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Properties and Functions of Melanin Pigment from *Klebsiella* sp. GSK

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Purified melanin pigment from *Klebsiella* sp. GSK was characterized by thermogravimetric, differential thermal, X-ray diffraction and elemental analysis. This melanin pigment is structurally amorphous in nature. It is thermally stable up to 300°C and emits a strong exothermic peak at 700°C. Its carbon, hydrogen and nitrogen composition is 47.9%, 6.9% and 12.0%, respectively. It was used to scavenge metal ions and free radicals. After immobilizing the pigment and using it to adsorb copper and lead ions, the metal ion adsorption capacity was evaluated by atomic absorption spectroscopy (AAS) and the identity of melanin functional groups involved in the binding of metal ions was determined by Fourier transform infrared (FT-IR) spectroscopy. Batch adsorption studies showed that 169 mg/g of copper and 280 mg/g of lead were adsorbed onto melanin-alginate beads. The metal ion adsorption capacity of the melanin-alginate beads was relatively significant compared to alginate beads. The metal ion desorption capacity of HCl was greater (81.5% and 99% for copper and lead, respectively) than that of EDTA (80% and 71% for copper and lead, respectively). The ability of the melanin pigment to scavenge free radicals was evaluated by inhibition of the oxidation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and was shown to be about 74% and 98%, respectively, compared with standard antioxidants.

Keywords: Adsorption, antioxidant, melanin, pigment, *Klebsiella*

Introduction

Melanins are an important class of natural pigments that have attracted great attention due to their biological role in photoprotection and technological applications [26]. These pigments are particularly intractable from an analytical perspective because they are photo-chemically very stable and are virtually insoluble in most organic solvents, acids and water [11]. Melanin pigment from microorganisms is a high molecular weight molecule that is decolorized by oxidizing agents. It has the ability to chelate metal ions and to strongly absorb uv-visible light [16]. However, despite significant scientific effort over the past 30 years, the basic functions of melanins are still a matter of controversy and speculation. This uncertainty results from the few and poorly defined structural and physicochemical properties that puzzle researchers even though their adaptive importance has already been proven [1, 25].

Environmental pollution by toxic metals occurs globally through different means such as industrial, military, agricultural and waste disposal activities. Many toxic heavy metals have been discharged into the environment as industrial wastes, causing serious soil and water pollution [18]. It is estimated that fuel and power industries generate 2.4 million tons of Pb, Cu, As, Cd, Cr, Hg, Ni, Se, V, and Zn annually [6]. Among them, Pb^{2+}, Cu^{2+}, Fe^{2+}, and Cr^{3+} are the most common metal ions that tend to accumulate in living organisms, causing numerous diseases and disorders [14]. Nonessential metal ions at high concentrations cause harm to living cells by potentially displacing essential metal ions in association with enzymes, competing with structurally related metal ions in cellular reactions, and by blocking

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functional groups in biomolecules [23]. The removal of heavy metals by adsorption is one of the most promising technologies for the removal of toxic metals from natural and industrial waste waters. It is a potential alternative technique for removing toxic metals in processes like ion exchange, precipitation and electro dialysis.

Adsorption of metal ions by cell wall components is one of the most important interaction mechanisms [37]. The principal mechanism of metallic cation sequestration involves the formation of complexes between a metal ion and different functional groups present on the surface or inside the porous structure of the biological material [9]. Biological pigments with different functional groups show different affinities for various metal ions [32]. Metal ions are normal constituents of natural melanins; in some cases, their role in the biosynthetic pathway of the pigment has been firmly established [19]. When in contact with solutions containing them, pigments are able to bind to them to maintain a proper metal ion balance [27]. It has been suggested that melanin polymers constitute the building blocks of melanin granules. The process of granule formation and their dimensions are strongly pH dependent wherein a low pH promotes aggregation and a high pH induces breakdown of granules to small particle-oligomers with a lower degree of polymerization. This process is a consequence of the polyelectrolytic nature of melanin, and is dependent on the ionization state of groups such as carboxylic, phenolic, and amine groups as well as on the ionic strength of the environment. These features make melanin a very complex adsorbing material [5, 41]. It has been suggested that microbial melanins possibly participate as precursors in the formation of soil humus. Similar to soil humic acids, the presence of the above-mentioned groups confers on these macromolecules a number of different potential binding sites for metal ions [10].

