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Research Article

Differential Influence of Supplements on Lignocellulolytic Enzyme Activities, Growth and Production of *Pleurotus Ostreatus*

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ABSTRACT

Lignocellulolytic enzymes are extracellular biocatalysts secreted by filamentous fungi and are involved in the breakdown of recalcitrant lignocellulose plant materials into useful products necessary for fungal growth and development. Even though several studies on filamentous fungi have reported the impact of different substrates on lignocellulolytic enzymes, there is limited information on how mushroom supplements affect secretion of the enzymes, growth, and yield of *Pleurotus ostreatus* when using the alkaline treatment method. In this study, we investigated the influence of cornmeal (T1) and coffee grounds (T2) supplements on lignocellulosic enzymes at different growth stages and the production of *P. ostreatus*. We found that lignolytic enzyme activity was significantly higher in the control (CK) and T2 during mycelial stages, while CK had the lowest hydrolytic enzyme activity during primordia and fruiting. Unlike T1 which had the best biological efficiency, T2 exhibited significantly higher levels of lignolytic enzymes during mycelial stage, whereas CMCase and xylanase activities were higher in supplemented treatments than in the control during primordia and fruiting. Taken together our results demonstrated that cornmeal and coffee ground supplements reduce mycelium growth rate, enhance the production of hydrolytic enzymes during fruiting, and remarkably increase the yield and protein content of *P. ostreatus*.

Keywords: *Pleurotus ostreatus*; Cornmeal; Coffee grounds; Substrate; Supplements; Mushroom production; Biological efficiency

INTRODUCTION

Pleurotus ostreatus is one of the most widely cultivated mushroom globally because of its flavour, good aroma, high nutritional and medicinal properties [1,2]. China produces more than 80% of the global mushroom yields, and cultivation of Pleurotus species is a very important industry in the nation [3]. For efficient growth and nutrient acquisition, *P. ostreatus* produces lignocellulolytic enzymes mainly laccase, Manganese Peroxidase (MnP), cellulases, and hemicellulases which break down lignocellulosic growth substrates into simpler and usable sugar molecules [4]. Essentially, lignocellulolytic enzymes are classified as either lignolytic or hydrolytic. Lignolytic enzymes include laccase, manganese, and versatile peroxidases, while the hydrolytic ones consist of mainly cellulase and hemicellulases. Several studies have appreciated lignolytic enzymes as key enzymes required for lignin degradation and mushroom development, especially during the vegetative growth of mycelia [5]. Besides enabling the fungus to mineralize recalcitrant lignin materials, laccase enzyme has been shown to provide defence against environmental compounds and toxic fungal metabolites such as aflatoxin [6,7].

On the other hand, cellulase and hemicellulase are hydrolytic enzymes that are respectively known to be involved in degradation of cellulose and hemicellulose plant polymers [8,9]. According to Xie et al. (2016), biological efficiency of a mushroom species is mainly attributed to its hydrolytic enzyme system. Because of the great bearing that lignocellulolytic enzymes have on both the mycelium growth and production of *P. ostreatus*, a number of studies in recent years have sought to understand how various growth substrates and supplements affect the secretion of these enzymes. For instance, activities of cellulase and laccase enzymes in mushrooms have been shown to be respectively responsible for mycelium colonization and sporophore formation [10,11]. In essence, the expressions of genes that encode these enzymes

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ultimately control the mycelia colonization, growth rate and yield of *P. ostreatus* [12,13]. The expression and activities of lignocellulolytic enzymes however depend on the composition of substrate, Carbon/Nitrogen ratio, and growth medium conditions, including pH and temperature [14]. Therefore, an appropriate choice of substrate, substrate preparation and sterilization method, and selection of suitable supplements is critical for optimum mushroom production.

Supplementation of diverse mushroom substrates has been shown to alter the carbon/nitrogen ratio and composition of the growth substrates. The changes in substrate content affect lignocellulolytic enzymes activities, which consequentially have effects on mycelia colonization period, fruiting body yield, biological efficiency and nutritional content of *P. ostreatus* mushroom [5,15,16]. Notably, previous studies have reported how supplements affect the production of *P. ostreatus* only when using conventional methods of substrate pasteurization or sterilization. However, recent studies have suggested alternative methods of substrate disinfection which involve use of low cost chemicals such as chlorinated water, formaldehyde, hydrogen peroxide and hydrated lime solution [17,18]. The use of these chemicals to disinfect mushroom substrate is referred to as alkaline treatment method, and according to [19], the success of this method is mainly determined by the concentration of chemical used, the origin as well as quality of the substrate and supplements used [19].

