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Research Article

Isolation, Morpho-Molecular Identification, and Partial Characterization of Melanin Pigment from two *Colletotrichum* species

Malika Suthar[1, 2] and Sanjay K. Singh[1, 2]

[1]National Fungal Culture Collection of India, Biodiversity and Palaeobiology Group, MACS-Agharkar Research Institute, G.G. Agarkar Road, 411004 5 Pune, India

[2]Savitribai Phule Pune University, Ganeshkhind Road, Ganeshkhind, 411007 Pune, India

Correspondence: email: sksingh@aripune.org; https://orcid.org/0000-0002-6329-630X

Abstract

Melanin is a ubiquitous natural biopolymer widely distributed across all kingdoms of life, where it serves multiple protective functions, including photoprotection, ultraviolet (UV) radiation shielding, metal ion chelation, protection against oxidative stress, and enhanced tolerance to environmental stresses such as heat and drought. In microorganisms, including fungi, actinomycetes, and bacteria, melanin biosynthesis is often triggered in response to adverse environmental conditions. Owing to its diverse biological properties and potential industrial applications, microbial melanin has attracted considerable research interest. In the present study, two species of *Colletotrichum*, *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043, were identified using an integrated morpho-molecular approach, and the melanin pigments produced by these isolates were extracted and characterized. The fungal isolates produced 323.8 mg melanin/g biomass and 41.8 mg melanin/g biomass from *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043, respectively, under the conditions employed. The identity of the extracted pigment as melanin was confirmed through ultraviolet spectroscopy, Fourier-transform infrared spectroscopy, and elemental composition analysis, which demonstrated characteristic physicochemical properties of melanin.

Keywords: Melanin, UV, FTIR, Elemental analysis, morphological, molecular studies.

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1. Introduction

When phenols are oxidized and then polymerized into intermediate phenols and quinones, a complex natural biopolymer called as melanin is produced. Based on its structural elements, it can be divided into three major categories: eumelanin, pheomelanin, and allomelanin (Singh *et al*., 2021). Eumelanin and allomelanin give a dark brown colour to the cells while pheomelanin, which is present in animals contributes to yellow or red pigmentation. Melanin is found in all living organisms, including fungi, plants, animals, and pathogenic bacteria (Ghadge *et al.,* 2021). However, the pigment possesses significant defensive mechanisms such as photo-protection and UV radiation shielding. Melanin can also enhance resilience to chelate metal ions, heat and drought and prevent oxidative stress caused by reactive oxygen species. It adds to the rigidity of the cell wall and can absorb heavy metals and contributes to the rigidity of the cell wall (Singh *et al*., 2021; El-Naggar and Saber 2022).

Therefore, investigating microbial bio-production as a potential source of melanin has attracted considerable attention. (Elsayis *et al*., 2022). Melanin is naturally produced by many micro-organisms in response to environmental conditions, including fungi, actinomycetes, and bacteria (Gessler *et al.,* 2014; Elsayis *et al*., 2022; Suthar and Singh, 2025). In unfavorable conditions, fungi are particularly known to produce melanin, which can protect them from UV radiation (Pandey *et al*., 2024). In the present study, we isolated and characterized melanin pigment produced by two fungal isolates, *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043. The two fungal cultures were identified based on morphological and molecular studies. Then, pigment was extracted from them using the alkaline-acid hydrolysis method and confirmed as melanin by spectral (UV and FTIR) tests and elemental analysis.

One of the most economically significant fungal genera is *Colletotrichum*, comprising saprobic, endophytic, and pathogenic species associated with numerous herbaceous and woody plants worldwide. The genus is a member of the family *Glomerellaceae* within the order *Glomerellales* (*Sordariomycetes, Ascomycota*). Species of *Colletotrichum* causes anthracnose diseases, fruit rots, leaf spots, stem cankers, and blossom blights in a wide range of economically important crops, including cereals, legumes, vegetables, fruits, ornamentals, and forest trees, causing significant reductions in yield and quality (Cannon *et al.,* 2012; Hyde *et al.,* 2014; Damm *et al*., 2012a).

The taxonomy of *Colletotrichum* has undergone substantial revisions over the past two decades. Traditionally, species identification was based primarily on morphological characteristics such as colony morphology, conidial size and shape, appressorial morphology, setae, and host association. However, these characteristics often overlap among species and are influenced by environmental conditions and culture media, making accurate species delimitation difficult (Sutton, 1992; Hyde *et al*., 2009). Consequently, many morphologically similar but genetically distinct taxa remained concealed within species complexes, leading to considerable taxonomic confusion.

Advances in molecular phylogenetics have revolutionised the classification of *Colletotrichum*. Multilocus sequence analyses have demonstrated that morphological characters and host specificity alone are insufficient for species identification. Currently, species are recognised through polyphasic taxonomic approach that integrates morphological observations, cultural characteristics, ecological information, pathogenicity, and multilocus phylogenetic analyses (Cai *et al.,* 2009; Weir *et al*., 2012; Cannon *et al.,* 2012).

Several protein-coding genes have been employed for species delimitation within the genus. Although the internal transcribed spacer (ITS) region is widely used as the universal fungal barcode, it often lacks sufficient resolution for distinguishing closely related *Colletotrichum* species. Therefore, additional loci including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin (TUB-2), actin (ACT), calmodulin (CAL), translation elongation factor 1-alpha (TEF1-α), are routinely combined to achieve reliable species-level identification (Cannon *et al.,* 2012; Damm *et al*., 2012a,b; Weir *et al.,* 2012; Liu *et al*., 2016; Talhinhas and Baroncelli, 2021).

Molecular phylogenetic studies have shown that the genus consists of numerous well-supported species complexes rather than a few morphologically defined species. Currently, more than 280 accepted species are recognised in *Colletotrichum*, grouped into approximately 16 major species complexes together with several singleton lineages. Among these, the *C. gloeosporioides, C. acutatum, C. boninense, C. dematium, C. destructivum, C. truncatum, C. graminicola, C. orbiculare, C. spaethianum, C. gigasporum,* and *C. caudatum* species complexes contain the majority of described taxa *(Marin-Felix et al., 2017; Talhinhas and Baroncelli, 2021; Bhunjun et al., 2021)*.

