



Melanin production by *Pseudomonas* sp. and *in silico* comparative analysis of tyrosinase gene sequences

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Abstract

Background. Melanin finds enormous applications in different industries for its unique photoprotective and anti-oxidant properties. Due to its emerging demand, scientific researchers are putting efforts to unravel more microorganisms with a potential of producing melanin on large scale. Hence, the present study was aimed at the isolation of extracellular melanin producing microorganisms from lime quarries of Karnataka, India. Besides this, the tyrosinase gene governing melanin synthesis in different organisms were compared *in silico* to understand its evolutionary aspects. **Material and methods.** Melanin producing microorganisms were screened on tyrosine gelatin beef extract agar medium. Potential isolate was explored for submerged production of melanin in broth containing L-tyrosine. Melanin was characterized by UV-Vis spectroscopy, thin layer and high performance liquid chromatographic techniques. Antibacterial activity of melanin was performed by agar well assay. Comparative tyrosinase gene sequence analysis was performed by using Geneious 2021.1 trial version software. **Results.** *Pseudomonas otitidis* DDB2 was found to be potential for melanin production. No antibacterial activity was exerted by the melanin against tested pathogens. The *in silico* studies showed that the common central domain of tyrosinase protein sequence of selected *Pseudomonas* sps. exhibited 100% identity with the common central domain of *Homo sapiens* tyrosinase (NP_000363.1). **Conclusions.** Our study shows the production of melanin in good quantities by the isolate *Pseudomonas otitidis* DDB2 which can be explored for scale-up process. Since the melanin formed is of eumelanin type and the tyrosinase gene sequence of several *Pseudomonas* sp. showed relatedness to humans, this molecule may be further developed for sunscreen formulations.

Key words: *Pseudomonas* sps., *Homo sapiens*, natural pigment, melanogenic genes, common central domain

Introduction

Microorganisms serve as natural resources of bioactive molecules. Among various bioactive entities, microbial pigments have recently gained wide research interest because of their natural origin and safe application. Melanins are one such group of pigments with complex structures and physicochemical properties (Banerjee et al., 2014). Most of the living organisms on the Earth produce melanin to protect themselves from detrimental effects of solar radiation (D'Ischia M et al., 2015). Although many microorganisms can produce melanin, their scaling up and extraction process require further optimization as melanin is produced inside melanosomes (Toledo et al., 2017). Some microorganisms

such as *Streptomyces*, *Nocardiopsis*, *Pseudomonas*, *Aspergillus*, *Auricularia*, and *Armillaria* have attracted the attention of scientists because of their ability to produce extracellular melanin which can be easily extracted and purified (Kiran et al., 2014; Raman et al., 2015; Sun et al., 2016; El Naggar and El-Ewasy, 2017; Kiran et al., 2017; Ribera et al., 2019). Jalmi et al. (2012) reported maximum melanin production (6.6 g/l) by the fungus *Gliocephalotrichum simplex* after 6 days of incubation. On the other hand, *Streptomyces kathirae* SC-1 produced 13.7 g/l melanin after 5 days of incubation (Guo et al., 2014). Most of these microorganisms depend on the exogenous supply of precursors such as mono-/diphenols for extracellular melanin synthesis (Tran-Ly

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et al., 2020). Melanin has gained importance because of its potential application in antioxidant and sunscreen products, organic semiconductor devices, biomedical devices, metal chelators, and dyeing agents (Ali Amal et al., 2011; Battistella et al., 2020; Tran-Ly et al., 2020). A study revealed that the thermal stability of the synthetic polymer polymethyl methacrylate was enhanced when eumelanin was added to the mixture. It was shown that melanin remarkably increased the decomposition temperature of the polymer by altering its radical initiated chain scission behavior (Shanmuganathan et al., 2011). A recent report showed the application of melanin as a colorant for soft contact lenses (Ahn et al., 2019). Another study described the use of melanin as a contrast agent in imaging application (Williams, 1994). Melanin extracted from *Streptomyces* sp. and *Pseudomonas balearica* shows significant antimicrobial activity against pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus vulgaris*, *Vibrio cholera*, *Proteus miralis*, *Salmonella typhae*, *Salmonella paratyphae*, and *Klebsiella oxytoca* (Vasanthabharathi et al., 2011; Zerrad et al., 2014). Because of its unique properties such as heterogeneity, hydrophobicity, and negative charge, the structural analysis of natural melanin is challenging (Pralea et al., 2019).

Many microorganisms can synthesize melanin through multiple pathways. For instance, microorganisms such as *Streptomyces glaucescens*, *Neurospora crassa*, and *Marinomonas mediterranea* can produce melanin by using a pathway similar to that of mammals, which involves the enzyme tyrosinase (Serrano et al., 2004; Kamal et al., 2014; Toledo et al., 2017), while other microorganisms such as *Aspergillus fumigatus*, *Talaromyces marneffeii*, and *Sporothrix schenckii* use the DHN pathway/malonyl coenzyme for melanin synthesis (Langfelder et al., 1998; Romero-Martinez et al., 2000; Woo et al., 2010; Cordero and Casadevall, 2017). Certain microorganisms such as *Cryptococcus neoformans* and *Bacillus weihenstephanensis* follow the laccase enzyme pathway, while bacteria such as *Vibrio cholerae*, *Hyphomonas* sp., and *Shewanella colwelliana* use the homogentisic acid pathway for melanin synthesis (Kotob et al., 1995; Eisenman et al., 2007; Wang et al., 2011; Drewnowska et al., 2015). Melanins produced through the DHN pathway are localized intracellularly; consequently, they are difficult to purify and hence not desirable for large-scale production (Toledo et al., 2017). Therefore, microorganisms that pro-

duce extracellular melanin within a short incubation period are of industrial interest and are relatively economical. An analysis of the melanogenic genes of different taxa would provide better understanding on the importance of their evolutionary aspects (Esposito et al., 2012). In view of this, the present study aimed to screen melanin-producing potential organisms isolated from harsh habitats such as lime quarries in Karnataka, India. The screening process was followed by characterization of melanin and assessment of its antibacterial property. In addition, an attempt was made for comparative analysis of tyrosinase gene, protein, and common central domain (CCD) sequences of selected organisms.

Materials and methods

Screening of soil samples for melanin-producing microorganisms

Eleven soil samples collected from lime quarries in Karnataka, India, were pretreated and initially isolated on starch casein agar medium. The isolates for extracellular production of tyrosinase and melanin were screened by the streak plate culture method on a tyrosine gelatin beef extract (TGB) agar medium containing tyrosine 0.5%, gelatin 0.5%, beef extract 0.3%, agar 2%, and distilled water with pH 8.0. The inoculated plates were incubated at 40 °C for 5 days. On the fifth day of incubation, the plates were visually assessed for zones of catalysis, and different intensities of brown color were recorded as positive for the synthesis of extracellular tyrosinase and melanin by the test isolates (Shivaveerakumar et al., 2013).

Identification of the potential isolate

The potential isolate for melanin production was identified preliminarily by Gram staining (Bartholomew and Mittwer, 1952) and motility test by the hanging drop method on a cavity slide. Molecular characterization of the isolate was performed by 16S rRNA gene sequencing using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGATCCAGCC-3') (Thorat and Dastager, 2018). The ability of the bacterial isolate to utilize different simple carbohydrates (0.3% w/v) such as glucose, fructose, sucrose, maltose, raffinose, and xylose as the sole source of carbon was assessed in M9 minimal medium at 37 °C for 24 h (Schleheck et al., 2009). The growth of bacteria was determined by measuring the optical density (OD) at 600 nm.

Optimization of physicochemical variables on melanin production

The influence of various factors on melanin production by a potential isolate was studied in L-tyrosine broth incubated at 40 °C for 24 h with agitation speed of 120 rpm (Madhusudhan et al., 2014). The effects of pH (6.0–10.0), temperature (30–50 °C), and L-tyrosine concentration (0–2 g/l) were studied. The effects of different nitrogen sources (0.5% each of beef extract, peptone, gelatin, yeast extract, and casein), metal salts (0.01% each of CuCl₂, CoCl₂, CuSO₄, MnCl₂, and MgCl₂), and salinity (0–2% NaCl) on the production of melanin by the potential isolate were also assessed. All the experiments were performed in triplicate, and the results are presented as mean ± standard deviation.

