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Antimicrobial potential of filamentous fungi growing on renewable substrates

Potencial antimicrobiano de fungos filamentosos cultivados em substratos renováveis

Potencial antimicrobiano de hongos filamentosos utilizando sustratos renovables

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Abstract
The bioprospecting of secondary metabolites with antimicrobial action produced by filamentous fungi isolated from various ecosystems using agro-industrial residues is of considerable importance, since it reconciles the discovery of new antimicrobial agents with the reduction of environmental impacts adding economic value to wastes. The present paper investigated the potential of filamentous fungi for antimicrobial metabolites production using alternative media based on corn steep liquor, cassava wastewater and glycerol as substrates in different combinations. From 15 filamentous fungi assayed in preliminary screening, *Paecilomyces variotii* UCP 0334, *Aspergillus flavus* UCP 0316, *Aspergillus foetidus* UCP 0360, *Aspergillus niger* UCP 1064 e *Aspergillus* sp. 74M4 showed higher activity against all tested bacteria. After submerged fermentation in media containing corn steep liquor, cassava wastewater and glycerol, *P. variotii* UCP 0334 exhibitted greater activity and spectrum of action with inhibition halos ranging from 7 to 28 mm in diameter against all Gram negative and positive bacteria tested regardless of the culture medium type. It is worth mentioning that all fungi in alternative medium containing cassava wastewater and glycerol (CWWG) showed inhibitory activity against 3 bacteria with inhibition halos ranging from 9 to 15 mm. On the other hand, the medium containing corn steep liquor and glycerol (CSLG) favored higher biomass yield for all fungi. These results stimulate further studies of identification and characterization of the secondary metabolites produced by the selected fungi with a view to discovering new antimicrobial drugs.
Keywords: Bioprospecting; Filamentous fungi; Metabolites; Antimicrobial activity; Agro-industrial waste.

Resumo
A bioprospecção de metabólitos secundários com ação antimicrobiana produzida por fungos filamentosos isolados de vários ecossistemas e cultivados em resíduos agroindustriais tem considerável importância, uma vez que, concilia a descoberta de novos agentes antimicrobianos com redução de impactos ambientais e agregação de valor econômico aos resíduos. A presente pesquisa investigou o potencial de produção de metabólitos antimicrobianos por 15 fungos filamentosos cultivados em meios alternativos a base dos resíduos agroindustriais milhocina, manipueira e glicerol em diferentes combinações. Dos 15 fungos filamentosos analisados no screening preliminar, foram selecionados *Paecilomyces variotii* UCP 0334, *Aspergillus flavus* UCP 0316, *Aspergillus foetidus* UCP 0360, *Aspergillus niger* UCP 1064 e *Aspergillus* sp. 74M4 por apresentarem atividade antibacteriana contra todas as bactérias testadas. Após fermentação submersa nos meios contendo milhocina, manipueira e glicerol, *P. variotii* UCP 0334 apresentou maior atividade e espectro de ação ao exibir os maiores halos (7 a 28 mm de diâmetro) de inibição contra todas as bactérias

Gram negativas e positivas testadas independentemente do tipo de meio de cultivo. Vale destacar que todos os fungos no meio alternativo contendo manipueira e glicerol (MAGLI) apresentaram atividade inibitória contra 3 bactérias com halos de inibição variando entre 9 e 15 mm. Por outro lado, o meio contendo milhocina e glicerol (MIGLI) favoreceu maior rendimento de biomassa em todos os fungos. Estes resultados estimulam mais estudos para identificação dos metabólitos secundários produzidos pelos fungos selecionados com vistas a descoberta de novas drogas antimicrobianas.
Palavras-chave: Bioprospecção; Fungos filamentosos; Metabólitos; Atividade antimicrobiana; Resíduos agroindustriais.

