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MICROBIAL MELANIN AND ITS BORAD-SPECTRUM SUNSCREEN

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Abstract: This study aim to isolate melanin producing bacteria from the garden soil sample and to optimize its compatibility as a broad spectrum cream. The duration of the research is six months. To carry out this study 32 research papers were referred from various journals. Till date many research has been carried out to obtain the melanin from bacteria, fungi, fruit extract, and many more. In previous studies the melanin pigment have been used for various purpose such as an antioxidant, anti-inflammatory activity, digestive systems protection, anti-cancer activity, antimicrobial action, neuroprotective agent, UV rays and X-ray protective agent and radio- protective agent. The purpose of this study was to extract the melanin from the soil organisms. The substrate used for the melanin production is L-tyrosine medium to enhance the production of melanin pigment. The obtain melanin pigment was further characterized for chemical and physical characteristics. Melanin pigment obtain is hydrophilic in nature and it also showed the antioxidant activity. Then the melanin based sunscreen was formulated by blending the melanin with the oil-based cream and it was evaluated for its board- spectrum activity. The sun protection factor of the melanin based sunscreen was found to be 35. Its physiochemical properties i.e. color,thermal stability, pH and homogeneity was also evaluated.

Keywords **– Melanin, Board spectrum activity, DPPH assay, Melanin based sunscreen, Sun protection factor**

1. INTRODUCTION

Melanin are group of naturally occurring dark colored pigment and it is produce by most organisms such as fungi, bacteria, plants, human as well as animals (Chakrabarty et al., 2018; Modi et al., 2016). The melanin term was first coined by Swedish chemist Jacob Berzelius in 1840, originated from ancient Greek melanos (means dark color) (Solano, 2014). It is located in melanosome organelles of animals, whereas in plants it is found in seed coat, while in fungi it is deposited in cell wall and bacteria secretes the melanin pigment extracellularly (Kurian, 2022). It is heterogeneous polymer obtain by oxidation of indolic compounds and polymerization of organic compound (Sajjan, 2010; Tran-Ly et al., 2020).

Melanin are classified into four different types based on their structural characteristics i.e. eumelanin (animal melanin, black or brown pigment), pheomelanin (animal melanin; yellow to brown pigment), allomelanin (plants, fungi, and bacteria, brown to black pigment) and neuromelanin (dark polymer pigment, produced in catecholaminergic neurons in the human brain) (Saud & Alaubydi, 2016).

Melanin are ubiquitous in nature, negatively charged hydrophobic, and high molecular weight compounds. The amino acid tyrosine and its hydroxylated DOPA product act as a starting point of biosynthesis of melanin pigments. The pheomelanin yellowish-reddish color, result from oxidative polymerization of the cysteinyldopa. Eumelanin, brown to black color, result from the oxidative polymerization of 5, 6-dihydroxyindole. The hydroxylation of the L-tyrosine at the L-DOPA is catalyzed by the tyrosine and a series of proteins related to tyrosinase (Maranduca et al., 2019).

The melanin pigments play a crucial role in defense and protection mechanisms that improve the survival and competitiveness of microorganisms. It is majorly known for its absorption capacity of radiation of all

wavelength and has an optimum absorbance at UV range which protects from photo induced damage. It also has biological activities such as radical scavenging, antioxidant, antitumor, anti-inflammatory and as immune stimulating agent. Microbial melanin has great advantages over melanin obtain form animals and plants. Microbes don't cause the problem of seasonal variation and can be modify according to the medium and conditions provided to them. Microbial melanin also shows metal chelating ability (Tarangini & Mishra, 2014). They can be also use as semiconductors in bioelectronics field (Zerrad A, 2014).

Melanogenesis in human skin absorbs 75% of UV radiations and its sun photoprotection value (SPF) are 1.5-2.0 and scavenges free radical generated by UV absorption. Thus melanin have been widely used in the dermo- cosmetic and biomedical fields. Microbial melanin can be promising candidate for a bioinspired sunscreen because it is environmentally sustainable and industrial scalable. It has no hazardous effect in the ecosystem afterits disposal and thus has great biocompatibility (Oh et al., 2021).

