Mushroom Production in the Circular Economy

Closing the loop from waste to food

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Abstract

Global food production is unsustainable and relies on agricultural practices that are linear and create abundant waste. Mushrooms, especially oyster mushrooms from the genus Pleurotus, produce enzymes that can break down lignocellulose in these wastes and upcycle them into edible mushrooms. However, current research is insufficient. Most studies only report mushroom growth based on typical substrate recipes using local waste and weakly correlate their results to the essential nutrients of the substrates like cellulose, lignin, and nitrogen (mostly from protein). While this does demonstrate that mushroom production on waste is possible, it makes it difficult for small-scale mushroom producers to translate these results to a different context where that waste stream is not available. Additionally, most studies are conducted in a lab setting where some data (e.g. infection rate) is not considered relevant, although it is a critical element of commercial mushroom production. To close these gaps, this research took a two-step approach, focused on the common commercial grey oyster (Pleurotus ostreatus) and king oyster (Pleurotus eryngii) mushrooms. First, a literature analysis was conducted to generalize findings from relevant studies that investigated mushroom production on various waste streams. Biological efficiency and the essential nutrients in the substrate were standardized across all the studies if possible. Second, experimental research was performed at a small-scale commercial mushroom production company in Utrecht, The Netherlands. Here a variety of agricultural and urban waste stream were used to produce both species of mushrooms. Biological parameters of mushroom growth including infection rate, mycelial colonization time, fruiting time, and biological efficiency were then reported. The effects that the essential nutrients in the substrate have on these biological parameters (from literature and experiments) were analyzed using generalized linear mixed models. Overall, the amount of cellulose and lignin, but not nitrogen, in the substrate had the most effect on mushroom growth. More cellulose and less lignin apparently increased the biological efficiency of both mushroom species according to the literature analysis. However, in the experimental part of this research more cellulose increased infection rates for P. eryngii, possibly masking the effect of cellulose on mushroom growth, although it did appear that increased lignin decreased biological efficiency for this species. Experimentally, for P. ostreatus, cellulose had the opposite effect and decreased biological efficiency, possibly due to the strain that was used. Future research should focus on reducing infection rates and increasing the cellulose component of substrates to see if results from the literature can be replicated. Overall, this research adds to current evidence that these mushrooms can be effectively used to upcycle waste streams and close the loop in a circular economy.

Layman's Summary

Global food production is unsustainable. Currently, farming practices require a lot of resources and create waste that does not get used. Mushrooms, especially oyster mushrooms from the genus Pleurotus, produce enzymes that can break down these waste streams and upcycle them into edible mushrooms. These wastes are mostly made up of different nutrients, especially cellulose, lignin, and nitrogen (mostly from protein) that can affect mushroom growth. However, research into this area is insufficient. Currently, most research only describes how various agricultural waste affects mushroom production, typically using local waste streams. However, they do not report the exact effects of cellulose, lignin, and nitrogen on mushroom production. While this does demonstrate that mushroom production on waste is possible, it makes it difficult for small-scale mushroom producers to use these results when that waste stream is not available in their local area. Additionally, most studies are conducted in a lab setting where some data, like infection rates, are not considered relevant, although it is very important for commercial mushroom production. To close these gaps, this research took a two-step approach to investigate the commercial grey oyster (Pleurotus ostreatus) and king oyster (Pleurotus eryngii) mushrooms. First, relevant literature was analyzed. From this literature I estimated the biological efficiency of the mushrooms (the grams of mushrooms produced based on the grams of substrate used) and the essential nutrients in the substrate. Second, I performed experiments at a small-scale commercial mushroom production company in Utrecht, The Netherlands. Here I used a variety of agricultural and urban waste streams to produce both species of mushrooms. I estimated the infection rate, time it took for the fungi to colonize the substrate, time between colonization and the growth of edible mushrooms, and again the biological efficiency. I then compared these biological data to the nutrients of the substrate they were grown on. Overall, my data showed that the amount of cellulose and lignin in the substrate had a large effect on the growth of the mushrooms. More cellulose and less lignin apparently increased the biological efficiency of both mushroom species according to the literature analysis. In contrast, in my experiments more cellulose increased infection rates for P. eryngii, although it did grow fewer mushrooms when more lignin was present. For P. ostreatus, and different from the literature, cellulose decreased the biological efficiency, possibly because of the strain I used. Overall, this research adds to the current evidence that these mushrooms can be effectively used to upcycle waste streams and close the loop in a circular economy.

1. Introduction

Global food production is unsustainable (Meadows et al., 1972). The current food system relies heavily on resource-intensive practices such as industrial agriculture, which contributes to soil degradation, water pollution, and climate change (Raworth, 2017; Röckstrom et al., 2009). This system is also linear, and large amounts of organic waste are created each year, including crop stalks, straw, and other post-processing by-products such as seed hulls or dried pulps (Figure 1)(Krausmann et al., 2008; Lauk & Erb, 2009). This waste is typically burned or left to decompose which releases greenhouse gases that contribute to air pollution and climate change. In some cases, agricultural residue such as sugar beet pulp or wheat bran may be used to feed livestock. However, this waste could be repurposed and used as a valuable resource for bioenergy production, biorefining, or other applications. Finding ways to utilize this abundant waste stream could help create a more sustainable and circular economy, where resources are used efficiently and waste is minimized (MacArthur, 2013).

As they do in nature, mushrooms can upcycle waste and close the loop of a linear food system (Figure 1). What is more, they can achieve this while producing sustainable food that is high in protein and other important nutrients for the human diet (Assan & Mpofu, 2014; Lavelli et al., 2018; Sadh et al., 2018). The *Pleurotus* genus, commonly referred to as oyster mushrooms, are a group of white-rot fungi that have been used as food for centuries. This genus is comprised of basidiomycete fungi that break down lignocellulose. Lignocellulose is a complex and rigid material found in plant cell walls, and it is notoriously difficult to break down. However, oyster mushrooms produce a number of enzymes that can efficiently decompose lignocellulose, making them potent agents for managing organic waste (Hadar et al., 1993). Therefore, when used in a circular food system, oyster mushrooms have the potential to upcycle a historically difficult-to-use waste stream into food or other useable products while creating high-quality compost in the process. As such, the *Pleurotus* genus offers an exciting opportunity to transition towards a circular economy.

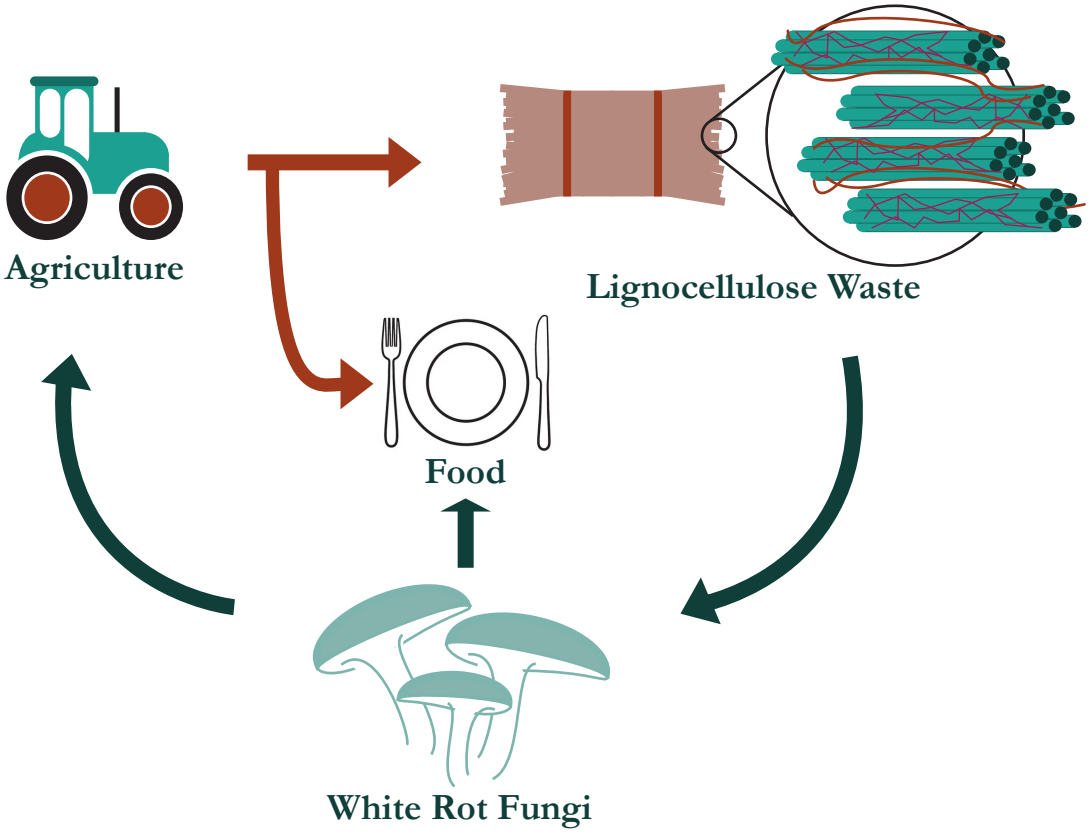

Figure 1. A circular model using white rot mushrooms to close the loop (green arrows) of linear agriculture and food production (brown arrows).

