

EXPLORING COLORANT PRODUCTION BY AMAZONIAN FILAMENTOUS FUNGI: STABILITY AND APPLICATIONS

João Vicente Braga de Souza¹, Luciana Oliveira², Michele Sanches², Walter Filho², Paulo Santiago³, Rodrigo Lima⁴, Ana Cortez¹, Érica Souza², Maria Lima¹, Emerson Silva³, Hector Koolen², and Laurent Dufossé⁵

¹National Institute of Amazonian Research

²Universidade do Estado do Amazonas

³Universidade Federal do Amazonas

⁴Centro Universitario do Norte

⁵Universite de La Reunion

August 9, 2023

Abstract

The aim of this study was to investigate the production, stability and applicability of colorants produced by filamentous fungi isolated from soil samples from the Amazon. Initially, the isolates were evaluated in a screening for the production of colorants. The influences of cultivation and nutritional conditions on the production of colorants by fungal isolates were investigated. The colorants produced by selected fungal isolates were chemically characterized using the LC-MS technique. The antimicrobial and cytotoxic activities, stability evaluation and applicability of the colorants were investigated. As results, we observed that the isolates *Penicillium sclerotiorum* P3SO224, *Clonostachys rosea* P2SO329 and *Penicillium gravinicasei* P3SO332 stood out since they produced the most intense colorants. Compounds produced by *Penicillium sclerotiorum* P3SO224 and *Clonostachys rosea* P2SO329 were identified as sclerotiorin and penicillic acid. The colorant fraction (EtOAc) produced by these species has antimicrobial activity, stability at temperature and at different pHs, stability when exposure to light and UV, and when exposed to different concentrations of salts, as well as being non-toxic and having the ability to dye fabrics and be used as a pigment in creams and soap. Considering the results found in this study, it was concluded that fungi from the soil in the Amazon have the potential to produce colorants with applications in the textile and pharmaceutical industries.

1. INTRODUCTION

A colorant is any substance added to a product for the purpose of changing its color, enhancing the existing color or restoring lost color. Natural colors are pigments made by living organisms (plants, lichens, algae, insects, bacteria and fungi) [1–3]. There is low consumer interest for the use of the synthetic colorants due to the fact that they are toxic to human health (mutagenic and carcinogenic potential) [4,5]. The current legislation of the European Union, the United States, Brazil, Norway and Sweden also limits the use of synthetic colorants as food additives, and the process used to produce synthetic colorants is toxic to the environment [6,7].

However, natural colorants extracted from plants have a drawback, i.e., they depend on seasonal availability[8]. As a potential bioprocess for the new bioindustry of colorants, the production of colorants using microbial fermentation is rising, since it is possible to reduce production costs by choosing the best growing conditions; in addition, unlike plants microorganisms do not depend on climatic conditions [9–11]. As well as these advantages, many fungi-based colorants have important biological properties, such as antimicrobial, antioxidant, immunosuppressive, antiviral, anticancer and cholesterol-lowering properties, which make them

important compounds for numerous industries. As a result, there is growing worldwide interest for the use of natural colorants[12–14] .

Studies have shown the potential of Amazonian fungi for colorant production. One study that can be highlighted isolated fungi-producing pigments [15] and obtained fractions with colors ranging from yellow to red. *Penicillium sclerotiorum* 2AV2 produced intensely colored pigments and the pigment was identified as sclerotiorin, which has specific biological activities such as the inhibition of aldose reductase enzymes, lipases, integrins and proteases of the human immunodeficiency virus HIV-1[16]. Teixeira et al. [17] evaluated the antibacterial activity and toxicity of organic extracts. The authors identified antimicrobial activity and lack of toxic action of the colorants produced by strains of the genus *Aspergillus* and *Penicillium*.

The Amazon Rainforest contains a rich diversity of microorganisms. Despite the richness of biological diversity in the Amazon, few works have been dedicated to researching fungal colorants. Therefore, the present study aimed to identify fungal isolates with the potential for production of colorants.

2. MATERIALS AND METHODS

2.1 Collection of samples and isolation of fungi

Superficial soil and litter were collected in September 2018 in a forest zone located in the Campina Biological Reserve (2°35'19.19" S, 60°1'59.91" W) Manaus, Brazil. The samples were collected at the following three points: point 1 (-2.5891030, -60.0319530), point 2 (-2.589249, -60.031893) and point 3 (-2.589368, -60.031712). Approximately 5 g of superficial soil and litter were collected with clean, dry and sterile polythene bags and a sterile spatula. From the collected samples, 1 g was added to 9 mL of sterile distilled water and serial dilution was performed to dilute the sample. The suspension (0.1 mL) was added to sterile Petri dishes in duplicates containing sterile Sabouraud agar and the spread plate technique was used. Plates were incubated at 28 °C±2 for 5-7 days [18].

2.2 Purification and identification of fungi

The isolated colonies were transferred to tubes containing Sabouraud agar plus chloranphenicol. The isolated colonies were purified on Petri plates containing potato dextrose agar (PDA) incubated at 28 °C±2 for 7 days. Pure cultures were transferred into tubes containing PDA. These pure cultures were stored in a refrigerator at 4 °C, with mineral oil and sterile distilled water. The isolated fungi were identified to the genus level by assessing the characteristics micro morphological characteristics using slide cultures [19].

The species of interest were identified via sequencing of the rDNA ITS region. Fungal DNA was extracted from mycelium the phenol:chloroform:isoamyl-alcohol method [20]. PCR reactions (3 µL of buffer (1X), 1.2 µL of MgCl₂ (2.5 mM), 3 µL of DNTPs (200 µM), 1.5 µL ITS1 (5'- TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5'- TCCTCCGCTTATTGATATGC- 3') [21] and ultrapure water to a final volume of 30 µL) were carried out in a thermocycler (SuperCycler™ SC-200, Kyrtec) using the following amplification conditions: 94 °C/5 min, 30 cycles of 94 °C/30 s, 53 °C/30 s, 72 °C/1 min and 72 °C/10 min. PCR products (8 µL) were visualized under UV light after electrophoresis (3 µL of SYBR® Safe (Invitrogen), 2 µL of Orange 6X (Fermentas), 10 µL 100-bp DNA Ladder, 40 min/100 V/100 A) on a 1.5% agarose gel in 1x TBE buffer. PCR products were purified via precipitation with polyethylene glycol (20% w/v PEG, 2.5 M NaCl). The sequencing reaction was performed using a commercial kit (BigColorant®, Applied Biosystems). Sequencing was carried out in a genetic analyzer (AB3130, Applied Biosystems). The sequences obtained were analyzed using BioEdit Sequence Alignment Editor and compared to those in the GenBank database. A phylogenetic tree was constructed using the program MEGA X v10.2.4 to verify the genetic and evolutionary relationship among the sequences.

2.3 Screening for colorant production

To identify which fungi isolated from the soil had the potential for extracellular colorant production, a visual evaluation of the colorants produced by the fungi in the culture medium was carried out. The fungus strains were transferred to tubes containing Czapeck-Dox-agar and incubated at room temperature for 14

days. After colony growth, the colorants present in the culture medium and soluble in ethyl acetate were observed. This solvent was transferred to tubes and shaken for 30 seconds in a tube shaker and maintained at room temperature for 30 min. Afterwards, the solvent was removed and the presence of color in the solvent was evaluated [15]. The eight fungal strains that showed the most intense colorants were submitted to a submerged bioprocess.