Sunscreens have used since decades to provide shield against UV radiations, specifically UV-A (320-400nm) and UV-B (280-320 nm) (Kapur et al., 2012). Currently, plant-based actives and bioinspired sunscreens are receiving increased attention (Lourith et al., 2017). Because synthetic chemical photo-protective compounds are more likely to be dangerous and carcinogenic, bioactive components are favor as major cosmetics ingredients due to their natural anti cancerous, anti-mutagenic, and non-toxic properties. Herbal elements in sunscreens are the least irritating to the skin, especially for sensitive skin, including natural components can regenerate the skinand give protection against UV radiation (Tiwari et al., 2022).

In this study, the microorganism producing melanin was isolated from the garden soil sample, and it was cultivated using the L-tyrosine medium. The purified melanin was further evaluated for its UV absorption activity by UV spectrophotometer and antioxidant activity by DPPH inhibition assay. Then the purified melanin pigment was used for formulation of biocompatible sunscreen. The sunscreen was characterized for its sun photo- protection factor (SPF) via UV spectrophotometer and further it was evaluated for its physical characteristics.

2. MATERIALS AND METHODS

2.1 Isolation of melanin producing bacteria

The soil sample was collected from the garden in localized areas. The 1g sample was enriched in 100ml tyrosine broth and incubated under shaker conditions at 37º C for 7 days. Then melanin pigment producing bacteria was isolated on tyrosine agar medium and incubated at 37º C for 7 days. After the incubation period colonies producing melanin pigment was observed for its characteristics. The color change from white to dark brown around the colonies indicates bacteria that produce melanin has utilize the tyrosine substrate for the melanin synthesis.

2.2 Purification of melanin pigment

The culture medium was centrifuged was centrifuged at 2000rpm for 20 min, to obtain the cell free medium. The obtain cell free medium that is the supernatant was treated with 5N NaOH and the pH was adjusted to 10 so that melanin gets precipitated. Then the medium was allow to stand for 5-10 min, so that melanin pigment polymerizes completely. The pH cell free medium was then adjusted to 2 with 2N HCl and allow it to stand for 1 week at room temperature, so that complete precipitation of melanin is obtained. The medium was boiled for 30 min at 100º C to avoid the formation of melanoids and it was followed by centrifugation for 20 minutes at 5000rpm. The obtain pellet was washed twice with 0.1N HCL and it was followed by distilled water. The pellet was then soaked with absolute ethanol and followed by boiling for 10 minutes at 100º C. The pellet was allowed to stand for a day. Then the pellet was washed ethanol to remove the impurities present in the pellet. Then the pellet was air dried and stored at -20º C for further analysis.

2.3 Characterization of melanin pigment

2.3.1 Chemical analysis

The solubility of the crude melanin extract was determine using solvents like 1M NaOH, 1M HCl, methanol,ethanol, chloroform, acetone, isopropyl ether and ethyl acetate, and also in distilled water.

2.3.2 UV Spectroscopic analysis

The UV absorption of crude melanin pigment was characterized by UV spectrophotometer within the range of 200-800 nm. The crude melanin was mix with 0.2N NaOH and was determine for UV absorption within the range of 200-800 nm at intervals of 50 nm.

2.3.3 FTIR analysis

The physical characteristics of crude melanin was determine by FTIR analysis. The crude melanin pigment was mixed with KBr and was scanned by FTIR spectrophotometer.

2.3.4 Antioxidant activity (DPPH assay)

The antioxidant activity of crude melanin pigment was determined by DPPH (2, 2-diphenyl-1picrylhydrazyl- hydrate) radical scavenging assay. The melanin extract (1 ml) was added to 2 ml DPPH solution and incubated for 30 min at 37º C. The absorbance was determined colorimetrically at 450 nm before and after the incubation period of 30 minutes.

2.4 Antibacterial activity of the melanin pigment

The antibacterial activity of the crude melanin pigment was studied against *Escherichia coli, Staphylococcus aureus, Bacillus spp., and Klebsiella pneumonia* by agar plate well diffusion method. The media used was Mueller-Hinton (MH) agar. The 1 ml cultures were inoculated in the cooled molten agar butts and was mixed properly and plated on the sterile plate. Then the melanin extract (1 ml) was in the wells and incubated at 37º C for 24 hrs.

