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Bacterial melanin with immense cosmetic potential produced by marine bacteria *Bacillus pumilus* MIN3

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Abstract

Melanins are phenolic polymers synthesized by most of the living organisms. This pigment is mainly attributed to provide photoprotection to the organism while it was found that pigment have immense bioactivities which could be utilized in day-to-day life ranging from sun screens lotions to solar cells. This pigment produced mainly via DOPA or homogentisate in bacteria. Melanin production is usually triggered by stress condition in bacteria. Marine bacteria have been reported as good melanin producers. In this study marine bacteria capable of melanin production were isolated from sea water of Kutch region, Gujarat using tyrosine basal media. The bacteria were identified using microscopic, biochemical and molecular techniques. Melanin produced by the bacteria is extracted and purified and further characterized using physicochemical techniques. Cosmetic properties of melanin like photoprotection, antioxidant and antimicrobial properties are evaluated.

Keywords: melanin, antimicrobial, antioxidant, photoprotection

Bacteria in the marine environment are often exposed to extreme conditions such as pressure, temperature, salinity and ability to produce biologically active compounds, often accompanied by depletion, survival and diffusion of micronutrients. marine bacteria produce biosurfactants that increase bioavailability, which aids in transport of soluble substrates in less hydrophobic water. The lipid composition of marine bacteria has been observed to adapt to environmental changes in pressure, temperature and salinity. Some fatty acids, including docosahexaenoic acid and ecological cyclopentaenoic acid, have been reported only in prokaryotes of deep-sea bacteria [1]. Melanin is a phenolic pigment which is reported to be produced by many marine bacteria living under extreme condition [2].

Melanin is a natural pigment of skin, hair and eyes in humans and can be divided into two main types: from black to brown eumelanin and from yellow to reddish brown pheomelanin. Melanin biosynthesis occurs in the inner ear, central nervous system and brain, as well as in

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the basal layer of the epidermis, in the eyeball, in melanocytes located in the hair follicles, in melanosomes, a specialized cytoplasmic organ of dendritic cells [3]. Melanogenesis is a multi-step process that begins with the conversion of the amino acid L-tyrosine to DOPAquinone. When cysteine or glutathione is added to DOPAquinone, an intermediate is formed, which is then converted and polymerized to the final product, pheomelanin. DOPAquinone undergoes intramolecular cycling and oxidation to form DOPAchrome, followed by 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyndol-2-carboxylic acid, is converted to eumelanin by the polymerization of DHI and DHICA and their quinones [3]. While in microbes' melanin biosynthesis follows many other alternative routes too resulting the production of heterogenous melanins called allomelanins. Fungi produce melanin predominantly by the polymerization of di or tetrahydrofolate resulting in the production of DHN-melanin while many bacteria follow the homogentisic acid pathway to produce melanin called pyomelanin [4]. The bacteria are one among the organisms producing allomelanin, which has produced significant quantities of all the types of melanin. The *Klebsiella sp.* a eumelanin producer, as well as a pheomelanin producer *Vibrio cholerae*, and *Azotobacter* produces catecholic melanin [5]. Though the melanin synthesized by different routes of biochemical conversions the final polymeric properties remain to be similar.

The ability of free-living microbes to produce melanin is likely to be related to their ability to survive in the environment. In this regard, many fungi constitutionally synthesize melanin, and even facultative melanotic microbes, such as *C. neoformansm* produce melanin considerably [6]. Melanized C. neoformans were less sensitive than non-melanized cells when exposed to ultraviolet rays [7]. Human skin is frequently exposed to UV, which affects the function and survival of many cell types and is thought to be a major factor in the development of skin cancer. The skin melanin not only acts as a broadband UV absorber, but also has antioxidant and free radical scavenging properties, and is considered to be the most important photoprotective factor. Additionally, many epidemiological studies have shown that dark-skinned people are less likely to develop skin cancer than fair-skinned people [8]. For people who are lightly pigmented, using sunscreens with photoprotective chemicals can provide extra protection. Melanin has proved to enhance sun protective property of sun screen lotions in various studies [5]. As the pigment is found to be cytotoxic in nature its addition to cosmetics could provide an enhanced protein to UV radiation.

Melanin pigments from natural sources such as microorganisms are attractive options for commercial scale production. In this study, marine bacteria capable of producing melanin from

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sea water were isolated and identified. The pigment was purified and characterized. The cosmetic properties of the purified pigment were evaluated.

Materials and Methods

Isolation and characterization of melanin producing bacteria

Water sample was taken from Narayan Sarovar Kutch region (23º 67' N. 68º 32'E) Gujarat. The serially diluted sample was plated in nutrient agar with 4% sodium chloride. Further purified colonies using quadrant streaking were subjected to melanin production in tyrosine basal broth (TBB) [9], (5 mL) taken in test tubes. After 8-10 days, melanin producing colonies were gram stained and biochemically tested [10,11]. The molecular identification of the bacteria done by 16S rDNA sequencing at NCIM, Pune, India. Sequence obtained was searched using BLAST tool [12] at NCBI to identify the bacteria. The sequence was submitted to GenBank and accession number was obtained. Phylogenetic tree of the melanin producing bacteria with related species was constructed using MEGA 7 software by neighbour joining method [13].