Phenolic compounds are a large and most interesting group of natural antioxidants that can undergo reversible oxidation and reduction and are involved in the exchange of electrons and protons. Animal and plant phenolic compounds such as tocopherols, naphtha quinones, ubiquinones, flavonoids, and pigments are used as antioxidants [21]. However, melanin pigments produced by living organisms have received little attention. Melanins are heteropolymers formed by the oxidative polymerization of tyrosine, dihydroxyphenylalanine and catecholamines [2, 17]. Melanins interact readily with free radicals and other reactive species because of the presence of unpaired electrons in their molecules. The melanin pigment from *Klebsiella* sp. GSK is very similar to typical melanins, which constitute a diverse group of aromatic polymers with many potential applications in the cosmetics and pharmaceutical industries. Bacterial synthesis of melanin pigment is an alternative option for commercial-scale production [30].

In this study, melanin pigment from *Klebsiella* sp. GSK was characterized by thermogravimetric analysis (TGA), differential thermal analysis (DTA), X-ray diffraction (XRD) and elemental analysis. It was immobilized in the form of melanin-alginate beads to remove Cu^{2+} and Pb^{2+} metal ions. FT-IR spectroscopy was used to explore the functional groups involved in binding metal ions. Metal ion adsorption was quantified using atomic absorption spectroscopy (AAS). Further, the free radical scavenging properties of the melanin pigment were studied using DPPH and ABTS.

Material and Methods

Chemicals and preparation of metal ion solutions

A multi-element standard metal ion solution for AAS analysis was procured from Fisher Scientific Chemicals, Mumbai, India. Standard metal ion solutions were prepared at 1000 mg/L in 0.5 M HNO_3. Working standards were prepared by appropriate dilution with water. ABTS and DPPH were procured from Fluka and Sigma Chemicals, respectively. All other chemicals and reagents used were of analytical reagent grade.

Bacterium

The melanin-producing bacterial strain *Klebsiella* sp. GSK used in this study was originally isolated from agriculture crop field soil. The bacterium was grown in mineral salt medium containing defined components, 55 mM glucose and L-tyrosine (1.4 mM), at pH 7.2. The medium was autoclaved at 15 psi (121°C) for 20 min and incubated at 37°C on a rotary shaker at 220 rpm for 72-96 h [30].

Extraction and purification of melanin pigment

Melanin pigment was extracted from bacterial spent medium and purified according to our previously reported method [30]. Briefly, spent medium was acidified with 1 N HCl to pH 2 and allowed to stand for a week at room temperature. Then, this suspension was boiled for 1 h and then

centrifuged. The black pigment pellet thus formed was washed three times with 15 ml of 0.1 N HCl followed by water. To this pellet, 10 ml of ethanol was added and the mixture was incubated in a boiling water bath for 10 min and then kept at room temperature for 1 d. The pellet was washed with ethanol two times and then dried in air. This purified melanin pigment was used for characterization and metal ion adsorption and free radical scavenging studies.

Thermal studies

TGA and DTA data were recorded using a Linseis STA PT-1600 (Germany) thermal analyzer under an atmosphere of air and at a heating rate of 5°C/min. The measurements were performed in ceramic crucibles under static air atmosphere using 10 mg of melanin pigment.

X-ray diffraction studies

Pure melanin powder was made into discs 1 cm in diameter and 1-2 mm thick. The melanin pigment was scanned using a Rigaku Ultima IV (Japan) X-ray diffractometer operating at a wavelength of 1.54056 Å with a step size of 0.02° and scanning rate of 2°/min X-ray beam at room temperature. Scattering intensity was recorded as a function of the scattering angle [7].

Elemental analysis

The percent composition of carbon, hydrogen, nitrogen and sulfur in the melanin pigment was analyzed with an Elementar Vario EL III CHN elemental analyzer (Germany). The operating conditions were: standard deviation of 0.1% and digestion temperatures of 950-1200°C. In this set-up, the analyzed products carbon, hydrogen, nitrogen and sulfur oxidize and form CO_2, H_2O, NO, and SO_2, respectively. These products were carefully collected and weighed. The weights were used to determine the elemental composition.

