J Ind Microbiol Biotechnol (2009) 36:1353–1362
DOI 10.1007/s10295-009-0620-1

ORIGINAL PAPER

Lignocellulolytic enzyme activity, substrate utilization, and mushroom yield by *Pleurotus ostreatus* cultivated on substrate containing anaerobic digester solids

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Received: 23 November 2008 / Accepted: 30 June 2009 / Published online: 18 July 2009
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Abstract Solid waste from anaerobic digestion of litter from the commercial production of broiler chickens has limited use as fertilizer. Its disposal is a major problem for digester operators who are seeking alternative use for anaerobic digester solids, also referred to as solid waste (SW). The use of SW as substrates for the cultivation of *Pleurotus ostreatus* strain MBFBL400 was investigated. Lignocellulolytic enzymes activity, substrate utilization, and mushroom yield were evaluated in ten different substrate combinations (SCs) containing varying amounts of solid waste, wheat straw, and millet. Nutritional content of mushrooms produced on the different substrates was also determined. Substrates containing 70–80% wheat straw, 10–20% SW, and 10–20% millet were found to produce the highest mushroom yield (874.8–958.3 g/kg). Loss of organic matter in all SCs tested varied from 45.8% to 56.2%, which had positive correlation with the biological efficiency. Laccase, peroxidase, and carboxymethylcellulase (CMCase) activities were higher before fruiting, whereas xylanase showed higher activities after mushroom fruiting. SW increased the nutritional content in mushrooms harvested, and the combination of wheat straw and SW with millet significantly improved mushroom yield. Our findings demonstrated the possibility of utilizing anaerobic digester solids in mushroom cultivation. The application of SW as such could improve the financial gains in the overall economy of anaerobic digester plants.

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Keywords *Pleurotus ostreatus* mushroom · Poultry litter · Lignocellulolytic enzymes · Lignocellulose degradation · White-rot fungi

Introduction

The cultivation of edible mushrooms on agricultural residues is one of most the efficient methods for recycling these waste products [25]. Animal beddings and residues have been used for mushroom cultivation [30, 37] but only to a limited extent. Animal wastes, poultry litter, and other agricultural residues have been used more frequently for mushroom cultivation. Supplementation of rice straw substrate with poultry litter is reported to be effective in increasing mushroom yield in *Pleurotus sajor-caju* [6]. In West Virginia, USA, poultry is the number one agricultural product measured by annual sales. The litter produced throughout the last 10 years averaged about 100,000 tons/year.

Anaerobic digestion has been used for decades to degrade wastes and produce effluents, which consist of a protein- and nutrient-rich slurry and solid waste (SW), high in organic matter that is suitable as fertilizer or soil amendment [39]. A more recent application of anaerobic digestion of animal waste is generating energy [15]. Digested sludge has been proven superior to farm manures or balanced nitrogen–phosphorous–potassium (NPK) chemical fertilizer in improving the growth of grass and potatoes [3]. The litter from the commercial production of broiler chickens, when subjected to anaerobic digestion, results in a liquid effluent and SW derived from the woodchip bedding that is rich in lignocellulose. SW is widely used in China and India for subsistence agriculture [39]. In Taiwan, digested effluent is used to grow crops and fruit

[10]. Liedl et al. [24] reported the use of liquid effluent from anaerobically digested poultry litter in the hydroponic cultivation of tomatoes and lettuce.

Pleurotus spp. are the third most commonly cultivated mushroom in the world and are known to have high nutritional values [43]. These fungi are able to colonize and degrade a wide variety of lignocellulosic residues and require a shorter cultivation period than other edible mushrooms. White-rot fungi, including *P. ostreatus,* produce a wide range of enzymes (laccase, peroxidases, cellulases, and xylanases) that degrade lignocellulose [23]. There are reports of lignocellulose-degrading enzymes in *P. ostreatus* [18] during cultivation on different lignocellulosic substrates. Enzyme activities change considerably during the shift between substrate colonization and fructification stages of mushroom growth, with the periodic flushing of *Agaricus bisporus* enzyme activities fluctuate in approximately weekly cycles [12, 31].

Various authors have tried to establish correlations between lignocellulose degradation and lignocellulolytic enzymes synthesis [14, 44], biological efficiency, and lignocellulose degradation [22, 46]. However, there is no clear understanding of how different substrates under different cultivation conditions affect lignin, cellulose, and hemicellulose degradation vis-à-vis enzyme production in *P. ostreatus*. There is limited knowledge on the use of SW from anaerobic digestion of poultry litter for *P. ostreatus* cultivation. The aim of this study was to investigate the applicability of substrate combinations (SCs) containing SW, wheat straw, and millet in *P. ostreatus* cultivation and the relationship between mushroom production and lignocellulolytic enzyme activities.

