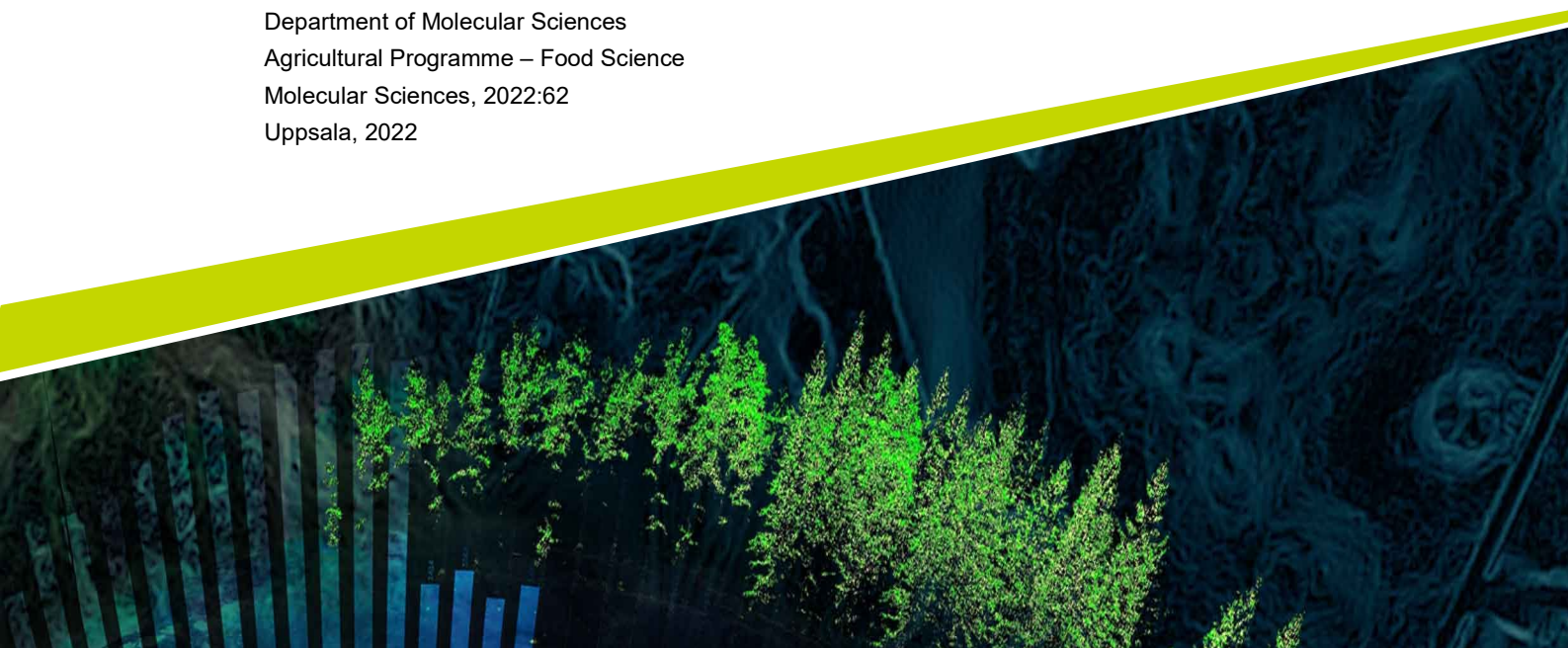
Exploration of ways to utilize wheat chaff through fungal substrate conversion

- A base for animal feed or substrate for cultivation of edible mushrooms

Caroline Sandberg

Independent project • 30 credits
Swedish University of Agricultural Sciences, SLU
Department of Molecular Sciences
Agricultural Programme – Food Science
Molecular Sciences, 2022:62
Uppsala, 2022



Exploration of ways to utilize wheat chaff through fungal substrate conversion
- A base for animal feed or substrate for cultivation of edible mushrooms

Utvärdering av svampars förmåga att växa på agnarna från vete för att omvandla substratet och möjliggöra för ytterligare tillämpningsområden – som foderingrediens eller substrat för odling av matsvampar

Caroline Sandberg

Supervisor:	Su-lin Hedén, Swedish University of Agricultural Sciences, Department of Molecular Sciences
Assistant supervisors:	Petter Melin & Xinmei Feng, Research Institute of Sweden (RISE)
Examiner:	Volkmar Passoth, Swedish University of Agricultural Sciences, Department of Molecular Sciences

Credits:	30 credits
Level:	A2E
Course title:	Master thesis in Food Science, A2E
Course code:	EX0877
Programme/education:	Agricultural Programme – Food Science (Livsmedelsagronomprogrammet)
Course coordination dept:	Department of Molecular Sciences
Place of publication:	Uppsala
Year of publication:	2022
Title of series:	Molecular Sciences
Part number:	2022:62

Keywords: harvest residues; chaff; oyster mushroom, agro-waste reduction; mushroom production

Abstract

The shift towards a more sustainable food system emphasises a need for increased circularity and valorisation of available resources. Chaff constitutes one of three main fractions obtained upon harvest of certain crops, during which it is generally discarded on the field. New technologies have enabled collection of the chaff, consisting of 'bait and chaff', which could potentially increase the harvested residues by 30% and thereby, strengthen the interest in utilizing this unexploited biomass resource. Due to the presence of lignin in the plant cell wall, which cannot be degraded by rumen microbiota, delignification is necessary to increase digestibility when used as a feed ingredient. *Pleurotus ostreatus* has been recognized for its efficient lignin degradation in addition to its ability to produce fruiting bodies (mushrooms) on various substrates. The aim of the study was to explore utilization of wheat chaff through fungal substrate conversion, as potential base for animal feed or substrate for edible mushroom production.

The trials were performed at three different scales. Firstly, the mycelial expansion of various filamentous fungi – *P. ostreatus*, *Pleurotus eryngii*, *Grifola frondosa*, *Lentinula edodes, Aspergillus oryzae* and *Rhizopus oligosporus* – was compared on wheat chaff with a moisture content (MC) of approximately 75% packed into petri dishes and incubated at 25°C. *P. ostreatus* displayed the fastest growth followed by *P. eryngii* which, together with *G. frondosa*, created the densest mycelium during extended incubation. After 77 days of incubation, the chaff inoculated with *P. ostreatus*, *P. eryngii*, *G. frondosa* had significantly increased in ash content in relation to uninoculated control demonstrating loss of organic material by fungal utilization. In the second trial, the moistened wheat chaff (75% MC) with pH adjusted to approximately 7.25 with $CaCO_3$ was inoculated with spawn of *P. ostreatus* strain M2191 and incubated in small boxes (5cm × 9 cm) for 46 days at 25°C to assess alterations in lignin, crude protein, and ash content in the wheat chaff. Lignin content decreased significantly from 12.2% to 10.8%, (of total solids), a decrease by 11.5%, in relation to the time zero control samples. Crude protein and ash content did however not show any significant difference. The wheat chaff exhibited structural alterations after treatment with *P. ostreatus* including bleached and fibrous appearance and granular slime sheets, all signs of ligninolytic activity by *P. ostreatus*. The third trial was performed with a larger substrate volume in bags (30 cm × 42 cm) to evaluate the potential of wheat chaff as a substrate for mushroom production. Three different bag trials were made with various adjusted pH in the range of 7.0-7.7. Bags were incubated at 25°C during the mycelial colonization and then subsequently moved to a pilot plant with conditions to induce fructification. Successful fruiting body production of *P. ostreatus* was established in bags from all the trials. From point of inoculation until first harvest a range of 62-85 days were required within the different trials. The study has contributed to the exploration of possible applications for fungal conversion of wheat chaff, as animal feed or mushroom substrate. To further evaluate the utilizations as feed, additional analysis on e.g., animal digestibility is required, as well as exploration of various strains to increase the efficiency and selectivity of the lignin degradation. The cultivation of fruiting bodies on wheat chaff also requires further optimization of culture conditions and substrate characteristics, possibly by supplementation, to reduce the time for the production cycle and increase the yields.

Keywords: harvest residues; chaff; oyster mushroom, agro-waste reduction; mushroom production

Sammanfattning

Skiftet mot ett mer hållbart livsmedelssystem understryker behovet av ökad cirkularitet och valorisering av tillgängliga resurser. Vid skörd av vissa sädesslag utgör fraktionen ”boss och agnar” en av tre huvudfraktioner, varpå den vanligtvis avlägsnas på fältet. Ny teknik har möjliggjort insamling av denna fraktion, vilket potentiellt skulle kunna öka de skördade resterna med 30% och därmed förstärks intresset att använda denna outnyttjade biomassaresurs. Eftersom lignin, som finns i växtmaterial, inte kan brytas ned av vommens mikrobiota, är ligninnedbrytning nödvändigt för att öka smältbarheten för att den sedan kunna utnyttjas som foderingrediens. *Pleurotus ostreatus* har erkänts för sin effektiva ligninnedbrytning, utöver sin förmåga att producera fruktkroppar (svampar) på varierande typer av substrat. Syftet med studien var att utforska potentiella användningsområden för veteagnar till följd av svampnedbrytning, som potentiell bas för djurfoder efter eller som substrat för produktion av matsvampar.

Experimenten utfördes på tre olika skalor: Det första; en screening för att utvärdera myceltillväxten av flera filamentösa svampar – *P. ostreatus, Pleurotus eryngii, Grifola frondosa, Lentinula edodes, Aspergillus oryzae* och *Rhizopus oligosporus* – på agnar med en vattenhalt på cirka 75 %, i petriskålar vilka inkuberades i 25°C. *P. ostreatus* uppvisade den snabbaste tillväxten följt av *P. eryngii* som tillsammans med *G. frondosa* skapade det tätaste mycelet efter förlängd inkubationstid. Efter 77 dagars inkubation hade substratet som var inokulerat med *P. ostreatus, P. eryngii, G. frondosa* ökat signifikant i askhalt i förhållande till kontrollen, vilket påvisar svampens nedbrytning av organiskt material i substratet. I det andra försöket inokulerades agnarna (75 % vattenhalt), med pH justerat till cirka 7,25 med $CaCO_3$, med ympmedel av *P. ostreatus* stam M2191. Därefter inkuberades det i små lådor (5 cm x 9 cm) i 46 dagar i 25 °C, följaktligen analyserades skillnader i lignin-, råprotein- och askhalt. Andelen lignin reducerades signifikant från 12,2% till 10,8%, en minskning på 11,5%, i förhållande till kontrollprovet. Råprotein- och askhalt visade dock ingen signifikant skillnad. Agnarna uppvisade strukturella förändringar efter inokulering med *P. ostreatus*. inklusive blekt och fibröst utseende och granulärt slemskikt, vilka alla är tecken på ligninolytisk aktivitet av *P. ostreatus.* Det tredje försöket utfördes med en större substratvolym i påsar (30 cm × 42 cm) för att utvärdera potentialen hos agnar som substrat för svampproduktion. Tre olika försök gjordes med varierande (justerade) pH-värden i intervallet 7,0–7,7. Påsarna inkuberades i 25°C under mycelet kolonisering av substratet och flyttades därefter till en pilotanläggning med en miljö för att inducera produktionen av fruktkroppar (svampar). Påsar från alla tre försök resulterade i fruktkroppar av *P. ostreatus* och sammanlagt krävdes ett tidsintervall på 62–85 dagar, från inokuleringspunkten till första skörden.

Den aktuella studien har bidragit till forskningen inom området för möjliga tillämpningar av agnar fermenterad med filamentösa svampar. För att vidare utvärdera dess potential som djurfoder krävs ytterligare analyser av exempelvis djurens smältbarhet samt utforskandet av olika svampstammar för att möjliggöra en mer effektiv och selektiv ligninnedbrytning. Odlingen av fruktkroppar på agnar behöver också ytterligare optimering av odlingsförhållanden och substrategenskaper, för att minska tiden för produktionscykeln och öka skördarna.

Table of contents

List of tables

List of figures

Abbreviations

ADL	Acid detergent lignin
BE	Biological efficiency ((FW of harvest/DW of substrate) ×100)
DW	Dry weight
FW	Fresh weight
MC	Moisture content
MEA	Malt Extract Agar
PPD	Polypropylene
RH	Relative humidity
SMS	Spent mushroom substrate
TS	Total solids

1. Introduction

A growing world population has led to an expanding agricultural sector and thus resulted in generation of great quantities of agro-industrial waste (Kumla et al. 2020). Much of the arable land in Sweden (33-40%) is used for cultivation of cereals, which translates to an annual production of approximately 2 723 000 tons of straw (Jordbruksverket 2018). The chaff, along with grain and straw, constitutes the three main fractions obtained during harvesting. Chaff is generally discarded on the field, despite available technology that enables collection and recovery of the chaff fraction, which could potentially increase harvested residues by 30% (Claesson 1980; Weiß & Glasner 2018).
Residual products originating from agriculture show great potential to be further utilized as biomass. Consequently, there is a growing research interest to find potential areas of application for this unexploited biomass resource. However, the presence of lignin in the plant cell wall limits the application of plant biomass as animal feed because lignin decreases the digestibility by rumen microbiota (Zadril 2000). Many microorganisms can degrade and utilize cellulose and hemicellulose as carbon and energy sources, but a smaller group of filamentous fungi has evolved with the ability degrade lignin (Sánchez 2009). The white-rot fungus *Pleurotus ostreatus* (Oyster mushroom) has been recognized as a primary agent of lignin degradation and for its ability to enable fruiting body (mushroom) production on various agro-waste substrates (Curvetto et al. 2002; Sánchez 2010). Cultivation of Oyster mushrooms on locally available residual products is suggested to play an important role in a circular food system as approximately 200 various residual materials could potentially be utilized as mushroom substrate (Hultberg et al. 2019).

