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A Laboratory-Scale Study on the Production of High-Value Products from Broiler Litter Involving Solid-State Anaerobic Digestion and Mushroom Cultivation

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A Laboratory-Scale Study on the Production of High-Value Products from Broiler Litter Involving Solid-State Anaerobic Digestion and Mushroom Cultivation

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Abstract

There is a need to investigate alternate uses and treatments of broiler litter that lessen environmental impacts and decrease costs associated with its disposal. Anaerobic digestion is a biological process in which organic material is converted to a renewable fuel source. However, the substrate for anaerobic digestion often requires some form or pretreatment. Certain types of fungus have been investigated as a pretreatment for anaerobic digestion, one of which is *Pleurotis ostreatus* or the oyster mushroom, which also produces an edible fruiting body. Thus, this study was performed to investigate the use of broiler litter for oyster mushroom cultivation and anaerobic digestion in terms of effects their effects on broiler litter characteristics, effects of mushroom cultivation on anaerobic digestion, and the yields associated with the two treatments.

It was found that the addition of 75% wheat straw was required to culture edible oyster mushrooms using broiler litter and that mushroom yields were larger than those for 100% wheat straw. Mushroom cultivation had either negative to no impacts on subsequent methane yields from anaerobic digestion. However, it was also found that lignin and soluble phosphorus contents could be reduced by mushroom cultivation while soluble nitrogen and extractives contents were increased. It was also found that nitrate concentrations were increased by mushroom cultivation, which could explain the decrease in yields from subsequent anaerobic digestion.

Although methane yields were not increased by fungal pretreatment, it was concluded that the cultivation of oyster mushrooms on broiler litter could have significant impacts by adding more value to the waste material through the production of edible mushrooms and the improvement of fertilizer value. It was also concluded that there is a need for further research to explain the decrease in methane production following the fungal pretreatment, and to possibly

find an additional pretreatment to account for this. Several questions were answered as to the general concept of utilizing broiler litter for both oyster mushroom cultivation and anaerobic digestion, but many other question must be answered before the findings discussed can be used to make recommendations to those involved in the poultry and mushroom industries.

Acknowledgments

I would like to think my major thesis advisor, Dr. Jun Zhu, and thesis advisory committee members, Dr. Thomas Costello and Dr. Wen Zhang, for their advice given throughout the project. I would also like to think Dr. Julie Carrier, Dr. Kalavathy Rajan, Dr. Angele Dijioleu, Ms. Gurshagan Kandhola, Ms. Leidy Santos, Mr. Leland Schrader, and Mr. Thomas Helvick for their technical and laboratory assistance. Thank you to the Biological and Agricultural Engineering Department for supporting this research and thank you to all my professors that prepared me for this achievement.

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1. Introduction

1-1. Broiler Production: Inputs and Impacts

In 2015, Arkansas ranked third in the nation for broiler production by producing a state total of approximately 962 million birds (USDA, 2016a). This amounts to approximately 11% of the nation's total number of broilers produced. Poultry production in Arkansas requires an average of 44 kWh of electricity, requires 5.8 gallons of propane, and results in the production of approximately 1 ton of litter for every 1000 birds produced (U of A Extension Services, 2015). Poultry litter is a byproduct from the production process that is composed of animal byproducts (manure, feathers, etc.) and bedding material (typically any combination of woodchips, rice hulls, straw, or some other woody material). Broiler litter typically forms a crusted layer in poultry houses where the chickens are raised, this layer (called "cake") is removed after every flock, is land-applied and can serve as a crop fertilizer and a soil amendment. However, land application is limited by transportation costs of the material, which requires large amounts of energy if the litter is to be transported great distances, and the accumulation of nutrients (mainly phosphorus) on areas of multiple applications.

If the litter is only applied in a small, localized area, nutrients can accumulate in the soils to a point that causes eutrophication of local waterways, which can have significant impacts on stream ecology, such as increased algal blooms, and can lead to increased risk to human health related to the production of safe drinking water (Conley et al., 2009). This is the cause of a lawsuit between the State of Oklahoma and 14 major poultry-producing companies based in Northwest Arkansas related to the alleged pollution of the Illinois River Watershed caused by the disposal of poultry litter resulting from the production process for these companies (*State of Okalahoma v. Tyson Foods, Inc. et al.*, 2005). Thus, to counteract the environmental and legal

problems associated with wastes resulting from poultry production, the Arkansas State Legislature developed Act 532 of 2007 titled the "Surplus Nutrient Removal Incentives Act" (Arkansas Code § 15-20-1201 through 1206), which established the surplus poultry litter removal incentives cost share program for promoting transport of poultry waste materials out of nutrient surplus areas. However, more could be done to reduce the monetary and environmental costs associated with the disposal of poultry litter by developing cost-effective alternative uses and treatment processes for the material.

More value could potentially be added to the waste litter generated by the large poultry industry in Arkansas by exploring alternative uses and treatment processes for this byproduct. Currently, poultry litter is most commonly used as a soil amendment for agricultural fields and pastures in the vicinity of where the birds are raised (Haggard, 2010). Although poultry litter produced in Arkansas is worth approximately \$62 ton^{-1} w.b.[1] based on nitrogen (N), phosphorus (P), and potassium (K) contents (Sharpley et al., 2009a), little to no profit is typically generated by this use of the material due to regulatory restrictions and transportation costs (Goodwin et al., 2005). These costs can be lowered in several ways, for instance, by utilizing practices to allow for higher application rates or by reducing the total mass of the waste material needing to be disposed of by converting the material into a high-value product. Practices that may allow for higher application rates include reducing water-soluble phosphorus (WEP) content of the material and implementing best management practices to reduce the amount of P present in runoff before entering the stream (Sharpley et al., 2009b). For instance, the addition of chemical amendments, such as alum, has been shown to decrease P concentrations present in runoff resulting from the first rain event following a litter application event (DeLaune et al., 2004). The

[1] suffix "w.b." signifies that values are normalized on the wet material basis

implementation of best management practices (BMPs), such as diversion structures, field borders, and riparian forest buffers can also be used to reduce increased P concentrations in streams resulting from adjacent litter application by either slowing the rate of enriched runoff reaching the stream or by promoting P uptake to take place before the runoff enters the stream.

Although common practices for the disposal of poultry litter in Arkansas are affective at preventing negative environmental impacts that could potentially be caused by the material, improvements could certainly be made related to the efficient and profitable use of the material. According to a study conducted by the University of Arkansas Division of Agriculture and published by the University's extension service which involved analyzing nearly 300 samples taken during the period of 2005 to 2007, the average carbon (C), nitrogen (N), and phosphorus (P) contents of broiler litter are 25.2%, 3.1%, and 1.5%, respectively. From an agricultural standpoint, N is the most valuable component of the material because fertilizer providing this macronutrient is in the highest demand; according to the USDA, N fertilizers accounted for 59% of total plant nutrient consumption in 2011, but P is also of value, accounting for approximately 20% of total plant nutrient consumption in 2011 (USDA, 2016b). Although the addition of C to soil can sometimes improve overall soil health and water retention capabilities, the presence of this element in soil is not essential for plant growth. Thus, removal of C from poultry litter should improve its value as a soil amendment by increasing the content of valuable macronutrients in the material. Removal of C from poultry litter could also potentially improve the economic viability for the use of the material as a fertilizer by reducing transportation costs. Removing C from broiler litter through processes that recover the element in a form that can be used as a carbon-based fuel source can also further improve the economic and environmental viability of the utilization of poultry litter, and thus, the poultry industry as a whole. Social and

environmental impacts of the land application of poultry litter could also be reduced by the removal of C by reducing the addition of BOD originating from the waste material to neighboring streams, which could lessen the restrictions placed on the local WWTPs.

1-2. Anaerobic Digestion

Various processes exist for the conversion of organic material, such as broiler litter, into a practical fuel source. In general, there are thermochemical and biochemical conversion processes. Typically, for wet organic waste materials, such as manure, biochemical conversions processes are more efficient than thermochemical processes due to the energy required in a thermochemical process to evaporate the moisture present in the material. Anaerobic digestion is a biochemical conversion process that results in the production of a renewable fuel called biogas (a mixture of approximately 60% methane (CH_4), 40% carbon dioxide (CO_2), and various trace gasses).

Although the use of anaerobic digestion appears to have the potential to offset the use of fossil fuels, there are issues limiting the application of the technology, such as biomass characteristics of available materials being responsible for either reduced efficiency or complete failure of the conversion process. Such negative biomass characteristics include low carbon to nitrogen (C/N) ratio and high lignin content (Chen et al, 2008). In general, a C/N ratio in the range of 20:1 – 30:1 is acceptable for anaerobic digestion. A C/N ratio below this range can result in the inhibition of methane-producing archaea (methanogens) by increased ammonia (NH_3) concentration, while a C/N ratio above this range can result in the accumulation of volatile fatty acids (VFAs), which can also have inhibitory effects on methanogens (Li et al., 2011). Lignin cannot be degraded in an anaerobic environment, and thus, a higher lignin content of a given material results in lower biogas yields for the material as a whole.

Untreated poultry litter is composed of animal waste and bedding material, which results in a material with a low C/N ratio (typically ~ 8:1) and a significant portion of the material being lignin from the bedding material. However, various pretreatment processes have been investigated to correct for unfavorable biomass characteristics and improve anaerobic digestion of non-ideal feedstocks, including poultry litter. One solution to the problem is codigestion or the digestion of multiple feedstocks mixed in ratios that result in a substrate with more ideal biomass characteristics. For instance, it has been found that anaerobic digestion of poultry litter can be improved by supplementing the litter with straw, which increases the C/N ratio of the feedstock (Zhang et al., 2014). Methods for improving the degradability of (i.e. lowering lignin content) include various thermal, chemical, and biological treatment processes (Zheng et al., 2014). For instance, Theuretzbacher et al. (2015) recently investigated a combined thermo-mechanical and biological pretreatment process to improve both ethanol and biogas production from wheat straw. Although the biological pretreatment process in this study did not significantly improve biogas production, other studies have shown success in the use of biological pretreatment to improve biogas production, which was also linked to a decrease in lignin content by pretreatment (Zheng et al., 2014; Yan et al., 2012; Zhong et al., 2011). Although, a large amount of research is being conducted on the use of biological pretreatment to improve the anaerobic digestion of lignocellulosic materials, not much has been done related to improving the anaerobic digestion of poultry litter through biological pretreatment.

The purpose of the pretreatment of biomass in an energy conversion process is typically to make organic compounds more biologically available to the microbes responsible for the conversion process. In general, structural carbohydrates such as cellulose, hemicellulose, and lignin are the compounds present in biomass that are the most resistant to biological degradation.

In anaerobic digestion, cellulose and hemicellulose are degraded through hydrolytic enzymes produced by the bacteria present, this step typically being the rate-limiting step for the entire conversion process. Lignin, however, cannot be biologically degraded in an anaerobic environment because ligninolytic enzymes are only produced by aerobic organisms, which, for the most part, are types of fungi. It has been found in recent years that the most effective ligninolytic enzymes that could be used for biotechnology (mainly for the production of biofuels) are types of laccase, manganese peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP), which are produced by various types of white-rot fungi, such as ascomycetes, basidiomycetes, and deuteromycetes, and the most common species being investigated are *Trametes versicolor, Phanerochaete chrysosporium, Pleurotus ostreatus, Dichomitus squalens, Lentinula edodes, Irpex lacteus, and Cerrena maxima* (Plácido et al., 2015). In this list, *P. ostreatus* is of special interest because of its use as a food source for humans.

1-3. *Pleurotus ostreatus:* The Oyster Mushroom

Pleurotus ostreatus, commonly referred to by the name "oyster mushroom", is an edible white-rot basidiomycete capable of producing various types of MiP and VP peroxidase enzymes along with laccase (Fernández-Fueyo et al., 2014). In the US, oyster mushrooms are also the most produced specialty mushroom (any edible mushroom not of the Agaricus variety), having just recently surpassed shiitake in 2016 (USDA, 2016c). Oyster mushrooms can be grown on a wide variety of substrate, but are typically grown on a substrate composed mostly of cereal straw or sawdust. However, it has been shown that oyster mushroom yields can be improved by supplementing the substrate with nitrogen-containing materials. For instance, Sonnenberg et al. (2015) found that the cultivation of oyster mushrooms on cassava waste could be improved by the addition of wheat or rice bran to the substrate. Naraian et al. (2009) also investigated the

effects of supplementing oyster mushroom substrate with nitrogen by adding varying levels of urea, ammonium sulfate, gram flour, soybean meal, ground nut cake, and molasses to corn cobs and found that each additive was capable of producing higher biological efficiencies over the control. Also, Isikhuemhen and Mikiashvilli (2009) found that the yields of oyster mushrooms grown on wheat straw could be increased by supplementing the wheat straw with millet and solid waste from an anaerobic digester. However, all of these studies also show that oyster mushroom yields decrease after a certain level of nitrogen is reached, meaning there is likely to be an optimum nitrogen content and/or C/N ratio in order to maximize the yields for the cultivation of oyster mushrooms. Yang et al. (2013) also performed a study investigating the yield of oyster mushrooms on various substrates and found that the maximum biological efficiency resulted from a substrate containing 80% cotton seed hull and 20% wheat bran, which had a C/N ratio of approximately 35:1.

