A Greener Recyclable Process for Extraction of Microbial Melanin using Ionic Liquid

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Melanins have multidimensional applications in medical and biomaterial science and thereby shooting up its demand. Conventional extraction techniques has many disadvantages like poor extraction efficiency, use of toxic solvents (acid and base), time-consuming, and consequent higher cost of the final product. To tackle this problem, an innovative ionic liquid (IL) based approach was used for the extraction and purification of melanin from the endophyte *Streptomyces hyderabadensis* strain 7VPT5-5R. ILs are furnished with best thermal, chemical, electrochemical stability, non flammability, trivial volatility, and broad range of solubility making them a promising medium for the extraction of melanin. Maximum yield 5.5 g/L was obtained by IL extraction method which is remarkably higher than conventional acid-precipitation method. The devised method also allows recyling of IL five times without loosing efficinecy, thus reducing use of fresh IL. Furthermore, the process is scalable, eco-friendly, rapid and produce superior quality melanin than that of the conventional method.

1. Introduction

Recently, natural pigments from microorganisms have gained attention due to their broad-spectrum biological activities and applications. Microorganisms are a felicitous source to obtain melanin at an industrial scale through environment-friendly and economically feasible processes. Melanins are dark black to brown coloured, hydrophobic, negatively charged, natural heterogeneous pigments which are constantly encountered in animals, plants, and in most of the microorganisms.[1] The increased interest in melanin research is justified by its numerous biofunctions, such as colouration, camouflage, photo- and radio-protection, energy transducer, antioxidant, thermoregulator, chemoprotectant, antitumor, antiviral, antimicrobial, immunostimulant, anti-inflammatory, and detoxification.[2] Microbial melanins have advantage over synthetic melanin and other natural melanins derived from plants and animals, in terms of easy and safe production, eco-friendliness, low cost, raw culture medium components, and biodegradablity.[3]

Melanin is formed by oxidative polymerization of phenolic- and indolic-compounds by the enzymes tyrosinase and laccase.[4] In the natural domain, three classes of melanin are frequently discerned by its colour and type of cyclic ring present. Eumelanins are black to brown in colour formed by oxidation of L-tyrosine (and/or phenylalanine) to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA).[5] Pheomelanins are yellow to red in colour and its synthesis is similar to eumelanins, wherein benzothiazine and benzothaizole units are formed from polymerization of cysteinyl DOPA.[6] Allomelanins, heterogenous group of polymers, are synthesized by oxidation/polymerization of 1,8-dihydroxynaphthalene (1,8-DHN) or 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) via the pentaketide pathway.[7] Melanin plays diverse protective roles in microbes for instance UV protection, antioxidant, bioremediation, and thermoregulation. In fungus it is called as "fungal armour" due to its role in protection against adverse environmental conditions.[8]

Among other natural sources, microbial melanin production receives much more attention due to its easier scalability and lack of seasonal variation.[9] The microbial melanin production was optimized by using statistical approaches such as response surface methodology (RSM), Central composite design (CCD), Plackett-Burman design (PBD) and one factor at a time (OFAT).[10,11,12] The genetic engineering approaches like cloning and overexpression of tyrosinase gene are also used to improve melanin yield for *Streptomyces kathirae*.[13]

Even though, there are a number of reported extraction methods on different types of sources the occurrence of melanin as bound form with cellular components and its insolubility in most of the organic solvent complicates their extraction procedures. Base dissolution and acid precipitation method generally employed abide issues like time consuming, multiple steps, and use of corrosive solvents increasing the production cost. Also, the use of acidic and basic solution will lead to destruction of the molecule. Various advanced methods are also reported such as ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), and enzymatic digestion. The chromatographic techniques are also reported for separation of melanin. IL is emerging as green solvent for extraction of biomacromolecules due to its excellent solvation property for various types of compounds and outstanding stability for protein, carbohydrates and Nucleic acid in the recent times. Use of IL is already reported for extraction of melanin from hair, Alpaca fibre.[14,15] Fungal polyketide pigment was also extracted by using quaternary and immidazolium based IL with aqueous two phase system.[16] Melanin extraction is still a challenging job on the grounds of its diversity and structural complexity. The selection of extraction method mainly depends on the primary raw material used. Conventionally, melanin is extracted by alkali dissolution and acid precipitation, but this foresaid method shows low extraction efficiency and is time consuming therefore, advanced extraction methods such as ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), and

