AN OSMAC STRATEGY FOR THE PRODUCTION OF ANTIMICROBIAL COMPOUNDS BY AMAZONIAN FUNGI

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ABSTRACT

This study aimed to assess the effectiveness of the OSMAC (One Strain Many Compounds) technique in influencing the production of secondary metabolites by Amazonian fungi and their antimicrobial activity. Two species of Amazonian fungi were used, each grown in different culture media. The produced metabolites were then extracted using ethyl-acetate, concentrated, and chemically characterized by TLC, FTIR, UV-vis, and HPLC. Additionally, they were tested for their antimicrobial activity against gram-positive and gram-negative bacteria, as well as yeasts. The results showed that all fungal extracts inhibited the growth of some of the tested pathogens. It was observed that the culture medium had a significant impact on the Minimum Inhibitory Concentration (MIC) value of the extracts. Chemical characterization indicated the presence of phenolic compounds, and the production profile was highly influenced by the culture medium used. The production of phenolic compounds may be associated with the observed antimicrobial activity. This study emphasizes the potential of Amazonian fungi as a rich source of new metabolites and highlights the importance of optimizing their production conditions using techniques such as OSMAC.

Keywords: OSMAC technique. Secondary metabolites. Antimicrobial activity. Phenolic compounds.

1 INTRODUCTION

Amazonian fungi are a rich source of new metabolites, and extracts containing these molecules can be a promising alternative to fight against pathogenic microorganisms [1,2]. To identify and optimize the production conditions of these bioactive components, several effective approaches can be used, such as the OSMAC (One Strain Many Compounds) technique [3]. This technique can be applied through co-cultivation, variation in cultivation conditions, and/or epigenetic modification, exposing the critical natural biogenetic capacity of fungi [4]. The OSMAC technique is a valuable tool to overcome the limitations of experiments performed under laboratory conditions. It allows the modulation of metabolite production by adjusting the culture medium and conditions [5].

The OSMAC technique is a powerful and effective strategy for discovering new metabolites with biological potential. Therefore, it deserves further exploration. In this sense, the objective of this research was to assess the variation in the production of secondary metabolites by Amazonian fungi and their antimicrobial activity using the OSMAC technique.

2 MATERIAL & METHODS

The strains of *Talaromyces pinophilus* CCM-UEA-F0414 (endophytic fungi isolated from *Myrcia guianensis*) and *Penicillium* sp. CCM-UEA-F0591 (epiphytic fungi isolated from *Aniba canelilla*), used in this study, are deposited in the Central Microbiological Collection of the Amazonas State University (CCM/UEA). The strains were reactivated on potato dextrose agar (PDA). To produce the fungal extract, 300 µL of the cell suspension (10^6 conidia/mL) was inoculated in Erlenmeyer flasks containing 150 mL of different culture media (Table 1), at pH 6.0. The cultivation was performed under static conditions, at 28 °C [6]. After 14 days, the metabolites were extracted with ethyl acetate P.A (1:1) for 4 h (28 °C, 120 rpm). After filtration, the ethyl acetate fraction was collected, the solvent was eliminated by a rotary evaporator, and the extracts obtained were stored in a refrigerator at 4 °C.

The antimicrobial activity of the fungal extracts was evaluated using the microdilution method, in the following concentrations: 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078 mg/mL. After incubation of the microplates, the growth of microorganisms was revealed using TTC for yeasts and resazurin for bacteria. The lowest concentration of the extracts capable of inhibiting microbial growth was considered the Minimum Inhibitory Concentration (MIC) [7]. Levofloxacin (0.25 mg/mL) and terbinafine (0.40 mg/mL) were used as reference antibiotics against bacteria and yeasts, respectively.

The fungal extracts were characterized by thin layer chromatography (TLC), 5 µL of extract (5.0 mg/mL in methanol), were applied to a silica gel chromatographic plate, then eluted with dichloromethane:methanol:formic acid (90:8:2). After chromatographic running, the chemical classes were detected by visualization in ultraviolet light (254 and 365 nm), and with chemical developers: $AlCl_3$, $FeCl_3$, Dragendorff reagent, vanillin/H_2SO_4, and ceric sulfate.

