Biolog Lab Services reveals innovative method for more successful identification of Filamentous Fungi

Introduction

Filamentous fungi, colloquially referred to as molds, are prevalent microorganisms found in most ecosystems, including soil, air, water, and plant surfaces. They play crucial roles in nutrient recycling, organic matter decomposition, and symbiotic interactions. Accurate identification of filamentous fungi in environmental samples is indispensable for comprehending ecological processes, appraising microbial diversity, and gauging potential risks associated with pathogenic species.

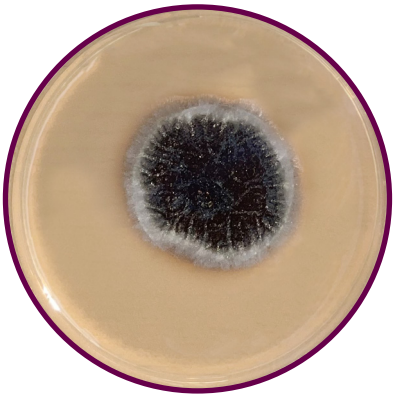

Figure 1. An *Aspergillus Brasiliensis* sample used for the study grown on FF proprietary media.

Traditional identification methods, reliant on morphology and other physical characteristics of cultured fungi, are not only labor-intensive but also prone to interpretation bias. In response to these challenges, Biolog has developed a novel MALDI-TOF-based approach that improves upon the commonly recommended extraction process. We have also developed an innovative media that reduces harvesting time while preventing contamination from bacteria, yeast, and agar. This proprietary media, a selective growth medium designed exclusively for filamentous fungi, effectively inhibits bacterial growth and enhances identification results.

Three different species of filamentous fungi were subjected to ID testing using our newly developed method compared to the current industry standard for fungal identification using MALDI-TOF. Here we show that our novel method enabled all three organisms to be successfully identified at the species level, in contrast to the existing method, which only yielded a passing score for one of the organisms.

Methods

The following cultures were selected for examination, and purchased from ATCC: *Penicillium chrysogenum 10106*, *Aspergillus brasiliensis 16404*, and *Purpureocillium lilacinum 10114*. The choices of these species were chosen because they are adaptable and found in many common environments. To facilitate the study, these fungi were sub-cultured on four distinct types of media, Biolog 2% Malt Extract media (B2ME), Biolog proprietary media (BPM), Millipore Malt Agar (MMA), and Sabouraud Dextrose agar broth (SAB,). These specific media were selected after experimenting with other industry-standard options tailored to filamentous fungi and in consideration of previously published research on filamentous fungi harvesting. The cultures were incubated at 28°C for both 24 and 48 hours.

The guidelines outlined in the Bruker MALDI Biotyper SOP for Cultivation and Sample Preparation for Filamentous Fungi were followed. For our novel method, extraction includes adding a lysing reagent to aid in cell lysis for protein recovery. For the extraction and harvesting procedures of the 24-hour and 48-hour cultures, a set of 12 samples were prepared from the following: *P. chrysogenum, P. lilacinum, and A. brasiliensis* in SAB; *P. chrysogenum, P. lilacinum, and A. brasiliensis* in B2ME; *P. chrysogenum, P. lilacinum, and A. brasiliensis* in BPM; and *P. chrysogenum, P. lilacinum, and A. brasiliensis* in MMA. *E.coli* was employed as a calibration standard (BTS) through Bruker. Three spots were utilized on the steel target for each extracted sample, with two spots designated for Bruker's calibration standard. The spots on the MALDI target were sealed with 1 µL of HCCA. Then, the target plate was placed into the

MALDI-TOF utilizing the MALDI Biotyper . To receive a species call, the score needs to be above 2.0 to obtain a secure genus identification and probable species identification. Any score above 2.3 is a highly probable species identification.

Results

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) provides a rapid and reliable method for the identification of fungi. This is particularly beneficial given the inherent difficulties with distinguishing morphologically similar fungi. Importantly, non-sporulating molds are unable to be identified based solely on morphology. Alternative methods, including mass spectrometry and genotyping, are needed, and we have developed an innovative method using MALDI-TOF that we have implemented to our Lab Services team in Newark, DE.

