ISSN 0012-4966, Doklady Biological Sciences, 2021, Vol. 500, pp. 159–169. © Pleiades Publishing, Ltd., 2021.

Functional and Structural Characterization of Melanin from *Brevibacillus invocatus* Strain IBA

Avinash Ammanagi[a,*], Shivasharana C. T.[a,*], Krishnaveni R.[b], Abhijeeth Badiger[c], and Vijayakumar Ramaraj[d]

Received April 22, 2021; revised May 18, 2021; accepted May 20, 2021

Abstract– Melanin is a polyphenol or indolic dark brown to black pigment of macromolecules that has a variety of biological functions including UV defence, desiccation, and oxidation. The pigment is classified as a heterogenic polymer. Analytical characterization of melanin can be difficult due to its heterogeneity. In this study, a newly isolated strain of *Brevibacillus invocatus* strain IBA capable of extracellular melanin production was grown on nutrient agar and the bacteria were molecularly identified. Chemical and physical methods were used to characterize melanin. The solubility of melanin in organic and inorganic solvents was used to characterise it chemically. According to the UV-visible wavelength scan, physical characterization revealed absorption in the UV region 200 to 300 nm, but declining towards the visible region. Functional group identification of extracted melanin was carried out by FTIR with different stretching vibrations at 3226, 2920, 2849, 1628, 1555, 1340 cm^{-1} and weak absorption bands at 1104 and 1015 cm^{-1}. Structural characterization was carried by SEM of extracted melanin which showed irregular shape and size at different magnifications. The crystallinity of melanin was studied using X-ray crystallography, with a lattice parameter of approximately $a = 8.54$. The XRD spectrum of the extracted melanin crystallographic pattern revealed peaks at $2\theta =$ 27.32, 31.66, 45.41, 53.84, 53.84, 56.44, 66.18, 73.10, 75.26, and 83.94, which correspond to reflections (111), (200), (220), (311), (222), (400), (331), (420), and (422), respectively. The analytical methods available for melanin analysis are largely complementary, providing detailed knowledge required to draw reliable conclusions about the sample under investigation.

Keywords: *Brevibacillus invocatus* strain IBA, fourier transform infrared spectroscopy, melanin, scanning electron microscopy, X-ray diffraction

DOI: 10.1134/S001249662105001X

INTRODUCTION

Melanin is a group of biopolymers with distinct physicochemical and biological properties [3]. They are macromolecules that are formed when phenolic or indolic compounds are oxidatively polymerized. The resulted pigments are frequently brown or black in color, but a wide variety of other colors have also been observed. Melanins are also negatively charged and hydrophobic in nature [4, 5]. It is one of the most commonly distributed pigments in the animal kingdom. However, melanin can be found in fungi, plants, and even bacteria, therefore its development is not limited to animals [3].

Melanins are available in three forms, Eumelanins, pheomelanins, and allomelanins. Eumelanins are pigments that appears in color from black to brown and are made by oxidative polymerization of tyrosine to L-DOPA, which is then converted to dopachrome and then to melanin [5]. Eumelanin is the most common pigment formed by humans and microbes [10]. Pheomelanins are red or yellow color pigments that are synthesized similarly to eumelanins, except that DOPA undergoes cysteinylation [10]. Allomelanins, which belong to the third class of pigments that are nitrogen-free heterogeneous polymers derived from a number of sources such as dihydrofolate, homogentisic acid, catechols, and others [4, 11].

The major precursor for melanin is tyrosine and phenylalanine [7]. The biosynthesis of melanin is catalysed by enzyme tyrosinase via a series of enzymatic and nonenzymatic reactions [6]. Tyrosinase (monophenol monooxygenase, EC 1.14.18.1) catalyses the conversion of L-tyrosine to L-3,4-dihydroxyphenyl alanine (L-DOPA), which is converted to dopa-

[a] *Department of Studies in Biotechnology and Microbiology, Karnatak University, Dharwad, Karnataka, India*

[b] *Department of Microbiology Vijayanagara Sri Krishna Devaraya University, Ballari, Karnataka, India*

[c] *Department of Biotechnology, Sri Dharmasthala Manjunatheshwara College, Ujire, Karnataka, India*

[d] *Shilpa Biologicals Private Limited, Dharwad, Karnataka, India*

**e-mail: avi.ammanagi7411@gmail.com*

chrome. A sequence of nonenzymatic oxidoreduction reactions transform dopachrome to melanin [7]. For the synthesis of L-DOPA and the removal of phenolic compounds from wastewaters, tyrosinases from various biological sources have been used [8]. Eumelanins are made by oxidative polymerizing tyrosine and/or phenylalanine to L-DOPA, which is then converted to dopachrome and then to melanin [5]. Pheomelanins are synthesized similarly to eumelanins, except that DOPA undergoes cysteinylation (incorporation of cysteine in the polymer) [10] and contain sulphur [4].

Melanization has been described as a defining characteristic of microorganisms that survive in extreme conditions, and it has helped pigmented species to colonize in such a harsh conditions. Since the pigmented organisms can colonize in such an extreme environments, the ecology of certain melanized species is especially fascinating. Their widespread distribution proves that the pigmentation supports the growth of such organisms in such harsh climatic environments [1, 2]. As per the previous literature different bacterial species have been identified such as *Streptomyces glaucescens, Proteus mirabilis, Pseudomonas* species and *Escherichia coli* which can also produce melanin [4, 66–69]. Melanin protect microorganisms from thermal, chemical (heavy metals and oxidizing agents), and biochemical stresses (reactive oxygen created by exposure to solar UV radiation) [12]. Microbe-produced melanin has the ability to chelate metal ions [13]. It also gives cell walls structural rigidity and assists in the storage of water and ions [4, 5].

Melanin can also provide resistance to antimicrobial drugs [14], and plants integrate melanin into their cell walls as a strengthening agent [15]. In Humans, melanin not only determines the skin color, but also protects against UV radiation [16], and its absence causes a variety of abnormalities and diseases. Melanoma is an increasingly common cancer that affects about 40000 people in the United States each year and an estimated 100000 people worldwide. Fungal (*Cryptococcus neoformans*) melanin has previously been used to produce monoclonal antibodies (mAb) against melanin for the treatment of human metastatic melanoma in mice. Since, the structure of fungal and human melanin is similar; monoclonal antibody generated against fungal melanin can also binds to human melanin [30, 31]. Melanin has physicochemical properties that allow them to act as ultraviolet absorbers, cation exchangers, drug carriers, amorphous semiconductors, and X-ray and γ-ray absorbers [17]. To provide higher UV protection, water soluble melanins are used in sun-screens, solid plastic films, lenses, paints, varnishes, and other surface protection formulations [18]. Melanins have a wide range of biological functions, including antimicrobial [19], antitumor [20], antivenin [21], and liver protection [22]. Bacterial melanins have been found to have anti-inflammatory properties [23]. Melanin protects melanocytes and keratinocytes from the induction of DNA strand breaks by hydrogen peroxide, according to Hoogduijn et al. [24], suggesting that this pigment plays an important antioxidant role in the skin [25]. AIDS treatment reveals synthetic soluble melanin's selective antiviral activity against the human immunodeficiency virus [26]. Microorganisms have recently gained attention as an environmental friendly and cost-effective alternative to chemical production [27]. Actinomycetes are biotechnologically important bacteria that are well-known for their secondary metabolites [28]. They can naturally produce and excrete dark soluble pigments known as melanins or melanoid pigments [4, 29].