Materials and methods

Fungus and inoculum preparation

Pleurotus ostreatus MBFBL400 was obtained from the Mushroom Biology and Fungal Biotechnology Laboratory (MBFBL) culture collection at North Carolina A&T University, NC, USA. Stock cultures of this fungus are maintained on potato-dextrose agar (PDA) at 4°C. Agar inoculum was made on PDA medium, prepared following the manufacturer's instruction (DIFCO, USA). Briefly, 39 g of PDA powder dissolved in 1 L distilled water, autoclaved at 121°C for 15 min, and poured into sterile petri dish plates (90 cm diameter) after cooling to 55°C and in a laminar flow hood. Each petri dish containing about 40 ml PDA medium was inoculated with test fungi and incubated at 25°C for 7 days before use. To make grain spawn, wheat grain was soaked in water for 6 h, drained, and mixed with 3 g/kg calcium carbonate ($CaCO_3$). The resulting substrate contained about 70% H_2O by weight. Grain was loaded (300 g) into micropore-fitted polyethylene bags (47 × 17 cm, type 4, Unicorn Bags, TX, USA), which were autoclaved for 3 h at 121°C. Each bag was inoculated with agar blocks cut out of the entire precolonized medium in a petri dish plate described above and incubated at 25°C for 7 days before use to inoculate the different SCs tested.

Preparation of substrates and experimental design

Solid waste, a component of anaerobic digester effluents, was collected from a thermophilic (56.6°C) anaerobic biodigester located on the campus of West Virginia State University. The feedstock for the anaerobic digester was broiler litter from wood-chip-based bedding [24]. The tank volume was 30 m^3. The daily feed rate was approximately 1 m^3/day of 8% solids [50–100 g/L chemical oxygen demand (COD)]. So a daily feed rate of 1 m^3 in a 30-m^3 reactor = 30-day retention time. Prior to use, the SW was air dried, during which time excess ammonia was volatilized (as indicated by smell). Table 1 shows other parameters of SW from the anaerobic digester used in the experiment. Wheat straw was obtained from the North Carolina A&T State University farm and was chopped to approximately 5-cm pieces using a common yard shredder. Millet was obtained from a local grain supply store.

The different combinations of SW, wheat straw, and millet used as cultivation substrates are shown in Table 2. The experimental design for this study was a single-factor randomized design with ten levels of the factor SCs. The ten factor levels were stratified into two groups: substrates without millet (SC1–SC6, with SC1: wheat straw 100%

Table 1 Characteristics of solid waste from anaerobic digester of poultry litter

Parameter	Mean value
Carbon oxygen demand	49.626 mg/L
Biochemical oxygen demand	12.478 mg/L
Volatile acids	3.681 mg/L
Ammonia	1.237 mg/L
Alkalinity ($CaCO_3$)	6.210 mg/L
Total solids	5.46%
Volatile solids	74.7%
Protein	13.8%
Crude fat	0.43%
Water-soluble carbohydrate	1.25%
Lignin	13.4%
Cellulose	22.5%
Hemicellulose	10.5%
pH	6.78

serving as the control for group 1) and substrates with millet (SC7–SC10, with SC7: wheat straw 80%, millet 20% serving as control for group 2). Substrates within each group had an increasing proportion of SW (ranging from 0% to 100%) for group 1, whereas, group 2 had SW increments of 0–20%. Each SC was replicated in four bags, and a random sample was taken from each bag to obtain a total of 40 experimental units. Type 422-l A cultivation bags (93 × 36 cm; Unicorn Bags) containing 1.5 kg each (dry weight) of the SCs were prepared. Each SC was mixed and moisture content adjusted to 70% before use. Substrate bags were autoclaved at 121°C for 3 h and allowed to cool. Each bag was inoculated with approximately 5% (w/w) of the grain spawn described above. Inoculated bags were incubated at 25 ± 2°C. Upon full colonization, holes (15 mm) were punched into the bags, and the bags were transferred to a fruiting room (80–85% relative humidity and 20–23°C). Mushroom fruit bodies in each bag were manually harvested from the substrate and weighed. After two flushes, the total mushroom yield was calculated. The mushrooms were dried at 50°C and analyzed for nutritional content. Lost organic matter (LOM) was calculated as the percent difference in dry weight between the test substrate and the control (un-inoculated substrate). Biological efficiency (BE) was determined by the ratio of fresh mushroom harvested to the substrate dry weight used in the study. A repeat experiment using one of the best performing substrate combinations (SC9) was conducted to determine extended fruit-body yield during five flushes.

Samples of substrates (50 g each) were taken from the cultivation bags before and after mushroom fruiting. Each sample was extracted with 300 ml sodium acetate buffer (50 mM, pH 5.0) for 2 h at 4°C and filtered through Whatman 1 paper. Samples of extracts obtained were stored in 4°C before analyses. The solid biomass (spent mushroom substrate) that remained after filtration was dried at 50°C until a constant mass was achieved and then weighed to obtain dry weight, after which the four replicates per treatment were combined, milled, and analyzed for protein and macromolecular contents (lignin, cellulose, and hemicellulose).

