International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2015): 6.391

Extraction and Analysis of Melanin Pigment Produced by *Clostridium tertium* Isolated from Water Sample of Saline Belt in West Vidardha Region

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Abstract: ***The aim of this study was to isolate a bacterium producing melanin pigment, its morphological, biochemical and molecular identification, studying effects of parameters like pH and temperature on the isolate and its melanin producing ability, extraction of melanin pigment from the isolate, chemical characterization of the melanin pigment and performing analytical techniques to confirm the pigment as melanin. A bacterium producing melanin pigment was isolated from a borewell water sample from a saline belt in west Vidarbha region of Maharashtra, India using nutrient agar medium enriched with L-tyrosine amino acid. It was identified as Clostridium tertium using molecular technique of 16S rRNA sequencing analysis. It was found to grow and produce melanin from pH-7 to 14 and it could tolerate and produce melanin at temperature range of 5°C to 55°C. The extracted crude melanin pigment was water soluble and techniques like Ultraviolet-Visible spectrum analysis and Fourier Transmission Infra-red spectrum analysis proved that the pigment extract is melanin.***

Keywords: melanin producing bacterium, *Clostridium tertium*, saline water, effect of pH, bacterial melanin

1. Introduction

Melanins form a diverse group of pigments synthesized in living organisms in the course of hydroxylation and polymerization of organic compounds. Melanin production is observed in all large taxa from both the Prokaryota and Eukaryota. Melanin is nearly a ubiquitous pigment. Animal melanins may be classified as black eumelanins and yellow-to-brown pheomelanins, whereas melanins from plants, fungi, and bacteria are brown-to-black allomelanins [2].

Melanins are found widely dispersed in the animal and plant kingdoms. They have a variety of biological functions, including protection against the UV radiation of the sunlight and energy transduction. Melanins influence human skin and hair colour and are found in the medulla and zona reticularis of the adrenal gland, the inner ear, and in pigment-bearing neurons within areas of the brain stem, such as the substantia nigra. Melanins can also protect microorganisms, such as bacteria and fungi, against thermal as well as chemical (e.g. heavy metals and oxidizing agents) and biochemical (e.g. host defenses against invading microbes) stresses that involve cell damage by the solar UV radiation through generation of reactive oxygen species. A potentially novel role of melanins as photosynthetic pigments in some fungi, enabling them to capture c-rays and harness their energy for growth, has recently been described [1].

2. Material and Methods

Chemicals used:
Nutrient agar media, L-tyrosine and all other media required for this study were purchased from HiMedia chemicals, Mumbai, India. Ethanol, HCL and all other chemicals used were of analytical reagent grade throughout the study. Ultrapure water used for the experiments and aseptic conditions were maintained wherever necessary.

Screening, isolation and identification of the melanin producing bacterium:
A borewell water sample from a saline belt in west Vidarbha region of Maharashtra, India was collected. Its geographical location is 20.890096N 77.001605W. By pour plate technique, the isolate was screened using nutrient agar medium enriched with amino acid L-tyrosine which is the initial precursor of melanin biosynthesis pathway. As it was able to utilize the l-tyrosine in the media, it was selected for further isolation. The same media was used to isolate the pure culture and culture maintenance. The isolate was named KRDB5 temporarily. The media and the glassware were autoclaved at 15 psi (121°C) for 20 mins prior to the experiment. The incubation was done at 37°C for 5 days. The bacterium was identified by performing morphological, biochemical and molecular technique (16S rRNA sequencing).

Optimization studies:
The effect of different parameters namely pH and temperature conditions on the bacterial isolate and on its melanin production ability were studied. The pH values taken into consideration in this study were 3, 5, 7, 8, 10, 12 and 14. The temperatures studied were 5°C, 15°C, 28°C, 37°C, 45°C and 55°C.

Pigment production and extraction of melanin:
Autoclaved 500ml nutrient broth media enriched with L-tyrosine, inoculated the isolate into the media and incubated at 37°C until the media became opaque due to the pigment production (3 -4 weeks approximately). The extraction process is explained in the flowchart in detail.

Chemical characterization of crude melanin extract:
The solubility of the crude melanin pigment extract in distilled deionized water, 1M NaOH, ethanol, acetone, chloroform, were checked. Reactions with oxidizing agents such as 6% sodium hypochlorite (NaOCl) and 30% hydrogen peroxide (H_2O_2) were determined and its precipitation in 3N HCl was observed.

UV-visible spectroscopy and FTIR spectrum analysis:
UV-visible spectrum of the melanin extract obtained from the bacterial isolate was analyzed from 200nm to 900 nm using UV-Visible spectrophotometer (Systronics-118 model). The FTIR analysis of pigment was carried out after mixing with IR grade KBr (1:10) using FTIR spectrophotometer (Shimadzu IR affinity1).

3. Results

The morphological observations and biochemical test results are shown in table no.1. and Fig.1 shows the bacterial isolate KRDB5.

Figure 1: bacterial isolate KRDB5

Table 1 - Morphological and Biochemical characteristics of KRDB5 strain:

Characteristics	*Results*
Colony shape	Circular
Colony size	1-3mm
Colony margin	Entire
Colony elevation	Umbonate
Colony texture	Smooth
Colony appearance	Shiny
Optical property	Opaque
Colony color	Brown
Pigmentation type	Non-diffusible
Gram character	Gram positive
Motility	Motile
Carbohydrate Fermentation:	
Lactose	+
D-Glucose	+
Sucrose	+
D-Mannitol	+
D-Fructose	+
D-Maltose	+
Starch	+

The molecular identification of the bacterial isolate was done using 16S rRNA sequencing technique and was identified as closest neighbour of *Clostridium tertium* (Genbank accession number- KX648433). Its phylogenetic tree is shown in fig. 2.

