Genetic validation and spectroscopic detailing of DHN-melanin extracted from an environmental fungus

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Accurate characterization of melanin using analytical methodologies has proved to be difficult due to its heterogeneity, insolubility in wide pH and broad range of solvents. The present study was undertaken to characterize melanin extracted from an environmental *Aspergillus fumigatus* AFGRD105 by studying its genes, chemical properties and spectral data. A gene based approach to confirm the type of melanin carried out indicated the extracted melanin to be of the dihydroxynaphthalene type. On comparison with synthetic melanin, UV–Vis and IR spectra of the extracted melanin revealed characteristic peaks that can be further used for confirmation of DHN-melanin extracted from any source. Solid state ^{13}C NMR spectroscopy established the presence of the hydroxyl-naphthalene moiety and validated the results obtained by genetic analysis. The correct assignment of the observed spectral frequency characteristic of functional groups can be further adapted in future works that deal with binding capacities and biomolecule systems involving melanin.

1. Introduction

The melanization systems in micro-organisms exhibit more heterogeneity than in animals. Melanin is a biopolymer synthesized from phenolic compounds and confers certain advantages to fungi such as increasing their survival potential in some environments and/or enhancing their virulence. Besides DOPA-melanin formed by the oxidation of tyrosine by tyrosinase among fungi, G-glutaminyl-3,4-dihydroxybenzene melanin, catechol melanin and 1,8-dihydroxynaphthalene (DHN) melanin derived from G-glutaminyl-4-hydroxybenzene, catechol and DHN respectively have also been observed [1]. The DHN-based pathway for melanin production has been termed to be common among fungi [2,3].

For more than a decade, studies regarding polymers have been interesting due to their chemical complexities, analysis of their physical and chemical properties such as identification of functional groups by spectroscopy [4]. Essential biopolymers such as proteins and carbohydrates have been successfully characterized and well-established by detailing their monomeric units, bond patterns and sequence determination, largely with specificity to their respective active site [5,6]. With respect to melanin, however, characterization of structure and its details have not yet been completely understood due to many limiting factors such as insolubility in broad range of organic solvents, purification strategies that may give rise to changes in its structural features and organization of melanin as a polymer [7].

Ultraviolet and visible (UV–Vis) absorption spectroscopy is the absorption measurement of a beam of light after it passes through a sample at a single wavelength or a wide spectral range and the concentration of the sample or analyte is determined by applying the Beer-Lambert law using a calibration-curve analysis [8]. UV–Vis spectroscopy is usually undertaken for quantitative analysis whereas vibration-based spectroscopy that includes infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy analyses the sample qualitatively. Vibration-based spectroscopy has been used widely to detail the structure of materials at molecular and chemical level including natural products [9].

IR spectroscopy involves the absorption of IR radiation by the sample and its conversion into energy of molecular vibration. As the mass of the atoms in the molecule and the bonds of the molecule determine the frequency of absorption, IR spectra provides information in the functional groups present in the molecule [10]. NMR spectroscopy, on the other hand, works by the absorption of radio frequency (RF) energy that occurs due to the presence of non-zero spin containing nuclei such as ^{1}H, ^{13}C, ^{31}P, ^{19}F, etc. The resulting absorption of the frequency plotted on a 2D-graph termed as NMR spectrum is considered as the fingerprint of the analyte [11,12].

This study was initiated to confirm whether or not the melanin obtained after extraction from the conidia of *A. fumigatus* AFGRD105 was DHN-melanin by characterizing its physical, chemical properties and spectral data. The results obtained were compared with synthetic

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melanin and data found in the literature related to fungal melanin after which the screening of antimicrobial potential was carried out.

2. Materials and methods

2.1. Genomic DNA extraction

Aspegillus fumigatus (AFGRD105) (GenBank:JX041523; NFCCI: 3826) isolated from endocarps of coconut was maintained on Sabourauds dextrose agar (SDA, Himedia, India) plates and slants. Five day old culture was grown in static conditions using Sabouraud dextrose broth (SDB, Himedia, India), harvested by filtration and kept overnight at −80 °C. This frozen mat was taken and milled using pestle and glass powder (200 mg of glass powder per 5 g of wet fungal mat) in order to break open the cell wall. The fungal mat specimens were ground using 2 ml extraction buffer containing 100 mM Tris HCl, 100 mM NaCl, 10 mM EDTA, 2% (w/v) polyvinylpolypyrrolidine (PVP), 0.1 M Na_2SO_4 at pH 8. An equal volume of tris saturated phenol: chloroform:isoamyl alcohol (25:24:1) (v/v/v) was added to the crude extract and inverted gently. The extract was then centrifuged at 10,000 g for 10 mins at 4 °C and the supernatant was carefully decanted and collected. An equal volume of isopropanol and 1/10th (v/v) of 3 M sodium acetate (pH 4.8) was added and mixed gently for 5 mins and made to stand for 5 mins. This mixture was centrifuged at 10,000 g for 10 mins at 4 °C. Recovered pellet was washed with 0.5 ml of 70% ethanol. Any trace of ethanol was removed by air drying and the pellet was re-suspended overnight at 4 °C in 100 µl TE Buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6) containing 5 µl of DNase free RNase (10 mg/ml).

2.2. Molecular confirmation for the type of melanin produced

The genomic DNA of *A. fumigatus* AFGRD105 was subjected to amplification of six genes involved in the pathway of DHN-melanin production (Table 1). Oligonucleotide primers [13] in this study were synthesized using standard phosphoramide chemistry on PCR-MATE DNA synthesizer (Applied Biosystems) at IDT Technologies Limited, India. The PCR conditions were two minutes of initial denaturation at 95 °C, followed by 30 cycles of 20 s at 95 °C for denaturation, 20 s at a temperature between 50 and 64 °C according to the melting temperature (T_m) of the primer for annealing and 30 s at 72 °C for elongation, and by a final elongation step of five minutes at 72 °C. The products were held at 4 °C until subjected to agarose gel electrophoresis using 2% agarose and visualized for products by using UVP GelDoc-It® Imager.

2.3. Extraction and chemical analysis of melanin from Aspergillus fumigatus (AFGRD105)

The extraction and chemical analysis of melanin was undertaken [14]. Chemical diagnostic tests were carried out in comparison with synthetic melanin (Myko Teck Pvt Ltd, Goa, India).

2.4. UV–Vis spectrophotometric analysis

Dilutions of 1:1, 1:3 and 1:9 of extracted melanin were made and adjusted to pH 12 by allowing dissolution in an aqueous substrate (1 N NaOH). Different concentrations of synthetic melanin at 0.05, 0.1, 0.25, 0.5, 1.0, 2.0 and 2.5 g/l were prepared using 1 N NaOH. Alkaline double distilled water of pH 12 was used as blank. Solutions were scanned in the UV and Visible wavelength (200–900 nm) by using a UV–Vis spectrophotometer (Hachs spectrophotometer). The relationship between log absorbance and wavelength was determined.

