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Amazonian Biodiversity: Pigments from *Aspergillus* and *Penicillium*-Characterizations, Antibacterial Activities and their Toxicities

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Abstract

The *Aspergillus* and *Penicillium* culture collection were screened for pigment production. The antimicrobial activity was measured by the agar diffusion methodology. The organic extracts (hexane, ethyl acetate and ethanol 95%) were tested for bioautography against yeasts and pathogenic bacteria. The extracts were also tested on *Artemia salina* toxicity. *Cryptococcus laurentii* and *Mycobacterium smegmatis* exhibited higher sensitivity to the pigments as test-microorganism. Pigments from *Penicillium simplicissimum* DPUA 1379 and *Penicillium janczewskii* DPUA 304 showed the highest degree of mortality for *Artemia salina* larvae. The other tested fungal strains of *Aspergillus* and *Penicillium* producing pigments, isolated from Amazonia, showed significant antimicrobial activities and total absence of toxicity.

Keywords: Colorants, Pigments, *Aspergillus, Penicillium,* Antagonism, Toxicity.

Introduction

In pigment industrialization (1,2), at present, there are many impediments such as the increasing cost of the raw materials and the energy sources for production that interfere economically in the process, besides the damage to the environment caused by effluent generation (3-9). Many fungal pigments were isolated in different environment exhibiting interesting medical applications (6-8, 10-21). An endophytic fungal pigment screening against human pathogenic bacteria was optimized to improve growth and antimicrobial pigment production. One of the most efficient isolated strains was identified as *Monodictys castaneae*. The best medium was Czapek yeast extract agar/Czapek yeast extract broth (CYA/CYB). Antimicrobial activity of the *M. castaneae* pigment significantly inhibited the growth of human pathogenic bacteria viz. *Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi* and *Vibrio cholerae*. The pigment was more active than streptomycin (22). Fungi isolated from fruits (23) and from fresh water (24) were able to produce pigments with antibacterial activities.

In this context, interests are addressed for the natural pigment sources due to the reduction of adverse effects to health and the largest consumer acceptability, when compared to the artificial pigments used in the food and cosmetics and by the pharmaceutical industries (25,26). Alternatives for production of natural pigments are microorganisms such as bacteria, filamentous fungi and yeasts. Among the filamentous fungi, *Aspergillus* and *Penicillium* are common in soil and foods, and are frequently mentioned in ecological studies (27,28). However, these fungi can cause pathologies such as breathing allergies, due to inhalation of the spores, and mycotoxicosis due to mycotoxin

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ingestion. Certain species have been the subject of much research because their potential application as sources of enzymes and antibiotics, among other natural products of industrial importance (28-34).

Thus the aim of this work was the screening of *Aspergillus and Penicillium* fungal species which produce non-toxic bioactive pigments with the best potential for possible industrial application.

Material and Methods

Microorganisms: To select pigment fungi producers, 30 cultures of *Aspergillus* and 30 of *Penicillium* were donated by the Cultures DPUA Collection of the Parasitology Department of the Federal University of Amazonas–UFAM (35) (Fig. 1). The species authentication was accomplished based on in the morphologic characteristics suggested by Raper and Thom (36), Raper and Fennel (37), Pitt (38), Samson et al. (39), Klick and Pitt (40) and Samson et al. (41).

Pigment production in solid medium: To identify the species of pigment producers, *Aspergillus* and *Penicillium* were cultivated in CYA medium and yeast extract sucrose agar (YES) medium, in Petri plates (90 mm x 10 mm), as described by Raper and Fennell (37) and Pitt (38). The cultures were maintained at 25°C for seven days and a positive result was determined by observation of pigment in the culture medium.

Pigment production in liquid medium in a bioreactor: *Penicillium melinii* DPUA-1391 cultured on YES liquid medium (20 g of yeast extract and 150 g of sucrose in 1000 mL of distilled water). The fungus was inoculated into a 1.5 L bioreactor (Bioflow-New-Brunswick) and grown with stirring at 25 °C for 7 days following a modified methods from as previously describe by Ariza et al. (42).

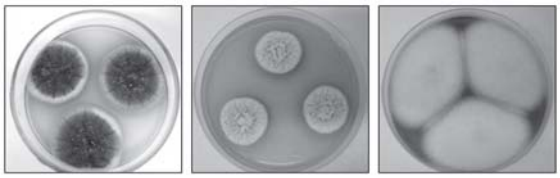

Fig. 1. Pigment production in CYA medium and YES medium for *Aspergillus* and *Penicillium* species, storage in Cultures DPUA'S Collection

Pigment extraction: After *Aspergillus* and *Penicillium* culture, 5x5 mm disks were withdrawn from the central area of the culture and were submitted to successive pigment extraction by hexane, ethyl acetate and 95% (v/v) ethanol for 48 h the solvents were evaporated at low pressure.

Preliminary chemical characterization of the most active and least toxic pigments: The extracted pigments were analyzed by liquid chromatography-mass spectrometry (LC-MS/MS, Waters UPLC Acquity - TQD Quattro Micro API).