Resumen
La bioprospección de metabolitos secundarios con acción antimicrobiana producidos por hongos filamentosos aislados de diversos ecosistemas y cultivados en residuos agroindustriales es de gran importancia, ya que compatibiliza el descubrimiento de nuevos agentes antimicrobianos con la reducción de impactos ambientales y la agregación de valor económico a los residuos. La presente trabajo investigó el potencial de producción de metabolitos antimicrobianos por 15 hongos filamentosos cultivados en medios alternativos a base de residuos agroindustriales de maíz, manipueira y glicerol en diferentes combinaciones. De los 15 hongos filamentosos analizados en la selección preliminar, *Paecilomyces variotii* UCP 0334, *Aspergillus flavus* UCP 0316, *Aspergillus foetidus* UCP 0360, *Aspergillus niger* UCP 1064 e *Aspergillus* sp. 74M4 mostraron actividad antibacteriana contra todas las bacterias probadas. Después de la fermentación sumergida en medios que contienen maíz, manipueira y glicerol, *P. variotii* UCP 0334 mostró la mayor actividad y espectro de acción al exhibir los mayores halos (de 7 a 28 mm de diámetro) de inhibición contra todas las bacterias Gram negativas y positivas probadas independientemente del tipo de medio de cultivo. Vale la pena señalar que todos los hongos en el medio alternativo que contenía manipueira y glicerol (MAGLI) mostraron actividad inhibidora contra 3 bacterias con halos de inhibición que oscilan entre 9 y 15 mm. Por otro lado, el medio con licor macerado de maíz y glicerol (LMGLI) favoreció un mayor rendimiento de biomasa en todos los hongos. Estos resultados estimulan más estudios para identificar los metabolitos secundarios producidos por los hongos seleccionados con miras a descubrir nuevos fármacos antimicrobianos.
Palabras clave: Bioprospección; Hongos filamentosos; Metabolitos; Actividad antimicrobiana; Residuos agroindustriales.

1. Introduction

Antibiotic resistance is a global public health problem as it reduces the effectiveness of antimicrobial therapy and tends to increase the severity, incidence, and costs of infection. It is estimated that by 2050, about 10 million lives will be at risk annually as a consequence of the increase in infections with multidrug-resistant bacteria. The source of the problem is the indiscriminate use of antimicrobials in various sectors such as human and veterinary clinical practice, as well as in the food production of the animal and plant origin. Therefore, there is an urgent need to search for new antibiotics with different mechanisms of action (Muzammil et al., 2018; Rodrigues et al., 2018; Clancy & Nguyen, 2019; Collignon & Mcewen, 2019; Hopman et al., 2019; WHO, 2020).

Thus, the Brazilian megadiversity justifies the prospection of new microorganisms' species or strains with biotechnological potential to produce numerous metabolites with applications in various sectors of the industry (Sá Filho et al, 2021). Microorganisms are an important source of natural antibiotics and filamentous fungi are responsible for about 20% of all antibiotics produced (Silber et al., 2016; Al-Fakih & Almaqtri, 2019). Species of the genera *Aspergillus* and *Paecilomyces* have been widely explored in studies of prospection and optimization of the production of new metabolites (Abdel-Azeem et al., 2019; Al-Fakih & Almaqtri, 2019). Among the species of the genus *Aspergillus* most used in the production of secondary metabolites with biological activity for application in the pharmaceutical industry, it can be mentioned *A. flavus*, *A. niger*, *A. awamori*, *A. oryzae*, *A. nidulans*, *A. fumigatus, A. clavatus, A. glaucus, A. ustus* e *A. versicolor* (Wang et al., 2018; Felipe et al., 2019; Xu et al., 2020). On the other hand, in the genus *Paecilomyces, Paecilomyces variotii* species is one of the most studied regarding the production of antimicrobial metabolites (Zhang et al., 2016; Cen et al., 2021).

Agro-industry generate a large quantity of wastes which are destined for landfills or incorrectly discarded. However, agro-industrial residues are rich in nutrients, allowing their reuse as raw material to obtain high added-value products. Therefore, the valorization of agro-industrial residues is the key strategy for the development of several products, promoting investment opportunities and economic and environmental gains (Ricardino et al., 2018; Gmoser et al., 2019; Nascimento; Torre & Kadowaki, 2020; Freitas et al., 2021). Thus, the aim of this work was evaluating the biotechnological potential for the secondary metabolites production with antimicrobial action by filamentous fungi using the agro-industrial wastes corn steep liquor, cassava wastewater and residual glycerol, as alternative substrates in submerged fermentation.