The aim of this study is to isolate and identify a *Brevibaccillus invocatus* strain IBA that produces effective extracellular melanin. This research study covers the isolation and molecular identification of bacteria, as well as the extraction and characterization of melanin using UV-visible spectroscopy, FTIR, SEM, and XRD, which are the most widely used techniques in melanin analysis. The structure of melanin is not confined, and this research is an attempt to determine the chemical and physical features of melanin. This is thought to be the first study of *Brevibacillus invocatus* strain IBA for melanin production and characterization. The overall goal is to assist specialists in choosing appropriate analytical methods for melanin analysis so that the findings can be correlated with existing literature and incredible conclusions can be drawn.

MATERIAL AND METHODS

Materials

All chemicals used were of highest purity and analytical grades were purchased from Sigma Aldrich, USA and SRL, India. Chemicals are mentioned below in the methods.

Media Preparation

Nutrient agar was prepared and plated. Briefly, the following ingredients were added in 90% of water in Erlenmeyer flask (peptone 0.5%, beef extract 0.3%, yeast extract 0.3%, NaCl 0.5%, agar 2%, $CuSO_4$ 0.004%, L-tyrosine 0.5%) and the pH is adjusted to 6.5. The flask is autoclaved at 121°C (15 psi) for 20 min. In hand bearable temperature (40–50°C) approximately 20–30 mL is poured on the 90 mm petriplates and allowed to solidify. After the plates were solidified, the plates were used.

Isolation and Screening of Bacteria

Melanin producing bacteria were isolated from soil loaded with water of paddy fields of Amblikoppa (15.357723° N 74.943763° E). Dharwad, India. A nutrient agar with L-tyrosine is used to isolate the melanin producing bacteria. Serial dilution techniques

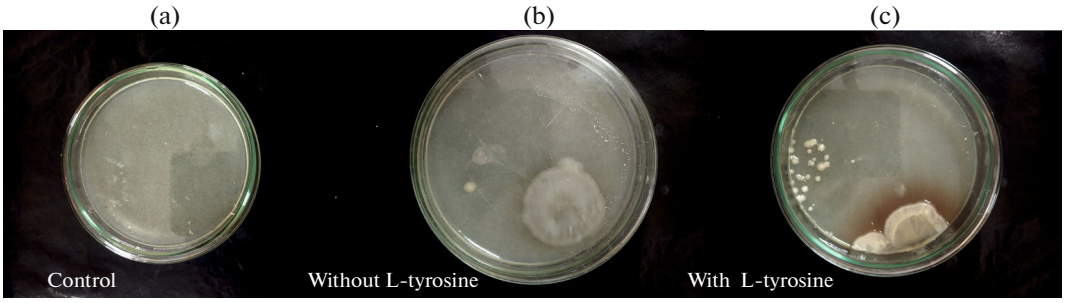

Fig. 1. Showing screening of *Brevibacillus invocatus* strain IBA (a) control, (b) bacterial growth without addition of substrate (L-tyrosine) because of this there was no any dark brown pigmentation around the colonies. (c) Bacterial growth with addition of substrate (L-tyrosine) so there is formation of dark brown pigmentation around the colonies.

(10 fold) with 0.85% saline were used to isolate bacteria. Dilution of 10^{-6} of 0.1 mL sample was spread over agar plates and incubated at 37°C for 48 h. Bacterial isolates with dark brown pigments surrounding colonies were selected and screened further for production of melanin (Fig. 1).

Molecular Identification of Bacteria

The genus and species of highest melanin producing bacteria was identified by 16S rRNA sequencing, carried out at BioEdge solutions Pvt. Ltd. Banglore, India.

The strain's genomic DNA was prepared using the CTAB method defined by Sambrook *et al* (33). The PCR amplification reaction was carried out according to El-Naggar et al. [34]. Primers used are 27F 5'-agagtttgatcctggctcag-3' at 53°C annealing temperature and 1492R 5'-ggttaccttgttacgactt-3' at 57°C annealing temperature. And PCR amplification condition used are: 1. initial denaturation 95°C for 2 min; 2. final denaturation 95°C for 30 s; 3. annealing 50°C for 30 s; 4. elongation 72 °C for 1 min; 5. repeat steps 2, 3, and 4 for 30 cycles; 6. final elongation 72°C for 10 min; 7. hold at 4°C forever.

Using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_ Sort= Blast Search) [35], the sequence was aligned against the sequences present in the NCBI database. The partial sequence was submitted in NCBI GenBank database under the accession number MW696201. By the sequences of representative members of the genus *Brevibacillus* was collected and Phylogenetic tree was constructed via the bootstrap test of neighbor-joining algorithm method [36] in MEGA 4 version 2.1 [37].

Molecular Identification of Bacteria

tccaccccgtaaaagatgagtgctaggtgttagg
ggtttcaatccctttagtgccgcagctaacgcaata
cgcactccgcctggggagtactgtcgcaagactgaa
actcaaaggaattgacgggggcccgcacaagcggtg
gagcatgtggtttaattagaagcaacgagaagaacc
ttcccaggtcttgacatcccgctgaccgctctggag
acagagtttcccttcggggcagcggtgacaggtggt
gcatggttgtcgtcagctcgtgtcgtgagatgttgg
gttaagtcccgcaacgagcgcaacccttatttttag
ttgccagcattcagttgggcactctagagagactgc
cgtcgacaagacggaggaaggcggggatgacgtcaa
atcatcatgcccctttatgacctgggctacacacgtg
ctacaatggttggtacaacgggatgctacctcgcga
gaggatgccaatctcttaaaaccaatctcagttcgg
attgtaggctgcaactcgcctacatgaagccggaat
cgctagtaatcgcggatcagcatgccgcggtgaata
cgttcccgggccttgtacacaccgcccgtcacacca
cgggagtttgcaacacccgaagtcggtgaggtcacc
acaacgtgccaacataacgcgtagtgtaatac

Bacterial sequence submitted to NCBI gene bank.

Melanin Production

In a 250 mL Erlenmeyer flask, nutrient broth with the following composition peptone 0.5%, beef extract 0.3%, yeast extract 0.3%, NaCl 0.5%, $CuSO_4$ 0.004%, L-tyrosine 0.5%, and pH 6.5 was used as the processing medium.

A loop full of culture was inoculated in nutrient broth flask and the flasks were incubated at 37°C, 120 rpm in an orbital shaking incubator for 48 h. After 48 h of incubation, melanin content in the broth was measured spectrophotometrically at 475 nm by using a standard melanin calibration curve (Sigma-Aldrich, St. Louis, USA) [32].

