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Research Article

Amorphous Crystalline DOPA- Melanin from Mineral Beach Sand Isolate *Bacillus cereus* BTSNGIST5

Jayaram Prasad S, Jayalakshmi M Menon, Vijil V V,
Aswathi P S, Nimitha K N, Athira L, Vijayalakshmi S V,
Sruthi P S, Sughi S and Noble K Kurian*
Department of Bioscience,
SNGIST Arts and Science College,
Manakkappady, N. Paravur
*Corresponding author: Email: kurianoble@gmail.com Tel: +919605293207

ABSTRACT:
Melanins are macromolecular pigments produced by most life forms which have significant importance commercially as well as therapeutically due to its protective property against UV radiation. Bacteria are a good producer of melanin which can be purified easily compared to other sources. A melanin producing bacterial strain BTSNGIST5 was isolated from stress prone mineral beach sand and identified as Bacillus cereus by biochemical and 16SrDNA sequencing. The bacteria produced 117.92±7.4µg/mL of melanin which had shown typical melanin like chemical and spectroscopic characteristics. Scanning electron micrographs revealed its amorphous crystallinity and found to be less toxic to fibroblast cell lines.

Keywords: *Bacillus, melanin, crystalline, amorphous*

[I] INTRODUCTION

Melanins are structurally diverse high molecular weight pigments synthesized by oxidative polymerisation involving quinones [1].which plays a major role in protecting of living forms from harmful radiations like UV rays, regulating body temperature, acting as electron acceptor etc. Melanin produced by both prokaryotes and eukaryotes belongs to the following three main types; Eumelanins which are black or brown pigments produced in the course of oxidation of tyrosine via DOPA, while Pheomelanins shares the same pathway with eumelanin up to DOPAquinone. Then DOPAquinone cysteinylation takes place to form cysteinyl DOPA, which gets further polymerized to form pheomelanin. Allomelanins are the least studied and most heterogeneous group of melanins, formed by the polymerization of di (DHN) or tetrahydrofolate via pentaketide pathway, leading to formation of various colored polymers including DHN-melanin, homogentisic acid (pyomelanins), γ- glutaminyl-4-hydroxybenzene, catechols, as well as of 4- hydroxyphenylacetic acid [2].

Besides its biological functions melanin especially plant and sepia origin are widely utilized in cosmetic formulations. But the melanin from these sources was highly subjected to batch variation. Most purified melanin without any batch variations can be obtained from bacterial sources [3]. So this makes the studies on bacterial melanin important.

Stress conditions trigger production of melanin in bacteria [4]. Mineral Beach Sand is characterized with its presence of ores of heavy metals such as zirconium, titanium, thorium, tungsten and rare earth elements [5]. Compared to animals and plants inhabiting in mineral beach sand, bacteria are more sensitive to heavy metal toxicity. This toxicity could create stress, which could make the bacteria from this environment melanogenic [6].

Present study explores the isolation and characterization a melanin producing bacteria BTSNGIST5 and characterization of the melanin produced.

[II] MATERIALS AND METHODS

2.1. Chemicals and bacterial isolates

Synthetic melanin (Sigma Chemicals Co, St Louis, USA), L-tyrosine (Himedia chemicals, Mumbai, India) and all other chemicals used were of analytical reagent grade. Bacteria was isolated from the sediment samples collected from the ore mining regions of mineral beach of Chavara (8° 58' N, 76° 32‘E), Kerala ,India.

2.2. Screening for melanin producers

Pure colonies of 15 isolates were initially screened on tyrosine basal agar plates [7] and then followed in tyrosine basal broth [8]. The bacteria were identified by biochemical screening and 16S rDNA sequencing using specific primers [9].

2.3. Production, Extraction and Purification of melanin

Tyrosine basal broth [8] containing 0.2% tyrosine served as the medium for melanin production. 5 mL of this culture suspension (OD_{600} = 1) was used as primary inoculum for 50 mL of production medium and kept in an environment shaker (Orbitek, Scigenics, India) at 140 rpm at 37±2^{o}C for 180 h. Melanin production was estimated spectrophotometrically at 400nm according to Turick et al. [10] .The melanin produced was extracted and purified by acid precipitation and subsequent washing with alcohol and water [11].

