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Kshitija R Deshmukh
P. G. Department of Microbiology, Shri Shivaji College of Arts, Commerce and Science, Akola - 444001, Maharashtra, India.

Archana S Pethe
P. G. Department of Microbiology, Shri Shivaji College of Arts, Commerce and Science, Akola - 444001, Maharashtra, India.

Correspondence
Archana S Pethe
P. G. Department of Microbiology, Shri Shivaji College of Arts, Commerce and Science, Akola - 444001, Maharashtra, India.

Isolation and identification of melanin producing bacterium from sewage water

Kshitija R Deshmukh, Archana S Pethe

Abstract
In this study, a melanin producing bacterium was isolated from sewage water sample collected from Akola region of Maharashtra using nutrient agar supplemented with L-tyrosine. The morphological and biochemical analysis showed that the bacterium was gram negative short rod shaped, highly motile, opaque irregularly shaped large colonies with lobate margin, raised elevation and smooth texture. Also, the isolate was oxidase, catalase and caseinase positive. It was citrate positive while indole, methyl red and voges-proskauer negative. Moreover, it can ferment glucose but not sucrose, starch and lactose. The isolated bacterium produced nearly black colored pigment which can be tentatively inferred as melanin due to its ability to utilize L-tyrosine. After phylogenetic identification, this bacterium was found to be closest neighbor of *Pseudomonas guezennei*

Keywords: melanin pigment, *Pseudomonas* spp., L-tyrosine

1. Introduction
Melanin is a biopolymer, a macromolecule with undefined structure and molecular weight. It performs many roles in the biosphere including as pigments and photo protectants. In addition, melanins have several functions like thermoregulation, acting as free radical sinks, cation chelators, and antibiotics. In plants, melanin acts as cell wall strengthener (Riley, 1997) [1], while in humans it determines the skin color and also plays a crucial role in protecting the skin against ultra violet light (Huang *et al*, 2012) [4].In the microbes, melanin acts as a protective agent against environmental stresses for example, melanin makes the bacteria resistant to antibiotics (Lin *et al*, 2005) [6]. Also, it shows antitumor activity (Hassib *et al,* 2006) [5]. antivenin activity (Huang *et al,* 2004) [7], anti-virus (Zhou *et al,* 1991) [8], liver protecting activity (Huang *et al,* 2003) [9] and radio protective (Casadevall *et al,* 2007) etc. They are widely used in medicine, pharmacology, cosmetics and other fields.
They have been shown to possess these several interesting physical and chemical properties. However, these properties are, in general, poorly understood. Because melanin is an aggregate of smaller component molecules, there area number of different types of melanins of various proportions and bondings. For example, Eumelanins are one of the types of melanins which are black or brown pigment produced in the course of oxidation of tyrosine (and/or phenylalanine) to *o*-dihydroxy- phenylalanine (DOPA) and then to dopaquinone, which further undergoes cyclization to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The other types are namely pheomelanin, allomelanins, sepia melanin and neuromelanin.
Several bacteria are reported to produce melanin. These include pyomelanin producers like *Pseudomonas aeruginosa***,** *Vibrio cholera*, etc., many marine bacteria also known to produce melanin which include *Vibrio cholerae* and *Shewanella colwelliana*. Several marine actinomycetes like *Streptomyces* strains are reported to use tyrosinase enzyme (one of the most studied) in the synthesis of melanin pigment. Another important melanin-synthesizing bacterium is *Marinomonas mediterranea*, which produces black eumelanin from L-tyrosine.

2. Material & method

2.1 Chemicals and Sample collection

L-tyrosine and media used for isolation were purchased from Himedia chemicals, Mumbai, India and all other chemicals used were of analytical reagent grade.

The sewage water sample was collected from a city drainage in Akola, Maharashtra (India).

