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Characterization and Enhanced Production of Fungal Melanin from Aspergillus Species Isolated from Soil

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Abstract:
This study aimed to isolate and identify fungi from soil samples, focusing on their ability to produce melanin. Eleven different genera of fungi were isolated and identified using lactophenol cotton blue staining and microscopic examination, referencing H.L. Barnett and Barry B. Hunter. Among these, only Aspergillus was found to produce melanin. Optimal melanin production conditions were determined to be a 1.5% tyrosine supplement in the growth medium, under shaking conditions (120 rpm) and dark incubation for three weeks, resulting in a yield of 21.08 mg/100 ml. Physiochemical characterization revealed that the extracted melanin was insoluble in organic solvents but soluble in alkaline solutions (NaOH, KOH), and partially soluble in DMSO. Spectrometric analysis using UV-Visible spectroscopy showed characteristic absorption peaks. FTIR indicated functional groups and Scanning Electron Microscopy (SEM) images displayed a granular and heterogeneous surface topology. The study also assessed the effects of different carbon and nitrogen sources, and trace elements on melanin production. Maltose and sucrose were the most effective carbon sources, while peptone was the most effective nitrogen source. Among trace elements, calcium significantly enhanced melanin yield, while copper and zinc had moderate effects. These findings provide valuable insights into optimizing fungal melanin production and its potential industrial applications. The study highlights the sustainable and scalable production of melanin from Aspergillus, contributing to the broader understanding of fungal metabolites and their commercial exploitation. Future research should focus on genetic and metabolic pathways to further enhance melanin biosynthesis and explore its diverse applications.

Keywords: Melanin, *Aspergillus*, soil fungi, FTIR, Optimization.

Introduction:

Melanin, a naturally occurring pigment, holds immense significance in both biological and industrial contexts. Biologically, melanin shields living organisms from harmful UV radiation, preventing DNA damage, mutations, and skin cancers in humans. It contributes to the diversity of pigmentation, determining skin and hair color, and may also play protective roles in the eyes, brain, and immune system (Vargas et al., 2015). Industrially, melanin finds applications in cosmetics, skincare, and bio-inspired sunscreens. Its properties are leveraged in biomedical fields for drug delivery and imaging (Tian et al., 2003). Additionally, melanin-based materials advance materials science, photovoltaics, and sustainable pigments for various industries. Melanin's versatile attributes continue to drive innovation across a range

of disciplines (Sánchez-Ferrer et al., 1995). The production of melanin remains a complex and tedious process, primarily occurring within living organisms such as humans, animals, and some microorganisms (Chornenka et al., 2018). However, advancements in biotechnology and synthetic biology have led to the development of alternative methods for melanin production. Alkaline hydrolysis is a common method used for melanin extraction from feathers and hair, involving treatment with a strong alkaline solution at an elevated temperature for an extended period (Rosas et al., 2000). Alternatively, acid hydrolysis is a good approach for melanin production from plant tissues and fungal cells, utilizing concentrated acids under controlled conditions (El-Naggar and El-Ewasy, 2017). Enzymatic digestion provides a gentle method for melanin extraction, where proteolytic enzymes digest the proteins surrounding melanin granules. This method can be applied for extracting melanin from tissues or cells (Rodrigues et al., 2008). Organic solvents like DMSO and DMF can also be used, where tissues are homogenized in the solvent to facilitate melanin extraction. Ultrasonication, combined with a mixture of solvents, can disrupt cell walls to release melanin (Tarangini and Mishra, 2014). Additionally, melanin can be produced by culturing melanin-producing bacteria or fungi, and it can be collected from the culture medium or microbial biomass (Kejžar et al., 2013). The extraction of melanin is sensitive to the source material, and the choice of method can be tailored to the intended downstream application of the extracted melanin.

