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Extraction and partial characterization of melanin pigment from *Alternaria burnsii* NFCCI 5753 and *Cladosporium tenuissimum* NFCCI 5754

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Abstract
Melanin is a natural pigment found in many living organisms, including humans, animals, bacteria, and fungi. It protects against UV radiation, binds metal ions, reduces oxidative stress, and helps organisms survive heat and drought. Fungi, actinomycetes, and bacteria produce melanin in response to environmental stress. This study identifies two fungal isolates, *Alternaria burnsii* NFCCI 5753 and *Cladosporium tenuissimum* NFCCI 5754, based on morpho-molecular characters and reports the extraction and characterization of melanin pigment produced by them. The amount of melanin produced was much higher than previously reported, with 153.97 ± 0.09 mg/100 ml from *A. burnsii* NFCCI 5753 and 73.50 ± 0.09 mg/100 ml from *C. tenuissimum* NFCCI 5754. The nature of the pigment was confirmed through spectral (UV and FTIR) tests and elemental analysis.

Keywords: Elemental analysis, FTIR, Molecular studies, Morphological, UV

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1. Introduction

The pigment melanin is a complex natural biopolymer that is formed when phenols are oxidized and then polymerized into intermediate phenols and quinones. Based on its structural elements, it can be divided into three major categories: eumelanin, pheomelanin, and allomelanin (Singh et al., 2021). While pheomelanin, which is present in animals, contributes to yellow or red pigmentation, eumelanin and allomelanin give a dark brown colour to the cells. Melanin is found in many living organisms, including plants, animals, fungi, and pathogenic bacteria (Ghadge et al., 2021). However, the pigment plays important protective roles, such as

photoprotection and UV radiation shielding. Melanin can also chelate metal ions, protect from reactive oxygen species-induced oxidative stress, and increase resistance to heat and drought. It can absorb heavy metals and contribute to the rigidity of the cell wall (El-Naggar and Saber, 2022; Singh et al., 2021). As a result, there has been a lot of interest in investigating microbial bioproduction as a potential source of melanin (Elsayis et al., 2022).

Melanin is naturally produced by a variety of micro-organisms in response to environmental conditions, including fungi, actinomycetes, and bacteria (Elsayis et al., 2022; Gessler et al., 2014). Fungi are particularly known to produce melanin in unfavourable circumstances and can protect them from UV rays (Pandey et al., 2024). In the present study, we isolated and characterized melanin pigment produced by two fungal isolates: *Alternaria burnsii* NFCCI 5753 and *Cladosporium tenuissimum* NFCCI 5754. The fungi were identified based on morphological as well as molecular characters. Then, the pigment was extracted from the fungal cultures by the alkaline-acid hydrolysis method and confirmed as melanin through spectral (UV and FTIR) tests and elemental analysis.

Alternaria (Pleosporaceae, Pleosporales) is a ubiquitous fungal genus that includes saprobic, endophytic, and pathogenic species associated with a wide variety of substrates. It has been divided into 29 sections and seven monotypic lineages based on molecular and morphological data. *Alternaria* section *Porri* is the largest section, containing the majority of large-spored *Alternaria* species, most of which are important plant pathogens (Lawrence et al., 2016; Zhao et al., 2022). There are now 366 recognised and accepted species in *Alternaria*, out of about 589 valid species epithets (Htun et al., 2022; Wijayawardene et al., 2020). These species are frequently encountered as pests on plants (Andersen et al., 2001). It is now necessary to use sophisticated analytical techniques based on molecular methodologies to detect and classify *Alternaria* to species level. The internal transcribed spacer regions of 5.8S rRNA gene (ITS), translation-elongation factor 1 (TEF1-α), and RNA polymerase second largest subunit (RPB2) are among the multiple gene loci used in these analyses (Hong et al., 2005; Htun et al., 2022). Species of *Alternaria* are extensively dispersed and infect a wide variety of commercially significant crops. Most *Alternaria* species are either saprobic or have been reported to occur on hosts with no economic significance.

Alternaria species have been identified and categorised using morphological traits such as conidial colour, size, form, and sporulation patterns. Nonetheless, certain traits are shared by different species and change based on environmental factors, including substrate and temperature. Molecular markers have been a common tool for classifying and identifying *Alternaria* species in recent years (Nasehi et al., 2014). The monophyly of *Alternaria* in Pleosporaceae has been robustly defined by molecular approaches (Chou and Wu, 2002; Woudenberg et al., 2013). In contemporary fungal systematics, morphological and molecular phylogenetic investigations are complementary in determining the taxonomic position of *Alternaria* species (Lawrence et al., 2016; Woudenberg et al., 2013).

Cladosporium (Cladosporiaceae, Cladosporiales) is one of the largest and most diverse genera of hyphomycetous fungi (Dugan et al., 2004; Xu et al., 2021). These are primarily plant pathogenic in nature, with some species being pathogenic to animals and humans as well, while some species serving as saprobes, endophytes, and hyperparasites on other fungi (Marin-Felix et al., 2017). Typically, *Cladosporium* is identified by its asexual form, which is typified by erect, straight, or bending conidiophores and branched acropetal chains of dry conidia that are smooth to rough in texture. These chains are formed by either mono- or polyblastic

conidiogenous cells (Crous et al., 2007; Iturrieta-González et al., 2021). However, due to the minute variations in conidial structures, the morphological categorization of this genus has become difficult, making it hard to completely study the taxa based on only microscopic and culture observations. Therefore, a multi-locus sequence analysis method has been applied to numerous evolutionary studies of *Cladosporium* species, ITS and two protein-coding genes, actin (act) and TEF1-α (Bensch et al., 2018; Iturrieta-González et al., 2021). Using morphological characteristics in conjunction with this molecular method has allowed for the identification of new species, widely dispersed into three species complexes: *C. cladosporioides*, *C. herbarum*, and *C. sphaerospermum* (Xu et al., 2021).

2. Materials and methods

2.1 Collection, isolation, and morphological identification of fungal isolates

Samples were collected from the garden soil of Agharkar Research Institute, Pune, Maharashtra, India, in April 2022. These samples were processed to isolate various fungal strains using serial dilution method on Potato Dextrose Agar (PDA). To prepare the initial stock solution for serial dilution, 1 gram of soil was mixed with 10 ml of sterile saline solution. From this stock solution, serial dilutions were made up to 10^{-7}. Then, a 0.1 ml sample from each dilution was spread-plated onto PDA plates. After serial dilution, the PDA plates were incubated at 25 °C and monitored for fungal growth over 7–10 days. Individual fungal colonies were picked and subcultured onto fresh PDA plates to obtain pure cultures. These pure fungal isolates were then transferred to freshly prepared agar slants and stored at 4 °C for preservation and future use. In total, 18 fungal strains were isolated from soil samples, among which two fungal isolates that were predominantly observed for their black colour were selected for further studies.