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enzymatic method have been used to extract melanin.[17] However, these advanced methods are also not without drawbacks; for example, sequential digestion steps with multiple specific enzymes would prolong the producing time that ultimately raise the production cost of melanin and rapid increase in temperature of the extraction mixture in microwave-assisted extraction method may terminate the extraction process early due to the boiling of the solvent.[18] Thus, the desired compounds are not sufficiently diffused from the material into the solvent and consequently the extraction yield is reduced.[19] The above insubstantial conventional production and extraction techniques result into limited melanin yield and subsequent high cost, which creates a barrier in its widespread industrial applicability. Therefore, there is an urgent need to search/develop a cromulent method to extract melanin pigment from microbial source. In the recent years, the concept of green solvents (Ionic liquids) has got recognition due to designable properties such as high thermal stability, dissolving ability, and low vapor pressures.[20] ILs have great solvation ability for broad range of biomolecules, thus allowing easier extraction of targeted compounds.[21] Due to solvation and stabilizing property, ILs have been evolved as potential solvent for dissolution, separation, and extraction of wide range compounds from natural resources.[22] Melanin production can be improved by optimizing production medium and efficient extraction methods resulting into better yields, reduced cost, and eco-friendly usage.

In the present study, extracellular melanin was extracted from the culture broth of endophytic actinomycete *Streptomyces hyderabadensis* 7VPT5-5R using IL TBAH. Melanin production in the microbial culture was increased by optimizing medium components. The obtained melanin was characterized as eumelanin using physico-chemical and spectroscopic methods. Finally, an eco-friendly, simple, high-yielding, and recyclable IL-based extraction method was developed for microbial melanin extraction.

2. Experimental

2.1 Materials Solvents were purchased from Qualigens (India). Microbial media components were purcahsed from HiMedia (India). Tetrabutylammonium hydroxide (TBAH) was procured from Molychem (India). Synthetic melanin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Isolation of endophytic actinomycetes

2.2.1 Sample collection and surface sterilization

Plant *Salicornia brachiata* was collected from Victor port, Amreli district, Gujarat on Feb 26, 2018 (N 20° 58′ 53.2″, E 071° 33′ 21.2 ″). Collected samples were packed in sterile Ziploc bags which was brought to laboratory in the ice-box for immediate processing as per reported method.[23,24] Briefly, sample was cleaned and washed with water and Tween 20 (0.1% v/v) followed by surface sterilization sequentially with 4% NaOCl, 2.5% $Na_2S_2O_3$, 70% alcohol, and sterile distilled water. The surface sterilized plant sample was subjected to pre-treatment.

2.2.2 Effectiveness of surface sterilization

The effectiveness of sterilization was confirmed by two methods; in first method, surface sterilized plant tissues were imprinted on ISP-4 agar plate composed of starch 10 g, K_2HPO_4 1 g, $MgSO_4{\cdot}7H_2O$ 1 g, NaCl 1 g, $(NH_4)_2SO_4$ 2 g, $CaCO_3$ 2 g, $FeSO_4{\cdot}7H_2O$ 0.001 g, $MnCl_2{\cdot}7H_2O$ 0.001 g, $ZnSO_4{\cdot}7H_2O$ 0.001 g, agar 20 g, pH 7.2±0.1 for 1 L and in second method, 100 µL water of last wash of surface sterilization was spread on ISP-4 agar plate and both these plates were incubated at 28 ℃ for 2-3 weeks.

2.2.3 Pre-treatment of surface sterilized plant sample

The tissues were subjected to continuous drying at 100 °C for 15 min and then plant parts were cut into small pieces and approximately 2 g of plant material was macerated in 1X PBS (pH 7). The plant extract was further treated with 1.5 % v/v phenol followed by an incubation of 15 min at 28 °C.[25] Dilutions were prepared up to 10^{-4} right after this step and 100 µL of these dilutions were spread on ISP-4 agar supplemented with nystatin and cycloheximide (60 µg mL^{-1} each). The inoculated plates were incubated at 28 °C for 2 to 4 weeks, following which colonies appearing on ISP-4 agar plates were picked up.

2.3 Microbial culture for production of melanin

2.3.1 Screening for melanin production

Isolates were inoculated into ISP-6 broth composed of peptic digest of animal tissue 15 g, proteose peptone 5 g, yeast extract 1 g, ferric ammonium citrate 0.5 g, K_2HPO_4 1 g, $Na_2S_2O_3$ 0.08 g, pH 6.7±0.2 for 1 L and tyrosine broth (ISP-7) composed of glycerol 15 g, L-asparagine 1 g, L-tyrosine 0.5 g, K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, NaCl 0.5 g, and trace salts ($FeSO_4{\cdot}7H_2O$ 1.36 mg, $CuCl_2{\cdot}2H_2O$ 0.027 mg, $CoCl_2{\cdot}6H_2O$ 0.04 mg, $Na_2MoO_4{\cdot}2H_2O$ 0.025 mg, $ZnCl_2$ 0.02 mg, H_3BO_3 2.85 mg, $MnCl_2{\cdot}4H_2O$ 1.8 mg, $C_4H_4Na_2O_6$ 1.77 mg), pH 7.3±0.1 for 1 L, incubated at 28 °C for 7 to 10 days. Production of brown to black pigment in the medium was scored as positive for melanin production. Melanin pigment production was quantified by measuring O.D. of the supernatant of culture broth spectrophotometrically at 400 nm.[10]

