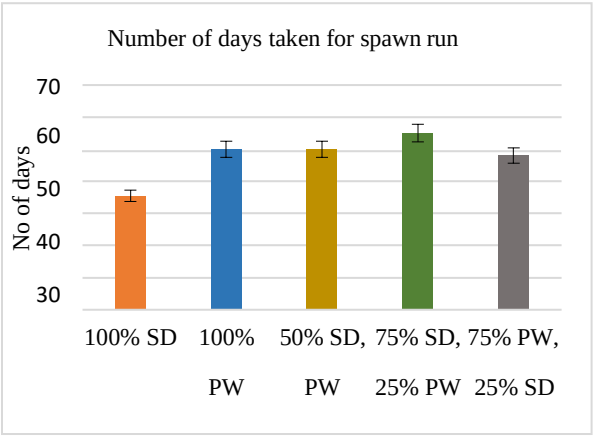


Figure 2. No of days taken for spawn run to complete.

100% SD took the least time to complete mycelial growth while 75% SD, 25% PW took the most time to complete mycelial growth (Figure. 2). A significant difference was

observed between the 100% SD group and all other groups.

3.4. Harvesting period

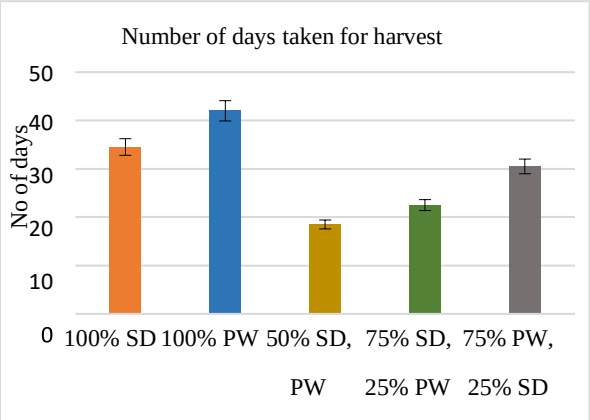


Figure 3. No of days taken for first harvest from incubation.

50:50 SD, PW took a significantly shorter period to produce fruiting bodies while 100% PW took a significantly longer period to produce fruiting bodies (Figure. 3).

3.5. Number of fruiting bodies

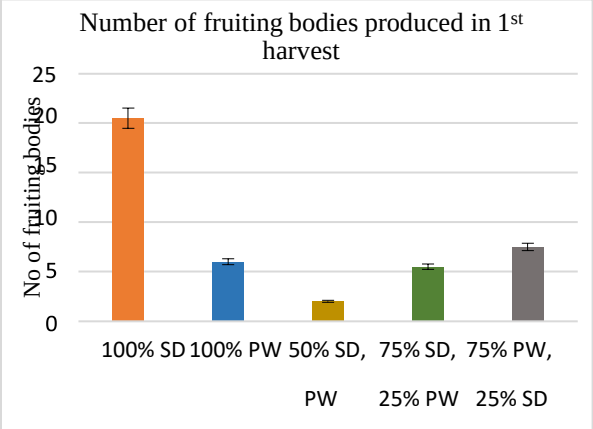


Figure 4. No of fruiting bodies produced by different substrate combinations.

100% SD produced the most fruiting bodies while 50% SD, PW produced the least number of fruiting bodies (Figure. 4). There is a significant difference between the 100% SD group and all other groups.

3.6. Fresh weight of mushrooms

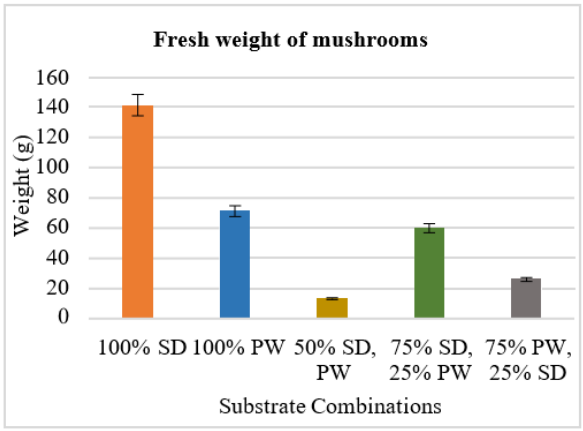


Figure 5. Fresh weight of mushrooms produced by each substrate combination.

