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Isolation and Characterization of Melanin from the Commercially Available Edible Mushrooms

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ABSTRACT

Melanin is a natural pigment with a wide range of biological and biomedical applications. It is currently a subject of great research interest. Its versatile uses span various fields and make it highly desirable. Melanin from the plant sources are difficult to separate from other phenolic compounds. Melanin from the animal derived sources is often contaminated with proteins, in the present investigation, three commercially available edible mushrooms, namely *Lentinula edodes, Agaricus bisporus* and *Pleurotus ostreatus* were used to isolate melanin pigment. The melanin pigment was successfully extracted from *Lentinus edodes* by hot alkali treatment followed by acid precipitation. Various tests carried out on the isolated pigment showed similar properties to melanin, insolubility in both water and organic solvents, while it is soluble in alkali. Examination of the pigment particles by scanning electron microscopy revealed that they have a spherical shape with a size of 20-50 nm. Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) spectroscopy were used for further characterization. These methods make possible a detailed analysis of the molecular structure of the pigment.

Key words: *Agaricus bisporus,* FT-IR, *Lentinula edodes,* Melanin, Mushrooms, NMR, *Pleurotus ostreatus.*

INTRODUCTION

Melanin is a common substance produced by animals, plants and micro-organisms (Selvakumar *et al.*, 2008) and a complex set of natural pigments (Ghattavi *et al*., 2022). They are the most prevalent pigments in nature. They can be found in large quantities in both the plant and animal kingdoms (Cao *et al.,* 2021). Melanin contains indoles and other intermediates of tyrosine oxidation (Liu and Nizet, 2009). Because of the presence of melanin in fossils (Wogelius *et al.,* 2011), they can be used as biomarkers in evolutionary research (Lindgren *et al.,* 2012). Melanins are found in all living organisms and are primarily used for defence. Melanins are the pigments that give animals, plants and microbes their black, gray and brown colours, and they have biochemical applications. In addition to strengthening plant cell walls, melanin is crucial for shielding human skin from UV rays (Schindler *et al.,* 2019; Kao *et al.,* 2021). It acts as a protective agent against environmental stress in microorganisms.

Melanin is best known for its photoprotective properties because of the ability to scavenge free radicals and shield the skin from UV radiation (Brenner and Hearing, 2008). It is frequently used in cosmetic formulations such as sunscreen lotions. Sunscreens containing water-soluble melanin protect against harmful UV radiation, which is one of the main causes of melanoma and other skin cancers. Melanin is also used as a substitute for paraaminobenzoic acid (PABA), another compound that absorbs UV light. The product bio-melanin, isolated from the fungus *Podospora anserina*, is used in various types of cosmetic products such as lotions, creams, balms, bath oils, gels, sprays, foams, powders, aerosols, sticks, pastes, sun protection products, skin and hair care products (Kalka *et al.,* 2000). Melanins are used as UV protectants in bioinsecticidal preparations such as the insecticidal crystals of *Bacillus thuringenesis* (Bt) (Zhang *et al.,* 2007).

Melanin research is in great demand due to its diverse applications, which are not limited to a specific area. Animal sources of melanin are usually contaminated with proteins. Plant sources of melanin are too complicated to separate from the other phenols of the plant. Plant melanin is also subject to batch variations. Mushroom sources can provide the purest melanin without batch variation (Tarangini and Mishra, 2014). Therefore, research on melanin from fungi is crucial. The current study attempts to investigate macrofungi as potential sources for effective melanin production. The work also included

the physical and chemical characterization of melanin.

MATERIALS AND METHODS

Collection and preparation of the samples

Dried basidiocarps of 3 commercially available edible mushrooms such as *Lentinula edodes* (Shitake mushroom), *Agaricus bisporus* (White button mushroom) and *Pleurotus ostreatus* (Oyster mushroom) were purchased from the market. The mushrooms were homogenised into fine powders using a mixer grinder. These powders were kept in airtight containers for future analysis.