Antibiotic Sensitivity analysis was done according to Kirby-Bauer disc diffusion method [14]. Placed an antibiotic disc on the bacteria lawn on Mueller-Hinton Agar plate, incubate for 24 hours at 37°C before checking. Results Interpreted on a resistant, moderate or sensitive basis of the size of the zone around each disc provided by the manufacturer. MAR (Multiple Antibiotic Resistance) index was calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics tested [15].

Inoculum preparation and melanin production

After initial screening in 5 mL TBB tubes, the characterized melanin producing bacteria was subjected to melanin production in 100 mL TBB [9] in conical flasks. 1 OD culture was inoculated (1 mL) to TBB broth and melanin production was monitored 12 hours interval. After 10 days of incubation the TBB is centrifuged and bacterial pellet was removed. Supernatant was read spectrophotometrically at 400 nm [16] and concentration of melanin produced was found out using a standard graph made of synthetic melanin.

Extraction and purification of melanin

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After 10 days of incubation, the TBB in the flask is centrifuged (BioEra, Japan). 6000 rpm, 10min, 28°c temperature to separate bacterial pellet from supernatant containing melanin. Supernatant was further acidified bellow pH 2 with 1N HCl. As the pH decreased, a black melanin precipitate was seen at the bottom of the flask. Again, performed centrifugation for 6000rpm, 10min, 37°c temperature for pelleting melanin out. The resulting pellet is washed two times each with absolute ethanol and distilled water to obtain purified melanin [17]. Purified melanin is dried in hot air oven (Equitron oven (stream series), India) for 2 days at 80°c temperature.

Characterization of melanin by physicochemical methods

Chemical properties of melanin

Solubility of melanin in water, Ethanol, Methanol, Isopropanol, Acetic Acid, HCl, H_2SO_4, DMSO, NaOH was checked. Added melanin powder and vortex for 5 min after centrifuge for 5000rpm /1min. Checked result whether the melanin dissolve or not [18].

Spectroscopic properties of melanin

UV-Visible Spectroscopy

UV-Vis spectra were generated by scanning the melanin solution at 190 nm to 890 nm using a UV-visible spectrophotometer (Jasco V-730 Spectrophotometer, Japan). Results were compared to that of synthetic melanin and earlier reports to confirm the pigment to be melanin [19].

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

The FT-IR spectrum is recorded at 4,000-400 cm-1 [20]. Characteristic peaks obtained were compared with earlier reports.

Cosmetic properties of melanin

Radical scavenging activity of melanin

The free-radical scavenging activity of melanin was determined using 2, 2- Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay as per Liyana and Shahidi, 2005 [21]. Ascorbic acid served as the positive control. Melanin at different concentrations were added

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to DPPH and incubated in dark for 30 minutes. Absorbance of the test samples were measured spectrophotometrically at 517nm.

The ability to scavenge DPPH radicals was calculated by the equation

Free radical scavenging activity (%) = $\frac{Acontrol-Asample}{Acontrol} x100$

Acontrol is the absorbance of the DPPH + methanol, and Asample is absorbance of the free radical solution with melanin/standard antioxidant.

UV photoprotection activity of melanin

The purpose of this investigation was to study the ultraviolet induced effects on melanin pigmentation. Melanin producing and non-melanin producing bacteria was subjected to UV radiation from a laminar airflow UV light (254 nm). The melanin (TBB) and non-melanin (nutrient broth) bacteria were kept 30 cm distance from the UV lamp of laminar air flow. The UV treatment was done in timings; 0min ,2min ,5min, 10min, 20min. 100µl of the treated broth was spread plated in nutrient agar plates. Incubate the plate for 24 hours at 37°C temperature. Next day compared the both samples for their difference in the growth of the bacteria plated [22].

Antibacterial activity of melanin: Minimum Inhibition Concentration

Antibacterial activity of the melanin was tested against the bacteria namely *Bacillus sp., E. coli, Salmonella sp., Shigella sp.* and *Staphylococcus aureus*. Well diffusion assay was used to find out the antibacterial activity. The above mentioned bacteria were swabbed on Mueller-Hinton agar plates, and melanin at different concentrations (10µg/mL, 20µg/mL, 40µg/ml, 60µg/ml , 80µg/ml , 100µg/ml) were poured on the well bored in the plate. Streptomycin is used as the control in the study. Plates were incubated at 37° C. for 24 hours and clear zone formation around the wells was checked [23].

