Purification and characterisation of a sulphur rich melanin from edible mushroom Termitomyces albuminosus Heim

Rosy Agnes De Souza, Nandkumar Mukund Kamat and Vishnu S. Nadkarni

Mycological Laboratory, Department of Botany, Goa University, Taleigao, Goa, India; Department of Chemistry, Goa University, Taleigao, Goa, India

ABSTRACT
Production, purification and characterisation of a black pigment from Termitomyces albuminosus as melanin is reported, for the first time, from shaken submerged culture condition using scanning electron microscopy (SEM), elemental analysis, ultraviolet–visible (UV-VIS), and Fourier transformed infrared spectroscopy (FTIR), electron paramagnetic resonance (EPR) and $^{13}$C (CP/MAS) NMR spectra. SEM results on T. albuminosus revealed nanogranular nature of melanin nanoparticles within size range of 400–100 nm with fractal dimension $D = 1.195–1.73$. Elemental analysis of melanin indicated 54.6% C, 3.5% H, 2.4% N, 26.9% O, and 12% S. UV-VIS and FTIR spectra confirmed to the characteristic of melanin and were identical to the reference commercial sepia melanin. Further validation of the identity of pigment as melanin was achieved by EPR analysis. Termitomyces albuminosus melanin is postulated to be DOPA-type melanin confirmed by $^{13}$C (CP/MAS) NMR spectral analysis showing chemical shift at 200–170 ppm carbonyl, 160–110 ppm aromatic region, and with high 40–30 ppm open chain aliphatic region. Chemical modification through oxidation and cysteinylation (Pheomelanin) is implied as indicated by relatively high sulphur content (12%).

ARTICLE HISTORY
Received 29 April 2018
Accepted 23 June 2018

KEYWORDS
Termitomyces; melanin; submerged fermentation; DOPA; purification

Introduction
Melanin biosynthesis is a common feature in kingdom fungi. The pigment not essential for hyphal growth appears as secondary metabolite. Melanins are most stable, amorphous polymers of phenolic compounds and can be classified into the following three types: eumelanins, pheomelanins and allomelanins. Melanin production helps in protection from extreme environmental conditions such as UV light, ionising radiation, resistance to heat or cold, phagocytosis, heavy metals, and oxidants and provides cell wall rigidity (Money et al. 1998; Plonka and Grabacka 2006; Pal et al. 2013; Casadevall et al. 2017). Despite its importance and ubiquity, many fundamental questions remain unanswered like details of its chemical structure and insolubility (Eisenman and Casadevall 2012). Some fungi undergo melanogenesis in response to certain environmental stress conditions such as extreme temperatures, dessiccation, hyperosmotic conditions, limited nutrients, pH changes, metal toxicity, UV or ionisation stress, action of antagonistic microbes. Melanisation in fungi mostly seen in hyaline hyphae, sclerotia, appressoria, reproductive structures or conidia (Cordero and Casadevall 2017). Hyphal melanin is often found to be deposited as the outermost layer or internal layer in cell wall only with age or other stress (Bell and Wheeler 1986; Henson et al. 1999; Butler et al. 2001). Melanogenesis in pathogenic fungi plays a key role in pathogenesis in species such as Cryptococcus neoformans (Polacheck and Kwon-Chung 1988), Gaeumannomyces graminis var. tritici, Magnaporthes grisea, Alternaria alternata, Colletotrichum lagenarium, Cochliobolus heterostrophus (Henson et al. 1999), Paecilomyces variotti (Babitskaya et al. 2000a), Rhizoctonia solani (Chen et al. 2015) and Aspergillus spp. (Babitskaya et al. 2000a; Schmaler-Ripcke et al. 2009; Gonçalves et al. 2012; Pal et al. 2013). Melanins are reported from mushrooms such as Agaricus bisporus (Mendoza et al. 1979), Inonotus obliquus (Babitskaya et al. 2000b; Babitskaya et al. 2002), and Schizophyllum commune (Arun et al. 2015). Plant-associated symbiotic ectomycorrhizal fungus, Cenococcum geophilum, produces melanin under dehydrated conditions...
(Fernandez and Koide 2013). Fungi synthesise melanin by one of the two synthetic pathways: 1,8-dihydroxynaphthalene (DHN) intermediate and 1,3,4-dihydroxyphenylalanine (L-DOPA). Melanin synthesis involves copper containing metalloenzymes such as laccase and tyrosinase and in fungi also shows involvement of chitin cross-links to other cell wall polysaccharides and proteins (Eisenman and Casadevall 2012). Studies on melanins in mushrooms are limited to edible mushrooms such as Pleurotus cystidiosus var. formosensis, P. australis, and P. purpureoolivaceus from which darkly pigmented arthroconidia forming black pigment on mycelium or basidiomata has been characterised (Selvakumar et al. 2008). According to Mendoza et al. (1979), the spore wall of Agaricus bisporus and Agaricus campestris contain 26–28% and 24–26% crude (dry weight cell wall) melanin. Mushroom fruitbody decolourisation is very common due to oxidation of phenolic substrates into quinones leading to the formation of brown-coloured melanin in species such as A. bisporus, thus decreasing its commercial value (Weijn et al. 2013). Exo- and endomelanin complex of Inonotus obliquus and Phellinus robustus in submerged conditions demonstrate high-antioxidant and genoprotective properties (Bisko et al. 2002, 2007). Melanin in Auricularia auricula has been studied extensively (Zou et al. 2010; Bin et al. 2012; Zhang et al. 2015; Sun et al. 2016a). Melanin is found useful in the field of material science as coating material in electronic/bioelectronics, drug delivery and cosmetics as sunscreens, emphasising the importance of finding good, nontoxic melanin sources (Blumenberg 2017).

