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Comparison of Techniques for Protein Extraction from Fungi Found on a Radiopharmaceutical Manufacturing Plant

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1. Introduction

The knowledge and control of microbial flora in radiopharmaceutical production areas is one of the main challenges, especially in the production of sterile medicines. The environmental monitoring of air, water and others surfaces in the manufacturing and adjacent areas is essential to check the effectiveness of to the procedures to control contamination by air or by mechanical transfer. Monitoring results are used to release pharmaceutical products, to investigate quality deviations and to take preventive actions against microbial contamination of products [1].

Environmental monitoring generally comprises the exposure of plates containing culture medium to the air in the monitored area and surfaces associated with the production process and counting airborne particles [2]. Monitoring viable particles (bacteria and fungi) generates quantitative results, analyzed against the alert and action limits established for each monitored area, according to its cleanliness grade [3]. Annex 1 is enhancing the need of qualitative analysis through identification of microorganisms that indicate deterioration in the state of cleanliness or that are difficult to contain, such as spore-forming bacteria and filamentous fungi [4].

The identification of filamentous fungi, in particular, benefits from advanced molecular biology techniques, such as mass spectrometry [5] and DNA fingerprinting [6]. However, in laboratories with limited technical, personnel and budgetary resources, advanced techniques are difficult to implement. In this context, we propose as an alternative the fingerprinting of whole-cell proteins, using simple and less expensive laboratory tools through Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique. Several studies have proven the potential and utility of whole-cell protein profiling by SDS-PAGE in bacterial identification [7, 8], while less is known about mold and yeast profiling [9].

The introduction of whole-cell protein fingerprinting into routine analysis follows the path: (i) selection of the most appropriate technique for protein extraction; (ii) application of the selected technique to samples of filamentous fungi isolated from environmental monitoring, previously identified by genus or species; (iii) assembly of a library with the protein profiles characteristic of each genus or species; (iv) application of the technique to new isolates, not yet characterized; (v) validation of the method, whose ability to identify new isolates must be proven. In this work, we carried out the first of those steps, using the mold *Aspergillus brasiliensis* as a reference microorganism. The genus *Aspergillus* is frequently found in radiopharmaceutical production areas, transported mainly by air.

2. Methodology

Three techniques for protein extraction from filamentous fungi were evaluated. For each technique, five replicate extractions were performed, using *Aspergillus brasiliensis* (NCPF 2275; Biomérieux) as reference microorganism, cultivated in 90 mm Petri dishes with 4% Sabouraud dextrose agar at 22.5 ± 2.5 °C for 4 days in the dark. Protein extracts were frozen at -20 °C until use. Extract samples were analyzed for protein content by Bradford method [10]. The proportion between the total mass of extracted proteins (m_p) and the initial mass of mycelium (m_m), m_p/m_m, was calculated for each replicate.

Technique M1 [11] –130-240 mg of mycelium were extracted from the culture plate into a microtube, to which extractor solution (TRIS 0.618 M, pH 6.8) was added in a proportion of 1 mL of solution to 300 mg of mycelium. While kept on ice bath, the suspension was crushed with a glass capillary for five minutes, and then maintained at 5.0 ± 3.0 °C for 12 hours. The suspension was centrifuged (40,000 g, 30 minutes, 5 °C), and the supernatant was collected as the protein extract.

Technique M2 [7] – From the culture plate, 240-290 mg of mycelium were extracted into a microtube. The mycelial mass was washed three times with the addition of 1 mL of 0.9% NaCl, followed by vortexing and centrifugation (12,000 g, 20 minutes, 4 °C). After adding 1 mL 0.1% SDS, the suspension was briefly vortexed and transferred to a glass bottle. The flask was subjected to four cycles with 30 s of sonication and 10 s of rest (Thornton GA 900 sonicator, INPEC), and the suspension was then transferred to a microtube, centrifuged (5,000 g, 10 minutes, 4 °C), and the supernatant collected as the final protein extract.

Technique M3 [12] —110-160 mg of mycelium were extracted from the culture plate into a microtube, to which 2 mL of deionized water was added. After centrifugation (5,000 g, 10 minutes, 20 °C), the supernatant was discarded and the mycelial mass was air-dried at 37 °C for 24 hours. The mycelium was then crushed with a glass capillary for five minutes, and deionized water was added in a proportion of 2 mL of water for 200 mg of mycelium. The suspension was briefly vortexed, kept at 5 °C for one hour and centrifuged (600 g, 10 minutes, 4 °C). The supernatant was collected as the protein extract.

