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ARTICLE

Food, cosmetic and biological applications of characterized DOPA-melanin from *Vibrio alginolyticus* strain BTKKS3

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Abstract Melanins are one of the most common pigments produced in nature and distributed throughout the biological kingdom. *Vibrio alginolyticus* strain BTKKS3 produced DOPA-melanin was used in the study. BTKKS3 melanin inhibited biofilm formation by pathogenic bacteria and effectively decreased the activity of four inflammatory enzymes tested viz. cyclooxygenase, lipoxygenase, myeloperoxidase and nitric oxide synthase. Melanin proved to be less cytotoxic to mouse fibroblast cells with an IC_{50} value of 134.98 µg/mL. The sun protection factor value of commercial sunscreens was enhanced by 3.42 units by DOPA-melanin.

Keywords *Vibrio alginolyticus* · Anti-biofilm · Anti-inflammatory · Sun protection factor

Introduction

Melanins are structurally diverse high-molecular weight pigments synthesized by oxidative polymerization involving quinones [1] and widely distributed among the living organisms. They were even observed in fossils from dinosaurs, early birds, non-avian theropod species [2, 3] and primitive cephalopods. Thus, they were distributed all over the living world as earlier as the time before life evolved and can serve as a biomarker in evolutionary studies [4]. Melanins basically are of three main types, the black eumelanins produced via *o*-dihydroxy phenylalanine(DOPA), red pheomelanins produced by the cysteinylation of DOPA quinone and allomelanins comprising a heterogeneous group of melanins including DHN-melanin, homogentisic acid (pyomelanins), γ- glutaminyl-4-hydroxybenzene, catechols, as well as 4-hydroxyphenylacetic acid [5].

Bacteria are commonly allomelanin producers, although they also produce considerable amounts of all other types of melanins. There is *Klebsiella* sp. a eumelanin producer [6] as well as pheomelanin-producing *Vibrio cholerae* [7]. *Azotobacter* makes catecholic melanin [8]. There are a large number of species utilizing homogentisic acid pathway for melanin synthesis like *Pseudomonas aeruginosa* [9], *Vibrio cholerae* [11], *Shewanella colweliana* [10] to name a few. Though there are reports regarding melanin production by many bacterial species, biosynthetic mechanism of melanin is least explored in most reported species.

Although there are only few commercial melanin-based products, the research utilizing melanin in many areas benefiting human beings is still in laboratory trials stage. Biofilms are a nuisance in food and dairy industry as biofilm on milking utensils and containers as well as spoilage biofilm-forming microflora from poultry, meat and its processing environments plays a significant role in determining the shelf life of these food products [12]. Melanin is effective as an anti-biofilm agent [13, 14] which can be utilized as a good food preservative if proved to be nontoxic.

Melanins are well known for their antioxidant property [15–18], which can help in reducing reactive oxygen species (ROS) generation [19]. ROS scavenging minimizes

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inflammation as these properties are interrelated to each other. It was reported that grape melanin interfered with the prostaglandin as well as the leukotriene- and/or complement system-mediated inflammation and thereby showing an anti-inflammatory potential [20]. Kurian et al. [21] reported *Bacillus* melanin inhibiting the activities of inflammatory enzymes. That reflects the importance of melanin for consideration as an anti-inflammatory drug.

Photoprotective role of melanin relies on its ability to serve as a physical barrier that scatters UVR and as an absorbent filter that reduces the penetration of UV through the epidermis [22]. The efficacy of melanin as a sunscreen was assumed to be about 1.5–2.0 sun protective factors (SPF), possibly as high as 4 SPF, implying that melanin absorbs 50–75% of UVR [23]. Huang et al. [24] reported the sun protection effect of melanin from berry of *Cinnamomum burmannii* and *Osmanthus fragrans.* Later, Tarangini and Mishra [25] also reported the profound enhancement SPF value by melanin produced by *Bacillus safensis.* Thus, studying the enhancement of SPF by melanin can determine its suitability in cosmetic applications.

The present study therefore explores the characterization of melanin produced by marine *Vibrio alginolyticus* strain BTKKS3, exploring the metabolic pathway involved in melanin biosynthesis and its effectiveness in food, cosmetic and therapeutic applications. To this end, the antibiofilm, the SPF enhancement and anti-inflammatory properties of DOPA-melanin were studied.

