JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

EVALUATION OF DIFFERENT PASTEURIZATION AND STERILIZATION METHODS FOR OYSTER MUSHROOM SUBSTRATES

Daniel Grimm[*1,2], *Enno Sonntag*[1,3], *Gerold Rahmann*[1,2]

Address(es):
[1] Thünen Institute for Organic Agriculture, Agroecology Group, LandLessFood-green project, Trenthorst 32, 23847 Westerau, Germany.
[2] University of Kassel, Faculty of Organic Agricultural Sciences, Steinstrasse 19, 37213 Witzenhausen, Germany.
[3] Wageningen University & Research, Farming Systems Ecology Group, Department of Plant Sciences, P.O. Box 9101, 6700 HB Wageningen, the Netherlands.

*Corresponding author: daniel.grimm@thuenen.de

https://doi.org/10.55251/jmbfs.10428

ARTICLE INFO

Received 2. 8. 2023
Revised 31. 1. 2024
Accepted 5. 2. 2024
Published 1. 4. 2024

Regular article

OPEN ACCESS

ABSTRACT

Oyster mushrooms can be cultivated with great spatial efficiency, on nutrient-poor plant materials, without light and under diverse climatic conditions. Their production therefore has a great potential for improving food security, especially in impoverished and overpopulated areas. However, the pasteurization or sterilization of mushroom substrates uses a lot of energy and water. This study investigates the impact of different pasteurization and sterilization techniques on the growth and yield of oyster mushrooms, and evaluates their water and energy usage. The efficacy of heat-based methods, including hot water, hot air, and pressurized steam, as well as a chemical method utilizing hydrated lime ($Ca(OH)_2$) were assessed. The results show that sterilizing mushroom substrates through autoclaving can significantly increase the dry yields, up to 50% compared to pasteurization methods. However, pasteurization methods also achieved excellent results compared to untreated substrates, with good harvests and low pest occurrence. The mushroom water content was significantly higher in pasteurization methods where the substrate is submerged in water. In terms of fresh yield, hot water pasteurization was as good as autoclaving and significantly better than the other pasteurization methods. Hot air pasteurization has, on balance, a better water and energy efficiency than autoclave sterilization (about 75 % less energy) or hot water pasteurization (about 85 % less water). When performed at an air temperature of 75° C, which was found to be sufficient for successful mushroom cultivation, as little as 1068 kJ was needed to pasteurize one kg of dry substrate (e.g. maize straw). While hydrated lime pasteurization could use as little as 270 kJ per kg of dry substrate, it is very wasteful of water, as is hot water pasteurization and could lead to nutrient leaking. The success of mushroom cultivation, especially with hot air pasteurization, could also be influenced by the duration of substrate soaking before treatment. The study provides slight evidence, although inconclusive, for a positive effect of prolonged soaking periods on yield. Lastly, the study discusses the applicability of different disinfection techniques at varying production scales and for different mushroom species, focusing on African countries, where comparably small mushroom economies are growing rapidly, but often through the use of unsustainable pasteurization technology.

Keywords: mushroom cultivation, *Pleurotus ostreatus*, pasteurization, sterilization, sustainable production, water usage, energy usage

INTRODUCTION

Grey oyster mushrooms (*Pleurotus ostreatus)* are easier to produce than most other edible mushrooms and can be cultivated on almost any agricultural plant waste **(Stamets, 2000)**. Moreover, they are a rich source of proteins, vitamins, minerals and dietary fibre **(Alam et al., 2008; Mattila et al., 2001)** and a potent tool for nutrient cycling in agricultural systems **(Cunha Zied, Sánchez, Noble, & Pardo-Giménez, 2020; Grimm, Kuenz, & Rahmann, 2021)**. Given the increasingly restricted availability of cropland, mushrooms, which can be cultivated without light or soil, could play an important role for guaranteeing food security in future agricultural systems **(Rahmann, Grimm, Kuenz, & Hessel, 2020)**. However, the environmental impact in terms of energy and water usage during the pasteurization or sterilization of mushroom substrates is considerable **(Dorr, Koegler, Gabrielle, & Aubry, 2021; Kurtzman, 2010)**. This impact could be mitigated by implementing more efficient technologies.
Mushroom producers who have enough investment capital, often choose to buy an expensive large-scale autoclave to sterilize substrates. This allows for the cultivation of less competitive mushroom species than the oyster mushroom, as virtually no living microorganism remains in a substrate after autoclaving. Mushroom farmers with less capital have to choose between different forms of pasteurization, which do not eradicate the spores of green moulds, such as species from the genus of *Aspergillus* and *Penicillum*, and some other microbes as efficiently **(González, Zafra, Albert, & Rodríguez-Porrata, 2022; Swenson et al., 2018)** but usually reduce the microbe count to a sufficient degree for mushroom cultivation. Hot water pasteurization (HWP) and calcium hydroxide (also known as hydrated lime or slaked lime) pasteurization (HLP) are among the most common methods **(Stamets, 2000)**. A less common method is hot air pasteurization (HAP), which requires an oven, rather than an ordinary steel drum, and is therefore more expensive than HWP and HLP, while still being cheaper than autoclaving. Also, according to **Wei et al. (2020)** HAP at 85°C requires an estimated 2.75 times less energy than autoclaving (A) while producing the same mushroom yields. Since their study is limited to shiitake mushrooms on a birchwood-based substrate, which could have had a relatively low microbial load (no control treatments in the experiment to confirm that pasteurization was necessary), we chose to conduct further studies, using oyster mushrooms and straw-based substrates and comparing HAP not only to A but also to HWP and HLP. **Kurtzman (2010)** notes that the function of pasteurization and sterilization is not only germ-reduction but also the soaking of the substrate. Since many plants have a thick cuticula, it can take several days to thoroughly soak a straw-based substrate in cold water **(Kurtzman, 2010)**. While HWP fulfills the soaking-function very well, HAP does not. It is therefore possible that soaking the substrate for a long period before HAP would improve mushroom growth and yield and decrease pest occurrence. While it could be that microbes proliferate during soaking time, it could also be that this induces spores to germinate, which would make the microorganisms more susceptible to heat.
To investigate these issues, we carried out two experiments. First, we looked at the soaking effect and air temperature in hot air pasteurization. Secondly, we compared the different pasteurization methods and autoclaving. In both experiments we measured mycelial growth, pest occurrence, fresh yield and dry yield. Apart from looking at the success of cultivation, we also make estimates on the energy and water usage of the different germ reduction techniques and discuss their applicability in different settings.

