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Production of Melanin from *Bacillus* Species Isolated from Rhizosphere Soil

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ABSTRACT

Melanin is an UV protective pigment which has shown its presence all across the living organisms except for viruses, for various functions such as UV-protection, Anti-microbial activity, free radical scavenging activity etc. The pigment is produced in specialized cells called the Melanocytes by the process of Melanogenesis in humans, while it is produced in cytoplasm in bacteria and other prokaryotes. The main enzyme that is responsible for catalyzing the production of melanin from L-tyrosine is 'Tyrosinase'. It is this enzyme which enables the bacteria to convert amino acid (L-tyrosine) into melanin. The melanin is a polymer which is made from the L-DOPA, DHI, and DHICA monomers. The current study deals with the production and extraction of melanin from *Bacillus* species isolated from rhizosphere soil of Neelgiri tree and Sagwan tree. The study further deals with melanin and its characterization using UV- visible spectroscopy, FT-IR spectroscopy and its solubility test in different solvents. Undertaking the industrial applications of bacterial melanin, the current study also deals with the UV-protection activity of the extracted melanin, and the antibacterial activity of the melanin extracted from the bacterial isolated.

Keywords - Melanin, UV, L-tyrosine, L-DOPA

I. INTRODUCTION

Melanin and its different types are the natural pigments which have their presence in all the five kingdoms of the living organisms. The compound melanin is predominantly Indolic in its nature and is found to be abundantly produced on the surface of the vertebrates, such as their skins. In the higher organisms this pigment is produced in specialized cells called the Melanocytes. The process for the formation of the Melanin is called as Melanogenesis, this process is generally triggered by the exposure of the melanocytes to the ultraviolet light. This pigment is also found to be secreted in microorganisms in their cytoplasmic organelles called the Melanosomes specifically. The origin of this word which is Melanin comes from Ancient Greek word called *melons* which means dark in the mentioned language. Based on the colour and the structural orientation of the atoms of the compound melanin, it can be broadly classified in

Three major classes which are: Eumelanin, Pheomelanin, and Allomelanin (Pathan and Pethe 2016; Simon *et al.*, 2001).

Microorganisms in marine as well as on terrestrial environment are often exposed to extreme conditions such as pressure, temperature, salinity etc and the ability to produce biologically active compounds are often accompanied by depletion and diffusion of micronutrients. Microorganisms such as bacteria produce different chemicals like biosurfactants which increases the bioavailability to over comes or survive in the harsh environmental conditions. One of such chemicals or pigments produced by bacteria is melanin. Melanin pigment is produced by the bacteria which enhances the ability of the bacteria to survive in the extreme environment. The ability of free-living microbes to produce melanin is likely related to their ability to survive in the environment. In regards many fungi also constitutionally synthesize melanin such as *Cryptococcus neoformansm.* Allomelanin is more common in microorganisms, some of the fungus known to produce melanin are *Sporothrix schenchii, Sepia officinalis, Aspergillus niger, Histoplasma capsulatum* etc. coming to bacteria that have shown the data of producing melanin are *Aeromonas salmonicida, Azotobacter spp, Mycobacterium spp, Pseudomonas aeruginosa, Legionella spp,* etc (Pathan and Pethe., 2016; Jigna *et al.*, 2022).

Microbial melanin has some distinct properties which will be discussed further, the first is the Physiochemical properties; solubility of melanin, as microbial melanin exhibits distinctive solubilities. Melanin is mostly insoluble in water and various inorganic solvents. But it shows solubility in some organic solvents such as DMSO, alkaline water (pH 10.0<), phosphate buffer etc. whereas, it gets precipitated in acidic conditions making the acidic condition more suitable for extraction processes. Decolorization of the pigment happens when reacted with hydrogen peroxide, mainly due to the degradation of the melanin. This is due to the attack of the hydrogen peroxide on the -COOH functional group (Singh *et al.*, 2021).

Melanin upholds diverse applications as a result of its environmentally sustainable and multifarious biological properties. Microorganisms have emerged as a great source of melanin pertaining to their easier upscaling and inoculum cultivation in very large quantities for industrial use. The complexity of the melanin biopolymer lies in its complex biosynthetic pathways both in prokaryotes as well as in eukaryotes. Considering all the aspects of the pigment; microbial melanin validates a promising future in the fields of dermatology, bioremediation, cosmetics, and environmental technology.