Materials and methods

Chemicals, cell lines and bacterial isolates

Synthetic melanin (Sigma Chemicals Co, St Louis, MO, USA), L-tyrosine (HiMedia Laboratories Pvt Ltd, Mumbai, Maharashtra, India) and all other chemicals used were of analytical reagent grade.

RAW 264.7 and L929 cell lines were maintained in Dulbecco's modified Eagle's media (HiMedia Laboratories Pvt Ltd, Mumbai, Maharashtra, India) supplemented with 10% FBS (fetal bovine serum) (Invitrogen, Carlsbad, CA, USA) and grown to confluence at 37 °C at 5% CO_2 in a CO_2 incubator (Eppendorf, Hamburg, Germany).

Melanin-producing *Vibrio alginolyticus* BTKKS3 was isolated from marine sediment from Kanyakumari (8°5′N, 77°32′E) coast of South India. Screening for melanin production was initially by a plate-based assay [26], followed by assay in tyrosine basal broth [9]. The bacteria were identified by biochemical and 16S rDNA sequencing and analysis [27, 28].

Production, extraction, purification and quantification of melanin

Tyrosine basal broth [9] containing 0.2% tyrosine served as the medium for melanin production. 5 mL of culture suspension ($OD_{600} = 1$) was used as primary inoculum for 50 mL of production medium, incubated in an environment shaker (Orbitek, Scigenics Biotech, Chennai, Tamil Nadu, India) at 140 rpm at 37 ± 2 °C for 180 h. Melanin production kinetics was studied by taking the samples at 12-h intervals and estimating the bacterial growth and melanin production spectrophotometrically [29].

After 180 h of incubation, the cell-free supernatant was acidified to pH 2 using 1 N HCl. Black melanin precipitate can be visualized at the bottom of the flask even as the pH is lowered. Further purification of melanin is done according to Sajjan et al. [16] as follows. The precipitate was allowed to stand for a week at room temperature (RT) for complete precipitation, followed by boiling for 1 h and centrifugation. 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 min, kept at RT for 1 day, washed twice in ethanol, air-dried and used for further analysis.

Quantification of melanin was done spectrophotometrically (Shimadzu, Kyoto, Japan) at 400 nm [29] using synthetic melanin (Sigma Chemicals Co, St Louis, MO, USA) as standard.

Characterization of melanin

Reactivity of melanin against various organic solvents, acidic and basic solutions, oxidizing and reducing agents was evaluated (Supplementary data) [30]. Spectroscopic techniques such as UV–visible (Supplementary data) [31], FT-IR [32], proton NMR [33] and EPR spectroscopy [34] were used to evaluate the biophysical properties of the pigment. Antioxidant and metal-chelating properties (Supplementary data) were evaluated using standard procedures [35, 36].

Exploring melanin biosynthesis metabolism in strain BTKKS3

Pheomelanin with high sulfur content was detected using the CHN (S) elemental analysis [16]. Melanin biosynthesis inhibitors kojic acid (50-400 μM) and sulcotrione (5-30 μM) were used to differentiate whether the biosynthetic pathway was DOPA-melanogenic or pyomelanogenic [6, 37].

Anti-biofilm activity of melanin against food-borne pathogens

Anti-biofilm activity of melanin against four food-borne strong biofilm-producing bacteria viz. *Bacillus altitudinus* BTMW1, *Pseudomonas aeruginosa* BTRY1, *Staphylococcus warneri* BTDF2, *Bacillus sp.* BTSD1 [38] was evaluated using microtiter plate assay [39].

Anti- inflammatory potential of melanin

RAW 264.7 cells were then grown to 60% confluence followed by activation with 1 µL lipopolysaccharide (LPS) (1 µg/mL). LPS-stimulated RAW cells were exposed to different concentrations (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 h. After incubation, the anti-inflammatory assays were performed using the cell lysate. Activities of four inflammatory enzymes namely cyclooxygenase (COX) [40], lipoxygenase (LOX) [41], myeloperoxidase (MPO) [42] and nitric oxide synthase (cellular nitrite levels) [43] were assayed using standard protocols.