2.4. Characterization of melanin

Reactivity of melanin against various organic solvents, acidic and basic solutions, oxidising and reducing agents were evaluated [12]. Spectroscopic techniques such as UV-Visible [13], FT-IR [14] and Scanning electron microscopy (SEM) [15] were used to characterize melanin.

2.5 Cytotoxicity of melanin

Cytotoxicity of melanin was tested on mouse fibroblast cell line L929 cells, which were maintained in Dulbecco’s modified eagles media (Himedia, India) supplemented with 10 % FBS (Fetal Bovine serum) (Invitrogen, USA) and grown to confluence at 37°C at 5 % CO_2 in a CO_2 incubator (Eppendorf, Germany). Different concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) of melanin were added to L929 cells and incubated for 24 hours. The percentage difference in viability was determined by standard 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay [16] after 24 hours of incubation.

[III] RESULTS AND DISCUSSION

3.1. Screening for melanin producers

Of the 15 pure colonies plated in tyrosine basal agar, only one had shown clear zones (Fig. 1 A) around the colonies indicating the utilization of tyrosine from the plates. The single isolate BTSNGIST5 was subjected to biochemical characterization which revealed the strain was Gram staining, Catalase and Oxidase tests positive in nature. The gram positive melanin producer was identified up to species level by partial 16S rDNA sequence analysis. 16S rRNA gene were amplified using specific primers and the amplified product of 1.5 Kb size was visualized by agarose gel electrophoresis (Fig 1 D).The amplicons were sequenced using Sanger’s dideoxy sequencing

method. After 16S rDNA sequencing, the identity of the sequence was determined using NCBI BLAST [17], where the sequences were searched against the GenBank database. BTSNGIST5 had shown similarity with *Bacillus cereus* and phylogenetic tree was also constructed (Fig 1 E) with the closely related sequences. The sequence of identified isolate was submitted to GenBank and accession number (KU598667) was obtained.

3.2. Production of melanin

BTSNGIST5 was then subjected to secondary screening (melanin production) on tyrosine basal broth which was initially white (Fig 1 B) in color at the time of incubation. The color turns to dark brown (Fig 1 C) in color gradually and got stabilized on the fifth day of incubation. On the fifth day, the concentration of melanin produced was estimated to be 117.92±7.4µg/mL which was comparable to that of earlier reports [18, 19].

3.3. Characterization of melanin

Melanin from BTSNGIST5 strain was soluble in alkaline solvents like sodium hydroxide, potassium hydroxide and dimethyl sulfoxide (DMSO). However, the pigment showed least solubility in water, acids and common organic solvents. This insoluble nature of melanin may be due to its macromolecular nature which is full of aromatic rings and carboxylic acids. Despite of this nature, melanins were solubilized in dimethyl sulfoxide (DMSO). This may be the result of thioalkylation of the phenolic units in melanins by DMSO which enables the solubility [20]. Oxidizing (30% H_2O_2) and reducing (Na_2SO_3) agents decolorized the pigment. This may be due to the existence of melanin in different oxidation states. Melanin existing in granular state fades when treated with oxidizing and reducing agents, which may depend on the actual number and disposition of the melanin granules [21]. UV–Visible spectrum (Fig. 2 A) showed maximum absorption at UV region which decreased considerably as it reached the visible region. The highly heterogeneous structure, probably also the coexistence of both reduced and oxidized polymer chain domains and aggregation of polymer chains contribute to this featureless