Nevertheless, despite the remarkable effects of alkaline treatment method on the production of *P. ostreatus*, there is limited information on the effects of supplementation on the secretion of lignocellulolytic enzymes and productivity of *P. ostreatus* when using substrates disinfected in robust alkaline solutions. Moreover, even with conventional methods of pasteurization, few studies have tried to elucidate the influence of readily available supplements on lignocellulolytic enzyme production at different growth stages of *P. ostreatus* and its amino acid contents. On this basis, the study investigated the effects of cornmeal and coffee grounds on *Pleurotus ostreatus* growth rate, lignocellulolytic enzyme activities and nutrition when using alkaline treated Arundo donax substrate. Results from the study indicated that the two supplements significantly induced hemicellulase enzymes and further enhanced the productivity of *P. ostreatus*. In addition, cornmeal gave the best biological efficiency although the fruiting bodies were a little bit smaller in size, while ligninolytic activity was higher in ground coffee supplements during the mycelia stage.

MATERIALS AND METHODS

Substrate and spawn preparation

Pleurotus ostreatus P969 strain of accession number MG819739.1 was obtained from the Juncao Research Institute at Fujian Agriculture and Forestry University, China. The strain was maintained on potato dextrose agar plate; a sterile mixture of saw dust and wheat bran was then used to prepare spawn at 22°C for 30 days as described [20]. Separately, fully grown Arundo donax plants were harvested, ground to 0.5-1.5 cm particle size, and then sun dried for 4 days. The dried A. donax substrate was disinfected in a robust alkaline solution according to Iossi et al, [19]. The substrate was submerged overnight in a 2% alkaline solution of calcium hydroxide for 12-15 hours. The alkaline solution was prepared by dissolving 20 g of solid calcium hydroxide into every 1 litre of pure water. It was then squeezed to remove excess water, followed by supplementation, and uniformly mixed for cultivation into growth bags.

Experimental design and Treatments

In this study, four sets of inoculation treatments were performed. In the first treatment (T1), the substrate formula was prepared by supplementing alkaline treated A. donax substrate with 25% dried cornmeal, a 0.1% urea, a 0.2% MgSO4.7H2O, and 2% lime powder. In the second treatment (T2), the alkaline treated A. donax substrate was supplemented with 25% dried coffee grounds, 0.1% urea, 0.2% MgSO4.7H2O, and 2% lime, whereas for T3 the substrate was supplemented with 25% dried cornmeal, 25% of dried coffee grounds, 0.1% urea, 0.2% MgSO4.7H2O, and 2% lime powder. We set up the control (CK) by adding 0.1% urea, 0.2% MgSO4.7H2O, and 2% of lime powder onto the alkaline treated Arundo substrate. The procedure for *P. ostreatus* cultivation was performed in transparent polythene bags based on the Juncao technology as described [21]. In each of the four treatments, around 460 g of each respective substrate formula was added to a cylindrical transparent polythene bag, tightly pressed, inoculated with 30 g-40 g of *P. ostreatus* spawn layer and the bags sealed with minimum aeration. The polythene bags were sealed with perforated caps that could allow slight aeration.

Each treatment had 15 replicates while each of the polythene bags had a total weight of 500 g substrate formula. The bags were then placed in a refrigerator with the temperature set at 22°C, 50% humidity and lights turned off for suitable mycelia running. The development and growth of mycelia in the bags were closely monitored to compare mycelium density and growth rate. Samples of mycelia were randomly collected from different colonized substrate points in each of the treatments after 10 and 20 days of mycelium growth and during primordia initiation, respectively. The number of days taken for mycelia to fully colonize each bag for primordia initiation (pin head formation) was recorded. After the appearance of fruiting bodies, the polythene bags were transferred in a controlled fruiting room with a temperature of 17°C and 90% humidity. Mature fruiting bodies were sampled, frozen in liquid nitrogen, and then stored at -80°C. The stored mycelia and fruiting body samples were later used to determine *P. ostreatus* enzyme activities in the four treatments.

The number of mature fruiting bodies in each bag was recorded, stipe length and pileus diameter measured, and fruiting bodies' total weight in each bag recorded. To establish the biological efficiency (BE) in each treatment, we used the formula;

BE=(Fresh weight of fruiting body ×100)/(Dry weight of the

substrate)

Analysis of substrate contents

Each of the four substrate formulae was subjected to compositional analysis of lignin, cellulose, hemicellulose, starch, ash, total carbon, and nitrogen content. In each of the substrate composition tests we employed three replicates. Total lignin content was quantified using the acetyl bromide procedure [22]. The ground substrate samples were crushed, and 20 g of each sample placed into test tubes fitted with a silicone screw cap. 0.08 ml of 70% perchloric acid was then added into the tubes followed by the addition of 2 ml of 25% acetyl bromide-glacial acetic acid (1:3, v/v) incubated at 70°C for 30 minutes with intermittent gentle shaking. After complete digestion, the sample was transferred to a volumetric flask containing 5 ml of 2 ml NaOH and 12 ml acetic acid and absorbance was taken at 280 nm as described [23].