Antimicrobial activity determination in solid media: *Aspergillus* and *Penicillium* cultures obtained in YES medium, 25°C/7 days, were analyzed against five test microorganisms [(*Candida albicans* DPUA 1336 and *Cryptococcus laurentii* DPUA 1501 cultivated in Sabouraud-agar medium, at 25°C/48 h) and (*Staphylococcus aureus* CCT 1352, *Escherichia coli* CCT 0547 and *Mycobacterium smegmatis* PDUFPE-71 cultivated in Müeller-Hinton agar medium, at 37°C/24 hours)]. From each test-microorganism an aliquot was removed to obtain a cellular suspension similar to concentration N°. 1 of MacFarland's scale. As standards, itraconazol and rifampicina (5 ug/mL) were used. For determination of the antimicrobial activity by the Block Gelose method for agar diffusion the Teixeira (35) methodology was followed. The antibacterial activity was determined by measuring the diameter of the halo around the culture disks.

Minimum Inhibitory Concentration (MIC): The microorganisms active against the fungal extracts (pigments) were tested and the MIC values were

measured by the microdilution method with the colorimetric indicator Alamar Blue (Invitrogen) in 96 wells microplates. The pigment extracts were added to the microplates up to 10 mg/mL (1% extract) following serial dilutions in the 9 successive wells (43).

Thin layer chromatograpy and bioautography: For the bioautography assay, *Aspergillus* and *Penicillium* were cultivated in YES medium at 25°C for seven days and extracted as previously described (44). The antimicrobial activity was determined by visualization of the inhibition area (44-46).

Bioassay with Artemia salina: The bioassays with *A. salina* were carried out following the modified Meyer et al. (47) method. *A. saline* eggs (60 mg) were added to Petri plates (90 mm x 10 mm) containing 30 mL of 3.5% (m/v) natural sea salt mix (Oceanic). For larvae outbreak, the plate was incubated at 25°C/48 hours, under constant brightness. The toxicity assay was carried out in multiwell plates (6x4), in triplicate. Each well was filled with 1800 µL of aqueous solution of 3.5% (m/v) sea salt, 10 larvae of *A. salina* and 20 µL of ethyl acetate extract diluted in dimethyl sulfoxide (DMSO) (concentration of 30 mg/mL), not exceeding 2000 µL of the total volume. In the control group, 20 µL of DMSO was used as substitute for the organic extract. After 24 hours the living and dead larvae were quantified. The degree of toxicity of the extracts was expressed in percent mortality and classified according to Harwig and Scott (48).

Statistical analysis: The data obtained were submitted to a descriptive statistical analysis using Microsoft Excel version 7.0.

Results and Discussion

Preservation in sterilized distilled water (49) became an option in Cultures DPUA Collection, being a simple method that provides the maintenance of the fungal cultures for long periods, preventing morpho-physiological modifications (50,51). The viability tests of the 30 cultures of *Aspergillus* and 30 of *Penicillium* strains, all preserved in sterilized distilled water from which over 90% were recovered in both cases. The presence of contaminants was observed in 3.3% of *Aspergillus, w*hile the phenomenon of pleomorphisms was only detected in 1.7% of *Aspergillus* and 5.0% of *Penicillium*. Morphologic alterations observed were reverted in the subcultures carried out in glycosated broth, consecutively in CYA medium. These data corroborate with the data obtained by Rodrigues et al. (52), Bueno and Gallardo (51) and Ulloa and Hamlin (53) with filamentous fungi. These results showed that the preservation process at the Cultures DPUA Collection of the Parasitology Department of the Federal University of Amazonas–UFAM was extremely efficient.

Beyond these factors, it is known that in microorganism diversity no standard technique exists that is capable to preserve them in an appropriate and widespread way (54). At this stage it is important to point out that the culture collections are conservation centers whose collection is constituted by live microorganisms, of interest for several scientific branches, besides being used in several biotechnological processes, under conditions that are established to preserve the species, in a way to maintain their vitality, specificity, activity and their immunogenic and other properties, in *ex situ* conditions (35,55). These aspects were well proven in this work.

The analysis of the cultures and of the microcultures obtained in CYA medium revealed expression of the characteristics of the *Aspergillus* and *Penicillium* genera, based on the growth rate, colony morphology, microstrutures and other characteristics compared to the specialized literature classification key (37,38,40,41) (data not shown).

The pre-screening in solid media of *Aspergillus* and *Penicillium* strains demonstrated that, among the 54 viable cultures, 13% (7 species) of the *Aspergillus* strains and 22% (12 species) of *Penicillium* strain showed pigments presence in CYA and YES media (data not

shown). The results in YES medium showed that, among *Aspergillus,* the brown pigment prevailed in relation to the others (yellow and red), the latter only being detected in *A. sparsus* DPUA 1542 cultured in CYA medium.

Table 1 summarizes the results of the *in vitro* assay of the antimicrobial activity of *Aspergillus* (n = 7) and *Penicillium* (n = 12) cultures producing soluble pigments in YES medium.