The current project was initiated from a research interest to explore the potential of wheat chaff as a main component in animal feed through fungal conversion of the substrate material. It was then expanded to also include the production of fruiting bodies (mushrooms) as an additional strategy to utilize wheat chaff.

1.1. Aim

The purpose of the study was to explore utilization of wheat chaff through fungal conversion, as potential base for animal feed or substrate for edible mushroom production.

The pilot study had the following aims:

- Firstly, to screen a selection of filamentous fungal species for growth on wheat chaff
- secondly, based on mycelial growth on the substrate, select a suitable species to observe alterations in the substrate following fungal growth for an extended period;
- and thirdly, to evaluate potential of wheat chaff as a substrate for mushroom production.

1.2. Limitations

- Only one wheat chaff sample was used for all trials.
- Only one strain of each tested fungal species was used, except for the two strains of *P. ostreatus* used in the first trial.

2. Background

2.1. Wheat chaff

Conventional grain harvesting practices includes a combined harvester where the harvested biomass is separated into grain, straw and chaff (Unger & Glasner 2019), with the approximate proportions of 50 %(w/w), 25 %(w/w) and 25 %(w/w), respectively (Weiß & Glasner 2018). Grain cropping in Europe lacks established practices for chaff collection upon harvest and therefore the majority of the chaff is currently being discarded on the field, where it serves as humus and nutrient supplier of the soil (Unger & Glasner 2019**;** Weiß & Glasner 2018). New technical developments have, however, enabled a more complete recovery of chaff by separating it from the straw during harvest (Petter Melin, personal communication; from application submitted to Formas 2021). This chaff fraction consists of glumes, hulls, parts of heads, short straw, leafy materials, weed seeds and cracked or whole kernels which are separated from the harvested grain (McCartney et al. 2006). It is an inconsistent material and therefore the composition varies depending on the crop it originates from, weather conditions during cultivation, moisture content as well as present morphological components (McCartney et al. 2006). Furthermore, chaff from cereal crops consist of smaller particles than straw and has been assumed to have a higher nutritive value due to the presence of seed, head parts and broken grains (McCartney et al. 2006). Utilizing this fraction, instead of discarding it on the field, could theoretically create concerns regarding the long-term consequences of the soil fertility. This effect may be limited by adjusting the rate of collection of the residual fraction depending on the farming practices and local conditions, such as climate and soil type (Weiß & Glasner 2018). Implementations, like improved plant succession or manure application, could also be used if compensation would be required (Formas application, as above). However, since much of the arable land in Sweden has soil with sufficient levels of organic matter, increased amounts are not expected to enhance productivity (Formas application, as above). Collecting the chaff may also prevent weeds from entering the soil bank, as the most part of the harvested weed seeds end up in the chaff fraction (Weiß & Glasner 2018).

2.2. Lignocellulose material as feed

Lignocellulose is the major component of plant biomass. It consist of three types of polymers: cellulose, hemicellulose, and lignin which are intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages (Martínez et al. 2005; Sánchez 2009). Lignin is constructed of a large group of aromatic polymers which constitutes its building blocks (Grabber 2005). The facilitation of the degradation of lignin is partly dependent on the structure and ratio between the different building blocks as well as the branching (Grabber 2005; van Kuijk et al. 2015a). Cellulose is an important substrate for rumen microbes and thus a primary constituent of the ruminant nutritional intake (van Kuijk et al. 2015a). Lignin on the other hand, has been shown to negatively affect digestibility in ruminants (Zadril 2000). Consequently, many agricultural by-products, rich in carbohydrates in the form of cellulose and hemicellulose, and with the potential to become feed, cannot be utilized by the ruminants due to the presence of lignin (van Kuijk et al. 2015b).

2.3. Fungi

2.3.1. Fungal treatment of lignocellulosic biomass

Wood decay fungi are typically classified into three categories: white-rot, brown-rot, and soft-rot fungi. The description of the action by white- and brown-rot fungi arises from the appearance of the wood following fungal degradation (Goodell et al. 2008). Brown-rot fungi typically make the wood brown and crumbly, and can rapidly depolymerize cellulosic materials, while only modifying lignin (Sánchez 2009; Goodell et al. 2008). White-rot fungi are instead associated with hard wood decay, where the wood, following degradation, normally exhibit a bleached appearance. If the white-rot fungi has performed a uniform degradation, the residues of the wood can be a soft, spongy and stringy mass which allows for separation (Goodell et al. 2008). The degradation process by white-rot fungi includes both cellulolytic and lignin degrading enzyme. They are able, under the right conditions, to degrade the entirety of the wood. (Goodell et al. 2008). The white-rot fungi are known to degrade lignin selectively, particularly early during colonization, thus making them very useful for treatment of lignocellulosic biomass (van Kuijk et al. 2015a). *Pleurotus* spp. are among those white-rot fungi that have been shown to preferentially delignify wood by degrading lignin more readily than hemicellulose and cellulose, enhancing the cellulose fraction (Abdel-Hamid et al. 2013). In addition to *P. ostreatus* (Oyster), *Pleurotus eryngii* (King oyster) and *Lentinula edodes* (Shiitake) have also been suggested as among the best candidates for selective delignification of wheat straw, among other substrates (van Kuijk et

al. 2015a). Another white-rot fungus known to enable successful colonization and degradation of various lignocellulosic materials is *Grifola frondosa* (Curvetto et al. 2002), an edible polypore mushroom, commonly known as Maitake (Dahlin 2021).

Upon degradation of lignocellulosic biomass, nitrogen is extracted and incorporated into the fungal proteins. Thus, enhanced crude protein content can be observed following the degradation of other nutrients (van Kuijk et al. 2015a; Fazaeli 2007). Degradation of lignocellulose requires synergistic action of multiple carbohydrate active enzymes because of the varying bonding fractions present in the macromolecule. This is enabled through cooperative activities of hydrolytic enzymes responsible for cellulose and hemicellulose degradation and the oxidative systems that participate in the degradation of lignin (Kumla et al. 2020). The composition of the enzymatic complexes by the white-rot fungi varies considerably. To enable lignin degradation one or more of following three are essential: lignin peroxidase, Mn-dependent peroxidase and laccase (Kachlishvili et al. 2006). Enzyme production is dependent on the composition of the substrate. Solid-state fermentation (SSF) supports greater production of lignocellulosic enzymes in comparison to synthetic liquid cultures, likely due to the fact that the growth conditions resembles the natural habitat of the fungi (Kachlishvili et al. 2006).

2.3.2. Cultivation of edible mushrooms

Wood-degrading mushrooms such as white- and brown- rot fungi are included in the broad classification of primary decomposers. Secondary decomposers are instead known as compost mushrooms (Ivarsson et al. 2021; Jellison & Jasalavich 2000). The classes indicate different criteria, such as substrate composition, necessary for cultivation (Cunha Zied et al. 2020). Primary decomposers require substrate materials with a higher carbon/nitrogen (C/N) ratio and lignin content, as well as a lower nitrogen content. Common substrates for primary decomposers include sugarcane, straw, and sawdust. Secondary decomposers or compost mushrooms instead required materials with lower C/N ratios and higher cellulose, hemicellulose and nitrogen contents, such as partly degraded plant material and manure (Cunha Zied et al. 2020; Ivarsson et al. 2021).

In recent years, the mushroom industry throughout the world has intensified. From 1978 to 2012, production increased from about 1 billion kg to 27 billion kg and China is currently responsible for 87% of the world's cultivated mushrooms (Royse et al. 2017).
Lentinula edodes (Shiitake) are the most cultivated mushrooms in the world. Historically they were grown on natural logs, whereas for commercial scale cultivation, sawdust-based substrates are most common due to the shorter production cycle (Royse et al. 2017). *Pleurotus eryngii* (King Oyster) is assumed

to produce the best tasting fruiting bodies within the genus of *Pleurotus.* It is easily cultivated and prefers hard wood substrates, although cultivation on wheat straw is possible (Stamets 2000). Oyster mushroom is regard as the easiest and least expensive mushroom to grow, mainly due to its rapid growth rate and ability to convert a large proportion of the substrate material into fruiting bodies (Sánchez 2010; Naraian et al. 2008)

2.3.3. Life cycle, important growth parameters and substrate characteristics

Cultivation of fruiting bodies of Oyster mushroom include different stages. Firstly, a pre-treatment of the substrate is made, usually by pasteurization, to reduce the level of competitive microorganisms. Subsequently, the substrate is inoculated with the Oyster mushroom, usually in the form of "spawn" (Ivarsson et al. 2021), an inoculum consisting of cereal grains e.g., wheat, rye or millet, coated in mycelia (Sánchez 2010). The fungi then colonize the substrate material and release extracellular enzymes that degrade lignocellulosic content. When the vegetative colonization by the fungi is completed, the temperature declines in response to the reduced growth rate and together with altered environmental stimuli, this triggers hyphal aggregates to form primordia, small fruiting bodies (Stamets 2000). Environmental stimuli involved in the initiation of the fruiting body formation (the fructification phase) include temperature reduction, increased humidity, increased CO_2 flow and greater light intensity. A successful fructification phase may then enable several flushes of harvested fruiting bodies (Ivarsson et al. 2021; Chang et al. 2004).

During mushroom cultivation a suitable substrate composition is essential. To promote fruiting body production of Oyster mushroom usually two or more substrate materials are used. One material constituting the base, with the additional materials considered supplements (Rodriguez Estrada & Pecchia 2017). Supplementation is commonly used to increase the nitrogen content of the substrate as most base materials used for mushroom cultivation contain a nitrogen content of less than 1% (Naraian et al. 2008; Rodriguez Estrada & Pecchia 2017); a C/N of 32-150 is recommended as most appropriate for *Pleurotus* spp. (Chang et al. 2004). Moreover, a peak in conversion of mushroom substrate to fruiting bodies by *P. ostreatus* was observed at a C/N of about 38 to 58% C/N (Cueva et al. 2017). Generally, slightly acidic pH is most suitable for fungal growth although growth within the range of pH 4 to pH 8 is common. Oyster mushroom is known to be less sensitive to substrates with various pH values and grows well during slightly alkali conditions. $CaCO_3$ (lime) is a frequently used to adjust pH of different substrates (Chang et al. 2004; Stamets 2000). Preferential physical characteristics of substrate materials includes a particle size that inhibits water accumulation at the bottom

whilst enabling gas exchange throughout the substrate (Naraian et al. 2008; Rodriguez Estrada & Pecchia 2017). The optimum moisture content (MC) varies depending on material, but generally a suitable MC is between 60 and 75% (Stamets 2000).

3. Materials and method

3.1. Materials

3.1.1. Wheat chaff

Wheat chaff of a winter wheat variety was obtained from a conventional farm. The wheat chaff, with a particle size of about 1-2 cm, was harvested Autumn 2020 and collected using a chaff collector mounted on a combine harvester (see appendix Figure A1). It was stored in plastic bags in ambient temperature prior to and during the experimental trial. The stored chaff, hereafter referred to as "dry" wheat chaff, had a MC of approximately 8%. This was measured by weighing the triplicate samples (~2g) prior to and after oven-drying them at 103 ± 2 °C for 24h.
MC was, throughout the experimental trial, calculated using the following equation:

$$MC = \frac{W_{\mathrm{w}} - W_{\mathrm{D}}}{W_{\mathrm{w}}} \times 100\ (\%)$$

where W_{w} is the wet weight of the chaff (g) and W_{D} is the dried weight of chaff.

3.1.2. Fungal strains

The experiment included four strains of mushrooms and two fungi frequently used in food fermentation. Fruiting bodies of *Pleurotus ostreatus* (Oyster), *Pleurotus eryngii* (King oyster) and *Lentinula edodes* (Shiitake) were bought in commercial food stores, isolated on Malt Extract Agar (MEA) and sub-cultured until isolated colonies were obtained. An active culture of *Grifola frondosa* (Maitake) strain M9827, on Malt Agar, was kindly provided by Malin Hultberg at the Department of Biosystems and Technology, SLU Alnarp. *Aspergillus oryzae* (Barley Koji spores) and *Rhizopus oligosporus,* (Tempeh spores) were purchased from Fermentacultureeu. The fungal cultures were maintained on Malt Extract Agar (MEA) by frequent sub-culturing of the active hyphae on new media and incubated in the dark at 25°C. Fresh grain spawn of the Oyster mushroom strain *P. ostreatus*

strain M2191 was purchased from Mycelia BVBA, Belgium. It was stored in 3°C ± 1°C throughout the time of the experimental trials.
The identity of the fungal strains was confirmed by sequencing the internal transcribed spacer region of the ribosomal DNA followed by comparison with NCBI database using BLAST, as described by Leong et al. (2012). To summarize, DNA was extracted from mycelium grown in broth culture by the method of (Cenis 1992) and PCR amplification of the ITS region was performed using primers ITS1F and ITS4 (White et al. 1990). Sanger sequencing of the PCR products was done by Macrogen Europe, and subsequently the sequences were compared with those in the NCBI database.

3.2. Growing fungi on wheat chaff

The trials were performed at three different scales with partly deviating objectives. First, a smaller trial in petri dishes was conducted to compare mycelial expansion of different fungal strains on the substrate. Thereafter, a larger trial was performed in autoclavable boxes inoculated with the fungal strain which exhibited the fastest mycelium expansion in the petri dish trial. This second trial aimed at looking at the fungal conversion of the substrate through two analytical methods, relevant in relation to feed value. Lastly, a trial was made to investigate the potential of the wheat chaff to support fruiting body production of Oyster mushroom. Initial experiments and learnings about handling the wheat chaff are described below, in the Preliminary trials and method development. (3.2.1)

3.2.1. Preliminary trials and method development

Preliminary experimental trials lead to findings which consequently formed the execution of the main trials and the substrate preparation. The pretrials showed that all the white-rot fungi in the experiment grew on the wheat chaff at a MC of approximately 75%, without supplementation or pH adjustment. The pH value of the substrate was estimated to be approximately 5 by litmus paper. For the substrate preparation, the preliminary trials showed that the water required massaging/mixing with the substrate to facilitate absorption. Following sterilization in the autoclave, some water was released from the substrate and therefore a period of cooling was required for distribution of the water to re-equilibrate prior to inoculation.