Isolation and Purification of Melanin

The broth was centrifuged at room temperature for 15 min at 10000 rpm to remove cells and debris in order to take supernatant which contains melanin. To ensure complex polymerization of melanin (aggregation), the supernatant was filtered through a 0.45-μm membrane and the pH was adjusted to 11 with 10 M NaOH. Since the solubility of melanin decreased within the pH range of 2.6–3.0, concentrated HCl was applied to the mixture to decrease the pH to 3, resulting in a dark brown precipitate known as crude melanoprotein. To deproteinize the pigment, these brown amorphous particles were dissolved in 10 M NaOH

Table 1. Sequences producing significant alignments for *Brevibacillus invocatus* strain IBA

Description	Max score	Total score	Query cover	E value	Per. ident	Accession
Brevibacillus invocatus SAR gene	1125	1125	96%	0.0	97.56	LC416189.1
Brevibacillus invocatus strain B32	1125	1125	96%	0.0	97.56	MH587029.1
Brevibacillus invocatus strain B25	1125	1125	96%	0.0	97.56	MH587028.1
Brevibacillus invocatus strain AB14	1125	1125	96%	0.0	97.56	MH587025.1
Brevibacillus invocatus strain ZLynn1000-20	1125	1125	96%	0.0	97.56	KY316485.1
Brevibacillus sp. strain invocatus	1125	1125	96%	0.0	97.56	KX863514.1
Brevibacillus sp. mkj-11	1125	1125	96%	0.0	97.56	KU159202.1
Brevibacillus sp. ADMK13	1125	1125	96%	0.0	97.56	KU850969.1
Brevibacillus invocatus strain CFPSW.5.1	1125	1125	96%	0.0	97.56	KT719835.1
Uncultured bacterium clone L54	1125	1125	96%	0.0	97.56	KJ554998.1
Brevibacillus invocatus strain SEPV-7	1125	1125	96%	0.0	97.56	KF228917.1

and then treated with 20% chloroform. The mixture was centrifuged for 15 min at 10000 rpm, and then the supernatant was precipitated by adding concentrated HCl until the pH was decreased to 3, then centrifuged. The base solubilization and acid precipitation steps were repeated five times more until the acid wash supernatant was almost colourless. The crude melanin was washed six times with distilled water, once with 100% methanol, 70% ethanol, and ether, and then air-dried and lyophilized. A dark brown powder is produced as a result of this process [38–41]. This pigment was collected and analyzed chemical based on solubility test and physically by Spectrophotometric, FTIR, SEM, and XRD analysis.

Chemical Characterization

Chemical nature of melanin was determined by its solubility test in water, inorganic solvents, such as NaOH, HCl, and organic solvents, as C_2H_6O and $CHCl_3$. Pigments were precipitated in HCl and C_2H_6O, and oxidized in H_2O_2. All these tests were carried out (Table 1) and compared with earlier studies done by Pacelli et al. [2].

PHYSICAL CHARACTERIZATION

Spectrophotometric Analysis

After extraction of melanin (0.1 g L^{-1}) pigments were dissolved in 10 M NaOH and its UV-visible spectrum was measured. 10 M NaOH was used as a blank and the instrument was set in a range of 200–800 nm for the analysis. The sample was scanned at the wavelength of 200–800 nm. The correlation between absorbance and wavelength was defined.

FTIR Analysis

The functional group of the molecule is determined by infrared spectroscopy analysis. Extracted pigment were analyzed with potassium bromide (KBr) as blank using FTIR spectrophotpmeter.

SEM Analysis

The scanning electron microscopy (SEM) is used for the morphological characterization and particle size distribution of extracted melanin. The extracted melanin was coated with gold-palladium (Au:Pd; 6O:40) layer using a coating sputter coater (Quorum Q 150 TES) to increase the conductivity of the sample. SEM was performed on same sample using imaging at 10 keV collected with JSM-IT 500 detector (Jeol) at 20 keV. SEM images showing the morphology of extracted melanin, at different magnifications, are presented in Fig. 2 [6].

XRD Analysis

The crystallinity of extracted melanin was determined by XRD. The extracted melanin was scanned using a SmartLab SE Rigaku corporation made in Japan X-ray diffractometer with CuK_α, radiation with wavelength, $\lambda(KK\alpha\alpha1) = 1.5405$ Å. The x-ray tube was powered with a current of 30 mA and a voltage of 40 kV. The step width was 0.01° and scanning speed was 5 deg/min. For extracted melanin X-ray scan range was kept at 10° to 90°. The thin films samples used for XRD measurement on clean glass slide. Sample is uniformly spread at the centre of slide and then analyzed.

RESULTS AND DISCUSSION

Isolation and Screening of Bacteria

Samples were taken from a paddy field in Amblikoppa village, India, near Dharwad. Soil was placed in a sterile container and transported to the laboratory right away. A total of 14 organisms were isolated from the soil, including 9 bacteria and 5 fungi. Nutrient agar

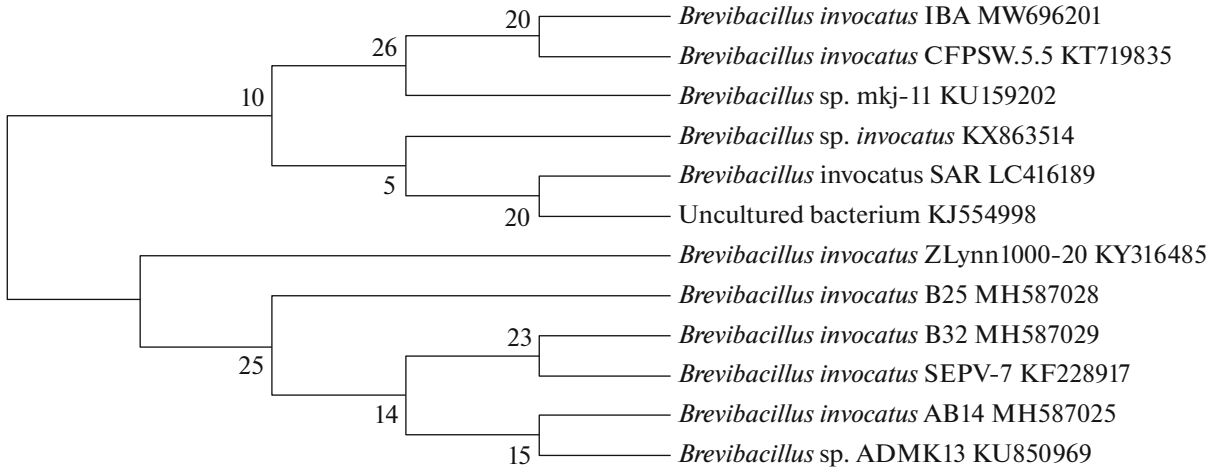

Fig. 2. Phylogenetic tree analysis of *Brevibacillus invocatus* strain IBA with the other similar genus of the *Brevibacillus* species.

and Potato dextrose agar were used as a base medium with L-tyrosine as a substrate for both bacteria and fungi respectively. Out of 9 bacteria, only 1 bacterium showed a change in media color from dark brown to black around the colonies (Fig. 1) after incubation period. The pigmentation was very less in fungi in comparison to the bacterial pigmentation. Hence, the bacterium which shown good pigmentation around the colony was taken forward for further study and the genus and species of the bacteria was identified by molecular method.