Symbiotic fungal species in Termitomyces Heim are found in Asian and African continents as exosymbionts cultivated by fungus growing termites belonging to subfamily – Macrotermiteinae in their nest as food (Wood and Sands 1978). During tropical monsoon, fruitbodies from subterranean fungus combs emerge by forcing their way through very hard layer of inert matter using a hard, melanised perforatorium (Heim 1977; Kendrick 2001). Traditionally, these species are known to be most popular and highly prized edible mushrooms in Asia and Africa. Taxonomists have reported dark pigmentation in fruitbodies especially in organs like hypogeeal pseudorhiza and epigeeal smooth or pointed umbo exhibiting brownish to greyish-black colouration, without commenting on chemical nature and role of such dark pigmentation, thus leaving the issue of its chemical identification and characterisation open (Otieno 1968; Pegler and Rayner 1969, 1969; Natarajan 1979; Van Der Westhuizen and Eicker 1990; Pegler and Vanhaecke 1994; Abdullah and Rusea 2009; De Kesel 2011; Srivastava et al. 2011; Tibuwa 2012; Karun and Sridhar 2013; Aryal et al. 2016).

In spite of extraction of melanin from several edible mushroom species, there is no knowledge regarding edible melanin obtained from a symbiotic mushroom which can provide better source of mushroom melanin as this Termitomyces species is well consumed in entire Asian and African continent for its delicacy. The present study thus aimed to produce the dark melanin-like pigment from pure culture under controlled conditions, purify it and verify its chemical identity as melanin and characterise it structurally.

Materials and methods

Source and growth conditions of melanin culture

Fresh, healthy Termitomyces albuminosus fruitbodies were collected from Mardol, Goa during monsoon season and taxonomically identified using standard published Termitomyces keys (Heim 1942, 1977). Several pure cultures were obtained from sterile context tissue explants of pileus on 2% Malt Extract Agar (MEA) medium (Malt extract refined bacteriological grade 2% and Agar bacteriological grade 2%) with 0.01 mg/mL concentration of nalidixic acid and neomycin (HiMedia Chemicals Ltd., Mumbai, India). Growth, morphology, and pigmentation in colonies were monitored and a promising strain showing dark melanin like pigmentation was selected and microscopically checked for purity. The melanic strain was deposited in Goa University Fungus Culture Collection (WFCC Reg. no. 946) bearing GUFCC No. 20002 and maintained on Czapek Dox Agar (CDA) medium (0.5% sucrose, 0.2% sodium nitrate, 0.1% dipotassium phosphate, 0.05% magnesium sulphate heptahydrate, 0.05% potassium chloride, 0.001% ferrous sulphate heptahydrate, and 2% agar bacteriological grade), pH 5.5 and was incubated in incubator (Modern Industrial Corporation, Mumbai, India) at 28 ± 1 °C in dark.
Production of melanin in shaken submerged culture condition

Ten identical culture plugs were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of Czapek Dox Solution (CDS) and were incubated on rotary shaker (Scigenics Biotech, Orbitek model LETT-A, Tamil Nadu, India) at 28 ± 1 °C, pH 5.5 for 1 week in dark with shaking at 150 rpm. Mycelial suspensions were obtained from pellets (Kalisz et al. 1986). Inoculum (10% v/v) was transferred into 2000 mL Erlenmeyer flasks containing 1000 mL of CDS having 5 g/L sucrose, pH 5.5 and incubated at 28 ± 1 °C for 20 days on rotary shaker at 150 rpm. Flasks were incubated at room temperature for 20 days. Insoluble melanin bound to mycelial biomass was extracted after 20 days.

Melanin extraction and purification

*Termitomyces albuminosus* pellet biomass was harvested using sterile stainless steel sieve of 100 µm mesh size, washed with sterile double distilled water three times, and oven dried at 70 °C overnight to a constant weight for estimation of mycelial dry weight. Melanin was extracted from the dry powdered fungal biomass using modification in previously described method (Sun et al. 2016b). Dry biomass powdered using mortar and pestle was subjected for melanin extraction in 100 mL 2 mol/L NaOH, in autoclave at 120 °C for 20 min. Extracts obtained were centrifuged at 5000 rpm for 5 min, supernatant was adjusted to pH 1.5 with 7 mol/L HCl, then kept at room temperature (RT) for 2 h and centrifuged at 8000 rpm for 20 min to collect the precipitate. The precipitate was washed three times with milliQ water, and dried and redisolved in 2 mol/L NaOH and supernatant was collected after centrifugation at 8000 for 20 min. The supernatant pH was readjusted to pH 1.5 with 7 mol/L HCl and then kept at RT for 2 h. The precipitate was collected by centrifugation at 8000 rpm for 20 min. The precipitates obtained of crude melanin were hydrolysed with 7 mol/L HCl at 100 °C for 2 h in order to remove bound carbohydrates and proteins. Then contents were cooled at RT and precipitate was collected by centrifugation at 8000 rpm for 20 min. The precipitate was washed three times with milliQ water to remove chloridion followed by drying at RT. The dried melanin was sequentially washed with chloroform, ethyl acetate and absolute ethanol in order to remove bound lipids, dried at RT and was transferred to a desiccator. Subsequently, the dried melanin was redissolved in 2.0 mol/L NaOH, followed by centrifugation at 8000 rpm for 20 min. The supernatant was adjusted to pH 1.5 and centrifuged at 8000 rpm for 20 min. The pure melanin was obtained after repeated washing of the precipitate with milliQ water and then drying to a constant weight in an oven at 60 °C. Purified melanin was stored in an air tight, moisture free amber bottle at −20 °C.

