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Exploration and characterization of melanin pigment from actinomycetes: A review

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ABSTRACT

Actinomycetes synthesize essential secondary metabolites that lead to the production of wide range of secondary metabolites of vital applications in every possible field. Pigments are one of those bioactive molecules. A bio-pigment produced by *Streptomyces*: melanin, a multifunctional biopolymer pigment, has gained significant attention due to its photoprotective, antioxidant, antimicrobial, and metal-chelating properties. This review explores the biosynthesis, isolation, characterization, and applications of melanin derived from actinomycetes, particularly *Streptomyces spp*. The study highlights the ecological sources, fermentation techniques, media, and analytical methods used to evaluate melanin's physicochemical traits. The potential of microbial melanin as a sustainable alternative to synthetic dyes in cosmetics, pharmaceuticals, and environmental remediation is emphasized.

KEY WORDS

Actinomycetes; *Streptomyces;* Secondary Metabolites; Melanin; Tyrosinase and ISP Medium

ARTICLE HISTORY

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Introduction

Actinobacteria, a unique group of bacteria belonging to the order Actinomycetes, are characterized by their filamentous structures and high G+C content in their genomes. Often referred to as "ray fungi," these microorganisms serve as a fascinating bridge between the bacterial and fungal kingdoms, showcasing a complex biology that is distinctly bacterial despite their fungal-like appearance. Among their notable features is the production of microbial pigments, secondary metabolites that arise in response to environmental stressors [1]. These pigments, which include carotenoids, melanin, and various other compounds, not only exhibit a vibrant colour spectrum but also possess significant biological activities, such as anticancer, antibacterial, and UV-protective properties [2].

Melanin is extractable and purifiable from both plant and animal tissues. While, this approach is cost-effective, ensuring consistent reproducibility in the chemical composition of the product presents challenges [3]. Microbes also serve as an additional source of melanin, possessing the inherent ability to synthesize these pigments. The microbial synthesis of melanin in liquid cultures under regulated conditions facilitates enhanced management of the product's composition and purity [4]. Actinobacterial melanin typically exhibit significant environmental and chemical stability while preserving inherent bioactive characteristics, including antioxidant and radical-scavenging activities, which can be sustained via gentle extraction methods [5].

As the demand for aesthetically pleasing products increases, the use of synthetic dyes has surged, raising concerns about their potential health and environmental hazards. Considering these challenges, there is a growing interest in harnessing natural pigments derived from actinobacteria as eco-friendly alternatives [6]. This review article aims to explore the multifaceted roles of melanin produced by actinomycetes, particularly in relation to its physicochemical characteristics, biological significance, and potential industrial applications [7]. By delving into the intricate relationship between actinobacteria and their pigments, we hope to illuminate the promising avenues for utilizing these natural compounds in various fields, including medicine, cosmetics, and environmental sustainability [2].

Streptomyces

The ability to produce melanin is a prevalent characteristic of *Streptomyces* and it serves as an identification tool of these filamentous, soil-dwelling bacteria [4]. Melanin pigments are just one of several bioactive chemicals made by *Streptomyces* species. *Streptomyces* is known to produce melanin, a dark pigment (found in many creatures), as a defense mechanism against environmental stress. Recent reports suggests that *Streptomyces*-derived melanin may have antioxidant and antibacterial properties [8].

Melanin pigments provide defense against physical and chemical stresses; they are present in many organisms, including microbes, plants, and animals. These pigments aid survival in extremely hot or bright environments. Pheomelanin may have mutagenic effects when exposed to ultraviolet light because of its poorer stability, whereas eumelanin is considered a useful agent because of its responsibilities in scavenging free radicals and providing radiation protection. Even though streptomyces has been acknowledged for its possible use in environmentally friendly pigment production [9].

The cosmetics, food, and dye industries have all given pigments the green light to use them as natural colorants. In the biomedical and industrial fields, these pigments' vivid and varied hues have been recognized as potential substitutes for synthetic dyes. Further scientific inquiry and technical developments are being pursued due to the bioactive features linked to these pigments, which include antioxidant, antibacterial, and anticancer capabilities [10].

DOPA vs DHN Pathways

Melanin biosynthesis in actinobacteria primarily occurs via the DOPA and DHN pathways, catalysed by distinct enzymatic systems and regulated by separate gene clusters. It represents a highly conserved yet mechanistically diverse process, primarily governed by two distinct biochemical routes the L-3,4-dihydroxyphenylalanine (DOPA) pathway and the 1,8-dihydroxynaphthalene (DHN) pathway. The DOPA pathway, catalysed by the copper-containing enzyme tyrosinase,

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involves the oxidation of L-tyrosine to L-DOPA and subsequently to DOPA-quinone, leading to the polymerization of indolic intermediates into dark, insoluble eumelanin pigments [11, 12]. In contrast, the DHN pathway follows a polyketide route that initiates from acetyl-CoA and malonyl-CoA via the action of a type I or type II polyketide synthase (PKS) complex, resulting in the formation of tetrahydroxynaphthalene (THN) and its subsequent reduction and dehydration to yield DHN, which polymerizes into allomelanin [13, 14].

Recent studies in Streptomyces, Nocardia, and other actinobacterial genera have demonstrated that both pathways contribute to pigment diversity and functional adaptation under environmental stress, including oxidativeand UV-induced damage [15, 16]. Moreover, actinobacterial melanin display considerable structural heterogeneity, reflecting the metabolic plasticity of these organisms and their ecological niches. Marine-derived actinomycetes, for instance, often exhibit enhanced melanin productivity, suggesting an evolutionary role in photoprotection and metal ion sequestration in saline environments. Collectively, comparative analyses indicate that while the DOPA pathway predominates in amino acid-derived systems, the DHN pathway is more prevalent in polyketide-rich actinobacteria, each serving convergent physiological roles in stress resistance and secondary metabolite regulation.