Production and purification of melanin

Submerged production of extracellular melanin by the potential isolate was accomplished in an optimized broth with agitation speed of 120 rpm for 24 h. The production broth was centrifuged after 24 h of incubation to separate the cell mass from other debris. The supernatant obtained was acidified with 1N HCl till it reached pH 2.0 and was allowed to stand for 96 h for melanin aggregation. The aggregate formed was separated and purified by centrifugation at 10 000 rpm for 20 min, followed by washing with ether, ethanol, acetone, and deionized water to remove impurities (Fava et al., 1993). The purified melanin was air-dried and stored at 4 °C for further use.

Characterization of melanin

Purified melanin was assessed for its solubility profile in different solvents such as water (pH 7.0), 1N HCl, alkaline solutions (1N NaOH and 1N NH₄OH) and organic solvents such as dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), methanol, ethanol, n-butanol, ethyl acetate, acetone, n-hexane, and diethyl ether. Other physical tests such as precipitation of melanin with 1% FeCl₃ and bleaching with alkaline H₂O₂ (10%) were performed as reported previously (Gibson and George, 1998). Melanin was characterized by spectroscopy and chromatographic methods. In brief, melanin was dissolved in 0.1N NaOH and scanned in the range of 200–800 nm UV-Vis spectroscopy. Thin layer chromatography (TLC) was performed by using ethyl acetate and n-hexane (7:3) along with 0.1% formic acid as the mobile phase. High-performance liquid chromatography (HPLC) was per-

formed by dissolving melanin (10 µg) in 10 µl of diluted KOH (0.01N) solution, and the volume was made up to 1 ml with methanol and filtered through 0.2 µm Millex membrane (MF-Millipore™). The mobile phase comprised 0.1% formic acid and methanol in the ratio of 85:15, and the detection was performed with a C18 Kinetex™ 5 µm, 260 × 4.6 mm column at the flow rate of 1 ml/min at 222 nm in the diode array detector (Singh et al., 2018).

Antibacterial activity of melanin

The agar well diffusion assay was performed to assess the antibacterial activity of the melanin extracted from the bacterial isolate against the test pathogenic bacterial species *E. coli*, *P. aeruginosa*, *Streptococcus entericus*, and *B. subtilis* (Arun et al., 2015). The culture medium of each test isolate incubated for 18 h in nutrient broth was swabbed separately on the surface of sterile nutrient agar plates. Eight wells of 10 mm diameter were punched on each plate and loaded with 100 µl of different concentrations of melanin (300, 250, 200, 150, 100, and 50 µg/well and labeled as A–F, respectively), streptomycin (1 mg/ml), and DMSO. All the plates were incubated at 35 °C for 24 h. Streptomycin was used as a positive control, while the solvent DMSO was added as a negative control. The diameter of the zones of inhibition were measured to assess the antibacterial activity.

Comparative analysis of tyrosinase gene and protein sequences from selected organisms

The sequences of the tyrosinase gene from selected organisms were compared and analyzed to find the closest match with the human tyrosinase gene. A total of 72 tyrosinase genes and their reference protein sequences from prokaryotic and eukaryotic organisms (Table 1) were retrieved from the National Center for Biotechnology Information (NCBI) in FASTA format. The reference protein sequences were analyzed to find conserved motifs by using the KEGG's Motif Search web tool allied with Pfam database. The selected gene sequences, their respective proteins, and conserved motif sequences were separately subjected to multiple alignment using Clustal Omega 1.2.2 in Geneious software 10.2. After the multiple alignment, a distance matrix of sequences and phylogenetic tree were constructed to comprehend the evolutionary relationship between the tyrosinase sequences of selected organisms and *Homo*

Table 1. List of selected organisms for the retrieval of tyrosinase gene and protein sequences

| <i>Pseudomonas</i> | <i>Streptomyces</i> | <i>Fungi</i> | Others |
|------------------------------------|---|--|----------------------------------|
| <i>P. putida</i> NBRC 14164 | <i>S. rimosus</i> ATCC 10970 | <i>Agaricus bisporus</i> | <i>Homo sapiens</i> (TYRP1 gene) |
| <i>P. helleri</i> DSM 29165 | <i>S. griseus griseus</i> NBRC 13350 | <i>Aspergillus niger</i> strain PA2 | <i>Sepia officinalis</i> |
| <i>P. frederiksbergensis</i> FW305 | <i>S. californicus</i> NRRL B-2988 | <i>Colletotrichum karsti</i> | <i>Vibrio nigripulchritudo</i> |
| <i>P. poae</i> LMG 21465 417 | <i>S. clavuligerus</i> F1D7 (plasmid) | <i>Lasiodiplodia theobromae</i> | |
| <i>P. lactis</i> SS101 | <i>S. scabei</i> 87.22 | <i>Geosmithia morbida</i> | |
| <i>P. chlororaphis</i> qlu-1 | <i>S. venezulae</i> ATCC 21113 | <i>Arthroderma uncinatum</i> | |
| <i>P. simiae</i> PCL1751 | <i>S. zinciresistens</i> K42 | <i>Daldinia childiae</i> | |
| <i>P. yamanorum</i> LBUM636 | <i>S. roseochromogenus</i> subsp. <i>oscitans</i> DS 12.976 | <i>Dothidotthia symphoricarpi</i> CBS 119687 | |
| <i>P. veronii</i> | <i>S. pluripotens</i> MUSC 135 | <i>Lindgomyces ingoldianus</i> | |
| <i>P. synxantha</i> R6-28-08 | <i>S. olivochromogenes</i> DSM 40451 | <i>Macroventuria anomochaeta</i> | |
| <i>P. proteolytica</i> LMG 22710 | <i>S. virginiae</i> NRRL ISP-5094 | <i>Pseudovirgaria hyperparasitica</i> | |
| <i>P. gingeri</i> A6001 | <i>S. subutilus</i> ATCC 27467 | <i>Aaosphaeria arxii</i> CBS 175.79 | |
| <i>P. moraviensis</i> TYU6 | <i>S. canus</i> DSM 40017 | <i>Didymella exigua</i> CBS 183.55 | |
| <i>P. asiatica</i> RYU5 | <i>S. avermitilis</i> MA-4680 | <i>Zymoseptoria tritici</i> IPO323 | |
| <i>P. orientalis</i> 8B | <i>S. flavovariabilis</i> NRRL B-16367 | <i>Grifola frondosa</i> | |
| <i>P. monteilii</i> SB3101 | <i>S. azureus</i> ATCC 14921 | <i>Talaromyces marneffeii</i> ATCC 18224 | |
| <i>P. azotoformans</i> | <i>S. atroolivaceus</i> NRRL ISP-5137 | <i>Blastomyces gilchristii</i> | |
| <i>P. fluorescens</i> Pf0-1 | <i>S. xanthophaeus</i> NRRL B-5414 | <i>Pyricularia oryzae</i> 70-15 | |
| <i>P. synxantha</i> NCTC 10696 | <i>S. hygrosopicus</i> subsp. <i>jinggansensis</i> TL01 | <i>Microsporum canis</i> CBS 113480 | |
| <i>P. proteolytica</i> WS 5126 | <i>S. cyaneus</i> CGMCC 4.1671 | <i>Sodiomyces alkalinus</i> F11 | |
| | <i>S. violaceoruber</i> S21 | | |
| | <i>S. katrae</i> S3 | | |
| | <i>S. fodineus</i> TW1S1 | | |
| | <i>S. glaucescens</i> GLA.O | | |
| | <i>S. fungicidicus</i> TXX3120 | | |
| | <i>S. lavendulae</i> subsp. <i>lavendulae</i> CCM 3239 | | |
| | <i>S. rubrolavendulae</i> MJM4426 | | |
| | <i>S. wuyuanensis</i> CGMCC 4.7042 | | |

