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Production, Characterization and Analysis of Melanin from Isolated Marine *Pseudomonas* sp. using Vegetable waste

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Abstract

Melanin pigment from natural sources like microorganisms was an attractive choice for commercial scale production. In this study, marine bacterium capable of melanin production on marine broth/agar was isolated and identified as Pseudomonas sp. (closely related to guinea) on phenotypic characterization. Melanin production activity of the isolate was studied in liquid mediums such as pure marine broth and vegetable cabbage waste. In pure marine broth, melanin yield was ~5.35 mg/mL and pigment production was absent in pure vegetable waste. However in the presence of marine broth (as starter culture) melanin yield increased to ~2.79 mg/mL. This indicates melanin production may be initiated austerely by marine broth. Pigment from the bacterium was purified and characterized using UV-visible and FTIR analysis. The morphology and size of the bacterium was visualized in scanning electron microscopy (SEM) and the pigment nature was identified by SEM/EDX analysis. The results indicated that the synthesized melanin was very near to synthetic dihydroxyphenylalanine (DOPA)-melanin in all aspects and possess antioxidant activity.

Keywords: Dihydroxyphenylalanine, marine bacterium, melanin, pigment, vegetable waste.

Introduction

Melanins are diverse group of macromolecules, synthesized ubiquitously in living organisms by oxidative polymerization of various phenolic substances in the process of adaption[1]. In nature, melanins act as photoprotectants (against UV and visible light), charge transport mediators, free-radical scavengers, antioxidants, metal ion balancers and etc[2]. Melanins also have applications in agriculture, medicine, cosmetic and pharmaceutical industries. In general, melanins are negatively charged, hydrophobic, high molecular weight compounds with amorphous nature. These are insoluble in common organic solvents, aqueous acids and water[1,3].

Based on color and structural classes primarily there are three types of melanins i.e. eumelanins, pheomelanins and allomelanins. Eumelanins are black to brown color pigments produced by melanisation by classic Mason-Raper pathway, which produce tyrosine intermediates or metabolites by the action of tyrosinases. Pheomelanins are brown, red or yellow color pigments which are produced in course of oxidation of tyrosine and/or phenylalanine to dihydroxyphenylalanine (DOPA) and dopaquinone. Pheomelanin results from cysteinylation of DOPA and these are sulphur containing compounds. Allomelanins include nitrogen free heterogeneous group of polymers formed from catechol precursors[3,4]. The eumelanins and pheomelanins commonly occur in animal species, while allomelanins can be seen in microorganisms and plants[5]. Some of the funguses known to produce melanins are *Cryptococcus neoformans*, *Sporothrix schenckii*, *Sepia officinalis*, *Aspergillus niger*, *Penicillium marneffei*, *Paracoccidioides brasiliensis*, *Histoplasmacapsulatum*, *C. neoforman*[6]. Coming to bacteria, some species of *Aeromonas salmonicida, Azotobacter*, *Mycobacterium*, *Micrococcus*, *Bacillus*, *Legionella*, *Streptomyces*, *Rhizobium*, *Vibrio*, *Proteus*, *Azospirillum*, *Pseudomonas aeruginosa*, *Hypomonas* sp, *Burkholderia cepacia*, *E. coli*, *Bordetella pertusis*, *Campylobacter jejuni*, *Yersinia pestis* etc[2,5].

Apart from terrestrial microorganisms, explorations of melanin production by marine microorganisms appear to be inadequate with limited literature. For instance, Kotob et al[7]. synthesized marine melanin from *Vibrio cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana*. They reported that the formed melanin was pyomelanin which result due to catabolism of tyrosine via Tyrosine degradation pathway. Another study by marine bacterium genus *Alteromonas* produced melanin in-vivo with the aid of tyrosine precursors[8]. Similarly, other marine bacteria capable of producing melanin are *Marinomonas mediterranea* MMB-1^T which belong to the phylum *Proteobacteria*[9] and thermo-alkaliphilic *Streptomyces* (from limestone quarries of the Deccan traps)[10]. Apart from bacteria, a study by obligate marine fungus *Cirrenalia pygmea* showed melanin production ability in its mycelium and conidia[11].