Species of *Colletotrichum* exhibit remarkable ecological diversity. Besides acting as destructive plant pathogens, several species occur as endophytes without producing visible disease symptoms, while others survive as saprobes on dead plant material, contributing to nutrient recycling in natural ecosystems (Hyde *et al.,* 2009; Cannon *et al.,* 2012). Many species display broad host ranges, whereas others exhibit varying degrees of host specificity, reflecting their complex evolutionary history and adaptation to diverse ecological niches.

In contemporary fungal taxonomy, the classification of *Colletotrichum* relies on an integrative approach that combines morphology with multilocus phylogenetic analyses. This polyphasic strategy has significantly improved species delimitation, clarified species complexes, resolved cryptic taxa, and enhanced our understanding of the evolutionary relationships within the genus, thereby facilitating accurate diagnosis, disease management, and biodiversity studies (Weir *et al.,* 2012; Damm *et al*., 2012a,b; Talhinhas and Baroncelli, 2021).

2. Materials and methods

2.1 Collection, isolation and morphological identification of fungal isolates

Soil samples were collected from the garden soil at Agharkar Research Institute, Pune, Maharashtra, India, in May 2021. Using the serial dilution method, these samples were processed to isolate various fungal strains on Potato Dextrose Agar (PDA). 10 ml of sterile saline solution was mixed with 1 gm of soil to prepare the initial stock solution for serial dilution. Serial dilutions were prepared from this stock solution to 10^-7. Then, 0.1 ml sample from each dilution was then spread-plated onto PDA plates. After serial dilution, the PDA plates were incubated at 25°C and checked for fungal growth over 7-10 days. Individual fungal colonies were selected and sub-cultured onto fresh PDA plates to obtain pure cultures. These pure fungal isolates were then transferred to freshly prepared agar slants and kept in storage at 4°C for further use. In total, 18 fungal strains were isolated from soil samples, among which two fungal isolates that were primarily observed for black colour were selected for further studies.

2.2 Molecular characterization of the fungal isolates

Genomic DNA was extracted from the two fungal isolates grown on PDA for 2 weeks following a rapid and simple DNA extraction protocol (Aamir *et al*. 2015). The ITS gene region (ITS1-5.8s-ITS2), actin (ACT) gene and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene for both *Colletotrichum* sp. 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. **Table 1** lists the gene regions involved along with the corresponding primers.

The thermocycling conditions involved are as follows:

For the ITS gene region, 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 the ACT gene region, 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.

For the GAPDH gene region, there was an initial denaturation at 94°C for 4 min, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, 2 min at 72°C, and a final extension at 72°C for 10 min.

The amplified PCR products were purified using FavorPrep™ PCR purification kit and analysed 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 Applied Biosystems SeqStudio Genetic Analyzer. The sequences were submitted to the NCBI nucleotide sequence database for GenBank accession numbers (Table 2).

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

Name of the Gene Region	Primer	Direction	Sequence (5'-3')	Reference
Internal transcribed spacer region of the nrDNA (ITS)	ITS5	Forward	GGAAGTAAAAGTCGTAACAAGG	White *et al*., 1990
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	White *et al*., 1990
Actin (ACT)	ACT-512F	Forward	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn 1999
	ACT-783R	Reverse	TACGAGTCC TTCTGGCCCAT	Carbone and Kohn 1999
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	GDF-F	Forward	GCCGTCAACGACCCCTTCATTGA	Templeton *et al.*, 1992
	GDF-R	Reverse	GGGTGGAGTCGTACTTGAGCATGT	Templeton *et al.,* 1992

A list of the selected strains that were used for the construction of phylogenetic trees along with their accession numbers and other pertinent information are provided in **Table 2**. Using MAFFT v. 6.864b, each gene region was separately aligned (Katoh & Standley 2013)**.** 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, Nguyen *et al.* (2015) employed the Windows version of the IQ-tree tool, version v.1.6.11. Reliability of the rebuilt branches was evaluated using 1000 replicate bootstrap analyses. FigTree v.1.4.4 displayed the phylogenetic trees that had been created.

Table 2. Strains used in the construction of phylogenetic tree for *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 for and their GenBank accession numbers.

Species	Accession No.	ITS	ACT	GAPDH
Colletotrichum aenigma	ICMP 18608	JX010244	JX009443	JX010044
Colletotrichum aeschynomenes	ICMP 17673	JX010176	JX009483	JX009930
Colletotrichum alatae	ICMP 17919	JX010190	JX009471	JX009990
Colletotrichum alienum	ICMP 12071	JX010251	JX009572	JX010028
Colletotrichum analogum	CGMCC 3.16079	OK030860	OK513599	OK513663
Colletotrichum aotearoa	ICMP 18537	JX010205	JX009564	JX010005
Colletotrichum arecicola	CGMCC 3.19667	MK914635	MK935374	MK935455
Colletotrichum artocarpicola	MFLUCC 18-1167	MN415991	MN435570	MN435568
Colletotrichum asianum	ICMP 18696	JX010196	JX009584	JX010053
Colletotrichum asianum	ICMP 18580	JX010192	JX009576	JX009915
Colletotrichum camelliae	CGMCC 3.14925	KJ955081	KJ954363	KJ954782
Colletotrichum cangyuanense	CGMCC 3.18969	OK030864	OK513603	OK513667
Colletotrichum changpingense	SA0016	KP683152	KP683093	KP852469
Colletotrichum chiangmaiense	MFLUCC 18-0945	MW346499	MW655578	MW548592
Colletotrichum chrysophilum	CMM 4268	KX094252	KX093982	KX094183
Colletotrichum cigarro	ICMP 18539	JX010230	JX009523	JX009966
Colletotrichum clidemiae	ICMP 18658	JX010265	JX009537	JX009989
Colletotrichum cobbittiense	BRIP 66219	MH087016	MH094134	MH094133
Colletotrichum cordylinicola	ICMP 18579	JX010226	JX009586	JX009975
Colletotrichum cycadis	BRIP 71326	MT439915	MT439919	-