2.4 Submerged bioprocesses for colorant production

Submerged bioprocesses were performed based on the protocol described by Celestino et al.[15] with modifications. The fungal strains were transferred to tubes containing PDA and incubated at room temperature for 72 h. The fungal spores of each strain were suspended in 5 mL of sterile distilled water and the density of the spore suspension was determined using a hemocytometer (Neubauer, Germany) under an optical microscope. This spore suspension (1×10^4 cell/mL) was inoculated into Erlenmeyer flasks (125 mL) containing 50 mL Czapeck broth plus yeast extract (3.0 g/L sodium nitrate, 1.0 g/L potassium phosphate, 0.5 g/L magnesium sulfate, 0.5 g/L potassium chloride, 0.01 g/L ferrous sulfate, 30.0 g/L of sucrose and 0.5 g/L yeast extract), at an initial pH of 5.0. The flasks were incubated in static conditions, in a dark place, and at room temperature (28 °C) for 14 days.

2.5 Extraction and evaluation of the solubility of the colorants

After 14 days of fermentation, each culture broth was separated from the mycelial mass by filtering through ordinary filter paper. The mycelial mass was washed with hexane (20 mL), ethyl acetate (20 mL) and methanol (20 mL). Solvents were added sequentially to the cultures and vigorously shaken in an orbital shaker (100 rpm/60 min). Extracts were filtered through filter paper for the removal of cell debris. The culture filtrates were subjected to successive extractions. The culture filtrate and solvent (hexane and ethyl acetate) were placed in a separating funnel (1:1, v/v) and mixed well. After separation, the solvent fraction was collected.

2.6 Effect of nutritional parameters on colorants production

To study the influence of nutritional factors in the production of colorants via the three selected fungi, univariate experiments were carried out to verify the influence of five carbon sources (sucrose, glucose, maize starch, maltose and carboxymethylcellulose - CMC) and four nitrogen sources (sodium nitrate, ammonium sulfate, peptone and yeast extract). In each experiment, only the carbon source (30 g/L) or nitrogen source (3 g/L) were modified separately. The production of colorants was estimated by analyzing the maximum absorbance (λ_{max}) in ethyl acetate via scanning (400 nm to 700 nm) in a UV/VIS spectrophotometer.

2.7 Kinetics of colorant production

In order to describe the kinetics of colorant production by the three selected isolates, submerged bioprocesses were performed (See *Submerged bioprocesses for colorant production*). Samples were collected in triplicate at intervals of 5 days for 20 days with the view to determination of dry cell weight (g/L) and extracellular colorants (Abs_{extra}). To determine the biomass, the mycelial masses were collected and filtered with filter paper previously dried in a desiccator and then weighed. After filtration, both were dried in an oven at 100 °C for 24 h. The biomass concentration was expressed by the dry weight of the mycelial mass using the difference between the final weight and the initial weight. The production of colorants was estimated by analyzing the maximum absorbance (400 nm) in ethyl acetate using a UV/VIS spectrophotometer.

2.8 Analysis of the colorants using liquid chromatography coupled to mass spectrometry (LC/MS-MS)

The extracted colorants were diluted in methanol (HPLC-Honeywell) to a concentration of 1 mg/mL. Then, the samples were analyzed using a liquid chromatograph (Prominence UFLC, Shimadzu), which was equipped with a C18 Shim-pack CLC-ODS(M)[®] column (5 μ m, 250 x 4.6 mm), and coupled to a mass spectrometer (Amazon Speed Ion trap, Brucker Daltonics) with an ESI source operating in positive and negative modes.

Samples were eluted in gradient of 20-100% methanol over 24 minutes at a flow rate of 1 mL/min. The data obtained were processed using the DataAnalysis program (Bruker Daltonics).

2.9 Stability of the Colorants

The investigation of the stability of the colorants and their resistance to degradation by temperature, as well as the pH value in the color of the colorants and salt concentrations was carried out as described [22] with modifications, whereby the colorants (0.1 mg/mL of DMSO) were incubated in a water bath for 1 hour at 40 °C, 60 °C, 80 °C, 100 °C or frozen at -20 °C before analysis. In another set of tubes, extracts were adjusted to pH 2, 4, 6, 8, 10 and incubated for 1 hour at room temperature. Test tubes containing the extracts maintained in 0.2%, 0.4%, 0.6%, 0.8% and 1% (v/v) NaCl solutions for 6 h of exposure.

For the investigation of their resistance to photodegradation, the colorants were left in a dark place (and covered with aluminum foil), exposed to white light (distance of 30 cm, 28W Cold White 5000K Tubular Fluorescent Lamp Brand/Manufacturer: Osram, Barueri-SP, Brazil) and ultraviolet light (distance of 30 cm, Ultraviolet Lamp 24W Pl Osram Germicidal Reactor, Barueri-SP, Brazil), for 6 h. Changes in absorbance were determined using a spectrophotometer at the maximum wavelength of the analyzed colorants (λ_{max}).

2.10 Antimicrobial activity

The ethyl acetate extracts from of the three isolates were subjected to determination of the minimum inhibitory concentration (MIC) using the broth microdilution method in 96-well microtiter plates, as described by the Clinical and Laboratory Standards Institute - CLSI (2012) [23], USA: standard M7-A9 (methodology of the antimicrobial dilution sensitivity tests for aerobic growth bacteria), with the modifications, and M27-A4 (susceptibility test for yeasts). Extracts were prepared at the initial concentration of 3.2 mg/mL and dissolved in 10% DMSO. Tests were performed against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 60193. Each microtiter plate was inoculated with a previously prepared standard bacterial (10^7 CFU/mL) and fungal suspension (2.5×10^3 CFU/mL). The microtiter plates were incubated at 35-37 °C for 16 to 20 hours for *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 and 35 °C for 24 h for *C. albicans* ATCC 60193. The lowest concentration of extract that inhibited visible growth was recorded as the MIC. Amoxicillin and oxacillin were used as standard antibacterial agents and fluconazole was used as the antifungal standard for positive inhibitory controls.

2.11 Toxicity of the colorants

The cytotoxicity of the colorants was evaluated in front of MRC-5 cells (human fibroblast)[24] grown in DMEM medium (Dulbecco's Modified Eagle's Medium), which was supplemented with 10% fetal bovine serum and kept in a CO₂ incubator at 37 °C and an atmosphere containing 5% CO₂. Cells were seeded in 96-well plates (0.5×10^4 cells per well). After 24 hours, extracts were dissolved in DMSO and added to each well (10 µg/mL) and incubated for 48 hours. Doxorubicin (5 µg/mL) was used as a positive control. Negative controls (blanks) received the same amount of DMSO and had the same final DMSO concentrations as the samples (0.1%). Two hours before the end of the incubation, 10 µL of alamarBlueTM were added to each well. The fluorescence signal was monitored with a multiplate reader using a range of 530-560 nm excitation wavelength and 590 nm emission wavelength. The fluorescence signal generated from the assay was proportional to the number of live cells in the sample.

2.12 Textile dyeing We conducted preliminary tests to evaluate the potential of crude colorant extracts for dyeing textiles. To prepare the coloring solutions, we dissolved the extracts from three studied isolates (*Clonostachys rosea* P2SO329, *Penicillium gravinicasei* P3SO332, and *Penicillium sclerotiorum* P3SO224) in 10% methanol, resulting in a concentration of 0.1 mg/mL. A control solution without added colorant was also prepared. For the dyeing process, we used polyester, silk, and cotton fabric fragments measuring 5 x 5 cm. The textile fragments were immersed in beakers containing 50 mL of the colorant solutions and left at 35 °C for 1 hour, following the method previous described [25,26]. After dyeing, the samples were washed with warm distilled water at 35 °C to remove any unfixed colorants. Next, the fabric fragments were soaked in a 0.1% CuSO₄ mordant solution for 10 minutes. Finally, the samples were rinsed with water and air dried

at room temperature.

Stability was visually assessed by observing the loss of color after exposing the fabric fragments to natural light for a period of five days.

