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Optimal Culture Conditions for the Enhanced Mycelial Growth and Cultivation of Shiitake Mushroom (*Lentinula edodes*)

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Abstract

Lentinula edodes (Berk.) Pegler, commonly known as shiitake mushroom, is one of the most popular edible mushrooms with valuable medicinal properties. Despite its global popularity, the cultivation of shiitake in Vietnam has been limited due to a lack of research on this macrofungus. This study aimed to optimize the culture conditions for the mycelial growth and fruiting body development of two shiitake strains, J1 and J2. The culture conditions were optimized by one-factor-at-a-time method. A temperature of 25°C was found to be optimal for the growth of both strains, while glucose was identified as the most effective carbon source. Yeast extract and ammonium chloride (NH_4Cl) were found to be the optimal nitrogen sources for favorable mycelial growth of strains J1 and J2, respectively. The most suitable spawning material for upscaling the mycelium was a mixture of 74% grain rice, 25% sawdust, and 1% $CaCO_3$. Additionally, the cultivation of strain J2 on a mixture of 69% corncobs, 20% sawdust, 10% wheat bran, and 1% $CaCO_3$ led to a higher biological yield (36.5%) than that of strain J1 (23.5%). These findings provide useful information for improving the cultivation technology of shiitake and expanding its cultivation in Vietnam.

Keywords

Shiitake, *Lentinula edodes*, cultivation, mushroom

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Introduction

Shiitake mushroom (*Lentinula edodes*), belonging to the order *Agaricales*, is a well-known edible and medicinal mushroom. This macrofungus is rich in nutrients and health-promoting substances, such as vitamins B, C, and D, phosphorus, zinc, iron, magnesium, manganese, alkaloids, phenols, and diterpenoids (Fukushima-Sakuno, 2020). Therefore, it has attracted the attention of researchers

for developing dietary supplements and medicines (Fukushima-Sakuno, 2020). Shiitake is used for medicinal purposes to treat diseases involving depressed immune function, environmental allergies, fungal infections, cancer, and heart disease (Bisen *et al.*, 2010; Zhang *et al.*, 2019a). Owing to its ability to degrade lignocellulose, shiitake can be used to exploit agricultural straw resources (Chen *et al.*, 2016). Additionally, shiitake can potentially be used for soil remediation (Ribas *et al.*, 2009).

Shiitake naturally thrives in temperate climates and grows on dead trees by degrading cellulose, hemicellulose, and lignin (Chen *et al.*, 2016). Although shiitake requires a longer incubation period than most mushrooms, including time for colonization, lumping, browning, and pinning (Alberti *et al.*, 2022), it can be grown on several substrates, such as sawdust (Ashrafuzzaman *et al*., 2009), corncobs (Yu *et al.*, 2022), and sunflower seed hulls (Curvetto *et al.*, 2002). For centuries, shiitake has been cultivated on artificial substrates or logs under conditions similar to its natural habitat for commercial purposes in Japan, Korea, and China (Koo *et al.*, 2016; Kwon *et al.*, 2018). By reducing the toxic effects of oxalate and facilitating the acidification of the sawdust medium, gypsum ($CaSO_4.2H_2O$) has been reported to improve shiitake growth and yield (Li *et al.*, 2022). The optimal temperature for mycelium growth of shiitake was found to be 25°C (Kumar *et al.*, 2019; Ishikawa *et al.*, 2001). Shiitake has also been shown to exhibit the best growth on a medium supplemented with glucose as a carbon source (Bisko *et al.*, 2020) and asparagine as a nitrogen source (Krupodorova *et al*., 2019).

Cultivation technology is critical in determining mushroom yield, the cultivation period, and contamination risk. Improved mushroom cultivation can increase the income of farmers by two- to three-folds (Thakur, 2020). The identification of the optimal culture conditions, such as temperature, cultural media, and substrate for mycelial growth and fruiting body formation, represents a pivotal initial stage in the development of mushroom cultivation technology (Nguyen *et al.*, 2021). Nonetheless, a limited number of studies have been conducted to optimize shiitake cultivation conditions in Vietnam, explaining why this macrofungus has not been widely cultivated. The suitable mother spawn medium for the mycelial growth of shiitake has been identified as 9% boiled grain, 90% sawdust, and 1% $CaCO_3$ (Nguyen *et al.*, 2020). Shiitake can form fruiting bodies when cultivated on a formula composed of 89% corncobs, 10% wheat bran, and 1% $CaCO_3$ (Nguyen *et al.*, 2020). Taken together, this study aimed to identify the optimal culture conditions for growing two shiitake strains, J1 and J2.