II. METHODS AND MATERIAL

❖ Isolation and identification of Melanin producing bacteria from the rhizosphere soil.

The bacteria were isolated from the rhizosphere of Neelgiri and Sagwan Trees using L-tyrosine Agar which constituted L-tyrosine as its main component, for screening of melanin producing bacteria. The bacterial isolates were then sub-cultured on the same L-tyrosine Agar, Microscopic analysis and Biochemical tests were performed.

❖ Inoculum Build-up for Melanin Production.

After identification of the bacterial strains, 2 test tubes containing 10ml L-tyrosine broths were inoculated in with the isolated bacterial isolates. Later the 10ml test tubes after incubation of 24 Hour at 37 ° C were subjected to melanin production in 250ml flasks containing 100ml L-tyrosine broth. The 100ml inoculum of the bacterial strains was later subjected to 1000ml flasks containing TBB media (Tyrosine Basal Broth) for the production of melanin. After the successful incubation of 15 days at 37 ° C, the 10ml sample from each flask was centrifuged and read spectrophotometrically at 400nm for the estimation of

the concentration of melanin produced (Jigna *et al.*, 2022; Turick *et al.*, 2002).

❖ Extraction and purification of Melanin

After the incubation period of 15 days the TTB in the 1000ml flasks is centrifuged using centrifuge machine. The TTB media was centrifuged at 6000 rpm at 28 ◦ C for 10 min, for the separation of bacterial pellets from the supernatant. The extracted supernatant was further acidified below the pH of 2 using 1N HCl. As the pH decreased black colored precipitate was observed at the bottom of the flask. The acidified supernatant was later subjected to further precipitation of 2 days. After this duration the precipitates were separated and centrifuged at 6000rpm at 28 ◦ C for 10 min, this process pellets the melanin out. The extracted pellets of melanin pigment were further washed with absolute ethanol and deionized water simultaneously two times to obtain purified melanin. The extracted and purified melanin was later dried in hot air oven (Sajjan *et al.*, 2013).

❖ Solubility test of the extracted melanin

For testing the solubility of the extracted melanin 20 test tubes were used in the set of 10 tubes for each extracted melanin pigment. Each set of test tubes had 1ml of the following compounds: Hydrochloric acid (HCl), Petroleum ether, Chloroform, Hydrogen peroxide (H_2O_2), Acetic acid, Sodium hydroxide (NaOH), Dimethyl sulfoxide (DMSO), Iso-propanol, Ethanol and Distilled Water. The extracted melanin was added in the test tubes and then the tubes were vortexed in the vortex machine for 5 min, then centrifuged at 5000rpm for 1min. The test tubes were observed for solubility of melanin (Fava *et al.*, 2013).

❖ Determination Of absorption spectrum of melanin by UV-Visible spectroscopy

The melanin pigments were read spectrophotometrically using the melanin solution at 200nm to 800nm at the interval gaps of 50nm (wavelength of the electromagnetic spectrum) in a UV-Visible spectrophotometer. Results were noted for further study.

❖ Fourier-Transform Infra-red (FT-IR) Spectroscopy

The FT-IR spectrum was recorded at the wavelength of 4000-400 cm-1. Characteristic peaks were obtained and the recorded report was analyzed. The extracted melanin samples were mixed with potassium bromide and grinded in a mortar and pestle, the melanin-KBr pellet was put in a laboratory hydraulic press to convert the pellet into discs, the discs were set into the FT-IR machine and then IR-radiations were bombarded on to the sample, of 4000-400 cm-1 wavelength to make the molecule of the sample to vibrate and on the basis of the molecular vibrations and molecular stretching of the functional groups we get the IR-spectra with characteristic peaks of different functional groups of the molecules.

❖ UV photoprotection activity of the melanin

Total of 22 test tubes were utilized for the test, in the set of 11, each for the isolated bacterial strain. Each test tube had 9ml of TBB media and 1ml of respective melanin producing bacterial cultures Neelgiri (NT) and Sagwan (ST) were inoculated in the tubes. The tubes were then subjected to 15 days of incubation at 37 ◦ C. TBB media in the test tubes turned brown indicating the production of melanin. These test tubes were then treated with UV radiations of 200nm in a laminar air flow for the time duration of 60min. Each subsequent test tube was subjected under UV radiation in the interval span of 5min, 10min, 15min, 20min, 25min, 30min, 35min, 40min, 45min, 50min, 55min, and 60min. 1 test tube from each set was set as control and was not treated under UV-radiation (0min). After the UV treatment 1ml of treated broth from each test tube was inoculated and spread uniformly on nutrient agar plates and the plates were kept for incubation in an incubator for 24 hours at 37 ◦

C. The bacterial growth on the plates was observed after the incubation and the photo protective property was estimated (Joshi *et al.*, 2021).

