Bioprospecting of Amazon soil fungi with the potential for pigment production

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A B S T R A C T
The aim of this study was to isolate fungi able to produce pigments. Fifty strains were isolated from the Amazon soil by the conventional technique of serial dilution. Submerged fermentation was performed in Czapek broth in order to select strains able to synthesise pigments. Five strains were able to produce pigments and were identified by sequencing the rDNA (ITS regions). These fungi were identified as Penicillium sclerotiorum 2AV2, Penicillium sclerotiorum 2AV6, Aspergillus calidoustus 4BV13, Penicillium citrinum 2AV18 and Penicillium purpurogenum 2BV41. P. sclerotiorum 2AV2 produced intensely coloured pigments and were therefore selected for chemical characterisation. NMR identified the pigment as sclerotiorin. In this work, the influence of nutrients on sclerotiorin yield was also studied and it was verified that rhamnose and peptone increased production when used separately. These results indicate that Amazonian fungi bioprospecting is a viable means to search for new sources of natural dyes.

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1. Introduction

Fungi are present in almost every environment on earth, with the greatest diversity found in tropical regions that have a hot and humid climate, which favours fungal multiplication [1]. Among the tropical biomes, the Amazon rainforest contains the richest biodiversity, with a large number of plants, animals and microorganisms that are not well known [2]. The soil of this forest, contrary to what one might imagine, is poor in nutrients, and the maintenance of this rich forest is ensured by the innumerable microbial diversity present in the soil, allowing the forest’s animals and plant components to feed by recycling organic matter [3–5]. Soil fungi are metabolically very active and are able to produce many substances of economic value, including enzymes of industrial interest [6], metabolites with pharmacological activity [7] and pigments [8].

Dyes derived from natural sources have been increasingly used by pharmaceutical, textile and food industries due to the dyes’ lower toxicities to the environment and to man when compared to synthetic dyes [9,10]. Several organisms, such as plants, animals, bacteria, fungi and algae, are capable of synthesising pigments, but fungi stand out for their potential to produce large amounts of dye in small spaces [11,12]. Most pigment-producing fungi are of the Aspergillus, Penicillium, Paeclomyces and Monascus species [8,13]. The majority of the pigments produced by fungi are quinones, flavonoids, melanins and azaphilones, which belong to the aromatic polyketide chemical group [14] and have been widely described for medicinal uses and potential use as dyes [15,16]. Given that many synthetic dyes used today are severely criticised for their long-term mutagenic and carcinogenic effects, legislation has imposed increasingly stringent restrictions on synthetic dyes, especially those that are food additives [11,17]. The chemical synthesis of natural products is not very viable because it generally leads to high costs and low yields; consequently, the search for biological sources that generate significant amounts of colourants has increased substantially [18].

In this context, with the aim of increasing the production capacity of natural dyes by fungi, submerged fermentation was carried out, and the factors that influence the biosynthesis of these...
metabolites, such as the nutritional composition of the culture medium, have been extensively studied [19]. The sources of carbon and nitrogen influence the growth of fungus, the type of pigment produced and the yield of the desired substance [20]. In this work, to identify Amazonian soil fungi that have the ability to produce pigments, it was (i) isolated and identified Amazon soil fungi that produce pigment, (ii) chemically characterised the pigment produced by a fungal isolate, and (iii) evaluated the effect some sugars and nitrogenous compounds had on the production of pigment.

2. Materials and methods

2.1. Isolation, identification and preservation of soil fungi

Four soil samples were collected from the surface (1–3 cm depth) of the forest located at the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil (Latitude-South 03°09’39” and Longitude West 59°98’77”). For isolation, 1 g of soil was transferred to a tube containing 9 mL of sterile distilled water, which was serially diluted from 10⁻² to 10⁻⁵ g/mL; 0.1 mL from each dilution was inoculated in Petri dishes containing Potato Dextrose Agar (PDA) with chloramphenicol (250 mg/L). The experiment was performed in triplicate. The plates were incubated at room temperature (25 ± 2 °C). Colonies that grew in 72 h were seeded onto PDA plates until individual colonies appeared. The genera of fungal species were identified based on the macro- and morphological characteristics, as suggested by Lacaz et al. [21] and Barnett and Hunter [22].