Table 1. Comparative overview of DOPA and DHN pathways of actinobacterial melanin synthesis.

Feature	DOPA Pathway (L-DOPA melanin / eumelanin type)	DHN Pathway (Allomelanin type)
Full name	3,4-Dihydroxyphenylalanine pathway	1,8-Dihydroxynaphthalene pathway
Type of melanin	DOPA-melanin / eumelanin	DHN-melanin / allomelanin
Major precursor	L-Tyrosine or L-DOPA	Acetyl-CoA or malonyl-CoA (via polyketide pathway)
Key enzyme initiating synthesis	Tyrosinase (monophenol monooxygenase, EC 1.14.18.1)	Polyketide synthase (PKS)
Intermediate compound(s)	L-DOPA → DOPA-quinone → DOPA-chrome → Indole-5,6-quinone	1,3,6,8-Tetrahydroxynaphthalene (THN) → Scytalone → 1,3,8-THN → Vermelone → 1,8-DHN
Final polymer	Indole-5,6-quinone polymers	Polymerized 1,8-DHN
Enzyme cofactors	Requires Cu^{2+} ions (for tyrosinase activity)	NADPH-dependent reductases & dehydratases in PKS pathway
Localization	Typically extracellular or cell-surface associated	Often cell-wall bound or intracellular
Inhibitors	Inhibited by tyrosinase inhibitors like kojic acid, phenylthiourea	Inhibited by tricyclazole (a reductase inhibitor)
Color of pigment	Brown to black	Dark brown to black
Genetic regulation	mel or tyr genes encode tyrosinase	pks, scd, arp genes encode PKS complex enzymes
Common in	Streptomyces glaucescens, Streptomyces kathirae, Nocardia spp.	Streptomyces griseus, Streptomyces avermitilis, Actinomyces spp.
Biological role	Protection from UV and oxidative stress; metal chelation	Similar protective roles; also enhances desiccation and antibiotic resistance
Nature of melanin	Derived from amino acid metabolism	Derived from polyketide metabolism

Sources and Isolation of Melanin Producing Actinobacteria

Sample collection

Marine sample

Marine sediment samples were collected from the coastal region near Thoothukudi, Tamil Nadu, and processed in sterile conditions. The sediments were dried for 48 hours, followed by 12 hours of sunlight exposure, and then ground into a fine powder [17]. Additionally, three soil samples from the Dharwad district, representing forest soil, lake soil, and the rhizosphere of a cornfield, were stored in zip lock bags under refrigeration for later processing to isolate pigment-producing actinomycetes. Notably, a marine *Streptomyces* strain was isolated from Versova beach sediments in India [18], and melanin-producing *Actinoalloteichu ssp.* was successfully isolated from Tuticorin coast sediments [3]. Further marine samples were obtained from the Arabian Sea at Alleppey beach, Kerala, and maintained at 4°C for analysis, employing three different approaches for actinobacteria isolation [19]. Additionally, actinomycetes associated with marine sponges were isolated from Pramuka Island, Indonesia [20].

Terrestrial soil sample

Soil samples were collected from diverse locations across India and other regions for the purpose of identifying melanin-producing actinomycetes. In India, samples were obtained from gardens, sugar manufacturing sites, agricultural lands, and mango orchards in Vapi and Valsad, as well as from various environments in Karnataka, including the rhizosphere of medicinal plants and forest habitats [21]. The collection process involved taking approximately 3 cm of surface soil with sterilized spatulas, placing them in sterilized polythene bags, and storing them at 4°C until further processing. Specific methodologies included discarding the top layer of soil, air-drying samples, and incubating them at elevated temperatures to facilitate the selective isolation of actinobacterial colonies.

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Additionally, samples were collected from the Pichavaram mangrove forest in Tamil Nadu [22]. The Wadi-Allaqui Biosphere Reserve in Egypt, where soil was aseptically collected and subjected to drying processes to enhance the recovery of spore-forming *Streptomyces*. The studies highlighted the importance of careful sampling and pretreatment techniques to ensure the successful isolation of actinomycetes from various soil types, contributing to the understanding of microbial diversity and potential applications in biotechnology [23].

Pretreatment

Soil sample collection involved discarding the top layer and collecting moist soil in sterile bags, which were then stored at 40°C for further processing. The soil was serially diluted in saline, and diluted tubes were subjected to a water bath at 45°C for spore separation.

Marine water samples were diluted in sterilized marine water to a concentration of 10^{-3} and subjected to dry heat treatment at 120°C for 60 minutes. Serial dilutions (10-fold) were prepared, and 0.1 ml of the 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions were spread on starch casein agar, Actinomycetes isolation agar, and tyrosine agar base. A pre-enrichment was conducted using sterilized Actinomycetes isolation broth, with samples incubated for 7 days at room temperature under shaking conditions of 120 rpm. Following enrichment, 0.1 ml aliquots were plated on the identical selective media, and growth on all plates was observed over a period of 21 days [19].

Media and Isolation of Melanin Producing Actinobacteria

Media and culturing actinobacteria

Selective media play a pivotal role in isolating and cultivating melanin-producing actinomycetes. The isolation of actinomycetes from soil samples using diverse media has proven effective in promoting growth and pigment production, with specific media formulations enhancing the selective isolation of these microorganisms.

Propagation of actinomycetes involve the use of various media for their isolation, including Actinomycetes Isolation Agar (AIA), starch casein agar (SCA), and yeast extract-malt extract agar (YEMA), among others. For marine actinobacteria, Kuster's agar (KUA) medium, enriched with cycloheximide and nalidixic acid to prevent contamination, is employed. Sediment samples are serially diluted and plated onto KUA, with colony-forming units per gram of sediment used to quantify the actinobacterial population [17]. Additionally, melanin synthesis is studied using a tyrosine agar-based medium supplemented with specific components, including glycerol, L-tyrosine, and seawater, followed by cultivation at 28°C with periodic sampling to monitor pigment production [3]. Soil samples are prepared by mixing 1g with 9 ml of sterile distilled water, agitating the mixture, and performing ten-fold dilutions for further analysis [16].