Cellulose content was determined using the Updegraff analysis method as described [24]. Around 20 mg of the substrate was measured into centrifuge tubes followed by the addition of a 3 ml mixture of nitric acid/water/acetic acid (1/2/8, v/v/v) and heating in a boiling water bath for 30 minutes. The suspension was centrifuged for 3 min at 2500 rpm and supernatant discarded. We re-suspended the pellets in tubes with 5 ml H2O, centrifuged and discarded the liquid. The remaining pellets were incubated with 2.5 ml of 72% sulphuric acid for 1 hour with vortex every 10 min. 10 ml of the solution obtained was diluted with water to 400 ml, 0.2 g anthrone in 100 ml of concentrated H2SO4 added, and the mixture heated in boiling water bath for 15 min. The reaction was then measured at 620 nm absorbance.

Hemicellulose was evaluated using one-step acid hydrolysis method described [25]. Ground substrate 0.01 g was hydrolysed in 87 ml of 4%wt sulphuric acid at 121°C for 1 hour. The liquid was drawn, then centrifuged and absorbance reading was taken at 540 nm for hemicellulose determination. The substrate's total nitrogen content was determined using the Kjeldahl colorimetric method [26] whereby a combination of selenium, CuSO4 and concentrated H2SO4 were used to digest the substrate sample for total nitrogen. Total carbon content was determined using a method described by [27]. Organic carbon was oxidised with a determined K2Cr2O7 oxidant in sulphuric acid medium in the method.

The analysis of enzyme assays

Assays of laccase, manganese peroxidase, FPase, CMCase, β-glucosidase, xylanase, xylosidase, and amylase enzymes were performed on each of the mycelium, primordia and fruiting body samples using the Elisa kit method. In each of the assays, 50 µL of the standard was added to micro-Elisa strip plates. 40 µL of sample dilution was then added to the testing sample wells followed by further addition of 10 µL of testing samples to the wells and gently mixed. The plates were closed with closure plate membranes and incubated for 30 minutes at 37°C before a wash solution was prepared, diluted 30-fold with distilled water and reserved. Closure plate membranes were then uncovered, liquid contents discarded, dried by swing, and washing buffer added to every well, left for 30 s, and then drained. Except for the blank, HRP conjugate reagent 50 µL was added to each well, incubated for 30 minutes at 37°C and washed with a buffer solution. 50 µL of chromogenic solutions A and B were added to each well, and light preservation evaded for 10 minutes at 37°C. The reaction was stopped by adding 50 µL sulphuric acid (stop) solution to each well, and when the colour changed from blue to yellow, absorbance was read at 450 nm within 15 minutes. A standard curve was drawn, and the OD value was used to determine the sample's corresponding density. Sample density from the graph was then multiplied by dilution factor to obtain the actual sample density of each enzyme.

Analysis of fruiting body composition

We carried out the analysis of fiber, polysaccharide, carbohydrate, fat, protein, amino acids, and heavy metal contents in each of the fruiting body samples. Phenol-sulphuric acid method [28] was used to evaluate the total polysaccharide content, whereby polysaccharide in the sample was extracted using water extraction and alcohol precipitation. Crushed sample (0.05 g) was weighed into test tube, and 1 ml water was added and homogenized in the water bath at 100°C for 2 hr. The mixture was centrifuged at 10,000 rpm for 10 min, the supernatant was then removed. 0.2 ml of supernatant was mixed with 0.8 ml of anhydrous ethanol and used to quantify polysaccharide at 490 nm. Fiber content was evaluated as described [29], while carbohydrate level was determined by phenol-acid method as described [30]. Protein content was determined using BCA kits method [31]. Standard working solution (SWR) was prepared by dissolving reagents A and B, and then the absorbance of known standards recorded at 562 nm. Fat content in the fruiting bodies was determined as described [32] while fiber was evaluated according to [33]. The composition of amino acids in the fruiting bodies was determined using the RP-HPLC method as described by [34]. About 0.2 g of the sample was hydrolysed by 6 M HCl, individual amino acids were separated and quantified using HPLC-1100 detector (DAD chromatographic column) at 36 0 nm. The level of Cd, Pb, As and Hg in the fruiting bodies were analysed by ion exchange method [35].

Statistical Analysis

We subjected the obtained data from three independent biological replicates to one-way Anova test using SPSS software version 25. The means were compared using the Duncan method at a 5% level of significance.

RESULTS

Chemical composition of supplemented substrate

The supplementation of Arundo donax substrate with cornmeal and coffee grounds significantly altered the composition and

texture of the substrate. Data from the analysis showed that cornmeal supplement considerably reduced the lignin, cellulose, C/N ratio and ash contents of the substrate. However, addition of the supplement to the substrate elevated the starch content while no change was recorded in the amount of hemicellulose (Table 1). When supplemented with coffee grounds, a reduction was noted in the levels of cellulose, hemicellulose, starch and C/N ratio, while lignin content slightly rose up. On the other hand, when we supplemented both cornmeal and coffee grounds as recorded in T3, all the substrate compositions that we analysed was shown to substantially decrease except for the ash content whose quantities were elevated (Table 1).

