**From waste to food: conversion of organic waste substrates into gourmet edible and medicinal mushrooms in Washington**

**Materials and Methods:**

The goal of this research will be to measure sporocarp production and biological efficiency during mushroom cultivation as functions of substrate mixture and mushroom species.

***Mushroom Species:***

*Collection of Samples:*

A total of 10 species were collected for this study. Seven species were collected from wild specimens in and around Pullman, WA; and three species were ordered from the commercial provider Mushroom Mountain, SC. All fungal strains were grown in laboratory conditions on PDA media. It is anticipated that nine of the collected species will be used in the research. The species cultivated will be *Agaricus augustus, Agaricus avrensis, Hericium americanum, Pleurotus ostreatus, Coprinus comatus, Ganoderma applanatum, Stropharia rugoso-annulata* (SRUG1), *Lentinula edodes* (LEDO2), and *Auricularia auriculara* (AAUR1) (Table 1).

*Species Identification:*

The species of collected samples will be determined by sequencing the Internal Transcribed Spacer (ITS) regions of each sample’s genome. PCR will be conducted using ITS-1 and ITS-4 primers.

***Substrate Materials:***

Four types of raw materials will be used as substrates: wheat straw (Four Star Supply, WA, locally produced), sawdust of *Pinus ponderosa* (Bennet Lumber Co, Clarkston WA), brewer’s spent grain (BSG) (Another Round Brewing, Pullman WA), and coffee grounds (various coffee shops, Pullman WA). These will be combined in different concentrations to create four different substrate mixture combinations (Table 2). Optimal substrate mixtures for most mushroom species consists of approximately 80% lignin/carbon source, 20% nitrogen-rich source, and other micronutrients such as gypsum and manure(Sokól *et al.* 2015, Stamets 2000, Sánchez 2004, Jang, Lee, Liu, & Ju. 2009). The substrate mixtures will be based around these parameters.

*Preparation:*

All substrates will be dried prior to use in order to accurately measure water content. Straw, Brewer’s Spent Grain (BSG), and coffee grounds will all be air dried in the university greenhouses. The sawdust will be composted in the WSU greenhouses prior to use as a substrate in order to break down substances that would hinder fungal growth (Oseni *et al.* 2012). The sawdust will be placed in plastic bins measuring 3 x 4 x 1 feet, watered to approximately 70% moisture, and covered in black plastic. The sawdust will be allowed to compost for approximately a month and turned once a week. At the end of a month, it will be ready to use as a mushroom substrate.

***Experimental Design:***

The design structure will take the form of a complete randomized design (CRD) using a nine-by-five factorial design with ten replicates. The two factors will consist of species (Table 1) with nine levels and substrate mixture (Table 2) with five levels. A control substrate mixture consisting of commercial mushroom substrate obtained from (X) will be used. There will be no negative control for species. The outcomes measured will consist of sporocarp individual size, sporocarp fresh weight, sporocarp dry weight, sporocarp nutrient content, and spent substrate mass post-harvest.

***Spawn Production:***

Grain spawn will be used to propagate all species samples for use in the cultivation experiments according to the methods recommended by Stamets (2000). A preliminary experiment testing the difference between using BSG and wheat berries will be conducted as the vigor of the spawn has a significant effect on colonization success of the bulk substrate. The spawn with the highest growth rate will be used as the inoculum for the bulk substrate. For each species, 2 spawn bags each containing 2.25 kg of substrate will be produced. Organic wheat berries obtained from the Moscow Food Co-op and SBG obtained from Another Round Brewing, WA will be used as substrates. Mushroom grow bags (Outgrow®, IL) will be used as spawning containers. Once a spawning substrate is selected, 4.5 kg of grain spawn will be produced for each species for a total of 40.5 kg. The final moisture content is expected to 50% by weight. The spawning run is expected to last two to three weeks.

***Substrate Inoculation:***

Five different substrate combinations will be tested for mushroom cultivation. Substrate materials will be mixed according to their dry weight ratios (Table 2). Each mixture will then be soaked in water for 12 hrs and the excess moisture allowed to run off until the substrate reaches a 65 ± 5% moisture content by weight. For each replicate, 3 kg of wet substate will be placed in a 1 L capacity, 50-micron polypropylene bag with linear ventilation filters (Outgrow®, IL) and autoclaved at 121°C for 15 minutes on wet cycle. Once cooled to room temperature, each bag will be inoculated with 50 g of grain spawn (5% inoculation). Each bag will then be sealed and mixed by gentle shaking to promote colonization.