For actinobacteria isolation, soil samples were pretreated and diluted up to a factor of 10^{-7}, with aliquots spread onto Actinomycetes Isolation Agar (AIA) and Bennett Agar (BA) plates, supplemented with amphotericin B and ampicillin to suppress fungal and bacterial growth. Incubation at 30°C for six days allowed for the selection of distinct colonies, which were then purified and stored for further analysis [24].

Actinomycetes were isolated using a peptone iron medium consisting of peptone (20 g/L), K_2HPO_4 (1 g/L), ferric ammonium citrate (0.5 g/L), sodium thiosulphate (0.8 g/L), and agar (32 g/L), adjusted to pH 7. Soil samples (1 g) were introduced into 250 ml Erlenmeyer flasks containing 100 ml of sterile peptone iron medium and incubated at 30°C for five days. Following incubation, serial dilutions were performed, and samples were spread onto tyrosine casein agar plates, which contained L-tyrosine (1 g), casein hydrolysate (25 g), sodium nitrate (10 g), and agar (32 g), also adjusted to pH 7. The inoculated plates were incubated at 30°C for 5-6 days, allowing for the identification and purification of pigment-producing colonies.

Various media, including Kuster's agar and glycerol casein medium, were employed for the selective isolation of actinobacteria, with micronutrients and inorganic salts in the media promoting actinobacteria growth while inhibiting unwanted microorganisms. Notably, several Streptomyces species capable of melanin synthesis were isolated from soil samples in regions such as Egypt, India, and Turkey, often linked to rhizosphere environments. Brown melanin was produced by Streptomyces species, while darker soluble pigments were derived from marine strains [25].

Additionally, various nutrient-rich media, including Peptone Yeast Extract Iron Agar (PYIA), Tyrosine Agar (TA), Glycerol Peptone Agar (GPA), and Starch Casein Agar (SCA), were tested for pigment production by isolated strains. Among these, PYIA was identified as the most effective medium for melanin production. Serial dilutions of soil samples were prepared, and aliquots were spread onto SCA plates, which were incubated at 28 ± 2°C for 15 days to monitor growth and pigment production.

Melanin synthesis was investigated using a tyrosine agar-based medium supplemented with glycerol, L-tyrosine, L-asparagine, and other components, with cultivation at 28°C under continuous shaking for seven days. Soil samples were mixed with sterile distilled water for dilution and further analysis [26].

Actinomycetes were isolated using a peptone iron medium, which included peptone, K_2HPO_4, ferric ammonium citrate, sodium thiosulphate, and agar, adjusted to pH 7. The isolation process involved introducing soil samples into sterile peptone iron medium and maintaining static conditions at 30°C for five days, followed by serial dilution and plating on tyrosine casein agar [21].

Different media, including Kuster's agar and glycerol casein medium, were employed for selective isolation, with micronutrients supporting actinobacteria growth while suppressing unwanted microorganisms. Isolates capable of melanin synthesis were documented from soil samples in various countries [16].

Role of Optimization

Melanin biosynthesis in actinobacteria is a growth-associated, enzyme-mediated process that is highly sensitive to environmental and nutritional parameters. The quantity, quality, and type of melanin (DOPA vs. DHN) produced can vary significantly depending on the cultural conditions, such as medium composition, pH, incubation temperature, and duration. These parameters regulate the expression and activity of key biosynthetic enzymes like

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tyrosinase, laccase, and polyketide synthase (PKS), thereby modulating the metabolic flux toward melanin precursors.

Media composition

The composition of the growth medium is the most influential factor determining melanin yield and type.

Carbon sources: Simple carbohydrates (e.g., glucose, sucrose, glycerol) serve as energy sources and influence carbon flux toward precursor molecules like acetyl-CoA and malonyl-CoA in the DHN pathway. However, excessive glucose can repress secondary metabolism due to catabolite repression.

Nitrogen sources: Organic nitrogen sources such as peptone, yeast extract, or casein hydrolysate enhance melanin production by supplying amino acids like L-tyrosine, which directly feeds into the DOPA pathway [27].

Amino acid precursors: Supplementation with L-tyrosine or L-DOPA can significantly boost DOPA-type melanin yields by directly providing substrate to tyrosinase [12].

Metal ions: Trace elements, especially Cu^{2+}, Fe^{2+}, and Mn^{2+}, serve as cofactors for tyrosinase and oxidoreductases. The presence of copper ions in the medium enhances the catalytic oxidation of L-tyrosine and L-DOPA, leading to higher pigment formation [27].

pH of the medium

The pH profoundly affects both enzyme stability and precursor oxidation during melanin biosynthesis.

- Neutral to slightly alkaline pH (6.5–8.0) generally favors tyrosinase activity and melanin polymerization in Streptomyces species [13].
- Acidic conditions (pH < 6.0) can denature key enzymes or hinder quinone polymerization, leading to lower yields or altered pigment hues.
- Additionally, pH regulates the solubility and redox potential of intermediates like DOPA-quinone and THN, which are essential for proper pigment polymerization [28].

Hence, maintaining a stable, buffered pH near the enzyme's optimum is critical for consistent pigment biosynthesis.

Incubation conditions

Temperature and incubation duration determine the kinetics of melanin synthesis.

Temperature: Most actinobacteria produce maximal melanin at 28–32°C, aligning with the optimal growth range for Streptomyces spp. Higher temperatures may cause enzyme denaturation, while lower ones slow metabolic rates.

Incubation time: Pigment production typically begins in the late exponential to stationary phase, coinciding with secondary metabolite synthesis. Extended incubation enhances pigment maturation and polymerization [29].