MATERIALS AND METHODS

Substrates

All straws used in the experiment were from certified organic agriculture (European Union). Therefore, no fungicides or other chemical treatments that could potentially influence microbial growth and affect the experimental results were applied to the plants during their lifecycle. The maize, faba bean and wheat

were cultivated on the land of the Thuenen Institute of Organic Agriculture in northern Germany, while the soy was cultivated at the Gladbacher Hof of the University Giessen in central Germany. After harvesting, the straws were chopped, dried in an oven at 40°C, and subsequently stored.
In the first experiment, maize straw (variety Saludo) was utilized. In the second experiment, a mixture consisting of equal parts of maize straw, faba bean straw (Tiffany variety), wheat straw (Faustus variety), and soy straw (a mix of varieties: Merlin, GL Melanie, Marquise, Aurelina, ES Favor, RGT Sphinxa, ES Comandor, Amarok, and Arcadia varieties) was employed. This diverse mixture of straws aimed to create a substrate with a broader range of microbial communities.

Model mushroom species

The grey oyster mushroom, *Pleurotus ostreatus*, was used as a model species. Grain spawn (strain number: P10001, type of grain: wheat) was acquired from Mushrooms & Equipment Shop, Münster Germany, and then used to produce more grain spawn (using wheat grain). The spawn used in the experiments was self-produced G3 (third generation) spawn from the culture that had been originally acquired.

Experimental design

The first experiment, as shown in Table 1, examined the influence of air temperature during HAP and the effect of soaking period length on mushroom growth and yield. The second experiment (In the second experiment (**Chyba! Odkaz na záložku nie je platný.**) it was necessary to include two hot air treatments, in order to account for differences in substrate moisture between treatments where the substrate was submerged in water (HWP and HLP) and treatments where water was added in the right amount to the substrate (HAP and A). Sterilized water was added to the HAP-heavy treatment so that it matched the 80% moisture content of HWP and HLP, rather than the moisture of 75% of the other treatments.

Table 2) compared different germ reduction methods: hot air pasteurization (HAP), hot water pasteurization (HWP), calcium hydroxide —hydrated lime— pasteurization (HLP), and autoclaving (A) (see section 2.3. Pasteurization and Sterilization Methods).
For both experiments, a 25x50 cm PVC mushroom grow bag with a micropore filter (EgBert brand) was utilized. The bags were filled with 800 g of moist substrate (200 g dry matter and 600 g water), to which oyster mushroom spawn was added following pasteurization/sterilization (see section 2.2, Substrates). The control treatments in both experiments did not undergo pasteurization or sterilization, but the mushroom spawn was added simultaneously with the other treatments. This approach allowed for the assessment of whether successful substrate colonization by the mushroom was possible without pasteurization or sterilization. In the first experiment, water was added to the dry substrate of the various treatments either immediately before or four days prior to pasteurization (0 hours vs. 96 hours of soaking time). The replicates were then placed in an oven for three hours at temperatures of 75°C, 85°C, or 100°C, excluding the control treatment replicates. After the substrate had cooled to room temperature, 20 g of fresh oyster mushroom spawn (equivalent to 5 g dry matter) was added to each replicate. The bags were sealed and transferred to the designated growing room (see section 2.4, Cultivation Conditions).

Table 1 Design of experiment 1 with different treatments of air temperature and soaking time. Each treatment with six replicates (n = 48).

Treatment name	Air Temperature	Soaking time
1 (Ctrl A)	-	0 h
2 (Ctrl B)	-	96 h
3 (75A)	75°C	0 h
4 (85A)	85°C	0 h
5 (100A)	100°C	0 h
6 (75B)	75°C	96 h
7 (85B)	85°C	96 h
8 (100B)	100°C	96 h

In the second experiment (**Chyba! Odkaz na záložku nie je platný.**) it was necessary to include two hot air treatments, in order to account for differences in substrate moisture between treatments where the substrate was submerged in water (HWP and HLP) and treatments where water was added in the right amount to the substrate (HAP and A). Sterilized water was added to the HAP-heavy treatment so that it matched the 80% moisture content of HWP and HLP, rather than the moisture of 75% of the other treatments.

Table 2 Design of experiment 2 with different treatments of disinfection and substrate moisture. Each treatment with six replicates (n = 36).

Treatment name	Disinfection method	Substrate moisture
1 (Ctrl)	None	75%
2 (HAP-1)	Hot air pasteurization	75%
3 (HAP-2)	Hot air pasteurization	80%
4 (HWP)	Hot water pasteurization	80%
5 (HLP)	Hydrated lime ($Ca(OH)_2$) pasteurization	80%
6 (A)	Autoclaving	75%