2.5 Formulation of sunscreen

The cream was prepared by using almond oil, jojoba oil, shea butter, zinc oxide and crude melanin extract with different concentrations (Table 1).

Table 1: Composition of different formulation of the sunscreen

Ingredients	F1	F2	F3	F4
Jojoba oil	0.2 ml	1 ml	5 ml	10 ml
Almond oil	10 ml	10 ml	5 ml	2 ml
Shea butter	5 gm	10 gm	5 gm	4 gm
Zinc oxide	1.25 gm	4 gm	10 gm	4gm
Melanin extract	2 ml	4 ml	-	10 ml

2.6 Characterization of the sunscreen

2.6.1 Physical parameter

The physical characteristics of the cream was determined by checking its appearance, color, and homogeneity.

2.6.2 pH analysis

The pH of the cream was determine by dissolving the 1 gm cream of different formulations in 20 ml distilledwater.

2.6.3 Thermal stability

The cream was applied to the wall of the beaker and the beaker was placed at 37º C for 5 hrs. The cream shouldnot show the oil separation after the incubation of 5 hrs.

2.7 Determination of SPF of the sunscreen

The SPF of the cream was evaluated by the UV spectrophotometer. The 0.05 g of the prepared sunscreen was dissolved in 10 ml of distilled water and the sample was scanned within the range of 290-320 nm at 5 nm intervals.The SPF was calculated by using the equation (Tiwari et al., 2022):

$$SPF = CF\sum_{290}^{320} EE(\lambda) \times I(\lambda) \times A(\lambda) \quad (1)$$

Where, CF = Correction factorEE = Erythemogenic effect
I = Intensity of solar light of wavelengthA = Absorbance

3. RESULTS AND DISCUSSION

3.1 Isolation of melanin producing bacteria

The brown pigmentation was observed by the melanin producing bacteria on the tyrosine agar medium after the incubation of 37º C for 7 days. The colony on the tyrosine agar plate producing the melanin pigment was observed to be white mucoid colony which produce the diffusible melanin pigment. Similarly the tyrosine broth turned dark brown in color after incubation of 37º C under shaker conditions for 7 days which indicates that the melanin producing bacteria had utilized the tyrosine for the synthesis of the melanin. However after 14 days of incubation under shaker conditions at 37º C maximum pigment production was observed.

Figure 1: (a) Enrichment of the sample in tyrosine broth resulting in brown color formation; **(b)** isolation ofmelanin producing bacteria on tyrosine plate resulting in diffusible brown color pigment

3.2 Chemical analysis of the pigment

The pigment was purified using acid hydrolysis using 2N HCl and the obtained pigment was in powder formand it was checked for its solubility in different solvents.

Table 2: Chemical characteristics of the crude melanin pigment

Tests	Results
H_2O	Soluble
NaOH (1M)	Soluble
HCl (1M)	Soluble
Methanol	Insoluble
Ethanol	Insoluble
Chloroform	Insoluble
Acetone	Insoluble
Isopropyl ether	Insoluble
Ethyl acetate	Insoluble

3.3 UV spectroscopic analysis

The UV visible wavelength of the crude melanin pigment was determined within the range 200-800 nm at intervals 50 nm. The obtained crude melanin pigment showed maximum absorbance in UV range 200-350 nm and then it decreased towards the visible spectra range. This is due to the presence of very complex structure in melanin (Sajjan, 2010). This property of melanin was confirmed by comparing with previous measurements (Chakrabarty et al., 2018; Deepthi et al., 2021; Gonçalves et al., 2012; Oh et al., 2021; Sajjan, 2010; Tarangini &Mishra, 2014; Toma et al., 2021).