Aeration and agitation: Since melanin formation involves oxidative reactions, sufficient oxygen availability is essential. Aerated shake-flask or bioreactor conditions promote higher melanin yields compared to static cultures [12].

Screening for Melanin Production

A screening procedure was conducted on actinomycetes isolates to evaluate their potential for melanin production across various media and to identify those capable of producing melanin pigment. To identify melanin diffusibility, isolates were initially cultured on yeast extract iron agar and ISP medium 6 plates supplemented with specific nutrients and incubated at 30°C for a duration of 2 to 6 days. The appearance of brown to black pigmentation around the colonies served as an indicator of melanin production [12].

Primary screening

Melanin pigment production by actinobacteria was assessed using ISP-1 and ISP-7 media to differentiate Streptomyces species based on their capacity to hydrolyze tyrosine, with pigmentation noted as either present or absent. The formation of pigment on the reverse side and in diffusible form was analyzed on ISP-7 medium, exhibiting colors from red to brown-black [23].

Reports suggests that pigment-producing actinobacteria are relatively prevalent in the soil. Most of them produced a brownish-black pigment when cultivated on PYIA and GM media. For example, when evaluated on PYI and glutamate medium (GM), isolates exhibited varying pigments or secondary metabolites based on the composition of the medium. Two isolates exhibited brown/black pigmentation on PYIA but transitioned to purple on GM. The alteration in color may result from variations between synthetic and semi-synthetic media.

The synthetic GM medium comprises precisely defined nutrients that may exclusively support specific metabolic pathways, potentially promoting the synthesis of purple pigment. Conversely, the semi-synthetic PYI medium, comprising peptone and yeast extract, provides a more intricate array of nutrients, including vitamins, amino acids, and growth factors. These supplementary components may augment pigment synthesis across a wider spectrum of colors [24].

To harness melanin-producing Streptomyces, screening was conducted on four media types: starch casein agar (SCA agar), oatmeal agar, mannitol soya bean agar, and glycerol-asparagine agar. A 0.5 ml aliquot from the 10^{-3} dilution of each soil sample was uniformly distributed onto these media using sterile glass spreaders. The plates were incubated at 30 °C for seven days. After incubation, the Streptomyces colonies were observed for melanin production, exo-pigment production in the medium was considered as positive with pigmentation varying from brown to black.

Secondary screening

The presence of brown pigments and clear catalytic zones on starch tyrosine agar indicated melanin production. These zones and pigment intensity were used to identify efficient producers [27].

The peptone yeast iron agar (PYI) (ISP 6) medium consistently enhanced pigment production. Adding peptone and yeast extract to the medium increases pigment levels. These components improve the environment, enabling microbes to produce more pigment. Both PYI and GP are semi-synthetic and contain peptone as a common ingredient. GP broth, with its abundant carbon sources like glycerol and beef extract, may support higher biomass. In addition to energy, these provide essential nutrients like vitamins, minerals, peptides, and amino acids for microbial growth. PYI's effectiveness may depend on the combination of carbohydrates, amino acids, and growth-promoting

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factors, particularly yeast extract. This combination boosts pigment production significantly.

Isolates on PYIA medium were screened for melanin production. After monitoring pigment formation daily for 5-7 days at 30°C, the isolate with the highest pigment was chosen for identification [28].

Melanin production was indicated by brown to black pigmentation around the colony. *Streptomyces puniceus* pigment production media. S. puniceus culture was incubated at 30°C for seven days in a Petri dish. A week of 24-hour pigment production was monitored [29]. Initially, isolates were spotted on yeast extract iron agar and ISP medium 6 plates with specific nutrients, incubated at 30°C for 2-6 days to identify melanin producers. The presence of brown to black pigmentation around colonies indicates melanin production [12].

The composition of the medium significantly impacts pigment production. Further research is needed to fully understand this relationship [28]. Researchers cultivated the strain on Kuster's agar at 28°C for a week, monitoring pigment changes. Melanin production was indicated by dark brown to black hues around the colony [3].

Media and fermentation techniques

A screening procedure was conducted on actinomycetes isolates to evaluate their potential for melanin production across various media and to identify those capable of producing higher pigment levels. The Streptomyces isolates were inoculated onto starch casein agar plates and incubated at 30°C for seven days. Sterile cork borers were subsequently employed to excise five discs [1].

Investigation and analysis of melanin pigment derived from Actinomycetes, measuring 6 mm in diameter, from the cultures. The discs were placed into flasks containing 50 ml of starch casein broth, adjusted to a pH of 7.2, to facilitate melanin synthesis. Incubation occurred at 30°C on a rotary shaker operating at 150 rpm for a duration of 7 days. Following incubation, cultures underwent centrifugation at 13,000 rpm for 15 minutes to isolate the biomass, which was subsequently discarded. The supernatant, which contained pigment, was collected and used for the quantitative analysis of melanin as outlined in the subsequent section [30].

Biosynthesis of Melanin Pigment

Melanin, distinguished by its black-brown hue, is produced by various microorganisms, including bacteria, fungi, and several marine strains like Streptomyces.Numerous studies have been conducted on these actinomycetes regarding their efficacy in melanin production.

The classification of melanin pigments is determined by their chemical composition, which encompasses eumelanin, pheomelanin, pyomelanin, and allomelanin. Eumelanin production has been documented in *Streptomyces* species. Pheomelanin is primarily produced by specific fungal species. The formation of pyomelanin is associated with bacteria including *Ralstoniapickettii*, *Pseudomonas aeruginosa*, and *Streptomyces avermitilis*. It is important to recognize that fungi can synthesize this pigment. *Streptomyces glaucescens* exhibits a melanin-like intense black pigment.

