Melanin production and biosynthetic pathways in *Aspergillus* section *Nigri* species cultured in liquid media

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As a result of being cost-friendly, consuming fewer chemicals and enabling simple purification, means for preparing the natural pigment melanin have become a dominant avenue. This study aimed to characterise and determine the amount and type of biosynthesis of fungal melanin from 8 species of *Aspergillus* section *Nigri* cultured in two liquid culture media, including potato dextrose broth (PDB) and L-tyrosine PDB, for 21 days. *Aspergillus* section *Nigri* species were identified by using Internal Transcribed Spacer (ITS), β-tubulin (*BenA*) and calmodulin (*CaM*). Qualitative and quantitative analyses of melanin using ultraviolet-visible analysis, Fourier transformed infrared (FTIR) analysis, scanning electron microscopy and atomic force microscopy. The results showed that the extracted melanin amount in powder form them were ranged from (0.3-2 g) at the end of incubation in culture media. The concentration of melanin extracted from species cultured in PDB was ranged from (37.40-134.28 ppm) while in L-tyrosine PDB liquid media, the findings indicate that the concentration range was (53.03-140.53ppm). Two inhibitors were used to determine the biosynthetic pathway, difenoconazole used for the first time, and kojic acid. l-3,4-dihydroxyphenylalanine was the pathway for melanin synthesis in *A. niger* and *A. brasiliensis*, and all the investigated species commonly produced melanin through the DHN pathway.

1. Introduction

Bioactive materials of microbial origin, also known as natural pigments, have attracted industry attention because of the increasing need to produce novel, safe and sustainable products [1]. Among the pigments produced by microbes, melanin has caught the attention of researchers due to its wide applications and interesting physical, chemical and biological characteristics [2]. Melanin is a secondary metabolite made up of complex heterogeneous polymers of phenol and/or indole monomers. It is a highly active molecule developed by plants, animals, fungi, bacteria and many species from several kingdoms. Melanin is negatively charged, hydrophobic and dark-coloured biological macromolecule of high molecular weight that is formed by the oxidative polymerization of phenol and indole compounds [3,4]. Melanin has been referred to as a fungal armor due to its ability to protect microorganisms from a broad range of toxic insults. This compound can be categorised into numerous classifications based on the type of substrate utilised in its biogenesis and synthetic process [5].

Many fungi synthesize melanin constitutively, others do so only under certain culture conditions and/or when specific precursors are available. Several classes of fungal melanin have been described based on their biochemical precursors, pathways and components. The two most significant types of melanin in section *Nigri* based on precursors and the pathway taken, are DHN-melanin (1,8-dihydroxy naphthalene) and DOPA melanin (L-3,4-dihydroxyphenylalanine) [6]. Melanin can be found widely in nature with a wide variety of functions in the biosystem. In microorganisms, it performs several roles, the primary one which is to safeguard host cells and organisms. This function involves resistance to physical alterations from the external environment, prevention against ultraviolet (UV) rays and energy absorption and physiological activity-based preservation of intracellular homeostasis [7]. Melanin absorbs UV-visible (UV-Vis) light, quenches free radicals and chelates metal ions. Given these qualities, the application of melanin compounds to new-age adhesive biomaterials and cost-effective bioelectronics is largely based on the combination of hybrid ionic–electronic conductance and redox reversibility properties [8].

The use of melanin has been proven to be the ideal method of its biosynthetic production, given that the technique is cost-effective and uses few chemicals and simpler purification processes. Numerous microorganisms produce melanin, a pigment in the cell wall that provides structural support and protects against environmental stressors [5,9]. Among them a group known as black Aspergilli, scientifically classified as *Aspergillus* section *Nigri*, is a group with a worldwide distribution. They are commonly present in soil, but can be found in a wide variety of environments and conditions [10]. *Aspergillus* section *Nigri*, is an important fungal species in biotechnology research, medical and food mycology [11].

Understanding the melanin synthesis pathway is important because it allows us to understand how melanin is produced and regulated, identify the enzymes responsible for its production, and helps in selecting conditions that increase melanin production for use in many fields such as drug development, improving industrial production, disease control, and environmental protection. Melanin synthesis pathway helps to explain why colors and properties differ between species, and this is achieved by identifying different inhibitors to target this process. To our knowledge, the study was the first of its kind in Basrah province. lack of comprehensive study on melanin type and quantity across multiple species in this section, so the current study aimed to determine the type and quantify melanin yield across eight species of *Aspergillus* section *Nigri* in PDB vs L-tyrosine PDB; determine pathway (DHN or DOPA) via inhibitor assays such as kojic acid and difenoconazole as specific inhibitors.

