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Isolation, Physiochemical Characterization and biological applications of Melanin Pigment from *Pseudomonas* sp. PNKS1

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Abstract- Melanin pigment produced by various microorganisms. In this study, soil isolate produce the melanin pigment in tyrosine medium which is identified Pseudomonas sp. closely related to stutzeri , the phenotypic characterization was identified. The purified pigment physical chemical properties was studied and characterized using UV-visible and FTIR analysis. The antibacterial and antioxidant assay were applicable in pharmacology.

Keywords- Pigment, DPPH, Antioxidant, *Pseudomonas* sp., Antimicrobial activity.

I. Introduction

Melanins are negatively charged, hydrophobic and ubiquitous pigment. Melanins are macromolecules, synthesized by many living organisms. These are hydrolyzed and polymerized the organic compounds such as various phenolic substances. Microbial melanins are act as a photoprotectants (against UV and visible light), charge transport mediators, free-radical scavengers, antioxidants, metal ion balancers and etc. Melanins are basically classified into three type's eumelanins, pheomelanins and allomelanins. Eumelanins are black to brown colour pigments and Pheomelanins are brown, red or yellow color pigments which are produced in course of oxidation of tyrosine and/or phenylalanine to dihydroxyphenylalanine (DOPA) and dopaquinone. Allomelanins include nitrogen free heterogeneous group of polymers formed from catechol precursors[1,2]. The fungal melanins are *Cryptococcus neoformans*, *Sporothrix schenckii*, *Sepia officinalis*, *Aspergillus niger*, *Penicillium marneffei*, *Paracoccidioides brasiliensis*, *Histoplasmacapsulatum* [3]. Bacterial melanins are *Aeromonassalmonicida*, *Azotobacter*, *Mycobacterium*, *Micrococcus*, *Bacillus*, *Legionella*, *Streptomyces*, *Rhizobium*, *Vibrio*, *Proteus*, *Azospirillum*, *Pseudomonas aeruginosa*, *Hypomonas* sp, *Burkholderia cepacia*, *E. coli*, *Bordetella pertusis*, *Campylobacter jejuni*, *Yersinia pestis* etc [4,5]. The production of melanin by living organisms at various conditions and different kinds of melanin represents the taxa group that are evolutionary importance[6].Microbial melanins have great applications in various fields such as agriculture, cosmetics, and pharmaceutical industries [7]. The biological functions of melanin were photoprotection, thermoregulation, action as free radical sinks, cation chelators, and antibiotics. The microbial melanins are protects against from the environmental stress. Bacterial melanins are more resistance to antibiotics [8], and melanins are also involved fungal pathogenesis of plants [9].Many researchers focused the melanin from Streptomycete showed photoprotection and mosquitocidal activity of Bacillus thuringiensis subsp. Israelensis [10]. Marine melanin from *Vibrio cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana* synthesized pyomelanin catabolisms of tyrosine via Tyrosine degradation pathway [11]. Marine bacteria *Marinomonas mediterranea* MMB-1T producing melanin's which belongs to the phylum *Proteobacteria* [12] and Thermo-alkaliphilic *Streptomyces* from limestone quarries of the Deccan traps [13]. In this study, the melanin producing *Pseudomonas* sp. PNKS1was characterized and screened. The isolate was tested on various temperature and pH. The extracted pigment from *Pseudomonas* sp. PNKS1was characterized by UV, FTIR and the biological application was further preceded.

II. Screening of Melanin Pigment Producing Bacteria

Melanin producing isolate was isolated from Nilgris soil sample and it was serially diluted. The 10^{-7} diluted sample was selected to isolate the pigment producing isolate and it was spread on melanin produing media ie. Serially diluted sample was spread over in LB agar (Tryptone 20 g/l, Yeast extract 5 g/l, Sodium chloride 10 g/l, Agar 15 g/l). Different isolates were selected and purified to be further used for the screening step over the M9 minimal medium (Na2HPO4.12 H2O, 15.02 g/l; KH2PO4, 3 g/l ; NH4Cl, 1 g/l ; NaCl, 0.5 g/l

