Research article

Bioproduction and characterization of melanin pigment produced by the fungal strain *Gliocephalotrichum* sp. DSGB2

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ABSTRACT

Introduction and Aim: The biosynthetic method of naturally occurring pigment melanin production has become a predominant technique in recent years owing to its cost-effectiveness, low chemical usage, and reduced purification procedure. This study aimed to investigate the significant production, characterization, and biological applications of fungal melanin pigment.

Materials and Methods: Melanin production in *Gliocephalotrichum* sp. DSGB2 strain was carried out by submerged fermentation in tyrosine broth and further purified by acidification and precipitation methods. The purified melanin was characterized by analytical methods such as ultraviolet-visible absorbance, TLC, FTIR spectroscopy, and LC-MS. The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging analysis was performed to evaluate antioxidant properties of melanin.

Results: Pigment was confirmed as melanin based on tyrosinase enzyme assay, UV-visible spectroscopy absorbance, TLC, FTIR, and LC-MS analysis. The biosynthesis of melanin was optimized by varying the culture conditions, and the highest yield was obtained under pH 6 at 30ºC. The strain produced about 3.82 gL^{-1} of melanin in 5 days under optimum conditions and exhibited antioxidant activity.

Conclusion: The study provides new ideas into the biosynthesis of water-soluble melanin by the fungal strain *Gliocephalotrichum* sp. DSGB2 has broad potential applications as an efficient biomaterial in the biopolymer, pharmaceutical sectors, cosmetic, and environmental.

Keywords: *Gliocephalotrichum* sp; antioxidant; tyrosinase; melanin.

INTRODUCTION

The bioactive materials of microbial origin known as natural pigments have attracted the industry attention because of the increasing need to produce novel, safe, and sustainable products (1). Among the pigments produced by microbes, melanin has gained the attention of researchers due to its wide applications and interesting physical, chemical, and biological characteristics (2). Melanin is a category of heterogeneous active polymeric molecules that are produced by animals, plants, fungi, bacteria, and other species from different kingdoms. Melanin is a hydrophobic, dark-colored biological macromolecule with molecular weight of 318.3 $gmol^{-1}$ (3, 4). Melanin pigments can be categorized into numerous classifications based on the type of substrate that is utilized for both the biogenesis and the synthetic process. In general terms, eumelanin is defined as dihydroxyphenylalanine (L-DOPA) and L-dopaquinone-based melanin. Additionally, it has been claimed that allomelanin comprises catechol-structured aromatic monomers with a like pheomelanin and caffeic acid (5). Tyrosinases are copper-dependent enzymes involved in the oxidation and ortho-specific hydroxylation of monophenols like tyrosine, key enzymes involved in the creation of melanin. Similar to tyrosinase, laccase is also a key enzyme that catalyzes oxidation of a wide variety of substrates, such as quinones and dihydroxy phenols. These enzymes are typically found in fungi, and plants than in bacteria. Therefore, fungal organisms can consider as potential candidates in melanin synthesis (6). Melanin has potential to bind with antibiotics and thus provides evolving resistance to pathogenic bacteria to antibiotics. As a result, in antimicrobial drug discovery, melanin and melanin synthesis pathways show significance (7). The primary function of producing melanin in organisms is to protect host cells and organisms. This involves resistance to physical alterations from the outside, prevention against UV rays and energy absorption, and physiological activity-based preservation of intracellular homeostasis (6). Melanin is an efficient UV-visible light absorber, free radical scavenger, and metal ion chelator. Due to its beneficial properties, melanin compounds are extensively used in the production of cutting-edge adhesive biomaterials and environmentally friendly bioelectronics. This is because of their hybrid ionic–electronic conductance and redox reversibility features (8). Melanin, can be utilized to make antibacterial medications, antiviral and anticancer therapies, nanoparticles, novel, biocompatible, and biodegradable devices for medicine, and also for antioxidant and radioprotective molecules (5,3). Numerous melanin producing microorganisms have been reported. Bacteria such as,

Pseudomonas stutzeri, *Nocardiopsis alba*, *Streptomyces glaucescens*, and fungi such as *Gliocephalotrichum simplex*, and *Aspergillus funigatus*, *Armillaria ostoyae*, have been reported to synthesize eumelanin from L-DOPA (5,9). The biosynthetic method of melanin production has become a predominant technique in recent years owing to its cost-effectiveness, low chemical usage, and reduced purification procedure. Hence, the objective of our study was to determine the significant synthesis, characterization, and biological applications of melanin from the newly isolated *Gliocephalotrichum* sp. DSGB2 strain.