Considering the halo average diameter tests for agar diffusion, the antimicrobial activity was classified as low activity (halo = 6.0 mm to 7. mm); moderate activity (halo =8.0 to 9.9 mm) and high activity (halo = 10.0 mm). The pigments of *A. sclerotiorum* DPUA 585 and *P. simplicissimum* DPUA 1379 exhibited a high affectivity to four of the tested microorganisms. Due to these results these two microorganism were selected to attempt pigment structure

Table 1. Antimicrobial activity of 19 *Aspergillus* and *Penicillium* cultures producing colorant, against five different microorganisms

Species	Antimicrobial activity				
	Ca	***Cl***	***Ec***	***Ms***	***Sa***
A. carneus DPUA 1290	R	R	R	[2]S16	R
A. sclerotiorum DPUA 585	[4]S9	R	[2]S9	[1]S8	[1]S8
A. sparsus DPUA 1542	R	R	R	R	R
A. sydowii DPUA 792	R	R	R	[1]S10	[1]S8
A. sydowii DPUA 796	R	R	R	R	2S8
A. terricola 1237	[3]S12	R	R	[2]S6	[2]S8
A. terricola var. American DPUA 1272	R	R	R	R	R
P. glabrum DPUA 1435	R	[3]S11	R	R	R
P. janczewskii DPUA 304	R	R	R	R	[2]S6
P. janthinellum DPUA 1381	R	R	R	R	[2]S11
P. melinii DPUA 1391	R	[4]S26	R	[2]S11	[2]S12
P. miczynskii DPUA 1406	R	[4]S31	R	[1]S12	[2]S9
P. montanenses DPUA 1533	R	R	R	[1]S11	R
P. paxilli DPUA 938	R	R	R	[1]S8	[2]S7
P. puberulum DPUA 1146	R	R	R	R	[2]S10
P. purpurogenum DPUA 1275	[3]S11	[3]S13	R	R	R
P. purpurogenum URM 5121	R	[3]S9	R	R	R
P. simplicissimum DPUA 1379	R	[4]S13	[2]S7	[2]S14	[2]S7
P. steckii DPUA 306	R	R	R	[2]S10	R

[1]Bacteriostatic, [2]Bactericide, [3]Fungistatic, [4]Fungicide, Ca = *Candida albicans* DPUA 1336, Cl = *Cryptococcus laurentii* DPUA 1501, Ec = *Escherichia coli* CCT 0547, Ms = *Mycobacterium smegmatis* PDUFPE-71, Sa = *Staphylococcus aureus* CCT 1352, R= resistant (there was no development of the inhibition halo), S=sensible (there was development of the inhibition halo) in mm, including the proper disk of 6 mm

characterization. From all the test-microorganisms, *C. laurentii* DPUA 1501 was the most sensitive yeast to the pigments of *P. miczynskii* DPUA 1406 (halo = 31 mm) and *P. melinii* DPUA 1391 (halo = 26 mm), thus, classified as high activity species. On the contrary, *S. aureus* CCT 1352 and *M. smegmatis* PDUFPE-71 demonstrated less sensitivity to the pigments of *P. janczewskii* DPUA 304 and *A. terricola* 1237; all exhibited inhibition areas of 6 mm, being classified as low activity species. *S. aureus* CCT 1352 was shown to be sensitive to the pigments from *A. sclerotiorum* DPUA 585 (halo = 8 mm), *A. sydowii* DPUA 792 (halo = 8 mm), *A. sydowii* DPUA 796 (halo = 8 mm), *A. terricola* 1237 (halo = 8 mm) and *P. miczynskii* DPUA 1406 (halo = 9 mm), though being considered species of moderate activity.

Related to the resistance test microorganisms, the *E. coli* CCT 0547 and *C. albicans* DPUA 1336 were the most resistant to the fungal pigments under the experimental conditions. The results demonstrated also that the pigments from *A. sparsus* DPUA 1542 and *A. terricola var. American* DPUA 1272 did not exhibit antimicrobial action against the test-microorganisms (Table 1).

Table 2. Antimicrobial activity for the bioautography technique of *Aspergillus* and *Penicillium* cultures storage in sterilized distilled water of the bioactive colorants (R_f by thin chromatography).

Species	R_f	Colour[a] (λ = 366 nm)	Antimicrobial activity			
			Ca	*Cl*	*Ms*	*Sa*
A. carneus DPUA 1290	0.8	Blue	R	R	S	R
A. sydowii DPUA 792	0.8	Green	R	R	S	R
	0.6	Green	R	R	S	R
	0.4	Blue	R	R	S	R
P. glabrum DPUA 1435	0.7	Blue	R	S	R	R
	0.6	Green	R	S	R	R
P. janthinellum DPUA 1381	0.8	Green	R	R	R	S
P. melinii DPUA 1391	0.8	Blue	R	S	S	S
	0.6	Green	R	S	S	S
	0.4	Green	R	S	S	S
P. miczynskii DPUA 1406	0.8	Blue	R	S	S	R
	0.6	Green	R	S	S	R
P. montanenses DPUA 1533	0.7	Green	R	R	S	R
P. purpurogenum DPUA 1275	0.7	Green	S	S	R	R
	0.5	Blue	S	S	R	R
P. simplicissimum DPUA 1379	0.7	Yellow-	R	S	S	R
	0.6	Green	R	S	S	R
		Blue	R	S	S	R
P. steckii DPUA 306	0.7	Green	R	R	S	R
A. sclerotiorum DPUA 585	0.5	Green	S	S	S	S

Ca = Candida albicans DPUA 1336*, Cl = Cryptococcus laurentii* DPUA 1501*, Ms = Mycobacterium smegmatis* PDUFPE-71*, Sa = Staphylococcus aureus* CCT 1352, R= resistant (there was no development of the inhibition halo), S= sensible (there was development of the inhibition halo) .