3.2.2. Comparison of mycelial growth rate on wheat chaff in petri dishes

The trial was made to compare the growth of different fungal strains on the substrate and thereafter select a suitable strain for further analysis.

3.2.2.1. Substrate preparation

200g of dry chaff was soaked in cold distilled water overnight in a permeable bag to enabling water to penetrate. The bag with substrate was placed in a beaker, water was added to cover the surface and a weight was placed on top. The wheat chaff was drained and massaged by hand until only a few drops of water could be expelled when squeezing. Wheat chaff (762g wet weight) was placed in a polypropylene bag (PPD), autoclaved in a CertoClav For 30 min at 121°C and thereafter left to cool for about 6h in ambient temperature.

The MC of the autoclaved substrate was estimated to be 75% through triplicate measurements from different parts of the bag and then calculated as described in 3.1.1.2.1

3.2.2.2 Inoculation and incubation

Petri dishes were filled with wet chaff, prepared as described above, and inoculated in the centre with one circular agar plug (8 mm diameter), taken from the active part of the mycelium. At least triplicates were made for each of the fungal strains: *P. ostreatus*, *P. eryngii*, *G. frondosa L. edodes, A. oryzae* and *R. oligosporus,* as well as reference plates without fungal inoculation. The remaining wheat chaff was stored overnight at 3°C and the following day used for an additional small trial to compare the mycelium expansion between the spawn of *P. ostreatus* and the isolated commercial strain of *P. ostreatus,* cultivated on MEA. The trial was also made to evaluate potential variation in fungal growth of *P. ostreatus* (Oyster mushroom) on the substrate left to cool overnight compared with cooling for a few hours at ambient temperature. After inoculation the plates were weighed and placed in plastic bags on trays in a dark incubation room at 25°C. The excess surface moisture was allowed to evaporate from the plates during the first 4 days. After that, parafilm was applied to the dishes and moist paper towels added to each bag to maintain a humid environment around the plates.

3.2.2.3 Growth assessment

Determination of the mycelium growth rate was estimated based on the days for the mycelium growth to reach the edge of the petri dish (~9cm diameter). When the fastest growing plates of fungi had reached the edge of the petri dish plate these substrates were dried at 103 ± 2°C to evaluate a potential decline in total solids (TS). No apparent difference in weight was observed, hence, the remaining plates were left to continue incubation, for a total of 77 days. Thereafter, the substrate in each petri dish was dried in aluminium beakers in 103 ± 2 °C for 24h and weighed to estimate TS. Ash content was then assessed by weighing after ashing for 1h at 350°C and then for 6h at 550°C. The ash content of the substrate was measured to calculate the loss of dry organic matter of the substrate and thus the effectiveness of fungal degradation.

3.2.2.4 Statistical analysis

A one-way ANOVA was performed in Excel, on the reference samples (incubated but not inoculated) and the three fungal strains with the highest ash content (triplicate samples): Oyster, Maitake and King Oyster. A significance level of 95% was used. Thereafter three separate two-way t-tests were made between the reference and the fungi named above. The significance level was therefore adjusted to 98.3% ($p<0.017$) since the increased number of comparisons enhances the probability of receiving a significant difference. The frequently used alpha level of 0.05 was adjusted by dividing it by three, the number of comparisons that were performed (0.05/3=0.017).

3.2.3. Characterisation of wheat chaff after fungal substrate conversion in boxes

The following trial was made with a selected fast-growing fungus to further analyse the fungal conversion of the substrate and its potential as feed component. This is because a good mycelial colonization of the substrate material is a prerequisite for effective fungal treatment for lignocellulosic activity (van Kuijk et al. 2015b).

3.2.3.1. Substrate preparation

30g of "dry" wheat chaff was weighed in beakers. $CaCO_3$ (lime) was added at a rate of 2.5% of the "dry chaff" weight and thereafter 90 ml of tap water was poured over the chaff. The pH of the tap water was measured with a pH meter to 7.8; tap water, rather than distilled water, was used to simulate practices on-farm. To distribute the water and lime evenly the substrate was mixed and then massaged by hand. Autoclavable polypropylene boxes (lid 11.8 cm diameter, base 9.0 cm diameter, hight 5.0 cm) made for mushroom production (Svamphuset), were tightly packed with the wet chaff from each beaker and allowed to equilibrate overnight at room temperature (16 boxes in total). The boxes were autoclaved for 20 min at 121°C in a CertoClav and thereafter placed at 25°C to cool and further equilibrate overnight.

MC was estimated to be 75% by triplicate measurements from the first and the last box of the trial, by drying the samples as described in 3.1.1. The pH value of the substrate was approximately 7.2-7.3, which was determined according to the standard EN13037; briefly, by adding 1:5 (sample: distilled water v/v), shaking for 30 min and measuring with a pH meter.

3.2.3.2 Inoculation and incubation

The boxes were inoculated on top of the substrate, in the centre, with 3g of spawn (10% of the "dry chaff" weight). Inoculated boxes and references without spawn

were put in plastic bags with a moist paper towel to maintain a humid environment and incubated in the dark at 25°C. To represent "time-zero" (T0) control samples, a set of inoculated boxes were frozen shortly after inoculation with spawn. Treatments were conducted with at least triplicate boxes.

3.2.3.3 Assessment of fungal substrate conversion

The effect of fungal growth on substrate pH was assessed a little over mid-way through the trial (in one box after 26 days and in two boxes after 33 days). Triplicate measurements were taken from different parts of each box as described in 3.1.1. Additionally, the mid-point samples were also used to estimate the MC-value of the substrate and for microscopic analysis.

After 46 days, the boxes were prepared for freeze-drying by removing the lids and instead covering the substrate with a Kleenex tissue to enable even evaporation. Following 48h of freeze-drying (Model Scanvac Coolsafe 110-4, Labogene ApS, Denmark) the samples still contained a core of ice (~1cm) and were therefore further dried at 55°C overnight. An overview of the control and treatment samples included in the analyses is shown in Figure 1.
In addition to the boxes with varying treatments, the stored "dry" wheat chaff and mushroom spawn were also analysed. The spawn as well as the wheat chaff (triplicates) used in the experiments were dried for 24h in 55°C. All samples were then ground in a Wiley Mini-Mill, at a mill grade of 20 mesh, equal to a sieve size of 0.85 mm.

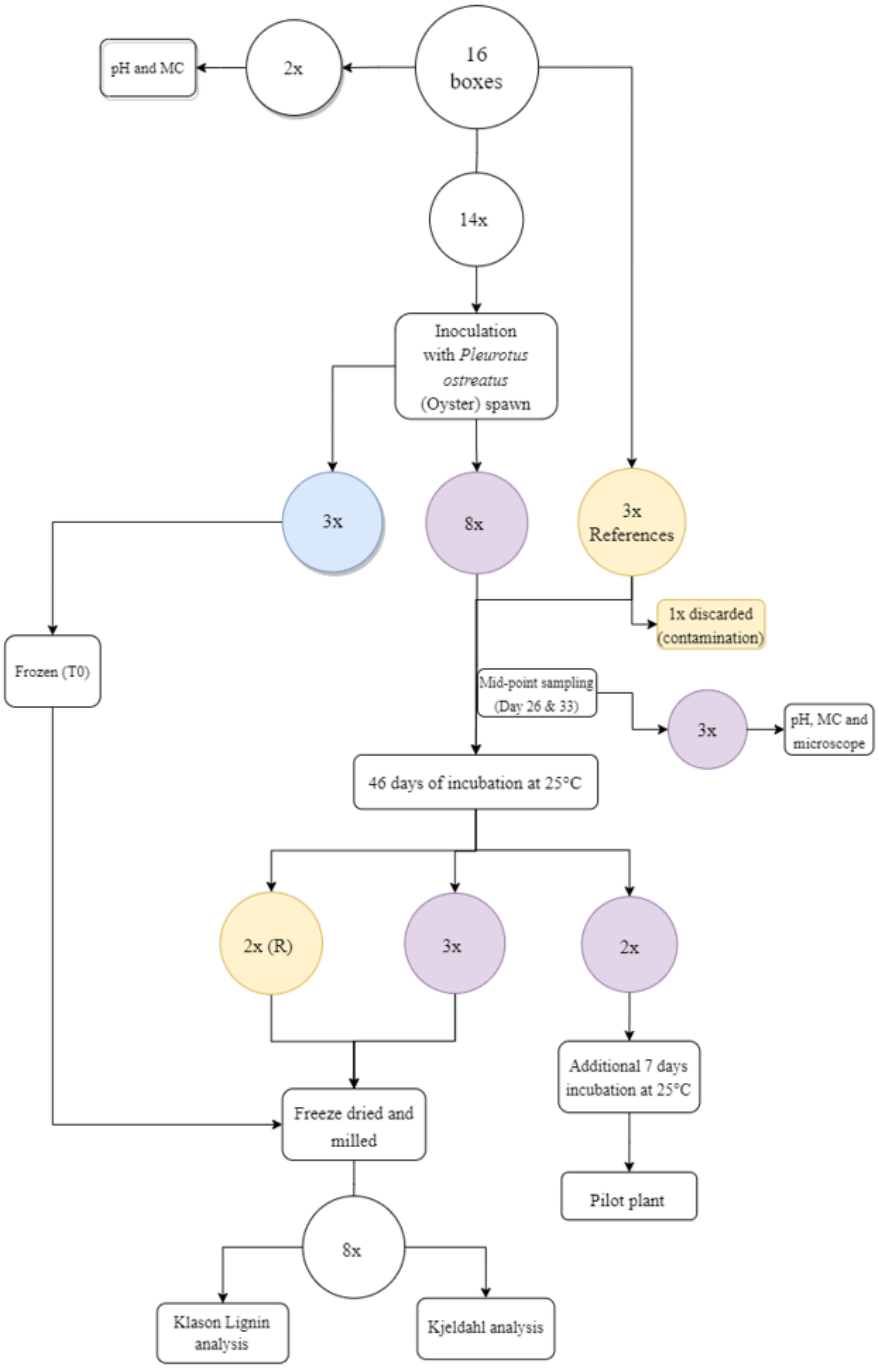


Figure 1. The flow chart visualises the trial where wheat chaff was incubated in boxes. The yellow circles represent the incubated references (R) without inoculation, the purple symbolizes the inoculated and incubated substrate and lastly the blue equals the inoculated and frozen sample (the time-zero (T0) reference).

The amount of Klason lignin present in the samples was determined gravimetrically as acid-insoluble material essentially according to Theander et al. (1995). Total solids (TS) of the samples were assessed by drying approximately 100-200mg

overnight in 105°C and followed by weighing. Ash content was measured as described in 3.2.2.3.

Crude protein was measured according to the Kjeldahl determination. The obtained total nitrogen content in the sample was then multiplied by conversion factor 6.25 to convert it to the crude protein content. Technical duplicates of 1g of sample were made; this analysis was performed by the SLU Department of Animal Nutrition and Management, Analysis Laboratory.

3.2.3.4 Statistical analysis

Each sample value was represented by the mean value of the technical duplicates from the Kjeldahl and Klason Lignin analyses. A one-way ANOVA was performed in Excel with a significance level of 95%. Two-way t-tests were made using three comparisons at a significance level of 98.3% *($p<0,017$).*

3.2.4. Fruiting body trial in bags

Three different trials were made to see if the substrate could support the production of fruiting bodies (see figure 2). Each trial was performed in triplicates in autoclavable (tolerate 123°C), polypropylene bags (PPD) (30 cm, 42 cm, thickness: 50 µm), with 4 filters (Svamphuset).

In trials nr 1 and 2, the wheat chaff was soaked overnight in the PPD-bags with 1.5% or 2.5% $CaCO_3$ (w/w "dry chaff"). However, in the third trial the chaff was instead soaked overnight in a permeable bag with excess of water. The wet chaff was subsequently divided into three bags and 1.5% of $CaCO_3$ was applied. Substrate used in all the trials were autoclaved for 45min at 121°C in a UNICLAVE (Sjukhusservice AB) and then left to cool overnight at 25°C. The MC and pH of the substrate were measured as described in 3.1.1 and 3.2.3.1. with duplicate samples from each bag. The spawn was inoculated at a ratio of 10% of the "dry chaff" weight.

After 47 days of incubation, the mycelial colonization in Trial 1 had declined considerably. The bags were therefore opened to inoculate the substrate with an additional 20g of spawn and to extract samples for MC and pH assessment.