2.13 Evaluation of fungal and commercial colorants in cream and cosmetic formulations A cream with a water-in-oil (W/O) emulsion was prepared using the following formulation. The oil phase consisted of 4 g of mineral oil and 10 g of a commercial base containing vegetal oil and an emulsifier, sourced from “Essências da Amazônia” (Manaus, AM-Brazil). The aqueous phase was prepared by combining 79 g of distilled water, 4 g of glycerin, and 2 mL of a commercial Aloe Vera extract, also obtained from Essências da Amazônia. To achieve emulsification, the oil phase was heated separately until it reached a temperature of 80 °C. Subsequently, the water phase was gradually added to the oil phase under constant stirring, while cooling the mixture to approximately 40 °C. This process resulted in the formation of a homogeneous cream. Fungal and commercial colorants were incorporated into the cream at a concentration of 0.05 mg per gram of the formulation. The cream was fortified with 1mL of a preservative solution (methyl paraben, propylparaben and propylene glycol)[27,28].

Colorant 1, from the *Penicillium sclerotiorum* P3SO224 strain, showed satisfactory results in the incorporation of the cream base a more attractive coloring, and was thus selected for the soap development test.

The soap formulation consisted of 250 g of glycerin soap base (Nossa Terra; composition: sodium babassuate, sodium tallow, propylene glycol, glycerin, sodium laureth sulfate, alcohol, water, sucrose, tetrasodium EDTA, dipropylene glycol), 20 mL of lauryl, 2-5 mL of essence, 5 mL of glycolic extract and the respective fungal colorant and commercial colorant in a final content of 0.05 mg of colorant per g of soup. All components were placed a hot plate for the complete casting of the base. The formulation was added to a silicone mold and cooled to -20 °C for solidification.

The developed cream formulation was subjected to a centrifugation test (3,000 rpm/30 min, Cosmetic products stability guide) in order to assess whether the product would maintain the miscible water and oil emulsion phases. The pH, appearance, color and odor parameters were evaluated for a period of 7 days. A 1:10 preparation (0.5 g cream/4.5 mL distilled water) was used for pH determination with analysis at time zero and every 48 hours for 7 days.

3. RESULTS

3.1 Isolation and identification of fungi

Samples of soil and litter were collected from the Campina Biological Reserve in order to isolate fungal strains to be screened for colorant production. The samples were subjected to the serial dilution method and plated in Sabouraud agar medium to obtain isolated colonies. We obtained 184 colonies of filamentous fungi (55 from litter and 129 from superficial soil). The micromorphological characteristics of the isolates were analyzed and a predominance of unidentified isolates was observed. However, among those that could be identified, the genera *Acremonium*, *Aspergillus* and *Penicillium* were the most frequent.

3.2 Screening for colorant production

Based on the visual evaluation of the colorants produced by fungi in the culture medium, eight isolates stood out for producing the most intense colorants and were submitted to submerged bioprocesses, extraction, and evaluation of the solubility of the colorant. In Fig. 1, more colorful fractions were obtained from the extraction with different solvents from the mycelial mass, and the fraction of the culture filtrate, which has a greater polarity, showed the most intense extracellular colorants.

The isolates *Penicillium* sp. P3SO332, *Clonostachys* sp. P2SO329 and *Penicillium* sp. P3SO224 were selected to evaluate the absorptivity of the colored extracts, which was obtained in the UV/VIS region. The colored fractions were scanned at 400–700 nm to determine the maximum absorption wavelength (λ_{max}) in ethyl acetate. Scanning spectrophotometry showed that the three colorants evaluated have a maximum absorbance around 400 nm.

The four fungi highlighted in the production of colorants (*Penicillium* sp. P3SO332, *Clonostachys* sp. P2SO329, *Penicillium* sp. P3SO224, *Monascus* sp. C02172R) were subjected to DNA sequencing of the targets ITS1-5.8S rDNA-ITS2 to confirm their genus and possible DNA identification. The phylogenetic tree constructed is shown in Fig. 2. The isolate *Penicillium* sp. P3SO332 was identified as *Penicillium gravinicaei* P3SO332 (99% accession: MG600581.1). *Clonostachys* sp. P2SO329 was identified as *Clonostachys rosea* P2SO329 (99% accession: MN452057.1). *Penicillium* sp. P3SO224 was identified as *Penicillium sclerotiorum* P3SO224 (99% accession: MN639705.1). *Monascus* sp. C02172R was identified as *Monascus purpureus* C02172R (99% accession: MT355839.1). The nucleotide sequences obtained after amplification and sequencing were submitted to NCBI GenBank and the accession numbers OP563023, OP563026, OP563024 and OP563023, respectively, were obtained.

3.3 Effect of nutritional parameters on colorant production

To study the effect of the alterations in the nutritional parameters on colorant production, the strains *Penicillium gravinicaei* P3SO332, *Clonostachys rosea* P2SO329 and *Penicillium sclerotiorum* P3SO224 were grown in Czapeck medium base that was modified with different sources of carbon or nitrogen (Fig. 3).

Colorant production was achieved when maize starch was used as a carbon source and peptone as a nitrogen source in the submerged bioprocess for *Penicillium gravinicaei* P3SO332 (Fig. 3 A, B). Glucose and maize starch were the carbon sources that allowed the highest absorbances and yeast extract was the best nitrogen source for *Clonostachys rosea* P2SO329 at the analyzed wavelength (Fig. 3 C, D). Maximum absorbance was observed in the submerged bioprocess that contained sucrose as a source of carbon and yeast extract as a source of nitrogen in *Penicillium sclerotiorum* P3SO224 (Fig. 3 E, F).

Based on the above results, glucose (*Clonostachys rosea* P2SO329), sucrose (*Penicillium sclerotiorum* P3SO224) and maize starch (*Penicillium gravinicaei* P3SO332) were selected as carbon sources and, as nitrogen sources, yeast extract (*Clonostachys rosea* P2SO329, *Penicillium sclerotiorum* P3SO224) and peptone (*Penicillium gravinicaei* P3SO332) were selected. The optimal production conditions for the three selected isolates were evaluated for 20 days.

Clonostachys rosea P2SO329, *Penicillium sclerotiorum* P3SO224 and *Penicillium gravinicaei* P3SO332 presented their maximum production of colorants at 15, 10 and 20 days, respectively. The maximum production of biomass was observed at 20 days by *Clonostachys rosea* P2SO329 (5.14 g/L), *Penicillium sclerotiorum* P3SO224 (8.62 g/L) and *Penicillium gravinicaei* P3SO332 (6.30 g/L).

3.4 Chemical characteristics of the color fractions of *Penicillium gravinicaei* P3SO332, *Clonostachys rosea* P2SO329 and *Penicillium gravinicaei* P3SO332

Fermentation was carried out in 125 mL Erlenmeyer flasks containing 50 mL of broth medium with the different sources of carbon or nitrogen that were selected in the previous results. This procedure was repeated 20 times and resulted in 1,000 mL of fungal cultivation. Broth containing plugs of mycelium was mixed with ethyl acetate (1:1 v/v) and shaken in an orbital shaker (100 rpm for 24 h). The extraction produced colorant yields of 105 mg/L by *Penicillium sclerotiorum* P3SO224, 83 mg/L by *Clonostachys rosea* P2SO329 and 142 mg/L by *Penicillium gravinicaei* P3SO332, and their metabolites were analyzed using LC/MS-MS.

The EtOAc extract of *P. sclerotiorum* P3SO224 showed a main peak eluting at 15 min (See Supplementary Material) and a molecular ion of m/z 391 $[M+H]^+$ with competitive losses of -42 (C_2H_2O) and -60 Da ($C_2H_4O_2$), related to neutral losses on the acetyl group. Moreover, after the loss of 60 Da, a carbon monoxide loss (-28 Da) yielding the ion m/z 303. These characteristics allowed us to propose that the this compound is the chlorinated azaphilone named sclerotiorin (Peres et al. 2023) (Fig. 4,1).

