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KF909003, Noble K Kurian, Harisree P Nair and Sarita G Bhat, 16S rDNA partial sequence *Pseudomonas stutzeri* strain BTCZ10.

KF494197, Noble K Kurian and Sarita G Bhat, 16S rDNA partial sequence *Vibrio alginolyticus* strain BTKKS3.

KF908996, Noble K Kurian and Sarita G Bhat, 16S rDNA partial sequence *Vibrio parahaemolyticus* strain BTKK10.

KF471760, Noble K Kurian and Sarita G Bhat, 16S rDNA partial sequence *Vibrio alginolyticus* strain BTKK16.
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KF515634, Noble K Kurian and Sarita G Bhat, 16S rDNA partial sequence Vibrio alginolyticus strain BTKK17.

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EVALUATION OF ANTI-INFLAMMATORY PROPERTY OF MELANIN FROM MARINE BACILLUS SPP. BTCE31

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ABSTRACT

Objectives: To evaluate the anti-inflammatory property of melanin from marine Bacillus spp. BTCE31.

Methods: Radical scavenging property of melanin was determined by 2,2-Diphenyl-1-picrylhydrazyl and metal-chelation assays, which was further confirmed by electron paramagnetic resonance (EPR) spectroscopy. Anti-inflammatory property of melanin was explored in vitro in RAW264.7 cell line using cyclooxygenase (COX), Lipoxygenase (LOX), Myeloperoxidase (MPO) and cellular nitrite inhibitory assays. Cytotoxicity of melanin was determined using trypan blue exclusion and lactate dehydrogenase (LDH) assay.

Results: BTCE31 melanin showed radical scavenging activity of 67.5% and ferrous ion chelating activity of 97.88%. EPR spectrum showed sharp peaks indicating the presence of unpaired electrons. Melanin inhibited the activity of COX and LOX enzymes with IC₅₀ values of 10.84 μg/mL and 10.53 μg/mL, respectively. It also reduced the activity of MPO and cellular nitrite levels. Cytotoxic concentration of melanin was found to be 105.4 μg/mL (IC₅₀).

Conclusion: Bacillus spp. BTCE31 melanin can be a potential anti-inflammatory agent. Further in vivo evaluations are needed for confirming the activity, leading to therapeutic applications.

Keywords: Anti-inflammatory, Antioxidant, Bacillus spp., Bacteria, Marine, Melanin.

INTRODUCTION

Free radicals are highly unstable, reactive species that contain one or more unpaired electrons. Reactive oxygen species (ROS) includes oxygen radicals like superoxide (O₂⁻), hydroxyl (OH⁻), peroxy (ROO⁻), hydroperoxy (ROOH) and certain non-radical existing agents like hydrogen peroxide (H₂O₂), nitric oxide (NO) and superoxide dismutase (SOD) [1]. ROS are understood to be involved in the etiology of many diseases such as aging, cancer, coronary heart disease, Alzheimer’s disease, neurodegenerative disorders, atherosclerosis, cataracts, and also have inﬂammation and oxidative stress [2]. Antioxidants act as physical barriers, which prevent ROS generation or access to important biological sites (e.g. enzymes, cell membranes); chemical traps/sinks that “absorb” energy and electrons, quenching ROS (carnosine, ascorbic acid); catalytic systems that neutralize or detoxify ROS (antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase); binding/oxidation of metal ions to prevent generation of ROS (ferrioxamine, glutathione, catecholamines); and chain breaking antioxidants, which scavenge and destroy ROS (ascorbic acid, tocopherols, uric acid, glutathione, and flavonoids) [3].

Inflammation is body’s natural response induced by tissue injury or infection and functions to combat external threats. During inflammatory response, the endothelial permeability increases, blood leukocytes infiltrate into the interstitium, oxidative burst, and release of cytokines (interleukins and tumor necrosis factor-α (TNF-α)) occlude. At the same time, there is also an induction of the activity of several enzymes like oxygenases, nitric oxide (NO) synthases, peroxidases as well as the arachidonic acid metabolism. These responses are also accompanied by an increase in expression of adhesion molecules like intercellular adhesion molecule and vascular cell adhesion molecules. An antioxidant molecule can minimize the ROS production and thereby help in reducing the inflammatory response [4]. Inflammation is also accompanied by increase in pain in arthritis, sprains, and other conditions and is commonly treated by non-steroidal anti-inflammatory drugs such as ibuprofen and naproxen, which can cause severe side-effects including heart attacks and stroke [5].