[a] Spot fluorescence at $\lambda_{exc.}$ 366 nm.

On the basis of the antimicrobial activity classification for the bioautography tests only 10 species were selected, including two *Aspergillus* and eight *Penicillium* species, all of which showed high antimicrobial activity (halo = 10.0 mm) (Table 2). The results of bioautography assays revealed the presence of bioactive compounds in all the fractions obtained after extraction with ethyl acetate with at least one of the test-microorganisms of the cultures obtained from YES medium. The test microorganisms, C. *laurentii* DPUA 1501 and *M. smegmatis* PDUFPE-71 were the most sensitive to bioactive components from the species of *Penicillium* and *Aspergillus,* respectively (Table 2).

A different result was observed with *C. albicans* DPUA 1336, test microorganisms that exhibited greater resistance to the compositions of the bioactive compound. The sensitivity was only certain in the biocompounds produced by *P. purpurogenum* DPUA1275 (Rf 0.5 and 0.7*). S. aureus* CCT 1352 was sensitive to the representative bioactives detected in the extracts of *P. janthinellum* DPUA 1381 (Rf 0.8), *P. melinii* DPUA 1391 (R_F 0.4, Rf 0.6 and R_F 0.8) and A. *sclerotiorum* DPUA 585 (R_F 0.5) (Table 2).In the minimum inhibitory concentration by the microdilution method three species of fungi were sensitive: *A. sclerotiorum* DPUA 585*, P. melinii* DPUA 1391 and *P. simplicissimum* DPUA 1379. The MIC values for *E. coli* were around 2.5 to 5 mg/mL and *M. smegmatis* was sensitive from 0.31 to 2.5 mg/mL. However, *S. aureus* demonstrated sensitivity to 5 to 10 mg/mL. These data showed the inhibitory potentiality of the pigments from *P. melinii* and *P. simplicissimum* against *M. smegmatis* and *E. coli*, respectively. The yeast were resistant to all the pigments tested (10 mg/mL) (Table 3).

Table 3. Minimum Inhibitory Concentration (MIC) by the Microdilusion Colorimetric Method by Alamar Blue

Species	Crude extracts	MIC (mg/mL)
E.coli	*A. sclerotiorum DPUA 585*	5.0 (0.50%)
	P. melinii DPUA 1391	5.0 (0.50%)
	P. simplicissimum DPUA 1379	2.5 (0.25%)
M. smegmatis	*A. sclerotiorum DPUA 585*	2.5 (0.25%)
	P. melinii DPUA 1391	0,3 (0.03%)
	P. simplicissimum DPUA 1379	2.5 (0.25%)
S. aureus	*A. sclerotiorum DPUA 585*	5.0 (0.50%)
	P. melinii DPUA 1391	10.0 (1.00%)
	P. simplicissimum DPUA 1379	5.0 (0.50%)
C. albicans	*A. sclerotiorum DPUA 585*	R
	P. melinii DPUA 1391	R
	P. simplicissimum DPUA 1379	R
C. laurentii	*A. sclerotiorum DPUA 585*	R
	P. melinii DPUA 1391	R
	P. simplicissimum DPUA 1379	R

R = Resistant

Results related to toxicity of the pigments from *Aspergillus* and *Penicillium* against *Artemia salina* larvae are exhibited in Table 4. In the experimental conditions, the ethyl acetate extracts from the *Aspergillus* and *Penicillium* cultures expressed different toxicity degrees when tested with A. *saline.* The toxic action of these extracts was expressed based on the classification done by Harwig and Scott (48) in microbial extracts as: not toxic (0 to 9%); slightly toxic (10 to 49%); toxic (50 to 89%) and very toxic (90 to 100%). In this way six ethyl acetate extracts were active and classified as not toxic or slightly toxic.

Table 4 demonstrates quantitatively the fungi classification in agreement with the toxicity level. The slightly toxic ones were P. *simplicissimum* DPUA 1379 and *P. janczewskii* DPUA 304, which promoted the largest rates of mortality of the *Artemia* larvas (26.7% and 20.0%, respectively*).* It is interesting to mention that in the *in vitro* assays of the antimicrobial activity, *P. simplicissimum* DPUA 1379 presented the larger spectrum, being toxic against four of the test-microorganisms (Table 2*).* The others (17 fungi) classified as non toxic were A. *sclerotiorum* DPUA 585*, A. sydowii* DPUA 796*, A. terricola* DPUA 1237*, A. terricola var. American* DPUA 1272, *A. carneus* DPUA 1290*, A. sparsus* DPUA 1542*, A. sydowii* DPUA 792*, P. glabrum* DPUA 1435*, P. janthinellum* DPUA 1381*, P. melinii* DPUA 1391, *P. miczynskii* DPUA 1406*, P. montanenses* DPUA 1533*, P. paxilli DPUA 938, P. puberulum* DPUA 1146*, P. purpurogenum* DPUA 1275*, P. purpurogenum* DPUA 1543 and *P. steckii* DPUA 306.