Materials and Methods

Mushroom strains

Shiitake strains J1 and J2 were obtained from the Research Institute of Edible and Medicinal Mushrooms, Vietnam National University of Agriculture. The two strains were preserved and maintained on potato dextrose agar (PDA) slants at 10°C and 25°C, respectively, for further use.

Effects of temperature, carbon source, and nitrogen source

The optimal culture conditions for the mycelial growth of the two strains were identified by the agar plate technique using the one-factor-at-a-time method. The experiment was conducted in triplicate. Mycelial discs 1 cm in diameter were cut and placed in the center of new agar plates for subculture. The strains were incubated under dark conditions. After six days, each colony diameter was measured.

Strains J1 and J2 were cultured on PGA medium (200g of potato, 20g of glucose, and 15g of agar/1 liter of medium) and incubated at four different temperatures: 15°C, 20°C, 25°C, and 30°C. The potato infusion was prepared as described previously (Nguyen *et al.*, 2021). To investigate the effect of carbon source on the mycelial growth, glucose, fructose, sucrose, maltose, lactose, or xylose were added to PA (200g of potato and 15g of agar/1 liter of medium) at a concentration of 20 g/L. Based on the results obtained, glucose was identified as the optimal carbon source and chosen to determine

the optimal nitrogen source. Strains J1 and J2 were cultured on PGA media supplemented with a nitrogen source at a concentration of 2 g/L. Six different nitrogen sources [i.e., yeast extract, peptone, casein, ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), and ammonium sulfate $(NH_4)_2SO_4$] were screened to identify the best nitrogen source for mycelial growth.

Effect of spawning material for upscaling of the mycelium

The ingredients for the different spawning material combinations used to upscale the mycelium were as follows: treatment 1 [99% (w/w) rice grain + 1% (w/w) $CaCO_3$], treatment 2 [74% (w/w) rice grain + 25% (w/w) sawdust + 1% (w/w) $CaCO_3$], treatment 3 [49% (w/w) rice grain + 50% (w/w) sawdust + 1% (w/w) $CaCO_3$], and treatment 4 [24% (w/w) rice grain + 75% (w/w) sawdust + 1% (w/w) $CaCO_3$]. The bottles were incubated at a temperature of 25°C.

Substrate preparation and cultivation

Strains J1 and J2 were cultured on a mixture of 69% (w/w) corncobs, 20% (w/w) sawdust, 10% (w/w) wheat bran, and 1% (w/w) $CaCO_3$. To prepare the substrate, a mixture of corncobs and sawdust was soaked in a 0.4% lime solution and then fermented for seven days (Nguyen *et al*., 2019). Subsequently, the resulting substrate was put into polyethylene bags and autoclaved at 121°C for 90 minutes. The culture bags were incubated at 25°C and a humidity of 60%. After the mycelial growth completely covered the bags, the culture bags were transferred to a growing room at a temperature of 15-20°C and a humidity of 85% to induce fruiting body formation.

Data collection and statistical analysis

The colony diameter and density of the shiitake were monitored to evaluate mycelial growth. The morphological characteristics of the mycelia, namely texture (cottony or floccose), density (high, moderate, or low), and color, were evaluated through visual observations. The mycelial growth rate was determined using the following formula:

$$V\ (mm/day) = D/T$$

where V represents the growth rate of the mycelium, and D and T represent the diameter growth (mm) and the incubation time (days), respectively.

The formula for calculating biological efficiency (BE) was as follows:

$$BE\ (\%) = (Fw/Dw).100$$

where Fw is the total fresh weight (grams) of mushroom yield, and Dw is the substrate dry weight (grams).

Data were analyzed and visualized using GraphPad Prism (version 9.0, GraphPad Software Inc., San Diego, CA). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison test.