Photoprotective nature of melanin

Photoprotective nature of melanin was expressed by its ability to enhance the sun protection factor (SPF) of commercial sun screens [44]. Commercial sunscreens of 0.1 g were added to 10 mL of absolute ethanol. Melanin was added at 0.005% concentration to this mixture. Absorbance in the UV range (290–320 nm) was taken at 5-nm intervals using ethanol as the blank.

SPFs were calculated, according to Mansur et al. [46], using the following formula

$$\mathrm{SPF} = \mathrm{CF} \times \sum_{290}^{320} \mathrm{EE}(\lambda) \times I(\lambda) \times \mathrm{Abs}(\lambda),$$

where CF (correction factor) = 10; EE (λ) = erythmogenic effect of radiation with wavelength k; Abs (λ) = spectrophotometric absorbance value of the solution; and I = solar intensity spectrum. EE (λ) × I is constant and was determined by Sayre et al. [45].

Cytotoxicity of melanin

Different concentrations (6.25, 12.5, 25, 50 and 100 µg/mL) of melanin were added to L929 cells and incubated for 24 h. The percentage variation in viability was determined by standard 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay [47] after 24 h of incubation

Statistical analysis

The statistical analysis was performed by ANOVA using GraphPad Prism (Ver. 6) computer program, where p values < 0.05 were considered significant and graphs were plotted using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). All the experiments were repeated thrice.

Results

Production of melanin

Vibrio alginolyticus strain BTKKS3 produced 158.59 ± 1.1 µg/mL (Fig. 1) of melanin after 8 days of incubation. Melanin production was considerable after 48 h and was up from 15.46 (60 h) to 103.85 µg/mL (72 h).

Characterization of melanin

BTKKS3 melanin had typical melanin-like solubility dissolving only with alkaline solvents and DMSO. The color of melanin on reaction with oxidizing and reducing agents faded (Supplementary data). Melanin also showed a featureless absorption without an absorption maximum in a UV–visible spectrum analysis (Supplementary data). The FT-IR spectrum showed a broad absorption around 3400 cm^{-1}, corresponding to the phenolic –OH and –NH stretching vibrations. Characteristic peaks observed between 1600 and 1400 cm^{-1} were attributed to aromatic ring C = C stretching (Fig. 2A). This confirmed the polyphenolic and aromatic nature of BTKKS3 melanin.