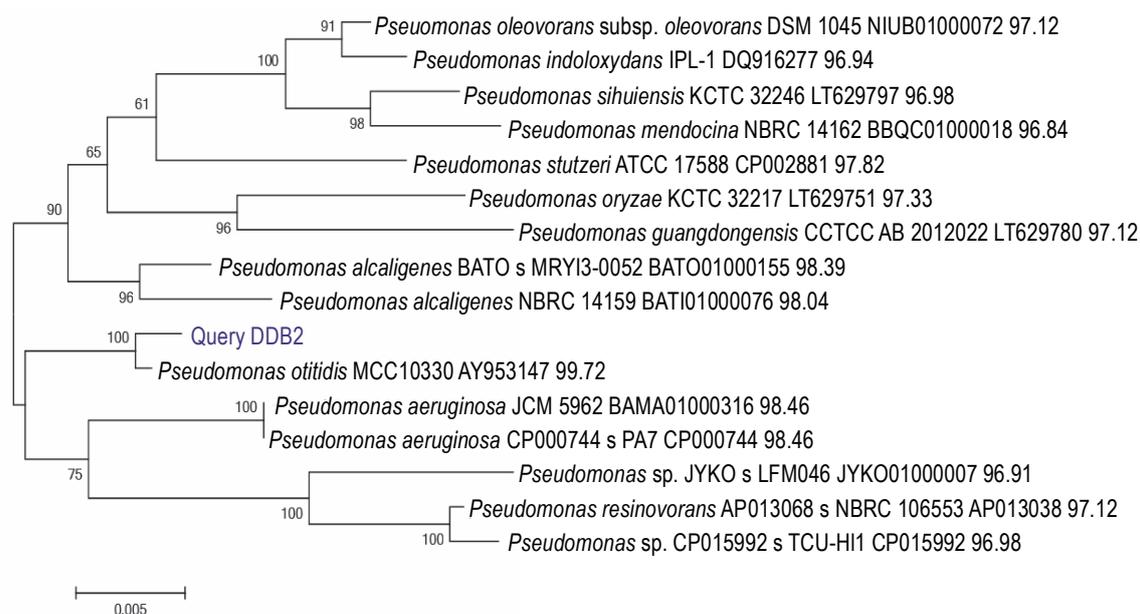


Fig. 1. Phylogenetic tree of *Pseudomonas otitidis* DDB2

sapiens (NM_000372.5) (Esposito et al., 2012). *H. sapiens* was selected as the out group while constructing the phylogenetic tree.

Results

A total of 58 isolates were obtained from lime quarry soil samples collected from Karnataka, India. All the 58 isolates were screened for melanin production, and only six isolates produced dark brown pigment on the TGB agar medium. Among the six positive isolates, the bacterial isolate named DDB2 (D: Name of Research Guide, Prof. Dayanand; D: Name of the Research Student, Deepthi; B: Type of isolate, bacteria; 2: number of the isolate) showed a large zone of pigmentation (38 ± 0.5 mm) after 24 h as compared to the other five isolates (supplementary Table 1), and hence, it was selected for further studies. The isolate DDB2 was gram-negative, motile, rod-shaped bacteria that produced melanin under alkaline conditions. The potential isolate DDB2 was identified by 16S rRNA gene sequencing, and the phylogenetic tree (Fig. 1) was constructed using the neighbor-joining method that showed a homology of 99% with *Pseudomonas otitidis*. The GenBank accession ID of *P. otitidis* DDB2 is MG585039. Figure 2 shows the extracellular dark brown pigment production by the isolate DDB2 on TGB agar. Carbohydrate utilization test showed that only glucose was utilized by the isolate



Fig. 2. Extracellular melanin produced by *P. otitidis* DDB2 on the TGB agar plate

DDB2, while other carbohydrate sources were not metabolized and no turbidity was observed (supplementary Table 2). The growth of DDB2 on M9 medium with glucose was compared with its growth in nutrient broth; the OD values of 0.24 ± 0.02 and 0.64 ± 0.02 were achieved in the former and latter media, indicating that the isolate poorly fermented sugars but actively utilized complex ingredients of nutrient broth.

Optimization of melanin production

The influence of physicochemical parameters such as pH, temperature, L-tyrosine concentration, metal salts,

Table 2. Solubility of melanin in different solvents tested

| Series number | Solvent | Solubility of melanin |
|---------------|-----------------------|-----------------------|
| 1 | Water (pH 7.0) | insoluble |
| 2 | 1N HCl | insoluble |
| 3 | 1N NaOH | soluble |
| 4 | 1N NH ₄ OH | soluble |
| 5 | DMSO | soluble |
| 6 | DMF | soluble |
| 7 | methanol | insoluble |
| 8 | ethanol | insoluble |
| 9 | n-butanol | insoluble |
| 10 | ethyl acetate | insoluble |
| 11 | acetone | insoluble |
| 12 | n-hexane | insoluble |
| 13 | diethyl ether | insoluble |

and salinity on melanin production was assessed. The amount of melanin produced increased with the increase in pH from 6.0 to 8.0, but gradually decreased from pH 9.0 to 10.0 (Fig. 3A). Melanin production exponentially increased from 30 to 40°C, and a further increase in temperature to 45°C slightly increased the production of melanin by 0.13 g/l (Fig. 3B). However, the growth of *P. otitidis* DDB2 was completely inhibited at 50°C. The use of the precursor L-tyrosine resulted in the maximum production of melanin (4.07 ± 0.07 g/l) at the concentration of 1.5 g/l (Fig. 3C). However, the absence of L-tyrosine resulted in no melanin formation (0.0 g/l), and hence, this precursor was found to be mandatory for the synthesis of melanin by *P. otitidis* DDB2. Among the different nitrogen sources tested, 0.3% of beef extract or gelatin was found to exert a similar positive effect on the amount of melanin production (4.6 ± 0.10 and 4.51 ± 0.10 g/l, respectively). Therefore, either beef extract or gelatin at the concentration of 0.3% was included in the final medium for melanin production. Casein was found to produce the least amount of melanin (1.56 ± 0.10 g/l) than the other nitrogen sources tested (Fig. 3D). The results also showed that 0.01% CuCl₂ significantly affects melanin production by *P. otitidis* DDB2 (Fig. 3E). The metal salts CoCl₂ and CuSO₄ showed moderate increment in melanin production, whereas MnCl₂ lowered melanin production by 2.54 ± 0.12 g/l. Complete inhibition of me-

lanin synthesis was observed with MgCl₂. The salinity range of 0–2% neither enhanced nor reduced melanin production. Because the presence of NaCl did not greatly influence the amount of melanin production, it was excluded from the final optimized medium. The optimized conditions for the maximum production of melanin were found to be pH 8.0, incubation temperature 45°C, L-tyrosine (1.5 g/l), beef extract (0.3%), and CuCl₂ (0.01%). Melanin production by *P. otitidis* DDB2 increased from 3.81 ± 0.1 g/l to 5.3 ± 0.1 g/l under the optimized conditions.

Characterization of melanin

Purified melanin was soluble in alkaline solutions and in solvents such as DMSO and DMF, but it was insoluble in all the other solvents tested (Table 2). Melanin was precipitated by FeCl₃ and turned colorless after reacting with H₂O₂. The requirement of L-tyrosine as the precursor, the dark brown color, and the solubility profile of the compound revealed that the pigment extracted from *P. otitidis* DDB2 belonged to the class eumelanin/DOPA melanin.

The UV-Vis absorption spectrum of melanin showed the maximum absorbance between the wavelength range of 215–350 nm (Fig. 4). The chromatogram developed on the TLC plate showed a broad spot with the retention factor (Rf) of 0.48. HPLC with 0.1% formic acid and methanol (in the ratio of 85:15 ratio) as the mobile phase showed three major peaks with retention time of 1.620, 2.273, and 2.693 min corresponding to 10.01%, 63.20%, and 17.42% of melanin constituents, respectively (supplementary Fig. 1).

Antibacterial activity of melanin

Purified melanin was assessed for its antibacterial activity (Fig. 5). A maximum diameter of 13 ± 2 mm for the zone of inhibition was observed for 300 µg of melanin against *S. entericus*, whereas the diameter of 12 ± 2 mm was observed for the zones of inhibition with 300 µg of melanin against *E. coli*, *P. aeruginosa*, and *B. subtilis* (supplementary Table 3). The zones of inhibition were 12 ± 1 mm in diameter for 250 µg of melanin against *E. coli*, *P. aeruginosa*, and *S. entericus*. For all the other tested concentrations of melanin, the diameter of zones of inhibition was 11 ± 1 mm against the tested pathogenic bacteria. The zones of inhibition for the positive control streptomycin were 22 ± 1, 24 ± 2, 19 ± 2,