For concentration determination of extracted melanin, synthetic melanin was prepared in 1 N NaOH at concentrations of 0.01, 0.05, 0.1, 0.25, 0.5 and 1.0 g/l. A standard curve at A_{650} was made. The sample melanin of the dilution 1:3 was taken, A_{650} was measured and pigment concentration for each sample was estimated using the A_{650} standard curve (www.graphpad.com).

2.5. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

For IR-spectroscopic investigation, ~2 mg of lyophilized melanin from *A. fumigatus* AFGRD105 and synthetic melanin was respectively mixed with 700 mg of FTIR grade KBr to form pellets by using hydraulic press. The spectral information was collected and studied at a resolution of 4 cm^{-1} using Perkin Elmer spectrophotometer in the wave number region of 400–4000 cm^{-1}.

2.6. NMR spectroscopy of extracted melanin

300 mg of sample as fine powder obtained on lyophilisation of melanin extracted from *A. fumigatus* AFGRD105 was subjected to one dimensional solid state ^{13}C NMR analysis using cross polarization, dipolar decoupling and magic angle spinning (CPMAS) in a ECX400-Jeol 100 MHz high resolution multinuclear FT NMR spectrometer. Samples were spun at a typical speed of 9.00 ± 0.01 kHz in a 1 mm probe and the signals were recorded.

2.7. Antimicrobial studies

2.7.1. Antibacterial assay of DHN-melanin and Synthetic melanin
Broth microdilution method for confirming minimum inhibitory concentration (MIC) of the extracted melanin was carried out. Clinically isolated cultures of *Escherichia coli, Streptococcus spp., Bacillus spp., Proteus spp., Enterobacter spp., Kliebsiella spp., Pseudomonas* spp. and *Staphylococcus* spp. were obtained from the Department of Microbiology, Aravind Eye Hospital and Postgraduate Institute of Ophthalmology, Coimbatore. Each of these bacterial suspensions was prepared to the turbidity of 0.5 McFarland standards (1×10^8 CFU/ml) from freshly subcultured strains in Mueller Hinton broth (Himedia Laboratories, India). The antibacterial agent cephalosporin and vancomycin (Himedia Laboratories, India) was dissolved in distilled water. Further dilutions were made using distilled water according to CLSI guidelines (CLSI document M100-S18). The stock solution of 1 mg/ml was prepared and used at concentrations of 0.125–64 µg/ml.

The MIC of cephalosporin, vancomycin, DHN-melanin (0.125–64 mg/ml) and Synthetic melanin (0.125–64 µg/ml) was

Table 1
Oligonucleotide primers used for the amplification of six genes that mediate the pathway of DHN-melanin production.

| Gene Product	Gene name	Forward primer (5′−3′)	Reverse primer (5′−3′)	Annealing temperature (°C)	Amplicon Size (bp)
Polyketide synthase	alb1	CAAACCACTCGCCATGGA	TCGGAGCAGAAGCTGAGGATA	56.5	921
Polyketide shortening	ayg1	ATGCCACGCTGGATCCTT	ATGATCAGCACGATGGGGA	61	695
Scytalone dehydratase	arp1	TCACACCACAATGGTCGAAA	CACATGAAATGGTACTTTTGC	54	725
Hydroxynaphthalene reductase	arp2	ATGGTGAACACCTGCACCTAT	TCAGCATTCCAAATCCCCA	57	895
Vermelone dehydratase	abr1	ATGTTCCATTCCAGGGCTCT	TCGTCGTCGTAGGCAAATG	54	495
Oxydase	abr2	ATACACGACAACAGGATGTGG	TCAATTCCTCGGGGTCGT	54	822
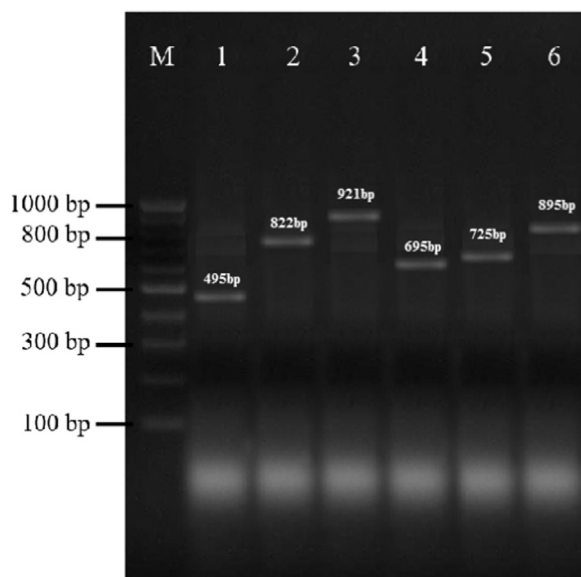
Fig. 1. Gene based confirmation of the biosynthetic pathway undertaken by *Aspergillus fumigatus* AFGRD105 for melanin production. Lane M: 100 bp ladder; Lane 1–6: Gene product obtained on using *alb1, ayg1, arp1, arp2, abr1* and *abr2* primers respectively.

determined using the broth microdilution method according to CLSI guidelines (2006). The adjusted bacterial inoculums were added at a concentration of 1×108 CFU/ml as 50 μl/well in a sterile 'U' based microtitre plate along with test concentration of cephalosporin, vancomycin, melanin and Synthetic melanin as prepared (50 μl/well). Accordingly the last inoculum concentration of 5×107 CFU/ml was obtained in each well and incubated for 24 h at 37 °C. The lowest concentration of antibiotic which inhibited the visible bacterial growth was determined as respective MIC of cephalosporin, vancomycin, DHN-melanin and Synthetic melanin for the isolate.

2.7.2. Antifungal assay of DHN-melanin and synthetic melanin

Clinically isolated cultures of *Aspergillus flavus, A. niger, A. fumigatus* and *A. tamarii* were obtained from the Department of Microbiology, Aravind Eye Hospital and Postgraduate Institute of Ophthalmology, Coimbatore. Seven day old cultures grown on potato dextrose agar (PDA) (Himedia Laboratories, India) were taken and the plate was covered with approximately 1 ml of 0.1% tween-20. Conidia were scraped gently and collected into a sterile tube. The suspension was vortexed for 15–20 s after which it was adjusted to a conidial concentration of $2–5 \times 106$ by adjusting the optical density between 0.15 and 0.18 at 530 nm and diluted to 1:50 using Roswell Park Memorial Institute (RPMI) 1640 (HiMedia Laboratories, India) before assaying for activity.