For morphological identification up to the genus level, each of the two selected fungi was cultured on four different media: PDA, malt extract agar (MEA), cornmeal agar (CMA), and potato carrot agar (PCA). After 7 days, colony characteristics such as color, texture, pigment production, and other observations were recorded. The Methuen Handbook of Color (Kornerup and Wanscher, 1978) was used to document the colony colors on agar media. Microscopic features were also examined using lactophenol-cotton blue stain with a Carl Zeiss Image Analyzer 2 microscope. Microphotographs of the fungal structures were obtained using AxioVision Rel 4.8 software and a Digi-Cam attached to the microscope. Based on these observations, the fungi were identified as *Alternaria* sp. and *Cladosporium* sp. Pure cultures were deposited in the National Fungal Culture Collection of India (*A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754).

2.2 Molecular characterization of fungal isolates

Genomic DNA was isolated from the two fungal isolates grown on PDA for 2 weeks following a rapid and simple DNA extraction protocol (Aamir et al., 2015). The study involved the amplification of ITS, TEF1-α, and RPB2 gene regions for *A. burnsii* NFCCI 5753, while for *C. tenuissimum* NFCCI 5754, ITS, TEF1-α, and act gene regions were amplified using specific primers. PCR was executed in a 25 µl reaction using 12.5 µl of EmeraldAmp GT PCR Master Mix, 2 µl template DNA (10–20 ng), 1.5 µl forward and reverse primer (10 pmol), and Sterile Ultra-Pure Water. The gene regions and their respective primers involved are listed in Table 1.

The thermocycling conditions involved are as follows: For ITS, there was an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 30 s at 52 °C, 1 min at 72 °C, and a final extension at 72 °C for 8 min. For TEF1-α, there was an initial denaturation

at 96 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C, 40 s at 72 °C, and a final extension at 72 °C for 7 min. For RPB2, there was an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 30 s at 55 °C, 2 min at 72 °C, and a final extension at 72 °C for 7 min. For ACT, there was an initial denaturation at 96 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 45 s at 60 °C, 1 min at 72 °C, and a final extension at 72 °C for 7 min.

The amplified PCR products were purified using the FavorPrep PCR purification kit and analyzed on 1.2 % agarose electrophoresis gels stained with ethidium bromide. The sequencing PCR reaction was performed using a Big Dye Terminator v3.1 Cycle Sequencing Kit. The PCR product was purified using Thermo Fisher Scientific's Big Dye X-terminator Purification Kit, and sequencing was performed using the Applied Biosystems SeqStudio Genetic Analyzer. The sequences were submitted to NCBI nucleotide sequence database for GenBank accession numbers (Tables 2 and 3).

Table 1. PCR primer sets used for amplification and sequencing in this study

Gene Region	Primer	Direction	Sequence (5'-3')	Reference
ITS	ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	White et al. (1990)
TEF 1-α	EF1-728F	Forward	CATCGAGAAGTTCGAGAAGG	Carbone and Kohn (1999)
	EF1-986R	Reverse	TACTTGAAGGAACCCTTACC	Carbone and Kohn (1999)
RPB2	RPB2-5F2	Forward	GAYGAYMGWGATCAYTTYGG	Liu et al. (2019)
	RPB2-7cR	Reverse	CCCATRGCTTGYTTRCCCAT	Liu et al. (2019)
ACT	ACT-512F	Forward	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn (1999)
	ACT-783R	Reverse	TACGAGTCC TTCTGGCCCAT	Carbone and Kohn (1999)

To determine the phylogenetic positions of the two fungal isolates, their respective gene regions were used to compare with the known taxa of the respective genera. Through ITS, *A. burnsii* NFCCI 5753 showed a similarity of 99.8 % with species of section *Alternaria*, while *C. tenuissimum* NFCCI 5754 revealed 100 % similarity with those of *Cladosporioides* complex species. Therefore, all the species of section *Alternaria* and those of *C. cladosporioides* species complex were used in the analysis and were aligned along with sequences of the two fungal isolates. *A. ipomoeae* CBS 219.79 of section *Porri* and *C. psychrotolerans* CBS 119412 of *C. sphaerospermum* species complex were selected as the outgroup taxa for *A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754, respectively.

A list of the selected strains that were used for the construction of phylogenetic trees, along with their accession numbers and other pertinent information, is provided in Tables 2 and 3. Using MAFFT v. 6.864b (Katoh and Standley, 2013), each gene region was separately aligned**.** Aliview was used to manually check and adjust the alignments (Larsson, 2014)**.** After alignments were concatenated, phylogenetic analyses were performed. To construct the phylogenetic trees, Windows version of IQ-tree v.1.6.11 was used (Nguyen et al., 2015). The

reliability of the rebuilt branches was evaluated using 1000 replicate bootstrap analyses. FigTree v.1.4.4 was used to display the phylogenetic trees (Rambaut, 2018).

Table 2. Strains used to build the phylogenetic tree for *Alternaria burnsii* NFCCI 5753 and their GenBank accession numbers

Section	Species	Culture accession no.	ITS	TEF 1-α	RPB2
Alternaria	*A. alternata*	CBS 877.95	KP124321	KP125097	KP124789
	A. alternata	CBS 916.96	AF347031	KC584634	KC584375
	A. arborescens	CBS 119544	KP124408	KP125186	KP124878
	A. arborescens	CBS 102605	AF347033	KC584636	KC584377
	A. arborescens	CBS 101.13	KP124392	KP125170	KP124862
	A. betae-kenyensis	CBS 118810	KP124419	KP125197	KP124888
	A. burnsii	CBS 879.95	KP124422	KP125200	KP124891
	A. burnsii	CBS 107.38	KP124420	KP125198	KP124889
	A. burnsii	CBS 108.27	KC584236	KC584727	KC584468
	A. burnsii	**NFCCI 5753**	**PP855652**	**PP861116**	**PP861115**
	A. eichhorniae	CBS 489.92	KC146356	KP125204	KP124895
	A. gaisen	CBS 118488	KP124427	KP125206	KP124897
	A. gaisen	CBS 632.93	KC584197	KC584658	KC584399
	A. gossypina	CBS 104.32	KP124430	KP125209	KP124900
	A. iridiaustralis	CBS 118486	KP124435	KP125214	KP124905
	A. jacinthicola	CBS 133751	KP124438	KP125217	KP124908
	A. longipes	CBS 121333	KP124444	KP125223	KP124914
	A. tomato	CBS 114.35	KP124446	KP125225	KP124916
	A. tomato	CBS 103.30	KP124445	KP125224	KP124915
Porri	*A. ipomoeae*	CBS 219.79	KJ718175	KJ718523	KJ718348

Table 3. Strains used to build the phylogenetic tree for *Cladosporium tenuissimum* NFCCI 5754 and their GenBank accession numbers

