

INFLUENCE OF RELATIVE HUMIDITY AND TEMPERATURE ON CULTIVATION OF *Pleurotus* SPECIES

Hamed Aghajani¹, Ehsan Bari^{2*}, Mohsen Bahmani³, Miha Humar⁴,
Mohammad Ali Tajick Ghanbary⁵, Darrel D. Nicholas⁶, Emran Zahedian²

ABSTRACT

Fungi exhibit different behavior under different conditions and react to light, temperature, moisture content etc. The objective of this study was to evaluate the degradation capability of three common white rot fungi, namely: *Pleurotus ostreatus*, *Pleurotus pulmonarius*, and *Lentinus sajor-caju*. The respective fungi were cultivated on rice straw under three different environmental conditions for 90 days. The fungi were collected, pure cultured, DNA extracted, and sequenced by ITS regions. The highest consumption of substrate occurred under the Cellar (dark) exposure condition with *Pleurotus pulmonarius* producing the least mass loss. The least amount of degradation occurred under the Air (daylight) condition for all of the fungi with the exception of *Pleurotus pulmonarius*. Exposure to light promoted the formation of fruiting bodies.

Keywords: *Lentinus sajor-caju*, mushroom cultivation, *Pleurotus ostreatus*, *Pleurotus pulmonarius*, wood fungi.

INTRODUCTION

Fungi exhibit different behavior under different environmental conditions such as moisture content, temperature, light, levels of oxygen and nitrogen, and pH, for a given substrate (Messner *et al.* 2003, Schwarze *et al.* 2004, Nikolouli *et al.* 2016). Many wood decay fungi are mesophilic and are able to grow at temperature ranges between 20-30 °C (Schmidt 2006). Some wood inhabiting fungi have the capability of colonizing wood at temperature levels around 50 °C (Eaton and Hale 1993). Fruiting bodies of some fungi respond to light due to their phototropic behavior. Many wood decaying fungi, Hymenomycetes, react to light by producing sporocarps (Schmidt 2006, Eaton and Hale 1993). Fungi may also respond to the direction from which the light is orientated (Jennings and Lysek 1999). The stipe grows towards the light source during the primordium growth of Basidiomycetes. Fungi of the genus *Pleurotus* have an important place among the commercially employed basidiomycetes because they have gastronomic, nutritional, biotechnological and medicinal properties and can be easily cultivated on a variety of substrates. In addition to studies on solid cultures focusing on the production of fruiting bodies, the submerged cultivation of the genus *Pleurotus* has also been studied by several authors with varied objectives including the production of: liquid inoculum (Rosado *et al.* 2002), extracellular enzymes (Garzillo *et al.* 1994), flavoring agents (Martin 1992), β -glucosidases (Morais *et al.* 2002),

¹Department of Forestry, Sari Agriculture Science and Natural Resources University, Sari, Iran.

²Department of Wood Science and Engineering, Technical Faculty of No. 2, Mazandaran Branch, Technical and Vocational University (TVU), Sari, Iran.

³Department of Natural Resources and Earth Science, Shahrekord University, Shahrekord, Iran.

⁴Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.

⁵Department of Mycology and Plant Pathology. College of Agronomic Sciences, Agriculture and Natural Resources University, Sari, Iran.

⁶Department of Sustainable Bioproducts, Mississippi State University, Mississippi State. USA.

*Corresponding author: bari_lenzites@yahoo.com

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antimicrobials (Beltran *et al.* 1997, Benkortbi *et al.* 2007) and vitamins (Solomko and Eliseeva 1998). However, solid state cultivation is still the main method of *Pleurotus* cultivation. The worldwide production of edible mushrooms cultivated on straw and wood is in the range of 2 million tons (fresh weight basis) per year. Thus, cultivation of mushrooms represents one of the economically most important microbial conversions of lignocelluloses (Chang and Hayes 1978). The Oyster mushroom, *Pleurotus ostreatus*, grows well on several substrates, including lignocellulosic residues (Pettipher 1978). In North America and Europe (predominately in Italy and Hungary), *Pleurotus* species such as *Pleurotus ostreatus* are grown on chopped wheat straw. Also, chopped lignocellulosics originating from agricultural residues and forestry residues are used by hobby breeders as well as commercially. Several *Pleurotus* species used for cultivation but nine species of the *Pleurotus* family are distributed in the forests of Iran (Ershad 2009). These species grow on *Fagus orientalis*, *Quercus* spp., *Populus* spp., *Ulmus* spp., and *Salix alba* and act in both saprophytes and wound parasites phases (Aghajani *et al.* 2017). Three species of *Pleurotus*: *Pleurotus ostreatus*, *L. sajor-caju*, and *Pleurotus pulmonarius* are of particular interest, as they can grow on a variety of woods, such as beech, oak, hornbeam, poplar, and alder. On the other hand, the ecology of these respective fungal species varies considerably. Hence, the aim of this study was to determine the effects of temperature, relative humidity, and light on the rate of fungal growth for these fungi. The performance criteria used in evaluating these fungi were the yield of fruiting bodies and their ability to degrade wood. These data are important from the fungal cultivation point of view as well as for assessment of the optimal conditions for fungal degradation. These data can serve as an input data for modeling of the service life of lingo-cellulosic materials in various climate regions.