Melanins are brown to black colored complex pigments produced mainly via amino acid tyrosine and are widely distributed in living beings, where they have several biological functions such as photo protection, thermoregulation, free radical-stores, cation-chelators, and antioxidants. In plants melanin is incorporated in their cell walls as strengthening [6], whereas in humans it not only determines the skin color but also plays an important role in protecting against UV radiation [7]. In the microbes, it protects against environmental stresses. Melanins are well known for its antiprostaglandin property, which can helps reduce ROS generation [8]. Minimizing ROS minimizes inflammation as both these properties are interrelated to each other. There are no reports of bacterial melanins with anti-inflammatory activity. In this study, we are exploring the antiprostaglandin and anti-inflammatory property of bacterial melanins produced by marine bacteria Bacillus spp. BTCE31.

METHODS

Cell lines and bacterial isolates

150/9 and RAW264.7 cell lines were maintained in Dulbecco's modified eagles media (Himedia, India) supplemented with 10% fetal bovine serum (Invitrogen, USA) and grown to confluence at 37°C at 5% CO₂ in a CO₂ incubator (Eppendorf, Germany).

Melanin producing Bacillus spp. BTCE31 was obtained from marine sediments collected from 96.87 as depth (93.95° N, 75.33° E) during the Sagar Sampaio cruise no 305 in the Arabian Sea on the west coast of India. The bacteria and its pigments were characterized (unpublished data).

Extraction and purification of melanin

Tyrosine basal broth [9] was used for melanin production. Melanin production was monitored spectrophotometrically at 400 nm [10] using synthetic melanin (Sigma, USA) as standard. The cell free supernatant
from the production broth was acidified to pH 2 with 1 N HCl, allowed to stand for a week at room temperature (RT) (28±0.5°C), followed by boiling for 1 h and centrifuged (Thermo Scientific, USA) at 7000 rpm for 10 minutes. Resultant black pellet was washed thrice with 15 mL of 0.1 N HCl, followed by water. To this pellet, 10 mL of ethanol was added and the mixture was incubated in a boiling water bath for 10 minutes, kept at RT for 1 day, washed twice in ethanol, air dried [11] and used for further analysis.

Antioxidant activities

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay
Free radical scavenging activity of melanin was determined using DPPH radical scavenging assay [12], wherein 1.6 mL of 0.135 mM DPPH in methanol was mixed with 10 mL of melanin at concentration ranging from 20 to 100 µg/mL, mixed well and left in the dark at RT for 30 minutes. Absorbance was measured spectrophotometrically (Shimadzu, Japan) at 517 nm. Acetate acid (standard antioxidant) served as positive control.

The ability to scavenge (DPPH) radical was calculated as follows:

Free radical scavenging activity (FSA) = \( \frac{A_{o} - A_{e}}{A_{o}} \times 100 \)

Where \( A_{o} \) is the absorbance of the DPPH + methanol, and \( A_{e} \) is the absorbance of the free radical solution with melanin/standard antioxidant.

Metal-chelating activity

Chelating potential of ferric ions (Fe**(3+)) by melanin was estimated as per Fossi et al. [13]. Melanin (25-100 µg/mL) and standard EDTA disodium tetra-acetate (EDTA) was added to a solution of 2 mM Fe**(3+) (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), mixture was shaken vigorously and left standing at RT for 10 minutes. Absorbance of the solution was read at 562 nm (Shimadzu).

The percentage inhibition of ferrozine–Fe**(3+) complex formation was calculated as:

\( \frac{A_{o} - A_{e}}{A_{o}} \times 100 \)

Where \( A_{o} \) is the absorbance of the control, and \( A_{e} \) is the absorbance of the melanin/standard.

Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra of melanin was obtained with JEOL, Model JES FA200 (X-band) EPR spectrometer. The EPR spectral conditions included: frequency 9.12 G; modulation frequency, 100.00 kHz; power, 0.9998 mW; field center, 326.296 mT and sweep time 2.9 minutes. Proportionality factor (g factor) was calculated by the following equation [14]:

\( \ln \gamma = \beta H \)

Where \( \ln \) is Planck’s constant, \( \gamma \) is microwave frequency, \( H \) is magnetic field and \( \beta \) is a constant, Bohr magneton.

Anti-inflammatory activities

RAW 264.7 cells were then grown to 60% confluence followed by activation with 1 µg/L lipopolysaccharide (LPS) (1ug/mL), LPS stimulated RAW cells were exposed with different concentration (6.25, 12.5, 25, 50, 100 µg/mL) of melanin solution. Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the sample was also added and incubated for 24 hours. After incubation the anti-inflammatory assays were performed using the cell lysate.

