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Micrococcus luteus Strain BAA2, A Novel Isolate Produces Carotenoid Pigment

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Research Article

Abstract

The present study describes the culture characteristics, isolation and partial purification of a yellow pigment produced by a novel bacterium, *Micrococcus luteus* strain BAA2. The growth conditions of *M. luteus* strain BAA2 were optimized. It showed the optimum growth (OD: 0.55 at λ_{660}) at 28ºC (pH 7, 24 h), while the maximum pigment (OD: 0.50 at λ_{466}) production was at 36 h (pH 7). The yellow pigment secreted in the medium was extracted in methanol, and purified using silica column chromatography, and the pigment was characterized using thin layer chromatography (2 spots: R_f 0.38 and 0.43), UV-visible and IR spectroscopic techniques; and both spectroscopic profiles showed the characteristic peaks of carotenoid pigment.

Keywords: *Micrococcus luteus* strain BAA2; Yellow pigment; Carotenoid.

1. 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 [1]. Owing to the stability of the pigments produced and easier availability of cultivation technology (production strategies), microbial colorants draw increased attention [2]. Production of natural food colorants for industrial use by microbial fermentation has several advantages; such as cheaper production, easier extraction, higher yields, availability of raw materials and lack of seasonal variations [3]. 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 [4]. 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. [5].

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 [6]. Among algae, *Dunaliella salina* and *Dunaliella bardawil* are well known producers of carotenoids [7]. *Haematococcus pluvialis*, a fresh water alga produces astaxanthin, an important and valuable *keto*-carotenoid [8]. *Haloferax alexandrinus* is one of the most promising microorganisms used for the commercial production of canthaxanthin, a di-ketocarotenoid [9]. Riboflavin, a yellow water soluble vitamin is produced from ascomycetes (*e.g*. *Ashbya gossypii)*, filamentous fungi (*e.g*., *Candida famata),* etc. [10]. Hence, work on the bacterial pigments should be intensified, especially in cheap and suitable growth media, which can reduce the production cost and increase its applicability in industry and medicine [11].

Micrococci are Gram-positive, non-sporulating and non-motile bacteria with spherical cells, and often found in tetrads. The genus Micrococcus has several species, all described as strict aerobes and *M. luteus,* the type species of the genus Micrococcus, is an obligate aerobe. Netzer et al. [12] characterized the major carotenoids synthesized by the *M. luteus* strain NCTC 2665 as sarcinaxanthin. Pigment produced by bacteria could be extracted in suitable solvents, 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 (FT-IR) and Nuclear Magnetic Resonance (NMR) spectroscopies [5].

Based on this background, the present work is aimed at: (1) identification of the pigment producing bacterium based on culture, morphological and molecular characteristics; (2) cultivation of the

selected bacterium in a suitable medium, and extraction of the pigment in suitable solvents; and (3) purification and characterization of the pigment produced using various analytical techniques.

2. Materials and Method

2.1 Growth conditions

The yellow pigmented bacterium *Micrococcus luteus* strain BAA2 (GenBank Accession No. KF550912) obtained from the culture collection of the Enzyme Technology Laboratory, Department of Botany, University of Calicut was used for this study.

2.2 Culture characterizations

Standard procedures were employed for the Gram-staining [13]. The photomicrographs were taken using image analyzer (Nikon Eclipse E 400, Towa optical, Japan) fitted with Nikon digital camera (DXM 1200F, Japan), and also by phase contrast microscope (Leica M80, Germany). The bacterial isolate was further characterized by the PCR amplification of 16S rRNA gene employing 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl genetic analyzer.

2.3 Growth profile and pH effect

The growth of the bacterium was observed by measuring the maximum absorbance (λ max) at 660 nm at every 3 h interval using UV-vis spectrophotometer (Shimadzu, Japan). To determine the optimum pH for pigment production, 10 ml nutrient broth (NB) with different pH (i.e., 6, 7 or 8) was incubated in a temperature-controlled shaker (Orbitech, India) at 28ºC. Growth of the bacterium, change in pH, biomass and production of the pigment (at 466 nm) were noted at every 6 h interval.