Table 1: Compositional analysis of the substrate formulae used to cultivate *P. ostreatus*

	Lignin	Cellulose	Hemicellulose	Starch	C/N ratio	Ash (%)
CK	114.21 ± 1.57b	355.96 ± 2.60a	363.49 ± 5.79a	58.84 ± 0.47b	38.15 ± 0.27a	5.50 ± 0.02b
T1	102.97 ± 2.09c	314.69 ± 2.59d	355.12 ± 3.60ab	61.96 ± 0.50a	25.84 ± 0.26c	5.17 ± 0.04c
T2	121.81 ± 1.95a	325.86 ± 1.33b	308.27 ± 3.38c	44.50 ± 0.56c	34.72 ± 0.23b	5.66 ± 0.03b
T3	92.37 ± 2.39d	316.83 ± 1.49c	341.15 ± 4.05b	46.45 ± 0.53c	21.37 ± 0.21d	6.29 ± 0.03a
Addition of cornmeal and coffee grounds to the substrate remarkably decreased cellulose, hemicellulose, and carbon/nitrogen ratio. The different letters within a column indicates significance difference (S.D) by Duncan's multiple range tests at $p \leq 0.05$, while mg/g was used as the unit of measurement.						

Influence of the supplements on growth, yield and fruiting body content

It took the shortest period of 21.9 days for *P. ostreatus* mycelium to colonise the non-supplemented Arundo substrate (CK). During mycelium growth, we recorded faster mycelia colonization in treatments supplemented with coffee grounds (T2) when compared to treatment with cornmeal (T1). The longest period required for complete mycelia colonization was however observed in T3 which had both supplements (Table 2). Primordial stage involves the transition of mycelia into mushroom pinheads that develop into mature fruiting bodies. Both mycelium and primordia development are important stages that have been shown to determine fruiting body development in fungi [36]. Primordia formation was first observed in treatment with coffee grounds, followed by cornmeal supplement (T1) and T3, while the control took the longest time. But in terms of productivity, cornmeal supplement produced the highest number of fruiting bodies as well as the best biological efficiency (BE) of 98.2%. Coffee grounds supplement was the second with BE of 84.6%, while a combination of both supplements (T3) generated 70.9% BE with the third most number of fruiting bodies. The smallest quantity of fruiting body weight, BE and fruiting body number was recorded in control, which had a BE of 57.3% (Table 2). However, the two supplements showed no significant difference between their stipe lengths and diameter of their fruiting body pilei. Moreover, the two supplements had no effect on the pileus diameter because no difference was recorded between T1/T2/T3 and the CK (Table 2).

Table 2: Effects of cornmeal and coffee ground on P. ostreatus growth and production

	Mycelia growth (days)	Primordia initiation (days)	Fruiting body weight (g)	Stipe length (cm)	Pileus diameter (cm)	No. of fruiting bodies	Biological Efficiency (%)
CK	21.9 ± 0.4a	54.9 ± 1.3c	114.5 ± 8.4d	6.6± 0.4c	7.1 ± 0.3a	9.5 ± 1.4c	57.3 ± 4.2d
T1	32.1 ± 0.7c	46.6 ± 0.8b	196.3 ± 10.0a	7.4 ± 0.3ab	7.4 ± 0.3a	28.1 ± 0.9a	98.2 ± 5.0a
T2	28.5 ± 0.4b	39.7 ± 0.4a	169.1 ± 8.9b	6.9 ± 0.3b	8.2 ± 0.4a	20.6 ±1.8b	84.6 ± 4.5b
T3	41.6 ± 0.4d	48.2 ± 0.3b	141.4 ± 9.4c	8.2 ± 0.3a	7.8 ± 0.7a	14.4 ± 1.0c	70.9 ± 4.7c
From the analysis, both supplements exhibited negative effects on the mycelia growth, whereas coffee grounds supplement promoted faster initiation of primordia. The letters within column shows S.D between the supplemented treatments by Duncan's multiple range tests at $p \leq 0.05$. Furthermore, coffee produced fruiting bodies with longest stipes while cornmeal generated the best B.E and highest number of fruiting bodies.							

Both cornmeal and coffee ground supplements had remarkable effects on *P. ostreatus* fruiting body contents. From the analysis of fruiting body samples taken, all the supplemented treatments T1, T2 and T3 had drastically lower polysaccharide contents as compared to the CK. Nevertheless, cornmeal produced fruiting bodies with higher polysaccharide content than coffee grounds (Table 3). In terms of fiber levels, no significance difference was noted between the treatments. However, coffee grounds generated the largest carbohydrate content while the cornmeal induced highest fat content in the fruiting bodies. The use of supplements

was indicated to significantly increase protein content *P. ostreatus* mushroom. When combined together, both supplements in T3 gave rise to fruiting bodies with largest quantity of protein levels, while cornmeal (T1) had significantly higher levels of protein than coffee ground (T2) (Table 3). Similarly, fruiting bodies in T3 had the highest number of amino acids (14) whose quantities were considerably higher than any of the other treatments. The comparison between the supplements in T1 and T2 showed that the quantities of Aspartate, Alanine, Threonine and Lysine were significantly higher in T2 than in T1, while the levels of Histidine, Arginine and Serine were greater in T1 than in T2 (Table 3). Apart from Valine which exhibited no difference between the treatments, all the amino acids we tested were distinctly lower in the CK compared to the supplemented treatments.

