Recent Insights in Melanin Research: From Extraction to Immense Applications of The Pigment

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Abstract
Melanins are black phenolic pigment which is produced by most of the organisms in nature. This pigment mainly provides protection from short wave radiation there by helping the organism to survive. The deficiency of melanin can lead to many dreadful diseases, which makes the pigment so important for the organism. Melanins differ in their structure as well as color based on the precursor molecules involved in biosynthesis. In animals eumelanin and pheomelanin are the predominant ones, while in bacteria pyomelanin and in fungi DHN-melanin been the most common melanin subtype. The pigment is widely extracted using acid precipitation but the procedures vary slightly depending on the source of the tissue. Extracted melanins could be used in wide variety of applications from medical to environmental which could be developed into future sustainable commercial applications.

Keywords: melanin, application, extraction, structure

1. Introduction

Melanin is a natural pigment obtained from bacteria, fungi, plants & animals. Mostly, they are insoluble and have a complex structure (1). In the early ages, a dark pigment was discovered and addressed as melanin, owning its significance to Greek name 'melaina' (2). These compounds are derived from the oxidation of phenols or indoles (1,3). Conversely, melanin compounds differentiate with respect to chemical structures & are named Eumelanin, Pheomelanin, Catechol, DHN melanin, Pyomelanin and Allomelanin (1). Some melanin represents significant colour difference such as Eumelanin appears to be black- brown, and Pheomelanin exists yellowish (1). Melanogenesis is natural pathway through which melanin is synthesized (4). As per various studies, tyrosinase is a key enzyme for the synthesis of melanin (4). Melanogenesis entails precursors to initiate the biochemical synthesis of melanin (1,4). For example, tyrosine, catechol, and homogentisic acid are few common precursors required for melanin synthesis (1). The depositories of melanin storage differ from bacteria to humans (5). In plants, they are mostly present in seeds (6). For example, in humans, melanocytes in the skin epidermis store melanin which provides prevention from UV dimer formation & further mutation of DNA nucleotides (5,6).

Melanin has unique roles in the species, they are found. Moreover, melanin produced from marine organisms has been found anti-microbial activity against opportunistic pathogens (8). In vivo studies have confirmed, melanin combat free radicals in rats (9). Conversely, in humans' melanin has found its significant not only in mutational repair, but it serves as an anti-oxidant agent (10). Furthermore, it lowers the enzymes involved in anti-inflammatory response such as Lipoxygenases, Dioxygenase (10). Its repression of lipid peroxidase enzyme in digestion system makes a suitable candidate for treating ulcers (10). Not only melanin in its natural form, but it aids in production of gold & silver nanoparticles, which are used as preservatives in the food industry (11). This altogether, makes melanin a valuable product.

Therefore, the focus of this review is to shed light on its extraction, purification methods and bioactivities of the pigment which can help in understanding the methods which help in gaining pure melanin and it emphasizes on the applications of melanin too

2. Melanin Structure and Biosynthesis

In comparison with its natural biosynthesis, its pathway differs with respect to eumelanin, pheomelanin, pyomelanin & allomelanin (12). As mentioned above, some of the precursors are the same but it diverges with respect to product formation (12). The synthesis is multi-step pathway starting from tyrosine or malonyl Co A as main precursor (12). As stated in Figure 1, Melanin can be secreted by fungus as well as bacteria. However, their products are different such as allomelanin, eumelanin, pheomelanin & pyomelanin (3). With respect to fungal melanin, Malonyl CoA is the starting precursor. Decarboxylation of malonyl CoA results in THN. Further reduces to DHN. This is followed by polymerisation and a series of reactions to yield allomelanin called DHN melanin (3).

Bacteria on the other hand can synthesize eumelanin, pheomelanin & pyomelanin through different pathways. Tyrosine is key precursor which oxidizes to give L-DOPA, followed by Dopaquinone via Laccase, to give Eumelanin (3). Additionally, the same pathway is observed in human melanocyte for eumelanin production (13). However, when DOPAquinone undergoes cysteinylation & polymerisation it gives pheomelanin (3). Another divergent pathway is for Pyomelanin synthesis. Deamination of tyrosine results in HPP (hydroxy phenyl pyruvate), which gives HMG. This HMG oxidizes to give Benzoquinone. After a series of polymerisation reactions, Pyomelanin is synthesized (3). Additionally, the same pathway is observed in human melanocyte for eumelanin production (13).