Although studies have been performed involving *P. ostreatus* in the context of producing the mushrooms as a food source and the context of utilizing the mushroom as a biological pretreatment for the production of biofuels from lignocellulosic waste materials, not many studies have been found that involve growing the mushroom on a substrate containing broiler litter. However, this topic is currently of interest because of the large supply of broiler litter in areas having heavy poultry production in which there is a need to derive alternative and potentially more profitable and environmentally-friendly uses of the waste material. Broiler litter contains a large component of lignocellulosic bedding material; meaning, the conversion of the waste material into biofuel could potentially be improved by a biological pretreatment involving *P. ostreatus*. Also, broiler litter, having a relatively low C/N ratio, could potentially be a beneficial supplement to the substrate for growing oyster mushrooms. However, both cases may

require the combination of broiler litter with another common waste material with a higher C/N ratio, such as wheat straw. Thus, this study was performed in order to investigate the anaerobic digestion and cultivation of oyster mushrooms on various combinations of broiler litter and wheat straw.

1-4. Hypothesis

The purpose of this study was to test the integration of anaerobic digestion and the cultivation of *P. ostreatus* as a means of utilizing broiler litter with the hypothesis that the cultivation of the fungus on a mixture of wheat straw and broiler litter would serve as a pretreatment to increase the yields of anaerobic digestion by reducing lignin content while also producing edible mushrooms as another high-value product. It was also expected that the addition of wheat straw to the broiler litter would be required for both biological systems to thrive. This hypothesis was to be tested by performing anaerobic digestion on mixtures of broiler litter and wheat straw before and after fungal pretreatment. Fungal treatment with and without fruiting of mushrooms was also performed to test the hypothesis that the production of mushrooms would be most beneficial. The hypothesis was also to be supported in terms of changes in chemical composition of the substrate materials.

2. Materials and Methods

2-1. General Methodology

Two anaerobic digestion batch assays and two fungal growth assays were performed to investigate the potential for producing high value products (biogas and mushrooms) from the agricultural waste materials (broiler litter "cake" and wheat straw). The broiler litter was obtained from D and L Farms located in Decatur, AR directly after cleanout from a pile composed of waste combined from three 12 m by 120 m houses. Approximately 20 kg of litter was collected from different locations in the pile and was stored in a cool room at 10^{o}C. A small square bale of wheat straw was purchased from a Farmer's Cooperative store located in Fayetteville, AR. A sub sample of litter weighing approximately 2 kg was slightly ground by hand, dried and stored in a freezer at -10^{o}C for later use. A similar amount of wheat straw was cut into 2 – 4 cm long pieces and treated in the same manner as the broiler litter sample. These materials were used for later chemical analyses and experiments. Five ratios of broiler litter to wheat straw were used in the experiments ranging from 100% broiler litter to 100% wheat straw and the combinations were performed based on volatile solids (VS).

2-2 Anaerobic Digestion Experiments

Two solid-state anaerobic digestion batch assays were performed during the study. The first was performed using combinations of the raw waste material in replicates of four, and the second was performed using combinations of untreated (raw) waste material and materials that had gone under two different fungal pretreatments in replicates of three. The anaerobic digestion assays were performed by combining the dried substrate materials, inoculum, and distilled water according to VS ratio, inoculum to substrate ratio (ISR), and moisture content. The ISR was also based on VS and was equal to 0.1 for the first assay and equal to 0.821 for the second assay. A

moisture content of 75% (25% TS) was used in the study. Blanks consisting of only inoculum were also performed in triplicate during both assays.

Inoculum for the study was obtained from a 250 gal experimental anaerobic digester operated by the Biological and Agricultural Engineering (BAEG) department at the University of Arkansas and was being fed a combination of swine wastewater, algae, senior equine feed, and ground wheat straw. This combination was based on materials available and the need to supply the necessary nutrients to the digester with a C/N ratio of 25:1. Inoculum for the experimental digester originated from the second-stage tank of a two-stage digester at the Fourche Creek WWTP in Little Rock, AR.

The materials for the assays were placed in glass jars, sparged with N_2 gas, and sealed with an air-tight cap vented through a soft PVC hose into a Tedlar sampling bag. The general setup for the anaerobic digestion experiments is shown in Figure 1. The volume of gas collected in the sampling bags was measured periodically using a drum-type gas flow meter and the time of collection was recorded along with the volume. Gas samples were also stored in Tedlar bags to later be analyzed for composition using gas chromatography (GC). The assays were allowed to run for 45 days, after which the jars were opened and the materials inside were removed to be weighed and processed for analysis.

Figure 1 An image showing the general setup used for the anaerobic digestion batch experiments, in which substrate was placed in jars capped with a lids modified to vent into a Tedlar gas sampling bag and placed in an incubator to be maintained at a temperature of 37ºC.

2-3 Fungal Growth Experiments

A 10 mL syringe of *P. ostreatus* mycelium was purchased from Gallboys through Amazon.com. Cultures of mycelium were maintained in 4% light malt extract medium in 150mL glass jars capped with lids that contained a rubber injection port for inoculation and a 1cm-diameter opening covered with a glass microfiber filter for air exchange. The medium was prepared in the jars, capped, sterilized in an autoclave at 121ºC for 20 minutes and allowed to cool to room temperature before being inoculated with 3mL of inoculum using a syringe and 4 gauge needle. The growth vessels were then placed in a water bath shaker set at 27ºC and 120

rpm and allowed to incubate for 10 – 14 days until mycelium had fully populated the growth medium. Finished cultures were briefly stored at 4ºC before being used as inoculum. An image of a finish mycelium culture is shown in Figure 2.

Figure 2 Image of a finished mycelium culture grown in a solution 4% light malt extract at a temperature of 27ºC to be used as inoculum for the fungal growth experiments. The liquid in the jar is the growth medium and the fibrous cotton-like substance in the jar is the mycelium.

Two fungal treatments were used in the study, one which involved allowing the substrate to be treated by mycelium only, and the other involved inducing the mushrooms to fruit and be harvested. The mycelium treatment was meant to represent the use of the fungus solely as a pretreatment for anaerobic digestion. The second treatment was meant to represent the use of the materials to grow mushrooms to be sold, and the spent mushroom substrate could then be tested as a substrate for anaerobic digestion. For both treatments, dried substrate materials were combined in a 150mL glass jar at the ratios previously described, were adjusted to a 75%w.b.

moisture content using distilled water, and jars were capped using the same type of lids described in the above paragraph. The substrate was then sterilized in an autoclave at 121^{o}C for 20 minutes and allowed to cool to room temperature before being inoculated at an ISR of 0.1 using a syringe and 4 gauge needle. The jars were then incubated at 27^{o}C. For the mycelium treatment, the jars were simply allowed to incubate for 50 days before ending the experiment and processing the materials for analysis and later anaerobic digestion experiments.

The fungal treatment with fruiting involved monitoring the weight of the jars during incubation and adding water as needed to maintain a moisture content of 75%w.b. After the mycelium had fully colonized the substrate (determined by direct visual observation), the jars were removed from the incubator, the caps were removed, the substrate was disturbed by sticking a 5mmOD glass rod through the surface of the substrate approximately five times, and the jars were placed randomly inside a growth chamber at 15^{o}C. The growth chamber was designed to supply the mushrooms with filtered air at an exchange rate of 4 – 6 exchanges per hour, maintain the temperature at 15 – 20^{o}C, maintain relative humidity at 95%, and irradiate the mushrooms with 2000 lux of light in the natural spectrum. The substrate was sprayed by hand with distilled water daily and mushrooms were harvested periodically after a cap diameter of 3 – 5cm had been reached. Mushrooms were weighed and dried directly after harvest. The duration of this second fungal treatment was 56 days.

2-4 Design of Mushroom Growth Chamber

In order to induce the formation of mushrooms (or fruiting) a growth chamber had to be designed and built. The design of the system was based on objectives of supplying fresh, clean, and moist air to the mushrooms while maintaining an optimal temperature for fruiting and providing periods of irradiation of light in the natural spectrum. The design parameters were

based on recommendation laid out by Stamets in his 1983 manual on mushroom cultivation titled *The Mushroom Cultivator*. These parameters are shown in Table 1.

Table 1 List of parameters and constrains that were considered when designing the mushroom growth chamber used in the fungal growth experiment which involved fruiting.

Parameter	**Constraint**
Air-to-Bed Ratio	> 5:1
Light Intensity	2000 lux
Air Exchange Rate	4 – 6 hr^{-1}
Relative Humidity	90 – 100%
Temperature	15 – 20^{o}C

The initial sizing of the growth chamber was determined by the size of jars that would contain the substrate and the air-to-bed ratio constraint. Air-to-bed ratio (ABR) is defined as the ratio of volume of free space in the growth chamber to the volume of the mushroom bed (in this case, total volume of jars). The chamber was expected to house 30 jars and it was decide that the jars would be placed in a vertical rack in three rows of ten. If approximately 5 cm of space was put between each 9 cm-in-diameter jar, and the geometry of the growth chamber was approximately a rectangular prism with the horizontal dimension of the square being 36 cm longer than the vertical dimension, this would correspond to an ABR of approximately 6:1. A sketch of the growth chamber is shown in Figure 3.

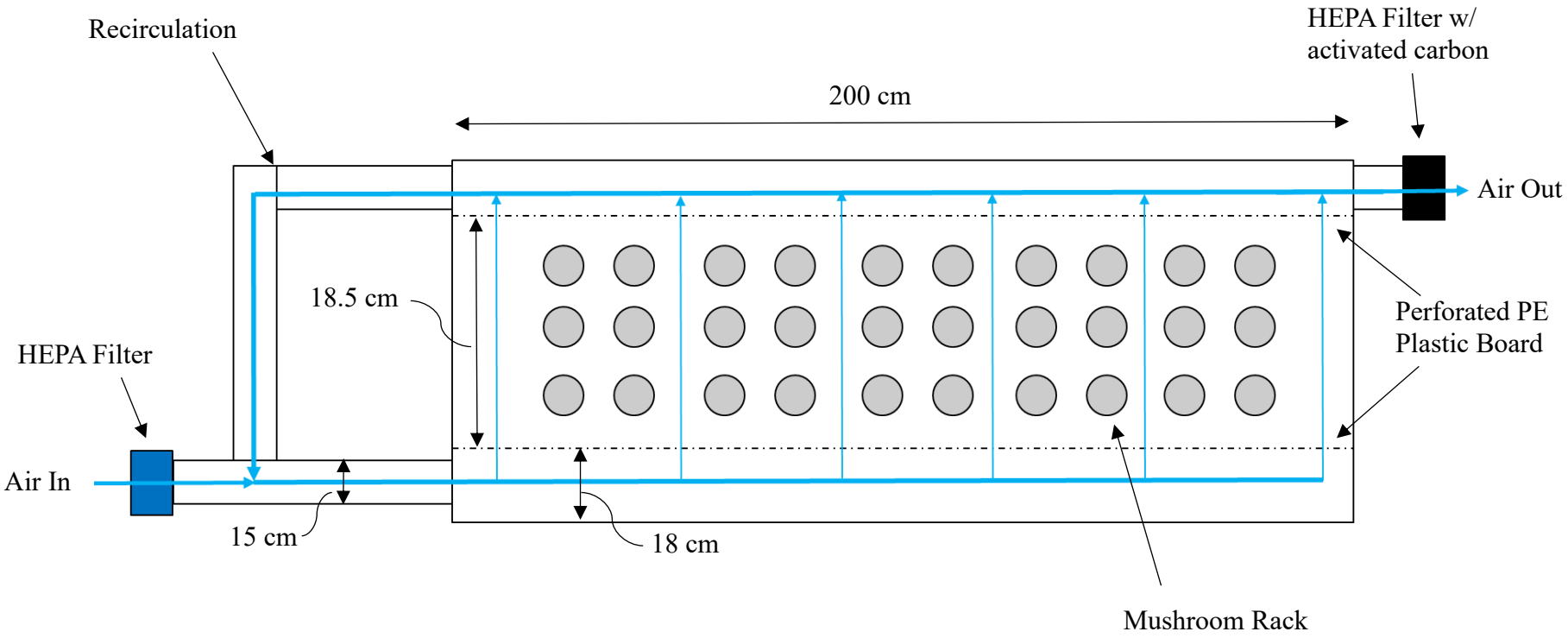

Figure 3 Sketch of mushroom growth chamber that shows dimensions, major components, and air flow.

The frame of the growth chamber was built using ¾ in. angle iron and the walls were made of 1 in. foam board. The circulation system was built using 4 in. duct and duct fan. HEPA filters were place on the inlet and outlet of the chamber to prevent both the contamination of air in the chamber and of the indoor environment that the chamber was placed in. The HEPA filter containing activated carbon was used to prevent the contamination of the ambient environment with both spores and smell.

Perforated polyethylene board was used to create plenums in the top and bottom of the growth chamber to promote uniform airflow across the mushroom growth rack. A humidification system (not shown in Figure 3) was installed into the system that circulated air through a closed bucket containing three 400 mL hr^{-1} atomizers (AGPtek B00P91ZFPA) using a 793 gph air pump (EcoPlus 728450). Light was provided using a 72 in. Lifegard Aquatics Ultra-Slim Freshwater LED Light. Air temperature was controlled using a strip heater installed into the air duct system and a 1500 BTU window air conditioner (Frigidaire). An image of the growth chamber built is shown in Figure 4 (next page).

Air temperature was controlled using an on-off temperature controller which controlled the power being supplied to the strip heater was programmed to have a set point at 17^{o}C. The air conditioner ran continuously on low setting. An on-off humidity controller was also used to control the power being supplied to the humidity system with a set point at 95%. Light was also set on a 12 hr on-off cycle using a programmable timer. The duct fan ran continuously to provide the system with a constant supply of fresh air.