Melanin extraction and purification

The melanin pigment was extracted according to the method of Suryanarayanan *et al*. (2004). Powdered mushroom (1 g) was simmered in 30 mL of distilled water for 5 min and centrifuged at 5000 g for 5 min. After washing and centrifuging the pellet, it was autoclaved with 10 mL of 1M KOH (20 min, 120°C) to extract the pigment. To precipitate the melanin, the alkaline pigment extract was acidified to pH 2 with concentrated HCl. To obtain the purified pigment, the melanin pellets were washed 3 times in distilled water. Then centrifuged at 9000 g for 15 min and dried overnight. Additional physicochemical characterization was performed with the dehydrated powder.

Characterization of melanin by physicochemical methods

Melanin was characterized both physically and chemically using the methods described by Suryanarayanan *et al*. (2004) and El-Naggar and El-Ewasy (2017). Melanin solubility was tested in deionized water, 1N KOH, HCl, acetone, benzene and methanol. All estimates were validated against the available literature.

Scanning Electron Microscopic (SEM) analysis

Dried pellets were gold coated (model: JFC1600) for 10 sec at 10mA of current and surface topography of melanin was performed using scanning electron microscope (JEOL, JSM 6390LA). SEM analysis was carried out at Sophisticated Test and Instrumentation Centre (STIC), Cochin University of Science and Technology, Kerala, India.

Fourier-Transform Infra-red (FT-IR) Spectroscopy

Using a pelletizer, the purified melanin was pressed into slices under high pressure and ground with infrared-capable KBr (1:10). A Thermo Nicolet Avatar 370 spectrophotometer with a KBr beam splitter and DTGS (Deuterated Triglycine Sulphate) detector (7800-350 cm^{-1}) was used to record the FT-IR spectrum at 4000-400 cm^{-1} and a resolution of 4 cm^{-1} (Suryanarayanan *et al.*, 2004). FT-IR analysis was performed at DBT, School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The NMR spectrum was recorded using a Jeol NMR spectrometer in 5^{-mm} NMR tubes at 250°C. The operating conditions were: Frequency 500.16 MHz, field strength 11.75 T (500 MHZ), resolution 0.57 Hz and acquisition time 1.75 s (El-Naggar and El-Ewasy, 2017). The NMR analysis was performed at SAIF, Cochin University of Science and Technology, Kerala State, India.

RESULTS

Out of the 3 mushrooms, only *Lentinula edodes* (Shitake mushroom), which was having black coloured fruiting bodies gave positive result with the procedure followed for the isolation of melanin. The isolated pigment was found to be black in colour. Isolated pigment showed positive results for the 5 biochemical tests for melanin characterization. The pigment was insoluble in water or organic solvents such as acetone, benzene or methanol. The pigment was readily soluble in 1M KOH at high temperature (100°C). The pigment reprecipitated in concentrated HCl. This confirms that the purification method employed in the investigation was sufficient and effective enough to yield pure melanin (**Table 1**).

Table 1: Behaviour of isolated melanin on treatment with different chemical reagents.

Chemicals used for the treatment	Observation
Water	Insoluble
Acetone	Insoluble
Benzene	Insoluble
Methanol	Insoluble
1 M KOH	Soluble
HCl	Reprecipitates

Scanning Electron Microscopy of melanin

The melanin particles isolated from *Lentinula edodes* were in the range of 20 nm to 50 nm and are spherical in shape. The SEM images of the fungal melanin sample is shown in **Figure 1**.