Overall, melanin pigment from various microbial species especially from marine species is an attractive option of research still in its infancy. In this study, we describe a procedure for the isolation of melanin producing marine microorganism (*Pseudomonas* sp.) and characterized biochemically. Melanin producing ability of the isolate was further tested on vegetable waste (pure and blended with marine broth) at ambient temperature and pH. The structure of the bacterium and pigment nature was identified by scanning

electron microscopy and the synthesized melanin was analyzed spectrophotometrically. The extracted and purified pigment was characterized using energy dispersive spectroscopy and Fourier infrared spectroscopy.

Material and methods

Chemicals used : Marine agar, marine broth, DPPH (2,2-diphenyl-1-picrylhydrazyl) were procured from HiMedia chemicals, Mumbai, India. Ethanol, NaCl, NaOH, HCL and all other chemicals used were of analytical reagent grade throughout the study. Ultrapure water used for the experiments and aseptic conditions were maintained wherever necessary.

Screening and isolation of the melanin producing strain: Microorganisms capable of producing melanin were isolated from the sea water samples collected from three different locations i.e. nearby rocks - (sample 1), shore - (sample 2) and from deep sea water (10 m away from shore) - (sample 3) of Vishakapatnam beach, Andhra Pradesh, India. 0.1 mL of diluted water samples (from 10^{-7} dilution) was individually spreaded on marine agar plates with pH 7.0. The media and the glassware was autoclaved at 15 psi (121^0C) for 20 min prior to the experiment; these agar plates with media and inoculum were incubated at 25^0C for 48 h. Melanin producing microorganisms were identified by the presence microbial colonies with dominant thick black color (diffusible) in agar plates. Selective colonies were separated out for subculturing and characterization.

Pigment production, extraction and purification: Marine broth medium was used for inoculum preparation and pigment production. About 10 µL (10^8 CFU/mL) culture suspension was added to 50 mL marine broth in 250 mL flasks. This medium was then incubated at 25 ^{0}C on a rotary shaker moving at 200 rpm for 48 to 72 h until the liquid medium becomes darkly pigmented and nearly opaque. All media used for the study were sterilized by autoclaving unless elsewhere stated. After the incubation time, the medium was centrifuged using REMI-RM12C, India centrifuge at 8000 rpm for 15 min to separate the broth (supernatant) and the cells. The solid pellet of cells was separated and suspended in distilled water. These cells again centrifuged to collect the supernatant. Melanin was extracted from the overall supernatant by acidification with 3 N HCl to pH-2 and allowed to stand for 48 h initially at room temperature. This process was repeated for 3 more days until no precipitation found. Then the obtained suspension was boiled for 5 min to prevent the formation of melanoidins[1]. As a final point, crude pigment pellet was collected after centrifugation at 4000 rpm for 15 min. In addition to marine broth medium, the pigment production ability of the isolate was tested by culturing them upon vegetable waste (from cabbage), blend of marine broth and vegetable waste (30:70) as nutrient source. Culture conditions and rest of the protocol maintained same as described above.

DPPH radical scavenging assay: DPPH radical scavenging activity of the produced melanin was tested according to the modified literature method[12]. Primarily, 0.1 mM of DPPH solution was prepared in 95% ethanol before use. To 1 mL of it, different volumes (10, 20 and 30 µL) of melanin suspension of strength 1.55 mg/mL was added along with 30, 20 and 10 µL water. Thus, final concentrations of melanin in melanin mixed DPPH solutions are 14.9, 29.8 and 44.7 µg/mL. The samples were incubated in dark at 40^0C throughout the study. The DPPH radical scavenging activity was evaluated by monitoring the absorbance decrease at 516 nm at various time intervals by keeping the respective melanin strengths dispersed in ethanol as a reference.