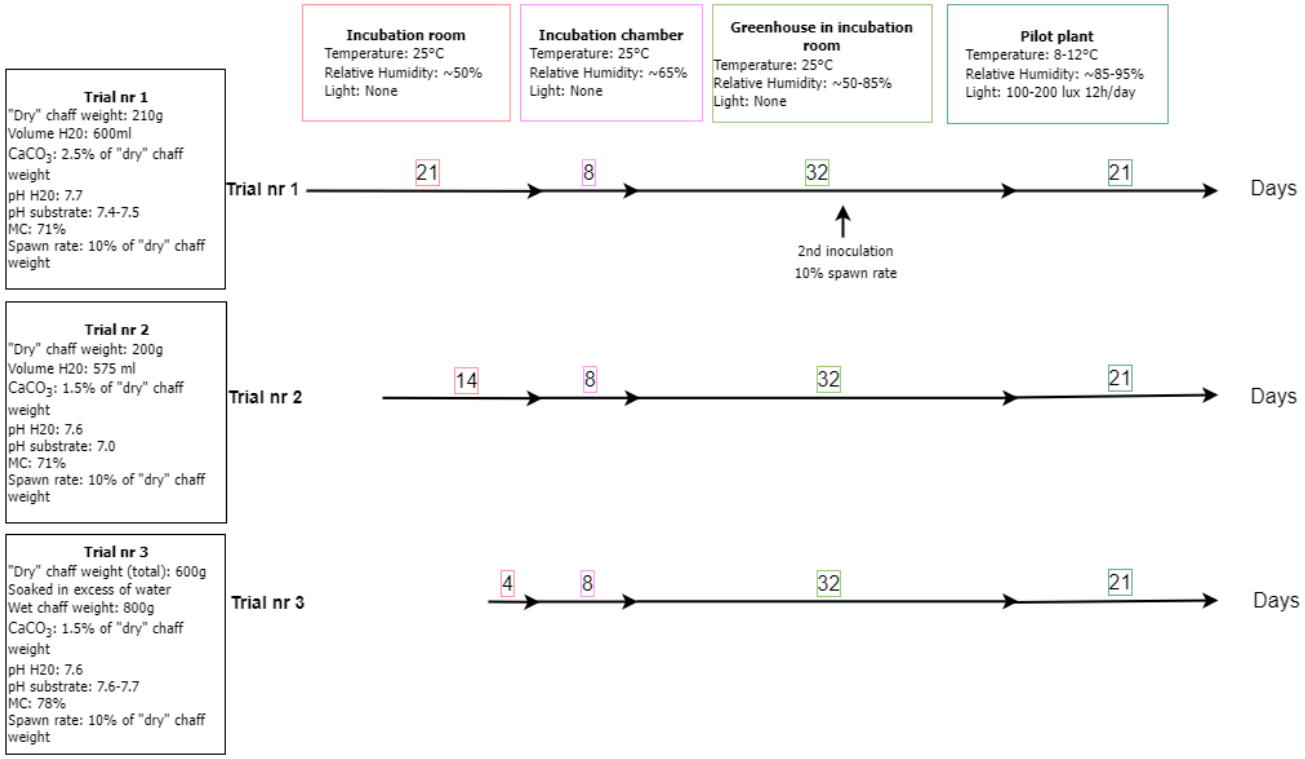


Figure 2. Schematics of the fruiting body trial consisting of three trials (each in triplicates). The figure illustrates the varying parameters within each trial, the different incubation times as well as the conditions in which the bags were stored in.

3.2.3.3 Fructification on chaff in boxes

After approximately 46 days, a coral shaped structure grew out of the filter in one of the boxes (see Figure 3). This was presumably a malformed fruiting body, which can arise in occurrence of light deficiency (Stamets 2000; Chang et al. 2004).

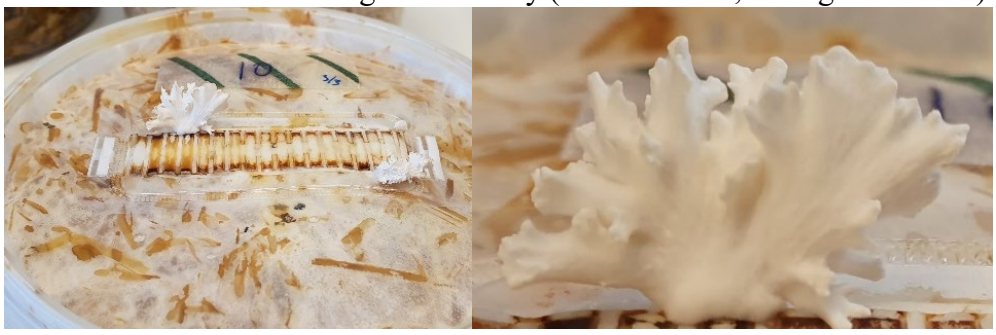


Figure 3. Malformed fruiting bodies emerged from one of the boxes after 46 days of incubation, the shape indicate light deficiency.

Two additional boxes from the same batch trial were incubated for another 7 days at 25°C, after which the lids were removed and, boxes placed in a condition to stimulate fructification. The temperature, RH% (Rubicson Kompakt digital hygrometer) and light intensity (Uni-T UT383 Ljusmätare mini) were monitored

twice a day. Light intensity was about 100-200 lux for 12h per day. To maintain a relative humidity (RH) above 85%, a humidifier (Crane Drop Humidifier, USA) was used. Concentration of the carbon dioxide (CO_2) was not monitored nor controlled instrumentally. The fruiting bodies were harvested and weighed at the point when the cap surface was flat to slightly up-rolled at the cap margins.

After 7 days of incubation in the pilot plant primordia appeared on the top layer of substrate in the boxes. The PPD bags with the most mycelial colonization were then also moved to the pilot plant, and subjected to the conditions mentioned above, to possibly induce fructification. Initially 8 holes with an approximate diameter of 6cm where were made on each bag to enable fruiting bodies to emerge. Subsequent fruiting bodies required additional holes to be made.

In order to compare the different trials, the initial dry weight (DW) of the substrate was estimated based on the MC of the “dry” wheat chaff for the following samples: Boxes, Bag Trial 1 and Bag Trial 2; and based on the MC of the wet chaff in Bag Trial 3

4. Results

4.1. Comparison of fungal growth rate on wheat chaff in petri dishes

4.1.1. Mycelial growth rate

Spawn of Oyster mushroom was the fastest strain to colonise the wheat chaff to the edge of the petri dish, as it required approximately 8 days (Figure 4). Thus, the spawn grew faster than the Oyster mushroom inoculated as MEA plugs, probably due to the additional nutrition provided by the grain kernels in the spawn. MEA agar plugs with Oyster mushroom grew faster on the substrate that had been cooled in the fridge overnight prior to inoculation, 10 days *c.f.* 12 days for substrate cooled for 6h at ambient temperature. The second fastest strain was the King Oyster, which finished mycelial growth on the substrate within 13 days. This was in line with the preliminary trials. Shiitake required about 15 days, again in accordance with the preliminary trials, and created a wispy mycelium. Maitake exhibited the slowest growth on the substrate (~28 days). This growth rate was comparable to the slow growth rate exhibited on MEA as well.

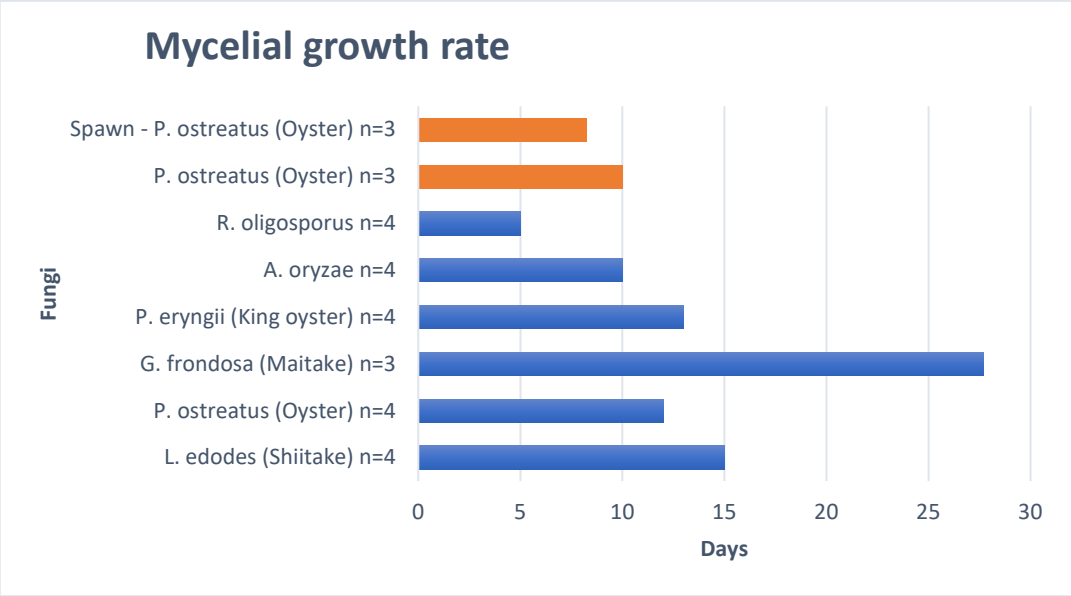


Figure 4. Mycelial expansion on the wheat chaff in the petri dishes inoculated with either spawn of P. ostreatus or plugs of MEA agar, obtained from the active part of the fungal hyphae. The blue bars represent fungi inoculated on the substrate material that was cooled for 6h at ambient temperature after sterilization; the orange bars represent fungal growth on substrate material that was cooled and stored at 3°C overnight after sterilization, with inoculation the following day.

The *R. oligosporus* strain was quick to radially colonise the substrate although over time, the growth decreased. The fast colonization could be partly due to the search for accessible nutrients on and outside the substrate, thus suggesting an inability to easily utilize nutrients in the wheat chaff. The sparse colonization of the substrate (see figure 5) may be seen in contrast to the dense mycelial formation attained upon tempeh fermentation by *R. oligosporus*. Neither did the strain of *A.oryzae* grow well on the substrate. Spore formation was observed after 4 days of incubation, potentially initiated as a stress response.
The Spawn and MEA plug of Oyster mushroom, inoculated on the substrate stored overnight at 3°C, were the fastest two strains; when the mycelium has reached the edge, the substrates were dried at 103 ± 2°C to evaluate a potential decline in TS. No apparent difference in weight was observed in relation to the estimated initial TS based on the MC upon inoculation.

4.1.2. Fungal substrate conversion following 77 days of incubation

Throughout incubation the colour of the substrate started to differ, depending on fungal inoculation. Wheat chaff inoculated with agar plugs of Oyster, King Oyster and Maitake mushrooms gradually became more yellow and pale. Substrate inoculated with Shiitake, however, did not bleach the substrate in a similar way but instead it became more orange.

Figure 5 illustrates the fungal growth on the wheat chaff in petri dishes after 77 days of incubation. The King Oyster and Maitake displayed the densest mycelium on the substrate compared to the other fungi. The mycelium expansion and density were homogenous throughout the substrate. Oyster mushroom displayed a sparse mycelial growth with only a few patches of greater density. However, the previously firm wheat chaff had become spongy and stringy: an appearance in line with the description of the degradation of wood by white rot-fungi. The Shiitake had a wispy mycelium throughout the entire substrate. A denser mycelium was only visible at the edge of the petri dish, possibly because it was searching for additional nutrients outside the substrate. When the plates were opened a significant fungal fragrance originated from the Maitake and the Oyster mushroom. Shiitake and King Oyster, however, only exuded a moderate, but noticeable, aroma when opened.

Figure 5. Petri dishes, following 77 days of incubation at 25°C, with wheat chaff inoculated with agar plugs of R. oligosporus, A. oryzae, G. frondosa (Maitake), P. ostreatus (Oyster), L. edodes (Shiitake), P. eryngii (King Oyster) and a reference plate, incubated without fungal inoculation.

Table 1 illustrates the ash content of the wheat chaff inoculated with the different fungal species following 77 days of incubation. The one-way ANOVA performed on the ash content revealed that there was a significant difference ($p<0.05$) among the reference, the King Oyster, Oyster and Maitake. The two-tailed t-tests also showed a significant increase ($p<0.017$) in the ash content between the substrate inoculated with Oyster, King Oyster and Maitake in relation to the uninoculated reference plates.

***Table 1**. Ash content of wheat chaff inoculated with different fungal species (n=3) in petri dishes incubated for 77 days. The reference representing incubated wheat chaff without fungal inoculation. Results are reported as % of total solids (i.e., % of dry weight), mean values ± standard deviation (SD).*

Parameter	Reference	*P. ostreatus* (Oyster)	*G. frondosa* (Maitake)	*P. eryngii (King Oyster)*	*L. edodes (Shiitake)*	*R. oligosporus*	*A. oryzae*
Ash (%TS)	11.1 ± 0.2	13.0 ± 0.5	12.9 ± 0.2	12.1 ± 0.3	11.6 ± 0.3	11.4 ± 0.1	11.7 ± 0.1

4.2. Characterisation of wheat chaff after fungal substrate conversion in boxes

4.2.1. Mid-point sampling

The MC obtained at the mid-point was approximately 76% and the pH was 5.5, hence, demonstrating an ability to retain moisture in the box and a decrease in pH from the initial adjusted pH of 7.2–7.3. The replicates within the pH measurements showed a greater variance within the boxes *(0.302)* than between the boxes *(0.0403)* (see Table A1 in Appendix), potentially due to similar fungal growth in the different boxes although slightly varying enzymatic activity throughout the substrate in the boxes. However, the standard method applied for pH measurements in the current experiment only provides a rough estimation and could also explain the variance.

During incubation, a granular slime sheath appeared on the surface of the wheat chaff particles. Figure 6 show a sample extracted from the granular sheath, in which a square crystal could be observed by light microscopy (see arrow).