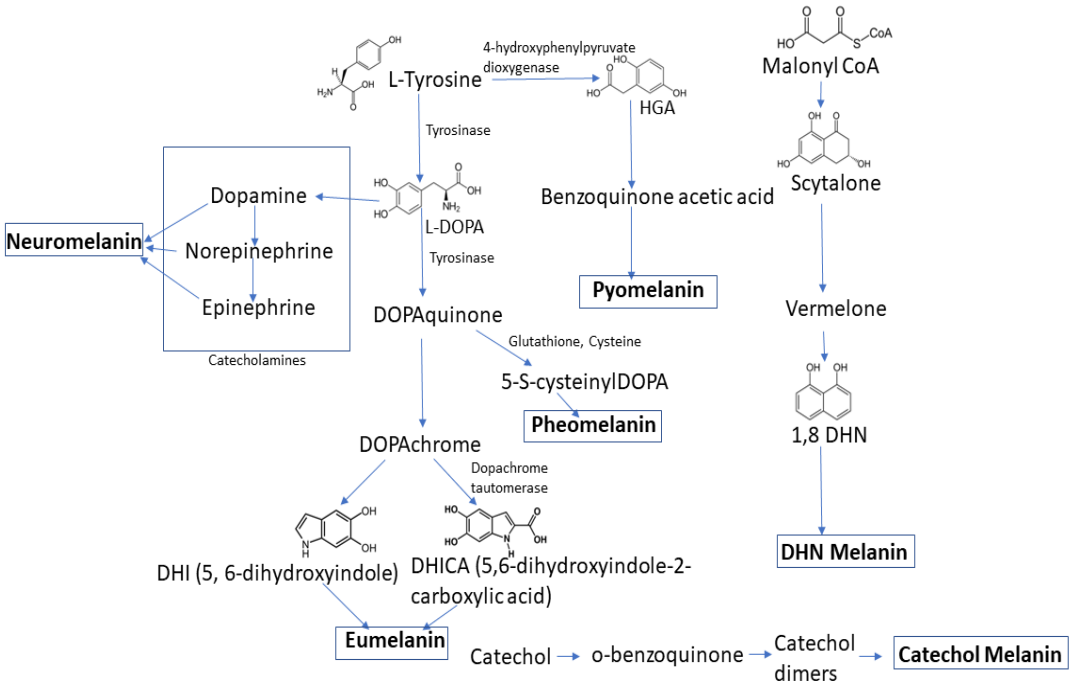

Figure 1: Biosynthesis of Melanin. Key: HMG: 2,5-dihydoxyphenylacetate, L-DOPA: L-3,4-dihydroxyphenylalanine, DHN: 1,8-dihydroxynapthalene

Basically, melanin structure consists of stacked polymeric layers made up of monomeric building blocks derived from 5, 6-dihydroxyindole (DHI) and 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) units in many possible oxidation states. These monomeric units get auto-polymerized with the formation of large number of conjugated double bonds which results in the formation of melanin pigment. The polymeric layers are arranged randomly further and they are connected by covalent bonds which makes the polymer water insoluble.

Our current knowledge on melanin structure still remains limited though advanced technologies are available now. There are many reasons that limit the determination of melanin structure. These include the following

Firstly, like protein, nucleic acid has, there is no standard methods available for determining melanin structure. Proteins and nucleic acids are arranged in a standard way with amino acid or nucleic acid subunits in an even pattern but in case of melanin the monomers are arranged randomly which makes the determination of structure difficult (14).

And the second reason, which is actually also the reason for the 1st reason - the atypical hierarchic structure of melanin. The case is rather about microscopic, mesoscopic and macroscopic structural organisation, than about primary, secondary, tertiary structures. There must be a special model generated, and every structural level corresponds to a different level of organization. While on the macroscopic level we can say about melanosome level and packing of melanin inside them – as agglomerates, and the microscopic structure represents the molecular level (biochemical) in which the primary and secondary, and to some degree even the tertiary structure is set, there are no examples of molecular organization of biological molecules corresponding to the mesoscopic structure, and this is probably the most characteristic and responsible for the unusual properties of melanin resembling the inorganic, solid body-like substance. There are no typical, periodical bonds (e.g., peptide bond, phosphodiester bond) in the amorphic structure of melanin. Limitation necessitates building the whole theory of structural organization of the polymer, from scratch (15).