Figure 4 Images of the external (left) and internal (right) of the mushroom growth chamber. The image of the internal of the chamber also shows jars in the mushroom rack, which would contain the substrate and the mushroom would grow out of.

2-5 Analytical Methods

GC analysis of gas samples was performed using a Shimadzu 2014-GC equipped with a Restek ShinCarbon ST Packed Column with a length of 2m and an inner diameter of 2mm. A thermal conductivity detector (TCD) was used and helium was used as the carrier gas and was supplied to the column at a rate of 40 mL/min. The column temperature was maintained at 40^{o}C for the first 2 minutes, was ramped up to 150^{o}C at a rate of 25^{o}C/min, and was maintained at 150^{o}C until the total time of the run reach 7.90 minutes. The temperatures of the detector and injector were both set at 185^{o}C and 180^{o}C, respectively. The method was calibrated for nitrogen (N_2), methane (CH_4), and carbon dioxide (CO_2). A calibration gas composed of 60% CH_4 and 40% CO_2 was also analyzed with each batch to continually ensure the accuracy of the results.

Analyses were performed to determine TS, VS, pH, chemical oxygen demand (COD), ammonia nitrogen (NH_3-N), total Kjeldahl nitrogen (TKN), total nitrogen (TN), nitrate nitrogen (NO_3^-), total phosphorus (TP), water extractives, ethanol extractives, and structural carbohydrates of the materials. TS and VS were determined gravimetrically by taking the initial weight of the material, the weight of the material after being dried in an oven at 105^{o}C, and the weight of the material after being combusted in a muffle furnace at 550^{o}C.

Cold extractions were performed to determine pH, COD, NH_3-N, TKN, TN, NO_3^-, and TP. The extractions involved adding approximately 2 g of undried material to 20 mL of distilled water in a centrifuge tube and storing at 4^{o}C for at least 24 hours. The extract was then separated into two 10 mL centrifuge tubes, centrifuged at 6500 rpm for 15 minutes, and filtered through a 0.45 μm nylon syringe filter. The analyses were then performed on the filtered extract. pH was measured using a Fisher Scientific accumet XL 600 probe while COD, NH_3-N, TKN, TN, NO_3^-,

and TP were measured using spectrophotometric methods developed by Hach Company (Hach Company, 2014; Hach Company, 2015; Hach Company, 2016; Hach Company, 2017).

Water extractives, ethanol extractives, and structural carbohydrates (cellulose, hemicellulose and lignin) contents of the materials studied were determined following the standard method according to NREL (Sluiter et al., 2012; Sluiter et al., 2008). In summary, extractives were determined using a Soxhlet extraction apparatus, first using HPLC grade Millipore water and then using 190 proof ethanol. The concentration of extractives in the water extract were determined gravimetrically by evaporating a known volume of extract in an oven at 105^{o}C, and the concentration of extractives in the ethanol extract were determined gravimetrically by evaporating a known volume of extract using a vacuum concentrator.

Structural carbohydrates were determined using a two-stage acid hydrolysis in which 3 mL of concentrated sulfuric acid was added to 300 mg of extractives-free biomass and kept at 35^{o}C for one hour. 84 mL of Millipore water were then added to the reaction vessels, which were then autoclaved at 121^{o}C for one hour. The hydrolysate was then filtered through a glass microfiber filter using vacuum filtration and the remaining solid material was recovered on the filter. The hydrolysate was brought to a pH of 5 – 6 using calcium carbonate and was then analyzed for glucose, xylose, arabinose, and galactose using HPLC, while the solids were dried and combusted to determine ash content. The volatile component of the solids remaining after hydrolysis was assumed to be lignin while the total mass of sugars measure by HPLC was assumed to originate from cellulose and hemicellulose.

2-6 Data Processing and Statistical Methods

Raw data was processed using Microsoft Excel to calculate methane contents of biogas produced and contents of the various chemical components of the biomass. All biogas produced was assumed to be composed entirely of methane and carbon dioxide with trace gasses constituting a negligible amount to the total volume. All nitrogen found in the biogas samples was assumed to originate from the gas used to sparge the reaction vessels at the beginning of the anaerobic digestion experiment. The methane contents of gas samples were used along with the gas production data to calculate cumulative methane productions. Yields of methane and mushrooms were normalized according to mass of substrate (TS and/or VS) present. Methane yield from blanks were also subtracted from total methane yields. All concentrations of chemical components determined through analyses of extracts and hydrolysates were corrected for dilution and used to determine the chemical composition of the materials in terms of milligrams of analyte per gram of material.

The statistical software program JMP 12.0 was used to performed descriptive statistics, construct graphs, and perform multiple-factorial ANOVA and Tukey post hoc analysis on the processed data. ANOVA and Tukey analysis was performed to determine the significance of the effects of the various experimental factors on their relevant response variables. They Tukey post hoc test used in JMP compares least square means in order to account for the variability of each factor when comparing means amongst the different levels of a single factor. Substrate ratios and fungal treatment factors were examined for effects on biomass characteristics, yields, and soluble nutrient contents immediately following fungal treatment and following anaerobic digestion. ISR was also investigated as a factor affecting anaerobic digestion.

3. Results and Discussion

3-1. Fungal Growth and Structural Carbohydrates

Two fungal growth experiments were performed during the study. One involved only allowing *P. ostreatus* mycelium to grow on combinations of broiler litter and wheat straw solely as a pretreatment for anaerobic digestion, while the other involved allowing the fungus to fruit and the mushrooms to be harvested (material treated with mushroom fruiting is referred to as "spent substrate"). The only observations that took place during the first fungal experiment were the visual inspections of the growth rate of mycelium and the subsequent effects of the treatment on the biomass characteristics of the materials. The same observations were made in the latter experiment along with quantification of mushroom yields that could be sold for profit in practice. Effects of the treatments on lignin content and biomass characteristics (including lignin content) are illustrated in Figure 5 and Figure 6, respectively, and mushroom yields are shown in Figure 7 and Figure 8.

A two-way ANOVA of biomass characteristics, the two experimental factors being substrate ratio and fungal treatment, showed that both fungal treatments and substrate ratio had significant effects on lignin, cellulose/hemicellulose, and extractives contents of the materials being studied. Lignin and cellulose/hemicellulose contents were both significantly reduced and extractives were significantly increased by the fungal treatments, the effects of the treatment involving fruiting being significantly higher than the treatment without fruiting. There were also significant interaction effects between fungal treatment and substrate ratio on the extractives contents. These results support the initial hypothesis that fungal treatment could have a significant effect on biomass characteristics of the substrate. The ANOVA tables for lignin, cellulose/hemicellulose, and extractives are shown in Table 2, Table 4, and Table 6, respectively.

The connecting characters reports showing significant differences for lignin, cellulose/hemicellulose, and extractives are shown in Table 3, Table 5, and Table 7, respectively.

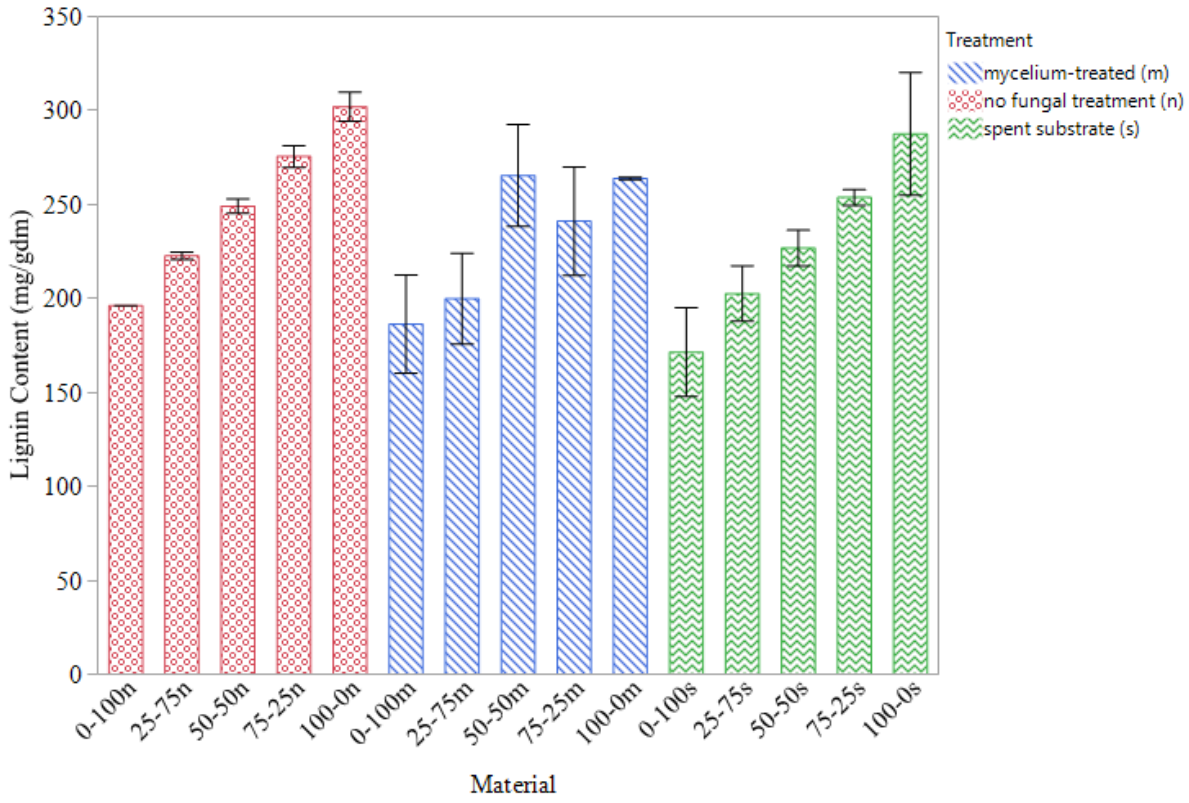

Figure 5 A bar plot of lignin contents of materials at the different levels of fungal treatment. The numbers on the x-axis represent the ratio of broiler litter to wheat straw, while the letters represent fungal treatment. For instance, the label 25-75m represents the material that was composed of 25% broiler litter and 75% wheat straw on a VS basis and had been mycelium fungal treatment (no fruiting). Error bars are constructed using one standard deviation from the mean.

Table 2 ANOVA effects test table for lignin. P-values less than 0.05 are marked with an asterisk to show significance of the corresponding factor on lignin content.

Factor	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	61884.099	54.6322	<.0001*
Fungal Treatment	2	2	4514.108	7.9702	0.0014*
Interaction	8	8	4550.574	2.0087	0.0743

Table 2 shows that both substrate ratio and fungal treatment had significant effects on lignin content of the substrate. However, there was not a significant interaction effect at $\alpha = 0.05$, but a p-value of less than 0.1 can be considered somewhat significant. After it was found that both substrate ratio and fungal treatment had effects on lignin content, a post hoc analysis was performed to determine which levels of each factor were significantly different. These results are shown in Table 2.

Table 3 Connecting letters report showing the results from the Tukey post hoc analysis of lignin contents of materials in units of milligrams of lignin per gram of dry mass of material at the various levels of substrate ratio and fungal treatment. The levels r, m, and s correspond to no fungal treatment (raw material), mycelium treatment (no fruiting), and spent substrate (fungal treatment with fruiting). The levels presented as numbers correspond to the ratio of broiler litter to wheat straw. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level					Least Sq Mean
100-0	A				284.5
75-25		B			256.9
50-50		B			247.2
25-75			C		208.4
0-100				D	184.7
Fungal Treatment Level					**Least Sq Mean**
r	A				249.2
m		B			231.4
s		B			228.5

Table 3 shows that when both fungal treatment and substrate ratio are taken into account, the lignin contents of untreated materials are significantly higher than the mycelium-treated materials and spent substrate, the two of which are not significantly different from one another. Also, lignin content gets progressively higher as the portion of broiler litter increases. This confirms the trends shown in Figure 1. Similar information regarding biomass characteristics of the material can be taken from Figure 2.