Molecular Identification of Bacteria

A BLAST scan of the GenBank database of with 678-bp 16 S rRNA partial gene sequence of unknown strain was identical to *Brevibacillus invocatus*. The strain had 97–100% identity to *Brevibacillus species* 16 S rRNA gene sequence. The phylogenetic tree depicts *Brevibacillus invocatus* strain IBA has close phylogenetic relationship with other *Brevibacillus invocatus* species. *Brevibacillus invocatus* strain IBA is consistently found in a clade with *Brevibacillus invocatus* SAR (GenBank/EMBL/DDBJ accession no. LC416189.1, similarity 97.56%), *Brevibacillus invocatus* B32 (GenBank/EMBL/DDBJ accession no. MH587029.1, similarity 97.56%), *Brevibacillus invocatus* B25 (GenBank/EMBL/DDBJ accession no. MH587028.1, similarity 97.56%), *Brevibacillus invocatus* AB14 (GenBank/EMBL/DDBJ accession no. MH587025.1, similarity 97.56%). Based on previously collected data and a comparison of the morphological, cultural, and physiological characteristics of the isolate *Brevibacillus invocatus* strain IBA in comparison to its nearest phylogenetic *Brevibacillus* genus neighbors (Table 1), in relation to its most closely related *Brevibacillus invocatus* SAR (GenBank/EMBL/DDBJ accession no. LC416189.1, the highest degree of similarity 97.56%). As a result, this strain was Identified as *Brevibacillus invocatus* strain IBA.

Melanin Production

Melanin production was checked in nutrient broth composition used as mentioned above. Change in the media color was observed from yellow to black color. As showed in Fig. 3. Melanin production was checked spectroscopically.

Chemical characterization of melanin produced by *Brevibacillus invocatus* strain IBA. The pigment extracted from *Brevibacillus invocatus* strain IBA was soluble in 10 and 1 M NaOH and insoluble in 1 M HCl and inorganic solvents, such as $C_2H_6O_4$ and $CHCl_3$ (Table 2). The extracted pigment was precipitated in HCl and C_2H_6O and oxidized in 30% H_2O_2. Therefore extracted pigment was assigned to be melanin based on its chemical properties as previously reported by Gadd [13] and Pacelli et al. [2]. Chemical properties of extracted melanin were compared with synthetic melanins DOPA and DHN as reported by the Pacelli et al. [2]. Based on this comparison, the melanin could not be typed. Hence the extracted melanin is purified and taken for further analysis.

Physical characterization of melanin produced by *Brevibacillus invocatus* strain IBA. *Spectrophotometric analysis.* The absorbance of the melanin in UV–Vis spectra was strongest in the UV region of 200 to 300 nm (Fig. 4a), but decreased towards the visible region (Fig. 4b). The linear curve was obtained after plotting the logarithm of absorbance against wavelength.

The UV–Vis measurement reveals UV–Vis absorbance in the 200–800 nm optical range. There are two distinct spectral regions: 200–300 nm and 300–800 nm. The absorption was strongest in the UV region of 200 to 300 nm, but decreased towards the vis-

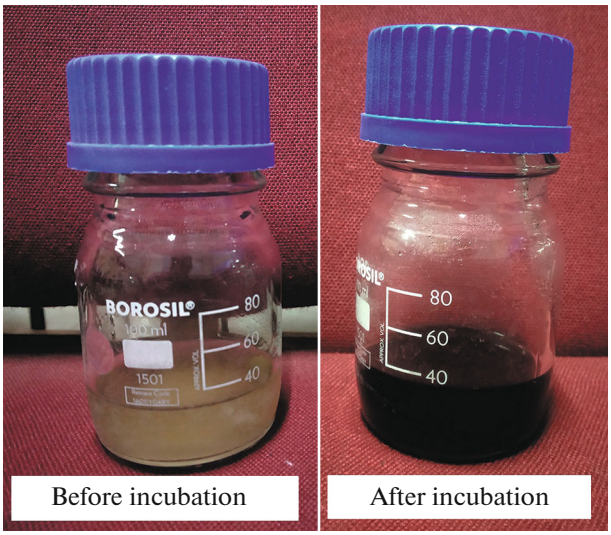

Fig. 3. Showing the production of melanin by *Brevibacillus invocatus* strain IBA. Before incubation there is no change in the colour and after incubation of around 12 to 18 h dark black pigmentation was formed with the indication of melanin formation.

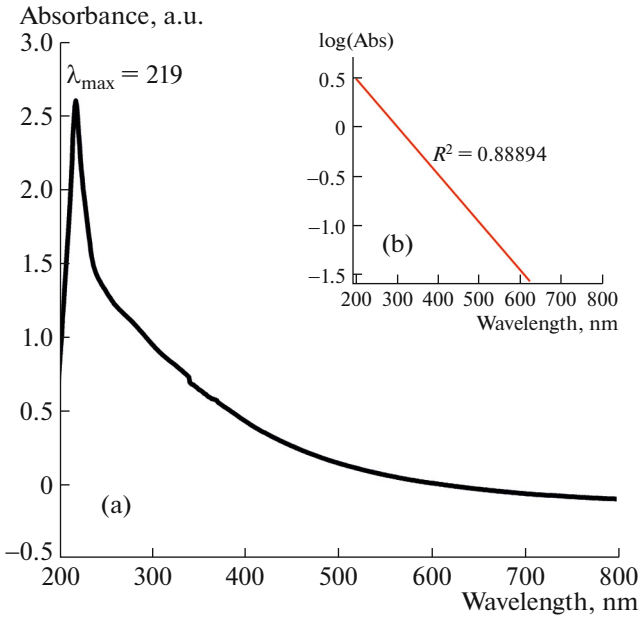

Fig. 4. (a) The UV–Vis spectra of extracted melanin of *Brevibacillus invocatus* strain IBA. (b) The linear curves obtained after plotting the logarithm of absorbance against the wavelength [3].

ible region. It has a single sharp peak that is centered at 219 nm. The UV–Vis spectrum of synthesized melanin nanoparticles [45, 46] has a similar overall structure. The UV–Vis spectrum of the melanin samples is shown in Fig. 4. According to Huang et al., melanin does not have distinct absorption peaks that differentiate it from other cutaneous chromophores. Instead, as wavelengths increase from 300 to 800 nm, melanin absorption decreases monotonically [45, 47].