3. Extraction and Purification of Melanin

Generally, *Sepia officinalis* (a cephalopod) serves as melanin source from commercial market (12,16). Although, there are disadvantages such as more-time & less yield from this cephalopod (16). Conversely, melanin from microbes is the best source due to high yield within less time (3). Additionally, bacterial melanin secretion is the far cheapest method adopted as compared to fungal melanin (1,3,17). In the case of bacterial melanin, tyrosine supplementation is required in the production media (12). However, studies have confirmed bacteria from sea water are able to synthesize melanin on

their own, without tyrosine (18). Moreover, only fruit and vegetable extracts are sufficient for melanin production and its yield comes within 24-48 hours (19,20).

3.1. Melanin from Bacteria

There are a number of treatment methods to extract melanin from bacterial species (12). The melanin extraction followed by a modified Sajjan et.al Method .The cultures are allowed to grow at room temperature ,followed by centrifugation at 10,000 ×g and cell -free supernatant is exposed to acid hydrolysis (1 N HCl) at room temperature at 1-week time duration .Furthermore ,the solution is boiled ,centrifuged at 8000 ×g for 10mins .Again ,pellet is treated with 10 ml 0.1N HCL ,ethanol treatment & stored at boiling water bath at 100 °C for 10mins .The subsequent melanin is observed followed by drying & stored for further use .The resultant melanin was pyomelanin (21) . Whereas, melanin from *Vibrio natriegens* was centrifuged, passed through Millipore size filter (0.2-µm), exposed to 6N HCL, washed with distilled water to attain neutrality & further lyophilized (17). The melanin type achieved was eumelanin (17).

Conversely, melanin without tyrosine supplement in media has also been extracted from *Pseudomonas stutzeri. Kumar* et. al centrifuged the cell-free extract with alkali (1 M NaOH), centrifuged at 5,000 g & collected supernatant. Moreover, it was subjected to precipitation with 1 N HCL. This melanin was again centrifuged at 12,000 g. To remove the excess acid, it was washed with distilled water & subjected to drying (18). In another method, the supernatant was given acid treatment at room temperature for 7 days. Further, boiled, and centrifuged at 10,000 ×g for 15 min. However, here it was again drenched with EtOH, boiled & further incubated for drying. Here, the *E. coli* was genetically modified for 4-hydroxyphenylpyruvate dioxygenase from *Flavobacterium kingsejongi* .4 Hydroxyphenypyruvate dioxygenase is essential enzyme required for melanin synthesis. As mentioned in figure 1, the resulting melanin was pyomelanin (3,22). However, the yield was 0.315 g/L (22) . Tarangi et.al adopted a technique where melanin was produced without tyrosine in medium ,the core source was fruit extract .In this ,the bacterial culture was incubated with fruit waste extract at shaker conditions .In addition ,it was centrifuged (9200 g ,15 mins) .The cells were further centrifuged ,supernatant was collected .This was treated with 3N HCL at room temperature .This incubation was essential to set free the precipitate in the supernatant .Again ,it was boiled & centrifuged for 15 mins at 4600g (19).

3.2. Melanin from Fungi

Jalmi et. al extracted melanin from *Gliocephalotrichum simplex.* The medium containing the fungus was centrifuged for 15 min at 12,000 g. Filtered, precipitated by using 1M acetic acid. Then, the filtrate was again centrifuged, resuspended in distilled water & centrifuged. Here, the melanin was given alkali treatment with 0.1M NaOH & further pH was adjusted using 0.1M HCl. The yield was 6.6 g/L (23). Ribera et. al purified melanin from *Armillaria cepistipes* .The medium contained tyrosine was subjected to shaking at 150 rpm ,followed by filtration using nitrocellulose membrane .The filtrate was dried & was called as 'raw melanin' .Further ,for its purification ,the powder was treated with 5 M HCL at shaker conditions .At this point, only the black pigment was used for further use. This black pigment was washed to achieve neutral pH. Moreover, ethanol was added, lyophilized & washed. In all, it took 161 days with yield 27.98 g/L (24). Another study by Oh et .al gave yield of eumelanin (4.5 g/Litre) with *Amorphotheca resinae* . The pigment was centrifuged, filtered using 0.45 µm glass ,followed by 1M Ammonia Hydroxide ,boiled for 120 mins at 80°C .The suspension was than monitored for acidic pH with the help of 6M HCL .Incubated at Room Temperature for 24 hr ,mixed with 6M HCL, boiled ,rinsed with distilled water . However, here the extraction is carried using CHCl3,EtOH (Ethidium Hydroxide).In this extraction method ,the strength of acid is far less than as prescribed by other methods ,this could be probable reason that more yield of melanin is achieved ,due the more melanin polymer due to not as much of HCL (25).