Morphology of melanin particles

**Bright field microscopy**

Culture from dark pigmented colonies of *T. albuminosus* and smaller melanised pellets were mounted in plain lactophenol. Pure melanin particles obtained by purification process were mounted in DPX on slides and examined using Nikon Eclipse E200 microscope with Nikon DS-fi2 camera and NIS element microscope imaging software.

**Scanning electro microscopy (SEM)**

Pure dried powdered melanin particles were fixed on carbon tape on aluminium stub and sputter coated with Palladium for 10 s (Quorum SC7620 Sputter Coater, UK) and examined by SEM at 5 kV (Vega 3 SB, TeScan, Advanced Scientific Equipment Pvt. Ltd., Bangalore, India).

**Fractal analysis**

SEM images of 10000× magnification were subjected to 11 different mathematical methods to compute fractal dimension using CMEIAS JFrad version 1.0 software freely available at http://cme.msu.edu/cmeias/ (Ji et al. 2015). The output data of melanin fractal dimensions were saved as *csv files and analysed statistically using the SYSTAT 13.

**Elemental composition of melanin**

The elemental composition CHN (O) of pure *T. albuminosus* melanin was determined with approximately 5 mg solid samples using elemental analyser (Thermo Finnigan, Italy model FLASH EA 1112 series, SAIF–IIT Bombay analytical laboratory, India) dispersed in water. The sulphur content was computed after addition of C, H, N, O percentages and qualitatively detected using Lassaigne’s test (Harki et al. 1997).
**Ultraviolet–visible (UV-VIS) and Fourier transform infrared spectroscopy (FTIR)**

UV-VIS spectrum was obtained in the range 190–750 nm using UV–VIS spectrophotometer (Shimadzu UV-2400) 0.1 mol/L NaOH as reference (Suryanarayanan et al. 2004; Selvakumar et al. 2008). A standard melanin spectrum was also obtained using *Sepia officinalis* melanin (Sigma, Aldrich Chemicals, India). For FTIR spectral analysis, the purified *T. albuminosus* melanin sample was mixed with KBr (1:10) and pressed into a 1 mm thin pellets. FTIR spectra were recorded between 4000 and 500 cm\(^{-1}\) in transmission/absorbance mode on FTIR spectrometer (Shimadzu IR Prestige 21, Japan) averaging of 40 scans. Spectral resolution was 4 cm\(^{-1}\), encoding interval 1 cm\(^{-1}\), Happ–Genzel apodisation and scanning speed 2.8 mm s\(^{-1}\) (Mbonyiryivuze et al. 2015).

**Electron paramagnetic resonance (EPR) spectroscopy**

EPR spectra were recorded using 25 mg samples at 77 K using ESR–JEOL, Japan model JES–FA200 ESR spectrometer for X band (SAIF–IIT Bombay analytical laboratory, India). Parameters used to acquire the spectra were as follows: modulation amplitude, 0.16 mT; modulation frequency 100 KHz; centre field, 325 mT; sweep width, 25 mT; sweep time, 2 min; microwave frequency, 9.1 GHz; microwave power, 0.1 mW; and temperature 77 K (Enochs et al. 1993).

**NMR studies**

Solid-state \(^{13}\)C (CP/MAS) NMR spectra were acquired on a Bruker Avance II 500 MHz spectrometer at Central Salt and Marine Chemicals Research Institute (CSMCRI) analytical laboratory, India.

**Results**

**Cultural growth and melanin production**

*Termitomyces albuminosus* colonies on CDA after 8 days showed 7.9 ± 0.17 cm diameter, initially cottony white but after 7–8 days of incubation, exhibited brownish to black pigmentation from central and older region. *Termitomyces albuminosus* hyphal growth characters were as per standard pure *Termitomyces* cultural descriptions (Botha and Eicker 1991). The pigmentation radiated towards the margin (Figure 1(a,b)). Repeated subcultures of melanogenic strain produced same results. In shaken submerged condition, *T. albuminosus* culture consistently produced spiky brown to black pellets (Figure 1(c,d)). Melanin yield from *T. albuminosus* in present study was found to be 0.0142 ± 0.005 g/L from pelletized biomass.

**Melanin deposition sites and morphology of melanin granules**

Micromorphologically *T. albuminosus* culture mat showed uniform deposition of brown–black pigment in hyphal cell wall and septa consistent with present knowledge (Figure 2(a)). Pellets showed central zone as dense black with brown peripheral spiky appendages (Figure 2(b)). Direct mount of purified melanin granules under bright field showed their polymorphic nature forming very thin, opaque amorphous black plates (Figure 2(c)). SEM images of purified sample showed the ultrafine structure of these thin amorphous plates comprising large clusters of almost spherical, compacted nanogranules. The plates show interesting but complex microtopography of nanogranules having 400–100 nm size (Figure 2(d,e,f)). Table 1 indicates the fractal analysis of pure melanin with fractal dimension \(D = 1.195–1.733\).
Elemental composition

Elemental analysis of *Termitomyces* melanin mainly indicated C:H:N:O:S composition percentage as 54.679%, 3.544%, 2.492%, 26.924%, and 12.361% as listed in Table 2. The sulphur content was not directly estimated due to lack of S detection probe but derived stoichiometrically which is an alternative method and presence of S was confirmed by the positive Lassaigne’s test.

**Table 2. Elemental composition of melanin.**

| Sample                                      | Content %          |
|---------------------------------------------|--------------------|
| Pure *Termitomyces albuminosus* melanin     | C: 54.679% H: 3.544% N: 2.492% O: 26.924% S: 12.361% |

Note. The sulphur content was calculated from the equation (Harki et al. 1997).