Results and Discussion

The effect of temperature on the mycelial growth of shiitake

Both strains were able to grow at all the tested temperatures. The mycelial growth of the strains tended to increase with an increase in the temperature from 15 to 25 °C (**Figure 1**). Strains J1 and J2 showed the highest growth rates at an incubation temperature of 25°C with rates of 1.57 mm/day and 2.88 mm/day, respectively. Low mycelial growth was found when the strains were incubated at 30°C (**Figures 1 and 2**). The mycelial density of the two strains appeared high at 25°C but low at 30°C.

Temperature is a critical factor that influences the growth of fungal mycelium (Nguyen *et al*., 2019). The growth of mushrooms is limited to a specific range of temperatures due to the direct effect of temperature on various metabolic processes, such as the assimilation and translocation of sugars and nitrogen, respiration, and biosynthesis (Krupodorova *et al.*, 2019; Dos Santos *et al.*, 2022). The optimal temperature for mycelial growth is species-specific. For example, mycelia of *Cantharellus cibarius* (Deshaware *et al.*, 2021), *Ganoderma sinense* (Nguyen *et al.*, 2023), *G. lucidum* (Nguyen *et al.*, 2019), and *Volvariella volvacea* (Zervakis *et al.*, 2001) grew fastest at temperatures of 22.5, 25-30, 30, and 35°C, respectively. In addition, the

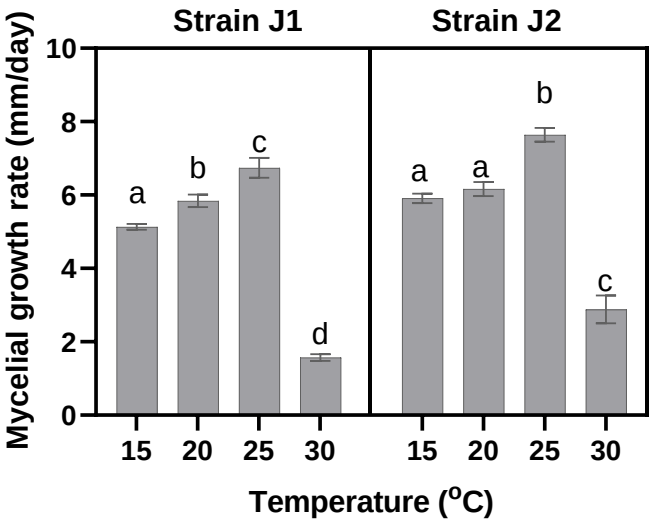

Figure 1. Effect of temperature on the growth rate of shiitake mycelium. Different letters represent significant differences (P <0.05).

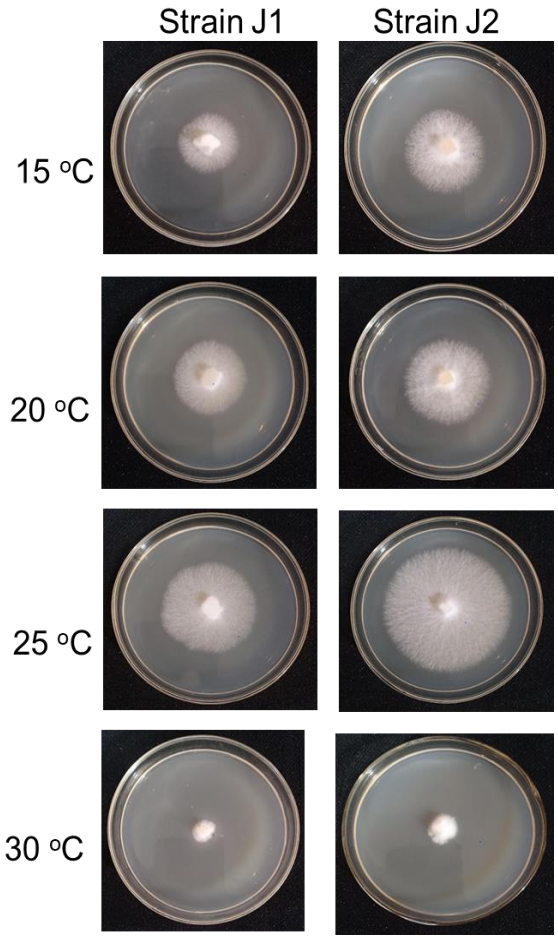

Figure 2. Effect of temperature on the mycelial density of shiitake after three days of incubation.