2. Materials and Methods

2.1. Sample collection

The collection of soil samples was completed in seven locations within Basrah Province, namely, Al-Hartha, Shatt Al-Arab, Al-Zubair, Safwan, Al-Zubair Oil Field, Hammar Mishrif Oil Field and Al-Faw, from Oct 2023 to Sep 2024. Six soil approximately 250g from each site was collected with the use of a sterile spatula and kept in sterile bags. The samples were then transported to the laboratory for the isolation of fungi [12].

2.2. Isolation of *Aspergillus* section *Nigri* species

The dilution method described by [12] was used for the isolation of fungi from each soil sample cultured in potato dextrose agar (PDA), malt extract agar (MEA), and dicloran rose Bengal chloramphenicol agar (DRCA) media.

2.3. Genomic DNA extraction and Sequencing of polymerase chain reaction (PCR) products

Genomic DNA from 134 fungal isolates was obtained in accordance with the guidelines provided by the manufacturer and using the Presto Mini gDNA yeast kit (Geneaid, Taiwan) after 7 days of growth on PDA. The quality of the extracted DNA was assessed through gel electrophoresis, and the samples were subsequently stored at –20 °C for future use. The primers listed in Table (1) were used for PCR amplification. The PCR reaction mixture had a total volume of 25 μL. The PCR amplification conditions for β-tubulin and calmodulin were as follows: an initial denaturation step of 95°C for 5 min followed by 35 cycles, denaturation of 95°C for 1min, annealing of 55°C for 1min and elongation at 72°C for 1min and final extension step at 72°C for 10 min [13]. The PCR amplification conditions for ITS were as follows: an initial denaturation step of 95°C for 1 min followed by 35 cycles, denaturation of 95°C for 1min, annealing of 56°C for 45s and elongation at 72°C for 1min and final extension step at 72°C for 10 min. The PCR products were measured by gel electrophoresis along with DNA ladder (Intronbio, South Korea) as the marker and visualized on a UV transilluminator and photographed. The amplified PCR products targeting the β-tubulin gene, calmodulin gene and internal transcribed spacer region were sent to Macrogen Inc. (South Korea) for purification and sequencing.

Table 1: Primers for Polymerase Chain Reaction (PCR).

Gene name	Primers	Sequence	Length	References
ITS region	ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	19 bases	[14]
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	20 bases	
β-tubulin	Ben2f	5′-TCCAGACTGGTCAGTGTGTAA-3′	21 bases	
	Bt2b	5′-ACCCTCAGTGTAGTGACCCTTGGC-3′	24 bases	
	cmd5	5′-CCGAGTACAAGGAGGCCTTC-3′	20 bases	[13]
Calmodulin	cmd6	5′-CCGATAGAGGTCATAACGTGG-3′	21 bases	

2.4. Extraction and purification of melanin from liquid medium after 21 days of incubation

Two liquid culture media were prepared in this experiment: potato dextrose broth (PDB) and PDB supplemented with L-tyrosine (1.5%, w/v). A total of 150 mL from each culture medium was placed in 250 mL conical flasks sterilised and supplemented with three mycelial plugs (5 mm) of a 7-day-old culture of *Aspergillus* Section *Nigri* species. Incubation was conducted under static and shaking conditions (130 rpm) at 28±2 °C for 21days. Once the period of incubation was completed, fungal biomass was separated from the broth by filtering and centrifuging at 8000 *g* for 10 min. The fungal mycelium obtained was collected and dried for 24 h at 60±1 °C in a hot-air oven. The mycelial biomass was evaluated for its ability to produce melanin [15].

The acid/alkali method [16,17] was used to extract and purify melanin partially. In this study, the dried fungal biomass was soaked in 10 ml 1 N KOH in a beaker overnight and autoclaved at 121 °C for 20 min. small amounts of double-distilled water were added to ground the fungal mycelium. Centrifugation was performed at 5000 *g* for 15 min, and the supernatant was collected. The supernatant was acidified using a10ml 3 N HCL solution until pH 2.5 was reached. Finally, the supernatant was precipitated and centrifuged again at 8000 *g* for 15 min to collect the pellets.

The pellets obtained were washed thrice with distilled water and then extracted with 50 ml (ethanol, chloroform and ethyl acetate at the ratio of 2:3:2). The resultant pellet was completely dried in a hot-air oven at 60 °C. The resulting melanin was collected in powdered form. An amount of melanin powder of each sample was used for SEM, FTIR, AFM analysis. The remaining melanin powder obtained was finally dissolved in 10% KOH for UV-Vis analysis [18].