Result and Discussion

Isolation and characterization of melanin producing bacteria

Thirty-two colonies were isolated from the water sample were tested for the melanin production in tyrosine basal broth. The bacteria MIN3 shown to produce the black melanin was used for

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the further studies. Characterization of MIN3 was done initially for its visual colony appearance. The colonies appeared white, medium sized, round shaped, margin entire and translucent. Gram staining revealed the bacteria as gram positive rods [Figure 1]

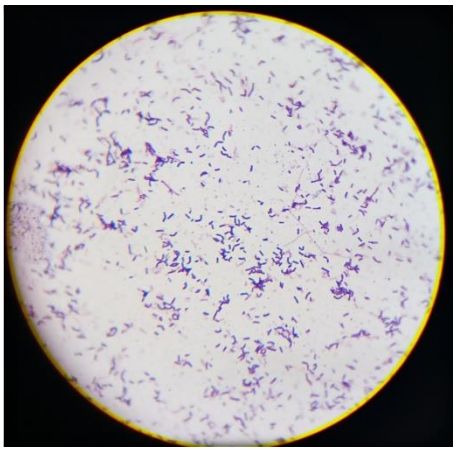

Figure. 1 Gram staining of strain MIN3 (Gram positive, rod shape)

After biochemical tests, the organism MIN3 was found to be Catalase, Indole, and Voges-Proskauer positive. Methyl red and Citrate utilization test was found to be negative [Table 1].

No.	Test	Results
1	Indole	Positive
2	MR	Negative
3	VP	Positive
4	Citrate	Negative
5	Catalase	Positive

Table. 1 Biochemical test results for MIN3

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The strain MIN3 was identified to species level by 16SrDNA sequencing and further analysis using BLAST search tool and identified as *Bacillus pumilus*. The sequence was submitted to GenBank and accession number was obtained (OM967457).

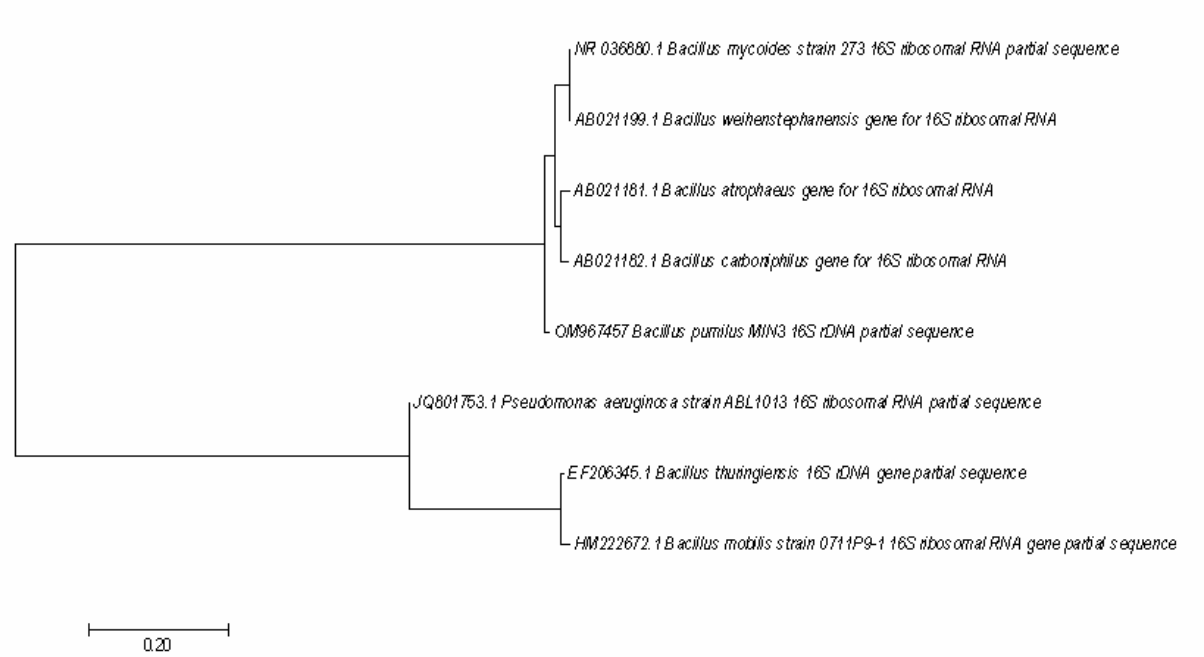

Figure 2. Phylogenetic tree showing the relationship of *Bacillus pumilus* MIN3 with the related species.