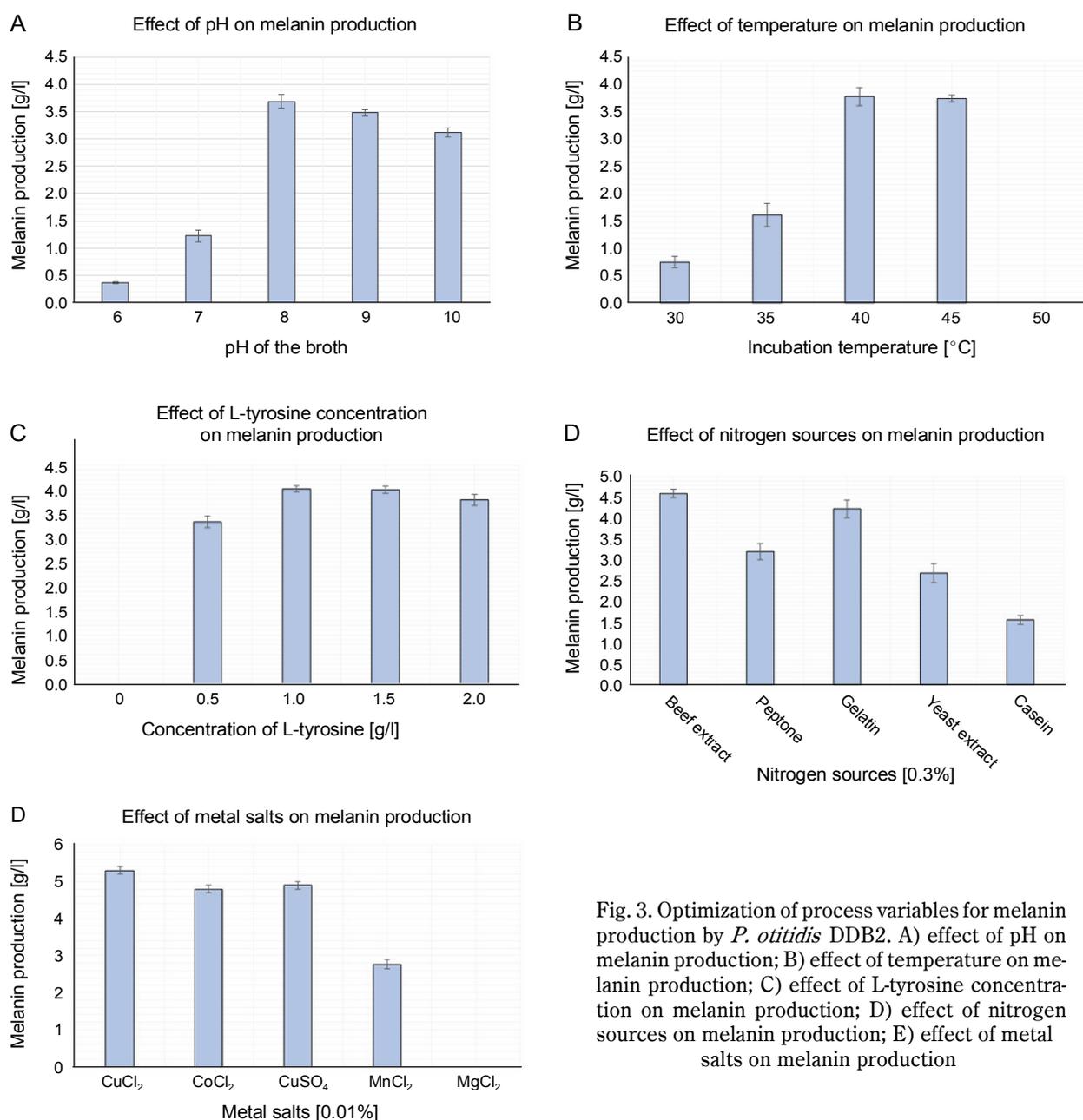


Fig. 3. Optimization of process variables for melanin production by *P. otitidis* DDB2. A) effect of pH on melanin production; B) effect of temperature on melanin production; C) effect of L-tyrosine concentration on melanin production; D) effect of nitrogen sources on melanin production; E) effect of metal salts on melanin production

and 18 ± 1 mm against *E. coli*, *P. aeruginosa*, *S. entericus*, and *B. subtilis*, respectively, whereas the negative control DMSO did not show any inhibition zone.

Comparative analysis of tyrosinase gene sequences

Tyrosinase gene sequences of selected organisms retrieved from NCBI were aligned, and a phylogenetic relationship was established to determine the relatedness of the tyrosinase gene of *H. sapiens* with that of other organisms such as *Pseudomonas* sp., *Streptomyces* sp., several fungal species, *S. officinalis*, *Vibrio*

sp., and tyrosinase-related protein 1 (TYRP1) of *H. sapiens*. The resultant phylogenetic tree (Fig. 6) showed that the difference in the tyrosinase gene sequences increased in clockwise direction with respect to *H. sapiens*. The distance matrix was generated by Geneious software for the tyrosinase gene, protein, and CCD sequences, which showed the distances between the sequences of organisms based on percentage identity (%) of the nitrogen bases or amino acid residues (supplementary Table 4).

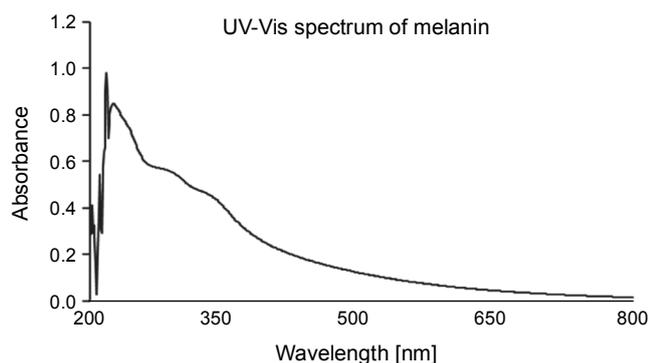


Fig. 4. UV-Vis absorption spectrum of melanin derived from *P. otitidis* DDB2

Comparative analysis of tyrosinase gene sequences of selected organisms with the tyrosinase gene sequence of *H. sapiens* (NM_000372.5)

Among all the selected organisms, the TYRP1 gene sequence of *H. sapiens* was found to be more related to the tyrosinase gene sequence of selected *H. sapiens* with 34.7% identical bases, whereas *S. officinalis* showed 21.9% and *V. nigripulchritudo* showed 14.6% identity. Among the selected fungi, *T. marneffeii* ATCC 18224 showed the highest similarity (20.5%) and *G. morbida* showed the lowest similarity (1.8%) with *H. sapiens*. Among the selected species of *Streptomyces*, *S. subutilis* and *S. fungicidicus* showed the maximum (12.4%) and the least (0.6%) percentage of identity, respectively. On the other hand, *P. proteolytica* and *P. frederiksborgensis* showed the highest (14.6%) and the least (6%) similarity with the tyrosinase gene sequence of *H. sapiens*, respectively.

Comparative analysis of tyrosinase protein sequences of selected organisms with the tyrosinase protein sequence of *H. sapiens* (NP_000363.1)

The TYRP1 sequence of *H. sapiens* showed 39.3% identity, while those of *S. officinalis* and *V. nigripulchritudo* showed 12.3% and 11.9% similarity, respectively, with the tyrosinase protein sequence of *H. sapiens*. *A. niger* showed the highest (12.4%) and *G. morbida* showed the lowest (7.9%) identical amino acid residues among the selected fungi. *S. azureus* showed the maximum (22.9%) and *S. fungicidicus* showed the least (3%) percentage of identity, whereas among the selected species of *Pseudomonas*, *P. fluorescens* exhibited the highest (20.8%) and *P. gingeri* showed the least (10.5%) percentage of identity with the tyrosinase protein sequence of *H. sapiens*.