In another method by using *Auricularia auricula*, the melanin yield was 2.97 g/L. *Sun et.al* filtered the broth containing mycelia with nylon mesh, acidic pH was maintained using HCL, stored at chilled conditions. It was centrifuged for 15 mins at 10,000 rpm. The excess of acid was washed with distilled water. The crude melanin was obtained by reducing the pressure conditions. Moreover, for purification, alkali treatment with NaOH with constant stirring. This resulted in a solution consisting of melanin, which was again centrifuged (10,000 rpm for 15 mins), given acid treatment to get the precipitate. This precipitate was subjected to distilled water to remove excess HCl. This process is repeated & melanin is dried (26). *Pacelli et.al* extracted & purified melanin from *Cryomyces antarcticus*, the cells were centrifuged & rinsed with PBS (Phosphate Buffer Saline) for neutral pH. Further, cells were treated with sodium citrate, citric acid & sorbitol as main ingredients. They were incubated at shaker conditions at room temperature. Further, PBS was added, the suspension was centrifuged at 16.100 RCF. Guanidine thiocyanate was added for removal of supernatant. Again, it was centrifuged twice at 16.100 RCF for 15 mins. Proteinase k, SDS, CaCl2 was added to denature proteins completely. Incubated at 37 for 4 hours, centrifuged at above mentioned conditions. Finally, washing with PBS followed by chloroform was given. For purification, they exposed with HCL, boiled & dialysis was followed (27). A method by Dong & Yao extracted & purified melanin from *Ophiocordyceps sinensis*. In this method, melanin was treated with 6M HCl, centrifuged at 6000 ,15 mins & washed with distilled water .Moreover it was purified by acid treatment ,washing with ethyl acetate ,ethanol to achieve the precipitate .Later ,again 7M HCl was added for at 100° C. Following this, it was filtered & subsequently washed to remove excess of acid. The washing was given with chloroform, ethyl acetate & ethanol to get precipitation. Finally, the precipitate was washed with 1M KOH. This protocol was repeated to get precipitation. This method supports the involvement of organic acids to eliminate lipids. The yield of melanin was 7.95% after purification (28).

Extraction of melanin from *Aspergillus nidulans* was carried out by Cassia et.al. In this method, acid hydrolysis is required with 6N HCL. After which, precipitation was observed by keeping it at room temperature. Further, it was centrifuged at 4500g & lyophilized. The mycelia were exposed with 2M NaOH. It was centrifuged at 4000g for 15min, acidified with 2M HCL. The centrifugation process was repeated once more & further acidified with 6M HCL. Finally, organic solvents such as chloroform, ethyl acetate, ethanol was used to get eliminate lipids (29).

3.3. Melanin from fishes and other animals

In animals, especially insects, they follow a developmental cycle where melanin is produced at the later stages which imbibe colour to the body, eyes etc. Apis *mellifera* has been used as source of melanin (30). In this method, the bee-corpse serves as melanin resource .The materials are crushed in small pieces up to 0.5 mm .Alkali treatment with 10% NaOH is given at 70°C . The liquid is filtered & subjected with petroleum to eliminate lipids. The precipitation is carried out using Con.HCL. This is followed by centrifugation, dialysis & lyophilisation. However, in this method, chitin-melanin complex is obtained (31). Similarly, melanin from *Hermetia illucens* was achieved. The corpse of this insect was subjected with 10% NaOH for 2-3 days. Followed by adding HCL, washed with water, centrifuged & dried. This process is repeated until the precipitate is formed sufficiently (32).