optimal temperature for cultivating different fungal strains within the same genus can differ (Dos Santos *et al.*, 2022). Considering the obtained mycelial density and average growth

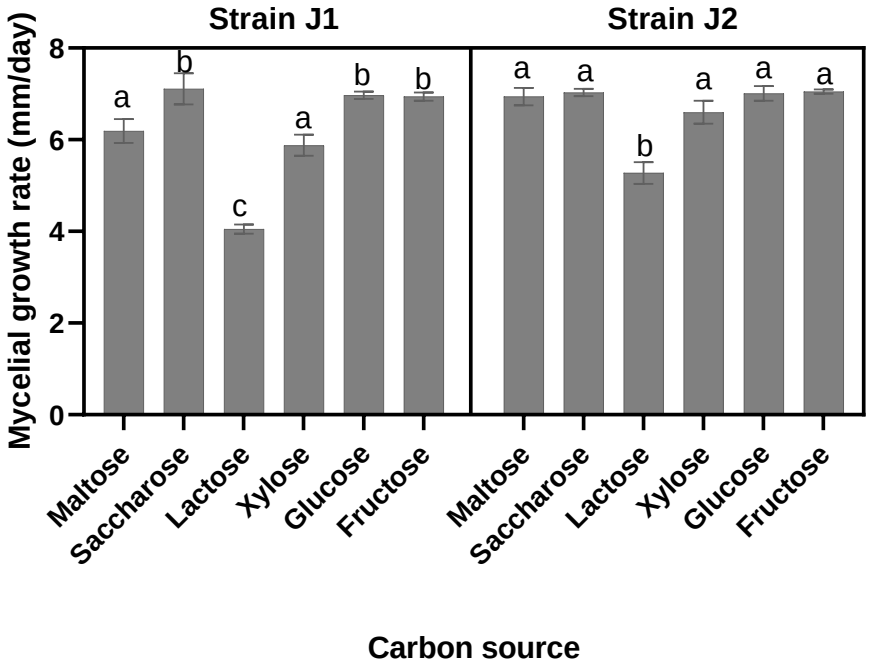

Figure 3. Effect of different carbon sources on the growth rate of shiitake mycelium. Different letters represent significant differences (P <0.05).

rate results, the optimal temperature for mycelium growth of strains J1 and J2 was determined to be 25°C, which aligns with previous studies conducted by Kumar *et al.* (2019) and Ishikawa *et al.* (2001). The growth of strains J1 and J2 was slow at 30°C, possibly due to the denaturation of essential enzymes responsible for catalyzing metabolic processes (Fetcher *et al.,* 2019) (**Figures 1 and 2**).

The influence of different carbon sources on the mycelial growth of shiitake mushroom

The carbon source significantly affected the mycelial growth of strains J1 and J2 (**Figure 3**). Saccharose was found to be the most favorable for the growth of strain J1, resulting in a growth rate of 7.11 mm/day. Fructose was identified as the best carbon source for strain J2, with a growth rate of 7.05 mm/day. Conversely, lactose was the least favorable carbon source for both strains, exhibiting the slowest growth rates. The mycelial density of shiitake varied depending on the strain and the carbon source used. For example, strain J1 grew rapidly with a high mycelial density on a saccharose-based medium but had comparatively lower densities on media supplied with glucose, fructose, or maltose as carbon sources. In contrast, strain J2 exhibited a high mycelial density on a glucose medium, but its densities were comparatively lower on media supplemented with fructose, saccharose, maltose, or xylose as carbon sources. Considering the average mycelial growth rate and density results, glucose was identified as the most suitable carbon source for the mycelial growth of strains J1 and J2The role of carbon sources in constructing essential cell components and providing energy for metabolic processes makes it crucial in the growth of mushrooms (Nguyen *et al.*, 2019). Accordingly, the carbon source used to culture mycelial mushrooms significantly affects their chemical, sensorial, and functional characteristics (Krupodorova *et al.*, 2021). To achieve maximum mycelial growth rates in fungi, the media must contain optimal carbon sources. Monosaccharides, oligosaccharides, and polysaccharides, such as glucose, saccharose, galactose, starch, and cellulose, are considered suitable carbon sources for fungal mycelial growth (Krupodorova *et al.*, 2021). The impact of the carbon source on the mycelial growth rate of mushrooms varies