Analysis of nutritional content in mushroom and substrate combinations

Dry mushrooms were analyzed for water-soluble carbohydrate (WSC) and crude fat content. The crude protein and fat were detected by official methods of analysis [1]. The conversion factor of total nitrogen to protein in the mushroom samples was 4.38 [7]. The C/N ratio in SCs was analyzed with Perkin Elmer 2400 Elemental Analyzer. Lignin, cellulose, and hemicellulose components were analyzed by the Van Soest et al. [45] method for dietary fiber. Determination of WSCs involved reacting extracts

Table 2 *Pleurotus ostreatus* MBFBL 400 mushroom production and biological efficiency, carbon/nitrogen (C/N) ratio and loss of dry matter in different substrate combinations

SCs no.	Substrate content	Total yield	Biological efficiency	C/N ratio	Loss of dry matter (%)	
					Before fruiting	After fruiting
Group 1						
SC1	Wheat straw 100	545.2 ± 79.3[a]	54.6 ± 7.2[a]	96/1	49.7 ± 1.1[a]	52.3 ± 3.8[a]
SC2	Wheat straw 90; SW 10	512.3 ± 67.3	51.2 ± 6.9	88/1	45.9 ± 1.6*	49.3 ± 0.7
SC3	Wheat straw 75; SW 25	511.9 ± 81.3	51.2 ± 7.5	75/1	48.9 ± 1.6	49.9 ± 0.8
SC4	Wheat straw 50; SW 50	193.3 ± 56.7*	19.4 ± 4.2*	56/1	47.9 ± 1.5*	48.1 ± 0.9*
SC5	Wheat straw 25; SW 75	7.7 ± 4.8*	0.78 ± 0.2*	30/1	48.7 ± 1.5*	45.8 ± 1.6*
SC6	SW 100	0	0	13/1	46.7 ± 1.9*	46.0 ± 2.4*
Group 2						
SC7	Wheat straw 80; millet 20	646.8 ± 65.3[b]	64.7 ± 4.3[b]	81/1	50.5 ± 1.6	51.6 ± 2.5[b]
SC8	Wheat straw 70; SW 10; millet 20	874.8 ± 150.7	87.5 ± 12.3	73/1	53.1 ± 2.3	53.2 ± 0.9
SC9	Wheat straw 70; SW 20; millet 10	958.3 ± 159.3*	95.8 ± 15.2*	72/1	53.4 ± 0.2	56.2 ± 1.4*
SC10	Wheat straw 80; SW 10; millet 10	931.0 ± 123.3*	93.2 ± 18.1*	81/1	49.1 ± 5.0	55.1 ± 1.3*

Total yield representing fresh weight (grams) of mushroom fruiting bodies produced per kilogram dry substrate. The data of C/N ratio, biological efficiency, and loss of dry matter are presented as percentage. Result are expressed as mean ± standard error (SE) of $n = 4$. Number in each substrate content in column 2 represents percentage (%) of substrate component

SCs substrate combinations, *SW* solid waste

[a, b] Controls

* Significantly different ($P = 0.05$) from respective controls

from test samples with 1 N sulfuric acid for 20 min in a boiling-water bath, followed by cooling and neutralizing [13]. The amount of reducing sugars was determined spectrophotometrically with potassium ferricyanide [8, 16]. Substrate and mushrooms materials used for analysis to determine C/N ratio, WSC, protein, fat, and macromolecules were pooled samples (equal proportions) from four replicates. Analysis for lignocellulose, crude protein, total fat, and WSC was conducted at the Rumen Fermentation Profiling Lab, West Virginia University, WV, USA.

Enzyme activity assay

The extract used for all enzyme assays was obtained as described above. Sample extracts were diluted five to ten times, depending on the concentration and color. When necessary to correct for color interference, un-inoculated control diluted as per sample extracts were measured and values obtained deducted from spectrophotometric values obtained for sample extracts. Laccase activity was determined following the oxidation of 2,2-azino-*bis*-ethylbenthiazoline (ABTS) as a substrate at 420 nm in 100 mM sodium acetate buffer (pH 5.0) [21]. The reaction mixture (1 ml) contained 0.1 ml diluted culture filtrate. Peroxidase activity was assayed by the oxidation of Phenol Red in sodium lactate-succinate buffer (pH 4.5) [19]. Absorbance was read at 610 nm. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of substrates at 24–25°C. The volume of culture extract used was 0.2 ml/1 ml reaction.

Carboxymethylcellulase (CMCase) activity was assayed with a 1% solution of carboxymethylcellulose as an enzyme substrate in 0.05 M citrate buffer (pH 5.0) according to the International Union of Pure and Applied Chemistry (IUPAC) recommendations [17]. The reaction mixture was incubated for 10 min in a buffer at a temperature of 50°C. Xylanase activity was assayed using a 1% solution of xylan from birch wood (Roth 7500) as a substrate in 0.05 M citrate buffer (pH 5.0). The release of glucose and xylose, respectively, in 10 min at 50°C were measured using the dinitrosalicylic acid method [2]. Culture extract (0.1 ml) was used in 1 ml enzyme reaction. One unit of enzyme activity is defined as 1 μmol of glucose or xylose equivalents released per minute under the given conditions.