The EtOAc extract of the fungus *Clonostachys rosea* P2SO329 showed four main chromatographic peaks eluting at 3.6, 5.5, 7.8 and 8.5 min. From these, only the peak eluting at 5.5 min was possible to annotate. The positive and negative modes displayed m/z of 171 $[M+H]^+$ and 169 $[M-H]^-$, both with consecutive losses of -18 Da (water), -15 Da (methyl) and -28 Da (CO), all consistent with the annotation of the compound is penicillic acid (Fig. 4, 2) [30] .

3.5 Stability of the colorants

The stability of the colorants was thoroughly assessed in this study, considering various environmental factors. The resistance of the colorants to temperature variations (-20°C, 40°C, 60°C, 80°C, and 100°C) for a duration of 1 hour revealed no noticeable color reduction. Additionally, the colorants displayed remarkable resistance to different pH levels (2, 4, 6, 8, and 10) during the same timeframe. However, out of the fungi-derived colorants, only those obtained from *P. sclerotiorum* P3SO224 exhibited 100% stability when incubated for 6 hours at different salt concentrations (0.2%, 0.4%, 0.6%, 0.8%, and 1% v/v). The other fungal colorants showed a significant reduction (50% color loss) starting from 0.2% NaCl. Furthermore, no significant color fading was observed in the fungal colorants after a 6-hour incubation under both white light and UV light exposure.

3.6 Antimicrobial activity

In order to investigate whether the colored fractions from the fungi *Penicillium graminicasei* P3SO332, *Clonostachys rosea* P2SO329 and *Penicillium sclerotiorum* P3SO224 showed antimicrobial activity, using the broth microdilution method, the ethyl acetate extract was analyzed for MIC determination. The color fractions demonstrated antimicrobial activity against the tested microorganisms (Table 1). The ethyl acetate extract from *Clonostachys rosea* P2SO329 had MIC value of 6.25 µg/mL against the strain *E. coli* ATCC 25922 and a MIC of 12.5 µg/mL against *S. aureus* ATCC 25923. Ethyl acetate extracts of the three isolates presented activity against *C. albicans* ATCC 60193 (400 µg/mL).

3.7 Cytotoxic activity of colorants

The cytotoxicity of the colorants was evaluated using the AlamarBlue™ assay against MRC-5 cells. The results obtained show the percentage of the number of live cells in the sample. The cytotoxicity of *Clonostachys rosea* P2SO329, *Penicillium graminicasei* P3SO332 and *Penicillium sclerotiorum* P3SO224 ethyl acetate extracts show an IC₅₀ > 100 µg/mL.

3.8 Textile dyeing

The ability of fungal colorants to dye materials, specifically fabrics, was evaluated. The results revealed that the colorant produced by *Penicillium sclerotiorum* P3SO224 was the most promising for this purpose (Fig. 5) due to the orange pigmentation that remained after washing the three types of fabrics used (polyester, silk and cotton). Polyester and silk fabrics dyed with the colorant produced by *Penicillium graminicasei* P3SO332 showed a slightly pink hue. The dyeing of fabrics with the colorant produced by *Clonostachys rosea* P2SO329 was not effective. It was found that all fabrics presented lighter shades after washing as well as after the period of exposure to light. Our study also indicated that the orange colorant produced by *Penicillium sclerotiorum* P3SO224 has a higher degree of pigmentation in polyester fabrics than in the other fabrics tested.

3.9 Formulation of fungal cosmetic colorants

The formulations were developed with the aim of evaluating whether the fungal extracts, in addition to presenting antimicrobial activity and low toxicity, could potentially be incorporated into products for cosmetic and pharmaceutical purposes. Five cream formulations were tested. The organoleptic characteristics (appearance, color, and odor) and pH were analyzed, and samples were also submitted to the pre-stability test.

When incorporated with the fungal extracts, they appeared as a cream with a shiny, homogeneous appearance, without lumps or fine granulations, and had an attractive color and light texture (Fig. 6). The formulation remained stable after the centrifugation test and presented no changes in color or odor during the period evaluated. The pH of the formulations did not change significantly during the period studied, and remained within a slightly acidic pH range, which is compatible with the pH of human skin.

Colorant 1, which was obtained from the *Penicillium sclerotiorum* P3SO224 strain, showed satisfactory results when incorporated into the cream base as it had a more attractive color and an inhibitory effect against *E.*

coli ATCC 25922, *S. aureus* ATCC 25923 and *C.albicans* ATCC 60193 strains. As a result, it was selected for a soap development test (Fig. 7) in order to assess its potential for incorporation into skin care products. The results of the tests involving the formulation showed to be promising with the incorporation of the colorant in a glycerin-base soap that had a homogeneous appearance, no lumps and production of adequate amounts of foam.

4. DISCUSSION

The findings of this study suggest that we can isolate fungal strains from the Campina Biological Reserve that are able to produce colorant. We highlight that the strains isolated in the present work (*Penicillium gravinicaei* P3SO332, *Clonostachys rosea*P2SO329 and *Penicillium sclerotiorum* P3SO224) were able to produce colorants via submerged fermentation and the colorant presented good stability, significant antibacterial activity and low toxicity. In addition, we observed that the colored extracts of the fungi were able to dye fabric and stain pharmaceutical formulations.

The current study demonstrates the investigation of fungi able to produce colorants from the Campina Biological Reserve. Campina Biological Reserve is a Neotropical ecoregion in the Amazon that contains vegetation that is adapted to the extremely poor soil found in the region [31]. The fungi in this biome are poorly studied; however, we believe that fungi play an important role in ecology for nutrient reutilization. In this environment, we found eight isolates from which we were able to synthesize colorants that resulted in yellow, orange and red-colored fractions.

In this study, the identification of the microorganisms present in the samples revealed a predominance of filamentous fungi of the following genera: *Penicillium* , *Clonostachys* , *Phoma* and *Monascus* . The fungi of these genera reveal the potential of species isolated from the soil of forest fragments to produce substances of biotechnological interest, such as colorants [12,15,32–34].

The present work highlights *Penicillium gravinicaei* P3SO332, *Clonostachys rosea* P2SO329 and *Penicillium sclerotiorum*P3SO224 as the main producers of colorants. *P.sclerotiorum* is known to produce colored compounds ranging from orange to yellow including pencolide, sclerotiorin and isochromophylone VI [12,35]. *P. gravinicaei* is known to produce an abundant yellowish exudate, which corroborates our findings [36]. The soil-derived fungus *Clonostachys rosea* (synonymous *Gliocadium roseum*) has been extensively studied for the production of secondary metabolites, including nitrogen-containing metabolites, polyketides, triterpenoids, and fatty acids that exhibit biological activities (cytotoxic, phytotoxic and antibacterial activity) [37]. However, in these occurrences, there are no reports of studies with this species as a producer of colorants, and the present study is also the first to record an Amazonian strain of this species as a producer of colored compounds, including one of a yellow color.

The metabolite sclerotiorin was identified in the EtOAc extract of *Penicillium sclerotiorum*P3SO224. This metabolite is known to have been originally isolated from this species, but it can be produced by other fungi such as *Cephalotheca faveolate* , *Penicillium frequentans* and *Penicillium mallochii* [38–40].

Penicillic acid was identified in the EtOAc extract of *Clonostachys rosea* P2SO329. Penicillic acid is a substance that is produced as a defense mechanism by a large number of fungi (48 documented species), including members of the genera *Aspergillus* and *Penicillium* [41].