Characterization/analytical methods: The morphology of the microorganism and the purified pigment was examined by scanning electron microscope (SEM) JEOL JSM-6480LV. The compositional pattern was determined by energy dispersive X-ray (EDX) spectroscopy which is coupled to SEM. UV-visible spectrum of the melanin was observed using UV-vis. spectrophotometer (UV-3600 Shimadzu). The FTIR analysis of pigment was carried out after mixing with KBr using FTIR spectrophotometer (Perkin Elmer, Model No.S2000, USA).

Results and Discussion

Strain selection and characterization of the microorganism: Inoculated sea water samples on marine agar plates were observed each day for melanin producing microorganism. At ambient conditions, during the end of second day (~ 48 h) visible black color colonies with diffused black color was evident in the agar plates inoculated with sea water sample 1 (figure 1).

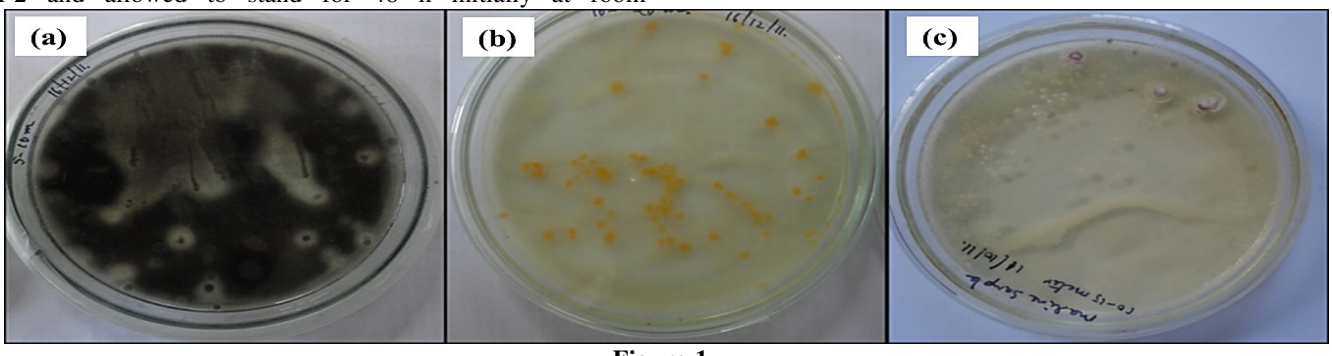

Figure-1
Screening of microbial strains obtained from various parts of a sea shore.
A) near stones, B) near shore and C) 10 m away from sea

Table-1
Colony characteristics of the isolated melanin producing bacterium

Characteristics	Result
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	Raised
Texture	Slimy
Opacity	Opaque
Gram's reaction	
Cell shape	Rods
Spore (s)	-
Motility	+
Growth temperatures	
4, 10, 25, 30, 37, 42, 55 ^{0}C	-, -, +, +, +, +, -
Growth pH's	
5, 6, 7, 8, 9, 10, 11, 12	-, +, +, +, +, +, +, -
Growth on NaCl (%)	
0.5, 2.0, 4.0, 6.0, 8.0, 10.0, 11.0, 12.0	+, +, +, +, -, -, -, -
Tests	
Catalase test	+
Oxidase test	+
Voges Proskauer test	-
Casein hydrolysis	-
Citrate	+
Nitrate	-
Arginine dihydrolase	-
Gelatin hydrolysis	-
Starch hydrolysis	-
Esculin hydrolysis	-
Tween 20	+
Tween 40	+
Tween 60	+
Tween 80	+
DNase	-
Acid Production from	
Cellobiose	-
Trehalose	-
Fructose	+
Maltose	+
Dulcitol	-
Sucrose	+
Dextrose	+
Raffinose	+

The other isolates devoid of black color were ignored for further study. Colonies form black color marine agar plates were transferred to new agar plates and allowed to grow for 2 days. The visible tiny colonies and morphology of the isolated microorganisms observed by SEM images (figure 2) states that the microbe might be of bacterial origin. The microbe size range was found to be from 1.2 to 1.7 µm and is rod shaped in appearance. For detail evidence regarding black pigment producing organism, isolated strain was sent for microbial phenotypic characterization to Institute of microbial technology (IMTECH), Chandigarh, India. The microorganism was identified as *Pseudomonas* sp. and is closely related to *Pseudomonas guinea*. Table 1 list out the key morphological and biochemical characteristics of the isolated bacterial strain.