; MgSO4, 1 mM ; CaCl2, 0.1 mM ; Agar, 20 g/l ; pH 7.4), and containing L-tyrosine at 1 g/l, and trace elements of CuSO4 and FeSO4 at 10 µM each with pH 7.0. The medium was autoclaved at 15 psi (120◦C) for 20 min. After autoclave the agar plates were prepared the inoculums was streaked. Then plates were incubated at 25◦C for 48h on rotary shaker, until the liquid medium becomes darkly pigmented and nearly opaque. The diffusible pigment melanin production was observed and the colonies were selected. The selected colonies are sub cultured and characterized. The isolate PNKS1 was identified based on the morphological and biochemical characterization based on the Bergey's Manual of Systematic Bacteriology [14], and 16S rRNA gene sequencing was performed using universal bacterial primers such as the forward primer 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and the reverse primer 1525r (5′-AAG GAG GTG WTC CAR CC-3′). The strain PNKS1 was sequenced and it was deposited in National Center for Biotechnology Information (NCBI) database under the Gene bank accession no. KU243132.The strain PNKS1 16S rRNA gene sequence was searched in nucleotide sequence databases by running the BLASTN program of National Centre for Biotechnology Information (NCBI) available at http://blast.ncbi.nih.gov [15]. The phylogenetic analysis was performed by based on the evolutionary relationships and closeness among the related genera. The evolutionary history was inferred using the neighbour-joining (NJ) method [16]. The identified sequences were aligned using CLUSTALX program of GenBank database [17].

III. Pigment production, extraction and purification

After the pigment production, the culture was centrifuge at 8000 rpm for 15 min to separate the cells and supernatant was discarded. The separated cells were suspended with distilled water again the cells were centrifuged. The extracted melanin pigment was adjusted to 10 with 5M NaOH then acidified with 3 N HCl to pH-2 and allowed to stand for 48 h initially at room temperature. This process was repeated until precipitation was not found, after the suspension was boiled at 5min. Then it was centrifuged at 4000 rpm for 15 min. After obtain the crude extract the equal volume of chloroform, ethyl acetate and methanol (1V:1V:1V) were added and mixed well. The steps repeated to 3-4 times. Finally the aqueous phase was concentrated by a rotary evaporator and the pigment was then collected [18].

Confirmation of melanin

After the fermentation, the kinetic of L-tyrosine uptake by medium was modified by this method [19]. 1ml of sulfate mercuric solution (15% in 5N sulphuric acid) was added to 1 ml of cell-free supernatant and left at 95°C for 10 minutes. Then, 1 ml of nitrite reagent (0,2% of sodium nitrite) was subsequently added and the total volume was made up to 5 ml with distilled water.

Spectroscopic Analysis

The extracted melanin UV-visible spectrum was performed in a solution of 50 mM Tris-HCl (pH 8.5) and was scanned from 200 to 1000 nm wavelengths. The spectra were determined using UV-Visible spectrophotometer. The extracted pigment was ground with IR grade KBr (1:10). The FTIR spectrum was recorded at 4000-400 cm-1 using a VERTEX 70 FT-IR spectrophotometer.

Chemical Analysis of the Pigment

Melanin pigment chemical analysis was carried out by a modified method [20]. The pigment solubility was tested with distilled deionized water, 1 N HCl, 1 N NaOH, ethanol, acetone; chloroform, benzene, and phenol were checked. Oxidizing agents such as 6% sodium hypochlorite (NaOCl) and 30% hydrogen peroxide (H2O2) were used to determine the reducing power of the pigment. The pigment was also precipitated with 1% FeCl3, ammoniacal silver nitrite, and potassium ferricyanide. The results were which help to identify the quality and physiochemical properties of the pigment.

Effect of *Pseudomonas* sp. PNKS1 Melanin production in different media

Different medium were selected for the *Pseudomonas* sp. PNKS1melanin production was observed.

Antimicrobial Activity of Extracted Pigment

Different pathogens were selected for antimicrobial activity of the pigment. The test pathogens, *Klebsiella pneumonia* ATCC 1388, *Entercoccus faecolis* ATCC 495, *Escherichia coli* ATCC 10536, *Proteus vulgaris* ATCC 33420, *Salmonella typhi* ATCC 13311 were cultivated in Muller-Hinton agar plates.10 µg of pigment extract was selected the antimicrobial activity by well diffusion method. The plates were incubated at 30°C or 37°C for 24 hrs. Observe the zone formation around the well and the antimicrobial activity of pigments was evaluated.