Table 1 Culture media used for the cultivation of *T. pinophilus* CCM-UEA-F0414 and *Penicillium* sp. CCM-UEA-F0591.

Culture Medium	Composition
BDL (B)	Potato (200 g/L), D-glucose (20 g/L), Yeast extract (2.0 g/L)
Malt (M)	Malt extract (20 g/L)
ISP2 Modified (I)	Corn starch (4.0 g/L), Yeast extract (4.0 g/L), Malt extract (10 g/L)
CZAPEK modified (C)	D-glucose (20 g/L), $Fe_2(SO_4)_3$ (10 mg/L), K_2HPO_4 (1.0 g/L); $MgSO_4$ (500 mg/L), KCl (500 mg/L)
YES (Y)	Sucrose (150 g/L), yeast extract (20 g/L), and $MgSO_4$ (0.5 g/L)

The infrared spectrum of the fungal extracts was obtained in between 400 and 4000 cm^{-1}, using the spectrophotometer IRAffinity-1S (Shimadzu). The UV-visible absorption spectrum of the fungal extracts was obtained using a UV-VIS spectrophotometer (UV-1800, Shimadzu), between 200–900 nm. The chromatographic profile of the extracts (1.0 mg/mL in methanol) was obtained by ultra-high performance liquid chromatography with a linear array diode detector (Shimadzu Nexera XR–SPD-M20A), using a column CLC-ODS(M), 4.6 mm x 15 cm, 5µ particles. Acetonitrile:formic acid (0.1%) was used as mobile phase, in the proportion of 80:20 (v/v), 0.7 mL/min flow, oven at 40 °C and 4 µL volume of injection. Samples and standards were analyzed in 320 nm.

3 RESULTS & DISCUSSION

Table 2 displays the MIC of the fungal extracts obtained from the cultivation of *T. pinophilus* CCM-UEA-F0414 (fungi 3) and *Penicillium* sp. CCM-UEA-F0591 (fungi 15) in different media. The extracts were found to inhibit the growth of gram-positive and gram-negative bacteria, and yeasts. *C. albicans* was the most sensitive pathogen, as the extract from *T. pinophilus* grown in YES (3Y) and the one from *Penicillium* sp. grown in BDL (15B) presented the lowest MIC (0.078 mg/mL). On the other hand, *K. pneumoniae* was the most resistant pathogen. It was observed that the MIC value was impacted by the culture medium used to grow the microorganism, leading to variations in the MIC value, depending on the medium composition.

Table 2 Minimal inhibition concentration, MIC (mg/mL) of the ethyl acetate extracts obtained from different fungal cultivation conditions.

Tested microorganisms	MIC of fungal extracts									
	3B	3I	3Y	3C	3M	15B	15I	15Y	15C	15M
Escherichia coli (CCCD-E005)	-	5.0	-	-	5.0	5.0	-	-	5.0	5.0
Bacillus subtilis (CCCD-B005)	-	1.25	5.0	5.0	5.0	5.0	-	2.5	-	2.5
Enterococcus faecalis (CCCD-E002)	-	-	-	5.0	-	5.0	-	-	-	2.5
Klebsiella pneumoniae (CCCD- K003)	-	-	-	5.0	-	-	-	-	-	5.0
Pseudomonas aeruginosa (CCCD-P004)	5.0	5.0	5.0	-	-	5.0	2.5	-	5.0	5.0
Staphylococcus aureus (CCCD-S009)	-	5.0	5.0	0.156	-	2.5	2.5	-	-	-
Salmonella enterica (CCCD-S003)	-	-	-	2.5	-	5.0	-	-	-	5.0
Staphylococcus epidermidis (CCCD-S010)	-	5.0	5.0	1.25	-	2.5	0.313	-	-	5.0
Streptococcus mutans (NCTC 10449)	-	-	-	2.5	-	-	5.0	5.0	-	5.0
Candida albicans (CCCD-CC001)	0.156	0.156	0.078	0.313	0.156	0.078	-	5.000	-	0.625
Candida parapsilosis (CCCD-C004)	-	-	-	5.0	-	-	-	2.5	5.0	5.0
Candida tropicalis (CCCD-C002)	2.5	5.0	5.0	2.5	5.0	2.5	0.625	1.25	5.0	2.5

B = BDL; I = ISP2 Modified; Y = YES; C = CZAPEK modified; M = Malt. 3 = *Talaromyces pinophilus* CCM-UEA-F0414; 15 = *Penicillium* sp. CCM-UEA-F0591. - = Absence of antimicrobial activity.