Stock solution of fluconazole (HiMedia Laboratories, India) and amphotericin B was prepared by dissolving 10 mg in 1 ml of sterile distilled water. Further dilutions were made to obtain concentrations ranging from 0.125 to 64 μg/ml and used as a control for all fungal strains mentioned. 100 μl of conidial suspensions diluted to 1:50 in RPMI 1640 medium and added to each well of 96-well microtitre plate containing 100 μl of each concentration of fluconazole, melanin (0.125–64 μg/ml) and Synthetic melanin (0.125–64 μg/ml). Additionally, 100 μl of PDA was added to each of the wells. A well containing fungal suspension with no drug (growth control) and a well containing only media (negative control) was included in each test condition. The plates were then incubated at 35 °C for 48 h.

2.7.3. Determination of minimum bactericidal and fungicidal concentration

Samples from bacterial cultures were taken from plates with no visible growth in the MIC assay, subcultured on freshly prepared nutrient agar plates and incubated at 37 °C for 48 h. The minimum

bactericidal concentration (MBC) was taken as the concentration from the well that did not show any growth on a new set of agar plates.

After the MIC for each strain was determined, 20 μl of fungal suspension from each well of microtitre plates that prevented the visible growth of a microorganism was subcultured onto PDA plates. The minimum fungicidal concentration (MFC) was defined as the lowest drug concentration at which no fungal colonies were observed after 72 h of incubation at 35 °C.

3. Results

3.1. Molecular analysis of genes involved in melanin biosynthesis in Aspergillus fumigatus AFGRD105

Although most members of class Ascomycetes produce DHN melanin, previous reports suggest that some aspergilli produce DOPA melanin. Prediction of the type of melanin only on the presence of polyketide synthase (PKS) would be speculative. It is only when the intermediate products are detected after inhibition of a particular melanin pathway that the type of melanin can be accurately assessed. Therefore, *A. fumigatus* AFGRD105 in this study was subjected to screening of the presence of six genes involved in production of DHN-melanin instead of confirmation of pathway using inhibitors. The gene products as mentioned in Table 1 represent the genes involved in melanin production by DHN pathway.

The DHN-pathway has been associated with the protein products such as scytalone and vermelone. Further, many intermediate products such as hydroxynaphthalenes are produced. The polyketide synthase (*alb1*) gene acts on malonyl-CoA and acetyl-CoA as substrate to yield a long chain of aromatic benezene heptaketide naphthopyrone which is further shortened to yield a tetrahydoxynaphathalene product with the presence of polyketide shortening enzyme (*ayg1*). The main intermediate of DHN-pathway scytalone is produced subsequently by scytalone dehydratase (*arp1*). Further, a trihydroxynaphthalene product is produced by hydroxy-naphthalene reductase (*arp2*) using scytalone as a substrate. Vermelone and dihydroxynaphthalene was produced consecutively by the action of hydroxynaphthalene reductase (*arp2*) and vermelone dehydratase (*abr1*). The final polymerized product of melanin from DHN pathway is obtained by the action of oxydase (*abr2*). The distribution of DHN-pathway genes in *A. fumigatus* AFGRD105, revealed the presence of *ayg1, arp1, arp2, abr1, abr2* and *alb1* at 695 bp, 725 bp, 895 bp, 495 bp, 822 bp and 921 bp respectively (Fig. 1). Further no variation in time taken for expression of these genes was observed as all the genes were expressed after a five day growth.

3.2. Chemical analysis

Results of chemical analysis of the extracted pigment are summarized in Table 2. The pigment could not be dissolved in water, acid, ethanol, warm chloroform, warm acetone or benzene. The pigment was soluble in phenol and excellently dissolved in concentrated alkaline solution. The dissolved, extracted black pigment was lightened by the oxidizing agents NaClO and H_2O_2, as well as by the reducing agents H_2S and $Na_2S_2O_4$ (sodium hydrosulfite).

3.3. UV–Vis spectroscopy based analysis of black pigment extracted from all strains of A. fumigatus

The absorbance decreased progressively as the wavelength was increased to the near red region. Furthermore, there was a linear relationship between log absorbance and wavelength from 400 to 600 nm which is one of the most important criteria for the characterization of melanin. When subjected to gradual dilution, the absorbance decreased unevenly, occurring in the near red ranges first, then in the UV range, and lastly in the far red ranges (Fig. 2). This can be explained by the fact that the percentage of absorption is greatest in the UV region. This

Table 2

Diagnostic properties of black pigment extracted from identified strains of *Aspergillus fumigatus* (AFGRD105).

Property	Treatment	Standard melanin	Melanin extracted from *A. fumigatus* (AFGRD105)
Colour	Naked eye	BB	BB
Solutibilty in	H_2O (pH 7)	–	–
inorganic solvent	1 N NaOH	+ +	+ +
	1 N HCl	–	–
Solubility in Organic	Ethanol	–	–
Solvents	Warm Chloroform	–	–
	Warm Acetone	–	–
	Benzene	–	–
	Phenol	+	+
Precipitation	1% $FeCl_3$	P	P
	1 N HCl	PP	PP
	1 N H_2SO_4	PP	PP
Oxidation	6% NaClO	OO	OO
	30% H_2O_2	OO	OO
Reduction	H_2S	R	R
	5% $Na_2S_2O_4$	RR	RR

Table 2: Indications in the table are as follows: – heavily insoluble; + + strongly soluble; + lightly soluble; p - light precipitation; pp- heavy precipitation; oo – strong oxidation (indicating clear solution); r – weakly reduced (solution gradually changes from dark brown to light yellowish brown); rr – strongly reduced (clear solution).
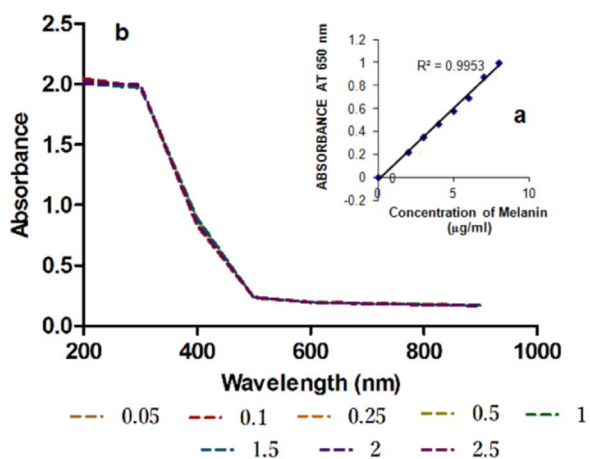

Fig. 2. Absorbance spectra of synthetic melanin at seven concentrations. a(inset)- Correlation between synthetic melanin concentration and absorbance at 650 nm used as a standard curve to estimate extracted melanin concentration from all identified *Aspergillus fumigatus* AFGRD105 (R^2 – gives the validity of the graph); b- the differential effect of melanin concentration on absorbance at varying wavelengths (0.05–2.5 – Standard melanin concentrations at µg/ml).

observation might resolve the question of why quite different descriptions of melanin UV–visible light absorption spectra exist in the literature. The melanin concentration at 1:3 dilutions was selected and strain *A. fumigatus* AFGRD105 yielded the maximum concentration at 3.87 µg/ml.

