Research, Society and Development, v. 11, n. 16, e242111638088, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i16.38088

Study of the culture medium variation in the production of secondary metabolite pyrophen by endophytic fungus *Aspergillus niger* from *Harconia speciosa*

Estudo da variação do meio de cultura na produção do metabólito secundário pirofen pelo fungo endofítico *Aspergillus niger* de *Harconia speciosa*

Estudio de la variación del medio de cultivo en la producción del metabolito secundario pirofen por el hongo endófito *Aspergillus niger* de *Harconia speciosa*

Received: 11/17/2022 | Revised: 11/27/2022 | Accepted: 11/29/2022 | Published: 12/06/2022

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Abstract
Pyrophen, a natural product, is a pyrone derivative from L-phenylalanine and isolated from different *Aspergillus* species. This compound exhibits promising anticancer activity, and various studies on its synthesis and biological activity have been reported. Thus, this study aimed to understand the substrate-related factors that influence the production of pyrophen and fungal biomass. So, the endophytic fungus *Aspergillus niger*, isolated from the plant species *Hancornia speciosa* (commonly known as mangabeira), was cultivated in different culture media (PDB, CZAPEK, and mangaba juice), ethyl acetate extracts were obtained for these different culture media. A high-performance liquid chromatography method was developed with a PDA detector to quantify pyrophen in these different extracts. This method evaluated the effect of other varying culture media and fungal growth times on the yield of this substance. The highest yield was obtained with the mangaba juice, thus revealing that this medium was better for cultivation. Finally, evaluation of the effect of cultivation time in PDB (Potato Dextrose Broth) medium reveals that the ideal fermentation period to produce pyrophen.
Keywords: Mangaba juice; Pyrophen; *Aspergillus niger*; HPLC.

Resumo
O pirofen, um produto natural derivado de pirona obtido do aminoácido L-fenilalanina e isolado de diferentes espécies de *Aspergillus*. Este composto apresenta atividade anticancerígena promissora, e vários estudos sobre sua síntese e atividade biológica têm sido relatados. Assim, o objetivo deste estudo foi entender os fatores relacionados ao substrato que influenciam a produção de pirofen e a biomassa fúngica. Então, o fungo endofítico *Aspergillus niger*, isolado da espécie vegetal *Hancornia speciosa* (vulgarmente conhecida como mangabeira), foi cultivado em diferentes meios de cultura (PDB, CZAPEK e suco de mangaba) sendo obtidos extratos acetato de etila para esses diferentes meios de cultura. Para quantificar o pirofen nesses diferentes extratos, foi desenvolvido um método por cromatografia líquida de alta eficiência com detector DAD. Este método avaliou o efeito dos diferentes meios de cultura e o tempo de crescimento de fungo no rendimento da substância. o maior rendimento foi obtido com o suco de mangaba, revelando que este meio

foi melhor para o cultivo. Por fim, a avaliação do efeito do tempo de cultivo em meio PDB revelou que o período ideal de fermentação para a produção de pirofen foi de 12 dias.
Palavras-chave: Suco de mangaba; Pirofen; *Aspergillus niger*; HPLC.

Resumen
Pirofen, producto natural derivado de la pirona obtenida del aminoácido L-fenilalanina y aislada de diferentes especies de *Aspergillus*. Este compuesto muestra una actividad anticancerígena prometedora y se han informado varios estudios sobre su síntesis y actividad biológica. Por lo tanto, el objetivo de este estudio fue comprender los factores relacionados con el sustrato que influyen en la producción de pirofeno y la biomasa fúngica. Después, en este estudio se cultivó el hongo endófito *Aspergillus niger*, aislado de la especie vegetal *Hancornia speciosa* (comúnmente conocida como mangabeira), en diferentes medios de cultivo (PDB, CZAPEK y jugo de mangaba), obteniendo extractos de acetato de etilo para estos diferentes medios de cultivo. Para cuantificar el pirofeno en estos diferentes extractos, se desarrolló un método de cromatografía líquida de alta resolución con detector DAD. Este método evaluó el efecto de diferentes medios de cultivo y el tiempo de crecimiento del hongo en el rendimiento de la sustancia. el mayor rendimiento se obtuvo con jugo de mangaba, revelando que este medio era mejor para el cultivo. Finalmente, la evaluación del efecto del tiempo de cultivo en medio PDB reveló que el período ideal de fermentación para la producción de pirofeno fue de 12 días.
Palabras clave: Jugo de mangaba; Pirofen; *Aspergillus niger*; HPLC.