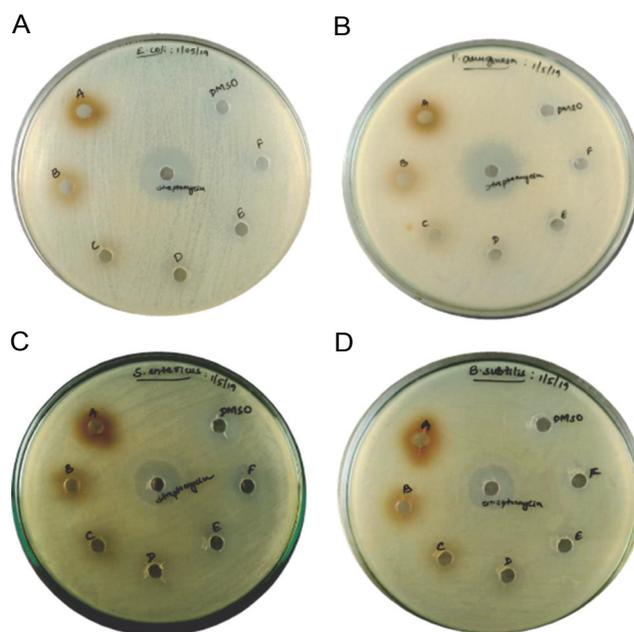


Fig. 5. Agar well assay for the antimicrobial activity of melanin (Plate A – *E. coli*; Plate B – *P. aeruginosa*; Plate C – *S. entericus*; Plate D – *B. subtilis*)

Comparative analysis of CCD of tyrosinase protein sequences

The CCD of the tyrosinase protein sequences of most selected organisms showed 100% identity with the CCD of *H. sapiens* (supplementary Table 3), and all these organisms exhibited common conserved residues at the positions 164–220 (PDQFRQNIPVLKNEVD RYL TNPSF VRFQADLDRGAHGAIHVFGGQNSSSPLPANS). The CCD of *P. putida*, *P. chlororaphis*, and *P. gingeri* showed 56.35, 61.4, and 74.2% identity, respectively, with that of *H. sapiens*, respectively. However, certain species of *Streptomyces* such as *S. zinciresistens*, *S. pluripotens*, *S. olivochromogenes*, *S. virginiae*, *S. canus*, *S. avermitilis*, *S. cyaneus*, *S. violaceoruber*, *S. katrae*, *S. fodineus*, *S. fungicidicus*, and *S. wuyuanensis* and the fungal species *G. morbida* exhibited no identity with the CCD of human tyrosinase protein sequence. Although these species did not harbor a CCD similar to that of *H. sapiens*, they were found to possess a conserved amino acid sequence (from position 31 to 73, AAMYEISELAVGDK RGYSALARAHGYDQDLCHDLDWAF L TWHR) that is commonly observed in other microorganisms selected in the present study. The multiple alignment of CCDs of the selected organisms is shown in Figure 7.

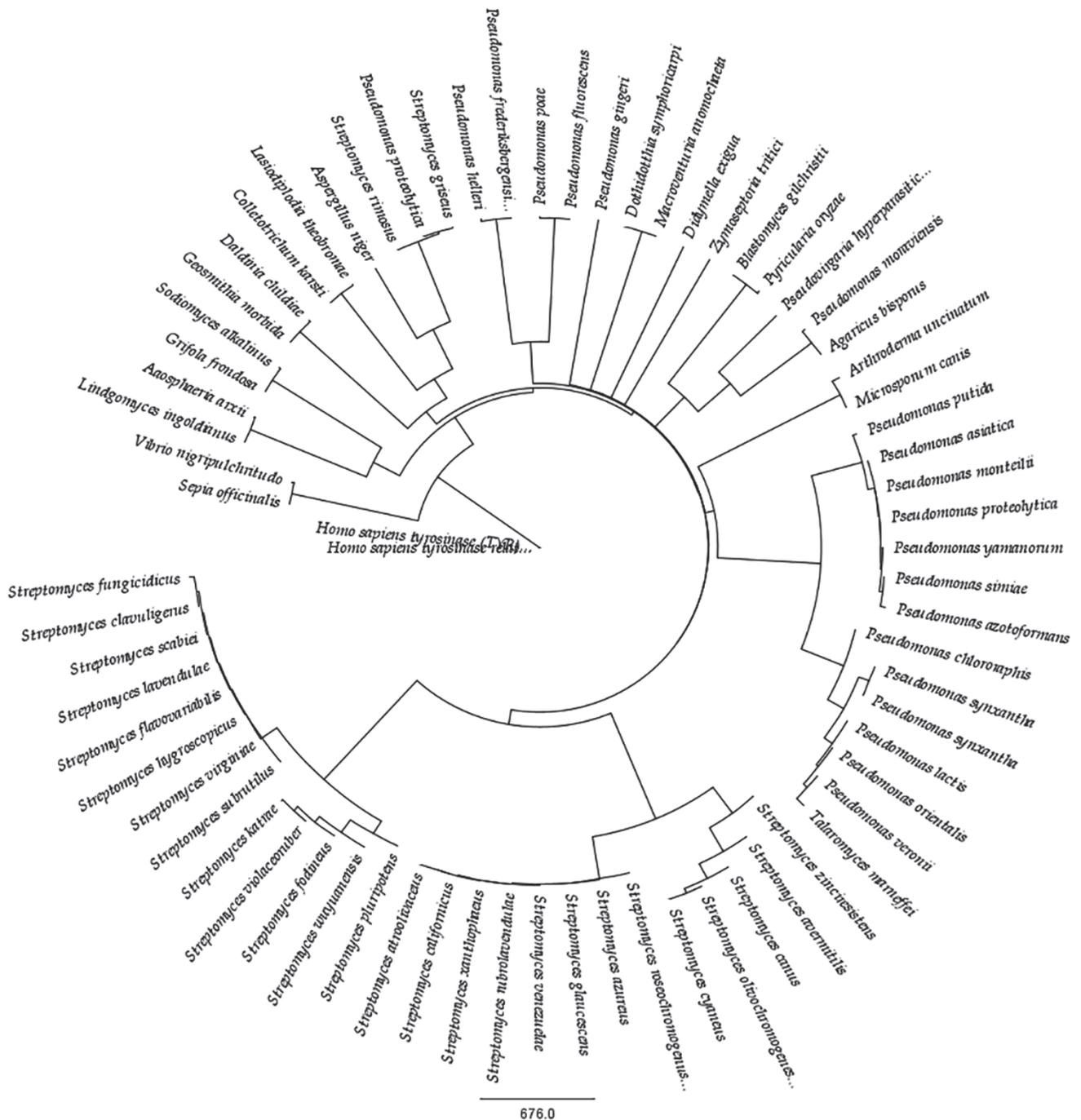


Fig. 6. Phylogenetic tree showing relatedness of tyrosinase gene sequences from selected organisms

Discussion

From preliminary screening, a single isolate named DDB2 showing the largest zone of extracellular melanin production was selected as the potential isolate and used for further studies. The other five isolates that produced maximum zones of pigmentation after 72 h of incubation were excluded from the study because the time of pig-

ment production was too long and the diameter of the zone of pigmentation was smaller than that for the isolate DDB2. The isolate *P. otitidis* DDB2 could not utilize the carbohydrates provided, except glucose. However, it was observed that DDB2 poorly utilized glucose, but exhibited a rich growth on nutrient agar medium, which indicated the organism's preference for complex nu-