A visual examination of the flasks was performed after 14 days of fermentation and only fungi that released pigments in Czapek medium during this period were submitted to identify the species by molecular biology. Specifically, fungal DNA was extracted from mycelium using the QiAamp Tissue and Blood (Qiagen®, Hilden, Germany) extraction kit according to the manufacturer’s recommendations. The Internal Transcribed Spacer (ITS) was amplified using the primers ITS1/ITS4 [23]. PCR products were purified with polyethylene glycol, based on the protocol described by Lis and Schleif [24], with modifications of Lis [25] and Paithankar and Prasad [26]. The sequencing reaction was performed with the Big-Dye terminator cycle sequencing reagents (BigDye®, Applied Biosystems, Foster City, CA, USA) kit, and sequencing was carried out in the ABI Prism® Seq 3130 Genetic Analyzer (Applied Biosystems). The sequences obtained were compared to those in the GenBank database (database incorporating DNA sequences of all publicly available sources).

A method developed by the INPA laboratory of Medical Microbiology was used to preserve the isolated strains capable of producing pigments. In this technique, 0.4 mL of distilled water, 0.025 mL of DMSO dimethylsulphoxide (cryoprotector), 0.050 mL of glycerol (cryoprotector) and 10 g of glass beads (with holes) were added to autoclaved cryotubes. Approximately 250 mg of small fragments collected from fungal cultures grown on PDA plates for 7 days, at room temperature (25 ± 2 °C), were transferred to microtubes (2 mL). This procedure was performed in triplicate. The vials were sent to the Mycology Collection Centre at INPA and were stored at −70 °C.

2.2. Screening for pigment production

To identify which fungi isolated from the soil had the potential for pigment production, submerged fermentation was performed. Initially, the fungal isolates were transferred to tubes containing PDA and incubated at room temperature (25 ± 2 °C) for 72 h. After 3 days, the fungus spores of each isolate were suspended in sterile distilled water (approximately 2 mL) and counted using a Neubauer chamber. This spore suspension was used to inoculate Erlenmeyer flasks (250 mL) with 50 mL of Czapek broth (3 g/L NaNO₃, 1 g/L KH₂PO₄, 0.5 g/L MgSO₄·7H₂O, 0.5 g/L KCl, 0.01 g/L FeSO₄·7H₂O and 30 g/L sucrose), pH of 5.0 and concentration of 1 × 10⁸ spores/mL medium. The flasks were incubated at room temperature (25 ± 2 °C) for 14 days and kept in a dark place, and fermentation occurred in static conditions. Culture media containing pigments (50 mL) were subjected to successive extractions with solvents of different polarities: hexane (30 mL), ethyl acetate (30 mL) and butanol (30 mL). After extraction three fractions of each solvent were obtained for each producing fungus. There was a visual analysis of fractions derived and a fungal strain was selected for further characterisation stage chemical, being chosen one that had the highest number of coloured fractions and also fractions containing pigments noticeably more intense. The selected strain was fermented in a volume of 4 L, and the pigment was extracted from the broth containing mycelium. Successive extractions with 100 mL ethyl acetate were performed until the total volume of 2 L. The extract was concentrated in a rotary evaporator (IKA, RV10 digital, Santa Clara, CA, USA) and then fractionated by column chromatography on a Sephadex LH-20 (h × Ø = 52.0 cm × 3.0 cm) column (Sigma–Aldrich Co, St. Louis, MO, USA), using 100% methanol as the eluent; 22 fractions were collected. The thin layer chromatography profile allowed to choose fraction 11 (197.3 mg) for further analysis by adding it to a microcrystalline cellulose column (h × Ø = 25.0 cm × 2.0 cm) (Merck, Darmstadt, Germany), eluted with a hexane:ethyl acetate gradient. The first 9–11 fractions, combined, yielded a precipitate that was purified with hexane:ethyl ether to give compound 1 (orange precipitate, 6 mg). The structural characterisation of the pigment was performed by NMR on a Bruker Fourier 300 apparatus; chemical shifts (δ) were expressed in ppm and coupling constants (J) in Hertz. A UV/vis spectrophotometer (Model No. UV-1102 SP) was used to identify the maximum absorbance (λmax) of the pigment, ranging from 320 to 700 nm.