2.3.2 Identification of melanin producer

Melanin producing endophytic isolate was characterized by 16S rRNA analysis. The genomic DNA was isolated using Genetix DNA isolation kit. 16S rRNA gene was amplified using universal primers (27F 5'-AGAGTTTGATCCTGGCTCAG-3', 1492R 5'GGTTACCTTGTTACGACTT-3'). PCR was performed using a thermal cycler (T100 Thermal Cycler BioRad) under the following optimized conditions: initial denaturation at 95 °C for 7 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, with a final extension temperature of 72 °C for 8 min. The 16S rRNA gene sequence was identified by a database search using

the BLAST program (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov).

2.3.3 Medium optimization for melanin production

2.3.3.1 One factor at a time (OFAT)

Medium components for melanin production were optimized by One factor at a time (OFAT) method. This method is used to identify the effect of individual component in which only one factor or a single variable is changed at a time while keeping all other variables constant.[11] Total 23 medium components were screened one by one to check the effect of each component on melanin production. Tyrosine broth was used as basal medium containing glycerol as carbon source, both L-tyrosine and L-asparagine as nitrogen source. To identify the best carbon source, glycerol was replaced with six other carbon sources (sucrose, starch, lactose, fructose, maltose, and glucose). Similarly, the nitrogen source (L-tyrosine and L-asparagine) was replaced with eight other nitrogen sources (L-tyrosine, peptone bacteriological, yeast extract, ammonium sulphate, L-asparagine, casein acid hydrolysate, beef extract, potassium nitrate, sodium nitrate) to identify best nitrogen source. Trace salts of tyrosine broth were completely replaced with eight individual trace salts ($ZnSO_4$, $Co(NO_3)_2$, $CuSO_4$, $MnCl_2$, $FeSO_4$, $NiCl_2$, $CuCl_2$ and $Ni(NO_3)_2$). The effect of the medium components on melanin production was quantified by measuring the absorbance of the respective culture supernatants at 400 nm.[23]

2.3.3.2 Production of melanin

The seed culture was prepared using the production medium composed of glycerol 20 g, $NaNO_3$ 10 g, $MgSO_4$ 1 g, K_2HPO_4 1 g, NaCl 1 g, $MnCl_2$ 0.004 g, pH 7.2. Then, 3 % (v/v) seed culture was transferred to 250 mL of production medium in a 1 L flask and the flask was incubated at 28 °C with shaking at 200 rpm for 8 days. Then, the culture broth was harvested, cell biomass was separated using centrifugation, and the supernatant was lyophilized.

2.4 Extraction of melanin

2.4.1 Conventional method

In the conventional method, 1 mL water was added to 20 mg lyophilized powder, the pH of the solution was adjusted to 12.0 with 1N NaOH and stirred for 1 h. Then, the pH of the solution was adjusted again to 2.0 using 6M HCL and allowed to stand for 4 h and then the solution was centrifuged at 13,000 rpm for 10 min. The obtained pellet was washed twice with ethanol followed by water to remove impurities. Finally, the purified melanin was obtained after lyophilizing the pellet collected in the previous step.

2.4.2 IL based extraction

To 20 mg of lyophilized powder, 1 mL water was added and mixed well followed by addition of 1 mL TBAH and stirred for 30 min. To this mixture, 1 mL ethanol was added, mixed well, and then allowed to stand for 1 h followed by centrifugation at 13,000 rpm for 10 min. Then, the pellet was separated and washed twice with ethanol to remove impurities and the remaining supernatant was used for recycling of IL. Eventually, the pellet was lyophilized to yield purified melanin.

2.4.3 Recycling of IL

To recycle the IL used in extraction, equal volume of chloroform (approx. 3 ml) was added to the supernatant remained after melanin precipitation. The addition of chloroform to the supernatant led to formation of two layers. The lower layer was then collected carefully and an equal volume of ethanol was added to facilitate easy evaporation of water using a rotary evaporator along with ethanol, thus, leaving behind only pure IL. Furthermore, recyclability was also determined using the recovered IL for extraction of melanin.

2.4.4 Melanin Synthesis Inhibition

In order to identify the enzyme involved in melanin biosynthesis in the present study, kojic acid was used as a well-known melanin-synthesis inhibitor. Concentrations of kojic acid ranging from 20 to 50 µg mL^{-1} were prepared in water and added to tyrosine broth. 1% (v/v) inoculum was added into above media and incubated at 28 °C, 160 rpm for 4–10 days and observed for pigment inhibition.[26]

2.5 Characterization of microbial melanin

2.5.1 UV/VIS spectrophotometry

The extracted pure melanin was dissolved in a 0.5 N NaOH solution and the UV spectrum was obtained by scanning at wavelength range of 200–800 nm using 0.5 N NaOH solution as blank control.[10]

2.5.2 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDX)

The EDX technique is helpful to determine the composition of samples based on the peak emission of X-rays resulting from the interaction between each element of a compound and the electron beam.[27] Hence, microbial melanin was dispersed in ethanol, sonicated for 5 min, and placed onto an SEM specimen stub. Then, the sample was dried in a desiccator overnight followed by visualization using SEM (JEOL).