DPPH radical scavenging assay

The DPPH radical scavenging activity of *Pseudomonas* sp. PNKS1 produced melanin was tested by modified method Anissi et al (2014). 0.1mg to 5mg melanin was mixed with 0.4mmol/l DPPH. Observe the reduced spectrum readings for free radical scavenging assay. UV absorbance was observed in 515nm. The antioxidant activity was determined by method.

IV. Result and Discussion

Strain characterization

The melanin producing strain PNKS1 was isolated from the soil sample and placed on L Tyrosine medium. The brown pigment producing strain was identified as *Pseudomonas* sp. PNKS1, based on the morphological characterization, molecular characterization such as16S rDNA sequence and

biochemical characteristics (Table 1). This bacterium was resistant to ampicillin, but sensitive to streptomycin, cefotaxime. Figure.1 showed the phylogenetic relationship of *Pseudomonas sp*. The strain PNKS1 showed 100% homology with *Pseudomonas stutzeri* SMG-8 NRB DRDO MP and 99% homology with *Pseudomonas stutzeri* SIITMB7. The molecular approaches of 16sr RNA secondary structure prediction (Figure.2) 32 stems are identified and using the NEB cutter restriction enzymes analysis (Figure.3) 53 enzymes was identified.

Table.1. *Pseudomonas* sp. PNKS1 morphological and biochemical characterization studies.

Characteristic	Result
Cell morphology	rod shape, slimy, opaque, motile
Growth Temperature	30,37,45,50
Growth at Nacl	6,7,8,9
Growth at pH	0.5,2,4,6
Biochemical tests	catalase, oxidase, urease, nitrate Positive

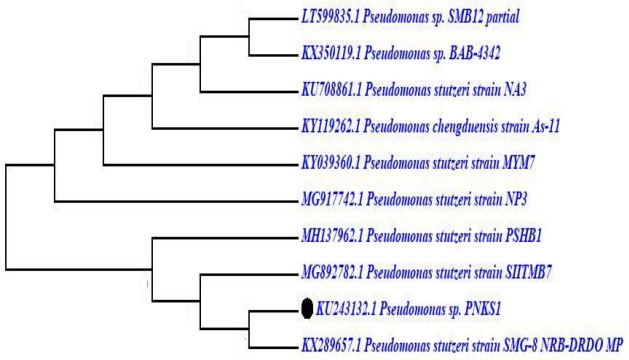

Figure .1. *Pseudomonas* sp. PNKS1 phylogenetic analysis

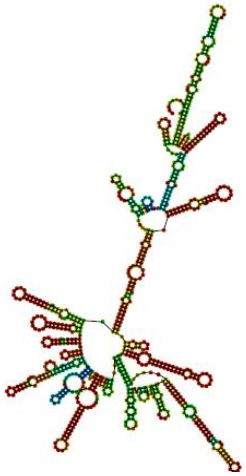

Figure .2. *Pseudomonas* sp. PNKS1 16srrna secondary structure

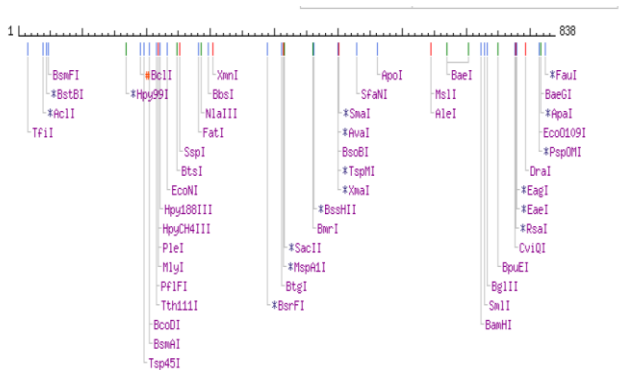

Figure .3. *Pseudomonas* sp. PNKS1 Restriction enzyme analysis

Spectroscopic Analysis of the Pigment

The *Pseudomonas* sp. PNKS1, UV / Visible spectrum of the pigment showing the strong absorbance in UV region was 350nm (Figure.4) and FTIR spectroscopy analysis (Figure.5) was revealed the functional groups were 3435,2391, 1637 and 1113 that indicates the N-H stretch (1,2 amines), C=N stretch (Nitriles), -C=C- stretches (alkenes), C-N stretches (aliphatic amines). The functional groups are moderate similar to *Pseudomonas balearica* strain.