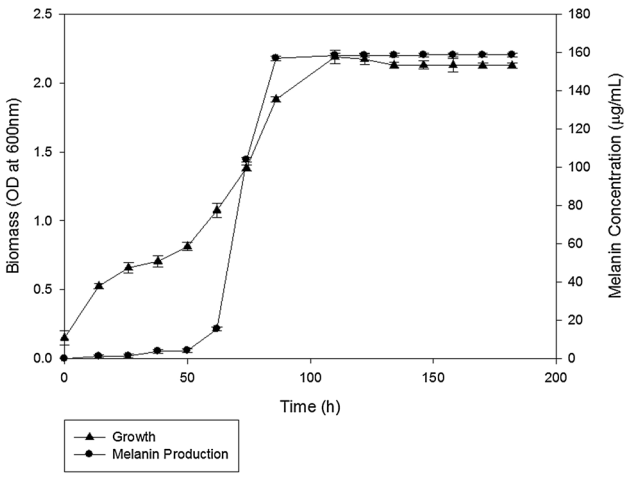

Fig. 1 Time course of melanin production by *Vibrio alginolyticus strain* BTKKS3

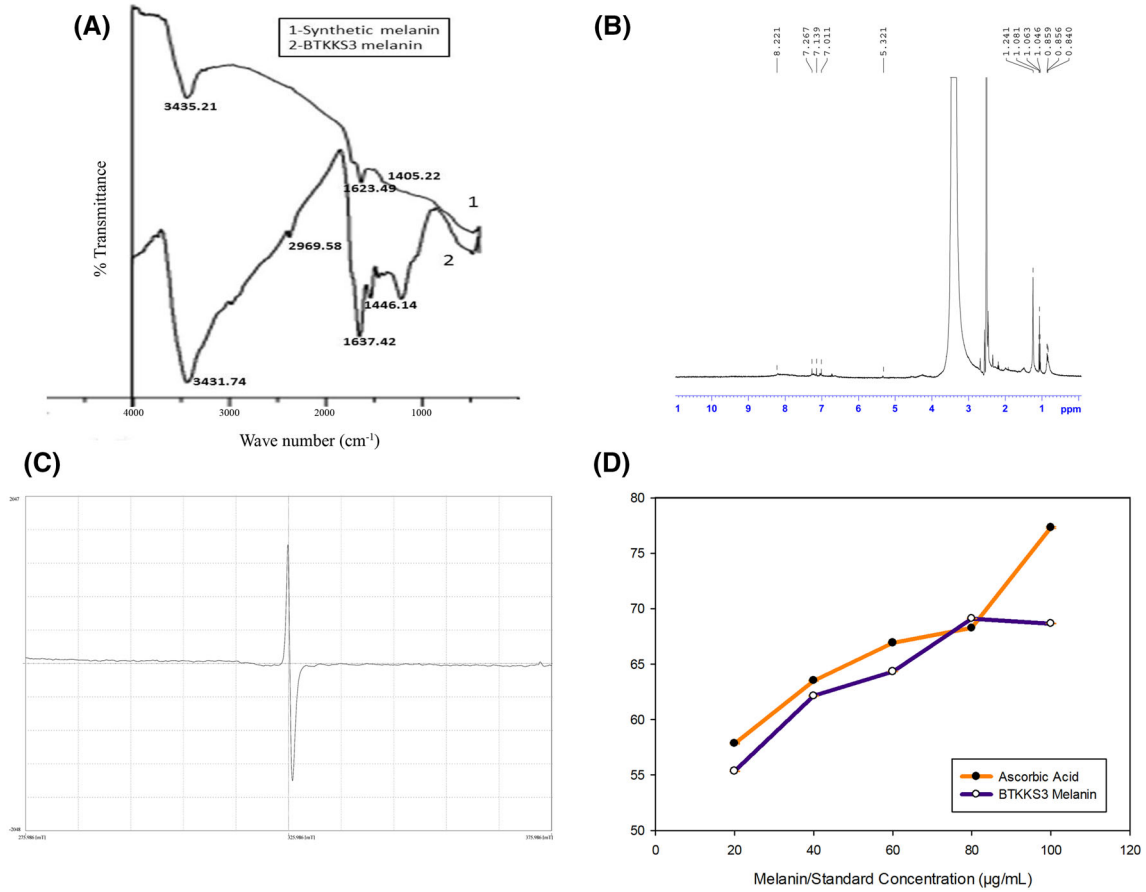

Fig. 2 Characterization of BTKKS3 melanin (**A**) FT-IR spectrum, (**B**) proton NMR spectrum, (**C**) EPR spectrum, (**D**) antioxidant activity

^{1}H NMR spectrum of melanin in DMSO showed signals in both the aromatic and aliphatic regions. Aliphatic region peaked at 0.84–0.85 ppm (parts per million), corresponding to the –CH3 groups of alkyl fragments, such as –CH_2CH_3 and –$CH(CH_3)_2$. Absorption at 1.240–1.241 ppm was attributable to –CHOH group, and at 6.9–7.2 ppm range probably corresponds to protons attached to different substituted aromatic or heteroaromatic rings (Fig. 2B). Sharp peaks in the EPR spectra of BTKKS3 melanin reflected the presence of unpaired electrons (Fig. 2C), which can help to trap free radicals. This was further confirmed by the profound radical scavenging activity (68.66%) (Fig. 2D). The pigment also showed good Fe^{2+} chelating potential (95.85%) compared to EDTA (99.64%) (Supplementary data).

Melanin biosynthetic metabolism

Elemental analysis of the BTKKS3 melanin showed only 0.69% sulfur, proving that the pigment is not pheomelanin (Supplementary data). Sulcotrione at a concentration of 30 µM decreased melanin production from 150.86 to 97.22 µg/mL; however, melanin production by kojic acid at 400 µM was much reduced, i.e., from 150.86 to 47.4 µg/mL. A combination of both inhibitors yielded only 19.02 µg/mL melanin (Fig. 3). Therefore, it is claimed that both DOPA and homogentisate pathways are involved in melanin production by *Vibrio alginolyticus* BTKKS3, but