UV -VIS and FTIR studies

UV-VIS spectrum showed absorption profile identical to standard sepia melanin. The absorption spectra of *T. albuminosus* melanin showed characteristic peak in the ultraviolet region at 233 nm and not in visible region (Figure 3(a)). Melanin from *T. albuminosus* culture also produced a linear form with a negative slope of −0.0026.

The infrared spectrum of melanin exhibited absorption band at 2964 cm\(^{-1}\) and 2891 cm\(^{-1}\), indicating the presence of CH\(_3\), CH\(_2\) aliphatic group. The 1724 cm\(^{-1}\), 1585 cm\(^{-1}\) and 1442 cm\(^{-1}\) bands indicate C = O, C = C and C = N / N–H group, whereas 1263 cm\(^{-1}\) indicates phenolic C–O–H band (Figure 3(b)). Table 3 provides a comparative view of FTIR spectral band analysis of *T. albuminosus* melanin with other fungal melanins. *Termitomyces albuminosus* melanin showed characteristic bands for aromatic rings and sulphur at 800 cm\(^{-1}\) and 678 cm\(^{-1}\).

EPR spectroscopy

In the present study, EPR spectrum showed the peak at 2.00968 (G-value) for *T. albuminosus* melanin (Figure 3(c)).

NMR spectroscopy

\(^{13}\)C (CP/MAS) NMR spectra are shown in Figure 3(d). Its spectral band assignments along with other
reported melanins are summarised in Table 4. Characteristic chemical shift at 70–30 ppm representing \( =\text{C}–\text{S} \) and \( \text{C}–\text{H} \) carbon of open-chain aliphatic carbons present in cysteine/DOPA was observed in \( ^{13}\text{C} \) NMR spectrum of \textit{Termitomyces}.

**Discussion**

This is first report on formation of a dark melanin like pigment in \textit{Termitomyces} colonies, a phenomenon noticed in natural fruitbodies and confirmation of the pigment as melanin. Despite taxonomic knowledge about universal occurrence of dark pigmentation in \textit{Termitomyces} fruitbodies, no attention has been paid to establish its chemical identity as melanin. In addition, no reports have been found on melanogenesis in pure cultures of \textit{Termitomyces} species. This may be due to availability of very few pure cultures available in world culture collections for scientific community to work. In spite of 90 total taxa recorded pending systematic revision and found listed in Index Fungorum mycological database (www.indexfungorum.org) indicating high diversity of \textit{Termitomyces} species in Asia and Africa, the catalogues in World Federation for culture collection have only 11 \textit{Termitomyces} strains listed globally. This may be due to relative lack of interest in high-frequency culturing of wild-edible \textit{Termitomyces} species or failure to get healthy fruitbodies and viable spores for isolating mycelial cultures. The present study overcame the problem by obtaining several mycelial cultures from different \textit{Termitomyces} species and zeroing down on a stable melanogenic strain of \textit{T. albuminosus} able to show excellent growth on solid medium as well as under submerged culture conditions. Previously (Siddiquee et al. 2012, 2015) reported dark grey to black colouration in \textit{T. heimii} and \textit{T. aurantiacus} culture grown on Potato Dextrose Agar medium after 7 days but failed to identify the melanogenesis process. Zhang et al. (2015) reported melanin from culture free filtrate of \textit{Auricularia auricula} in submerged culture conditions yielding 0.124–0.558 g/L. However, Sun et al. (2016b) reported yield of 2.22 g/L melanin in culture filtrate of \textit{A. auricula} in complete medium containing lactose, yeast extract, tyrosine, calcium chloride and sodium chloride, but not estimated melanin bound to cultural biomass. In the present study, the final product of melanin accounted for about 0.012% (w/w) of dry biomass. Relatively \textit{T. albuminosus} strain used in the present study yielded less melanin probably due to choice of the medium, being a symbiotic mushroom or many other physiological parameters which need to tested in future.

In melanised fungi, pigment is known to be localised in the cell wall, in the outermost layer or embedded within the wall as granules, layered in fibrils, or bound to cell wall chitin (Butler and Day 2017).
In this study, *Termitomyces* melanin was microscopically detected to be present in cell wall or septa. Nanoparticle nature of melanin has been studied (Beltrán-García et al. 2014) and our results are consistent with the same. Consistent with the latest development in understanding the properties of such complex surfaces in topological quantum chemistry it would be interesting to see whether melanin nanogranules could also be subjected to topochemical studies (Bradlyn et al. 2017; Fiete 2017) which might explain some interesting properties. Melanins fractal dimensions results clearly implying that assembly of melanin nanogranules may occur in fractal pattern (Bridelli 1998; Eom et al. 2017). It has been known that melanin purification steps lead to dehydration thus making the polymer more aggregated and it results in loss of capacity for physiological interactions (Nicolaus 1968; Prota 1992). The aggregated structure of melanin is postulated to prevent reactive oxygen species formation because photoactive residues are less exposed (Beltrán-García et al. 2014) however the function of *T. albuminosus* melanin may be more complex as it is a mutualistic species with hypogeal anamorph and epigeal teleomorph (Piearce 1987).