100% SD produced fruiting bodies with the highest weight while 50% SD, PW produced fruiting bodies with the least weight (Figure. 5). A significant difference was observed between the groups.

3.7. Morphological Parameters of the largest mushroom in a bunch

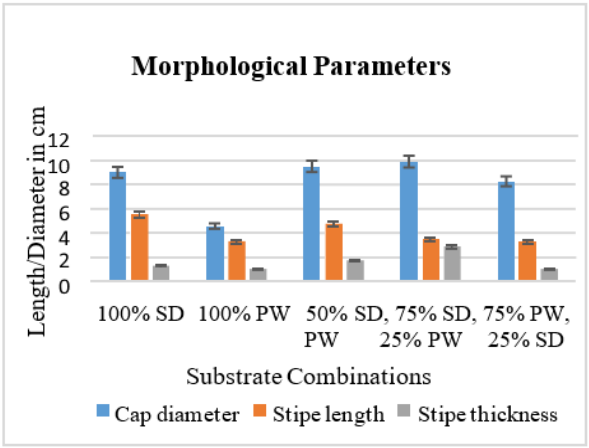


Figure 6. Parameters of the largest mushroom produced by each substrate combination in the first harvest.

A significantly high cap diameter was, and a significantly high stipe thickness was observed in

75% SD, 25% PW. The highest stipe length was recorded in 100% SD and it was significantly different compared to other groups. 100% PW displayed a significantly low cap diameter and stipe length. The lowest stipe thickness was observed in 75% PW, 25% SD (Figure. 6).

3.8 Total protein content

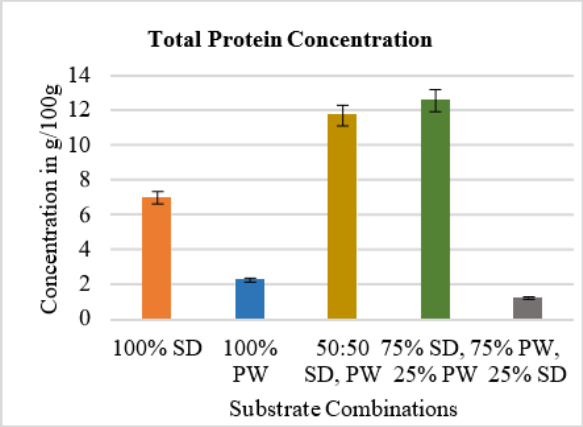


Figure 7. Protein content of *Pleurotus ostreatus* species grown in different substrate combinations.

The highest protein content was observed in 75% SD, 25% PW combination and the lowest concentration was present in 75% PW, 25% SD (Figure. 7). A significant difference was observed between the groups.

3.9 Total carbohydrate content

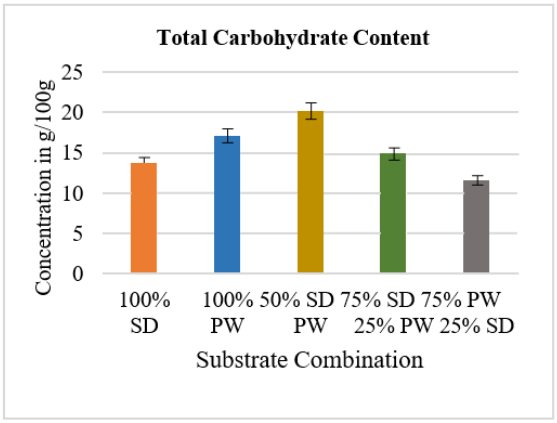


Figure 8. Total carbohydrate content in *Pleurtous ostretatus* species grown in different substrate combinations.

The highest carbohydrate content was present in 50:50 SD, PW and the lowest carbohydrate content was present in 75%PW, 25% SD (Figure. 8). A significant difference was observed between the groups.