Immobilization of melanin pigment using sodium alginate

Sodium alginate (3%) was dissolved in water, autoclaved and allowed to cool to 40°C. To this, 1 mg/ml of pure melanin pigment was added with constant stirring on a magnetic stirrer. The alginate-melanin mixture was extruded dropwise through a burette fixed with a tapered pipette into a cold, sterile 2% calcium chloride solution. The beads thus formed were spherical in shape and their size was maintained at about 2.5-3 mm in diameter. Melanin-free alginate beads served as a control.

Metal ion adsorption studies

Adsorption studies in batch experiments were conducted for each metal ion as a function of a constant pH of 7.0, a temperature of 37°C and the respective metal ion concentrations. One gram of melanin alginate beads were taken in 50-ml conical flasks with different metal ion solutions (5 ml) and kept for 1 h at 37°C in an incubated shaker (100 rpm). After adsorption of metal ions, the suspensions were centrifuged at 6000×*g* for 10 min. The supernatant was used for subsequent metal ion analysis by flame AAS.

Desorption of metal ions

Metal ion desorption studies were carried out using EDTA and HCl. Metal ion-bound melanin-alginate beads were separated from the metal ion solution and 1 g of beads was mixed with either 5 ml of 1 mM EDTA or 0.1 M HCl for 1 h in an incubated shaker. Then, the contents were centrifuged and the supernatant was analyzed by AAS and the beads by FT-IR. The concentration of metal ion adsorbed or desorbed by the melanin-alginate beads in each sample was calculated by subtracting the amount of metal ions remaining in the supernatant from the original concentrations.

Atomic absorption spectrometry

A Thermo Scientific iCE 3000 series AAS fitted with a Beckman total-consumption burner for sample atomization with SOLAAR series software was used. An air/acetylene-type flame was used throughout the experiment at a wavelength of 324.8 nm for Cu^{2+} and 283.3 nm for Pb^{2+} using a hollow cathode lamp. Each time the AAS was aspirated with distilled water. A standard calibration curve was prepared for each metal ion before analysis. To read each solution, the AAS was aspirated for 1 min. Three readings were taken and an average reading was considered for the study.

Fourier transform infrared (FT-IR) spectroscopy

Metal ions adsorbed to melanin-alginate beads were analyzed by FT-IR spectroscopy. Each sample was dried and mixed with pure potassium bromide and then ground in an agate mortar. The resulting mixture was pressed at 10 tons pressure for 5 min to form pellets. These pellets were characterized with infrared transmission spectra using a

Perkin-Elmer FT-IR spectrometer (Waltham, MA, USA). The spectra were collected within a range of 400-4000 cm^{-1}. All spectra were recorded and plotted on the same scale on the transmittance axis.

Batch adsorption kinetic studies for metal ions

All adsorption experiments were carried out at a uniform concentration for each metal ion (20 ppm for Cu^{2+} and 40 ppm for Pb^{2+}). The amount of metal ion adsorbed, q_t (mg/g), was calculated using the mass balance equation as follows:

$$q_t = \frac{(C_o - C_t)V}{m}$$

where C_o and C_t are the concentration of the metal ion (mg/L) in the solution at time t = 0 and at a given time t (h), respectively. V is the volume of the metal ion solution (ml) and m, the weight of the sorbent (g).

DPPH radical scavenging activity

DPPH radical scavenging by purified melanin was estimated using the method of Liyana-Pathirana and Shahidi [20]. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 10 μl of melanin pigment at two different concentrations (25 and 50 μg were selected using the IC_{50} value). Ten micrograms of each standard antioxidant was used as positive controls. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the test samples was measured spectrophotometrically at 517 nm. Standard antioxidants were used as positive controls. The ability to scavenge DPPH radical was calculated by the following equation:

Free radical scavenging activity (%)

$$= \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the free radical solution (DPPH/ABTS) + methanol, and A_{sample} is the absorbance of the free radical solution with melanin/standard antioxidant.

ABTS radical scavenging assay

Stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate were prepared according to the method of Re *et al.* [29]. A fresh working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react at room temperature in the dark for 12 h. One milliliter of solution was then diluted with nearly 60 ml of methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Melanin pigment (10 μl) was allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 30 min using the spectrophotometer. The ABTS scavenging capacity of the melanin was calculated using the formula described earlier.