Cytotoxicity assay (CCK-8 activity)

The CCK-8 activity was assayed by the method of Walker and Giese [15].

The cell lysate was incubated in Tris- HCl buffer (pH 8), glutathione-5 mM/L, and hemoglobin 5 mM/L for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 µM/L and terminated after 20 minutes incubation at 37°C, by the addition of 10% trichloroacetic acid in 4 N hydrochloric acid. After the centrifugal separation and the addition of 1% thionin, CCK activity was determined by reading absorbance at 540 nm (Shimadzu). Percentage inhibition of the enzyme was calculated as:

\( \% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100 \)

Lipoxigenase (LOX) activity

The determination of LOX activity was as per Jandeleit et al. [16]. Briefly the reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL). The LOX activity was monitored as an increase of absorbance at 234 nm (Shimadzu), which reflects the formation of 5-hydroxyeicosatetraenoic acid.

Percentage inhibition of the enzyme was calculated using the formula:

\( \% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100 \)

Myeloperoxidase (MPO) activity

Cell lysate was homogenized in a solution containing 50 mM potassium phosphate buffer and 0.1% hexadecyltrimethyl ammonium bromide (HDTMA). Homogenized mixture was frozen in liquid nitrogen and thawed. After freeze thawing 3 times, the samples were centrifuged at 2000 g (Sigma, Germany) for 10 minutes at 4°C, and supernatant was assayed for MPO activity. MPO in the sample was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/mL guaiacol and 0.0005% H2O2. [17]. The change in absorbance at 460 nm (Shimadzu) was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µmol of peroxidase per minute at 25°C.

Cellular nitrite levels

Cellular nitrite level was estimated by the method of Legrand et al. [18]. To 0.5 mL of cell lysate, 0.1 mL of sodium nitroprusside and 8 was added, warmed for 30 minutes and then centrifuged at 5000 rpm (Sigma) for 15 minutes. The protein-free supernatant was used for nitrite estimation. To 200 µL of the supernatant, 30 µL of 1% NaOH was added, followed by 300 µL of Tris- HCl buffer and mixed well. To this, 530 µL of Griess reagent was added, incubated in the dark for 10-15 minutes, and the absorbance was read at 540 nm (Shimadzu) against a Greiss reagent blank. Sodium nitrite solution was used as standard. The amount of nitrite present in the samples was estimated from the standard curve.

Cytotoxicity studies

Different concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) of B. thuringiensis melanin were added to L929 cells and incubated for 24 hours. The percentage difference in viability was determined by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [19] after 24 hours of incubation. The cells were washed with phosphate buffer saline (PBS), added 30 µL of MTT solution, and incubated at 37°C for 3 hrs. MTT was removed by washing with PBS and 240 µL of dimethyl sulfoxide (DMSO) was added to the culture. Incubation was at RT for 30 minutes until the cell were losed and color was obtained was read at 540 nm using DMSO as blank in a microplate reader (Ehruasdiium II, Germany).

Statistical analysis

The data were expressed as Mean ± standard deviation (n=3). Statistical significance was determined by one-way ANOVA using GraphPad Prism Software. At 95% confidence interval, p<0.05 were considered to be significant.

RESULTS

Purified Bacillus spp. B. thuringiensis melanin was previously characterized using chemical and spectroscopic characterizations (unpublished data).
to confirm its identity and used for further studies. The DPPH radical scavenging activity of pure melanin from Bacillus spp. BTC231 was comparable with the activity of standard antioxidant ascorbic acid. The results revealed that 100 μg/mL bacterial melanin exhibited 59.55% radical scavenging activity, which was comparable to that of standard antioxidant ascorbic acid showing an activity of 77.52% (Fig. 1a). Sharp peaks in the EPR spectra (Fig. 1c) of melanin indicated the presence of unpaired electrons, which can trap free radicals. Proportionality factor (g-factor) of BTC231 melanin was 1.96, which was as good as that of organic radicals. Antioxidant efficiency of BTC231 melanin was also reflected in its metal scavenging property. Melanin even at lower concentration of 25 μg/mL had shown 72.79% e⁺-scavenging activity (Fig. 1b), which increased as the concentration increased. 100 μg/mL of melanin showed a scavenging activity of 97.64% which was equivalent to that of the standard EDTA (99.64%).