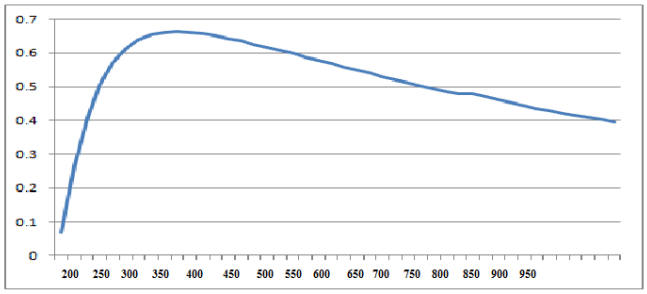

Figure .4. *Pseudomonas* sp. PNKS1 melanin UV spectrum

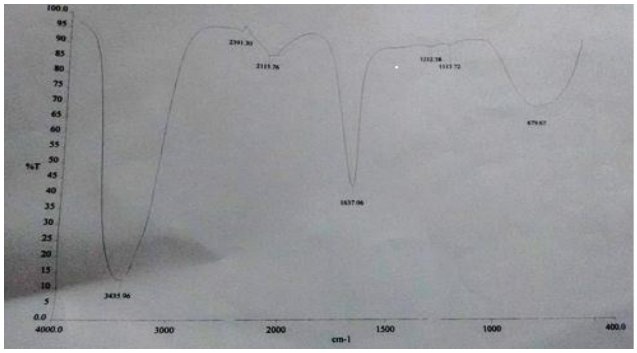

Figure .5. *Pseudomonas* sp. PNKS1 melanin FTIR spectrum

Chemical Analysis of the Pigment

The chemical analysis of Pseudomonas sp. PNKS1 melanin was tested by solubility, solubility in various organic solvents. The Pseudomonas sp. PNKS1Melanin was soluble in water, good soluble in ethanol, methanol, ethyl acetate

and the precipitations with alkane solutions are KOH,NaOH, HCl. Reactions with oxidizing agents such H_2O_2, $Fecl_3$,$NaoBH_4$, KMnO4.

Pseudomonas sp. PNKS1 Melanin production in different media

The growth pattern of the Pseudomonas sp. PNKS1 and the melanin production were determined in different media. The positive results for melanin production in Tryptone yeast extract broth(ISP-1), Yeast extract malt extract agar medium (ISP-2), Oatmeal agar medium (ISP-3), Peptone yeast extract–iron agar medium (ISP-6).

Antimicrobial Activity of Extracted Pigment

The melanin pigment antimicrobial activity was tested against different pathogens. The results showed that strong antibacterial activity against *Entercoccus faecolis* ATCC 495, *Escherichia coli* ATCC 10536. The zone inhibition was higher in *Escherichia coli* ATCC 10536 (23mm) and *Entercoccus faecolis* ATCC 495 (19mm).

DPPH radical scavenging assay

The DPPH radical scavenging activity of *Pseudomonas* sp. PNKS1was determined by various melanin concentrations. The reduction of melanin activity based on concentrations and compare to standard *Pseudomonas* sp. PNKS1melanin strong activity with DPPH molecule reduction.

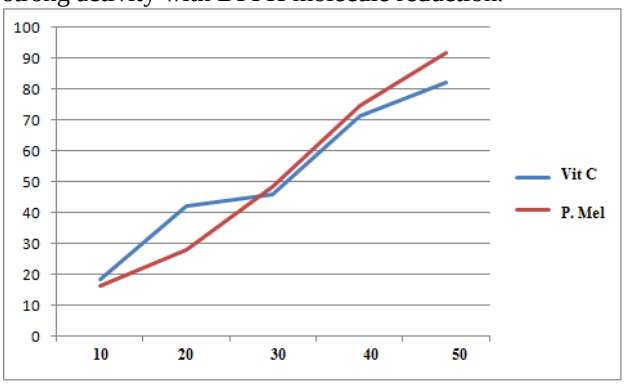

Figure. 6

V. Conclusion

Different soil bacteria were isolated but only isolate produced the melanin pigment. Different mediums were used to produce a melanin, the maximum production was observed in 350nm and functional groups of *Pseudomonas* sp. PNKS1melanin was analysed by FTIR. Finally biological applications of PKN1 melanin were confirmed by antibacterial activity and antioxidant assay.