Pigment production and characteristics of the produced melanin: The isolated bacterium was incubated in marine broth and vegetable waste up to 48 h for melanin production. Here, vegetable waste from cabbage leftovers is selected as a growth medium as it satisfies the major micronutrient append as that of marine broth. Prior to the addition of inoculum, vegetable waste was supplemented with 1.9 % NaCl to maintain salinity as that of marine broth. Additionally marine broth – vegetable waste blend in 30:70 ratio also used as melanin production medium for comparison.

The dark brown to black color (figure-3a) of the marine broth medium indicates more melanin production by the bacterial isolate than the marine broth - vegetable waste blended medium (figure 3b). The sole vegetable waste was not given any color but has cream slimy growth appearance after incubation of 2 days. The observed behaviors indicate melanin production was strictly medium dependent (i.e. marine broth here). The influence of medium was clearly evident from figure 3a and 3b where a 30:70 ratio of marine broth - vegetable waste blend gave less intense melanin when compared to marine broth alone. The melanin produced from pure marine broth and marine broth - vegetable waste blend was found to be 5.35 ± 0.4 and 2.79 ± 0.2 mg/mL after 72 h of incubation. The melanin from blended medium was found to be ~ 0.52 times lesser than pure marine broth.

However melanin from both sources after purification (by acid treatment) looked alike in appearance. The physical appearance of the purified melanin was also shown in Fig. 4 with a true black color typical of melanins in general[13]. The produced melanin was insoluble in water, ethanol, chloroform, acetone, benzene and slightly soluble in phenol and 1N NaOH. The melanin was precipitated with 6 N HCl and decolorized with the addition of H_2O_2. The observed features when compared with previous reports indicate the synthesized melanin was similar to that of bacterial melanin in properties[1].

Spectroscopy, SEM/EDX and IR analysis of melanin: For a detail inference and structural elucidation UV-visible spectroscopy, SEM/EDX and FTIR analysis were performed for the purified melanin pigments from two different media. The UV-visible wavelength scan showed the absorption was highest at the UV region of 200 to 300 nm, but diminished towards the visible region (figure 5) for both the melanins obtained. This phenomenon is characteristic to melanin and was due to actual complex structure of melanin[1].
Figure 6 shows the SEM image of the purified melanin. The

appearance from the figure suggests that the material was an amorphous deposit with no differentiable structures, similar to past reports of purified bacterial melanin[3]. Further to know the compositional examination, EDX analysis was performed and shown in Figure-7. The analyses revealed that the majority composition of the purified melanin from marine broth alone is of C, O with ~ 66 and 30 weight % and minor S content with ~3.85 %. While, melanin from blended medium showed C, O with ~ 35.62, 50.29 weight % and minor Ca content with ~14.09 %. Some peaks of Figure-7 are undetectable as EDX may not be a reliable method to quantify elements in low weight %[3]. This result serve as an additional support which reflects the purity of the melanin produced. The compositional variation of melanin pigments might be due to the change in medium compositions.

FTIR spectroscopic analysis was performed on the acid treated purified melanin pigments to know the information about functional groups and structure. Figure 8 shows the IR spectrum of melanins pressed into KBr disks. Similar spectral pattern from figure 8a and 8b indicate both melanin pigments obtained are having similar peaks corresponding to equivalent functional groups. The details of both spectra are as follows: A broad absorption at 3373 cm^{-1} indicate the presence of – OH and NH_2 groups and small band at 2918 cm^{-1} can be assigned to stretching vibration of aliphatic C-H group[14]. The characteristic strong band at 1625 cm^{-1} (between 1650 - 1620 cm^{-1}) attributed to vibrations of aromatic ring C=C of amide I C=O and/or of COO- groups. Bands at ~1400 to 1500 cm^{-1} can be due to aliphatic C-H groups and weak bands below 700 cm^{-1} ascribed to alkene C-H substitution in the melanin pigment[1]. The observed IR patterns for the purified melanins were similar to the earlier reported DOPA-melanin study[4].