Phylogenetic analysis of *Bacillus pumilus* MIN3 16S rDNA partial sequence [Figure 2] shown to be most similar to *Bacillus carboriphilus*. *Pseudomonas aeruginosa* strain ABL1013 served as the outgroup in this analysis

MIN3 had shown sensitive to most of the antibiotic tested. The strain had shown resistance to two antibiotics tested namely, Cefotaxime and Ceftizoxime. MAR index of MIN3 was found to be 0.16, which indicates the sampling area was not much contaminated with antibiotics

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Figure 3. Antibiotic sensitivity test plate showing clear zones

Antibiotics	Sensitive (+) Resistant (-)
AS: Ampicillin	+
BA: Co-Trimoxazole	+
CF: Cefotaxime	-
PC: Piperacillin	+
CH: Chloramphenicol	+
RC: Ciprofloxacin	+
Cl: Ceftizoxime	-
TE: Tetracycline	+
ZN: Ofloxacin	+
GM: Gentamicin	+
AK: Amikacin	+
GF: Gatifloxacin	+

Table 2. Antibiotic sensitivity of strain MIN3

Production of melanin

After inoculation the colour of medium slightly got changed from white to pale as the day progresses. On 7th day considerable colour change in the medium was observed. At the end of the 10th day, medium got completely changed into black in colour [Figure 4 b]. Amount of melanin produced at the 10th day was spectrophotometrically found to be $239.44 \pm 9.03 mg/L$. The amount of melanin produced is comparable to that of earlier reports. *Bacillus sp*. have been reported to produce melanin in many cases. *Bacillus* sp. BTCZ31 was reported to produce 32.63±0.4 µg/mL [24] of melanin while, *Bacillus subtilis* 4NP-BL produced 1.5 g dry wt L^{-1} of melanin [25]. A *Bacillus thuringiensis* BMB181 found to produce 8.55 mg/mL of melanin

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[26]. Compared to these reported *Bacillus* sp., MIN3 was shown to produce moderate amount of melanin without optimization of media components. Optimization of media components could improve the melanin production by the bacteria.

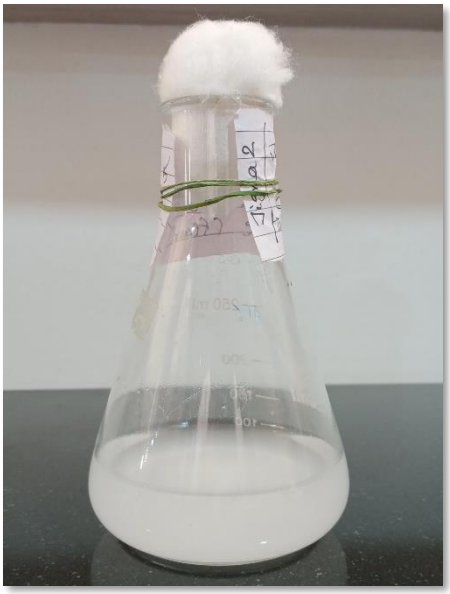
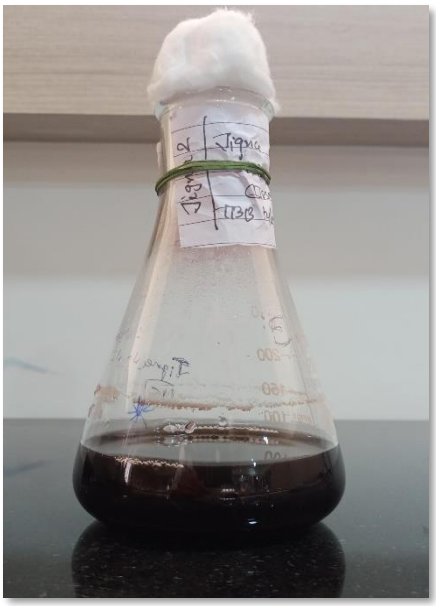
(a)  (b) 

Figure 4: TBB flask before inoculation (a) and with Melanin production (b) after 10 days of incubation

Characterization of melanin by physicochemical methods

Chemical properties of melanin

MIN3 melanin was found to be insoluble in water. The non-polar nature of melanin is considered to be a general characteristic of the pigment [27]. In organic solvents such as Ethanol, Methanol, Isopropanol, Acetic Acid, HCL and H_2SO_4 melanin was found to be insoluble. In DMSO, melanin was found to be sparingly soluble. Though the colour of the DMSO turned black some particles were remained insoluble after more than 15 minutes of vortex. Melanin was found to be completely soluble only in alkali, sodium hydroxide [Table 3]. These chemical characteristics of MIN3 melanin was found to be similar to most of the common bacterial melanin produced by different bacteria [27,28].

No.	Solvent	Results

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1	Water	Insoluble
2	Ethanol	Insoluble
3	Methanol	Insoluble
4	Isopropanol	Insoluble
5	Acetic Acid	Insoluble
6	HCL	Insoluble
7	H_2SO_4	Insoluble
8	DMSO	Sparingly Soluble
9	NaOH	Soluble

Table 3: Chemical properties of melanin

UV-Visible Spectroscopic properties of melanin

The MIN3 melanin had shown the characteristics UV-Visible spectrum of melanin. The pigment had shown higher absorption in the UV region and the absorption decreases when it reaches the visible spectrum [Figure 5]. This type of featureless absorption without an absorption maxima peak is generally uncommon among organic chromophores. The spectrum of MIN3 pigment had shown similarity with earlier reports [29,5}.

