Research in
AGRICULTURE, LIVESTOCK and FISHERIES
ISSN : P-2409-0603, E-2409-9325
An Open Access Peer-Reviewed International Journal

Article Code: 466/2024/RALF
Article Type: Research Article

Res. Agric. Livest. Fish.
Vol. 11, No. 3, December 2024: 383-388.

Establishment of a Simple and Efficient Single Spore Isolation Method for Gilled Mushroom

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ARTICLE INFO

Received
07 October, 2024
Revised
08 November, 2024
Accepted
14 November, 2024

Key words:
Single spore
Cotton swab
Serial dilution
Genetic improvement
Mushroom

ABSTRACT

Single-spore isolation is a fundamental technique for exploring genetic diversity in fungi, particularly in heterothallic species like *Pleurotus ostreatus*. This study outlines a method for isolating single spores from fruiting bodies, aimed at facilitating genetic improvement of mushrooms. The approach is especially suitable for the laboratories of mushrooms which are not necessarily well-funded, requiring only basic laboratory equipment. It is simple, resistant to contamination, and highly efficient. Under sterile conditions, spores were collected from mature mushroom fruiting bodies using a sterilized cotton swab and subjected to serial dilutions in distilled water. Each dilution was plated on Potato Dextrose Agar (PDA) and then incubated, producing a consistent source of genetically uniform mycelium for further study. Further, microscopic examinations revealed the presence of clamp connections, specialized structures that ensure genetic stability and facilitate cell division during hyphal growth. This method is broadly applicable, though fungi that do not germinate on artificial media may require an alternative approach.

To cite this article: Islam S., M. A. H. M. Niloy, S. Yesmin, M. H. Mahi and J. Khandakar, 2024. Establishment of a simple and efficient single spore isolation method for gilled mushroom. Res. Agric. Livest. Fish. 11(3): 383-388.

DOI: https://doi.org/10.3329/ralf.v11i3.78896

INTRODUCTION

Mushroom farming is a prime example of circular agriculture, utilizing low-value agricultural and forestry by-products to produce nutrient-rich food (Ahmed et al., 2024). In recent years, commercially cultivated mushroom species are *Agaricus sp*. *Lentinus* sp. *Pleurotus* ap. *Grifola* sp. *Calocybe* sp, *Ganoderma sp.* Among them, the genus *Pleurotus*, comprising various commercially significant edible species, has gained global recognition due to its nutritional value (Raman et.al, 2020). Consequently, there is a growing demand for improved strains with enhanced productivity. The considerable diversity within species such as *P. eryngii*, *P. ostreatus*, *P. pulmonarius*, and *P. djamor* presents opportunities for the development of region-specific strains through hybridization or cross-mating. Fungi display a remarkable variety of sexual life cycles including heterothallic, and homothallic systems. *Pleurotus* species exemplify heterothallism, requiring two genetically distinct compatible mating types for reproduction (Barh et al., 2019). This process reduces the likelihood of inbreeding to about 25% while promoting outbreeding opportunities at around 75% (Neaves et al., 2015).

Generally, three primary approaches have been employed in the strain improvement of mushrooms, specifically recombinant breeding, transgenic breeding, and mutation breeding (Dong et al., 2022). Recombination breeding focuses on developing new varieties and strains through traditional breeding approaches such as selection and hybridization. *Pleurotus* spp improvement mainly relies on selection, which can involve direct selection from germplasm gathered during fungal expeditions or from cultures produced by multispore germination. Another method is to select and mate genetically diverse parents to take advantage of hybrid vigor through hybridization. Therefore, for studies on fungal variation, mutation, and segregation, it's essential to establish an efficient technique for isolating homokaryotic single spore isolates from fungi. Furthermore, in mycological research, obtaining pure cultures is essential. While tissue culture from desired mushroom fruiting bodies is a typical approach, it doesn't fully capture meiosis outcomes and isn't suitable for homothallic organisms, limiting genetic analysis and breeding efforts. Therefore, two key methods that are widely used for acquiring pure cultures include single spore separation and hyphal tip isolation (Leyronas et al., 2012). The latter is especially useful for obtaining pure cultures from sterile mycelial isolates or those unsuitable for single-spore isolations, as well as for addressing contamination or mutant colony issues. For spore-forming fungi like *Pleurotus spp*, single spore methods on culture media such as Potato Dextrose Agar (PDA) is commonly used, though it requires meticulous skill and a clean environment to prevent contamination, making it a time-consuming and complex process.