As oyster mushrooms have gained attention for their potential use within a circular economy, scientific research on this genus has increased. Various studies have shown that members of the *Pleurotus* genus can grow mushrooms (sporocarps) from a diverse range of waste substrates including straw, coffee husks, spent coffee grounds, waste paper, rice straw, rice bran, corn cobs, corn husks, coconut husks, pressed cotton waste, peanut shells, banana leaves, pal oil fronds, and sawdust from various species of tree (Baysal et al., 2003; Dedousi et al., 2023; Economou et al., 2020; Mandeel et al., 2005; Melanouri et al., 2022a; Melanouri et al., 2022b; Naraian et al., 2009; Philippoussis et al. 2001; Rizki & Tomai, 2011; Sardar et al., 2022; Zárate-Salazar et al., 2020). Furthermore, other waste streams, such as vegetable oil, calcified oyster shells, and wheat bran, have been utilized as nutrient-rich supplements to enhance the growth of oyster mushrooms (Ayodele & Okhuoya, 2007; Naraian et al., 2014; Sánchez, 2010; Wanzenböck et al., 2017).

Various waste streams and supplements have shown great promise for producing high quality mushrooms, however the actual essential elements that *Pleurotus* species need for growth are not well understood. In a nutrient limitation study, Tshinyangu and Hennebert (1995) grew grey oyster mushrooms (*Pleurotus ostreatus*) on wheat straw and compared it to the growth of mushrooms on a synthetic substrate created using cellulose, sugars, nitrogen, and minerals to mimic concentrations found in wheat straw. Their research showed that *P. ostreatus* could only produce mushrooms when the synthetic substrate contained cellulose, nitrogen, and minerals. However, interestingly, the synthetic substrate, even with concentrations of these essential elements similar to wheat straw, could not out-perform sporocarp yield on wheat straw alone (Tshinyangu & Hennebert, 1995). Therefore, they concluded that lignin and/or other minerals are essential elements that enhance mushroom growth (see also Hadar et al. 1992).

Commercially, nutrient requirements are met by mixing substrates into different "recipes" to produce fresh mushrooms. While some studies do point to key characteristics of the substrates that limit or enhance mushroom growth, for example the ratio of carbon to nitrogen (Sozbir et al., 2015) or the amount of nitrogen alone (Rizki & Tomai, 2011), others report unclear and/or correlative findings (e.g., see Philippoussis et al., 2001). Further, many of the recipes are designed and disseminated by popular or trade sources. While these mixtures ostensibly optimize different essential elements in the substrate mixture the reasoning for the specific ratios is not always clear or based on scientific methodology (e.g. see Stamets, 2011). For example, literature sources and commercial growers promote the use of "nitrogen supplementation" to increase mushroom yields but have little research supporting these claims or prescribing specific amounts of nitrogen. For example, Wanzenböck et al. (2017) used wheat bran as a nitrogen supplement but perform a coarse correlative analysis that only assumes that more wheat bran produces more nitrogen and does not account for confounding factors, e.g., the higher cellulose: lignin ratio of wheat bran as compared to the base substrate of beech wood used in the experiment. Other studies state that they "control for" specific ratios or nutrient elements, but do so poorly. For example, Dedousi et al. (2023) amended their substrate with soy flour to control for the carbon:nitrogen ratio, but report that it was standardized within a wide range (between 20-30:1). Overall, it is difficult to know what nutritive component of various substrate mixes increases yields. Further, in the interest of sustainability, understanding nutrient profiles of typical substrate mixes and their effects on mushroom production would allow mushroom growers to translate results to other geographic areas where different waste streams are available to replicate this circular model within other food systems.

One such typical substrate mixture is the so-called "master's mix" that consists of 50% hardwood sawdust (typically in a pelletized form used for fuel) and 50% soy hulls (a by-product of the soy bean processing) that is hydrated to 60%. This substrate mix is widely used by small-scale mushroom growers, many of which report beneficial results, although they are not reported empirically (GroCycle, n.d.; Urban Spore, n.d.). While this substrate is theoretically comprised of multiple waste streams, the hardwood fuel pellets are a "high-value" byproduct that already has a sufficient use, and soybean hulls are not readily available worldwide. Therefore, understanding what elements of this mixture (and other typical substrate mixes) are beneficial for mushroom growth, and reproducing these nutrient profiles with other, more circular and local,

waste streams is of interest to mushroom growers in a variety of contexts.

Small-scale and local mushroom production companies not only have the potential to upcycle waste streams into food, but they can do so sustainably with limited emissions. These companies also have the ability to utilize smaller and otherwise wasted urban waste streams, such as spent coffee from cafes or the waste grains at the end of the beer brewing process (Sánchez, 2010; Wang et al., 2001). However, apart from the treatment of essential nutrients in the literature, the results of academic and empirical studies are difficult to reproduce for small and local mushroom farms. Current research has been mostly conducted in lab settings, which is not easily translated to small-scale commercial farms. In particular, lab settings can be easily maintained in an extremely sterile state to reduce infections, but this degree of sterility is simply out of reach financially and logistically for most small-scale mushroom producers. Therefore, while some studies report significant increases in sporocarp yield using various waste resources, they may not take into account how some correlating results (e.g., slow mycelium growth rate, or favorability of nutrients for other infecting fungi) may make these growing processes more susceptible to infection and thus not applicable in the real world (Dedousi et al., 2023; Melanouri et al., 2022a; Melanouri et al., 2022b).

In this study I examined the effects of different waste streams and their principal components on the grey oyster (*Pleurotus ostreatus*) and king oyster (*Pleurotus eryngii*) mushrooms. *P. ostreatus* mushrooms are commonly and successfully grown in low-tech environments, especially due to recent genetic variants becoming widely available (Baars et al., 2000). Conversely, *P. eryngii* mushrooms have proven to be more difficult to grow, but this makes them more commercially attractive because they can be sold for a higher price. Therefore, optimizing the growth of these mushroom species is particularly interesting in the context of small-scale and local mushroom producers. To examine the gaps in the current research I took two approaches in this study. First, I collected data from the current literature to conduct a new analysis that used essential elements of substrates to focus on translating results into real world contexts, particularly for small-scale mushroom producers to re-create ideal substrate mixtures with local waste streams. Second, I conducted empirical research at a small-scale mushroom production company in Utrecht, The Netherlands. Here, I experimentally tested different substrate mixtures that were relevant to the Dutch context and compared them to the "master's mix" that is widely used by similar companies. I then modeled various parameters of sporocarp production based on the different nutrient profiles to relate mushroom growth to reproducible recipes based on major substrate components. Here I also report directly on infection rate as a dependent variable and do not treat it as a source of error. Ultimately, my aim is to provide greater insight into enhancing mushroom yields that are reproducible for small-scale mushroom producers. This will allow these results to be replicated in many more food systems and close small urban waste stream loops globally.

2. Methods

2.1 Research setting

This study was conducted at The Fungi Factory, Utrecht, The Netherlands (TFF). TFF is a small-scale mushroom farm that produces fresh mushrooms to be sold to local markets, restaurants, and individual consumers. As a small-scale and for-profit producer, the facilities can often be ad-hoc and while efforts are made to maintain a sterile and controlled environment, this is often only done to an extent where mushrooms can still be profitably produced and is not done to maintain a scientific standard. Therefore, while the conditions are not necessarily suitable for academic research, they are representative of many companies in the Netherlands and abroad attempting to function as circular businesses upcycling local waste stream flows into edible mushrooms.

2.2 Strains and storage conditions

Pleurotus ostreatus (Strain SPOPPO 830321, Sylvan Inc., Horst, The Netherlands) and *Pleurotus eryngii* (Strain M2603-5LSR-2, Mycelia, Deinze, Belgium) were used in this study. The species *P. ostreatus* was chosen as it was already used regularly for production at TFF, and *P. eryngii* was used because it is a gourmet mushroom that can be sold for a higher price and was desirable to be grown at TFF for commercial purposes. Mushroom strains were stored as mycelium inoculated grain spawn in a refrigerator at 4 C. To reduce costs, spawn was shipped in batches and stored for long periods of

time, thus, the age of spawn was not controlled for in this research. However, the same batch of spawn was used to inoculate the bags of the same experimental run.

