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Optimising Fungal Spore Cultivation and Inoculation Methods for Enhanced Antifungal Performance Testing of Textiles

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Article

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ABSTRACT

To improve the precision and operability of antifungal performance testing, this study systematically examines the cultivation time, spore suspension preparation, and inoculation protocols for fungal spores commonly used in mildew and rot resistance testing. Key optimisations were achieved in inoculum time, centrifugation conditions, and spray inoculation methods, enhancing experimental reliability. The findings indicate that Aspergillus brasiliensis and Trichoderma lignorum spores form abundantly within 5–7 days, while Chaetomium globosum requires 17–20 days. Penicillium funiculosum, Aureobasidium pullulans, Paecilomyces variotii, and Trichoderma virens reach sufficient spore production in 13–15 days. Effective spore separation was achieved via centrifugation at 6000 r/min for 10 minutes. Uniform and quantitative spore inoculation was ensured using 20–50 µm spray nozzles. Quantitative methods, including specific turbidity, hemocytometer counting, and the plate count method, were evaluated, with turbidity and hemocytometer methods offering efficiency and simplicity, while plate counts provided verification. These findings contribute to establishing standardised and reproducible antifungal testing procedures, addressing operational guidelines gaps and supporting the development of antifungal technologies.

KEYWORDS

antifungal activity, fungal spore preparation, quantitative inoculation, spore counting methods

INTRODUCTION

Fungi are ubiquitous organisms capable of colonising a wide range of substrates, including plants, animals, and humans, due to their versatility in metabolising diverse organic materials. This adaptability enables fungi to thrive in various environments, posing significant challenges to material preservation and public health. Fungal growth not only alters the aesthetic and sensory properties of materials, such as texture, colour, and odour but also introduces safety concerns through the production of mycotoxins by specific

species [1,2]. There has been a notable increase in severe fungal infections in recent years, driven by the growing population of immunocompromised individuals and the emergence of antifungal-resistant pathogenic fungi [3]. Among the most drug-resistant forms are species belonging to the genera Candida and Aspergillus [3]. These challenges have led to increased efforts in antifungal research, focusing on developing more effective treatments and preventive measures, including antifungal textiles and coatings [4,5].

The application of antifungal agents, including essential oils, natural plant extracts, and synthesised compounds, has shown promise in controlling fungal growth [6-9]. Additionally, compounds like econazole nitrate have been embedded in textiles, demonstrating sustained antifungal activity and durability over multiple washes [10]. Similarly, 1-monocaprylin has been applied to textile materials, effectively reducing microbial contamination [5]. Such innovations provide sustainable solutions for safeguarding materials and enhancing public health.

Fungal spores are widely used in laboratory settings as biological media for evaluating antifungal performance [11]. Current research has explored various aspects of fungal growth and spore germination, including optimising medium composition, assessing the impact of surfactants and temperature, and studying the physiological state of spores under different environmental conditions [11-13]. However, gaps remain in standardising spore preparation and inoculation methods for mildew and rot resistance testing. Existing standards, such as ASTM G21-15 and AATCC TM30-2017e, provide general guidelines for spore preparation but lack specific operational details, including centrifugation conditions and spray nozzle parameters [14,15]. These ambiguities result in inconsistencies in test outcomes across laboratories.

To address these challenges, this study aims to improve the operability and reliability of fungal spore preparation and inoculation procedures for antifungal testing. Specifically, this work focuses on identifying optimal inoculum times for commonly used fungal strains, including *Aspergillus brasiliensis, Trichoderma lignorum, Penicillium funiculosum*, and *Chaetomium globosum*. It also refines centrifugation techniques to achieve effective spore separation and purification. Furthermore, the study evaluates the suitability of spray nozzle sizes for uniform and quantitative spore inoculation and investigates spore concentration control methods, including specific turbidity, hemocytometer counting, and plate count techniques, to ensure reproducibility and accuracy [16-18].

This study builds on foundational research, such as Korra's application of aloe vera extracts for fungal resistance in textiles, which highlights the importance of precise and eco-friendly methods in antifungal applications [19]. By addressing operational issues in fungal spore preparation and inoculation, this work

provides a robust technical foundation for antifungal performance testing, contributing to advancements in material preservation and public health.

This research aims to establish standardized, reproducible, and accurate protocols for fungal spore preparation and inoculation, thereby enhancing the reliability of antifungal testing and supporting the development of innovative antifungal technologies.

MATERIALS AND METHODS

Test standards and preparation of fungal spores

Two widely utilised test standards, ASTM G21-15 and AATCC TM30-2017e, were referenced for evaluating antifungal performance in materials, particularly focusing on mildew and rot resistance. These standards outline the procedures for cultivating fungal spores, preparing inoculum, and inoculating test specimens. Despite their broad adoption, several inconsistencies and operational gaps were identified within the current protocols, particularly concerning the incubation times, centrifugation and filtration techniques, and inoculation methods, all of which can influence the reliability and reproducibility of results across laboratories.