Species	Culture accession no.	ITS	TEF 1-α	ACT
C. acalyphae	CBS 125982	HM147994	HM148235	HM148481
C. alboflavescens	CBS 140690	LN834420	LN834516	LN834604
C. angulosum	CBS 140692	LN834425	LN834521	LN834609
C. angustisporum	CBS 125983	HM147995	HM148236	HM148482
C. angustiterminale	CBS 140480	KT600379	KT600476	KT600575
C. anthropophilum	CBS 140685	LN834437	LN834533	LN834621
C. asperulatum	CBS 126339	HM147997	HM148238	HM148484
C. asperulatum	CBS 126340	HM147998	HM148239	HM148485
C. australiense	CBS 125984	HM147999	HM148240	HM148486
C. austroafricanum	CBS 140481	KT600381	KT600478	KT600577
C. chalastosporioides	CBS 125985	HM148001	HM148242	HM148488
C. chubutense	CBS 124457	FJ936158	FJ936161	FJ936165
C. cladosporioides	CBS 113738	HM148004	HM148245	HM148491
C. cladosporioides	CBS 112388	HM148003	HM148244	HM148490
C. cladosporioides	CPC 14292	HM148046	HM148287	HM148533
C. colocasiae	CBS 386.64	HM148067	HM148310	HM148555
C. colocasiae	CBS 119542	HM148066	HM148309	HM148554
C. colombiae	CBS 274.80	FJ936159	FJ936163	FJ936166
C. crousii	CBS 140686	LN834431	LN834527	LN834615
C. cucumerinum	CBS 171.52	HM148072	HM148316	HM148561
C. cucumerinum	CBS 173.54	HM148074	HM148318	HM148563
C. delicatulum	CBS 126342	HM148079	HM148323	HM148568
C. delicatulum	CBS 126344	HM148081	HM148325	HM148570
C. exile	CBS 125986	HM148090	HM148334	HM148579
C. exile	CBS 125987	HM148091	HM148335	HM148580

C. flabelliforme	CBS 126345	HM148092	HM148336	HM148581
C. flavovirens	CBS 140462	LN834440	LN834536	LN834624
C. funiculosum	CBS 122128	HM148093	HM148337	HM148582
C. gamsianum	CBS 125989	HM148095	HM148339	HM148584
C. globisporum	CBS 812.96	HM148096	HM148340	HM148585
C. grevilleae	CBS 114271	JF770450	JF770472	JF770473
C. hillianum	CBS 125988	HM148097	HM148341	HM148586
C. inversicolor	CBS 143.65	HM148100	HM148344	HM148589
C. ipereniae	CBS 140483	KT600394	KT600491	KT600589
C. iranicum	CBS 126346	HM148110	HM148354	HM148599
C. licheniphilum	CBS 125990	HM148111	HM148355	HM148600
C. longicatenatum	CBS 140485	KT600403	KT600500	KT600598
C. lycoperdinum	CBS 574.78	HM148115	HM148359	HM148604
C. lycoperdinum	CBS 126347	HM148112	HM148356	HM148601
C. montecillanum	CBS 140486	KT600406	KT600504	KT600602
C. montecillanum	CPC 15605	KT600407	KT600505	KT600603
C. myrtacearum	CBS 126350	HM148117	HM148361	HM148606
C. oxysporum	CBS 125991	HM148118	HM148362	HM148607
C. oxysporum	CBS 126351	HM148119	HM148363	HM148608
C. paracladosporioides	CBS 171.54	HM148120	HM148364	HM148609
C. parapenidielloides	CBS 140487	KT600410	KT600508	KT600606
C. perangustum	CBS 125996	HM148121	HM148365	HM148610
C. perangustum	CBS 126365	HM148123	HM148367	HM148612
C. phaenocomae	CBS 128769	JF499837	JF499875	JF499881
C. phyllactiniicola	CBS 126353	HM148151	HM148395	HM148640
C. phyllactiniicola	CBS 126355	HM148153	HM148397	HM148642
C. phyllophilum	CBS 125992	HM148154	HM148398	HM148643
C. pini-ponderosae	CBS 124456	FJ936160	FJ936164	FJ936167
C. pseudochalastosporioides	CBS 140490	KT600415	KT600513	KT600611
C. pseudocladosporioides	CBS 667.80	HM148165	HM148409	HM148654
C. pseudocladosporioides	CPC 14020	HM148185	HM148429	HM148674
C. rectoides	CBS 125994	HM148193	HM148438	HM148683
C. rugulovarians	CBS 140495	KT600459	KT600558	KT600656
C. scabrellum	CBS 126358	HM148195	HM148440	HM148685
C. silenes	CBS 109082	EF679354	EF679429	EF679506
C. subuliforme	CBS 126500	HM148196	HM148441	HM148686
C. tenuissimum	CPC 10882	HM148204	HM148449	HM148694
C. tenuissimum	CPC 11555	HM148205	HM148450	HM148695
C. tenuissimum	CPC 12795	HM148209	HM148454	HM148699
C. tenuissimum	**NFCCI 5754**	**PP855653**	**PP861114**	**PP861113**
C. varians	CBS 126362	HM148224	HM148470	HM148715
C. verrucocladosporioides	CBS 126363	HM148226	HM148472	HM148717
C. xantochromaticum	CBS 140691	LN834415	LN834511	LN834599
C. xantochromaticum	CPC 12792	HM148136	HM148380	HM148625
C. xylophilum	CBS 125997	HM148230	HM148476	HM148721
C. psychrotolerans	CBS 119412	DQ780386	JN906992	EF101365

2.3 Extraction and purification of melanin pigment

Melanin pigment was extracted from *A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754 following the standard alkali-acid method by Gadd (1982). In brief, 10 mm diameter mycelial plugs were taken from freshly growing colonies on PDA and inoculated into 500 ml of sterile Potato Dextrose Broth in 1000 ml Erlenmeyer flasks. These flasks were incubated at 28 ± 2 ºC with shaking at 120 rpm for 14 days. After incubation, the fermented culture broth was filtered through Whatman filter paper to collect the biomass and dried in an oven at 60 ºC overnight.

The dried biomass was dissolved in 50 ml of 1M NaOH and ground using a mortar and pestle. To enhance melanin extraction efficiency, the crushed biomass was sonicated for 30 minutes and then autoclaved (20 min at 120 ºC). Following autoclavation, the biomass was centrifuged at 10000×g for 10 minutes. The supernatant was collected, and the pelleted biomass was discarded (Liu et al., 2018; Suthar et al., 2023).

Melanin in the supernatant was precipitated by acidifying with 2M HCl (pH 2.0) and leaving at room temperature overnight. Then, it was centrifuged at 10000×g for 10 min to pellet the black precipitated melanin. The resulting pellet, referred to as "crude melanin," was collected in petri plates and dried overnight (Selvakumar et al., 2008)**.**

The crude melanin was purified using the acid hydrolysis method. For this process, 2 ml of 7M HCl was added to the crude melanin and boiled in a water bath (100 ℃ for 2 hr), followed by centrifugation at 10000×g for 10 min. The supernatant was discarded, and the pellet was washed with distilled water to remove acid residues. Subsequent washing with organic solvents (chloroform, ethyl acetate, and ethanol) was performed to remove impurities such as lipids and other residues. The purified melanin was then dried and stored at -20 ºC until further analysis (De Souza et al., 2018; Zhan et al., 2011).

2.4 UV- spectroscopy

The UV absorption spectra of pure melanin of *A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754 in comparison to standard melanin (Sigma M8361-100 mg) dissolved in 1M KOH were taken from 200–800 nm wavelength range using Shimadzu UV-2450 UV-Vis Spectrophotometer (Surendirakumar et al., 2022).

2.5 FTIR analysis

The pure melanin of *A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754, along with standard melanin, were subjected to Fourier Transform Infra-Red (FTIR) spectroscopy. For this, samples were mixed individually with KBr and pressed into a pellet. Then, FTIR spectra of melanin from the two fungal isolates and standard melanin were taken from the 4000–400 cm^{-1} absorbance range using the IR Affinity-1 Spectrophotometer (Suwannarach et al., 2019).

