DEVELOPMENT OF HPLC METHOD FOR A FUNGAL ANTAGONIST

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ABSTRACT

Fungi are considered promising sources in terms of biotechnological potential; they have symbiotic associations that can be considered beneficial or not. The association of fungi with plants is still little explored, including fungi having the ability to benefit the plant by producing secondary metabolites. In the search for new techniques to study microorganisms, it is of great importance to add HPLC, seeking efficient and faster processes. Thus, this research aimed to perform a chemical prospecting study of the fungal antagonistic, carrying out comparative studies between two culture mediums, Potato dextrose in one and yeast extract, peptone and glucose in the other, studying their behavior and seeking to characterize the compound(s) through extraction with ethyl acetate and as the mobile phase water plus trifluoroacetic acid (0.5%) and acetonitrile plus trifluoroacetic acid (0.5%) were used as solvents. The fungal antagonist used was cultivated in PD broth for seven days at 25°C in a shaker rotating at 150 rpm. After extraction, it was possible to observe that the fungus evaluated presented three major substances with retention times of 11 min, 16.9 min and 19 min. This result demonstrated the importance of the fungal antagonist as a promising source of bioactive molecules.

Keywords: Keyword 1. HPLC. Keyword 2. Antagonist. Keyword 3. Secondary metabolites.

1 INTRODUCTION

Studying the culture medium is extremely important for the development of biotechnological processes as it is through it that essential nutrients are supplied for the growth and development of microorganisms. Understanding the culture medium's composition and the quantity of its components is essential to guarantee the efficiency of microorganism growth.

For a more specific study to know the identity of the compounds in the mixture, chromatographic techniques are necessary, such as high performance liquid chromatography (HPLC). This technique allows the separation of compounds and their identification by comparing the retention times in the column of synthetic standards of these compounds, analyzed by at least two different mobile phases, with the construction of calibration curves for each of the standards[2]. According to Vibha et al. (2012), many different strategies for developing chromatographic methods are used and for this, a more suitable mobile phase, a column, a column temperature, a wavelength and an appropriate gradient must be found that provide compatibility and stability for the sample being analyzed.

Historically, fungi are among the microorganisms that have contributed most to products and processes of great importance for the population's well-being. This fact has its origin in the ability of these beings to produce molecules of great biological value that arise from their secondary metabolism. These molecules are thus called secondary metabolites[5]. Secondary metabolites are characterized as low molecular weight substances produced by a wide spectrum of organisms and capable of presenting various biological potentialities, therefore being the target of studies around the world[4].

Thus, this research aimed to perform a chemical prospecting study of the fungal antagonistic, carrying out comparative studies between two culture mediums, Potato dextrose in one and yeast extract, peptone and glucose in the other, studying their behavior.

2 MATERIAL & METHODS

Preparation of the culture medium: The culture medium, Yeast Extract, Peptone and Glucose broth (YPD) and potato dextrose broth (PD), were weighed in a 250 mL Erlenmeyer flask and after dilution, they were autoclaved at 120º C for 20 min. After sterilization, the broths were shaken at 150 rpm for 7 days.

Extraction[7]: The broths were then filtered through filter paper and approximately 150 mL of the filtrate was placed in a volumetric flask and 10 mL of ethyl acetate (Neon 99.5% PA) was added for extraction. After shaking, the supernatant was separated and reserved in a glass bottle and left at room temperature for the sample to dry completely. After drying, 2 ml of 50% acetonitrile was added and filtered with a syringe filter (PES 30 mm X 0.45 µm) and the samples were stored at 2.5º C until analysis.

Analysis: The samples were analyzed using high-performance liquid chromatography (HPLC - PerkinElmer). The column was maintained at 45°C throughout the analysis and detection was at 330 and 225 nm. For the gradient, the mobile phase used was a mixture of + UV/HPLC trifluoroacetic acid (TFA - 0.05%) and Acetonitrile + TFA (0.05%) with a flow rate of 0.5 mLmin-1 and an injection volume of 10 µL.

