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Isolation, Characterization and Antimicrobial Evaluation of Melanin Produced by *Streptomyces thermocarboxydus* (RSMLAC01)

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Abstract

Actinomycetes are often said to produce all kinds of pigmentation species on various media. They are abundant in soil and hot regions like hot water springs. Water samples for microbial isolations were taken from hot spring Ganpatipule, Ratnagiri district of Maharashtra State. Isolation of microorganisms was carried out with a minimal salt medium. These isolates were cultured on iron rich yeast extract peptone agar to determine their potential for melanin pigment preparation. The isolated strain was then sent for 16-S RNA sequencing. Actinomycetes producing dark black pigments were kept in spores in a 10% glucose solution. These spores were inoculated into seed producing broth. Subsequently, this seed medium was inoculated in large production medium for 2-3 days at 30°C. UV-Vis spectroscopy was made after the melanin pigment produced to check if it is able to absorb the UV radiations. PDA plates and nutrient agar plates of different microbial cultures were prepared to assess the antimicrobial activity of the pigment. With the growing significance of melanin for pharmaceutical and cosmetic products, this study aimed to obtain pigment-producing Actinomycetes isolated in extreme environments. New strain RSMLAC01, isolated from the hot springs of Ganpatipule, Maharashtra, was isolated from the soil and confirmed as Streptomyces thermocarboxydus by 16S-rRNA sequencing. The strain showed well-defined extracellular melanin production through L-DOPA pathway. The purified melanin had typical UV-Vis absorption peaks at 221 nm and showed significant antimicrobial activity against several fungal and bacterial pathogens, indicating a promising bioactive action for cosmeceutical and biomedical applications.

Keywords: *Actinomycetes, Hot springs, 16S rRNA sequencing, UV-Vis spectroscopy, Antimicrobial activity.*

1. Introduction

Actinomycetes are Gram-positive, filamentous bacteria known for their complex life cycles and prolific production of secondary metabolites. Among these metabolites, melanin—an ancient biological polymer—serves as a high-performance shield against UV radiation and oxidative stress [1]. While melanin is traditionally sourced from animal or synthetic origins, microbial production offers a scalable, eco-friendly alternative. This research explores the potential of a thermo-tolerant Streptomyces strain to serve as a biological factory for melanin [2]. Melanin is a pigment formed by organisms in every aspect of life. In most studies, melanin was chemically prepared or obtained from animals. In some previous studies, DOPA melanin demonstrated a functional activity as an antibacterial natural product against 12 pathogenic bacteria from hospital isolations. Nowadays the growing antibiotic resistance of microorganisms with increasing bacterial resistance to antibiotics is a serious problem [3]. Actinomycetes, is one of the most colorful microbes, has the ability to produce a wide variety of pigments through various natural and synthetic media. Thousands of substances, both antibacterial and antifungal, are generated by marine organisms along with many. As for synthesizing melanin, microbial mediated production is the most feasible method. The scalability and yield of melanin are advantages of this method [4]. The melanin color varies from yellow to black and is also dependent on the metabolic pathways which induce the production of the melanin. Melanins are widely used in many spheres of life-in everything from cosmetics, optical lenses, pharmaceuticals, batteries to many other things [5].

Streptomyces are all Gram-positive aerobic members of the order Actinomycetales within the class Actinobacteria and have a high DNA G-C content of 69 ± 78 mol%. They primarily appear as spores in soil, spores which germinate into the substrate and production of the aerial mycelia in good nutritional conditions. They have a substantial impact to the microbial community of the soil environment responsible for degrading and recycling of cellulose, lignin and chitin, natural biopolymers, etc [6]. Actinomycetales are important producers of biologically active compounds: two-thirds of microbially

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obtained antibiotics are from actinomycetales, particularly Streptomyces spp [7]. The actinomycins are chromopeptide lactone antibiotics, a crucial category of natural products that although discovered more than 60 years ago, continue to be the subject of much of the research attention, especially in biological and medicinal sciences [8]. Among the actinomycins, Streptomyces thermocarboxydus has been investigated most extensively and is used to treat malignant tumors and childhood rhabdomyosarcoma. It has been shown to possess anti- cancer activity (e.g., against HeLa or MCF7 cell lines) with low toxicity to normal cells [9]. Melanin form Streptomyces shows significant free radical scavenging (DPPH method), often >80% at low concentrations. Streptomyces thermocarboxydus is produced from a variety of Streptomyces species as part of a cocktail of actinomycins and from certain strains of Micromonospora [10]. This paper described the isolation of modern Streptomyces spp. isolated from the hot springs of Ganpatipule, Maharashtra, and identified as Streptomyces thermocarboxydus via 16S-rRNA sequencing.