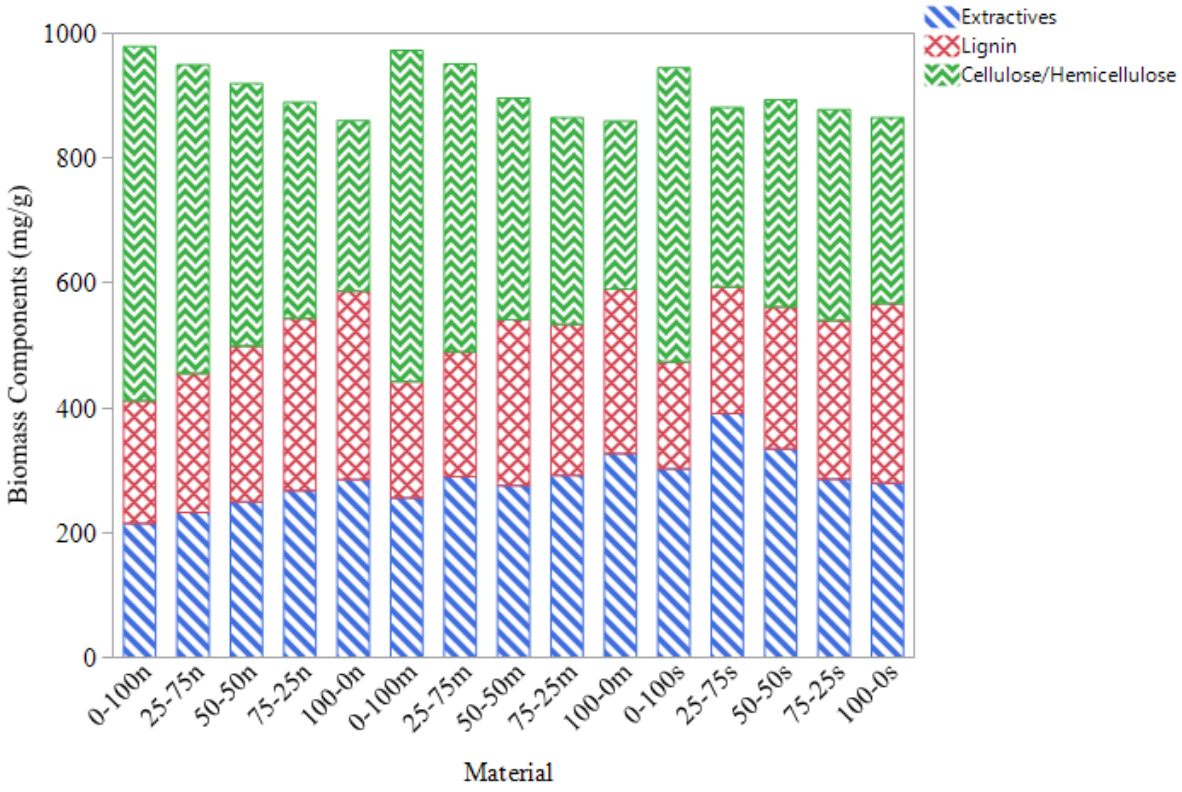

Figure 6 A stacked bar plot showing the biomass characteristics (extractives, cellulose/hemicellulose, and lignin) contents of the materials before and after fungal pretreatment. The numbers on the x-axis represent the ratio of broiler litter to wheat straw, while the letters represent fungal treatment; n, m, and s meaning no fungal treatment, mycelium treatment (no fruiting), and spent substrate (fungal treatment with fruiting) (see legend in Figure 1). For instance, the label 25-75m represents the material that was composed of 25% broiler litter and 75% wheat straw on a VS basis and had been mycelium fungal treatment (no fruiting).

Figure 6 shows how the different biomass components changed with mixing of substrates and fungal treatment. With no fungal treatment, lignin and extractives increase and cellulose/hemicellulos decreases as the portion of broiler litter in the material increases. Also, the total height of the stacked bars decreases with increasing broiler litter because the broiler litter had a higher ash content than the wheat straw. Fungal treatment resulted in less distinct trends in the biomass components, signifying that the fungus had varying effects on the biomass depending on portion of broiler litter. Thus, statistical analyses were required to determine the effects of fungal treatment on the biomass components while taking substrate ratio into account.

Table 4 ANOVA effects test table for cellulose/hemicellulose. P-values less than 0.05 are marked with an asterisk to show significance of the corresponding factor on cellulose/hemicellulose content.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	327249.00	144.1382	<.0001*
Fungal Treatment	2	2	48496.42	42.7209	<.0001*
Interaction	8	8	64493.03	14.2031	<.0001*

Table 4 shows that both substrate ratio and fungal treatment had significant effects on cellulose/hemicellulose content of the substrate. There was also a significant interaction effect between the two factors, which confirms the notion that fungal treatment varied depending on substrate ratio. Post hoc analysis was performed to determine which levels of each factor were significantly different from one another. These results are shown in Table 5.

Table 5 Connecting letters report showing the results from the Tukey post hoc analysis of cellulose/hemicellulose contents of materials in units of milligrams of cellulose/hemicellulose per gram of dry mass of material at the various levels of substrate ratio and fungal treatment. The levels r, m, and s correspond to no fungal treatment (raw materials), mycelium treatment (no fruiting), and spent substrate (fungal treatment with fruiting). The levels presented as numbers correspond to the ratio of broiler litter to wheat straw. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level					**Least Sq Mean**
0-100	A				523.2
25-75		B			414.4
50-50			C		369.4
75-25			C		339.0
100-0				D	280.1
Fungal Treatment Level					**Least Sq Mean**
r	A				420.6
m		B			389.6
s			C		345.4

Table 5 shows that when both fungal treatment and substrate ratio are taken into account, the cellulose/hemicellulose content of untreated materials are significantly higher than the mycelium-treated materials, which are significantly higher than the spent substrate. Also, cellulose/hemicellulose gets progressively higher as the portion of wheat straw increases. The decrease in cellulose/hemicellulose and lignin upon fungal treatment could signify that the biomass was made more degradable through fungal treatment. This could also be supported if extractives contents of the biomass were to increase upon fungal treatment. The results from the statistical analyses of extractives are shown in Table 6 and Table 7.

Table 6 ANOVA effects test table for extractives. P-values less than 0.05 are marked with an asterisk to show significance of the corresponding factor on extractives content.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	12601.015	7.7767	0.0001*
Fungal Treatment	2	2	41342.595	51.0291	<.0001*
Interaction	8	8	32956.108	10.1694	<.0001*

Table 6 shows that both substrate ratio and fungal treatment had significant effects on extractives content of the substrate. There was also a significant interaction effect. Post hoc analysis was used to determine which treatments had a significant impact on extractives content, which could be used to make predictions about subsequent anaerobic digestion of the materials.

Table 7 Connecting letters report showing the results from the Tukey post hoc analysis of extractives contents of materials in units of milligrams of extractives per gram of dry mass of material at the various levels of substrate ratio and fungal treatment. The levels r, m, and s correspond to no fungal treatment (raw materials), mycelium treatment (no fruiting), and spent substrate (fungal treatment with fruiting). The levels presented as numbers correspond to the ratio of broiler litter to wheat straw. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level				Least Sq Mean
25-75	A			304.6
100-0	A			297.0
50-50	A			286.8
75-25	A	B		281.9
0-100		B		257.7
Fungal Treatment Level				**Least Sq Mean**
s	A			318.8
m		B		288.1
r			C	250.0

Table 7 shows that when both fungal treatment and substrate ratio are taken into account, the extractives content of untreated materials are significantly higher than the mycelium-treated materials, which are significantly higher than the spent substrate. Higher extractives content could potentially lead to a higher rate of methane production by effectively decreasing the rate of hydrolysis; extractives supply substrate that does not require hydrolysis to the microbial community. The results regarding the effects of substrate ratio and fungal treatment on biomass characteristics suggested that materials that had undergone fungal treatment should result in higher methane yields and faster methane production rates upon anaerobic digestion due to a decrease in lignin and cellulose/hemicellulose and an increase in extractives.

The potential for the production of edible mushrooms from substrate containing broiler litter was also investigated as an alternative, potentially more profitable and sustainable use of the waste material. This was assessed through the fungal growth experiment which involved fruiting, during which, mushroom produced were harvested, weighed and dried to determine biological efficiency (BE; shown in Figure 3) and yield (shown in Figure 4) of mushrooms from each substrate. BE is defined as the wet weight of mushrooms produced divided by the dry weight of substrate used, while yield is defined as the dry weight of mushrooms produced divided by the dry weight of substrate used. It was found that only the substrate which contained 100% wheat straw and that which contained 25% broiler litter and 75% wheat straw were capable of producing edible mushrooms.[2]

[2] The substrate containing 50% broiler litter and 50% wheat straw produced some discolored and misshapen fruiting bodies, which were judged to not be edible mushrooms.

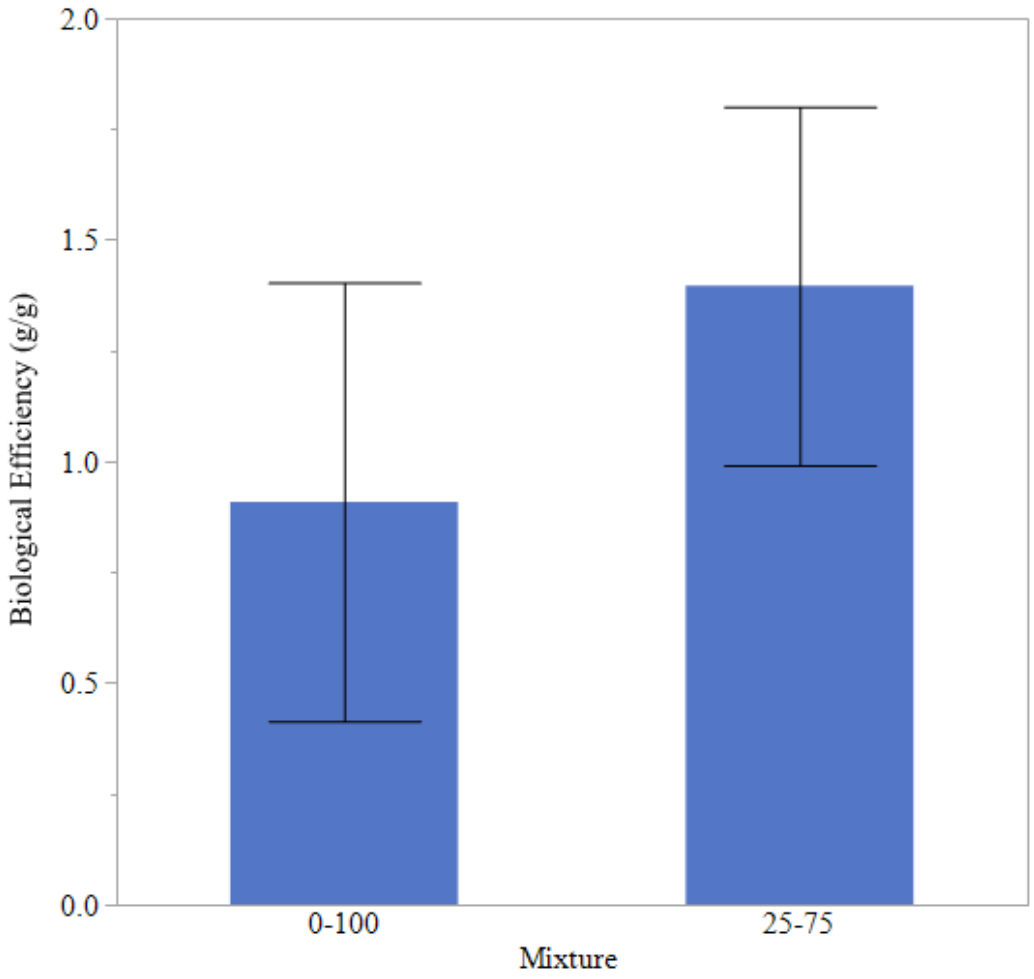

Figure 7 A bar plot showing the mean biological efficiencies and standard deviations for mushroom production from the 0-100 and 25-75 substrates.

Figure 7 illustrates how the 25-75 substrate resulted in a larger average BE than the 0-100 substrate, but that there was too much variation to be a significant difference in BE. However, this variation can be attributed to variation in moisture content of the mushrooms, which is made apparent when looking at the mushroom yields shown in Figure 8.

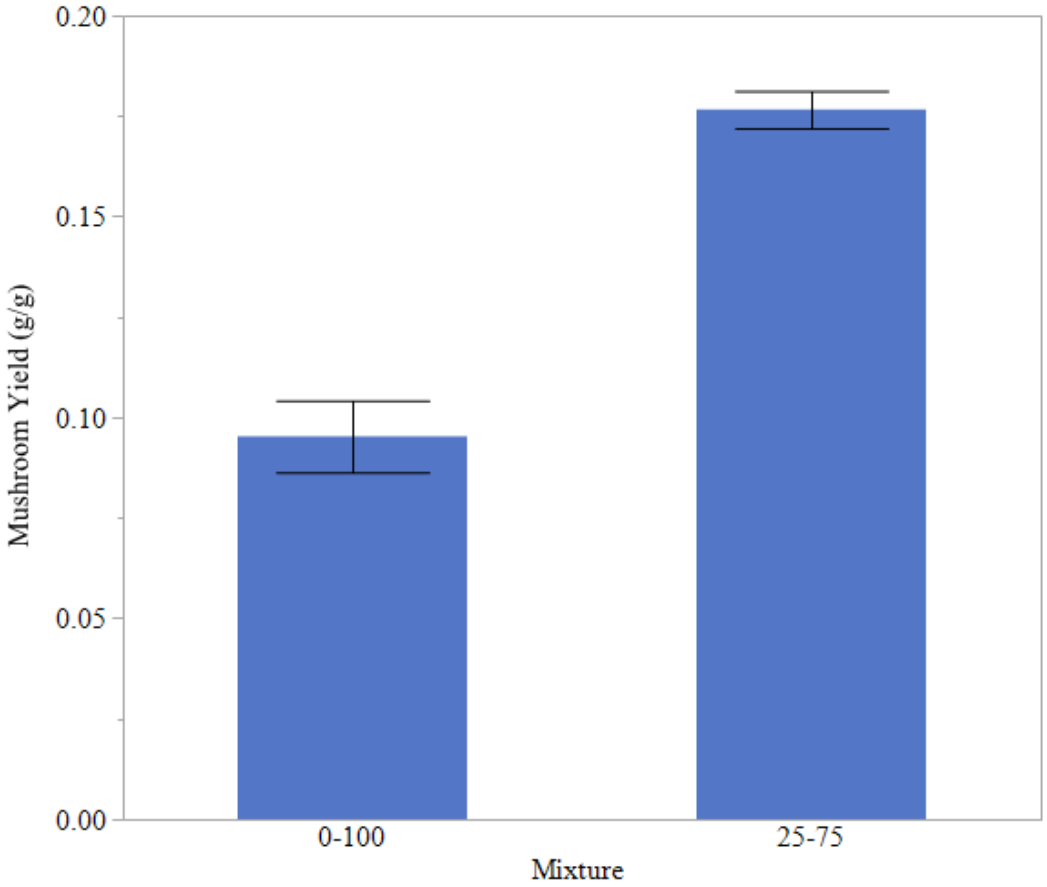

Figure 8 A bar plot showing the mean yields and standard deviations for mushroom production from the 0-100 and 25-75 substrates.