The activities of exo-1,4-β-glucanase (EC 3.2.1.4), 1,4-β-glucosidase (EC 3.2.1.2), and 1,4-β-xylosidase (EC 3.2.1.37) were determined by measuring the rate of hydrolysis of *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-glucopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside as described by Poutanen and Puls [34]. The reaction mixture containing 1.8 ml of 2.5 mM substrate and 0.2 ml of the culture extract filtrate was incubated at 50°C for 10 min. The reaction was stopped by the addition of 1 ml of 1 M sodium carbonate (Na_2CO_3) into the reaction mixture. The enzyme quantity catalyzing the release of 1 μmol of *p*-nitrophenol per minute under these conditions was considered to be a unit of enzyme activity.

Statistical analysis method

The following variables were measured in the cultivation of *P. ostreatus* 400 under the ten treatment conditions (SCs containing wheat straw, SW, and millet): total mushroom yield, BE, LOM, and enzyme activity in the substrates before and after mushroom fruiting. All results were obtained in four replicates, and data are expressed as means. The data were analyzed using analysis of variance (ANOVA) to determine significant differences in each group. The statistical analysis was done using the SAS System (the SAS System for Windows 8.0). When the overall F ratio was found to be statistically significant ($\alpha = 0.05$; $P < 0.05$) for both groups, only then were multiple comparison t tests [Fisher's protected least significant difference (LSD)] conducted within each group to compare each treatment mean with the control for that respective group. A correlation analysis was calculated using Excel (Microsoft).

Results and discussion

Mushroom yield, biological efficiency, C/N ratio, and loss of organic matter

The content of different SCs tested, mushroom yield, biological efficiency, C/N, and loss of organic matter are presented in Table 2. Vigorous mycelia growth was observed in all SC with 25% SW or less (SC1–SC3, SC7–SC10), with mycelia achieving full colonization within 28 ± 2 days. It took 33 ± 1 days to achieve full colonization in SC containing 50% SW or more (SC4–SC6), and mycelial growth was less vigorous (visual estimation) in SC5 and SC6 than in SC4 (Table 2).

Substrates containing wheat straw and SW only (SC2–SC5; group 1) produced a lower mushroom yield than the control (SC1). BE in this group decreased with increasing SW content in the substrate. However, in group 2, a significantly higher mushroom yield was obtained in SC8, 9, and 10 compared with the control in this group (SC7). SC9 gave highest yield (958.3 g/kg), which was 32.5% higher than SC7 (Table 2). The LOM after mushroom fruiting ranged from 45.8% to 56.2%. The maximum loss was in SC9 (56.2%), followed by SC10 and SC8 (55.1% and 53.2%, respectively); the least LOM occurred in SC5 and SC6 (Table 2). The repeat mushroom production

Table 3 Nutritional content of mushroom and substrate combinations of *Pleurotus ostreatus* 400

SC no.	Crude protein			Mushroom		
	Untreated substrate	Treated substrates		Crude protein	Water-soluble carbohydrates	Crude fat
		Before fruiting	After fruiting			
SC1	3.67	7.9	9.6	30.0	24.8	0.31
SC2	4.67	10.1	10.6	30.3	25.3	1.95
SC3	6.16	10.3	9.5	31.5	27.1	1.04
SC4	8.65	12.3	12.8	35.1	24.2	0.87
SC5	11.14	12.4	14.1	–	–	–
SC6	13.63	14.6	16.2	–	–	–
SC7	5.23	6.5	7.5	27.5	9.9	1.00
SC8	6.23	10.4	10.4	33.6	24.7	1.29
SC9	5.44	10.1	11.9	31.2	17.7	0.71
SC10	5.45	9.4	10.5	29.2	22.7	1.04

The data on crude protein, water-soluble carbohydrates, and crude fat content are presented as percentages in substrates or mushroom dry weight

SCs substrate combinations

experiment conducted using SC9 over a 115-day fruiting cycle produced a total yield (five flushes) of 1,815.1/ kg substrate and BE of 181.5%.

Zhang et al. [47] showed 128% BE for *P. sajor-caju* cultivated on rice straw and 97% on wheat straw substrates. The combined harvest from two flushes of *P. ostreatus* grown on grass and coffee pulp produced a BE that varied between 59.8% and 93% [20]. Banik and Nandi [6] observed a much higher yield (186% BE) in *P. sajor-caju* cultivated on biogas residual slurry manure mixed with rice straw in a 1:1 ratio.