2.5.3 Elemental Analysis

The percent elemental composition of carbon, hydrogen, nitrogen, and sulfur in the purified melanin was determined with 5 mg of melanin powder using an elemental analyzer.[23]

2.5.4 Fourier transform infrared spectroscopy (FTIR)

FTIR is mainly used to identify functional groups to aid structure elucidation of melanin. The purified melanin was ground with KBr powder in an agate mortar to break it up and the mixed disc was scanned at 4000–400 cm^{-1} using an FTIR spectrophotometer.[28]

2.5.5 Electron Paramagnetic Resonance Spectroscopy (EPR)

The presence of free radical in melanin was analyzed by EPR spectroscopy. 2 mg melanin powder was added into a thin-walled glass tube and EPR signals were recorded. The parameters used to acquire the spectra were as follows; modulation amplitude: 0.16 mT; modulation frequency: 100 kHz; center field: 325 mT; sweep width: 25 mT; sweep time: 2 min; microwave frequency: 9.1 GHz; microwave power: 0.1 mW; at room temperature.[2]

2.5.6 NMR Spectroscopy

10 mg of dark brown coloured melanin was added into 1 mL of 40% sodium deuteroxide solution (Sigma-Aldrich) and sonicated for 2 to 3 min. The obtained solution was centrifuged at 13,000 rpm for 5 min and the dark brown coloured supernatant was used for NMR analysis.[29] Bruker Avance II 500 MHz and Jeol 600 MHz NMR spectrometers were used to acquire ^{1}H-NMR and ^{13}C-NMR spectra, respectively.

3. Results and discussion

3.1 Isolation of entophytic Streptomyces

The strain 7VPT5-5R was isolated on ISP-4 media from the halophyte *Salicornia brachiata*. The pre-treatments helped to eliminate fast-growing gram-negative and other bacteria which allowed to obtain comparatively slow-growing actinomycetes on ISP-4 medium. After 7 days of incubation the spore like colonies were appeared which were picked up and purified on ISP-4 agar plate.

3.2 Microbial culture for production of melanin

3.2.1 Screening for melanin production

The isolate 7VPT5-5R produced dark brown colour after 4 days of incubation. The absorbance of culture supernatant measured by UV/VIS spectroscopy showed increase in absorbance in the UV region while decreased absorbance towards the visible region, which is the characteristic of melanin.[10]

3.2.2 Identification and phylogenetic analysis

The sequence of 16S rRNA (1364 bp) was amplified and sequenced at Macrogen (Seoul, South Korea). Based on BLAST result, endophytic isolate 7VPT5-5R was found to be belonged to genus *Streptomyces* and showed maximum sequence similarity with *S. hyderabadensis* strain OU-40 (99.55 %), *S. rubrogriseus* strain NBRC 15455 (98.72 %), and *S. rubrogriseus* strain DSM 41477 (98.72 %). Based on phylogenetic tree (Fig. 1) analysis, the strain *S. hyderabadensis* strain 7VPT5-5R fall into a clade together with *S. hyderabadensis* strain OU-40 and formed a distinct phyletic line together with the strains *S. olivaceus* NBRC 12805, *S. pactum* NBRC 13433, and *S. litmocidini* NRRL B-3635.