Figure 4 shows that the yield of oyster mushrooms from the 25-75 substrate was nearly double that of the 0-100 substrate. Also, there is much less variation in the yield compared to that of BE, which makes the difference in yield more significant than the difference in BE. The reason for the discrepancy between the variation in BE and yield is due to a large variation in moisture content of the mushrooms. This shows how mushrooms can easily absorb and release moisture depending on the environmental conditions. The large variation in moisture most likely can be explained by the inability of the growth chamber to maintain a constant relative humidity. This, however, should not be an issue in an industrial mushroom production facility in which

properly sized and designed equipment can be used for the larger growth chambers. If the average yields are used to calculate BE, assuming a moisture content of 85%, the expected BE for the 25-75 substrate would be ~120% while that for the 0-100 substrate would be ~65%. This means that production of oyster mushrooms from wheat straw, which is a common substrate, could be nearly doubled by supplementing the straw with 25% broiler litter.

It was found through visual observation of both fungal experiments that mycelium grew noticeably faster in materials which contained higher amounts of wheat straw. This, however, could be because more mass was present in jars containing a higher percentage of broiler litter, which is denser than wheat straw. It was also found that the mycelium colonized the 75-25 and 100-0 substrates more quickly and fully in the no-fruiting experiment. This could be because the no-fruiting experiment allowed for the materials to dry as the experiment progressed and that the broiler litter had a lower saturated moisture content than the wheat straw. This suggests that mycelium growth rate may depend on the level of water saturation of the material rather than moisture content. An image showing mycelium growth in the different substrates is shown in Figure 9.

Figure 9 Image of mycelium growth on one replicate of every substrate 10 days after inoculation. The jars contain increasing amounts of broiler litter going from right to left with the jar on the far left being 100% wheat straw and the jar on the right being 100% broiler litter. The white substance in the jars is the mycelium.

3-2. Anaerobic Digestion

Anaerobic digestion was carried out in two different batches with differing inoculum to substrate ratios (ISRs). The first batch consisted of the raw waste materials being digested at the five substrate ratios under consideration at an ISR of 0.1. The second batch involved the digestion of raw and fungal-treated materials at an ISR of 0.821. The cumulative gas production curves are shown in Figures 10, 11, 12, and 13 and the methane yields resulting from these anaerobic digestion batch experiments are shown in Figure 14.

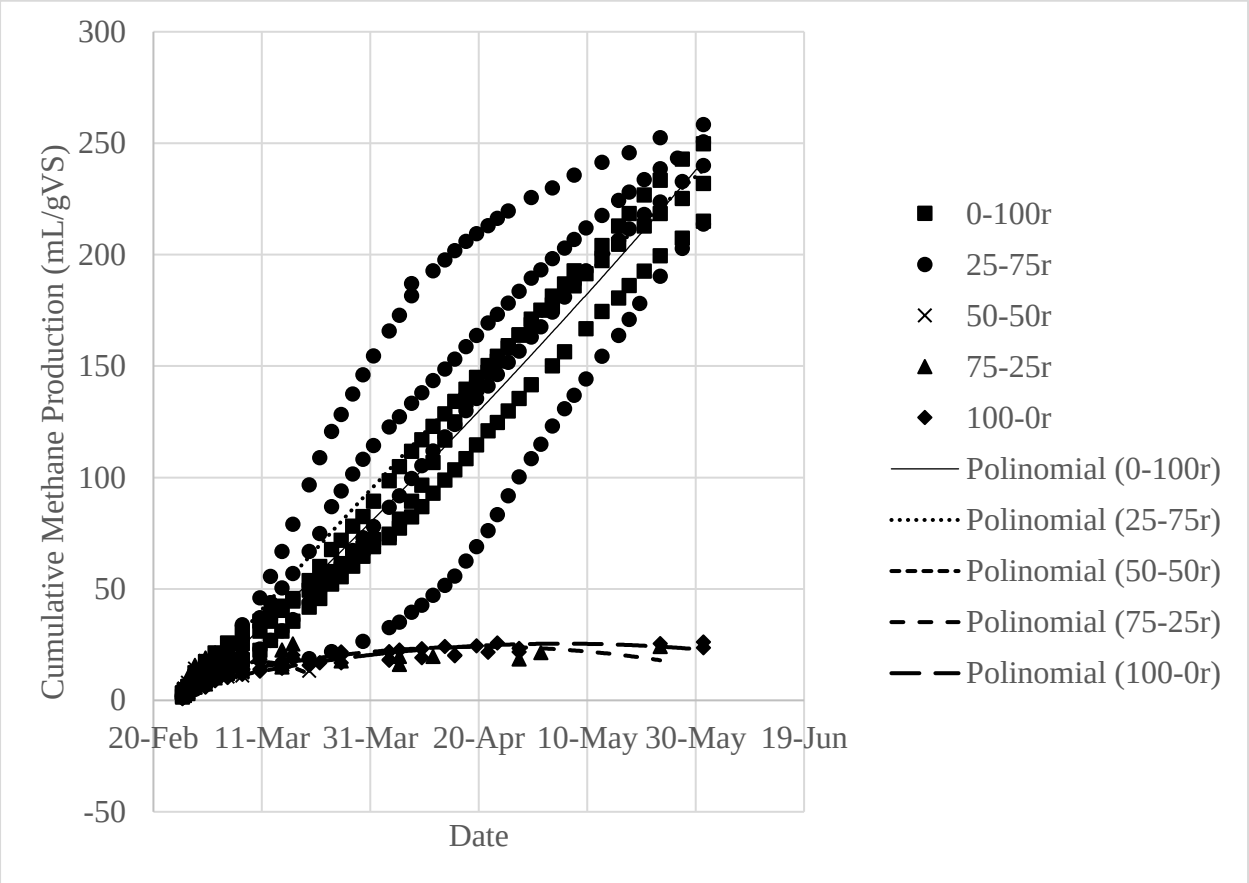

Figure 10 A plot of cumulative gas production versus time resulting from the anaerobic digestion of raw materials at an ISR of 0.1. A second-degree polynomial was fit to gas production data for each substrate ratio to show the general trends of gas production.

Figure 10 shows that the 0-100 and 25-75 substrates had almost equal gas production trends, but that the variability amongst the 25-75 replicates was much larger than that for the 0-100 replicates. It is also shown that the gas productions for the 50-50, 75-25, and 100-0 substrates were much smaller than those for the 0-100 and 25-75 substrates.

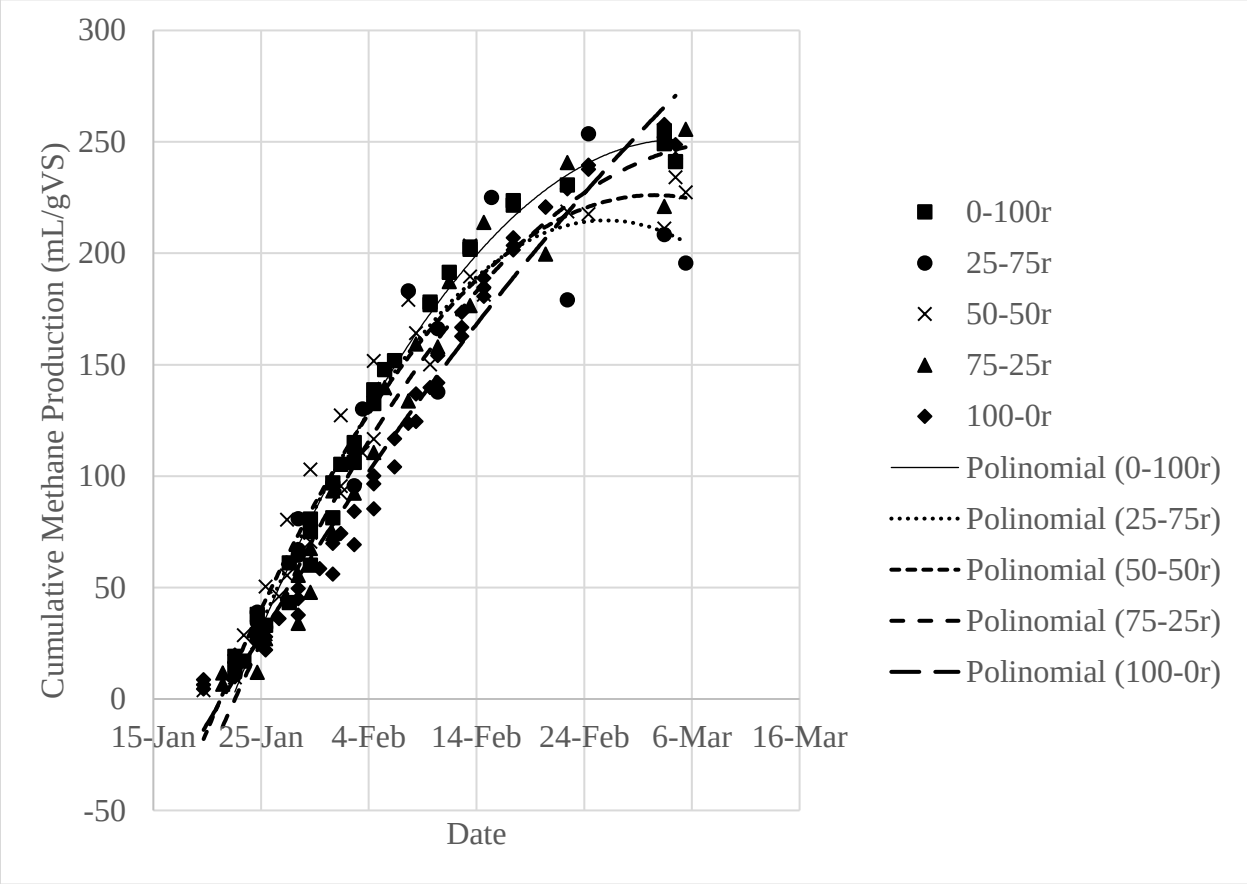

Figure 11 A plot of cumulative gas production versus time resulting from the anaerobic digestion of raw materials at an ISR of 0.821. A second-degree polynomial was fit to gas production data for each substrate ratio to show the general trends of gas production.

Figure 11 shows that the anaerobic digestion of the raw substrates at an ISR of 0.821 resulted in very similar gas production curves for all substrates ratios. However, close observation of the trend lines shows that the 100-0 substrate had the most linear gas production curve, which suggests that it had been digesting to a lesser extent than all others and thus, would

have a higher ultimate methane yield. This is somewhat perplexing because the broiler litter contained a higher lignin content than the wheat straw, which would suggest a lower ultimate gas production yield for substrates containing higher amounts of broiler litter. Perhaps this could be due to the cellulose and hemicellulose present in the wheat straw being bound by lignin and thus, less available for hydrolysis. Comparison of Figures 11 and 12 shows that ISR has a large impact on ultimate gas yields especially for substrates with more than 25% broiler litter. Also, the rate of gas production was much slower at an ISR of 0.1, taking nearly twice as long for the same yields to be reached by the 0-100 and 25-75 substrates as at an ISR of 0.821.

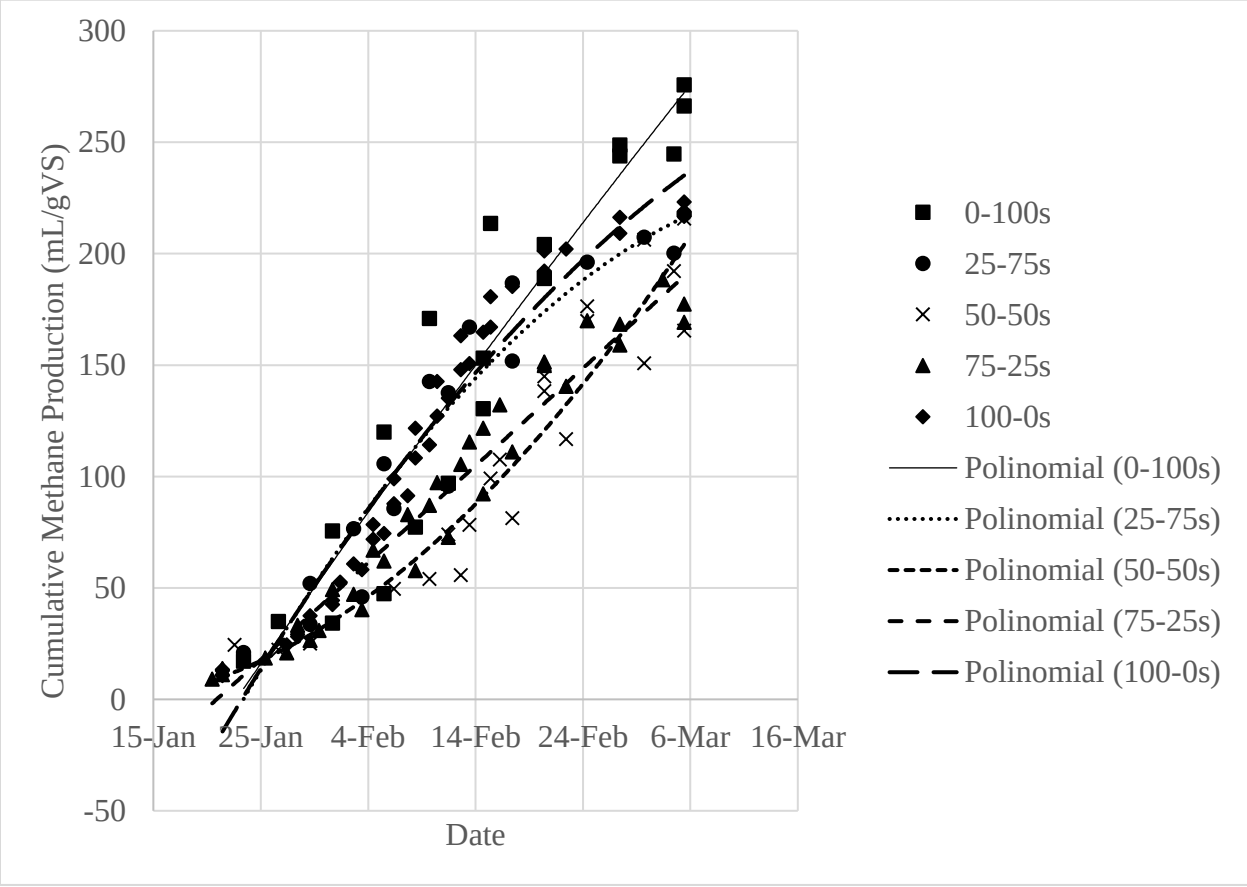

Figure 12 A plot of cumulative gas production versus time resulting from the anaerobic digestion of spent substrate at an ISR of 0.821. A second-degree polynomial was fit to gas production data for each substrate ratio to show the general trends of gas production.

