Halophilic bacteria *Bacillus altitudinis* MIM2 producing bioactive melanin isolated from Mundra port, Kutch, Gujarat, India

Pankhaniya Mira Gordhanbhai, Chavda Jigna, Noble K Kurian*

Department of Microbiology, Atmiya University, Rajkot, Gujarat

*Corresponding author: kurianoble@gmail.com , noble.kurian@atmiyauni.ac.in

Abstract

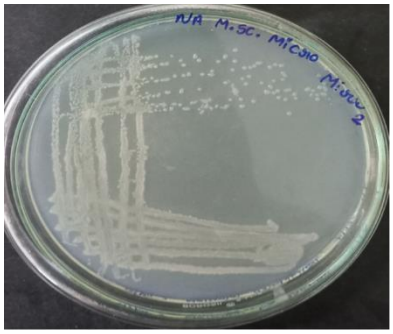
Melanins are ubiquitous black or brown color pigments exhibiting a wide variety of bioactivities. They are stable and insoluble in nature. Melanin are industrially important pigment currently used in cosmetics, medicine and pharmaceuticals, industries. Bacteria mainly produce three types of melanin namely, Eumelanin, pheomelanin and pyomelanin which is usually extracellularly secreted. This makes the downstream processing of bacterial melanin easier. Stress conditions like salt stress, radiation stress etc. triggers the bacteria to produce melanin and several bacterial species were found to produce melanin under different stress induced conditions. In this present study we had isolated melanin producing bacteria from saline sediment sample of Mundra port, Kutch region of Gujarat state of India. The melanin producing bacteria was characterized using staining, biochemical and molecular methods. The melanin produced was extracted and analyzed using physicochemical techniques. The extracted melanin had shown good anti-bacterial and radical scavenging activity. To our knowledge, this is the first report on *Bacillus altitudinis* producing melanin.

Keywords: melanin, bacteria, halophilic, saline soil

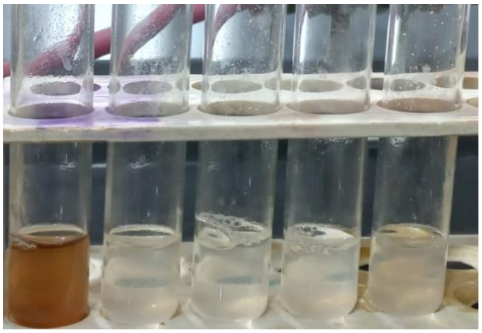
Melanins are bioactive pigments produced most of the living organism. Its function mainly ranges from photoprotection to contributing in virulence in these organisms (Kurian & Bhat, 2017). These black pigments can be classified mainly into 3 types based on their difference in biosynthetic process. They are eumelanins which synthesized via DOPA, while cysteine is incooperated to DOPA in second type of melanin i.e., pheomelanin. All other remaining types of melanin are classified under allomelanins which are synthesized from a wide variety of substrates. Allomelanins include bacterial pyomelanin, fungal DHN melanins, plant catechol melanin and so on (Kurian & Bhat, 2014). Though the melanins originate via different metabolic pathway they exhibit similar physicochemical properties. The properties include their nonpolar nature, effective antioxidant activity, efficient metal chelating and radioprotective activity makes them a good candidate in many useful applications. Extraction of melanin is complex process in many organisms as contamination of cellular materials could occur in the pigment especially protein contamination. Bacteria produce melanin extracellularly in culture medium makes it easy for the downstream processing of the pigment compared to melanin from other organisms. This makes bacterial melanin preferred over melanin from other sources. Major drawback about bacterial melanin is that bacteria only produce low quantity of the pigment. So, the search for better melanin producing bacteria is still a major research topic in bioprocess technology (Singh et al, 2021). Stress usually induces bacteria to produce melanin. Bacteria isolated from stress prone areas are considered to be good candidate strains to be screened for melanin production. In the present research melanin

producing bacteria was isolated from the saline soil of Kutch region and the melanin produced was characterized.