Under the experimental conditions, colored extracts of the fungi *Penicillium gravinicaei* P3SO332, *Clonostachys rosea*P2SO329 and *Penicillium sclerotiorum* P3SO224 showed good stability, significant antibacterial activity and low toxicity. These result agree with previous works from the literature: a) The red-orange colorant from *Penicillium mallochii* BAU-TACB-16 showed no color changes to the naked eye after 1 h of exposure to different temperatures and pH values [42]; b) The antibacterial activity of the crude extract obtained from the soil-derived fungus *Clonostachys rosea*YRS-06 exhibited a MIC of 25 µg/mL against *E. coli* [43], c) Pigments produced by *Penicillium mallochii* ARA1 and *Penicillium purpurogenum* Li-3 demonstrated activity against *Staphylococcus aureus* using the disk diffusion method with the zone of inhibition ranging from 6 to 13 mm [44,45]; d) The dark-yellow pigment produced by *Penicillium sclerotiorum* AK-1

has no cytotoxic effect against the yeast *Saccharomyces cerevisiae* [46] and *Penicillium murcianum* yellow pigment had no toxic effect on HEK-293 ATCC® CRL-1573 and NIH/3T3 ATCC^(r) CRL-1658 cells [47].

In the present work, the colored extracts of *Penicillium gravinicasei* P3SO332, *Clonostachys rosea* P2SO329 and *Penicillium sclerotiorum* P3SO224 were able to be used to dye fabrics and stain pharmaceutical formulations. The natural colorant isolated from *Penicillium sclerotiorum* AK-1 revealed a high potential for dyeing cotton fabrics on an industrial scale [46]. The colorant obtained from *Penicillium minioluteum* was used in the dyeing of wet blue goat nappa skin [48]. The dyeing of wool fabric samples by the yellow pigment produced by *Penicillium murcianum* was considered promising [47]. Red and yellow pigments from fungi were evaluated to enhance sun protection factor (SPF) of commercial sunscreens and *Aloe vera* extract [49].

Among the limitations in the study, one of them was that it was not possible to carry out the purification step of the colored extracts, which limited the chemical characterization of these substances via nuclear magnetic resonance (NMR), which would have permitted the isolation and elucidation of all the pigmented and non-pigmented molecules of these extracts.

The present work presents the screening of colorant production by fungi from forest soil and litter and our findings contribute to the development of technologies for production, isolation, chemical characterization, stability and application of pigments due to the few existing works in the literature that have investigated the production of these compounds using fungi. Our findings may contribute to the process of including colorants of microbial origin in all industrial sectors, whether as a food additive or a nutritional supplement or in textile and cosmetic pigmentation, which benefits both the producer and the consumer due to the various promising characteristics of these compounds. The study of compounds produced by fungi found in Amazonian soils is also of great contribution to the knowledge and development of the region.

Considering the results found in this study, it was concluded that: a) the Campinas Biological Reserve could be a source of colorant-producing fungi; b) the genera of the colorant-producing isolates were *Penicillium*, *Clonostachys*, *Phoma* and *Monascus*; c) *Penicillium gravinicasei* P3SO332, *Clonostachys rosea* P2SO329 and *Penicillium sclerotiorum* P3SO224 were the main colorant producers; d) the sclerotiorin metabolite was identified in the EtOAc extract of *Penicillium sclerotiorum* P3SO224; e) the metabolite penicillic acid was identified in the EtOAc extract of *Clonostachys rosea* P2SO329; f) under the experimental conditions, colored extracts of the fungi showed good stability, significant antibacterial activity and low toxicity; g) under the experimental conditions, the colored extracts of the fungi were able to dye fabrics and stain pharmaceutical formulations.

Authors' contributions:

L.A.O was responsible for conceptualization, methodology, investigation, data curation, and writing in both the original draft and review/editing stages. M.A.S contributed to the investigation phase. W.O.P.F.S was involved in investigation, data curation, and writing review/editing. P.A.L.S focused on data curation and writing review/editing. R.Q.L contributed to investigation and data curation. A.C.A.C was responsible for investigation. E.S.S was involved in writing review/editing. M.P.L also contributed to writing review/editing. E.S.L handled investigation and writing review/editing. H.H.F.K was responsible for writing review/editing. L.D. was involved in writing review/editing. Finally, J.V.B.S took on multiple responsibilities including conceptualization, methodology, supervision, writing review/editing, and project administration.

Funding

We would like to thank Fundacao de Amparo a Pesquisa do Estado do Amazonas (FAPEAM) for the funding of the research by Luciana Aires de Oliveira under the Universal Call FAPEAM-006/2019 and for the POSGRAD 2018 grant.

This work was funded by Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior (Finance code 001) and Fundacao de Amparo a Pesquisa do Estado do Amazonas (FAPEAM) via the project POSGRAD 2021/2022.

Statements and Declarations

The authors declare that they have no conflict of interest. This research was conducted independently, and the views and opinions expressed in this article are solely those of the authors and do not represent those of any institution or organization.

Data Availability Statement:

The data supporting the findings of this article are available within the article itself, as well as in the National Center for Biotechnology Information (NCBI) website and the supplementary material referenced in the text of the article.

5. REFERENCES

- [1] Caro, Y., Venkatachalam, M., Lebeau, J., Fouillaud, M., & Dufosse, L., 2017. Pigments and colorants from filamentous fungi. *Fungal Metab.*, 2017, 499–568.
- [2] Yusuf, M., Shabbir, M., Mohammad, F., 2017. Natural Colorants: Historical, Processing and Sustainable Prospects. *Nat. Products Bioprospect.*, 2017, **7** , 123–145.
- [3] Narsing Rao, M.P., Xiao, M., Li, W.J., 2017. Fungal and bacterial pigments: Secondary metabolites with wide applications. *Front. Microbiol.*, 2017, **8** , 1–13.
- [4] Ogbonna, C.N., 2016. Production of food colourants by filamentous fungi. *African J. Microbiol. Res.*, 2016, **10** , 960–971.
- [5] Freire, A.K.L., dos Santos Bentes, A., de Lima Sampaio, I., Matsuura, A.B.J., et al., 2012. Molecular characterisation of the causative agents of Cryptococcosis in patients of a tertiary healthcare facility in the state of Amazonas-Brazil. *Mycoses*, 2012, **55** .
- [6] Pankaj, V.P., Kumar, R., 2016. 4. Microbial pigment as a potential natural colorant for contributing to mankind. *Res. Signpost India Res. Trends Mol. Biol.*, 2016, **37661** , 85–98.
- [7] Venkatachalam, M., Magalon, H., Dufosse, L., Fouillaud, M., 2018. Journal of Food Composition and Analysis Production of pigments from the tropical marine-derived fungi *Talaromyces albobiverticillius* : New resources for natural red-colored metabolites. *J. Food Compos. Anal.*, 2018, **70** , 35–48.
- [8] Sigurdson, G.T., Tang, P., Giusti, M.M., 2017. Natural Colorants: Food Colorants from Natural Sources. *Annu. Rev. Food Sci. Technol.*, 2017, **8** , 261–280.
- [9] Dufosse, L., Fouillaud, M., Caro, Y., Mapari, S.A.S., Sutthiwong, N., 2014. Filamentous fungi are large-scale producers of pigments and colorants for the food industry. *Curr. Opin. Biotechnol.*, 2014, **26** , 56–61.
- [10] Dufosse, L., 2018. Red colourants from filamentous fungi: Are they ready for the food industry? *J. Food Compos. Anal.*, 2018, **69** , 156–161.
- [11] Kalra, R., Conlan, X.A., Goel, M., 2020. Fungi as a Potential Source of Pigments: Harnessing Filamentous Fungi. *Front. Chem.*, 2020, **8** , 1–23.
- [12] Souza, Patricia Nirlane da Costa; Grigoletto, T.L.B., Moraes, L.A.B., Abreu, L.M., Guimaraes, C.S., et al., 2016. Production and chemical characterization of pigments in filamentous fungi. *Microbiology*, 2016, **162** , 12–22.
- [13] Akilandeswari, P., Pradeep, B. V., 2016. Exploration of industrially important pigments from soil fungi. *Appl. Microbiol. Biotechnol.*, 2016, **100** , 1631–1643.
- [14] Anugraha, A.C; Thomas, T., 2021. A review on pigment producing soil fungi and its applications 2021, **4** , 89–112.