Figure 12 shows that the anaerobic digestion of spent substrate resulted in very similar initial gas production curves for the 0-100, 25-75, and 100-0 substrates. However, gas production for the 25-75 and 100-0 substrates began to slow towards of the end of the experiment while that for the 0-100 substrate remain quite constant. Also, although the 75-25 substrate initially out-performed the 50-50 substrate, gas production for the 50-50 substrate surpassed that for the 75-25 substrate the overall trend of the gas production from the 50-50 substrate was concave-up; meaning, the rate was still increasing towards the end of the experiment.

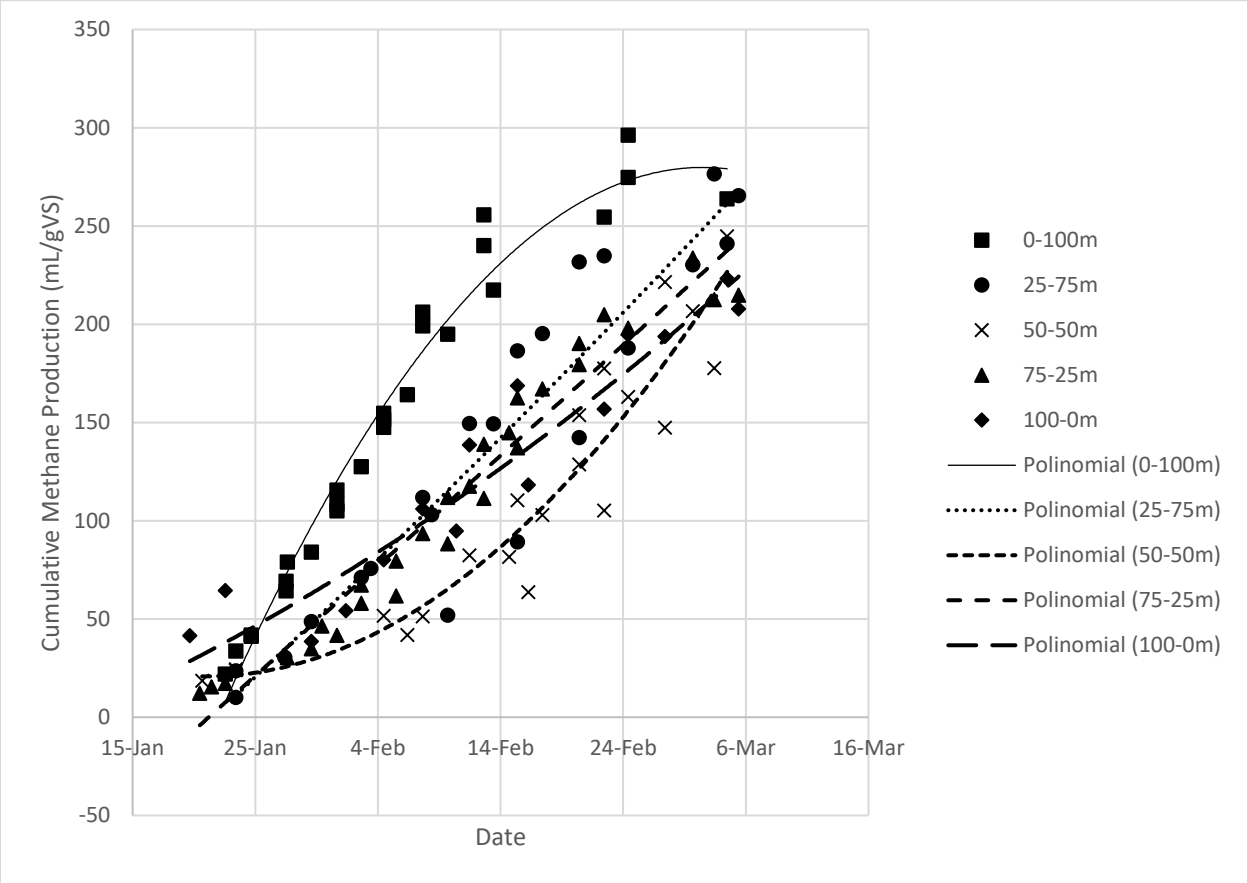

Figure 13 A plot of cumulative gas production versus time resulting from the anaerobic digestion of mycelium-treated materials at an ISR of 0.821. A second-degree polynomial was fit to gas production data for each substrate ratio to show the general trends of gas production.

Figure 13 shows that the anaerobic digestion of mycelium-treated materials resulted in gas production curves from the 25-75, 75-25, and 100-0 substrates being quite similar, but with the 25-75 substrate having a slightly steeper slope that the other two. The 0-100 substrate had the steepest gas production curve but had slowed to a rate of approximately zero by the end of the experiment. The 50-50 substrate had the slowest initial rate, but had reached a cumulative gas production similar to that of the 100-0 substrate by the end of the experiment. The general trend for the 50-50 substrate was also concave-up, signifying an increasing gas production rate.

Analysis of the gas production curves shows that ISR, substrate ratio, and fungal pretreatment could all have potential impacts on yields. The significance of the impacts of ISR on yield seemed apparent; however, the rate at which this yield was attained was also a factor. The effects of substrate ratio and fungal pretreatment on yields were less apparent. Thus, statistical analyses were performed on 45-day yields resulting from each experiment in order to determine which factors had significant impacts and the extent of these impacts.

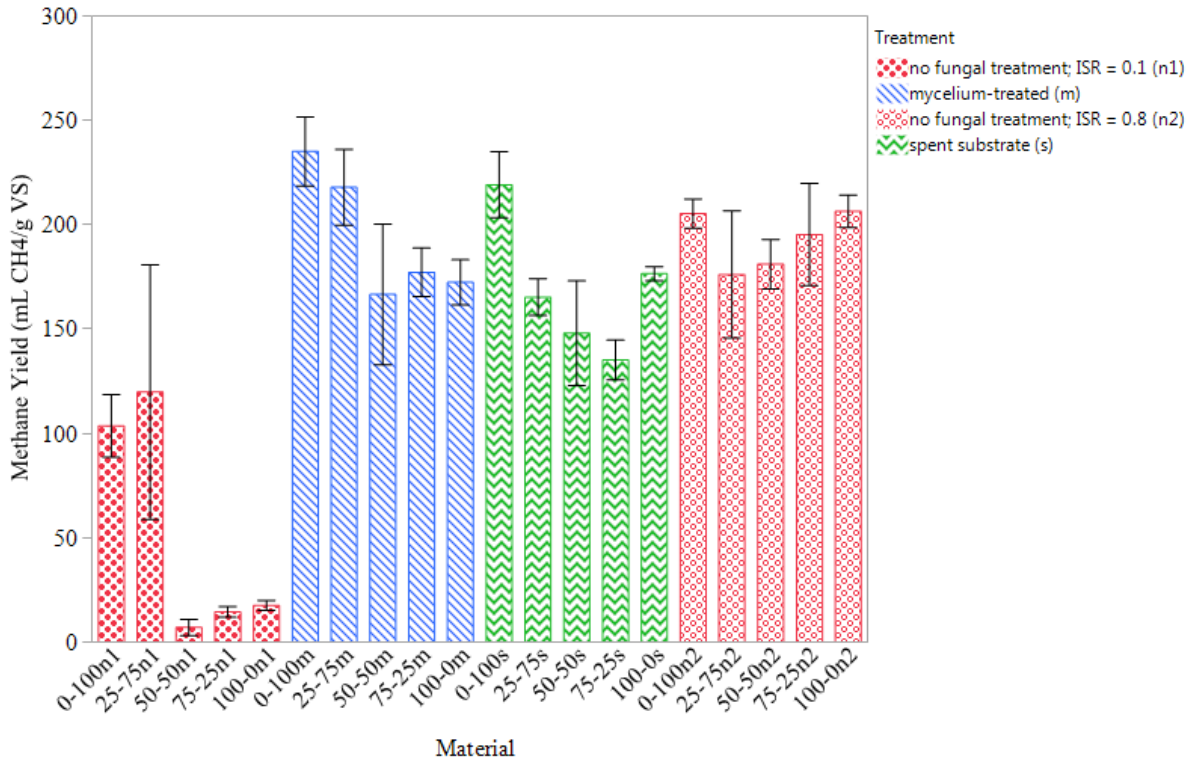

Figure 14 A bar plot showing methane yields resulting from the anaerobic digestion batch experiments for all materials that were digested throughout the course of the project. Methane yields are expressed in terms of volume of methane produced divided by the initial mass of volatile solids introduced. Bars are distinguished by either fungal treatment or ISR through color and pattern. Error bars are constructed using one standard deviation from the mean.

One result from the anaerobic digestion experiments that is most apparent in Figure 14 is that ISR had a significant effect on methane yields. The results from a two-way ANOVA and post hoc analysis of yields resulting from only the untreated materials at the two different ISR's with the factors being ISR and substrate ratio show that both ISR and substrate ratio had significant effects on methane yield with yields resulting from an ISR of 0.821 being significantly higher than those from an ISR of 0.1 and yields from the 0-100 and 25-75 substrate ratios being significantly higher than all others. There were also significant interaction effects between the two factors. These results are also expressed in Table 8 and Table 9.

Table 8 ANOVA effects test table for methane yields of materials not treated by fungus and digested at the two different ISR's of 0.1 and 0.821. P-values less than 0.05 are marked with an asterisk to show significance of the corresponding factor on extractives content.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	18536.36	7.2744	0.0006*
ISR	1	1	155529.96	244.1454	<.0001*
Interaction	4	4	22509.50	8.8337	0.0002*

Table 8 shows that both substrate ratio and ISR had significant effects on methane yields of the raw materials. There was also a significant interaction effect. After it was found that the ANOVA test for substrate ratio and ISR as factors of methane yield showed significance. Post hoc analysis was used to determine which levels of each factor were significantly different from one another (shown in Table 9).

Table 9 Connecting letters report showing the results from the Tukey post hoc analysis of methane yields for raw materials digested in units of milliliters of methane produced per gram VS of substrate at the various levels of substrate ratio and ISR. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level				Least Sq Mean
0-100	A			154.5
25-75	A	B		148.0
100-0		B	C	112.1
75-25			C	104.9
50-50			C	94.09
ISR Level				**Least Sq Mean**
0.821	A			192.9
0.1		B		52.54

Table 9 shows that when both ISR and substrate ratio are taken into account, yields resulting from 0-100 and 25-75 substrates were significantly higher than those for the 75-25 and 50-50 substrates. The 100-0 substrate (100% broiler litter), however, was only significantly lower than the 0-100 substrate (100% wheat straw). Also, the table shows that yields resulting from an ISR of 0.821 were significantly higher than yields resulting from an ISR of 0.1.

The results regarding the effects of ISR on methane yields were to be expected because they conform to established concepts related to anaerobic digestion. As ISR increases, the inhibitory effects of intermediates such as ammonia and VFA's becomes less pronounced because the material as a whole is composed of less substrate at a higher ISR, and thus, the substrate has less influence on the chemical environment of the microbial community. Also, from the kinetics perspective, a lower ISR implies a lower biomass concentration, which lowers the reaction rate for methane formation. Although this set of statistical analyses showed significantly higher yields for the 0-100 and 25-75 substrate ratios, this is mainly due to the results from the lower ISR experiment. Results from the statistical analyses of the higher ISR experiment differ.

A two-way ANOVA was performed on all yields resulting from the anaerobic digestion experiment which involved an ISR of 0.821 with substrate ratio and fungal treatment being the two factors. The results from this set of analyses are shown in Table 10 and Table 11. Results from these statistical analyses show that both substrate ratio and treatment had significant effects on methane yields. The 0-100 substrate resulted in significantly higher yields than all other substrate ratios, all of which were not significantly different from one another. It was also found that the mycelium treatment had no effect on methane yields and spent substrate exhibited

significantly lower yields compared to the untreated material in spite of the two fungal-treated materials having a significantly lower lignin content.