2.6 Elemental analysis

The elemental composition of melanin from *A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754 in comparison to standard melanin were assessed by using Elemental analyzer (Suwannarach et al., 2019).

3. Results

3.1 Taxonomy

Alternaria burnsii **Uppal BN, Patel MK, Kamat MN,** ***Indian J. Agric. Sci.*** **8: 49 (1938)**

Culture characteristics: Colonies on PDA reaching 34–36 mm diam. after 7 days at 25 °C; from obverse white (8A1), circular, velvety, raised, umbonate, margins undulated, smooth, and entire; from reverse brownish grey in centre (8F3) and greyish brown in the periphery (7E3), sulcate. Colonies on MEA reaching 50–53 mm diam. after 7 days at 25 °C; from obverse white in the centre (7A1) and dark brown in the periphery (7F6), circular, raised, umbonate, margins regular, smooth, and entire; from reverse dark brown in centre (7F8) and white in the periphery (7A1), sulcate from both sides. Colonies on PCA reaching 21–22 mm diam. after 7 days at 25

°C, from obverse white (1A1), circular, velvety, raised, margins irregular, smooth, entire; from reverse reddish brown in the centre (8E4) and dull red in the periphery (8C4). Colonies on CMA reaching 32–35 mm diam. after 7 days at 25 °C; from obverse white (1A1), circular, moist, flat, margins irregular, entire; from reverse grey in the centre (8B1) and white in the periphery (8A1).

Morphology: White aerial mycelium observed with a dark reverse colour on PDA was observed when cultured on PDA. Conidiophores are single, erect, simple or branched, arising laterally from hyphae or terminals either straight or curved, smooth-walled; sometimes slightly swollen at the apex. Conidia were in short or moderately long chains; occasionally branched, normally ellipsoid or ovoid with transverse septa, tapering towards the apex, and elongating into a beak; mostly smooth in the conidial wall.

Material collected: M. Suthar; Garden soil of Agharkar Research Institute, Pune, Maharashtra, India; 4 May, 2021.

Material examined: S.K. Singh and M. Suthar; 22 June 2021; fungal culture was deposited in the National Fungal Culture Collection of India (*A. burnsii* NFCCI 5753); GenBank Accession Numbers: ITS=PP855652, TEF=PP861116, RPB2=PP861115.

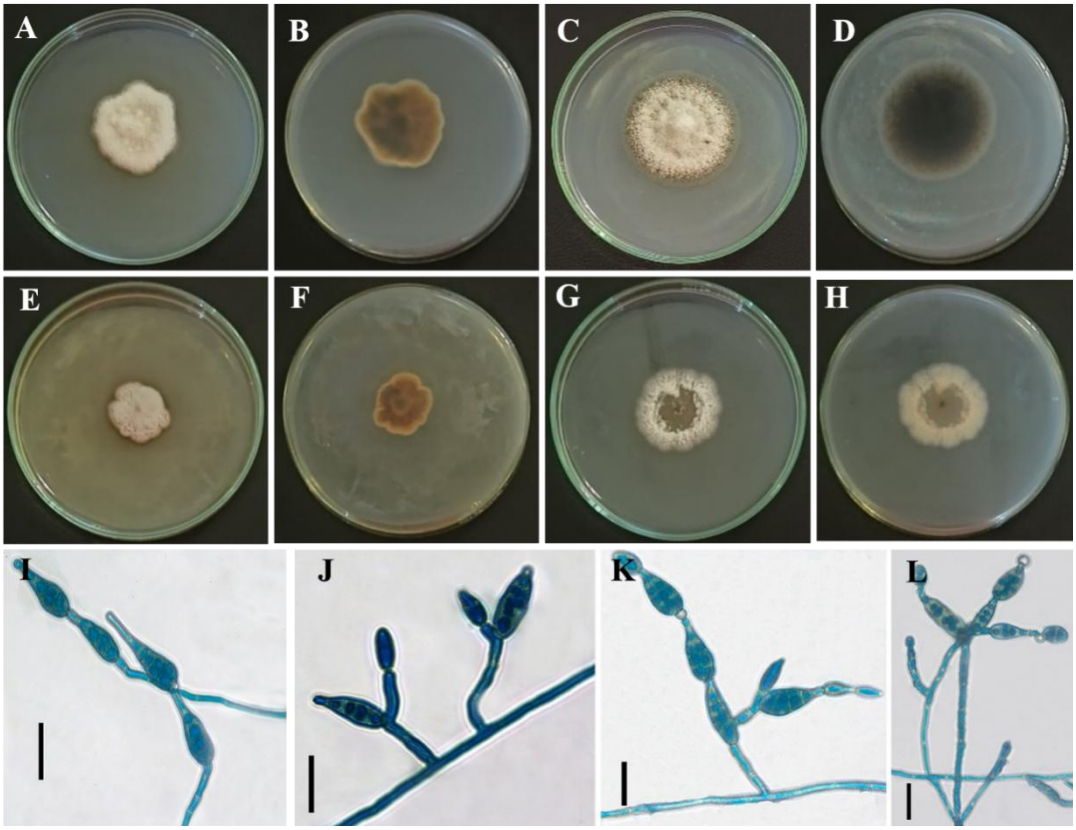

Fig. 1 - *Alternaria burnsii* NFCCI 5753; **A–B** Colonies on PDA, obverse and reverse views. **C–D** Colonies on MEA, obverse and reverse views; **E–F** Colonies on PCA; obverse and reverse views. **G–H** Colonies on CMA, obverse and reverse views; **I** –conidial branches; **J**-conidial growth patterns**; K–L** conidiophores and chains of conidia; Scale bars (**I–L**) – 20 µm.

***Cladosporium tenuissimum* Cooke, *Grevillea* 6 (no. 40): 140 (1878)**

Culture characteristics: Colonies on PDA reaching 29–30 mm diam. after 7 days at 25 °C; from obverse greenish grey (28F2), circular, velvety, raised, margins regular, smooth and entire; from reverse dark brown (8F6). Colonies on MEA reaching 30–32 mm diam. after 7 days at 25 °C; from obverse greyish green (28D4), circular, raised, cottony, umbonate, margins regular, smooth, and entire; from deep green (28E8). Colonies on PCA reaching 17–22 mm diam. after 7 days at 25 °C, from obverse greyish white (1B1), circular, slightly cottony, umbonate, raised, margins lobate, smooth, entire; from reverse brown in the centre (5E3) and greyish brown in the periphery (5D3), sulcated from both sides. Colonies on CMA reaching 22–23 mm diam. after 7 days at 25 °C; from obverse orange grey (5B2), circular, moist, flat, umbonate, margins regular, entire; from reverse ash grey (4B1).

Morphology: White aerial mycelium abundantly found, superficial or submerged, regular or symmetrical and feathery; hyphae are usually straight, septate, branching, and smooth-walled. Conidiophores are erect, branching, smooth and thin-walled; Conidia aseptate, mostly smooth, and thin-walled, globose to subglobose in shape; usually found without septa; no chlamydospores are found in this species.