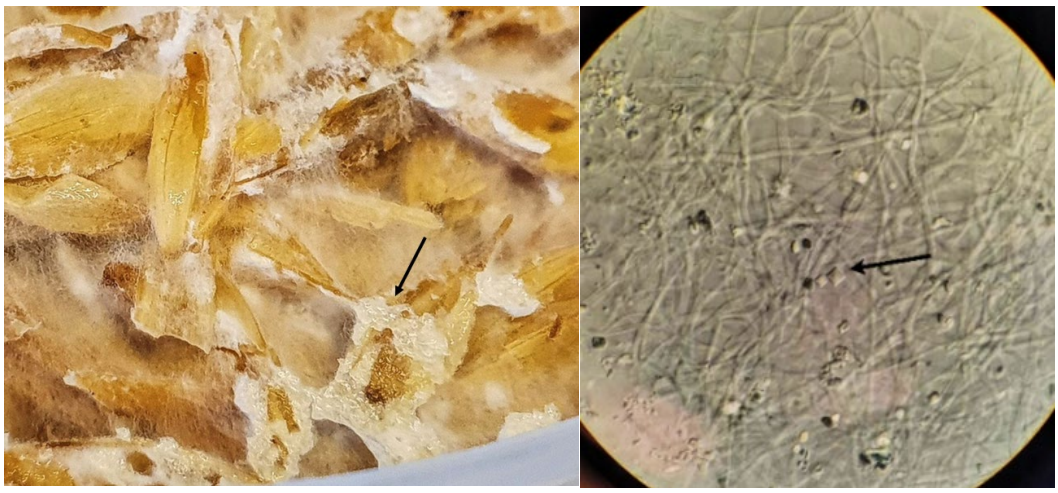


Figure 6. Left: Wheat chaff inoculated with P. ostreatus (Oyster). Arrow indicating granular/crystalline material, which was subjected to microscopic analysis. Right: Picture of sample studied by light microscopy. Magnification ×1000. Arrow pointing towards a square crystal.

4.2.2. Characterisation of wheat chaff after 46 days of incubation

The one-way ANOVA of the crude protein content revealed that there was a significant difference ($p<0.05$) among the samples (Table 2). However, the two-tailed t-tests only illustrated a significant difference ($p<0.017$) in the crude protein content of the *Inoculated* samples in comparison to the W*heat chaff* and the *Uninoculated* (incubated but not inoculated). No significant difference was observed between the *Inoculated* and the *Frozen* samples, i.e., the two samples to which spawn had been added. The results obtained from the gravimetric determination of the acid-insoluble material (see Table 2) followed by the one-way ANOVA revealed a significant difference in the Klason Lignin content among the samples ($p<0.05$). Moreover, the t-tests also indicated a significant decrease ($p<0.017$) in Klason lignin content between the *Inoculated* boxes in relation to the *Frozen* control and the *Wheat chaff*. Between the *Frozen* control (T0 reference sample) and the *Inoculated* sample, the Klason lignin decreased from 12.2% (of DW) to 10.8%, representing a decrease of approximately 11.5% in Klason lignin content. As the ash content of the two *Uninoculated* boxes varied notably (~7.8%) they were excluded from the one-way ANOVA analysis. No significant difference ($p<0.05$) in ash content was observed between the *Wheat chaff*, *Inoculated* and *Frozen samples*.

The crude protein of the spawn was 11.8% of TS and the Klason lignin content 16.1% of TS (mean values calculated on technical duplicates from one replicate).

Table 2. *Characterisation of wheat chaff in boxes after incubation for 46 days with spawn of P. ostreatus (Oyster) mushroom assessed in relation to control samples. Samples were characterised for crude protein content (Kjeldahl method), gravimetric determination of the acid-insoluble material (Klason lignin) and ash content. Each replicate was calculated as the mean value of two technical duplicates. Results are reported as % of total solids, mean value ± SD (n=3, except for uninoculated samples where n=2).*

Sample content	Wheat chaff	Uninoculated (Incubated 46 days)	Frozen T0 sample (Incubated 0 days)	Inoculated (Incubated 46 days)
Crude protein (%TS)	3.1 ± 0.2	3.0 ± 0.3	3.6 ± 0.3	4.0 ± 0.1
Klason lignin (%TS)	12.3 ± 0.2	11.7 ± 0.5	12.2 ± 0.2	10.8 ± 0.3
Ash (%TS)	13.6 ± 0.4	14.1 ± 1.5	13.9 ± 1.2	15.1 ± 1.3

4.1. Fruiting body trial in bags

Similar trends as previously observed were also seen in the bags of the fruiting body trial. Following approximately 10 days of incubation, the bags demonstrated signs of a crystalline/granular coating, as observed in the boxes (4.2.1). When the coating

was rubbed on the substrate surface it appeared slimy. After about 15 days of incubation the substrate became considerably bleached and turned yellow. Following inoculation, an initial quick colonization of the mycelium was seen, which then declined after about a week of incubation. As the inoculum (spawn) included nutrients, an initial rapid growth was predicted. Likewise, the subsequent lag phase during acclimatization of the new media was expected. However, throughout the incubation period the mycelium growth was not dense, nor did it lead to an expansive growth over the substrate, but instead appeared in fragmented mycelium clusters. These temporary mycelial developments seemed to be triggered by factors such as fluctuating RH. Clusters of colonization and subsequent decline appeared rapidly, as a response to either too elevated or low RH, assessed by appearance or disappearance of moisture droplets accumulating inside the bag.

After 47 days of incubation, bags from trial nr 1 were opened due to lack of visual mycelial colonization on the wheat chaff. The MC of the substrate was approximately 77% and the pH 5.8. The structure of the chaff appeared to have altered as it was stringier and less cohesive in comparison to soaked chaff prior to incubation, which was evaluated by massaging it by hand.

The time required for the different samples in the pilot plant to produce fruiting bodies is summarised in Table 3. It shows the incubation time from inoculation to primordia formation, days from appearance of primordia until first harvest, the mushroom weight and the initial dry weight of the substrate which supported mushroom production, as estimated based on MC. The first signs of primordia formations in the PDD bags (from Trial nr 2) were observed after 10 days in the pilot plant, or 64 days following inoculation. The first fruiting body was subsequently harvested 4 days later. Upon 12 days of incubation in the pilot plant, one PDD bag from trial nr 3 had formed primordia, equivalent to 56 days after fungal inoculation of the substrate. After 14 days at the pilot plant, 75 days after inoculation, primordia were also exhibited in the PDD bags from trial 1. Trial nr 3 required the shortest incubation time to induce primordia formation and, in relation to the other PDD trials, created the first harvested mushroom with the greatest weight (WW) per initial substrate weight (DW) (Table 3). Following the first mushroom harvest, the PDD bags continued to support the formation of additional fruiting bodies, although the size decreased notably following the harvest of the initial two fruiting bodies, potentially suggesting limited available nutrients. It was also notable that although the wheat chaff was not densely coated in mycelia, the fungi were still able to produce fruiting bodies.

Table 3. Time frame from inoculation to primordia formation, days from primordia formation until first harvest, the fresh weight of the first harvested mushroom and the initial amount of substrate in dry weight, for different batches incubated in the pilot plant for fruiting body production. Values are shown for the replicate from each batch which first initiated fructification.

Sample	Days until primordia formation	Days until first harvest	First mushroom weight (g) (WW)	Initial substrate (g) (DW)
Boxes	60	10	12.5	27.6
Bag Trial 1	75	5	7.0	193.2
Bag Trial 2	64	4	13.5	184
Bag Trial 3	56	6	15.5	176

Figure 7. Left: Fruiting body growing out of a PDD bag from Trial nr 3, prior to harvesting. This occurred 6 days after primordia formation and 62 days from inoculation. Right: Pilot plant.

3.2.3.3 Fructification on chaff in boxes

The malformed fruiting body appeared after 46 days and may therefore be seen as an indication of the approximate time frame required for fruiting body production. After being transferred to the pilot plant, 7 days (60 days after inoculation) were required before primordia formation could be observed in the boxes. The first mushroom was harvested 10 days following the first sign of primordia formation. After this first harvest (one fruiting body) (see Figure 9), the growth of the initiated, notably smaller, fruiting bodies declined and eventually stopped.

Figure 8. Left: Primordia of P. ostreatus after 9 days of incubation in pilot plant, and 62 days after inoculation. Right: The fruiting body at the point of harvest, 10 days after the first signs of primordia formation.

5. Discussion

5.1. Comparison of fungal growth rate on wheat chaff in petri dishes

As mentioned above, degradation of woody materials by the white-rot fungi results in a bleached appearance. Therefore, the change in substrate colour upon inoculation with Oyster, King Oyster and Maitake may indicate fungal degradation of lignin. Although Shiitake classifies as a white-rot fungi, it did not bleach or grow particularly well on the substrate. One explanation could be due to the porous structure of the wheat chaff as Shiitake is usually cultivated on compact substrate materials with higher density, such as wood logs and sawdust (Stamets 2000). Neither was a dense mycelium coating of the substrate exhibited by *R. oligosporus,* as seen in tempeh production. The current experiment did thus not indicate that *R. oligosporus* was a suitable organism for conversion of wheat chaff, without, for example, prior treatment, or potentially through co-culturing with other microorganisms. Another well-known fungus within food fermentation, *A. oryzae* has attracted interest as an alternative to the known ligninolytic degrading fungi due to extensive production and secretion of various enzymes, but limited studies have shown production of lignin degrading enzymes. A study conducted by Zhang et al. (2015) found that a known strain of *A. oryzae* with ligninolytic enzymatic production did degrade lignin in cornstalk pre-treated with H202. Thus, although the strain in the current experiment did not exhibit successful colonization of the substrate, future research with selected strains known to secrete lignin degrading enzymes could be of interest, particularly in combination with pre-treatment of the substrate (Zhang et al. 2015).

The results indicated that the substrate inoculated with Oyster mushroom, Maitake and King Oyster had a significant difference in the ash content in relation to the reference plates. This suggests fungal respiration, meaning that organic material was converted to CO_2 which consequently decreased the proportion of TS and increased the relative proportion of ash content in the substrate. Oyster mushroom appeared to grow well on the substrate initially but did not continue to form a dense

mycelium. However, both the mentioned visual observations of substrate alterations and increased ash content, points towards successful fungal digestion of the substrate. The dense mycelial growth exhibited by Maitake and King Oyster, in addition to the significant ash content shows an ability to utilize the nutrients in the substrate material. For that reason, it would be interesting continue investigating their conversion of wheat chaff and potential to produce fruiting bodies.

Despite the significant increase in ash content, exhibited by the inoculation with the above-mentioned fungi, the increase in ash content was only moderate. The small margins consequently make the results more sensitive to minor errors and may affect considerably. Additional replicates would thus be required to increase the statistical accuracy.

5.2. Characterisation of wheat chaff after fungal substrate conversion in boxes

5.2.1. Mid-point sampling

The MC of 76% suggests that the substrate was able to retain moisture in the box. The decrease in pH from the initial adjusted pH of 7.2-7.3 to pH 5.5 suggested fungal fermentation had occurred.

The observed crystalline slime sheets that appeared during incubation may be explained by the known feature of white-rot fungi to produce extracellular hyphal sheaths, primarily consisting of polysaccharides. A mucilaginous extracellular matrix is assumed to facilitate action of ligninolytic enzymatic activity (Gutiérrez et al. 1995). Furthermore, calcium oxalate has been observed in the extracellular mucilage of several fungal species, which could potentially explain the square crystal observed by microscope in Figure 6 (Baldrian 2003). More research is required to further characterize the slime sheath and the square crystal, to identify the purpose of the production and potential areas of application.

5.2.2. Chaff characterisation after 46 days of incubation

According to the results from the Kjeldahl analysis the *Inoculated* substrate showed a significant difference in crude protein in relation to the *Uninoculated* sample as well as the *Wheat chaff*. However, the *Wheat chaff* and the *Uninoculated* boxes did not included added spawn, which itself had a crude protein content of approximately 11.8% of TS. This is the likely explanation for the elevated crude protein content in the *Inoculated* samples, and consequently, the comparison of the crude protein content between the *Frozen* and the *Inoculated* samples is the most

representative for demonstrating fungal conversion. Table 2 suggests a slightly higher crude protein content in the *Inoculated* sample in relation to the *Frozen*, however the difference is not significant. The ash content showed substantial variation among the replicates; no increase in ash content due to fungal conversion was observed. Note that both crude protein and ash content are reported as % of TS. Thus, both results suggest that fungal digestion of organic material (resulting in net loss of CO_2 via respiration) did not occur to the extent that relative increases in crude protein and ash could be observed. It was hoped that fungal respiration would significantly increase the relative proportion of crude protein (as % of TS), though the total amount of nitrogen is unchanged. However, this was not the case. The result in the current study may be interpreted as an indication that a longer incubation time may be required to decrease the amount of organic material in the substrate. Greater fungal degradation could potentially also increase bioavailability of the nitrogen present in the substrate e.g., bound to the lignocellulosic material, however this may not be observed with the Kjeldahl method. Moreover, the addition of spawn in certain samples made the assessment between the treatments difficult. This comparison could be facilitated by for example reducing the spawn applied or by instead inoculating the substrate with fungal spores which would not increase the crude protein content.

In contrast to the crude protein analysis, the significant difference in Klason lignin content between the *Inoculated* sample and the *Wheat chaff* and *Frozen* control samples is a clear sign of the desired fungal conversion. As spawn has a slightly higher Klason lignin content than *Wheat chaff* (16% *c.f.* 12%), the fungal inoculation with spawn could be assumed to increase the Klason lignin content of the samples somewhat, in contrast to the samples not containing the spawn. However, the Klason lignin content in the *Inoculated* sample was significantly decreased compared to the *Wheat chaff* and the *Frozen* boxes, which supports the hypothesis that this results from lignin degradation by the fungi. The lack of significant difference between the *Inoculated* and the *Uninoculated* samples could be due to the added lignin in the spawn, the greater SD between the duplicates of the *Uninoculated* boxes or the limited number of replicates. It should also be noted that, the Klason lignin content in the experiment is estimated as a percentage of TS. Thus, if the fungi would have utilized a considerable part of the organic material in the substrate in addition to Klason lignin, this could challenge the comparison between the fungal fermented samples and the controls as the proportion of Klason lignin could potentially appear to be a similar or higher relative to the substrate material, despite the actual amount of Klason lignin being reduced. However, this type of reduction in organic material would then be observable in the ash content, which in current experiment was not significantly different among the samples, implying that this skewed effect had not occurred. The slight increase in ash content

of the *Inoculated* sample (see Table 2), may, however, point out a risk for potentially misleading assessments of the amount of Klason lignin, when comparing the *Inoculated* substrate to the samples which were not incubated with fungi. Consequently, this suggests that the methodological development should be considered for future trials, as the comparison of percentage of Klason lignin might be misleading depending on fungal metabolization.