2.4. Effect of carbon and nitrogen sources on pigment production

To investigate the influence of the culture medium on pigment production, the Czapek broth was prepared with modified carbon (30 g/L) and nitrogen (3 g/L) sources. The carbohydrates evaluated were sucrose, glucose, fructose, lactose, galactose, rhamnose and xylose. The nitrogen sources analysed were sodium nitrate, potassium nitrate, peptone (12% total nitrogen), yeast extract (8% total nitrogen), malt extract (2% total nitrogen) and monosodium glutamate. All these substances had analytical grade and were obtained by Sigma–Aldrich, USA. The experiments were performed in triplicate, where the bioprocess was conducted as described in section 2.2 [250 mL Erlenmeyer flasks, 50 mL of Czapek broth, in static conditions, kept in the dark at room temperature (±25 °C), with 14 days of fermentation]. To extract the pigment, 30 mL of ethyl acetate was added to the flask being collected after only 24 h. Pigment production was measured using a UV/vis spectrophotometer (Model No. UV-1102 SP) through the maximum absorbance of the pigment analysis (λmax).

3. Results

3.1. Isolation and identification of Amazon soil fungi

To isolate microorganisms to be screened for pigment production, soil samples were serially diluted and plated on BDA to obtain isolated colonies. The isolated cultures were determined to belong to the Ascomycota phylum. It was obtained 50 filamentous fungi
Table 1
Coding and genus identification of fungi isolated from samples of the Amazon soil.

<table>
<thead>
<tr>
<th>Number</th>
<th>Code</th>
<th>Genus</th>
<th>Number</th>
<th>Code</th>
<th>Genus</th>
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<td>Trichoderma sp.</td>
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<th>Fractions</th>
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<tr>
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<td>3.3</td>
<td></td>
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<tr>
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<td>7.5</td>
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</tr>
<tr>
<td><strong>Penicillium citrinum</strong> 2AV18</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td><strong>Penicillium purpurogenum</strong> 2BV41</td>
<td>5.4</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Pigments produced by Penicillium sclerotiorum 2AV2, Penicillium sclerotiorum 2AV6, Aspergillus calidoustus 4BV13, Penicillium citrinum 2AV18 and Penicillium purpurogenum 2BV41 extracted with solvents of different polarities (hexane, ethyl acetate and butanol) (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

3.2. Screening of pigment production by soil fungi and molecular identification of dyes

To screen strains for pigment production, the strains in Table 1 were subjected to submerged fermentation in Czapek broth. As
a result, it was found that 5 of the 50 fungi were able to synthesise pigments. The pigments were extracted after fermentation by successive partition with hexane, ethyl acetate and butanol. Fractions with colours ranging from yellow to red, as shown in Fig. 1, were collected. The five pigment-producing fungi were identified by ribosomal DNA sequencing (rDNA region) and showed similarity with the species *Penicillium sclerotiorum* (100%, strains 2AV2 and 2AV6), *Aspergillus calidoustus* (99%, strain 4BV13), *Penicillium citrinum* (99%, strain 2AV18) and *Penicillium purpurogenum* (99%, strain 2BV41).

### 3.2.1. Chemical characterisation

*Penicillium sclerotiorum* 2AV2 released intensely coloured substances in three of the used solvents; consequently, it was selected for further chemical colourant characterisation. Purification procedures resulted in a yellow–orange pigment. NMR analysis allowed the structural elucidation of the molecule. Carbons were assigned based on HSQC and HMBC data. The long-range correlations observed (HMBC experiment) confirmed the attribution of all the carbon signals of the molecule. The spectral data of 1H and 13C NMR (Fig. 2; Table 2) were compared with (+)-sclerotiorin identified by Paired et al. [27], and confirmed the identification of the pigment sclerotiorin (Fig. 3). Scanning spectrophotometry showed that the sclerotiorin pigment has a maximum absorbance (\( \lambda_{\text{max}} \)) in ethyl acetate at 350 nm.