2. Methodology

2.1 Microorganisms

For the screening of the production potential of antimicrobial compounds, 15 filamentous fungi isolated from Igarassu River, Municipality of Igarassu/PE, Brazil, from Caatinga soil - PE, Brazil and from mangrove sediments, Municipality of Rio Formoso/PE, Brazil were selected. For antimicrobial activity assays, *Escherichia coli* UCP 1575 and *Salmonella enterica* UCP 1550 were used as examples of Gram negative test bacteria and *Staphylococcus aureus* UCP 1576 and *Bacillus subtilis* UCP 1593 were used as Gram negative test bacteria. All microorganisms were kindly provided by the Culture Collection of the Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Brazil. The Culture Collection is registered in the World Federation for Culture Collection (WFCC). The fungi were maintained on potato dextrose agar medium and bacteria on nutrient agar medium at 5 °C.

2.2 Agroindustrial substrates

The corn steep liquor, nitrogen source, was provided by Ingredion Brasil Ingredientes Industriais LTDA, municipality of Cabo de Santo Agostinho-PE, Brazil. The glycerol, carbon source, was obtained by CETENE-PE, MCT, Brazil. The cassava wastewater, carbon source, came from the indigenous village Pankará, in Carnaubeira da Penha-PE, Brazil.

2.3 Agroindustrial culture media

In this work were formulated three agroindustrial media through mixture of the corn steep liquor, glycerol, and cassava wastewater according to Table 1.

Table 1. Composition of the alternative media for metabolites production with antimicrobial activity.

Compostos	CSLG	CWWG	CWW
Saline base * (mL)	87	87	87
Glycerol (mL)	10	10	-
Cassava wastewater (mL)	-	3	13
Corn steep liquor (mL)	3	-	-

* Saline base composition: KH_2PO_4: 0.23%, K_2HPO_4: 0.25%, $MgSO_4$: 0.1% e $FESO_4$ 0.1%. Source: Authors.

2.4 Preliminary antimicrobial assay

For the primary screening of antimicrobial activity, filamentous fungi were submitted to an antimicrobial assay in solid medium which allows a qualitative and rapid selection of the microorganisms with antimicrobial action (Ichikawa et al., 1971). Thus, 100 µL of test bacteria suspension at a concentration of 1.5×10^8 UFC/mL (MacFarland scale 0.5) were seeded into Petri dishes containing Muller Hinton Agar. Then, 6 mm diameter mycelium discs of filamentous fungi with 7 days of growth in Sabouraud medium were transferred to the center of Petri dishes, which were incubated at 37°C for 24 hours. Antibacterial activity was expressed by the diameter of the inhibition zones (mm) of bacterial growth. The tests were carried out in triplicate.

2.5 Fermentation of agroindustrial culture media

The selected fungi in the preliminary screening of antimicrobial activity were cultivated in alternative media CSLG, CWWG and CWW. As a control, the fungi were cultivated in Sabouraud medium (SAB). Thus, 10 discs (6 mm in diameter) of mycelium of each fungus were inoculated in an Erlenmeyer flask with the alternative and control media and incubated under agitation at 100 rpm for 21 days at 30 ºC. At the end of the growth period, the culture supernatants were separated by centrifugation at 10.000 rpm for 15 minutes, followed by filtration using qualitative sterile cellulose-type filter paper (Whatman n.4) (Lyra et al., 1964). Finally, the lyophilized biomass was estimated by gravimetry, and the pH determined by potentiometry, using the Electron Orion pH meter (model 310). The culture supernatants or fermented broths were used in the secondary antimicrobial assay.