2.5. Fungal melanin characterization

2.5.1. Physiochemical characterization:

The tests were conducted following the methodology of [19] to characterise the fungal melanin extracted from *Aspergillus* section *Nigri* species and synthetic melanin. The initial physiochemical properties of fungal melanin, including solubility in distilled water and common organic solvents. such as acetone, chloroform, dimethyl sulfoxide, ethanol, methanol and ethyl acetate, and 1 M KOH were determined by preparing 0.5 mg/mL solutions of the extracts and synthetic melanin, dissolving them and vortexing for 10 s each. Solubility was evaluated visually. In addition, bleaching and precipitation tests were performed using H_2O_2 and $FeCl_3$ [20].

2.5.2. UV-Vis Analysis:

Synthetic melanin was dissolved in 1 M KOH to create and prepare four different concentrations (10, 50, 100, and 150 ppm) whose absorption spectra were recorded. Similarly, fungal melanin extracts were dissolved in 1 M KOH as per [18] protocol. The UV-Vis absorption spectrum of fungal melanin was scanned at a wavelength range of 200–700 nm on a UV visible spectrophotometer (Shimadzu, Japan) and compared with synthetic melanin, the maximum absorption value was recorded [21].

2.5.3. Fourier-transform infrared (FTIR) analysis:

FTIR spectroscopic analysis was performed in the College of Science, University of Shiraz, Iran. The synthetic melanin standard and the melanin extracted from each fungal isolate were ground with analytical-grade KBr and then pressed into pellets to be analysed. The FTIR spectra were recorded using a Bruker FTIR spectrometer (USA) at a resolution of 4 cm^{-1} over the wavenumber range of 400–4000 cm^{-1}.

2.5.4. Scanning electron microscopy (SEM):

The dried melanin powder obtained from the extracted samples was used for surface imaging through SEM. The samples were first lyophilised and then coated with a thin layer of gold to enhance conductivity. Surface topography analysis was performed using an analytical field-emission scanning electron microscope (TESCAN, Czech Republic) at the Taban Laboratory in Tehran, Iran.

2.5.5. Atomic Force Microscopy (AFM):

Surface roughness and tri-dimensional images of melanin were assessed via AFM dimension. The two-dimensional (2D) and 3D AFM micrographs of melanin were captured in tapping mode using an AFM JPK Nano Wizard II (BRUKER, Germany) in the laboratories of the Faculty of Chemistry, University of Tehran.

2.6. Melanin Synthesis Pathway

To distinguish the biosynthesis pathways of melanin, the study evaluated the relevance of DHN melanin pathway inhibitor (to our knowledge, difenoconazole was used for the first time in this study as an inhibitor for DHN melanin) and DOPA-melanin pathway inhibitor (kojic acid) in the pigmentation and growth of colonies. The compounds were dissolved in ethanol, incorporated into the autoclaved and cooled PDA medium and dispensed in Petri dishes (90 mm diameter) to achieve final concentrations of 60 and 120 ppm for difenoconazole or kojic acid (Sigma Aldrich, USA). The control plates consisted of PDA only. Samples were prepared in triplicate. PDA plates were inoculated with a 3 mm diameter mycelium plug cut from the margins of an actively growing colony and incubated for 10 days at 25 °C in the dark [22].

2.7. Statistical analysis

For statistical analysis, one-way analysis of variance was applied using Minitab version 16.

3. Results

3.1. Identification of *Aspergillus* section *Nigri*

Eight fungal species belonging to *Aspergillus* section *Nigri* was isolated and identified using molecular methods (Table 2). All identified species were registered in the DNA Data Bank of Japan and assigned accession numbers.

Table 2: *Aspergillus* section *Nigri* species isolated during the study and their accession numbers.

Species name	GenBank/DDBJ Accession Nos.		
	ITS	β-tubulin	Calmodulin
Aspergillus awamori	LC884984	LC884800	LC884808
A. brasiliensis	LC884986	-	-
A. costaricaensis	LC884987	LC884804	LC884811
A. luchuensis	LC884988	LC884805	LC884812
A. niger	LC884985	LC884801	LC884809
A. piperis	-	LC884802	LC884810
A. tubingensis	LC884982	LC884798	LC884806
A. welwitschiae	LC884983	LC884799	LC884807

3.2. Extraction of Melanin from Liquid Media at 21 days

Table 3 shows the amount of melanin in powder form extracted from eight species of *Aspergillus* section *Nigri*. The maximum amount of melanin was 2g which was recorded by *Aspergillus niger* in L-tyrosine PDB, and the maximum amount in the PDB culture media 1.8g was attained by *A. tubingensis* at 21days. One-way ANOVA analysis indicated significant differences ($p < 0.01$) were observed in melanin production across species at 21days of incubation on both media.