#### 3.2.1.1. Effect of carbon and nitrogen sources on pigment synthesis

To study the effect of the culture medium on sclerotiorin production, the selected fungus was grown in Czapec medium base modified with different sources of carbon or nitrogen in each experiment. The carbon sources were composed of carbohydrates and nitrogen sources were represented by inorganic salts or nitrogenated organics. The maximum absorbance at 350 nm was achieved when rhamnose was used as sugar in the fermentation base medium (data are shown in Fig. 4). Peptone and yeast extract were the nitrogen sources that allowed the greatest absorbances at the analysed wavelengths (\( \lambda_{\text{max}} \times 350 \text{ nm} \)) (data are shown in Fig. 4).

![Fig. 2](image-url)  
**Fig. 2.** 1H(300 MHz) NMR spectral data (a), HSQC (Heteronuclear single-quantum correlation spectroscopy) (b) and HMBC (Heteronuclear Multiple Bond Correlation) (c) spectrum of the pigment produced by *Penicillium sclerotiorum* 2AV2.
4. Discussion

Soil fungi have been widely isolated from nature for their ability to produce pharmacologically active molecules [7]. These microorganisms have also been noted to produce natural pigments that could be used in the dye industry, given the variety and intensity of colours produced by different strains of environmental fungi and the great value to natural colourants [8,28]. Despite the Amazon rainforest being a home to a great diversity of microorganisms, there are few published studies that explore the ability of these fungi to produce pigments. Therefore, this work aimed to isolate pigment-producing soil fungi from the Amazon.

In this work, the identification of microorganisms in soil samples revealed the existence of 50 fungi of the genera *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, *Paecilomyces*, *Verticillium* and *Scedosporium*. These fungi have been isolated from environmental samples of soil [6,29], water [30,31], interior plants (endophytic fungi) [32,33], marine organisms [34,35] and even arctic glacial ice [36,37]. Serial dilution was used for the isolation of soil fungi. This is a very usual techniques for isolating microorganisms from environmental samples, since these often harbour a large number of viable microbial cells. Thus, it was possible to obtain isolated colonies, Amazonian soil samples had to be diluted before plating on culture media. On the other hand, the dilution restricted the number of fungal strains and allow only a small fraction of fungi present in the soil were identified where the samples were collected.

Of the all isolates, some fungi produce pigments in Czapeck broth during submerged fermentation. Producing fungi were identified by rDNA ITS region as *P. sclerotiorum* 2AV2, *P. sclerotiorum* 2AV6, *A. calidoustus* 4BV13, *P. citrinum* 2AV18 and *P. purpurogenum* 2BV41.

*P. sclerotiorum* is a recognised producer of azaphilones, including rotiorin, isochromaphilone and sclerotiorin, with the latter being the most reported for these species [38,39]. The literature on *A. calidoustus* describes it as a rare pathogen that can cause infection mainly in those immunocompromised [40] but can also produce metabolites of industrial interest [41]. *P. citrinum* is a producer of anthraquinone chromophores [42] and is often described by mycotoxin citrinin production, a yellow metabolite that induces nephrotoxicity and hepatotoxicity in humans [43]. *P. purpurogenum* is a producer of azaphilones, such as mitorubrin and mitorubrinol, and quinones as purpurgenone and purpurquinones [44,45]. Amazon strains of *P. purpurogenum* have also already been described for the production of coloured substances [16].

In this work, the pigments produced by *P. sclerotiorum* 2AV2 were chosen for chemical characterisation due to their intense colouration in the three solvents, indicating a fungus with good production capacity. The substance was identified by one-dimensional and two-dimensional NMR as sclerotiorin, a yellow–orange pigment that is soluble in organic solvents such as ethyl acetate and ether but insoluble in water, being first reported its synthesis by fungi of the Amazon.

Sclerotiorin was originally isolated from *P. sclerotiorum* [46]; however, it can also be produced by other fungi [47,48]. It has important biological activities, including endothelin receptor binding activity [27], aldose reductase inhibition [48], antimicrobial activity [39,48], antifungal activity [50], inducing apoptosis of HCT-116 cancer cells [47] and inhibitory effect on human immunodeficiency virus HIV-1 integrase and protease [49].