1. Introduction

Researchers worldwide have investigated endophytic fungi because these microorganisms produce biologically active compounds (Ióca et al., 2016; Macedo et al., 2018). For example, Aspergillus produces various bioactive compounds that exhibit antimicrobial and anticancer activities as pyrophen (**1**). Therefore, the natural product pyrophen (**1**), a pyrone derivative from L-phenylalanine, was first isolated from *Aspergillus niger* (Barnes et al., 2003). Pyrophen (**1**) has been isolated from other *Aspergillus* species, such as *Alternaria alternata* (Astuti et al., 2016; Astuti et al., 2020; Hai et al., 2020). This compound has shown anticancer activity, and various studies on its synthesis and biological activity have been previously reported (Reber et al., 2018; Quang et al., 2022). In recent decades, the search for bioactive secondary metabolites from renewable sources has intensified (Strobel et al., 2004; Gunatilaka et al., 2006; Reber et al., 2018; Oladipo et al., 2022), with microorganisms, particularly fungi, as alternatives. One of the main advantages of working with microorganisms is the control of operational processes because compared with plants, fungi grow faster in a shorter period and space, where the cultivation conditions can be changed to direct the production of metabolites of interest (Pearce et al., 1997; El-Bondkly et al., 2021). Fermentation processes, for example, can be optimized by changing the substrate and controlling the temperature, pH, and cultivation time, thus leading to a significant increase in the production of the target substance (Alcano et al., 1994).

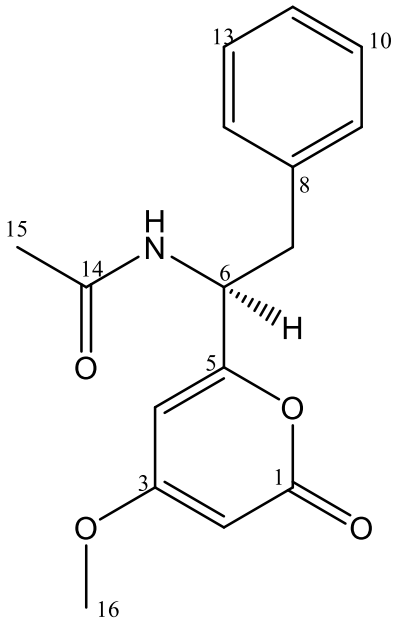
Production of compounds isolated from fungi can be optimized by adjusting the fermentation process and substrate, which leads to a better output of the target compound (Demain et al., 2000), thus being a renewable source. In addition, the production of fungal biomass must be optimized. Fungal biomass has several advantages for industrial exploitation owing to its ability to grow in cheap media and ease in harvesting. In addition, industries use fungal biomass to remove effluent dyes (Andrade et al., 2008). Aspergillus is the most widespread saprophytic fungus in terrestrial environments, which renders it an excellent choice. Previous work performed by our research group demonstrated that the fungus *A. niger* isolated from *H. speciosa* produced pyrophen (**1**) as the main metabolite when grown in PDB for 28 d. The aim of this study was to understand the substrate-related factors that influence the production of this compound and fungal biomass. Finally, the influence of the fermentation period was investigated. The media chosen for the study were PDB, Czapek, and mangaba juice. Mangaba juice was selected because it was isolated from this plant.

2. Methodology

2.1 General experimental procedures and reagents

The liquid chromatography system consisted of a SHIMADZU PROMINENCE LC-6A chromatograph model equipped with an M2DA 330 PhotoDiodo-Array (PDA) and a communications module. The LC-Solution Multi-PDA software was used to process the data. The analyses were conducted on a Shim-pack ODS column (250 mm × 4.6 mm; internal diameter (i.d.), 5 μm; Shimadzu). Acetonitrile (chromatographic grade) was supplied by Mallinkrodt Baker, Inc. (Phillipsburg, NJ, USA). Water was purified using a Milli-Q Plus filter system (Millipore, Billerica, MA, USA). High-resolution electrospray mass spectrometry (HRESI-MS) was performed by direct injection into a quadrupole time-of-flight mass spectrometer ESI-TOF system (Waters-XEVO-G2XS-QTOF). NMR spectra were recorded on a Varian Inova 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). The samples were dissolved in $CDCl_3$, and the spectra were calibrated using solvent signals. Pyrophen (**1**) (Figure 1), used as a standard, was isolated from the PDB culture medium.