Fungal melanin, a unique and versatile biopolymer, has garnered attention for its diverse biological and industrial applications. In a recent study, fungal melanin was isolated from soil samples collected from a specific regional area, highlighting the untapped potential of local microbial biodiversity (Verma and Karkun Sur, 2017). The extraction and optimization of melanin from these soil-derived fungi involved a series of innovative methodologies aimed at maximizing yield and purity (Karkun Sur and Verma, 2016). Initially, the soil samples were subjected to microbial isolation techniques to identify melanin-producing fungal strains. Once isolated, these strains underwent a rigorous extraction process utilizing both chemical and enzymatic methods to efficiently release melanin from the fungal cells (Karkun Sur et al., 2016). The extraction protocols were carefully optimized, taking into consideration factors such as pH, temperature, and solvent concentration, to enhance the quality and quantity of melanin produced. The optimized extraction process not only improved the efficiency of melanin recovery but also ensured the preservation of its key properties, making it suitable for various applications. This study underscores the importance of exploring local soil microbiota for novel sources of melanin and paves the way for sustainable and scalable production methods that can be tailored to meet specific industrial needs (Nosanchuk et al., 2015).

Materials and Methods:

Sample collection & Isolation of fungi: The soil sample was procured from garden of Amity University Chhattisgarh, Raipur (Latitude-21.250000 & longitude-81.629997) on June 2022. The collected soil sample was serially diluted from 10^-1 to 10^-6, and fungi were isolated by plating the diluted soil onto potato Czapek dox agar media (CDA) supplemented with 0.01% w/v chloramphenicol. These plates, placed in 90mm petri dishes, were sealed with double parafilm and incubated at 28 ± 1 ℃ for 15 days under dark conditions. The plates were regularly monitored every 48 hours for fungal growth. The emerging fungi were subsequently sub-cultured onto fresh CDA. Only representative isolates were preserved in glycerol stock (40%) as axenic cultures for further use, while repetitive isolates were discarded (Karkun Sur and Verma, 2016; Karkun Sur et al., 2016).

Screening of Melanin Producing Fungi: Melanin production by the isolated fungal species was evaluated using previously described methods with minor modifications. Briefly, a 5mm mycelial plug

from a seven to eight days-old fungal culture was inoculated onto L-tyrosine (1.5%) CDA medium. The plates were sealed with double parafilm and incubated in dark conditions for 15 days. Fungal isolates exhibiting brownish black pigmentation were selected and sub-cultured through three generations to verify the stability of the pigmentation. The fungal isolate that demonstrated the most stable and intense pigmentation was chosen for further identification and characterization (Kejžar et al., 2013).

Microscopic Identification of melanin producing fungi: Well, grown fungal mycelium were picked from CDA plates with the help of sterile scalpel and cut down the small portion of mycelium. One drop of lactophenol cotton blue stain was placed on a clean glass slide and small portion of fungal mycelium was transferred on it. The sample was covered with cover slip ensuring there was not any air bubble present. The slide was then observed in microscope.

Production of melanin pigment and biomass production: Culture of Czapek dox broth (CDB) was prepared by growing the fungus in pre-sterilized CDB. Concisely, the process involved aseptic inoculation of 100 ml pre-sterilized CDB in a 250 ml Erlenmeyer flask (Borosil, India), supplemented with L-tyrosine (1.5%, w/v). Mycelial plugs (5 mm) from a seven to eight days-old culture of *Aspergillus species* were added to the medium. The cultures were incubated at 28 ± 1 ℃ under both static and shaking conditions (120 rpm) for three weeks. During incubation, the broth color changed to brownish black, indicating the onset of pigment production by the fungal strain. After the incubation period, the fungal biomass was separated from the broth by filtration, followed by centrifugation at 7000 g for 15 minutes. The obtained fungal mycelium was collected and dried in a hot air oven at 60 ± 1 ℃ for 26 hours. The dried mycelial biomass was then evaluated for melanin production (Singla et al., 2021).

Extraction and Partial purification of melanin: The extraction of melanin pigment extracellularly was conducted using the acid/alkali method described by De la Rosa et al. (2017) and Gadd (1982), with minor modifications. The dried fungal biomass was soaked in a beaker containing 1N KOH solution overnight and then autoclaved at 121°C for 20 minutes. The mycelium was ground into fine particles by adding a small amount of double-distilled water using a mortar and pestle. The solution was centrifuged at 5000 g for 15 minutes to collect the supernatant. The supernatant was acidified with 3N HCl until the pH reached 2.5, causing precipitation. This mixture was then centrifuged at 8000 g for 15 minutes to collect the pellets. The pellets were washed three times with double-distilled water and extracted using a mixture of ethanol, chloroform, and ethyl acetate in a ratio of 2:3:2. The resultant pellets were thoroughly dried in a hot air oven at 60°C, yielding melanin in powdered form. The melanin powder was finally dissolved in a 10% KOH solution (Rajagopal et al., 2011).