The results show that the concentration of melanin extracted from eight species of *Aspergillus* section *Nigri* cultured in PDB culture media was range from 37.40ppm which achieved by *A. piperis* to 134.28ppm which attained by *Aspergillus tubingensis*. At the end of the incubation period in the L-tyrosine PDB liquid media, the findings indicate that the concentration range of melanin extracted from *Aspergillus* section *Nigri* species was (53.03-140.53ppm) *A. niger* recorded 140.53ppm while 40.53ppm was achieved by *A. piperis* (Table 4). One-way ANOVA analysis revealed statistically significant differences ($p < 0.01$) in melanin concentrations among the species in both culture media at 21days.

Table 3: Amount (g) of melanin powder extracted from *Aspergillus* section *Nigri* species in two liquid media at 21days ($p < 0.01$).

Species name	Liquid Media Potato dextrose broth	 L-tyrosine Potato dextrose broth
Aspergillus awamori	1	0.8
A.brasiliensis	1.2	1.8
A.costaricaensis	1.4	1.1
A.luchuensis	1.3	0.6
A. niger	1.2	2
A.piperis	0.4	0.3
A. tubingensis	1.8	1.5
A.welwitschiae	0.6	0.4

Table 4: Absorbance measurement, concentration(ppm) of melanin extracted from *Aspergillus* section *Nigri* species from two liquid culture media at 21days of incubation ($p < 0.01$).

Species	Absorbance	Concentration(ppm)	Absorbance	Concentration (ppm)
	In PDB		In L-tyrosine PDB	
Aspergillus awamori	1.95	121.78	1.84	87.40
A.brasiliensis	1.89	103.03	1.98	131.15
A.costaricaensis	1.97	128.03	1.92	112.40
A.luchuensis	1.94	118.65	1.79	71.78
A. niger	1.62	112.40	2.01	140.53
A.piperis	1.68	37.40	1.69	40.53
A. tubingensis	1.99	134.28	1.95	121.78
A.welwitschiae	1.75	59.28	1.73	53.03

3.3. Physiochemical characterization

Table (5) shows the physical and chemical properties of fungal and synthetic melanin.

Table 5: Physiochemical characterization of Fungal and Synthetic melanin.

Test	Fungal melanin	Synthetic melanin
Solubility in water	insoluble	insoluble
Solubility in organic solvents: methanol, ethanol, ethyl acetate, chloroform, and acetone.	insoluble	insoluble
Solubility in inorganic solvents: KOH and NaOH	soluble	soluble
Solubility in DMSO	partial solubility	partial solubility
precipitation in 1% $FeCl_3$	Precipitate	Precipitate
Decolorization by 30% H_2O_2	decolorize	decolorize

3.4. Spectrophotometric characterization

The fungal melanin extracts were analyzed spectrophotometrically and compared with the synthetic melanin standard at a wavelength (200 700nm) spectrum. The fungal extracts and synthetic standard exhibited similar UV–Vis absorption spectra, characterised by a strong absorption peak in the UV region and a gradual decline in absorbance toward the visible range. The maximum absorption for both samples was recorded at 217 nm, which indicates their comparable optical properties. (Figure 1) presents the standard curve for the synthetic melanin.

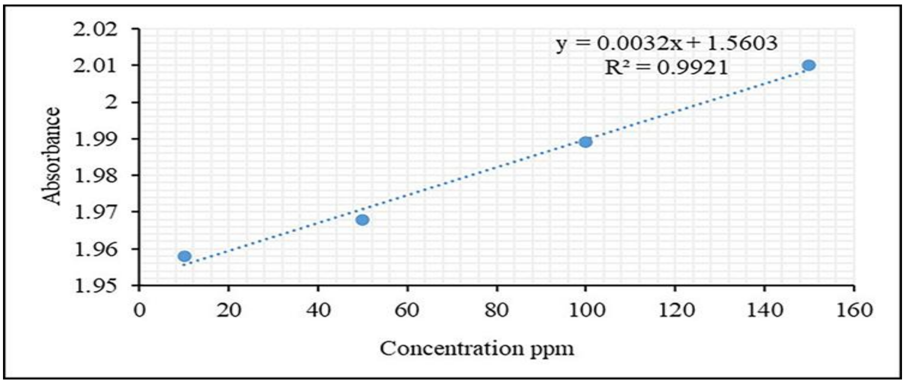

Figure 1: Synthetic melanin standard curve concentration measurement.