❖ **Determination of antibacterial activity of the melanin pigments**

Antibacterial activity of the extracted melanin pigments was tested against different pathogenic strains of bacteria: *Escherichia coli, Pseudomonas aeruginosa, Klebsiella spp.* Solutions of 100mg/ml of concentration of melanin were prepared in DMSO solvent. Agar well diffusion assay was used for testing the antibacterial activity. Test bacterial cultures were inoculated by swabbing on the Mueller-Hinton Agar plates. Wells were prepared by sterile cork borer in each plate. The Melanin solutions and DMSO (control) were poured in the wells under a sterile environment. The plates were then incubated for 24 hours at 37 °C, and clear zone formation around the wells was checked for determining the antibacterial activity.

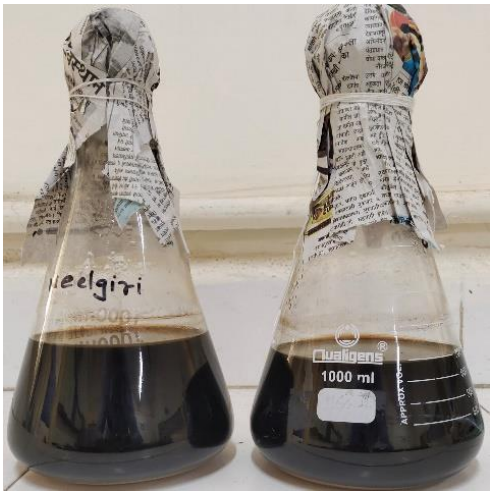
III. RESULTS AND DISCUSSION

The morphological, cultural and biochemical characteristics of melanin producing bacterial isolates ST and NT which were isolated from the rhizosphere of Sagwan and Neelgiri trees respectively were determined using different morphological, cultural, and biochemical methods.. Different tests performed confirmed that the bacterial isolates ST and NT belongs to *Bacillus spp.*

The bacterial isolates (ST and NT) were inoculated in 1000ml TBB media in separate conical flasks and incubated at 37 °C for 15 days. After the incubation period the TBB media in the conical flasks turned completely dark, the media then were centrifuged at 6000 rpm at 28 °C for 10 min. This process separated the bacterial pellets from the melanin containing supernatants. The supernatants were acidified below the pH of 2 using 1N HCl, which would precipitate melanin at the bottom of the flask. After two days the precipitates were centrifuged at 6000 rpm at 28 °C for 10 min to obtain melanin. The extracted melanin pigments were washed twice using absolute ethanol and deionized water to obtain purified melanin. The TBB media inoculated with ST isolate gave 2.68 gm of purified melanin and the TBB media inoculated with NT isolate gave 3.28 gm of purified melanin.

NT Melanin (Extracted)

ST Melanin (Extracted)



Melanin production in L-tyrosine broth after 15 days of incubation

The solubility of the extracted melanin pigments from the isolates (ST and NT) was tested (Table 1). It was found that the melanin pigment extracted from ST isolate was soluble in Ethanol, Sodium hydroxide (NaOH), Acetic acid and Dimethyl sulfoxide (DMSO). The pigment was found to be insoluble in Hydrochloric acid (HCl), Petroleum ether, Chloroform, and Water. While it was found that the pigment was partially soluble in Hydrogen peroxide (H_2O_2) and Iso-propanol. The extracted melanin pigment form the NT isolate was found to be soluble in Ethanol, Hydrogen peroxide (H_2O_2), Sodium hydroxide (NaOH), Dimethy sulfoxide (DMSO), and Iso-propanol. The pigment was found to be insoluble in Hydrochloric acid (HCl), Petroleum ether, Chloroform, and Water. While it was found that the pigment was partially soluble in Acetic acid.