The DOPA pathway is the principal mechanism for microbial melanin biosynthesis. Tyrosine serves as the initial substrate in this process.Tyrosinase and laccase are enzymes that convert tyrosine into L-DOPA, which is then oxidized to dopaquinone.The DHN pathway entails the synthesis of malonyl coenzyme A through the action of polyketide synthases. This intermediate is subjected to a sequential enzymatic process that produces 1,3,6,8-tetrahydroxynaphthalene (THN).

Microorganisms that employ the DOPA pathway are preferred for large-scale melanin production due to their superior pigment yields. Research has concentrated on identifying different microorganisms that can synthesize melanin, classifying the types of melanin according to their chemical structure, and examining the primary pathways involved in microbial pigment biosynthesis [9].

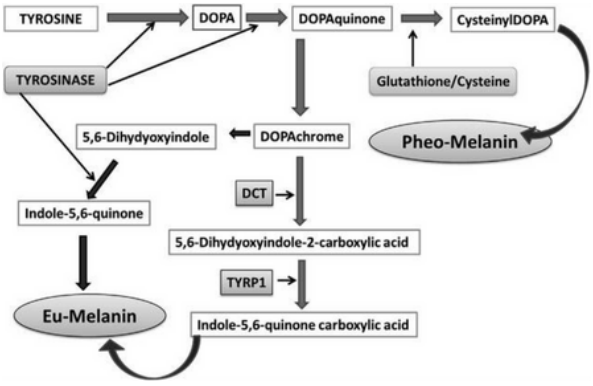

Figure 1. Schematic representation of the melanin synthesis pathway and the role of melanogenic enzymes. The initial synthesis of melanin is catalyzed by tyrosinase and subsequently categorized into eumelanogenesis or pheomelanogenesis. Other melanogenic enzymes, specifically l-3,4-dihydroxyphenylalanine (DOPA) chrome tautomerase (DCT) and tyrosinase-related protein 1 (TYRP1), play a role in eumelanogenesis [1].

Extraction and Purification

Melanin is easier to purify from Streptomyces strains because they produce it extracellularly. The initial purification process can extract the pigment from clarified broth supernatant. Melanin isolation and purification require many steps and methods. Researchers have mentioned precipitation, extraction, dialysis, and chromatography techniques in purification of exo-pigment.

To extract and purify the cell-free supernatant, the production broth was centrifuged at 8000 rpm for 20 minutes. The supernatant was acidified with 6M HCl to 2.0 pH to precipitate melanin pigment for 4 hours. The precipitate was centrifuged at 9000 rpm for 15 minutes and washed three to four times with distilled water. Each wash was centrifuged under the same conditions to preserve melanin (19). A simple two-step acidic precipitation was shown. Start by adding 5 M HCl to 400 mL of supernatant to pH 1.5. To settle pigment, samples were left at 4°C overnight after adjustment. Next day, centrifuged at 4°C for 20 minutes at 5000 rpm.

Washing precipitated melanin three times. After each wash, centrifugation recovered pigment. Purified melanin was air-dried at room temperature after the final wash. To refine dried melanin, 15% was washed again. The pigment was treated with 5 M HCl at room temperature for 5 hours under constant stirring using a modified protocol. Extra steps were taken to remove any remaining proteins or nucleic acids from the pigment.

After treatment, the sample was centrifuged at 4°C for 20 minutes at 4500 rpm. The melanin was washed and dried again. The final product was ready for characterization [31].

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Centrifuges are used to separate pigments from biomass and recover clarified broth supernatant. This step removes most cell debris and metabolites.

Melanin is purified using multiple methods. Common methods include acidic precipitation and liquid-liquid solvent extractions. Acidic pH, especially below 2.0, promotes pigment precipitation. Methanol, ethanol, and hexane are good solvents for testing melanin solubility. For liquid-liquid melanin extraction from the supernatant, different polar solvents are used.

Sonication increased extraction efficiency. Purification may follow with membrane filtration, dialysis, or chromatography. Purity is often achieved through multiple crystallization and freeze-drying rounds.

Because melanin is insoluble in organic solvents and acidic aqueous solutions below pH 2.0, scientists struggle to design purification protocols. Melanin Pigment from Actinomycetes was isolated and characterized. Melanin is usually separated from biomass by centrifugation after Streptomyces syntheiszes it into the culture broth [3].

Some studies have mentioned the use of 3 mol/L HCl into the supernatant to acidify at pH 3 and left at room temperature for 24 hours to precipitate crude melanin. Second centrifugation followed the same procedure. Washing the melanin pellets four times with distilled water and centrifuging again purified the pigment. To preserve the pigment for physicochemical and spectroscopic analysis, it was freeze-dried and stored at 20 °C after purification. For confirmation, TLC is performed. The purified melanin was compared to a synthetic standard using silica gel chromatography.

Silica gel plates are employed for TLC analysis. The solvent system's mobile phase was petroleum ether, ethyl acetate, ethanol, and ammonia at 4:4:6:1 (v/v). A standard melanin sample and the purified pigment were carefully spotted on TLC plates. To fix spots, plates were oven-dried after developing. A UV chamber revealed the spots. The retention factor (Rf) of purified melanin was compared to standard melanin for validation. After scraping melanin bands from TLC plates, physicochemical and spectroscopic analyses confirmed its identity and purity [23].

Extraction and purification of microbial melanin, particularly from actinobacterial sources, pose significant challenges due to its complex polymeric structure, hydrophobicity, and tight binding to cell walls or extracellular matrices. Among the various reported protocols, the alkali precipitation followed by dialysis method remains the most reliable, reproducible, and chemically non-destructive approach for isolating high-purity melanin from culture broths and biomass [32].