Table 4. Percentage of mortality rate of the *A. salina* larvae and the toxicity level of the ethyl acetate extracts from the *Aspergillus* e *Penicillium* species

Toxicity level	Species	Mortality (%)
Slightly toxic	*P. simplicissimum* DPUA 1379	26.7
	P. janczewskii DPUA 304	20.0
Non toxic	*A. sclerotiorum* DPUA 585	6.67
	A. sydowii DPUA 796	
	A. terricola DPUA 1237	3.33
	A. terricola var. *americana* DPUA 1272	
	A. carneus DPUA 1290	0
	A. sparsus	
	A. sydowii DPUA 792	
	P. glabrum DPUA 1435	
	P. janthinellum DPUA 1381	
	P. melinii DPUA 1391	
	P. miczynskii DPUA 1406	
	P. montanenses DPUA 1533	
	P. paxilli DPUA 938	
	P. puberulum DPUA 1146	
	P..purpurogenum DPUA 1275	
	P. purpurogenum URM 5121	
	P. steckii DPUA 306	

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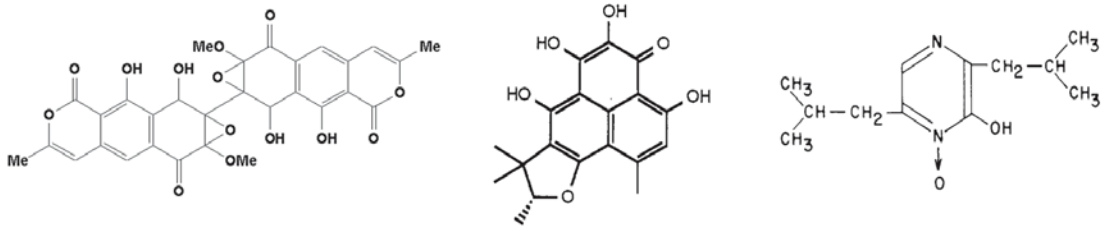
These data are different from those obtained by Sallenave-Namont et al. (56), accomplished with marine anamorph fungi. With this test 14% of *Penicillium spp.* and 15% of the *Aspergillus spp*. were classified as highly toxic. The observed level of toxicity of *Penicillium* and *Aspergillus* in our research probably are associated with the origin of microorganisms, since they are terrestrial fungi under different methods of preservation from 1 to 16 years.

From the strains non–toxic to *Artemia salina* it is worth to mention that *A. sclerotiorum* DPUA 585 and *P. melinii* DPUA 1391, which were active against 4 and 3 microorganisms (Table 4) and had no toxicity to *A. salina,* together with *P. simplicissimum* DPUA 1379, which exhibited a slight toxicity and high antimicrobial activity, were selected for an attempt to characterize the pigment structures. *P. miczynskii* DPUA 1406 that produced two pigments of blue and green fluorescence and both were no non-toxic to *A. salina* are now under analysis of its structures. It is known the production of a deep yellow-gold or yellow-brown in CYA medium by *P. miczynskii* (12).

Among *Penicillium* the yellow pigment was of greater occurrence, in both culture media, also being observed red, brown and lilac pigments. The production of these soluble pigments in the fungi culture occurred due to the influence of the composition of the culture medium, such as carbon and nitrogen sources and pH changes (57). The yellow one was produced by *P. simplicissimum DPUA 1379*, cultured in CYA medium. It is known from the literature that the main pigment from this strain is xanthoepocin (Fig.2), with antibiotic activity (58). In our strain the same color was observed (main peak at R_F 0.7 with 6:4 v/v ethylacetate:hexane and yellow-green fluorescence, mass spectrometry analyses gave the molar mass of xanthoepocin, among others components (not shown). *Penicillium melinii* DPUA-1391 also produced a yellow extract (R_F 0.6 in 6:4 and ethylacetate:hexane with a green fluorescence). It is known that *Penicillium melinii* (formerly *Penicillium atrovenetum)* produced the yellow pigment atrovenetin (Fig.2) (59) with antibiotic activity (60) and excellent antioxidant properties (61). Mass spectrometry analyses of various extracts gave the molar mass of atrovenetin, among other components. *Aspergillus sclerotiorum* DPUA-585 produced a yellow-green pigment. Probably is a similar pigment as formed from *Aspergillus sclerotiorum Huber* (CBS 549.65 strain), that produces neoaspergillic acid (Fig.2) as a yellow pigment (62) with antibiotic activity (63). This yellow pigment, when treated with ferric chloride, gives a red pigment, presumably ferrineo-aspergillin, which is red pigment. Further work is in progress in order to chemically characterize all these non-toxic pigments by NMR, FTIR and MS techniques.