Tested methodologies[8]: chromatographic conditions, culture media were analyzed by four different methods. Method 1: The mobile phase was a gradient of water +TFA and Acetonitrile + TFA from 85:15 to 0:100% (v/v) in 40 minutes, followed by 10 min under the same conditions and returned 3 min to 85:15 , using C18 Analytical Column, Particle size: 5um, Length: 150mm, Internal diameter 4.6mm. Method 2: The mobile phase was a gradient of water + TFA and Acetonitrile + TFA from 85:15 to 0:100% (v/v) in 20 minutes, followed by 3 min under the same conditions and returned 3 min to 85:15 , using C18 Analytical Column under the same conditions as Method 1. Method 3: The mobile phase was a gradient of water + TFA and Acetonitrile + TFA, 5 min from 85:15 to 0:100% (v/v) in 20 minutes, followed by 10 min under the same conditions and returned 3 min at 85:15, using Analytical Column C18, under the same conditions as Method 1 and Method 4: The mobile phase was a gradient of water + TFA and Acetonitrile + TFA 5 min of 85: 15, followed by the same conditions for another 10 min (85:15) and to 0:100% (v/v) in 17 minutes, followed by 10 min in the same conditions (0:100) and returned 3 min to 85:15 , using the column Column: Nucleodur C18, Particle size 5um, internal diameter 4.0mm, length 250mm.

Fungus Test: The standard fungal antagonist was inoculated into the PD broth and incubated under agitation at 150 rpm for 7 days at 25º C. After growth, extraction was carried out by ethyl acetate, using Method 4.

3 RESULTS & DISCUSSION

Evaluating the YPD broth and the PD broth using Method 1, it was possible to observe that the YPD broth contains a lot of residue, which could interfere with the reading of the microorganisms inoculated in this medium. Therefore, to choose the best culture medium, which would not interfere too much with a microorganism, the tests were continued in PD broth. In the PD broth, methods 2, 3 and 4 were tested and it was possible to observe that the best method was four (4), where it was possible to observe a smaller amount of medium residue. The selected standard fungal antagonist showed the presence of medium absorption compounds (between 12 to 17 min and between 21 to 23 min) and high absorption in the extracts (between 11 to 11.5 min, between 16.9 to 17 min and between 19 to 20 min.

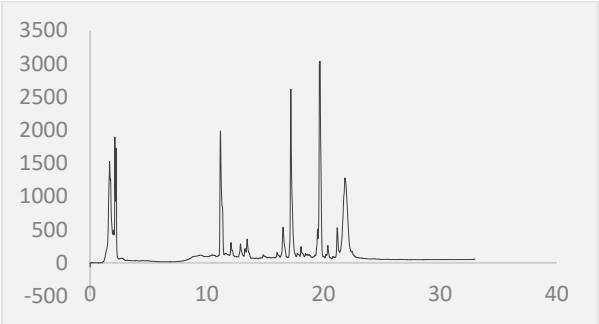

Figure 1. Chromatogram of the fungus extract after 7 days of growth in potato dextrose broth (PD).

By analyzing the chromatogram referring to the fungal extract (Figure 1), the presence of three major substances was verified with retention times of 11 min, 16.9 min and 19 min. Therefore, in future studies, another purification step will be carried out to isolate and identify these biocompounds.

Fungi are organisms known to produce a wide range of bioactive compounds, which is why research from years ago still continues to be required to isolate and identify new metabolites[1]. This is a challenge that can bring substantial benefits to several productive sectors[3]. Hence the importance of opening this area of interest in laboratories focused on the conservation and characterization of microbial biodiversity[6].

Gil and Vasundhara (2019) studied an endophytic fungus isolated from a non-Taxus Terminalia plant and the fungus was cultivated in potato dextrose broth and extracted with ethyl acetate, reading on HPLC, under conditions similar to our work. The authors observed that the HPLC readings were quite similar obtained by UV spectroscopy and FTIR analysis, corroborating that our work is heading in the right direction.

CONCLUSION

We concluded that the best methodology tested was methodology four, where the wavelength was 225 nm and the C18 column had a particle size of 5um and an internal diameter of 4.0mm. Further research is necessary to identify and separate the biocompounds obtained by the standard fungal antagonist adopted in this work.

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