Lin et.al extracted melanin from octopus, sepia & silkie-fowl. The ink sac of these animals 20 g of was blended with 200ml of 1M KOH, followed by ultrasonication. Further, it was subjected to 1M KOH for 5 hours. This extract was then filtered, acidified & precipitate was collected by centrifugation at 10,000 rom for 10mins. Again, acid treatment was given using 7M HCL at 100°C. Furthermore, chloroform is added & butanol to remove lipid particles. This procedure was repeated until precipitate is obtained & washed with distilled water to remove excess alkali & acid (33)

4 Applications of melanin

4.1. Cosmetics & Pharmaceutical

Melanin from *Amorphotheca resinae* had shown to block UV-B radiations. In addition, this research showed it has no ill effects on keratinocytes after 72-hour exposure. Moreover, sunscreen had a SPF (Sun Protection Factor) of 2.5 with 5% melanin content. Contrastingly, it was found this sunscreen is more blended with dark skin-coloured individuals because of melanin naturally being black- brown in colour (34). This study entails a future sunscreen melanin formulation against UV light as protection for skin disorders (34). Melanin from *Vibrio alginolyticus strain BTKKS3* formulated in sunscreen showed increased SPF Factor at 0.005 % w/w. In addition, there was 3.42 increase in SPF as compared with available manufacturer sunscreens. The more the SPF, the more is the shielding effect by melanin. Hence. more SPF in sunscreens can save skin from erythema & sunburn (35). Additionally, extract from *Cinnamomum burmannii* & *Osmanthus fragrans* showed higher SPF in melanin containing gel (36). Since melanin is known to have photoprotective properties, it can be used in sunscreen against UV-A light. UV-A light has a potential to cause skin cancer. Eventually, humans suffering from Xeroderma pigmentosum have a defective repair mechanism which makes them susceptible to skin cancer. As per a study, melanin from *Pseudomonas maltophilia AT18* have been found as a protectant barrier against UV-A light in human fibroblasts from Xeroderma pigmentosum patients (37).

4.2. Health & Medicine

Research emphasizes melanin being used as radioprotectant when ingested orally. Mice ingested with black edible mushroom *Auricularia auricila-judae* possessed antioxidant activity. This research has shown the ability of melanin in enhancement of cell repair with respect to Gastrointestinal tract infection (38). In another study, melanin from *Auricularia auricula* showed positive effects against more alcohol utilization in animal cell culture experiments. The melanin extracted & purified from this fungus was able to lower Reactive Oxygen Species (ROS) created by more alcohol consumption. This research shed more light on melanin used for treating alcohol liver injury which deteriorate the liver cells & tissues (39). Moreover, melanin has been proven to balance the pro-inflammatory cytokines in rat models such as Tumour Necrosis Factor. The study by Kunwar *et.al* showed melanin had less toxic effect on these rats purified from *Gliocephalotrichum simplex* (MTCC 5489). It also reduced the radiation induced mutation in DNA of the peripheral leucocytes exposed to radiation (40). Another research focused on anti-diabetic activity of melanin purified from *Inonotus obliquus* (41). Melanin has also shown anti-cancer activity from *Inonotus obliquus* (42,43,44) .In one study ,melanosomes B16-F10 were able to decrease tumor formation in mice , isolated from the same fungi (43) .Melanin from *Auricularia auricula* was found to have anti-biofilm activity against some of the pathogenic microbes. According to this study, the pigment was able to adversely affect the biofilm formed by E. *coli K-12, P. aeruginosa PAO1 and P. fluorescens P-3*. Melanin upto 80 mcg/ml was able to diminish the biofilm growth by these bacterial species. This research emphasizes the antagonistic activity of melanin against bacterial species (45). The main aspect of biofilm production is due to quorum sensing which have a potential role in speeding up pathogenesis. Moreover, *Auricularia auricula* melanin was found to combat quorum sensing by *Chromobacterium violaceum* (46). This two research focuses on melanin as anti-biofilm agent & thus it can attribute to human pathogenic infections driven by biofilm formation (45,46). Furthermore, bacterial melanin has also restrained biofilm production. *Vibrio alginolyticus strain BTKKS3* suppressed biofilm growth of *Pseudomonas aeruginosa BTRY1, Bacillus altitudinus BTMW1, Staphylococcus warneri BTDF2 and Bacillus sp. BTSD1*. Above all melanin upto 100 mcg/ml showed 80 % inhibition against *Pseudomonas aeruginosa*. *This* research implies to tackle nosocomial infections which are at an alarming rate. Likewise, it also hindered the production of cyclooxygenase & lipoxygenase by these microbes, which are key modulators in inflammation (35). Additionally, an elevated nitric oxide

is a key regulator in inflammation. Melanin at 100 mcg/ml lowered nitric oxide production in RAW 264.7 animal cells (35)