| Table 4. $^{13}$C NMR spectroscopic characteristics of melanin. |
|-----------------------------------------------|-----------------|-----------------------------------------------|
| Source and type of melanin                     | Chemical shift range (ppm) | Possible assignments                              | References |
| Oidiodendron tenuissimum,                      | 220–160          | Carboxyl/carbonyl groups                       | Knicker et al. (1995) |
| *Trichoderma harzianum*, Ulocladium atrum,     | 160–140          | Aromatic COR or CNR groups                     |               |
| Hendersonula toruloidea, Eurotium echinulatum  | 140–110          | Aromatic C–H carbons, guaiacyl C-2/C-6         |               |
|                                                | 110–90           | Olefinic carbons                               |               |
|                                                | 90–60            | Carbohydrate- derived structures (C-2 to C-5)  |               |
|                                                | 60–45            | Methoxy groups, C-6 of carbohydrates, C-2      |               |
|                                                | 45–0             | Methylene groups in aliphatic rings & chains,  |               |
|                                                |                  | methyl groups bound to carbon                   |               |
| Dopa melanin                                  | 172              | Carboxyl carbon                                | Duff et al. (1988) |
|                                                | 143, 118         | Aromatic carbons                               |               |
|                                                | 55, 35           | Aliphatic carbons                              |               |
| Melanoma melanin                              | 173              | Carboxyl carbon                                |               |
|                                                | 125              | Aromatic carbons                               |               |
|                                                | 53,33            | Aliphatic carbons                              |               |
| Sepia melanin                                 | 173              | Carboxyl carbon                                |               |
|                                                | 140–110          | Aromatic carbons                               |               |
|                                                | 70–30            | Aliphatic carbons                              |               |
| Sepia melanin Free acid (MFA)                 | 200–160          | Carboxyl carbon                                | Adhyaru et al. (2003) |
|                                                | 160–135          | Aromatic & Indolic Cq (non-protonated)         |               |
|                                                | 135–90           | Aromatic & Indolic CH (protonated)             |               |
|                                                | 95–10            | Aliphatic carbons                              |               |
| Sepia melanin                                 | 200–160          | Carboxyl carbon                                | Adhyaru et al. (2003) |
|                                                | 165–135          | Aromatic & Indolic Cq (non-protonated)         |               |
|                                                | 135–100          | Aromatic & Indolic CH (protonated)             |               |
|                                                | 95–10, 50–0      | Aliphatic carbons                              |               |
| Sepia melanin                                 | 200–187, 167, 164| Carboxyl carbon                                | Hervé et al. (1994) |
|                                                | 147–110          | Aromatic & ethylenic Cq (non-protonated)       |               |
|                                                | 131–127, 119–95  | Aromatic & ethylenic CH (protonated)           |               |
|                                                | 75–15            | Aliphatic carbons                              |               |
| T. albuminosus melanin                        | 200–170          | Carboxyl carbon                                | Present study |
|                                                | 160–110          | Aromatic carbons                               |               |
|                                                | 45–40            | $=\text{C}=\text{S}$                         |               |
|                                                | 71, 56, 52, 33, 30| Aliphatic carbons in cysteine/DOPA             |               |
and benzothiazol derivatives. Generally, pheomelans or DOPA melanin chemically modified by amino acids such as cys–DOPA melanins are known to have approx. 9–16% sulphur content. These findings are in accordance with those reported by Harki et al. (1997; Costa et al. 2015; Sun et al. 2016b). According to Ye et al. (2014), about 14.83% sulphur content was determined by elemental analysis from Lachnum YM404 strain. Also the effect of medium composition on melanin composition is known. According to Bull (1970), in Aspergillus nidulans melanin pigment varied in composition with response to growth medium and the most significant finding was the widely varying nitrogen content of the melanin in response to the growth medium. Bull (1970) reported percentage composition of melanin in Czapek Dox Medium as C = 56.40%, H = 6.55%, and N = 3.92–1.78% (on addition of DOPA & Catechol), indicating that melanin composition can vary from medium to medium. High sulphur content of melanin in Termitomyces is possible due to availability of sulphur-containing amino acids and sulphite reductase enzymes. Previously, Alofe (1991; Botha and Eicker 1992; Ijeh et al. 2016; Sun et al. 2017) reported sulphur-containing (methionine, cysteine) amino acids from Termitomyces umkowaani, T. sagittaeformis T. reticulatus, T. robustus, and T. microcarpus fruitbodies. These amino-acid compositions vary from one geographic region to another. Laccase enzyme which is known to play a key role in biosynthesis of melanin has been also reported from Termitomyces (Bose et al. 2007; Gangwar et al. 2016). Rahmad et al. (2014) identified sulphite reductase enzyme from T. heimii which plays a key role in sulphur assimilation. Our results indicate that Termitomyces species may have efficient sulphur metabolism involving an unidentified pathway linked to O-acetylsersine to form cysteine (Leustek et al. 2000; Kopriva and Koprivova 2003). According to Plonka and Grabacka (2006), the possible melanin synthesis pathway in Termitomyces using laccase enzyme and source of sulphur pool as amino acids can be written as

DOPA→DOPAQuinone→CysteinylDOPA→1,4-Benzothiazylalanine→pheomelanin.

which is required to be tested in future as the present study only aimed at the characterisation of melanin pigment from genus Termitomyces.

The linear decrease in the absorption with increasing wavelength was observed for Termitomyces melanin similar to that reported by (Zhang et al. 2015). Absorption peaks in UV regions occur due to the presence of many conjugated structures in melanin molecule (Ou-Yang et al. 2004). The log of optical density of a melanin solution when plotted against wavelength produces a linear curve with negative slopes. Such characteristic straight lines with negative slopes have been obtained from some melanogenic fungi such as Phyllosticta capitalensis and Auricularia auricula with slope ranging −0.0015 to −0.0030 (Ellis and Griffiths 1974; Suryanarayanan et al. 2004; Bin et al. 2012; Zhang et al. 2015). The slopes of linear plots are often used to identify melanins and matching spectral features in the present work confirms the identity of T. albuminosus melanin.

TFTIR studies carried out by Sava et al. (2001) reported that absorption is reduced at 3450 cm\(^{-1}\) and 1650 cm\(^{-1}\), after acid hydrolysis treatment undertaken during purification step due to formation of reactions between phenolic and carboxylic groups to form lactones. Also treatment with chloroform and ethyl acetate could have reduced absorption at 2900–2850 cm\(^{-1}\) in spectra.