2.4 Pigment extraction

The pigment was extracted according to the method of Jagannadham et al. [14], with some modifications. Briefly, 1 ml of the culture broth was taken in a microcentrifuge tube and centrifuged at 9,440 × *g* for 15 min at 4ºC. 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 × *g* for 15 min at 4ºC, 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.

2.5 Purification by column chromatography

Pigment produced by *M. luteus* BAA2 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.

2.6 Characterization of the pigment

Thin layer chromatography (TLC): 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_f) was calculated subsequently.

UV-Vis spectrophotometry: Absorption spectra of both the crude and purified pigments were taken [14] using a UV-vis biospectrophotometer (Elico double beam BL 200 bio-spectrophotometer).

FT-IR spectroscopy: The purified pigment was characterized by FT-IR spectroscopy, as described by Song et al. [16]. The methanolic extract of the pigment was concentrated, pelleted with potassium bromide (KBr) and analyzed using Jasco FTIR 400 Series (Jasco, Japan). The relative intensity of transmitted light was measured against the wavelength of absorption in the region 400-4000 cm^{-1}.

3. Results and Discussion

3.1 Culture characterizations

M. luteus strain BAA2 on nutrient agar plates (after 3 d of incubation at 37ºC) 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 BAA2 was identified as Gram-positive non-sporulating coccus.

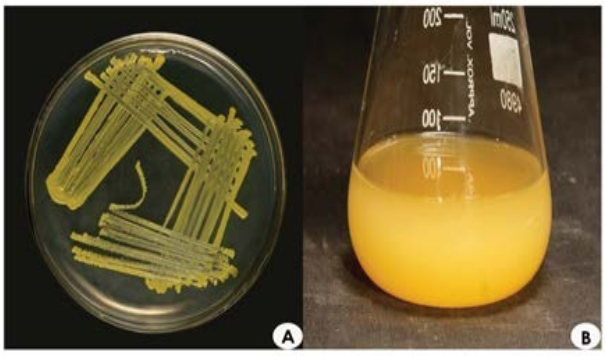

Figure 1. Culture characteristics of *M. luteus* strain BAA2. (A) Growth on nutrient agar plates (3 d old). (B) Culture growing in nutrient broth (3 d old).

The genomic DNA was isolated from *M. luteus* strain BAA2 and subjected to PCR amplification. A consensus sequence of 1321 bp of 16S rRNA gene was generated to carry out BLAST with the non-redundant database of NCBI GenBank. Based on the maximum identity score, first ten sequences were selected and aligned using multiple alignment software programmes Clustal W, and the phylogenetic tree was constructed using tree view. *M. luteus* strain BAA2 showed 100% similarity with all the ten accessions analyzed. From the phylogenetic tree (Figure 2), it is evident that *M. luteus* strain BAA2 is closely related to *M. luteus* strain Z382 (GenBank accession number: KC2119951). The genus, Micrococcus now harbors only five species: *M. luteus, M. lylae, M. antarticus, M. endophyticus* and *M. flavus* [17,18].

3.2 Growth profile

The change in pH was observed at every 3 h interval during the growth of *M. luteus* strain BAA2 (Figure 3A). The pH of the bacterial culture was increased at every 3 h interval till 27 h, subsequently the culture became stable (Figure 3A). From the growth profile, *M. luteus* strain BAA2 attained the maximum growth at 30 h of incubation (Figure 3B).

3.3 Effect of pH

The pH change during the optimization of pH was noted (Figures 4A-4D); the maximum growth (OD, 0.55 at 660nm) was found at pH 7 in 24 h of incubation (Figure 4B), and the maximum pigment production was also observed at pH 7 (OD, 0.502 at 466 nm), but in 36 h of incubation (Figure 4D).