2.3 Substrate preparation and inoculation

A variety of substrates and supplements were secured from different sources (Table 1). While some (e.g. sawdust from city tree maintenance) were sourced from local waste streams, others (e.g. soybean hulls) were purchased as secondary agricultural byproducts (typically already used commercially for other purposes) to represent waste streams found in other geographic regions. Base substrates and supplements were then mixed to make a number of final substrate mixes to either represent typical "recipes" from other small-scale farmers or growers, or experimental mixes. To create final substrate mixes substrates and supplements were first weighed on a scale (Vida XL, HABA Trading B.V., Venray, The Netherlands) and then mixed using a cement mixer until the mixture was homogenous. All substrate mixes were prepared using dry weight ratios (d/w). To calculate this, triplicate samples of all substrates were dried in a food dehydrator (SD-P9150 Sedon Dehydrator, Tribtest, California, USA) at 60 C until they maintained a constant weight. Water was then added to the mixture to achieve a final moisture content. The mixed substrate was then added into polypropylene bags with a 5-micron filter (Type 4b, Unicorn Bags, Texas, USA). Bags were then folded (but not closed) and stacked in a hand built 50-gallon drum steamer-sterilizer; a tool that is typical of other small-scale mushroom producers (Cactus Hat Mushrooms, 2021).

Experiments were run in groups of 40 bags. Each experimental run was conducted with four different batches, all batches had different substrate mixes, each batch comprised of 10 bags. All 40 bags were placed in the steamer and left overnight, for at least 16 hours. In this steamer, bags were in a closed chamber that was filled with steam with only a small outlet where steam escapes slowly. Thus, they were exposed to temperatures near 100˚C. After steaming overnight, bags were removed and then left to cool below 27C.

Bags were opened and inoculated with 100 ± 20 g of grain spawn. 5 bags from each batch were inoculated with *P. ostreatus*, and another five were inoculated with *P. eryngii*. In each experimental run, one batch was made with the master's mix 75:25 oak sawdust: soy hulls (d/w) and was treated as the standard in this experiment. While the original intention was to use a master's mix of 50:50 oak sawdust: soy hulls to, initial experimentation showed that only the 75:25 mix produced fruit, so this was chosen as the standard.

Note that inoculation took place in the open air inside a shipping container, although efforts were made to sterilize all surfaces and reduce air flow during inoculation (e.g. closing doors). Bags were then sealed using an impulse heat sealer. The bags were then manually shaken until the grain spawn was reasonably well mixed into the substrate.

Substrate	*Form*	*Source*	*d/w*	*Cellulose*	*Lignin*	*Protein*	*Data source*
Oak sawdust	Fuel pellets, soaked overnight	European timber byproduct	0.95	0.41	0.2571	0.0499	1
Soy hulls	Small sand-like grains	European agro-byproduct	0.89	0.441	0.024	0.128	2
Wheat straw	Finely cut, ~0.5-2cm long	European agro-byproduct	0.91	0.3073	0.0565	0.0406	3
Hemp straw	Cut, ~1cm-3cm long	Dutch agro-byproduct	0.87	0.377801	0.123799	0.097332	4
Wheat bran	Flakes	European agro-byproduct	0.92	0.2502	0.0763	0.2106	3
Spent beer grain	Soaked barley grains	Local beer breweries	0.21	0.165	0.056	0.262	2
Waste sawdust	Fine sawdust	Local tree maintenence	0.55	0.41	0.2571	0.0499	2
Beet root pulp	Feed pellets	European agro-byproduct	0.85	0.23	0.018	0.087	2
Coffe waste	Finely ground and brewed	Local coffee shops	0.47	0.251	0.1782	0.1188	3
Walnut oil cake	Ground and pressed	Local walnut oil producer	0.98	0	0.07	0.406	5
Flax seed	Whole	European food product	0.92	0.225	0.083	0.058	3
Rye seed	Whole	European food product	0.87	0.022	0.011	0.098	4

Table 1. Substrates used in experiments conducted at TFF. Dry weight (d/w) and percentage of essential nutrients by d/w for cellulose, lignin, and protein were taken from relevant data sources 1. Sozbir et al. (2015), 2. Tran et al. (2021) , 3. Melanouri et al. (2022), 4. Wang et al. (2022), 5. Archimède et al. (n.d.).

2.4 Growth conditions

Spawning – During spawning bags were kept sealed and in the dark in a shipping container. While the container was not temperature controlled, efforts were made to keep temperatures below 28C by either opening doors during the day or using a small air cooling unit. Bags were left in the spawning container until mycelium completely covered the outside surface of the substrate as seen through the bags. Once the substrate was colonized the bags were moved to another shipping container for fruiting.

Fruiting – During fruiting, bags were opened and kept in a different shipping container exposed to 12 hours of light per day. The climate of the container was measured using a home weather station (Netatmo, Boulogne-Billancourt, France). Efforts were made to maintain a favorable climate, although these were done manually and adhoc. During the experimental period the air was kept below 1200 ppm of CO2. The climate was typically between 8 and 23C although for short durations the temperature dropped lower, but never below 2.8C. The humidity was also typically kept between 80-100%, although it was lower for some short periods, however it never dropped below 58%. To open the bags for fruiting, two different methods were used. Bags of *P. ostreatus* were opened by means of cutting two X-shaped cuts (~5cm x 5cm) in the side of the bag to allow mushrooms to grow from the side and mimic the natural side-fruiting behavior, excess air was then pressed out of the bag. Bags of *P. eryngii* were cut open at the very top of the bag and then left until primordia developed to create a more humid microclimate in the bag (Earth Angel Mushrooms, 2018). For both species, primordia usually developed on the mycelial surface that was exposed to air, however, if this did not occur and primordia formed where they could not escape the bad, the bag would be opened where primordia did develop to allow them to develop into mushrooms.

Harvesting—Mushrooms were harvested when they had reached maturity and when the caps of the mushrooms had fully opened (Stamets, 2011). Yields were only reported for the first flush of mushrooms as this is typically the highest yielding flush, and therefore the most time and resource efficient for small businesses. Additionally, while some other research does report on subsequent flushes, the first flush is always reported in other studies. Mushrooms were harvested as close to the substrate as possible, without including any substrate within the mushroom stem. Mushrooms were then weighed with a digital kitchen scale (Item 1920108, Blokker, Amsterdam-Dulvendrecht, The Netherlands) to an accuracy of ±1 g.

2.5 Literature screening for analysis

To better understand the effects of different essential elements on mushroom production for *P. ostreatus* and *P. eryngii* I conducted a post hoc data analysis on literature reviewed in this research. Relevant literature used in this report were screened for use in this analysis and were included if they reported (1) a relevant experiment on *P. ostreatus* and/or *P. eryngii*, (2) sporocarp yields, (3) sufficient information to analyze the biological efficiency of the first flush of mushrooms alone based on wet weight (w/w) of mushrooms divided by dry weight of substrate (as this is the easiest to standardize among studies and the most commercially relevant), and (4) the proportions of various components used in the substrates such that the nutrient profiles could be calculated from the substrate dry weight.

2.6 Substrate nutrient analysis

Values for nutritional components (cellulose, lignin, and protein) for various substrates were taken from INRAE-CIRAD-AFZ Feed Tables when possible (Tran et al. 2021), or were otherwise taken from the literature (Table 1, Table 2). When the value of cellulose and lignin were not directly reported, but detergent fiber analysis was conducted, cellulose was reported as the value of acid detergent fiber minus the value of acid detergent lignin, and lignin was reported as the cell fraction determined by acid detergent lignin analysis (Van Soest et al., 1991). Crude protein was used for analysis where possible, but if only nitrogen was reported, then a correction factor of 6.25 was used to estimate the total protein (Mandeel et al., 2005).

2.7 Data analysis

2.7.1 Literature data

From all papers that met all four screening criteria biological efficiency was calculated for the first flush of harvested mushrooms. Biological efficiency was always calculated as the amount of wet weight of mushrooms divided by the dry weight of the substrate. Nutrient values of the substrate were taken from their

Substrate	Cellulose	Lignin	Protein	Data source
Barley oat straw	0.2822	0.175	0.0294	3
Rice husk	0.306	0.103	0.0035	3
Sunflower oil	0	0	0	2
Corn oil	0	0	0	2
Peptone	0	0	0.906	6
Yeast extract	0.017	0.007	0.476	2
Beech wood shaving	0.4592	0.1645	0.0144	3
Palm oil empty fruit bunch	0.397	0.2794	0.130625	7
Palm oil trunk	0.355	0.3454	0.0375	7
Acacia bark	0.404	0.2739	0.091875	7
Cogongrass	0.451	0.2641	0.0475	7
Palm oil frond	0.468	0.2152	0.021875	7
Cotton seed hull	0.472	0.181	0.052	2
Walnut shell	0.256	0.523	0.0644	1
Peanut shell	0.3836	0.2905	0.09125	8
Moringa leaf powder	0.119	0.034	0.268	5
Cotton waste	0.5252	0.1015	0.06875	8

Table 2. Substrates and their nutrient profiles used from the literature or from relevant data sources 1. Sozbir et al. (2015), 2. Tran et al. (2021) , 3. Melanouri et al. (2022), 4. Wang et al. (2022), 5. Archimède et al. (n.d.), 6. Yinqiang (2020), 7. Rizki & Tomai (2011), 8. Philippoussis et al. (2001).

respective literature article where possible, but otherwise they were retrieved from relevant sources or literature (Table 2).