***Growing Procedures:***

**Note: Growth chambers are in high demand, limiting our ability to adjust temperature for different species.**

Growing conditions will be the same for all experimental groups and will be kept at a constant temperature and humidity throughout the growing procedure. The bags will be kept in an environmentally controlled growing room at the WSU Pullman campus. The temperature of the growing room will be 25 ± 2 °C and humidity will be approximately 95 - 100% during the colonization period. Full colonization is expected to be achieved in approximately three weeks for most species. Once full colonization is achieved, the bags will be opened at intervals of 24 inches. Temperature will then be kept at 25 ± 2 °C, relative humidity at 75 ± 5%, and air CO2 concentrations below 1000 ppm. The growing chamber will receive dim light on a 12-hour day cycle.

***Data Collection:***

Sporocarps will be harvested at maturity for all species except for *C. comates*. *C. comates* sporocarps will be harvested just before maturity, while the gills are still veiled to prevent rapid decomposition. Sporocarps will be cut from the substrate at the growing bag surface.

*Sporocarp Size:*

At the end of growth, the sporocarps and substrate mass will be measured. Sporocarp production will be measured by mean individual diameter, mean fresh weight, and mean dry weight per experimental unit.

*Spent Substrate Mass:*

The substrate remaining in each replicate will be weighted after each harvest. This and the weight of the harvested sporocarps will be used to obtain the biological efficiency.

**Data Analysis:**

The means and standard deviations will be calculated for wet and dry yields, biological effeciency, and mean sporocarp sizes for each treatment. Significant differences between the means of substrate mixtures for each species will be estimated using a Tukey’s test at *p* < 0.05. In addition, linear models will be applied in order to estimate the effects of each substrate mixture across species

***Evaluate Assumptions of Linear Models:***

Four linear models will be constructed to describe the responses of each of the dependent variables to species and substrate mixture. Each model will use binary indicator variables to represent the means of each species, each substrate mixture, and the interactions between the two. This will result in 9, 5, and 40 terms respectively for a total of 54 terms in each model. The β0 will represent the mean response to the control substrate treatment across all species.

Various plots of the residuals will be created in the software program R to evaluate the assumptions of randomness, normal distribution, and equal variance of the means. Diagnostic tests will be performed with a significance level of α = 0.05. A Durban-Watson test will be used to evaluate the randomness of the residuals in regard to factors not accounted for in the model: the flush the sporocarps were harvested from and a bag’s sequential location in the growing chamber. A significant correlation would indicate that these factors had an impact on the data. A Shapiro-Wilk test will be used to test for a normal distribution of the residuals for each treatment group as well as the pooled residuals across groups. A significant difference from a normal distribution would indicate that the residual may be skewed and/or that there may be an outlier. A Breusch-Pagan test will be used to evaluate the variance of the residuals across the treatment groups. A significant difference in the variance could indicate that one or more species has a higher degree of variability in its response to different substrate mixtures or that different substrate mixtures cause yields to have a higher variance. A Correlation test will be used to test for the presence of outliers in the data. Any significant outliers will be investigated further.

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| **Table 1: Fungal Species Used** | | |
| **Common Name** | **Latin Name** | **Source** |
| Prince Agaricus | *Agaricus augustus* | Whitman County, WA |
| Horse Mushroom | *Agaricus arvensis* | Whitman County, WA |
| Shaggy Mane | *Coprinus comatus* | Whitman County, WA |
| Artist's Conk | *Ganoderma applanatum* |  |
| Bear's Head | *Hericium americanum* | Bonner County, ID |
| Oyster Mushroom | *Pleurotus ostreatus* | Hoh Rainforest, Olympic National Park WA |
| Winecap | *SRUG1 - Stropharia rugoso-annulata* | Retailer - Mushroom Mtn. NC |
| Shiitake | *LEDO2 - Lentinula edodes - Cold Outdoor* | Retailer - Mushroom Mtn. NC |
| Wood-ear | *AAUR1 - Auricularia auricula - Wood Ear - Wild type, SC* | Retailer - Mushroom Mtn. NC |

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| **Table 2: Substrate Mixtures** | | | | | |
| **Mixture:** | **Substrates (% dry weight)** | | | | **Additives** |
|  | **Sawdust** | **Straw** | **Brewer's Grain** | **Coffee Grounds** | **Gypsum** |
| **A** | 40 | 40 | 20 | 0 | 3 |
| **B** | 40 | 40 | 0 | 20 | 3 |
| **C** | 80 | 0 | 15 | 5 | 3 |
| **D** | 0 | 80 | 15 | 5 | 3 |
| **Industry Control** | N/A | N/A | N/A | N/A | N/A |