Characterization of Extracted Melanin:

Physiochemical analysis: Tests were conducted following the methods described by Nakarin Suwannarach et al. (2019) to characterize the fungal melanin obtained from Aspergillus species. The primary physicochemical properties of the fungal melanin, such as solubility in distilled water and common organic solvents like acetone, chloroform, dimethyl sulfoxide, ethanol, methanol, and ethyl acetate, as well as inorganic solvents such as KOH and NaOH, were determined (Zaidi et al., 2014).

Spectrometric analysis: The obtained fungal melanin (1 mg) was dissolved in 20 ml of 10% 1N KOH following the method described by Rajagopal et al. (2011). The UV-visible absorption spectrum of the fungal melanin was scanned over a wavelength range of 200-420 nm using a UV-visible spectrophotometer (UV-Vis spectrophotometer Evolution™ 201/220). The 10% 1N KOH solution served

as the reference blank. The maximum absorption levels of the fungal melanin were recorded (Harki et al., 1997).

Fourier infrared transformed spectrum analysis: The purified fungal melanin was homogenized with analytical grade potassium bromide (KBr) and processed for FTIR analysis. The samples were pressed into discs under vacuum using a KBr press (Singla et al., 2021). The FTIR spectra were recorded using a Benchtop LT4100 Labtronics spectrometer. The spectra were measured at a resolution of 4 cm^{-1} over the range of 400-4000 cm^{-1}. The FTIR analysis was performed at Kalinga University, Raipur, Chhattisgarh, India.

Scanning Electron Microscopy: Dried powder melanin sample was used in imaging for scanning electron microscopy. This imaging was performed at Kalinga University, Raipur, Chhattisgarh, India.

Effect of Carbon source on production rate: To assess the effect of different carbon source on melanin production 2% Glucose, Fructose, Sucrose, Cellulose and Maltose were added in Cdapek dox medium (CDA) under optimum pH and temperature. It was ensured that all the inoculation were done under sterile condition and incubated for 15 days.

Effect of Nitrogen source on production rate: To assess the effect of different Nitrogen source on melanin production 2% Yeast extract, Beef extract, Urea, Peptone and Ammonium sulphate were added in Cdapek dox medium (CDA) under optimum pH and temperature.

Effect of Trace elements on production rate: To assess the effect of different Trace elements on melanin production 2% Copper, Zinc, Calcium, Iron and Potassium were added in Cdapek dox medium (CDA) under optimum pH and temperature.

Results:

Isolation of fungi from soil: The soil sample collected from the regional area were carried out. 11 different genera of fungi were isolated. Each of the genus of the fungi were identified *via* lactophenol cotton blue. The microscopic images obtained after staining were refered from H.L Barnett & Barry B. Hunter.

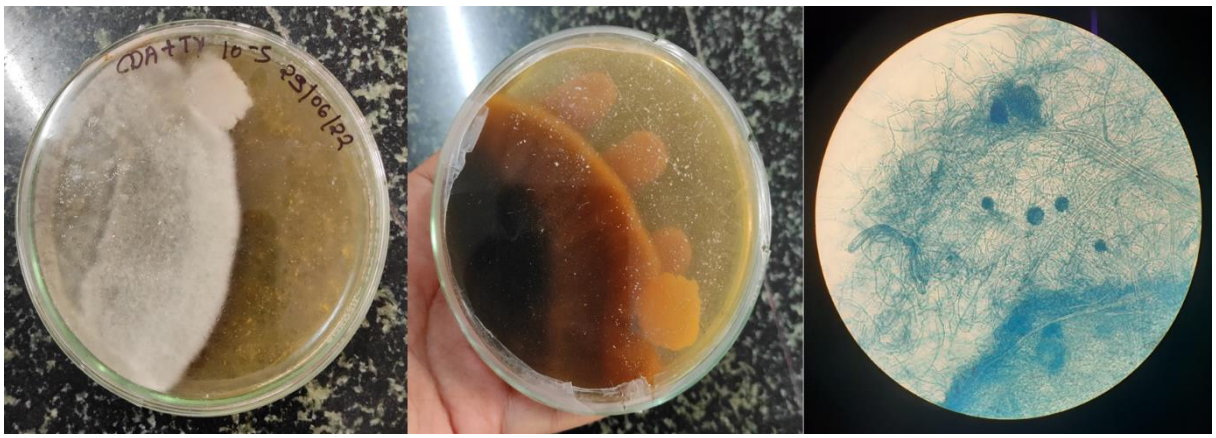

Figure 1. Showing front and back view of fungal plate and microscopic view of lactophenol cotton blue.