MATERIALS AND METHODS

Fungal collection, isolation, and identification

The fruiting bodies of three fungi, *Pleurotus ostreatus* (MG835874), *Pleurotus pulmonarius* (MG835875), and *Lentinus sajor-caju* (MG835876) were collected from fallen beech and oak trees in Alamdardeh forest (located in Sari (36°22'34"N, 53°14'59"E), stored in a cold chamber (-10°C) and immediately transported to the laboratory. About 20 mg of the mushroom tissue was taken from the internal part of the cap of each fungus, placed on potato dextrose agar (PDA) and incubated at 25°C for 3 days. After sufficient fungal hyphae development, the single tip of several hyphae were isolated and transferred onto new malt extract agar (MEA) and incubated at 25°C for 5 days. Thereafter, subcultures of all three fungi was transferred to 4% MEA and kept at 4°C until used.

Molecular identification of the fungi was performed in accordance with methods described by Bari (2014) and Bari *et al.* (2017). Mycelium was washed with sterile distilled water. Dehydrated, the Mycelium was reduced to powder in a sterile mortar with nitrogen. About 10 mg of the powdered mycelium was placed in a vial (Eppendorf tube). 600- μ l of CTAB solution and 1- μ l β -Mercapto-ethanol (2%) were added to the vial, and transferred to a hot water chamber (Ben-Murry) 65°C for 45 min, and then the vials were inverted for 15 min. Next, 600- μ l of chloroform: isoamylalcohol (24:1) was added to the vials prior to centrifuging at 12000 rpm for 10 min. Thereafter, 500- μ l of supernatants containing DNA were transferred to new vials and about the same volume of cold isopropanol (-20°C) was added. The vials were kept in the freezer at -20°C for 30 min. Afterward the vials were centrifuged at 13000 rpm for 15 min, the liquid phase was removed and the vials were washed with 1 ml of 70% ethanol for 1 min. After removing the ethanol, 70- μ l of sterile distilled water was added and the vials were refrigerated for 30 min before storing in a freezer at -20°C.

For the polymerase chain reaction (PCR), ITS4 and ITS5 primers were chosen. Two replicates were used. The PCR protocol was as follows: an initial denaturization of 4 min at 98°C, then 35 cycles of 30 sec at 94°C for denaturization, 30 sec at 58°C for annealing, 1 min at 72°C for extension, and a final extension of 7 min at 72°C. Aliquots of PCR products were examined on 2% agarose gel. Gels were stained with GelStar Nucleic Acid Gel Stain (Lonza Rockland, Inc, USA) and examined using UV light. PCR products were sequenced at Takapozist Co. (Bioneer- Korea). Sequence identification

was done by comparison with sequences deposited in the DNA databases using the BLAST program Schmidt *et al.* (2012).

Sample preparation and incubation

The rice straw was cut into 20-25 cm long pieces and then washed in hot water at 100°C for 15 min. before oven drying at 103±2°C for 24 h. Afterward, about 50 g of rice straw was placed in a plastic bag (common nylon) and autoclaved (1,5 bar) at 121°C for 20 min. The rice straw had an average moisture content of about 25%. The bags were then inoculated with the purified fungal mycelia. Three environment conditions were selected for incubation (Table 1).

Table 1. Environmental conditions for the three exposure conditions used during incubation of the samples.

Type of Exposure	Cellar	Room	Air
Lighting	Dark	lamp (200 watt)	Daylight
Relative Humidity (%)	80-85	75-85	65-75
Temperature Range (°C)	29-31	19-21	26-28

Selected micro-climate conditions were chosen, as they represent the most typical conditions where cultivation of the fungi could take place, or potential building infestation could appear. Three replicates were used for each fungus as well as for the environment conditions. The samples were incubated for 90 days. Mass loss was determined and was calculated in accordance with the following formula Equation 1:

$$ML(\%) = \frac{M_i - M_d}{M_i} \times 100 \quad (1)$$

Where ML is mass loss (%); M_i : dry mass before decay (g); M_d : dry mass after decay (g).