Pigment synthesis by fungi is directly influenced by the nutrients available in the medium, such as carbon and nitrogen, as well as the selection of the fungal strain due to interspecies variation [19]. Some carbohydrates or compounds containing nitrogen can be more easily assimilated by certain strains than for others, which justifies the higher or lower yields of the desired product [20]. In this study, synthesis of sclerotiorin by *Penicillium sclerotiorum* 2AV2 was on average three times higher when the medium was prepared with rhamnose sugar compared to sucrose (the usual sugar in Czapeck medium). Galactose was the second choice to obtain higher concentrations of the bioprocess. Lactose is a convenient carbon source for many microorganisms [50], but it caused a large decrease in the production of sclerotiorin for this strain.

![Fig. 4. Effect of different carbon (a) and nitrogen (b) sources in production of Sclerotiorin (λ<sub>max</sub> in ethyl acetate at 350 nm) by *Penicillium sclerotiorum* 2AV2.](image-url)
These results show that unusual sources in culture media available can significantly increase the synthesis of this pigment by the *P. sclerotium* 2AV2 fungal strain isolated from an Amazonian soil sample.

The results indicate that the effect of nitrogen sources on the synthesis of sclerotiorin was far greater than that exerted by carbohydrate sources. The literature describes nitrogen sources as very important in the synthesis of pigments for regulating the expression of genes of interest and activating important pathways in the production of these compounds [51]. In the present study, nitrogenated peptone and yeast extract most favourled sclerotiorin production when compared to sodium nitrate (the usual nitrogen source in Czapek), with a yield more than six times higher in conventional Czapek. Peptone is commonly used in various culture media, and many fungi appear to have the ability to use it during metabolism, leading to increased production of pigments [52]. In addition to nitrogen, the organic nitrogen compounds provide carbon and sulphur to the culture medium and enable the fungus to obtain energy. Yeast extract and malt also provide vitamins and coenzymes, favouring the growth of the fastidious microorganisms. However, it is known that pigment production is favoured when the medium contains nitrogen sources, especially of organic origin [53]. In the present study, it was found that yeast extract showed results consistent with peptone, increasing pigment synthesis. However, the use of malt extract caused suppression of pigment production for the fungal strain studied. In fermentation, the use of malt, peptone, and rice as a nitrogen source for *P. sclerotium* LAB18 isolated from Brazilian cerrado soil, peptone was also the nutrient that caused the greatest increase in sclerotiorin pigment production, while the others showed low levels of production [54].

In this work, when a new submersed fermentation was prepared with peptone and rhamnose (the sources of carbon and nitrogen that increased production of sclerotiorin when the modified base medium Czapek), under the same conditions as previously described fermentation, the maximum absorbance ($A_{\text{max}}$) at 350 nm was 69 ± 0.15, showing that the combination repressed the synthesis of sclerotiorin when compared with the bioprocess in which the peptone was used without modifying the usual way Czapeck sugar (sucrose). As mentioned above, peptone is an organic nitrogen source; therefore, it also provides carbon molecules, providing the culture medium with many nutrients. The use of rhamnose in this case may have caused the phenomenon of carbon catabolite repression, in which the presence of two sources easily assimilable by the fungus can reduce or even suppress the production of the metabolite of interest.

The aim of these experiments with different carbon and nitrogen sources was to start a discussion about the nutritional conditions related to production of the pigment. It is well known that for the industrial purposes it is necessary inexpensive substrates and the results presented in the present work can help selection of these substrates.

Considering the results found in this study, it was concluded that fungi from Amazon soil may be potential pigment producers. Of the 50 fungal isolates, 5 species stood out by the synthesis of coloured compounds in Czapek broth, whether they belong to the genera *Penicillium* sp. and *Aspergillus* sp. Sclerotiorin, which is an important pigment that has been isolated previously, was isolated by *P. sclerotium* 2AV2. Through the standardisation of carbon sources and nitrogen, it was possible to increase the production of this compound, though more studies are needed to optimise the production by the selected strain. Given these findings, it was concluded that the vast diversity of Amazon microorganisms with biotechnological potential and the increasing need for new bio-colourants demonstrate the importance of further bioprospecting studies.

References