MATERIALS AND METHODS

Chemicals

L-asparagine, sodium chloride, dipotassium hydrogen phosphate, and magnesium sulfate heptahydrate were purchased from SD Fine Chemicals, India. Ferrous sulfate heptahydrate, sodium tartrate, sodium molybdate, manganese chloride tetrahydrate, cobalt chloride hexahydrate, zinc chloride, and boric acid were procured from SRL India. L-DOPA, L-tyrosine, Agar, and media ingredients were purchased from HiMedia Chemicals (Mumbai, India), all other chemicals utilized in the present research were analytical grade.

Optimization of parameters to produce melanin pigment

The fungal strain *Gliocephalotrichum* sp. DSGB2 was previously isolated, identified, and used for nitrile degradation. The fungus was maintained on potato dextrose agar (PDA) media and produced brown color pigment in PDA media. Further, the strain was inoculated in various experimental media such as Minimal mineral salt (MM1) media, malt extract medium, tyrosinase broth, and Czapek Dox broth for pigment production. Melanin production from the DSGB2 strain was carried out using submerged fermentation in tyrosine broth. A fresh culture of 5% inoculum was inoculated in 250 ml flasks with 100 mL of tyrosine broth, which contained 1 gL^{-1} L-asparagine, 5 gL^{-1} L-tyrosine, 0.5 gL^{-1} sodium chloride, and 0.5 gL^{-1} magnesium sulfate hexahydrate (2). Additionally, one milliliter of a trace salt solution including sodium molybdate, zinc chloride, boric acid, manganese chloride tetrahydrate, cobalt chloride hexahydrate, ferrous sulfate heptahydrate and sodium tartrate in distilled water (2) was used and grown on static condition at a temperature of 30°C. The production parameters were optimized by inoculating in media with different pH ranges from 5.0-9.0, incubation at different temperatures from 25-50°C, and continued incubation time up to 12 days.

Tyrosinase assay

The tyrosinase activity of the crude sample was determined spectrophotometrically using modified tyrosinase enzyme assay method of Rudrappa *et al*. (2), which measures the conversion of L-DOPA to dopachrome. The reaction mixture comprised enzyme solution, 50 mM sodium phosphate buffer (pH 6.5), and L-DOPA (2 mg ml^{-1}). The absorbance of the reaction was measured at 500 nm at zero min and the reaction mixture was then incubated for 5 min at 35℃. The reaction mixture of enzyme solution, sodium phosphate buffer, and water was used as a blank. The enzyme activity is equal to the variation in absorbance. The enzyme activity was measured at different intervals and all the assays were performed in triplicate. One unit of enzyme is equivalent to the amount of enzyme needed to catalyze the bioconversion of one µmol of the tyrosine to produce per minute.

Purification of melanin

Pigment produced by *Gliocephalotrichum* sp. DSGB2 in submerged fermentation broth was extracted and purified by methods such as precipitation and acidification (5,10). The fermented broth was centrifuged at 6000 rpm for 10 min to remove cell debris. After adjusting culture supernatant pH to acidic condition (pH 2.0) with a 6N hydrochloric acid, the pigment was precipitated, and the resulting solution was subsequently kept for 4 hours. The precipitated melanin was then centrifuged at 10000 rpm for 15 min and the precipitate with melanin was kept at 60 ℃ for 24 hours.

UV-Vis spectrophotometric and thin-layer chromatography analysis

By dissolving the extracted pigment in DMSO, the UV-Vis absorption spectra of the extracted melanin were examined using a UV-Vis spectrophotometer (SYSTRONICS UV-VIS spectrophotometer 119) from 200 nm to 800 nm range of wavelength, with the DMSO working as a blank. TLC analysis was performed using pigment dissolved in DMSO, take out the sample into a capillary tube, loading it up on a TLC plate, and performing TLC with a solvent solution of ethanol, 75% butanol, and water (1:4:1 v/v), accompanied by iodine staining (11, 12).