Results and Discussion

Properties of melanin pigment

Melanin pigment from *Klebsiella* sp. GSK, a natural polymer, was purified and characterized. The characteristic property of the melanin is its high thermal stability (Fig. 1), with a mass loss up to 220°C caused by the weight of water; approximately 20% of the initial mass of the melanin was strongly bound water. Between 220 and 350°C, the weight loss was 40%, a high percentage of which could be explained by considering melanin degradation because the purification processes removed all cellular molecules. A gradual weight loss was observed up to 1000°C, indicating the complete combustion of melanin pigment. A strong exothermic peak was attributed to the beginning of rapid polymer decomposition and an endothermic peak at 700°C was specified as weakly bound water [4, 24]. The TGA of this bacterial melanin is quite similar to that of a previously reported result for melanin [8]. Herein, the mass that was lost is greater than that lost by L-DOPA melanin during
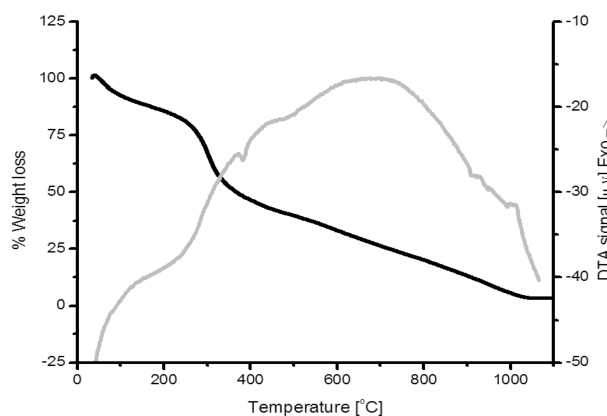

Fig. 1. TGA (black line) and DTA (grey line) spectra of melanin pigment isolated from *Klebsiella* sp. GSK.

electro-oxidation for 9 d and auto-oxidation for 21 d. In those cases, 40 and 55% of the total mass, respectively, was retained at temperatures up to 500ºC [8]. However, other works with different synthetic and natural melanins have reported retention of 12-26% of the initial weight [34]. Some authors have proposed a relationship between melanin resistance to thermal degradation and the origin of the molecule [31]. The only structure capable of withstanding such high temperatures would be graphite-like sheets [8]. *Klebsiella* sp. GSK bacterial melanin is thermally more stable than melanins produced by other organisms. However, one study has found that 17.5% of the initial mass of the L-DOPA melanin, obtained by auto-oxidation over 48 h, remains stable up to 500ºC [34], suggesting a close relationship between thermal stability and the degree of polymerization of the melanin.

The scattering of X-rays by crystalline structures produces sharp peaks in the diffraction spectrum that serve as a signature for the crystal that is analyzed. In contrast, the amorphous compound melanin produces broad features in a diffraction spectrum, known as non-Bragg features, resulting in the absence of coherent scattering from regular and repeating structures as observed in crystals. A consistent finding with all the samples is the lack of structure in the diffraction pattern corresponding to any significant crystallinity in the melanin preparations. The XRD spectra of the black melanin pigment present a broad diffraction peak ($2\theta=10-80º$) that can typically be attributed to amorphousness (Fig. 2). Melanin structures are uncertain [21] due to the amorphous, heterogeneous and insoluble nature of these pigments. Amorphous melanins are not amenable to study by crystallography. The utility of X-ray diffraction te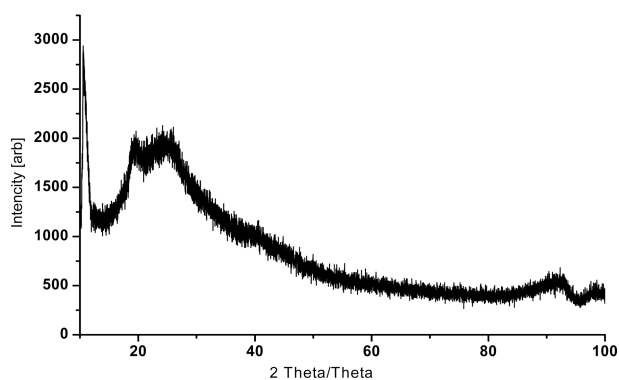chniques for the analysis of the structure of amorphous materials is limited [7]. In such materials, the orientations of the structural elements are random and the resulting spectrum represents an integrated spatial average. Such diffraction spectra can provide a template for iterative testing of proposed structures by rejecting those structures for which the calculated coherent scattering spectra are in significant disagreement with the measured spectrum after the background is removed [7]. In the general classification of melanins, eumelanin and pheomelanin are two types of pigments that are broadly accepted. Eumelanin does not contain sulfur while the mixed reddish brown pigment pheomelanin contains a variable percentage of sulfur [15]. Percentage elemental analysis of the *Klebsiella* sp. GSK melanin pigment showed it to be 47.9% carbon, 12.0% nitrogen, 6.3% hydrogen, and 0.8% sulfur (Table 1). This melanin belongs to the eumelanin subclass due to the considerable CHN content and the relatively very low concentration of sulfur, where the starting material used was L-tyrosine rather than cysteine. The small amount of sulfur may be retained in purification steps. Similar observations regarding the elemental composition of melanin pigments were made by several researchers [11-13, 15, 35, 36] (Table 1).