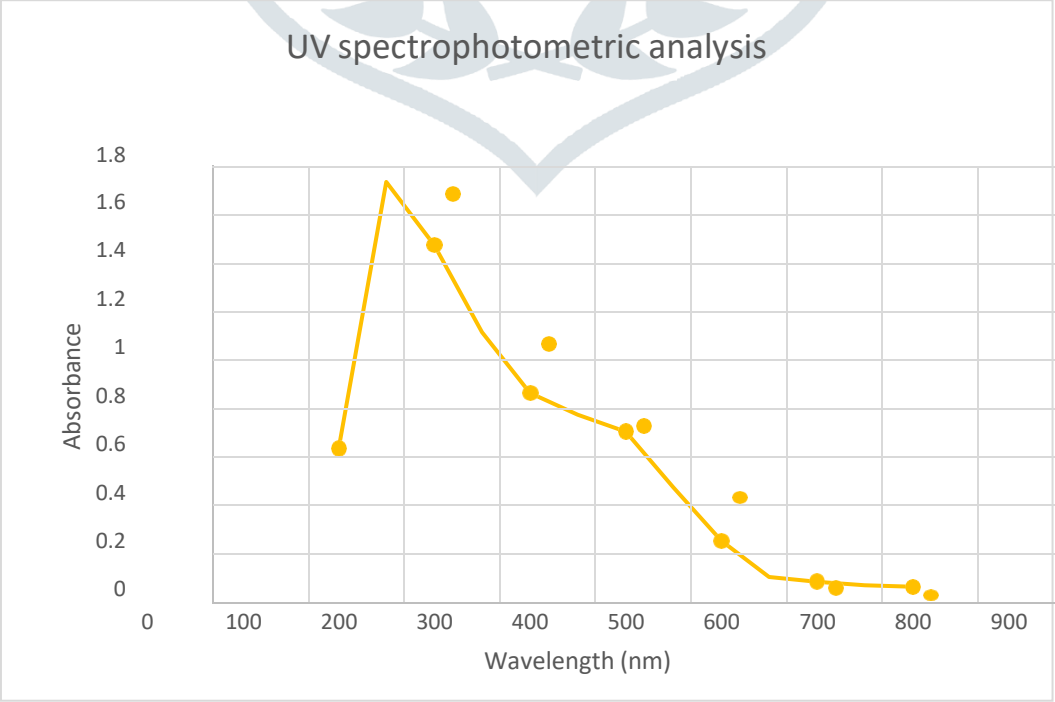

Figure 2: UV spectroscopic analysis of the pigment

3.4 FTIR analysis of the pigment

To study the structure of the melanin FTIR analysis was carried out. The board absorption was observed after 3000 cm^{-1} and it indicates the presence of amine group and C-N bonds, which supports the indolic group in melanin. The smaller peaks at 2854 and 2927 cm^{-1} indicates the presence of alkyl chains, which are bounded to indole (Kiran et al., 2017). The peaks around 1797 cm^{-1} indicates the presence of carbonyl group (C=O). The peak at 1287 cm^{-1} shows the presence of the phenolic groups in the melanin.