2.7.2 Experimental data

Infection rate—The infection rate was calculated for each batch by species (infections per 5 bags). These rates were then subtracted from the infection rate of the standard run of the same day to control for errors and inconsistencies that could arise throughout the inoculation and incubation process for each experimental run (e.g. time in the sterilizing barrel, temperature of the incubation chamber). Infection rate is reported as infections per batch, but they are modeled as the infection percent difference from the standard.

Other parameters— Colonization time was calculated as the number of days it took for the substrate to be visually completely colonized by the mycelium after it was inoculated. Fruit time was calculated as the number of days between the complete colonization of the substrate, and when the mushrooms were harvested. Biological efficiency was calculated as the amount of wet weight of mushrooms from the first flush divided by the initial dry weight of the substrate.

To analyze and compare these parameters, they were not pooled by batch. These values were calculated individually per bag of substrate. This value was then subtracted from a pooled average for the standard batch run on the same day to again control for inconsistencies between experimental runs. Additionally, any bags that were infected during the experiment and did not produce mushrooms were not included in these analyses.

Each experimental batch was compared to the standard batch of the same experimental run to strengthen the data. The intention is to control for inconsistencies in a non-lab environment where some things cannot be controlled over the long-term, e.g. changing humidity due to open and closing doors, infection rates due to inoculation environment. But that they can reasonably be assumed to be similar on the same day. Therefore, it is more important and rigorous to compare, for example, the infection rate of a batch to another batch mixed on the same day, and not one mixed one month earlier. Overall, analyzing the data in this way will better show the effects of the nutrients in the substrates, and will remove some of the inconsistencies that would be expected in this research setting.

2.7.3 Modelling

In general, the modeling approach here can be described in five steps. (1) Choose a biological parameter to analyze (infection rate, colonization time, fruiting time, or biological efficiency). (2) propose different potential relationships between the biological parameter and essential nutrients in the substrate. These are deemed "candidate models" and are detailed below. (3) Compare all of the candidate models to each other using statistical criteria, which details how much of the data can be explained by the model. (4) Choose the model(s) that best explain the data. (5) Look at key elements of the best model(s) to analyze the quality of the model and discuss how well it explains the data.

Step One— Each biological parameter is described above in Sections 2.7.1 and 2.7.2. Note that infection rate, colonization time, fruiting time, or biological efficiency are all determined for the experimental data, but only biological efficiency is analyzed for the literature data.

Step Two— For each biological parameter under consideration, six candidate models were proposed based

on literature and common hypothesis of how mushroom growth is affected by primary substrate nutrients. The relationships described by the models are that the biological parameters were affected by (1) the percent of cellulose in the substrate, (2) the percent of lignin in the substrate, (3) the percent of protein in the substrate, (4) the ratio of cellulose to lignin in the substrate, (5) the ratio of cellulose to protein in the substrate (as a proxy for the carbon to nitrogen ratio) or (6) a single fixed value (intercept-only model). All of these models were assessed as linear effects, because there was no evidence or a compelling argument to suggest that any of these nutrients would have a threshold of their concentrations that significantly affected any biological parameters such that it would require the assessment of non-linear models. i.e. Cellulose concentration promotes growth up to a threshold at which point it limits growth (quadratic model).

Step Three— To compare the candidate models I used Akaike's information criterion adjusted for sample size (AICc). This criterion assesses the quality of each model, compared to the other models. It also "rewards" models for being more simplistic. This is important for this study because it is easy to overfit models with small sample sizes. All modelling analysis was performed using R (R Core Team, 2019). Candidate models for different variables were developed and fit using the package lme4 (Bates et al., 2015). AICc was calculated using the package AICcmodavg (Mazerolle, 2016).

Step Four— Models with the highest, or a reasonably high "weight" according to AICc were chosen as the most likely model to fit the data. i.e. The essential nutrient with the strongest effect on the biological paremeter. The AICc weight measures the likelihood that the given model is the strongest of the models.

Step Five—The most likely model is plotted and analyzed using different metrics. Here the coefficient, the significance of the coefficient, and the adjusted r-squared of the model are reported. The coefficient indicates the slope of the line in the model, i.e., how the essential nutrients are related to the biological parameter. The significance of the coefficient (p-value) indicates whether the relationship in the linear model is actually significant. If it is significant then the relationship is stronger. If it is not significant then, while the relationship does exist, it may just be a trend and not an actual cause and effect relationship between the nutrients and the biological parameter. Finally, the adjusted r-squared describes how well the model fits the data. A higher adjusted r-squared would indicate that this model alone explains most of the data, and a lot of the data points fall within a reasonable distance of the line. A lower adjusted r-squared means that there is a lot of variance in the data and that the linear model does not explain all of the variance in the data. Adjusted r-squared is reported here over other similar metrics because it is the most conservative estimate.

Overall, when interpreting these models, all of these outputs need to be taken together. Not one value (AICc, linear coefficients, p-value, or adjusted r-squared) can be used on it's own to determine whether a relationship between the nutrients in the substrate and mushroom growth is important. All plots were generated using the package ggplot2 (Wickham, 2016). For these analyses data for *P. ostreatus* and *P. eryngii* were always treated separately.

3. Results

3.1 Literature analysis

All 23 papers deemed relevant to the introduction of this paper were screened based on four criteria for inclusion in a post-hoc literature analysis (Table 3). This analysis aimed to generalize findings from previous research and reveal larger patterns of how mushroom production is affected by different essential nutrients in the substrate. Many papers that met the screening criteria supplemented substrates with nitrogen, so I hypothesized that the amount of nitrogen (protein) in the substrate would significantly influence mushroom production. Only seven of 23 papers met the criteria to be included in this analysis (Table 3). From these seven papers together 63 experiments for *P. eryngii* and 77 experiments for *P. ostreatus* were reported and analyzed.

The ratio of cellulose: lignin in the substrate played a significant role in determining the biological efficiency of both *P. eryngii* and *P. ostreatus*. Out of all the candidate models tested, the linear model including the cellusose: lignin ratio was the most likely model explaining biological efficiency. According to AICc the cellulose: lignin ratio accounted for 98% and 92% of the weight of all models considered for *P. eryngii* and

P. ostreatus respectively. In both models the cellulose: lignin ratio had a significant positive correlation with biological efficiency (coefficient = 7.155, $p<0.001$; coefficient = 6.828, $p<0.001$) (Figure 2). Therefore, a higher ratio of cellulose to lignin, i.e. more cellulose and less lignin in the substrate, leads to more mushrooms of both species. However, the linear models were not well fitted with an adjusted r-squared of 0.3751 and 0.2332 respectively. While there is a significant relationship between the cellulose: lignin ratio and biological efficiency, there is still a lot of variability in the data. Therefore we cannot assume that this variable alone causes biological efficiency to change. There are likely other nutrient (e.g. minerals or lipids) or experimental (e.g. amount of spawn used or small sample sizes) components influencing these data.

	Source	Criteria			
		1	2	3	4
1.	Assan & Mpofu (2014)	x			
2.	Lavelli et al. (2018)	x			
3.	Sadh et al. (2018)	x			
4.	Hadar et al. (1993)	x			x
5.	Baysal et al. (2003)	x	x		
6.	Dedousi et al. (2023)	x	x	x	x
7.	Economou et al. (2020)	x	x	x	
8.	Mandeel et al. (2005)	x	x		
9.	Melanouri et al. (2022a)	x			x
10.	Melanouri et al. (2022b)	x	x	x	x
11.	Philippoussis et al. (2001)	x	x	x	x
12.	Naraian et al. (2009)		x	x	x
13.	Rizki & Tomai (2011)	x	x	x	x
14.	Sardar et al. (2022)	x	x	x	x
15.	Zárate-Salazar et al. (2020)	x	x		x
16.	Ayodele & Okhuoya (2007)		x		x
17.	Naraian et al. (2014)		x		x
18.	Sánchez (2010)	x			
19.	Wanzenböck et al. (2017)	x	x	x	x
20.	Tshinyangu & Hennebert (1995)	x	x		x
21.	Sozbir et al. (2015)	x	x	x	x
22.	Wang et al. (2001)	x	x		x
23.	Baars et al. (2000)	x	x		

Table 3. Literature sources screened for inclusion in data analysis based on four criteria. (1) Paper was relevant to these species, (2) paper reported sporocarp yields, (3) paper included sufficient information to analyze the biological efficiency of the first flush of mushrooms alone based on wet weight of mushrooms divided by dry weight of substrate, and (4) paper included the proportions of various components used in the substrate mixes such that the nutrient profiles could be calculated from the substrate dry weight. "x" indicates if the paper met the criteria. Papers that met all four criteria were included in the analysis.(2001).

3.2 Experimental data

In this experiment 35 batches of various substrates were mixed and inoculated over 9 experimental runsfor a total of 9 standard batches and 26 experimental batches. Overall, more bags of *P. ostreatus* (n=74) produced fruit than *P. eryngii* (n=31).