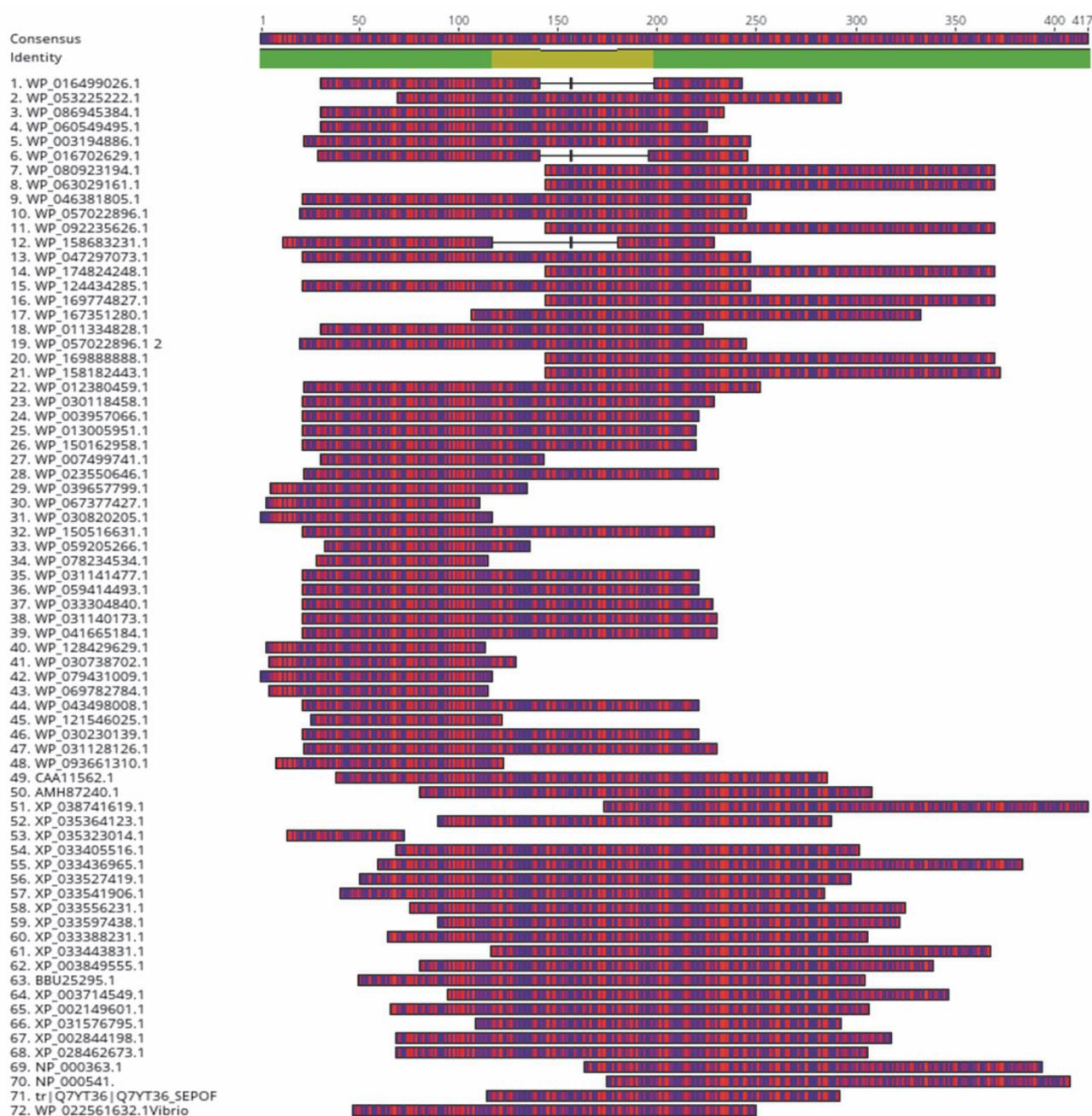


Fig. 7. Aligned sequences of CCD of the tyrosinase protein from selected organisms

trients. The genus *Pseudomonas* prefers organic acids or amino acids over sugars as their carbon source (Daniels et al., 2010). *P. otitidis* DDB2 produced the maximum amount of melanin at an alkaline pH of 8.0 (3.81 ± 0.1 g/l) and a high temperature of 45°C (3.85 ± 0.1 g/l). A similar requirement of alkaline pH (9.0) and temperature 45°C was observed for melanin production by *Streptomyces* sp. isolated from limestone quarries in Deccan traps (Quadri and Agsar, 2012). Previous studies have shown that microorganisms produce melanin under certain stress

conditions such as poor nutrient supply, high temperature, or high osmotic pressure (Fogarty and Tobin, 1996; Tran-Ly et al., 2020). This may be the reason for the requirement of high temperature ($40 \pm 5^\circ\text{C}$) and high pH ≥ 8.0 for melanin production by the isolated *Pseudomonas* sp. in this study. The isolate *P. otitidis* DDB2 showed an obligatory requirement of the precursor L-tyrosine for melanin production. Previous reports show that *Pseudomonas stutzeri* HMGM-7 and *Pseudomonas* sp. WH001 55 also produced melanin in the pre-

sence of L-tyrosine (Ganesh Kumar et al., 2013; Kiran et al., 2017). The prerequisite of L-tyrosine as the precursor is a common feature of eumelanin-synthesizing organisms (Tran-Ly et al., 2020). It was observed that beef extract was consumed more readily by the isolate and facilitated the high titer of melanin production (4.6 ± 0.1 g/l). A similar requirement of beef extract for enhanced melanin production (5.29 g/l) by *S. lusitanus* DMZ3 was reported previously (Madhusudhan et al., 2014). Copper ions are important cofactors for the functioning of melanogenic enzymes such as tyrosinases and laccases (Sendovski et al., 2011; Yang et al., 2017). The addition of CuCl_2 (0.01%) significantly increased the yield of melanin produced by the isolate *P. otitidis* DDB2 by 0.7 g/l. Enhanced production of melanin can also be achieved with other metals by inducing stress in microorganisms (Gowri and Srivastava, 1996). For instance, *Auricularia auricula* and *Streptomyces glaucescens* NEAE-H require magnesium and iron salts, respectively, for enhanced melanin production (Sun et al., 2016; El Naggari and El-Ewasy, 2017). Surwase et al. (2013) reported improved production of melanin (6.8 g/l) by *Brevundimonas* sp. SGJ in the presence of L-tyrosine, tryptone, and CuSO_4 after 54 h of incubation. In another study, the fungus *Armillaria cepistipes* produced the maximum amount of melanin (27.98 g/l) after 161 days of incubation and in the presence of carbon-nitrogen sources such as glucose and yeast extract along with metal salts of copper, iron, and magnesium (Ribera et al., 2019). The present study showed the maximum production of melanin (5.3 ± 0.1 g/l) by *P. otitidis* DDB2 with minimum medium ingredients and a short period of incubation (24 h) as compared to those reported earlier.

The extracted and purified melanin from *P. otitidis* DDB2 was found to be insoluble in most of the solvents tested, and this property of poor solubility is peculiar to melanin. The precipitation of melanin after reacting with FeCl_3 indicated the presence of polyphenolic groups (Pralea et al., 2019). The melanin obtained in this study was degraded by alkaline H_2O_2 , which was indicated by its bleaching. Purified melanin showed higher absorbance in UV range (Fig. 4), which decreased gradually with further increase in wavelength toward the visible region; this attribute is unique to melanin (Meredith and Sarna, 2006; Al Khatib et al., 2018). We characterized melanin extracted from *P. otitidis* DDB2 by other methods such as thermal stability, elemental analysis,

FT-IR, and NMR as reported earlier (Seelam et al., 2021). Our previous studies on the characterization of melanin revealed its basic constituent as 5,6-dihydroxy indole, 2-carboxylic acid (Seelam et al., 2021). The three peaks observed in chromatogram of HPLC may be attributed to the oligomers of melanin with varying number of monomeric units. The broad spot developed on chromatogram of TLC and multiple peaks observed in HPLC show the heterogeneous nature of melanin under study. It is a common feature of natural melanin to exhibit the property of heterogeneity and insolubility due to the complex arrangement of its molecular structure (Pralea et al., 2019).

In the present study, no antibacterial activity of melanin was detected against the tested pathogens *E. coli*, *P. aeruginosa*, *S. entericus*, and *B. subtilis*. The diameters of inhibition were very low as compared to the positive control streptomycin. Antibacterial activity of melanin in the present study showed contrasting results to earlier reports. For instance, melanin obtained from the fungus *Schizophyllum commune* exhibited antibacterial activity against *E. coli*, *Klebsiella pneumoniae*, *Proteus* sp., and *Pseudomonas fluorescens* at the concentration range of 50–200 μg (Arun et al., 2015). In another study, melanin (30 $\mu\text{g}/\text{disc}$) from *Streptomyces* sp. showed antibacterial activity against *P. aeruginosa* and *Vibrio parahaemolyticus* (Sivaperumal et al., 2014). Similarly, purified melanin from *Actinobolus* sp. MA-32 exhibited antibacterial activity against *B. subtilis* and *E. coli* at the concentration of 30 and 50 $\mu\text{g}/\text{disc}$ (Manivasagan et al., 2013). Lopusiewicz (2018) reported the antibacterial activity of purified melanin extracted from *Armillaria* sp. against *B. cereus*, *B. subtilis*, *Enterococcus faecalis*, and *P. aeruginosa*. However, no activity was observed against *B. anthracis*, *E. coli*, *Micrococcus luteus*, *P. putida*, and *S. aureus*. These incongruities in the antibacterial activity of melanin may result from the differences in the composition and molecular structure of melanin (Correa et al., 2017).

Recent advances in gene sequencing approaches and the development of computational biology tools have enabled the researchers to study the diversity of genes and understand the evolutionary relationship among different species. In the present study, an attempt was made to comparatively analyze tyrosinase gene and protein sequences of selected organisms with *H. sapiens*. The rationale underlying the selection of organisms for

this study was that the potential organism in the present study was identified as *Pseudomonas* sp., and hence, different species of the same genus were selected for comparative analysis. Several studies have shown that the other common microbial sources for tyrosinase or melanin are *Streptomyces* (Madhusudhan et al., 2014; El Naggar and El-Ewasy 2017), *Vibrio* (Vijayan et al., 2017; Kurian and Bhat, 2018), or fungal species (Mattoon et al., 2021), and hence, they were also included in the study. *S. officinalis* was selected because this is the only source of commercially available natural melanin (Guo et al., 2014). The TYRP1 sequences of *H. sapiens* involved in melanin synthesis, maintenance of melanosome structure, and stabilization of tyrosinase were also selected as parameters to determine how likely TYRP1 and tyrosinase sequences of *H. sapiens* are related despite their location on different chromosomes (Zheng et al., 2010). However, the sequences of *H. sapiens* used as the out group were arbitrarily selected.