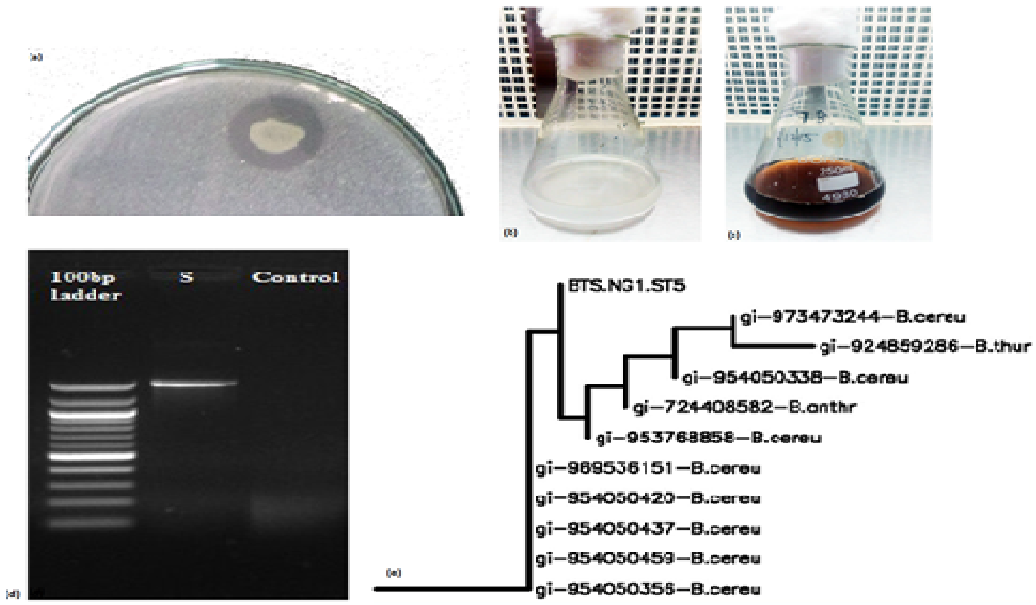

Fig: 1. Screening for melanin producers (a) BTSNGIST5 colony showing clear zone around it in tyrosine basal agar (b) tyrosine broth before inoculation and (c) after melanin production (d) 16S rDNA amplicons on lane 2 marked 'S', lane 1 – 100bp ladder and negative control on lane 3 (e) Phylogenetic tree of BTSNGIST5

absorption [22]. The spectrum was comparable with that of the previous reports [23]. FTIR spectrum of *Bacillus cereus* BTSNGIST5 melanin (Fig. 2 B) showed considerable similarity to those in earlier reports [18, 24]. The spectrum showed a broad absorption around 3399 cm^{-1}, corresponding to phenolic –OH and –NH stretching vibrations. Characteristic peak observed between 1634 cm^{-1} was attributed to aromatic ring C=C stretching, while the peak at 1049 cm^{-1} accords with the phenolic C–O stretching vibration. This confirmed the polyphenolic and aromatic nature of BTSNGIST5 melanin.

Scanning electron micrographs of BTSNGIST5 melanin (Fig. 2 C,D and E) reflected an amorphous crystaline nature.From the SEM images it was clear that the melanin showed a blocky crystaline structure, having irregular edges and diffeernt sizes distributed in an amorphous mesh. This nature of melanin had shown similiarty with fungal melanin from *Lachnum sp.* [25], while it differed a lot from the reported uniform spherical struture of melanin