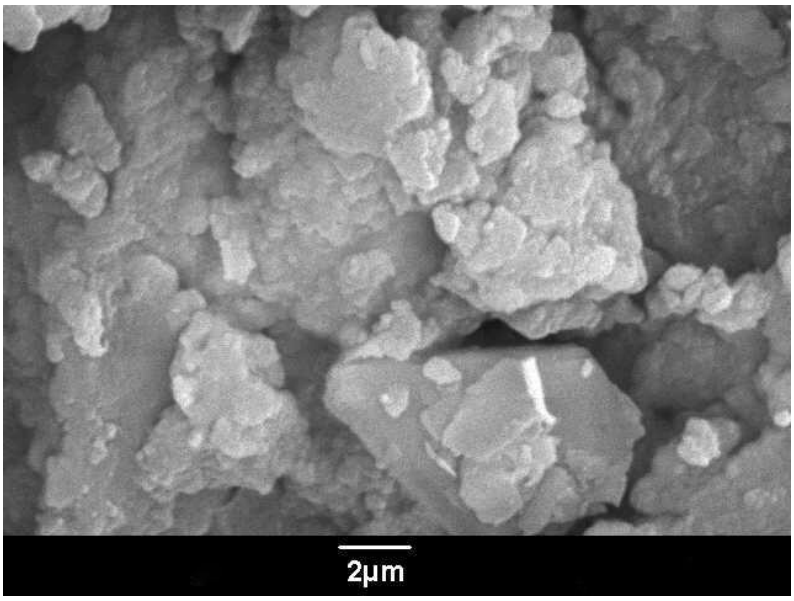

Figure 1: Scanning electron microscope micrographs of the melanin pigment granules extracted from *Lentinula edodes.*

Fourier-Transform Infra-red (FT-IR) Spectrum

The FTIR spectrum of melanin (**Figure 2**) revealed a strong resemblance to those from previous studies (Guo *et al*., 2014; Suryanarayan *et al*., 2004; El-Naggar and El-Ewasy, 2017). A broad absorption was observed in the spectrum at 3469.64 cm^{-1}, which is indicative of the stretching vibrations of phenolic –OH and –NH. The aromatic ring C=C stretching was identified as the cause of the characteristic peaks seen between 1650 and 1620 cm^{-1} (1633.71 cm^{-1}). This demonstrated that melanins are aromatic and polyphenolic. Weak bands were seen below 700 cm^{-1}, and some extra peaks were also seen at 1047.35 at 898.83 cm^{-1}.

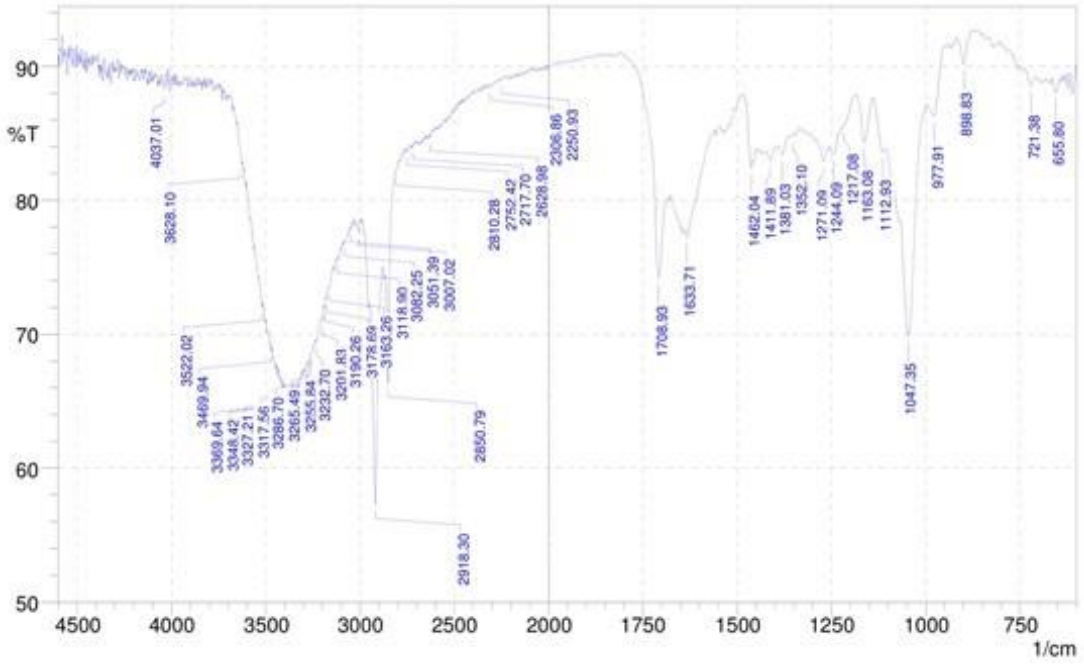

Figure 2: FT-IR Spectrum of the melanin pigment extracted from *Lentinula edodes*.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The pigment was also characterized by nuclear magnetic resonance (NMR) spectroscopy. The NMR peaks of the melanin (**Figure 3**) were consistent with previous reports (Guo *et al.,* 2014; El-Naggar and El-Ewasy *et al.,* 2017). The ^{1}H NMR spectrum of melanin in DMSO revealed the presence of aromatic and aliphatic regions. The peaks located at 0.813 ppm in the aliphatic region are associated with the -CH3 groups of alkyl fragments, including -CH2CH3 and -CH(CH3)2. The absorbance at 1.0 ppm is due to the -CHOH group, while the absorbance at 3.2 - 4.054 ppm is probably due to protons bound to various substituted aromatic or heteroaromatic rings (Katritzky *et al.,* 2002). These data can be very helpful in determining the structure of the melanin produced.