Pasteurization and Sterilization Methods

Prior to pasteurization or sterilization, the moisture content of the substrate ingredients was determined. For the HAP+A treatments, the substrate was moistened to 75%, as recommended by **Stamets (2020)** for straw-base mushroom substrates, using tap water. The substrate was then filled into plastic mushroom bags and sealed with reusable zip-ties beneath the microfilter to prevent water from evaporating or entering during the pasteurization or autoclaving process. In the case of the HWP and HLP treatments, a different approach was followed since pasteurization inside plastic bags was not feasible. Instead, a self-made bag of gauze fabric was used, and the substrate was submerged in water. This allowed the substrate to be fully immersed without losing smaller particles of the chopped straw. The substrate was filled into mushroom grow bags only after the pasteurizing and drenching the substrate.
For the HWP treatment, a clean oil barrel was set up with a propane-gas fire underneath. The water temperature was monitored using a thermometer placed 20 cm beneath the surface. Once the water temperature reached 63°C, the substrate was submerged and kept inside the barrel for one and a half hours, with the temperature maintained between 63°C and 70°C.
In the HLP treatment, calcium hydroxide was added to the water at a rate of approximately 5 g per liter to achieve a pH of 9.5 before submerging the substrate. The substrate remained in the barrel for 8 hours. After pasteurization, the substrate of HLP and HWP was drenched overnight. To minimize spore entry during this period, the substrate was transferred to large plastic bags while still hot. To allow water to drip off, holes were punctured in the undersides of the bags, which were then hung from the ceiling using strings.
At this stage, samples were taken, and the bags were weighed. The samples were dried in an oven at 105°C overnight to determine the moisture content. The following day, the bags were re-weighed to calculate the water loss overnight. This information was used to calculate the precise amount of substrate required for each mushroom bag, ensuring that each bag contained exactly 200 g of dry matter, as in all other treatments. Additionally, this approach enabled the calculation of the amount of water needed for the HAP-2 treatment, which was adjusted to achieve a moisture content of 80% to match that of the HWP and HLP treatments.
By carefully monitoring and adjusting the moisture content of the substrate, the study aimed to ensure consistent conditions across the different germ reduction treatments.

Mushroom cultivation conditions

The mushroom cultivation period followed the parameters described in Stamets (2000) for the grey oyster mushroom, as outlined in Table 3. Once the substrate was sterilized or pasteurized and distributed into mushroom filter bags, mushroom spawn was added to the top of the substrate. The spawn was spread by shaking and twisting the bag between the hands for approximately ten seconds. In the first experiment, 20 grams (fresh weight) of spawn were added to each bag, while in the second experiment, only 10 grams of spawn were added. The cultivation took place in growing boxes equipped with automatic humidity control, within a temperature-controlled room. To ensure randomness, the replicates were rotated twice weekly. When the mushroom mycelium had fully colonized the substrates in all treatments except the control group, the bags were opened, and the climate settings were adjusted to induce primordia formation. Once the mushrooms were harvested, the climate settings were reverted to those used during the initial spawn run for a period of three days. Afterward, the settings were adjusted again to promote primordia formation for subsequent flushes of mushrooms. This cycle of adjusting climate settings for fruiting and returning to spawn run conditions was followed throughout the cultivation period.

Table 3 Cultivation parameters for *Pleurotus ostreatus* according to Stamets (2000)

Spawn run (colonization phase):	**Primordia formation:**	**Fruitbody development:**
Duration: 12 – 21 days	Duration: 3 – 5 days	Duration: 4 – 7 days
Temperature: 24°C	Temperature: 15°C	Temperature: 20°C
Relative humidity: 85 %	Relative humidity: 95 %	Relative humidity: 90%

After opening the bags, yellow traps were set up, to reduce the number of flies laying eggs into the substrate – a problem that tends to increase with time. We used yellow sticky traps to mitigate this problem. In the first experiment, several flushes were harvested. In the second experiment only the first flush was harvested, due to time constraints and because the first harvest most accurately reflects the success of germ reduction, as germs can easily enter the substrate once the bags have been opened.

Data collection

Grey oyster mushrooms were harvested at the stage of fully maturity (Figure 1), following **(Stamets, 2000)**.
Upon harvest, the fresh weight of the mushrooms was measured using a scale to determine their weight before any moisture loss occurred. Then the dry weight was determined, by drying for 24 hours in an oven at 105°C. From the yield data, the biological efficiency and the biomass conversion rate were calculated. The biological efficiency is a commonly used expression of yield in mushroom cultivation, which gives the amount of fresh mushroom harvested per dry substrate **(Stamets, 2000)**. According to the biological efficiency formula, a 100% biological efficiency is achieved when one pound of fresh mushroom is harvested from one pound of dry substrate. The biomass conversion rate is a similar measure, which gives the amount of dry yield as a percentage of the dry substrate. According to this formula, a 10 % conversion rate is achieved when 10 g of dry mushrooms are harvested from 100 g of dry substrate. The visually discernible occurrence of pests (e.g. bacteria, moulds and other fungi) in each mushroom bag was documented weekly during the cultivation process. Mycelial growth was regularly checked and estimated (from 0% of substrate colonized to 100%). The weight of the bags was measured once a week. Substrate pH was measured before and after sterilization/pasteurization, and after cultivation was completed.

Figure 1 Photo of fully mature grey oyster mushroom P. ostreatus, ready for being harvested

Table 4 Chemical composition of the straws used for substrate formulation in the experiments

	Straw type				
Parameter	Maize	Wheat	Faba bean	Soy	Mixture
Ash (% dm)	6.64	7.02	7.50	6.21	6.84
C (% dm)	47.07	47.12	47.12	47.18	47.12
N (% dm)	0.68	0.36	1.05	0.59	0.67
C/N ratio	69.2	130.9	44.9	80	70.3
K (% dm)	1.50	0.52	2.32	1,61	1.49
P (% dm)	0.40	0.22	0.14	0.07	0.21
ADF (% dm)	45.29	53.84	60.91	53.18	53.30
NDF (% dm)	74.82	75.74	71.24	66.65	72.11

Legend: The mixture was an equal-parts mix of the four different straws. All values are given as percentage of dry matter (% dm). C is carbon, N is nitrogen, K is potassium, P is phosphorous, ADF stands for acid detergent fibre (lignin and cellulose) and NDF stands for neutral detergent fibre (lignin, cellulose and hemicellulose).

Chemical analysis

The straws used as mushroom substrates in the experiments were chemically analysed in the laboratory at the Thünen Institute of Organic Farming (Table 4). Most of the analyses were carried out as described in the Commission Regulation No 152/2009, Annex III (EC, 2009) and method numbers are given below. The dry matter content was determined by oven-drying at 103°C (Annex III, A). Ash, crude fat and starch content were determined using methods M, H and L. Phosphorous content was determined photometrically (Annex III, P). The nitrogen and carbon-content were determined with the DUMAS-method (Naumann & Bassler, 2004).

Data analysis

For statistical analysis, Microsoft "Excel" and the freeware "R" were used. One-way analysis of variance (ANOVA) and the post-hoc Tukey's test were used to compare different treatments.