3.10 Total phenolic content

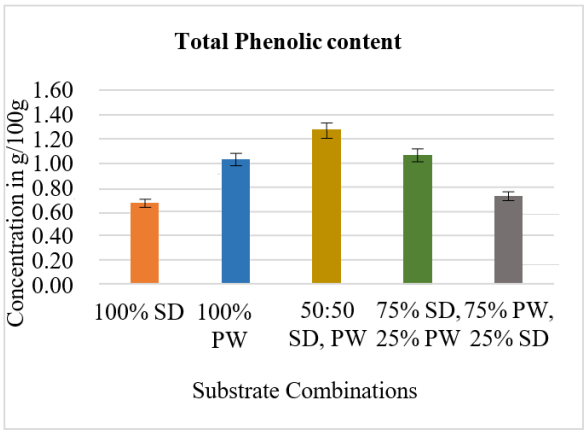


Figure 9. Total phenolic content of *Pleurotus ostreatus* species grown in different substrates.

50:50 SD, PW contained a significantly high TPC while 100% SD contained a significantly low TPC compared to other groups (Figure. 9).

3.11 DPPH Radical Scavenging Activity – IC_{50}

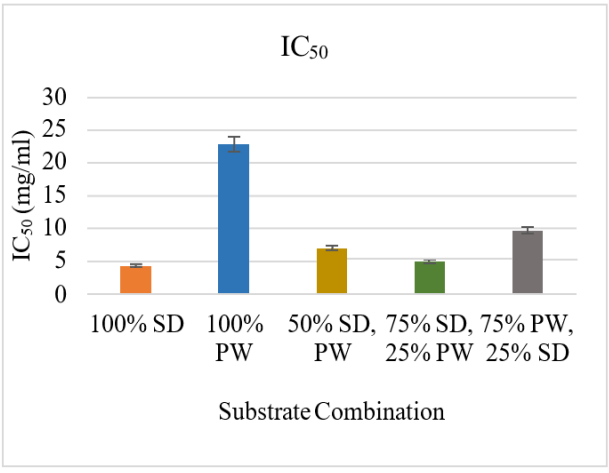


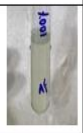
Figure 10. IC_{50} values of *Pleurotus ostreatus* species grownin different substrates.

The highest IC_{50} value was seen in 100% PW and

the lowestvalue was seen in 100% SD (Figure. 10). There is a significant difference between 100% PW and all other groups.

3.12 Qualitative tests for bioactive compounds

Table 4. Test results of the qualitative tests

Bioactive compound	100% SD	100% PW	50% SD, PW	75% SD, 25% PW	75% PW, 25% SD
Saponins					
	√	√	√	√	√
Flavonoids					
	X	X	X	X	X
Polyphenols					
	√	√	√	√	√
Tannins					
	X	X	X	X	X
Terpenoids					
	√	√	√	√	√
Anthraquinones					
	X	X	X	X	X
Steroids					
	√	√	√	√	√

4. Discussion

Pleurotus ostreatus species are one of the most widely cultivated mushrooms mainly due to their health benefits and ease of cultivation. The following study focused on using rubber sawdust and paper waste purchased locally to cultivate *Pleurotus ostreatus* species and to analyze the impact of these substrates on the nutrient composition, bioactive compound levels and antioxidant capacity of oyster mushrooms.

In this study, supplementing rice bran to the substrates, provides the mushrooms with added nutrients to support its growth, to increase its yield and to reduce the time taken for spawn run.[39] Moreover, the use of $CaCO_3$ and $MgSO_4$ serves as a buffer to maintain the pH of the substrate medium.[40]

The substrate combinations were all analyzed for their water holding capacity. None of them eluted any amount of water indicating that both SD and PW have a 100% WHC in both its combined and uncombined form.

As depicted in Figure 2, the fastest mycelial growth was observed in 100% SD bags (35 days) while the slowest mycelial growth was seen in 75% SD, 25% PW (55 days). These results were not in line with the results of Girmay *et al*., 2016 in which mycelial growth in 100% SD was seen within 19 days while PW without supplementing other materials, showed mycelia growth within 14 days.[41] With regards to the results obtained on the rate at which the first harvest was obtained after incubation (Figure 3); 100% PW took the longest period (42 days) while 50% SD, PW was the fastest (18 days). This can be supported with the results obtained by Tesfay *et al*., 2020 which proved that PW without supplementary materials took a longer period to show both mycelial growth and pinhead formation. These results were similar to the study conducted by Baysal *et al*., in 2003 which stated that supplementing PW with other lignocellulose rich substances like rice husk (80:20 or 50:50)

reduced the number of days for pinhead formation.[13,15]