Screening of melanin producing fungi: Only one fungal species was able to produce melanin under the normal circumstances. We have obtained maximum melanin production on 1.5% tyrosine supplement. The broth culture was found to be more efficient in shaking condition (120rpm) as compared to static condition

at the incubation period of 3 weeks in dark. All the fungal strains were studied for melanin production, above all only one species showed the maximum production of melanin 21.08mg/100ml.

Table 1. List of fungi obtained from soil sample and Tyrosine concentration for melanin production showed in individual fungi

Name of Fungal species	Tyrosine concentration			
	0.5%	1%	1.5%	2%
Acremonium species	-	-	-	-
Alternaria species	-	-	-	-
Aspergillus species	+	++	+++	+
Curvularia species	-	-	-	-
Dimeriella species	-	-	-	-
Drechslera species	-	-	-	-
Fusarium species	-	-	-	-
Gliocephalotrichum species	-	-	-	-
Penicillium species	-	-	-	-
Russula species	-	-	-	-
Trichoderma species	-	-	-	-

Physiochemical characterization: To check the solubility of extracted melanin in various organic and inorganic solvents. Every solvent was prepared in 10%(w/v) concentration to dissolve extracted melanin.

Table 2. Listing the name of solvents and their solubility in extracted melanin

Name of Solvent	Solubility
Methanol	Insoluble
Ethanol	Insoluble
Ethyl acetate	Insoluble
Chloroform	Insoluble
Hexane	Insoluble
NaOH	Soluble
KOH	Soluble
DMSO	Partially soluble
HCL (3N)	Precipitated

Spectrometric analysis: The extracted melanin was characterized via UV-Visible spectroscopy (200-500nm). It has shown the highest peak around 240 and 270nm as the maximum point range of UV absorption.

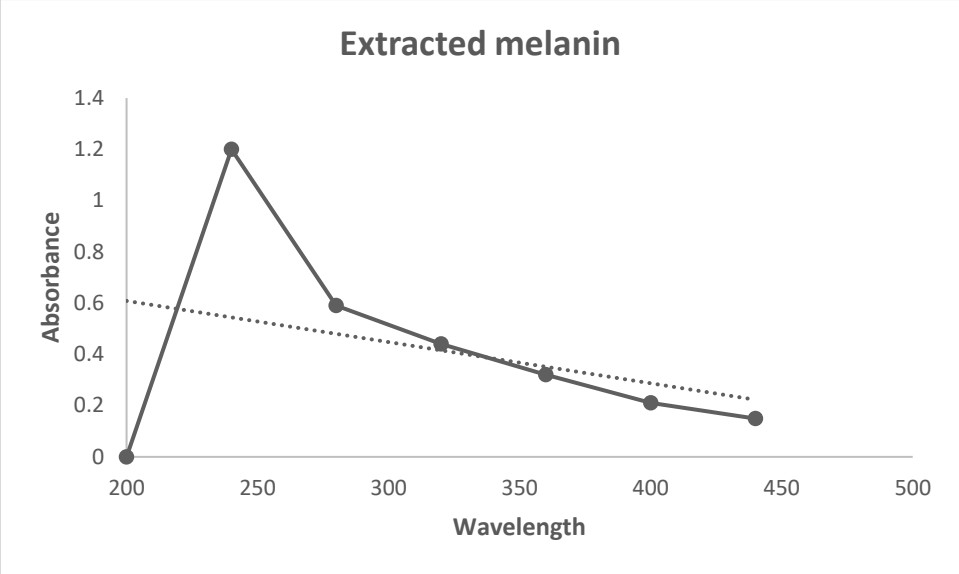

Figure 2. Graph showing peaks of extracted melanin in UV-VIS Spectroscopy

Fourier-transformed infrared spectroscopy: The obtained data of the extracted melanin showed peaks near 3388cm^{-1} showing OH-stretching and aliphatic primary amines, 3259cm^{-1} showing CH-stretching, 2966cm^{-1} showing NH-stretching, 1654cm^{-1} showing C=C stretching, 1288cm^{-1} Showing C-O Stretching, 632cm^{-1} Showing C-H Bending and 449cm^{-1} Showing C-I Stretching.