Metal ion adsorption onto melanin-alginate beads

FT-IR absorption spectra were interpreted on the basis of the vibration of the melanin-alginate beads. The band at 3445.18-3329.08 cm^{-1} is due to the asymmetric and symmetric stretching vibration of the melanin-alginate beads and the melanin pigment [30], which is mainly due to the presence of an -O-H group, usually H-bonded, and the presence of an aldehyde -C-H group indicated by two bands at 2850-2925 cm^{-1}. The band with two peaks at 1637.57-1424.60 cm^{-1} is due to the stretching of -C-C/C=N and -C=C, respectively. The peaks at 1026.08 cm^{-1} and 875 cm^{-1} are correlated to the asymmetric stretching vibration of -C-N and aromatic -C-H groups, respectively. In the melanin-alginate beads, a strong sharp peak at 875 cm^{-1} indicates the presence of a =CH$_2$ group, which had disappeared after binding to metal ions, whereas in the HCl-chelated FT-IR spectra, a new peak near 1740 cm^{-1} is due to the exposure of an anhydride carboxylic acid group. The binding of metal ions to the melanin pigment causes a shift in the FT-IR vibration and depends on the percent adsorption. The changes in binding properties correlated with those of the AAS studies, giving an accurate comparison of

Fig. 2. XRD analysis of *Klebsiella* sp. GSK melanin pigment showing its amorphous state.

Table 1. Elemental analysis of melanin pigment from *Klebsiella* sp. GSK compared with other melanins.

Precursors/origin of melanin pigment	% Carbon	% Hydrogen	% Nitrogen	% Sulfur	Reference
Klebsiella sp. GSK	47.9	6.9	12.0	0.9	Present study
Baccilus subtilis	70.0	–	12.0	4.1	[11]
Sepia	43.3	3.3	7.6	–	[12]
*Dopamine	48.2	3.4	7.1	–	[13]
*L-Cysteine, L-Dopa	33.5	4.9	10.8	22.3	[13]
†Tyrosine (Sigma)	48.0	–	6.6	–	[13]
Synthetic dopa melanin	56.5	3.2	8.5	0.1	[15]
Pheomelanin	46.2	4.5	9.4	9.8	[15]
Electrochemical	47.0	4.0	7.0	–	[35]
TSBF	59.5	4.2	6.6	0.6	[36]