Figure 1 - Pyrophen isolated from *A. niger*.



Source: Authors.

2.2 Plant material

Plant samples *of H. speciosa* leaves were collected from the village of Santa Rita Park, São Cristóvão-SE, Brazil (11º 00'53" S; 37º12'23" W). The exsiccate of the plant species was deposited in the herbarium of the Federal University of Sergipe-UFS. The leaves of *H. speciosa* were processed at the Department of Chemistry, Federal University of Sergipe (UFS).

2.3 Fungal strain isolation

The surface of the leaves was sterilized by dipping the leaves into NaClO solution (2 %) for 5 min and 70 % ethanol for 1 min. The leaves were washed twice in sterile water for 10 min to eliminate epiphytic microorganisms, and subsequently dried. The endophytic fungi were isolated from fragments of the leaves (3−4 pieces) by placing them in Petri dishes containing potato dextrose agar (PDA) and antibiotics (gentamicin sulfate 1 mL/250 mL) to prevent bacterial growth (Silva et al., 2022).

Fungal growth was monitored daily until each mold reached 1–2 cm in diameter. The colonies were successively sub-cultured to obtain pure PDA strains. Pieces were grown continuously until a pure *A. niger* culture was obtained. The *A. niger*

strain was filed in the mycology collection of the Chemistry Department of the Federal University of Sergipe. The fungus was identified and classified by Dr. João Basílio Mesquita, professor at the Department of Agricola Engineering at the UFS and deposited in the library of the Department of Chemistry-UFS with the deposit code of P3P6F2.

2.4 Pyrophen isolation of AcOEt extract obtained from PDB liquid medium.

Aspergillus niger was cultivated in PDA for 7 d at room temperature, during which it was inoculated in 3 L of PDB liquid medium. The cultures were maintained at room temperature for 28 d. After the liquid fermentation period, the broth was separated from the mycelium by filtration, extracted three times with half the crop volume of ethyl acetate (AcOEt), and dried in a rotary evaporator to obtain the crude extract AcOEt-PDB (371.2 mg). The AcOEt extract was fractionated on a chromatographic column (column 15 cm × 3 cm silica gel) using a gradient of 200 mL of AcOEt/MeOH in the proportions of: 95/5, 90/10, 80/20, 50/50 and 0/100; 82 fractions were collected. The fractions were analyzed by TLC, and fractions with a single spot and even Rf were collected, thus resulting in pyrophen (fractions 11–17; 90/10 AcOEt/MeOH). The pyrophen (**1**), purified in the laboratory by column chromatography, was recrystallized. It has a melting point of 178 °C and it was used to prepare the standard solution at different concentrations.

2.5 Development of the analytical method and quantification of pyrophen (1) in the extracts

Chromatographic analyses were performed through RP-HPLC-UV using a Shim-pack ODS column (250 mm × 4.6 mm; i.d., 5 μm; Shimadzu) and an isocratic system consisting of methanol and water, with 0.1 % acetic acid as mobile phase (50/50 in 10 min). The flow rate was 1.0 mL/min, injected volume was 20.0 μL, and detection wavelength was 254 nm. The extracts were diluted in methanol to a concentration of 1.0 mg/mL, and 20 μL was injected into the analytical column in triplicate. The fermented broths were only filtered for injection, and the analytical conditions were the same as those used to construct the standard curve for analysis of the fermented broth; directly, without extraction, the volume injected was 50 μL, while maintaining the other chromatographic conditions. An excellent chromatographic resolution (above 1.5) was obtained for the peak corresponding to pyrophen (**1**).

2.6 Preparation of sample solutions, construction of linear analytical curves, and determination of limits of detection (LOD) and quantification (LOQ)

Analytical solutions were prepared by dissolving 5.0 mg of the sample in 5 mL of methanol, followed by filtration through a UNIFLO 25/0.2 PTFE syringe filter (Whatman, Schleicher & Schuell, Maidstone, UK). A dilution of the metabolite pyrophen (**1**) (0.0625 to 1.0 mg/mL) was prepared in methanol. Subsequently, triplicate injected 20.0 μL aliquots of these solutions were injected into the HPLC equipment. Linear analytical curves were obtained by plotting the areas of individual chromatographic standards. Correlation coefficients were calculated using Microsoft Excel. The LOD and LOQ values were determined based on the response (σ) standard deviation and the analytical curve (S) slope standard, using LOD = 3.3 σ/S and LOQ = 10 σ/S expressions (Carneiro et al., 2022).