LC-MS and FTIR analysis

The purified melanin is further analyzed on an LC-MS equipped with an ESI interface. A PerkinElmer Series 200 HPLC system interfaced with the mass spectrometer API 3000 series (Agilent Technologies) controlled by Analyst 1.4 software was used for analyzing the mass of the melanin. The instrument was equipped with a reverse phase C18 column, and the analytes were eluted with a mobile phase of 1% formic acid in water and methanol (1:1) with a flow rate of 1 ml min^{-1}. The molecular structure of melanin was studied using a Bruker alpha II FTIR spectrometer at University Scientific Instrumentation Centre, Davangere University, Karnataka, India. A 5 mg powdered melanin sample was directly measured with

attenuated total reflection (ATR) technique Spectra were recorded in the wavenumber range of 600–4000 cm^{-1} by using resolution of 2 cm^{-1}.

Antioxidant activity

Purified melanin was tested for antioxidant activity based on the free radical scavenging effect of DPPH by a previously reported method with modifications (13). By dissolving 0.24 % of DPPH in methanol, a DPPH solution was prepared, which was used in about 3.9 ml along with 0.1 ml (4 mg ml^{-1}) of melanin solution for the test. Reaction mixture shaken continuously, allowed to stand for 30 min in the dark at room temperature. The absorbance after incubation is measured at 517 nm. Ascorbic acid (4 mg ml^{-1}) is considered as positive control. The decrease in absorbance of DPPH indicates the radical scavenging activity. DPPH radical scavenging activity was calculated by following formula (14), all the tests were done for three trials.

$$DPPH\ radical\ scavenging\ activity\% = \frac{[(Absorbance\ of\ control)-(Absorbance\ of\ test\ sample)]}{(Absorbance\ of\ control)} \times 100$$

RESULTS

Production, purification, and characterization of melanin

Gliocephalotrichum sp. DSGB2, which had been previously isolated and identified for the biodegradation of benzonitrile, was used to produce melanin under submerged fermentation, at various media, optimized pH, temperature, and time intervals. The organisms grew to their maximal capacity and produced 3.82 gL^{-1} of melanin at a temperature of 30 ℃, pH 6.5, and 7 days of incubation in tyrosinase broth. Fig. 1 shows the color change due to pigment production in the PDA plate and tyrosine broth medium. Centrifugation was used to collect the pigmented supernatant from *Gliocephalotrichum* sp. DSGB2, which was acidified with 6N hydrochloric acid, and melanin gets precipitated. The melanin extracted from the medium by *Gliocephalotrichum* sp. DSGB2 was capable of dissolving in water, sodium hydroxide, phenol, DMSO, potassium hydroxide, methanol, and ethanol, but insoluble in acetone, chloroform, sodium chloride, acetonitrile, petroleum ether, n-propanol ethyl acetate, n-butanol, and benzene, and it was bleached by reacting with 30 % hydrogen peroxide, 10% sodium hypochlorite solution.

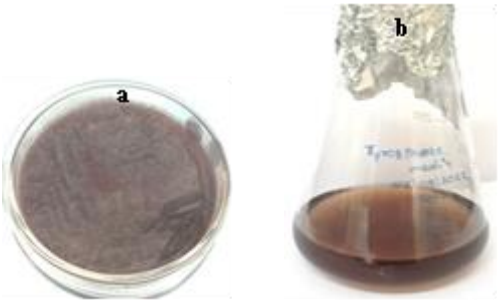

Fig. 1: Production of melanin pigment in (a) PDA plate, (b) tyrosinase broth by *Gliocephalotrichum* sp. DSGB2.

UV-visible and thin-layer chromatography studies

UV-visible spectroscopy of extracted melanin shows a maximum absorption peak at 257 nm in the UV range; the absorbance peak of produced melanin is shown in Fig. 2. Melanin from Sepia, human and bovine black hair, and retinal pigment epithelium (RPE) shows absorbance at 282 nm (15). TLC of the extracted melanin with a single band and RF value of 0.68 on the silica gel with mobile phase, water: ethanol: 75% butanol and staining with iodine (2) is shown in Fig. 3.