The main obstacles to preparing suitable filamentous fungi cultures for these methods are harvesting a sufficient quality and quantity of pure sample, and breaking through the chitin cell walls for the extraction of proteins. These challenges stem from several factors. Filamentous fungi are characterized by their hyphae, which exhibit a branching, thread-like structure. These hyphae extensively permeate the agar surface and penetrate deep into the growth medium, making the task of harvesting them difficult without disturbing the agar substrate and contaminating the sample with components from the agar. Moreover, these fungi adhere to the agar surface firmly, and their mycelium forms intricate networks, complicating detachment without causing harm. Their delicate nature adds to the challenge, as the hyphae can easily break during the harvest from Sabouraud agar broth, leading to the loss of sediment formed.

In addition to these complexities, filamentous fungi are highly susceptible to contamination by other microorganisms, including bacteria and yeast. During the harvesting process, contaminants can infiltrate the culture, adversely affecting the sample's purity. To mitigate these challenges, we implemented various techniques and precautions. These include sterile tools, gentle scraping or lifting of fungal growth, and the careful transfer of fungi to suitable containers or media with minimal disturbance. Furthermore, upholding proper aseptic techniques and working in a clean environment is imperative to prevent contamination.

For this work, *Penicillium chrysogenum, Aspergillus brasiliensis*, and *Purpureocillium lilacinum* were subjected to testing using both the MALDI Biotyper SOP for Cultivation and Sample Preparation for Filamentous Fungi and Biolog's novel method and proprietary media. Our novel method enabled all three organisms to be successfully identified at the species level, in contrast to the standard Bruker method, which only yielded a passing score for *A. brasiliensis*.

We compared the growth of these molds on all media and concluded our BPM media works best when extracting the filamentous fungi organisms to avoid possibly sampling any agar for the sensitive extraction the MALDI-TOF requires.

Using the common Bruker extraction method, the species identification threshold was met by only *A. brasiliensis* (Figure 1) when plated on MMA and incubated for 24 hours. However, the other two organisms exhibited visible agar contaminants, leading to their classification as not entirely pure and thus failing to meet the species identification threshold, as indicated in Figures 1 and 2.

After the 48-hour incubation, the test organisms again failed to achieve scores that surpassed the required threshold with the standard Bruker method. Interestingly, *A. brasiliensis* was again the only organism that had a nearly passing score, but on a different media than at 24 hours (B2ME). Although a visual pellet was obtained for all three organisms after lysis and washing, they did not have sufficient purity due to agar contaminants.

Very little to no growth can lead to less precise identification and reduced biomass for sampling. Acquiring enough sample from the plate can be challenging due to the need to avoid agar contamination. The presence of contaminants within the sample can result in low match scores. Furthermore, filamentous fungi may embed within the agar as it grows, making it challenging to scrape off without introducing agar contaminants. The use

of broth media presented a different challenge, primarily due to difficulties in the sample cleansing process. This challenge stemmed from the inability to prevent broth contamination while preserving the sample's viability. The absence of a lysing agent in the Bruker protocol also underscores the need to adequately break down chitin cell walls for sufficient protein extraction.

The newly developed Biolog method, assisted by the addition of using a lysing reagent to facilitate cell lysis, outperformed the Bruker MALDI Biotyper SOP for Cultivation and Sample Preparation for Filamentous Fungi. The initial non-passing scores transitioned to species-level scores in a significantly shorter processing time, specifically within 24 hours of incubation, as depicted in Figure 1. This demonstrates the potential of our new in-house method to expedite and enhance the identification process for filamentous fungi.

In summary, applying Biolog's innovative method for harvesting filamentous fungi on proprietary media greatly improves species identification with a significantly accelerated extraction process compared to the common Bruker method.