2.2 Bacterial isolation

Bacterium was screened by pour plating followed by quadrant streaking technique to purify the isolate. The agar medium used to grow the isolate contained peptone, 5g/L; yeast extract, 3g/L; sodium chloride, 5g/L; L-tyrosine, 2g/L and distilled water and was incubated at 37 °C for 7 days. The brown to black colored bacterial colony producing diffusible brown to black colored pigment were isolated from the mix cultured plate. The pure culture was maintained on the above mentioned agar medium plates and slants.

2.3 Morphological and Biochemical Studies

The morphological characteristics of the bacterial colony such as colony shape, size, margin, elevation, optical property, gram character, motility were studied. Then, the biochemical studies of the bacterium were also performed including enzyme tests (oxidase, catalase, urease, caseinase, amylase and gelatinase), IMViC tests (Indole, Methyl red, Voges-Proskeuar and citrate utilization) and carbohydrate utilization tests (glucose, lactose, fructose, sucrose, mannitol, starch, xylose).

2.4 Molecular identification of melanin producing bacteria DNA Extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505). The DNA isolated from bacteria was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The size obtained was approx. 850bp for 16S rRNA region. The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI).

2.5 Phylogenetic characterization of the bacteria

The BLAST results were used to find out evolutionary relationship of bacteria. Altogether twenty sequences, including sample were used to generate phylogenetic tree. The tree was constructed by Neighbor-Joining method using MEGA 5 software (Saitou N. and Nei M., 1987; Felsenstein J. 1985)

3. Results & Discussion

Table 1: Morphological characteristics of KRDB4.

Characteristic \ Isolates	KRDB4
Colony Shape	Irregular/Spreading
Colony size (in mm)	5-15
Colony margin	Lobate
Colony elevation	Raised
Optical property/ Density	Opaque
Texture	Smooth
Appearance	Shiny
Motility	Motile
Gram Character	Gram negative rods
Pigmentation	Dark brown, Diffusible

Table 2: Biochemical characteristics of KRDB4.

Tests \ Isolates	KRDB4
Enzyme Utilization:	
Oxidase	+ve
Catalase	+ve
Amylase	-ve
Gelatinase	-ve
Urease	-ve
Caseinase	+ve
IMViC:	
Indole	-ve
Methyl red	-ve
Voges-Proskeur	-ve
Citrate Utilization	+ve
Carbohydrate Fermentation:	
Lactose	-ve
Glucose	+ve
Sucrose	-ve
Mannitol	+ve
Fructose	+ve
Maltose	-ve
Starch	-ve

A melanin producing bacterium was isolated from sewage water sample when grown on nutrient agar medium supplemented with L-tyrosine for 2-3 days. The colony capable of producing brown to black colored pigment were picked (labelled temporarily as KRDB4) and identified as Pseudomonas *spp.* based on its morphological (Table 1) and biochemical characteristics (Table 2). According to the phylogenetic identification using 16S rDNA sequencing technique, the closest neighbor of KRDB4 was found to be *Pseudomonas guezennei.* The phylogenetic relationship of this strain is shown in Fig.1.

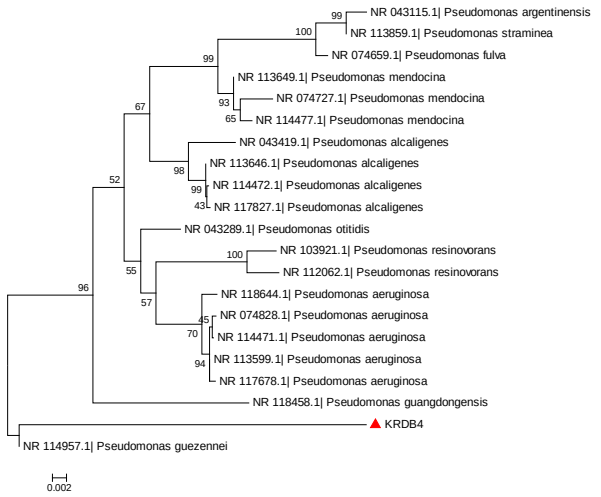

Fig 1: Phylogenetic tree showing the position of isolate KRDB4 with reference to related strains.

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