The distance matrix of the aligned sequences showed that the TYRP1 gene of *H. sapiens* (34.7%) is closer to tyrosinase of *H. sapiens*, followed by *S. officinalis* (21.9%) and *T. marneffeii* ATCC18224 (20.5%). The protein sequences of TYRP1 of *H. sapiens* showed 39.3% identity, followed by *S. azureus* with 22.9% identity and *S. clavuligerus* with 22.7% identity with the tyrosinase protein of *H. sapiens*. The central domains of most of the species in the present study showed 100% identity with that of CCD of *H. sapiens*. Although a large variation was observed in the tyrosinase gene sequences of selected organisms, a 100% similarity in their central domains strongly implicate that the functional domains of the tyrosinase protein are highly conserved irrespective of their taxonomic positions. *In silico* studies provided insights on the variability of sequences and applicability of melanin to closely related species. Researchers from China have performed a similar analysis of TYRP1 among different species and showed that there were two evolutionarily conserved noncoding regions present upstream to the initiation sites of translation. They also reported the amino acids mutated within the species and the amino acids highly conserved among the species (Zheng et al., 2010). Therefore, the use of bioinformatics tools in scientific research aid in better understanding of evolutionary changes in a particular gene sequence and its directionality.

Conclusions

Melanin has recently gained significance for application in green technology and material science as an additive that can substantially enhance the performance of other materials. In light of its increasing demand, in the present study, we report the screening, isolation, and production of melanin by a potential strain of *P. otitidis* DDB2. The isolated melanin belonged to the eumelanin class with the maximum absorption range in the ultraviolet region, and hence, it could be used as a natural pigment in sunscreen products. Furthermore, *in silico* studies of melanogenic gene sequences provided us insights into the applicability of melanin to humans due to the evolutionary relatedness between the tyrosinase genes of humans and the selected *Pseudomonas* sp.

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References

- Ahn S.Y., Choi M., Jeong D.W., Park S., Park H., Jang K.S., Choi K.Y. (2019) *Synthesis and chemical composition analysis of protocatechualdehyde-based novel melanin dye by 15T FT-ICR: high dyeing performance on soft contact lens*. Dyes Pigments 160: 546–554.
- Al Khatib M., Harir M., Costa J., Baratto M.C., et al. (2018) *Spectroscopic characterization of natural melanin from a Streptomyces cyaneofuscatus strain and comparison with melanin enzymatically synthesized by tyrosinase and laccase*. Molecules 23(8): 1916.
- Ali Amal M., Keera Abeer A., Helmy Samia M., Abd El-Nasser Nadia H., Ahmed K.A., El-Hennawi H.M. (2011) *Selection of pigment (melanin) production in Streptomyces and their application in printing and dyeing of wool fabrics*. Res. J. Chem. Sci. 1(5): 22–28.
- Arun G., Eyini M., Gunasekaran (2015) *Characterization and biological activities of extracellular melanin produced by Schizophyllum commune (Fries)*. IJEB. 53: 380–387.
- Banerjee A., Supakar S., Banerjee R. (2014) *Melanin from the nitrogen-fixing bacterium Azotobacter chroococcum: a spectroscopic characterization*. PLoS One. 9(1): e84574.
- Bartholomew J.W., Mittwer T. (1952) *The Gram stain*. Bacteriol. Rev. 16(1): 1–29.
- Battistella C., Naneki Mc C.C., Gnanasekaran K., Zhou X., Caponetti V., Montalti M., Nathan Gianneschi C. (2020)

- Mimicking natural human hair pigmentation with synthetic melanin*. ACS Cent. Sci. 6(7): 1179–1188.
- Cordero R.J.B, Casadevall. (2017) *A functions of fungal melanin beyond virulence*. Fungal Biol. Rev. 31: 99–112.
- Correa N., Covarrubias C., Rodas P.I., Hermosilla G., Olate V.R., Valdés C., Meyer W., Magne F., Tapia C.V. (2017) *Differential antifungal activity of human and Cryptococcal melanins with structural discrepancies*. Front. Microb. 8: 1–13. <https://doi.org/10.3389/fmicb.2017.01292>
- D'Ischia M., Pezzella A., Napolitano A., et al. (2015) *Melanins and melanogenesis: from pigment cells to human health and technological applications*. Pigment Cell Melanoma Res. 28: 520–544.
- Daniels C., Godoy P., Duque E., Molina-Henares M.A., De La Torre J., Del Arco J.M., Herrera C., Segura A., Guazzaroni M.E., Ferrer M. et al. (2010) *Global regulation of food supply by Pseudomonas putida DOT-T1E*. J. Bacteriol. 192: 2169–2181
- Drewnowska J.M., Zambrzycka M., Kalska-Szostko B., Fiedoruk K., Swiecicka I. (2015) *Melanin-like pigment synthesis by soil bacillus weihenstephanensis isolates from northeastern Poland*. PLoS One 10(4): e0125428.
- Eisenman H.C., Mues M., Weber S.E., Frases S., Chaskes S., Gerfen G., et al. (2007) *Cryptococcus neoformans laccase catalyzes melanin synthesis from both D- and L-DOPA*. Microbiology 153: 3954–3962.
- El-Naggar N.E.A, El-Ewasy S.M. (2017) *Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories Streptomyces glaucescens NEAE-H*. Sci. Rep. 7: 42129.
- Esposito R., D'Aniello S., Squarzone P., Pezzotti M.R., Ristrotore F., Spagnuolo A. (2012) *New insights into the evolution of metazoan tyrosinase gene Family*. PLoS One 7(4): e35731.
- Fava F., Gioia D.D., Marchetti L. (1993) *Characterization of a pigment produced by Pseudomonas florescens during 3-chlorobenzoate co-metabolism*. Chemosphere 27(5): 825–835.
- Fogarty R.V., Tobin J.M. (1996) *Fungal melanins and their interactions with metals*. Enzyme Microbiol. Technol. 19: 311–317.
- Ganesh Kumar C., Sahu N., Narendra Reddy G., Prasad R.B.N., Nagesh N., Kamal A. (2013) *Production of melanin pigment from Pseudomonas stutzeri isolated from red seaweed Hypnea musciformis*. Lett. Appl. Microbiol. 57: 295–302.
- Gibson L.F., George A.M. (1998) *Melanin and novel melanin precursors from Aeromonas media*. FEMS Microbiol. Lett. 169(2): 261–268.
- Gowri P.M., Srivastava S. (1996) *Encapsulation as a response of Azospirillum brasilense sp7 to zinc stress*. World J. Microbiol. Biotechnol. 12: 319–322. <https://doi.org/10.1007/BF00340207>
- Guo J., Rao Z., Yang T., Man Z., Xu M., Zhang X. (2014) *High-level production of melanin by a novel isolate of Streptomyces kathirae*. FEMS Microbiol. Lett. 357: 85–91.
- Jalmi P., Bodke P., Wahidullah S., Raghukumar S. (2012) *The fungus Glioccephalotrichum simplex as a source of abundant, extracellular melanin for biotechnological applications*. World J. Microbiol. Biotechnol. 28: 505–512. <https://doi.org/10.1007/s11274-011-0841-0>
- Kamal U.Z., Ayesha Ali S., Sharique Ali A., Ishrat N. (2014) *Microbial tyrosinases: promising enzymes for pharmaceutical, food bioprocessing, and environmental industry*. Biochem. Res. Int. 014: 854687.
- Kiran G.S., Dhasayan A., Lipton A.N., Selvin J., Arasu M.V., Al-Dhabi N.A. (2014) *Melanin-templated rapid synthesis of silver nanostructures*. J. Nanobiotechnol. 12: 1.
- Kiran G.S., Jackson S.A., Priyadharsini S., Alan Dobson D.W., Selvin J. (2017) *Synthesis of Nm-PHB (nanomelanin-polyhydroxy butyrate) nanocomposite film and its protective effect against biofilm-forming multi drug resistant Staphylococcus aureus*. Sci. Rep. 7: 9167.
- Kotob S.I., Coon S.L., Quintero E.J., Weiner R.M. (1995) *Homogentisic acid is the primary precursor of melanin synthesis in Vibrio cholerae, a Hyphomonas strain, and Schwenella colwelliana*. Appl. Environ. Microbiol. 61: 1620–1622.
- Kurian N.K., Bhat S.G. (2018) *Food, cosmetic and biological applications of characterized DOPA-melanin from Vibrio alginolyticus strain BTKKS3*. Appl. Biol. Chem. 61(2): 163–171.
- Langfelder K., Jahn B., Gehringer H., Schmidt A., Wanner G., and Brakhage A.A. (1998) *Identification of a polyketide synthase gene (pksP) of Aspergillus fumigatus involved in conidial pigment biosynthesis and virulence*. Med. Microbiol. Immunol. 187: 79–89.
- Lopusiewicz L. (2018) *The isolation, purification and analysis of the melanin pigment extracted from Armillaria mellea rhizomorphs*. World Sci. News 100: 135–153.
- Madhusudhan D.N., Bi Bi Zainab M., Syed D.G., Dayanand A. (2014) *Production and cytotoxicity of extracellular insoluble and droplets of soluble melanin by Streptomyces lusitanus DMZ-3*. J. Biomed. Biotechnol. 5: 306895.
- Manivasagan P., Venkatesan J., Senthilkumar K., Sivakumar K., Kim S.K. (2013) *Isolation and characterization of biologically active melanin from Actinoalloteichus sp. MA-32*. Int. J. Biol. Macromol. 58: 263–274. <https://doi.org/10.1016/j.ijbiomac.2013.04.041>
- Mattoon E.R., Cordero R.J.B., Casadevall A. (2021) *Fungal melanins and applications in healthcare, bioremediation and industry*. J. Fungi. 7: 488. <https://doi.org/10.3390/jof7060488>
- Meredith P., Sarna T. (2006) *The physical and chemical properties of eumelanin*. Pigment Cell Res. 19: 572–594.
- Pralea I.E., Moldovan R.C., Petrache A.M. et al. (2019) *From extraction to advanced analytical methods: the challenges of melanin analysis*. Int. J. Mol. Sci. 20: 3943.
- Quadri S.R., Agsar D. (2012) *Detection of melanin producing thermo-alkaliphilic Streptomyces from limestone quarries of the Deccan traps*. World J. Sci. Technol. 2(2): 08–12.
- Raman N.M., Shah P.H., Mohan M., Ramasamy S. (2015) *Improved production of melanin from Aspergillus fumigatus*