In general, the results indicate that SW supplementation of wheat straw alone (group 1) may not result in increased mushroom yield of *P. ostreatus*, despite the fact that SW introduced nutrients to improve substrate degradation and utilization. In fact, any substrate that contained wheat straw and SW (group 1) had a lower yield compared with the control (SC1), and the yield gradually decreased to almost zero at 75% SW in substrate (SC5). It appears that the inclusion of millet (group 2) is needed for the effective use of substrate components (wheat straw and SW) to achieve high BE. It is possible that more time is needed for better utilization and conversion of the available nutrients in SCs in group 2 in order to obtain maximum yield and BE, as represented in repeat cultivation with SC9.

Adequate C/N ratio is critical to the rate of lignocellulose degradation and mushroom production [26, 33]. SW consists of 3.1% nitrogen, and SCs with higher content of SW resulted in low C/N ratio (13/1–30/1). The SCs that produced highest yields (SC9 and SC10) had C/N ratios of 72/1 and 81/1, respectively (Table 2). These values are within the C/N ratio 32/1–150/1 range reported by Chang and Miles [9] to be effective for primordial induction in *Pleurotus* spp. It is clear that higher amounts of supplementation with SW can drive the C/N ratio and nutrient contents to levels that are counterproductive for fruit body production in *P. ostreatus.*

Changes in nutritional parameters in substrates and fruit bodies

Crude protein in SCs tested and mushrooms harvested varied depending on SW content. The crude protein level (total nitrogen content) in the spent substrate increased with increasing SW content. The highest increase in crude protein was recorded in SC1, SC2, and SC9, with values that were 2.6, 2.3, and 2.1 times higher, respectively, than the untreated substrate. Mushrooms (fruit bodies) from SCs 8–10 contained 29.2–33.6% crude protein, which is within the range (18–35%) reported by other investigators [11, 27, 29]. However, Wang et al. [46] obtained 53.3% crude protein in *P. ostreatus* cultivated on spent beer grain.

WSC was highest in mushrooms from group 1 (SC1–SC4). Mushrooms from SC7 in group 2 had the lowest WSC (9.9%), as shown in Table 3. The carbohydrate contents obtained in our study were lower than reported by other investigators and data from the Standard Tables of Food Composition of Japan [29, 40].

Solid waste content did not significantly affect the lipid content of mushrooms (Table 3). Mushrooms from SC2 had the highest lipid content (1.95%). Reported lipid contents in oyster mushrooms range from 1.0% to 2.4% [5], although much higher lipid content in *P. ostreatus* (4.6%) was reported by Wang et al. [46].

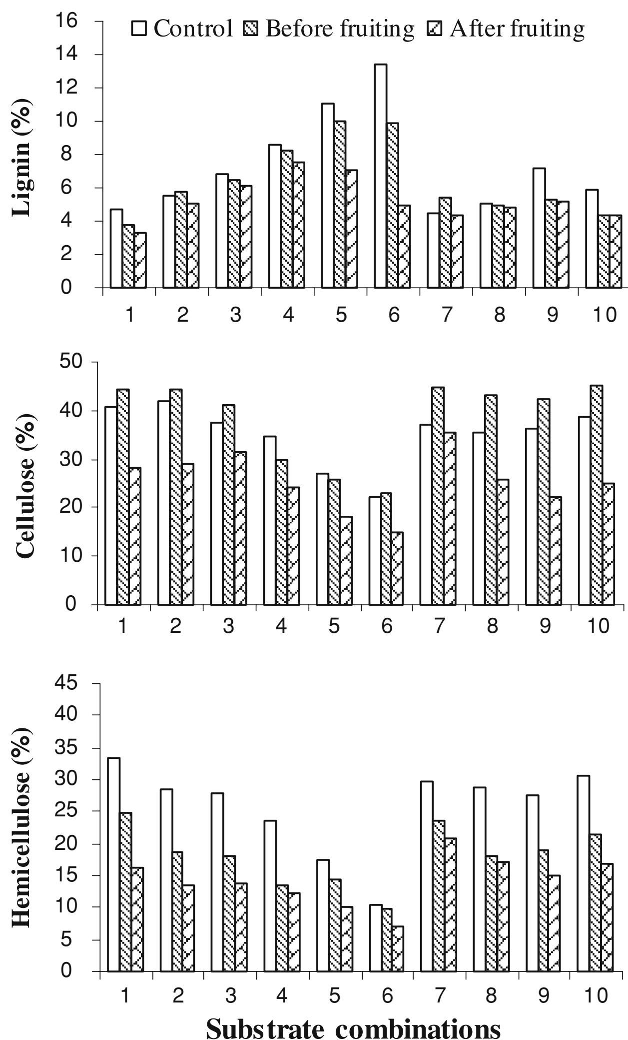

Fig. 1 Lignin, cellulose, and hemicellulose contents in substrate combinations: control (un-inoculated), before and after mushroom fruiting (percent dry weight)

Lignin, cellulose, and hemicellulose degradation

The lignin, cellulose, and hemicellulose composition in SCs is shown in Fig. 1. Lignin degradation varied widely among the SCs tested, ranging from 2.2% to 46.4%. An increase in SW content increased lignin degradation. The highest lignin loss occurred in SC5 (36%) and SC6 (46.4%), although these SCs had the lowest (7.7 g/kg) or zero mushroom yield, respectively. A negative correlation was recorded between BE and lignin degradation. Nicolini et al. [28] observed that *P. ostreatus* degraded 50–60% of the lignin in orange peels and grape stalks. Our results are consistent with the findings of Wang et al. [46], which indicated that a rapid and high degree of lignin degradation did not support the highest yield in *P. ostreatus*.