Fig. 2: Characterization of melanin using UV-Vis spectrophotometer

Fig.3: Thin layer chromatography of melanin observed under UV light

Characterization of the melanin pigment from the FTIR spectrum

Purified melanin was subjected to FTIR analysis to examine the compound's functional groups. The –OH bond stretching is linked to the broadband between 2500 and 3500 cm^{-1} (16). The stretching of C–O from

carboxylic acid and phenol C=C are linked to the bands at 1600 and 1430 cm^{-1}. At approximately 1724-1486 cm^{-1}, the carbonyl group (-COOH), also known as semiquinones or 5, 6-indole quinones, is visible through stretching. All the above functional groups confirm the melanin (Fig.4)

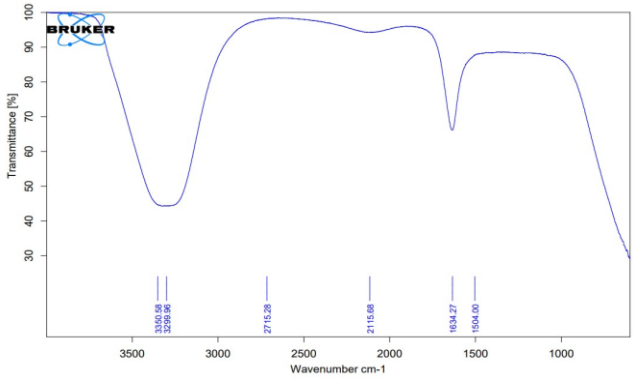

Fig. 4: FTIR spectrum of extracted melanin from *Gliocephalotrichum* sp. DSGB2.

LC MS analysis

The LC MS analysis of purified melanin pigment produced by *Gliocephalotrichum* sp. DSGB2 was carried out as described in the methods. The purified melanin pigment in the fermentation broth was identified based on the mass-to-charge ratio of molecular ions. The extracted melanin pigment showed a corresponding molecular ion peak at m⁄z 319.50 $[M + H]^+$, indicating the strain produced melanin pigment (18; Fig. 5).

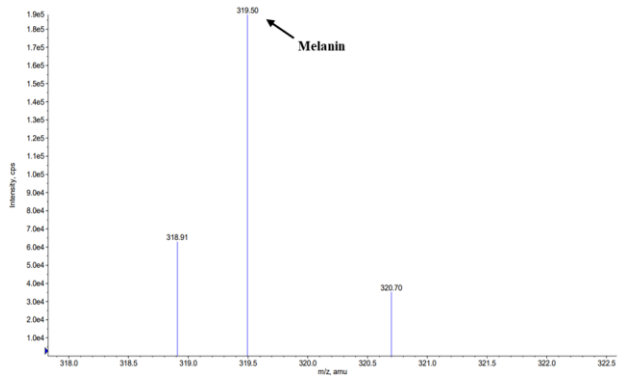

Fig. 5: LC-MS analysis of the extracted melanin produced by *Gliocephalotrichum* sp. DSGB2.

Antioxidant activity of melanin pigment

Melanin pigment is capable of accepting or donating electrons. Melanin molecules contain unpaired electrons, react with free radicals and other reactive species efficiently, and function as an antioxidant. The extracted melanin pigment from DSGB2 exhibited considerable radical scavenging activity EC_{50} of 43.28 µg ml^{-1}, while ascorbic acid showed higher radical scavenging activity EC_{50} of 48.38 µg ml^{-1}.