Table 3: Compositional analysis of the fruiting bodies

	Treatments			
Composition	**CK**	**T1**	**T2**	**T3**
Polysaccharide	62.3 ± 0.8a	56.77 ± 0.4b	40.8 ± 0.4d	47.0 ± 0.3c
Fiber	36.61 ± 1.26a	37.83 ± 0.77a	40.53 ± 0.42a	36.39 ± 1.50a
Carbohydrate	13.86 ± 0.31b	14.53 ± 0.23b	15.74 ± 0.30a	12.87 ± 0.25c
Fat	3.29 ± 0.03c	4.17 ± 0.30a	3.84 ± 0.03b	3.23 ± 0.01c
Protein	34.9 ± 0.4d	45.5 ± 0.5b	39.5 ± 0.6c	49.8 ± 1.1a
Amino acids				
Asp	5.68 ± 0.07c	7.10 ± 0.17b	8.24 ± 0.35a	8.80 ± 0.14a
Glu	12.41 ± 0.38c	16.01 ± 0.37b	18.28 ± 0.42b	19.48 ± 0.40a
Ser	4.98 ± 0.16b	6.52 ± 0.45a	6.44 ± 0.29b	7.76 ± 0.41a
Arg	4.44 ± 0.40b	7.42 ± 0.27a	5.57 ± 0.38b	8.33 ± 0.24a
Gly	3.82 ± 0.18c	5.33 ± 0.12b	4.76 ± 0.06b	6.04 ± 0.23a
Pro	5.58 ± 0.38c	7.34 ± 0.35bc	6.29 ± 0.24ab	8.31 ± 0.21a
Ala	6.24 ± 0.29d	7.54 ± 0.15c	8.60 ± 0.25b	9.55 ± 0.16a
Thr	3.82 ± 0.18c	4.60 ± 0.22b	5.70 ± 0.99a	4.78 ± 0.19b
Val	1.24 ± 0.16a	1.58 ± 0.18a	1.20 ± 0.10a	1.83 ± 0.20a
Met	3.64 ± 0.21c	4.91 ± 0.07b	5.13 ± 0.16b	6.05 ± 0.24a
Cys-Cys	1.62 ± 0.03b	1.50 ± 0.03a	0.84 ± 0.15a	1.43 ± 0.17a
Ile	4.05 ± 0.11c	4.98 ± 0.23b	5.09 ± 0.08b	5.86 ± 0.09a
Leu	6.14 ± 0.11c	7.42 ± 0.08b	7.72 ± 0.18b	8.40 ± 0.05a
Phe	5.22 ± 0.22c	6.45 ± 0.11b	6.17 ± 0.08b	7.16 ± 0.14a
His	1.42 ± 0.07b	1.86 ± 0.04a	1.43 ± 0.08b	2.15 ± 0.12a
Lys	3.13 ± 0.12d	3.65 ± 0.07c	4.80 ± 0.10a	4.38 ± .12b
Tyr	1.20 ± 0.18b	1.73 ± 0.12a	1.95 ± 0.05a	2.07 ± 0.08a
Cornmeal induced significantly higher levels of protein and fat contents compared to coffee grounds. However, coffee grounds produced high levels of carbohydrate and more number of amino acids that were significantly higher compared to cornmeal.				

Effects of supplements on enzymes during mycelia, primordia and fruiting stages

We carried out assays of lignocellulolytic enzymes at days 10 and 20 of mycelia growth, during primordia and fruiting body development. From the results obtained, it was evident that supplementation of the substrate with cornmeal and coffee grounds had varied effects on *P. ostreatus* enzyme activities. After 10 and 20 days of mycelia growth, the activity of laccase enzyme was significantly higher in CK than in the supplemented treatments T1, T2 and T3. However, during primordial and fruiting stages, laccase activity was relatively higher in both T1 and T2 than CK (Table 4 and 5). The level of manganese peroxidase was higher in T2 than T1 during all the growth stages showing the positive influence of coffee grounds on the enzyme compared to cornmeal. In all the treatments, xylanase was the most active enzyme, and then followed by xylosidase and CMCase. Besides, majority of enzymes in T3 had lower activities than T1 and T2, demonstrating that excessive addition of supplements does not necessarily enhance enzyme secretion in *P. ostreatus*. In fact, T3 generated the lowest activities of laccase, MnP, FPase, and xylanase at days 10 and 20, whereas Bgl activity was the lowest in T3 at day 10 of mycelia development.