Cellulose content in un-inoculated SCs ranged between 22.1% and 41.9% (Fig. 1). Degradation of cellulose after mushroom fruiting varied from 4% (SC7) to 39% (SC9). Hemicellulose content in un-inoculated SCs ranged between 10.5% and 33.4%. Hemicellulose degradation varied from 30.0% (SC7) to 52.1% (SC2) among all SCs tested. In SC9, which gave the highest yield, cellulose and hemicellulose losses were 39% and 46%, respectively. In general, it appears that hemicellulose is more easily utilized than cellulose (Fig. 1). Our results are consistent with those reported previously, wherein hemicellulose was also degraded preferentially with respect to cellulose during the cultivation of *P. ostreatus* on wheat straw [42]. Similarly, Wang et al. [46] reported that cellulose was poorly degraded by *P. ostreatus* cultivated on spent beer grains. Data obtained showed that there was a positive correlation between BE and cellulose and hemicellulose degradation, whereas a negative relationship was observed between BE and lignin degradation.

Ligninolytic enzyme activity

Laccase and peroxidase activities were detected in all SCs tested and were highest before (28–33 days after inoculation) than after (58 days) fruiting, independent of the substrate used for cultivation (Table 4). In group 1, SC2–SC5 SW content was positively correlated with laccase activity ($P < 0.05$), and the highest activity occurred in SC5 (164.6 U/L). In group 2, SC9 produced the highest laccase activity 147.8 U/L. Manganese peroxidase (MnP) and peroxidase enzymes activity showed trends similar to laccase before fruiting: MnP activity varied from 5.6 U/L (SC8) to 28.8 U/L (SC6) and peroxidase activity from 2.2 U/L (SC2) to 40.8 U/L (SC6). SC7 had the lowest enzyme activity of all three enzymes. After mushroom fruiting, both peroxidase activities, except those in SC1, SC2, and SC7, decreased (Table 4).

Lignocellulolytic enzyme activities were affected significantly by changes in the SW content of SCs tested; increasing SW content resulted in increased laccase and peroxidases enzymes production. Our results are consistent with the findings of Rajarathnam et al. [36], who reported that laccase reached maximum activity at the beginning of fructification and decreased thereafter. However, by comparing laccase and peroxidase enzyme activities (Table 4) with lignin degradation (Fig. 1), it is obvious that changes in laccase and peroxidase enzymes were associated with lignin degradation. There was a negative correlation between enzyme activity and fruit body production ($P < 0.05$).

Hemicellulase and cellulase activity

Xylanase activity varied from 0.51 to 1.89 U/ml. An increase in SW content, between 10% and 100%, did not

Table 4 Ligninolytic enzymes activities of *Pleurotus ostreatus* 400 before and after fruiting periods

SCs no.	Laccase		Manganese peroxidase (MnP)		Peroxidase	
	Before fruiting	After fruiting	Before fruiting	After fruiting	Before fruiting	After fruiting
SC1	100.3 ± 8.2[a]	53.8 ± 19.5[a]	4.9 ± 2.7[a]	6.6 ± 2.1[a]	2.6 ± 0.8[a]	5.8 ± 2.4[a]
SC2	111.4 ± 15.8	39.4 ± 3.7	5.6 ± 1.9	7.1 ± 1.0	8.0 ± 1.2	12.1 ± 1.6*
SC3	127.6 ± 44.2*	11.6 ± 4.3*	16.7 ± 3.9*	2.4 ± 0.9*	23.9 ± 4.8*	5.5 ± 2.1
SC4	133.1 ± 16.9*	67.1 ± 9.4	19.6 ± 2.5*	5.4 ± 1.5	28.2 ± 4.1*	12.1 ± 3.8*
SC5	164.6 ± 20.2*	23.5 ± 2.4*	21.9 ± 4.1*	4.3 ± 1.2	34.3 ± 6.7*	8.4 ± 1.6
SC6	142.9 ± 13.4*	52.2 ± 9.6	28.8 ± 9.2*	4.9 ± 2.6	40.8 ± 11.1*	2.7 ± 1.3*
SC7	65.3 ± 8.3[b]	37.7 ± 1.2	1.7 ± 1.1[b]	5.7 ± 2.9	2.2 ± 0.8[b]	2.4 ± 1.2
SC8	55.3 ± 15.3	35.1 ± 3.4	6.1 ± 1.9*	4.1 ± 1.5	15.2 ± 5.1*	2.5 ± 1.7
SC9	147.8 ± 7.9*	49.3 ± 14.0	8.2 ± 1.2*	4.1 ± 0.7	19.6 ± 2.7*	2.8 ± 0.3
SC10	63.3 ± 15.0	50.5 ± 12.3	10.8 ± 5.3*	4.4 ± 1.5	27.8 ± 14.5*	3.7 ± 1.2

