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Bioprospecting of Amazon soil fungi with the potential for pigment production

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ABSTRACT

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 Czapeck 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.

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 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*, *Paecilomyces* 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

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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 micromorphological 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 Czapeck 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 Czapeck broth (3 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4{\cdot}7H_2O$ and 30 g/L sucrose), pH of 5.0 and concentration of 1×10^4 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.

2.3. Isolation and chemical characterisation of pigment produced by selected fungus

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 ($h \times$ 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 ($h \times$ 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 (λ_{max}) 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 Czapeck 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 Czapeck 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 (λ_{max}).

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