The ASTM G21-15 standard outlines the method for determining the resistance of synthetic polymeric materials to fungi, specifically focusing on fungal growth in controlled environments. Similarly, AATCC TM30-2017e provides a framework for assessing mildew and rot resistance in textile materials using a range of fungal species. Both standards describe general processes for fungal spore cultivation and harvesting but fail to provide specific operational details that are critical for ensuring reproducibility. The incubation periods specified in the standards, for instance, lack specificity for different fungal species, which may lead to variations in spore production across experiments. Fungal species such as *Aspergillus brasiliensis* and *Trichoderma lignorum* require as few as 5-7 days for abundant spore formation, while others, such as *Chaetomium globosum*, may require 17-20 days. The existing guidelines do not address these temporal differences, which could result in inconsistent spore yields, especially when handling multiple species simultaneously.

The current standards also inadequately describe the centrifugation and filtration techniques, which are essential for ensuring clean and viable spore suspensions. The standards provide only broad instructions, such as centrifuging at unspecified speeds for unspecified durations, which leaves room for experimentation and potential inconsistencies in spore recovery.

Inoculation procedures, including spray nozzle specifications, were another area identified for improvement. Both standards recommend spraying a spore suspension onto test specimens but provide little guidance on the spray nozzle size or spore concentration.

The need to optimise these aspects of the standards became evident, and the primary objective of the current study was to address these gaps by refining the operational protocols. Specific changes targeted include defining the optimal incubation periods for different fungal species, standardising centrifugation conditions for spore purity, and providing more precise recommendations for spray nozzle sizes and spore concentrations.

To provide a comparative framework, Table 1 summarises the test methods used for antifungal performance testing, detailing the fungal species, inoculum preparation, harvesting techniques, and inoculation procedures as outlined in the standards. The table also highlights the inconsistencies and gaps in the existing protocols, illustrating the areas where modifications were made to enhance reproducibility and accuracy.

Table 1. Summary of test methods, fungal species, inoculum preparation, harvesting, and inoculation procedures

Test method	Fungal species	Inoculum	Harvesting and washing the spores	Inoculation
ASTM G21-15	*Aspergillus brasiliensis, Penicillium funiculosum, Chaetomium globosum, Trichoderma virens, Aureobasidium pullulans*	Potato dextrose agar, 7–20 days	Shake to release spores, filter through glass wool to remove mycelium. Centrifuge and discard supernatant [14].	Spray inoculation with a sterilized atomizer to cover the entire surface [14].
AATCC TM30-2017e (Test II)	*Chaetomium globosum*	Mineral salts agar, 10–14 days	Shake vigorously to suspend spores, and filter through sterile gauze [15].	Apply 1.0 ± 0.1 mL of inoculum evenly over specimens using a sterile pipette [15].
AATCC TM30-2017e (Test III)	*Aspergillus niger*	Mineral salts agar, 7–14 days	Shake to release spores, and filter through sterile gauze [15].	Apply 0.5 ± 0.1 mL evenly over the agar surface [15].
AATCC TM30-2017e (Test IV)	*Aspergillus niger, Penicillium varians, Trichoderma viride*	Potato dextrose agar (A. niger, P. varians), malt extract agar (T. viride), 7–10 days	Shake to break up spore clumps, and filter through sterile cotton [15].	Distribute 1.0 ± 0.1 mL of inoculum on both sides of each specimen using a spray or pipette [15].

Test method	Fungal species	Inoculum	Harvesting and washing the spores	Inoculation
AATCC TM174-2011 (2016)e (III)	*Aspergillus niger*	Sabouraud dextrose agar, 7–10 days	Shake to release spores, and filter through sterile cotton [20].	Apply 1.0 ± 0.1 mL of inoculum over the agar surface, and distribute 0.2 mL per disc using a sterile pipette [20].

The revised protocols were designed to standardize the antifungal testing process by targeting the specific issues of incubation, centrifugation, and inoculation methods, thereby increasing the accuracy and consistency of the results. This work provides a technical foundation for future studies in fungal resistance, ensuring that the developed antifungal textiles and materials perform consistently across different laboratory settings. Moreover, these improvements contribute to a more efficient and reproducible approach to evaluating antifungal treatments, supporting the development of more effective and reliable antifungal technologies.