3.5. FTIR spectrum analysis

The analysed FTIR spectra were used to ascertain and confirm that the fungal pigment was melanin. Figure 2 illustrates the FTIR for the synthetic and extracted fungal pigment.

The obtained data on the fungal pigment after 21 days of incubation in PDB culture media showed peaks at 3735–3400 cm^{-1}, which displayed OH-stretching, and aliphatic primary amines, (3332-3270cm^{-1}), which unveiled CH-stretching (2915–2855cm^{-1}), NH-stretching, (2167–2083 cm^{-1}), C≡C stretching, (1990–1909cm^{-1}), C=C=C stretching (1703–1710 cm^{-1}), C=O unconjugated with benzene ring, (1640–1602 cm^{-1}), C=O; C=C aromatic (from conjugated quinone structures), N-O stretching (1375–1200 cm−1), C-OH (alcoholic, carboxylic, and phenolic groups); alcoholic C-O; C-H in-plane of aliphatic structure (1034–1021cm^{-1}), C-H stretching (790–576 cm^{-1}), C-H out-of-plane in aromatic structure and C-I stretching (461–446 cm^{-1}). The data on synthetic melanin displayed similar peaks with some noticeable differences at 1255 and 755 cm^{-1} (Table 6).

The obtained data on the extracted melanin after 21 days of incubation in L-Tyrosine PDB revealed peaks near 3728–3625, 3274–3268, 2920–2853, 2190–2102, 1989–1905, 1708–1710, 1652–1618, 1375–1202, 1077–1019, 805–699, and 552–461 cm^{-1}. The data showed similar peaks with synthetic melanin (Table 7).

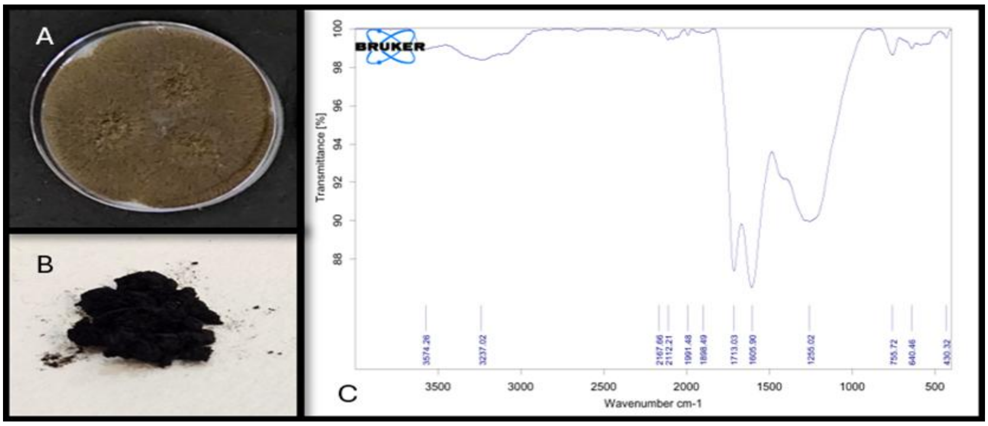

Figure 2: (A) *Aspergillus tubingensis* colony (B)melanin powder extracted (C)Spectra obtained by FTIR showing the functional groups of extracted melanin generated.

3.6. SEM

SEM was employed to investigate the surface morphology of the extracted melanin. The analysis revealed that the melanin exhibited a granular and heterogeneous surface topology (Figure 3). SEM images were captured at magnifications corresponding to 1 and 2 µm scales.

3.7. AFM micrographs

Melanin was subsequently visualised via AFM in addition to the above spectroscopic techniques. The micrographs created from this work are found in Figure 3. Melanin formed spherical-shaped particles or aggregates upon examination by SEM and AFM. As a result of this microscopic imaging, spherical-shaped particles of varying sizes at the nanoscale were observed in AFM and SEM micrographs, in accordance with what was already published in literature.