greatly depending on the species, growth conditions, and the medium used (Itoo & Reshi, 2014). For instance, the most beneficial carbon sources for the radial growth of *Trametes versicolor* (Nguyen *et al.*, 2021), *Hericium erinaceus* (Gonkhom *et al.*, 2022), and *Laccaria laccata* (Itoo & Reshi, 2014) were fructose, molasses, and trehalose, respectively. Bisko *et al.* (2020) reported that shiitake strain IBK 2541 grew best on a medium supplemented with glucose. This finding is in line with our study, which identified glucose as the most effective carbon source for enhancing the mycelial growth of strains J1 and J2. Glucose is the most suitable carbon source for mycelial growth in most basidiomycetes because it is a fundamental building block of various saccharides (Krupodorova *et al.*, 2021).

Mycelial growth of shiitake on different nitrogen-supplemented media

Given that the nitrogen source is a crucial element for the mycelial growth of fungi, we investigated the effect of six nitrogen sources on the mycelial growth of strains J1 and J2. The highest mycelial growth rates of strains J1 and J2 were recorded for NH_4Cl, exhibiting average growth rates of 6.43 mm/day and 6.72 mm/day, respectively (**Figure 4**). Strain J1 also showed robust growth on a medium enriched with NH_4NO_3, with an average growth rate of 6.32 mm/day, while the mycelial growth rate of strain J2 was lower on this medium (5.67 mm/day). There were significant differences in mycelial density among the various media tested. For instance, the mycelial densities of strain J1 grown on NH_4Cl, NH_4NO_3, or casein-supplemented media were relatively low, whereas significantly higher mycelial densities were observed on media containing malt extract or $(NH_4)_2SO_4$. The mycelial densities of strain J2 were highest on malt extract, peptone, and NH_4Cl-supplemented media but lower on casein, NH_4NO_3, and $(NH_4)_2SO_4$-supplemented media. Collectively, malt extract and NH_4Cl were the most suitable nitrogen sources for the mycelial growth of strains J1 and J2, respectively. The growth culture medium must contain a nitrogen source, a vital nutrient for producing nitrogen-containing compounds and chitin cell wall components (Miles & Chang, 1997). Both organic and inorganic sources of nitrogen can be used for mycelial growth (Miles & Chang, 1997). This study employed organic and inorganic nitrogen sources to assess their impact on mycelium growth. Most mushrooms prefer to

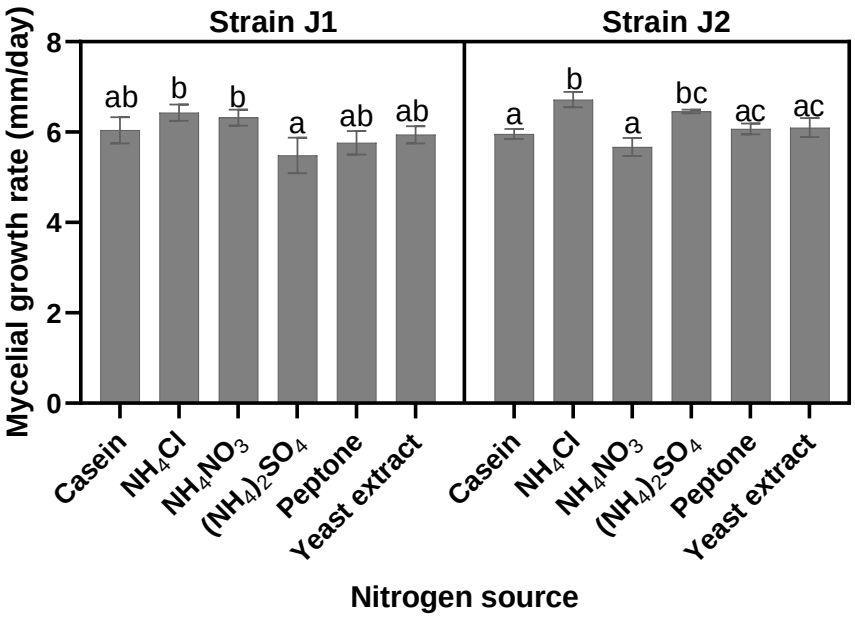

Figure 4. Effect of different nitrogen sources on the growth rate of shiitake mycelium. Different letters represent significant differences (P <0.05).

utilize complex organic nitrogen sources rather than inorganic nitrogen sources (Nguyen *et al.*, 2021). Yeast extract has been considered an optimal nitrogen source for the growth of *G. sinense* (Nguyen *et al.*, 2023). The two strains in this study grew well on media containing both organic and inorganic nitrogen, which was in line with the study by Krupodorova *et al.* (2019). Notably, the optimal nitrogen source for the growth of shiitake depends on each strain (Krupodorova *et al.*, 2019). Consistently, we observed that strains J1 and J2 were found to have different optimal nitrogen sources for mycelium growth.