Penicillium melinii was cultivated in a YES medium in a preliminary process in a pilot scale in order to study the economical feasibility of the atrovenetin production from our Amazonian strain. The preliminary study showed a production



Xanthoepocin Atrovenetin Neoaspergillic acid

Fig. 2. Chemical Structures of pigments

of atrovenetin around 1.5 mg/mL of culture medium at 25°C for 7 days. When *Penicillium atrovenetum* strain S.M. 683 was cultivated on Czapek-Dox glucose solution for 21 days at 24°C a residual yellow-brown coloring matter was purified gave around 0.7 mg/ml of a crystalline atrovenetin (64). This shows that the Amazonian strain apparently is more efficient than the previous process studied by other strain and probably might be economical feasible. Optimization of the process is under studying.

Conclusion

This pioneering investigation on pigments from *Aspergillus* and *Penicillium* strains from the Amazon forest, identifying the antimicrobial activity and the toxic action of pigment is unique. This is an important step for a feasible application of these renewable materials in the pharmaceutical and nutritional industries in a friendly and sustainable use of Brazilian resources.

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References

1. Herbst, W., Hunger, K. (2004). Industrial Organic Pigments: Production, Properties, Applications. 4th Ed. Weinheim, Wiley-VCH, Germany.
2. Saron, C., Felisberti, M.S. (2006). Ação de colorantes na degradação e estabilização de polímeros. Quim Nova 29: 124-128.
3. Hobson, D.K., Edwards, R.L., Wales, D.S. (1997). Cyanodotin: A secondary metebolite and dyestuff intermediate. J Chem Technol Biotechnol. 70: 343-348.
4. Gill, M. (1999). Pigments of fungi (Macromycetes). Nat Prod Rep. 16: 301-317.
5. Mapari, S.A.S., Nielsen, K.F., Larsen, T.O. Frisvad, J.C., Meyer, A.S., Thrane, U. (2005). Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants. Curr Opinion Biotechnol. 16: 231-238
6. Mapari, S.A.S., Meyer, A.S., Thrane, U. (2006). Colorimetric characterization for comparative analysis of fungal pigments and natural food colorants. J Agric Food Chem. 54: 7027-7035.
7. Mapari, S.A.S., Hansen, M.E., Meyer, A.S., Thrane, U. (2008). Computerized screening for novel producers of *Monascus*-like food pigments in *Penicillium* Species. J Agri. Food Chem. 56: 9981-9989.
8. Mapari, M.A.S., Meyer, A.S., Thrane, U., Frisvad, J.C. (2009). Identification of potentially safe promising fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale. Microbial Cell Factories 8:24 doi:10.1186/1475-2859-8-24.
9. Sivakumar, V., Anna, J.L., Vijayeeswarri, J., Swaminathan, G. (2009). Ultrasound assisted enhancement in natural dye extraction from beetroot for industrial applications and natural dyeing of leather. Ultrason Sonochem. 16: 782-789.
10. Hiort, J., Maksimenka, K., Reichert, M., Perovic-Ottstadt, S., Lin, W.H., Wray, V., Steube, K., Schaumann, K., Weber, H., Proksch, P., Ebel, R., Muiller, W.E.G., Bringmann, G. (2004). New natural products from the sponge-derived fungus *Aspergillus niger*. J Nat Prod. 67: 1532-1543.
11. Fang, L.Z., Qing, C., Shao, H.J., Yang, Y.D., Dong, Z.J., Wang, F., Zhao, W., Yang, W.Q., Liu, J.K. (2006). Hypocrellin D, a cytotoxic fungal pigment from fruiting bodies of the ascomycete *Shiraia bambusicola.* J Antibiotics 59: 351-354.
12. Christensen, M., Frisvad, J.C., Tuthill, D. (1999). Taxonomy of the *Penicillium miczynskii* group based on morphology and

secondary metabolites. Mycol Res. 103: 527-541.

13. Frisvad, J.C., Andersen, B., Thrane, U. (2008). The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol Res 112: 231-240.

14. Mapari, S.A.S., Thrane, U., Meyer, A.S. (2010). Fungal polyketide azaphilone pigments as future natural food colorants?. Trends Biotechnol. 28: 300-307.

15. Nielsen, K.F., Smedsgaard, J. (2003). Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography–UV–mass spectrometry methodology. J Chromatogr A. 1002:111-136.

16. Koch, B., Kilpert, C., Steglich, W. (2010). Cristatomentin, a green pigment of mixed biogenetic origin from *Albatrellus cristatus* (Basidiomycetes). Eur J Org Chem. 2: 359-362.

17. Muangsin, N., Wisetsakdakorn, W., Chaichit, N., Sihanonth, P., Petsom, A., Sangvanich, P. (2008). Austrocortinin: Crystal structure of a natural anthraquinone pigment from fungi. Dyes Pigments 77: 653-656.