3.4 Pigment extraction

The yellow pigment produced by *M. luteus* strain BAA2 was extracted using different solvents after centrifugation at 9,440 × g. 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.

3.5 Purification by column chromatography

The solvent system used for column chromatography of the crude pigment (Figure 5A) comprised of hexane and ethyl acetate (50 ml of *n*-hexane was used as initial elution solvent and colorless fractions were eluted out). The polarity of the solvent was increased by adding ethyl acetate. The major fractions of yellow pigments (Figure 5B) were separated collected when the second elution solvent (ethyl acetate: n-hexane; 15:35; v/v), which was concentrated and used for characterization studies (Figures 5A-5B).

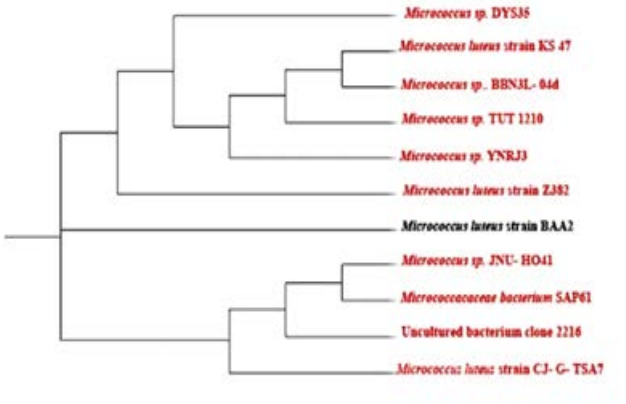

Figure 2. Phylogenetic tree of *M. luteus* strain BAA2.

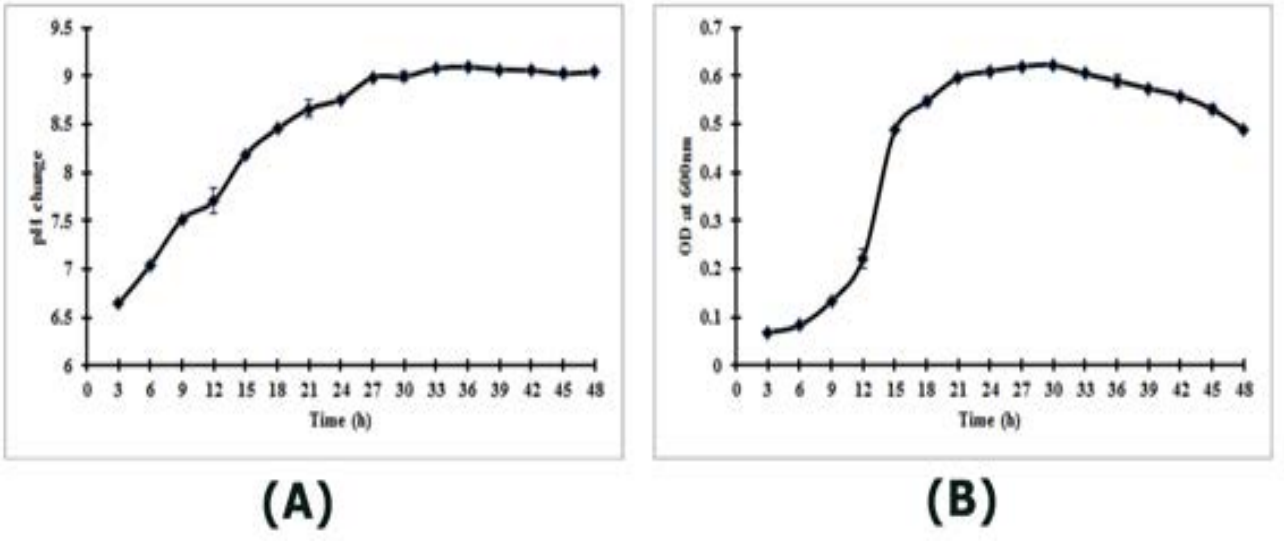

Figure 3. (A) The change in pH during the growth of *M. luteus* strain BAA2. (B) Growth curve of *Micrococcus luteus* strain BAA2 observed optimum growth at 30 h.