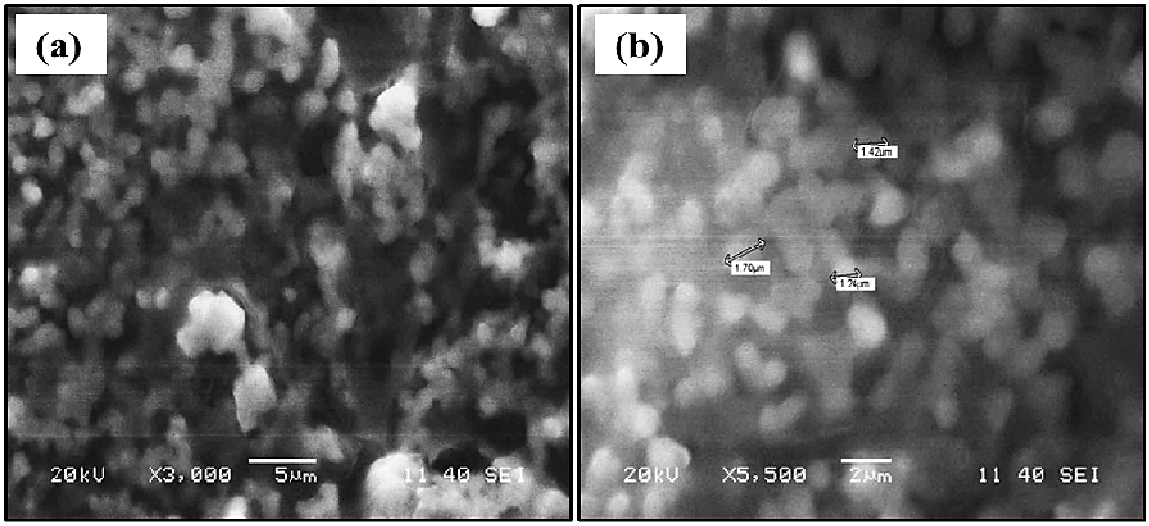

Figure-2
Low (a) and high (b) magnification SEM images of the isolated microorganism with black colonies on agar plates

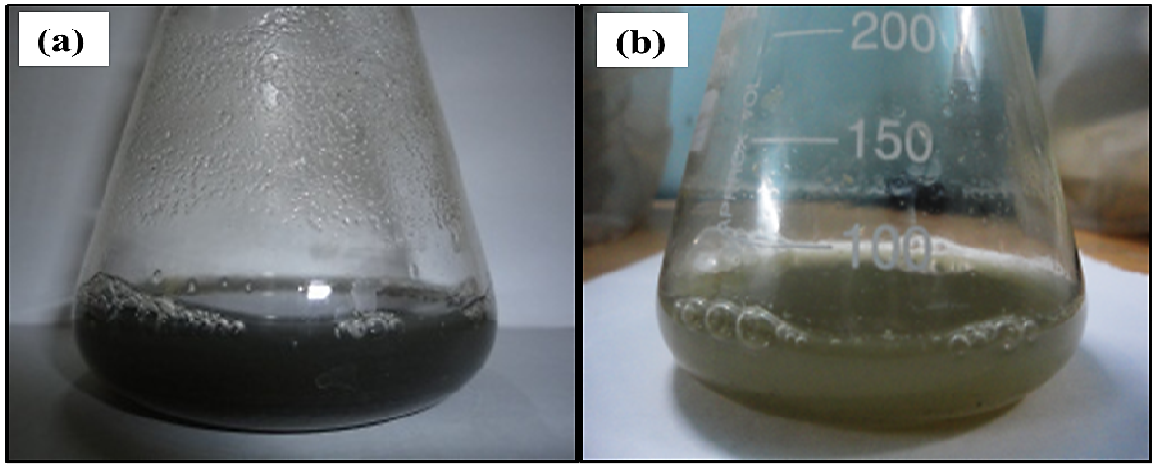

Figure-3
Dark brownish black colonies due to melanin production in sole marine broth (a) and in marine broth – vegetable wate blend medium (b)