- [15] Celestino, J.D.R., Carvalho, L.E. De, Lima, M.D.P., Lima, A.M., et al., 2014. Bioprospecting of Amazon soil fungi with the potential for pigment production. *Process Biochem.*, 2014, **49** , 569–575.
- [16] Osmanova, N., Schultze, W., Ayoub, N., 2010. Azaphilones: a class of fungal metabolites with diverse biological activities. *Phytochem. Rev.*, 2010, **9** , 315–342.
- [17] Teixeira, M.F.S., Martins, M.S., da Silva, J.C., Kirsch, L.S., et al., 2012. Amazonian biodiversity: Pigments from *Aspergillus* and *Penicillium*-characterizations, antibacterial activities and their Toxicities. *Curr. Trends Biotechnol. Pharm.*, 2012, **6** , 300–311.
- [18] CLARK, F., 1965. *Actinomyces* .
- [19] Riddell, R.W., 1950. Permanent Stained Mycological Preparations Obtained by Slide Culture. *Mycologia*, 1950, **42** , 265–270.
- [20] Ferrer, C., Colom, F., Frase, S., Mulet, E., et al., 2001. Detection and Identification of Fungal Pathogens by PCR and by ITS2 and 5 . 8S Ribosomal DNA Typing in Ocular Infections. *J. Clin. Microbiol.*, 2001, **39** , 2873–2879.
- [21] White, T.J., T. Bruns, S. Lee, and J.W.T., 1990. In: Innis, M.A., Gelfand, D.H., Sninsky J.J, WTJ (Ed.), *PCR Protoc. A Guid. to Methods Appl.* , Academic in Press, New York, pp. 315–322.
- [22] Velmurugan, P., Hur, H., Balachandar, V., Kamala-kannan, S., et al., 2011. Monascus pigment production by solid-state fermentation with corn cob substrate. *JBIOSC*, 2011, **112** , 590–594.
- [23] CLSI, 2010. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts ; Approved Standard — Third Edition.vol. 28.
- [24] Cruz, K.S., Lima, E.S., Silva, M.D.J.A.D., Souza, E.S.D., et al., 2019. Screening and antifungal activity of a β -carboline derivative against *Cryptococcus neoformans* and *C. gattii*. *Int. J. Microbiol.*, 2019,**2019** .
- [25] Sharma, D., Gupta, C., Aggarwal, S., Nagpal, N., 2012. Pigment extraction from fungus for textile dyeing 2012, **37** , 68–73.
- [26] Kramar, A., Ilic-Tomic, T., Petkovic, M., Radulović, N., et al., 2014. Crude bacterial extracts of two new *Streptomyces* sp. isolates as bio-colorants for textile dyeing. *World J. Microbiol. Biotechnol.*, 2014, **30** , 2231–2240.
- [27] Leonardi, G.R., Gaspar, L.R., Maia Campos, P.M.B.G., 2002. Study of pH variation on the skin using cosmetic formulations with and without vitamins A, E or ceramide: By a non-invasive method. *An. Bras. Dermatol.*, 2002, **77** , 563–569.
- [28] Libório, J.F.D.A., 2016. CARACTERIZAÇÃO FÍSICO-QUÍMICA DO EXTRATO SECO DAS SEMENTES DE MYRCIARIA DUBIA (KUNTH) MCVAUGH E DESENVOLVIMENTO DE FORMULAÇÃO SEMISSÓLIDA. 2016.
- [29] Peres, E.G; Souza, M.P; Sousa, T.F; Silva, C.V.A; Barros, A.L; Silva, F.M.A; Costa, E.V; Medeiros, L.S; Forim, MR; Souza, A.D.L; Paz, W.H.P; Silva, G.F; Souza, A.Q.L; Koolen, H.H., 2023. Dereplication of Sclerotiorin-Like Azaphilones Produced by *J. Braz. Chem. Soc.*, 2023,**00** , 1–15.
- [30] Vansteelandt, M., Blanchet, E., Egorov, M., Petit, F., et al., 2013. Ligerin, an antiproliferative chlorinated sesquiterpenoid from a marine-derived *penicillium* strain. *J. Nat. Prod.*, 2013, **76** , 297–301.
- [31] Guimarães, F.S., Bueno, G.T., De Sena Oliveira Mendes, D., Do Nascimento, N.R., et al., 2018. Vegetation dynamics and landscape evolution in the contact between campinarana and campina on spodosols - Demini River basin-AM (Brazil). *Rev. Bras. Geomorfol.*, 2018,**19** , 587–600.
- [32] Narendrababu, B., Shishupala, S., 2017. Spectrophotometric detection of Pigments from *Aspergillus* and *Penicillium* isolates. *J. Appl. Biol. Biotechnol.*, 2017, **5** , 053–058.

- [33] Darwesh, O.M., Matter, I.A., Almoallim, H.S., Alharbi, S.A., Oh, Y.K., 2020. Isolation and optimization of *Monascus ruber* OMNRC45 for red pigment production and evaluation of the pigment as a food colorant. *Appl. Sci.*, 2020, **10** , 1–15.
- [34] Surendirakumar, K., Pandey, R.R., Muthukumar, T., Sathiyaseelan, A., et al., 2022. Characterization and biological activities of melanin pigment from root endophytic fungus, *Phoma* sp. RDSE17. *Arch. Microbiol.*, 2022, **204** , 1–15.
- [35] Lucas, E.M.F., De Castro, M.C.M., Takahashi, J.A., 2007. Antimicrobial properties of sclerotiorin, isochromophilone VI and pencolide, metabolites from a Brazilian cerrado isolate of *Penicillium sclerotiorum* Van Beyma. *Brazilian J. Microbiol.*, 2007, **38** , 785–789.
- [36] Anelli, P., Peterson, S.W., Haidukowski, M., Logrieco, A.F., et al., 2018. *Penicillium gravinicaei*, a new species isolated from cave cheese in Apulia, Italy. *Int. J. Food Microbiol.*, 2018, **282** , 66–70.
- [37] Han, P., Zhang, X., Xu, D., Zhang, B., et al., 2020. Metabolites from *clonostachys* fungi and their biological activities. *J. Fungi*, 2020, **6** , 1–30.
- [38] Chidananda, C., Rao, L.J.M., Sattur, A.P., 2006. Sclerotiorin, from *Penicillium frequentans*, a potent inhibitor of aldose reductase. *Biotechnol. Lett.*, 2006, **28** , 1633–1636.
- [39] O. dos Santos, P., Ferraz, C.G., Ribeiro, P.R., Miranda, F.M., et al., 2019. Antioxidant and antibacterial activities of the chlorine pigment sclerotiorin from *Penicillium mallochii* and its chemotaxonomic significance. *Biochem. Syst. Ecol.*, 2019, **86** , 103915.
- [40] Falade, A.O., Adewole, K.E., Ekundayo, T.C., 2021. Therapeutic potentials of endophytes for healthcare sustainability. *Egypt. J. Basic Appl. Sci.*, 2021, **8** , 117–135.
- [41] Frisvad, J.C., 2018. A critical review of producers of small lactone mycotoxins: Patulin, penicillic acid and moniliformin. *World Mycotoxin J.*, 2018, **11** , 73–100.
- [42] Bouhri, Y., Askun, T., Tunca, B., Deniz, G., et al., 2020. The orange-red pigment from *Penicillium mallochii*: Pigment production, optimization, and pigment efficacy against Glioblastoma cell lines. *Biocatal. Agric. Biotechnol.*, 2020, **23** .
- [43] Zhai, M.M., Qi, F.M., Li, J., Jiang, C.X., et al., 2016. Isolation of Secondary Metabolites from the Soil-Derived Fungus *Clonostachys rosea* YRS-06, a Biological Control Agent, and Evaluation of Antibacterial Activity. *J. Agric. Food Chem.*, 2016, **64** , 2298–2306.
- [44] Ayyolath, A., Kallingal, A., Thachan Kundil, V., Variyar, E.J., 2020. Studies on the bioactive properties of *Penicillium mallochii* ARA-1 pigment isolated from coffee plantation. *Biocatal. Agric. Biotechnol.*, 2020, **30** , 101841.
- [45] Jin, H.J., Zhang, X., Cao, H., Niu, Y.J., et al., 2018. Chemical Composition, Security and Bioactivity of the Red Pigment from *Penicillium purpurogenum* Li-3. *Chem. Biodivers.*, 2018, **15** .
- [46] Kallingal, A., Ayyolath, A., Thachan Kundil, V., Joseph, T.M., et al., 2021. Extraction and optimization of *Penicillium sclerotiorum* strain AK-1 pigment for fabric dyeing. *J. Basic Microbiol.*, 2021.
- [47] Hernández, V.A., Machuca, Á., Saavedra, I., Chavez, D., et al., 2019. *Talaromyces australis* and *Penicillium murcianum* pigment production in optimized liquid cultures and evaluation of their cytotoxicity in textile applications. *World J. Microbiol. Biotechnol.*, 2019, **35** , 1–9.
- [48] Sudha, Gupta, C., Aggarwal, S., 2016. Dyeing wet blue goat nappa skin with a microbial colorant obtained from *Penicillium minioluteum*. *J. Clean. Prod.*, 2016, **127** , 585–590.
- [49] Koli, S.H., Suryawanshi, R.K., Mohite, B. V., Patil, S. V., 2019. Prospective of *Monascus* Pigments as an Additive to Commercial Sunscreens. *Nat. Prod. Commun.*, 2019, **14** .

