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

**Materials and Methods:**

The goal of this experiment 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 in SC. All fungal strains were grown in laboratory conditions on PDA media. It is anticipated that eight of the collected species will be used in the research.

*Species Identification:*

The species of fungal isolates used in this research will be verified using PCR amplification and sequencing of the ITS genomic region.

***Substrate Materials:***

Four types of raw materials will be used as substrates: wheat straw, sawdust, spent brewer’s grain, and coffee grounds. These will be combined in different concentrations to create five 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 loosely based around these parameters.

***Experimental Design:***

The treatment structure will consist of an eight-by-five factorial design with ten replicates. The two factors will consist of species (Table 1) with eight 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 design structure will take the form of a complete randomized blocked design (CRBD) experiment. The experimental units will be sorted into forty blocks of ten units each with each block representing one species x substrate mixture treatment combination. There will be a total of 400 experimental units. 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) for 1-liter jars. Organic wheat/rye will be used for the grain. For each species, 125 g of grain spawn will be used for the experiment in total.

***Treatment/Preparation of Substrate and Inoculation:***

The substrate mixtures will be made by thoroughly mixing substrates according to their dry weight ratios in Table 2. Water will then be added to each substrate mixture to bring it to 70% moisture. For each replicate, 1 kg of substate mixture will be added to a 1 L capacity, 50-micron polypropylene bag with linear ventilation filters. The bags will then be sealed and autoclaved at 121°C for 15 minutes and left to fully cool to room temperature (25°C). Once cooled, 5 g of grain spawn will be added to each bag. Each replicate bag will then be sealed, and the spawn mixed evenly by gentle shaking to promote colonization.

***Growing Procedures:***

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 60-70% during the colonization period. Full colonization is expected to be achieved in approximately three weeks on average depending on the species and will be determined through visual checks of the spread of mycelium through its bag. Once full colonization is achieved, the bags will be opened at intervals of 24 inches to allow for fruiting. During fruiting, the conditions will be kept constant at 25 ± 2 °C and 70-80% humidity. The bags will be watered by hand for the duration of fruiting to maintain ideal growing conditions. Fruiting will be allowed to continue for approximately two weeks, or until full sporocarp size is achieved.

***Data Collection:***

*Sporocarp Size:*

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

*Nutrient Content:*

*Spent Substrate Mass:*

After harvest, the spent substrate remaining in each replicate will be weighted before and after drying. This and the weight of the harvested sporocarps will be used to obtain the biological efficiency.

**Data Analysis:**

The outcomes will be analyzed using analysis of variance (ANOVA) and linear models. Two-way ANOVAs for randomized blocks with forty treatment groups will be applied for each of the three dependent variables. A level of significance α = 0.05 will be used.

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

The linear model used to describe this experiment will use binary indicator variables to represent the treatment group, resulting in a model with forty variables. All treatment groups will be represented as independent additive effects. Using a linear model to analyze the results will allow for comparison between subsets of multiple treatment groups (e.g., comparing sporocarp production between two substrate treatments across all species).

Linear models are based on the assumptions that the predictor and dependent variables have a linear relationship and that the residuals are independent, normally distributed, and have equal variance. If the model does not meet these assumptions, it could indicate that of factors not accounted for in the experiment are having and effect on the dependent variables. Evaluation of the assumptions for a two-way ANOVA will be achieved by creating additional plots of the residuals and identifying any deviation from expected distributions. A plot of the residuals fitted on the predictor variable in sequential order helps reveal independence and a plot of residuals fitted on the predictor variable(s) helps reveal equal variance. A linear model that satisfies our assumptions will result in plots that show no relationship or trend in the residuals. Likewise, histogram of the residuals should have a normal distribution centered on zero.

*Linearity:*

The variables used in linear models are assumed to have a linear additive relationship. To test for linearity, the residuals are plotted against one predictor variable at a time. If the resulting plot(s) forms a pattern other than a straight line, then it is likely that the true relationship between the variables is nonlinear, and a new model may be needed. In this experiment, it would be difficult to test for linearity because each the treatments are categorical.

*Independence – Breusch-Pagan Test:*

The Breusch-Pagan Test is used to test the independence (randomness) of the residuals. Residuals are independent if they do not follow any pattern with a sequential order (such as time or location) that is not accounted for in the model. This test is performed by forming a chi-squared distribution of the residuals from n \* R2 and comparing it to the observed distribution. The null hypothesis is that the residuals are independent of any sequential order. In this experiment, a Breusch-Pagan Test will be used to test if variables such as the location of experimental units in the growing room had any effect on the data.

*Normal Distribution – Shapiro-Wilk Test:*

The values of residuals should follow a normal distribution. Shapiro-Wilk Test is used to evaluate the normality of a population. The null hypothesis is that the population is normally distributed. In this experiment, a Shapiro-Wilk test will be run on the pooled residuals of all treatment groups as well as the residuals of each individual treatment group to test for normal distribution.

*Equal Variance - Brown-Forsythe Test & Breusch-Pagan Test:*

The variance of residuals should be constant across the values of the predictor variable(s) or the predicted value. A Breusch-Pagan Test can be used to evaluate this assumption, as residuals that are independent are also equal in variance. A Brown-Forsythe Test can be used to test equal variance. In this case, data the is split into two equal sized subsets between high and low predictor variable values. The variances of each subset are then compared using a two-sample *t*-test to check if they are significantly different. The null hypothesis is that the residuals have equal variance. In this experiment, a Brown-Forsythe Test will consist of t-tests between the treatment groups. A significant difference would between one or more groups could indicate a higher variance associated with a species or treatment group, or a data collection error.

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| **Table 1: Fungi Species** | |
| **Species:** | **Source:** |
| *Agaricus augustus* | Wild |
| *Agaricus avrensis* | Wild |
| *Hericium americanum* | Wild |
| *Pleurotus* | Wild |
| *Pisolithus* | Wild |
| *Coprinus comatus* | Wild |
| *Ganoderma applanatum* | Wild |
| SRUG1 *- Stropharia rugoso-annulata* | Mushroom Mountain |
| LEDO2 *- Lentinula edodes - Cold Outdoor* | Mushroom Mountain |
| AAUR1 *- Auricularia auricula - Wood Ear - Wild type, SC* | Mushroom Mountain |

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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 |  |
| **B** | 40 | 40 | 0 | 20 |  |
| **C** | 80 | 0 | 15 | 5 |  |
| **D** | 0 | 80 | 15 | 5 |  |
| **Industry Control** | N/A | N/A | N/A | N/A | N/A |