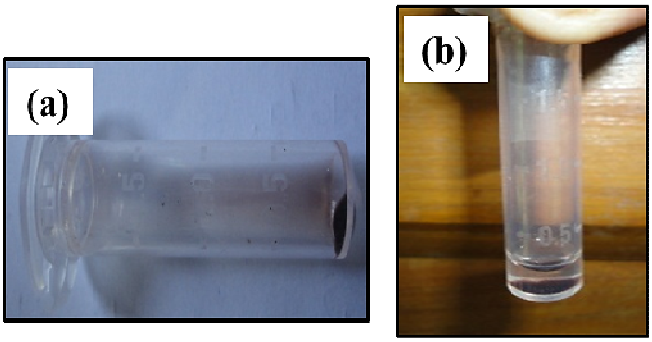

Figure-4
Acid treated (a) and purified melanin (b) after centrifugation

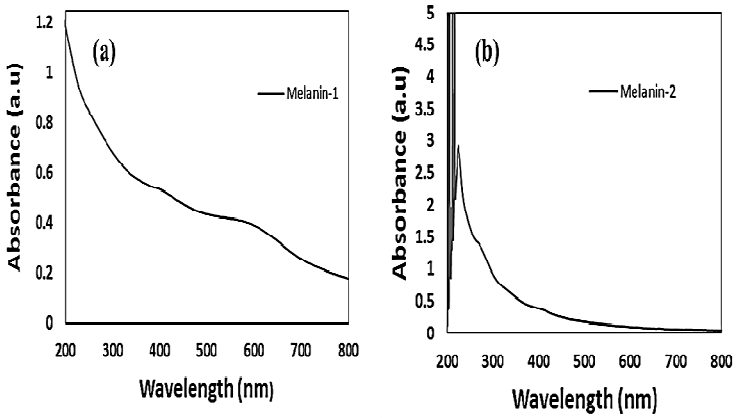

Figure-5
UV-visible spectral properties of melanin pigment obtained from marine broth (a) and marine broth –vegetable waste medium (b)

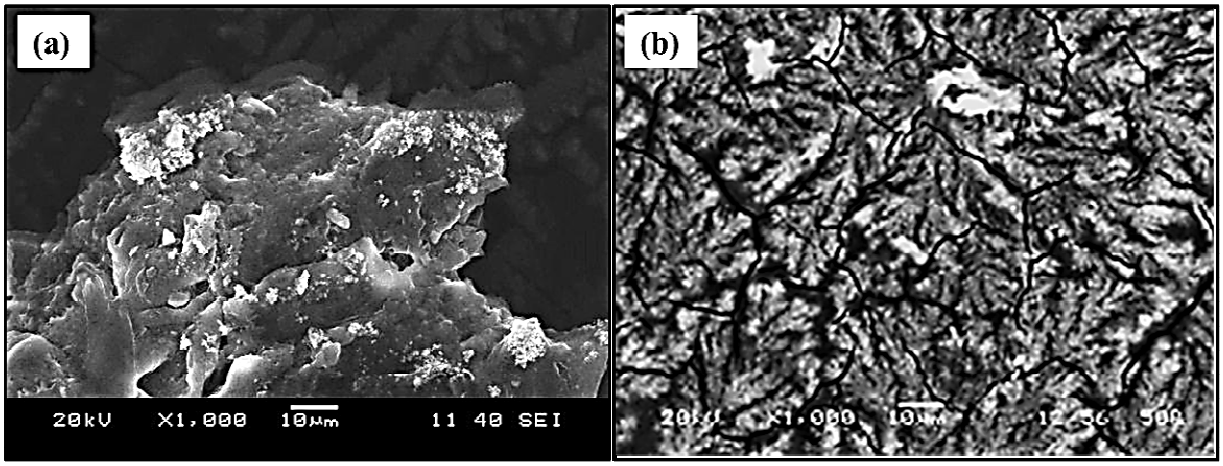

Figure-6
SEM images of purified bacterial melanin from a) marine broth and b) marine broth – vegetable waste medium