Material collected: M. Suthar; Garden soil of Agharkar Research Institute, Pune, Maharashtra, India; 4 May, 2021

Material examined: S.K. Singh and M. Suthar; 22 June 2021; fungal culture was deposited in National Fungal Culture Collection of India (*C. tenuissimum* NFCCI 5754); GenBank Accession Numbers: ITS=PP855653, TEF=PP861114, ACT=PP861113.

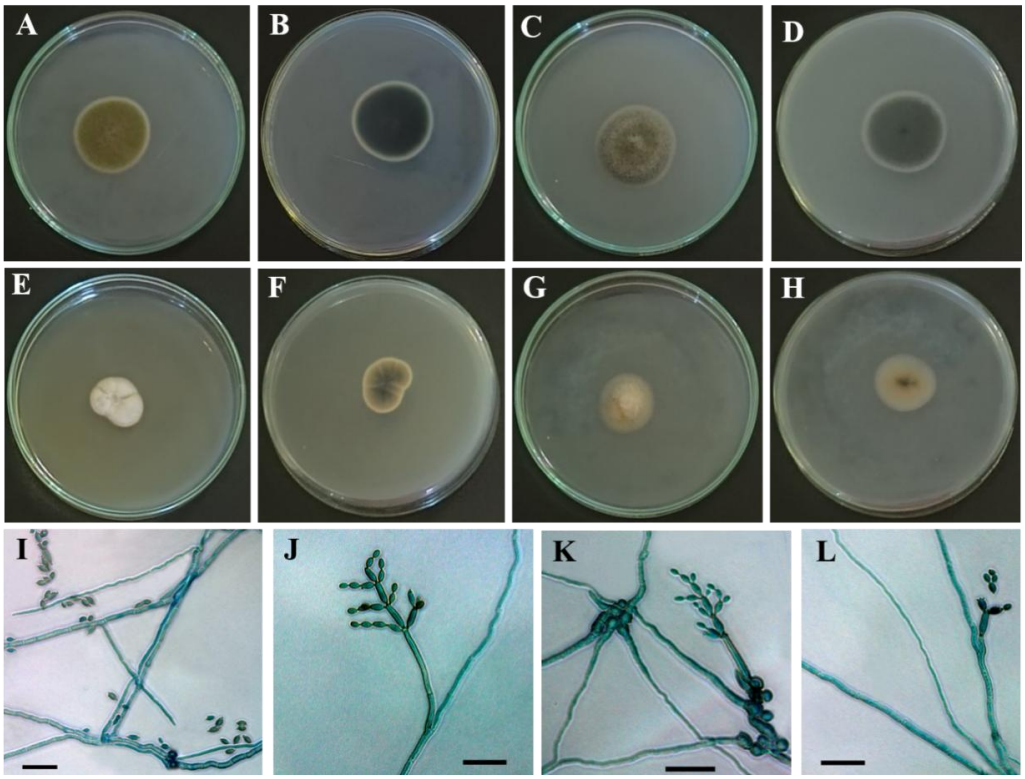

Fig. 2 - *Cladosporium tenuissimum*, NFCCI 5754; **A–B** Colonies on PDA, obverse and reverse views. **C–D** colonies on MEA, obverse and reverse views; **E–F** colonies on PCA; obverse and

reverse views. **G–H** Colonies on CMA, obverse and reverse views; **I** – scattered conidia; **J–L** conidiophores and chains of conidia; scale bars (**I–L**): 20 µm.

3.2 Phylogenetic analysis

Given the recent research and revisions to the taxonomy of *Cladosporium* as well as *Alternaria*, it was inferred that morphological studies are not reliable for accurate recognition of these two genera at the species level. Henceforth, multi-gene phylogenetic analysis was considered essential to resolve the species. The ITS, TEF1-α and RPB2 sequence alignments were together used to confirm the identity of *A. burnsii* NFCCI 5753 (Fig. 3) ITS, TEF1-α and act gene regions were used to resolve *C. tenuissimum* NFCCI 5754 (Fig. 4). The accession numbers of the related strains used to build the two phylogenetic trees are listed in Tables 2 and 3. The sequences of *A. burnsii* (NFCCI 5753) and *C. tenuissimum* (NFCCI 5754) were submitted to GenBank for future taxonomic analysis.

Sequence data from 20 strains was used in the concatenated file for the phylogenetic analysis of *A. burnsii* (NFCCI 5753). There were 1407 constant sites, 45 parsimony-informative sites, and 100 distinct site patterns in the alignment. After investigating 88 models, TNe+1 was determined to be the best-fit model and selected using the Bayesian Information Criterion (BIC). Based on this model, the phylogeny was inferred using the Maximum Likelihood Method. The log-likelihood of the consensus tree was -3193.033896. The rate parameters are as follows: 1.0000 for A-C, 3.5021 for A-G, 1.0000 for A-T, 1.0000 for C-G, 8.9626 for C-T, and 1.00000 for G-T. Similarly, the concatenated file of *C. tenuissimum* NFCCI 5754 included sequence data for 72 strains. The alignment contained 3944 constant sites, 2049 parsimony-informative sites, and 3815 distinct site patterns. Based on the Bayesian Information Criterion (BIC), TPM2+F+I+G4 was determined to be the best-fit model out of the 88 models that were examined. The log-likelihood of the consensus tree was –4522.0488. The rate parameters are as follows: A-C: 1.7124, A-G: 3.8965, A-T: 1.7124, C-G: 1.0000, C-T: 3.8965, and G-T: 1.0000.