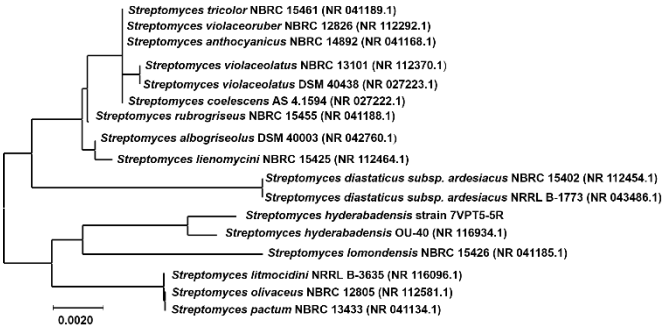

Fig. 1. Phylogenetic tree of *S. hydrabbadensis* strain 7VPT5-5R

3.2.3 Medium optimization for melanin production

3.2.3.1 One factor at a time (OFAT)

Since culture media components influence the melanin production, screening of critical components are important for yield improvement.[11] Among the six carbon sources, glycerol showed maximum melanin production. Similarly, the maximum melanin production was also observed in the culture containing sodium nitrate as nitrogen source. Among the trace elements, $MnCl_2$ was found to show maximum melanin production. The ability to utilise various media components to achieve better melanin production varies from species to species. Various studies have reported that the yield of microbial melanin is significantly influenced by the nutrient components and culture conditions.[10-12,23,30] Melanin production was reported to be greatly influenced by tyrosine as nitrogen source in case of *S. lusitanus* DMZ-3.[30] In addition, yeast and beef extract were used as nitrogen sources for melanin production.[31] Many *Streptomyces* isolates have been reported to produce brown colour when cultured on peptone-yeast extract iron agar.[10] Protease peptone was reported to result the maximum production of melanin by *S. glaucescens* NEAE-H.[10] Similarly, soy peptone was reported to yield the maximum production of melanin by *Streptomyces* sp. ZL-24.[11] Since the ability to utilize nitrogen source for melanin production also changes from species to species, the identification of suitable nitrogen source is important to improve the melanin yield. Metal ions play a crucial role in melanin production as well as in the role of the melanin synthesizing enzymes.[11] The enhancement of melanin production due to copper ion was previously observed for *Streptomyces kathirae*, *Streptomyces kathirae* SC-1, *Brevundimonas* sp. SGJ, *Streptomyces lusitanus* DMZ-3.[12,13,30,32] While other metal ions such as Ni and Fe were also reported to increase the melanin production in *S. glaucescens* NEAE-H and *Streptomyces* sp. ZL-24 repectively.[10,11]

3.2.3.2 Production of melanin

After 8 days of incubation, culture broth was harvested and then it was centrifuged at 8000 rpm for 15 min and supernatant

was collected. Then culture supernatant was freeze dried to obtain crude powder. Crude powder was extracted using above IL liquid method. 5.504 g pure melanin was obtained from 1 L of production medium.

3.3 Extraction of melanin

3.3.1 Conventional and IL extraction

From 1 L of culture 12.511 g lyophilized crude powder was obtained from which 20 mg powder was individually used in the present study. The IL method utilized in this study resulted in a final quantity of 8.8 mg pure melanin from 20 mg lyophilized powder; thus, giving the melanin yield at 5.504 g L^{-1} after 196 hr incubation. In comparison to IL method, conventional method yielded less quantity (5.3 mg) of pure melanin from 20 mg lyophilized powder, thus, resulting the yield of 3.315 g melanin from 1 L culture broth after 196 hr incubation. The reduced melanin yield through the conventional method can be asserted to the very low solubility of the extract containing melanin in water. Due to this insolubility of melanin, other associated proteins and components also get precipitated with melanin when conventional method is used. It is also noticed that the melanin obtained in conventional extraction method is difficult to separate by washing with solvents.

3.3.2 Recycling of IL

The IL liquid was recycled by liquid-liquid extraction method. The biphasic system was formed after addition of equal volume of chloroform. The upper layer was made up of chloroform and the remained components after extraction of melanin whereas lower layer contained IL, water, and ethanol. The water and ethanol from IL was evaporated by rotary evaporator and this recycled IL was used for extraction of melanin. This use of recycled IL for extraction process was repeated for 5 times and extraction efficiency was found to be constant (Table 1). The melanin extraction was not affected by the use of recycled IL. Here complete recycling of IL was done by aqueous biphasic system of IL and chloroform.

Table 1 Recycling of IL and respective melanin yield.

Numbers of recycles	yield/ 10 mg of crude extract
1	4.45 mg
2	4.40 mg
3	4.43 mg
4	4.39 mg
5	4.24 mg
6	4.29 mg
7	4.25 mg
8	4.21 mg
9	4.18 mg
10	4.15 mg

3.3.3 Melanin Synthesis Inhibition

The purpose of this study was to traverse the biosynthetic pathway of melanin using kojic acid which is a DOPA melanin pathway inhibitor. The DOPA melanin is synthesized by the enzyme tyrosinase.[26] The inhibition of pigment production (Fig. 2) was observed after treatment with kojic acid in the culture medium indicating that the melanin synthesized is DOPA melanin.

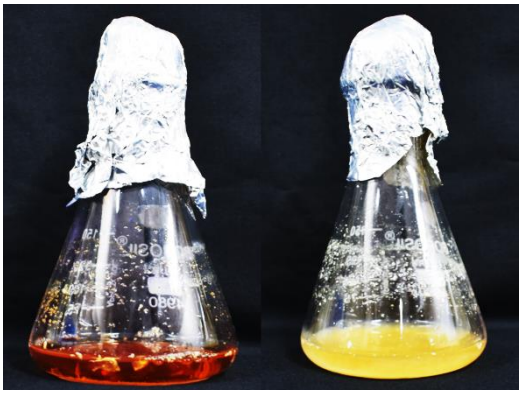

Fig. 2. Effect of melanin synthesis inhibitor. Right side (Control), Left side (Kojic acid).