Table 10 ANOVA effects test table for methane yields of raw and fungal-treated materials digested at an ISR of 0.821. P-values less than 0.05 are marked with an asterisk to show significance of the effects of the corresponding factor.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	16379.852	12.8263	<.0001*
Fungal Treatment	2	2	5824.341	9.1216	0.0009*
Interaction	8	8	8413.103	3.2940	0.0089*

Table 10 shows that both substrate ratio and fungal treatment had significant effects on methane yield. There was also a significant interaction effect. After significant effects of substrate ratio and fungal treatment on methane yields were established, post hoc analysis was performed to determine which levels of each factor were significantly different (shown in Table 11).

Table 11 Connecting letters report showing the results from the Tukey post hoc analysis of methane yields for raw and fungal-treated materials digested at an ISR of 0.821 in units of milliliters of methane produced per gram VS of substrate at the various levels of substrate ratio and fungal treatment. The levels r, m, and s correspond to no fungal treatment (raw materials), mycelium treatment (no fruiting), and spent substrate (fungal treatment with fruiting). The levels presented as numbers correspond to the ratio of broiler litter to wheat straw. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level			**Least Sq Mean**
0-100	A		219.9
25-75		B	186.5
100-0		B	185.2
75-25		B	169.2
50-50		B	165.3
Fungal Treatment Level			**Least Sq Mean**
m	A		193.9
r	A		192.9
s		B	168.9

Table 11 shows that when both fungal treatment and substrate ratio are taken into account, yields resulting from the 0-100 substrate were significantly higher than those for all other substrate ratios, the rest of which were not significantly different from each other. The table also shows how yields resulting from substrates treated by fungus with fruiting (spent substrate or s) were significantly lower than those for materials treated by mycelium only and for raw materials.

These results do not support the initial hypothesis that lowering lignin content of the substrate would lead to higher methane yields. This is believed to be due to other effects that fungal treatment can have on a material that make the material less suitable as a substrate for anaerobic digestion. This is believed to be related with the changes in the portions of the various nitrogen species present in the material caused by fungal treatment, which will be discussed in the next section.

3-3 Chemical Analyses

COD, TN, nitrate, ammonia, TKN, TP, and pH of cold extracts were analyzed for the materials being studied. However, TN, nitrate, TKN, and TP were only measured on the raw waste materials, spent substrate, inoculum, and materials digested with an ISR of 0.821. Figure 15 shows the nitrogen species contents for the materials measured and Figure 16 shows TP. Also, pH was not measured on extracts for the materials treated with fungus. It was found that all digested materials had a pH in the neutral range except for the substrate ratios 50-50, 75-25, and 100-0 that were digested at an ISR of 0.1. The acidification of these materials indicates that materials containing more than 25% broiler litter may not be suitable for digestion, especially at high loading rates corresponding to an ISR of 0.1

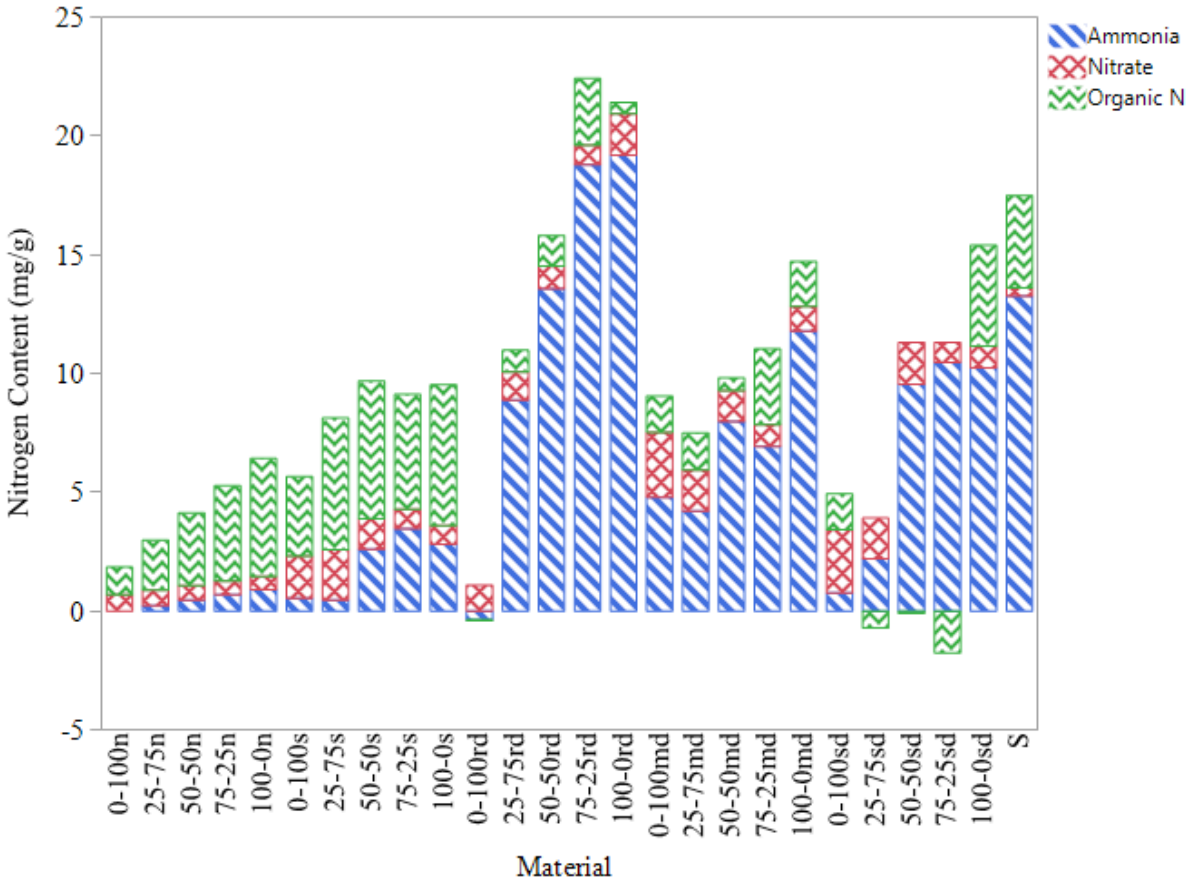

Figure 15 A bar plot showing concentration of various soluble nitrogen species as a portion of biomass including sludge used for inoculum (S). Organic nitrogen was calculated by subtracting ammonia from TKN and bars with negative values may be assumed to be zero. The labels md, n, rd, S, and sd represent digested and mycelium-treated material, no treatment (raw material), digested raw material, sludge (inoculum), and digested spent substrate.

As illustrated in Figure 15 and confirmed using a two-way ANOVA and post hoc analysis of various nitrogen concentrations with treatment (fungus and anaerobic digestion) and substrate ratio being the two factors, it was found that total soluble nitrogen was significantly increased by both fungal treatment and digestion. However, TN was not significantly increased upon digestion of spent substrate, signifying that most nitrogen species were already made soluble by the fungal treatment with fruiting. Results for the statistical analysis of TN are shown in Table 11 and Table 12.

Another thing to note from Figure 15 is that some of the materials containing higher levels of broiler litter had TN and ammonia levels that were comparable and in some cases higher than those for the inoculum, meaning more nitrogen was released into the environment than what was initially present in the healthy inoculum. This could indicate that if a digester were designed to be fed materials with more than 25% broiler litter, measures would have to be taken to prevent the buildup of ammonia in the reactor which could potentially result in reactor failure due to inhibition of methanogenesis by ammonia.

Table 12 ANOVA effects test table for TN contents of raw and fungal-treated materials before and after being digested at an ISR of 0.821. P-values less than 0.05 are marked with an asterisk to show significance of the corresponding factor on extractives content.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	1536.2092	28.5578	<.0001*
Fungal Treatment	4	4	1240.7426	23.0651	<.0001*
Interaction	16	16	1401.7655	6.5146	<.0001*

Table 12 shows that both substrate ratio and fungal treatment had significant effects on TN. There was also a significant interaction effect. After it was found that both substrate ratio and fungal treatment had significant effects on TN, a post hoc analysis was performed to determine which levels of each factor were significantly different from one another in order to draw further conclusions.

Table 13 Connecting letters report showing the results from the Tukey post hoc analysis of TN contents for raw and fungal-treated materials before and after being digested at an ISR of 0.821 in units of milligrams of TN per gram of dry matter of substrate at the various levels of substrate ratio and fungal treatment. The levels rd, md, and sd correspond to digested raw material, digested mycelium-treated material (no fruiting), and digested spent substrate (fungal treatment with fruiting), respectively. The levels r and s correspond to materials not treated by fungus (raw materials) and spent substrate before digestion. The levels presented as numbers correspond to the ratio of broiler litter to wheat straw. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level				**Least Sq Mean**
100-0	A			13.50
75-25	A	B		11.09
50-50		B		10.14
25-75			C	6.571
0-100			C	4.052
Fungal Treatment Level				**Least Sq Mean**
rd	A			13.88
md		B		10.43
sd		B		8.473
s		B		8.433
r			C	4.141

Table 13 shows that when both fungal treatment and substrate ratio are taken into account, TN increases progressively as the portion of broiler litter in the substrate increases. Also, although extracts of the spent substrate had a higher TN content than that for the raw material before digestion, that for the digested raw material was significantly higher than those for the digested fungal-treated material, which were not significantly higher than that for the spent substrate before digestion.

Also, ammonia was significantly increased upon digestion, and both ammonia and TN levels for the materials not treated by fungus and digested at an ISR of 0.821 were significantly higher than those for materials that had undergone fungal pretreatment; results for the statistical

analysis of ammonia are shown in Table 14 and Table 15. This could signify that the fungal-treated materials had been digested to a lesser extent than the raw materials.

Table 14 ANOVA effects test table for ammonia contents of raw and fungal-treated materials before and after being digested at an ISR of 0.821. P-values less than 0.05 are marked with an asterisk to show significance of the corresponding factor on extractives content.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	1173.5237	29.7523	<.0001*
Fungal Treatment	4	4	2225.5923	56.4253	<.0001*
Interaction	16	16	954.7447	6.0514	<.0001*

Table 14 shows that both substrate ratio and fungal treatment had significant effects on ammonia. There was also a significant interaction effect. After it was found that both substrate ratio and fungal treatment had significant effects on ammonia, a post hoc analysis was performed to determine which levels of each factor were significantly different from one another in order to draw further conclusions.

Table 15 Connecting letters report showing the results from the Tukey post hoc analysis of ammonia contents for raw and fungal-treated materials before and after being digested at an ISR of 0.821 in units of milligrams of ammonia nitrogen per gram of dry matter of substrate at the various levels of substrate ratio and fungal treatment. The levels rd, md, and sd correspond to digested raw material, digested mycelium-treated material (no fruiting), and digested spent substrate (fungal treatment with fruiting), respectively. The levels r and s correspond to materials not treated by fungus (raw materials) and spent substrate before digestion. The levels presented as numbers correspond to the ratio of broiler litter to wheat straw. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level				**Least Sq Mean**
100-0	A			8.992
75-25	A			7.700
50-50	A			6.831
25-75		B		3.198
0-100		B		1.146
Fungal Treatment Level				**Least Sq Mean**
rd	A			12.02
md		B		7.137
sd		B		6.278
s			C	1.980
r			C	0.4563

Table 15 shows that when both fungal treatment and substrate ratio are taken into account, ammonia increases progressively as the portion of broiler litter in the substrate increases with two significantly different groups. Also, all digested material had significantly higher ammonia levels than all undigested material. However, digested raw material had significantly higher ammonia levels than the digested fungal-treated materials.

Another notable observation made from the analysis of nitrogen species of the materials is that the fungal treatment with fruiting had significant effects on nitrate concentration, especially for materials with substrate ratios of 0-100 and 25-75 which had average percent increases of 156% and 223%, respectively. Results for the statistical analysis of nitrate are shown in Table 16 and Table 17. It is well-known that nitrate can have inhibitory effects on methanogenesis due to the ability of denitrifying bacteria to outcompete methanogens for acetate and proprionate (Tugtat et al., 2006). Thus, this is a likely explanation for the lower methane yields exhibited by materials treated by fungus compared to that of the untreated material.

Table 16 ANOVA effects test table for nitrate contents of raw and fungal-treated materials before and after being digested at an ISR of 0.821. P-values less than 0.05 are marked with an asterisk to show significance of the corresponding factor on extractives content.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	17.277567	17.8926	<.0001*
Fungal Treatment	4	4	13.899850	14.3947	<.0001*
Interaction	16	16	18.385775	4.7601	<.0001*

Table 16 shows that both substrate ratio and fungal treatment had significant effects on nitrate. There was also a significant interaction effect. After it was found that both substrate ratio and fungal treatment had significant effects on nitrate, a post hoc analysis was performed to determine which levels of each factor were significantly different from one another in order to draw further conclusions.