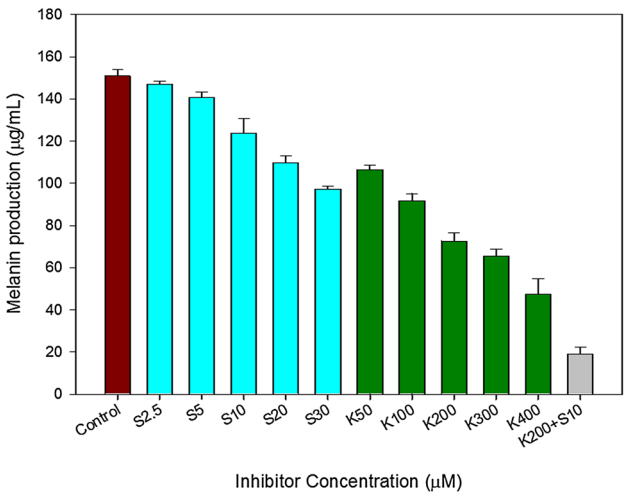

Fig. 3 Effect of sulcotrione and kojic acid on melanin metabolism of *Vibrio alginolyticus* BTKKS3 (S—sulcotrione and K—kojic acid)

melanin derived via tyrosinase catalyzed pathway, i.e., DOPA-melanin, is predominant.

Anti-biofilm activity of melanin

Melanin from strain BTKKS3 showed profound biofilm inhibitory action against all the four biofilm formers tested, with biofilm reduced to 38.31, 79.81, 79.99 and 58.02%, respectively (Fig. 4) in case of *Bacillus altitudinus* BTMW1, *Pseudomonas aeruginosa* BTRY1, *Staphylococcus warneri* BTDF2 *and Bacillus sp.* BTSD1.

Anti-inflammatory potential of melanin

At 100 µg/mL, melanin from *Vibrio alginolyticus* BTKKS3 reduced COX and LOX activity to 45.80 and 63.67%, respectively. The pigment significantly ($p \leq 0.0001$) reduced the MPO activity at 0.0014U/mL of cell lysate. Melanin reduced the cellular nitrite levels to 648.05 µg/mL cell lysate at the highest dose (100 µg/mL) (Fig. 5).

Photoprotective nature of melanin

BTKKS3 melanin showed excellent sun protective ability as it increased sun protection factor (SPF) of commercial sunscreens by an average of 3.42 units (Table 1).

Cytotoxicity of melanin

At the highest concentration (100 µg/mL) tested, *Vibrio alginolyticus* BTKKS3 melanin inhibited 37.04% of L929 cells (Fig. 6A). The half maximal inhibitory concentration (IC_{50}) was estimated to be 134.98 µg/mL. Majority of the cells retained their morphology, while some cell debris formed as result of toxicity of melanin was too observed with live cells under phase-contrast microscope (Fig. 6B).

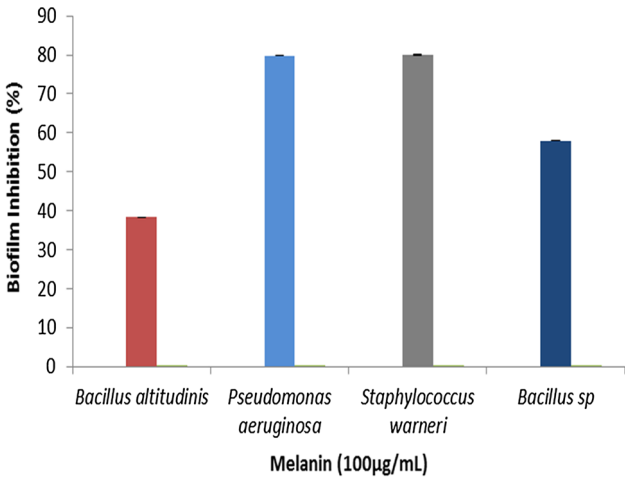

Fig. 4 Anti-biofilm activity of BTKKS3 melanin against food-borne strong biofilm producers