For *P. ostreatus*, 10 batches either had lower infection rates than the standard batch from the same experiment, or also had a 0% infection rate when the standard was the same. Five batches (0413-3, 0413-4, 0425-2, 0425-4, and 0511-4) outperformed the standard in terms of biological efficiency. However, two of these batches were more susceptible to infection than the standard (0413-4, 0511-4). Therefore, while they did produce more mushrooms than the master's mix, fewer bags made it from inoculation to fruiting. Additionally, only 0425-2 and 0425-4 had shorter colonization times than the standard, but all five of these batches, except 0413-4, had shorter fruit times than the standard (Table 4).

For *P. eryngii* infection rates were significantly higher. Only three batches (0511-2, 0511-3, and 0511-4) had lower infection rates than the standard. Six batches outperformed the standard in terms of biological efficiency (0316-4, 0406-4, 0413-3, 0413-4, 0420-2, and 0425-4). However, none of these batches had a shorter colonization time, and only 0413-3 and 0425-4 had shorter fruiting times (Table 4).

3.2.1 Infection rate

The percentage of cellulose in the substrate had the strongest influence on infection rates for *P. eryngii*. The model including the percentage of cellulose in the substrate carried nearly the entire weight of all candidate models based on AICc (75%). However, for *P. ostreatus* no model outcompeted the intercept only model (AICc weight protein percentage 34%). The linear model analyzing the relationship between the percentage of cellulose in the substrate and the infection rate of *P. eryngii* revealed a significant positive correlation (coefficient = 2.7769, $p < 0.05$)(Figure 3). Therefore, it is likely higher percentages of cellulose in the substrate lead to higher rates of infection. However, the adjusted R-squared value indicated that a low proportion of variance explained by the model (adjusted R-squared = 0.1891), and other factors may also play an important role. The intercept-only model for the infection rate of

			P. ostreatus				*P. eryngii*				Nutrient Profile (%)		
Batch ID	***Substrates***	***Ratio***	***IR%***	***CT***	***FT***	***BE%***	***IR%***	***CT***	***FT***	***BE%***	***Cellulose***	***Lignin***	***Protein***
0302-1	OS:SH	50:50	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	0.43	0.14	0.09
0302-2 *	OS:SH	75:25	0 *	13 *	16.8 *	63.05 ± 29.34 *	0 *	15 *	18.4 *	67.56 ± 34.41 *	0.42	0.20	0.07
0302-3	OS:SB	83:17	0 =	13 =	22.2 +	20.38 ± 3.57 -	0.4 +	18.67 +	19.33 +	15.23 ± 9.53 -	0.37	0.22	0.09
0302-4	SB:WS	25:75	0 =	13 =	17.2 +	59.13 ± 23.92 -	0.4 +	17.67 +	21.33 +	41.63 ± 27.26 -	0.35	0.21	0.07
0316-1 *	OS:SH	75:25	0 *	13 *	14.8 *	56.83 ± 9.68 *	0.2 *	13 *	22 *	37.16 ± 22.36 *	0.42	0.20	0.07
0316-2	OS:SB	89:11	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	0.38	0.24	0.07
0316-3	SB:WS	11:89	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	0.38	0.24	0.03
0316-4	SH:ST	25:75	0.2 +	13 =	21.5 +	17.32 ± 13.13 -	0.8 +	13 =	22 =	42.08 ± 18.82 +	0.34	0.05	0.06
0323-1 *	OS:SH	75:25	0 *	11 *	20.4 *	68.10 ± 5.98 *	0.8 *	11 *	21 *	41.05 ± 18.36 *	0.42	0.20	0.07
0323-2	OS:SB	89:11	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	0.38	0.24	0.07
0323-3	SH:ST	25:75	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	0.34	0.05	0.06
0323-4	SB:WS	11:89	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	0.38	0.24	0.03
0330-1 *	OS:SH	75:25	0.4 *	19 *	26.7 *	16.60 ± 11.11 *	1 *	*dnf*	*dnf*	0.00 ± 0.00 *	0.42	0.20	0.07
0330-2	OS:SB	75:25	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.35	0.21	0.10
0330-3	ST:SB	89:11	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.29	0.06	0.06
0406-1 *	OS:SH	75:25	0.6 *	12 *	29 *	43.97 ± 29.66 *	1 *	dnf	dnf	0.00 ± 0.00 *	0.42	0.20	0.07
0406-2	OS:BP	75:25	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.37	0.20	0.06
0406-3	ST:SB	75:25	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.27	0.06	0.10
0406-4	SH:WS	25:75	0.4 -	16.67 +	17 -	42.85 ± 24.83 -	0.8 -	12 +	22 +	60.01 ± 26.84 +	0.42	0.20	0.03
0413-1 *	OS:SH	75:25	0 *	7 *	16.4 *	70.32 ± 9.25 *	0 *	10.2 *	18.6 *	49.40 ± 17.71 *	0.42	0.20	0.07
0413-2	SH:HS	25:75	0 =	8.6 +	20 +	30.55 ± 9.80 -	0.6 +	9 -	18 -	29.49 ± 17.15 -	0.39	0.10	0.10
0413-3	HS:WB:SB	80:5:15	0 =	*11* +	14 -	77.92 ± 25.80 +	0.8 +	*11* +	18 -	115.05 ± 51.45 +	0.34	0.11	0.13
0413-4	SH:ST:CW	25:37.5:37.5	0.4 +	11 +	18 +	71.44 ± 45.78 +	0.6 +	11 +	29.5 +	73.91 ± 40.84 +	0.32	0.09	0.09
0420-1 *	OS:SH	75:25	0.2 *	19 *	16.5 *	44.84 ± 21.04 *	1 *	*dnf*	*dnf*	0.00 ± 0.00 *	0.42	0.20	0.07
0420-2	OS:WC	75:25	0 -	19 =	17.5 +	40.45 ± 23.59 -	0.8 -	19 +	21 +	40.35 ± 18.04 +	0.31	0.21	0.14
0420-3	OS:WB:FS:RS	75:10:5:10	0.4 +	*19* =	22.3 +	33.49 ± 18.58 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.35	0.21	0.07
0420-4	OS:WB:WC:RS	75:10:5:10	0 -	19 =	22 +	33.92 ± 18.92 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.33	0.21	0.09
0425-1 *	OS:SH	75:25	0.2 *	14.75 *	18.7 *	36.90 ± 23.56 *	0.8 *	14 *	23 *	28.35 ± 12.68 *	0.42	0.20	0.07
0425-2	ST:CW	50:50	0.2 =	14 -	17.5 -	51.89 ± 29.73 +	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	0.28	0.12	0.08
0425-3	SH:ST:CW	25:37.5:37.5	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	0.32	0.09	0.09
0425-4	ST:CW:WC	37.5:37.5:25	0 -	14 -	27 +	50.10 ± 22.41 +	0.8 =	14 =	21 -	44.48 ± 19.89 +	0.21	0.11	0.16
0511-1 *	OS:SH	75:25	0 *	11.8 *	15.2 *	43.61 ± 13.98 *	1 *	*dnf*	*dnf*	0.00 ± 0.00 *	0.42	0.20	0.07
0511-2	ST:CW:WC	37.5:37.5:25	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.21	0.11	0.16
0511-3	ST:CW:WC	15:75:10	1 +	*dnf*	*dnf*	0.00 ± 0.00 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.23	0.15	0.14
0511-4	ST:CW:WC	90:5:5	0.2 +	19.5 +	11.3 -	41.50 ± 28.01 -	1 =	*dnf*	*dnf*	0.00 ± 0.00 =	0.29	0.06	0.06

Table 4. The composition of the substrate for standard (marked *) and experimental batches, including the substrate mix (OS = oak sawdust, SH=soy hulls, ST = wheat straw, HS = hemp straw, WB = wheat bran, SB = spent beer grain, WS = waste sawdust, BP = beet pulp, CW = coffee waste, WC = walnut cake, FS = flax seed, RS = rye seed), the infection rate (%) (IR%), colonization time (CT) in days, fruiting time (FT) in days and biological efficiency (BE%) with the standard deviation reported. Symbols following the values indicate positive (+), negative (-), or no (=) change from the standard (*). Dnf denotes batches where the parameter was not calculated because it did not fruit.