4.3. Bioremediation & Environment

Volatile Organic Compounds (VOCs) are a threat to the environment. These include hydrocarbons persisting in the atmosphere & are unable to naturally degrade by natural process. Hence an alternative is needed to solve indoor air pollution. It was found that black fungi were able to absorb VOCs by 95% by *Cladosporium cladosporioides, Neohortaea acidophila, Cladophialophora psammophila.* This study emphasizes the role of black fungi in directly absorbing volatile compounds through air (47). Melanin has also been found to adsorb metals in order to tackle heavy metal pollution. *Armillaria cepistipes* dark extract was able to absorb metal ions such as lead, chromium, nickel, cadmium, zinc & calcium in water. This study emphasizes melanin could be used against heavy metal contamination ,which possess hazardous role in environment (48) . Moreover, melanin from *Amorphotheca resinae* at acidic conditions were able to absorb metal ions such as copper, lead, cadmium & zinc within less than 2.5 hours (49). Not only this, but it was found that fungal melanin from *Rhizopus arrhizus* & *Aspergillus niger* were able to eliminate thorium from radiation. Besides, it also signifies the characteristic of melanin as radioprotectant (50).

Due to Melanin being black- brown in colour, it has attained much implication in dying industry. Melanin from *Lasiodiplodia theobromae* has been used to dye poplar veneer wood (51). Furthermore, artificial allomelanin nanoparticles synthesized using dihydroxy naphthalene were able to imbibe dark colour to nylon cotton swatches. This research broadens the application of melanin being used in the textile industry (52). In another study, *Streptomyces virginiae* melanin was able to dye wool fabrics. This has subsequently gained much interest in dyeing of wool & other cotton fabricated materials (53)

Table 1: Applications of melanin

Sr.No.	Broad Application	Organism Name	Type	Direct purpose	Reference
1.	Cosmetics & Pharmaceutical	*Amorphotheca resinae*	Fungi	Sunscreens	Oh et.al (34)
		Vibrio alginolyticus strain BTKKS3	Bacteria		Kurian et.al (35)
		Pseudomonas maltophilia AT18			Geng et.al (37)
		Cinnamomum burmannii & *Osmanthus fragrans*	Plants		Huang et.al (36)
2.	Health & Medicine	*Auricularia auricila-judae*	Fungi	Gastro-intestinal tract infection	Revskaya et.al (38)
		Auricularia auricula	Fungi	Alcohol liver injury, Antibiofilm, Quorum sensor quencher	Hou et.al (39), Li Bin et.al (45), Zhu et.al (46)
		Gliocephalotrichum simplex	Fungi	Anti-tumour	Kunwar et.al (40)
		Inonotus obliquus	Fungi	Anti-cancer, Anti-diabetic	Duru et.al (41), Youn, et.al (43), Babitskaya, et.al (44)
		Vibrio alginolyticus strain BTKKS3	Bacteria	Anti-Biofilm,Anti-inflammatory	Kurian et.al (35)
3.	Bioremediation & Environment	*Cladosporium cladosporioides, Neohortaea acidophila, Cladophialophora psammophila*	Fungi	For treating VOCs	Prenafeta et.al (47)
		Armillaria cepistipes	Fungi	Heavy Metal Degradation	Tran-ly et.al (48)
		Amorphotheca resinae	Fungi	Heavy Metal Degradation	Oh et.al (49)
		Rhizopus arrhizus & *Aspergillus niger*	Fungi	Radioprotectant on lands	White & Gaddg (50)
		Lasiodiplodia theobromae	Fungi	Wood Industry	Liu et. al (51)
		Streptomyces virginiae	Bacteria	Textile Industry	Amal et.al (53)

Conclusion

In conclusion, the dark pigment melanin which is ubiquitous in nature could be used in wide number of sustainable applications. Though purification of the pigment is done using different methods for different sources, principle of it remains more or less similar. Contamination of the pigment from impurities like proteins, lipids etc remain a major problem in getting the purified pigment. More cost-effective technologies for purifying the pigment is needed. More standard methods to determine the structure of the pigment will help us to utilize the melanin pigment in wide variety of commercial products and applications.

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