The bacteria were isolated from saline soil of Mundra Port, Kutch Region (22.74ºN, 69.7ºE), Gujarat, India. Saline soil was collected in autoclaved polythene bags and brought to the laboratory. One gram of the soil is serially diluted and dilutions were spread plated on nutrient agar containing 4% sodium chloride for selecting halophilic bacteria. Total of 30 colonies were obtained which are subjected to quadrant streaking to obtain pure colonies (Figure 1 (a)). These colonies were subjected to melanin production in 10 mL tyrosine basal broth in test tubes. After 10 days of incubation one tube (MIM2) had shown good colour change from white to dark brown in colour (Figure 1 (b)).



(a)



(b)

Figure 1 (a) Nutrient agar plates showing MIM2 colonies (b) tyrosine basal broth tube with MIM2 showing melanin production

Characterization of bacteria was done using gram staining and biochemical techniques. The bacteria MIM2 was found to be gram positive rods (Figure 2) with Indole, Voges-Proskauer and Citrate tests found to be negative while Methyl Red and catalase tests had shown to positive (Table 1). The genotypic characterization of MIM2 was done using 16S rDNA sequencing and sequence analysis was done using bioinformatics tools. After sequencing at National Center for Microbial Resource- NCCS, Pune the sequence identity was determined using NCBI Nucleotide BLAST tool (Altschul *et al*., 1990). The organism MIM2 was identified as *Bacillus altitudinis* and the sequence is submitted to GenBank and accession number (OM967420) was obtained. Phylogenetic tree (Figure 3) was constructed using the Neighbor Joining method (Saitou and Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and nonsynonymous nucleotide substitutions using the software MEGA 7. Statistical support for branching was estimated using 1000 bootstrap steps.

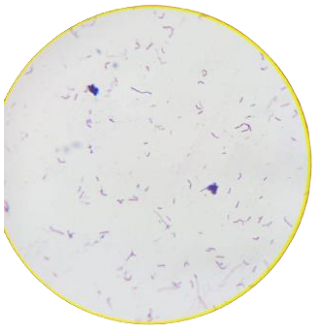


Figure 2: Gram staining image of MIM2 (40X) showing gram positive rods

Biochemical test	Results
Indole	-
Methyl Red	+
Voges-Proskauer	-
Simmons Citrate	-
Catalase	+