In addition, the activities of FPase, Bgl and xylanase enzymes remained relatively higher both in T1 and T2 than the control during primordia and fruiting body formation (Table 4 and 5). Cornmeal supplement in T1 generated fruiting bodies with the highest concentrations of FPase, xylanase, xylosidase and amylase. Similarly, we also observed higher laccase activities in both T1 and T2 during primordial and fruiting stages. Activity levels of MnP and CMCase enzymes were consistently higher in T2 than T1 during mycelia, primordia and fruiting stages. Amylase levels were consistently highest in T1 during mycelial, primordial and fruiting stages. We recorded significance difference between the enzymes within each of the treatments with xylanase being recorded as the most active enzyme during all the growth and developmental stages (Tables 4 and 5). Except for manganese peroxidase, the levels of tested enzymes were relatively lower in non-supplemented treatment (CK) during both primordia and fruiting stages.

Table 4: Enzyme activities during mycelium stage of P. ostreatus

Activity after 10 days of mycelium growth								
	Laccase	**MnP**	**FPase**	**CMCase**	**Glucosidase**	**Xylanase**	**Xylosidase**	**Amylase**
b	95.6 ± 2.0a	51.6 ± 0.9b	124.1 ± 2.1a	245.7 ± 17.5a	45.2 ± 1.8a	358.9 ± 12.5a	231.1 ± 6.1b	75.7 ± 1.8a
T1	74.2 ± 1.5c	53.5 ± 0.5b	113.2 ± 1.4a	219.2 ± 7.7a	42.5 ± 0.4a	354.3 ± 16.5a	277.6 ± 9.7a	71.8 ± 5.0ab
T2	89.3 ± 2.4b	59.1 ± 1.1a	120.6 ± 4.8a	206.1 ± 16.9a	44.5 ± 1.1a	355.4 ± 6.0a	286.3 ± 12.0a	70.4 ± 2.4ab
T3	60.5 ± 0.4d	48.3 ± 1.3c	104.9 ± 5.8b	209.6 ± 19.8a	38.2 ± 1.3b	314.0 ± 9.7b	271.8 ± 18.8a	64.6 ± 0.5b
Activity after 20 days of mycelia growth								
CK	91.2 ± 3.3a	58.3 ± 3.4a	114.1 ± 2.1bc	201.4 ± 15.6b	38.0 ± 2.9b	306.1±18.2a	244.5 ± 11.9a	68.4 ± 1.3b
T1	77.3 ± 1.4b	53.8 ± 2.3b	122.4 ± 4.3ab	288.3 ± 21.5a	47.9 ± 1.8a	329.3 ± 9.0a	282.4 ± 14.3a	79.5 ± 6.6a
T2	80.7 ± 2.2b	57.5 ± 0.3a	128.0 ± 3.4a	254.9 ± 10.6a	40.6 ± 1.6b	276.3 ± 24.6a	276.3 ± 22.8a	66.4 ± 2.1b
T3	75.5 ± 4.5b	51.6 ± 2.3b	107.2 ± 2.4c	191.6 ± 8.9b	39.7 ± 1.8b	249.3 ± 25.9a	249.3 ± 9.1a	69.7 ± 0.9b

The table above shows comparative differences of laccase, manganese peroxidase, FPase, CMCase, *β*-glucosidase, xylanase, xylosidase, and amylase activities between the treatments supplemented with cornmeal and coffee grounds after 10 and 20 days of mycelia growth. Different letters within a column indicate significant difference (S.D) between treatments by Duncan's multiple range tests at $p \leq 0.05$, while unit of measurement are in Iu/L. Lignolytic enzymes were relatively higher in CK and T2, while FPase, glucosidase and xylanase activities were generally lower in T3. From the analysis, significance difference was recorded between the treatments except for xylanase and xylosidase enzyme activities.

Table 4: Enzyme activities during mycelium stage of P. ostreatus

Activity during primordial stage								
	Laccase	**MnP**	**FPase**	**CMCase**	**Glucosidase**	**Xylanase**	**Xylosidase**	**Amylase**
CK	72.7 ± 2.3b	53.7 ± 1.8b	128.7 ± 1.3a	210.0 ± 5.8c	38.2 ± 2.2b	268.2 ± 4.9b	232.6 ± 5.7a	65.5 ± 1.7b
T1	88.5 ± 1.3a	54.9 ± 0.5b	125.4 ± 5.8a	250.1 ± 8.7b	48.3 ± 0.7a	307.9 ± 9.1a	250.4 ± 8.5a	79.8 ± 1.6a
T2	91.3 ± 4.5a	60.6 ± 1.4a	134.2 ± 2.5a	285.5 ± 13.9a	44.2 ± 1.2a	305.2 ± 13.2a	251.0 ± 3.4a	71.9 ± 1.7b
T3	78.3 ± 2.3b	59.4 ± 1.6a	132.4 ± 3.0a	239.4 ± 10.4b	45.3 ± 1.8a	320.2 ± 9.3a	240.8 ± 6.3a	75.0 ± 2.6a