The UV–Vis spectrum revealed the typical absorption profile of melanin, which is characterized by heavy absorption in the UV–Vis spectral range, a nearly featureless line form, and absorbance values that decrease monotonically from the UV–Vis spectral area [45, 47–49]. The $\pi\pi \rightarrow \pi\pi^*$ and $n \rightarrow \pi\pi^*$ of the amino, carboxylic, and aromatic moieties are responsible for the high UV absorption in the 200–300 nm range of the spectrum [48]. The UV–Blue energy transitions relates to the transition after the nonbonding orbital n to the anti-bonding orbital $\pi\pi^*$ ($n \rightarrow \pi\pi^*$), which follows mainly in carbonyl (C=O) bonds that are very abundant in the melanin. Region 2 (300–800 nm) shows a quasi-constant and large absorbance which definitely is the source of the black color of the extracted melanin itself. This wide-ranging and large spectral absorption is accredited to strong absorption transitions containing the orbital energy of the anti-bonding $\pi\pi^*$ and the bonding $\pi\pi$ ($\pi\pi \rightarrow \pi\pi^*$). This later occurs in the aromatic-unsaturated C bonds [48, 50]. The measure of delocalized electron in the extracted melanin structure and the high degree of conjugation enable the transitions to the anti-bonding

Table 2. Chemical characterization of *Brevibacillus invocatus* strain IBA extracted melanin compared with DHN and DOPA melanins [2]

	Chemical tests	DHN melanin	DOPA melanin	Melanin extracted from *Brevibacillus invocatus* strain IBA
Solubility in inorganic solvents	NaOH 1 M	+	+	+
	HCl 1 M	–	–	–
Solubility in inorganic solvents	C_2H_6O	–	–	–
	$CHCl_3$		–	–
Precipitation	HCl	Light	Light	Light
	C_2H_6O	Heavy	Heavy	Heavy
Oxidation	30% H_2O_2	$+^O$	$+^O$	$+^O$

Soluble = +, insoluble = –, low precipitation = light, high precipitation = heavy, high oxidation = $+^O$, light oxidation = $-^O$.

ππ* orbitals. A strong absorption of extracted melanin in the red part of visible extracted melanin is mainly due to many carbonyl groups present in its indolic groups [45, 48]. This is the cradle of the black color of extracted melanin. After plotting the logarithm of absorbance against the wavelength, negative slope of the extracted melanin (Fig. 4b) was (–0.00487). Similar results with little difference were obtained [3].

FTIR Analysis

FTIR spectroscopy is measurement of the wavelength and intensity of the absorption of mid-infrared light by a sample. Mid-infrared light (4000–500 cm^{-1}) is energetic enough to excite molecular vibrations to higher energy states (42). The wavelength of many IR absorption bands is characteristic of specific types of chemical bonds, and IR spectroscopy finds its greatest utility for qualitative analysis of organic and organometallic molecules. IR spectroscopy is used to identify particular compound or a newly synthesized molecule (43, 44).

Melanin FTIR spectra typically have three distinct bands: 3600–3000, 1650–1600, and 1500–1400 cm^{-1} [51–55]. Figure 5 shows the FT-IR spectrum of extracted Melanin. The stretching vibration of hydroxyl (O–H) and amine (N–H) groups was due to the characteristic peak at 3226 cm^{-1} [56].

The symmetric and asymmetric C–H stretching vibrations of methylene ($–CH_2$) groups are aligned with the peaks at 2920 and 2849 cm^{-1}, respectively. The presence of a sharp characteristic peak at 1628 cm^{-1} corresponded to the stretching vibration of carbonyl (C=O). The C–N stretching vibration of aromatic amine was attributed to the peak at 1340 cm^{-1}. According to previous research papers, unique stretches 1500–1400 cm^{-1} were found in some melanin and were due to the bending vibration of N–H and the stretching vibration of C–N (secondary amine) of an indolic system. Based on this, a characteristic pick appears at 1555 cm^{-1}, indicating the existence of N–H and C–N (secondary amine) groups. As a result, the extracted melanin FT-IR result was very similar to previously recorded melanin [3, 52, 55, 57]. The stretching vibration of phenolic –OH groups causes absorption bands between 1250 and 1180 cm^{-1} in several melanin spectra [58, 59]. The weak absorption bands found between 1150 and 1100 cm^{-1} may be due to the C–O–C bond's symmetric contraction vibration [60]. Absorption bands below 1100 cm^{-1} were normally weak and were attributed to C–H bond in-plane deformation, aromatic CH groups, or alkene CH substitution/conjugated systems [58, 61–63]. Other separate bands have been established depending on the source of the melanin [59].

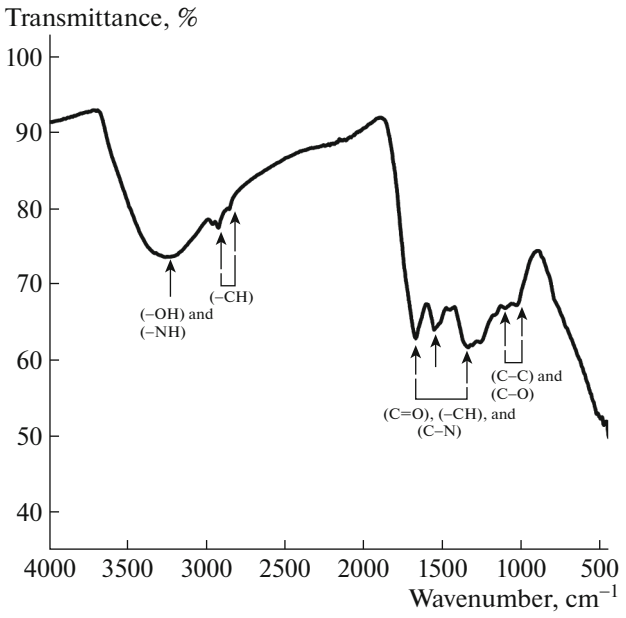

Fig. 5. FTIR spectra of extracted melanin from *Brevibacillus invocatus* strain IBA.

SEM Analysis

Scanning electron microscopy (SEM) is a powerful technique for the structural characterization and particle size distribution of different types of melanin [64, 65]. Based on this structural characterization is carried out which is shown the extracted melanin showed the irregular shape and size at different magnifications (Fig. 6).

The magnifications at 10, 5, 2, and 1 μm showed the irregular structure of extracted melanin. Depending on the melanin source, the granule morphology and size range between 30–1000 nm and melanin granules are usually amorphous with irregular shape as per the previous studies [59, 66–69].

XRD Analysis

Peaks at 2θ angles are visible in a standard XRD spectrum. The type of crystals present on the sample can be determined by the location of the peaks. This is achieved by conducting qualitative phase analysis (search match) with the ICDD PDF-2 2021 database for evaluation in order to identify the crystal phase. The absence of structure in the diffraction pattern corresponding to any important crystallinity in this extracted melanin was observed in the results from the extracted melanin sample. A strong non-Bragg diffraction pattern dominated the spectrum. XRD spectra of a highly concentrated extracted melanin solution drop coated onto 2 × 2 cm cleaned glass substrates are shown in Fig. 7. Sharp peaks in the diffraction spectrum caused by X-ray scattering by crystalline structures serve as a signature for the crystal being studied.