Table 1 : Determination of solubility of extracted melanin from the bacterial isolates

Sr. No	Solvent	Solubility	
		ST	NT
1	Hydrochloric acid	Insoluble	Insoluble
2	Petroleum ether	Insoluble	Insoluble
3	Chloroform	Insoluble	Insoluble
4	Ethanol	Soluble	Soluble
5	Water	Insoluble	Insoluble
6	Hydrogen peroxide	Partially soluble	Soluble
7	Acetic acid	Soluble	Partially soluble
8	Sodium hydroxide	Soluble	Soluble
9	Dimethyl sulfoxide	soluble	Soluble
10	Iso-propanol	Partially soluble	Soluble

The UV-Visible spectroscopy of the extracted melanin pigments was performed. The melanin pigment isolated from NT isolate showed the maximum absorbance of 1.327 at 200nm followed by 1.321 at 250nm, 1.307 at 300nm, 1.208 at 350nm, 1.012 at 400nm, 0.921 at 450nm, 0.915 at 500nm, 0.777 at 550nm, 0.569 at 600nm, 0.250 at 650nm, 0.098 at 700nm, -0.25 at 750nm, and -0.25 at 800nm. The

graph was plot considering the absorbance at the particular wavelength of NT melanin (Figure 1). The melanin pigment extracted from ST isolate showed maximum absorbance of 1.253 at 200 nm followed by 1.208 at 250 nm, 1.168 at 300nm, 1.115 at 350nm, 0.995 at 400nm, 0.881 at 450nm, 0.716 at 500nm, 0.650 at 550nm, 0.448 at 600nm, 0.030 at 650nm, 0.007 at 700nm, -0.25 at 750nm, and -0.25 at 800nm. The graph was plot considering absorbance at particular wavelength of the ST melanin (Figure 2).

Figure 1 : Absorption spectrum of Melanin extracted from isolate NT by UV-Visible Spectroscopy

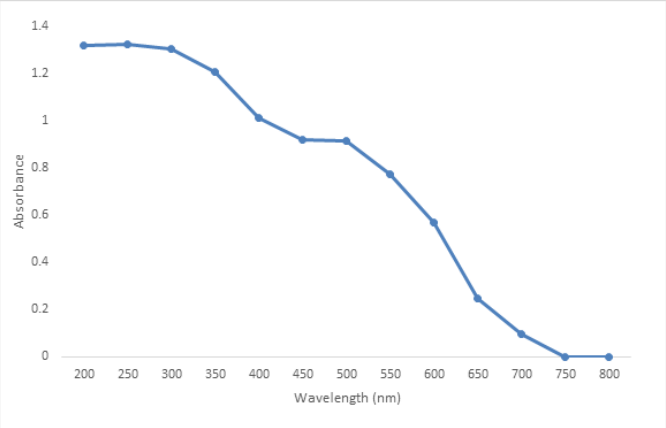
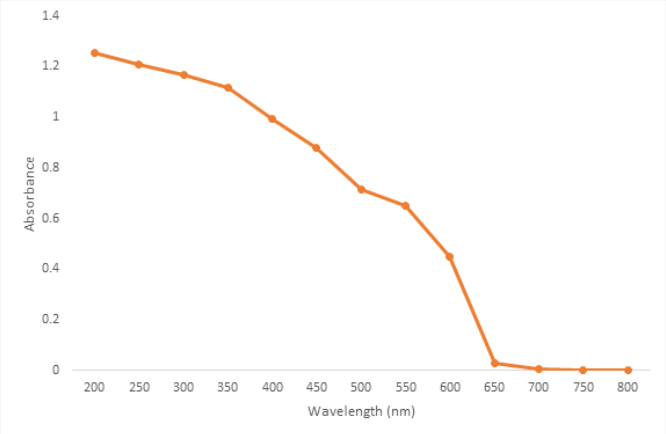


Figure 2: Absorption spectrum of Melanin extracted from isolate ST by UV-Visible Spectroscopy.