3.4 Characterization of microbial melanin

3.4.1 UV/VIS spectrophotometry

The maximum absorbance was observed at 229 nm, which was decreased towards the visible region as seen below (Fig. 3) which is the characteristic property of melanin. The λmax of microbial melanin varies from species to species in the range UV region.[10] The ratio of A_{650}/A_{500} helps to calculate the amount of eumelanin in the total melanin.[23] The ratio A_{650}/A_{500} was calculated to be 0.35 and indicated that extracted melanin was belonging to eumelanin class whereas, a ratio below 0.15 is observed for pheomelanin.

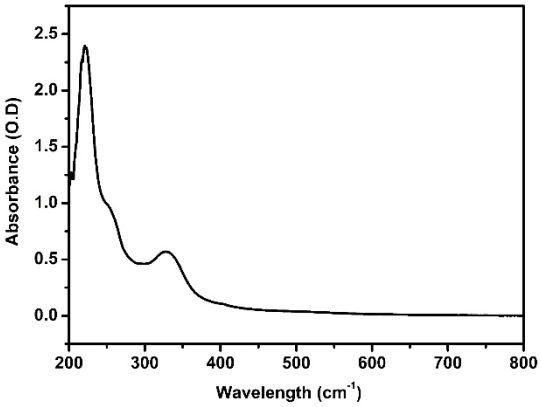

Fig. 3. UV/VIS spectroscopy of melanin.

3.4.2 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDX)

In the SEM study, purified melanin showed an amorphous (irregular) shape (Fig. 4) pattern in comparison with reported bacterial purified melanin. The major elemental composition of the melanin surface revealed by EDX analysis includes carbon,

nitrogen, oxygen, and sulfur with approximate weight as 32.95%, 5.10%, 50%, and 0.1%, respectively.

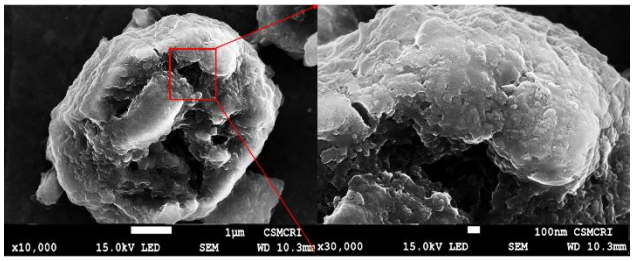

Fig. 4. SEM image of purified melanin at different magnification.

3.4.3 Elemental Analysis

Elemental analysis of the isolated melanin showed percent composition of C, H, N, and S as 27.38, 4.50, 5.08, and 0.88%, respectively. The presence of large proportion of C, H, N and the absence of Sulphur, indicated that the obtained melanin from endopytic actinomycete *S. hyderabadensis* strain 7VPT5-5R can be classified as eumelanin.

3.4.4 Fourier transform infrared spectroscopy (FTIR)

In order to investigate the structure of purified microbial melanin, the FTIR spectrum was obtained (Fig. 5). The FTIR spectrum of purified melanin exhibited a broad band at 3422.23 cm^{-1} due to the stretching vibrations of O-H and N-H groups, a small band at 2964.23 cm^{-1} due to stretching vibration of aliphatic C-H groups.[10] The characteristic bands appeared at 1661.74 cm^{-1} corresponds to the C=O stretching or aromatic C=C stretching.[33] The peaks at 1452.56 cm^{-1} and 1405.90 cm^{-1} is attributed to a C-N stretching peak, indicating that it has the typical indole structure of melanin.[26] The peak at 1042.76 cm^{-1} indicated the presence of aliphatic CH group in plane, which shows characteristic of melanin pigment. The presence of aromatic C-H group corresponds to band at 877.13 cm^{-1}. The band appeared at 707.62 cm^{-1} corresponds to alkene C-H substitution in the melanin pigment.[34]

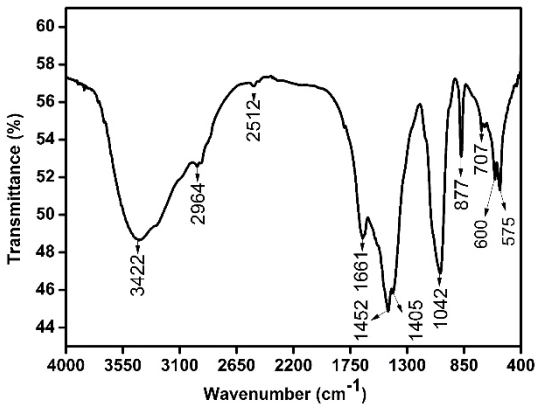

Fig. 5. FTIR spectrum of melanin.