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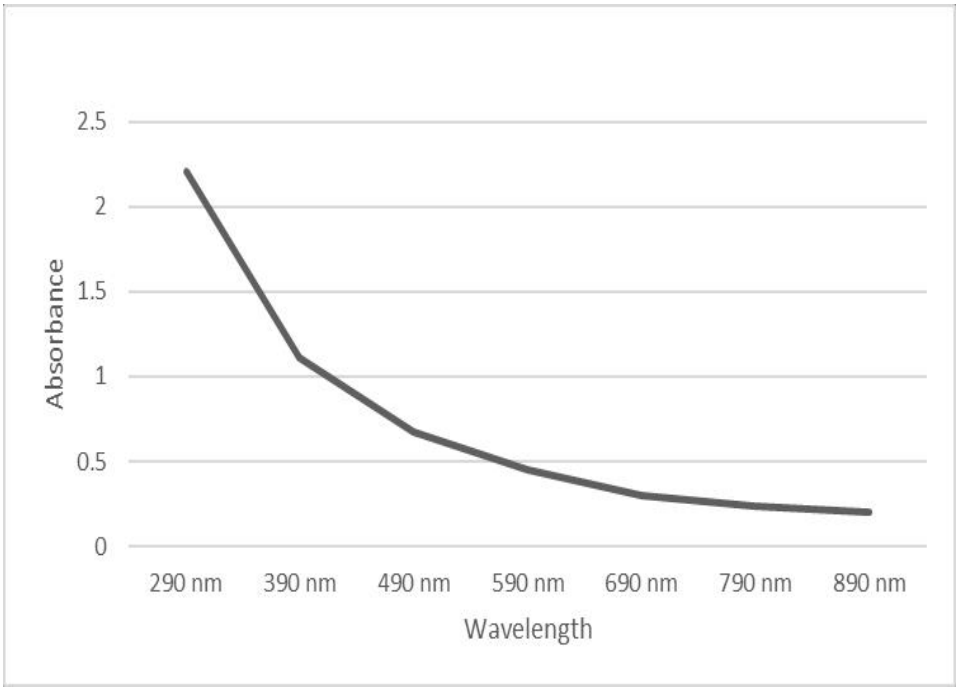

Figure 5: UV-Visible spectrum of melanin

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

FT-IR spectrum confirmed the pigment produced by strain MIN3 is melanin. The infrared spectroscopy of melanin in which a prominent peak was observed at 3368.64 cm-1 corresponds to the phenolic–OH and–NH stretching vibrations. Peaks seen between 1600 and 1400 cm-1 were attributed to aromatic ring C = C stretching [Figure 6]. This confirmed the aromatic nature of the MIN3 pigment. The FTIR peaks had shown similarity with synthetic melanin as well as earlier reports [30, 5]. This confirm the MIN3 pigment is melanin.

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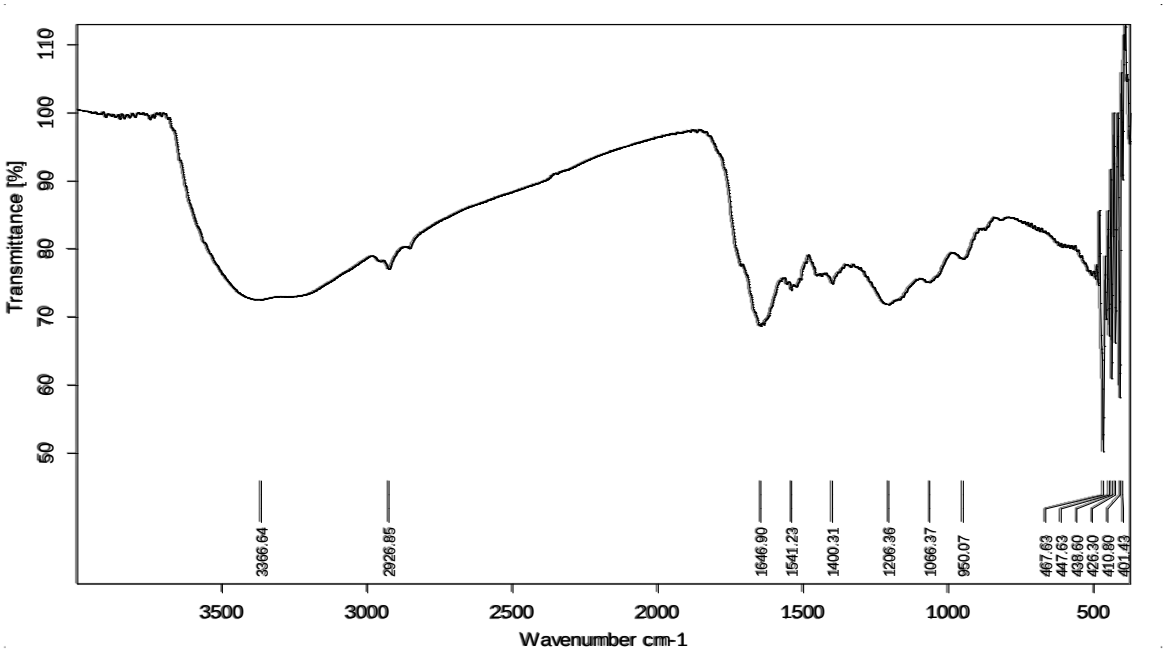