3.4. FTIR spectral analysis of extracted melanin in comparison with synthetic melanin

In the present analysis the FTIR spectrum of the extracted melanin from *A. fumigatus* AFGRD105 and synthetic melanin obtained from Myko Teck Pvt. Ltd. were analyzed at resolution of 4 cm^{-1} in transmission mode (4000–400 cm-1) (Fig. 3). Characteristic peaks were observed at 3402 cm^{-1}, 2924 cm^{-1}, 2854 cm^{-1}, 2376 cm^{-1}, 1627 cm^{-1}, 1458 cm^{-1}, 1373 cm^{-1}, 1381 cm^{-1}, 1072 cm^{-1}, 1048 cm^{-1}, 678 cm^{-1}, 617 cm^{-1} and 601 cm^{-1}.

3.5. NMR analysis for chemical groups in extracted melanin

The NMR spectrum of the extracted melanin from *A. fumigatus* AFGRD105 (Fig. 4) shows the expected olefinic carbon resonance at 105 ppm, sharp signals for long open chain aliphatic methylene groups (20–40 ppm), aliphatic oxygenated carbons (60–80 ppm) and presence of COO groups at 170–175 ppm.

3.6. Antibacterial assay

Vancomycin and cephalosporin were the antibiotics used in this study against Gram-positive and Gram-negative bacteria respectively. The results obtained indicate decreased susceptibility towards DHN-melanin extracted from *A. fumigatus* for all Gram-positive and Gram-negative bacteria when compared to both antibiotics and Synthetic melanin. The Synthetic melanin, however, showed similar activity when compared to the antibiotics used. The MIC and MBC concentrations of DHN-melanin were effective at double the concentration of Synthetic melanin therefore *E.coli, Proteus spp., Enterobacter spp., Klebsiella* spp., *Pseudomonas spp., Bacillus spp., Streptococccus* spp., and *Staphylococcus* spp. showed decreased susceptibility to DHN-melanin.

On treatment with vancomycin the MIC concentration was observed at 0.5 µg/ml for Bacillus spp. whereas *Streptococccus* spp., and *Staphylococcus* spp. was observed to be susceptible at 0.25 µg/ml. On comparison the DHN-melanin showed susceptibility at higher concentration (≥ 4 µg/ml) whereas Synthetic melanin rendered inhibition at ≥ 1 µg/ml. Among Gram-negative bacteria, the antibiotic cephalosporin used as a standard rendered susceptibility at 0.25 µg/ml for *E.coli* and *Proteus* spp., 0.5 µg/ml for *Pseudomonas* spp. and *Enterobacter* spp., and 1 µg/ml against *Klebsiella* spp. Synthetic melanin were effective inhibitors of growth towards *E.coli, Proteus spp., Enterobacter spp., Klebsiells* spp. and *Pseudomonas* spp. at 0.5 µg/ml. The inhibition of DHN-melanin extracted and identified from *A.fumigatus* was effective only at concentrations ≥ 8 µg/ml for all Gram-negative isolates in this study.

3.7. Antifungal assay

The highest dilution of the drug which inhibited the fungal growth was taken as the MIC. MIC50 was calculated by taking the drug concentration, where fifty percent of isolates are inhibited. Similarly MIC90 was noted with drug concentration where ninety percent of the isolates were inhibited The MIC values were noted based on the rate of growth inhibition. The ranges of the minimal inhibitory concentration, MIC50 and MIC90 values of the antifungal drugs, DHN-melanin and synthetic melanin for the clinical isolates of *Aspergillus flavus, A. niger, A. fumigatus, A. tamarii* have been detailed in Table 4. A majority of the aspergilli could be inhibited only at a concentration of 512 µg/ml of fluconazole similar to DHN-melanin when compared to synthetic melanin which demonstrated antifungal activity at much lower concentrations.