TLC was performed to identify the main classes of metabolites present in each extract. The TLC plates stained with $AlCl_3$, $FeCl_3$, and vanillin indicate that the extracts are rich in phenolic compounds. This suggests a significant bioactive potential for these extracts, such as antioxidant, cytotoxic, and antimicrobial [9]. The presence of alkaloids was not observed in TLC plates stained with Dragendorff reagent, which is interesting considering the application of the extract to produce antimicrobials, as alkaloids are closely associated with cytotoxicity [10]. The presence of terpenes, revealed with ceric sulfate, was also not observed.

The infrared spectra obtained for both fungal extracts corroborate the results obtained by TLC, revealing a profile that indicates the presence of phenolic compounds. The infrared spectra show similarities with the spectrum presented by Pereira et al. [2]. An intense band at 3300 cm^{-1}, which indicates the presence of hydroxyl groups, a characteristic of phenolic compounds, is observed. This band is more clearly visible in the C medium for fungi 3, and in the Y medium for fungi 15, but it is present in all samples, except for 3M. Another prominent peak observed in both studies is at 1600 cm^{-1}, commonly associated with an aromatic ring stretch, another characteristic of phenolic compounds. This peak is observed in all samples, except for 15M.

All fungi extracts evaluated in this study showed absorption in the UV region. This corroborates the presence of phenolic compounds, as multiple conjugated bonds in their structure make them chromophores that absorves in this region[11]. The maximum absorption value for each extract is listed in Table 3. The variations in the FTIR spectra, TLC profile, and UV-visible spectrum for extracts obtained from the same fungus grown in different culture media suggest that the medium strongly influences the production of phenolic compounds.

Table 3 Maximum absorbance wavelength (λ_{max}) for each fungal extract.

Fungal extract	λ_{max} (nm)	Fungal extract	λ_{max} (nm)
3B	260	15B	270
3M	266	15M	263 and 350
3I	260	15I	260 and 340
3C	260 and 340	15C	260 and 350
3Y	251 and 288	15Y	270

The chromatographic profiles of the fungi extracts, determined by UHPLC-DAD, corroborate the results obtained by TLC, FTIR, and UV-visible, indicating the presence of phenolic compounds. The chromatograms of the extracts were compared with standards of phenolic compounds, such as caffeic acid, chlorogenic acid, quercetin, shaftoside, and vicenin-2.

The HPLC analysis results can be seen in Table 4. For each fungus, the culture medium that presented the highest value for each compound was highlighted in bold. It was also observed other peaks in the chromatograms for all extracts evaluated, indicating the presence of several other compounds in addition to the standards used in this work. The difference in the obtained results demonstrate that the culture medium influences the metabolite profile of the fungi.

Table 4 Peak area of phenolic compounds present in the fungal extracts, analyzed by UHPLC-DAD with detection at 320 nm.

Fungal extract	Peak area				
	Caffeic acid	Chlorogenic acid	Quercetin	Shaftoside	Vicenin-2
3B	19483	**8684**	0	**60645**	**878**
3M	0	0	0	1582	0
3I	0	0	0	0	0
3C	**60406**	0	0	0	0
3Y	0	1373	**15465**	1741	0
15B	**10270**	5514	0	2441	1953
15M	8399	**27412**	0	**32586**	**47790**
15I	3825	220	0	10491	0
15C	1322	1187	0	2785	0
15Y	2088	0	0	2496	0

4 CONCLUSION

The study's findings demonstrate the significance of the OSMAC strategy in influencing the variety of metabolites produced by fungi and their corresponding antimicrobial activities. This underscores the technique's importance in facilitating the discovery of new bioactive fungal metabolites. Additionally, the observed antimicrobial activity may be attributed to the presence of phenolic compounds in the extracts. These insights emphasize the potential for further use of OSMAC-guided approaches in the search for bioactive molecules with therapeutic implications.

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