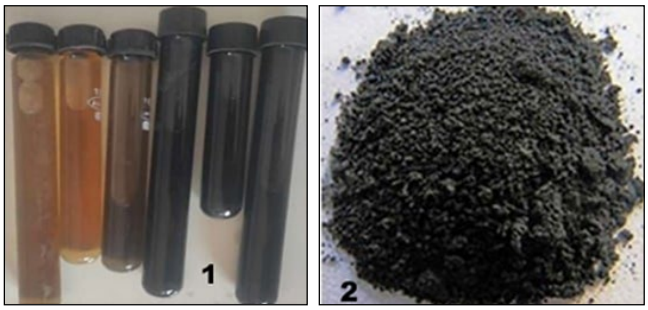
Actinomycetes comprise a varied group of Gram-positive filamentous bacteria classified under the order Actinomycetales within the Phylum Actinobacteria, Class Actinobacteria, and Subclass Actinobacteria [11]. These saprophytic microorganisms thrive in both natural and artificial ecosystems, significantly contributing to decomposition processes [12]. They are vital prokaryotes from an economic and biotechnological standpoint, known for their ability to generate an extensive array of bioactive secondary metabolites, including antibiotics, antitumor agents, immunosuppressants, and enzymes [13]. Remarkably, more than 10,000 of the roughly 23,000 recognized bioactive secondary metabolites originate from actinomycetes. Their impact on society is substantial; they supply crucial pharmaceuticals like antibiotics and anticancer treatments while also serving as important sources for novel discoveries in the pharmaceutical industry [14]. Pigments can be categorized into naturally occurring and synthetic types. Organic pigments produced by microbes are present in various organisms including bacteria, fungi, and actinomycetes. Significant natural pigments encompass carotenoids, flavonoids, tetrapyrroles, as well as xanthophylls such as astaxanthin, melanin, violacein, and riboflavin [15] emphasize that actinomycetes are key microorganisms with considerable economic relevance due to their capability to produce significant quantities of bioactive compounds and natural pigments. The increasing resistance among microbial pathogens globally poses a severe challenge, thus prompting the need for innovative medications that reduce side effects. Research has thoroughly investigated the antibacterial properties of marine actinobacteria [16]. Studies have characterized antifungal peptides (AFPs) generated by actinomycetes for their beneficial effects against plant diseases. Furthermore, research indicates promising antioxidant properties found in Streptomyces species isolated from soil and marine samples [17]. Marine environments provide unique opportunities for discovering or extracting various strains of Streptomyces with diverse chemical profiles capable of combating numerous microorganisms as well as diseases related to living organisms and cancer [18].

Marine actinomycetes show promise as sources of new bioactive metabolites noted that extracts from these bacteria exhibited protective effects against viruses such as influenza virus, Sendai virus, and Newcastle Disease Virus through antiviral activity. Breast cancer remains one of the foremost causes of cancer mortality among women worldwide and continues to present significant health challenges on a global scale [19]. Metabolites obtained from marine drugs frequently derive from marine actinobacteria; these compounds are essential for uncovering new pharmaceutical agents [20]. This study intends to assess the antimicrobial, antiviral, antioxidant, and antitumor properties of pigments extracted from marine Streptomyces thermocarboxydus (RSMLAC01) for potential clinical applications.

2. Materials and Methods

i). Sample Collection: Water samples were collected from the hot spring of Ganpatipule, situated in the Ratnagiri district of Maharashtra state, 375 km away from South Mumbai. Hot water was collected in a thermos. Samples were carried to the laboratory for further studies.

ii). Isolation of Microorganisms: Collected water samples were spread on the minimal salt agar medium and incubated at 30 degrees Celsius for 5 to 6 days. Obtained cultures were then isolated on sterile nutrient agar slants.