Method principle

Melanin exhibits amphoteric properties, being insoluble in water and most organic solvents, but soluble in strong alkaline conditions (e.g., NaOH, KOH). During extraction, cell pellets or supernatants are treated with an alkaline solution to solubilize the melanin polymer. This solubilized melanin is then precipitated by acidification (usually using HCl to pH 2.0), which induces aggregation of the pigment molecules. The resulting precipitate is separated by centrifugation, washed repeatedly with distilled water, and subjected to dialysis to remove residual salts, proteins, and low-molecular-weight impurities [15, 12, 23, 33]. This process exploits melanin's pH-dependent solubility and ionic charge characteristics, providing a clean separation without chemical degradation.

Reliability and efficiency

Selective solubilization and recovery: Alkali treatment specifically dissolves melanin without affecting most other cellular components. Acidic reprecipitation ensures selective recovery due to the pigment's low isoelectric point (~pH 4).

Preservation of chemical integrity: Unlike oxidative or solvent extraction methods, the precipitation–dialysis approach does not involve harsh oxidants or organic solvents, minimizing structural alteration of the melanin polymer [11,13].

Scalability: The method is simple and scalable for both analytical and preparative quantities, making it suitable for large-scale pigment recovery from Streptomyces or Nocardia fermentations. Reproducibility: Because it relies on fundamental solubility behavior rather than organism-specific reagents, it can be universally applied to DOPA- and DHN-type melanin [16].

Role of dialysis in purification

Dialysis acts as a post-precipitation purification step, removing inorganic ions, unreacted alkali, proteins, and small organic contaminants that co-precipitate with melanin. The semi-permeable membrane allows diffusion of low-molecular-weight impurities (Na^+, Cl^-, small peptides) while retaining high-molecular-weight melanin polymers. Dialysis under constant stirring or against deionized water for 48–72 hours result in high-purity melanin, confirmed by stable UV-Vis absorption, FTIR spectra, and elemental composition consistent with reference melanin [12, 15]. The process ensures removal of ionic impurities, which is essential for accurate downstream spectroscopic and physicochemical characterization.

Comparison with other methods

Alternative extraction methods, such as solvent extraction or oxidative degradation, often lead to partial depolymerization, metal loss, or color alteration of melanin. Enzymatic extraction, while gentle, is less cost-effective and organism specific. In contrast, precipitation–dialysis offers an ideal balance between purity, yield, and structural preservation [13].

Characterization Techniques

Melanin characterization relies on a combination of spectroscopic and analytical techniques to confirm its structural identity, owing to its amorphous and heterogeneous nature. UV–Visible spectroscopy reveals a broad, featureless absorption spectrum with a monotonic decline from UV to visible wavelengths, confirming the presence of conjugated aromatic systems. FTIR spectroscopy identifies key functional groups such as hydroxyl, carboxyl, and indolic moieties, while EPR (or ESR) detects the stable free radicals characteristic of melanin's semiquinone structure. Elemental (CHNS) analysis differentiates between nitrogen-rich DOPA melanin and nitrogen-poor DHN melanin, whereas XRD confirms their amorphous carbon-like nature. Thermogravimetric (TGA/DSC) and microscopic (SEM/TEM) analyses further establish the pigment's thermal stability and morphology. Complementary HPLC and mass spectrometry reveal monomeric degradation

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products, enabling pathway identification (DOPA vs. DHN type). Collectively, these methods particularly UV–Vis, FTIR, and EPR form a reliable analytical framework for validating the chemical authenticity and structural composition of actinobacterial melanin [34].

UV-spectrophotometer

The pigment was analyzed using a UV-Visible spectrophotometer to record its absorption range of 200-800 nm. Observing the highest absorption peak (λmax) helps identify the pigment's presence and properties. UV-visible spectrophotometric analyses followed specific purification procedures. Melanin from *Streptomyces roseochromogenes* showed two major absorption peaks at 222 and 254 nm, and a minor peak at 332 nm. Melanin from *Streptomyces* sp. Had a maximum absorption at 300 nm. In another instance, *Streptomyces cavourensis* melanin showed a stronger absorption peak at 260 nm after precipitation and purification.

The UV-visible spectrophotometric analysis of purified melanin pigment was conducted from 200-700 nm. A strong UV absorption at 250 nm was observed, gradually decreasing towards the visible range, indicating a typical melanin property. This 250-nm peak Melanin pigment from Actinomycetes was found to be comparable to that of *Phyllostictacapitalensis*. Other melanin sources had varying absorption maxima. Additionally, *Streptomyces bikiniensis* melanin showed exceptional UV absorption [20].

FTIR spectroscopy

FT-IR spectroscopy examined melanin chemical bonds and structures at 4000 to 500 cm^{-1}. FTIR analyses raw and purified melanin; to determine structural similarities, test results were compared to standard melanin profiles. Melanin from exhibited strong absorption bands at 3399 and 1633 cm^{-1}, with a broad range of 3300-3500 cm^{-1}. This is due to stretching vibrations of –OH and $–NH_2$ groups. Also observed were absorption at 2925 cm^{-1} and characteristic bands at 1633.71 cm^{-1} (C=C, $–COO^-$) and 1075.44 cm^{-1} (C–O, phenolic or carboxylic groups). The extracted powdered pigment was analyzed using FTIR. It showed functional groups. The analysis found stretching frequencies for O-H, N-H, C=C, C-H, C-N, and C=O.IR bands were found by comparing symmetric and asymmetric stretching. Alkanes are identified by the peak at 2902 cm^{-1}, linked to C-H stretching, and alkenes by bands at 3000-3100 cm^{-1}.Peaks at 3267-3333 cm^{-1} indicate alkynes. 2024-25 found aromatic C=C bonding at 1310-1250 cm^{-1}, particularly at 1292 cm^{-1}, and a matching band at 1388 cm^{-1}.