Melanin polymers are known to have paramagnetic character and o-semiquinone free radical with spin (S = 1/2). These unpaired electrons of free radicals obey EPR effect (Pilawa et al. 2017). Enochs et al. (1993) described a standardised and effective test for the identification of melanin pigment by identifying the presence of stable population of organic free radical signal. The G-value of fungal melanin is reported to be 2.0012 (Selvakumar et al. 2008). Termitomyces albuminosus melanin G-value is found to be somewhat higher which could be due to O-semiquinone free radicals. Bin et al. (2012) also showed higher G-value of 2.0042 for Auricularia auricula melanin. It has been known that sulphur-containing radicals show high G-value (Bolman et al. 1970); therefore, incorporation of a sulphur-rich scaffold in melanin of T. albuminosus may result in a high G-value.

TAalphatic amine structural elements are proposed to arise in \(^{13}\)C NMR spectrum from coupling of dopamine/quinone structural units which are unique to dopamine melanins (Della Vecchia et al. 2013; Chatterjee et al. 2014). Tian et al. (2003) reported that carbon-near sulphur shows chemical shift at 45–40 ppm and CH\(_2\)
melanin needs further exploration as it has been seen around 40–35 ppm in $^{13}$C NMR spectrum which is consistent with our sulphur-containing melanin claim.

**Conclusions**

The present study successfully established the chemical identity of the dark pigment as a unique form of fungal melanin with high sulphur content. The exact structure of melanin polymers is difficult to elucidate and the benefit of incorporation of a sulphur scaffold in Termitomyces melanin needs further exploration as it may play functionally important roles at crucial and critical stages in the natural life cycle of Termitomyces holomorph in protecting the species from injury and damage.

**Acknowledgments**

The authors thank Goa University Fungus Culture Collection and Research Unit (GUFCCRU) for culture, Department of Botany, Goa University for providing Scanning Electron Microscope facility and University Science Instrumentation Centre (USIC), Goa University for sputter coating facility. The authors also thank SAIF IIT, Bombay for providing elemental analysis and CSMCRI for $^{13}$C NMR facility. A word of thanks also goes to Department of Chemistry, Goa University for providing FTIR facility. Special thanks to Senior sales Azhar Ullah Khan for providing Systat 13 software. RA De Souza acknowledges DST-Inspire Fellowship (SRF), Govt. of India.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**ORCID**

Rosy Agnes De Souza [http://orcid.org/0000-0002-2812-8458](http://orcid.org/0000-0002-2812-8458)
Nandkumar Mukund Kamat [http://orcid.org/0000-0003-1070-0492](http://orcid.org/0000-0003-1070-0492)
Vishnu S. Nadkarni [http://orcid.org/0000-0002-6440-9687](http://orcid.org/0000-0002-6440-9687)

**References**

Abdullah F, Rusea G. 2009. Documentation of inherited knowledge on wild edible fungi from Malaysia. Blumea-Biodiversity, Evol Biogeophy Plants. 54:35–38.

Index Fungorum mycological database. Available from: [www.indexfungorum.org](http://www.indexfungorum.org). Accessed May 31, 2018.

Adhyaru BB, Akhmedov NG, Katritzky AR, Bowers CR. 2003. Solid-state cross-polarization magic angle spinning $^{13}$C and $^{15}$N NMR characterization of sepiad melanin, sepiad melanin free acid and human hair melanins in comparison with several model compounds. Magn Reson Chem. 41(6):466–474.

Alofe FV. 1991. Amino acids and trace minerals of three edible wild mushrooms from Nigeria. J Food Comp Anal. 4(2):167–174.

Arun G, Eyini M, Gunasekaran P. 2015. Characterization and biological activities of extracellular melanin produced by *Schizophyllum commune* (Fries). Indian J Exp Biol. 53:380–387.

Aryal HP, Ghimire SK, Budathoki U. 2016. *Termitomyces*: New to the Science. J Plant Sci Res. 3(1):148–150.

Batistaia VG, Shcherba VV, Ikonnikova NV. 2000b. Melanin complex of the fungus *Inonotus obliquus*. Appl Biochem Microbiol. 36:377–381.

Batistaia VG, Shcherba VV, Ikonnikova NV, Bisko NA, Mitropolskaya NY. 2002. Melanin complex from medicinal mushroom *Inonotus obliquus* (Pers.: Fr.) Pilat (Chaga) (Aphyllophoromycetidae). Int J Med Mushrooms. 4:139–145.

Batistaia VG, Shcherba VV, Filimonova TV, Grigorochuk EA. 2000a. Melanin pigments from the fungi *Paecilomyces variotii* and *Aspergillus carbonarius*. Appl Biochem Microbiol. 36(2):128–133.

Bell AA, Wheeler MH. 1986. Biosynthesis and functions of fungal melanins. Annu Rev Phytopathol. 24(1):411–451.

Beltrán-García MJ, Prado FM, Oliveira MS, Ortiz-Mendoza D, Scalfi AC, Jr A P, Medeiros MH, White JF, Di Mascio P. 2014. Singlelet molecular oxygen generation by light-activated DHN-melanin of the fungal pathogen *Mycosphaerella fijiensis* in black Sigatoka disease of bananas. PloS one. 9:e91616.

Bin L, Wei L, Xiaohong C, Mei J, Mingsheng D. 2012. In vitro antibiofilm activity of the melanin from *Auricularia auricula*, an edible jelly mushroom. Ann Microbiol. 62(4):1523–1530.

Bisko NA, Mitropolskaya NY, Ikonnikova NV. 2002. Melanin complex from medicinal mushroom *Inonotus obliquus* (Pers.: Fr.) Pilat (Chaga) (Aphyllophoromycetidae). Int J Med Mushrooms. 4(2):139–145.