Plates 1–2: Melanin pigment production in culture tubes and dried melanin powder obtained after extraction.

Minimal Salt Medium (MSM): Minimal Salt Medium is a chemically defined medium used primarily for the isolation and cultivation of microorganisms capable of surviving under nutrient stress, specifically in environments lacking complex carbon and nitrogen sources.

Table 1: Composition of Minimal Salt Agar Medium

Ingredients	Amount (g/100 mL)
Potassium Nitrate KNO3	2.0
Dipotassium Phosphate K2HPO4	0.2
Magnesium Sulfate Heptahydrate MgSO4. 7H2O	0.1
Sodium Chloride NaCl	0.05
Calcium Carbonate CaCO3	0.05
Ferrous Sulfate Heptahydrate FeSO4 7H2O	0.3
Agar	0.001
Final pH	2.0

iii). Screening of Isolates for Melanin production: Isolates were streaked on the tyrosine agar (ISP medium 7) to detect melanin-producing ability. Cultured plates were incubated at 30 degrees Celsius for 2 to 3 days. After incubation time it was observed that among all isolates, only one white-colored isolate was able to produce dark black-colored extracellular pigment. That one strain was then named RSMLAC01.

Peptone Yeast Extract Iron Agar (ISP Medium): This differential medium is utilized to screen for microorganisms capable of synthesizing melanin pigments via the L-DOPA pathway. The presence of ferric ammonium citrate serves as an indicator for hydrogen sulfide production and pigment enhancement.

Table 2: Composition of Peptone Yeast Extract Iron Agar (ISP 6)

Ingredients	Amount (g/100 mL)
Peptone	1.5
Protease Peptone	0.5
Ferric Ammonium Citrate	0.05
Dipotassium Phosphate (K2HPO4)	0.1
Sodium Thiosulphate	0.008
Yeast Extract	0.1
Agar	2.0
Distilled Water (D/W)	100 mL
Final pH (at 25°C)	7.0 ± 0.2

Tyrosine Agar (ISP Medium 7): Tyrosine Agar is used for the differentiation of Streptomyces species based on their ability to produce tyrosinase. This enzyme catalyzes the production of melanin from L-tyrosine, resulting in a dark brown to black pigment in the medium.

Table 3: Composition of Tyrosine Agar (ISP Medium 7)

Ingredients	Amount (g/100 mL)
Glycerol	1.5
L-Tyrosine	0.05
L-Asparagine	0.05
Dipotassium Phosphate (K2HPO4)	0.05
Magnesium Sulfate Heptahydrate (MgSO4 . 7H2O)	0.05
Ferrous Sulfate Heptahydrate (FeSO4. 7H2O)	0.001
Agar	2.0
Distilled Water (D/W)	100 mL
Final pH (at 25°C)	7.0 ± 0.2

Peptone Yeast Extract Iron Broth: This broth is specifically formulated for the detection of hydrogen sulfide (H_2S) production and the extracellular production of melanin pigments in liquid culture.

Table 4: Composition of Peptone Yeast Extract Iron Broth

Ingredients	Amount (g/100 mL)
Peptone	15.0
Protease Peptone	5.0
Ferric Ammonium Citrate	0.5
Dipotassium Phosphate (K2HPO4)	1.0
Sodium Thiosulphate	0.08
Yeast Extract	1.0
Final pH (at 25°C)	7.0 ± 0.2

iv). Identification: The lead isolate, RSMLAC01, underwent Gram's staining and 16S-rRNA sequencing for taxonomic classification. 16S-rRNA sequencing of RSMLAC01 was done, and it was found that the strain had its genome very similar to that of Streptomyces thermocarboxydus (98.6%).

v). Production of Melanin: Spores of the strain RSMLAC01 was inoculated in the peptone tyrosine broth (ISP medium 7) and then it was incubated on rotatory shaking incubator at 300 rpm for 3 to 5 days at 30 degrees Celsius.