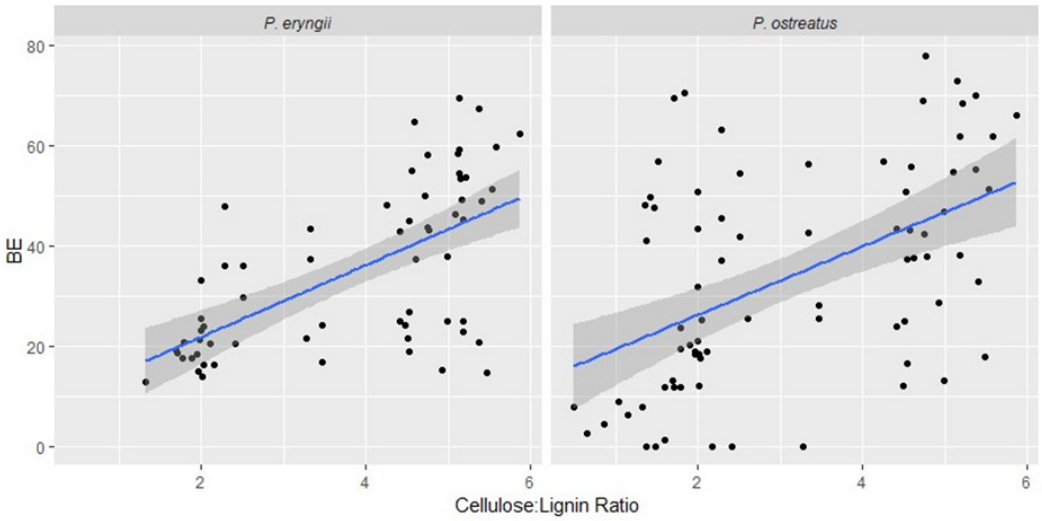

Figure 2. The biological efficiency (w/w mushrooms divided by d/w of substrate) of *P. eryngii* and *P. ostreatus* mushrooms using datapoints from literature compared to the relative percent of lignin in the substrate. Blue line represents the most competitive linear model and gray shading indicates the 95% confidence interval of the line.

P. ostreatus revealed a significant coefficient of 0.42 ($p < 0.001$)(Figure 3). Because the intercept-only model was the most competitive of the candidate models, it is likely that the infection rate of *P. ostreatus* substrate is simply based on a fixed percentage (42% infection rate) and does not change due to any of the essential elements of the substrate included in the analysis.

3.2.2 Colonization time

For the colonization time in days of *P. eryngii* bags compared to the standard, no model outcompeted the intercept only model. Therefore, the most competitive model showed that the colonization time for experimental substrates did not vary based on essential nutrients in the substrate, but instead was different from the standard based on a single fixed value (+3.33 days). However, this linear model was not significant ($p<0.1$) (Figure 4). On the contrary, the model including lignin percentage carried the most weight (82%) for the colonization time of *P. ostreatus*. The linear model containing percentage of lignin had a negative coefficient (-17.98) and was significant ($p<0.01$), however the adjusted r-squared was low (0.1496) (Figure 4). Therefore, it is likely that the colonization time of *P. ostreatus* decreases when more lignin is present in the substrate.

3.2.3 Fruiting time

For both *P. eryngii* and *P. ostreatus* the cellulose:protein ratio was the most important factor influencing the number of days between when the bags were opened and fruit was harvested. The models containing the cellulose:protein ratio out competed the intercept-only model (36% compared to 31%, and 97% compared to 1% respectively). For *P. eryngii* this linear model had a positive coefficient, but was not significant (coefficient= 1.374, $p=0.0872$) and had a very low adjusted r-squared (0.1474)(Figure 5). For *P. ostreatus* there was a negative coefficient, and it was significant (coefficient= -1.18, $p<0.001$) with a low adjusted r-squared (0.20) (Figure 5). Therefore, while the cellulose: protein ratio likely does affect the fruiting time of both species, other factors are also likely responsible for changes in fruiting time in the experimental substrates.

3.2.4 Biological efficiency

The essential nutrients of the substrate did not affect the biological efficiency of *P. eryngii* mushroom growth. The model containing the lignin percentage of the substrate was narrowly out-competed by the intercept only model (AICc weight 28%, 34%, respectively). The intercept-only model had a coefficient of -0.02 but it was not significant ($p=0.80$) (Figure 6). The linear model for lignin percentage had a negative coefficient but it was not significant (coefficient=-2.62, $p=0.13$). Therefore, while biological efficiency did generally decrease when there was more lignin in the substrate, this was not the strongest model. The biological efficiency of *P. ostreatus* was negatively affected by the percentage of cellulose in the substrate. The model containing cellulose percentage of the substrate carried 95% of the model weight according to AICc. The linear model had a negative coefficient (-2.70) that was significant ($p<0.01$) (Figure 6). However, the adjusted r-squared was still low (0.18) which indicates

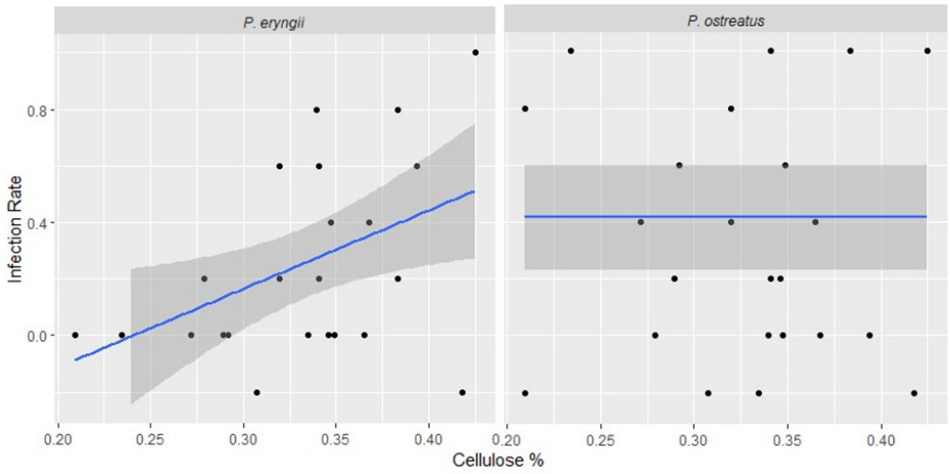

Figure 3. Infection rate of *P. eryngii* and *P. ostreatus* based on the percentage of cellulose in the substrate. Infection rate is corrected by the infection rate of the standard batch from the same experimental run. Blue line represents the most competitive linear model and gray shading indicates the 95% confidence interval of the line.

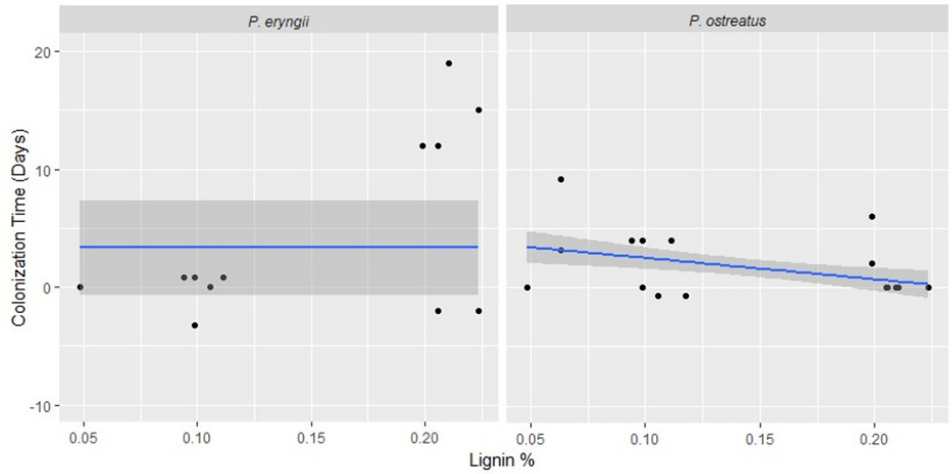

Figure 4. Colonization time (days) of *P. eryngii* and *P. ostreatus* based on the percentage of lignin in the substrate. Colonization time is corrected by the colonization time of the standard substrate. Blue line represents the most competitive linear model and gray shading indicates the 95% confidence interval of the line.

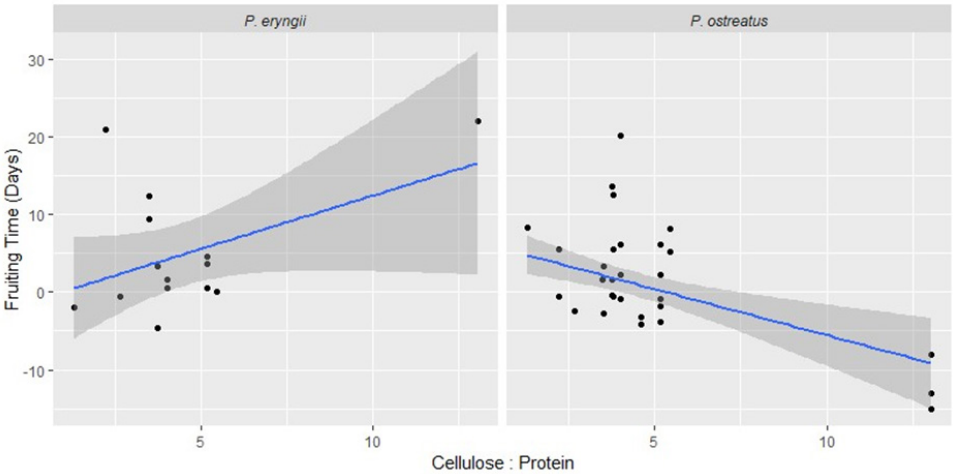

Figure 5. Fruiting time (days) of *P. eryngii* and *P. ostreatus* mushrooms by percentage cellulose in the substrate. Fruiting time is corrected by the fruiting time of the standard batch from the same experimental run. Blue line represents the most competitive model and gray shading indicates the 95% confidence interval of the line.