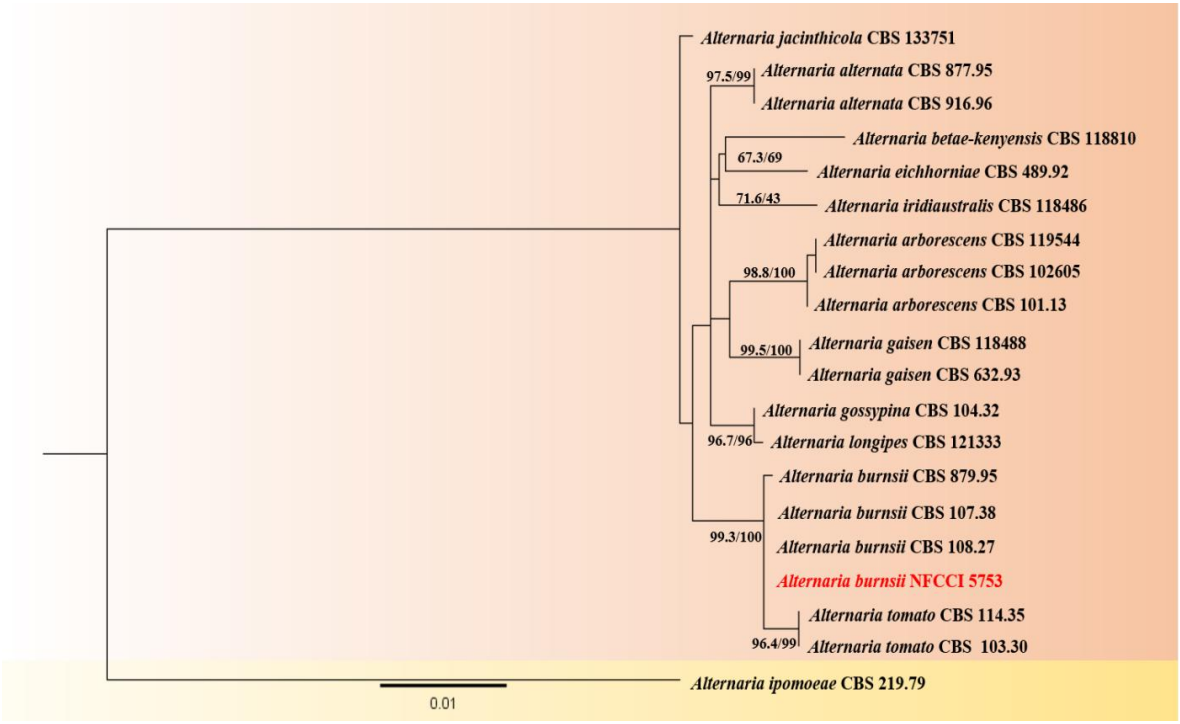

Fig. 3 - Maximum likelihood (ML) tree based on combined ITS, TEF1-α, and RPB2 sequences from 20 strains representing 12 *Alternaria* species in the *Alternaria* section. The tree is rooted with *Alternaria ipomoeae* (CBS 219.79) belonging to the *Porri* section. The numbers on the branches represent ML bootstrap values. A new sequence, *A. burnsii* (NFCCI 5753), is highlighted in red.

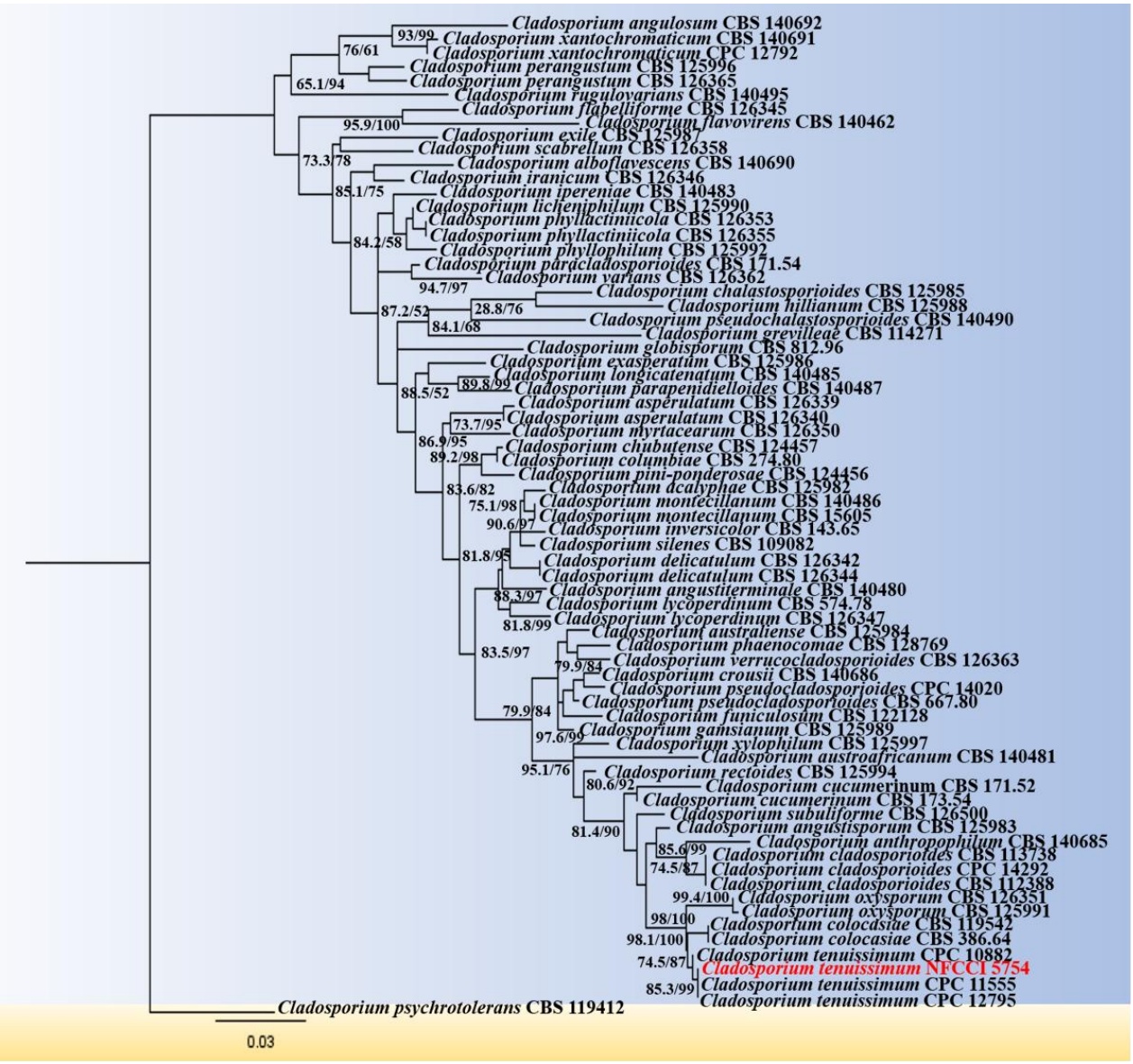

Fig. 4 - Maximum likelihood (ML) tree based on combined ITS, TEF1-α, and ACT sequences from 71 strains representing 55 *Cladosporium* species within *C. cladosporioides* species complex. The tree is rooted with *C. psychrotolerans* CBS 119412, belonging to *C. sphaerospermum* species complex. Numbers on the branches represent ML bootstrap values. The new sequence, *Cladosporium tenuissimum* (NFCCI 5754), is highlighted in red.

3.3 Extraction and purification of melanin pigment

Upon completion of 14 days of incubation, filtration of the fermented culture broth of *A. burnsii* NFCCI 5753 resulted in 0.50 ± 0.01 gm of dry biomass, and that of *C. tenuissimum* NFCCI

5754 produced 0.68 ± 0.07 gm of dry biomass. The extraction from the dried biomass of *A. burnsii* NFCCI 5753 yielded 153.97 ± 0.09 mg/100 ml of crude melanin, while *C. tenuissimum* NFCCI 5754 produced 73.50 ± 0.09 mg/100 ml of crude melanin.

3.3.1 UV spectroscopy

The UV spectra of melanin pigment from the two fungal isolates in comparison to standard melanin are as shown in Fig. 5 (*A. burnsii* NFCCI 5753) and Fig. 6 (*C. tenuissimum* NFCCI 5754).