2.6 Secondary antimicrobial assay

The crude culture supernatants of the fungi cultivated in CSLG, CWWG and CWW and SAB media was used to qualitatively determine the antibacterial activity by the disk-diffusion method (Bauer et al., 1966). For this purpose, suspensions of test cultures of *E. coli*, *S. aureus*, *B. subtilis* and *S. enterica* were made at a concentration of 1.5×10^8 CFU/mL (MacFarland scale 0.5). Then 100 µL of the suspensions were seeded on Petri dishes containing Muller Hinton agar medium. Soon after, 6 mm filter paper discs impregnated with 50 µL of the metabolic liquid of each fungus were added to the center of the Petri dishes, which were incubated at 28°C for 24 to 48 h. Antibacterial activity was expressed as the diameter of the inhibition zone in millimeter. The broad spectrum antibiotic norfoxacin was used as a positive control and culture media as a negative control. The assay was performed in triplicate.

3. Results and Discussion

3.1 Preliminary screening of antimicrobial potential

The antimicrobial activity of 15 filamentous fungi against 4 test bacteria is presented in Table 2. An inhibitory activity dependent on the test bacterium can be observed. Among the 15 filamentous fungi tested, 8 (53.3%) showed antimicrobial activity against at least two test bacteria and majority demonstrated broad-spectrum antibacterial activity by inhibiting Gram negative and positive bacteria. Of these 8 filamentous fungi with antibacterial activity, all of them inhibited Gram negative bacteria (*E. coli* and *S. enterica*). In turn, *B. subtilis* and *S. aureus* were inhibited by 87.5% (7/8) and 62.5% (5/8) of the active filamentous fungi. *A. niger* UCP 1064, *A. foetidus* UCP 0360, *P. variotii* UCP 0334, *A. flavus* UCP 0316 and *Aspergillus* sp. 74M4, stood out by showing antimicrobial action against all bacteria tested, exhibiting inhibition zones from 10 to 23 mm in diameter. It is also noteworthy that *A. flavus* UCP 0316 presented the largest inhibition zone with 23 mm in diameter for *B. subtilis*. Frighetto & Melo (2007) reported that the preliminary assay is important because it avoids the extraction procedure without prior knowledge of the ability of a microorganism to produce or not metabolites with antimicrobial activity. Santos et al. (2015) in preliminary screening also demonstrated broad-spectrum antibacterial action of endophytic fungi against Gram positive and negative bacteria and reported inhibition zones ranging from 0 to 36 mm in diameter. Additionally, Zhang et al. (2009) reported that marine epiphytic and endophytic fungi showed inhibitory action against 62.8% of Gram negative bacteria and 79.1% of Gram positive bacteria tested.

Table 2. Preliminary screening of the antimicrobial potential of 15 filamentous fungi against pathogenic bacteria.

Code	Fungi strains	Isolation source	Inhibition diameter zone (mm)			
			E. coli	***S. enterica***	***S. aureus***	***B. subtilis***
UCP 1461	*Aspergillus* sp.	Caatinga/PE	14±0.46	12.5±0.23	-	14.3±0.60
UCP 1132	*Aspergillus* sp.	Caatinga/PE	-	-	-	-
UCP 1064	*Aspergillus niger*	Caatinga/PE	19.3±0.22	14±0.0	17.4±0.30	12±0.0
UCP 0360	*Aspergillus foetidus*	Igarassu river/PE	11±0.0	17.5±0.25	15±0.0	13±0.0
UCP 0334	*Paecilomyces variotii*	Igarassu river/PE	18.3±0.32	18±0.0	18±0.0	17.3±0.40
UCP 0316	*Aspergillus flavus*	Igarassu river/PE	15.5±0.3	20.5±0.34	10±0.0	23±0.24
74M	*Aspergillus* sp.	Rio Formoso/PE	-	-	-	-
74M4	*Aspergillus* sp.	Rio Formoso/PE	13±0.0	13.5±0.94	10.5±0.0	15±0.0
4YGPD	*Aspergillus* sp.	Rio Formoso/PE	-	-	-	-
5M1	*Aspergillus* sp.	Rio Formoso/PE	-	-	-	-
8M	*Aspergillus* sp.	Rio Formoso/PE	-	-	-	-
35AB	*Aspergillus* sp.	Rio Formoso/PE	-	-	-	-
37YGPD	*Aspergillus* sp.	Rio Formoso/PE	11±0.0	14.8±0.62	-	-
14YGPD	*Aspergillus* sp.	Rio Formoso/PE	7.5±0.46	11.5±0.20	-	10±0.0
85M	*Aspergillus* sp.	Rio Formoso/PE	-	-	-	-

-: No inhibition. Source: Authors.