4. Discussion

A variation in the type of melanin has been observed among fungi as L-3,4-dihydroxy phenylalanine (L-DOPA) melanin pathway is mostly seen in class Basidiomycetes whereas members of class Ascomycetes produced melanin through 1,8-dihydroxy naphthalene (DHN) pathway [1]. The determination of the type of melanin produced by members of genus *Aspergillus* has usually been determined by using DHN pathway inhibitors such as phthalide, fenoxalin, and tricyclazole that target tetra- and tri-hydroxylnaphthalene reductases and DOPA pathway inhibitors such as tropolone and kojic acid that target enzyme tyrosinase. However, confirmation of the type of melanin production using these inhibitors has led to new reports that members of class Ascomycetes also reveal the production of DOPA-melanin.
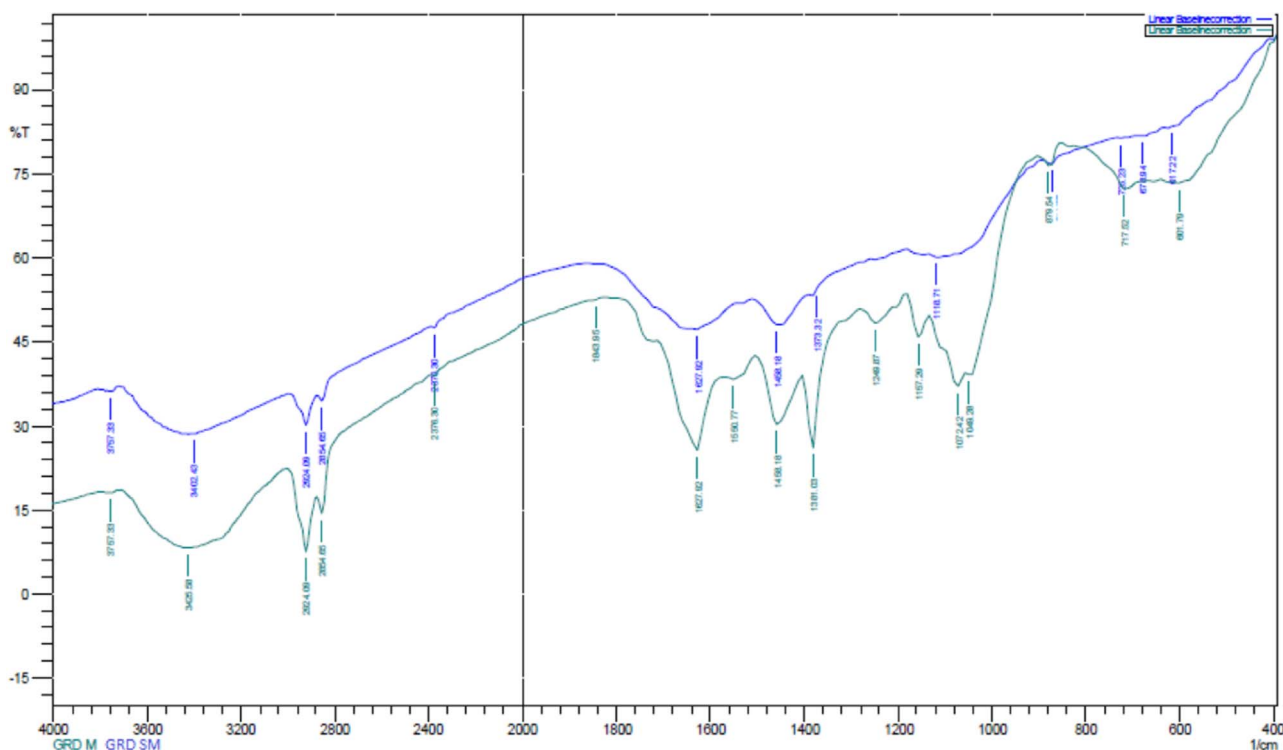
Fig. 3. Fourier transform infra red spectroscopy (FT-IR) spectra of extracted melanin obtained from *Aspergillus fumigatus* AFGRD105 and synthetic melanin. GRD M denotes the spectrum obtained on analysis of extracted melanin whereas GRD SM denotes the IR spectrum obtained on analysis of synthetic melanin.
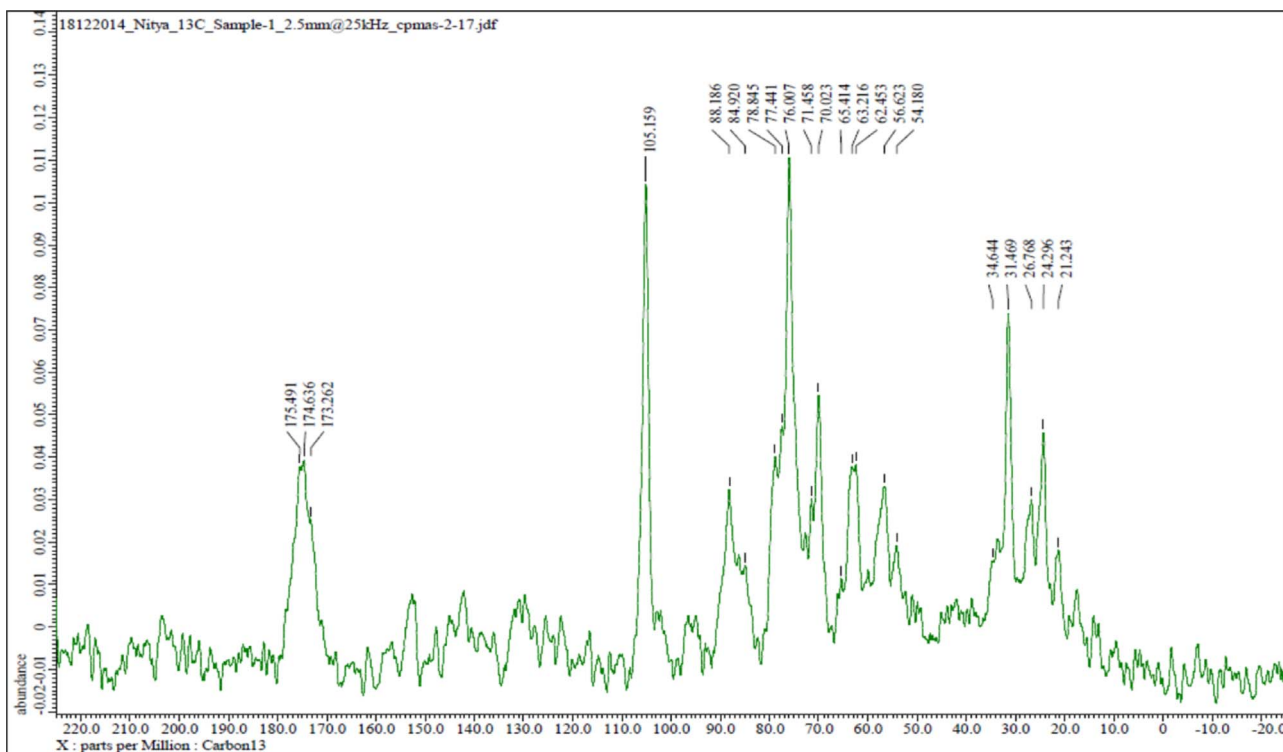

Fig. 4. Nuclear magnetic resonance characterization obtained by using cross-polarization magic-angle spinning (CPMAS) solid state approach using ^{13}C nuclei for melanin extracted from *Aspergillus fumigatus* AFGRD105.

Results by Pal et al. [15] show that DOPA-melanin pathway was present in *A. niger, A. flavus* and *A. tamarii* whereas DHN-pathway melanin was found in *A. tubingensis*. However, San-Blas et al. [16] revealed the melanin pathway in *A. flavus, A. terreus* and *A. nidulans* was not inhibited by tricyclazole. The authors also studied the polyketide synthase gene (the first enzyme in the DHN melanin pathway) and concluded that *A. flavus* contained melanin similar to DHN melanin, whereas *A. terreus* lacked the DHN melanin genes. In another study, it was validated that *A. terreus* not only lacks polyketide synthase, but also the production of naphthopyrone [17].

Table 3

MBC, MIC and number of bacterial colonies obtained on treatment with DHN-melanin extracted from *A. fumigatus* (AFGRD105), synthetic melanin and respective antibiotics.

Clinical isolates	Concentrations of potential growth inhibitors (µg/ml)									
	0.125	0.25	0.5	1	2	4	8	16	32	64
Vancomycin										
Bacillus spp.,	13	7	1							
Streptococccus spp.,	15	2								
Staphylococcus spp.	16	2								
Cephalosporin										
E.coli	11	1								
Proteus spp.	12	1								
Pseudomonas spp.	13	6	2							
Enterobacter spp.	12	7	2							
Klebsiella spp.	11	9	4	1						
DHN-melanin extracted from *A. fumigatus* (AFGRD105)										
Bacillus spp.,	24	19	15	11	8	5	3			
Streptococccus spp.,	33	17	13	10	10	1				
Staphylococcus spp.	24	18	12	11	9	7	1			
E.coli	36	29	22	19	13	11	4	2		
Proteus spp.	23	21	17	15	14	10	3	1		
Pseudomonas spp.	28	23	17	14	12	8	2			
Enterobacter spp.	32	29	21	18	15	11	9	4	2	
Klebsiella spp.	34	29	23	20	16	13	11	5	2	
Synthetic melanin										
Bacillus spp.,	16	13	11	5	2					
Streptococccus spp.,	11	5	3	1						
Staphylococcus spp.	12	8	4	1						
E.coli	11	5	1							
Proteus spp.	10	3	1							
Pseudomonas spp.	8	5	1							
Enterobacter spp.	9	4	1							
Klebsiella spp.	13	6	1							

Table 3 The three vertical lines indicate the MBC value of the potential growth inhibitor; The double lines indicate the MIC value of the potential growth inhibitor; the numbers indicate the number of bacterial colonies with decreased susceptibility counted after plating on nutrient agar.