Fungal strains and reagents

The selection of fungal strains for antifungal performance testing is crucial to ensure the reliability and reproducibility of results. This study chose seven fungal species commonly associated with mildew and rot based on their prevalence in textile degradation and relevance to industry standards. These species include *Aspergillus brasiliensis, Penicillium funiculosum, Chaetomium globosum, Trichoderma lignorum, Aureobasidium pullulans, Paecilomyces variotii*, and *Trichoderma virens*. The selected species were obtained from reputable sources such as the American Type Culture Collection (ATCC) and the China General Microbiological Culture Collection Center (CGMCC), ensuring their genetic consistency and authenticity for laboratory use.

In addition to the fungal strains, several reagents were used to facilitate the preparation of spore suspensions. Potato Dextrose Agar (PDA), sourced from Beijing Land Bridge Technology Co., Ltd., was the primary growth medium. PDA has been widely adopted due to its ability to support the growth of a broad range of fungi, providing an ideal environment for fungal development and spore production. Tween 80, a surfactant obtained from Tianjin Fuyu Fine Chemical Co., Ltd., was incorporated at a concentration of 0.05% (v/v) to aid in preparing spore suspensions. This surfactant was used to reduce surface tension, facilitating the dispersion of fungal spores and ensuring uniform suspension. Additionally, a sterile 0.9% saline solution was used to wash and resuspend the fungal spores during centrifugation and filtration.

Table 2 summarises the fungal strains used in this study, their sources, and the culture details, along with the reagents and materials involved in spore suspension preparation.

Table 2. List of fungal strains, sources, and culture details

Fungal strain	Strain No.	Source	Culture medium
Aspergillus brasiliensis	ATCC 16404	American Type Culture Collection (ATCC)	Potato Dextrose Agar
Trichoderma lignorum	AS 3.2941	China General Microbiological Culture Collection Center (CGMCC)	Potato Dextrose Agar
Penicillium funiculosum	ATCC 11797	American Type Culture Collection (ATCC)	Potato Dextrose Agar
Chaetomium globosum	ATCC 6205	American Type Culture Collection (ATCC)	Potato Dextrose Agar
Aureobasidium pullulans	ATCC 15233	American Type Culture Collection (ATCC)	Potato Dextrose Agar
Paecilomyces variotii	ATCC 18502	American Type Culture Collection (ATCC)	Potato Dextrose Agar
Trichoderma virens	ATCC 9645	American Type Culture Collection (ATCC)	Potato Dextrose Agar

Experimental setup and instruments

The experimental setup utilised in this study was designed to meet the specific requirements of antifungal testing, focusing on fungal spore cultivation, inoculum preparation, and the evaluation of antifungal performance. Several key instruments were employed to ensure precise control of environmental conditions, accurate measurement of spore concentration, and the effective inoculation of fungal spores onto test specimens. The main instruments used in the test included the MJ-150-II type constant temperature and humidity fungal incubator, the ESCO biological safety cabinet, the XSP-8CA optical microscope, the TGL-16G benchtop centrifuge and the WGZ-XT bacterial turbidimeter used in the test. The combination of these instruments provided precise control over the experimental conditions, ensuring that all variables were accounted for, thereby improving the reliability and reproducibility of the results.

EXPERIMENTAL PROCEDURE

Inoculation

The inoculation procedure was optimised to ensure consistent spore preparation, which is essential for accurate antifungal performance testing. Fungal strains were initially cultivated on Potato Dextrose Agar (PDA), a widely used medium for the growth of various fungal species. Throughout the incubation period, the cultures were regularly monitored for spore development. Examinations were performed to assess the morphology and viability of the fungal spores. This process was essential to determine the precise

timing for spore harvesting, ensuring that the spores were mature and viable for inoculation. The incubation times varied depending on the species. For instance*, Aspergillus brasiliensis* and *Trichoderma lignorum* produced abundant spores within 5–7 days, while species such as *Penicillium funiculosum*, *Aureobasidium pullulans*, *Paecilomyces* variotii, and *Trichoderma virens* required 13–15 days for sufficient spore formation. *Chaetomium globosum*, known for its slower growth rate, needed 17–20 days to yield rich spore populations.

Figure 1 illustrates the growth of the maintained fungal cultures on PDA, showing the different fungal species' distinct growth patterns and spore formation. These visual observations were critical in determining the optimal harvesting time for each strain.

a) *Aspergillus brasiliensis*

b) *Trichoderma lignorum*

c) *Penicillium funiculosum*

d) *Chaetomium globosum*

e) *Aureobasidium pullulans*

f) *Paecilomyces variotii*

g) *Trichoderma virens*

Figure 1. The growth of maintained cultures of test fungi separately on potato dextrose agar

The harvested spores were processed into spore suspensions for subsequent inoculation procedures. The data on the incubation durations and the consistency of spore development across strains are summarised in Table 3, which presents the incubation durations required for each fungal species to produce abundant, viable spore populations.