3.2 Secondary screening of antimicrobial activity

The five filamentous fungi with the highest activity and spectrum of action in the preliminary screening of antimicrobial activity were selected for the secondary assay. Therefore, antibacterial activity of the crude culture supernatants of the selected active fungi and cultivated in the agroindustrial media (CWWG, CWW, CSLG) and standard medium (SAB), are presented in Table 3. The antimicrobial activity varied according to the test bacteria and the culture medium. All fungi in alternative CWWG medium showed inhibitory activity against *E. coli*, *S. enterica* and *S. subtilis* with inhibition zones between 9 and 15 mm in diameter. However, when cultivated in SAB medium none of the fungi presented antimicrobial action against test bacteria. *P. variotii* UCP 0334 showed the best activity and spectrum of action by exhibiting the highest inhibition zones against all Gram negative and positive bacteria tested, regardless of the culture medium, except for *S. aureus*. In addition, the crude culture supernatants of *P. variotii* UCP 0334 showed the largest inhibition zone (28 mm in diameter) against *E. coli* in the CSLG medium consisting of corn steep liquor and glycerol. Although the highest inhibition zone was observed in the CSLG medium, the antimicrobial activity in this medium was quite limited, with only two fungi showing an inhibitory effect when cultivated in this culture medium. Different bioactive compounds with different mechanisms of action or different concentrations of the same compound produced by the different filamentous fungi evaluated may explain the variation in antimicrobial action (Mahapatra & Banerjee, 2010).

Table 3. Antimicrobial activity in the secondary assay of active filamentous fungi grown in agroindustrial media. CWWG: cassava wastewater and glycerol; CWW: cassava wastewater; CSLG: corn steep liquor and glycerol; SAB: Sabouraud medium. Positive control: norfloxacin (NOR); Negative control: culture media.

Teste Bacteria	Alternative Media	Inhibition diameter zone (mm)						
		P.variotii	*A. flavus*	*A. foetidus*	*A. niger*	*Aspegillus* sp	NOR	Control (-)
E. coli UCP 1575	CWWG	14±0.94	10±0.48	10±0.0	11±0.0	11±0.43	29±0.37	-
	CWW	07±0.49	05±0.24	05±0.23	05±0.88	05±0.47	28±0.0	-
	CSLG	28±0.94	10±0.23	-	-	-	28±0.0	-
	SAB	-	-	-	-	-	30±0.0	-
S. enterica UCP 1550	CWWG	15±0.88	15±0.0	14±0.75	11±0.0	14±0.0	26.5±0.0	-
	CWW	10±0.41	-	10±0.23	06±0.37	06±0.48	26.5±0.0	-
	CSLG	10±0.0	08±0.47	-	-	-	34.5±0.24	-
	SAB	-	-	-	-	-	30±0.0	-
S. aureus UCP 1576	CWWG	-	-	-	-	-	27.5±0.47	-
	CWW	-	07±0.81	07±0.48	-	-	29.5±0.0	-
	CSLG	10±0.0	-	-	-	-	34±0.0	-
	SAB	-	-	-	-	-	30±0.23	-
B. subtilis UCP 1593	CWWG	12±0.0	10±0.56	09±0.0	11±0.23	10±0.88	29.5±0.23	-
	CWW	-	06±0.94	07±0.49	-	-	29±0.0	-
	CSLG	10±0.0	-	-	-	-	31±0.0	-
	SAB	-	-	-	-	-	35±0.0	-

-: No inhibition. Source: Authors.