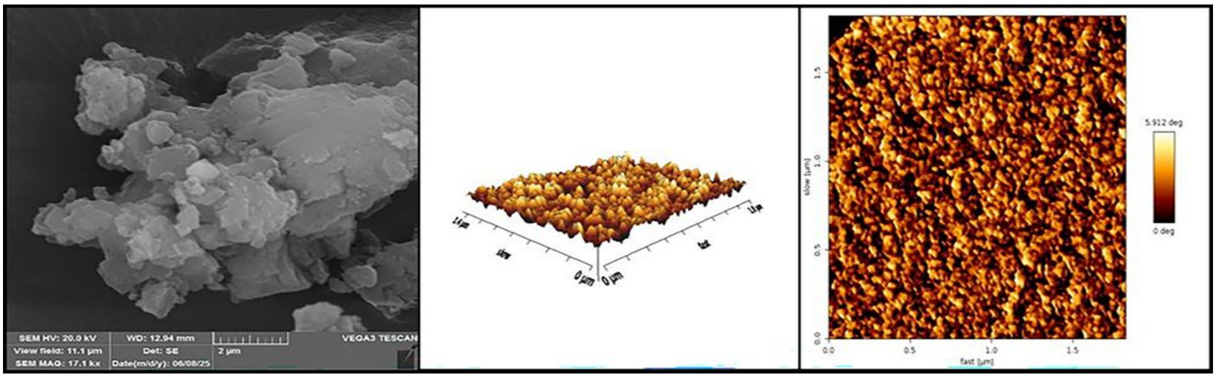

Figure 3: Surface topology image of extracted melanin analyzed by SEM and AFM.

Table 6: Melanin of *Aspergillus* section *Nigri* species IR signals in PDB.

Synthetic melanin Frequency(cm^{-1}) [a]	***Aspergillus* section *Nigri* species Frequency(cm^{-1})** [b]								**Assignment**
	A.awamori	***A.brasiliensis***	***A.costaricaensis***	***A.luchuensis***	***A. niger***	***A.piperis***	***A. tubingensis***	***A.welwitschiae***	
3574	3732		3735			3620		3400	O-H stretch
			3640						
3237	3271	3272	3332	3275	3271	3270	3271	3300	O-H stretch and primary amines NH, C-H stretch
	2918	2922	2915	2922	2920	2922	2918	2917	
	2850	2852	2847	2853	2854	2855	2851	2849	
2167	2167	2167	2166	2167	2165	2166	2165	2165	C≡C stretch
2112	2107	2114	2102	2083	2110	2103	2110	2110	C≡C stretch
1991	1988	1986	1987		1988	1987	1989	1990	C=C=C stretch
1898	1924	1936	1921	1919	1909	1929	1930	1928	C=C=C stretch
1713	1703	1710	1705		1706			1703	C=O unconjugated with benzene ring
1605	1615	1625	1601	1640	1625	1602	1626	1602	C=O; C=C aromatic (from conjugated quinone structures), N-O stretch
1255	1374	1372	1373	1373	1373	1373	1375	1375	C-OH (alcoholic, carboxylic, phenolic groups); Alcoholic C-O; C-H in-plane of aliphatic structure
	1203	1258	1218	1258	1202	1200	1258	1203	
	1029	1030	1034	1029	1030	1027	1026	1021	C-H stretch
755	743		783	798	729	734	740	790	C-H out-of-plane in aromatic structure
640	696	698	690	698	690	692	680	576	C-H stretch
430	461	550	453	552	460	446	456		C-I stretch

[a, b] Spectra signals of synthetic and extracted melanin after 21days of incubation in PDB. Identification based on [23].

Table 7: Melanin of *Aspergillus* section *Nigri* species IR signals in L-tyrosine PDB.

Synthetic melanin Frequency(cm^{-1}) a	*Aspergillus* section *Nigri* species Frequency(cm^{-1}) b								Assignment
	A.awamori	*A.brasiliensis*	*A.costaricaensis*	*A.luchuensis*	*A. niger*	*A.piperis*	*A. tubingensis*	*A.welwitschiae*	
3574						3728			O-H stretch
						3625			
3237	3271	3272	3268	3274	3271	3272	3270	3268	O-H stretch and primary amines
	2924	2920	2921	2923	2922	2926	2920	2920	NH, C-H stretch
		2851	2852	2853	2852	2870	2851	2852	
2167	2168	2167	2184	2190	2165	2167	2167	2167	C≡C stretch
2112	2111	2109	2105	2102	2112	2103	2113	2112	C≡C stretch
1991	1982	1982	1989	1986	1984	1985	1983	1984	C=C=C stretch
1898	1916	1905	1903	1915		1936	1933	1935	C=C=C stretch
1713		1710	1708		1709			1709	C=O unconjugated with benzene ring
1605	1625	1626	1652	1645	1625	1618	1626	1624	C=O; C=C aromatic (from conjugated quinone structures), N-O stretch
1255	1374	1371	1373	1374	1375	1373	1375	1375	C-OH (alcoholic, carboxylic, phenolic groups); Alcoholic C-O; C-H in-plane of aliphatic structure
	1256	1215	1258	1258	1202	1205	1258	1258	
	1027	1027	1019	1030	1077	1027	1028	1024	C-H stretch
755	744	805	716	797	720	744	744	743	C-H out-of-plane in aromatic structure
640	697	698			699	696	698	697	C-H stretch
430	461	552	455	553	462	462	456	455	C-I stretch

[a, b] Spectra signals of synthetic and extracted melanin after 21days of incubation in L-tyrosine PDB. Identification based on [23].