3.4.5 Electron Paramagnetic Resonance Spectroscopy (EPR)

EPR spectrum revealed that melanin particles obtained contained a stable free radical compound, which is characteristic physiological property. The g-factors were in the range 2.003 and the signal peak at 337 mT in the EPR spectrum (Fig. 6) of the pigment indicated the presence of free radicals. Furthermore, EPR spectroscopy has been successfully used to identify the type of melanin on the basis of g value and EPR line shapes.[35] In this study, the obtained melanin was identified as eumelanin by comparison of EPR line shapes to the line shapes obtained from the synthetic eumelanin.

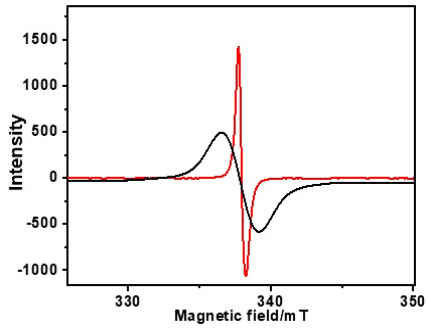

Fig. 6. Overlay of EPR spectra of purified melanin (Black) and synthetic melanin (Red).

3.5.6 Nuclear magnetic resonance (NMR)

The ^{1}H NMR spectrum (Fig. 7) of isolated melanin from *S. hyderabadensis* strain 7VPT5-5R was found to display signals for similar broad functional groups. Thus, the peaks observed at δ_H 0.5 to δ_H 1.5 were assigned to CH_3 and CH_2 groups of alkyl fragments. Peaks from δ_H 3.2 to δ_H 4.2 were assigned to protons on carbons attached to oxygen or nitrogen atoms. In the aromatic region, the peaks at δ_H 6.64 and δ_H 6.73 were assigned to the benzene-CH group of indole. The single signal observed at δ_H 8.46 indicated the existence of pyrrole-CH group of a carboxyl substituted indole.[36]

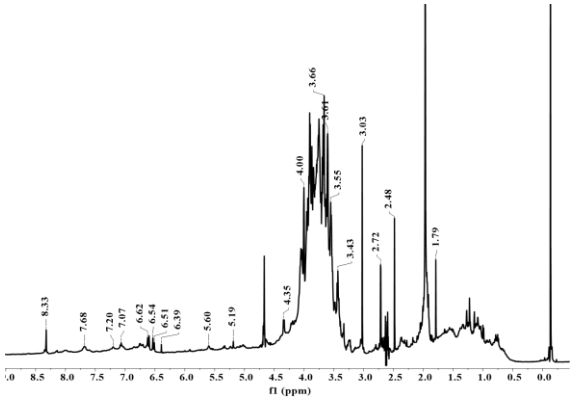

Fig. 7. ^{1}H NMR spectrum of isolated melanin.

4. Conclusions

In conclusion, the endophytic actinomycete isolated from the halophyte *Salicornia brachiata* was identified as *Streptomyces hyderabadensis* strain 7VPT5-5R that is closely related to *Streptomyces hyderabadensis* strain OU-40. The ability to utilize various nutrient components and thereby augmented melanin production is attributed to the natural and genetic diversity of the microbial strain thus varies from species to species. Hence,

screening for the most efficient nutrient is inevitable and therefore, the present work focused on improvement of production yield of melanin. In the previously reported data, the production yield was achieved by traditional (OFAT), Statistical (RSM, CCD, PBD etc.), and genetic engineering approaches. In the present study, screening for melanin production was accomplished by OFAT and the production yield was optimized. In this study, an IL (TBAH) was used for extraction of melanin and IL was effectively recycled by using biphasic system. The yield of pure melanin obtained using the present method was found to be higher in comparison to conventional extraction process and could achieve elite quality melanin in comparison to conventional methods. Furthermore, the process is scalable and IL was recycled and reused in the same process.

Conflicts of interest

There are no conflicts to declare.