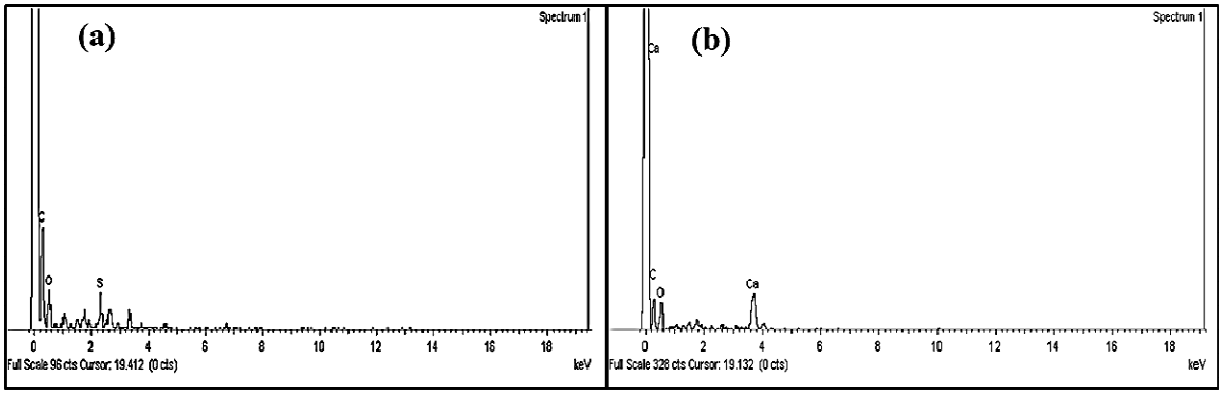

Figure-7
EDX analysis of elemental composition of melanin from a) marine broth and b) marine broth – vegetable waste medium

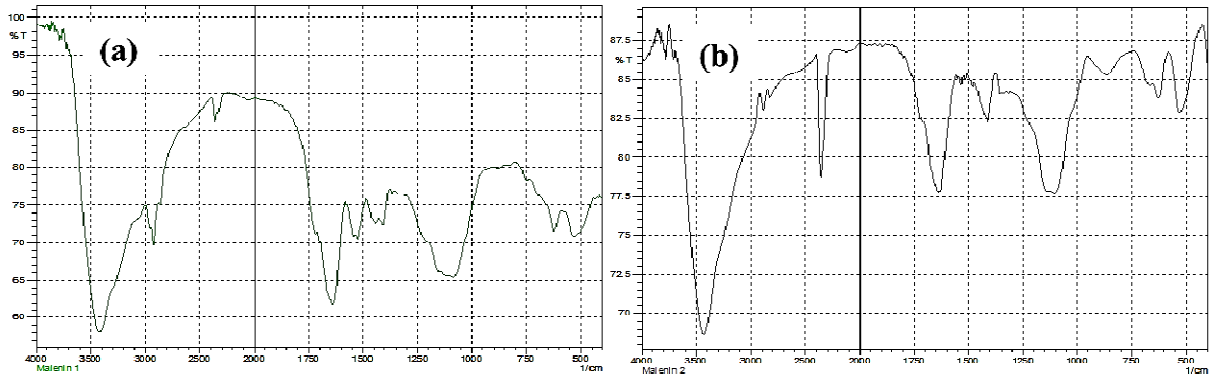

Figure-8

FTIR spectrum of the melanin pigment obtained from a) marine broth and b) marine broth – vegetable waste medium