TABLES

Table 1

Minimum inhibitory concentration (MIC in $\mu\text{g/mL}$) of ethyl acetate extract from three isolates using the broth dilution method.

Μινιμουμ Ινιhibιτορψ δνξεντρατιον (μγ/μΛ)

Microorganisms	Microorganisms	Microorganisms	Microorganisms
Fungi	<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC 25923	<i>C. albicans</i> ATCC 60193
<i>Penicillium sclerotiorum</i> P3SO224	25 $\mu\text{g/mL}^c$	25 $\mu\text{g/mL}^t$	400 $\mu\text{g/mL}^{ft}$
<i>Penicillium graminicasei</i> P3SO332	12.5 $\mu\text{g/mL}^c$	100 $\mu\text{g/mL}^t$	400 $\mu\text{g/mL}^{ft}$
<i>Clonostachys rosea</i> P2SO329	6.25 $\mu\text{g/mL}^c$	12.5 $\mu\text{g/mL}^c$	400 $\mu\text{g/mL}^{ft}$
Positive control	Amoxilin (3.125 $\mu\text{g/mL}$)	Oxacillin (0.39 $\mu\text{g/mL}$)	Fluconazole (0.125 $\mu\text{g/mL}$)

c: bactericidal t: bacteriostatic ft: fungistatic

FIGURES LEGENDS

Fig. 1. Phases of the extracts of different polarities (hexane, ethyl acetate, methanol) using the mycelial mass and broth of the eight isolates that produce the most intense colorants (*Penicillium* sp. P3SO332, *Clonostachys* sp. P2SO329, *Penicillium* sp. P3SO224, *Penicillium* sp. P3SO322, *Phoma* sp. P2SO127, *Penicillium* sp. P3SO133, *Penicillium* sp. P2SO334, *Monascus* sp. C02172R).

Fig. 2. The evolutionary history was inferred by using the maximum-Likelihood likelihood method and the Tamura-Nei (1993) model. The percentage of trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 12 nucleotide sequences. The green dots indicate the isolates from this study and the others are ITS sequences of colorant-producing fungi. There was a total of 738 positions in the final dataset. Evolutionary analyses were conducted in MEGA X v10.2.4.

Fig. 3. Effect of different carbon and nitrogen sources in the production of colorants with the three selected fungi. *Penicillium graminicasei* P3SO332 (A, B); *Clonostachys rosea* P2SO329 (C, D); *Penicillium sclerotiorum* P3SO224 (E, F). Different letters represent significant differences according to the Student's t-test ($p < 0.05$).

Fig. 4. Structure of sclerotiorin present in the EtOAc extract of the fungi *P. sclerotiorum* P3SO224 (1) and penicillic acid (2) present in the EtOAc extract of *Clonostachys rosea* P2SO329. *Structures designed using Chemdraw 19.0

Fig. 5. Polyester, silk and cotton fabrics dyed with crude colorants extracted from *Clonostachys rosea* P2SO329 (CR9), *Penicillium graminicasei* P3SO332 (PG2) and *Penicillium sclerotiorum* P3SO224 (PS4).

Fig. 6. Color and aspect of cream formulations comprising the colorant extracts (0.05 mg per gram of the formulation) produced by *Penicillium sclerotiorum* P3SO224 (PS4 10 mg), *Clonostachys rosea* P2SO329 (CR9 14 mg), *Penicillium graminicasei* P3SO332 (PG2 14 mg) and commercial colorant (yolk-yellow liquid food coloring - MIX).

Fig. 7. Soap formulation using colorant 1 (0.05 mg per gram) obtained from *Penicillium sclerotiorum* P3SO224. Commercial colorant (yolk-yellow liquid food coloring - MIX).

FIGURES

















































Fungal isolates	Phases					
	Mycelial Mass			Broth		
	Hexane	Ethyl Acetate	Methanol	Hexane	Ethyl Acetate	Water
<i>Penicillium</i> sp. P3SO332						
<i>Clonostachys</i> sp. P2SO329						
<i>Penicillium</i> sp. P3SO224						
<i>Penicillium</i> sp. P3SO322						
<i>Phoma</i> sp. P2SO127						
<i>Penicillium</i> sp. P3SO133						
<i>Penicillium</i> sp. P2SO334						
<i>Monascus</i> sp. C02172R						

Fig. 1. Phases of the extracts of different polarities (hexane, ethyl acetate, methanol) using the mycelial mass and broth of the eight isolates that produce the most intense colorants (*Penicillium* sp. P3SO332, *Clonostachys* sp. P2SO329, *Penicillium* sp. P3SO224, *Penicillium* sp. P3SO322, *Phoma* sp. P2SO127, *Penicillium* sp. P3SO133, *Penicillium* sp. P2SO334, *Monascus* sp. C02172R).

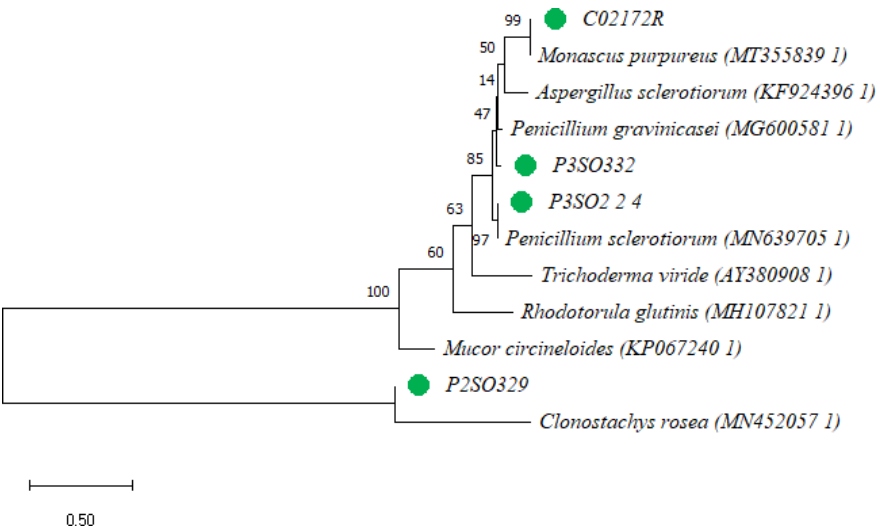


Fig. 2. The evolutionary history was inferred by using the maximum-Likelihood likelihood method and the Tamura-Nei (1993) model. The percentage of trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 12 nucleotide sequences. The green dots indicate the isolates from this study and the others are ITS sequences of colorant-producing fungi. There was a total of 738 positions in the final dataset. Evolutionary analyses were conducted in MEGA X v10.2.4.