Estimation of energy and water efficiency

The water usage of the different pasteurization and sterilization techniques was determined by considering the percentage of water in the substrate (75%) and the water needed for transferring that heat and/or submerging the substrate. We measured that 18 kg of water were needed to submerge 1 kg of the dry substrate used in experiment 2. With a substrate that is more finely chopped, less water might be needed, but it could also lead to higher nutrient losses, which are a general disadvantage of HWP **(González et al., 2022)**. The amount of water needed to transfer heat via steam, during autoclaving depends on the energy content of saturated steam at 2 bar atmospheric pressure, which is 2202 kJ/kg **(Wei et al. 2020)**.
To estimate the energy usage per kg of substrate and kg of produced mushroom, we used thermodynamic equations and literature. The energy (Qt) that is needed to heat a given mass (m) from starting temperature (Ti) to final temperature (Tf) depends on the specific heat capacity (c) of the substances that are heated and can be calculated with the following formula: $Qt = c * m * (Tf - Ti)$. The specific heat capacity of water is ca. 4.19 kJ/kg. For the straw, we assumed a specific heat capacity of 1.4 kJ/kg, as did Wei et al (2020) for their ligno-cellulosic raw materials. The specific heat capacity of the substrate (75% water, 25% straw) was therefore assumed to be 3.49 kJ/kg and the specific heat capacity of 18 kg of water and 1 kg of straw was assumed to be 4.04 kJ/kg. For autoclaving, we assumed that 2706 kJ/kg are needed to turn water in to saturated steam (Wei et al., 2020). Assuming a starting temperature of 10°C, it takes 1823 kJ to heat 1 kg of the substrate to 121°C. Therefore 828 g of steam would be needed to transfer that energy. In the HLP method, 5 g of calcium hydroxide were added per kg of water. The amount needed per kg of substrate was therefore 90 g. The amount of energy needed to produce calcium hydroxide varies from 3000 – 9000 kJ/kg, depending on such factors as the quality of raw materials and the type of fuel and kiln used for production **(European Commission, 2013; Laveglia, Sambataro, Ukrainczyk, Belie, & Koenders, 2022)**. For example, parallel flow regenerative kilns generally use less energy than annular shaft kilns and larger ones are more efficient than small ones **(European Commission, 2013).**

RESULTS AND DISCUSSION

Effect of air temperature and soaking time (Experiment 1)

The duration of the experiment was 72 days. In this time, most of the pasteurized replicates produced two or three harvests and one replicate in treatment 100B produced four harvests, while replicates in non-pasteurized treatments (CtrlA and CtrlB) produced no, or one harvest. The pasteurized treatments took on average 24 to 25 days until the first harvest. Those bags, which produced mushrooms in the non-pasteurized treatments (5 of 6 in CtrlA and 3 of 6 in CtrlB) took on average 48 days (CtrlA) and 52 days (CtrlB) until the first harvest. Relevant harvest data are given in Table 5.

Table 5 Mean fresh yield (biological efficiency), dry yield (biomass conversion rate), water content and days until first harvest in the different treatments of experiment 1

Treatment	Biological Efficiency (%)	Biomass Conversion Rate (%)	Water content (%)	Days to first harvest
CtrlA	63.6 (+/- 19.9)	6.2 (+/- 3.6)	90.2 (+/- 0.8)	47.8 (+/- 4)
CtrlB	15.4 (+/- 9.1)	1.5 (+/- 1.7)	90.4 (+/- 1.2)	52.3 (+/- 9.3)
75A	98.5 (+/- 5.7)	9.3 (+/- 1.1)	90.5 (+/- 0.5)	25 (+/- 1.5)
75B	110.9 (+/- 4)	10.5 (+/- 1.3)	90.5 (+/- 0.8)	25.3 (+/- 1.5)
85A	114.6 (+/- 5.8)	10 (+/- 0.7)	91.3 (+/- 0.4)	24.7 (+/- 2)
85B	116 (+/- 8.5)	10.6 (+/- 1.5)	90.9 (+/- 0.3)	24.3 (+/- 2.1)
100A	111.3 (+/- 9)	10.1 (+/- 1.9)	91 (+/- 0.3)	24.7 (+/- 0.9)
100B	115.1 (+/- 12)	10.9 (+/- 2.3)	90.5 (+/- 0.9)	25.2 (+/- 3.5)

Legend: The standard deviation is given in brackets behind the means. Biological efficiency refers to the percentage of substrate dry matter converted to mushroom fresh matter. Biomass conversion rate refers to the percentage of substrate dry matter converted to mushroom dry matter.

Growth of green mould (*Aspergillus spec*.) was found in 5 out of 6 of the replicates of both CtrlA and CtrlB after two weeks and in all replicates of these treatments after three weeks. Fruit bodies of the snowy inkcap mushroom *Coprinopsis nivea* were observed in two replicates of CtrlA and four replicates of CtrlB after three weeks. This mushroom is very common on the fields of our research station, which explains its appearance in the control treatments, though it is remarkable, as it is usually associated with cow dung, which was not present in our substrates. No competitor microbes were found in any of the pasteurized treatments, except for a small occurrence of green mould on the replicate 100B1, which however did not spread or grow in size. The mycelial growth (spawn run) of the oyster mushroom was similar in all treatments, except CtrlA and CtrlB, where it was much slower. After two weeks, all replicates in all treatments were fully colonized, except for the non-pasteurized treatments, where none were fully colonized. Only one replicate of the non-pasteurized treatment (replicate CtrlA1) was fully colonized during the experiment.
The total dry yields that were obtained from the different treatments during the experiment are shown in Figure 2.