Purified melanin from Bacillus spp. BTC231 inhibited COX and LOX enzymes effectively at increasing concentrations. Melanin inhibited COX enzyme with an IC₅₀ value of 164.84 μg/mL, while LOX enzyme was inhibited with an IC₅₀ value of 18.5 μg/mL. COX-1 and LOX enzymes at 47.92 and 69.48% (p<0.0001), respectively. MPO activity was released significantly (p<0.0001) as the concentration of melanin increased from 6.25-100 μg/mL. Cellular nitrite levels, an indicator of NO produced during inflammation had reduced (647.54 μg/mL) as melanin concentration increased (Fig. 2). MTT assay revealed that BTC231 melanin inhibited growth of L929 cells, with IC₅₀ being 105.4 μg/mL (Fig. 3).

**DISCUSSION**

In this study, the evaluation of melanin as a potent anti-inflammatory agent was carried out. Antioxidant ability of melanin is the major factor determining its ability to act as a good anti-inflammatory agent. Many previous reports [11, 20] discussed the immense antioxidant potential of bacterial melanin. Mathurwase et al. [20] reported 90% radical scavenging activity for melanin (3.3 mg/mL) from Actinomyces thaliana spp. Xyloepathy spp. Melanin [11] was reported to have 796 (50 μg/mL) free radical scavenging activity. Here, Bacillus spp. BTC231 melanin (100 μg/mL) showed 67.55% scavenging activity which was comparable with the earlier reports. Like lutein, cornelianchys, echinacea etc., melanin also binds to metal ions and minimizes the generation of ROS [3]. Therefore, antioxidant potential of melanin can also be related to its metal chelating ability. Moreover BTC231 melanin had shown nearly cent percent Fe⁺-chelating activity, which further reflects its ability to scavenge free radicals. EPR peaks of melanin indicated the presence of unpaired electrons, which can trap free radicals. Proportionality factor (g) of BTC231 melanin (1.96) was similar to that of melanin from Aeropyrgillus bradleyi (1.90) [21] and free electron in vacuo (2.002) [22]. This also could lead to the radical scavenging ability of BTC231 melanin.

COX and LOX enzymes have significant roles to play in the regulation of inflammatory responses [21]. Anti-inflammatory drugs and agents decrease this response by suppressing the production pathway of the inflammatory mediators, which in turn block the initiation and progression of inflammation-associated diseases [23]. In the present study, the in vitro experiments showed the dose-dependent inhibition of COX, LOX, MPO and NO activity in RAW 264.7 cell lines by BTC231 melanin. COX is a rate limiting enzyme in inflammation as it is involved in the conversion of arachidonic acid to prostaglandins, which are associated with many inflammatory diseases. The inhibition of COX by BTC231 melanin was significant. LOX enzyme which produces leukotrienes is important in the pathophysiology of inflammatory diseases [24]. At lower concentration, BTC231 melanin inhibited LOX activity. Myeloperoxidase catalyzes the formation of HOCI by the oxidation of HO⁻ during inflammatory response [25]. The addition of BTC231 melanin decreased the MPO activity considerably. The decrease in cellular nitrite level [26] after BTC231 melanin treatment indicates its capability for use as a potential anti-inflammatory agent. There are only few reports on anti-inflammatory properties of melanin. Arramied et al. [27] reported that grape melanin interacted with the prostaglandins as well as the leukotrienes and/or complement system mediated inflammation. This supports our findings. Bacillus spp. BTC231 melanin was also less toxic to cells, which suggests its feasibility in therapeutic applications.

![Fig. 1](image_url)

- (a) Radical scavenging activity of facultis spp. BTC231 melanin compared to standard antioxidant ascorbic acid
- (b) Fe⁺-chelating potential melanin compared to standard ethylene diamine tetra acetic acid
- (c) Electron paramagnetic resonance spectrum of melanin
Fig. 2: Effect of RTC231 melatonin on the activities of (a) Cyclooxygenase (b) Lipooxygenase (c) Molybdenum and (d) Cellular nitrite levels

Fig. 3: Cytotoxic effect of Bacillus spp. RTC231 melatonin (a) Phase contrast micrographs (+20 magnification) showing the cytotoxic effect (b) Control (c) Treated (100 µg/mL)

The inhibition of key enzymes, which participate in inflammatory responses.