18. Cretu, R., Bahrim, G., Stefan, D., Olteanu, M. (2008). Evaluation of physical and chemical characteristics of yellow colorant produced by *Epicoccum nigrum* MIUG 2.15 in crude extracts and emulsions. Romanian Biotechnol Lett. 13: (SI Suppl. S) 59-68.

19. Gunasekaran, S., Poorniammal, R. (2008). Optimization of fermentation conditions for red pigment production from *Penicillium* sp under submerged cultivation. Afric J Biotechnol. 7: 1894-1898.

20. Velmurugan, P., Chae, J.C., Lakshmana-perumalsamy, P., Yun, B.S., Lee, K.J., Oh, B.T. (2009). Assessment of the dyeing properties of pigments from five fungi and anti-bacterial activity of dyed cotton fabric and leather. Color Technol. 125: 334-341.

21. Velmurugan, P., Kamala-Kannan, S., Balachandar, V., Lakshmanaperumalsamy, P., Chae, J.C., Oh, B.T. (2010). Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather. Carbohydr Polym 79: 262-268.

22. Visalakchi, S., Muthumary, J. (2010). Antimicrobial activity of the new endophytic *Monodictys castaneae* SVJM139 pigment and its optimization. Afric J Microbiol Res. 4: 38-44.

23. Qiu, M., Xie, R.S., Shi, Y., Zhang, H., Chen, H.M. (2010). Isolation and identification of two flavonoid-producing endophytic fungi from Ginkgo biloba L. Ann Microbiol. 60:143-150.

24. Wang, L., Dong, J.Y., Song, H.C., Shen, K.Z., Wang, M., Sun, R., Wang, C.R., Li, G.H., Li, L., Zhang, K.Q. (2008). Screening and isolation of antibacterial activities of the fermentative extracts of freshwater fungi from Yunnan Province, China. Ann Microbiol. 58: 579-584.

25. Dziezak, J.D. (1987) Applications of food colorants. Food Technol. 41: 78-80.

26. Dufosse, L., Galaupa, P., Yaronb, A., Arad, S.M., Blanc, P., Murthy, K.N.C., Ravi shankar, G.A. (2005) Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? Trends Food Sci Technol. 16: 389-406.

27. Asan, A. (2000). Check list of *Aspergillus* and *Penicillium* species reported from Turkey. Turkish J Bot. 24: 151-167.

28. Frisvad, J.C., Larsen, T.O., de Vries, R., Meijer, M., Houbaraken, J., Cabañes, F.J., Ehrlich, K., Samson, R.A. (2007). Secondary metabolite profiling, growth profiles and other tools for species

recognition and important *Aspergillus* mycotoxins. Studies Mycol. 59: 31-37.

29. Hedayati, M.T., Pasqualoto, A.C., Warn, P.A., Bowyer, P., Denning, D.W. (2007). *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiology 153: 1677-1692.

30. Durán, N., Teixeira, M.F.S., De Conti, R., Esposito, E. (2002). Ecological-friendly pigments from fungi. Crit Rev Food Sci Nutr. 42: 53-66.

31. Durán, N., Teixeiran, M.F.S., Esposito, E. (2002). Fungal pigments-Legislation and biosafety. In Biosafety (Biossegurança) (E. Esposito and J.L. Azevedo, Eds.) ESALQ-Editora, Piracicaba, S.P., Brazil, Cap.IX, 81-192.

32. Durán, N., Teixeira, M.F.S., Espósito, E. (2004). Fungal pigments and their biological potentiality. In Biology, Biochemistry and Biotechnology in fungi (Biologia, Bioquímica e Biotecnologia de Fungos) (E. Esposito e J.L. Azevedo, Eds.) Univ. Mogi das Cruzes Editora, Mogi das Cruzes, S.P., Brazil, Editora EDUCS, Caxias do Sul, ISBN 85-7061244-3, RG, Cap. 9, 289-310.

33. Durán, N., De Conti, R., Teixeira, M.F.S. (2009). Pigments from fungi: Industrial perspectives. In Advances in Fungal Biotechnology (M.Rai, ed.) I.K. Intern. Press, Bangalore, Índia.Ch. 7, pp185-225.

34. Dawson, T.L. (2009). Biosynthesis and synthesis of natural colours. Color Technol. 125: 6173.

35. Teixeira, M.F.S. (2006). Cultures collections: Organization and contribution to the biotechnological process. Conf. at First Workshop of Environment, Science and Technology, together for the future of the Catholic University at Pernambuco, August 15-18, Recife/PE, Brasil.

36. Raper, K.B., Thom, C.A. (1968). A Manual of the Penicillia. New York, Hafner Publishing.

37. Raper, K.B., Fennell, D.I. (1977). The genus *Aspergillus.* Huntingdon, New York. Robert E. Krieger Publishing Co.

38. Pitt, J.I. (1985). A laboratory guide to common *Penicillium* species. CSIRO: Austria

39. Samson, R.A., Varga, J. (2007). *Aspergillus* systematics in the genomic era. Stud. Mycol. 59: 1-206.

40. Klick, M.A., Pitt, J.I. (1988). A laboratory guide to common *Aspergillus* species and their teleomorphs. CSIRO, Division of Food Processing, Canberra, Australia.

41. Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O. (1995). Introduction to food-borne fungi. In Samson RA, Hoekstra ES, Frisvad JC and Filtenborg O (Eds.). In Identification of the common food-borne fungi. Wageningen: Centraalbureau Voor Schimmelcultures. The Netherlands.p. 322.

42. Ariza, M.R., Larsen, T.O., Petersen, B.O., Duus, J.O., Barrero, A.F. (2002). *Penicillium digitatum* metabolites on synthetic media and citrus fruits. J Agric Food Chem. 50: 6361-6363.

43. Ribeiro, M.O., Gomes, M.S., Senna, S.G., Rossetti, M.L.R., Fonseca, L.S. (2004). A rapid assay of evaluation in microplates using a cellular viability indicators to determine the subseptibility of *Mycobacterium tuberculosis* strains to isoniazide and rifampicin. J Bras Pneumol. 30: 455-460.

44. Schmourlo, G., Mendonça-filho, R.R., Alvino, C.S., Costa, S.S. (2005). Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants. J Ethnopharmacol. 96: 563-568.

45. Balinova, A. (1995). Extension of the bioautograph technique for multiresidue determination of fungicide residues in plants and water. Anal Chim Acta 311: 423-427.

46. Ahmad, I., Beg, A.Z. (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against mult-drug resistant human pathogens. J Ethnopharmacol. 74: 113-123.

47. Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E. (1982). Brine Shrimp: A convenient general bioassay for active plant constituents. J Med Plant Res. 45: 31-34.

48. Harwig. J., Scott, P.M. (1971). Brine Shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. Appl Microbiol. 21:1011-1016.

49. Castellani, A. (1939). Viability of some pathogenic fungi in distilled water. J Trop Med Hyg. 42: 225-226.

50. Smith, D., Onions, A.H.S. (1983). The preservation and maintenance of living fungi. Kew (UK) Commonwealth Mycological Institute, England.

51. Bueno, L., Gallardo, R. (1998). Filamentous fungi preservation in sterile distilled water. Rev Iberoamer Micol. 15: 166-168.

52. Rodrigues, E.G., Lírio, V.A., Lacaz, C.S. (1992). Fungi and actinomycetes of medical interest preservation in distilled water. Rev Inst Med Trop São Paulo 34: 159-165.

53. Ulloa, M., Hanlin, R.T. (2000). Illustrated Dictionary of Mycology. The American Phytopathological Society, St. Paul, Minnesota, APS Press.

54. Girão, M.D., Prado, M.R., Brilhante, R.S.N., Cordeiro, R.A., Monteiro, A.J., Sidrim, J.J.C., Rocha, M.F.G. (2004). Viability of *Malassezia pachydermatis* strain maintained in different conservation methods. Rev Soc Brasil Med Trop. 37: 229-233.

55. Uzunova-Doneva, T., Donev, T. (2005). Anabiosis and conservation of microorganisms. J Culture Collect. 4: 17-28.

56. Sallenave-Namont, C., Pouchus, Y.F., Robiou du Pont, T., Lassus, P., Verbist, J.F. (2000). Toxigenic saprophytic fungi in marine shellfish farming areas. Mycopathologia 149: 21-25.

57. Cho, Y.J., Park, J.P., Hwang, H.J., Kim, S.W., Choi, J.W., Yun, J.W. (2002). Production of red pigment by submerged culture of *Paecilomyces sinclairii*. Appl Microbiol. 35: 195-202.

58. Igarashi, Y., Kuwamori, Y., Takagi, K., Ando, T., Fudou, R., Furumai, T., Okic, T. (2000). Xanthoepocin, a New Antibiotic from Penicillium simplicissimum IFO5762. J Antibiot (Tokyo) 53: 928-933.

59. Buchi, G., Leung, J.C. (1986). Total Syntheses of Atrovenetin and Scleroderodione. J Org Chem. 51: 4813-4818.

60. Narasimhachari, N., Gopalkrishnan, K.S., Haskins, R., Vining, L.C. (1963). Production of antibiotic atrovenetin by a strain of *Penicillium herquei* Bainier and Sartory. Can J Microbiol. 9: 134-136.

61. Ishikawa, Y., Morimoto, K., Iseki, S. (1991). Atrovenetin as a potent antioxidant compound from penicillium species. J Amer Oil Chem Soc. 68: 666-668.

62. Assante, G., Camarda, L., Locci, R., Merlini, L., Nasini, G., Papadopoulos, E. (1981). Isolation and structure of red pigments from *Aspergillus flavus* and related species grown on a differential medium. J Agric Food Chem. 29: 785-787.

63. MacDonald, J.C., Micetich, R.G., Haskins, R.H. (1964). Antibiotic activity of neoaspergillic acid. Can J Microbiol. 10:90-95.

64. Neill, K.G., Raistrick, H. (1957). Studies in the biochemistry of micro-organisms 100. Metabolites of *Penicillium atrovenetum* G. Smith. Part I. Atrovenetin, a new crystalline colouring matter. Biochem J. 65: 166-176.