Table 4

Comparison of DHN-melanin and Synthetic melanin with fluconazole and Amphotericin B for its potential antifungal activity.

Fungal isolate (n = 10 each)	MIC (µg/ml)	Fluconazole[a]	Amphotericin B[a]	DHN-melanin[a]	Synthetic melanin[a]
A. flavus	MIC_{50}	512	2	128	16
	MIC_{90}	512	8	256	32
	Range	256–512	0.5–16		
A. niger	MIC_{50}	512	4	64	8
	MIC_{90}	512	16	256	32
	Range	256–512	0.5–16		
A. fumigatus	MIC_{50}	512	0.5	256	1
	MIC_{90}	512	1	512	4
	Range	256–512	0.25–1		
A.tamarii	MIC_{50}	512	2	512	4
	MIC_{90}	512	8	512	16
	Range	256–512	0.5–16		

Table 4 The range of the antifungal agents has been given according to the CLSI guidelines.

[a] All the concentrations have been presented as µg/ml.

Polyketide synthases are enzyme complexes that produce a large class of intermediates called polyketides including melanin. The prediction of DHN melanin, on the basis of characterization and identification of polyketide synthase genes, has been reported from *A. parasiticus* [18] and *A. nidulans* [19]. However, a detailed characterization of melanin from *A. nidulans* showed that it is DOPA melanin [20] although Taborda et al. [21] revealed no inhibition of melanin production by tricyclazole in *A. nidulans*. Exceptions like these were also reported in dermatophytes in which neither tricyclazole nor kojic acid was able to inhibit melanin synthesis [22].

Numerous reports evaluating the presence of PKS genes in *A. niger* been concluded that the melanin is of the DHN type [23]. Pal et al. [15] found that kojic acid inhibits melanin production by *A. niger* and therefore the melanin is of DOPA type. A similar observation was made in an earlier study where kojic acid inhibited melanin production by *A. niger* [24]. Although pigmentation in *A. niger*, *A. flavus* and *A. tamarii* was susceptible only to kojic acid, there is no definite proof that these species did not produce the pigment via the DHN pathway leading to conflicting conclusions.

The genes involved in the pathway have been abbreviated for laboratory purposes by adapting Pihet et al. [13] and this can be used further by other researchers interested to pursue molecular based practices with respect to DHN-pathway of melanin production. In this study, BLAST tool was used to indicate the expected size of the resultant PCR product [25,26]. However, due to the heuristic nature of BLAST and removal of low complexity data, queries for short sequences like primers often return incomplete data [27] Therefore, the assay was tested to demonstrate the presence of amplified products in comparison with the amplified size obtained by subjecting to Genomic BLAST analysis using *A. fumigatus* (taxid: 746128) assembled genome. The respective forward and reverse primer sequences were then concatenated into one sequence separated by ~20 nucleotides and entered into BLAST sequence box and the resultant amplicon size was obtained for each gene (Table 3.1).

The chemical analysis of the black pigment in this study was similar to the diagnostic characteristics of melanins [1] because the unique structure allows them to act as either proton donors (reductants) or receivers (oxidants). The black pigment also reacted positively in a test for polyphenols with $FeCl_3$, producing a flocculent brown precipitate. All the properties of melanin [28] obtained for melanin extracted from *A. fumigatus* AFGRD105 reacted identically to synthetic melanin. No variation has been observed with respect to the diagnostic features under laboratory conditions between melanin obtained by L-DOPA and

DHN mediated pathway. Similar observations are made from studies involving melanin from melanocytes [29], cerebrospinal fluid [30,31], malignant tissues [32], bacteria [33] and fungi [34,35].

Absorption of the black pigment as 200–900 nm wavelength scan from all strains of *A. fumigatus* AFGRD105 occurred at all wavelengths with no specific peaks. This is consistent with the colour black because this colour results from the absorption of all visible wavelengths. The percentage of absorption was greatest in the UV region, which is a property of aromatic organic compounds. Slopes of these plots are not useful for distinguishing among different types of melanins because oxidation will change the slopes appreciably [1]. Dilution effect, which has not been previously reported, was observed on the light absorbance of both the black pigment from strains of *A. fumigatus* and synthetic melanin. For example, two peaks (223 and 269 nm) have been found to occur for synthetic melanin [36], while monotonic increases in the absorbance with decreasing wavelength, with a barely detectable shoulder between 290 and 320 nm, have been described by others [37].

Different concentrations might have been obtained if measurements had been made at different concentrations. Spectral methods based on melanin solubilisation try to overcome the insolubility of melanin by drastic treatment but even if the solubilisation is unsuccessful, melanin (as a finely divided black particulate material) could produce scattering and absorption effects in any colorimetric procedure [38]. The spectroscopic properties of the black pigment obtained from the identified *A. fumigatus* AFGRD105 confirmed with those of melanin reported previously [39,40] as well as with the properties of synthetic melanin. Therefore, this pigment is likely a melanin.

Among spectroscopic techniques FTIR spectroscopy has been the most preferred technique to analyze the assignment of the spectral composition of the functional groups present in the sample by corresponding them to the respective absorption bands [41]. Additionally, FTIR spectroscopy has been termed to be non-destructive, rapid and requires only a small quantity of the sample [42]. The spectrum of radiation energy absorbed helps the research community to characterize the sample as the chemical bonds present in the sample vibrate to a characteristic frequency; thus revealing information regarding bond length and bond angle [43]. With effect to the incident radiation absorbed at specific wavelengths, absorption peaks and individual chemical bonds can be identified. This data can further qualitatively identify individual compounds in complex systems [6].

In the absence of water, Naumann et al. [44] recommended analysis of five major absorbance regions in IR spectra for the identification of functional groups: the 3000–2800 cm^{-1} spectral region is the fatty acid region (region I); 1700–1500 cm^{-1} contains the amide I and II bands of proteins and peptides (region II); 1500–1200 cm^{-1} is a mixed region of fatty acid bending vibrations, proteins, and phosphate-carrying compounds (region III); 1200–900 cm^{-1} contains absorption bands of the carbohydrates in microbial cell walls (region IV); and 900–700 cm^{-1} is the 'fingerprint region' that contains weak but very unique absorbance that are characteristic (region V). Shift in absorbance of the bands were observed on a comparative degree among any two test conditions. These bands indicate the decrease or increase of frequency corresponding to each bond present [45].