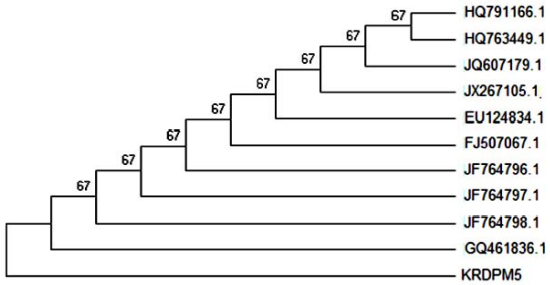


Figure 2: Phylogenetic tree for KRDB5 on basis of 16S rRNA sequencing analysis

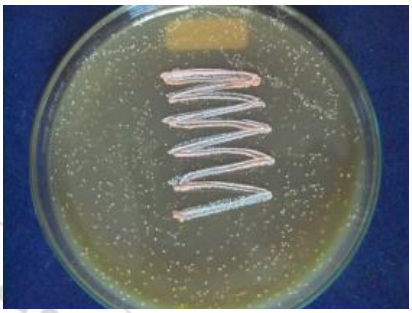


Figure 3: Effect of temperature 5°C on growth of *Clostridium tertium* and its melanin production

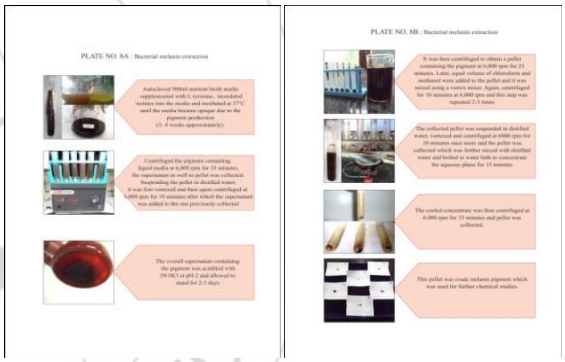
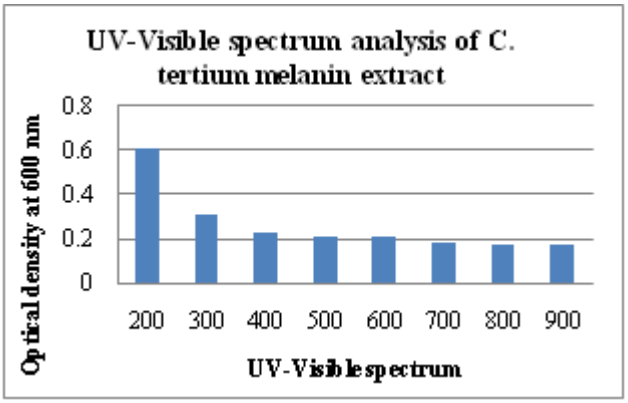


Figure 4: flowchart for extraction of melanin extract from *Clostridium tertium*

Table 2: Chemical characterization of crude melanin extract from *Clostridium tertium*

Characteristics	*Clostridium tertium crude melanin extract*
Solubility	
Water	Soluble
Ethanol	soluble
Chloroform	Insoluble
Acetone	Insoluble
1MNaoH	Soluble
Color	Dark brown
Precipitation in 3N HCl	Precipitated readily
Reaction with oxidizing agent H2O2	Decolorized
Reaction with NaOCl	Decolorized



Graph 1: UV-Visible spectrum of *Clostridium tertium* melanin extract

The UV-visible spectrum of *Clostridium tertium* melanin extract was compared with earlier reports and found the same pattern i.e. higher absorption in the UV region of 200 - 300 nm and decreasing towards the visible region.

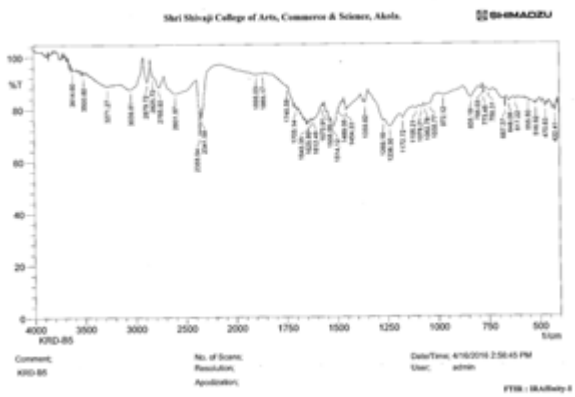


Figure 5: FTIR spectrum analysis of *Clostridium tertium* melanin extract

4. Conclusion

The bacterial isolate, *Clostridium tertium*, was isolated from an environmental sample and basic media was used to isolate and culture maintenance. *C. tertium* could grow and produce melanin at pH conditions 7 to 14. It could tolerate and produce melanin at temperature range of 5°C to 55°C which is a very wide range. The crude melanin extract obtained from *Clostridium tertium* was soluble in water, ethanol and 1M NaOH but insoluble in chloroform and acetone; readily precipitated in 3N HCl and decolorized while reacting with H_2O_2 and NaOCl. UV-Visible spectrophotometry and FTIR spectrum analysis techniques were used to confirm the extracted pigment as melanin.

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