During the gravimetric determination of the acid insoluble fraction, the *Inoculated* sample was perceived to have a slower retention upon vacuum filtration, in relation to other samples. Although merely based on this observation, it could potentially be due to initiated degradation of the lignin polymer into smaller fragments however not to the extent to which it solubilizes in the sulphuric acid. Moreover, the method used to evaluate the Klason lignin fraction was calibrated to the gravimetric determination according to Theander et al. (1995) and therefore included relatively small sample sizes (~250mg). This, as well as the fact that the method only provides a rough estimation of the Klason lignin content, may affect the accuracy of the results. A small sample size also enhances the need for homogenous samples. So, a risk of heterogeneity within the ground samples in addition to uneven enzymatic activity throughout the substrate enhances the probability of misleading results. Conducting a trial with greater sample size would presumably reduce the risk of inaccurate results and strengthen the conclusions which could be made. There are also additional ways to measure the lignin fraction, by for example Acid Detergent Lignin (ADL) analysis which is frequently used within animal feed analysis. However, in relation to quantification of the total lignin concentrations in forage plants, especially for grass species, the Klason lignin analysis has been suggested as more accurate than ADL (Hatfield et al. 1994).

Length of incubation in the box trial was based on practical time constraints. Nevertheless, the appearance of the malformed fruiting body signalled that the time frame was suitable for feed conversion, as lignin degradation has been suggested to be less pronounced during fruiting body production and ligninolytic enzymes most active just before the fructification phase is initiated (Bano et al. 1993). Fermentation of Oyster on wheat straw also indicated a peak in enzymatic activity earlier on during incubation than lignin degradation, which reached a significant rate after 40 days (Knežević et al. 2013). Additionally, wood decay fungi are known to selectively degrade lignin particularly in the early colonization phase, following an increase in degradation of cellulose and hemicellulose upon fructification, as carbon and energy are required to produce the fruiting bodies (van Kuijk et al. 2015a). Thus, the fruiting body in addition to the assumed peak in ligninolytic activity prior to fructification indicates a suitable time frame for incubation in relation to the purpose of the trial. Continuous incubation would hypothetically

primarily stimulate the degradation of cellulose and hemicellulose, which are the desirable components of animal feed to ruminants.

According to the results, there was an approximate reduction in lignin content of 11.5%, between the *Frozen* T0 reference and the *Inoculated* sample. This decrease was significant, however, not as great as has been previously reported for other substrate/fungal strain combinations. Various studies have shown a ~2-40% reduction in lignin content (of TS) upon cultivation of Oyster mushroom on wheat straw up to 30 days of incubation (Adamović et al. 1998; Shrivastava et al. 2011; Knežević et al. 2013; Shabtay et al. 2009). Although this depends on the percentage of lignin present in the original sample, it also suggests that increased efficiency of the delignification is possible, as the current results of the lignin degradation are within the lower part of the range, despite the comparatively extended incubation time.

5.2.3. Fungal transformed wheat chaff as feed

The following parameters will be brought up as important to consider in future explorations of the potential of fungal-transformed wheat chaff as a feed ingredient: strain characteristics; trace elements; differences in chaff material; physical pre-treatment; farm scale production; additional analysis; and sensitivity to chitin.

The strain of Oyster mushroom in the trial was not selected based on known ligninolytic activity or selective lignin degradation. Instead, it was selected for producing spawn intended for productive fruiting body formation. As fructification has been shown to stimulate the degradation of cellulose and hemicellulose, this strain was potentially not the ideal strain for selective delignification. However, no conclusions can be made regarding the strains' ligninolytic activity. For future research it would although be of interest to include additional strains, and preferably strains known for efficient and selective lignin degradation. Several studies have been made to evaluate Oyster mushrooms preferential delignification, although, due to variations in the selected fungal strains as well as divergent executions of the tests, this ultimately complicates comparisons (van Kuijk et al. 2015a). Finding suitable microorganisms to degrade lignin selectively has been recognized as one of the main problems before achieving large scale production of bioconversion of lignocellulosic plant material, i.e., to enable liberation of lignin from the complex without further metabolization of the cellulose and hemicellulose (Zadril 2000; Sharma & Arora 2010). Further exploration of a wide range of different strains on the current substrate is therefore relevant.

The ligninolytic activity of Oyster mushroom is closely related to the enzymatic production of laccase, as it is the dominant degrading enzyme when cultivated on

lignocellulosic substrates. Peak in laccase activity has been seen just prior to initiation of fruiting body production (Hultberg et al. 2020). However laccase production has also been seen to not necessary correlate with increased lignin degradation (Knežević et al. 2013). Copper (Cu) is a cofactor in the catalytic centre of laccase and is in addition to Manganese (Mn), an example of essential heavy metals which are involved in lignin degradation. Mn is present in the reaction cycle of Mn-dependent peroxidase and also assumed to be an important part of selective delignification by the white-rot fungi (Baldrian 2003;Kerem & Hadar 1995). Addition of Mn to substrate materials has been shown to elevate levels of ligninolytic enzymes and boost lignin degradation by Oyster mushroom (Curvetto et al. 2002). Also, the nitrogen content present in the substrate affects the lignocellulosic enzyme production. As lignin degradation in nature often occurs under nitrogen deficient conditions, the delignification activity of some fungal strains might be supressed by addition of nitrogen to the culture media (van Kuijk et al. 2015a). Oyster mushroom has for example shown a suppressed ligninolytic activity by excess nitrogen, whereas enhanced degradation was observed in growth-limiting nitrogen levels (Commanday & Macy 1985). Results from various studies on the effect of the nitrogen accessibility on lignin degradation are, however, at times contradictory, and trials have been executed differently. This consequently blurs the trends although suggesting that nitrogen and carbon content, as well as the form in which they are present in the substrate, does indeed affect lignin degradation.

The current trial only included one sort of wheat chaff variety, cultivated, and harvested under one set of conditions. The structure of the lignin influences the degradation; the content and form of lignin in plant biomass varies among different species and the complexity is enhanced upon maturation (Grabber 2005). Linear lignin is more easily degraded than branched, and certain building blocks are assumed to be more resistant to degradation by white-rot fungi (Grabber 2005; van Kuijk et al. 2015a). The lack of lignin degradation could thus potentially be partly due to the structure of the lignin, in creating impediments for fungal degradation. This accentuates the difficulty in transcribing the lignin content and achieved rate of degradation in the one variety of wheat chaff used in the current experiment with other wheat chaffs. Consequently, evaluating fungal delignification on a variety of chaff samples would be beneficial for future trials. The inclusion of other chaff materials is also of interest in relation to the inconsistency of the chaff fraction and the observed correlation between CP content in the grain and the CP content in the chaff (McCartney et al. 2006). This is a relevant consideration in relation to animal feed applications, as one of the main limiting factors in the utilization of certain lignocellulosic materials (e.g., straw) as feed to ruminants lies in the low crude

protein content (van Kuijk et al. 2015a); thus, an elevated crude protein content in the chaff would be interesting in terms of feed value.

Physical pre-treatment, like grinding or pelleting of the wheat chaff prior to fungal inoculation may also assist lignin degradation. In the current experiment, the wheat chaff was used in the state which it was collected upon harvest, as an on-farm solution would presumably be facilitated by limited steps of pre-treatment. However, depending on the proposed utilization of the wheat chaff, additional treatments could be an alternative. The low density of the substrate material consequently makes it costly to transport and could therefore actualize pelleting as an alternative (Weiß & Glasner 2018). Regarding pre-treatment to reduce microflora at a large scale on farm level, pasteurization would be preferred as supposed to sterilization, which has a greater energy demand. Generally the cultivation of Oyster mushroom only requires pasteurization, due to its rapid growth rate in comparison to other edible fungi (Sánchez 2010). Pasteurization was tested in the current project, however, there were difficulties in obtaining an even heat penetration and an increased risk of contamination was observed (Unpublished results). Hence, autoclaving the substrate was the preferred alternative for the current small-scale experiments. On farm- applications may also want to explore different methods for pre-treatment of the substrate. Cold pasteurization by addition of hydrated lime ($Ca(OH)_2$) instead reduces microflora by raising the pH of the substrate for about 12-24 hours. This method is gaining interest among commercial and low-tech mushroom farm cultivators due to comparatively reduced energy requirements in relation to autoclaving and heat pasteurization (Balan et al. 2022; Shields 2018).

Another aspect to be considered in relation to fungal conversion of chaff as a potential base for animal feed is the chitin that is present in the cell wall of fungi. Omnivores, herbivores and carnivores have varying gene expressions for the production of chitinase, a chitin degrading enzyme (Tabata et al. 2018). Ruminants have shown decreased capacity to degrade chitin in relation to omnivores such as pigs and poultry. The study by Tabata et al. (2018) also demonstrated that the animal's gene expression was governed by feeding behaviour. However, due to ruminants' frequent ingestion of insects (chitin-containing organisms), from e.g., grass, in contrast to the low gene expression, they hypothesized a possible symbiotic activity with bacteria present in the gastrointestinal tracts, i.e., that bacterial chitinases could potentially play a central role in chitin digestion. Prior to introducing chitin-containing organisms, such as fungi, to animal feed, further research is required to assess the impact of chitin on nutritional quality. Addition of chitinase-producing microorganisms could potentially be an alternative (Tabata et al. 2018).

Such thoughts further accentuate the need for additional analyses and a wider scope of assessments regarding the potential to utilize fungal transformed wheat chaff as a base for animal feed. For example, by including analysis of *in vitro* digestibility and nutritional composition of the substrate, as the current study only assessed alterations in crude protein, Klason lignin and ash content.

5.1. Fruiting body trial in bags

The results show that the substrate material inoculated with Oyster mushroom spawn was able to support production of fruiting bodies and gave an approximate time frame from inoculation to primordia formation and mushroom harvest (see Figure 7). Different samples exhibited a vast range of different incubation times required, e.g., initial signs of primordia formation after 46 days in the box trial, as indicated by the malformed fruiting body, up to 75 days required for primordia formation in the PPD bags from trial 1. The summary of the varying times, within the different trials, from point of inoculation until first mushroom harvest, resulted in a time frame of 62-80 days. For Oyster mushroom, this time frame is, however, usually approximately 19-33 days (Stamets 2000). The time required for development of the fruiting bodies, from primordia formation until harvest, also varied between the trials. This typically occurs within 3-7 days (Chang et al. 2004), and in the box trial 10 days were required until harvest, whereas the first fruiting body in the PPD trial 2 was harvested after only 4 days. The time of harvest was determined based on the appearance of the mushroom, with a slightly up-rolled at the cap margins. Because of the notable difference in structure and appearance of the mushrooms this complicated a standardised assessment of suitable point of harvest. Some of the mushrooms were therefore harvested although still comparably small, which can be seen in the varying weight of the fruiting bodies. This should therefore be considered when comparing the results in Figure 7, by regarding both weight and time required for development. As an example, the PPD bag from trial nr 2 exhibited a fast growth rate as well as one of the heaviest mushrooms. Due to limited time, the data obtained in this experiment is only based on the first harvested mushroom from each trial, which ultimately reduces the accuracy and needs to be considered when looking at the results. On the other hand, it provides a certain indication of the substrate's ability to support fructification and the varying times among the trials suggests that optimization of culture conditions may decrease the crop production time.

In the substrate in both boxes, it could also be observed that following the first mushroom harvest, the growth of the initiated fruiting bodies was reduced and then stopped. The PPD bags, with greater substrate volume, did however manage to continue producing fruiting bodies after the first harvested mushroom, although the

size and growth declined notably after about two fruiting bodies were harvested. The staggered growth and inability to support further growth in the boxes may therefore be due to nutrient depletion. The comparatively long time required from inoculation to first harvest could be partly explained by the spawn (inoculation) rate, as greater application of spawn accelerates the mycelial colonization and fruiting body development. Reduced spawn rate may significantly decrease yields and prolong the crop cycle. In a study conducted by Zhang et al. (2002) a significant difference was observed at 12% (of DW) spawn rate, in contrast to 16% and 18% upon cultivation on rice and wheat straw. The current trial applied spawn at 10% of "dry" chaff weight, equivalent to approximately 11% of chaff (DW), determined based on similar trials of Oyster cultivation on residual fractions. However, the primary objective in the current trial was to observe and evaluate wheat chaff as mushroom substrate material – an assessment which was facilitated by limiting the spawn fraction, as it provides additional nutrients to the substrate.

5.1.1. Observations and suggestions for optimized production

The results from the MC and pH sampling upon second inoculation of trial 1 demonstrated a drop from the initial adjusted pH of 7.4-7.5 to pH 5.8, which was observed in different parts of the PPD-bags. This suggests that the substrate material enabled fungal growth throughout the entire mass, by, for example, not limiting the gas exchange which is an important feature for substrate materials. The MC of 77% showed that the substrate was able to retain moisture.

Oyster mushroom's ability to grow in slightly alkaline environments in addition to the uncertainty regarding the extent of the pH drop that would occur in the substrate upon fungal growth was the reason that a slightly alkaline initial pH was aimed for in current trial. Varying MC in the substrates and pH values in the tap water did, however, contribute to differences and challenged uniformity. Trial nr 3 had a slightly higher initial pH value (pH 7.7) although the substrate still enabled successful fructification. Due to the varying parameters that may have affected the results, the effect of the pH on the fructification cannot be interpreted easily. Additional analysis is therefore required to find the optimal pH for Oyster mushroom cultivation on wheat chaff.