Colletotrichum dimorphum	CGMCC 3.16083	OK030867	OK513606	OK513670
Colletotrichum dracaenigenum	MFLUCC 19-0430	MN921250	MT313686	MT215577
Colletotrichum endophyticum	MFLUCC 13-0418	KC633854	KF306258	KC832854
Colletotrichum fructicola	ICMP 18581	JX010165	JX009501	JX010033
Colletotrichum fructicola	ICMP 18613	JX010167	JX009491	JX009998
Colletotrichum fructivorum	CBS 133125	JX145145	MZ664126	MZ664047
Colletotrichum gloeosporioides	ICMP 17821	JX010152	JX009531	JX010056
Colletotrichum gracile	CGMCC 3.16075	OK030868	OK513607	OK513671
Colletotrichum grevilleae	CBS 132879	KC297078	KC296941	KC297010
Colletotrichum hebeiense	MFLUCC 13-0726	KF156863	KF377532	KF377495
Colletotrichum hederiicola	MFLU 15-0689	MN631384	MN635795	-
Colletotrichum helleniense	CBS 142418	KY856446	KY856019	KY856270
Colletotrichum henanense	CGMCC 3.17354	KJ955109	KM023257	KJ954810
Colletotrichum horii	ICMP 10492	GQ329690	JX009438	GQ329681
Colletotrichum hystricis	CBS 142411	KY856450	KY856023	KY856274
Colletotrichum jiangxiense	CGMCC 3.17361	KJ955149	KJ954427	KJ954850
Colletotrichum kahawae	ICMP 17816	JX010231	JX009452	JX010012
Colletotrichum makassarense	CBS 143664	MH728812	MH781480	MH728820
Colletotrichum musae	CBS 116870	HQ596292	HQ596284	HQ596299
Colletotrichum nanhuaense	CGMCC 3.18962	OK030870	OK513609	OK513673
Colletotrichum	CGMCC 3.16080	OK030872	OK513611	OK513675

nullisetosum				
Colletotrichum nupharicola	ICMP 18187	JX010187	JX009437	JX009972
Colletotrichum oblongisporum	CGMCC 3.16074	OK030874	-	OK513677
Colletotrichum pandanicola	MFLUCC 22-0159	OP802369	OP801689	OP801724
Colletotrichum pandanicola	MFLUCC 22-0164	OP802373	OP801692	OP801727
Colletotrichum perseae	CBS 141365	KX620308	KX620145	KX620242
Colletotrichum proteae	CBS 132882	KC297079	KC296940	KC297009
Colletotrichum pseudotheobromicola	MFLUCC 18-1602	MH817395	MH853681	MH853675
Colletotrichum psidii	ICMP 19120	JX010219	JX009515	JX009967
Colletotrichum queenslandicum	ICMP 1778	JX010276	JX009447	JX009934
Colletotrichum rhexiae	CBS 133134	JX145128	MZ664127	MZ664046
Colletotrichum rhizophorae	MFLUCC 17-1927	OR828933	OR840847	OR840870
Colletotrichum rhizophorae	MFLUCC 17-1911	OR828934	OR840848	OR840871
Colletotrichum salsolae	ICMP 19051	JX010242	JX009562	JX009916
Colletotrichum siamense	ICMP 18121	JX010171	JX009518	JX009924
Colletotrichum siamense	ICMP 12567	JX010250	JX009541	JX009940
Colletotrichum siamense	**NFCCI 6046**	**PX945828**	**PZ351383**	**PZ351386**
Colletotrichum subhenanense	CGMCC 3.16073	OK030883	OK513618	OK513684
Colletotrichum syzygiicola	MFLUCC 10-0624	KF242094	KF157801	KF242156
Colletotrichum tainanense	CBS 143666	MH728818	MH781475	MH728823
Colletotrichum	CBS 133122	JX145159	MZ664125	MZ664045

temperatum				
Colletotrichum tengchongense	YMF 1.04950	OL842169	OL981238	OL981264
Colletotrichum thailandica	MFLUCC 17-1924	OR828935	OR840849	OR840872
Colletotrichum theobromicola	ICMP 18649	JX010294	JX009444	JX010006
Colletotrichum ti	ICMP 4832	JX010269	JX009520	JX009952
Colletotrichum tropicale	ICMP 18653	JX010264	JX009489	JX010007
Colletotrichum viniferum	CBS 130643	JN412804	JN412795	JN412798
Colletotrichum wuxiense	CGMCC 3.17894	KU251591	KU251672	KU252045
Colletotrichum xanthorrhoeae	ICMP 17903	JX010261	JX009478	JX009927
Colletotrichum xishuangbannaense	MFLUCC 1- 0107	MW346469	MW652294	MW537586
Colletotrichum yuanjiangense	CGMCC 3.18964	OK030885	OK513620	OK513686
Colletotrichum yulongense	CFCC 50818	MH751507	MH777394	MK108986
Colletotrichum arecacearum	LC13850	MZ595867	-	-
Colletotrichum arecacearum	LC13851	MZ595868	-	-
Colletotrichum arxii	CBS 169.59	KF687717	KF687784	KF687824
Colletotrichum chiagraiense	MFLUCC 14-0119	MH448522	MH376383	-
Colletotrichum gigasporum	CBS 125730	MH863654	KF687793	KF687840
Colletotrichum gigasporum	CBS 125731	MH863655	KF687794	KF687837
Colletotrichum gigasporum	**NFCCI 6043**	**PX945827**	**PZ351382**	**PZ351385**
Colletotrichum	GZU HJ2-G3	NR172036	MH708135	MH681658

jishouense				
Colletotrichum magnisporum	CBS 398.84	NR1332056	KF687803	KF687842
Colletotrichum pseudomajus	CBS 571.88	NR_132059	KF687801	JX009422
Colletotrichum radicis	CBS 529.93	NR132057	KF687785	KF687825
Colletotrichum serranegrense	COAD 2100	KY400111	KY407892	-
Colletotrichum subvariabile	NN040649	NR177594	MZ664181	MZ664054
Colletotrichum variabile	NN040656	NR177595	MZ664182	MZ664055
Colletotrichum vietnamense	CBS 125477	MH863699	KF687791	KF687831
Colletotrichum zhaoqingense	NN071036	-	MZ664205	MZ664067
Monilochaetes infuscans	CBS 869.96	JQ005780	JQ005843	JX546612

2.3 Extraction and purification of melanin pigment

Melanin pigment was extracted from *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 following the standard alkali-acid method by Gadd (1982). In brief, 500 ml of sterile Potato Dextrose Broth in 1000 ml Erlenmeyer flasks were inoculated with 10 mm diameter mycelial plugs from freshly growing colonies on PDA. For 14 days, these flasks were shaken at 120 rpm and incubated at 28 ± 2ºC. Following incubation, the biomass from the fermented culture broth was filtered through Whatmann filter paper and dried in oven at 60ºC overnight.