Table 3. Incubation durations for rich spore formation across strains

Fungal Species	Strain No.	Incubation Duration (Days)
Aspergillus brasiliensis	ATCC 16404	5–7
Trichoderma lignorum	AS 3.2941	5–7
Penicillium funiculosum	ATCC 11797	13-15
Chaetomium globosum	ATCC 6205	17–20
Aureobasidium pullulans	ATCC 15233	13–15
Paecilomyces variotii	ATCC 18502	13–15
Trichoderma virens	ATCC 9645	13–15

By understanding the specific growth conditions and spore production timelines of each fungal species, more reliable and efficient inoculation methods were developed, which enhanced the accuracy and reproducibility of antifungal performance assays.

Spore suspension preparation

Preparing fungal spore suspensions involved a series of critical steps, which were optimised to ensure uniformity and high viability of spores for antifungal testing. Initially, fungal spores were harvested by scraping the surface of mature fungal cultures using an inoculation loop. This method facilitated the collection of spores from the surface of the cultures grown on Potato Dextrose Agar (PDA) plates. The collected spores were then suspended in a saline solution containing 0.05% (v/v) Tween 80, a surfactant that helped disperse the spores and prevent clumping.

The spore mixture was filtered after the initial suspension to remove any mycelial fragments or particulate matter. Sterile glass wool was employed for filtration, as it effectively trapped larger mycelial pieces while allowing the spore suspension to pass through. This filtration process ensured the spore suspension was free from contaminants that could interfere with subsequent inoculation steps.

Centrifugation was performed to further purify the spore suspension and achieve a concentrated spore solution. The centrifugation conditions were optimised to balance effective sedimentation with minimal damage to the spores. Centrifugation was carried out at varying speeds and durations to determine the ideal conditions for uniform spore sedimentation. The conditions were refined through a series of trials,

with 6000 r/min for 10 minutes emerging as an efficient setting for obtaining well-separated spores. This protocol minimised spore damage and yielded a high-quality suspension suitable for inoculation.

Table 4 presents the centrifugation conditions and results, detailing the speed and duration used for each trial and the effectiveness in achieving clear spore separation. These optimised conditions were crucial for ensuring the reproducibility and accuracy of the antifungal testing procedures.

Table 4. Centrifugation conditions and results for fungal spore separation

Centrifugation condition	*Aspergillus brasiliensis*	*Trichoderma lignorum*	*Penicillium funiculosum*	*Chaetomium globosum*	*Aureobasidium pullulans*	*Paecilomyces variotii*	*Trichoderma virens*
7000 r/min, 10 min	√	√	√	√	√	√	√
6000 r/min, 10 min	√	√	√	√	√	√	√
5000 r/min, 10 min	√	×	×	×	√	√	√
4000 r/min, 30 min	×	×	×	×	√	√	√
3000 r/min, 30 min	×	×	×	×	√	×	×

Note: "√" represents a good centrifugation effect, while "×" represents loose sediment that is difficult to decant, resulting in incomplete separation.

The results indicated that 6000 r/min for 10 minutes was optimal for spore separation across all fungal species tested, ensuring high-quality suspensions for inoculation. This centrifugation step was integral to preparing spores for the subsequent antifungal performance testing on textile materials.

Observation and characterisation of spores

Following the centrifugation process, the morphology of the fungal spores was examined using a microscope to ensure that the spores were intact and suitable for antifungal testing. A small aliquot of the spore suspension was placed on a microscope slide, and the sample was observed under a light microscope at a magnification of 100x. The spore morphology was carefully documented, with particular attention given to the shape, size, and any potential damage or abnormalities resulting from the preparation process.

In addition to qualitative observation, the size of the fungal spores was measured to characterise the spore populations. A calibrated micrometre was used to measure the diameter of individual spores under

the microscope. The spore size distribution was recorded, and the average diameter for each species was calculated. This step was essential for confirming the specifications of the spray nozzle used in the inoculation process, which is vital for ensuring consistent inoculation and reproducible results in antifungal testing.

Table 5 summarises the morphological characteristics and size measurements for the seven fungal species used in the study. These data provided essential insights into the spore characteristics, helping to further refine the inoculation protocols.