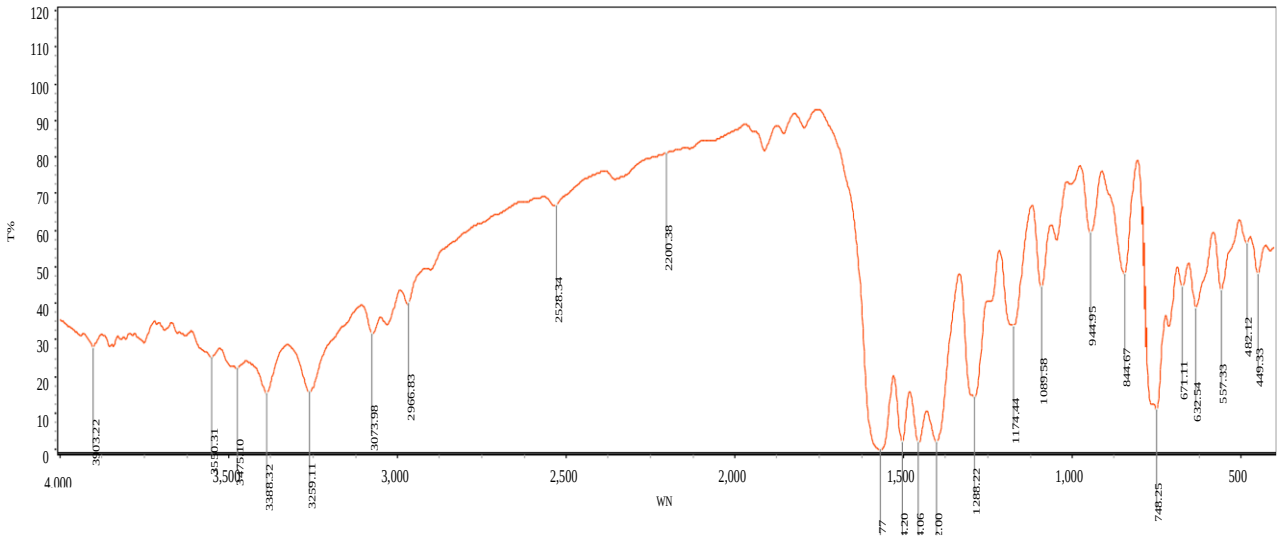

Figure 3. Graph showing various peaks of Extracted melanin showing functional group generated via FTIR

Scanning electron microscopy: The surface topology of the extracted melanin has been shown here. The images taken at 100nm and 50nm.

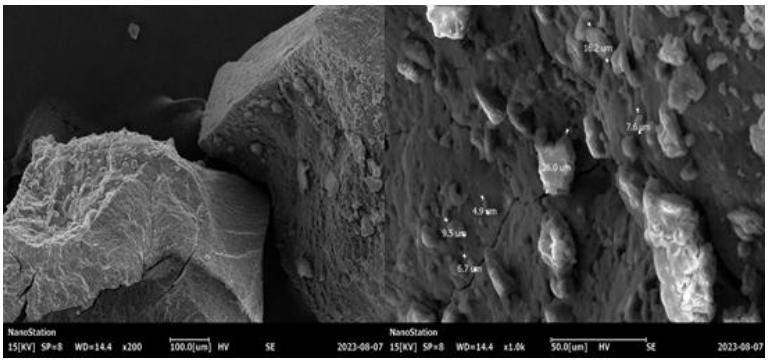

Figure 4. Showing surface topology image of extracted melanin in Scanning electron microscopy

Effect of Carbon source on production rate:

Carbon Source	Biomass Production/100ml	Melanin Yield/100ml
Glucose	0.99gm	1.2mg
Fructose	0.00gm	0mg
Maltose	2.03gm	1.9mg
Sucrose	0.94gm	2mg
Cellulose	0.53gm	1.4mg

Effect of Nitrogen source on production rate:

Nitrogen Source	Biomass Production/100ml	Melanin Yield/100ml
Beef extract	0.64gm	1mg
Yeast extract	0.26gm	1.06mg
Urea	0.14gm	1mg
Peptone	0.58gm	2mg
Ammonium sulphate	0.21gm	0.8mg