DISCUSSION

Several microbial strains producing melanin have been identified and characterized because melanin is a promising biomedical application in the pharmaceutical industry and several biotechnological applications. In the present study, we focused on the production of pigment in PDA medium by DSGB2, followed by purification and characterization of a blackish brown pigment from the fungal strain *Gliocephalotrichum* sp. DGSB2 using different media. The fungus culture filtrates that were cultured in a medium supplemented with tyrosine produced melanin and exhibited a blackish-brown color. Additionally, the blackish-brown pigment confirmed positive for melanin in each qualitative test. Consequently, pigment was completely decolorized in an H_2O_2 solution, and when potassium permanganate was added, a green precipitate was produced. Based on these findings, it was preliminarily determined that the produced pigment was melanin. The melanin pigment from the submerged fermentation broth was extracted by centrifugation, the supernatant obtained was precipitated using 6 N hydrochloric acid followed by centrifugation to precipitate out melanin. Melanin has a considerable electron affinity for proteins in the culture medium during acidification. The precipitated melanin was allowed to stand in 6N hydrochloric acid for 4 hours to break down the bound protein (2). Melanin from the DSGB2 strain can be dissolved in water and alkali solutions but insoluble in most of the organic solvents (17-19). UV absorption peak at 257 nm was maximum and decreased gradually when wavelength increased, because of variation in a complex structure of melanin. The FTIR of melanin pigment exhibited band peak values in between 3600 and 3000 cm^{-1}, and 1650–1600 cm^{-1}, the characteristic features of the melanin (20). Melanin from the DSGB2 matches with the FTIR peaks of cuttlefish and synthetic melanin (21). The vibrations caused by stretching in amides, carboxylic acid, amines, and aromatic functional groups essential functional groups in the indole and pyrrole structures of melanin are represented by the infrared peaks that span from 3600 to 2800 cm^{-1}. The key elements of the melanin structure are functional groups C-C, C-N, C-O, C-H, and O-H (13, 22). RF values of melanin from different organisms exhibited RF of 0.62, and 0.68 (2), similar to the melanin of the DSGB2 strain. Many investigators reported melanin synthesis from many microorganisms, animals, and plants. Melanin from cuttlefish, sepia is widely reported. Fungal organisms such as *Aspergillus* sp., and *Gliocephalotrichum simplex*, utilize the DOPA pathway for the synthesis of melanin (9, 23). The transformation of tyrosine into L-DOPA and then to dopachrome by the action of tyrosinase enzymatic activity produces the reddish-brown color in the broth. DSGB2 strain also follows the DOPA pathway for melanin biosynthesis, which was confirmed by tyrosinase enzyme assay. Jalmi *et al.*, reported that *Gliocephalotrichum simplex* produced 0.66 g/100 ml of melanin when conditions were optimized using a full factorial design using 3 levels of tyrosine and peptone in 8 days of incubation

duration (9). In tyrosine-containing medium *Armillaria cepistipes* produced 27.98 g/L eumelanin on 161 days of incubation (24). An *E. coli* strain was cloned and modified to express the *mel*A gene, derived bacteria *Rhizobium etli* (24). When the resultant recombinant strain was grown in solid or liquid media containing tyrosine and copper, it produced a dark pigment known as melanin. After 36 hours of culture, the melanin concentration in this culture was 0.06 gL^{-1}. To increase the amount of melanin produced, this strain's growth conditions were further improved (25). In the study, DSGB2 strain produced 0.382 g melanin in 100 ml tyrosinase broth i.e., 3.82 gL^{-1} in the absence of peptone within 5 days of incubation. The extracted melanin exhibited radical scavenging activity of EC_{50} of 43.28 µg ml^{-1}, we can consider this as a significant antioxidant activity compared with ascorbic acid which shows EC_{50} of 48.38 µg ml^{-1}. Kumar *et al.*, reported about radical scavenging activity of DPPH i.e., EC_{50} of 54.12 µg ml^{-1} whereas synthetic melanin exhibited 40.28 µg ml^{-1} (13). This shows that extracted melanin is a significant antioxidant.

As per the literature survey, several microorganisms have been reported for melanin synthesis using various mediums. The DSGB2 strain showed maximum production of 3.82 gL^{-1} under optimum conditions. Characterization confirms melanin in various applications as antioxidant, cytotoxicity, antimicrobial, drug binding activities and as a textile martial tint. It appeared that the isolated melanin pigment from DSGB2 was just as effective as manufactured melanin at scavenging peroxide free radicals. Based on our experimental analysis, the study shows that DSGB2 strain has the effectiveness to yield melanin.

CONCLUSION

Natural melanin is one of the most abundant pigments in the environment. Due to its good biocompatibility and various activities, it is widely used as a biomaterial in cosmetics, medicine, biotechnological applications, and the environment. In this study, melanin was produced, characterized, and analyzed by various methods. The biotransformation of tyrosine and synthesis of water-soluble melanin is catalyzed by the tyrosinase enzyme of *Gliocephalotrichum* sp. DSGB2. The extracted melanin has antioxidant properties as it can donate hydrogen and reacts with DPPH to form the antioxidant compound, 1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine. These fungi can be used for massive production of commercially important melanin.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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