Table 5. Morphological and dimensional characteristics of fungal spores

Fungal Species	Shape	Size (μm)	Specific turbidity (MCF)	Hemocytometer count (spores/mL)	Plate count (CFU/mL)
Aspergillus brasiliensis	Spherical	~5	3.85	2.0×10^7	3.8×10^7
Trichoderma lignorum	Oval to near-spherical	~3 × 5	4.88	7.0×10^7	1.3×10^8
Penicillium funiculosum	Oval, rounded at both ends	~2 × 3 to ~3 × 5	0.07	1.1×10^6	1.2×10^6
Chaetomium globosum	Oval, slightly pointed ends	~8 × 10 to ~9 × 11	0.12	1.0×10^5	1.3×10^5
Aureobasidium pullulans	Irregular shape	~3 × 5 to ~5 × 15	0.65	5.8×10^6	2.5×10^6
Paecilomyces variotii	Irregular, nearly spherical	~2 × 3 to ~3 × 10	1.04	3.9×10^7	1.7×10^7

Inoculation optimisation

The influence of spray nozzle size was evaluated to ensure the uniform distribution of fungal spores during the inoculation process. Nozzles with apertures of 20 μm to 50 μm were tested for their ability to produce a consistent spray pattern. The objective was to achieve even inoculum distribution across the test surface, as non-uniform spore coverage can result in unreliable outcomes in antifungal performance testing.

In addition to confirming the spray nozzle size, several methods were used to control spore concentration. These included turbidity measurements, hemocytometer counting, and the plate count method. Turbidity measurement was employed to provide a quick and efficient estimate of spore concentration, while hemocytometer counting allowed for more precise quantification of spore density. The plate count method was used as a final verification step to ensure accuracy in the spore concentration.

The turbidity of the spore suspension was measured using a WGZ-XT bacterial turbidimeter, and the results were correlated with the actual spore concentration determined by the hemocytometer and plate count methods. The plate count results confirmed that both the turbidity and hemocytometer methods provided approximate estimates of spore concentration, with slight variations due to the inherent differences in each technique.

The optimisation of both inoculation techniques and spore concentration control methods was critical for ensuring the reproducibility and accuracy of the antifungal tests. These improvements enhanced the experimental reliability, which is essential for drawing meaningful conclusions in antifungal performance evaluations.

RESULTS AND DISCUSSION

Incubation time analysis

The incubation times for the various fungal species were carefully analysed to determine the optimal conditions for obtaining abundant spore formation. Each of the seven fungal strains used in the study exhibited different growth rates, significantly impacting the time required to reach the stage of spore production suitable for inoculation. *Aspergillus brasiliensis* and *Trichoderma lignorum* produced abundant spores within 5 to 7 days, whereas species such as *Penicillium funiculosum*, *Aureobasidium pullulans, Paecilomyces variotii*, and *Trichoderma virens* required approximately 13 to 15 days for sufficient spore formation. *Chaetomium globosum* displayed the slowest growth, with abundant spore formation taking between 17 and 20 days.

The histogram in Figure 2 illustrates the distribution of incubation times for each fungal species, providing a clear visual representation of the differences in growth rates. These findings are essential for optimising the experimental setup for antifungal performance testing, as they allow for the adjustment of inoculum preparation protocols to match the optimal spore maturation periods for each strain.

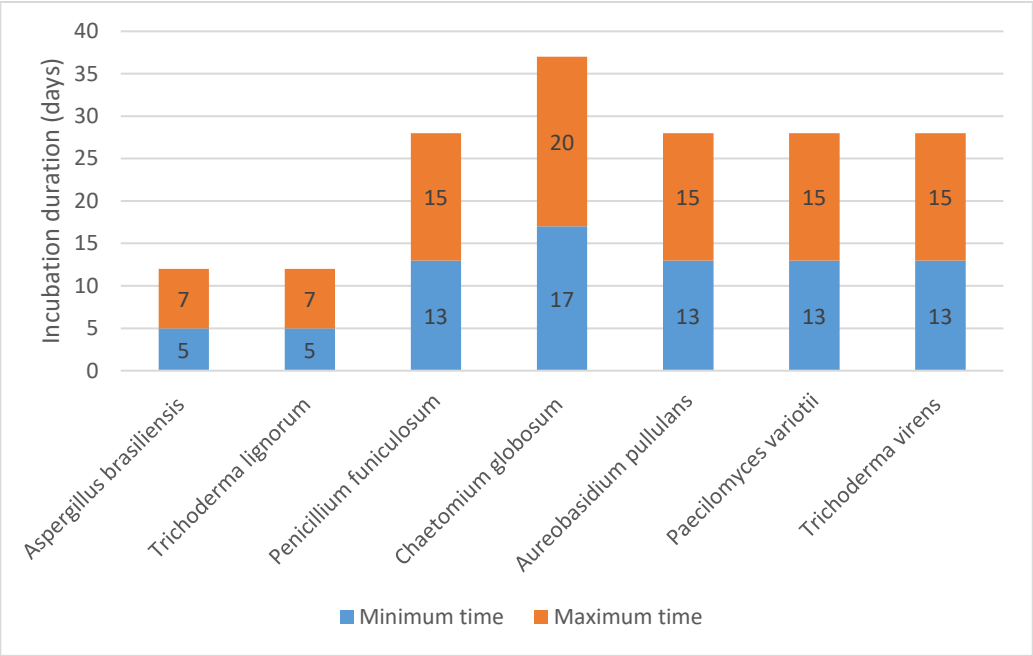


Figure 2. Histogram of incubation times for each fungal species

Overall, the data confirmed that appropriate adjustments in incubation time are necessary for each fungal species to ensure the reliable formation of viable spores for inoculation. This optimisation directly influences the reproducibility and accuracy of antifungal performance assays, underscoring the importance of considering fungal growth characteristics when designing experiments.