ACKNOWLEDGMENTS

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A melanin producing bacterial strain BTCZ10 was isolated from marine sediments at more than 96 m depth. Phylogenetic analysis revealed 100% homology of the 16S rDNA sequence with Pseudomonas stutzeri strains in NCBI database. BTCZ10 melanin polymerization occurred outside the cell wall, which could be visualized as melanin ghosts by light microscopy. The pigment was soluble in alkali, but insoluble in water, acid and organic solvents. Melanin turned colorless by the treatment with oxidizing and reducing agents. UV visible spectrum revealed a higher absorption at the UV region which decreased as it reached the visible region. Strain BTCZ10 produced 47.47±0.2 µg/ml of melanin.
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Figure 1: Screening for melanin production (a) tyrosine basal agar plates with *Pseudomonas stutzeri* BTCZ10 showing clear zones around the colonies (b) Dark brown melanin produced in tyrosine basal broth.

Figure 2: Molecular Identification and Phylogenetic characterization of BTCZ10 (a) Agarose gel showing 16 rDNA amplicon of BTCZ10 in lane 1; lane 2 has the Thermus Scientific Gene Ruler 1kb Ladder (b) Phylogenetic tree showing the position of *Pseudomonas stutzeri* BTCZ10 with reference to the related strains.
Figure 3: UV–Visible spectrum of melanin pigment (a) *Pseudomonas stutzeri* BTCZ10 melanin (b) synthetic melanin (c) sepi melanin

Figure 4: Melanin ghosts encircled under light microscope (40X).
Materials and Methods

Chemicals and Bacterial Isolates

Synthetic melanin and Sepia melanin (Sigma Chemicals Co., St Louis, USA). L-tryosine (Jenmeda chemicals, Mumbai, India) and all other chemicals used were of analytical reagent grade.

Marine sediment samples were collected from 96.47°N at depth 5.960’N, 75.392’E during the Sagar Sampada cruise no 305 in the Arabian Sea on the West coast of India. Bacteria were isolated after serial dilution and pour plating, followed by quadrant streaking to purify them.

Screening for melanin producers

Primary screening involved spot inoculating on tyrosine basal agar plates with 2g/L L-tyrosine added as the sole source of carbon and nitrogen (Yabuchi and Ohyaama, 1972). Selection was based on clear zone formation around the colonies.

Melanin production was quantitatively analyzed by feeding the isolates selected after the primary screening into the melanin production media containing KH2PO4, 2.0 g; NaCl, 5.0 g; MgSO4·7H2O, 0.4 g and distilled water 1000 mL with 2g/L L-tyrosine serving as carbon and nitrogen sole source (Yabuchi and Ohyaama, 1972). Melanin production was monitored spectrophotometrically at 400nm (Turuck, et al., 2002) using synthetic melanin (Sigma, USA) as standard.

Molecular identification of melanin producing bacteria

Genomic DNA was isolated and purified (Sambrook et al., 2000); a portion of the 16S rDNA was amplified using a primer pair for 16S rDNA (Roky et al., 2002). The products after PCR amplification were purified by gene clean kit (Bangalore Genei, Bangalore, India) and the nucleotide sequence was determined by the ABI Prism 310 Genetic analyzer using the big dye terminator kit (Applied Biosystems, USA). The identity of the sequences was cross checked using the 16S rDNA sequence with the sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using their World Wide Web site (http://www.ncbi.nlm.nih.gov) and the BLAST (Basic Local Alignment Search Tool) algorithms (Altschul et al., 1990) and the nucleotide sequence was submitted to NCBI Genbank.

Phylogenetic characterization of the bacteria

The nucleotide sequence of the most similar sequences obtained by BLAST search using Bacterial Sequence Alignment Editor (Balle, 1999) and the phylogenetic tree was constructed using the neighbor-joining method using MEGA5: Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura et al., 2011).

Extraction and Purification of melanin

Melanin was extracted from the cell free supernatant, following acidification with 1 N HCl to pH 2 and standing for a week at room temperature. The resulting suspension was boiled for 1 h and then centrifuged (Sigma 3K30, Germany). The black pigment thus formed was washed three times with 15 mL of 0.1 N HCl, followed by water. To this pellet, 10 mL of ethanol was added and the mixture was incubated in a boiling water bath for 10 min and kept at room temperature for a day. The pellet was washed with ethanol twice and then air dried (Sajjan et al., 2013) and this purified pigment was used for further analysis.

Chemical analysis of the pigment

Solubility of melanin in deionized water, IN HCl, IN NaOH, IN KNO3, ethanol, acetone, chloroform, benzene, xylene, hexane and acetone was evaluated. Reaction with oxidizing agent hydrogen peroxide (H2O2) and reducing agent sodium sulfite (Na2SO3) was determined (Faria et al., 1993). All reactions were compared with synthetic melanin as standard.