2.7 Selectivity

The selectivity of the method was evaluated by comparing the UV spectra in the regions of the respective pyrophen (**1**) peaks in the linear analytical curve and the sample. All spectra matched, thereby confirming that no other metabolites were co-eluted with the target compound (Carneiro et al., 2022).

2.8 Production of pyrophen in function of culture medium and time

The fungal isolate was cultivated in PDA for 7 d at room temperature, during which it was inoculated in liquid medium PDB (2000 mL), Czapek (2000 mL), and mangaba juice (2000 mL), contained in 500 mL Erlenmeyer flask filled with 250 mL of medium. The culture was kept static at room temperature for 28 d, and was subsequently filtered and extracted three times with ethyl acetate (AcOEt) and dried in a rotary evaporator, thus providing the respective AcOEt extracts with 177.3 mg of extract for Czapek, 123.1 mg for PDB and 506.5 mg for mangaba juice. Fungal cultivation to obtain the growth curve in PDB medium fungus was inoculated in a 36 500 mL Erlenmeyer flask containing 250 mL of PDB medium. Every 3 d of cultivation, four Erlenmeyer flasks were filtered and extracted with AcOEt. The dry weight of the mycelium was determined, and the samples were filtered with filter paper to separate the mycelium from the fermented broth. The mycelia were then dried in a ventilated greenhouse for 72 h at 60 °C.

3. Results and Discussion

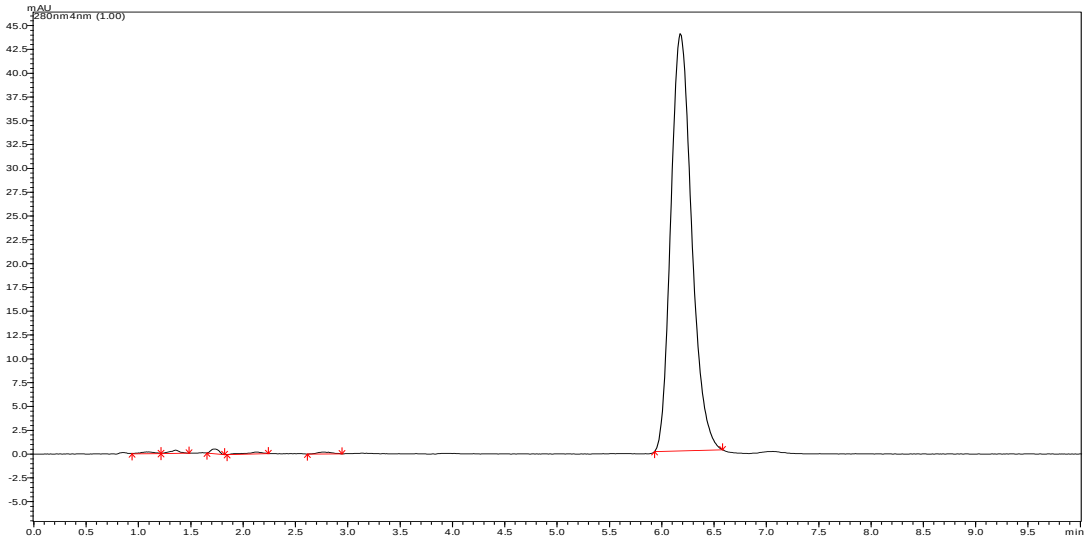
3.1 Identification and quantification of pyrophen (1) in PDB, CZAPEK, and mangaba juice extract