Figure 6: FTIR spectrum of MIN3 melanin

Cosmetic properties of melanin

DPPH Radical scavenging activity of melanin

Melanin showed immense DPPH radical scavenging activity compared to the standard scavenger ascorbic acid. This graph is concentration of MIN3 melanin (µg/mL) vs DPPH radical scavenging activity (%). Concentration of MIN3 melanin 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml showed 59.46±2.35%, 59.79±3.05%, 60.8±2.02%, 61.81±1.65%, 63.71±2.35% scavenging activity respectively with highest scavenging activity of 63.71% [Figure 7]. The good antioxidant activity of MIN3 melanin indicates it could be utilized in cosmetic products which could scavenge the ROS generated by UV radiation or chronic damage [31]. Ultimately the MIN3 melanin containing cosmetics could be used as a antiaging solution.

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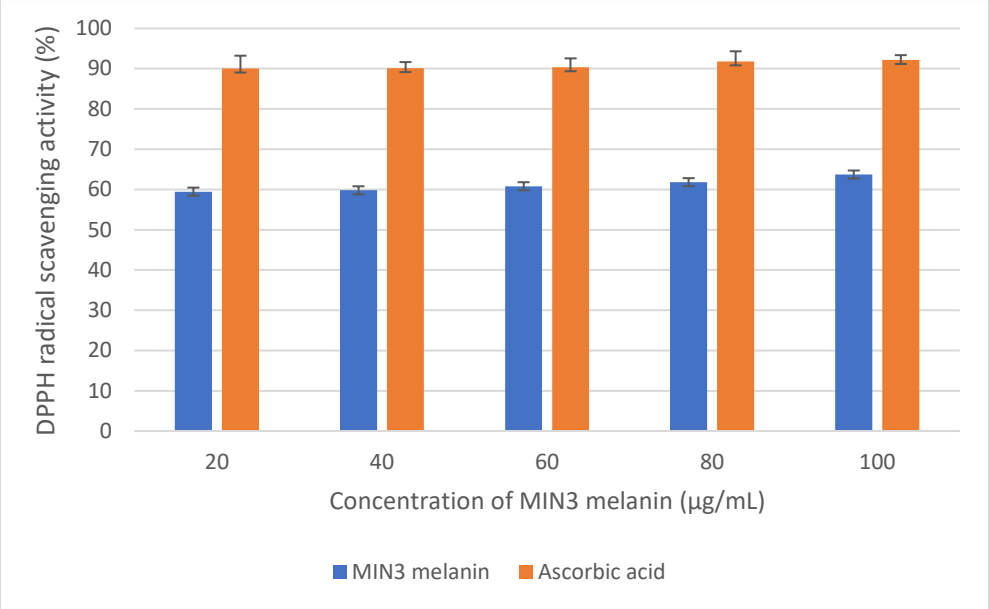

Figure 7: Concentration of MIN3 melanin µg/ml vs DPPH Radical scavenging activity (%) graph

UV Photoprotection Analysis

After the 24 hours of incubation melanized colony plate had shown higher number of colonies compared to non-melanized strain MIN3. The number of the colonies was beyond the countable limit but had shown considerable difference in the counts, i.e., after 20 min of UV treatment in non-melanized bacterial plate 222 colonies were observed [Figure 8 b] while melanized bacterial plates contain colonies beyond countable limit. This type of variation is observed in all plates from all durations of UV treatment. Qualitatively the change was evaluated from the Petri plates [Figure 8] that melanized colonies are more protected to UV radiation denoted by the increase colony count. The assay needs to be modified so that to quantify this change more accurately.

According to Joshi *et al*, 2021 [22] up to 15 minutes melanized cells have resisted UV treatment and survived after treatment. But in the present study melanized MIN3 strain had survived even after 20 min of UV treatment. Melanin had shown to enhance the SPF value of sun screen cosmetics in many earlier reports [5, 32]. Photoprotection is an important property for most of the cosmetic products, MIN3 melanin with this immense potential could be a good ingredient in cosmetic solutions.