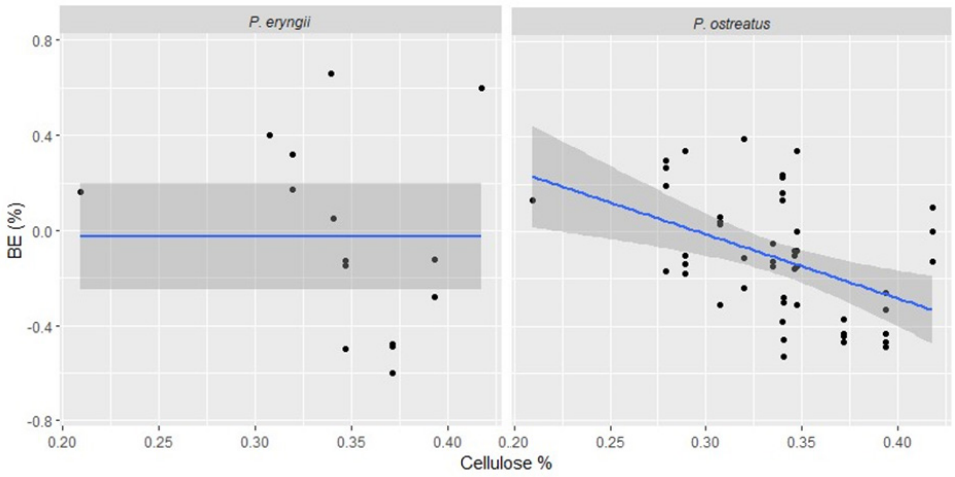

Figure 6. Biological efficiency of *P. eryngii* and *P. ostreatus* based on the percentage of cellulose. Biological efficiency is corrected by the biological efficiency of the standard batch of the same experimental run. Blue line represents the most competitive model and gray shading indicates the 95% confidence interval of the line.

that there is a lot of variability in the data that could be due to experimental error, a small sample size, or other experimental factors besides cellulose percentage influencing the biological efficiency.

4. Conclusion

In this research I demonstrated that local waste streams can be successfully utilized to grow edible mushrooms. Notably, the biological efficiency of mushrooms grown on some of these waste streams surpassed those observed with more conventional, and less circular substrates. Further, trends were observed in both experimental and literature data that highlight the relationships between various essential nutrients in growth substrates and key growth parameters of the mushrooms. These findings have important implications for the FF and for other circular mushroom companies globally as they can help them explore other suitable waste streams and incorporate them into their production to increase mushroom yields and continue fostering more sustainable practices.

Experimentally, the amount of mushrooms grown per gram of substrate (biological efficiency) of *P. eryngii* did not change based on the substrate. However, generally speaking, *P. eryngii* mushrooms had a higher biological efficiency when less lignin was present. Lignin is a complex and three-dimensional aromatic biopolymer that is difficult for fungi to degrade. Specifically, compared to cellulose, lignin does not have any hydrolysable linkages, and therefore requires oxidative enzymes for degradation (Hatakka & Hammel, 2011). Only some basidiomycetes (those belonging to the group of white-rot fungi) are able to degrade this complex component of the plant cell wall (Blanchette, 1995; Buswell Et al., 1987). The ability of white-rot fungi to degrade lignin varies widely based on species, and those of the *Pleurotus* genus are generally believed to be effective agents of lignin degradation (Ander & Eriksson, 1977; Hadar et al. 1992, Hakala et al., 2004). In a comparative study between 86 different isolates of white-rot fungi, one strain of *P. ostreatus* was found to degrade lignin in a 1:1 ratio with cellulose (Kajar et al., 2004). However, even within this genus, there is a great amount of variability. Many studies show that the production of lignin degrading enzymes such as laccase and peroxidase can vary based on a wide variety of environmental factors (such as the concentration of copper and manganese), even the type of lignin present in the substrate (Buswell et al. 1987; Elisashvili et al. 2018; Kerem & Hadar, 1995, Melanouri et al. 2022a; Philippoussis et al. 2001). And one study of different strains *P. eryngii* collected from Iran showed that different genotypes can have different rates of lignin and cellulose degradation (Sonnenberg et al., 2016). Therefore, it is very difficult to generalize claims such as "*Pleurotus* mushrooms are good at degrading lignin." In this experiment the two experimental batches with the highest biological efficiency were 0413-3 and 0413-4 which were comprised of 80:5:15 Hemp straw: Wheat bran: Spent beer grain and 25:37.5:37.5 Soy Hulls: Straw: Coffee waste. These substrates had relatively low lignin concentrations. Therefore, while other factors are important to consider, it is likely that this strain of *P. eryngii* cannot efficiently degrade lignin or prefers substrates that are less complex.

Colonization times of *P. eryngii* were not significantly influenced by the essential nutrients of the substrate. Presumably, the relatively difficult lignin degradation process would cause longer colonization times. However, this did not appear to be the case. Interestingly, a higher percentage of lignin in the substrate also did not lead to higher infection rates. In general, in a non-lab environment like the TFF, substrate bags are often contaminated with other fungal species other than the target species. Consequently, an argument could be made that longer colonization times would allow infective species to establish themselves in the substrate and outcompete the target species (Soto-Cruz et al., 1999). However, this was not observed for *P. eryngii*, and only higher concentrations of cellulose were significantly correlated with higher infection rates. Therefore, while lignin rich substrates yield fewer mushrooms, they may be more effective in preventing infections.

A similar relationship between lower lignin concentrations and higher biological efficiency was found in the literature. The collected data from the seven selected literature sources (Dedousi et al. 2023, Melanouri et al., 2022b; Philippoussis et al., 2001; Rizki & Tomai, 2011; Sardar et al., 2022; Sozbir et al., 2015; Wanzenböck et al., 2017) showed that the cellulose: lignin ratio was the strongest model for estimating the biological efficiency of both *P. eryngii* and *P. ostreatus*. The analysis on this data showed that both fungi could efficiently produce more mushrooms on substrate with a higher cellulose: lignin ratio. This means the fungal strains can grow on substrates with more cellulose and less lignin. As described earlier, nutrient degradation varies greatly between species (Kajar et al., 2004), and even between the strains of the same species (Sonnenberg et al., 2016). Therefore, it is particularly interesting that this relationship was strong for two species of mushroom and across multiple studies where a variety of strains were used. This relationship, presumably caused by the differential degradability of cellulose and lignin is well reported in the literature as discussed throughout this research. However, this ratio never emerged as a strong model describing my experimental results. It is possible that this relationship was difficult to observe due to the positive correlation between cellulose percentage and infection rates, at least for *P. eryngii*. Stated differently, while the data collected and presented here does potentially demonstrate the negative impact of lignin on the biological efficiency of *P. eryngii*, the high infection rate of more cellulosic waste streams could have masked the positive effects of cellulose.

Interestingly, the obtained experimental results for *P. ostreatus* contradicted these findings and showed that higher cellulose concentrations in the substrate lead to lower rates of biological efficiency. The linear model containing cellulose percentage of the substrate carried 95% of the model weight and had a coefficient that was significant. However, as described above, cellulose theoretically is a more available source of glucose (Hatakka & Hammel, 2011). The availability of cellulose to fungi may differ based on different factors such as the degree of crystallinity of the cellulose, or its arrangement within the matrix of hemicellulose and lignin the species of plant (Blanchette, 1995; Coughlan, 1990; Hakala et al., 2004). However, the sources of cellulose (woody and grass species) and the variety of cellulose:lignin ratios of the substrates used here were diverse, but still contributed to the overall trend. Cellulose concentration was also positively correlated with colonization time, such that more cellulose in the substrate lead to longer periods for the mycelium to colonize the substrate. However, pure cellulose was never added to substrates, and so we cannot say what effect it has on mushroom growth alone, only in ratio to the other primary components of the substrate. Thus, many confounding factors exist, and some of the highest producing substrates are likely low in other essential nutrients. Additionally, some substrates that stimulated mushroom growth, such a spent beer grain, contain simple sugars that are readily available in a form other than cellulose (Jackowski et al. 2020, Wang et al. 2001). Therefore, while the crude percentages of these primary substrate components are important, other factors, especially the pre-processing of lignocellulosic waste substrates may play a significant role.