Although the cultures adhered to the same chronological timeline, their physiological ages diverged. This is exemplified by *A. brasiliensis*, which achieved a passing score after 24 hours (as depicted in Figure 2), while the other two cultures did not. This disparity suggests that they follow distinct physiological timelines. It is essential to recognize, through visual observation by an experienced laboratory scientist, when an organism has matured sufficiently to be removed from the incubator and subjected to identification.

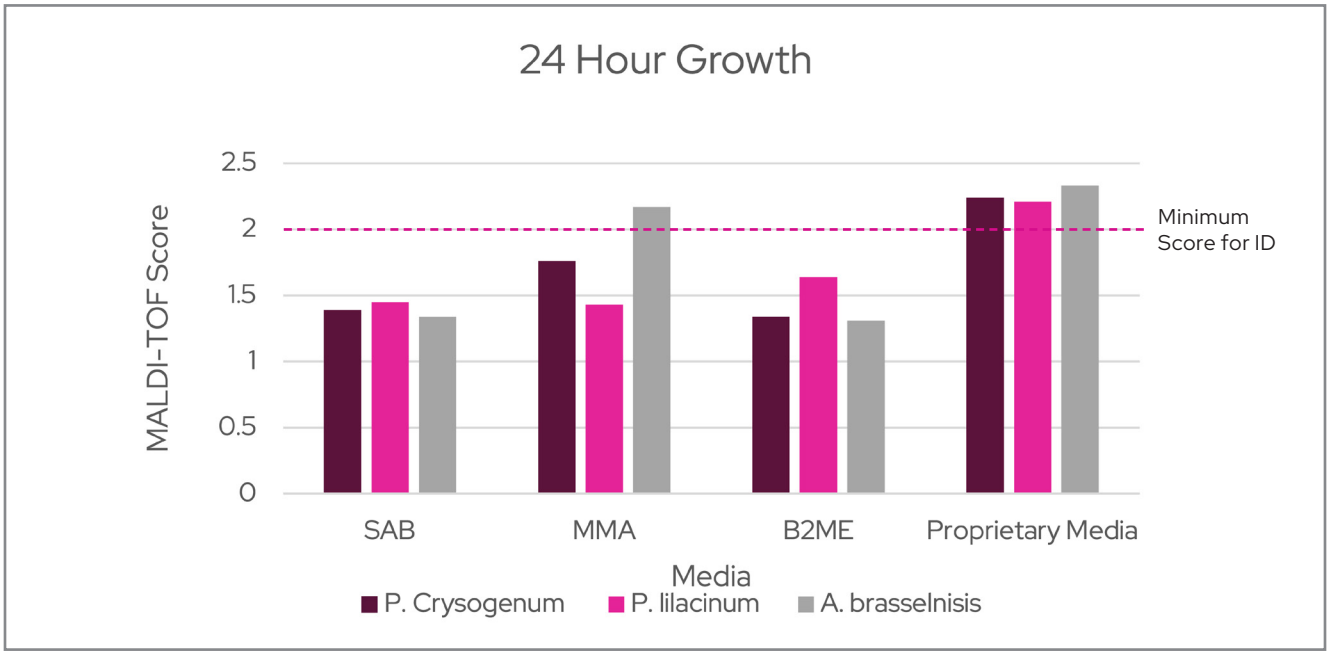

Figure 2. 24-hour filamentous fungi culture growth results on different media vs their MALDI-TOF Score. The score above 2.0 is required to obtain a secure genus identification and probable species identification and any score above 2.3 is a highly probable species identification.