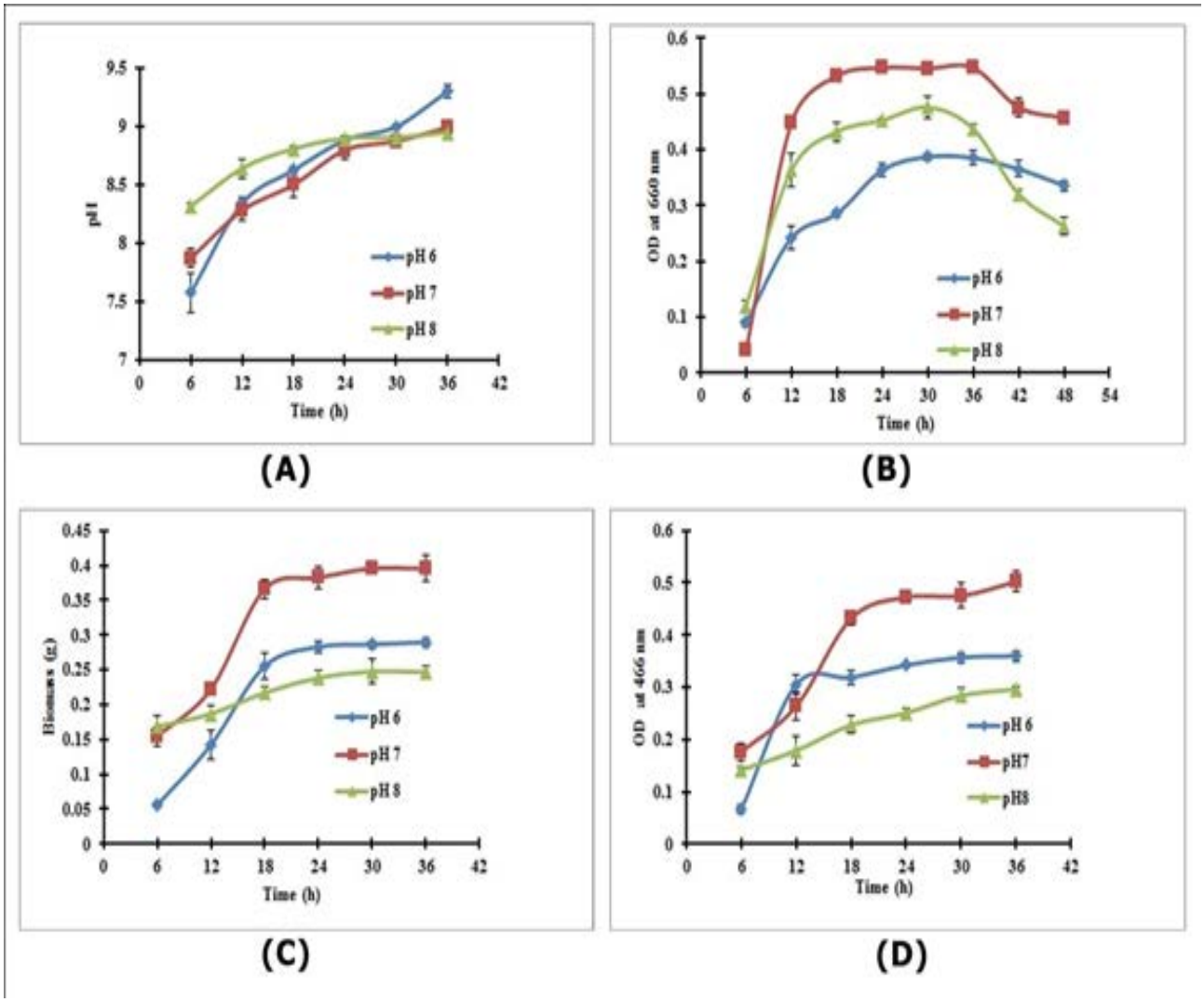

Figure 4. Optimization of pH on pigment production. (A) Change in pH of the culture broth. (B) Growth of the bacterium at 600 nm. (C) Biomass of the bacterium at pH 6, 7 and 8. (D) Production of the pigment at 466 nm.