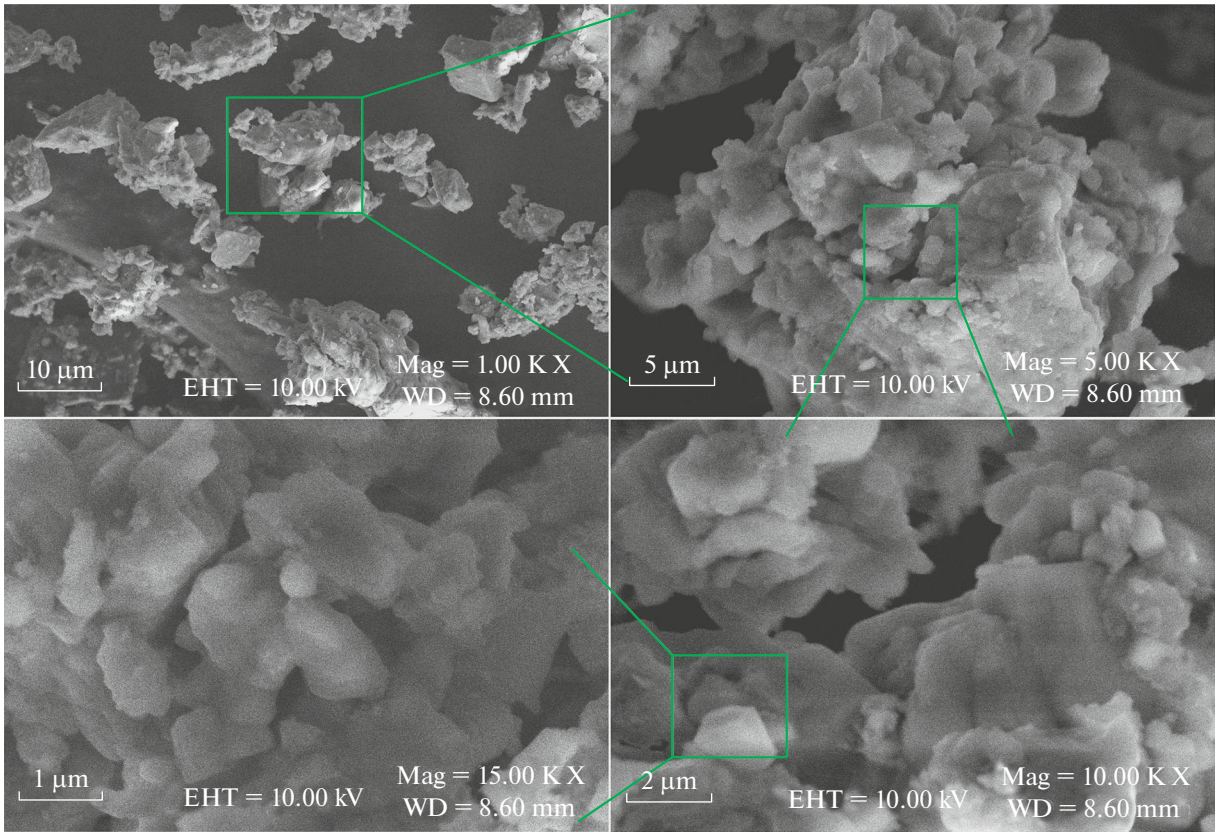

Fig. 6. SEM image showing the multi-scale morphology of extracted melanin at different magnifications.

Amorphous compounds such as extracted melanin, on the other hand create large features in a diffraction spectrum known as non-Bragg features as a result of the absence of coherent scattering from normal and repeated structures as seen in crystals [45, 70, 71].

The sharp diffraction peaks visible on the spectrum (except for the first peak, which was identified by the software) are caused by NaCl molecules, which have been reported by EDS of Mbonyiryivuze et al., 2015 to be a component of sepia melanin. As mentioned above the extracted melanin crystallographic patterns showed peaks at $2\theta = 27.32, 31.66, 45.41, 53.84, 56.44, 66.18, 73.10, 75.26$, and 83.94, which correspond to reflections (111), (200), (220), (311), (222), (400), (331), (420), and (422), respectively, as shown in the XRD spectra shown in Fig. 7. XRD spectra of extracted melanin is similar with little difference of Mbonyiryivuze et al., 2015 sepia melanin. This crystallographic structure of extracted melanin lattice parameter is approximately $a = 8.54$ (JCPDS 00-001-0993) and Mbonyiryivuze et al., 2015 sepia melanin was to be found lattice parameter is $a = 5.6$.

On the XRD spectra of extracted melanin, a broad diffraction peak ($2\theta = 10^{o}$–90^{o}) reveals its amorphous nature. Melanin structures are unclear due to its amorphous, insoluble, and heterogeneous existence [71]. The application of XRD techniques to the structure analysis of amorphous materials like extracted melanin is limited. The extracted melanin XRD result was very similar to Mbonyiryivuze et al. [2, 45, 71, 72]. XRD pattern of sepia melanin was carried out by Mbonyiryivuze et al., 2015, and the findings of sepia melanin are used as a standard in several research publications.

CONCLUSION

The main objective of this study is to demonstrate isolation of melanin producing *Brevibacillus invocatus* strain and to establish various analytical methods which can be used to characterize melanin from various matrices, either qualitatively or quantitatively.

This is the first research on the extraction of melanin from *Brevibacillus invocatus* strain. The strain was isolated from soil of paddy fields of Amblikoppa (15.357723° N 74.943763° E). Dharwad, India. The strain was identified by molecular method and confirmed. An attempt was made to characterize the melanin with various analytical methods in this study. Chemical characterization of melanin based on its solubility in different organic and inorganic solvents was done, according to the results melanin dissolved in NaOH Physical characterization of melanin was carried out by UV–visible spectra, FTIR for functional

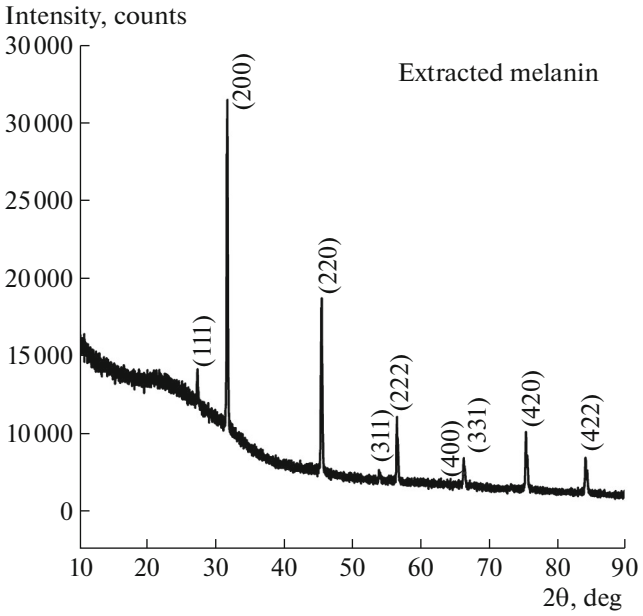

Fig. 7. XRD spectrum of extracted melanin with scan range from 10° to 90°.

group identification of melanin at different Strong and weak absorption can be determined, SEM for morphology of melanin at different magnifications and XRD for crystallographic study of melanin.