For further characterization of the pigment produced by the bacterial isolates was indeed melanin, FT-IR analysis of the pigments was performed. For this analysis IRAffinity-1 spectrophotometer from SHIMADZU was used. The melanin pigments from the isolates were mixed with KBr (Potassium bromide) and grinded with mortar and pestle. The formed pellet was pressed in a laboratory hydraulic press converting the pellets into discs. These discs were then loaded into the FR-IR spectrophotometer. The IR spectra and the data table was obtained for the respective melanin pigments. The NT melanin pigment showed peak at 3396.79 which was a broad peak which is for -O-H (acid group stretching), -COOH stretching and a potential -NH stretching. A peak at wavelength 2938.68 confirmed the presence of -CH stretching and another peak at 1641.49 confirms the presence of vibration of aromatic C=C group, and -C=O stretching (Figure 3). The ST melanin pigment showed a broad peak at 3383.08 which confirms the presence of -NH. Broad peak corresponding to -NH group at 2938.68 confirms the presence of aliphatic -CH stretching with some association with the aromatic C=C and -C=O stretching confirming with the peak at 1630.88 and 1692.61 (Figure 4). The presence of the functional groups like -NH, -COOH, -CH, C=C, -C=O, -O-H (acid group) confirms the pigments indeed to be melanin.

Figure 3: FT-IR Spectra of Melanin from NT bacterial isolate

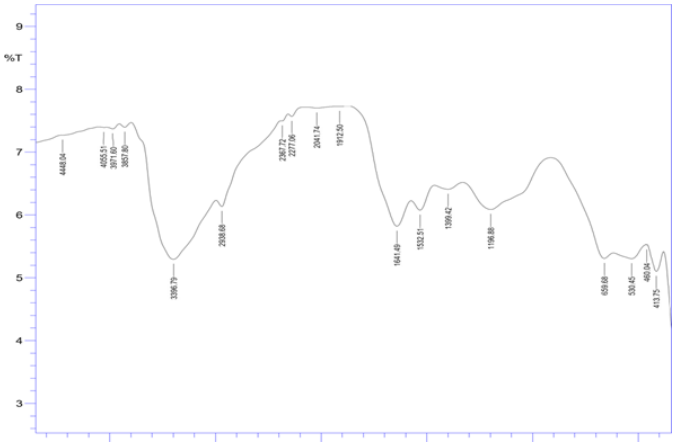
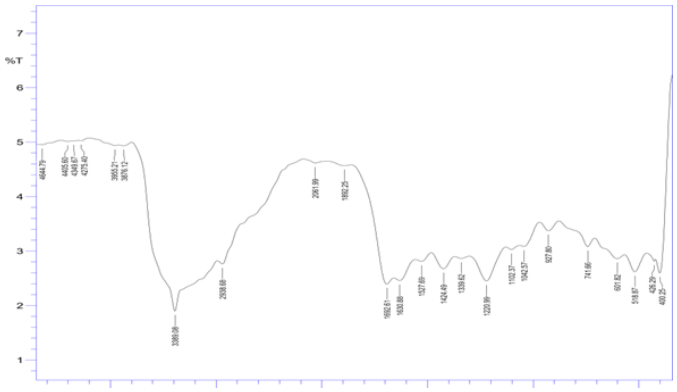
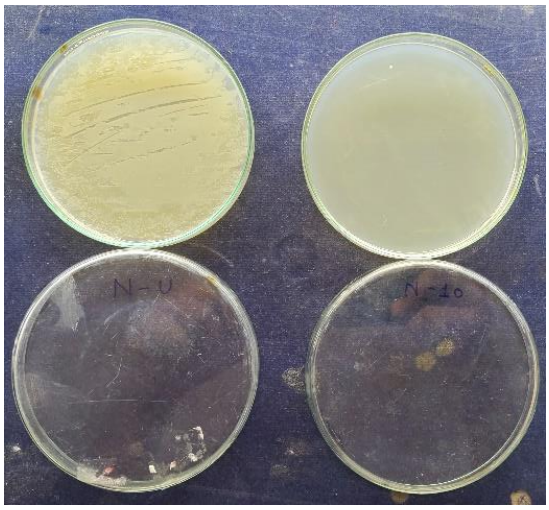


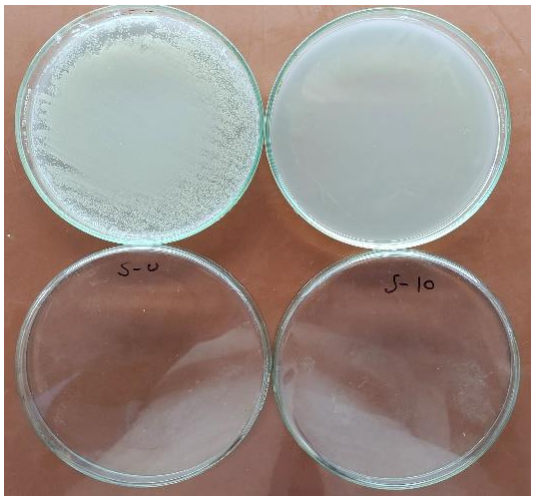
Figure 4: FT-IR Spectra melanin extracted from ST bacterial isolate