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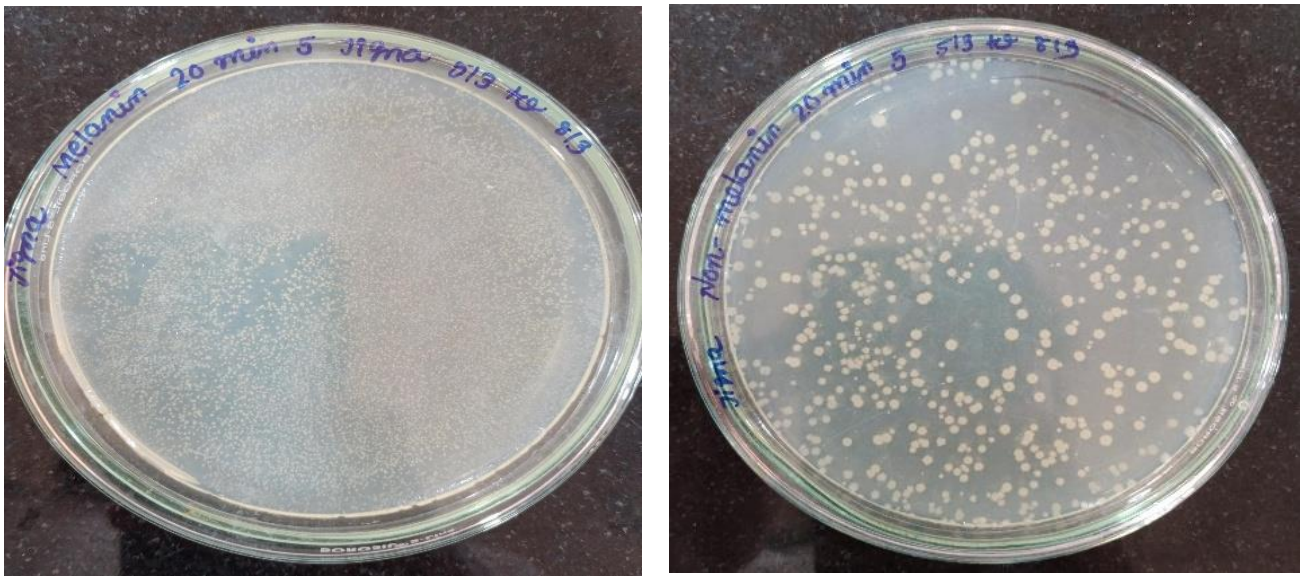

Figure 8: Photoprotection of melanin against UV radiation (a) melanized bacteria showing more colonies (b) non melanized bacteria showing less colonies

Antimicrobial activity: Minimum Inhibition Concentration

Melanin exhibits significant antibacterial activity against all the organism tested even in very lower concentrations. The minimum inhibitory concentration against *Bacillus sp.* was found to be 40µg/mL. While *E. coli*, *Salmonella sp.*, and *Shigella sp.* the MIC was found to be 20µg/mL. The minimum inhibitory concentration of MIN3 melanin against *Staphylococcus aureus* was found to be 60µg/mL [Table 4]. Anti-microbial ingredients can act as preservative booster in cosmetic properties [33]. Effectiveness of MIN3 melanin against pathogenic microbes indicates it could provide an additional preservative potential apart from other efficient cosmetic properties.

Bacteria	Minimum Inhibitory Concentration of MIN3 melanin
Bacillus sp.	40µg/mL
E. coli	20µg/mL
Salmonella sp.	20µg/mL
Shigella sp.	20µg/mL
Staphylococcus aureus	60µg/mL

Table 4: Minimum inhibitory concentration of MIN3 melanin against bacteria

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In Conclusion, the halophilic bacteria Bacillus *pumilus* MIN3 has shown to produce considerable good amount of melanin. Further optimization of media components could improve the melanin production by the strain. The immense bioactivities shown by the pigment makes it an important candidate ingredient which could be used in cosmetic products. The cytotoxicity analysis of pigment need to analysed before using it in cosmetic products.

References

1. De Carvalho CC, Fernandes P. Production of metabolites as bacterial responses to the marine environment. Marine drugs. 2010;8(3):705-27.

2. Wang Z, Tschirhart T, Schultzhaus Z, Kelly EE, Chen A, Oh E, Nag O, Glaser ER, Kim E, Lloyd PF, Charles PT. Melanin produced by the fast-growing marine bacterium Vibrio natriegens through heterologous biosynthesis: characterization and application. Applied and environmental microbiology. 2020;18;86(5):e02749-19.

3. Rzepka Z, Buszman E, Beberok A, Wrześniok D. From tyrosine to melanin: Signaling pathways and factors regulating melanogenesis. Postepy higieny i medycyny doswiadczalnej (Online). 2016; 30;70:695-708.

4. Płonka P, Grabacka M. Melanin synthesis in microorganisms: biotechnological and medical aspects. Acta Biochimica Polonica. 2006;53(3).

5. Kurian NK, Bhat SG. Food, cosmetic and biological applications of characterized DOPA-melanin from Vibrio alginolyticus strain BTKKS3. Applied Biological Chemistry. 2018;61(2):163-71.

6. Nosanchuk JD, Valadon P, Feldmesser M, Casadevall A. Melanization of Cryptococcus neoformans in murine infection. Molecular and cellular biology. 1999;1;19(1):745-50.

7. Wang Y, Casadevall A. Decreased susceptibility of melanized Cryptococcus neoformans to UV light. Applied and environmental microbiology. 1994;60(10):3864-6.

8. Brenner M, Hearing VJ. The protective role of melanin against UV damage in human skin. Photochemistry and photobiology. 2008;84(3):539-49.