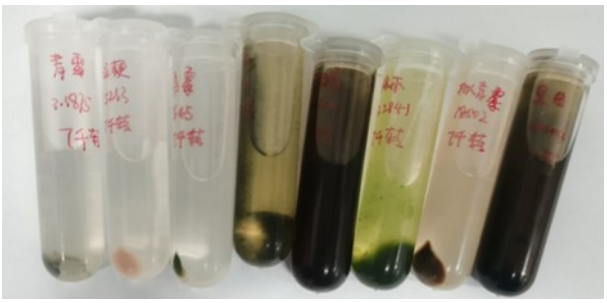
Effectiveness of centrifugation

The centrifugation process was systematically evaluated to determine optimal conditions for efficient fungal spore separation. Different centrifugation speeds and durations were tested to obtain well-defined and compact spore sediments while minimizing contamination with mycelial fragments. Various centrifugation protocols were implemented, ranging from 3000 r/min to 7000 r/min for durations of 10 to 30 minutes, to assess their effectiveness in producing uniform spore separation.

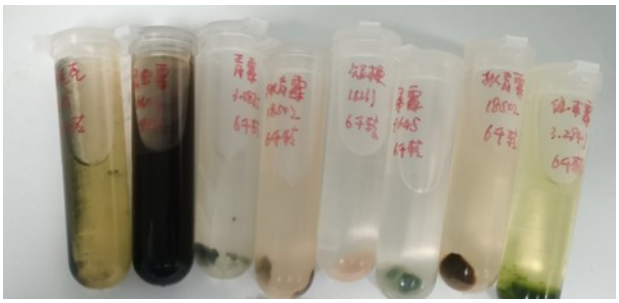
At higher centrifugation speeds (6000 r/min and 7000 r/min), satisfactory sedimentation was achieved, with clear and compact spore pellets observed. In contrast, lower speeds (4000 r/min and below) resulted in less effective separation, leading to looser sediments that were challenging to decant without additional washing steps. The centrifugation conditions that yielded the most consistent and reliable results were determined to be 6000 r/min for 10 minutes. Under these conditions, the fungal spores were

efficiently separated from the surrounding mycelial debris, ensuring a high-quality inoculum for subsequent inoculation procedures.

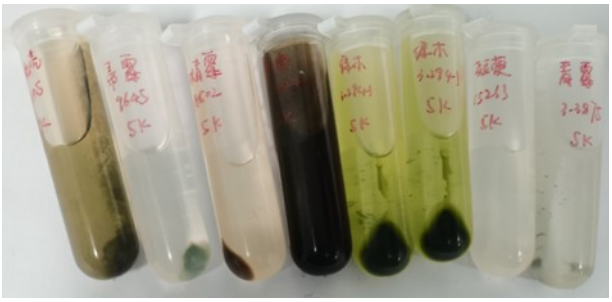
The results were visually documented and analysed through microscopic examination to confirm the quality of the spore suspension. Figure 3 illustrates the effectiveness of centrifugation at different speeds and durations, providing a clear comparison of spore sedimentation at various conditions. This figure highlights the impact of centrifugation speed and duration on the quality of the spore separation, with optimal conditions producing well-defined pellets and minimal contamination.



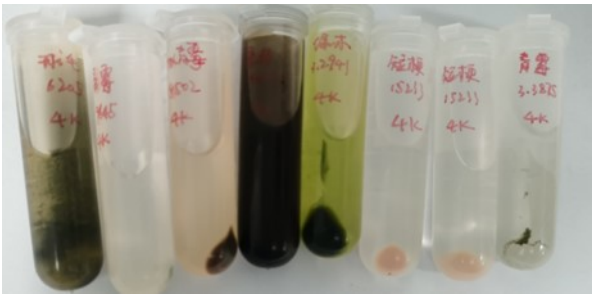
a) 7000 r/min, 10 min



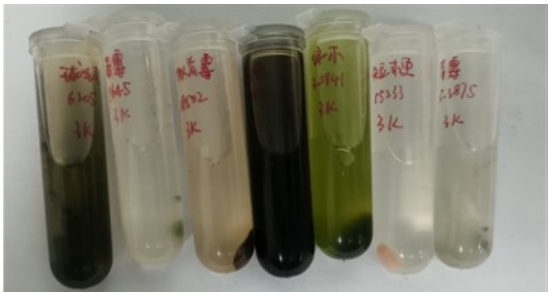
b) 6000 r/min, 10 min



c) 5000 r/min, 10 min



d) 4000 r/min, 30 min



e) 3000 r/min, 30 min

Figure 3. Visualization of Centrifugation Effects at Different Speeds and Durations