Activity during fruiting stage								
CK	66.9 ± 2.5c	52.0 ± 0.4a	98.0 ± 0.9c	218.9 ± 3.7c	36.3 ± 1.5b	235.4 ± 14.8b	224.2 ± 7.7c	51.5 ± 0.5c
T1	89.8 ± 1.1a	50.3 ± 2.3fa	114.9 ± 0.3a	238.4 ± 7.2b	37.3 ± 2.9b	344.1 ± 19.4a	305.3 ± 11.7a	67.9 ± 0.3a
T2	87.5 ± 0.8a	51.7 ± 0.6a	108.2 ± 0.4b	259.0 ± 4.4a	47.3 ± 3.8a	315.0 ± 18.6a	285.5 ± 3.4b	63.2 ± 0.6b
T3	74.2 ± 2.0b	49.5 ± 1.2a	103.3 ± 1.0c	269.5 ± 2.1a	36.9 ± 1.4b	306.2 ± 17.8a	216.6 ± 8.8d	64.5 ± 0.6b

The table indicates enzyme activity differences between the treatments supplemented with cornmeal and coffee grounds in primordia and fruiting bodies. Different letters within a column shows S.D between treatments while the SI units of the assays are in Iu/L. Majority of the enzymes at primordia were significantly higher in the supplemented treatments T1, T2, and T3 while activities in the control remained relatively lower. At fruiting stage, most of the enzyme activities in T1 and T2 were significantly higher than activities in control.

DISCUSSION

Supplementation disparately altered the growth, productivity and composition of *P. ostreatus*

From the results obtained, it was evident that supplementation of Arundo donax substrate with cornmeal and coffee ground wastes significantly altered the composition and texture of the growth substrate formulation used in T1, T2, and T3 (Table 1). We inferred that these changes in substrate composition could have had substantive influence on growth rate, yield, BE, stipe length, protein content and the number of fruiting bodies recorded in the treatments. This is in relation to a study by Soto-Cruz et al, (1999), which reported that substrate composition has a strong influence on mycelial growth and enzyme activities of *P. ostreatus* [37]. The growth rate of *P. ostreatus* mycelium was indicated to significantly differ between the treatments. Supplementation of the substrate with cornmeal and coffee grounds was indicated to increase the number of days taken for mycelia to fully colonize the bags. The slower growth rate in T1 than T2 was associated with the lower C/N in T1 as compared to T2. Similarly, the increased nitrogen contents in the supplemented treatments T1, T2, and T3 might have greatly contributed to their slow mycelia growth as compared to the CK (Table 1). The impact of the supplements on primordia formation was however contrasting, and we observed appearance of pin heads to first occur in supplemented bags. This observation correlated with [38], who reported various agricultural supplements including maize bran to improve biological efficiency (BE) and shorten period required for primordia formation in Calocybe indica.

Highest number of fruiting bodies was recorded in T1 followed by T2, while the CK gave the smallest number of fruiting bodies. This translated to the significantly higher BE in T1 and T2 as compared to CK and T3. The high percentage of supplementation in T3 resulted to a low C/N. The low C/N T3 could have considerably contributed to the lower BE and the longer mycelial colonization period. Although the addition of nitrogen supplements is generally known to increase *P. ostreatus* yield, [39] observed that the addition of large quantities of supplements and carbon/nitrogen ratio below 30 decrease mycelial growth. We made similar observations in this study whereby the mycelium took a significantly longer period to colonize supplemented substrate than the control. Nevertheless, it is worth noting that contamination in T3 after the first harvest was highly conspicuous and could have contributed to its lower biological efficiency. It took the longest period (41.6 days) to colonize substrate in T3, which had both supplements added to it and its C/N ratio was ideally below 30 (table 1 and 2). The length of fruiting body stipes was higher in all treatments with supplements than the control, which had no supplement. The longest fruiting body stipes was observed in T3, whose substrate formula had the highest nitrogen content (Table 2). Analysis of the protein content indicated cornmeal to generate higher protein levels in the *P. ostreatus* fruiting bodies than coffee grounds. A combination of both supplements in T3 however, gave fruiting bodies with the highest protein content while the control gave the lowest (Table 3).

Surprisingly, the highest quantity of polysaccharide content was recorded in CK (without supplement), followed by T1, T3 and then T2 treatments. This suggests that both supplements could have interfered with polysaccharide synthesis, although the content in T1 was preferably higher than in T2. The high polysaccharide content in CK could be attributed to the fibrous nature of non-supplemented Arundo substrate, and its high cellulose and starch content. Coffee grounds supplement exhibited coarse particulate nature with low starch content, thus the lowest polysaccharide content in T2 fruiting bodies. Although cornmeal had coarse particles similar to coffee grounds, their differences in starch, fiber and other chemical contents could explain why higher polysaccharide content was recorded in T1 than T2. In fact, the lowest polysaccharide content was from T2. In this study, we recorded no significance difference between the pileus diameter and fiber contents of the fruiting bodies. Just like the protein levels, amino acids content was highest in T3, followed by T2 and T1, and lowest in CK. Therefore, supplementation could be one

of the suitable ways to enhance amino acid content of *P. ostreatus*.