vi). Harvesting of Melanin: Black pigment produced was then transferred into the centrifugation tubes and centrifuged at 15000 rpm for 10 minutes. Supernatant was then collected into the clean and sterile flask and the cell pallets were discarded. Supernatant was then treated with 1N HCl for 3 days for polymerization of melanin pigment. Then it was again centrifuged at 10000 rpm for 10 minutes. Sediment black colored complex were collected and dried. After drying, purified melanin was stored in powdery form for further studies.

vii). UV-Vis Spectrophotometry: 1N NaOH was used as a solvent for melanin. Baseline was set by using 1N NaOH and the UV-visible spectrophotometer spectrum (200-500 nm) of purified melanin was obtained.

viii). Antimicrobial Activity: The well-diffusion method was employed on PDA and Nutrient Agar plates to test activity against various pathogens.

- **Antifungal Activity:** 4 sterile PDA plates were prepared. Fungal cultures were spread on them in sterile condition. Then wells were made in the middle of the plates using a cork-borer. After that 2 drops of melanin pigment were added in the wells. Plates were incubated at room temperature for 4 to 6 days. After incubation period plates were observed for zone of inhibition.
- **Antibacterial Activity:** 4 sterile Nutrient agar plates were prepared. Different bacterial cultures were spread on them, maintaining sterile conditions. Wells were made inn the middle of the plates using cork-borer. Then 2 drops of melanin pigment were added in the wells. Plates were incubated at room temperature for 24 to 48 hours. After incubation period plates were observed for zone of inhibition.

3. Results

Isolation: Different types of microbes were obtained on minimal salt medium.

Screening for Tyrosinase Activity: Tyrosine agar plate showing growth of RSMLAC01 and diffused black pigment secreted by strain RSMLAC01.

16S-rRNA Sequencing: After 16S-rRNA sequencing it was found that strain RSMLAC01 is most closely related to *Streptomyces thermocarboxydus* (Accession: MZ389913), sharing a direct node with a bootstrap value of 98, which indicates a very high level of statistical confidence in this specific grouping. Image 3 clearly shows that RSMLAC01 is part of a distinct lower clade that includes: -*Streptomyces thermocarboxydus, Streptomyces sp. SXHMA, Streptomyces sp. 5-1457, Streptomyces sp. 3860.* Scale bar at the bottom (0.005) in image 3 represents the number of nucleotide substitutions per site. The horizontal branch length between RSMLAC01 and its neighbour is very short; it suggests a high degree of sequence similarity (likely > 99%).

Gram's Staining: After gram's staining it was observed that the strain is gram-positive filamentous bacilli. They were having small deep purple-colored small granular structures on the branching filaments which is characteristic property of Actinomycetes.

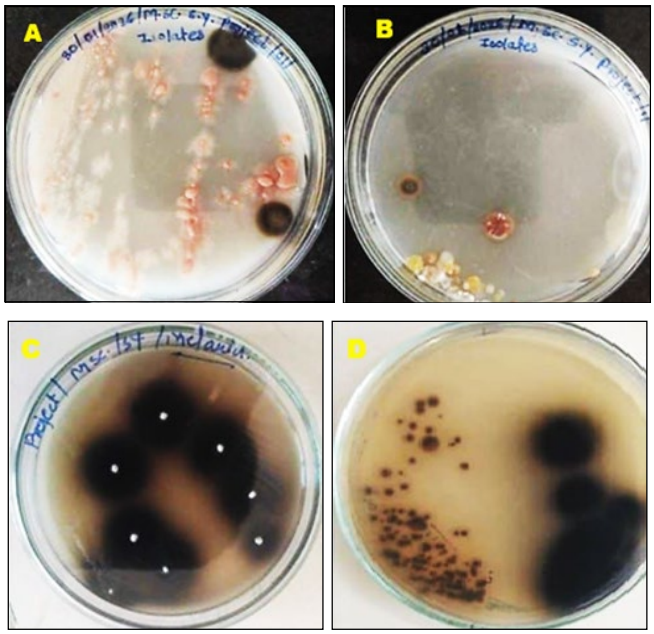
Production of Melanin: After the incubation period, color of tyrosine broth was changed to deep black indicating the strong tyrosinase activity of the strain RSMLAC01. The color change was due to accumulation of produced melanin. The production of melanin was done using the L-DOPA pathway because only L-tyrosine (Precursor amino acid for L-DOPA pathway) and Copper ions (as a cofactor for the tyrosinase enzyme) was provided in the medium. As there was only amino acid (i.e., L-tyrosine) was present, inoculated strain produced the tyrosinase enzyme which oxidised the tyrosine into L-DOPA, and then rapidly oxidized L-DOPA into dopaquinone.