Standard pyrophen (**1**) was isolated from AcOEt extract, the fungus *A. niger*, cultivated in PDB culture medium. The substance was recrystallized and its ^{1}H and ^{13}C NMR spectra was analyzed, which shows the characteristic signals of pyrophen (**1**) (Figures S1 and S2, and Table S1) (Barnes et al., 1990). The purity of the pyrophen standard was confirmed by HPLC analysis (Figure 2). For quantification by HPLC-PDA of pyrophen (**1**) in different culture media, a calibration curve was obtained using the chromatographic method described, and an external standard at concentrations of 0.0625–1.0 mg/mL and injecting 20 µL, thereby obtaining the following equation for straight Y; Y = 0.000000251379–0.003574716871 (r^2 = 0.999). The limit of quantification (LOQ) was 0.002749 mg/mL, and the detection limit (LOD) was 0.0008248 mg/mL, which indicated that the developed method is adequate for determining the pyrophen (**1**) content in the analyzed extracts. Figure 2 shows typical HPLC-PDA chromatograms for the analytical standard pyrophen (**1**). The results of three extracts analyzed (PDB, CZAPEK, and mangaba juice extracts) by HPLC-PDA are presented in Table 1. The contents were 9.50, 11.5, and 23.5 % in PDB, CZAPEK, and mangaba juice extract, respectively. This confirms the previously reported high content of these compounds in different species of Aspergillus. The RSD values for pyrophen (**1**) concentrations were below 5 %, and the means obtained on different days did not differ significantly ($p < 0.05$). These results show that *A. niger* is promising for commercial production of pyrophen (**1**) in different growth media. The data presented in Table 1 reveals that the best medium to produce pyrophen (**1**) was mangaba juice. This may be related to the interaction between the fungus and mangabeira (*H. speciosa*). To evaluate the production of fungal biomass and the concentration of pyrophen (**1**) as a function of time, PDB and the standard commercial media were used as the culture media in this investigation.

3.2 Evaluation of dry mass production (biomass) by A. niger as a function of cultivation time in PDB

Evaluating biomass production data is essential in developing studies for various reasons, including optimizing cultivation conditions, production planning, and bioreactor design. The results obtained for biomass production of this fungus were consistent, thus exhibiting an increase in mycelial biomass until the 15th day of cultivation. After the 15th day of cultivation, a decrease was observed until the end of growth, as shown in the production biomass curve as a time function (Figure 3). Mycelial growth varies according to fungal species, genetic traits of the strain, culture medium, supplementation, light, and temperature (Zadrazil, 1998; Miles, 1997). The growth curve can be divided into several stages of fungal cultures; it begins with the adaptation of cells to the environment to which they are exposed and continues until their death (Andrade et al., 2008).

Figure 2 - Chromatogram of the pyrophen (**1**).



Source: Authors.

The chromatogram shows the peak of pyrophen (**1**) isolated from the endophytic fungus. This chromatogram shows the purity of the isolated compound, which was used in constructing the calibration curve for quantifying the metabolite in the culture medium and extracts.

Table 1 - Quantification of pyrophen (**1**) in the different extracts obtained from *A. niger*.

liquid growth medium	Extract mass (g/L)	Percentage of pyrophen in the extract
CZAPEK	0.0886 ±0.007	11.5 %
PDB	0.0615 ±0.003	9.5 %
Mangaba juice	0.2532 ±0.007	23.5 %

Source: Authors.

Table 1 shows mangaba juice as the most suitable substrate for fungus cultivation when the objective is the production of pyrophen (**1**) since the substance corresponds to 23.5% of AcOEt extract.

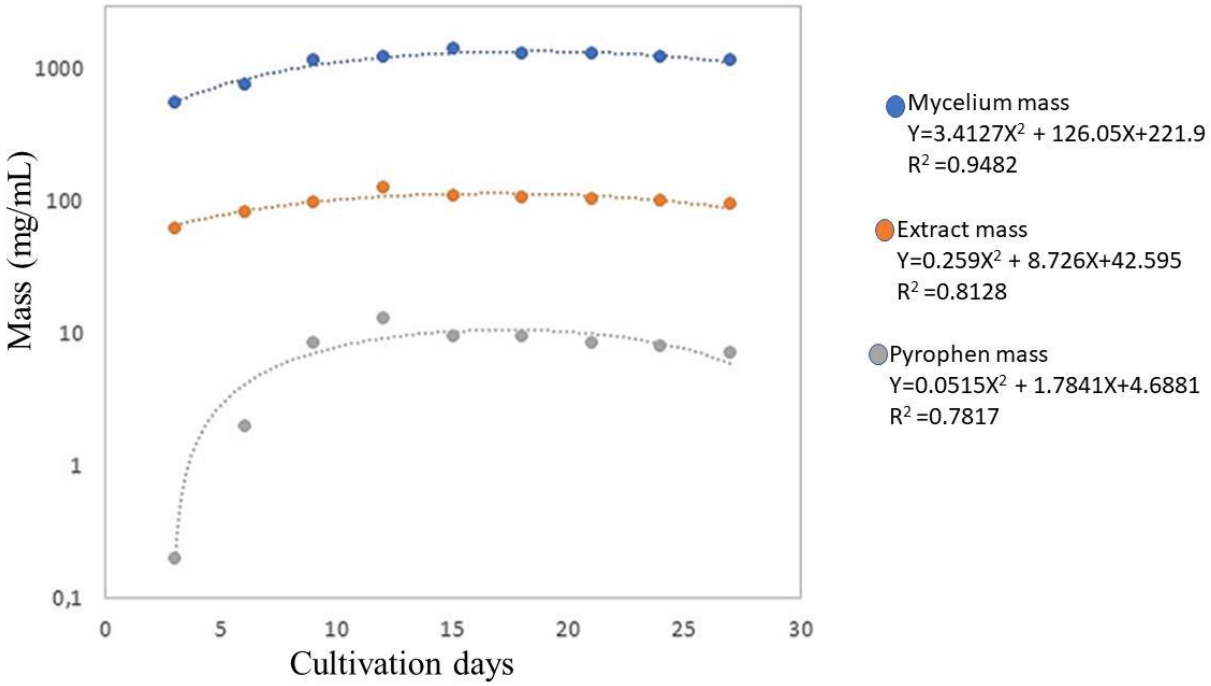
3.3 Evaluation of crude extract production and pyrophen (1) concentration as a function of cultivation time in PDB medium