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

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

The crude melanin was then purified using the acid hydrolysis method. This procedure involved adding 2 mL of 7 M HCl to the crude melanin, boiling it for 2 hours at 100°C in a water bath, and then centrifugation at 10,000 × g for 10 minutes. The supernatant was discarded, and the pellet was washed with distilled water to remove acid residues. Subsequent washing with organic solvents (ethanol, ethyl acetate, and chloroform) was performed to remove impurities such as lipids and other residues. The purified melanin was then dried and kept at -20ºC until further analysis (Zhan *et al*., 2011; De Souza *et al.*, 2018). All quantitative experiments were performed in triplicate (n = 3), and results are expressed as mean ± SD.

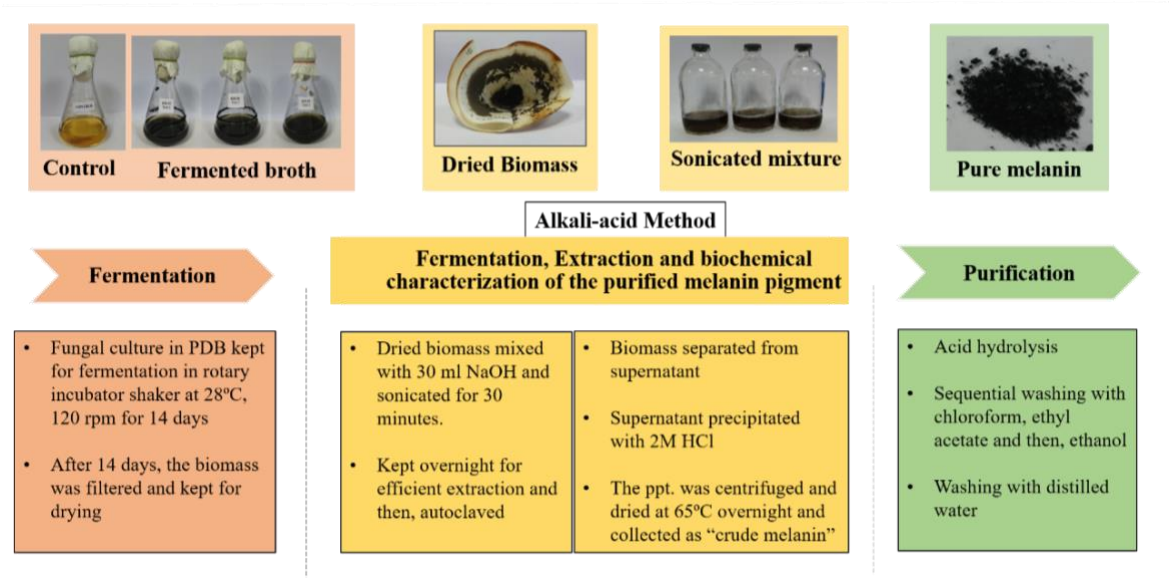


Fig.1. Schematic representation of the alkali–acid method for extraction and purification of fungal melanin.

2.4 UV- spectroscopy

The UV absorption spectra of pure melanin of *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 in comparison to standard melanin (Sigma) dissolved in 1M KOH was 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 *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 alongwith standard melanin were subjected to Fourier Transform Infra-Red (FTIR) spectroscopy. For this, each sample was mixed with KBr and pressed into a pellet. Then, using IR Affinity-1 Spectrophotometer, the FTIR spectra of melanin from the two fungal isolates and standard melanin were obtained from 4000 - 400 cm^{-1} absorbance range **(**Suwannarach *et al*., 2019**).**

2.6 Elemental analysis

The elemental composition of melanin from *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 in comparison to standard melanin were assessed by using Elemental analyzer (Suwannarach *et al*., 2019).

2.7 Physico-chemical Characterization

A range of physicochemical tests was conducted to compare the properties of melanin extracted from the two *Colletotrichum* sp. with those of the standard melanin pigment. These tests included evaluation of solubility in alkaline solutions, distilled water, and various organic solvents. In addition, characteristic diagnostic features of melanin, such as precipitation upon acidification with hydrochloric acid and reactivity toward oxidizing agents including 30% hydrogen peroxide, were assessed to further confirm the identity of the extracted pigment (El-Naggar & El-Ewasy 2017).

3. Results

3.1 Taxonomy

***Colletotrichum siamense* Prihast., L. Cai & K.D. Hyde, *Fungal Diversity* 39: 98 (2009)**
Isolate code: MS-40

Cultural characteristics: Colonies on PDA reaching 65–75 mm diam. after 7 days at 25 °C; from obverse pale grey (1D1), cottony, dense, with abundant aerial mycelium; colony surface smooth to slightly zonate; margins regular and entire; from reverse olivaceous grey (4F4), becoming darker with age, sometimes showing concentric rings.

Morphology: White aerial mycelium develops on PDA with a darker colony reverse. Acervuli form abundantly, producing orange conidial masses. Conidiophores are short, hyaline, septate, bearing phialidic ampulliform to cylindrical conidiogenous cells (10–25 × 2–4 µm). Conidia are hyaline, aseptate, straight, cylindrical to narrowly ellipsoid, measuring 13–18 × 4–6 µm. Appressoria are brown, thick-walled, ovoid to clavate, 5–10 × 4–7 µm. Setae, when present, are brown, septate, tapering, and 60–140 × 3–5 µm.