Screening of cosmetic property of extracted melanin from bacterial isolates ST and NT was performed. 2 sets of 11 test tubes containing 10 ml of TBB media with respective bacterial inoculum of ST and NT after the incubation of 15 days were treated with UV light in a laminar air flow. UV treatment to the test tubes was done in the interval span of 5min, 10min, 15min, 20min, 25min, 30min, 35min, 40min, 45min, 50min, 55min, 60min. After the UV treatment the broth from each test tube was inoculated on the nutrient agar plate. The ST isolate showed the growth in the nutrient agar plates till the time duration of 50min when treated under UV light, while it did not show any growth beyond the time duration of 50min, 55min and 60min.The NT isolate showed the growth in the nutrient agar plates till the time duration of 55min when treated under UV-light. It did not show any growth beyond the time duration of 55min, and 60mins. The test confirms that the ST and NT bacterial isolates can survive the UV radiations for the duration of 50min and 55min respectively.



NT isolate under UV radiation for 0min vs 60min



ST isolate under UV radiation for 0min vs 60min

The antibacterial activity of the extracted melanin from the bacterial isolates ST and NT was performed using Agar well diffusion assay. The activity was tested against the test bacteria: *Escherichia coli, Pseudomonas aeruginosa, Klebsiella spp*. The ST and NT melanin pigments showed the maximum antibacterial activity on *Pseudomonas aeruginosa* with the zone of inhibition of 23mm and 25mm respectively. Which was followed by the zone of inhibition of 20mm each (ST and NT melanin) against *Escherichia coli*. The least antibacterial activity was shown against *Klebsiella spp* with the zone of

inhibition of 13mm for ST melanin and 15 mm for NT melanin.

Table 2 : Antibacterial activity of extracted melanin from bacterial isolates

Sr.No.	Extracted Melanin	Zone of inhibition against test bacteria (in mm)		
		Escherichia coli	*Pseudomonas aeruginosa*	*Klebsiella spp*
1	ST	20	23	13
2	NT	20	25	15

Discussion:

In the present study two prominent *Bacillus* species were selected which were capable of melanin production. Similar results were seen in the study conducted by Barate and Sonar (2023) where they reported melanin producing *Bacillus* species which they isolated from rhizosphere soil of Sagwan and Neelgiri.

The current study dealt with the melanin pigments isolated from the isolates ST and NT. The melanin from these isolates was extracted and partially purified. It was found that the NT isolate produced more quantity of melanin pigment in comparison to the ST isolate. The extracted melanin was characterized, in which solubility test was performed for the extracted melanin for checking the chemical properties. In the present study It was found that the melanin pigment extracted from ST isolate was soluble in Ethanol, Sodium hydroxide (NaOH), Acetic acid and Dimethyl sulfoxide (DMSO). The pigment was found to be insoluble in Hydrochloric acid (HCl), Petroleum ether, Chloroform, and Water. While it was found that the pigment was partially soluble in Hydrogen peroxide (H_2O_2) and Iso-propanol. The extracted melanin pigment form the NT isolate was found to be soluble in Ethanol, Hydrogen peroxide (H_2O_2), Sodium hydroxide (NaOH), Dimethy sulfoxide (DMSO), and Iso-propanol. The pigment was found to be insoluble in Hydrochloric acid (HCl), Petroleum ether, Chloroform, and Water. While it was found that the pigment was partially soluble in Acetic acid. Study conducted by Jigna *et al.*, (2022) reported that melanin pigment produced by *Bacillus pumilius* isolated from sea water sample was soluble in NaOH, while insoluble in water, ethanol, methanol, iso-propanol, acetic acid, HCl and sparingly soluble in DMSO. Likewise, Korumilli and Mishra (2013) reported melanin produced from *Pseudomonas spp* found to be insoluble in water, ethanol, chloroform, acetone and benzene and slightly soluble in phenol and NaOH.