Table 1: Biochemical Characterization of MIM2

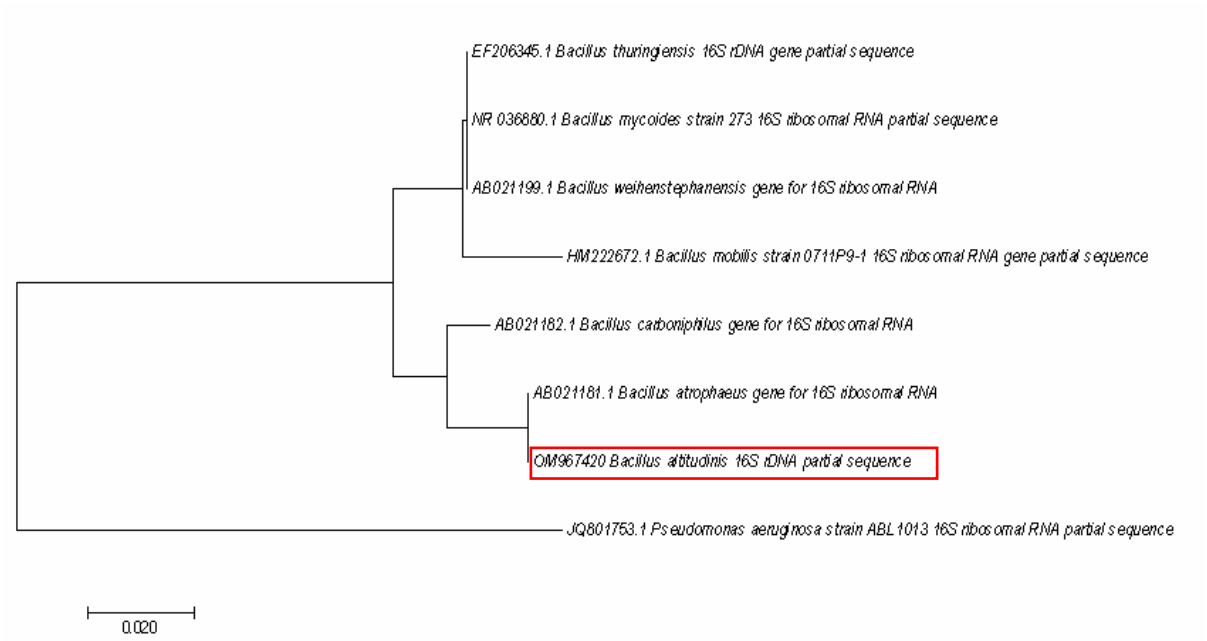


Figure 3: The phylogenetic tree of MIM2 inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.23991073 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 262 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Strain MIM2 had shown sensitive to majority of the antibiotics tested. The strain had shown resistant to CF: Cefotaxime and Cl: Ceftizoxime only (Figure 4, Table 2).

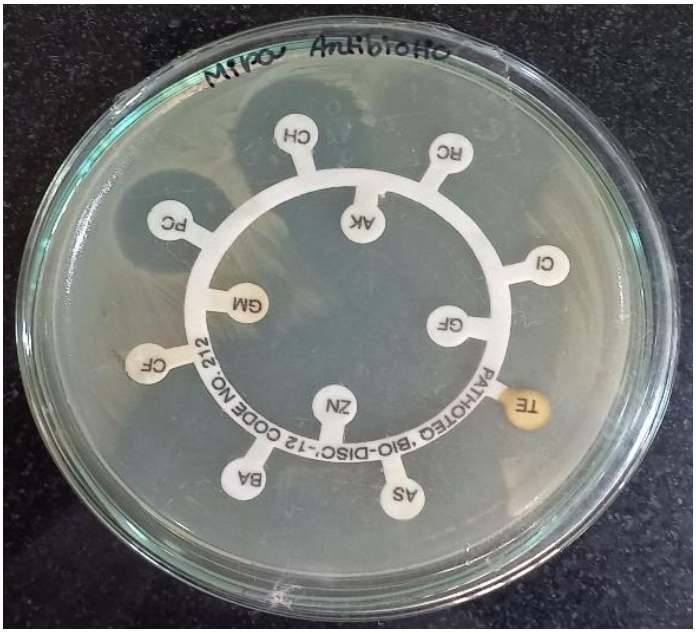


Figure 4: Antibiotic sensitive tests for the strain MIM2

Antibiotics	**Sensitive (+) Resistant (-)**
AS: Ampicillin	+
BA: Co-Trimoxazole	+
CF: Cefotaxime	-
PC: Piperacillin	+
CH: Chloramphenicol	+
RC: Ciprofloxacin	+
Cl: Ceftizoxime	-
TE: Tetracycline	+
ZN: Ofloxacin	+
GM: Gentamicin	+
AK: Amikacin	+
GF: Gatifloxacin	+

Table 2: Antibiotic sensitivity profile of strain MIM2

For scaling up the melanin production 1 OD culture of MIM2 was inoculated in tyrosine basal broth and melanin production was monitored every 12 hours intervals. Color change was observed from the 6th day of incubation and it increased up to 10 days of incubation. After 10th day, the amount of melanin (Figure 5 (a)) produced was estimated spectrophotometrically at 400nm and the concentration of melanin produced was quantified using synthetic melanin standard curve. The amount of melanin produced by MIM2 was found to be 245.67±8.7 mg/L. Melanin produced is extracted by acid precipitation and subsequent washing in organic solvents to get purified melanin.