3.6 Characterization of the pigment

TLC was used for analyzing, identifying or separating mixtures of the crude pigment. Two spots (Figure 6) with 0.43 and 0.38 R_f values were obtained after developing the silica gel plates with chloroform:methanol:water (65:25:4, v/v) system. The chemical nature of pigments has been determined only for two mesophilic species of the genus Micrococcus so far. The pigment produced by *M. luteus* was a dihydroxy C_{50} carotenoid [19-21]; while *α* or *β* carotene derivative with canthaxanthin as the main pigment was produced by *M. roseus* [22,23]. Pigments purified from the strain of *M. arborescens* by TLC using petroleum ether and ethyl acetate (90:10 v/v) as solvent system showed four spots with different R_f values: yellow (0.15), pink (0.20), light yellow (0.47) and dark yellow (0.77) [24]. UV-vis spectroscopy helped to determine the λ_{max} of the pigment present in both crude and purified fractions (from column chromatography). In the

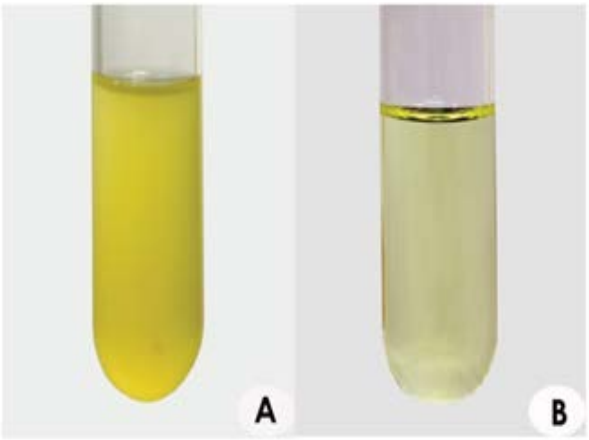

Figure 5. (A) Crude extract of the pigment. (B) Purified fraction of the pigment eluted from the column.

absorption spectrum of crude pigment (scanning range λ_{350}-$_{550}$), 3 peaks were seen i.e., one major

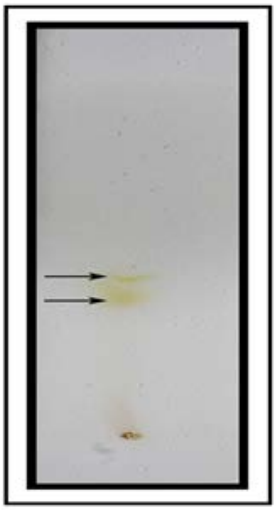

Figure 6. TLC profile of the pigment. Two visible spots with R_f value 0.43 and 0.38 obtained after developing the TLC plates with chloroform:methanol:water (65:25:4;v/v) solvent system.

peak and two minor peaks; and both the minor peaks showed absorbance at $\lambda_{412.5}$ (0.780) and $\lambda_{467.5}$ (0.745). Among the 3 peaks obtained, the middle peak showed the maximum absorbance (0.834), i.e., at λ_{440} (Figure 7A). The absorption spectrum of purified pigment by column chromatography also showed 3 peaks, and the middle peak showed the maximum absorbance (0.117) at λ_{439} (Figure 7B). The absorption spectrum of the pigment saproxanthin produced by the bacterial strain 040KA-13-27 belongs to the family Flavobacteriaceae also showed 3 peaks [25] (Figures 7A-7B). UV-Vis absorption spectra of carotenoid pigments are of immense importance, since they aid a great deal in determining the structure of carotenoids [26]. The UV-Vis absorption spectra of 5 pigments isolated from the psychotrophic *M. roseus* strain by HPLC appeared to be identical and exhibited a fine structure, with three absorption maxima, i.e., at $\lambda_{466,493 \text{ and } 523}$; and one of them was characteristic of carotenoids [14].