Chemical analysis

Cell wall components of the straw were determined by TPPI methods. Lignin and cellulose determination was carried out using T-222 om-98 (TAPPI 1998) and T 17 wd-70 (TAPPI 1997) procedures, respectively.

For Klason lignin determination one g of straw powder (free from extractives) was mixed with 72% sulfuric acid (15 ml) for 2 h at room temperature. The mixture was then diluted with 560 ml of boiling distilled water to obtain a 3% solution in sulfuric acid, which was heated under reflux for 4 h and then filtered. The Klason lignin residue was washed with hot distilled water and dried at 103°C to constant weight. The Klason lignin content was calculated in accordance with the following formula Equation 2:

$$KL(\%) = \frac{Sd - KL}{Sd} \times 100 \quad (2)$$

where: Sd is the dried weight of sawdust and KL is the dried weight of Extracted Klason lignin.

Cellulose content was determined by mixing 2 g of extractive free sawdust with 96% EtOH (100 ml) and then diluted with 65% nitric acid to 50 ml. The mixture was heated under reflux for 1 h and filtered. The cellulose residue was then washed with warm distilled water, dried at 103°C to constant weight. Cellulose content was calculated in accordance with the following formula Equation 3:

$$\text{Cellulose(\%)} = \frac{Sd - EC}{Sd} \times 100 \quad (3)$$

where: Sd is the dried weight of sawdust and EC is the dried weight of extracted cellulose

Statistical analysis

A Student t-test for each exposure period (95% level of confidence) was used to compare the mass loss and chemical components of decayed and non-decayed specimens. A Two-way ANOVA was conducted to examine the effect of decay condition on mass and chemical losses. All statistical analyses were performed using SPSS version 17 (2010).

RESULTS AND DISCUSSION

Mass loss determination

Figure 1 shows the mass loss of rice straw caused by the fungi after 90 days incubation. The rice straw was white to colorless after incubation and had a light and almost stringy consistency. The fungus appears to degrade the majority of the lignin, which resulted in the bleaching and whitening due to the abundance of the cellulose. This presumption is connected based on the fact that *Pleurotus* is a white rot decay fungus (Eaton and Hale 1993, Martínez *et al.* 2001). Several white-rot fungi can cause selective rot in most wood species (Camarero *et al.* 1994, Martínez *et al.* 2005). However, non-selective white rot is also reported and in general *Pleurotus* causes simultaneous type of white rot (Bari *et al.* 2015a). The highest mass losses were observed for *Pleurotus ostreatus* and *L. sajor-caju* incubated straw under Cellar conditions (Figure 1).

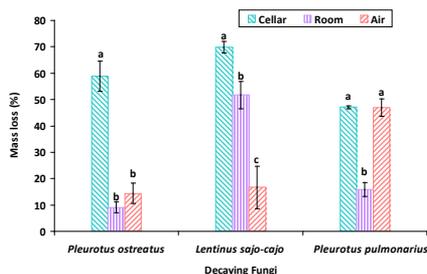


Figure 1. Mass losses of rice straw exposed to white rot fungi under different environmental conditions after 90 days incubation.

It is also apparent that the mass loss caused by *Pleurotus pulmonarius* was comparable for straw incubated under both Air and Cellar conditions. On the other hand, mass loss of rice straw after exposure to *Pleurotus ostreatus* in the air and Room conditions was negligible compared to other fungi. For example, rice straw exposed to *Pleurotus pulmonarius* in air was 46,88% while mass losses for straw exposed to *Pleurotus ostreatus* under the same conditions was (14,40%) (Figure 1). The lowest mass losses of rice straw were obtained after exposure to *L. sajor-caju*, and *Pleurotus pulmonarius* incubated in Air and Room conditions, respectively. Temperature appeared to play an important role in the degradation ability of *L. sajor-caju* and *Pleurotus ostreatus* in contrast to *Pleurotus pulmonarius*. The influence of temperature and relative humidity on fungal activity is well known (Cartwright and Findlay 1934, Viitanen 1997).

Changes in the chemical composition of rice straw after incubation

An overview of the chemical changes in rice straw due to the fungal degradation under the three environmental conditions is shown in Figure 2. The fungi caused considerable chemical degradation which was influenced by the environmental conditions (Figure 2).

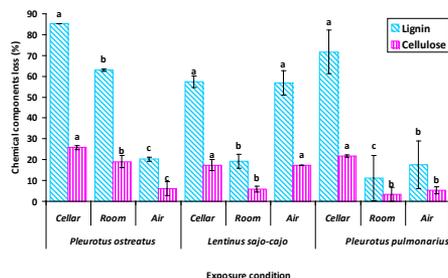


Figure 2. Chemical component losses of rice straw after exposure to white rot fungi under different environmental conditions after 90 days incubation.