Oxidation by *Tyrosinase and †H_2O_2, melanin from the muscles of Taihe Black-bone silky fowl (TSBF).
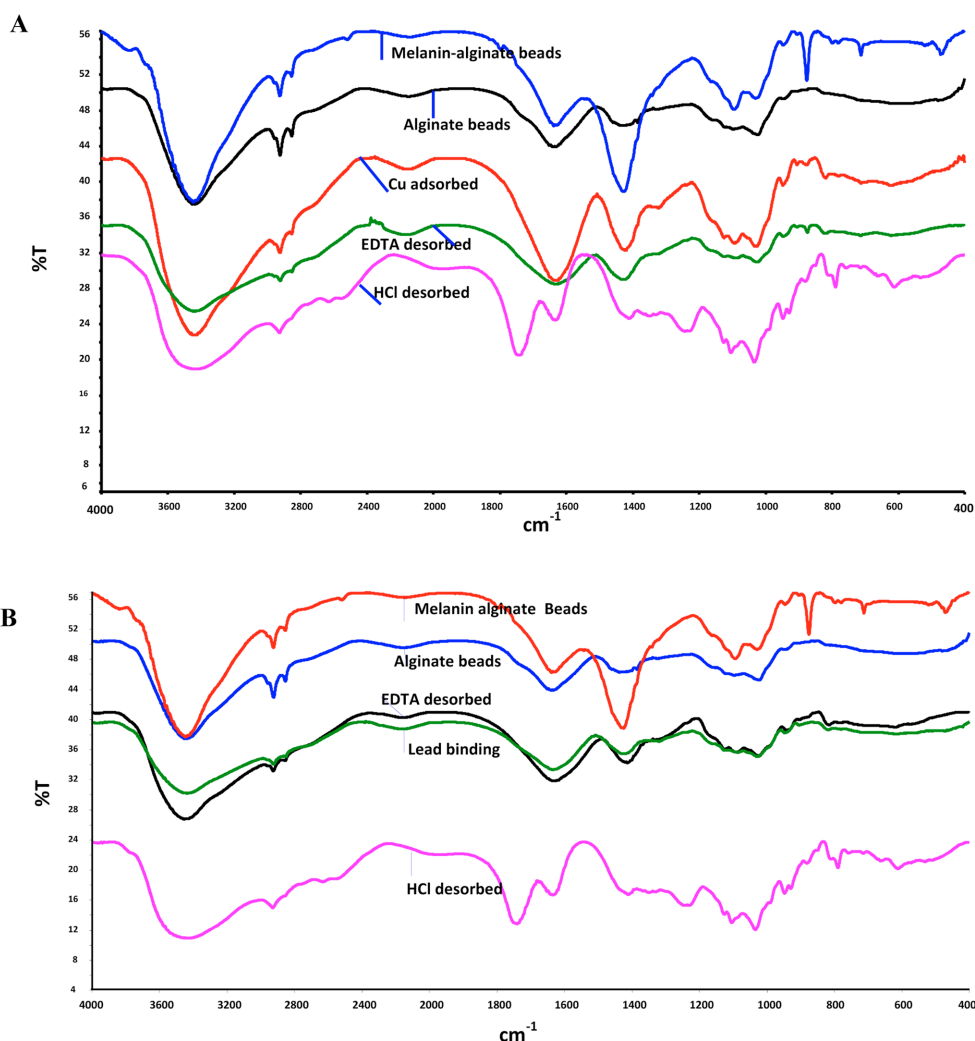

Fig. 3. FT-IR spectra for metal ion adsorption to melanin-alginate beads and desorption by HCL and EDTA.
Spectra for copper metal ions (A) and lead metal ions (B).

the results in both adsorption and desorption studies. FT-IR analysis of metal ion-enriched squid melanin has demonstrated that the possible functional groups responsible for metal ion binding are phenolic hydroxyl (-OH), carboxyl (-COOH) and amine groups (-NH) [33]. The FT-IR spectral studies show that the changes in the absorption bands of the surface functional groups of the melanin pigment are due to metal ion adsorption. The stretching vibrations of the functional groups -N-H, -O-H, -C-H, -C-C, -C=C, -C=N, -C=O and -C-N usually form a broad band region within 400-4000 cm^{-1}. The maximum binding of most of the metal ions occurs within the first 10-15 min and remains fairly uniform throughout for 120 minutes [3]. Metal ion chelation was also carried out for 1 h under similar conditions with EDTA and HCl. The FT-IR studies show that the adsorption of Cu^{2+} on the melanin surface resulted in the shifting of a peak from 3445.18 cm^{-1} to 3432.20 cm^{-1}. The decrease in the wavenumber of the peak is attributed to the attachment of Cu^{2+} to -O-H and -N-H groups. Another noticeable feature is the shift in the frequency of the adsorption bands of both -C=O and -C-O in carboxyl groups (-COOH). The -C-O bond shifts to the lower frequency, from 1424.60 cm^{-1} to 1419.72 cm^{-1}, which can be attributed to the association of Cu^{2+} with hydroxyl groups. A shift of the -C=O bond to the lower frequency, from 1637.57 cm^{-1} to 1633.47 cm^{-1} (Fig. 3A), was observed. This result may be due to the high electron density induced by the adsorption of Cu^{2+} onto an adjacent hydroxyl group. In the lead ion adsorption study, we observed a noticeable shift in FT-IR vibrations to the higher band from 3445.18 cm^{-1} to 3455.54 cm^{-1}, which is attributed to bound amine groups. The shift from 1637.57 cm^{-1} to 1632.66 cm^{-1} indicates binding to -C=N and -C-C groups, and the shift from 1424.60 cm^{-1} to 1412.84 cm^{-1} shows the binding of Pb^{2+} ions to aromatic -C=C (Fig. 3B). Later, the beads were chelated with EDTA and HCl; the bound metal ions (Cu^{2+} and Pb^{2+}) were released from the melanin-beads, causing a backshift in the FT-IR adsorption peaks almost to their original positions (Fig. 3).