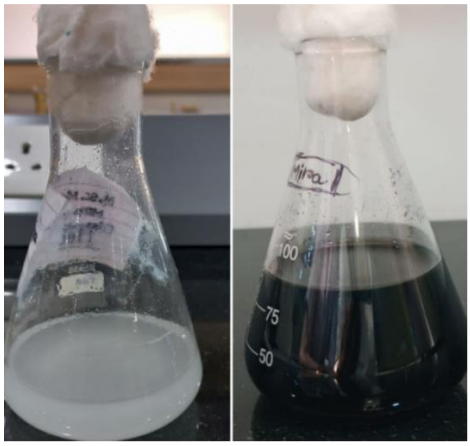
The chemical reactivity of MIM2 melanin was tested by testing its solubility in acidic, alkaline and organic solvents (Table 3). Melanin had found to be soluble in 1 N NaOH only, and in DMSO it was found to be sparingly soluble with some particles remaining.

Solvent	**Solubility**
Distilled Water	Insoluble
1N NaOH	Soluble
Ethanol	Insoluble
Methanol	Insoluble
DMSO	Sparingly Soluble
Isopropanol	Insoluble
Acetic Acid	Insoluble
HCl	Insoluble
H_2SO_4	Insoluble

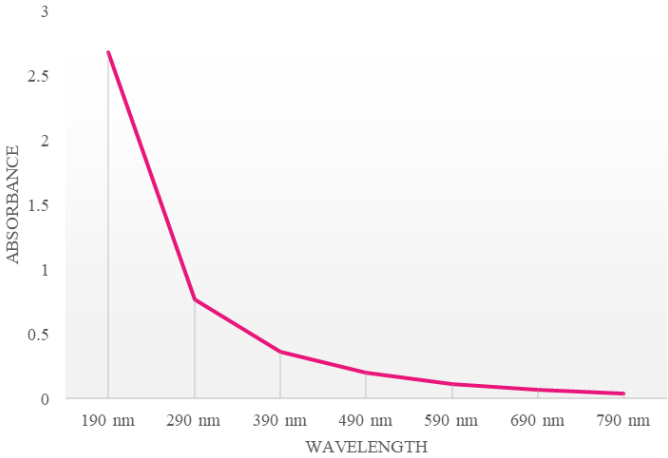
Table 3: Solubility of melanin in various solvents

Purified melanin is analyzed by UV visible spectrophotometry and FTIR spectrophotometry to confirm the pigment as melanin. The pigment has shown characteristic spectrum of melanin with maximum absorption at the UV region which significantly decrease as it reaches the visible region (Figure 5 (b)).

FTIR spectrum of melanins (Figure 5 (c)) had shown considerable similarity with synthetic melanin and earlier reports. (Sajjan et al., 2010, Selvakumar et al., 2008). The spectrum showed a broad absorption around 3392 cm^{-1}, corresponds to phenolic –OH and –NH stretching vibrations. Characteristic peaks observed between 1600-1400 cm^{-1} was attributed to aromatic ring C=C stretching. This confirmed the polyphenolic and aromatic nature of MIM2 melanin.



(a)



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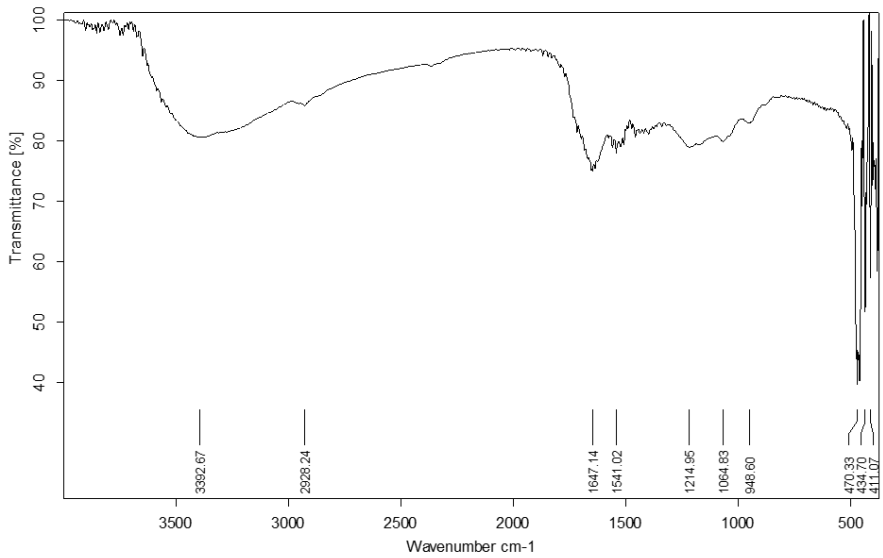

(c)

Figure 5: (a) Melanin production in tyrosine basal broth by MIM2 (b) UV Visible spectrum MIM2 melanin (c) FTIR spectrum of MIM2 melanin

The pigment produced by the halophilic bacteria *Bacillus altitudinis* MIM2 was found to be melanin by spectroscopic characterization. The pigment needs to be characterized more to use it for application studies.