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References

1 K. Tarangini and S. Mishra, *Res. J. Engineering Sci.,* 2013, **2**, 40–46.
2 N. Suwannarach, J. Kumla, B. Watanabe, K. Matsui and S. Lumyong, *PLoS One*, 2019, **14**, 1–15.
3 S. R. Pombeiro-Sponchiado, G. S. Sousa, J. C. Andrade, H. F. Lisboa, and R. C. Gonçalves, *Melanin*, 2017, 48–75.
4 E. A. Elsayed and E. N. Danial, *J. Appl. Pharm. Sci*., 2018, **8**, 93–101.
5 P. M. Plonka and M. Grabacka, *Acta Biochim. Pol*., 2006, **53**, 429–443.
6 A. J. Nappi and E. Ottaviani, *BioEssays*, 2000, **22**, 469–480.
7 M. Varga, O. Berkesi, Z. Darula, N. V. May and A. Palagyi, *Phytochemistry*, 2016, **130**, 313–320.
8 B. L. Gomez and J. D. Nosanchuk, *Curr Opin Infect Dis*., 2003, **16**, 91–96.
9 A. N. Tran-Ly, C. Reyes, F. W. Schwarze and J. Ribera, *World J. Microbiol. Biotechnol*., 2020, **36**, 1–9.
10 N. E. A. El-Naggar and S. M. El-Ewasy, *Sci. Rep.,* 2017, **7**, 1–19.
11 L. Wang and Y. Li, *Int. J. Biol. Macromol*., 2019, **123**, 521–530.
12 J. Guo, Z. Rao, T. Yang, Z. Man, M. Xu and X. Zhang, *FEMS Microbiol. Lett*., 2014, **357**, 85–91.
13 J. Guo, Z. Rao, T. Yang, Z. Man, M. Xu and X. Zhang, FEMS *Microbiol. Lett*., 2015, **362**, 1–7.
14 N. Singh and K. Prasad, Green Chem., 2019, **21**, 3328–3333.
15 Y. Liang, Q. Han, N. Byrne, L. Sun and X. Wang, Fibers Polym., 2018, **19**, 1640–1646.
16 J. Lebeau, M. Venkatachalam, M. Fouillaud, L. Dufosse and Y. Caro, *8th International Conference of Pigments in Food, Coloured foods for health benefits*., Cluj-Napoca, Romania, 11, 2016.
17 Y. Lu, M. Ye, S. Song, L. Li, F. Shaikh and J. Li, *Appl. Biochem. Biotechnol*., 2014, **174**, 762–771.
18 Y. Liang, Q. Han, N. Byrne, L. Sun and X. Wang, *Fibers and Polymers*, 2018, **19**, 1640–1646.
19 H. V. Chuyen, M. H. Nguyen, Roach, P. D. Nguyen, J. B. Golding and S. E. Parks, *Nutr. Food Sci*., 2018, **6**, 189–196.
20 S. P. Ventura, F. A. e Silva, M. V. Quental, D. Mondal, M. G. Freire and J. A. Coutinho, Chem. Rev., 2017, **117**, 2117–2121.
21 D. A. Fort, R. C. Remsing, R. P. Swatloski, P. Moyna, G. Moyna and R. D. Rogers, Green Chem., 2007, **9**, 63–69.
22 H. Passos, M. G. Freire and J. A. Coutinho, Green Chem., 2014, **16**, 4786–4815.
23 V. Ghadge, P. Kumar, S. Singh, D. E. Mathew, S. Bhattacharya, S. B. Nimse and P. B. Shinde, *J. Agric. Food Chem*., 2020, **68**, 6854–6863.
24 S. Singh, V. Ghadge, P. Kumar, D. E. Mathew, A. Dhimmar, H. Sahastrabudhe, Y. Nalli, M. R. Rathod and P. B. Shinde, Anton Leeuw. Int. J. G., 2021, 114, 591–608.
25 M. Hayakawa, T. Sadakata, T. Kajiura and H. Nonomura, *J. Ferment. Bioeng*., 1991, **72**, 320–326.
26 P. Manivasagan, J. Venkatesan, K. Senthilkumar, K. Sivakumar and S. K. Kim, *Int. J. Biol. Macromol*., 2013, **58**, 263–274.
27 N. Correa, C. Covarrubias, P. I. Rodas, G. Hermosilla, V. R. Olate, C. Valdes and C. V. Tapia, *Front. Microbiol*., 2017, **8**, 1–13.
28 M. Al Khatib, M. Harir, J. Costa, M. C. Baratto, I. Schiavo, L. Trabalzini, S. Pollini, G. M. Rossolini, R. Basosi and R. Pogni, *Molecules*, 2018, **23**, 2–12.
29 A. R. Katritzky, N. G. Akhmedov, S. N. Denisenko and O. V. Denisko, *Pigment Cell Res*., 2002, **15**, 93–97.
30 D. N. Madhusudhan, B. B. Z. Mazhari, S. G. Dastager and D. Agsar, *BioMed Res. Int*., 2014, 1–11.
31 S. Sun, X. Zhang, S. Sun, L. Zhang, S. Shan and H. Zhu, *Food Chem*., 2016, **190**, 801–807.
32 S. N. Surwase, S. B. Jadhav, S. S. Phugare and J. P. Jadhav*, 3 Biotech.,* 2013, **3**, 187–194.
33 R. Hou, X. Liu, K. Xiang, L. Chen, X. Wu, W. Lin and M. Zheng, *Food chem*., 2019, **277**, 533–542.
34 S. Sajjan, G. Kulkarni, V. Yaligara, L. Kyoung and T. B. Karegoudar, *J. Microbiol. Biotechnol*., 2010, **20**, 1513–1520.
35 M. Zdybel, B. Pilawa, J. M. Drewnowska and I. Swiecicka, *Chem. Phys. Lett.*, 2017, **679**, 185–192.
36 M. S. El-Gamal, H. A. El-Bialy, M. A. Elsayed and M. A. Khalifa, *J. Nucl. Sci. Technol*., 2017, **5**, 57–72.