The AAS study showed that metal ions adsorb strongly to melanin-alginate beads, Cu^{2+} and Pb^{2+} much more strongly so (81.5% and 99%, respectively). Melanin-free beads were able to adsorb only 5% and 14.3% of Cu^{2+} and Pb^{2+} ions, respectively (Fig. 4), indicating that the melanin pigment is a good adsorbent of copper and lead metal ions. Metal ion chelated by HCl was about 88.6% and 107.2%, respectively, for Cu^{2+} and Pb^{2+} from *Solanum elaeagnifolium*
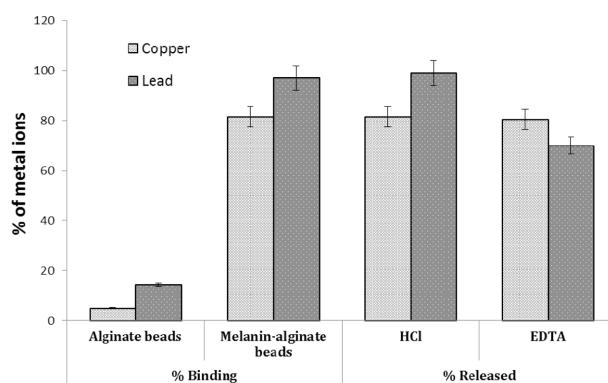

Fig. 4. Comparison of different metal ion adsorption and release capacities of melanin-alginate beads and alginate beads.
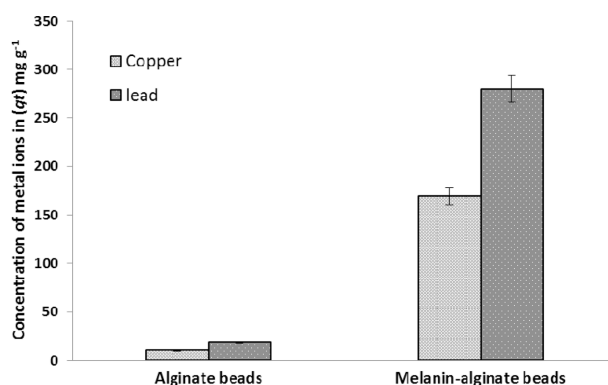The data obtained by AAS analysis.

Fig. 5. Metal ion batch adsorption studies.
The adsorption capacity q_t (mg/g) of melanin-alginate beads for each metal ion was compared to that of alginate beads.