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References

[1]. Gómez-Marín A. M., and Sánchez C. I., "Thermal and mass spectroscopic characterization of a sulphur-containing bacterial melanin from Bacillus subtilis", J. Non-Cryst.Solids., 356, 1576–1580,2010.
[2]. Harki E., Talou T., and Dargent R., "Purification, characterisation and analysis of melanin extracted from Tuber melanosporum", Food Chem., 58, 69–73, 1997.
[3]. Youngchim S., Morris-Jones R., Hay R. J., and Hamilton A. J.," Production of melanin by Aspergillus fumigates", J. Med. Microbiol., 53, 175–181, 2004.
[4]. Geng J., Yuan P., Shao C., Yu S. B., Zhou B., Zhou P., and Chen X. D., "Bacterial melanin interacts with doublestranded DNA with high affinity and may inhibit cell metabolism in vivo", Arch. Microbiol., 192, 321–329 , 2010.
[5]. Coyne V. E., and Al-Harthi L., Induction of melanin biosynthesis in Vibrio cholera, Appl. Environ Microbiol., 58, 2861–2865,1992.
[6]. Wood, J. M., K. Jimbow, R. E. Boissy, A. Slominski, P. M. Plonka, J. Slawinski, J. Wortsman, and J. Tosk. "What is the use of generating melanin?" Exp. Dermatol. 8: 153-164, 1999.
[7]. Dastager S, Li WJ, Dayanand A, Tang SK, Tian XP, Zhi XY, Xu LH,Jiang CL , " Separation, identification and analysis of pigment (melanin) production in Streptomyces". Afr J Biotech 5:1131–1134, 2009.
[8]. Lin WP, Lai HL, Liu YL, Chiung YM, Shiau CY, Han JM, Yang CM,Liu YT " Effect of melanin produced by a recombinant Escherichia coli on antibacterial activity of antibiotics". J Microbiol Immunol Infect 38:320–326, 2005.
[9]. Butler M, Day A, "Fungal melanins: a review". Can J Microbiol 44:1115–1136, 1998.
[10]. Liu, Y. T., M. J. Sui, D. D. Ji, I. H. Wu, C. Chou, and C. C. Chen."Protection from UV irradiation by melanin of mosquitocidal activity of Bt. var. Israeliensis" . J. Invertebr. Pathol. 62: 131-136, 1993
[11]. Kotob S. I., Coon S. L., Quintero E. J., and Weiner E. M., "Homogentisic acid is the primary precursor of melanin synthesis in Vibrio cholerae, a Hyphomonas strain, and Shewanella colwelliana", Appl. Environ. Microbiol., 61,1620–1622 (1995).
[12]. Lucas-Elío P., Goodwin L., Woyke T., Pitluck S., Nolan M.,Kyrpides N. C., Detter J. C., Copeland A., Teshima H., Bruce D., Detter C., Tapia R., Han S., Land M. L., Ivanova N., Mikhailova N., Johnston A. W. B., and Sanchez-Amat A., "Complete genome sequence of the melanogenic marine bacterium Marinomonas mediterranea type strain (MMB- 1T)", Stand. Genomic. Sci., 6, 63–73, 2012.
[13]. Quadri S. R., and Agsar D., "Detection of melanin producing thermo-alkaliphilic Streptomyces from limestone quarries of the Deccan traps", World J. Sci. Technol., 2, 8-12, 2012.
[14]. Krieg, N.R. and Holt, J.G. "Gram negative rods and cocci. In Bergey's Manual of Systematic Bacteriology ed. Holt J.G. pp. 140–219. Baltimore, MD: Williams and Wilkins.1989.
[15]. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. "Gapped BLAST and PSI-BLAST: a new generation of protein database search program".. Nucl Acids Res 25, 3389–3402, 1997.
[16]. Saitou, N. and Nei, M. "The neighbor-joining method: a new method for constructing phylogenetic trees". Mol Biol Evol 4, 406–425, 1987.

[17]. Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F.and Higguns, D.G. "The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools". Nucl Acids Res 24, 4876–4882, 1997.
[18]. Vasanthabharathi V., Lakshminarayanan R. & Jayalakshmi S." Melanin production from marine Streptomyces" African Journal of Biotechnology, 54, 11224-11234, 2011.
[19]. Fava F., Gioia D.D. & Merchetti LChemosphere, 27, 825-835, 1993.
[20]. Zerrad A., Anissi J., Ghanam J., Sendide K. & EL Hassouni M. "Antioxidant and antimicrobial activities of melanin produced by a pseudomonas balearica strain", Journal of Biotechnology Letters ISSN: 0976-7045, 2014.

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