Effect of Trace elements on production rate:

Trace elements	Biomass Production/100ml	Melanin Yield/100ml
Copper	0.034gm	0.5mg
Zinc	0.04gm	0.16mg
Calcium	0.64gm	1.4mg
Iron	0.0gm	0.0mg
Potassium	0.0gm	0.0mg

Discussion:

In this study, 11 different genera of fungi were successfully isolated from soil samples collected from a regional area. The identification of these fungi was conducted using lactophenol cotton blue staining and microscopic examination, with reference to H.L. Barnett and Barry B. Hunter. The genera identified were Acremonium, Alternaria, Aspergillus, Curvularia, Dimeriella, Drechslera, Fusarium, Gliocephalotrichum, Penicillium, Russula, and Trichoderma. This diversity reflects the rich microbial ecosystem present in the soil and underscores the potential for discovering fungi with unique metabolic capabilities, such as melanin production. Among the isolated fungi, only one species, Aspergillus, demonstrated the ability to

produce melanin under normal circumstances. The optimal conditions for melanin production by Aspergillus were found to be in a broth culture with 1.5% tyrosine supplementation, under shaking conditions at 120 rpm, and incubated in the dark for three weeks. This species produced a maximum of 21.08 mg/100 ml of melanin, indicating its potential as a significant source of fungal melanin. The enhancement of melanin production with shaking suggests that oxygen availability plays a crucial role in the biosynthesis process. The solubility tests revealed that the extracted melanin was insoluble in organic solvents such as methanol, ethanol, ethyl acetate, chloroform, and hexane. However, it was soluble in alkaline solutions like NaOH and KOH, and partially soluble in DMSO. The melanin precipitated in HCl (3N), indicating its sensitivity to acidic environments. This solubility profile is consistent with the characteristics of melanin, which is typically insoluble in most organic solvents but soluble in alkaline conditions. UV-Visible spectroscopy of the extracted melanin showed prominent absorption peaks around 240 and 270 nm, which is characteristic of melanin pigments. These peaks correspond to the aromatic structures and conjugated double bonds within the melanin polymer, confirming the presence of melanin. The FTIR analysis of the extracted melanin revealed several functional groups: OH-stretching and aliphatic primary amines (3388 cm^{-1}), CH-stretching (3259 cm^{-1}), NH-stretching (2966 cm^{-1}), C=C stretching (1654 cm^{-1}), C-O stretching (1288 cm^{-1}), C-H bending (632 cm^{-1}), and C-I stretching (449 cm^{-1}). These functional groups are indicative of the complex molecular structure of melanin, which includes various functional groups contributing to its biological and chemical properties. The SEM images at 100 nm and 50 nm resolution displayed the surface topology of the extracted melanin. The morphology showed a granular and heterogeneous structure, which is typical of melanin aggregates. These images provide insight into the physical structure of melanin and its potential applications in various fields, such as biomedicine and materials science. The study investigated the impact of different carbon and nitrogen sources on biomass production and melanin yield. Among the carbon sources tested, maltose and sucrose were found to be the most effective, with melanin yields of 1.9 mg and 2 mg per 100 ml, respectively. This suggests that disaccharides may provide a more favorable energy source for melanin biosynthesis compared to monosaccharides like glucose and fructose. In terms of nitrogen sources, peptone yielded the highest melanin production (2 mg/100 ml), followed by yeast extract (1.06 mg/100 ml). This indicates that complex nitrogen sources, which provide a variety of amino acids and peptides, are more conducive to melanin production than simpler nitrogen compounds like ammonium sulfate. The influence of trace elements on melanin production was also examined. Calcium was the most effective, enhancing melanin yield to 1.4 mg/100 ml. Copper and zinc had a moderate impact, while iron and potassium did not promote melanin production. This highlights the role of specific trace elements in the enzymatic processes involved in melanin biosynthesis.

Conclusion:

The study successfully isolated and identified 11 genera of fungi from soil samples, with Aspergillus emerging as a significant melanin producer. The physiochemical, spectrometric, and structural analyses confirmed the characteristics of the extracted melanin. The findings on the effects of different carbon, nitrogen, and trace elements on melanin production provide valuable insights for optimizing melanin biosynthesis in fungal cultures. This research contributes to the broader understanding of fungal melanin production and its potential applications in various industries.

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