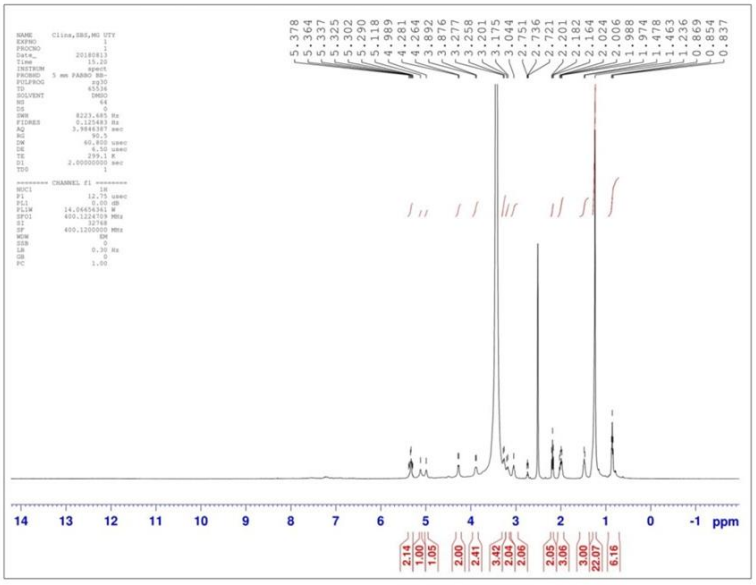

Figure 3: NMR spectrum of the melanin pigment extracted from *Lentinula edodes*

DISCUSSION

The basidiocarps of the *Lentinula edodes* were dark brown in colour indicating the wall bound melanin production. Selvakumar *et al.* (2008) have reported the characterization of black coloured melanin obtained from *Antromycopsis macrocarpa* (anamorph of *Pleurotus cystidiosus*). Melanin aids in the survival and long life of fungi in the environment (Michael *et al.*, 2023). The melanized hyphae help the host to survive stress conditions because cell wall melanin can trap and eliminate oxygen radicals generated during abiotic stress (Richier *et al.*, 2005). The isolated pigment was characterized by biochemical tests showed positive response for melanin. The chemical characteristics that characterize a fungal pigment as melanin include its dark colour, insolubility in cold or boiling water and organic solvents, resistance to degradation by hot or cold concentrated acids, bleaching by oxidizing agents such as hydrogen peroxide, and solubilization and degradation by hot alkali solutions. The pigment isolated from *Lentinula edodes* responded positively to all the biochemical tests for melanin. The pigment was extracted using the alkali procedure of Suryanarayanan *et al*., (2004). The pigment could not be extracted with organic solvents and dissolved only in 1M KOH (Selvakumar et al., 2008; Rajagopal *et al*., 2011). Fungal melanins are identified by their black color, solubility in KOH, and insolubility in water and organic solvents (Suryanarayanan *et al*., 2004; Selvakumar et al., 2008).

CONCLUSION

In the present study, the basidiocarps of 3 edible mushrooms such as *Lentinula edodes*, *Agaricus bisporus* and *Pleurotus ostreatus* were used to isolate melanin. Only one mushroom, *Lentinula edodes*, was found to be capable of synthesising melanin. Physical and chemical characterization of the isolated pigment was performed. The physical characterization was carried out using SEM analysis. For the chemical characterization, solubility studies were performed. The molecular structure of the isolated compound was deduced from the analysis of FT-IR spectrum and NMR spectrum. All these data were compared with previous studies on fungal melanin. From these analyses and comparisons, it was concluded that the isolated compound is melanin, i.e. wall-bound melanin.

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