Thus, the objective of our study was to develop a straightforward protocol for single spore isolation from gill mushrooms. Our approach offers a method that is not only simple and cost-effective but also significantly more reliable, efficient, and less susceptible to contamination. In addition, it requires basic equipment for tissue culture, making spore collection more accessible to mushroom farmers.

Equipment required

The technical equipment utilized in this protocol comprises Petri dishes (90mm), cotton swabs, test tubes, distilled water, a spreader, a media plate (Potato Dextrose Agar), and a micropipette with tips, as illustrated in Figure 1. Additionally, essential tools such as a bright field microscope, stereomicroscope, and a laminar flow cabinet are vital components of the setup, although not depicted in the figure.

Materials and Methods

Germplasm collection and maintenance

The germplasm of the Oyster mushroom (*Pleurotus spp*) was sourced from the Mushroom Development Institute (MDI).

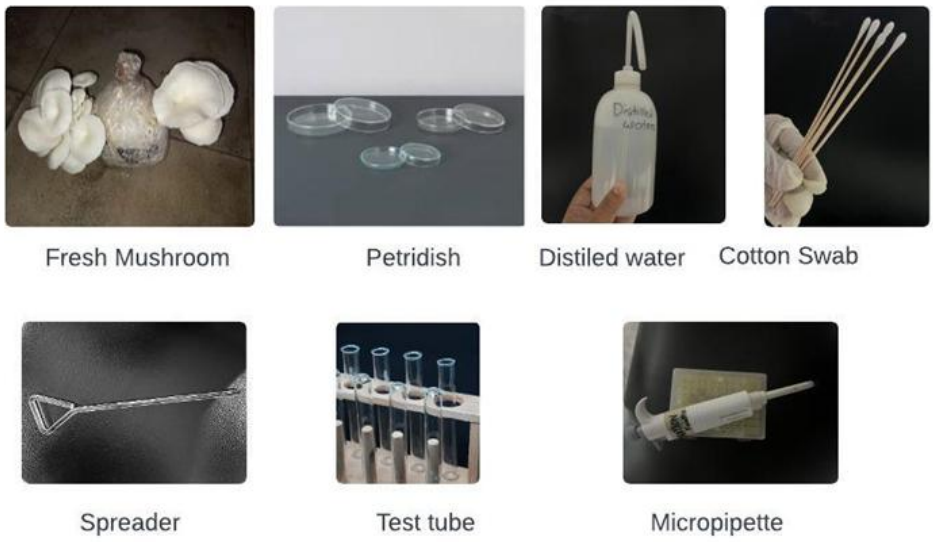

Figure 1. Materials required for single spore isolation

Methodology

Collection of spores from the mushroom fruiting body

To ensure the integrity of the collected mushroom spores and to optimize success rates, it is imperative to adhere to stringent sterile procedures throughout the process. In summary, a robust and mature mushroom fruiting body was selected, and then delicate surface sterilization of the mushroom fruiting body was done using 75% ethanol. For gilled mushrooms such as *Pleurotus*, spore retrieval was accomplished by gently swirling a cotton swab over the gill surface. Afterward, the spore-laden cotton swab was submerged into a test tube containing 6 ml distilled water. All activities have been conducted within a laminar flow cabinet to ensure aseptic conditions and minimize the risk of contamination.

Preparation of spore suspension by serial dilution method

After collecting spores using a cotton swab and suspending them in sterile distilled water, further dilutions were made up to 10-3. Subsequently, 0.1 ml of each spore suspension was spread onto separate petri plates containing PDA media and incubated at 25±2°C to promote germination. Following 3-4 days of incubation, single spore isolates (monokaryons) emerged as small colony heads. These monokaryon colonies were carefully transferred onto PDA media and then incubated at 25±20°C for 7-10 days. Confirmation of the monokaryotic nature of these cultures was conducted under a bright light microscope at 40x magnification.