Mycelial growth of shiitake mushrooms on different spawning material

It is crucial to explore new spawning materials to enhance mushroom cultivation efficiency. In Vietnam, rice, corn, wheat, sawdust, and barley are commonly used as spawning materials for upscaling mycelium (Nguyen *et al.*, 2021). Therefore, we utilized grain rice as the primary component of the culture medium to grow the mother spawn. Both strains started colonizing the spawn substrates three days after inoculation. Strain J1 exhibited the highest mycelial growth rate in treatment 2 (2.97 mm/day), followed by treatment 3 (2.27 mm/day). In contrast, strain J2 displayed robust growth in all the treatments, with no statistically significant differences in the mycelial growth rate observed among the tested spawning materials (**Figure 5**).

The mycelial density and morphology of the shiitake were significantly affected by the spawning material (**Figure 6**). Strain J1 exhibited weak growth, uneven morphology, and a low mycelial density in treatment 4. Conversely, when grown on the treatment 2 spawning materials, the strain showed vigorous, uniform growth and a very high mycelial density. Strain J2 exhibited vigorous, uniform growth with high mycelial density in treatments 2 and 3. In contrast, in treatment 4, strain J2 showed poor and uneven growth with a low mycelial density. Based on the mycelial growth rate and density results, the treatment 2 spawning materials were identified as the most suitable for scaling up the mycelium of strains J1 and J2.

Spawn is crucial for the cultivation of mushrooms, determining the biological efficiency. Using grains as a spawning material can lead to higher production costs (Zhang *et al.*, 2019b). To address this issue, we employed a combination of sawdust and rice grains at different ratios to decrease costs and minimize contamination rates (Nguyen *et al.*, 2021). Our results demonstrated that mixtures of rice grains and sawdust significantly increased the mycelial growth rate of both strains, especially in treatment 2 (74% rice grain + 25% sawdust + 1% CaCO3) (**Figure 5**). These findings are consistent with previous studies by Nguyen *et al.* (2021, 2023).

Cultivation of strains J1 and J2

The cultivation technology of shiitake mushrooms has evolved from log-based to bag-based production, resulting in a significant reduction of the cropping period from 3.0 to 1.5 months (Thakur, 2020). In Vietnam, corn is the primary material resource for fulfilling 90% of the demand for livestock and poultry feed (Dang & Nguyen, 2020). Due to the significant growth observed in animal husbandry, the demand for corn is increasing (Dang & Nguyen, 2020), leading to corncobs becoming a major agricultural waste product. A recent study by Yu *et al.* (2022) demonstrated that corncobs could replace sawdust as the primary cultivation substrate for shiitake mushrooms, but a nitrogen source is required to achieve a high biological efficiency. Accordingly, in this study, we cultivated strains J1 and J2 using corncobs as the primary substrate and wheat bran as a nitrogen source within a bag-based system.

There was no significant difference in the timing of fruiting body formation between the two strains, which occurred after 100-105 days of mycelium incubation. However, the density of the mushroom fruiting bodies of strain J2 was higher than that of strain J1, with an average of 8.00 fruiting bodies per bag (**Table 1**). The average weight of the fruiting bodies per bag of strain J2 was also higher than that of strain J1, reaching 74.77 g and 67.09 g, respectively. Due to the higher number of fruiting bodies, the average size of the fruiting bodies of strain J2