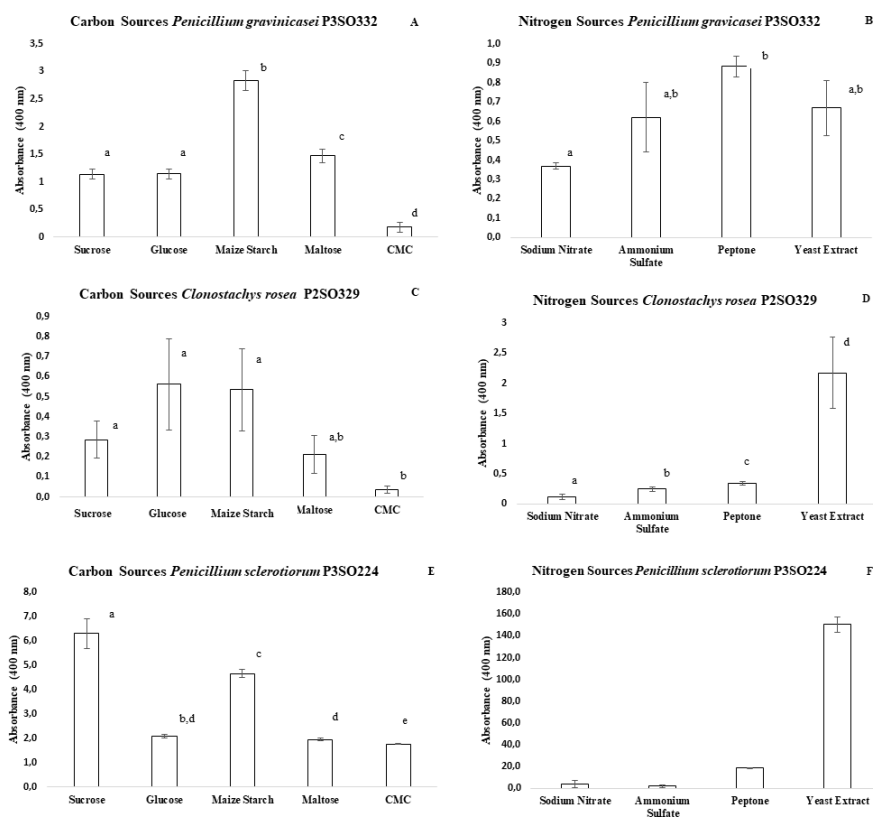


Fig. 3. Effect of different carbon and nitrogen sources in the production of colorants with the three selected fungi. *Penicillium graminicasei* P3SO332 (A, B); *Clonostachys rosea* P2SO329 (C, D); *Penicillium sclerotiorum* P3SO224 (E, F). Different letters represent significant differences according to the Student's t-test ($p < 0.05$).

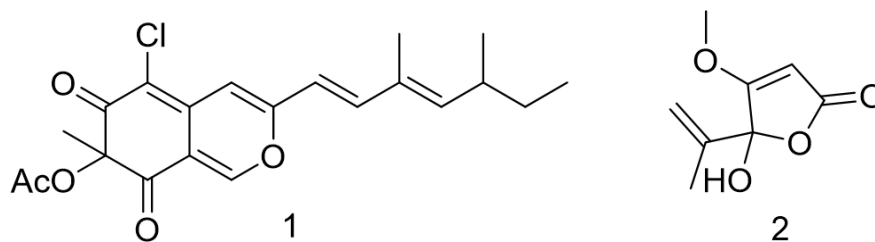


Fig. 4. Structure of sclerotiorin present in the EtOAc extract of the fungi *P. sclerotiorum* P3SO224 (1) and penicillic acid (2) present in the EtOAc extract of *Clonostachys rosea* P2SO329. *Structures designed using Chemdraw 19.0

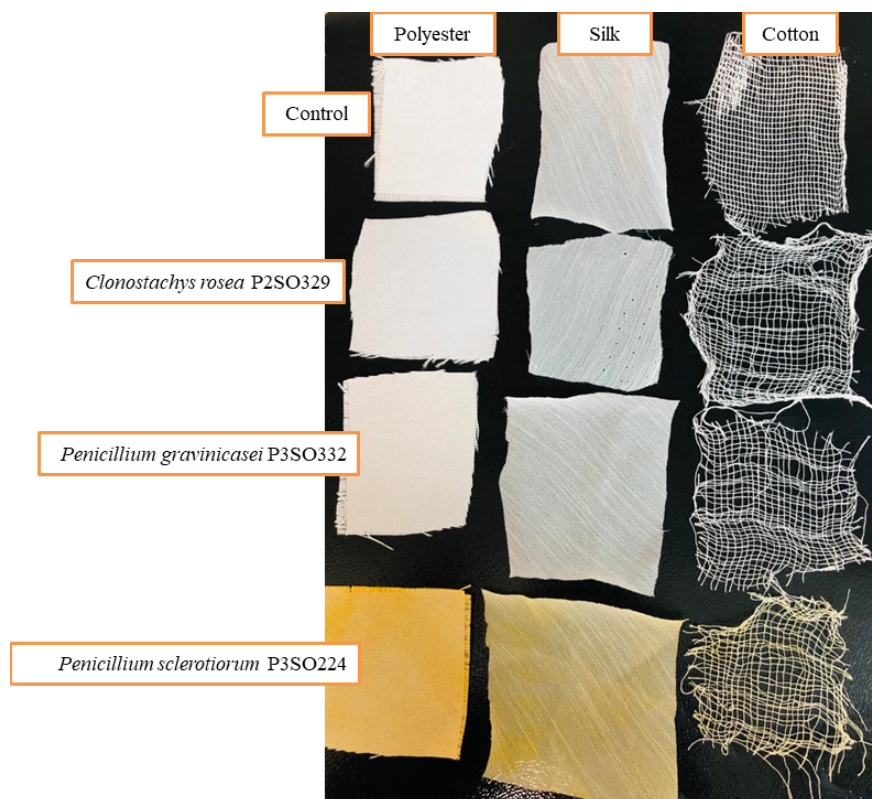


Fig. 5. Polyester, silk and cotton fabrics dyed with crude colorants extracted from *Clonostachys rosea* P2SO329 (CR9), *Penicillium gravinicasei* P3SO332 (PG2) and *Penicillium sclerotiorum* P3SO224 (PS4).



Fig. 6. Color and aspect of cream formulations comprising the colorant extracts (0.05 mg per gram of the formulation) produced by *Penicillium sclerotiorum* P3SO224, *Clonostachys rosea* P2SO329, *Penicillium gravinicasei* P3SO332 and commercial colorant (yolk-yellow liquid food coloring - MIX).



Fig. 7. Soap formulation using colorant 1 (0.05 mg per gram) obtained from *Penicillium sclerotiorum* P3SO224. Commercial colorant (yolk-yellow liquid food coloring - MIX).