Enzyme data are presented in units per liter (U/L). Results are expressed as mean ± standard error (SE) of $n = 4$

SCs substrate combinations

[a, b] Controls

* Significantly different ($P = 0.05$) from respective controls

Table 5 Hemicellulolytic enzyme activities in *Pleurotus ostreatus* MBFBL 400 before and after fruiting periods

SC no.	Xylanase		1,4-β-xylosidase	
	Before fruiting	After fruiting	Before fruiting	After fruiting
SC1	1.89 ± 0.28[a]	1.12 ± 0.34[a]	1.7 ± 0.3[a]	2.8 ± 0.8[a]
SC2	0.71 ± 0.21*	2.71 ± 1.49	1.8 ± 0.4	4.9 ± 1.1*
SC3	0.51 ± 0.23*	8.30 ± 2.83*	1.3 ± 0.4	3.8 ± 0.8*
SC4	1.16 ± 0.24*	7.46 ± 1.43*	2.1 ± 0.3	2.9 ± 0.5
SC5	1.01 ± 0.22*	2.94 ± 1.53*	2.2 ± 0.5	3.7 ± 0.7
SC6	1.07 ± 0.21*	0.67 ± 0.30	2.6 ± 0.6*	1.0 ± 0.5*
SC7	0.82 ± 0.17[b]	1.46 ± 0.37[b]	2.2 ± 0.7	2.9 ± 0.4
SC8	1.74 ± 0.50*	4.62 ± 1.62*	1.7 ± 0.3	3.1 ± 0.7
SC9	1.70 ± 0.34*	3.96 ± 1.43*	1.4 ± 0.7	2.8 ± 0.6
SC10	1.64 ± 0.22*	2.82 ± 1.02	1.3 ± 0.4	2.6 ± 0.6

Enzyme data are presented in units per milliliter (U/ml). Result are expressed as mean ± standard error (SE) of $n = 4$

SCs substrate combinations

[a, b] Controls

* Significantly different ($P = 0.05$) from respective controls

result in a linear increase in enzymatic activities ($P = 0.05$; Table 5). Enzyme activities increased in most of the SCs tested after mushroom fruiting; SC3 and SC4 had the highest activities (8.3 and 7.5 U/ml, respectively), whereas SC6 had the lowest levels (0.67 U/ml). SC9 and SC10 (group 2) had twice the xylanase activities than control (SC7), and after mushroom fruiting, enzyme activity increased 1.7–2.7 times over the values obtained before fruiting. In all SCs tested, 1,4-β-xylosidase enzyme activity varied between 1.3 and 2.6 U/ml before fruiting. However, except in SC6, enzyme activities in all other SCs increased 1.3–2.9 times by the end of the second flush. Positive correlation was observed between fruit body production and xylanase activity in most SCs ($P = 0.05$). Our results from xylanase activities support the finding of Rajarathnam [35] and Terashita et al. [41], who observed that xylanase activity increased during vegetative mycelial growth and reached its maximum activity after cropping of *P. flabellatus* and *P. sajor-caju*.

Higher CMCase activities were observed before fruiting (Table 6); SC1 had the highest activity (1.08 U/ml), and the least activity occurred in SC6 (0.26 U/ml). Increasing the amount of SW between 20% and 100% resulted in decreasing CMCase production (group 1; $P = 0.05$). In group 2, SC9 and SC10 with 10% millet had 2.2 and 1.9 times higher CMCase activities, respectively, compared with the control (SC7). After mushroom fruiting (58 days), enzyme activities decreased considerably. Production of 1,4-β-glucosidase was also affected by the percentage of SW content in the substrate. Furthermore, 1,4-β-glucosidase activities increased after mushroom fruiting: 3.2-fold higher in SC8 and SC9, and 3.4-fold higher in SC10 than the control (SC7). Exo-1,4-β-glucanase activities did not appear to be affected by the amount of SW content in the substrate (Table 6).