3.8. Melanin Synthesis Pathway

Complete discoloration of the fungus in the presence of an inhibitor during the test for all isolates directly indicated the effective inhibition of melanin synthesis (Table 8). Two inhibitors each from the DOPA and DHN pathways were tested against all isolates. Melanin synthesis by *A. niger* and *A. brasiliensis* was inhibited by kojic acid, which indicates a DOPA melanin pathway, whereas melanin synthesis by all other tested species were inhibited by difenoconazole, which demonstrates the DHN melanin pathway. *A. niger* and *A. brasiliensis* demonstrated the two pathways. The effective concentration of kojic acid was 120 ppm. The same effective concentration (120 ppm) of difenoconazole, no pigment was observed for all tested species. The concentration 60ppm of each inhibitor did not gave complete discoloration for all isolates.

Table 8: Effect of melanin inhibiting compounds(120ppm) on pigmentation of *Aspergillus* section *Nigri* species grown on PDA medium ($p < 0.01$). a - = No inhibition of pigmentation, b + =Inhibition of pigmentation**.**

Species name	Kojic acid	Difenoconazole
Aspergillus awamori	-[a]	+
A. brasiliensis	+[b]	+
A. costaricaensis	-	+
A. luchuensis	-	+
A. niger	+	+
A. piperis	-	+
A. tubingensis	-	+
A. welwitschiae	-	+

4. Discussion

Aspergillus section *Nigri* isolated from soils has been investigated as a novel source during melanin production. Given its good biocompatibility and various activities, melanin is widely used as a biomaterial in cosmetics, medicine and biotechnological applications [7, 24, 25].

This study focused on the melanin content of eight different species of *Aspergillus* section *Nigri* and determined whether differences exist in the amount of melanin produced in two liquid culture media after 21 days of incubation. Some studies have focused on determining the quantity and quality of melanin in *Aspergillus* section *Nigri* species. However, results have been reported only for *A. carbonarius*, *A. niger* and *A. tubengensis* [21].

To confirm the characteristics of melanin, we executed spectral studies on the isolates. The solubility profile is consistent with the characteristics of melanin, which is typically insoluble in most organic solvents but soluble in alkaline conditions. Our results completely agree with those of several studies that dealt with the characteristics of melanin [19, 20, 26].

UV–Vis spectroscopy of the extracted melanin showed prominent absorption peaks around 217 nm, which is characteristic of melanin pigments. These peaks corresponded to the aromatic structures and conjugated double bonds within the melanin polymer and thus confirmed the presence of melanin. Our finding is similar to those of [27, 19, 26, 25]

FTIR analysis of purified melanin was performed to analyse functional groups of the compound. Some of the functional groups detected through FTIR analysis were O-H bond stretching, C-O stretching due to carboxylic acid and phenol C=C, coupled carbonyl group (-COOH), which are also known as semiquinones, or 5,6-indole quinones visible through stretching. All of these functional groups confirmed the presence of melanin. This absorption correlated with those identified in synthetic melanin standards and melanin found in various fungal species in previous studies [28,19, 29, 26, 30, 31].

The SEM and AFM images displayed the surface topology of the extracted melanin. The morphology showed spherical, granular and heterogeneous structure, which is typical of melanin aggregates [20, 25].

Melanin from all isolates grown in PDB and L-tyrosine PDB were purified via alkali extraction coupled with pH adjustment with acid [32]. Both culture media produce intense pigmentation and were used for growth of isolates [18, 20, 33]

The kinetics of colony differentiation and melanin synthesis varied depending on the medium used, which resulted in a clear quantitative difference in melanin concentration between the two-culture media used for species in section *Nigri* [34, 35]. Furthermore, different melanin production may result from differences in natural pigment production among species and strains within a species [19, 26].