9. Yabuuchi E, Ohyama A. Characterization of “pyomelanin”-producing strains of Pseudomonas aeruginosa. International Journal of Systematic and Evolutionary Microbiology. 1972;1;22(2):53-64.

10. Taylor WI, Achanzar D. Catalase test as an aid to the identification of Enterobacteriaceae. Applied microbiology. 1972;24(1):58-61.

11. Faddin M. JF Biochemical tests for identification of medical bacteria. The Williams & Wilkins Co, Baltimore

12. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990 Oct 5;215(3):403-10.

13. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular biology and evolution. 2007 Aug 1;24(8):1596-9.

14. Bauer AW. Antibiotic susceptibility testing by a standardized single disc method. Am J clin pathol. 1966;45:149-58.

15. Krumperman PH. Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. Applied and environmental microbiology. 1983;46(1):165-70.

16. Turick CE, Tisa LS, Caccavo Jr F. Melanin production and use as a soluble electron shuttle for Fe (III) oxide reduction and as a terminal electron acceptor by Shewanella algae BrY. Applied and environmental microbiology. 2002;68(5):2436-44.

17. Sajjan SS, Anjaneya O, Kulkarni GB, Nayak AS, Mashetty SB, Karegoudar TB. Properties and functions of melanin pigment from Klebsiella sp. GSK. Microbiology and Biotechnology Letters. 2013;41(1):60-9.

18. Fava F, Di Gioia D, Marchetti L. Characterization of a pigment produced by Pseudomonas fluorescens during 3-chlorobenzoate co-metabolism. Chemosphere. 1993 Sep 1;27(5):825-35.

19. Yuan W, Burleigh SH, Dawson JO. Melanin biosynthesis by Frankia strain CeI5. Physiologia plantarum. 2007 Oct;131(2):180-90.

20. Ravishankar JP, Muruganandam V, Suryanarayanan TS. Isolation and characterization of melanin from a marine fungus. Botanica Marina,1995;38: 413-416.

21. Liyana-Pathirana CM, Shahidi F. Antioxidant activity of commercial soft and hard wheat (Triticum aestivum L.) as affected by gastric pH conditions. Journal of agricultural and food chemistry. 2005 Apr 6;53(7):2433-40.

22. Joshi MH, Patil AA, Adivarekar RV. Characterization of brown-black pigment isolated from soil bacteria, Beijerinckia fluminensis. bioRxiv. 2021 Jan 1.

23. Łopusiewicz Ł. Scleroderma citrinum melanin: isolation, purification, spectroscopic studies with characterization of antioxidant, antibacterial and light barrier properties. World Scientific News. 2018;94(2):115-30.

24. Kurian NK. Melanins from marine bacteria Characterization production and applications. 2015, http://hdl.handle.net/10603/102159

25. Ghadge V, Kumar P, Singh S, Mathew DE, Bhattacharya S, Nimse SB, Shinde PB. Natural melanin produced by the endophytic Bacillus subtilis 4NP-BL Associated with the Halophyte Salicornia brachiata. Journal of Agricultural and Food Chemistry. 2020;4;68(25):6854-63.

26. Liu F, Yang W, Ruan L, Sun M. A Bacillus thuringiensis host strain with high melanin production for preparation of light-stable biopesticides. Annals of Microbiology. 2013;63(3):1131-5.

27. Kurian NK, Bhat SG. Photoprotection and Anti-Inflammatory Properties of Non–Cytotoxic Melanin from Marine Isolate Providencia rettgeri Strain BTKKS1. Biosciences Biotechnology Research Asia. 2017;25;14(4):1475-84.

28. Pavan ME, López NI, Pettinari MJ. Melanin biosynthesis in bacteria, regulation and production perspectives. Applied Microbiology and Biotechnology. 2020;104(4):1357-70.

29. Tarangini K, Mishra S. Production, characterization and analysis of melanin from isolated marine Pseudomonas sp. using vegetable waste. Res J Eng Sci. 2013;2278:9472.

 doi:10.20944/preprints202205.0296.v1

30. Sajjan S, Kulkarni G, Yaligara V, Lee K, Karegoudar TB. Purification and physiochemical characterization of melanin pigment from Klebsiella sp. GSK. Journal of Microbiology and Biotechnology. 2010;20(11):1513-20.

31. Ribet V, Nobile V, Rossi AB. In situ antioxidant activity of a dermo-cosmetic product: A randomized controlled clinical study. Experimental dermatology. 2019;28(11):1219-26.

32. Huang S, Pan Y, Gan D, Ouyang X, Tang S, Ekunwe SI, Wang H. Antioxidant activities and UV-protective properties of melanin from the berry of Cinnamomum burmannii and Osmanthus fragrans. Medicinal Chemistry Research. 2011;20(4):475-81.

33. Herman A. Antimicrobial ingredients as preservative booster and components of self-preserving cosmetic products. Current microbiology. 2019;76(6):744-54.