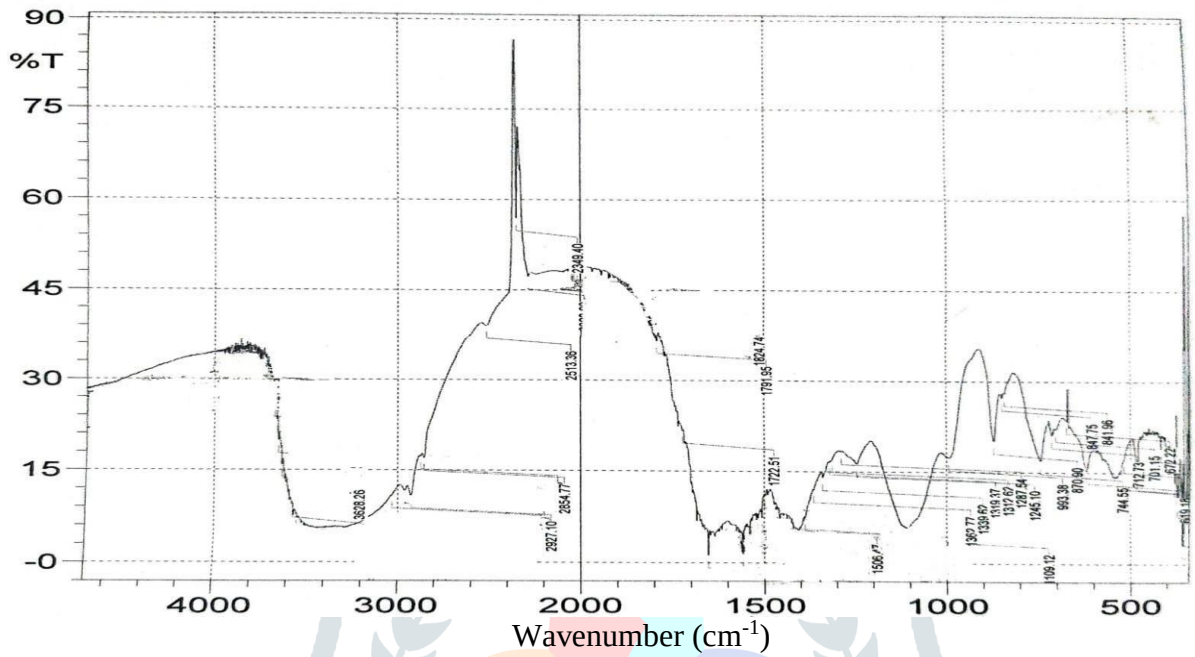

Figure 3: FTIR analysis of the pigment

3.5 Antibacterial activity

The antioxidant activity of the crude melanin pigment was studied against the *Escherichia coli, Staphylococcus aureus, Bacillus spp.*, and *Klebsiella pneumonia.* The pigment showed antibacterial activity only against *S. aureus and Bacillus spp.* and it did not showed any activity against *E. coli,* and *K. pneumonia*. The pigment showed maximum inhibition against *Bacillus spp.*, and it is followed by the *S. aureus*. The results are presented in table 3.

Table 3: Antibacterial activity of the pigment

Bacterial strain	Zone of inhibition (mm)
Escherichia coli	-
Staphylococcus aureus	14
Bacillus spp.	25
Klebsiella pneumonia	-

3.6 Antioxidant activity

The free radical in cells can damage the proteins and DNA due to endogenous radical formation. This can lead to tissue damage and ageing. This can also results in the oxidative stress and cancer. The antioxidant molecules can help to neutralize the reactive oxygen species (ROS) (Oh et al., 2021; Tiwari et al., 2022).

The DPPH assay was carried out to determine the free radical scavenging activity of the crude melanin pigment. The melanin pigment consists of H atom which gets bound to the free radical present in 2, 2-diphenyl- 1-picrylhydrazyl-hydrate (DPPH). Thus DPPH gets reduced in presence of this H atom, resulting in the color change from deep violet to pale yellow color. The antioxidant activity of the crude melanin pigment was measured using colorimeter at 450 nm. The crude melanin pigment showed increased inhibition of DPPH after incubation of 30 min at 37º C. The antioxidant activity of the melanin was found to be 65% after the incubation period, whereas it showed 33% of DPPH inhibition.

3.7 Characteristics of formulated sunscreen

3.7.1 pH analysis

The pH of the sunscreen cream were found within the range of 6.5-6.8. An acidic pH generates an effective barrier. The improved defense against invading organisms in an acidic range rather than an alkaline or neutral environment is one of the important topic that is use when analyzing the scientific literature on skin surface pH(Tiwari et al., 2022).

3.7.2 Thermal stability

Thermal stability of the cream was determined by allowing the cream to stand at 37º C for 6 hrs. F1 to F4 formulations were stable as they did not any phase separation at 37º C.