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

Pure culture examined: S.K. Singh and M. Suthar; 22 June 2021; fungal culture was deposited in the National Fungal Culture Collection of India (*C. siamense* NFCCI 6046).

GenBank Accession Numbers: ITS= PX945828, GAPDH= PZ351386, ACT= PZ351383.

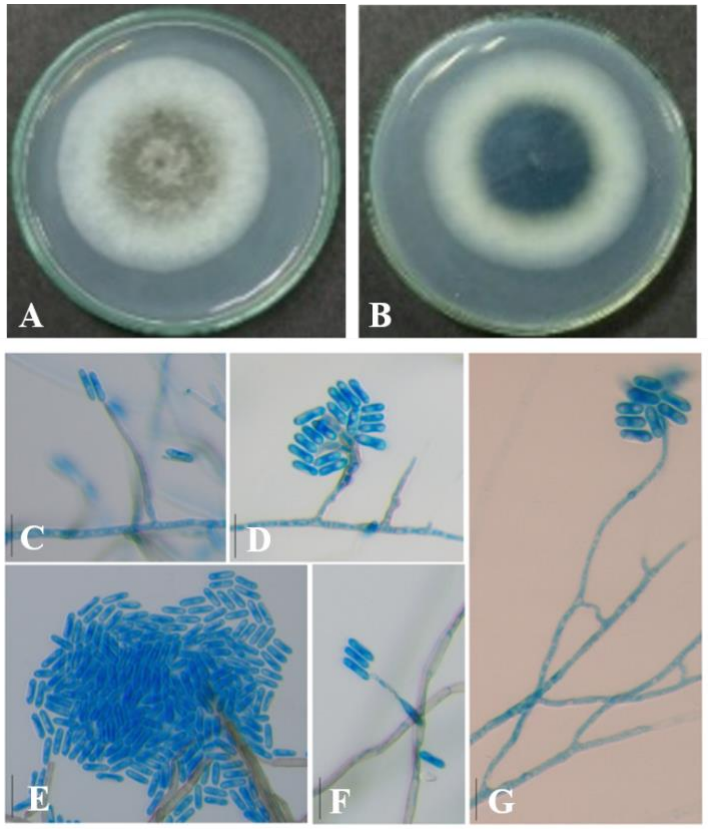


Fig. 2. ***Colletotrichum siamense*** **NFCCI 6046**; (A) Septate, hyaline hyphae bearing developing conidiophores, (B) Cluster of conidiogenous cells producing conidia at the apex (C) Elongated conidiophore with terminal conidial mass (D) Dense aggregation of hyaline, smooth-walled, cylindrical conidia (E) Individual conidia attached to short conidiogenous cells along the hyphae. Scale = 20 µm.

Colletotrichum gigasporum **Rakotonir & Munaut, *Mycol. Progress* 12(2): 407 (2012)**

Isolate code: MS-33

Culture characteristics: Colonies on PDA reaching 65–75 mm diam. after 7 days at 25 °C**;** from obverse white (1A1)**,** circular, cottony to velvety, moderately raised, with abundant aerial mycelium; margins regular, smooth and entire; from reverse dark olivaceous grey (4F4)**;** acervuli forming abundant conidial masses, mainly towards the centre of the colony.

Morphology: White to pale-grey aerial mycelium develops on PDA, becoming denser with age and showing a grey-green to dark olivaceous reverse. Acervuli form abundantly on the colony surface, appearing as orange to salmon-coloured conidial masses. Conidiophores are hyaline, smooth-walled, septate and generally short, arising directly from hyphae or within acervuli; conidiogenous cells are phialidic, ampulliform to cylindrical, measuring 10–25 × 2–4 µm. Conidia are hyaline, smooth-walled, aseptate, straight, cylindrical to narrowly ellipsoid with rounded ends, measuring 13–18 × 4–6 µm. Appressoria are brown, thick-walled,

solitary, ovoid to clavate or slightly lobed, typically 5–10 × 4–7 µm. Setae may be present, dark brown, septate, tapering to an acute apex, measuring 60–140 × 3–5 µm.

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

Pure culture examined: S.K. Singh and M. Suthar; 22 June 2021; fungal culture was deposited in the National Fungal Culture Collection of India (*C. gigasporum* NFCCI 6043)

GenBank Accession Numbers: ITS= PX945827, GAPDH= PZ351385, ACT= PZ351382.

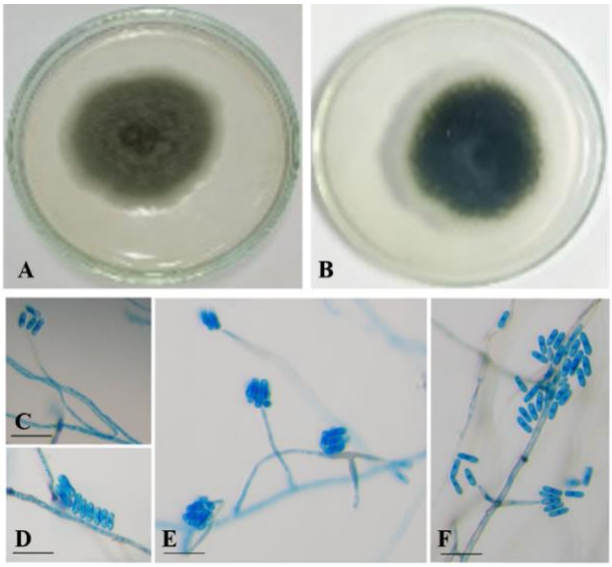


Fig. 3. ***Colletotrichum gigasporum*** **NFCCI 6043**; (A) Hyaline, septate hyphae with emerging conidiophores, (B) Conidiogenous cells producing clusters of conidia, (C) Enlarged, smooth-walled, hyaline conidia characteristic of the species, (D) Mass of mature conidia aggregated at the conidiophore apex. Scale = 20 µm.

Phylogenetic analysis

In light of recent research and revisions to the taxonomy of *Colletotrichum*, it was inferred that morphological studies are insufficient for precise recognition for these two species.