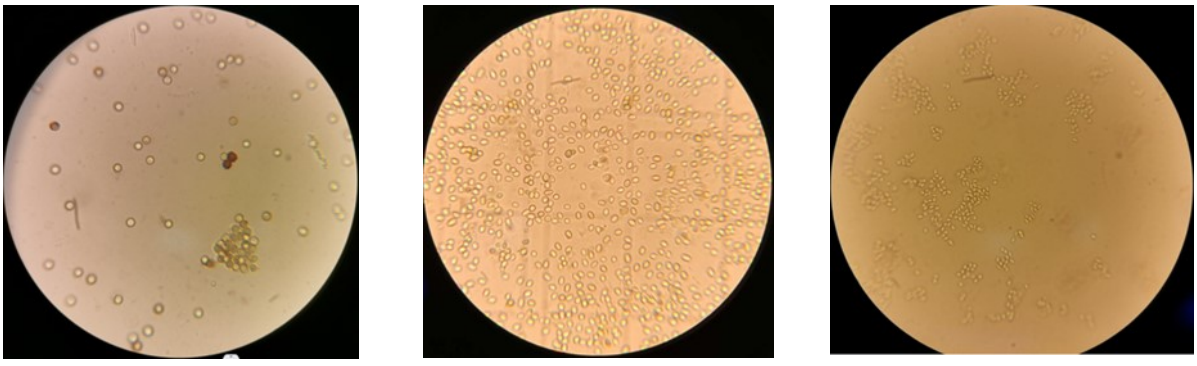
These findings underscore the importance of optimising centrifugation parameters to improve the reproducibility and accuracy of spore inoculation, which is crucial for reliable antifungal performance testing. The centrifugation conditions tested in this study have provided a robust framework for preparing fungal spore suspensions, ensuring that the inoculum is of consistent quality for use in subsequent antifungal assays.

Spore size and morphology

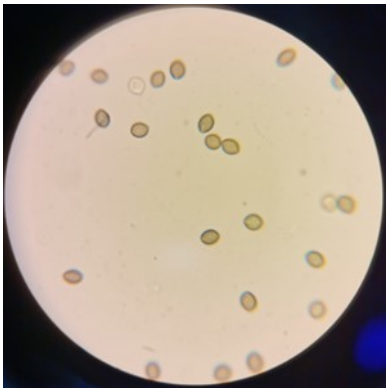
The morphological characteristics and size distribution of fungal spores were assessed to determine their consistency and suitability for inoculation. After centrifugation and suspension preparation, the spores were examined under a microscope to observe their overall shape, size, and any potential morphological aberrations. The spores from each fungal species exhibited distinct shapes and sizes, with notable differences observed between strains.

In general, the spores of *Aspergillus brasiliensis* were spherical with an average diameter of approximately 5 µm. *Trichoderma lignorum* spores were slightly elongated, measuring about 3 µm × 5 µm, while *Penicillium funiculosum* spores were oval, with dimensions ranging from 2 to 3 µm by 3 to 5 µm. Larger spores were observed in *Chaetomium globosum* (8-9 µm by 10-11 µm), while *Aureobasidium pullulans* and *Paecilomyces variotii* had irregular shapes, typically ranging from 3 µm to 15 µm in size. *Trichoderma virens* spores were nearly spherical, ranging from 2 µm to 4 µm in diameter.

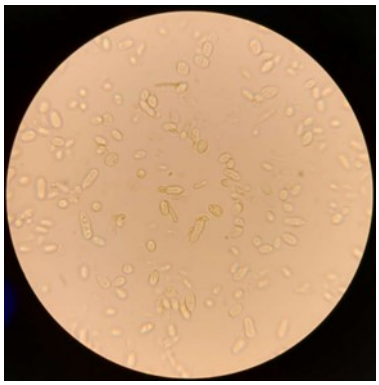
Figure 4 presents the microscopic images of fungal spores post-centrifugation, visually representing their morphological characteristics.