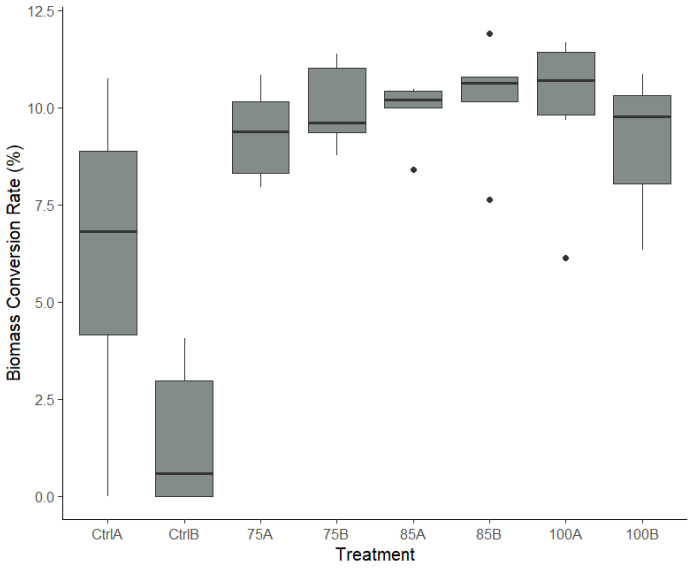

Figure 2 Dry yield in different treatments
Total dry yield, given as the biomass conversion rate (the percentage of substrate dry matter converted to mushroom dry matter), per different treatments of air temperature and soaking time (Experiment 1). N = 48 (8 treatments with six replicates each).

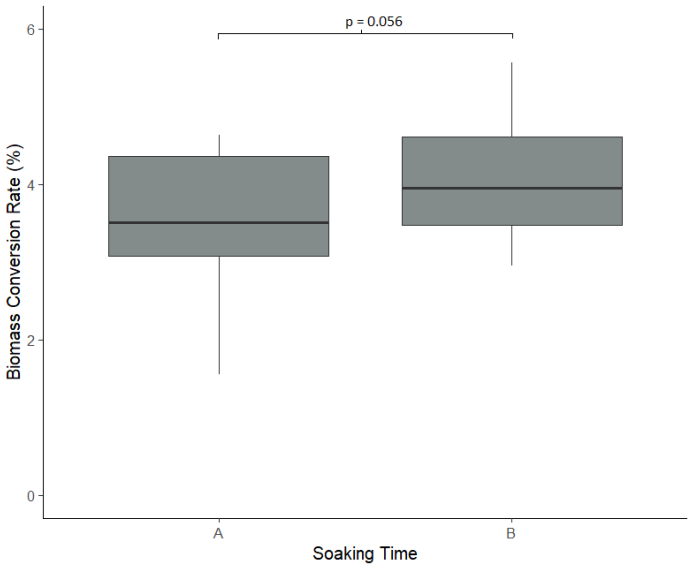

Figure 3 Effect of soaking on dry yield

First harvest dry yield, given as the biomass conversion rate rate (the percentage of substrate dry matter converted to mushroom dry matter), in treatments soaked directly before (A) or 96 hours before hot air pasteurization (B) in experiment 1. N = 36 (18 replicates in group A and 18 in group B).

The non-parametric, Kruskal Wallis test revealed a significant difference between treatments in terms of dry yield ($p = 0.001$). The post-hoc Dunn's test, revealed that this difference was only between the non-pasteurized and pasteurized treatments, with the former (CtrlA and CtrlB) producing significantly less mushrooms than the latter. Also, CtrlA produced significantly more mushrooms than CtrlB. When removing these two treatments from the data pool and only looking at the pasteurized treatments, it was possible to perform parametric tests, since the assumptions of normal distribution of data and variation were met. ANOVA revealed no significant difference in terms of total dry ($p = 0.7$) and fresh yield ($p = 0.54$) between the pasteurized treatments. Likewise, no statistical significance was found for the factors temperature ($p = 0.45$) and soaking ($p = 0.12$). When looking only at the first harvest, no difference between pasteurized treatments was found either but, as shown in Figure 3, a trend ($p = 0.056$) toward higher dry yields in treatments soaked for 96 hours than in treatments soaked directly before pasteurization could be detected.
The first experiment confirmed that hot air pasteurization is an efficient form of disinfecting mushroom substrates. While non-pasteurized replicates often failed to produce any mushrooms or took twice as long until the first harvest, the pasteurized replicates produced very good yields in a short time, with a biological efficiency of more than 110 % in most treatments and biomass conversion rates of more than 10 %. This compares well to the oyster mushroom yields reported by **González et al. (2022)** using various pasteurization techniques on straw- and wood-based substrates. In an experiment where maize straw was used, like in the one we conducted, a lower biological efficiency of only 97% was reported, after using the hot water bath method **(Fanadzo, Zireva, Dube, & Mashingaidze, 2010)**. This result agrees with **Stamets (2000)** that a "good grower" should operate in the range of 75 % -125 % range of biological efficiency.
We did not find statistically significant differences between the different temperatures, so that 75°C might be as good as 100°C, although when looking at Figure 2, there seems to a small trend towards higher yields at higher temperatures. In an experiment with larger sample sizes, statistically significant differences might be found, but this remains unclear.
Interesting results were found regarding the effect of soaking time. As was expected, in the control treatments (CtrlA and CtrlB), a longer soaking time led to smaller yields, because the microbiota in the substrate was able to grow before the mushroom spawn was added, which is a competitive disadvantage for the mushroom. But looking at the pasteurized treatments, the effect was reversed, with a trend towards higher first harvest yields in the treatments that where soaked for a longer time. Since this effect was just barely below statistical significance, more experiments, with larger sample sizes, should be conducted. Also, the difference between treatments with different soaking times became smaller when looking at total yields, rather than just the first harvest. This could be explained by the fact that the mushroom faces less microbial competition once it has colonized the entire substrate. The first harvest, which occurs shortly after full colonization, is thus more likely to be affected by microbial competition, than the second harvest, which occurs several weeks after the mushroom has "taken control" of the entire substrate. However, whether the observed beneficial effect of soaking on the first harvest is due to better moisture distribution, as **Kurtzman (2010)** describes, or due to a better elimination of microorganisms, remains unclear.

Effect of different techniques of pasteurization and sterilization (Experiment 2)

The duration of the experiment was 45 days. When it ended, only the autoclaved mushroom bags had produced two harvests, while all pasteurized treatments had produced just one harvest and the non-pasteurized control treatment had produced no harvest, because the oyster mushroom had failed to colonize the substrate. The occurrence of pests was higher in the control treatment, with green mould (*Apergillus spec*.) and other species, such as *Coprinopsis nivea* and slime moulds, occurring in all replicates. In the other treatments no pests were observed, except for the HLP treatment, where a slime mould was found in one replicate and green mould in another one, although both pest occurrences remained small and contained.
Relevant harvest data is depicted in Table 6.