The broad absorption peak characteristic of N–H or O–H stretching vibration modes was observed for both extracted and synthetic melanin at 3402 cm^{-1}. Centeno and Shamir [46] and Bernsmann et al. [47] had attributed the broad absorption spectral region between 3600 and 3200 cm^{-1} to vibrations of phenolics, carboxylic acid and aromatic amino functions mainly present in pyrrolic and indolic molecules. Two absorption peaks observed at 2924 cm^{-1} and 2854 cm^{-1} may be assigned to the stretching vibration of C–H aliphatic group in both extracted and synthetic melanin. These peaks have been reported in melanin from previous studies [6,41,47]. The observation of two frequencies recorded with slight variation may be due to the hydrogen atoms present in components of different planes [47] suggesting a polymeric structure of melanin extracted in this study. The strong band

at 1627 cm^{-1} (1647 cm^{-1} – 1531 cm^{-1}) is characteristic to the bending vibrations of aromatic ring of C=N and C=C with a C=O of carboxylic (COOH) function [6]. The oxidation of the hydroxyl groups to carboxyl groups has been ascertained to enhance the colour intensity. Melanin molecules previously reported a high content of carboxylic groups (COOH) due to the presence of conjugated complex structural information in a melanin molecule [3].

Another characteristic bond within the same frequency range was recorded at 1550 cm^{-1} for extracted melanin alone. The recording frequency of 1458 cm^{-1} (1468–1330 cm^{-1}) is attributed to aliphatic C–H groups in both synthetic and extracted melanin whereas other weak bands ascribed to alkene C–H substitution are depicted in bands below 700 cm^{-1}. These results obtained on analysis of both extracted and synthetic melanin are in accordance with previous studies undertaken to detail the functional groups of melanin extracted from non-fungal sources [46,48].

The hydroxyl group (O-H) bending of the carboxylic and phenolic groups present in the 1400 cm^{-1} – 1300 cm^{-1} area centered at 1373 cm^{-1} and 1381 cm^{-1} for synthetic and extracted melanin respectively indicates the CNC stretching (or) vibration of indole ring [41]. The peak centered at 1118 cm^{-1} for synthetic melanin and 1072 cm^{-1} and 1048 cm^{-1} for extracted melanin indicates the C–H bond in-plane/out-of-plane deformation. Weak bonds 678 cm^{-1} and 617 cm^{-1} and 601 cm^{-1} for extracted melanin are in accordance with the out-of-plane bending of aromatic alkene (C–H) bond reported earlier [40]. The absorbance at these ranges proves the pigments as melanin and the peaks in the insoluble melanin indicate purity [49,50]. The obtained spectrum from FTIR radio frequency is in agreement for closely related compounds such as substituted pyrrole, indole and pyrole; this revealed the conservation of functional groups in both extracted and synthetic melanin with slight variations in the exact frequency.

Although the significance of melanin, especially in their natural form has been reiterated in many studies [51–53] these pigments have defied advanced structural and chemical analysis due to their amorphous nature, heterogeneity and insolubility. Additionally their amorphous nature makes it quite difficult to determine a complete analysis of its planar and molecular structures as they are resistant to crystallization. Several research groups have challenged these inhibitions by using ^{13}C and ^{15}N solid state NMR spectroscopic technique for characterization of melanin from fungal and/or animal sources [54,55]. As the melanin extracted from *A. fumigatus* AFGRD105 in this study has proved to be insoluble in organic solvents and water, cross-polarization magic-angle spinning (CPMAS) solid state approach of NMR spectroscopy was taken with reference to ^{13}C nuclei.

The spectrum obtained (Fig. 4) can be divided or deconvoluted into three parts (i) 165–220 ppm; (ii) 50–120 ppm; and (iii) 10–50 ppm. Each of these spectral frequencies can be attributed to aromatic, carbonyl and aliphatic carbon functionalities [33,56,57]. The ^{13}C chemical shift value at ~175 ppm shows that there are structural changes associated with the carbonyl group from the quinine tautomer of DHI-melanin that are associated with the L-DOPA melanin. This hypothesis is inferred from the theoretically calculated chemical shift values of different tautomers of 5,6-dihydroxyindole and 5,6-dihydroxyindolecarboxylic acid according to Banerjee et al. [33]. The appearance of peaks at 30–60 ppm establishes the presence of carbon atoms involved in aliphatic structures and matches well with the chemical shift values of similar carbon structures such as dopamine, L-DOPA, 5-dimethoxyindole-2-arboxylate used in the elucidation of chemical structure of melanin by previous studies [56,57] This is also in accordance with the contributions of Nosanchuk et al. [58] who confirmed the application of NMR techniques to reveal a chemically resistant aliphatic matrix in fungi for deposition of indole-based pigments.

The appearance of a broad olefinic region (110–160 ppm) may be attributed to the aryl groups present in close proximity to unpaired electrons, overlap of signals from polymeric building blocks and/or any

structural disorder that is characteristic of an amorphous sample [59]. The sharp aromatic spectral feature at 10 ppm supports the presence of single indole like moiety possibly converted from an aromatic precursor [56,60]. Specific resonances at 24 ppm and 31 ppm indicates the typical pattern of alkyl $(CH_2)_n$-CH_2CH_3 chains [61] The spectrum revealed no presence of phenolic moieties (58 ppm and 150 ppm) although the peak at ~72 ppm may be due to the presence of conidial polysaccharides. However, the signal intensities obtained as a spectrum with a function of cross-polarization indicates a composition of rigid carbons present in the melanin sample. The chemical shifts obtained could possibly confirm the presence of CH_3COO at ~34 ppm, distinct long chain methyl groups at 21 ppm and 31 ppm, $CH_2CH=CH$ at 26 ppm, CH_2COO at 21.2 ppm and a secondary alcohol group at 63 ppm.

The overall ^{13}C spectrum obtained from melanin extracted from *A. fumigatus* AFGRD105 was relatively dissimilar to melanin obtained from *Sepia officinalis*, human hair and melanoma [33] although a rich assortment of alkenes, alkanes, esters, alcohols and indolic functional groups were present in melanin obtained from both DHN and DOPA mediated pathways [61]. Absence of peaks from ~90–105 ppm found in the spectrum under study confirmed the substitutions in aromatic carbons suggesting a hetero-polymerization of the indole moiety responsible for the polymeric structure of DHN-melanin. Additionally, the faint signal-noise ratio in the spectrum may be attributed to the dipolar line broadening due to the presence of unpaired electrons.