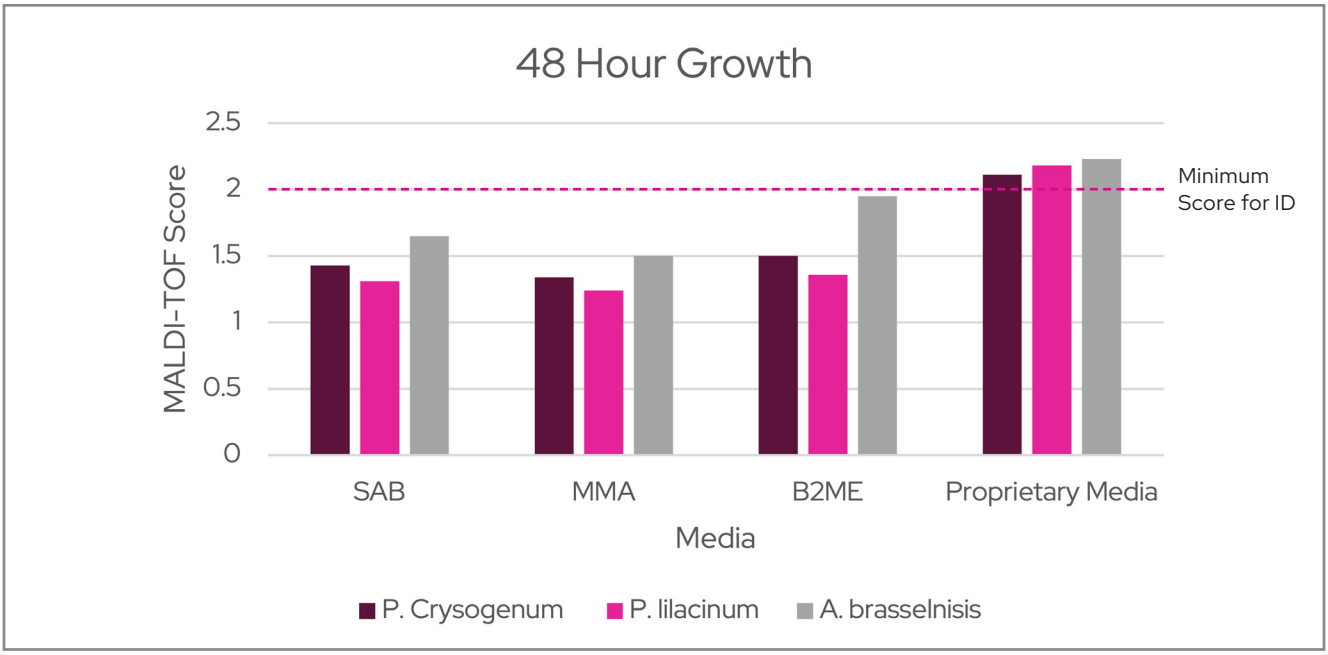

Figure 3. 48-hour filamentous fungi culture growth results on different media vs their MALDI-TOF Score. The score above 2.0 is required to obtain a secure genus identification and probable species identification and any score above 2.3 is a highly probable species identification.

Conclusions

Biolog Lab Services has developed a new method to overcome the limitations of existing protocols, due to the challenges of acquiring sufficient and high-quality sample from the filamentous fungi. Obtaining a substantial sample from a plate can be difficult, as it is essential to prevent agar contamination, due to the instrument requiring a pure sample of the mold. The use of broth media also poses its challenges, as it is necessary to cleanse the sample thoroughly to avoid any broth contamination while maintaining sample viability. Using a lysing agent is beneficial for breaking down the chitin cell walls more effectively, extracting proteins.

The Biolog method was created to expedite production processing and increase the likelihood of achieving species identification. The plates incorporate antibiotics to inhibit bacterial growth as well as a membrane to prevent agar contamination. Biolog's novel method, featuring enhanced lysing capabilities, effectively breaks down the chitin cell walls, enabling the retrieval of essential proteins for accurate identification. Furthermore, the utilization of proprietary media reduces harvesting time and assures the absence of agar, yeast, or bacterial contamination during the identification of filamentous fungi organisms.

While the choice of materials holds significance, the employed technique carries equal weight. Scientists at Biolog's Lab Services undergo comprehensive training in various identification methods such as MALDI-TOF, Sanger Sequencing, cellular metabolic characterization, growth kinetics, and phenotype identification using Biolog's Odin instrument. and Biolog scientists regularly perform these methods to help customers accurately and rapidly identify microbial species in a multitude of sample types. We have developed this new method for mold identification to ensure our customers get the most accurate ID possible.