The free radical scavenging activity of MIM2 melanin was assayed using DPPH method (Liyana & Shahidi, 2005). MIM2 melanin had shown to have very good radical scavenging activity of 66.4±1.1% (100 µg/mL) compared to control ascorbic acid it was 92.16±1.8% for the same concentration (Figure 6).

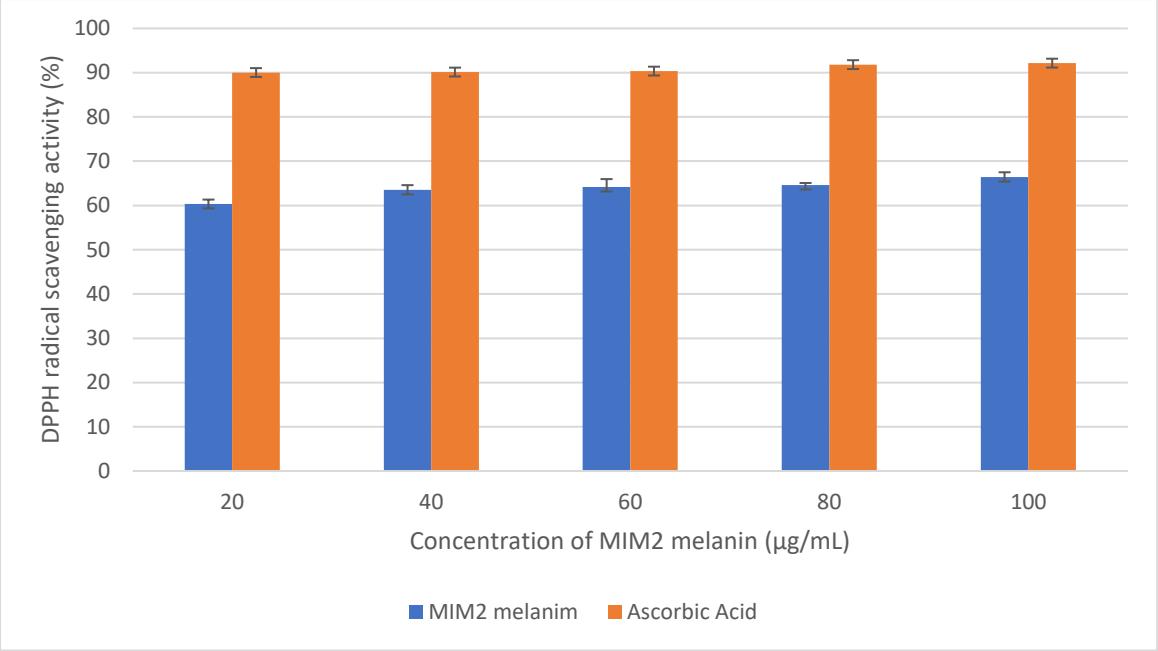

Figure 6: Radical scavenging activity of MIM2 melanin

Antibacterial activity of the melanin was evaluated using well diffusion assay (Łopusiewicz, 2018). MIM2 melanin had shown good antibacterial activity at even very lower concentrations against potential pathogens. The minimum inhibitory concentration was between the range of 20-40µg/mL against all bacteria tested (Table 4).

Bacteria	Minimum Inhibitory Concentration of MIM2 melanin
Bacillus sp.	40µg/mL
E. coli	20µg/mL
Salmonella sp.	40µg/mL
Shigella sp.	20µg/mL
Staphylococcus aureus	20µg/mL

Table 4: Antibacterial activity of MIM2 melanin

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