The protein content of the substrate did not appear to have a significant influence on any of the biological parameters for either *P. eryngii* or *P. ostreatus*, based on the literature and my experimental results. Only fruiting time showed a weak correlation with protein content, but it was in ratio to cellulose, and had a very low adjusted r-squared value. Therefore, while it may have affected fruiting time some, it did not affect it alone. Further, because the adjusted r-squared value was low, the fit of the model was weak, and therefore the cellulose: nitrogen ratio did not have a strong influence

on fruiting time. Nitrogen supplementation, for which protein is the main source, is commonly prescribed in various academic, popular, and trade sources to increase biological efficiency (e.g. Ayodele et al., 2007; Stamets, 2011; Naraian et al., 2009). Nitrogen is a critical component of various metabolic pathways, particularly in synthesizing enzymes used for lignin and cellulose degradation (Bellettini et al., 2019; Buswell et al., 1987; Membrillo et al., 2008). However, studies focusing on these pathways show that nitrogen supplementation does not always directly increase enzyme production. Studies showed that the source of the nitrogen or carbon (i.e. the cellulose, lignin, or hemicellulose) can have a greater impact to enzyme production than the amount of nitrogen (Mikiashvili et al., 2006; Stajić et al., 2007). However, existing research on mushroom production rarely controls for confounding factors of the nitrogen supplements, and often rely on correlative treatment of the data. For example, it is often suggested that adding soy hulls to oak sawdust in the master's mix acts as a nitrogen supplement, but they also have the highest cellulose:lignin ratio of all substrates that were used in either my research or in the literature I reviewed (Figure 7). And wheat bran, another common nitrogen supplement, also has a relatively high cellulose:lignin ratio compared to substrates such as wood sawdust. In the literature analysis, nitrogen did not show a specific effect on the models of biological efficiency despite including studies that ostensibly manipulated nitrogen levels in the substrate. However, these post-hoc data analyses are limited, and the models explained only a small portion of the data variance. Therefore, my findings likely do not capture the full picture of the dynamics observed in those studies.

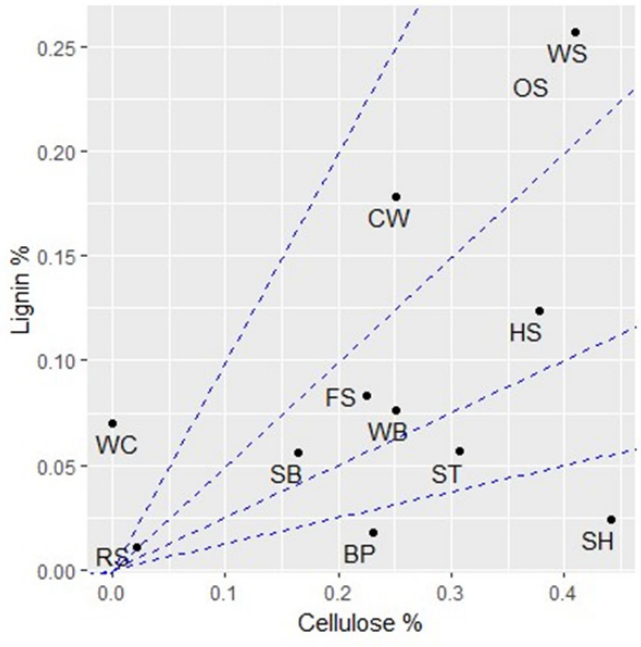

Figure 7. The distribution of the substrates currently in use at TFF based on the percent composition of cellulose and lignin. (OS = oak sawdust, SH=soy hulls, ST = wheat straw, HS = hemp straw, WB = wheat bran, SB = spent beer grain, WS = waste sawdust, BP = beet pulp, CW = coffee waste, WC = walnut cake, FS = flax seed, RS = rye seed). Blue dotted lines represent 1:1, 1:2, 1:4, and 1:8 lignin: cellulose ratios.

While the research conducted here has not been able to add clarity to the exact effects that these essential nutrients have on mushroom growth, it has successfully demonstrated that, if chosen correctly, local waste streams can be effectively substituted for less circular substrates. For both *P. ostreatus* and *P. eryngii* one of the substrate mixtures with the highest biological efficiency (0413-4) was composed of a 25:37.5:37.5 mixture of Soy hulls: Straw: Coffee waste. This mixture was composed of one local waste stream (coffee waste), and two agricultural by-products that have other potential uses. However, in another experimental run this same mixture was used (0425-3), but it was also grown alongside a mixture where the soy hulls were replaced by walnut cake based on dry weight (0425-4). In this experimental run the walnut cake substrate outperformed the soy hull mixture in the same run because it was less susceptible to infection. Overall, this demonstrates that local waste streams can be effectively used to enhance mushroom production over less circular alternatives.

Within the Dutch context, and for the Fungi Factory specifically, many circular substrates are available that efficiently produced mushrooms for both species in this study. For both *P. eryngii* and *P. ostreatus*, a mixture of Hemp straw: Wheat bran: Spent beer in a ratio of 80:5:15 had the highest biological efficiency of any substrate studied here (115.05%, 77.92%, respectively). This mixture is comprised of one Dutch agricultural byproduct (hemp straw), one European agricultural byproduct (wheat bran), and one local waste stream. While this substrate is both efficient and sustainable, there is potential to make it more circular. A future experiment could replace at least some of the hemp straw with more spent beer grain as they have similar

cellulose: lignin ratios and would make the substrate more circular and local (Figure 7). Further, according to my results, this may have the potential to reduce infection rates by avoiding the use of a waste stream with a high cellulose component.

Overall, it is beneficial to choose waste streams that are lower in lignin to increase biological efficiency, but lower in cellulose to reduce infections. This is especially applicable to *P. eryngii*. Both beet pulp and walnut cake have low cellulose content compared to soy hulls, and have relatively low lignin content, and therefore may be a sufficient replacement in the master's mix. As mentioned above a substrate mixture of 25: 37.5: 37.5 Walnut cake: Straw: Coffee waste (0425-4) grew more mushrooms than the standard batch for both *P. ostreatus* and *P. eryngii*. It also had a lower infection rate than the same mixture when soy hulls were used instead of walnut cake (0425-3), when the same mixture (0413-4) had previously performed very well. As demonstrated by these batches, reducing the amount of cellulose can reduce infections, and maintaining a low percentage of lignin can increase biological efficiency. Therefore, using walnut cake (or other substrates similarly low in cellulose and lignin) as a replacement for other substrates is a recommended course of future research.

According to the results of the literature analysis, it is likely that *P. eryngii* and *P. ostreatus* will produce more mushrooms on more cellulosic substrates. The results were not observed in the experimental research done here, due to the high infection rates within the experimental set up that has been discussed in this paper. Therefore, it would be beneficial to take effort to decrease infection rates at TFF. While there was a relationship between more cellulose in the substrate and more infections for *P. eryngii* it is not clear why this exists. It is possible that these substrates already have a higher rate of infection before they are used, and thus need to be better sterilized before inoculation. It is also possible that substrate sterilization is sufficient and *P. eryngii* has difficulty outcompeting infective fungi introduced during inoculation. Therefore, in order to reduce infection rates, it is recommended to both use more sterile inoculation procedures (e.g. using a cross flow or other air filtration) as well as increasing the effectiveness of the substrate sterilization. If infection rates can be reduced then, based on the literature, it is advisable to investigate mushroom production on substrates rich in cellulose. To accomplish this, substrates such as soy hulls, wheat straw, or hemp straw could be used. Moreover, other waste streams can be available locally that are also high in cellulose. For instance, SCOBY, a waste stream of kombucha brewing comprised of bacterial cellulose, may be an excellent cellulose source that is more sustainable and more bio-available as the polymerization of bacterial cellulose is 4-fold lower than plant cellulose (Coughlan, 1990).

Overall, this research points to a greater need for standardization in studies investigating mushroom production. Future research in this field should prioritize the comprehensive reporting and analysis of results that results, taking into account the full nutrient profile of substrates and their supplements. It is recommended that studies always report on the first flush of mushrooms to best standardize results between papers. Moreover, it should be standard practice to report sufficient information to determine the biological efficiency calculated as the wet weight of mushrooms divided by dry weight of substrate, as this is the most widely used formula. Ultimately, it would be beneficial to conduct a more systematic review of the literature and a more intensive data analysis. It would be particularly interesting to include a wider range of nutrients, such as fat and minerals (such as calcium and manganese)—factors that were outside the scope of this research. Further, a more extensive and rigorous literature review could reveal additional papers that meet the screening criteria. Conducting this review, and performing further analysis, could add greater clarity and strengthen the results of this study.

In conclusion, both *P. ostreatus* and *P. eryngii* have great potential for cultivation on local and sustainable waste streams within the Dutch context and globally. However, this research demonstrates that more standardization in mushroom production studies is greatly needed for results to be translated from the local contexts in which they are conducted. By considering the complete nutrient profile of substrates, and adopting common reporting standards, future research can improve the reliability and comparability of findings. This approach could also shed light on the effect of nitrogen supplementation on biological efficiency, which, contrary to prevailing beliefs, did emerge as a significant factor in my literature analysis. Overall, for

small-scale mushroom producers cultivating *P. eryngii* and *P. ostreatus* mushroom the best course of action is to prioritize waste streams rich in cellulose and low in lignin, but this will require minimizing infection risks. Following these guidelines can help enhance sustainable mushroom production and grow a more circular economy.

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