Dikaryotization of mycelium

Dikaryotization was achieved through a dual culture method. In this approach, two individual single spore colonies were positioned diametrically opposite within a 90mm diameter Petri dish, maintaining a distance of 4 cm between them (Figure 3). Subsequently, the plates were incubated at 25±2°C for 5-7 days.

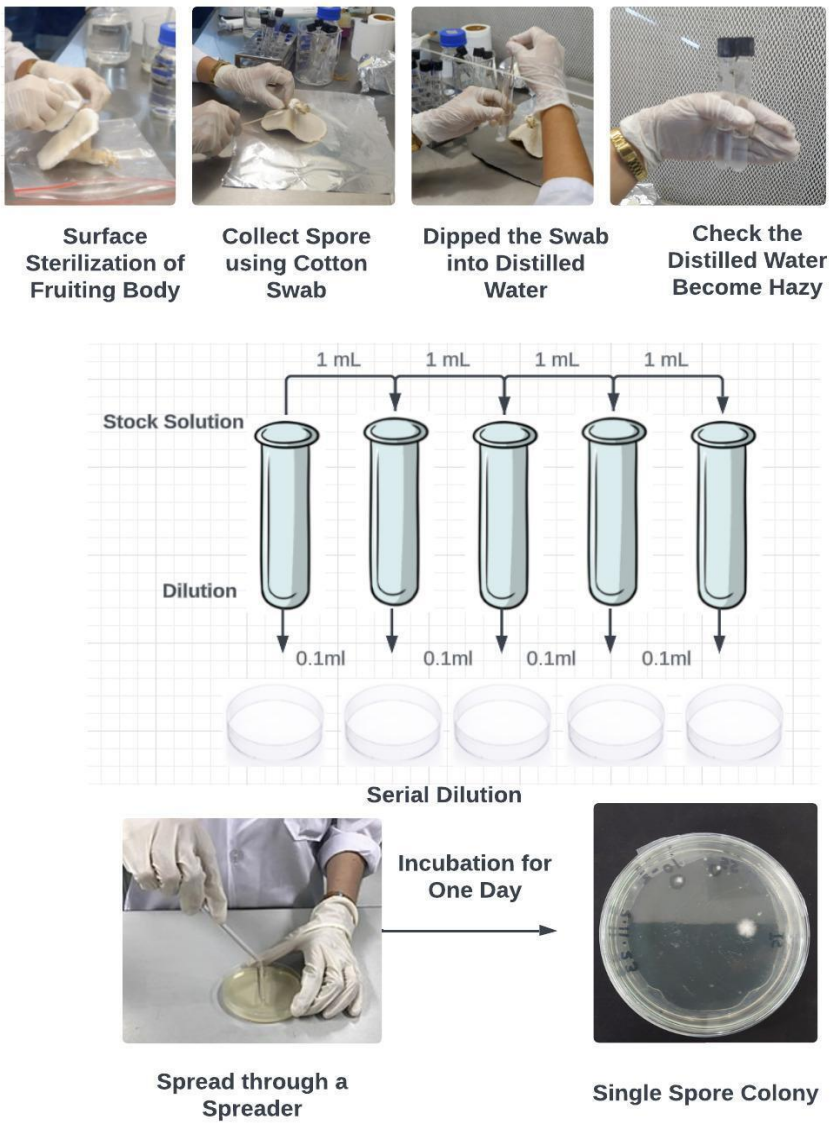

Figure 2. Single Spore Isolation Techniques

Results and Discussion

The monokaryotic nature of these cultures was confirmed under a bright light microscope at 40x magnification, where no clamp connections were observed. Furthermore, a field experiment was also conducted to assess the ability of these cultures to produce fruiting bodies during cultivation. Both mother culture and commercial spawn packets were prepared from monokaryotic pure culture. Despite observing intermingled hyphae of the

monosporous pairs indicating potential mating, subsequent testing revealed sterility which means they did not produce any fruiting bodies. While some monokaryons showed initial primordial development, they were abortive and did not progress further in growth. According to Jaswal et al., 2013, monospores of *Pleurotus* are incubated at 25^{0}C for 7-10 days. These monosporus cultures are confirmed to be monokaryotic when examined under a microscope, as they lack clamp connections.