Melanin pigment in Actinomycetes shows S=O stretching, suggesting sulfate groups.Additionally, the 2902 cm^{-1} signal showed CH_2 group vibrations [30]. FTIR spectra for crude and purified melanin were nearly identical, and the transmission pattern confirmed functional groups. Although fragments during acid precipitation caused narrow, sharp peaks in the crude sample, the molecular structure matched the purified standard. Due to its functional group-related absorption bands, Fourier-transform infrared spectroscopy (FT-IR) is used to analyze melanin structures from various sources. Broad FT-IR peaks at 3400-3200 cm^{-1} suggest O-H and N-H stretching vibrations, typically from indolic and pyrrolic rings. Absorption bands at 2964-2850 cm^{-1} indicate aliphatic C-H groups, while peaks at 2300-2800 cm^{-1} indicate amines and amides. C=O stretches from uinines or carboxylic acids usually occur at 1700 cm^{-1}.Other signals around 1660-1520 cm^{-1} are caused by aromatic C=C bonds and N-H bending.

Additionally, bands at 1530-1515 cm^{-1} or 1450-1400 cm^{-1} indicate CH_2-CH_3 or indole structures, while those at 1300-1250 cm^{-1} indicate aromatic estersPhenolic C-OH groups are observed at 1260-1150 cm^{-1}, while C-N stretches are at 1120 cm^{-1}. Signals from 1050-800 cm^{-1} typically indicate aliphatic and aromatic C-H bending. Melanin with sulfur displays S=O and C–S signals at 1420-1380 and 662 cm^{-1} [9].

NMR spectrum

The H NMR spectrum in DMSO-d_6 showed resonances between 0.6 and 8.0 ppm. The signals at 6.66, 7.28, and 7.18 ppm were linked to aromatic hydrogens in indole and pyrrole groups, which are common in melanin structure.A peak at 3.46 ppm indicates methyl groups on oxygen or nitrogen atoms. Resonances at 2.5 and 3.3 ppm are due to the solvent used to dissolve the compound. The spectrum confirms $–CH_3$ groups from various alkyl fragments at 0.6-1.0 ppm.A sharp peak at 1.8 ppm suggests protons from a $–CH_3$ group connected to an ethylene carbon atom [5].

SEM scanning

Scanning Electron Microscopy (SEM) is effective in the examination of the surface morphology and structure of purified melanin. It also allows for size distribution analysis of melanin particles, revealing their microstructural characteristics. In addition to SEM, EDX spectroscopy identified the elemental composition of the extracted pigment [5]. A small amount of melanin powder was gently applied to SEM sample plates. The samples were coated with a thin layer of gold particles through sputtering before imaging. The prepared samples were examined under a scanning electron microscope at 5,000× and 15,000× magnification levels. Clear and detailed images were captured using a 5 µm working distance and 15 MV acceleration voltage [20].

Table 2. Comprehensive table of analytical techniques in characterization of Melanin.

Technique	Principle	Key Findings / Diagnostic Features	Purpose in Melanin Confirmation
UV–Visible (UV–Vis) Spectroscopy	Measures light absorption across UV–visible range	Broad absorption decreasing from 200–800 nm; linear decay in semi-log plot	Confirms conjugated aromatic systems and optical properties of melanin
Fourier Transform Infrared (FTIR) Spectroscopy	Detects molecular vibrations and functional groups	Peaks at 3400 cm^{-1} (–OH, –NH), 1620 cm^{-1} (C=O, C=C), 1380 cm^{-1} (C–N)	Identifies functional groups (indolic, phenolic, carboxylic) confirming melanin chemistry
Electron Paramagnetic Resonance (EPR/ESR)	Detects unpaired electrons in free radicals	Single symmetric signal, g ≈ 2.003–2.005	Confirms presence of semiquinone-type radicals — definitive evidence of melanin

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Elemental (CHNS) Analysis	Quantifies C, H, N, S content	DOPA-melanin: high N (~8–12%); DHN-melanin: low N (<3%)	Differentiates melanin types (DOPA vs. DHN) and verifies biogenic origin
X-Ray Diffraction (XRD)	Examines crystalline vs. amorphous structure	Broad diffuse peak around 2θ = 20–25°	Confirms amorphous, non-crystalline carbon-like polymeric structure
Thermogravimetric / Differential Scanning Calorimetry (TGA/DSC)	Measures thermal degradation and stability	Gradual weight loss up to 800 °C; no sharp melting point	Confirms thermal stability and polymeric nature of melanin
Scanning/Transmission Electron Microscopy (SEM/TEM)	Visualizes morphology and surface topology	Irregular, amorphous, granular or aggregated particles	Confirms pigment morphology and homogeneity post-purification
High-Performance Liquid Chromatography (HPLC) & Mass Spectrometry (MS)	Separates and identifies degradation products	Detection of indolic acids (DHI, DHICA) or DHN derivatives	Confirms precursor pathway and chemical identity (DOPA vs. DHN origin)

Melanin Applications

The use of microbial pigments is seen as safer compared to manufactured colors. The pigments made by microbes serve as a defense mechanism against potentially dangerous environmental factors. There is a vast array of industrial uses for microbial pigments, which are biodegradable, non-toxic, and non-carcinogenic. Melanin or melanoid is a dark pigment that these microbes may generate and release. Melanin can be used in food packaging to extend shelf life due to its natural antibacterial, anti-radiation, and antioxidant properties. Melanin has potential as a natural antioxidant in the food, cosmetic, and pharmaceutical industries [35].