Degradation of both lignin and cellulose was greatest for *Pleurotus ostreatus* under all three environmental conditions with the degradation being greatest under the Cellar and Room conditions. Overall, the consumption of polymeric components of the cell wall of rice straw after exposure to *Pleurotus pulmonarius* and *L. sajor-caju* was lower in comparison to that of *Pleurotus ostreatus*. Moreover, lignin was heavily degraded by the fungi during incubation. The Cellar incubation condition appears to have the greatest effect on lignin degradation caused by *Pleurotus ostreatus*. During the cellar incubation, lignin consumption was 71,73 and 57,41% by *Pleurotus pulmonarius* and *L. sajor-caju* fungi, respectively, while it was 85,19% for *Pleurotus ostreatus*. The highest amount of lignin degradation for rice straw was observed under Air and Room incubation. Our data clearly confirmed previous report showing that the *Pleurotus* species readily degrade lignin (Martinez *et al.* 2001). Cellulose was less degraded than lignin for all of the three fungi investigated, indicating that these fungi preferentially degraded lignin under the conditions investigated. The lowest cellulose degradation was obtained for straw exposed to *Pleurotus pulmonarius* and *L. sajor-caju* under Air conditions. Severe reduction in the rice straw cellulose content due to the *Pleurotus pulmonarius* degradation was observed by Adaskaveg *et al.* (1995). It showed that temperature was one of the most important factors influenced fungal behavior. These authors stated that when the temperature changed from 40 to 45 °C, some wood decay fungi such as *Ganoderma* species switched from the selective to simultaneous rot. However, in another it was shown that the white rot fungus *Pleurotus ostreatus* switched from simultaneous rot to selective degradation when the wood moisture content decreased from 65 to 45% (Bari *et al.* 2015a, Bari *et al.* 2015b, Bari *et al.* 2016; Bari *et al.* 2018). On one hand, Messner *et al.* (2003) indicated that moisture content was important for was appeared to expression of fungal enzymes and subsequent degradation of lignin bonds in wood cell walls. On the other hand, fungi use a hydrolysis system for degradation of cellulose bonds (Kubicek 2013).

Yield of fungal fruit-bodies

One of the key objectives of this research was to elucidate the influence of the growth conditions on the yield of the fruiting bodies by *Pleurotus* fungi. Production of fruiting bodies for the three *Pleurotus* fungi grown on rice straw are shown in Figure 3. Results indicated that *L. sajor-caju* (Figure 3 E&F) produced abundant fruiting bodies more rapidly compared to the *Pleurotus ostreatus* (Figure 3 C&D) and *Pleurotus pulmonarius* (Figure 3 A&B), respectively. The *Pleurotus osteratus* samples were degraded due to the sensitivity to humid conditions. On the other hand, *L. sajor-caju* produced more fruiting bodies under the Air incubation in comparison to the Room and Cellar conditions. *Pleurotus pulmonarius* also produced fruiting bodies under Air and Room incubation while *Pleurotus ostreatus* was not able to produce fruiting bodies under any of the incubation conditions tested. Light has been shown to affect formation of fungi fruiting bodies (Butin 1956). Light is the signal indicating that the mycelium has reached the surface, where the spores can be produced in an environment suitable for spore release (Jennings and Lysek 1999). Short wavelengths (blue light) stimulate fruiting bodies while longer wavelengths are ineffective. The light acceptor for the photons hitting the mycelium is riboflavin, which then reduces a cytochrome. The required light quantities are low, below those of the full moonlight in a clear sky (Schmidt 2006). Moisture content of the substrate is an important factor in the growth and metabolic activity of fungi (Schmidt 2006, Stienen *et al.* 2014). White rot fungi attack the cell wall of plants by a hydrolysis and hydrogen peroxide production is important in the degradation

of lignin bonds (Eriksson *et al.* 1990). The optimum moisture content of the substrate for the growth of *Pleurotus* mushroom is between 50% and 80% (Mohammadi Goltapeh and Pourjam 2010).



Figure 3. Fungal growing and production of fruit body on the rice straw after 90 days incubation in (A&B) *Pleurotus pulmonarius*, (C&D) *Pleurotus ostreatus*, and (E&F) *L. sajor-cajo* in air incubation.

CONCLUSIONS

The highest consumption of substrate occurred under Cellar conditions, where the fungi were incubated in the dark under high humidity and temperature conditions. *Pleurotus pulmonarius* produced the lowest degradation of rice straw. The lowest amount of degradation for all fungi was observed for samples exposed in the air (daylight) condition. The highest yield was obtained in the samples exposed to *L. sajor-cajo*. Moreover, light was found to be the most important variable affecting formation of fruiting bodies.

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