Bisko NA, Shcherba VV, Mitropolskaya NY. 2007. Study of melanin complex from medicinal mushroom *Phellinus robustus* (P. Karst.) Bourd. et Galz. (Aphyllophoromycetidae). Int J Med Mushrooms. 9(2):177–184.

Blumenberg M. 2017. Introductory Chapter: Melanin, a Versatile Guardian. In Melanin. InTechOpen, Rijeka.

Bolman PS, Safarik I, Stiles DA, Tyerman WJ, Strausz OP. 1970. Electron paramagnetic resonance spectra of some sulfur-containing radicals. Can J Chem. 48(24):3872–3876.

Bose S, Mazumder S, Mukherjee M. 2007. Laccase production by the white rot fungus *Termitomyces clypeatus*. J Basic Microbiol. 47(2):127–131.

Botha WJ, Eicker A. 1991. Cultural studies on the genus *Termitomyces* in South Africa. I. Macro-and microscopic characters of basidiome context cultures. Mycol Res. 95(4):435–443.

Botha WJ, Eicker A. 1992. Nutritional value of *Termitomyces* mycelial protein and growth of mycelium on natural substrates. Mycol Res. 96(5):350–354.
Streptomyces glaucescens harvested. World J Microbiol Biotechnol. 28(6):1908–1917.

Fernandez CW, Koide RT. 2013. The function of melanin in the ectomycorrhizal fungus Cenococcum geophilum under water stress. Fungal Ecol. 6(6):479–486.

Fiete GA. 2017. Materials science: chemistry and physics happily wed. Nature. 547:287–288.

Mbonyiriyuze A, Mwakikunga B, Dhlamini SM, Maaza M. 2015. Fourier transform infrared spectroscopy for sepioid melanins. Phys Mater Chem. 3(2):25–29.

Gangwar R, Rasool S, Mishra S. 2016. Evaluation of cellulose dehydrogenase and laccase containing culture fluids of Termotomycy sp. OE147 for degradation of Reactive blue 21. Biotechnol Rep. 12:52–61.

Gonçalves RC, Lisboa HC, Pombeiro-Sponchiado SR. 2012. Characterization of melanin pigment produced by Aspergillus nidulans. World J Microbiol Biotechnol. 28(4):1467–1474.

Harki E, Talou T, Dargent R. 1997. Purification, characterisation and analysis of melanin extracted from Tuber melanosporum. Vitt. Food Chem. 58(1–2):69–73.

Heim R. 1942. Nouvelles etudes descriptives sur les agarics termitephiles d’Afrique tropicale. Arch Mus Natl Hist Nat ser. 6(18):107–166.

Heim R. 1977. Termites et champignons. Les champignons termitephiles d’Afrique noire et d’Asie meridionale. Soc. Nouv. Edit. Paris: Boubee.

Henson JM, Butler MJ, Day AW. 1999. The dark side of the mycelium: melanins of phytopathogenic fungi. Annu Rev Phytopathol. 37(1):447–471.

Hervé M, Hirschinger J, Granger P, Gilard P, Deflandre A, Goetz N. 1994. A 13C solid-state NMR study of the structure and auto-oxidation process of natural and synthetic melanins. Biochim Biophys Acta Protein Struct Mol Enzymol. 1204(1):19–27.

Ijhe II, Eke IN, Ugwu CC, Ejike EC. 2016. Myco-nourishment from the wild: chemical analyses of the nutritional and amino acid profile of Termotomycy robustus harvested from Uzuakoli, Nigeria. Nat Prod Chem Res. 4:225.

Ji Z, Card KJ, Dazzo FB. 2015. CMEIAS JFrad: a digital computing tool to discriminate the fractal geometry of landscape architectures and spatial patterns of individual cells in microbial biofilms. Microb Ecol. 69(3):710–720.

Kalisz HM, Wood DA, Moore D. 1986. Regulation of extracellular laccase production of Agaricus bisporus by nitrogen sources in the medium. FEMS Microbiol Lett. 34(1):19–27.

Karun NC, Sridhar KR. 2013. Occurrence and distribution of Termotomycy (Basidiomycota, Agaricales) in the Western Ghats and on the west coast of India. Czech Mycol. 65(2):233–254.

Kendrick B. 2001. The fifth kingdom, 3rd:Sidney, BC, Canada. Mycologue Publisher.

Knicker H, Almedros G, Gonzalez-Vila FJ, Lüdemann HD, Martin F. 1995. 13C and 15N NMR analysis of some fungal melanins in comparison with soil organic matter. Org Geochem. 23:1023–1028.
Kopriva S, Koprivova A. 2003. Sulphate assimilation: a pathway which likes to surprise. In: Abrol VP, Ahmad A, eds. Sulphur in Plants. Dordrecht: Springer; p. 87–112.

Leustek T, Martin MN, Bick JA, Davies JP. 2000. Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Biol. 51:141–165.

Mendoza CG, Leal JA, Novaes-Ledieu M. 1979. Studies of the spore walls of Agaricus bisporus and Agaricus campestris. Can J Microbiol. 25(1):32–39.

Money NP, Caesar-TonThat TC, Frederick B, Henson JM. 1998. Melanin synthesis is associated with changes in hyphal-tidal turgor, permeability, and wall rigidity in Gaeumannomyces graminis var. graminis. Fungal Genet Biol. 24(1–2):240–251.

Natarajan K. 1979. South Indian Agaricales V: Termitomyces heimii. Mycologia. 71(4):853–855.

Nicolaus RA. 1968. Melanins. Paris: Hermann.