Harvesting the Melanin Pigment: After centrifugation

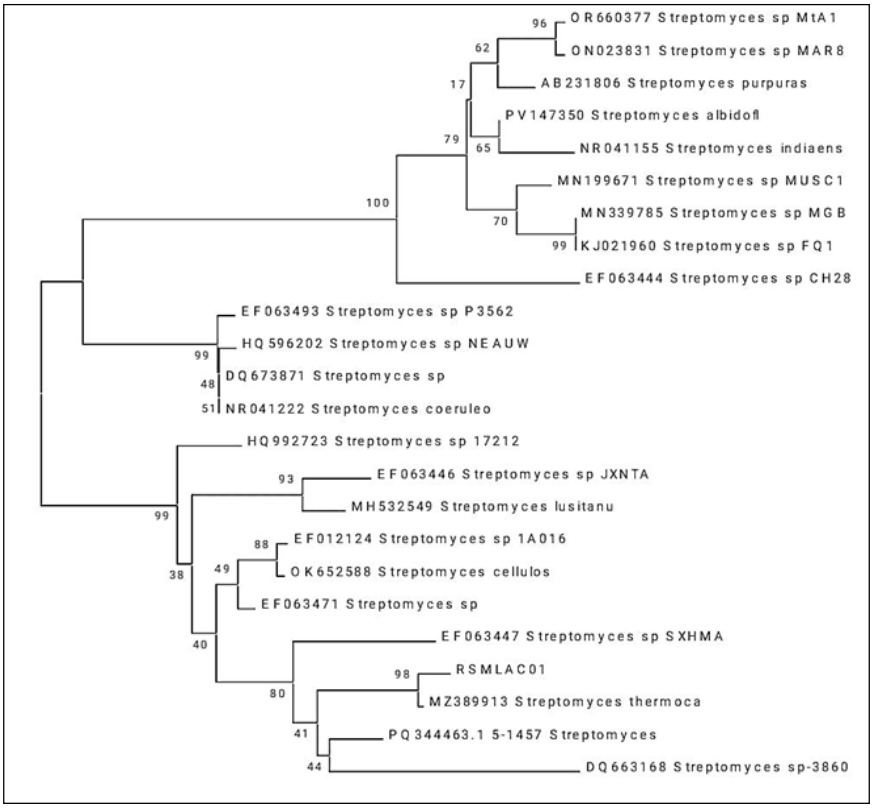
supernatant was collected in screw cap tubes and the cell pallets were discarded. Collected supernatant was then acidified using conc. HCl for about 3 days for polymerisation because at acidic conditions melanin gelts polymerized which helps to settle it down. Then this acidified pigment was centrifuged at high speed. Pallets of the melanin was collected and this process continued till the complete dissolved melanin got recovered from medium. After complete recovery collected melanin was dried and stored.

Solubility Test: After solubility test it was observed that it is only soluble in bases like NaOH and insoluble in water and organic compounds.

