



# ISOLATION AND CHARACTERIZATION OF PIGMENTS FROM AGAVE RHIZOSPHERE *MICROCOCCLUS LUTEUS*

Nagamani T.S.<sup>1</sup>, Raju H<sup>2</sup>

<sup>1</sup>Department of PG studies in Biotechnology, Government Science College (autonomous) Bengaluru.

<sup>2</sup>Department of Biotechnology, R V College of Engineering (autonomous) Bengaluru  
[naguts@gmail.com](mailto:naguts@gmail.com)<sup>1</sup>, [raju22aybt@rvce.edu.in](mailto:raju22aybt@rvce.edu.in)<sup>2</sup>

## Abstract

The present study elucidates the culture characterization, isolation and partial purification of novel yellow pigment produced from group of *Micrococcus* bacterial strains. Growth conditions were optimized during the study. It showed optimum growth at optical density 0.55 at 660nm at 280C (pH7.0 for 48 hours), while the maximum growth and pigment (OD: 0.50 at  $\lambda$ 466) production was at 72 h (pH 7). The yellow pigment present in the medium was extracted in methanol, and purified using silica column, and thus produced pigment was characterized using (TLC) thin layer chromatography (2 spots: Rf 0.38 and 0.43), UV-visible and IR spectroscopic techniques; and both spectroscopic profiles showed the characteristic peaks of carotenoid pigment.

**Index Terms:** Agave plant, *Micrococcus luteus*, Carotenoid pigment, Methanol, Silica column, TLC

## I INTRODUCTION

Demand for natural pigments is increasing day-by-day, because of its environmental safety as well as beneficial effects on human health. It is essential to explore various natural sources of food grade colorants and their potentials. Microbial colorants draw increased attention towards the production of natural food colorants for industrial use. Microbial fermentation has several advantages such as inexpensive production, easier extraction, higher yields and availability of raw materials and lack of seasonal variations. Microbial pigment production has

two fundamental approaches: first is to find out new strains of efficient microbes producing pigments and the other approach is to obtain enhanced and consistent yields either through optimizing the process parameters for the better yield or through strain improvement. Due to biodegradability and higher compatibility with the environment, bacterial pigments offer promising avenues for various applications in industries like food, pharmaceuticals, cosmetics, textiles, etc.

Carotenoids are the most important pigment group comprising of yellow to orange-red variants, which are ubiquitous in nature with proven anti-carcinogenic and immune-modulation properties. Among algae, *Dunaliella salina* and *Dunaliella bardawil* are well known producers of carotenoids. *Haematococcus pluvialis*, a fresh water alga produces astaxanthin, an important and valuable keto-carotenoid. *Haloferax alexandrinus* is one of the most promising microorganisms used for the commercial production of canthaxanthin, a di-ketocarotenoid. Riboflavin, a yellow water soluble vitamin is produced from ascomycetes (e.g. *Ashbya gossypii*), filamentous fungi (e.g., *Candida famata*), etc. Hence, bacterial pigments production can be intensified which can reduce the production cost and increasing applicability in industry and medicine.

*Micrococci* are Gram-positive, non-sporulating and non-motile bacteria with spherical cells, which are often found in tetrads. The genus *Micrococcus* has several species, as all described as strict aerobes the *M. luteus* is an obligate aerobe. Netzer et al. Characterized the major carotenoids synthesized by the *M. luteus*

strain NCTC 2665 as sarcinaxanthin. Pigment produced by bacteria could be extracted in suitable solvent and be characterized using various analytical techniques such as thin layer chromatography (TLC); gel permeation chromatography; High Performance Liquid Chromatography (HPLC); UV-Vis, Fourier Transform Infra-Red (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy.

Based on this review, the present work was aimed at the identification of the pigment producing bacterium based on culture, morphological and molecular characteristics. Cultivation of the selected bacterium in a suitable medium, extraction and characterization of pigment in suitable solvents.

## II MATERIALS AND METHODS

Soil rhizosphere of Agave plant at Karahalli village, Devanahalli taluk, Bengaluru rural, Karnataka was collected. The Agave roots were soaked in distilled water and rinsed. The obtained water is serially diluted with different aliquots of H<sub>2</sub>O with the pH 7.2

**Growth profile and pH effect :** The obtained yellow colony was examined by carrying out Gram's reaction. HiMedia Grams Stain Kit is used for differentiation of bacteria on the basis of their gram nature. The bacterium was also subjected to biochemical test of catalase fermentation of carbohydrates. The species was confirmed by 16srRNA sequencing.

**Growth Conditions:** Bacterial strains were grown on Luria Bertani broth (LB) medium, (Hi Media, Cat no M1245-500G). Broth cultures grown from plate cultures were inoculated from a single colony using a plastic loop. Bacterial strains were routinely cultured in LB medium. Liquid cultures were grown in 250ml Erlenmeyer flask in a BOD shaker incubator (225rpm) at 37°C unless otherwise stated.

### Pigment extraction

The pigment was extracted according to the method of Jagannadham et al., with some modifications. Briefly, 1 ml of the culture broth was taken in a microcentrifuge tube and centrifuged at  $9,440 \times g$  for 15 min at 4°C. The pellet was washed with distilled water and again subjected to centrifugation as above. Methanol (0.5 ml) was added to the pellet and then mixed well until the methanol layer turned yellow. The entire suspension was centrifuged at  $9440 \times g$  for

15 min at 4°C, and the methanol layer containing the crude pigment was recovered. The yellow pigmented cell pellet was extracted once again with methanol, and the methanol layer was recovered as described above. All methanol fractions were pooled, and used for further analyses.