Because of logistical hurdles that were encountered throughout the incubation of the fruiting bodies trial, the fungi and substrate were exposed to a vast range of different environmental conditions. Nevertheless, these deviating circumstances enabled several opportunities to observe the substrate response to different environmental stimuli. As an example, the fluctuating RH% revealed certain substrate characteristics, such as, quick absorption and evaporation and thus underlining the importance of a stable RH% during fungal cultivation on wheat

chaff. In the PPD-bags containing the substrate the water successively migrated to the bottom of the bag whilst the substrate surface on the top slowly dried out, giving rise to a moisture gradient within the substrate, possibly due to the physical nature and high porosity of the wheat chaff. This suggests that the particle size of the wheat chaff was not optimal as a substrate material. The petri dish trial as well as the box trial was not affected by a moisture gradient, but with increased substrate volume, the uneven distribution became more apparent. The moisture gradient in the substrate could therefore create obstacles when scaling up the production. Moreover, the moisture gradient created an unsuitable environment for the mycelial growth as the chaff at the top became too dry whilst the bottom was too wet. The bags, which were made for a greater substrate volume than what was actually used, contained filters for gas exchange, which consequently might have altered the sensitivity for fluctuations in RH and increase the ability to absorb water. Smaller bags with tightly packed substrate would have been preferable to enable an optimal gas exchange and water absorption and desorption.

Initially the substrate was mixed about 2 times a week to redistribute the mycelium and water throughout the bag. However, instead of boosting growth this appeared to separate the substrate and decrease mycelial expansion, potentially because of applying stress on the fungi and increasing space between the substrate particles. When fungi are cultivated on substrate materials with large spacing, and lack of interconnection, a lot of cellular energy is utilized in the bridging of these particles. The result is a dispersed and fluffy fungal mycelium which may support vegetative mycelial growth, however not fruiting bodies as the substrate lacks mass density (Stamets 2000). Low substrate density, mixing, and bag volume presumably all together contributed to a lack of interconnected particles, which likely obstructed successful colonization. Despite this, the substrate in the PDD bags did enable fruiting body formation although not densely colonized with mycelia. However, greater mycelial coverage facilitates fungal nutritional utilization, and the lack of density could also indicate a lack of nitrogen within the substrate as the production of Oyster mushroom is greatly dependant on the C/N ratio. Given wheat chaffs' porous physical structure and low density, application of additional substrate materials might be beneficial to enhance substrate properties. Indeed, cultivation of edible mushroom are generally promoted by mixing different substrate materials (Stamets 2000). Seed hulls, particularly cotton seed hulls, are known for their appropriate particle size, ability to retain water and a nitrogen content greater than most cereal straws (Stamets 2000). An increase in yield was observed when rice straw was supplemented with cotton seed hulls (Yang et al. 2013). Okara, the residual fraction obtained from the production of tofu or soy milk, may also be a potential supplement for wheat chaff (Li et al. 2012). As a substrate material, it enables quick colonization by a variety of mushrooms, including *Pleurotus* spp.

(Stamets 2000). A small trial was performed using wheat chaff and fresh okara (obtained from Nordic Green Food AB) where wheat chaff was supplemented with 10% okara (DW/DW); the density of the initial mycelial formation increased notably in comparison to the unsupplemented reference (Unpublished results). Further research is needed to find a suitable substrate mixture that utilizes the water holding capacity and additional nitrogen content provided by the okara and the porous structure of the wheat chaff.

5.1.2. Wheat chaff as substrate for mushroom production.

The following section will include additional assessments to be considered regarding potential usage of wheat chaff as a mushroom substrate, such as estimation of biological efficiency, and aspects of quality and food safety.

To facilitate the evaluation and comparison between wheat chaff and alternative or additional substrate materials, it would be valuable if future research estimated the biological efficiency (BE). In short, it reflects the efficiency of the mushroom strain regarding substrate conversion by measuring the fresh weight of the mushrooms in relation to the dry weight of the substrate. Subsequently, this would enable a greater understanding for the substrates' ability to produce fruiting bodies and thus help optimize growth parameters to increase the yields. In addition to yield, the composition of the substrate should also be considered as it alters the nutritional composition of the fruiting bodies. This has led to the suggestion that not only BE should be assessed, but also the protein levels in the fruiting bodies, if the aim is to produce high quality nutritious mushroom for human food consumption (Hultberg et al. 2019). Furthermore, heavy metal accumulation is also relevant to consider prior to proposing a certain wheat chaff as mushroom substrate because white-rot fungi are known to accumulate heavy metals from substrate material in the fruiting bodies, sometimes at levels unfit for human consumption (Laursen 2018; Baldrian 2003). Whilst certain heavy metals are essential for fungal metabolism, others are seen as nonessential, without proven biological role; when present in excess, both essential and nonessential heavy metals may be toxic to the fungi (Baldrian 2003). This accumulative ability might therefore be important to consider whilst researching different variations in wheat chaffs, on national and international level, and their potential as substrate materials.

5.1.3. Utilizing spent mushroom substrate as feed

From a processing perspective, it may be hypothesised to integrate the two mentioned potential areas of application for fungal-transformed wheat chaff: first, as mushroom substrate, and subsequently as feed.

The agro-residues and the fungal mycelium that is left after the mushrooms have been harvested is called Spent mushroom substrate (SMS) (Antunes et al. 2020). The cultivation of 1 kg of mushroom produces approximately 5kg of SMS, a material rich in trace elements, cellulose, hemicellulose, lignin, crude protein, and fat (Antunes et al. 2020; Finney et al. 2009). Thus, actualizes the potential integration of feed and fruiting body production into one cycle, by utilizing the SMS of wheat chaff as feed, an alternative application for SMS which is currently receiving great attention. However, as previously described, the lignin degradation by white-rot fungi has been shown to be most effective and selective prior to fruiting body production. Fructification instead stimulates increased utilization of cellulose and hemicellulose. This decrease in total organic material conversely increases the ash content, which may, in excessive amounts, cause digestive difficulties for the animal (Sharma & Arora 2010). Consequently, this poses challenge for integration of SMS and feed, in consideration to the current substrate material. Further research on the SMS as potential feed would be of interest, as well as other potential applications, e.g., biofuel production or as compost, particularly since waste management practises and proper utilization of SMS are emphasised as major barriers to the increasing mushroom production throughout the world (Prasad et al. 2021; Finney et al. 2009).

5.2. Conclusion

The aims of the current pilot study were to investigate the potential growth of different filamentous fungi on wheat chaff, and to select a suitable fungus for further analysis of its conversion of the substrate in relation to feed value. Furthermore, to explore wheat chaff as a potential substrate for fruiting body production The exploration of wheat chaff substrate characteristics in the study contributed to hands-on knowledge about how to prepare the substrate in order to facilitate fungal colonization. The results showed successful colonization by Oyster, King Oyster and Maitake, further demonstrated by increased ash content due to fungal metabolization of organic material. Screening of additional fungal species/strains that can grow well on the substrate material is therefore a topic of interest.

Cultivation of Oyster mushroom for 46 days significantly decreased the lignin content of the substrate from approximately 12.2%-10.8% resulting in a decrease of about 11.5%, but no significance difference in crude protein or ash content was observed. The presence of ligninolytic activity by the Oyster mushroom was also exhibited by the substrates' altered structure and appearance, namely, a bleached and fibrous wheat chaff coated in a granular slime layer. Fungal fermentation by white-rot fungi hence exhibits potential for successful alteration of the substrate. Further studies aimed to optimize the fermentation process and screening of additional strains would likely result in increased delignification. Moreover, additional analysis on e.g., animal digestibility and nutritional composition, would be required to evaluate the potential of fungal-transformed wheat chaff as a base for animal feed. The study also showed that wheat chaff was able to support fruiting body production of Oyster mushroom, without additional supplementation. However, further research is needed to optimize growth conditions and to evaluate the nutritional composition of the fruiting bodies. Supplementation with additional substrate material, to optimize nutritional composition and physical structure, may also be required to increase yield and reduce the time of the crop cycle.

In conclusion, the current pilot study has contributed to increased knowledge within potential areas of application of the unexploited biomass resource, chaff. Although extensive research is still required prior to plausible implementation, the results indicate possibilities to utilize wheat chaff through fungal conversion which in turn would contribute to circularity within food production system.

References

Abdel-Hamid, A.M., Solbiati, J.O. & Cann, I.K.O. (2013). Chapter One - Insights into Lignin Degradation and its Potential Industrial Applications. In: Sariaslani, S. & Gadd, G.M. (eds.) *Advances in Applied Microbiology*. Academic Press, 1–28. https://doi.org/10.1016/B978-0-12-407679-2.00001-6

Adamović, M., Grubić, G., Milenković, I., Jovanović, R., Protić, R., Sretenović, L. & Stoićević, L. (1998). The biodegradation of wheat straw by Pleurotus ostreatus mushrooms and its use in cattle feeding. *Animal Feed Science and Technology*, 71 (3), 357–362. https://doi.org/10.1016/S0377-8401(97)00150-8

Antunes, F., Marçal, S., Taofiq, O., M. M. B. Morais, A., Freitas, A.C., C. F. R. Ferreira, I. & Pintado, M. (2020). Valorization of Mushroom By-Products as a Source of Value-Added Compounds and Potential Applications. *Molecules*, 25 (11), 2672. https://doi.org/10.3390/molecules25112672

Balan, V., Zhu, W., Krishnamoorthy, H., Benhaddou, D., Mowrer, J., Husain, H. & Eskandari, A. (2022). Challenges and opportunities in producing high-quality edible mushrooms from lignocellulosic biomass in a small scale. *Applied Microbiology and Biotechnology*, 106 (4), 1355–1374. https://doi.org/10.1007/s00253-021-11749-2

Baldrian, P. (2003). Interactions of heavy metals with white-rot fungi. *Enzyme and Microbial Technology*, 32 (1), 78–91. https://doi.org/10.1016/S0141-0229(02)00245-4

Bano, Z., Shashirekha, M.N. & Rajarathnam, S. (1993). Improvement of the bioconversion and biotransformation efficiencies of the oyster mushroom (Pleurotus sajor-caju) by supplementation of its rice straw substrate with oil seed cakes. *Enzyme and Microbial Technology*, 15 (11), 985–989. https://doi.org/10.1016/0141-0229(93)90176-3

Cenis, J.L. (1992). Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research*, 20 (9), 2380. https://doi.org/10.1093/nar/20.9.2380

Chang, S.T., Miles, P.G. & Chang, S.T. (2004). *Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact*. 2nd ed. Boca Raton, Fla: CRC Press.

Claesson, S. (1980). *Kan halmen utnyttjas bättre? [Can the Straw be Better Utilized?]*. (Konsulentavdelningens rapporter, Allmänt 23). Uppsala: Sveriges Lantbruksuniversitet.

Commanday, F. & Macy, J.M. (1985). Effect of substrate nitrogen on lignin degradation by Pleurotus ostreatus. *Archives of Microbiology*, 142 (1), 61–65. https://doi.org/10.1007/BF00409238

Cunha Zied, D., Sánchez, J.E., Noble, R. & Pardo-Giménez, A. (2020). Use of Spent Mushroom Substrate in New Mushroom Crops to Promote the Transition towards A Circular Economy. *Agronomy*, 10 (9), 1239. https://doi.org/10.3390/agronomy10091239

Curvetto, N.R., Figlas, D., Devalis, R. & Delmastro, S. (2002). Growth and productivity of different Pleurotus ostreatus strains on sunflower seed hulls

supplemented with N–NH4+ and/or Mn(II). *Bioresource Technology*, 84 (2), 171–176. https://doi.org/10.1016/S0960-8524(02)00013-5
Dahlin, A. (2021). Swedish substrates suited for circular production of the edible gourmet mushroom maitake. 54
Fazaeli, H. (2007). Nutritive value index of treated wheat straw with Pleurotus fungi. *Biotechnology in Animal Husbandry*, 23 (5-6–1), 169–180. https://doi.org/10.2298/BAH0701169F
Finney, K., Sharifi, V. & Swithenbank, J. (Univ of S. (United K. (2009). The reuse of spent mushroom compost and coal tailings for energy recovery. https://www.osti.gov/etdeweb/biblio/1027560 [2022-05-06]
Goodell, B., Quian, Y. & Jellison, J. (2008). Fungal Decay of Wood: Soft Rot-Brown Rot-White Rot. In: Schultz, T.P., Militz, H., Freeman, M.H., & Nicholas, D.D. (eds.) *Development of Commercial Wood Preservatives: Efficacy, Environmental, and Health Issues*. Washington, DC: American Chemical Society, 22. https://doi.org/10.1021/bk-2008-0982
Grabber, J.H. (2005). How Do Lignin Composition, Structure, and Cross-Linking Affect Degradability? A Review of Cell Wall Model Studies. *Crop Science*, 45 (3), 820–831. https://doi.org/10.2135/cropsci2004.0191
Gutiérrez, A., Martínez, M.J., Almendros, G., González-Vila, F.J. & Martínez, A.T. (1995). Hyphal-sheath polysaccharides in fungal deterioration. *Science of The Total Environment*, 167 (1), 315–328. https://doi.org/10.1016/0048-9697(95)04592-O
Hatfield, R.D., Jung, H.-J.G., Ralph, J., Buxton, D.R. & Weimer, P.J. (1994). A comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. *Journal of the Science of Food and Agriculture*, 65 (1), 51–58. https://doi.org/10.1002/jsfa.2740650109
Hultberg, M., Ahrens, L. & Golovko, O. (2020). Use of lignocellulosic substrate colonized by oyster mushroom (Pleurotus ostreatus) for removal of organic micropollutants from water. *Journal of Environmental Management*, 272, 111087. https://doi.org/10.1016/j.jenvman.2020.111087
Hultberg, M., Nordström Nilsson, K., Jonsson, E. & Persson, C. (2019). *Odling av ostronskivling på restprodukter*. [Faktablad]. https://pub.epsilon.slu.se/16316/ [2022-04-28]
Ivarsson, E., Grudén, M., Södergren, J. & Hultberg, M. (2021). Use of faba bean (Vicia faba L.) hulls as substrate for Pleurotus ostreatus – Potential for combined mushroom and feed production. *Journal of Cleaner Production*, 313, 127969. https://doi.org/10.1016/j.jclepro.2021.127969
Jellison, J. & Jasalavich, C. (2000). A review of selected methods for the detection of degradative fungi. *International Biodeterioration & Biodegradation*, 46 (3), 241–244. https://doi.org/10.1016/S0964-8305(00)00075-5
Jordbruksverket (2018). *Jordbruksmarkens användning 2018. Slutlig statistik*. (JO0104). Jordbruksverket, Jönköping. https://jordbruksverket.se/om-jordbruksverket/jordbruksverkets-officiella-statistik/jordbruksverkets-statistikrapporter/statistik/2020-06-22-jordbruksmarkens-anvandning-2018.--slutlig-statistik#h-Rapport [2022-05-24]
Kachlishvili, E., Penninckx, M.J., Tsiklauri, N. & Elisashvili, V. (2006). Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *World Journal of Microbiology and Biotechnology*, 22 (4), 391–397. https://doi.org/10.1007/s11274-005-9046-8
Kerem, Z. & Hadar, Y. (1995). Effect of manganese on preferential degradation of lignin by Pleurotus ostreatus during solid-state fermentation. *Applied and Environmental Microbiology*, 61 (8), 3057–3062
Knežević, A., Milovanović, I., Stajić, M., Lončar, N., Brčeski, I., Vukojević, J. & Ćilerdžić, J. (2013). Lignin degradation by selected fungal species.