Henceforth, multi-gene phylogenetic analysis was considered essential to resolve the species. Sequence alignments of the internal transcribed spacer (ITS), actin (ACT), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loci were used together to confirm the identity of the two Colletotrichum species. (Fig. 4). All the accession numbers of the related strains obtained from NCBI for the construction of the two phylogenetic trees have been listed in **Table 2**. The sequences of *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 were submitted to GenBank for the purpose of facilitating future taxonomic analysis.

Sequence data from 90 strains were used in the concatenated file for the phylogenetic analysis of *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043. There were 806

distinct site patterns in the alignment, including 363 parsimony-informative sites, 214 singleton sites, and 687 conserved sites. The concatenated sequences of ITS, ACT and GAPDH were utilized to robustly resolve the phylogenetic placement of the two fungal isolates. Model selection conducted across 88 nucleotide substitution models using the Bayesian Information Criterion (BIC) identified TN+F+G4 as the best-fit model, which was subsequently applied for tree reconstruction. The final Maximum Likelihood tree yielded a log-likelihood value of -9431.116. Estimated nucleotide substitution rates were A–C = 1.00000, A–G = 2.65284, A–T = 1.00000, C–G = 1.00000, C–T = 3.82142, and G–T = 1.00000. The proportion of invariable sites was estimated at 0.615, and rate heterogeneity among sites was modelled using a gamma distribution with a shape parameter (α) of 0.426. Branch support was evaluated using 1,000 ultrafast bootstrap replicates, yielding a bootstrap correlation coefficient of 0.996, indicating highly consistent and reliable support values across replicates.

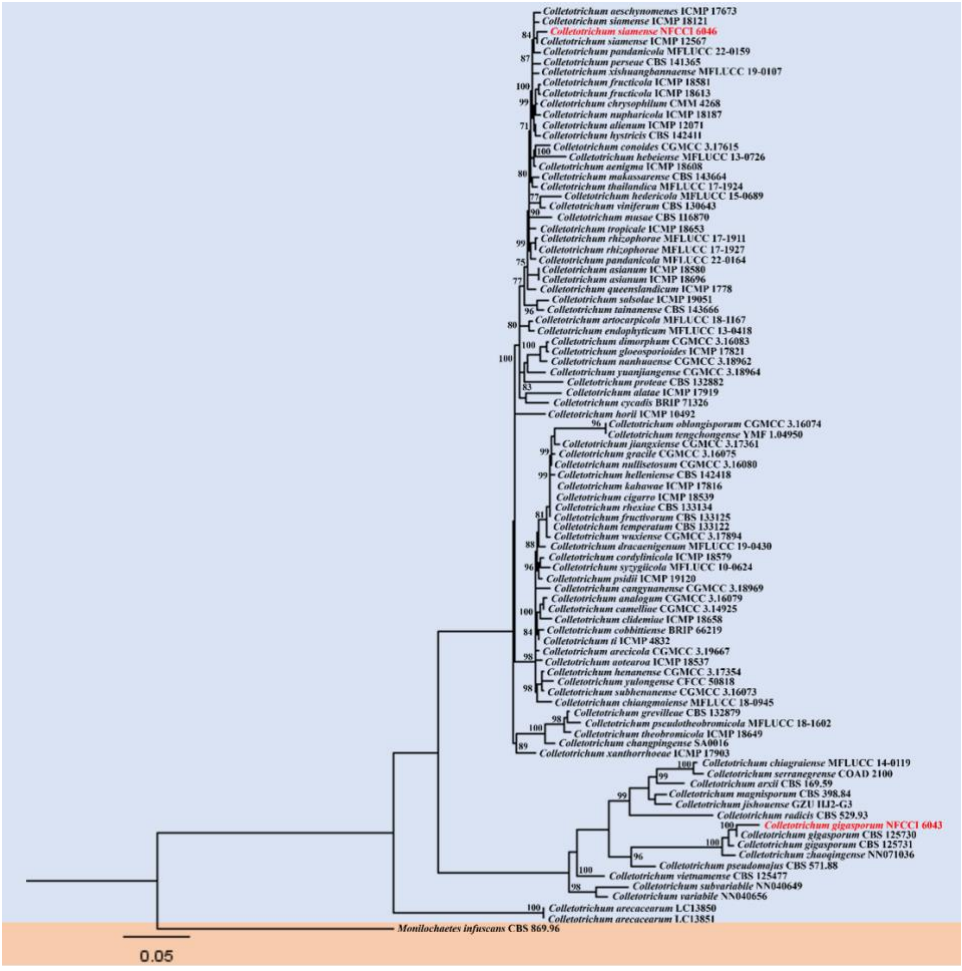


Fig. 4. Phylogenetic tree of *Colletotrichum siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 inferred from maximum likelihood (ML) analysis based on combined ITS, ACT and GAPDH sequences. *Monilochaetes infuscans* CBS 869.96 was used as outgroup. Newly generated sequences are in red. One thousand bootstrap replicates were analyzed to get ultrafast bootstrap values, and the values above 50% were represented on nodes in the tree.

3.2 Extraction and Purification of Melanin Pigment

After the 14 days of incubation period, the extraction from *C. siamense* NFCCI 6046 yields 323.8 mg melanin/g biomass while *C. gigasporum* NFCCI 6043 produces 41.8 mg melanin/g biomass. *C. siamense* had considerably higher pigment production relative to biomass, despite producing less biomass All quantitative experiments were performed in triplicate (n = 3), and results are expressed as mean ± SD.

3.3 UV- spectroscopy

The UV spectra of melanin pigment from the two fungal isolates in comparison to standard melanin are as shown in the **Fig. 5**.