Otieno NC. 1968. Further contributions to a knowledge of termite fungi in East Africa: the genus Termitomyces Heim. Sydowia. 22:160–165.

Ou-Yang H, Stamatgas G, Kollias N. 2004. Spectral responses of melanin to ultraviolet A irradiation. J Investig Dermatol. 122(2):492–496.

Pal AK, Gajjar DU, Vasavada AR. 2013. DOPA and DHN pathway orchestrate melanin synthesis in Aspergillus species. Med Mycol. 52(1):10–18.

Pegler DN, Rayner RW. 1969. A contribution to the Agaric flora of Kenya. Kew Bull. 23(3):347–412.

Pegler DN, Vanhaecke M. 1994. Termitomyces of Southeast Asia. Kew Bull. 49(4):717–736.

Pierce GD. 1987. The genus Termitomyces in Zambia. Mycologist. 1(3):111–116.

Pilawa B, Zdybel M, Chodurek E. 2017. Application of electron paramagnetic resonance spectroscopy to examine free radicals in melanin polymers and the human melanoma malignum cells. In Melanin. InTechOpen, Rijeka.

Plonka PM, Grabacka M. 2006. Melanin synthesis in microorganisms-biotechnological and medical aspects. Acta Biochim Pol. 53:429–443.

Polacheck I, Kwon-Chung KJ. 1988. Melanogenesis in Cryptococcus neoformans. Microbiology. 134(4):1037–1041.

Prota G. 1992. Melanins and melanogenesis. San Diego, CA: Academic Press.

Rahmad N, Al-Obaidi JR, Rashid NM, Zean NB, Yusoff MH, Shaharuddin NS, Jamil NA, Saleh NM. 2014. Comparative proteomic analysis of different developmental stages of the edible mushroom Termitomyces heimii. Biol Res. 47:30.

Sava VM, Galkin BN, Hong MY, Yang PC, Huang GS. 2001. A novel melanin-like pigment derived from black tea leaves with immuno-stimulating activity. Food Res Int. 34(4):337–343.

Schmaler-Ripcke J, Sugareva V, Gebhardt P, Winkler R, Kniemeyer O, Heinke T, Brakhage AA. 2009. Production of pyomelanin, a second type of melanin, via the tyrosine degradation pathway in Aspergillus fumigatus. Appl Environ Microbiol. 75(2):493–503.

Selvakumar P, Rajasekar S, Periasamy K, Raaman N. 2008. Isolation and characterization of melanin pigment from Pleurotus cystidiosus (telomorph of Antromycopsis macrocarpa). World J Microbiol Biotechnol. 24(10):2125–2131.

Siddiquie S, Rovina K, Naher L, Rodrigues KF, Uzzaman MA. 2015. Phylogenetic relationships of Termitomyces aurantia investigated from internal transcribed spacers DNA sequences. Adv Biosci Biotechnol. 6:358–367.

Siddiquie S, Yee WY, Taslima K, Fatihah NH, Kumar SV, Hasan MM. 2012. Sequence analysis of the ribosomal DNA internal transcribed spacer regions in Termitomyces heimii species. Ann Microbiol. 62(2):797–803.

Srivastava B, Dwivedi AK, Pandey VN. 2011. Morphological characterization and yield potential of Termitomyces spp. mushroom in Gorakhpur forest division. Bull Env Pharmacol Life Sci. 1(1):54–56.

Sun L, Liu Q, Bao C, Fan J. 2017. Comparison of free total amino acid compositions and their functional classifications in 13 wild edible mushrooms. Molecules. 22(3):350.

Sun S, Zhang X, Chen W, Zhang L, Zhu H. 2016a. Production of natural edible melanin by Auricularia auricula and its physicochemical properties. Food Chem. 196:486–492.

Sun S, Zhang X, Sun S, Zhang L, Shan S, Zhu H. 2016b. Production of natural melanin by Auricularia auricula and study on its molecular structure. Food Chem. 190:801–807.

Suryanarayanan TS, Ravishankar JP, Venkatesan G, Murali TS. 2004. Characterization of the melanin pigment of a cosmopolitan fungal endophyte. Mycol Res. 108(8):974–978.

Tian S, Garcia-Rivera J, Yan B, Casadevall A, Stark RE. 2003. Unlocking the molecular structure of fungal melanin using 13C biosynthetic labeling and solid-state NMR. Biochem. 42(27):8105–8109.

Tibuhwa DD. 2012. Termitomyces species from Tanzania, their cultural properties and unequalled basidiospores. J Biol Life Sci. 3:1.

Van Der Westhuizen GC, Eicker A. 1990. Species of Termitomyces occurring in South Africa. Mycol Res. 94(7):923–937.

Weijn A, Bastiaan-Net S, Wichers HJ, Mes JJ. 2013. Melanin biosynthesis pathway in Agaricus bisporus mushrooms. Fungal Genet Biol. 55:42–53.

Wood TG, Sands WA. 1978. The role of termites in ecosystems. In: Brian MV, Ed. Production ecology of ants and termites. Cambridge, UK: Cambridge University Press; p. 245–292.

Ye M, Guo GY, Lu Y, Song S, Wang HY, Yang L. 2014. Purification, structure and anti-radiation activity of melanin from Lachnum YM404. Int J Biol Macromol. 63:170–176.

Zhang M, Xiao G, Thring RW, Chen W, Zhou H, Yang H. 2015. Production and characterization of melanin by submerged culture of culinary and medicinal fungi Auricularia auricula. Appl Biochem Biotechnol. 176(1):253–266.

Zou Y, Xie C, Fan G, Gu Z, Han Y. 2010. Optimization of ultrasound-assisted extraction of melanin from Auricularia auricula fruit bodies. Innov Food Sci Emerg Technol. 11(4):611–615.