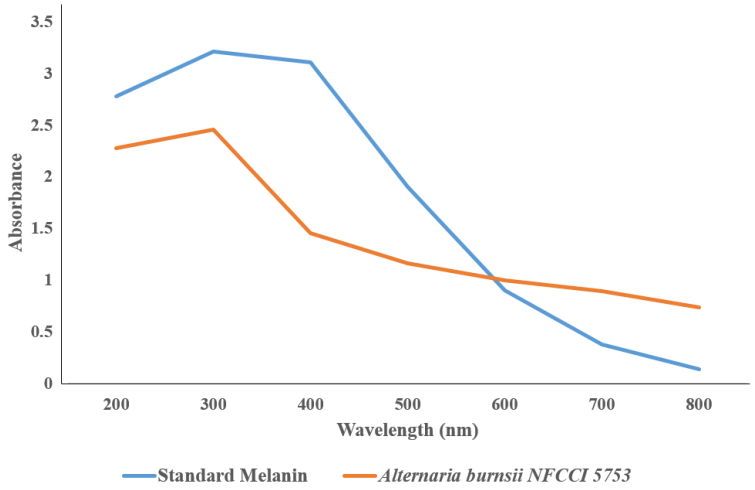

Fig. 5 - UV spectra of melanin from *Alternaria burnsii* NFCCI 5753 and standard melanin

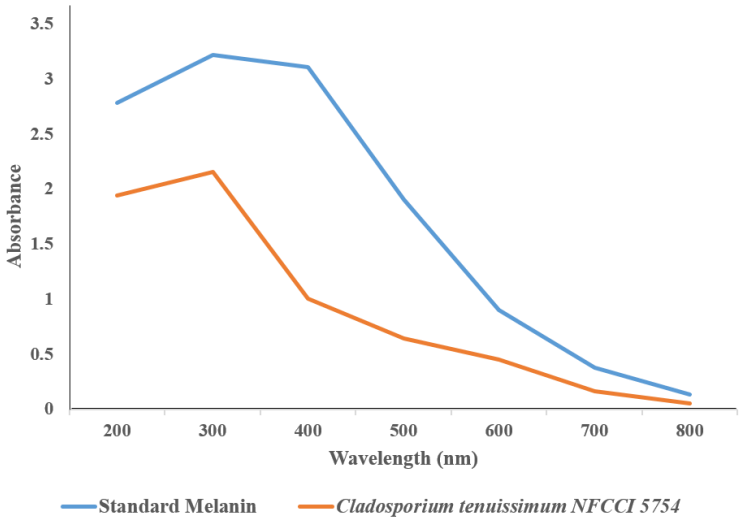

Fig. 6 - UV spectra of melanin from *Cladosporium tenuissimum* NFCCI 5754 and standard melanin

The UV-visible spectrum (200–800 nm) of fungal melanin demonstrates significant optical absorption in the UV-Vis region; as the wavelength increases, the absorbance tends to decrease

in a directly proportional manner. Spectroscopic studies suggest that the patterns of melanin in *A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754 (Figs. 5 and 6) are comparable to those of standard melanin, which confirms the nature of the pigment.

3.3.2 FTIR analysis

The FTIR spectra of melanin pigment from the two fungal isolates in comparison to standard melanin are as shown in Fig. 7 (*A. burnsii* NFCCI 5753) and Fig. 8 (*C. tenuissimum* NFCCI 5754).

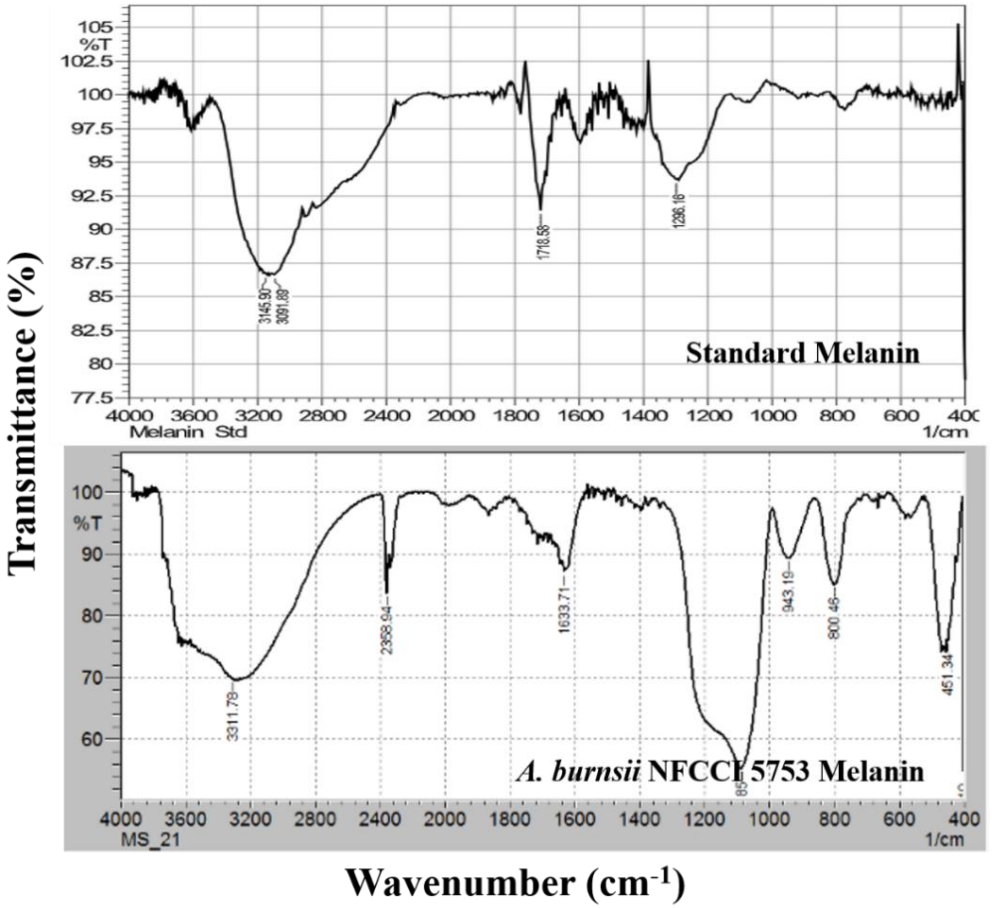

Fig. 7 - Infra-red spectra of melanin extracted from *Alternaria burnsii* NFCCI 5753 in comparison with standard melanin