Since the chemical synthesis of melanin is cost effective, a biotechnological approach to melanin production by bacteria, fungi, and actinomycetes will lower production costs. The analytical methods available for melanin analysis are largely complementary, providing detailed knowledge required to draw reliable conclusions about the sample under investigation. The most optimal work flow for qualitative and quantitative approaches will be GC-MS, MALDI, and LC-MS, respectively. The best method for structural elucidation will be microscopic techniques (SEM, TEM, and AFM), as well as FTIR, XRD, and other techniques. Basic determinations such as solubility and UV-spectra, which can provide useful knowledge in a short amount of time, should not be overlooked.

This is an attempt to produce melanin from a bacteria *Brevibacillus invocatus* and characterize the melanin by using different analytical tools. However, more information about the structure, properties, and functional relationships is needed. Melanin research may aid in the development of creative and long-term solutions for human health and the environment, as well as long-term exploration.

ACKNOWLEDGMENTS

We are grateful to Karnatak University Dharwad, for providing us University Researcher Scholar Fellowship. We thankful to P.G Department of studies in Biotechnology and Microbiology for providing necessary laboratory facilities to carry out the research work. We extend our deepest gratitude to University Scientific Instrumentation Centre-Sophisticated Analytical Instrument Facility under DST for letting us to use the analytical instruments. We would like to extend our special thanks to technical assistant Pavan Bennur, Akash Badami, and Rajanagouda Shivalli for providing the data through various analytical instruments.

REFERENCES

1. Selbmann, L., Zucconi, L., Isola, D., and Onofri, S., *Curr. Genet.,* 2015, vol. 61, no. 3, pp. 335–345.
2. Pacelli, C., Cassaro, A., Maturilli, A., Timperio, A.M., Gevi, F., Cavalazzi, B., Stefan, M., Ghica, D., and Onofri, S., *Appl. Microbiol. Biotechnol.,* 2020, vol. 104, pp. 6385–6395.
3. Pralea, I.-E., Moldovan, R.-C., Petrache, A.-M., Ilies, M., Heghes, S.-C., Ielciu, I., Nicoara, R., Moldovan, M., Ene, M., Radu, M., Uifalean, A., and Iuga, C.-A., *Int. J. Mol. Sci.,* 2019, vol. 20, p. 3943.
4. El-Ahmady El-Naggar, N. El-Ewasy, S.M., *Sci. Rep.,* 2017, vol. **7**, p. 42129.
5. Langfelder, K., Streibel, M., Jahn, B., Haase, G., and Brakhage, A.A., *Fungal Genet. Biol.,* 2003, vol. 38, pp. 143–158.
6. Ikeda, K. et al., *FEMS Microbiol. Lett.,* 1998, vol. 168, pp. 195–199.
7. Marino, S.M. et al., *Arch. Biochem. Biophys.,* 2011, vol. 505, pp. 67–74.
8. Seetharam, G.B. and Saville, B.A., *Water Res.,* 2003, vol. 37, 436–440.
9. Tarangini, K. and Mishra, S., *Res. J. Eng. Sci.,* 2013, vol. 2, no. 5, pp. 40–46.
10. Nappi, A. J. and Ottaviani, E., *Bioessays,* 2000, vol. 22, pp. 469–480.
11. Plonka, P.M. and Grabacka, M., *Acta Biochim. Pol.,* 2006, vol. 53, pp. 429–443.
12. Allam, N.G. and El-Zaher, E.A., *Afr. J. Biotechnol.,* 2012, vol. 11, pp. 6566–6577.
13. Gadd, G.M. and de Rome, L., *Appl. Microbiol. Biot.,* 1988, vol. 29, pp. 610–617.
14. Casadevall, A., Rosas, A.L., and Nosanchuk, J.D., *Curr. Opin. Microbiol.,* 2000, vol. 3, pp. 354–358.
15. Riley, P., *Int. J. Biochem. Cell Biol.,* 1997, vol. 29, pp. 1235–1239.
16. Huang, H.C. and Chang, T.M., *World J. Microb. Biot.,* 2012, vol. 28, pp. 2903–2912.
17. Ye, M. et al., *Int. J. Biol. Macromol.,* 2014, vol. 63, pp. 170–176.
18. Gallas, J. and Eisner, M., *US Patent 7029758,* 2006.
19. Manivasagan, P., Venkatesan, J., Senthilkumar, K., Sivakumar, K., and Kim, S.K., *Int. J. Biol. Macromol.,* 2013, vol. 58, pp. 263–274.

20. El-Obeid, A., Al-Harbi, S., Al-Jomah, N., and Hassib, A., *Phytomedicine,* 2006, vol. 13, pp. 324–333.

21. Hung, Y.C., Sava, V., Hong, M.Y., and Huang, G.S., *Life Sci.,* 2004, vol. 74, pp. 2037–2047.

22. Sava, V., Hung, Y., Blagodarsky, V., Hong, M.Y., and Huang, G., *Food Res. Int.,* 2003, vol. 36, pp. 505–511.

23. Kurian, N.K., Nair, H.P. and Bhat, S.G., *Asian J. Pharm. Clin. Res.,* 2015, vol. 8, pp. 251–255.

24. Hoogduijn, M., Cemeli, E., Anderson, D., Wood, J., and Thody, A., *Exp. Cell Res.,* 2003, vol. 294, pp. 60–67.

25. Kalka, K., Mukhtar, H., Turowski-Wanke, A., and Merk, H., *Skin Pharmacol. Phys.,* 2000, vol. 13, pp. 143–149.

26. Montefiori, D.C. and Zhou, J., *Antiviral Res.,* 1991, vol. 15, pp. 11–25.

27. Wan, X. et al., *J. Appl. Microbiol.,* 2007, vol. 103, pp. 2533–2541.

28. Balagurunathan, R. and Radhakrishnan, M., in *Industrial Exploitation of Microorganisms,* Maheshwari, D.K., Dubey, R.C., and Saravanamuthu, R., Eds., New Delhi: International Publishing House, 2010, pp. 302–321.

29. Dastager, S. et al., *Afr. J. Biotechnol.,* 2006, vol. 5, no. 8, pp. 1131–1134.

30. Dadachova, E., Revskaya, E., Sesay, M.A., Damania, H., Boucher, R., Sellers, R.S., Howell, R.C., Burns, L., Thornton, G.B, Natarajan, A., Mirick, G.R., DeNardo, S.J., DeNardo, G.L., and Casadevall, A., *Cancer Biol. Ther.,* 2008, vol. 7, pp. 1116–1127.

31. Surwase, S.N., Jadhav, S.B., Phugare, S.S., and Jadhav, J.P., *3 Biotech,* 2013, vol. 3, pp. 187–194.

32. Hoti, S. and Balaraman, K., *J. Gen. Microbiol.,* 1993, vol. 139, pp. 2365–2369.

33. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning,* New York: Cold Spring Harbor Laboratory Press, 1989.