Our results indicate that *P. variotii* UCP 0334 has a greater potential for metabolites produce with antibacterial action. However, the composition of the culture media, especially the C:N ratio, can influence the production of microbial metabolites (Garbayo et al., 2003). Culture media formulated with the agro-industrial residues apple bagasse, yeast waterwaste, fish flour, cheese whey and foraged oats improved the antimicrobial activity of lactic acid bacteria (Linares-Morales et al., 2022). Santos et al. (2015) demonstrated that *N. sphaerica* URM-6060 showed greater antimicrobial action when cultivated in potato dextrose broth and rice-based medium. Similar to our research, they also reported that the metabolic liquid from the SAB medium only inhibited *E. coli*. In turn, Lopes et al. (2013) reported the production of antimicrobial metabolites by *Penicillium chrysogenum* IFL1 grown in grape wastes and whey. Coetzee et al. (2007) obtained an optimal level of production of the broad-spectrum bacteriocin (bacST4SA) by the bacterium *Enterococcus mundtii* ST4SA in a corn-based medium. In this work, greater inhibitory activity of fungi cultivated in the agroindustrial media was observed, mainly in the CWWG medium, based on cassava wastewater and glycerol, suggesting the potential of these wastes as substrates to produce antimicrobial compounds by filamentous fungi.

3.3 Biomass yield and pH on the agroindustrial media

The biomass production and final pH in the agroindustrial and standard media by the selected filamentous fungi is presented in Table 4. All fungi showed higher biomass yield in the CSLG medium, composed of corn steep liquor and glycerol, but the best yield was shown by *A. niger* UCP 1064 (38.31 g/L). However, the lowest biomass yield was obtained in cassava wastewater-based CWW and CWWG media. On the other hand, the final pH varied with the fungus and the culture medium used. The greatest final pH variation was observed in *A. foeditus* UCP 0360 with values of 3, 4, 4 and 8, in SAB, CSLG, CWW and CWWG media, respectively.

Table 4. Biomass yield and final pH of the filamentous fungi with antimicrobial potential cultivated in agroindustrial and Sabouraud media.

Fungi Strain	**Agroindustriais media**							
	CWWG		**CWW**		**CSLG**		**SAB**	
	Biomass (g/L)	pH (final)	Biomass (g/L)	pH (final)	Biomass (g/L)	pH (final)	Biomass (g/L)	pH (final)
P. variotii	1.0	6.5	3.48	6.0	33.55	6.0	26.48	7.0
A. flavus	7.43	7.7	2.92	7.5	35.00	7.5	14.69	4.0
A. foeditus	1.19	8.0	2.98	4.0	37.82	4.0	16.04	3.0
A. niger	1.01	7.5	2.78	4.0	38.31	4.0	14.69	3.0
Aspergillus sp.	13.71	6.0	1.89	7.5	35.57	7.0	15.13	5.0

Source: Authors.

Studies have shown that corn steep liquor and cassava wastewater are efficient alternative substrates for biomass production by Mucorales order fungi (Berger et al., 2014; Souza et al., 2020; Silva et al., 2022). In this study, the alternative medium containing corn steep liquor induced greater growth of filamentous fungi than cassava wastewater. Thus, it is possible to deduce that cassava wastewater induces the metabolites production more than biomass production. Machado & Burkert (2015), after optimizing the culture medium constituted by the association of corn steep liquor and glycerol for cultivation of the yeast *Sporidiobolus pararoseus*, obtained a maximum biomass concentration of 6.47 g/L. We obtained a maximum biomass yield of 38.31 g/L by *A. niger* using a medium with corn steep liquor and glycerol.

4. Conclusions

The present study showed that *P. variotii* UCP 0334 has biotechnological potential to produce antimicrobials in submerged fermentation using agro-industrial residues as substrates. The alternative medium constituted by the association of cassava wastewater and glycerol wastes is promising for sustainable and low-cost production of secondary metabolites with strong inhibitory effect and broad spectrum of action against Gram positive and negative bacteria. More studies are needed to identify the antimicrobial metabolites produced by *P. variotii* UCP 0334 and define the optimal C:N ratio for improve production.

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