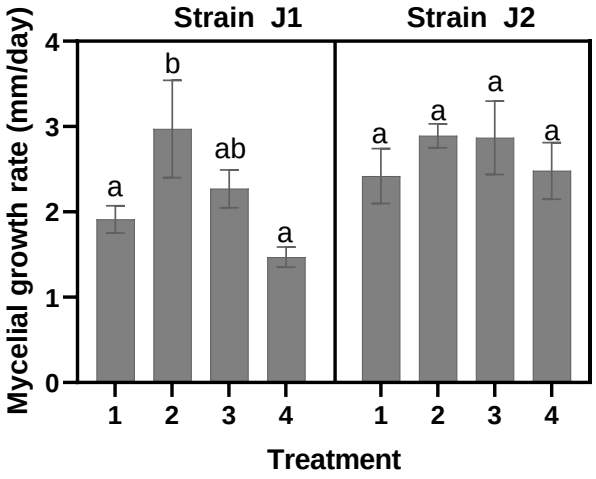

Figure 5. Effect of different spawning materials on the growth rate of shiitake mycelium. Different letters represent significant differences (P <0.05).

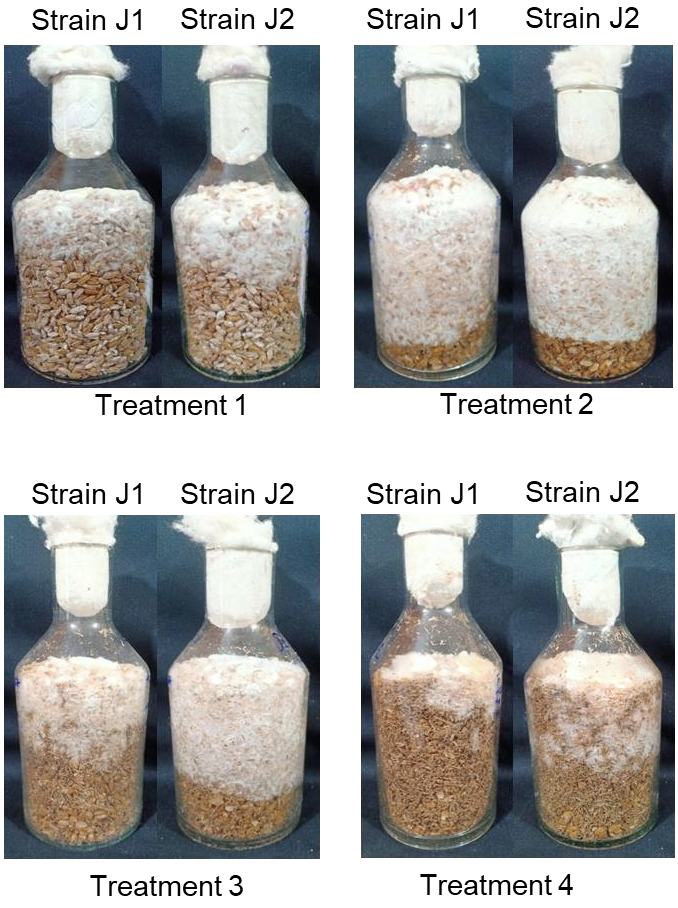

Figure 6. Mycelial growth of strains J1 and J2 grown on different spawning materials after 40 days at 25 ^{o}C.

was smaller than that of strain J1 (**Figure 7**). The first flush biological efficiencies of strains J1 and J2 were 20.33% and 22.66%, respectively. In the second flush, both strains exhibited a lower yield and density of fruiting bodies. Compared to the first flush, the yield of strain J1 decreased by 6.70 times, and the number of fruiting bodies harvested decreased by 7.52 times. Similarly, the