Discussion

There are numerous reports on melanin production by *Vibrio* species, but the majority focused only on qualitative estimation [11, 48, 49] or indicated melanin production based on spectroscopic absorbance [50], making difficult an effective comparison of the actual concentration of melanin produced. Recently, Vijayan et al. [60] reported a sponge-associated *Vibrio alginolyticus* producing 50 mg ml^{-1} of melanin and comparing with the report BTKKS3 produce less melanin. Characterization of BTKKS3 melanin revealed typical melanin-like characteristics, while the similarity of the IR spectrum to that of the synthetic melanin reflects the purity. Melanins are reportedly good radical scavengers, BTKKS3 melanin too showed profound radical scavenging property as reflected in EPR spectrum and DPPH radical scavenging assay. Manivasagan et al. [15] reported 90% free radical scavenging activity for melanin (3.5 mg/mL) from *Actinoalloteichus* spp., while *Klebsiella* spp. melanin [16] showed 70% (50 µg/mL) activity. Blarzino et al. [51] reported DHICA-melanin, DHI-melanin and DOPA-melanin with scavenging capacity of > 90% at 100 µg/mL. Wang et al. [52] indicated that a significant decrease in singlet oxygen production was observed in the presence of eumelanin extracted from pig retinal pigment epithelium cells. Melanin interacted with free radicals via the simple one-electron transfer processes [53]. Many reports had indicated the superior ability of melanin to scavenge-free radicals, and our experimental data also support this. Jara et al. [61] pointed out that functional group of melanin could have cross-linking with ion conferring chelating power. Riley [62] stated that in melanin structure there was 2-carboxyl indole that could link with metal ion that made melanin to have Fe^{2+} chelating power. Earlier, a soil microbial isolate showed Fe^{2+} chelating activity of 64% at a concentration of 0.2 mg/mL [25]. Fungal melanin from *Ophiocordyceps sinensis* had shown a chelating activity of 94.74% at the concentration of 10 µg/mL [63]. Comparing with these reports, BTKKS3 melanin had shown a very good potential in chelating Fe^{2+} ions.

Earlier studies in *Vibrio cholerae* revealed that melanin was produced via homogentisate pathway [48, 54]. Yoshida et al. [55] reported the production L-DOPA, an intermediate in melanin biosynthetic pathway by *Vibrio tyrosinaticus.* As L-DOPA is the precursor of melanin [5], it can be correlated with the present study that *Vibrio* sp. can produce melanin via L-DOPA.