Table 6 First harvest fresh yield (biological efficiency), dry yield (biomass conversion rate), water content and days until first harvest in the different treatments of experiment 2.

Treatment	Biological Efficiency (%)	Biomass Conversion Rate (%)	Mushroom Water content (%)	Days to first harvest
A	66.8 (+/- 4.5) a	6.9 (+/- 0.6) a	89.6 (+/- 0.9) b	23.3 (+/- 0.5) a
HAP1	45.4 (+/- 3.9) b	4.6 (+/- 0.7) b	89.9 (+/- 1.4) b	27.5 (+/-2.3) b
HAP2	43.9 (+/- 11.1) b	4.1 (+/- 1.1) b	90.6 (+/- 0.8) b	28.8 (+/-4.2) b

HLP	52 (+/- 11.4) b	4 (+/- 0.8) b	92.3 (+/- 0.3) a	28.2 (+/- 1.1) b
HWP	75.7 (+/- 4.3) a	5.2 (+/- 0.2) b	93.1 (+/-0.3) a	26.8 (+/-0.9) ab

Legend: The standard deviation is given in brackets behind the means. The letters a and b display significant differences between the treatments found with Tukey's test. Biological efficiency refers to the percentage of substrate dry matter converted to mushroom fresh matter. Biomass conversion rate refers to the percentage of substrate dry matter converted to mushroom dry matter.

The autoclaved replicates (treatment A) colonized the substrate faster and produced significantly more mushroom dry yield in the first harvest, in significantly less time than all other treatments except HWP ($p < 0.05$), as ANOVA and Tukey's test revealed. The average first harvest dry yield of the sterilized treatment (A) was 54% higher than the combined average of the pasteurized treatments. This difference is visible in Figure 4. No significant differences were detected in terms of dry yield or time to harvest between other treatments, though there was a trend towards higher dry yields in HWP than in HLP ($p = 0.09$).

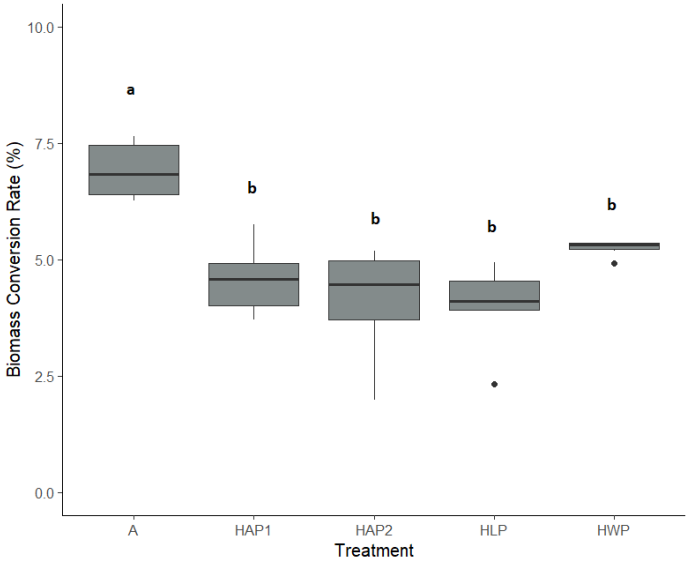

Figure 4: Effect of sterilization or pasteurization methods on dry yield
First harvest dry yield of experiment 2, given as the biomass conversion rate (the percentage of substrate dry matter converted to mushroom dry matter). The letters above the boxplots display significant differences found with Tukey's test. N = 30 (five treatments with six replicates each. Ctrl treatment not included, as it produced no yields).

The picture was different when looking at the fresh yields, where the biological efficiency of the HWP treatment was the same as of the A treatment and significantly higher than the other treatments. This can be seen in Figure 5. Also, as can be seen in table 6, the mushrooms in HWP and HLP contained significantly more water than those of the other treatments.

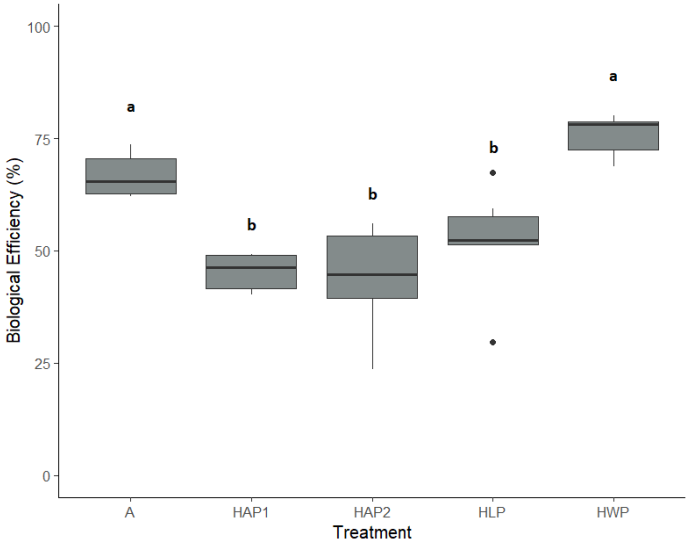

Figure 5: Effect of sterilization or pasteurization methods on fresh yield
First harvest fresh yield of experiment 2, given as the biological efficiency (the percentage of substrate dry matter converted to mushroom fresh matter). The letters above the boxplots display significant differences found with Tukey's test. N = 30 (five treatments with six replicates each. Ctrl treatment not included, as it produced no yields).