Kinetic fungal growth can be defined as an increase in the number of microbial cells in a population, which is an exothermic process. The heat generated must be dissipated because high temperatures are not favorable for the growth of microorganisms and product formation (Mitchell et al., 1992; Ghildyal, et al., 1994). Because they are relatively simple in organization, endophytic fungi have a great power to multiply and adapt to different nutritional situations, thus modifying their metabolism with a lack of supply of nutrients to the culture medium. The kinetics of fermentation originates from chemical and enzymatic kinetics, consisting of a mathematical description of the metabolic processes of microorganisms grown under controlled conditions (Nielsen et al., 1992; Bamaalabong et al., 2020). Kinetic growth is an expression par excellence of the dynamic nature of microorganisms. Among the general methods available for the scientific investigation of fungal growth dynamics, the most commonly used are those based on kinetic aspects (Antinori et al., 2020; Van Niel, 1949).

The production of the AcOEt extract obtained from the PDB medium by *A. niger* followed the pattern of biomass

production, and the extracted mass reached the maximum value on the 12th day of cultivation. In contrast, the mycelial mass increased until the 15th day of cultivation. This explains the consumption of some substances (such as organic acids) present in the extracted biomass and the energy consumption (Figure 3). Pyrophen (**1**) production also reached the maximum point after 12 d of culture and the production of extracts; a decrease was observed at 27 d (Figure 3). Using the kinetic data obtained in the first quantification (Figure 3), we proposed a new method to better visualize the production of pyrophen (**1**) by *A. niger*.

Figure 3 - Production of micellium, AcOEt extract, and Pyrophen (1) in the cultivation of *fungus A. niger* in PDB medium for 27 days, analysis every 3 days.



Source: Authors.

Figure 3 relates the fungal biomass, extract AcOEt, and pyrofen (**1**) production to fungus cultivation days.

4. Conclusion

This study demonstrated that *A. niger* produces pyrophen (**1**) as the major metabolite. Pyrophen (**1**) was identified in the three culture medium used, PDB, mangaba juice, and CZAPEK, with mangaba juice being the best production method. The mangaba juice probably provides the fungus with the necessary nutrients for the production of pyrophen (**1**), which opens up a study perspective for different culture media for the production of this compound. The ideal period for the cultivation of *A. niger* in a PDB liquid medium was 15 d for biomass production and 12 d for the production of AcOEt extract and pyrophen (**1**) when inoculated with culture discs. The analytical method developed to quantify pyrophen (**1**) proved efficient because it enabled the identification and quantification of pyrophen (**1**) and the analysis of both the extract and fermented medium.

Supplementary material

Supplementary material related to this article can be found at https://rsdjournal.org/

Acknowledgments

The authors thank the National Council for Science and Technology (Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq –CNpq/INCT Process 465637/2014–0), Coordination for the Improvement of Higher Education Personnel

(Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES) and Inct Bionat for financial support and the researchers from Natural Products and Organic Synthesis Research Group (GEAPS-CNPq-UFES).

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