On comparison with L-DOPA melanin where peaks of the aromatic rings resonated at 35.2 ppm and 55.7 ppm with the predominant spectral features of aromatic residues that corresponded to 116 ppm and 124 ppm along with peaks at 154 ppm and 161 ppm for side products [60], none of these specific peaks were observed in the ^{13}C NMR spectrum obtained from melanin extracted from *A. fumigatus* AFGRD105. According to the Masor-Raper scheme for melanin biosynthesis in *Cryptococcus neoformans*, the molecular architecture resembled 5,6-indolequinone (IQ) or 5,6-dihyroxyindole (DHI) biosynthetic intermediates at 116 ppm and 124 ppm [62,63] which is not observed in the melanin extracted from *A. fumigatus* AFGRD105; thus confirming the L-DOPA based intermediates are less likely to be present in the sample used in this study.

Presence of proteinaceous matter in the extracted melanin from *A. fumigatus* AFGRD105 can be inferred from the peaks obtained from ~165–200 ppm in ^{13}C spectra and the appearance of strong band along ~1650 cm^{-1} − 1600 cm^{-1} in FTIR absorption spectrum due to the presence of amide carbonyls similar to Banerjee et al. [33]. However, these peaks may be established to have minor significance in comparison to the peak intensity ratio obtained to the aromatic and aliphatic signals in the ^{13}C spectrum. With an overall comparison of the FTIR spectrum with the ^{13}C NMR study, the presence of hydroxyl-naphthalene moiety can be emphasized to be present in its tautomeric form as reported in previous studies [64–67]. Further, as the extracted compound is confirmed to be chemically similar to the melanin obtained from several different sources using complementary techniques, melanin constituted by the hydroxy-naphthalene moiety is reported for melanin extracted from *A. fumigatus* AFGRD105. The molecular composition of polymerized DHN-melanin has always been considered to be the bonding of bulky methylene groups with unsaturated residues. By augmenting the tentative structural assignments made traditionally from ^{13}C chemical shift trends, functional groups such as alkanes, alkenes, alcohols, ketones, and carboxylic acid esters revealed the melanin synthesis proceeds by the polymerization of aromatic rings in accordance with previous studies [61].

The membrane and outer wall of Gram-negative is different from Gram-positive as the inner cellular membrane and cell wall in Gram-positive organisms consists of peptidoglycan and is thick. A large role in resistance of Gram-negative microorganisms is mainly attributed to an inner cell membrane, peptidoglycan layer and a thick lipopolysaccharide complex [68]. The increasing resistance among Gram-

negative bacteria is most likely to be a consequence of the presence of outer membrane that acts as a barrier to several environmental or foreign substances including antibiotics. The range of the antibiotic compound used as the standard and potential growth inhibitors are usually carried out by serially diluting concentration greater or lower than 1 mg/ml. The MIC is defined as the lowest concentration that will inhibit the visible growth of the organism after overnight incubation whereas MBC indicates the complete death of the organism. For further confirmation apart from the visual observations, about 50 µl of the test condition was plates on nutrient agar plates in this study to confirm if any colonies do show any growth. The number of colonies obtained on plating the test organisms was indicated in Table 3 with the respective MIC and MBC values. In previous studies it has been widely observed that the addition of silver has always shown a much more effective inhibition of both Gram-negative and Gram-positive microorganisms. Among the many inorganic antibacterial agents, silver has been confirmed to have antibacterial activity even at minute concentrations usually by catalytic oxidation [69]. This effective nature of synthetic melanin on par with DHN-melanin has been observed in the present study.

The lower curative rates of antifungal drugs at the treatment levels have been hampered more when compared to antibacterial agents due to the eukaryotic nature of fungal cell wall [70]. In NCCLS/CLSI M38-A, spectrophotometric adjustment of conidial suspensions is recommended. To reduce the time required for antifungal susceptibility testing of filamentous fungi, SAAS method uses inoculum suspensions that can be readily prepared from the original pure plate. The test can even be set up as soon as the mold is isolated because only mycelial growth, the invasive form, can also be used as the inoculum unlike NCCLS/CLSI method, in which a calibrated conidial suspension only is recommended [71] and used in the present study.

As genus Aspergillus has been widely recognised to be intrinsically resistant [72], only aspergilli were used in the present study. 50% of the *A.flavus*, *A. niger* and *A. tamarii* strains were susceptible to amphotericin B when compared to *A.fumigatus* in the present study. These observations are comparable to previous studies that report higher MIC's for *A. flavus* when compared to *A. fumigatus* among other species of aspergilli [73]. The inefficacy of lower amphotericin B concentrations against Aspergillus strains is known from the literature [74]. The unresponsiveness to amphotericin B confirmed in a fraction of the isolates may in part explain the poor clinical response seen among some patients. Similarly, Nayak et al. [75] reported higher MICs against *A. flavus* and *A. niger* compared to *A. fumigatus* isolated from infectious keratitis and suggested a high index of suspicion for amphotericin B resistance.

It was found that synthetic melanin was notably active against the clinically isolated aspergilli within the range of amphotericin B and was a more promising anti-aspergillic agent than the extracted form of DHN-melanin. A majority of the aspergilli could be inhibited only at a concentration of 512 µg/ml of fluconazole similar to DHN-melanin when compared to AgMPs which demonstrated antifungal activity at much lower concentrations. However, these results were not in accordance with our study as the efficiency of synthetic melanin was slightly lesser than amphotericin B.

5. Conclusion

In conclusion, the confirmation of the type of melanin production was undertaken by screening the presence of genes involved in the DHN-pathway instead of using inhibitors. The distribution of pathway genes in *A. fumigatus* AFGRD105 confirmed that the strain produced DHN-melanin. On chemical analysis and UV–Vis spectroscopy, it was found that strain AFGRD105 showed production of melanin within five days of growth. The analysis of melanin from *A. fumigatus* AFGRD105 by FTIR spectroscopy revealed the existence of phenolics, carboxylic acid and aromatic amino functional groups mainly present in indolic molecules. The presence of a rich assortment of alkenes, alkanes, esters,

alcohols and indolic functional groups constituted by the hydroxy-naphthalene moiety based on NMR spectral intensities confirmed the L-DOPA based intermediates are less likely to be present; thus, establishing the DHN pathway for production of melanin extracted from *A. fumigatus* AFGRD105. The antimicrobial assays also revealed synthetic melanin to have a more effective although DHN-melanin was on par with the standards used. The study for DHN-melanin has been carried out for the first time to the best of our knowledge that provides a molecular gene based and biophysical chemistry based approach for studying and confirming the type of melanin.

Acknowledgements

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.08.008.

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