The second experiment revealed significant differences in mycelial growth and in yields between autoclaving and different pasteurization techniques. The autoclaved treatment produced significantly higher dry yields than the other treatments, as reflected by the biomass conversion rate. The results therefore contradict the findings of **Wei et al. (2020)**, where hot air pasteurization yielded the same results as autoclaving. This could be because we used a substrate with a higher microbial load than they did – we deliberately mixed four different types of straw, to get a broad spectrum of microorganism, while they used sawdust from birchwood. Another contributing factor could have been that we used a very low spawn rate. If a higher spawn rate had been used, the difference might have been less dramatic. A less likely explanation for the different findings is that we used a different mushroom species, since the oyster mushroom is more competitive and resilient than shiitake, which **Wei et al. (2020)** cultivated. In our experiment, autoclaved replicates also manifested significantly faster mycelial growth and needed less time until the first and second harvest than pasteurized replicates. Only the HWP treatment was comparable to autoclaving, in terms of biological efficiency. This was because the mushroom water content of HWP was significantly higher (93.1 % vs. 89.6%). HLP also had a higher water content than the other treatments. This suggests that methods where the substrate is submerged lead to higher mushroom water content. Interestingly, the HAP2 treatment, where the substrate water content was adjusted to that of HWP and HLP, had a significantly lower water content than those two treatments. This suggests, that the water, which we added after pasteurization, did not soak into the substrate and was less available to the mushroom. While the biological efficiency might be economically interesting for farmers, since mushroom producers mostly sell fresh rather than dry mushrooms, it plays less of a role in terms of food security and nutrition. For this reason, researchers should not only focus on biological efficiency, but also the biomass conversion rate. In terms of this measure, the experiment clearly showed that autoclaving was better than pasteurization, while the three different pasteurization techniques that we investigated were equally good. This also counters the hypothesis that pasteurization could have an advantage over sterilization by letting "beneficial" microbes survive, which is sometimes made **(González et al., 2022; Kurtzman, 2010)**. While we are aware of the existence of beneficial bacteria in button mushroom production **(Cochet, Gillman, & Lebeault, 1992)**, we have not seen convincing evidence for the existence of beneficial bacteria in oyster mushroom cultivation.

Energy and water efficiency of different methods

The amount of energy and water needed for the different pasteurization and sterilization methods are presented in Table 7. The energy and water usage per kg of produced mushrooms takes into account the mushrooms produced in the different treatments in experiment 2, where only the first harvest was taken into account. HLP uses the least energy of all methods, even though the range of energy use is quite large, due to differences in the production process of calcium hydroxide (European Commission, 2013). HAP uses the second least energy, even when using the most energy intensive treatment. Autoclaving is slightly less energy intensive than HWP, which is the most energy intensive treatment. In terms of water use, HWP and HLP use the most water. HAP has the lowest water footprint of all treatments.

Table 7 Estimations of energy and water efficiency of different Pasteurization and Sterilization techniques used in the experiments

Method	**Energy usage per kg of substrate (dm)**	**Energy per kg of mushroom (dm)**	**Water usage per kg substrate (dm)**
Hot Air Pasteurization			
100°C	**1478 kJ**	**32220 kJ**	**3 kg**
85°C	**1232 kJ**	**26858 kJ**	**3 kg**
75°C	**1068 kJ**	**23282 kJ**	**3 kg**
Autoclaving	**4050 kJ**	**58523 kJ**	**4 kg**
Hot Water Pasteurization	**4148 kJ**	**78812 kJ**	**18 kg**
Hydrated Lime ($Ca(OH)_2$) Pasteurization	**270 - 810 kJ**	**6750 – 20250 kJ**	**18 kg**

In terms of environmental impact (energy and water usage), those pasteurization and sterilization methods which produced the highest yields in our experiments are not the most sustainable ones. Especially hot water pasteurization has a very high energy and water usage. Though the water usage could be reduced by using a substrate that takes up less volume, e.g. by chopping it more finely, this could also lead to increased leaching of nutrients from the substrate. Autoclaving needs only

marginally more water than hot air pasteurization and much less than hot water or hydrated lime pasteurization. But since autoclaves are very expensive, they are not a solution for many farmers, especially in developing nations. Hot air pasteurization has, on balance, a better water and energy efficiency than autoclave sterilization (about 75 % less energy) or hot water pasteurization (about 85 % less water). When performed at an air temperature of 75° C, which was found to be sufficient for successful mushroom cultivation, as little as 1068 kJ was needed to pasteurize one kilo of dry substrate (e.g. maize straw). While hydrated lime pasteurization could use as little as 270 kJ per kilo of dry substrate, it is uses as much water as hot water pasteurization and could have the same problems of nutrient leaking. In addition, water that is mixed with hydrated lime ($Ca(OH)_2$) should not be released into the environment in large amount, especially in urban areas, as it could lead to water pollution **(Laveglia et al., 2022)**.

Oyster mushroom cultivation has tremendous potential for sustainable food production. Especially densely populated regions of the world, which have too little available farm land to achieve self-sufficiency, could profit greatly from increasing mushroom production **(Grimm et al., 2021)**. This is especially the case in many African countries, where the mushroom economy is still very small compared to Europe, America and above all Asia (Royse, Baars, & Tan, 2017). But if unsustainable pasteurization methods are used, a scale-up of production could also have very negative environmental consequences. Since oyster mushroom production in Africa is often carried out by small-scale farmers **(Atikpo et al., 2008; Fanadzo et al., 2010)**, simple methods such as hot water pasteurization or similar scalding techniques are the most common. During a trip to Uganda, we interviewed several women in and around Kampala, who produced oyster mushrooms. Four out of five of them used a variety of the hot water method with wood as a fuel source. Only one of them performed hot air pasteurization, utilizing the same oven in which she prepares meals for her family. While the oven is also heated with firewood, it is built to use the heat more efficiently.

While countries such as Uganda could greatly profit from an upscaling of mushroom production, since protein-rich food is much needed and agricultural residues are underutilised, it would be unsustainable to do so without providing the mushroom farmers with the means for water- and energy-efficient pasteurization methods. The sustainable development goals by the United Nations **(UN DESA, 2023)** provide a useful framework for what to focus on. While the goals number 1 (no poverty) and 2 (no hunger) would be positively impacted by increased mushroom production, one needs to be more careful to be in full concordance with the goals 6 (clean water and sanitation), 12 (responsible production and consumption), 13 (climate action) and 15 (life on land). Choosing which pasteurization or sterilization techniques to use for mushroom production touches on all of these different areas. In our view, to maximize sustainability, electrical devises should be used, so that solar energy could be used for substrate disinfection, whether by hot air pasteurization (which would be most sustainable) or autoclaving (which would produce the highest yields). Autoclaving would require more investment capital, but would also enable the cultivation of less competitive mushrooms than the grey oyster mushroom. For oyster mushroom cultivation however, hot air pasteurization would probably be the best choice.