biomass [3]. Cu^{2+} and Pb^{2+} were easily chelated by HCl to about 81.5% and 99%, and by EDTA to about 80.5% and 70%, respectively (Fig. 4). Wuyep *et al.* [39] reported that calcium alginate-immobilized mycelia of *Polyporus squamosus* adsorbed Cu^{2+} and Pb^{2+} at 31.6 ± 1.29 and 26.52 ± 1.10 mg/g, respectively, from untreated wastewater. The metal ion adsorption capacity of *Solanum elaeagnifolium* biomass was 20.6 mg/g of Pb^{2+} and 13.14 mg/g of Cu^{2+} [3]. Keratin powder prepared from Algerian sheep hoofs adsorbed 65 mg/g of Pb^{2+} [28]. A maximum of 134 mg/g of Pb^{2+} ions was adsorbed onto squid (*Ommastrephes bartrami*) melanin irrespective of temperature and pH [33], whereas herein the melanin-alginate beads adsorbed 169 mg/g of Cu^{2+} and 280 mg/g of Pb^{2+}, levels that are much higher than those in the earlier reports (Fig. 5).
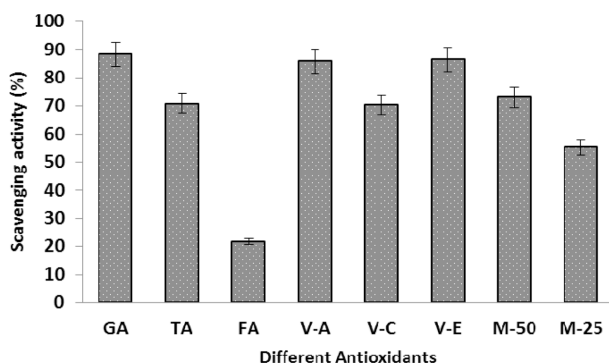
Fig. 6. DPPH assay using standard antioxidants and melanin pigment.
GA: gallic acid, TA: tannic acid, FA: ferullic acid, V-A: vitamin A, V-C: vitamin C, V-E: vitamin E, M-50: melanin (50 μg), M-25: melanin (25 μg).
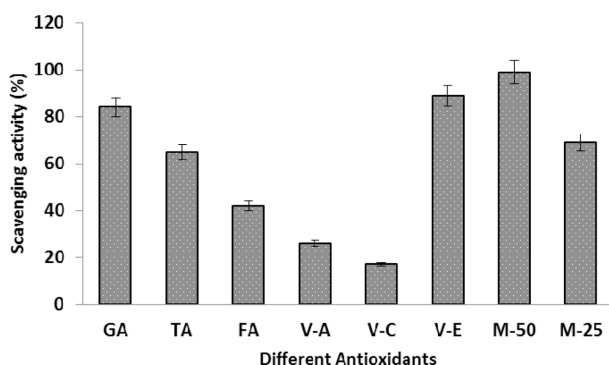

Fig. 7. ABTS assay using standard antioxidants and melanin pigment.
GA: gallic acid, TA: tannic acid, FA: ferullic acid, V-A: vitamin A, V-C: vitamin C, V-E: vitamin E, M-50: melanin (50 μg), M-25: melanin (25 μg).

Free radical scavenging activities

The DPPH radical scavenging activity of the pure melanin from *Klebsiella* sp. GSK compares well with that of standard antioxidants. The results reveal that the melanin has a higher scavenging activity at 50 μg/ml (74%) than at 25 μg/ml (55%). Ferullic and gallic acids showed 20% and 88% scavenging activity, respectively (Fig. 6). ABTS radical was quickly and effectively scavenged by the melanin pigment. The percentage inhibition was 98% and 48.5% for melanin at 50 μg/ml and 25 μg/ml, respectively (Fig. 7). In contrast, vitamin C, vitamin A, ferullic, tannic and gallic acids, and vitamin E showed actvity in the increasing order of 18, 24, 40, 65, 84 and 89%, respectively. Higher concentrations of melanin pigment were more effective at quenching free radicals in both systems. The melanin scavenged more ABTS radical than DPPH radical. Factors such as the ste-

reoselectivity of the radicals or the diffusiblity of melanin in different testing systems have been reported to affect its capacity to react with and quench different radicals [40]. Wang *et al.* [38] found that some compounds that have ABTS scavenging activity did not show DPPH scavenging activity. Melanin pigment, which scavenges different free radicals in different systems, shows strong scavenging activity against both DPPH and ABTS radicals, indicating that it may be a useful therapeutic agent for treating radical-related pathological damage. Melanin pigment interacts with free radicals and other reactive species readily due to the presence of unpaired electrons in its molecules and acts as an antioxidant, suggesting its use as a raw cosmetic material to minimize toxin-induced tissue destruction.

In conclusion, melanin pigment from *Klebsiella* sp. GSK is analogous to typical melanins, which together form quite a heterogeneous group of biopolymers. It is amorphous, thermally stable and is classified as eumelanin type. The results of this study provide the first evidence that bacterial melanins share the basic stacked planar sheet structure and can be identified by their stacking peak parameters. Bacterial synthesis of this type of pigment has the potential to be used effectively in thermal insulation. Melanin pigment is a good adsorbent of metal ions, and HCl is considered the best chelator for metal ions bound to melanin pigment as compared to EDTA. Metal ion interaction with melanin-alginate beads may be valuable for the development and optimization of bioremediation processes. Its antioxidant properties indicate that the melanin pigment could serve as a broadly specific free radical scavenger. As a consequence, melanogenesis in bacteria can serve as an example of evolutionary convergence, as well as providing protection against extreme temperatures and free radicals and enabling the maintenance of a proper balance of metal ions.

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