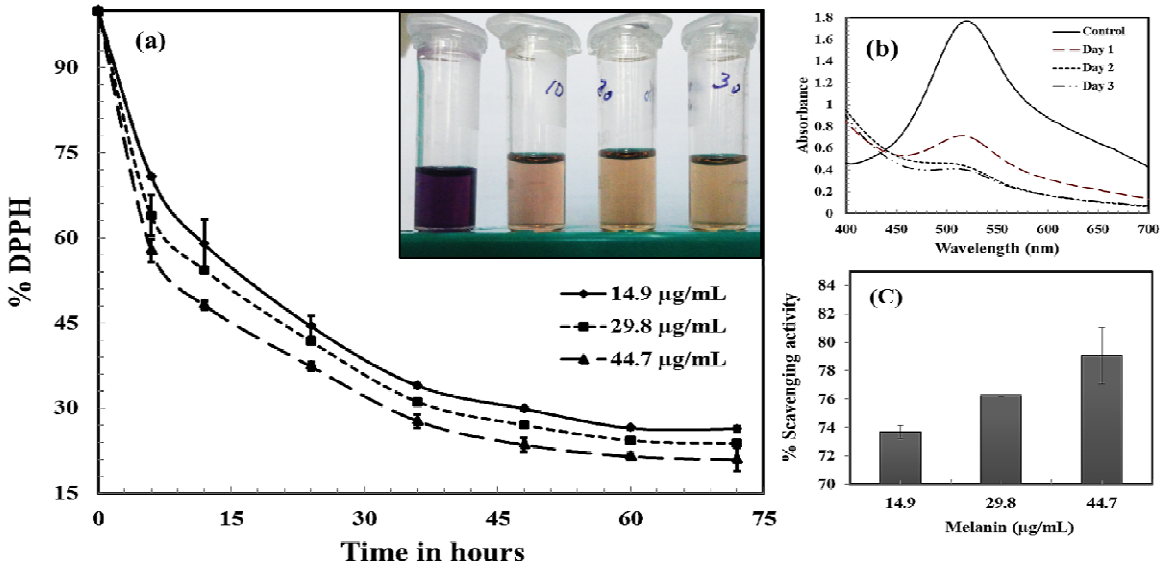

Figure-9

DPPH radical scavenging activity of synthasized melanin pigment with various doses (a) and UV-vis absorption spectrum of melanin (44.7 µg/mL) - DPPH at different days along with control containing no melanin (b). Dose dependent scavenging activity of the synthasized melanin (c). Insert of (a) shows the melanin doses 0, 14.9, 29.8 and 44.7 µg/mL to 0.1 mM DPPH from left to right

DPPH assay: Melanin particle were found to possess antioxidant property in biological systems. It can scavenge free radicals and has the ability to sequester redox active metal ions[12]. Free radical scavenging activity was evaluated by performing *in-vitro* DPPH assay. Reduction of absorbance at 516 nm supplied with different melanin doses was shown in figure 9a. The colored DPPH solution faded and turned dull during the course of incubation of 3 days.

This may be due to the reduction of the DPPH molecules and electron transfer from melanin suspension. Figure 9a also indicates a nonlinear pattern of DPPH reduction for various melanin doses used. A sharp change in absorbance up to 48 h for the used melanin concentrations indicates that the rate of reduction is rapid at initial stages. The diminished behavior (from figure. 9a) beyond 48 h indicates the maximum threshold reduction by a particular melanin dose used. The reduction in spectral behavior at various time intervals (in days) was shown in Figure 9b for a melanin dose of 44.7 µg/mL. Moreover from figure 9a, we can plot % scavenging activity with respect to melanin dosage after a residual period of 72 h (figure 9c). Henceforth, the minimum time period required by the melanin molecules for the maximum DPPH reduction and dose dependent scavenging activity were successfully valued in the present study.

Conclusion

Though different marine isolates were used, it was found that the sample 1 contains the melanin producing bacterium and was

found to be *Pseudomonas guinea*. Upon investigation for the melanin production in different media, 5.35 ± 0.4 and 2.79 ± 0.2 mg/mL pigment was produced when cultured in marine broth alone and blended medium (vegetable waste and marine broth), respectively. Pigments produced from the different nutrient sources have shown different elemental compositions with varying weight %. Moreover, the FTIR analysis revealed that functional groups were conserved in both the melanins and were appeared to be same. The compositional variation in the pigments might be due to the change in the media composition although *P.guinea*, only was used in both the cases. Furthermore, the produced melanin noticed to have efficient free radical scavenging activity of a model DPPH radical. This study confirms that the antioxidant melanin pigment can be produced from the cheaper substrates without any functional variation.

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