### Purification by column chromatography

Pigment produced by *M. luteus* was purified by column chromatography using silica gel (60-120 mesh size), and eluted initially with n-hexane (flow rate 1 ml/min). The polarity of the solvent was increased subsequently by adding ethyl acetate (5-100%), and the yellow colored fractions were collected from the column.

### Characterization of the pigment

The procedure for TLC (silica gel GF234) was as described by Basker et al. [15] with slight modifications. A suitable solvent system containing chloroform:methanol:water in the ratio 65:25:4 was designed by trial and error method. The retention factor (R<sub>f</sub>) was calculated subsequently. UV-Vis spectrophotometry: Absorption spectra of both the crude and purified pigments were taken [14] using a UV-vis bio spectrophotometer (Elico double beam BL 200 bio-spectrophotometer).

### Results and Discussion

Culture characterizations *M. luteus* strain on nutrient agar plates (after two days of incubation at 37°C) showed yellowish colonies. It grew well in the complex nutrient medium, both on agar plates and in broth (Figures 1A and 1B). Upon Gram-staining, the bacterium appeared as cocci; the colonies were seen arranged in tetrads and also in irregular clumps of tetrads. Thus, the bacterium *M. luteus* strain was identified as Gram-positive non-sporulating coccus.

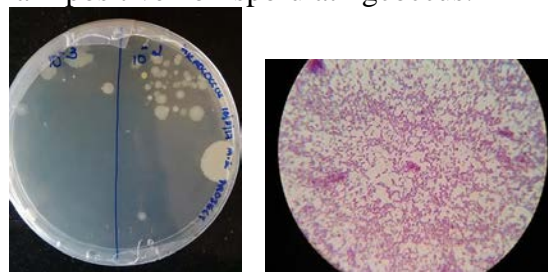


Fig: soil sample cultured on plate: bacterial colonies and gram nature

It was noted that the pigment was insoluble in distilled water, and showed the maximum solubility in other solvents such as acetone, methanol, chloroform and hexane. From the

results, it was also inferred that methanol is an ideal solvent for extracting this water insoluble pigment.



### Pigment extracted and separated by column chromatography

Thin layer chromatography is used to separate mixtures of substances into their components. The silica gel plates with chloroform:methanol:water (65:25:4, v/v) system. The pigment produced by *Micrococcus luteus* was a dihydroxy C50 carotenoid. The Rf value of the pigment is 0.68.

In comparison to the known characteristics of the pigments produced by various species of *Micrococcus* and the characteristics of the pigment produced by *M. luteus* strain, it seems that the water insoluble and extracellular yellow colored pigment secreted by *M. luteus* strain BAA2 seems to be a carotenoid. It was produced inexpensively in nutrient broth upon incubating for 3 days in a shaker at 28°C and 150 rpm, and showed the maximum growth, biomass and pigment production at pH 7.

### CONCLUSION

In the present study the known characteristics of pigment produced by various *Micrococcus* from the soil sample of agave plant seems to be water insoluble and extracellular yellow coloured pigment called carotenoid and it was produced efficiently and cost effectively in LB broth which was incubated for 48h in a shaker incubator at varying temperatures and varying pH at 150rpm which showed the maximum growth of bacterial biomass and pigment production at pH 8 and at incubation temperature of 37°C. Moreover the pigment produced was partially elucidated using TLC and Column Chromatographic techniques. Efficacy of the pigment need to be confirmed by bioinformatics aided computational tools.

### REFERENCES

- [1] Dufossé, L., Galaup, P., Yaron, A., Arad, S. M., Blanc, P., Murthy, K. N. C., & Ravishankar, G. A. (2005). Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality. *Trends in Food Science & Technology*, 16(9), 389-406.
- [2] George Y.Liu and Victor Nizet. (Sep2009). Color me bad: microbial pigments as virulence factors. *Trends Microbiol*; 17(9): 406–413.
- [3] Greenblatt, C. L., Baum, J., Klein, B. Y., Nachshon, S., Koltunov, V., & Cano, R.
- [4] J. (2004). *Micrococcus luteus*-survival in amber. *Microbial ecology*, 48(1), 120-127.
- [5] Jagannadham, M. V., Rao, V. J., & Shivaji, S. (1991). The major carotenoid pigment of a psychrotrophic *Micrococcus roseus* strain: purification, structure, and interaction with synthetic membranes. *Journal of bacteriology*, 173(24), 7911-7917.
- [6] Johnson, E. A., & Schroeder, W. A. (1995). Microbial carotenoids. Downstream processing biosurfactants carotenoids, 119-178.
- [7] Joshi V. K., Devender Attri, Anju Bala and Shashi Bhushan (2003). Microbial Pigments. *Indian Journal of Biotechnology*. Vol 2, pp 362-369.
- [8] Kocur, M., Kloos, W. E., & Schleifer, K. H. (2006). The genus *Micrococcus*. In *The prokaryotes*. Springer New York. (pp. 961-971).
- [9] Thirkell, D., Strang, R. H. C., & Chapman, J. R. (1967). The pigments of *Sarcina flava*: a new series of C50 carotenoids. *Microbiology*, 49(1), 157-164.
- [10] Tuli, H. S., Chaudhary, P., Beniwal, V., & Sharma, A. K. (2015). Microbial pigments as natural color sources: current trends and future perspectives. *Journal of food science and technology*, 52(8), 4669-4678
- [11] Umadevi. K., Krishnaveni. M(2013). Antibacterial activity of pigment produced from *Micrococcus luteus* KF532949, *International Journal of Chemical and Analytical Science* 4,149-152.
- [12] Venil C. K., Zainul Akmar Zakaria, Wan Azlina Ahmad(June 2013). Bacterial pigments and their applications. *Process Biochemistry*(48), pp1065–1079.