Table 17 Connecting letters report showing the results from the Tukey post hoc analysis of nitrate contents for raw and fungal-treated materials before and after being digested at an ISR of 0.821 in units of milligrams of nitrate nitrogen per gram of dry matter of substrate at the various levels of substrate ratio and fungal treatment. The levels rd, md, and sd correspond to digested raw material, digested mycelium-treated material (no fruiting), and digested spent substrate (fungal treatment with fruiting), respectively. The levels r and s correspond to materials not treated by fungus (raw materials) and spent substrate before digestion. The levels presented as numbers correspond to the ratio of broiler litter to wheat straw. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level					**Least Sq Mean**
0-100	A				1.793
25-75	A	B			1.481
50-50		B	C		1.184
100-0			C	D	0.9971
75-25				D	0.7842
Fungal Treatment Level					**Least Sq Mean**
sd	A				1.563
md	A				1.548
s	A	B			1.353
rd		B			1.156
r			C		0.6188

Table 17 shows that when both fungal treatment and substrate ratio are taken into account, nitrate increases progressively as the portion of wheat straw in the substrate increases with every other step increase being significantly differ; the exception being that 100-0 had significantly higher nitrate level than 75-25. Also, digested fungal-treated materials had significantly higher nitrate levels than digested raw materials, and the same goes for those materials before digestion.

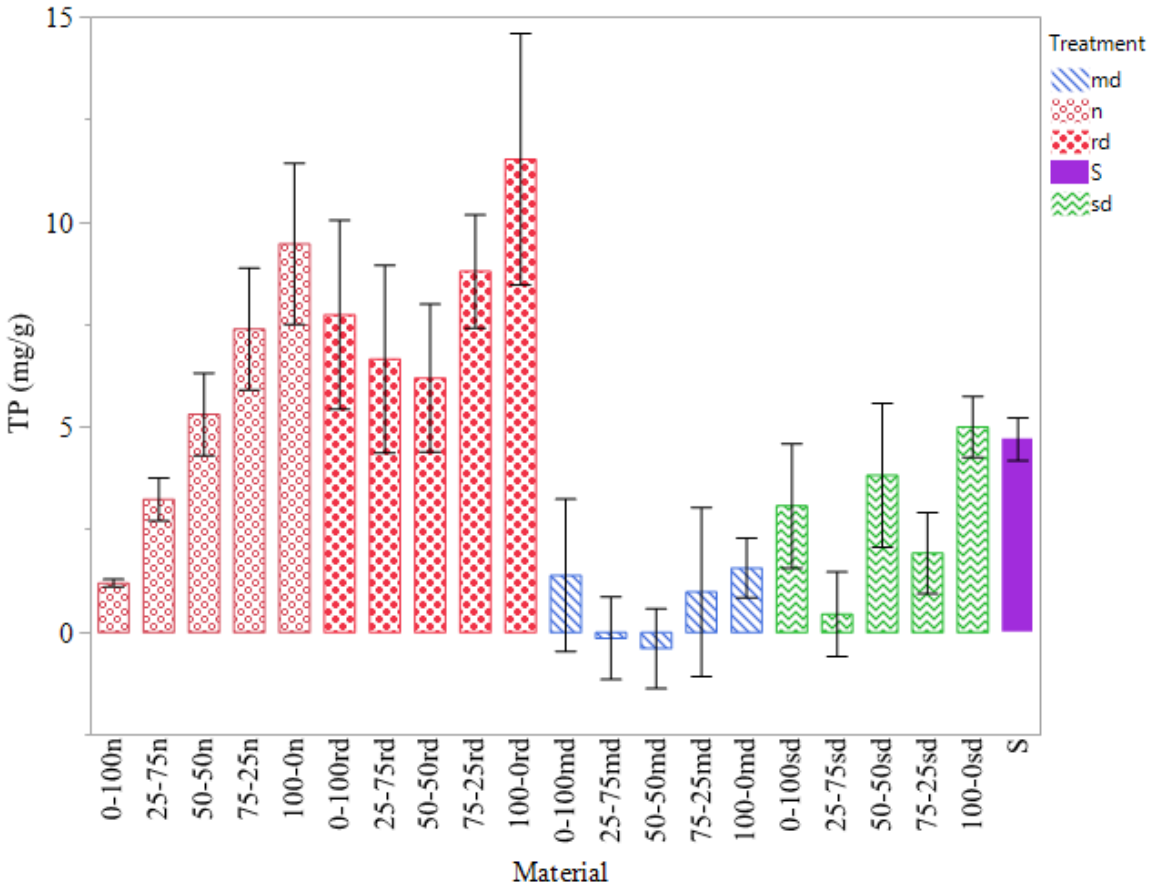

Figure 16 A bar plot showing TP of cold extracts of biomass. The labels md, n, rd, S, and sd represent digested and mycelium-treated material, no treatment (raw material), digested raw material, sludge (inoculum), and digested spent substrate.

As shown in Figure 16 and supported using a two-way ANOVA and post hoc analysis of TP with substrate ratio and fungal treatment being the two factors, the results of which are shown in Table 18 and Table 19, it was found that both substrate ratio and treatment had an effect on the phosphorus content of the materials. Addition of wheat straw, having a lower TP content, was able to lower the TP content of the material as a whole. More importantly, digested raw materials had significantly higher TP than untreated raw materials; however, digested materials that had undergone fungal pretreatment had significantly lower TP contents.

Table 18 ANOVA effects test table for phosphorus contents of raw and fungal-treated materials before and after being digested at an ISR of 0.821. P-values less than 0.05 are marked with an asterisk to show significance of the corresponding factor on extractives content.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Substrate Ratio	4	4	217.20741	20.2355	<.0001*
Fungal Treatment	3	3	921.52183	114.4677	<.0001*
Interaction	12	12	148.29476	4.6051	<.0001*

Table 18 shows that both substrate ratio and fungal treatment had significant effects on phosphorus. There was also a significant interaction effect. After it was found that both substrate ratio and fungal treatment had significant effects on phosphorus, a post hoc analysis was performed to determine which levels of each factor were significantly different from one another in order to draw further conclusions.

Table 19 Connecting letters report showing the results from the Tukey post hoc analysis of phosphorus contents for raw and fungal-treated materials before and after being digested at an ISR of 0.821 in units of milligrams of phosphate per gram of dry matter of substrate at the various levels of substrate ratio and fungal treatment. The levels rd, md, and sd correspond to digested raw material, digested mycelium-treated material (no fruiting), and digested spent substrate (fungal treatment with fruiting), respectively. The levels r and s correspond to materials not treated by fungus (raw materials) and spent substrate before digestion. The levels presented as numbers correspond to the ratio of broiler litter to wheat straw. Factors not connected by the same letters are significantly different ($p < 0.05$). Letters do not signify a comparison between levels of two different factors.

Substrate Ratio Level					Least Sq Mean
100-0	A				6.899
75-25		B			4.783
50-50		B	C		3.744
0-100		B	C		3.359
25-75			C		2.555
Fungal Treatment Level					**Least Sq Mean**
rd	A				8.192
r		B			5.329
sd			C		2.864
md				D	0.6865

Table 19 shows that when both fungal treatment and substrate ratio are taken into account, phosphorus increases progressively as the portion of broiler litter in the substrate increases with the exception of the 25-75 substrate which had a lower average phosphorus level than the 0-100. The 100-0 substrate had significantly higher TP levels than all other substrate ratios and the 25-75 substrate was only significantly lower than the 75-25 and 100-0 substrates. Also, every treatment factor tested was significantly different than all other with the digested raw materials having the highest TP levels and digested mycelium-treated material having the lowest.

The decrease in TP upon fungal treatment could possibly be due to the incorporation of P into macromolecules such as proteins, DNA, and RNA, but more information would be required to determine this. Although it is not well understood why fungal treatment would result in lower soluble TP content of the biomass (keep in mind that all chemical analyses were performed on extracts; meaning, only soluble components were measured), this effect along with other results discussed could have significant economic, social, and environmental impacts.

3-4 Potential Applications and Impacts

The discoveries made during this project have some significant implications that could have direct economic, social, and environmental impacts related to agriculture. For one, it was found that the yield of oyster mushrooms could be significantly increased by an average of 85% using a mixture of 25% broiler litter and 75% wheat straw as opposed to 100% wheat straw as a substrate. This provides an alternate use for broiler litter, which leads to the production of a more high-value product; raw broiler litter is sold for approximately $25/ton whereas, using the yields exhibited in the study and the average 2016 market value of oyster mushrooms being $3.60/lb, approximately $4,000 worth of oyster mushrooms could be produced per ton of substrate

composed of 25% broiler litter. This could have positive economic impacts on both the mushroom and poultry industries.

It was also found that the lignin contents of the materials were decreased by fungal treatment. Although this did not lead to higher methane yields, likely due to an increase in nitrate concentration, the spent mushroom substrate was still capable of being utilized through solid-state anaerobic digestion for the production of methane, which could potentially be used to offset energy usage associated with the mushroom and poultry production supply chains. The decrease in lignin content may also mean that the spent mushroom substrate could be more useful as a feed supplement for livestock compared to raw wheat straw.

It was also found that both fungal pretreatments resulted in a significant reduction in soluble phosphorus for all substrate ratios after digestion, whereas materials that had not previously been treated by fungus exhibit higher TP contents after digestion compared to the raw materials. This could have many potential implications. For one, these results suggest that fungal treatment could be used solely for the purpose of reducing soluble TP (or WEP) in broiler litter, which would allow for higher land application rates. This could in turn reduce the environmental impacts of the poultry industry and thus, reduce the legal disputes, regulatory restrictions on industry, and other negative social factors resulting from environmental impacts of agriculture. The same implications apply to the scenario where the broiler litter would be combined with wheat straw for mushroom production.

While fungal treatment was capable of effectively lowering soluble TP content, it and solid-state anaerobic digestion were both effective at increasing soluble nitrogen content of the materials. This implies that the fertilizer value of waste materials would be increased by the two biological treatments by effectively increasing the N:P ratios of the materials. This is another

implication that would have positive effects on agricultural supply chains, especially related to mushroom and poultry production and crop production in the same vicinity.

4. Conclusion: Summary and Future Research

Given the need for increased agricultural productivity and reduced environmental impacts to ensure the sustainability of our civilization, innovators need to be thinking of new ways to improve efficiency by reducing waste and increasing yields through the integration of agricultural supply chains in a way that mimics natural, ecological principles. In this manner, we can use biological resources to transform unwanted organic materials into products that are useful to us and continuously recirculate macronutrients needed for biological processes. If we look at unwanted agricultural byproducts as waste, the energy and macronutrients (or biological potential) of these materials could potentially be made less available to us and could directly cause significant negative impacts on the environment. This research exemplifies the application of these principles to create a real-world solution to an instance where a biological resource is not being utilized to its fullest potential.

Broiler litter, although it is often thought of a soil amendment, is an under-utilized and misused organic resource that is currently causing significant environmental and social problems in certain parts of the world. The oyster mushroom, thought of a specialty mushroom, is a highly productive and versatile fungus that in recent years has gained popularity as both a food source and a useful tool for bio-product engineering. Thus, the integration of these two biological resources was investigated and found to have significant implications related to increased mushroom productivity, increased utilization of broiler litter, the offset of energy usage, and improved downstream usage of macronutrients for other agricultural processes. Although many

questions were answered over the course of this study, several others have arisen along with unexpected results.

Although it was found that mushroom yields could be increased by supplementing wheat straw with broiler litter, only the lowest substrate ratio was capable of producing edible mushrooms. This leaves the question as to what the ideal ratio would be for oyster mushroom cultivation. Thus, a study could be performed to investigate the cultivation of oyster mushrooms with a smaller range of broiler litter addition. For instance, another study could be performed on ten substrate ratios ranging from 100 to 50 percent wheat straw in increments of 5 percent. A possible outcome of this experiment would be to find the amount of broiler litter that maximizes oyster mushroom yield. Also, the construction of the experimental growth chamber could be improved so that humidity could be controlled more precisely to see if this would reduce the variability in moisture content and biological efficiency.

Larger scale mushroom production experiments could also be performed in order to produce results that would be more representative of what would be expected in practice. More substrates could also be investigated to determine what the best recipe incorporating broiler litter and other waste materials available in the same area. The composition of broiler litter could also change depending on location. Thus, a large number of experiments would have to be performed and the data would have to be analyzed with source of broiler litter in mind in order to create results that could be highly useful to potential mushroom growers by accurately predicting yields.

It was also found that the fungus could improve the degradability of the materials, with and without mushroom fruiting by lowering lignin content and increasing extractives contents. This, however, did not improve methane yields likely due to the unexpected increase in nitrate

concentration. Yet, further experimentation could be performed to confirm this observation. If this is confirmed to be the case, possible solutions could be investigated and a more detailed analysis of potential impacts, which dictate the necessity of a solution, could be performed. It is quite possible that another biological, physical, or chemical process may need to be added prior to anaerobic digestion in order to reduce nitrate concentration. Perhaps the material could be "rinsed" before anaerobic digestion to remove soluble nitrogen. The leachate resulting from this process could potentially be used as nutrient-rich aqueous solution for green house operations.

Once more is understood about the relationships between the materials and the biological systems that were studied, more could be done to make these discoveries more applicable to industry. As previously discussed, an improved mushroom cultivation experiment could be performed to generate more consistent biological efficiencies that could be referenced by potential producers to make business decisions. Also, more extensive anaerobic digestion experiments could be performed to determine the operating parameters (i.e. loading rate, retention time, moisture content, pretreatments, etc.) needed to maximize methane yields and/or profits. However, all information generated needs to ultimately be related to the actual impacts that the proposed processes would have in practice, which could be determined through life cycle assessment (LCA) studies. It is often the case that proposed processes intuitively seem would reduce impacts, but the contrary is found to be the actual case. Thus, great care should be taken to makes insure that adverse effects of recommendations do not outweigh the benefits. This would help to insure that the general goal of improving the sustainability of our civilization is being improved.

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