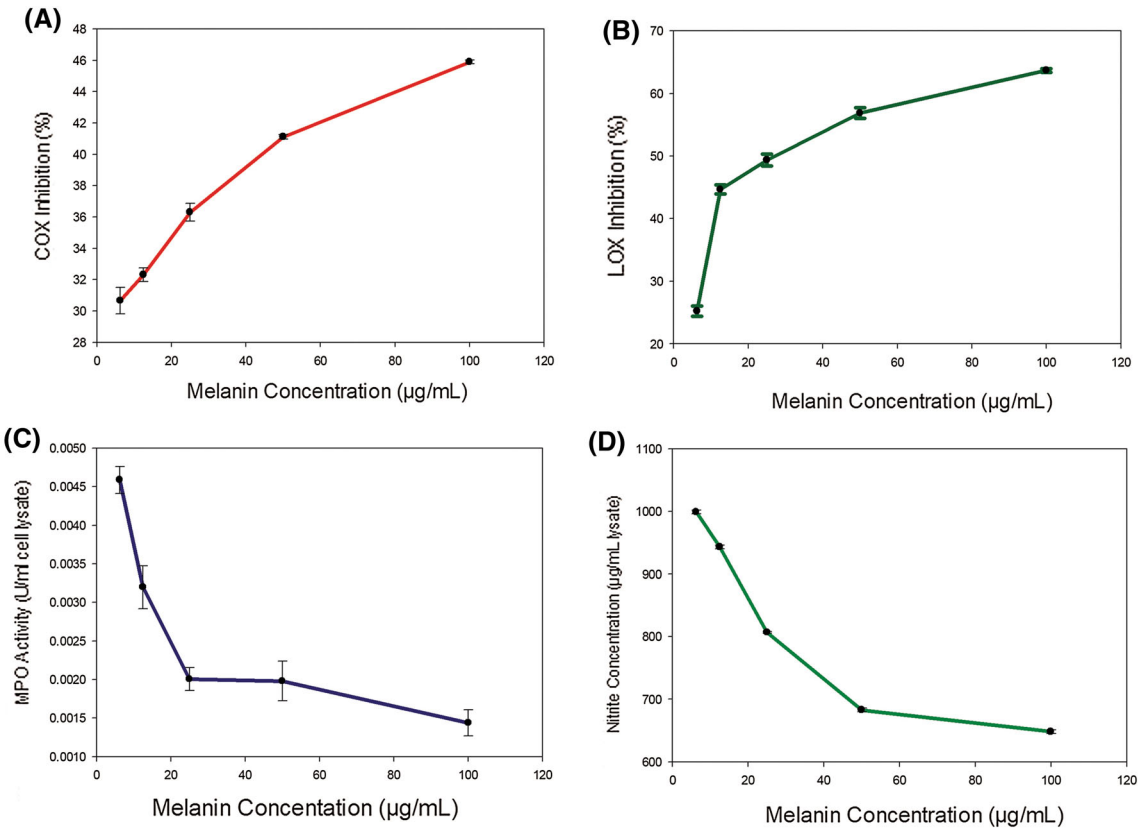

Fig. 5 Effect of BTKKS3 melanin on inflammatory enzymes and cellular nitrite levels (**A**) COX, (**B**) LOX, (**C**) MPO, (**D**) cellular nitrite

Table 1 Sun protection factors (SPFs) for commercial sunscreen preparations before and after supplementation with BTKKS3 melanin

Commercial sunscreen	SPF value stated by the manufacturer	SPF value determined empirically during the current study	+BTKKS3 Melanin (0.005% w/w) SPF
Sunscreen 1	15	14.24 ± 0.007	18.05 ± 0.03
Sunscreen 2	15	14.61 ± 0.01	18.98 ± 0.01
Sunscreen 3	15	14.77 ± 0.05	18.95 ± 0.06
Sunscreen 4	17	16.47 ± 0.04	20.51 ± 0.1
Sunscreen 5	30	26.26 ± 0.04	29.68 ± 0.08

Bacteria in biofilms are generally more resistant to stress such as disinfection and mechanical shear than their free-living counterparts [56]. From the study, it was evident that BTKKS3 melanin was profoundly effective against biofilm formers like *Pseudomonas aeruginosa* BTRY1 and *Staphylococcus warneri* BTDF2. The significance of this study is that both species are well-known strong biofilm formers in clinical environs [57] and food samples [56].

BTKKS3 melanin effectively and significantly ($p \leq 0.0001$) inhibited the inflammatory enzymes, but the exact mechanism behind this activity is still unknown. There were some earlier reports regarding the phenolic extracts from plants like *Dioscorea opposita* [58] and *Gastrodia elata* [59] that inhibited inflammatory enzymes. As a phenolic compound, BTKKS3 melanin may also be employing a similar mechanism of action, which needs to be proved. There are only few reports regarding the anti-inflammatory properties of melanin so far. Avramidis et al. [20] reported that grape melanin interfered with prostaglandin as well as leukotriene- and/or complement system-mediated inflammation. Kurian et al. [21] reported that *Bacillus* sp. melanin inhibited inflammatory enzymes showing anti-inflammatory property. This supports the present findings. Thus, this study suggests that BTKKS3 melanin can be utilized as an effective anti-inflammatory drug after further trials.