Increasing SW content in the substrates resulted in decreased CMCase activity, indicating that SW may inhibit the production of this enzyme. CMCase activities also decreased after mushroom fruiting in most SCs tested. It is known that high CMCase activities are associated with the fruiting period, and Ohga et al. [32] reported that cellulase messenger RNA (mRNA) transcripts were maximally

Table 6 Cellulolytic enzyme activities in *Pleurotus ostreatus* 400 before and after fruiting periods

SCs no.	CMCase		Exo-1,4-β-glucanase		1,4-β-glucosidase	
	Before fruiting	After fruiting	Before fruiting	After fruiting	Before fruiting	After fruiting
SC1	1.08 ± 0.21[a]	0.78 ± 0.17[a]	1.9 ± 0.5[a]	2.8 ± 1.2[a]	3.2 ± 0.7[a]	6.9 ± 1.2[a]
SC2	0.41 ± 0.10[b]*	0.35 ± 0.09*	1.8 ± 0.4	4.7 ± 0.7*	6.2 ± 1.6*	10.2 ± 1.1*
SC3	0.56 ± 0.26*	0.44 ± 0.14*	1.3 ± 0.3	3.3 ± 0.8	6.1 ± 1.4*	11.9 ± 1.2*
SC4	0.54 ± 0.16*	0.46 ± 0.12*	3.1 ± 0.9*	2.1 ± 0.3	6.7 ± 2.1*	12.3 ± 2.3*
SC5	0.28 ± 0.13*	0.18 ± 0.09*	2.2 ± 0.9	2.2 ± 0.5	8.3 ± 3.6*	15.1 ± 4.0*
SC6	0.26 ± 0.23*	0.19 ± 0.08*	2.8 ± 1.1*	1.0 ± 0.4	4.6 ± 2.9	7.7 ± 1.4
SC7	0.46 ± 0.20[b]	0.30 ± 0.12[b]	2.4 ± 1.0	1.8 ± 0.4	3.3 ± 1.6	8.6 ± 1.1[b]
SC8	0.61 ± 0.16	0.46 ± 0.14	1.8 ± 0.3	1.5 ± 0.3	2.9 ± 0.6	9.4 ± 0.8
SC9	0.99 ± 0.20*	0.57 ± 0.13*	2.1 ± 0.5	1.9 ± 0.5	2.7 ± 0.8	9.3 ± 1.2
SC10	0.89 ± 0.06*	0.56 ± 0.06*	2.2 ± 0.6	1.7 ± 0.5	3.1 ± 0.9	10.2 ± 0.5*

Enzymes data are presented in units per milliliter (U/ml). Results are mean ± standard error (SE) of $n = 4$

SCs substrate combinations, *CMCase* carboxymethylcellulase

[a, b] Controls

* Significantly different ($P = 0.05$) from respective controls

expressed at the veil-break stage of fruiting body development. Singh et al. [38] also reported high cellulase productivity in the second month, which occurred during the fruiting phase of the *Pleurotus* spp. There was no correlation between CMCase activities and fruit-body production. Exo-1,4-β-glucanase activity showed positive correlation with fruit-body production, but the results of statistical analysis in the different SCs (group 2) were not significantly different from control ($P = 0.05$). During long-term monitoring of CMCase in lignocellulosic substrates, the activity of this enzyme had undulating peaks of activity that coincided with fruiting. We assume that a 58-day measurement of CMCase, with decreased activity compared with before mushroom fruiting, coincided with a period in the cultivation process when there was no fruiting.

Conclusions

This is the first report that compares the effect of SW supplementation of wheat straw, with or without millet, on lignocellulolytic enzyme production, substrate degradation, and mushroom production in *P. ostreatus*. The results of this study demonstrate that SW from anaerobic digestion of poultry litter can be used as an effective supplement for increasing mushroom yield in *P. ostreatus* 400. The results also suggest that the addition of SW is most effective in combination with millet. Considered together, the results indicate that the use of a lignocellulose-rich SW supplement can improve the nutrient content in substrate composition substantially to benefit the commercial production of oyster mushrooms, particularly when substrates are formulated with millet. Fruiting or mushroom production affected enzyme activities differently: after mushroom fruiting, laccase, peroxidases, and CMCase enzyme activities decreased; xylanase activities increased in the period between before and after fruiting. A recent report on the attempt to apply spent substrate from *P. ostreatus* cultivation points to the potential for additional use of white-rot fungi in resolving the delignification necessary to achieve success in biomass conversion for cellulosic ethanol production [4]. This report demonstrates the enhancement of ligninolytic enzyme production and delignification due to the inclusion of SW in the substrate. Our results also confirm previous observations that *P. ostreatus* selectively use hemicellulose over cellulose in biomass. These findings and observations could be exploited in further research on the possibility of combining SW and white-rot fungi in bioconversion of lignocellulose-rich biomass intended for bioenergy applications.

Acknowledgments This research was supported by USDA grants entitled "Organic Waste Treatment Using Thermophilic Anaerobic Digestion (Bioplex) Phase 5 (grant #2005-38850-02292)" and the WVSU Gus R. Douglass Land-Grant Institute of Agricultural, Consumer, Environmental and Outreach Programs. We are grateful to Monica Haddix for help with statistical analysis, Eric Lopez for experimental work, David LeBauer and the North Carolina A&T Agricultural Research program for technical review, and to the Ag Communications team at NC A&T State University for editorial review.

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