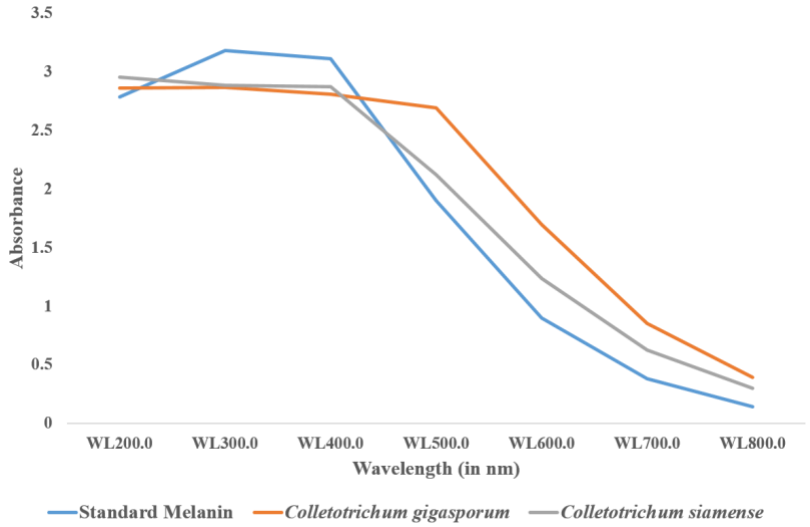


Fig. 5. UV spectra of melanin from *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 in comparison with standard melanin.

The UV-visible spectrum (200–800 nm) of fungal melanin exhibits notable optical absorption. As the wavelength increases, absorbance tends to decrease linearly. This study establishes important criteria for identifying the pigment as melanin. Spectroscopic studies suggest that the melanin patterns of *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 (**Fig. 5**) are like that of standard melanin which, confirms the identity of the pigment.

3.4 FTIR analysis

The FTIR spectra of melanin pigment from the two fungal isolates in comparison to standard melanin are as shown in the **Fig. 6**.

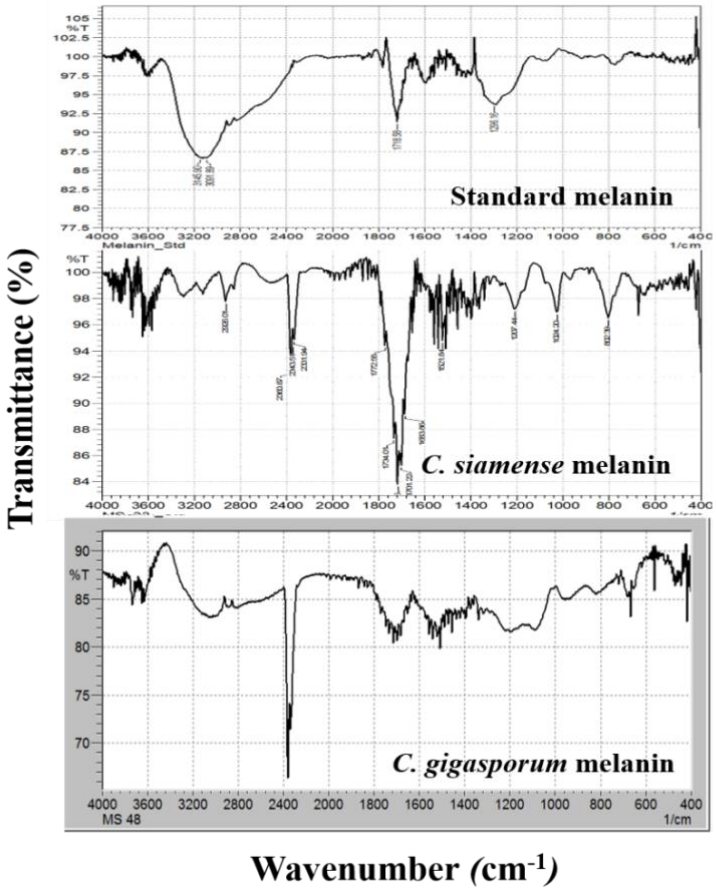


Fig. 6. Infra-red spectra of melanin extracted from *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 in comparison with standard melanin.

The Fourier Transform Infrared (FTIR) spectra of the pigments extracted from *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043 exhibited absorption patterns comparable to those of the standard melanin, confirming their melanin-like chemical nature (**Fig. 6**). A broad absorption band was observed around 3311.78 cm^{-1} in *C. siamense*, whereas the *C. gigasporum* spectrum showed a band at approximately 3626.17 cm^{-1}. These absorptions were considered as part of the hydroxyl/amine-associated spectral features, although the exact assignment of individual FTIR bands should be interpreted cautiously. The broadness of this band is attributed to extensive hydrogen bonding between hydroxyl and amine functionalities, a characteristic feature of melanin polymers. The absorption bands around 2340–2360 cm^{-1} are likely associated with atmospheric CO_2 and were therefore not used as diagnostic evidence for melanin functional groups. Furthermore, distinct peaks at 1633.71 cm^{-1} and 1697.36 cm^{-1}, comparable to the 1718.58 cm^{-1} band of the standard melanin, correspond to the stretching vibrations of conjugated carbonyl (C=O), imine (C=N), and aromatic carbon-carbon (C=C) groups. These bands are indicative of the quinone and aromatic indole structures that constitute the melanin backbone. Additional absorption bands observed in the fingerprint region (1500–1000 cm^{-1}) are attributed to aromatic ring vibrations, further supporting the complex heterogeneous structure of melanin. Overall, the

close resemblance of the FTIR spectra of both fungal pigments to that of the standard melanin, together with the presence of characteristic functional groups, confirms the extracted pigments as melanin.

3.5 Elemental analysis

The elemental % of melanin pigment from the two fungal isolates in comparison to standard melanin are as shown in the **Table 3**.

Table 3. Element composition (%) of melanin from *C. siamense* NFCCI 6046 and *C. gigasporum* NFCCI 6043.