- AFGRD105* by optimization of media factors. *AMB Express* 5: 72.
- Ribera J., Panzarasa G., Stobbe A., Osypova A., Rupper P., Klose D., Francis Schwarze W.M.R. (2019) *Scalable biosynthesis of melanin by the basidiomycete Armillaria cepistipes*. *J. Agric. Food Chem.* 67: 132–139.
- Romero-Martinez R., Wheeler M., Guerrero-Plata A., Rico G., Torres-Guerrero H. (2000) *Biosynthesis and functions of melanin in Sporothrix schenckii*. *Infect. Immun.* 68: 3696–3703.
- Schleheck D., Barraud N., Klebensberger J., Webb J.S., Mc Dougald D., Rice S.A., Kjelleberg S. (2009) *Pseudomonas aeruginosa PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation*. *PLoS One* 4(5): e5513. <https://doi.org/10.1371/journal.pone.0005513>
- Seelam S.D., Agsar D., Sampath Kumar H.M., Prakasham R.S., Sravanthi V., Mohan Reddy K., Umesh M.K., Rajitha C.H. (2021) *Characterization and photoprotective potentiality of lime dwelling Pseudomonas mediated melanin as sunscreen agent against UV-b radiations*. *J. Photochem. Photobiol. B: Biol.* 216: 112126.
- Sendovski M., Kanteev M., Ben-Yosef V.S., Adir N., Fishman A. (2011) *First structures of an active bacterial tyrosinase reveal copper plasticity*. *J. Mol. Biol.* 405: 227–237. <https://doi.org/10.1016/j.jmb.2010.10.048>
- Serrano D.L., Solano F., Sanchez-Amat A., (2004) *Identification of an operon involved in tyrosinase activity and melanin synthesis in Marinomonas mediterranea*. *Gene* 342: 179–187.
- Shanmuganathan K., Cho J.H., Iyer P., Baranowitz S., Ellison C.J. (2011) *Thermooxidative stabilization of polymers using natural and synthetic melanins*. *Macromolecules* 44: 9499–9507. <https://doi.org/10.1021/ma202170n>
- Shivaveerakumar S., Madhusudhan D.N., Agsar D., Kontro M. (2013) *Catalytic zone, a novel screening approach for the extracellular tyrosinase by Streptomyces vinaceusdrappus DSV5*. *J. Pure Appl. Microbiol.* 7: 2799–2808.
- Singh D., Kumar J., Kumar A. (2018) *Isolation of pyromelanin from bacteria and evidences showing its synthesis by 4-hydroxyphenylpyruvate dioxygenase enzyme encoded by hppD gene*. *Intl. J. Biol. Macromol.* 119: 864–873.
- Sivaperumal P., Kamala K., Rajaram R. (2014) *Bioactive DOPA melanin isolated and characterised from a marine actinobacterium Streptomyces sp. MVCS6 from Versova coast*. *Nat. Prod. Res.* 29: 2117–2121.
- Sun S., Zhang X., Chen W., Zhang L., Zhu H. (2016) *Production of natural edible melanin by Auricularia auricula and its physicochemical properties*. *Food Chem.* 196: 486–492.
- Surwase S.N., Jadhav S.B., Phugare S.S., Jadhav J.P. (2013) *Optimization of melanin production by Brevundimonas sp. SGJ using response surface methodology*. *3 Biotech* 3: 187–194.
- Thorat M.N. and Dastager S.G. (2018) *High yield production of cellulose by a Komagataeibacter rhaeticus PG2 strain isolated from pomegranate as a new host*. *RSC advances.* 8: 29797–29805. <https://doi.org/10.1039/C8RA05295F>
- Toledo A.V., Franco M.E.E., Yanil Lopez S.M., Troncozo M.I., Saparrat M.C.N., Balatti P.A. (2017) *Melanins in fungi: types, localization and putative biological roles*. *Physiol. Mol. Plant Pathol.* 9: 2–6.
- Tran-Ly A.N., Reyes C., Francis Schwarze W.M.R., Ribera J. (2020) *Microbial production of melanin and its various applications*. *World J. Microbiol. Biotechnol.* 36: 170.
- Vasanthabharathi V., Lakshminarayanan R., Jayalakshmi S. (2011) *Melanin production from marine Streptomyces*. *Afr. J. Biotech.* 10(54): 11224–11234.
- Vijayan V., Jasmin C., Anas A., Parakkaparambil Kuttan S., Vinothkumar S., Perunnikulath Subrayan P., Nair S. (2017) *Sponge-associated bacteria produce non-cytotoxic melanin which protects animal cells from photo-toxicity*. *Appl. Biochem. Biotechnol.* 183(1): 396–411.
- Wang R., Wang H., Zhou H., Wang Y., Yue J., Diao B., Kan B. (2011) *Characters of homogentisate oxygenase gene mutation and high clonality of the natural pigment-producing Vibrio cholerae strains*. *BMC Microbiol.* 11: 109.
- Williams R.F. (1994) *Melanin-based agents for image enhancement*. U.S. Patent No. 5310539.
- Woo P.C., Tam E.W., Chong K.T., Cai J.J., Tung E.T., Ngan A.H., Lau S.K., Yuen K.Y. (2010) *High diversity of polyketide synthase genes and the melanin biosynthesis gene cluster in Penicillium marneffeii*. *FEBS J.* 277: 3750–3758.
- Yang J., Li W., Bun Ng T., Dend X., Lin J., Ye X. (2017) *Laccases: production, expression regulation, and applications in pharmaceutical biodegradation*. *Front. Microbiol.* 8: 832. <https://doi.org/10.3389/fmicb.2017.00832>
- Zerrad A., Anissi J., Ghanam J., Sendide K., El Hassouni M. (2014) *Antioxidant and antimicrobial activities of melanin produced by a Pseudomonas balearica strain*. *J. Biotech. Lett.* 5(1): 087–094.
- Zheng H., Li X., Zhou R., Li L., Guo X., Kang J., Li D. (2010) *Bioinformatics analysis of tyrosinase-related protein 1 gene (TYRP1) from different species*. *Front. Agric. China.* 4(1): 109–115.