Cornmeal and coffee grounds induced significant variations in lignocellulolytic enzymes activities at different growth stages of *P. ostreatus*

The addition of cornmeal and coffee grounds had varied effects on *P. ostreatus* growth and enzyme activities during mycelial stage. Lignolytic enzymes (laccase and manganese peroxidase) are essentially known to drive mycelia colonization during vegetative growth of *P. ostreatus* [9]. The lower levels of laccase in the supplemented treatments during the vegetative mycelium growth (10 and 20 days) imply the negative effect of supplementation on laccase enzyme during mycelial stage (Table 4). The activity of laccase enzyme was however relatively higher in T1 and T2 during primordial and fruiting phases. Besides, manganese peroxidase activity was higher in T2 than T1 treatment during all the growth stages of *P. ostreatus*, indicating the positive effect of coffee grounds on the enzyme. Activity levels of lignocellulolytic enzymes significantly varied within the each of the treatments with xylanase being the most active enzyme in all the treatments (Table 4 and 5). It is worth noting that at day 20 of mycelia growth, activities of the FPase, CMCase, and glucosidase were significantly higher in both T1 and T2. This indicates the inductive effect of the cornmeal and coffee supplements on cellulases during mycelial growth of *P. ostreatus*. The level of majority of enzymes in T3 was lower as compared to T1 and T2, demonstrating that excessive supplementation does not necessary enhances enzyme secretion, vegetative growth of mycelium or even productivity of *P. ostreatus*. In fact, T3 generated the lowest activities of laccase, manganese peroxidase, FPase, and xylanase at days 10 and 20, whereas β-glucosidase was lowest in T3 at day 10 of mycelia development. The lower levels of these enzymes in T3 could have contributed to reduced mycelium growth rate and thus taking longest period to colonize substrate in T3.

Primordia formation is an important developmental stage in *P. ostreatus* that encompasses a wide range of metabolic processes and enzymatic activities. During primordial stage the mycelium transitions to pin heads which develop into mature fruiting bodies. The enzyme activities of FPase, β-glucosidase, xylanase, and amylase enzymes remained relatively higher in T1, T2 and T3 during primordia and fruiting body formation (Table 5). Corn supplementation (T1) generated fruiting bodies with highest concentrations of FPase, xylanase, xylosidase and amylase. It is the same treatment (T1) that produced highest number of fruiting bodies and best yield. The high concentrations of hydrolytic enzymes during fruiting were consistent with findings of [40], who reported significant levels of these enzymes when P. ostreatus was cultivated on wheat straw and tree leaves. Activity of laccase, CMCase, Bgl and xylosidase was generally lower in fruiting bodies from CK when compared to supplemented treatments (Supp. 2). We also observed high levels of MnP and CMCase in mycelia, primordia and fruiting from coffee supplemented substrate, this suggests that ground coffee induces these enzymes during *P. ostreatus* growth. Amylase levels were consistently highest in T1 during mycelial, primordial and fruiting stages attributed to high starch contents from corn. Except for manganese peroxidase, the enzyme levels were relatively lower in non-supplemented treatment (CK) during both primordia and fruiting stages. This suggests that supplementation of substrates with agricultural products differently affects lignocellulolytic enzymes in *P. ostreatus*. Various agricultural supplements have different effects on the mycelial, primordia and fruiting body due to different contents. In addition the general observation is that supplements may generate different yields and composition of *P. ostreatus*. This study provides useful insights on how readily available agro-wastes can be tapped for mushroom production. Furthermore, understanding on how different supplements influence lignocellulolytic enzymes can be employed in biotechnological applications to optimize enzyme production and P. ostreatus yield. The non-sterile method of preparing substrates that were used in this study could also be of great benefit to small scale mushroom farmers.

CONCLUSION

In summary, supplements used in this study differentially affected lignocellulolytic enzyme activities, mycelium growth, primordia, fruiting development, and biological efficiency of *P. ostreatus*. During mycelia stage, lignolytic enzymes activity was significantly low in cornmeal supplementation although laccase activities increased during reproductive stage. High level of xylanase in the treatments was attributed to large content of hemicellulose in the substrate and supplements. The supplements prolonged the mycelia colonization period and decreased the polysaccharide content of the fruiting bodies. Cornmeal supplement induced amylase activity in all growth stages of *P. ostreatus*. Compared to ground coffee, cornmeal gave the best results in terms of biological efficiency and polysaccharide content.

AUTHOR CONTRIBUTIONS

E.J.O. and L.H. and R.Y conceived the study design. E.J.O., I.D.L., and Y.Z. conducted all experiments. E.J.O. drafted the first manuscript and W.J.N., L.J, improved the manuscript, while L.Z provided supervision and fund acquisition. All authors have read and agreed to the published version of the manuscript

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare

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