Elements	*C. siamense* NFCCI 6046	*C. gigasporum* NFCCI 6043	Standard Melanin
Carbon (C)	54.34 ± 4.5	49.65 ± 2.6	48.73 ± 2.08
Hydrogen (H)	5.526 ± 0.7	5.247 ± 0.6	2.95 ± 0.11
Nitrogen (N)	1.717 ± 0.4	3.645 ± 0.2	6.54 ± 0.45
Sulfur (S)	1.098 ± 0.3	2.076 ± 0.7	0.10 ± 0.09
Oxygen (O)[a]	37.319 ± 0.5	39.382 ± 0.8	41.68 ± 2.33

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

Conventionally, DOPA melanin is low in sulphur (0-1%) and nitrogen (4-6%) elements because eumelanins are produced by oxidizing L-dopa or L-tyrosine. Conversely, pheomelanins are composed of cysteinyl conjugates of DOPA and have high concentrations of nitrogen (8−11%) and sulphur (9–12%) levels. Therefore, the presence of sulphur levels becomes a characteristic differentiating feature between the two types of melanin. However, melanin produced via DHN pathway contains trace quantities of nitrogen.

In the present study, the elemental composition of the melanin extracted from the two *Colletotrichum* isolates revealed distinct differences. The pigment from *C. siamense* NFCCI 6046 contained 1.72% nitrogen and 1.10% sulphur, whereas *C. gigasporum* NFCCI 6043 contained 3.65% nitrogen and 2.08% sulphur (Table **3)**. The comparatively lower nitrogen content of both fungal pigments than the synthetic melanin standard is consistent with fungal melanin-like pigment composition; however, elemental analysis alone does not permit definitive assignment of the biosynthetic pathway as DHN melanin (Suwannarach *et al.,* 2019; Belozerskaya *et al.,* 2017).

3.6 Physico-chemical Characterization

Table 4. Physico-chemical properties of melanin from the two *Colletotrichum* species and synthetic melanin.

Test	Result		
	C. siamense **NFCCI 6046**	***C. gigasporum*** **NFCCI 6043**	**Synthetic melanin**
	Colour observation		
Colour of pigment	Black	Black	Black
	Solubility test		
A. Distilled water	Insoluble	Insoluble	Insoluble
B. Organic solvents	**Solubility test**		
1. Acetone	Insoluble	Insoluble	Insoluble
2. Chloroform	Insoluble	Insoluble	Insoluble
3. DMSO	Soluble	Soluble	Soluble
4. Ethanol	Insoluble	Insoluble	Insoluble
5. Ethyl acetate	Insoluble	Insoluble	Insoluble
6. Methanol	Insoluble	Insoluble	Insoluble
C. Inorganic alkaline solutions	**Solubility test**		
1. NaOH	Soluble	Soluble	Soluble
2. KOH	Soluble	Soluble	Soluble
3. Borate buffer	Soluble	Soluble	Soluble
	Precipitation test		
HCl (3 M)	Precipitate formed	Precipitate formed	Precipitate formed
	Reaction with oxidizing agent		
30 % H_2O_2	Decolorization	Decolorization	Decolorization

The pigment is characterized by its hydrophobic nature, negative charge, and typically black appearance. In the present study, the physical and chemical properties of melanin extracted from the two *Colletotrichum* species exhibited solubility patterns comparable to those of the standard melanin (Table 4). The positive results from the diagnostic tests described by Thomas (1955) further confirmed that the extracted pigment was melanin. The pigment was insoluble in distilled water and common organic solvents, including methanol, acetone, chloroform, ethanol, and ethyl acetate, with dimethyl sulfoxide (DMSO) being the only exception. In contrast, it readily dissolved in alkaline inorganic solutions such as sodium hydroxide (NaOH), potassium hydroxide (KOH), and borate buffer, demonstrating melanin's characteristic alkali solubility.

The alkaline solubility of fungal melanin facilitates its efficient extraction on a large scale using alkaline reagents such as NaOH or KOH, making the process economically cost-effective for industrial production. Nevertheless, its poor solubility in water and most organic solvents restricts its direct utilization in biomedical, cosmetic, and coating applications. Although alkaline hydrolysis is an effective method for melanin recovery, prolonged exposure to strong alkali may induce structural alterations that can influence its functional properties. To overcome these limitations, chemical modification strategies, including carboxylation, sulfonation, and esterification, have been explored to enhance its dispersibility in aqueous and organic media. Furthermore, the development of nanoparticle-based delivery systems and enzymatic modification approaches offers promising opportunities to improve its applicability, particularly in pharmaceutical formulations. Broadening the solubility profile of fungal melanin through such modifications could significantly expand its potential across a wide range of industrial and biomedical applications.

4. Discussion

For many years, species of the genus *Colletotrichum* have been recognized primarily as destructive plant pathogens with a worldwide distribution. In the present study, however, we demonstrate that members of this genus also possess considerable biotechnological potential owing to their ability to synthesize melanin, a valuable secondary metabolite known for its diverse biological and industrial applications. Based on macroscopic and microscopic characteristics, the two fungal isolates were initially identified as *Colletotrichum* species. Their identities were subsequently confirmed through multilocus phylogenetic analyses using combined ITS, ACT, and GAPDH gene sequences, which identified the isolates as *C. siamense* and *C. gigasporum*, respectively. The two isolates demonstrated measurable melanin production under the culture conditions employed, with *C. siamense* NFCCI 6046 producing a higher amount of melanin than *C. gigasporum* NFCCI 6043. The extracted pigments were characterized using UV–visible spectroscopy, FTIR spectroscopy, elemental analysis and physicochemical tests, and the combined results supported their identification as fungal melanin. Although the elemental composition and spectroscopic characteristics were consistent with fungal melanin, the present study does not provide sufficient pathway-specific evidence to definitively classify the pigments as DHN melanin. Confirmation of the

biosynthetic pathway would require additional approaches such as pathway-specific inhibition, targeted chemical analysis or structural characterization. The present study was based on two representative *Colletotrichum* isolates obtained from a single soil sampling location, and therefore the findings should not be extrapolated to the genus. The study was primarily intended to establish the identity of the isolates and provide a preliminary characterization of their melanin pigments. Culture conditions were not systematically optimized, and functional assays such as antioxidant, antimicrobial or UV-protection studies were not included in the present work. Consequently, the current results should be considered a preliminary characterization and baseline for subsequent investigations involving strain screening, optimization of pigment production and evaluation of biological or functional properties. Furthermore, the findings provide a preliminary basis for further investigation of melanin production by *Colletotrichum* isolates, including optimization of culture conditions and evaluation of the functional properties of the extracted pigments.

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Statement 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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