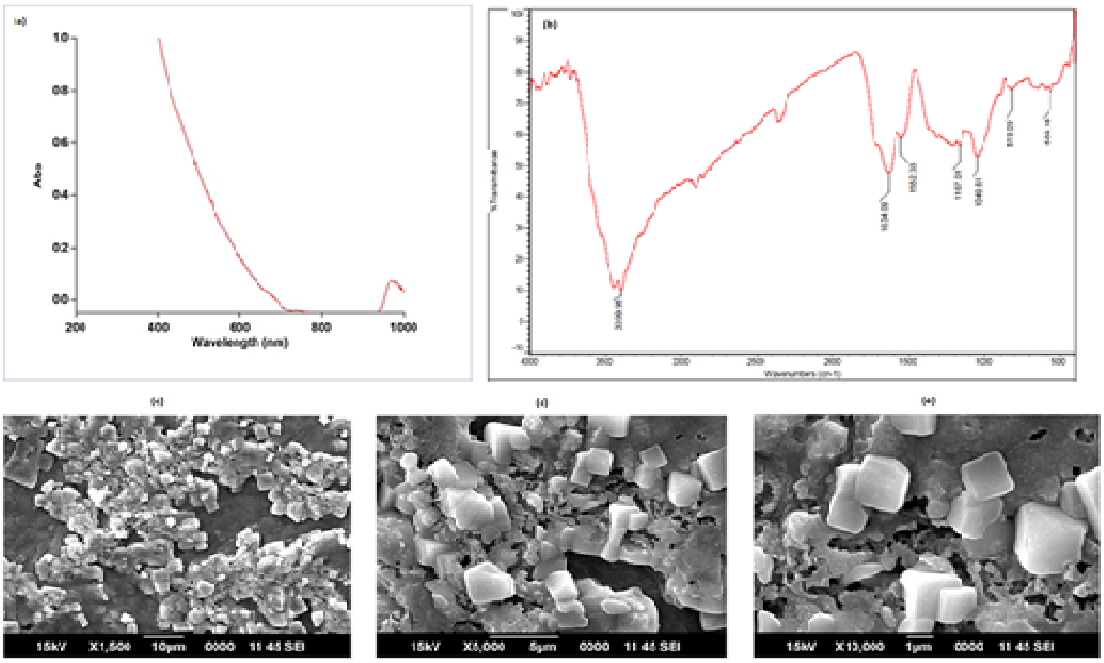

Fig: 2. Characterization of BTSNGIST5 melanin (a) UV Visible spectrum of melanin (b) FT-IR peaks of melanin (c), (d),and (e) Scanning electron micrographs of melanin showing crystals and amorphous matter in its microscopic morphology.

[26]. More X-ray diffraction studies are needed to confirm the amorphous crystaline nature of melanin.

3.4. Cytotoxicity of melanin

Bacillus cereus BTSNGIST5 melanin was found to be less toxic to L929 cells with an IC_{50} value of 199.6 ug/mL (Fig. 3 B and C). Highest concentration tested (100µg/mL) had shown only 25.49±1.33% inhibition (Fig. 3A). Arun et al (2015) [27] demonstrated that in HEP2 cells, *Schizophyllum commune* melanin had inhibited cell growth 53% at a concentration of 60 µg. While Madhusudhan et al (2014) [28] reported *Streptomyces lusitanus* melanin showing a

cytotoxicity of LC_{50} 0.80 µg/mL while testing in brine shrimps. BTSNGIST5 melanin outperform many of the earlier reports with respect to its less cytotoxicity.

[IV] CONCLUSION

In conclusion the amorphous crystaline melanin from *Bacillus cereus* strain BTSNGIST5 had showed typical melanin like characteristics with a difference in its microscopic morphology which needs to be explored further. Its less toxicity of fibroblast cells makes it a potential candidate for applications in cosmetics and therapeuticals.

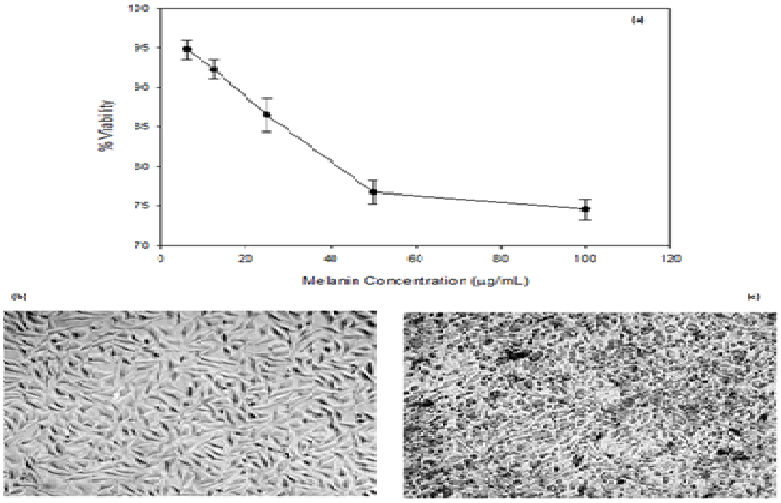

Fig: 3. Cytotoxicity of melanin (A) graph showing the dose dependent toxicity of BTSNGIST5 melanin (B) Phase contrast micrographs (×20 magnification) of control (without melanin treatment) (C) melanin treated cells (100µg/mL).

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