UV-Visible spectrum of purified melanin

UV-Visible spectrum of the purified melanin at the wavelength ranging from 250nm to 800nm was generated. Melanin was dissolved in 0.1N NaOH, which served as the blank for evaluation (Wendin et al., 2007). Spectrum obtained was compared with synthetic and septa melanin.

Preparation of Melanin ghosts

BTCZ10 was cultured in tyrosine basal broth at 37°C in a shaking incubator for 5 days. Melanin ghosts were isolated from the spent medium by boiling in HCl and ethanol extraction (Eisenman et al., 2007). Ghosts were fixed on glass slides by gently heating for 1-2 s and viewed under a light microscope (Radical, India) (40X) without staining.

Results

Selection of strain and Pigment production

Bacteria (N=40) isolated from the deep sea sediment samples were spotted on tyrosine agar medium to select for tyrosinase producers. Strain BTCZ10 which formed clear zones (Fig 1a) around their colony was selected. Secondary screening showed significant pigment production by strain BTCZ10 from the 20th hour itself. From 146th hour the production medium turn dark brown (Fig 1b) and the production entered the stationary phase, with 4.74µg/mL of melanin.

After DNA isolation from strain BTCZ10, the 16S rRNA gene was sequenced (Fig 2a). The 1.5 kbp gene was sequenced, submitted to NCBI database and accession number was obtained (KT909093).

Phylogenetic characterization of BTCZ10

16S rRNA sequence of strain BTCZ10 showed 100% similarity with Pseudomonas stutzeri sequences from the NCBI database. Phylogenetic tree is shown in Figure 2b. The sequence also showed 99% similarity with other Pseudomonas species like P. frutetii, P. aeruginosa and P. putida. Bacillus subtilis M55008 served as the outgroup in the phylogenetic analysis.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.421 12096 is shown. The tree was drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 442 positions in the final dataset. Evolutionary analyses were conducted in MEGAS (Tamura et al., 2011).

Chemical characterization of purified melanin

The dark brown melanin was purified and characterized. It was insoluble in water and most of the organic solvents tested. It could be precipitated by addition of acids. Only
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Alkaline solvents like sodium hydroxide (NaOH) and potassium hydroxide (KOH) solubilized the pigment. The pigment turned colorless on reacting with oxidizing and reducing agents like hydrogen peroxide (H₂O₂) and sodium sulfite (Na₂SO₃) respectively.

Spectroscopic Analysis of the Pigment
UV-Visible spectrum of melanin from 250-800nm was generated and is as shown in Fig. 3. Higher absorption was observed at the UV region (200-300nm) which then decreased towards the visible region. It was noted that the melanin spectrum of BTCZ10 was similar to that of the synthetic and sepia melanin, and also to those previously reported (Ronai et al., 2000).

Melanin Ghosts
Melanin ghosts obtained after the removal of unwanted cell lumen material (Fig. 4) were visualized under a light microscope (40X) as dark spots.

Discussion
Marine sediments from approximately 97 m depth in the Arabian Sea were screened for melanin producers on tyrosine based agar plates. Bacteria forming clear zones on utilizing tyrosine was selected as a novel screening method, as not only it was fast and effective, but even colorless bacteria that formed clear zones on the agar plates produced melanin in the production media. Strain BTCZ10 secreted an extracellular dark pigment. Production medium which was initially white in color turned dark brown by the 6th day, and stabilized thereafter.

Strain BTCZ10 was identified based on the homology match of the 16S rDNA sequence with the reference strains from NCBI database as Pseudomonas stutzeri. Pigment produced by strain BTCZ10 was identified as melanin by physical and chemical characterization. Presence of conjugated complex structures in pigment makes its absorbance maxima fall in far UV region. This property reflects the ability of melanin in protecting the skin from harmful UV radiation. BTCZ10 melanin also showed typical melanin like chemical properties, especially in their solubility. Melanins are soluble in alkaline solvents only. This limitation makes it difficult in the elucidation of chemical structure of the pigment.

Conclusion
Marine melanin producing sources are little explored. A marine pigment producer Pseudomonas stutzeri BTCZ10 was identified from the depths of the sea. The purified BTCZ10 melanin exhibited the physical and chemical properties of typical melanin. With its high UV absorption property, it can be utilized further in optical lenses and in sunscreen lotions. More studies are required to understand the other properties of melanin.

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