CONCLUSION

Hot air pasteurization is a sustainable method of substrate pasteurization for oyster mushroom production. It uses less water and energy than most other methods of substrate pasteurization while producing the same amount of dry yield. Soaking the substrate for several days before hot air pasteurization could increase yields. Sterilization by means of autoclaving, while requiring about four times as much energy as hot air pasteurization, can lead to more than 50 % better dry yields in the first harvest and to faster mycelial growth.

REFERENCES

Alam, N., Amin, R., Khan, A., Ara, I., Shim, M. J., Lee, M. W., & Lee, T. S. (2008). Nutritional Analysis of Cultivated Mushrooms in Bangladesh -Pleurotus ostreatus,Pleurotus sajor-caju,Pleurotus floridaandCalocybe indica. Mycobiology, 36(4), 228. https://doi.org/10.4489/myco.2008.36.4.228

Atikpo, M., Onokpise, O., Abzinge, M., Louime, C., Dzomeku, M., Boateng, L., & Awumbilla, B. (2008). Sustainable mushroom production in Africa: A case study in Ghana. African Journal of Biotechnology. (7), 249–253.

Cochet, N., Gillman, A., & Lebeault, J. -M. (1992). Some biological characteristics of the casing soil and their effect during Agaricus bisporus fructification. Acta Biotechnologica, 12(5), 411–419. Portico. https://doi.org/10.1002/abio.370120510

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

Dorr, E., Koegler, M., Gabrielle, B., & Aubry, C. (2021). Life cycle assessment of a circular, urban mushroom farm. Journal of Cleaner Production, 288, 125668. https://doi.org/10.1016/j.jclepro.2020.125668

European Commission (2009). Commission Regulation No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Off. J. Eur. Union. (54), 1–169. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32009R0152

European Commission (2013). Best Available Techniques (BAT) Reference Document for the Production of Cement, Lime and Magnesium Oxide. Retrieved from https://eippcb.jrc.ec.europa.eu/sites/default/files/2019-11/CLM_Published_def_0.pdf

European Union. Regulation 2018/848 of the European Parliament and of the Council of 30 May 2018 on Organic Production and Labelling of Organic Products and Repealing Council Regulation (EC). (EU Regulation 834/2007). https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32018R0848

Fanadzo, M., Zireva, D. T., Dube, E., & Mashingaidze, A. B. (2010). Evaluation of various substrates and supplements for biological efficiency of Pleurotus sajor-caju and Pleurotus ostreatus. African Journal of Biotechnology, 9(19), 2756–2761.

González, A. A. M., Zafra, L. M. C., Albert, B., & Rodríguez-Porrata, B. (2022). Pasteurization of agricultural substrates for edible mushroom production. Journal of Microbiology, Biotechnology and Food Sciences, 12(1), 1 - 7. https://doi.org/10.55251/jmbfs.5729

Grimm, D., Kuenz, A., & Rahmann, G. (2021). Integration of mushroom production into circular food chains. Organic Agriculture, 11(2), 309–317. https://doi.org/10.1007/s13165-020-00318-y

Kurtzman, R. H., JR. (2010). Pasteurization of mushroom substrate and other solids. African Journal of Environmental Science and Technology. (4), 936–941.

Laveglia, A., Sambataro, L., Ukrainczyk, N., Belie, N. de, & Koenders, E. (2022). Hydrated lime life-cycle assessment: Current and future scenarios in four EU countries. Journal of Cleaner Production, 369, 133224. https://doi.org/10.1016/j.jclepro.2022.133224

Mattila, P., Könkö, K., Eurola, M., Pihlava, J.-M., Astola, J., Vahteristo, L., Hietaniemi, V., Kumpulainen, J., Valtonen, M., & Piironen, V. (2001). Contents of Vitamins, Mineral Elements, and Some Phenolic Compounds in Cultivated Mushrooms. Journal of Agricultural and Food Chemistry, 49(5), 2343–2348. https://doi.org/10.1021/jf001525d

Naumann, K., & Bassler, R. (Eds.) (2004). Methodenbuch, Band 3, Bestimmung von Rohproteinen Mittels Dumas-Verbrennungsmethode. Bonn, Germany: VDLUFA Verlag.

UN DESA (2023). The Sustainable Development Goals Report 2023: Special Edition - July 2023. New York, USA: UN DESA. © UN DESA. https://unstats.un.org/sdgs/report/2023/

Rahmann, G., Grimm, D., Kuenz, A., & Hessel, E. (2020). Combining land-based organic and landless food production: a concept for a circular and sustainable food chain for Africa in 2100. Organic Agriculture, 10(1), 9–21. https://doi.org/10.1007/s13165-019-00247-5

Royse, D. J., Baars, J., & Tan, Q. (2017). Current Overview of Mushroom Production in the World. Edible and Medicinal Mushrooms: Technology and Applications, 5–13. https://doi.org/10.1002/9781119149446.ch2

Stamets, P. (2000). Growing gourmet and medicinal mushrooms (3rd ed.). Crown Publishing Group, New York.

Swenson, V. A., Stacy, A. D., Gaylor, M. O., Ushijima, B., Philmus, B., Cozy, L. M., Videau, N. M., Videau, P. (2018). Assessment and verification of commercially available pressure cookers for laboratory sterilization. PloS One, 13(12), e0208769. https://doi.org/10.1371/journal.pone.0208769

Wei, M., Xiong, S., Chen, F., Geladi, P., Eilertsen, L., Myronycheva, O., Lestander, T. A., Thyrel, M. (2020). Energy smart hot-air pasteurisation as effective as energy intense autoclaving for fungal preprocessing of lignocellulose feedstock for bioethanol fuel production. Renewable Energy, 155, 237–247. https://doi.org/10.1016/j.renene.2020.03.154