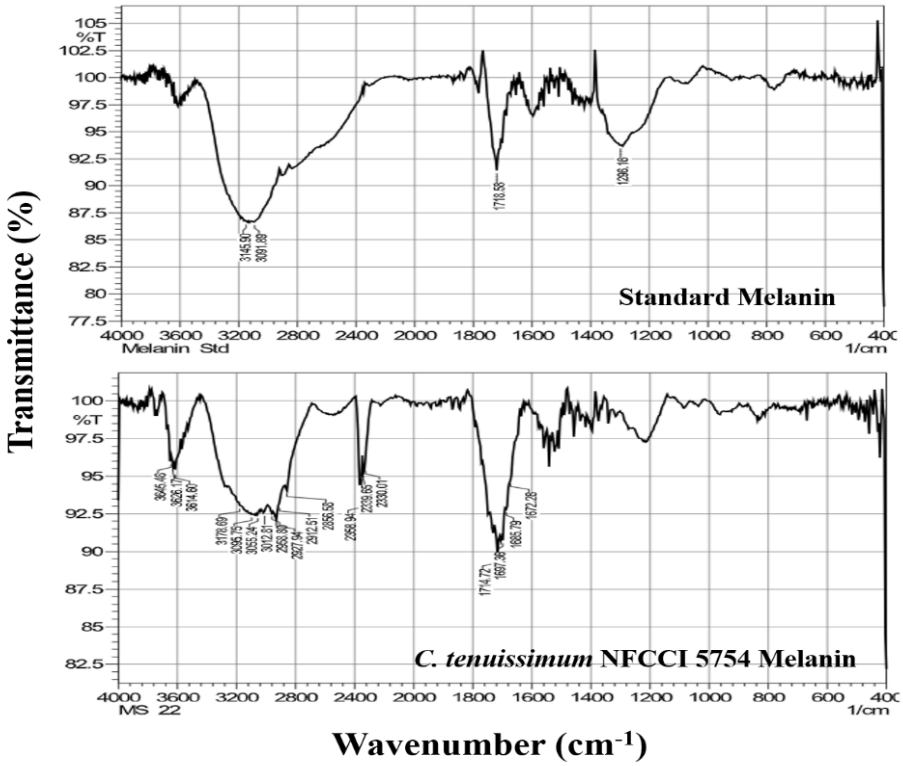

Fig. 8 - Infrared spectra of melanin extracted from *Cladosporium tenuissimum* NFCCI 5754 in comparison with standard melanin

The distinct functional groups found in melanin pigment are revealed by the FTIR spectra. Therefore, when FTIR spectra of melanin extracted from *A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754 were compared with those of standard melanin, they were found to be quite similar, i.e., a peak at 3311.78 cm^{-1} and 3626.17 cm^{-1} for the two fungal isolates, similar to the peak at 3145.90 cm^{-1} for standard melanin, attributes to the –OH functional group, and a broad spectrum band with a peak at 2358.94 cm^{-1} and 2339.65 cm^{-1} were for –NH functional group_stretching. The expansion of the band could be due to hydrogen bonding of the -OH group with amine groups. Peaks located at 1633.71 cm-1 and 1697.36 cm^{-1} signify the stretching of double bonds (C=O, C=N, and C=C), similar to 1718.58 cm^{-1} in the case of standard melanin. This band is a conjugated quinoid structure and, therefore, becomes important for the identification of melanin. The evidence of absorbance shift across wavelengths and the functional groups of chemical structures confirms the pigment as melanin.

3.3.3 Elemental analysis

The elemental percent of melanin pigment from the two fungal isolates in comparison to standard melanin is shown in Table 4.

Elemental analysis of the pigment enables us to identify the type of melanin based on elemental composition and their ratios. Considering DOPA melanin, since eumelanins are produced by oxidizing L-dopa or L-tyrosine, they are low in sulphur (0–1 %) and nitrogen (4–6 %) elements. Pheomelanins, on the other hand, are made from the cysteinyl conjugates of DOPA and contain significant sulphur (9–12 %) and nitrogen (8−11 %) levels. Therefore, the presence of sulphur levels becomes a characteristic differentiating feature between the two types of

melanins. However, melanin produced via the DHN pathway contains trace quantities of nitrogen (Suwannarach et al., 2019; Belozerskaya et al., 2017).

Table 4. Element composition (%) of melanin from *Alternaria burnsii* NFCCI 5753 and *Cladosporium tenuissimum* NFCCI 5754

Elements	*Alternaria burnsii* NFCCI 5754	*Cladosporium tenuissimum* NFCCI 5754	Standard Melanin
Carbon (C)	2.63 ± 2.03	56.83 ± 0.74	48.73 ± 2.08
Hydrogen (H)	0.45 ± 0.47	6.46 ± 0.18	2.95 ± 0.11
Nitrogen (N)	0.47 ± 0.78	4.15 ± 0.09	6.54 ± 0.45
Sulfur (S)	0.79 ± 0.10	1.72 ± 0.67	0.10 ± 0.09
Oxygen (O)[a]	95.66 ± 0.19	30.84 ± 1.50	41.68 ± 2.33

[a] The percent of oxygen (O) was calculated as follows: O%=100% − (C% + H% + N% + S%)

In the present study, the elemental distribution of the two fungal isolates is such that *A. burnsii* NFCCI 5753 contains 0.47 % Nitrogen (N) and *C. tenuissimum* NFCCI 5754 consists of 1.72 % Sulphur (S) and 4.15 % Nitrogen (N) (Table 4). As a result, it implies that while *A. burnsii* NFCCI 5753 may be a DHN melanin, *C. tenuissimum* NFCCI 5754 might fall as a eumelanin in the DOPA category of the pigment.

4. Discussion

For centuries, *Alternaria* and *Cladosporium* have been well-known for their pathogenic behaviour worldwide. However, in this study, we attempted to demonstrate that the two genera might be a veritable treasure because of their capability to produce an intriguing secondary metabolite called “melanin”, known for its multifarious properties. Based on their macro- and microscopic investigations, the two fungal isolates were identified as *Alternaria* sp. and *Cladosporium* sp. The phylogenetic results from combined datasets of ITS, TEF 1-α, and RPB2 gene sequences for *Alternaria* sp. and ITS, TEF1-α, and ACT sequences for *Cladosporium* sp. confirmed the fungal cultures as *A. burnsii* NFCCI 5753 and *C. tenuissimum* NFCCI 5754, respectively. Further, melanin pigment was extracted from the two fungi, and it was discovered that the pigment was significantly more abundant than had been reported in earlier studies on melanin production (Eisenman & Casadevall, 2012; Prasetyo et al., 2009), where typical yields ranged from 20–60 mg/100 ml. In contrast, extraction from the dried biomass of *A. burnsii* NFCCI 5753 yielded 153.97 ± 0.09 mg/100 ml of crude melanin, and *C. tenuissimum* NFCCI 5754 produced 73.50 ± 0.09 mg/100 ml. Analytical studies of the extracted pigment using spectral studies (UV-visible spectroscopy and FTIR) and elemental studies confirmed it to be melanin. Hence, this study opens avenues for further research to explore the pigment's biological activities and industrial applications from such opportunistic genera. This study provides a foundation for understanding and harnessing the capabilities of *Alternaria* species and *Cladosporium* species in melanin production.

According to previous studies, *A. alternata* and *C. sphaerospermum* have been observed to withstand increasing levels of radiation (Dadachova and Casadevall, 2008; Pombeiro et al., 2017). This suggests that melanogenesis enables these fungi to survive in extreme radiation-tolerant environments. Therefore, melanin from these two genera is considered a promising biosorbent for the effective removal of toxic metal ions from wastewater and soil because of its metal binding and stability. The future potential of the pigment in these two genera can be

extended to its use in the textile and dyeing industry as well. Unlike synthetic pigments, which pose risks to both human health and the environment, fungal melanin offers a sustainable, biodegradable alternative. Its use in textiles can protect the skin due to its photoprotective, antioxidant, and UV-blocking properties (Venil et al., 2020). Further, the development of eco-friendly inks that take full advantage of the biodegradability of fungal melanin has potential application. Hair dyes, and cosmetics with natural melanin are already on sale in the market, but the market for fungal melanin products is likely to be larger than that for natural melanin products (Panzella et al., 2018).

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Statements and Declarations:

Author contributions
All the authors contributed to the conception and design of the research article. MS: Experimental data analysis; Writing - original draft preparation, S.K.S.; Conceptualization, review, and editing.

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Conflict of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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