34. El-Naggar, N.E., Sherief, A., and Hamza, S.S., *Afr. J. Microbiol. Res.,* 2011, vol. 5, pp. 5308–5315.

35. Altschul, S.F., et al., *Nucleic Acids Res.,* 1997, vol. 25, pp. 3389–3402.

36. Saitou, N. and Nei, M., *Mol. Biol. Evol.,* 1987, vol. 4, pp. 406–425.

37. Tamura, K., Dudley, J., Nei, M., and Kumar, S., *Mol. Boil. Evol.,* 2007, vol. 24, pp. 1596–1599.

38. Liu, Y., Kempf, V., Nofsinger, B., Weinert, E., Rudnicki, M., Wakamastu, K., Ito, S., and Simon, J., *Pigm. Cell Melan. Res.,* 2003, vol. 16, pp. 355–365.

39. Wan, X., Liu, Y., Su, Y., Geng, J., Yang, M., Chen, X., and Shen, P., *J. Appl. Microbiol.,* 2007, vol. 103, pp. 2533–2541.

40. Zhang, J., Cai, J., Deng, Y., Chen, Y., and Ren, G., *Front. Biol. China,* 2007, vol. 2, pp. 26–29.

41. Aghajanyan, A., Hambardzumyan, A., Hovsepyan, A., Asaturian, R., Vardanyan, A., and Saghiyan, A., *Pigm. Cell Melan. Res.,* 2005, vol. 18, pp. 130–135.

42. Patwardhan, S.V. and Swann, G.E.A., *Clim. Past,* 2011, vol. 7, pp. 65–74.

43. D'Amico, F., Control of structural conformation and electronic properties of organic semiconductors thin films, *PhD Thesis,* Camerino University, 2008.

44. Mbonyiryivuze, A., Mwakikunga, B., Dhlamini, S.M., and Maaza, M., *Phys. Mat. Chem.,* 2015, vol. 3, pp. 25–29.

45. Mbonyiryivuze, A., Omollo, I., Ngom, B.D., Mwakikunga, B., Dhlamini, S.M., Park, E., and Maaza, M., *Phys. Mat. Chem.,* 2015, vol. 3, pp. 1–6.

46. Kim, D.J., Ju., K.-Y., and Lee, J.K., *Kor. Chem. Soc.,* 2012, vol. 33, no. 11, pp. 3788–3792.

47. Huang, H.Lui., Chen, A., Alajlan, D.I., Mclean, D.I., and Zeng, H., *J. Biomed. Optics,* 2004, vol. 9, pp. 1198–1205.

48. Magarelli, M., Purification, characterization, and photodegradation studies of modified sepia melanin Sepia (*Sepia officinalis*). Determination of eumelanin content in fibers from Alpaca (*Vicugna pacos*), *Doctoral Thesis.,* Macerata: University of Camerino, 2011.

49. Perna, M., Lasalvia, C., Gallo, G., Quartucci, G., and Capozzi, V., *Open Surface Sci. J.,* 2013, vol. 5, pp. 1–8.

50. Magarelli, P., Passamonti, P., and Renieri, C., *Rev. CES Med. Vet. Zootec.,* 2010, vol. 5, no. 2, pp. 18–28.

51. Haining, R.L. and Achat-Mendes, C., *Neur. Regen. Res.,* 2017, vol. 12, pp. 372–375.

52. Selvakumar, P., Rajasekar, S., Periasamy, K., and Raaman, N., *J. Microbiol. Biotechnol.,* 2008, vol. 24, pp. 2125–2131.

53. Ye, M, Guo, G.Y, Lu, Y, Song, S, Wang, H.Y, and Yang, L., *Int. J. Biol. Macromol.,* 2014, vol. 63, pp. 170–176.

54. Hu, W, Dai, D, Huang, G, and Zhang, Z., *Am. J. Food Technol.,* 2015, vol. 10, pp. 68–77.

55. Dadachova, E., Bryan, R.A., Huang, X., Moadel, T., Schweitzer, A.D., Aisen, P., Nosanchuk, J.D., and Casadevall, A., *PLoS ONE,* 2007, vol. 2, p. e457.

56. Cordero, R.J. and Casadevall, A., *Fungal Biol. Rev.,* 2017, vol. 31, pp. 99–112.

57. Gómez-Marín, A.M. and Sánchez, C.I., *J. Non Cryst. Solids,* 2010, vol. 356, pp. 1576–1580.

58. Wang, L.F. and Rhim, J.W., *LWT,* 2019, vol. 99, pp. 17–23.

59. El-Naggar, N.E.A. and El-Ewasy, S.M., *Sci. Rep.,* 2017, vol. 7, p. 42129.

60. Ye, Z., Lu, Y., Zong, S. Yang, L., Shaikh, F., Li, J., and Ye, M., *Proc. Biochem.,* 2019, vol. 76, pp. 203–212.

61. Xin, C., Ma, J.H., Tan, C.J., Yang, Z., Ye, F., Long, C., Ye, S., and Hou, D.B., *J. Biosci. Bioeng.,* 2015, vol. 119, pp. 446–454.

62. Hou, R., Liu, X., Xiang, K., Chen, L., Wu, X., Lin, W., Zheng, M., Fu, J., and Lia, W., *Food Chem.,* 2019, vol. 277, pp. 533–542.

63. Raman, N.M. and Ramasamy, S., *Biochem. Biophys. Rep.,* 2017, vol. 12, pp. 98–107.

64. Mbonyiryivuze, A., Nuru, Z.Y., Ngom, B.D. Mwakikunga, B., Dhlamini, S.M., Park, E., and Maaza, M., *Am. J. Nanomater.,* 2015, vol. 3, pp. 22–27.

65. Strube, O.I., Bungeler, A., and Bremser, W., *Bio Macromol.,* 2015, vol. 16, pp. 1608–1613.

66. Li, C., Ji, C., and Tang, B., *FEMS Microbiol Lett.,* 2018, vol. 365, pp. 1–8.

67. Agodi, A., Stefani, S., Corsaro, C., Campanile, F.R.M., Gribaldo, S., and Sichel, G., *Res. Microbiol.,* 1996, vol. 147, pp. 167–174.

68. Tarangini, K. and Mishra, S., *Res. J. Eng. Sci.,* 2013, vol. 2, pp. 40–46.

69. Amin, S., Rastogi, R.P., Sonani, R.R., Ray, A., Sharma, R., and Madamwar, D., *Sci. Total. Environ.,* 2018, vol. 635, pp. 323–332.

70. Casadevall, A. and Nakouzi, P.R., Crippa, P.R., and Eisner, M., *PLoS ONE,* 2012, vol. 7, no. 2, p. e30299.

71. Sajjan, G.B., Kulkarni, A.S., and Nayak, S.B., *Kor. J. Microbiol. Biotechnol.,* 2013, vol. 41, pp. 60–69.

72. Subianto, S., Electrochemical synthesis of melanin-like polyindolequinone, *PhD. Thesis,* Queensland University of Technology, 2006.