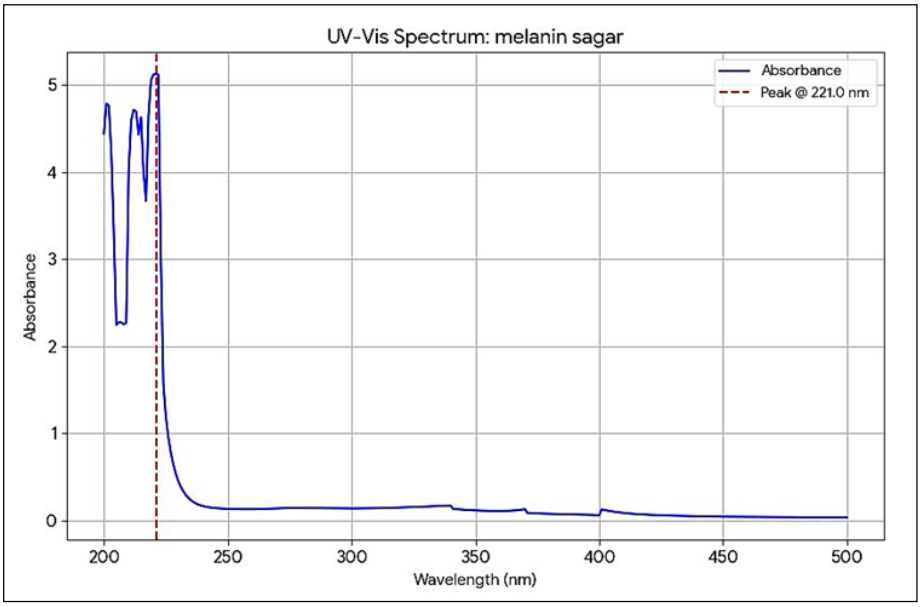
UV-Visible Spectrophotometry: As melanin was soluble in NaOH, 1N NaOH solution was used as a solvent and also the baseline was set using 1N NaOH. UV-Vis absorbance spectrum (200 nm to 500 nm) was obtained using JASCO V-730 spectrophotometer. The obtained data shows a general trend where absorbance remains relatively low (below 0.2) from 500 nm down to approximately 235 nm, before spiking sharply as it approaches the lower UV range, peaking at 221 nm. As the data follows the general trend, with absorbance is higher at 278 nm wavelength and decreased significantly as it moves from 200 nm to 500 nm. Data is consistent with biological extract likely melanin.



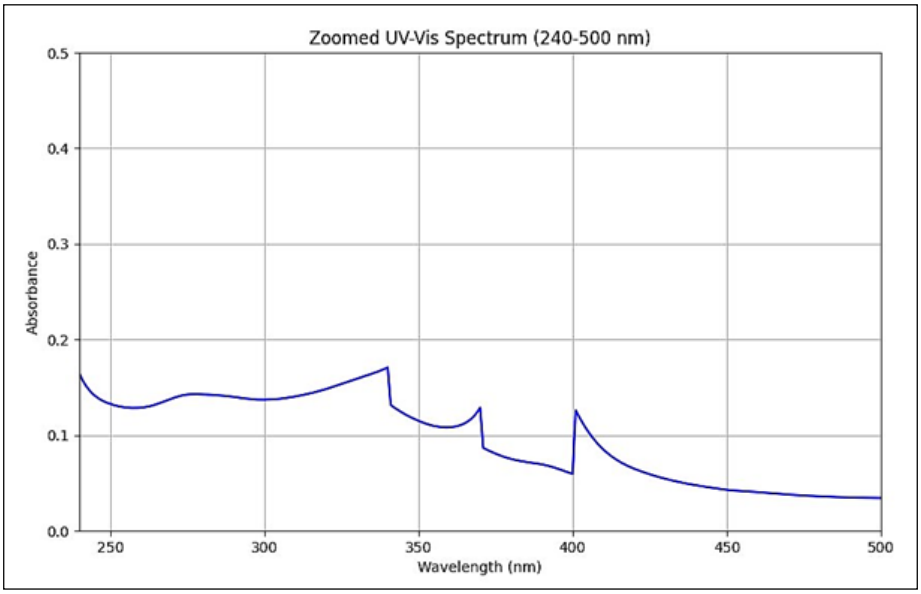
Plates A–D: Isolation and screening of microbial isolates on different media showing colony variation and melanin-producing strains (dark pigmented colonies).