Whether the pathway DHN or DOPA produced melanin was determined using kojic acid, which is a DOPA pathway inhibitor, and difenoconazole, which is a DHN pathway inhibitor [21]. Results of the present study indicate that the DOPA melanin pathway is present in *A. niger* and *A. brasiliensis*, whereas the DHN pathway was found in all tested species. The present of two pathways in *A. niger* and *A. brasiliensis* maybe related to the fact that, some fungi are capable of synthesizing both types of melanin (DOPA-melanin and DHN-melanin), and this dual use provides them with important adaptive advantages because each melanin type serves a distinct protective role. Therefore, when a fungus is exposed to multiple stress factors simultaneously (e.g., living in harsh environmental conditions), it may activate both pathways to achieve broader protection. In addition, *Aspergillus* spp contain both gene sets (Tyrosinase genes for the DOPA pathway, and Polyketide synthase (PKS) genes for the DHN pathway). Usually, only one pathway operates under normal conditions. However, under environmental stress or nutrient limitation, both pathways may be activated simultaneously. The ability of Aspergillus spp to produce the two types of melanin was a strategy for adaptation of to Changing Environment [25, 36].

Our finding agrees with those of [21, 16] *A. niger* and *A. brasiliensis* demonstrated the two pathways, which was in agreement with the study of [22] who reported that *A. brasiliensis* is strongly pigmentated by melanin in the cell wall that supports the cell wall structure and confers resistance to a range of environmental stressors, which causes it to behave in the two pathways for melanin synthesis. The types of melanin pathways have not been previously reported for most species in section *Nigri*, to our knowledge this work being the first study of its kind in Basrah province. Our findings are in accordance with those of previous studies [37, 38, 34, 39, 40] which suggest *Aspergillus* species as prolific producers of melanin pigment.

5. Conclusion

Aspergillus section *Nigri* can synthesize a diverse range of melanin, which has significantly advanced green and sustainable manufacturing properties with appropriate physicochemical and biological characteristics and is used through its various applications in health, environmental, energy and industrial spheres. In the current study, production, characterization and various methods, such as UV–visible spectroscopy, FTIR, SEM and AFM, were used to analyze melanin and confirm it to be a pigment. *A. tubingensis* and *A. niger* showed well production of melanin in both culture media used. The findings on the effects of different inhibitors on melanin production provide valuable insights into melanin biosynthesis pathway in all tested species. The present work revealed differences in the types and amounts of melanin produced by various species of *Aspergillus* section *Nigri*. Additional genetic studies must be carried out on the characterization of genes that encode enzymes of both pathways, the use of other inhibitors and the detection of intermediate products of the pathways, studying the optimal factors for enhancing melanin production, especially when other species in section *Nigri* or various sections were identified. Such fungi can also be used for massive production of commercially important melanin which can be applied in industrial scale and environmental protection.

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أنتاج ومسارات التخليق الحيوي للميلانين في انواع الفطر *Aspergillus* section *Nigri* المزروعة في أوساط سائلة

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المستخلص

نتيجة لأنخفاض تكلفتها، واستهلاكها كميات أقل من المواد الكيميائية، وسهولة تنقيتها، أصبحت طرق تحضير صبغة الميلانين الطبيعية خيارًا شائعًا. هدفت هذه الدراسة إلى تشخيص وتحديد كمية ونوع التخليق الحيوي للميلانين الفطري في ثمانية أنواع عائدة الى *Aspergillus* section *Nigri* في وسطين سائلين هما potato dextrose broth (PDB) و L-tyrosine PDB لمدة 21 يومًا. شخصت الانواع الفطرية من خلال تسلسل جينات ITS, β-tubulin gene, calmodulin. أجريت التحليلات النوعية والكمية للميلانين باستخدام التحليل فوق البنفسجي المرئي، وتحليل الأشعة تحت الحمراء (FTIR)، والمجهر الإلكتروني الماسح، ومجهر القوة الذرية. أظهرت النتائج أن كمية الميلانين المستخلص على شكل مسحوق تراوحت ما بين 0.3 -2 غرام بعد انتهاء فترة الحضن في الأوساط السائلة. وتراوح تركيز الميلانين المستخلص من الأنواع المزروعة في وسط PDB بين 37.40 -134.28 جزءًا في المليون، بينما في وسط L-tyrosine PDB أشارت النتائج إلى أن التركيز يتراوح بين 53.03 -140.53 جزءًا في المليون. استُخدم مثبطان لتحديد مسار التخليق الحيوي للميلانين، وهما ديفينوكونازول الذي استخدم لأول مرة في هذه الدراسة وحمض الكوجيك. وكان مسار تخليق الميلانين في كل من *A. niger* و *A. brasiliensi*s هو DOBA بينما أنتجت جميع الأنواع المدروسة الميلانين بشكل عام عبر مسار DHN.

الكلمات المفتاحية : DOBA ميلانين , DHN ميلانين , ديفنوكونازول , حمض الكوجك , الاوساط السائلة.