When two single spore colonies of *Pleurotus ostreatus* were crossed on sterilized PDA, the hyphae exhibited compatibility upon contact, leading to the formation of dikaryons. This was evidenced by the dense, fluffy aerial growth at the point where the colonies met, indicating potential dikaryon formation. Subsequently, to confirm the dikaryotic nature of the hybrids, a microscopic examination at 45x magnification was conducted to observe the presence of clamp connections (see Figure 3). Using this mycelium we obtain fruiting bodies. Similar findings were reported by Sharma (2002), who tested fifty-seven *Pleurotus* hybrids to confirm dikaryotic nature of mycelium. Out of these, thirty-five hybrids successfully produced fruiting bodies and early spawn running, which occurred between 26 and 65 days after inoculation.

For obtaining a pure culture of monokaryotic mycelium our described methods contrast to commonly used spore printing methods. In traditional spore printing methods, contamination risk is quite higher as the spores are exposed to the surrounding environment during the printing process. Contamination such as mold or bacteria can interfere with spore viability and lead to failed cultivation attempts.

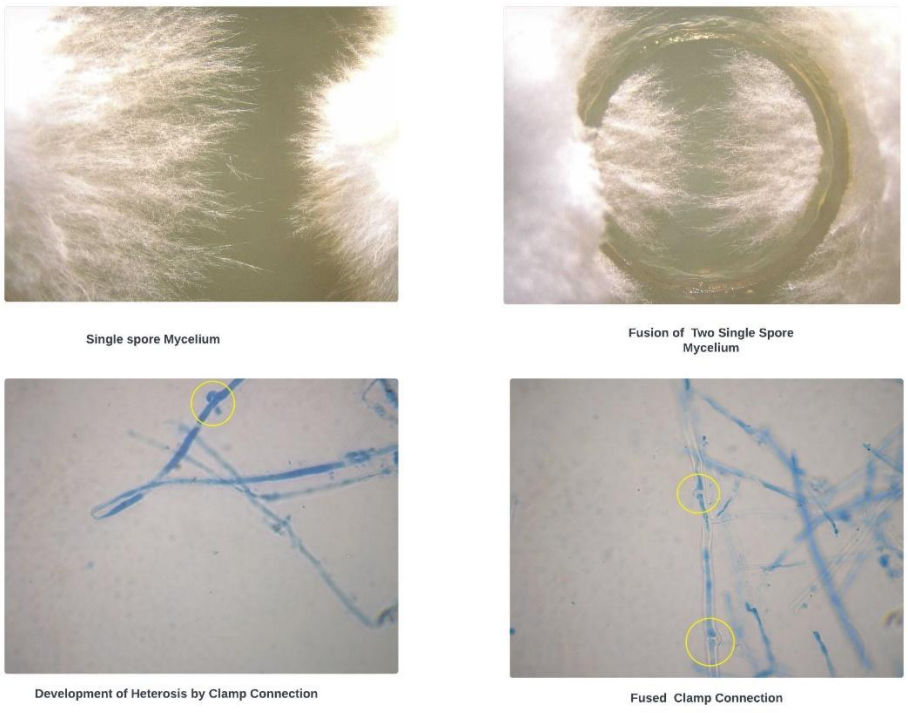

Figure 3. Development of Heterosis Using Monokaryotic Mycelium Fusion

Conclusion

The technique we developed successfully produced monokaryotic mycelium, confirmed by the absence of clamp connections. Although there were initial signs of mating between monokaryotic hyphae and the formation of abortive primordia, the resulting cultures were sterile and did not produce any fruiting bodies. These results emphasize the difficulties in cultivating monokaryotic cultures and the critical role of successful mating in

achieving productive growth. Furthermore, our method for obtaining pure monokaryotic cultures was more effective than traditional spore printing, minimizing contamination risks and improving the chances of successful cultivation. However, a limitation of the study is that key characteristics such as yield, biological efficiency, and the shape, texture, and taste of the fruiting bodies were not assessed.

Competing of Interest

The authors stated that they have no competing interests.

Acknowledgment

The authors extend their appreciation to Krishi Gobesona Foundation (KGF) Bangladesh for funding the research. The grant number is (CN/FRPP): TF141-C/23. The authors also thank Mushroom Development Institute (MDI), Savar, Dhaka for the germplasm of mushroom.

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