In the current study, to find out the maximum absorbance of the melanin pigments, UV- visible spectroscopy was performed. It was found that the melanin extracted from isolate ST showed maximum absorbance of 1.253 at 200 nm, and the melanin extracted from isolate NT showed the maximum absorbance of 1.321 at 200nm. As the wavelength of light increased towards the visible spectrum both the melanin pigments showed a steep drop in the absorbance and above the wavelength of 750nm both the melanin pigments showed the absorbance of -0.25. Study conducted by Jigna *et al.*, (2022) reported that the melanin pigment produced by *Bacillus pumilius* showed the maximum absorbance at the wavelength of 290nm, similar pattern in the current study of decrease in absorbance as the wavelength reaches visible spectrum is seen in this study. Singh *et al.*, (2021) reported the melanin produced by *Pseudomonas spp* also show the maximum absorbance around UV spectrum of 230nm.

The current study FT-IR spectroscopy was utilized for further characterization of melanin pigments. Both the melanin pigments isolated from ST and NT

isolates showed the presence of -NH, -OH (acidic), -COOH -CH and possible C=C (aromatic) stretching, which was interpretated from the spectra and the data obtain from the FT-IR. The NT melanin pigment showed peak at 3396.79 which was a broad peak which is for -O-H (acid group stretching), -COOH stretching and a potential -NH stretching. A peak at wavelength 2938.68 confirmed the presence of -CH stretching and another peak at 1641.49 confirms the presence of vibration of aromatic C=C group, and -C=O stretching. The ST melanin pigment showed a broad peak at 3383.08 which confirms the presence of -NH. Broad peak corresponding to -NH group at 2938.68 confirms the presence of aliphatic -CH stretching with some association with the aromatic C=C and -C=O stretching confirming with the peak at 1630.88 and 1692.61. Similarly, Jigna *et al.*, (2022) reported the results of FT-IR from melanin extracted from *Bacillus pumilius*, in which prominent peak was observed at 3368.64 which corresponds to -OH and -NH stretching and peaks seen between 1600-1400 confirms C=C aromatic stretching. Korumilli and Mishra (2013) reported the FT-IR report and spectrum of melanin which was extracted from *Pseudomonas spp*, show the absorption peak at 3373 indicating the presence of -OH and -NH stretching and the sharp peak of 1650 confirms the presence of C=C aromatic carbons.

Utilization of melanin in cosmetics industry depends upon the ability of the melanin to protect skin tissues from UV-light for a particular duration of time. The current study dealt with the characterization of cosmetic properties of melanin pigments produced by isolate ST and NT. It was found that the melanized isolate ST survived up to 50min in UV radiation, while the isolate NT showed survival till 55min in the UV radiation. According to Joshi *et al.*, (2021) melanized marine bacteria *Providencia rettgiri* survived the UV radiation till 15 min. While in the recent study conducted by Jigna *et al.*, (2022) it was reported that melanized isolate *Bacillus pumilius* can survive UV radiation up to 20 min of time duration.

The bacteria due to evolution and natural genetic transformation are getting resistant to modern antibacterial drugs, the current study dealt with the antibacterial properties of the extracted melanin from the two isolates. It was found that the ST and NT melanin pigments showed the maximum antibacterial activity on *Pseudomonas aeruginosa* with the zone of inhibition of 23mm and 25mm respectively. Which was followed by the zone of inhibition of 20mm each (ST and NT melanin) against *Escherichia coli*. The least antibacterial activity was shown against *Klebsiella spp* with the zone of inhibition of 13mm for ST melanin and 15 mm for NT melanin. Corresponding study published by Vasanthabharathi *et al.*, (2013) reported that the melanin produced and extracted from marine *streptomyces* showed prominent antibacterial activity against *Escherichia coli, Salmonella typhi, Salmonella paratyphi,* and *Vibrio cholerae.*

IV. CONCLUSION

In the present study the two *Bacillus* species showed the ability to produce melanin in good quantity. The melanin pigment produced by the two isolates NT and ST showed predominant photo-protective activity which make the melanin pigments of these isolates an excellent ingredient in cosmetics industry aiding in the manufacturing of sunscreens and lotions if further used and studied. The melanin pigments in the study also showed predominant antibacterial activity which make these pigments an important alternative in the field of medicine against different drug resistant bacteria.

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