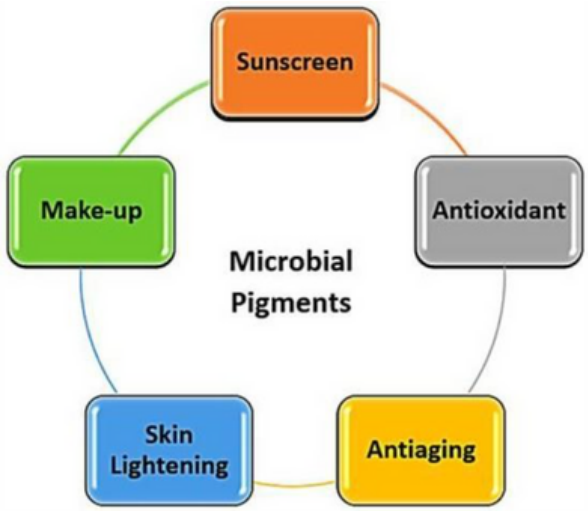

Figure 2. Applications of Microbial Pigments in cosmetic industries.

Bio-medical applications

Actinobacterial melanin confers a dual-functional defense system by absorbing harmful ultraviolet (UV) and visible radiation and scavenging reactive oxygen species (ROS), thereby offering photoprotection and antioxidant resilience. The pigment's broadband absorption enables it to act as a natural UV filter, dissipating incident photon energy and attenuating penetration into underlying biological components [36]. Simultaneously, its structure harbors stable semiquinone radicals capable of reversible redox cycling, which neutralizes ROS and prevents oxidative damage. In microbial and material contexts, this redox buffering property is harnessed in biomedical applications such as UV-protective coatings, radical-scavenging additives, and bio-compatible antioxidant materials [37]. Because actinobacteria-derived melanin combines strong light absorbance, radical quenching, and structural stability, it is ideally suited for deployment in fields requiring robust photoprotection and oxidative stress mitigation.

Bio-medical applications

Actinobacterial melanin holds promising utility in environmental remediation and sustainable material science owing to its high affinity for metal ions, redox capacity, and biocompatibility. First, melanin's chelation and sorption properties make it an effective biosorbent for removal of heavy metals and organic pollutants from water and soil its polymeric matrix binds cations, dyes, and aromatic compounds, thus reducing contaminant loads [20, 38, 39]. Moreover, microbial melanin have been demonstrated in bioremediation and radioprotection applications: they can scavenge reactive species generated by radiation and shield biomolecules from oxidative damage, enabling their use in treating radioactive waste or contaminated sites [40, 41]. In the agricultural domain, melanin-producing microbes or melanin amendments can enhance soil remediation, degrade synthetic dyes, and improve plant resilience to abiotic stress [29]. Additionally, actinobacterial melanin can substitute synthetic chemical dyes in textile wastewater treatment, acting both as pigments and as adsorbents to sequester residual dye molecules [42]. Finally, melanin's electronic and redox-active nature suggests potential roles in bioelectronics, electron-shuttling in microbial fuel cells, and catalytic interfaces for environmental sensing [37]. Together, these capabilities point to actinobacterial melanin as a multifunctional, environmentally friendly tool for pollution control, soil and water rehabilitation, and sustainable technologies.

Melanin in cosmetics

Recent research highlights the potential of melanin-inspired materials in cosmetic formulations to regulate pigmentation. Synthetic melanin nanoparticles and plant or microbial-derived melanin have shown antioxidant and photoprotective properties that can reduce UV-induced melanogenesis, thereby preventing hyperpigmentation and indirectly contributing to skin lightening effects [43].

Challenges and Biotechnological Approaches

Scaling up melanin production from Streptomyces faces

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several technical and biological challenges, primarily linked to strain variability, low yield, and process inconsistency during large-scale fermentation. The biosynthesis of melanin in Streptomyces is a secondary metabolic process, tightly regulated by growth phase and environmental cues, leading to fluctuating pigment titers under non-optimized conditions [37]. Additionally, tyrosinase and polyketide synthase (PKS) activities are sensitive to nutrient composition, oxygen transfer rates, and metal ion availability, making it difficult to maintain consistent enzyme expression in bioreactors [44]. Another limitation is product recovery—melanin's strong cell-wall association and insolubility complicate downstream extraction and purification, often reducing overall process efficiency [45].

Biotechnological innovations, however, are increasingly addressing these bottlenecks. Metabolic and genetic engineering approaches have been employed to overexpress key biosynthetic genes (melC2, tyrA, and pks clusters) and to develop regulatory knockouts that redirect metabolic flux toward pigment synthesis [46]. Furthermore, systems biology tools and omics-guided strain improvement enable fine-tuning of precursor supply (L-tyrosine, acetyl-CoA) and cofactor balance, enhancing pigment productivity. Optimization of bioprocess parameters using response surface methodology (RSM) and fed-batch strategies improves aeration, pH stability, and nutrient feed control, thereby scaling production more predictably [47]. Emerging synthetic biology and bioreactor engineering techniques such as CRISPR-based gene regulation, immobilized cell systems, and continuous fermentation are further enhancing yield, reproducibility, and downstream recovery efficiency. Collectively, these integrated biotechnological advances provide a scalable framework for industrial melanin biosynthesis using Streptomyces as a robust microbial platform.

Conclusions

Actinobacteria possess sophisticated pathway in their cell physiology that ensures them to undergo complex biosynthetic pathways in-order to produce bioactive molecules. The bioactive molecules are of a varied array of compounds ranging from antibiotics, enzymes and pigments. Highlighting the bio-pigments, melanin is one such example restricted to genus Streptomyces. Melanin of Streptomyces origin have many biological benefits. They exhibit potential in various fields due to their antioxidant, antimicrobial, antitumor, antiviral, and radioprotective properties. Melanin plays a crucial role in protecting living organisms from harmful UV and ionizing radiation. It protects cells from damage from extreme temperatures and radiation exposure, like sunscreen. Products that use melanin's radical-scavenging properties can protect skin from the harmful effects of sunlight. Melanin, known for its UV-absorbing properties, finds applications in sunscreens and skincare products. Additionally, its ability to filter high-energy blue and violet light makes it a promising anti-aging ingredient, slowing premature skin aging.

Disclosure Statement

The author does not have conflict of interest in this research.

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