The comparison between the techniques was carried out by applying the Kruskal-Wallis test, with Dunnett's post-test for multiple comparisons (GraphPad Prism 5.0 software) on the sets of m_p/m_m values from the three techniques.

3. Results and Discussion

We selected three techniques for protein extraction from filamentous fungi based on molds and bacteria literature review. As shown in Fig. 1, extraction with 0.618 M TRIS (pH 6.8) provided the highest yield among the three techniques, with an average of 1.2 µg of total proteins per 1 mg of mycelium, with adequate reproducibility (variation coefficient of 14.3% in the quintuplicate).

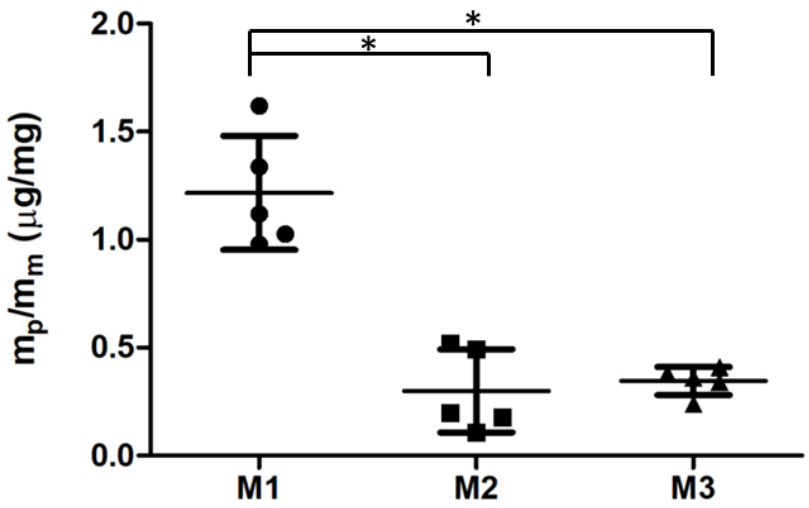

Figure 1: Protein extraction yield (m_p/m_m) using three different techniques. M1: extraction with 0.618 M TRIS (pH 6.8); M2: extraction with 0.1% SDS/sonication; M3: extraction with deionized water; mp/mm: total mass of extracted proteins/mass of mycelium. The graph shows the means and standard deviations of the five replicates. Statistically significant differences between techniques ($P < 0.05$) are marked with (*)

Extractions with 0.1% SDS/sonication or deionized water showed much lower yields than the first technique (respectively, 0.30 µg and a maximum of 0.35 µg per 1 mg of mycelium). Regarding Technique M3, there is a reference [12] to the extraction of proteins from fungi of the genus *Alternaria* with deionized water, which resulted in a protein concentration between 2.2 and 4.5 $mg.mL^{-1}$, higher than that obtained in this work (maximum 0.062 $mg.mL^{-1}$). The morphological differences between *Alternaria* and *Aspergillus* genera may contribute to the discrepancy between extraction yields, a hypothesis that extends to other techniques. This hypothesis will be tested in a later stage of this work by using a single extraction technique against twenty two species of filamentous fungi, which were isolated from environmental monitoring.

The low extraction yield using Technique M2 may be due to sonication being unable to break the fungal cell wall during mycelium disruption, with only partial release of the protein content of the cells [13]. Since the ideal protein extraction protocol, aiming at fungal proteomics, would imply the reproducible capture of all protein species from the proteome with low contamination by other molecules [14], we conclude that Technique M1, with a higher ratio m_p/m_m, turns out to be the most appropriate for the whole set of the experiments with filamentous fungi isolated from environmental monitoring. Furthermore, the buffering capacity of the TRIS solution at physiological pH, unlike the other extraction solutions, favors the use of the protein extract in other biotechnological applications.

4. Conclusions

The evaluated techniques were ease of execution after making some adaptations to laboratory conditions and availability of reagents and equipment. The extraction of proteins with TRIS 0.618 M pH 6.8 from *Aspergillus brasiliensis*, a filamentous fungus model, was the most appropriate technique.

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