Bioresource Technology, 138, 117–123. https://doi.org/10.1016/j.biortech.2013.03.182
van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H. & Cone, J.W. (2015a). Fungal treated lignocellulosic biomass as ruminant feed ingredient: A review. *Biotechnology Advances*, 33 (1), 191–202. https://doi.org/10.1016/j.biotechadv.2014.10.014
van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H. & Cone, J.W. (2015b). Fungal treatment of lignocellulosic biomass: Importance of fungal species, colonization and time on chemical composition and in vitro rumen degradability. *Animal Feed Science and Technology*, 209, 40–50. https://doi.org/10.1016/j.anifeedsci.2015.07.026
Kumla, J., Suwannarach, N., Sujarit, K., Penkhrue, W., Kakumyan, P., Jatuwong, K., Vadthanarat, S. & Lumyong, S. (2020). Cultivation of Mushrooms and Their Lignocellulolytic Enzyme Production Through the Utilization of Agro-Industrial Waste. *Molecules*, 25 (12), 2811. https://doi.org/10.3390/molecules25122811
Laursen, A. (2018). *The effect of different nitrogen sources on mycelial growth of oyster mushroom, Pleurotus ostreatus*. [Grundnivå, G2E]. https://stud.epsilon.slu.se/14016/ [2022-05-17]
Leong, S.L., Niba, A.T., Ny, S. & Olstorpe, M. (2012). Microbial populations during maize storage in Cameroon. *African Journal of Biotechnology*, 11 (35), 8692–8697. https://doi.org/10.4314/ajb.v11i35
Li, B., Qiao, M. & Lu, F. (2012). Composition, Nutrition, and Utilization of Okara (Soybean Residue). *Food Reviews International*, 28 (3), 231–252. https://doi.org/10.1080/87559129.2011.595023
Martínez, A.T., Speranza, M., Ruiz-Dueñas, F.J., Ferreira, P., Camarero, S., Guillén, F., Martínez, M.J., Gutiérrez, A. & del Río, J.C. (2005). Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology: The Official Journal of the Spanish Society for Microbiology*, 8 (3), 195–204
McCartney, D.H., Block, H.C., Dubeski, P.L. & Ohama, A.J. (2006). Review: The composition and availability of straw and chaff from small grain cereals for beef cattle in western Canada. *Canadian Journal of Animal Science*, 86 (4), 443–455. https://doi.org/10.4141/A05-092
Naraian, R., Sahu, R., Kumar, S., Garg, S., Singh, C. & Kanaujia, R. (2008). Influence of different nitrogen rich supplements during cultivation of Pleurotus florida on corn cob substrate. *The Environmentalist*, Volume 29, 1–7. https://doi.org/10.1007/s10669-008-9174-4
Prasad, R., Lisiecka, J., Antala, M. & Rastogi, A. (2021). Influence of Different Spent Mushroom Substrates on Yield, Morphological and Photosynthetic Parameters of Strawberry (Fragaria × ananassa Duch.). *Agronomy*, 11 (10), 2086. https://doi.org/10.3390/agronomy11102086
Rodriguez Estrada, A., E. & Pecchia, J. (2017). Cultivation of Pleurotus ostreatus. In: Pardo-Gimnez, A. & Cunha Zied, D. (eds.) *Edible and Medicinal Mushrooms: Technology and Applications*. Hoboken, UNITED KINGDOM: John Wiley & Sons, Incorporated, 22. http://ebookcentral.proquest.com/lib/slub-ebooks/detail.action?docID=4914175 [2022-05-21]
Royse, D., J., Baars, J. & Tan, Q. (2017). Current Overview of Mushroom Production in the World. In: Pardo-Gimnez, A. & Cunha Zied, D. (eds.) *Edible and Medicinal Mushrooms: Technology and Applications*. Hoboken, UNITED KINGDOM: John Wiley & Sons, Incorporated, 10. http://ebookcentral.proquest.com/lib/slub-ebooks/detail.action?docID=4914175 [2022-05-21]

Sánchez, C. (2009). Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*, 27 (2), 185–194. https://doi.org/10.1016/j.biotechadv.2008.11.001

Sánchez, C. (2010). Cultivation of Pleurotus ostreatus and other edible mushrooms. *Applied Microbiology and Biotechnology*, 85 (5), 1321–1337. https://doi.org/10.1007/s00253-009-2343-7

Shabtay, A., Hadar, Y., Eitam, H., Brosh, A., Orlov, A., Tadmor, Y., Izhaki, I. & Kerem, Z. (2009). The potential of Pleurotus-treated olive mill solid waste as cattle feed. *Bioresource Technology*, 100 (24), 6457–6464. https://doi.org/10.1016/j.biortech.2009.07.044

Sharma, R. k. & Arora, D. s. (2010). Changes in biochemical constituents of paddy straw during degradation by white rot fungi and its impact on in vitro digestibility. *Journal of Applied Microbiology*, 109 (2), 679–686. https://doi.org/10.1111/j.1365-2672.2010.04695.x

Shields, T. (2018). *Cold Water Lime Pasteurization For Growing Mushrooms*. *FreshCap Mushrooms*. https://learn.freshcap.com/growing/lime-pasteurization/ [2022-05-20]

Shrivastava, B., Thakur, S., Khasa, Y.P., Gupte, A., Puniya, A.K. & Kuhad, R.C. (2011). White-rot fungal conversion of wheat straw to energy rich cattle feed. *Biodegradation*, 22 (4), 823–831. https://doi.org/10.1007/s10532-010-9408-2

Stamets, P. (2000). *Growing gourmet and medicinal mushrooms*. Berkeley: Berkeley: Ten Speed Press, CA, USA.

Tabata, E., Kashimura, A., Kikuchi, A., Masuda, H., Miyahara, R., Hiruma, Y., Wakita, S., Ohno, M., Sakaguchi, M., Sugahara, Y., Matoska, V., Bauer, P.O. & Oyama, F. (2018). Chitin digestibility is dependent on feeding behaviors, which determine acidic chitinase mRNA levels in mammalian and poultry stomachs. *Scientific Reports*, 8 (1), 1461. https://doi.org/10.1038/s41598-018-19940-8

Theander, O., Åman, P., Westerlund, E., Andersson, R. & Pettersson, D. (1995). Total Dietary Fiber Determined as Neutral Sugar Residues, Uronic Acid Residues, and Klason Lignin (The Uppsala Method): Collaborative Study. *Journal of AOAC INTERNATIONAL*, 78 (4), 1030–1044. https://doi.org/10.1093/jaoac/78.4.1030

Unger, J.S. & Glasner, C. (2019). Cost Analysis of Chaff Harvesting Concepts in Germany. *Agronomy*, 9 (10), 579. https://doi.org/10.3390/agronomy9100579

Weiß, B.D. & Glasner, C. (2018). Evaluation of the Process Steps of Pretreatment, Pellet Production and Combustion for an Energetic Utilization of Wheat Chaff. *Frontiers in Environmental Science*, 6. https://www.frontiersin.org/article/10.3389/fenvs.2018.00036 [2022-05-18]

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., & White, T.J. (eds.) *PCR Protocols: A Guide to Methods and Applications*. San Diego: Academic Press, 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Yang, W., Guo, F. & Wan, Z. (2013). Yield and size of oyster mushroom grown on rice/wheat straw basal substrate supplemented with cotton seed hull. *Saudi Journal of Biological Sciences*, 20 (4), 333–338. https://doi.org/10.1016/j.sjbs.2013.02.006

Zadril, F. (2000). Is conversion of lignocellulosics into feed with white rot fungi realizable? Practical problems of scale-up and technology transfer. In: Griensven, L.J.L.D. van (ed.) *Science and Cultivation of Edible Fungi: Proceedings of the 15th International Congress on the Science and*

Cultivation of Edible Fungi, Maastricht/Netherlands/15-19 May 2000. A.A. Balkema, 9

Zhang, Z., Xia, L., Wang, F., Lv, P., Zhu, M., Li, J. & Chen, K. (2015). Lignin degradation in corn stalk by combined method of H2O2 hydrolysis and Aspergillus oryzae CGMCC5992 liquid-state fermentation. *Biotechnology for Biofuels*, 8 (1), 183. https://doi.org/10.1186/s13068-015-0362-4

Popular science summary

The shift towards a more sustainable food system emphasises a need for increased circularity and valorisation of available resources. During harvest of certain cereal crops, chaff constitutes one of three main fractions but is generally discarded on the field. New technologies have enabled collection of the chaff, and this could potentially increase the harvested residues by 30%. Hence, there is a research interest in making use of this, currently, unused resource as e.g., animal feed. Although a problem in using chaff, and other residues from agricultural production, as feed to ruminant animals is due to difficulties in digesting it. Ruminant animals cannot digest certain rigid and complex structures that are present in these plant materials, one of them being lignin. Growing certain filamentous fungi on these plant materials can however increase the digestibility, due to the ability of the fungi to break down lignin into more easily digestible components. Growing these fungi on residues from agricultural production is therefore a potential way to enable further applications of these residual materials. Hence, the current project was initiated from a research interest to explore the potential of wheat chaff as a main component in animal feed following fungal growth. It was then expanded to also include the potential to cultivate edible mushrooms on wheat chaff, as an additional way to utilize it.

First, a trial was made to find suitable fungi, able to grow well on moistened wheat chaff. Following that, a few analyses were made to see how a selected fungus changed the structure of the substrate, in ways that could be valuable in terms of using it as animal feed. Lastly it was explored if wheat chaff could be used as a base for cultivation of edible mushrooms.

The results of the study showed that Oyster mushroom, King Oyster and Maitake grew well on the wheat chaff. The Oyster mushroom was selected for further analysis, and these showed that the fungus enabled successful break down of lignin, reducing the amounts by approximately 11.5%. However, the other two analysis showed that the Oyster mushroom did not metabolize a lot of the organic material in the substrate, indicating that it did not grow that well on the substrate material. Despite that, the third trial, made at larger scale, did show that Oyster mushroom was able to produce edible mushrooms when cultivated on the wheat chaff. However, in relation to how long it generally takes for Oyster mushroom to produce the mushrooms, the required time in the current experiment was notably longer. This suggests that more trials are needed to improve the production, and that this potentially could be done by mixing wheat chaff with other substrate materials. Additional research is also required before fungi grown on wheat chaff can be used as a potential base for feed, although the decrease in lignin seen in the experiment does show potential for this sort of fermentation of wheat chaff. To summarise, the current experiment contributed with increased knowledge in how to prepare the

wheat chaff to enable fungi to grow on it; it showed that the Oyster mushroom could degrade the rigid structured lignin and produce mushrooms when cultivated on wheat chaff.

Acknowledgements

I want to thank SLU and RISE for giving me the opportunity to engage myself in this interesting project. I would like to pay my special regards to Su-lin. Thank you for the continuous support and guidance along the process. I would like to thank Xinmei for introducing me to the world of mushroom cultivation and Petter for arranging so that I could be a part of this project and for multiple times running up to the lab with additional bags of wheat chaff. Thank you to everyone at the Department who has supported me in the lab, I would not have accomplished this without your help. A special thanks to Annica Andersson.
I would also like to show my appreciation towards Malin Hultberg at SLU Alnarp for sending me the Maitake and for answering my questions. Finally, I want to thank my parents, not only for all their support, but especially for letting me transform your garage into a small low-tech mushroom farm.

Appendix 1

Figure A1. Typical chaff collector mounted on a combine harvester.

Table A1. pH-measurement during mid-point sampling in boxes.

Replicate	pH in box nr 7 (26 days of incubation)	pH in box nr 3 (33 days of incubation)	pH box nr 4 (33 days of incubation)
1	5.68	5.48	5.46
2	5.36	5.62	5.95
3	5.3	5.35	5.4
Mean value	5.4	5.5	5.6