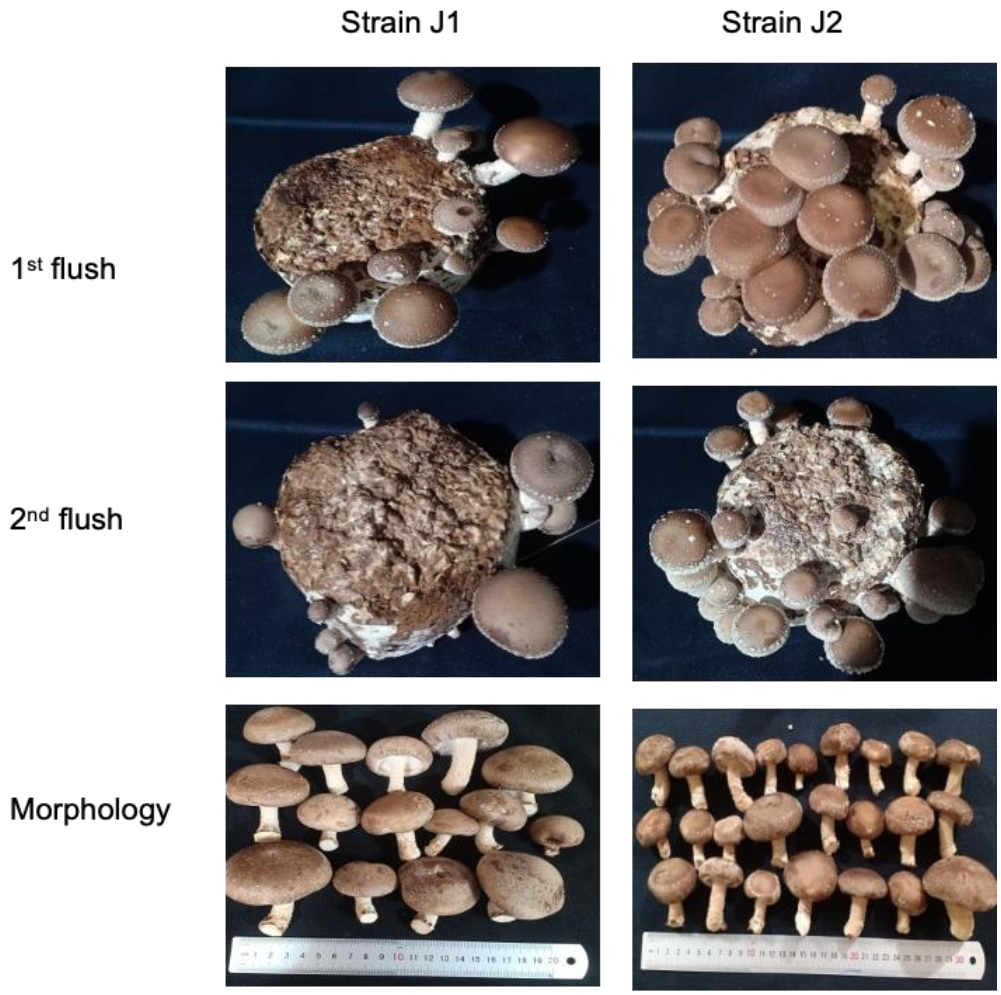

Figure 7. Fruiting body morphology of the shiitake strains J1 and J2

Table 1. The biological yields of the shiitake strains J1 and J2

Flush	Strain	Fruiting body number/bag	Fresh weight fruiting bodies/bag(g)	Average fresh weight fruiting bodies (g)	Biological efficiency (%)
1st flush	J1	6.20	67.09	10.82	20.33
	J2	8.00	74.77	9.35	22.66
2nd flush	J1	0.89	10.82	12.13	3.28
	J2	3.53	44.66	12.67	13.54

yield of strain J2 decreased by 1.67 times, and the number of fruiting bodies harvested decreased by 2.27 times. These observations agree with the study by Dissasa (2022), which reported that 91 to 96% of the fresh weight came from the first three consecutive flushes, while 4 to 9% came from the last flush. Mushroom yield is highly related to the content of carbohydrates, nitrogen, macronutrients, and micronutrients in the substrate (Yu *et al.*, 2022). The reduction in yield during the second flush may be due to the decline in the nutrition content in the substrate after the first harvest. Overall, strain J2 exhibited a higher biological efficiency than strain J1, indicating

that the biological efficiency of shiitake is strain-dependent. The high performance of strain J2 makes it a promising candidate for the commercial cultivation of shiitake.

Conclusions

Both strains J1 and J2 grew best at 25°C, with glucose being the most effective carbon source for favorable mycelial growth. The ideal nitrogen sources for strains J1 and J2 were identified as yeast extract and NH_4Cl, respectively. For upscaling mycelium growth, the best mixture was found to consist of 74% grain rice, 25% sawdust, and 1% $CaCO_3$. When cultivating strain J2 with 69% corncobs, 20% sawdust, 10% wheat bran, and 1% $CaCO_3$, a higher biological yield (36.5%) was achieved than strain J1 (23.5%). These findings offer valuable insights for enhancing shiitake cultivation techniques.

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