The above results can be further supported by a study done by Oei and Nieuwenhuijzen in 2005, which found that substrates with high lignin and cellulose content start pinning more slowly than substrates with lower lignin and cellulose content.[42] In this study, combining two lignin and cellulose rich substrates like rubber sawdust and paper waste might have led to the delayed appearance of pinheads.[41] However, these results contradict with the findings of Girmay *et al*., 2016 which stated that 100% PW took about 32 days for pinhead formation.[41] Hence it was thought that the variations in every study could be due to environmental conditions and the nature of the substrate combined, in this case rubber sawdust.[43]

The number of fruiting bodies (Figure 4) and the fresh weight (Figure 5) was highest in the mushrooms produced by the 100% SD combination. The number of fruiting bodies observed in our study, very well matched with the findings of Hoa and Wang, 2015 which recorded the highest number of fruiting bodies that were grown in 100% SD. However, our findings associated with the weight of the mushrooms, did contradict with Hoa and Wang's study conducted in 2015 which stated that 100% SD produced mushrooms with lowest weight.[6]

Moreover, the results obtained for the cap diameter and stipe length (Figure 7), 75% SD, 25% PW showed the highest number. Contrary, 50:50 SD, PW in our study showed the highest stipe thickness (Figure 6) than the other combinations. These results were also relating to the findings of Hoa and Wang, 2015 which stated that the lowest cap diameter (70.62 mm) was observed in 100% SD, but 50% or 80% SD combined with 20% or 50% sugarcane bagasse or corncob, produced mushrooms with a higher cap diameter (> 80 mm). Similar findings were seen for the stipe length thickness, indicating both 50% and 80% SD combined with other substrates produced mushrooms with good stripe length and thickness.[6]

The protein content was analyzed using the Lowry method. It was noted that mushrooms grown in 75% SD,25% PW substrates showed the highest protein content (Figure 7). Again, these results were close to previous findings which identified highest protein content when 80% SD was combined with other substrates. On the other hand, in this study the total carbohydrate content and TPC was highest in the mushrooms grown using the 50:50 SD, PW combination (Figure 8 and Figure 9). These results were also matching with Hoa and Wang's study in 2015, which stated that 80% SD combination produced mushrooms with the highest carbohydrate content followed by the 50% SD combination.[6]

DPPH is an antioxidant assay which relies on the transfer of electrons to produce a violet solution.[44] In this study, DPPH assay was performed to identify the antioxidant activity of mushrooms and to recognize the IC_{50} value, the concentration of the mushroom required to scavenge 50% of the DPPH radicals.[45] Based on the results obtained from our study, 100% PW showed the highest IC_{50} values and the lowest IC50 values were seen in 100% SD (Figure 10). This implies that the mushrooms grown in 100% SD has a higher antioxidant capacity while the mushrooms grown in 100% PW has the lowest antioxidant activity. These results did not match with the study conducted by Hoa and Wang, 2015 which concluded that 100% SD showed the lowest antioxidant activity.[6]

Moreover, the bioactive compounds present in Pleurotus ostreatus were not affected by the type of substrates. Qualitative results performed indicated that all the mushrooms contained saponins, polyphenols, terpenoids and steroids. These results were somewhat similar to the ones obtained by Rahimah *et al*., 2019 which proved the presence of saponins, phenolic compounds and steroids in *Pleurotus ostreatus* species. However, this study also identified the

presence of flavonoids and tannins in the oyster mushrooms which were not identified in our study. This might have been due to the use of different protocols in the previous study.[46]

Conclusion

To conclude, with regards to the period of incubation, harvesting, number of fruiting bodies and yield; 100% SD combination appears to be an ideal substrate to grow *Pleurotus ostreatus*. However, combining it with 25-50% of paper waste, has shown to produce mushrooms with better cap diameter, stipe length and thickness. Also, the protein, carbohydrate and phenolic content appear to be greater when sawdust is combined with paper waste. Based on the antioxidant activity, 100% SD appears to be the best substrate. Therefore, it can be recommended that *Pleurotus ostreatus* grown in 100% SD and SD combined with paper waste (50:50, 75:25) can be used to have a well-balanced diet which can be used in preventing diseases and promoting better human health.

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