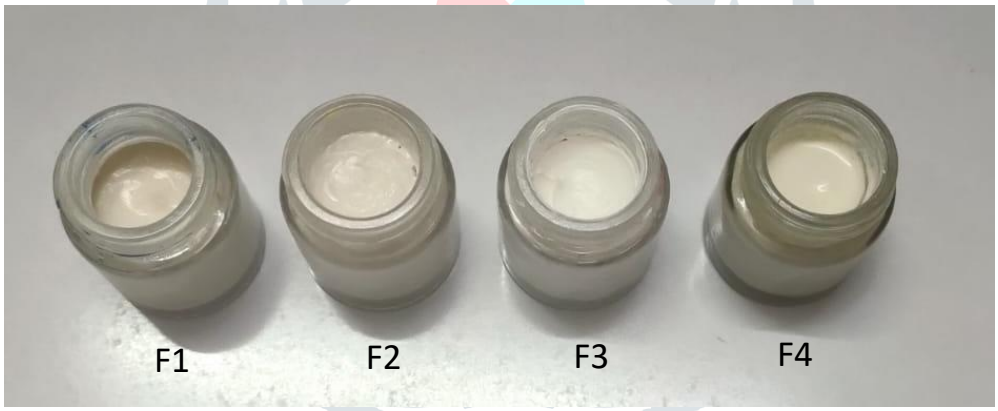

Figure 4: Different formulations of the cream

Table 4: Characteristics of sunscreen

Characteristics	F1	F2	F3	F4
Color	Apricot white	Apricot white	White	Apricot white
Appearance	Cream like	Cream like	Cream like	Liquid lotion
pH	6.5	6.7	6.6	6.8
Thermal stability	Stable	Stable	Stable	Stable
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous
SPF	19.3	12.5	27.4	35.5

3.8 Determination of SPF by UV spectrophotometer

The sun protection factor (SPF) of the sunscreen was evaluated using UV spectrophotometer. The absorbance of sunscreen cream was measured within the range of 290-320 nm at the intervals of 5 nm. The SPF of the sunscreen of F4 formulation was found to be highest than the other formulations, which was followed by the F3,F1, and F2 formulations.

Table 5: SPF determination of the cream

Wavelength	EE(λ) × I(λ)	F1		F2		F3		F4	
		Abs	Abs × EE(λ) × I(λ)	Abs	Abs × EE(λ) × I(λ)	Abs	Abs × EE(λ) × I(λ)	Abs	Abs × EE(λ) × I(λ)
290	0.015	2.21	0.03315	2.04	0.0306	3.01	0.04515	4.01	0.06015
295	0.0817	2.15	0.175655	1.09	0.089053	3.19	0.260623	3.1	0.25327
300	0.2874	2.01	0.577674	1.1	0.31614	3.59	1.031766	3.8	1.09212
305	0.3278	1.82	0.596596	1.5	0.4917	2.09	0.685102	3.9	1.27842
310	0.1864	1.91	0.356024	1.14	0.212496	2.19	0.408216	2.9	0.54056
315	0.0837	2.02	0.169074	1.09	0.091233	3.09	0.258633	3.2	0.26784
320	0.018	1.4	0.0252	1.04	0.01872	3.12	0.05616	3.3	0.0594
	TOTAL		1.933373		1.249942		2.74565		3.55176
	SPF		19.3		12.5		27.5		35.5

4. CONCLUSION

The melanin pigment was extracted by acid hydrolysis method and melanin obtained was 1.85g per 100 ml of tyrosine broth. The pigment was further studied for its chemical and physical characteristics. The pigment was soluble in distilled and organic solvents such as NaOH and HCl. The pigment showed maximum absorbance in the UV range 200-350 nm. The FTIR analysis of the melanin pigment was studied and it showed the presence of the amine groups, alky chains, phenolic groups, and carbonyl group which confirms the melanin pigment. The pigment was used to prepare the sunscreen with different formulations (F1 to F4). The sunscreen showed the pH range between the 6.5-6.8, which satisfies the requirement for topical sunscreen. The formulation F4 showed the highest SPF value of 35.5 which was followed by formulation F2 which showed SPF value of 27.5. Whereas the formulation F1 and F2 showed the SPF value of 19.3 and 12.5 respectively. All the formulations are thermally stable at 37º C. Thus the formulation F4 of the sunscreen showed suitable SPF value and other physical characteristics and it further evaluated for cytotoxicity study and for its non-mutagenic characteristics.

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