Phylogenetic tree of strain RSMLAC01



Graph 1: *UV-Vis Spectrum (200-500 nm) of melanin.*



Graph 2: *Zoomed UV- Vis Spectrum (240-500 nm).*

Antimicrobial Activity: After incubation period of PDA and Nutrient agar plates it was observed that melanin had shown zones of inhibition indicating antimicrobial activity against fungi like: *Fusarium, Trichoderma, Aspergilus fumigatus*, and against bacteria like: *Bacillus subtilis, Actinomycetes, an Staphylococcus sp* but, not showing antimicrobial activity against *Aspergillus niger* (fungus). RSMLAC01 was identified as *Streptomyces thermocarboxydus* (98.6% similarity), which is consistent with previous reports highlighting the diversity and bioactive potential of *Streptomyces* species [9, 10]. Morphologically, it appeared as Gram-positive filamentous bacilli with characteristic granular structures, a typical feature of Actinomycetes [11]. The broth transitioned to a deep black color, confirming extracellular melanin synthesis through the oxidation of L-tyrosine by the tyrosinase enzyme, which aligns with earlier studies on microbial melanin production via the L-DOPA pathway [13, 19].

The pigment was soluble in bases (NaOH) but insoluble in water and organic solvents, which is a well-established property of eumelanin pigments [15, 16]. UV-Vis analysis showed a sharp peak at 221 nm and a high absorbance range between 200–280 nm, consistent with characteristic absorption patterns reported for microbial melanin [14, 18].

Melanin exhibited clear antimicrobial activity with zones of inhibition against fungi such as *Fusarium*, *Trichoderma*, and *Aspergillus fumigatus*, and bacteria including *Bacillus subtilis*, *Staphylococcus* sp., and other Actinomycetes. These findings are in agreement with previous studies reporting the broad-spectrum antimicrobial and antioxidant properties of melanin derived from *Streptomyces* species [8, 17, 20]. However, no activity was observed against *Aspergillus niger*, which may be attributed to inherent resistance mechanisms or structural differences in the fungal cell wall, as also noted in earlier reports [9].

Conclusion

The study successfully demonstrated that Streptomyces thermocarboxydus RSMLAC01 is an efficient source of concentrated, biocompatible melanin. The pigment's strong photoprotective properties in the UV-C and UV-B ranges, combined with its broad-spectrum antimicrobial activity, highlight its potential for use in natural sunscreens and

biomedical treatments.

Research on "Isolation, Characterization, and Antimicrobial Evaluation of Melanin Produced by Streptomyces thermocarboxydus (RSMLAC01)" lead to several key conclusions regarding the characterization and potential of this biopolymer have spectral Identity and Consistency where UV spectrum obtained from the sample shows a characteristic profile for microbial melanin. The significant spike in absorbance as the wavelength decreases into the UV range—peaking at 221 nm and remaining high through the 200–280 nm range is consistent with the recorded data for eumelanin. The relative transparency in the visible light region (400–500 nm) further distinguishes it from other pigments like chlorophyll or carotenoids, which would show prominent peaks in the visible spectrum.

The Concentration and Measurement Thresholds where melanin has maximum absorbance recorded ($MAXY = 5.12$) indicates that the sample was highly concentrated. In spectrophotometry, absorbance values above 2.0 or 3.0 often exceed the linear range of the instrument (Beer-Lambert Law), leading to increased noise and potential inaccuracies at the peak. For precise quantification in future studies, a serial dilution would be necessary to bring the peak absorbance into a more reliable range (typically between 0.1 and 1.5). The microbial Production Potential have successful isolation of melanin from Actinomycetes (specifically Streptomyces) underscores the value of these microorganisms as bio-factories. Unlike synthetic or animal-derived melanins, microbial melanin is eco-friendly and Biocompatible: Produced through natural oxidative polymerisation of phenolic compounds (like L-tyrosine). It is scalable and suited for submerged fermentation, which allows for controlled, large scale production. It also have some broad Application Value that particularly have data supports the conclusion that the extracted pigment possesses strong photo protective properties, particularly in the UV-C and UV-B ranges. This makes it a viable candidate for cosmeceuticals (as a natural active ingredient in sunscreens), Biomedicine (for its antioxidant and free-radical scavenging capabilities), Bioremediation (as future approach) that Utilising its ability to bind heavy metals or mitigate environmental stressors.

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