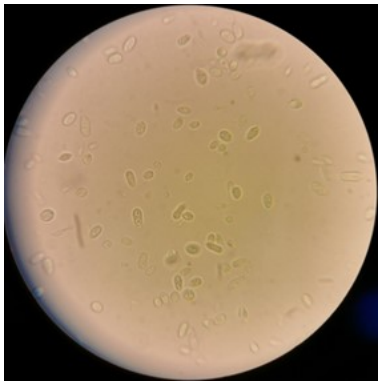
a) Aspergillus brasiliensis b) *Trichoderma lignorum* c) *Penicillium funiculosum*



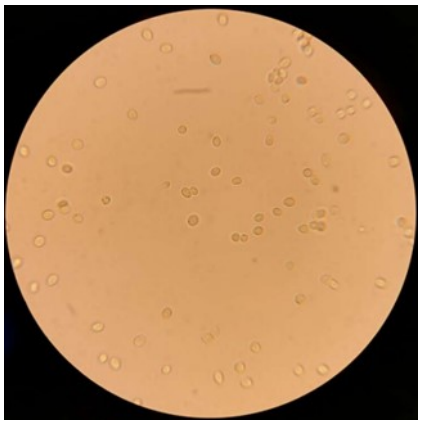
d) *Chaetomium globosum*



e) *Aureobasidium pullulans*



f) *Paecilomyces variotii*



g) *Trichoderma virens*

Figure 4. Microscopic observation of spore morphology

Spray inoculation and spore concentration

The optimization of spray inoculation and spore concentration measurement was carried out to ensure uniform inoculum distribution and reproducibility across experiments. Spray nozzles with aperture sizes ranging from 20 to 50 µm were employed, as this range demonstrated the ability to produce consistent and uniform spore distribution. Nozzles with apertures smaller than 20 µm were prone to clogging, while those larger than 50 µm often resulted in uneven spore distribution.

Spore concentration was measured using three methods: turbidity measurement, hemocytometer counting, and plate count method. Turbidity provided a quick and approximate estimate of spore concentration, but it was less reliable for low spore concentrations. Hemocytometer counting offered higher precision, and the plate count method was used as a verification step. A strong correlation was observed between the hemocytometer and plate count methods, with the turbidity measurement showing more variability, particularly for fungal species with lower spore concentrations.

CONCLUSION

A preliminary examination of key experimental parameters successfully optimised fungal spore cultivation and inoculation methods. The incubation times for seven commonly used fungal species were optimised, with precise durations established for the abundant formation of spores. The results indicated that shorter incubation periods were sufficient for species like *Aspergillus brasiliensis* and *Trichoderma lignorum*, while slower-growing species such as *Chaetomium globosum* required significantly longer periods for spore maturation. These findings contribute to more consistent and reliable fungal spore production protocols.

Furthermore, centrifugation conditions were optimised to improve spore separation and purification. The study identified centrifugation speeds of 6000 r/min for 10 minutes as the most effective for all tested fungal species, resulting in uniform spore sedimentation and minimal contamination with mycelial fragments. This optimised centrifugation process supports the creation of high-quality spore suspensions essential for antifungal performance testing.

The inoculation protocol was standardised by utilising spray nozzles in the 20–50 μm range. This nozzle size was found to ensure even and reproducible spore distribution across experimental surfaces. The use of this optimised spray method contributes significantly to improving the reliability of inoculation procedures, particularly for antifungal testing applications.

Spore concentration control was successfully achieved using turbidity measurement, hemocytometer counting, and the plate count method. The results confirmed that hemocytometer counting and plate counting were the most reliable techniques for accurate spore quantification, while turbidity measurements were most effective for high-concentration suspensions. This finding underscores the importance of employing multiple methods to ensure precise spore concentration determination in antifungal testing.

These optimised methods and standardised protocols hold important implications for the field of antifungal performance testing. They offer a more robust and reproducible approach to spore preparation, inoculation, and concentration measurement, which could enhance the accuracy and comparability of antifungal activity assessments across different research settings. Future studies could explore the application of these methods to different textile materials and further refine the testing protocols to account for diverse environmental and experimental variables.

Author Contributions

Conceptualization, methodology and formal analysis – Deng H and Xu X; investigation – Deng H, Xu X, Zhang Y, Chen Q, and He X; resources – Yang C, Deng H, Xu X; writing-original draft preparation –Deng H, Zhang Z; writing-review and editing –Yang C; visualization – Yang C; supervision – Yang C. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Hazards

Some of the fungi used in these tests are allergenic and pathogenic; i.e., capable of infecting humans and producing disease. Therefore, every necessary and reasonable precaution must be taken to eliminate this risk to the laboratory personnel and personnel in the associated environment. Wear protective clothing, respiratory protection, and impervious gloves when working with the organisms. Choose respiratory protection that prevents penetration by the spores. Sterilize all contaminated samples and test materials before disposal [7].

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