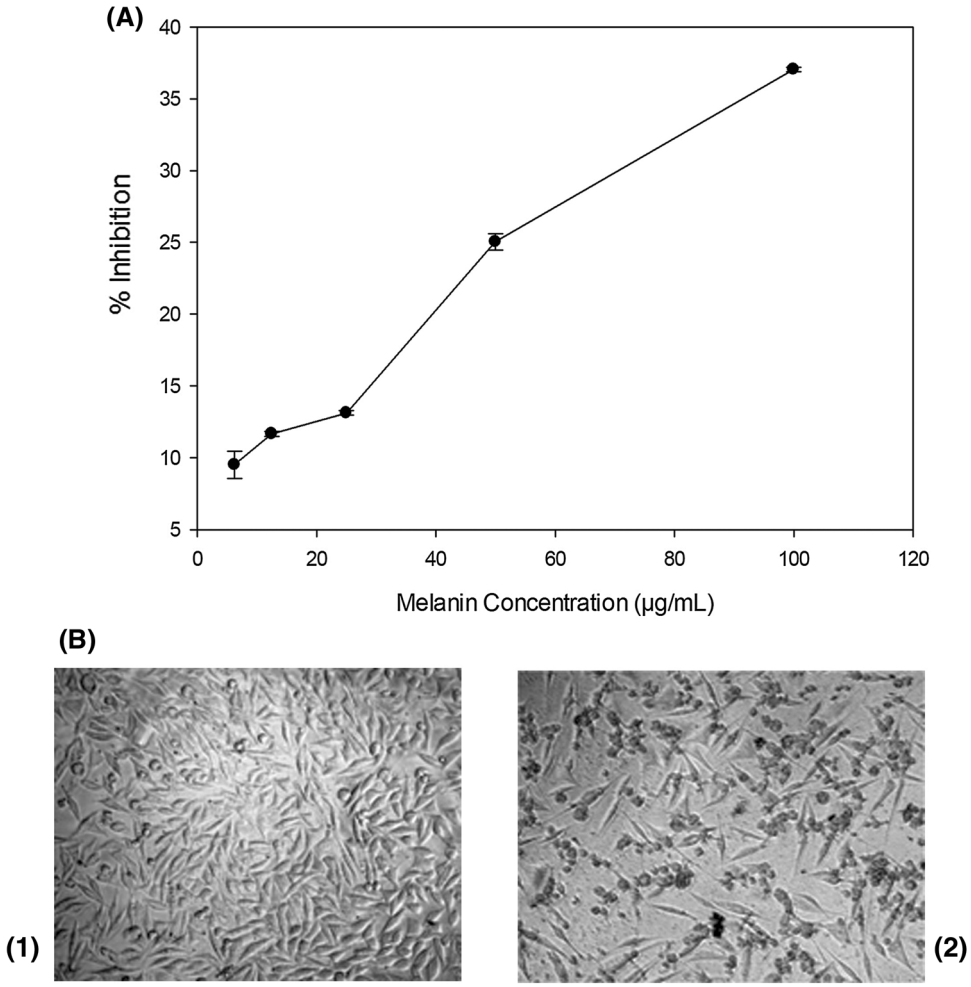

Fig. 6 (**A**) Cytotoxicity of melanins, (**B**) phase-contrast micrographs (×20 magnification) showing the cytotoxic effect of *Vibrio alginolyticus* BTKKS3 melanin [1]. Control [2] melanin treated (100 µg/mL)

The efficacy of melanin as a sunscreen was 1.5–2.0 sun protective factors (SPF), possibly as high as 4 SPF, implying that melanin absorbs 50–75% of UVR. An SPF of 2 indicates doubling of protection of skin against sunburn [23]. BTKKS3 melanin enhanced SPF value of sun screen by 3.42 units predicting excellent photoprotective ability. Melanins absorb UV rays by bringing the molecule to an excited state. Release of energy as heat and light radiation occurs as it returns back to the ground state and can excite again. This UV absorptive property of melanin makes it an excellent SPF enhancer. And free radical scavenging activity of BTKKS3 melanin can prevent UVA-mediated damage by reactive oxygen species adds to its photoprotective role [64]. The sun protective effect of melanin from *Cinnamomum burmannii* berry and *Osmanthus fragrans* has been reported [24]. Tarangini and Mishra [25] also reported the profound enhancement in SPF value by melanin produced by *Bacillus safensis.* Our results also support the reports. Despite the properties like anti-biofilm and inhibition of inflammatory enzymes, BTKKS3 melanin was proved to be less cytotoxic. This may due to its cytoprotective properties like radical scavenging and iron chelation potential. Thus, the melanin can be used effectively as an ingredient in commercial sunscreens to enhance the sun protection after sufficient trails.

A bacterial strain *(Vibrio alginolyticus* BTKKS3) producing good amount of melanin, with the pigment having profound bioactivities, was identified. The pigment with its anti-biofilm activity against food-borne pathogens can be made use of in food processing industries. Anti-inflammatory activity of BTKKS3 melanin can be further studied for its therapeutic benefits in treating inflammation associated with diseases, especially arthritis and edema, while its SPF enhancing property in sun screens makes it an ideal and essential ingredient in cosmetic formulations. More *in vivo* and clinical trials are necessary further to confirm its utility.

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