FT-IR absorption of the yellow pigment showed strong and broad peaks (Figure 8) at 3421.1 cm^{-1}. The spectrum obtained after analysis also showed medium peaks at 1636.3 and at 1404 cm^{-1}. The peaks at 3421.1, 1636.3 and 1404.89 cm^{-1} correspond to different functional groups (Table 1). FT-IR helps to determine the functional groups in the sample, and different functional groups absorb characteristic frequencies of IR radiations differently [16]. Ahmad et al. [11] isolated and characterized a crude violet pigment, which showed characteristic frequencies at a broad range (3700 to 3000 cm^{-1}), corresponding to –OH stretching in the violet pigment. Prodigiosin, a red pigment isolated and characterized from *Serratia marcescens* showed characteristic vibrations at 3445.45, 2926.23, 1718, 1650.96 and 1559.85cm^{-1} [11].

The appropriate use of the fermentation physiology together with the metabolic engineering could allow mass production of valuable pigments from bacteria efficiently. Optimization of fermentation processes is an important strategy required to achieve the higher production of pigments [2]. Bacteria belong to the genus Micrococcus may be yellow, yellowish green or orange (*M. luteus*); dark yellow (*M. varians*); pink or red (*M. roseus* and *M. radiodurans*); dark rose-red (*M. agilis*); and cream or white (*M. lylae*, *M. kristinae*, *M. nishinomiyensis*, *M. sedentarius* and *M. halobius*) [27,28].

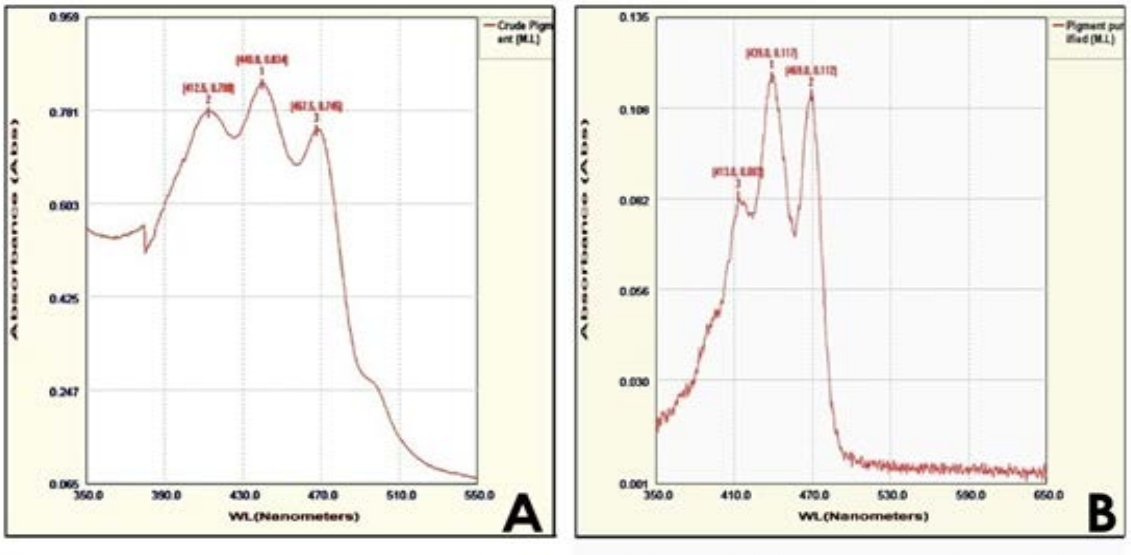

Figure 7. UV-vis profile of the pigment. (A) Absorption spectrum of crude pigment. (B) Absorption spectrum of purified pigment.

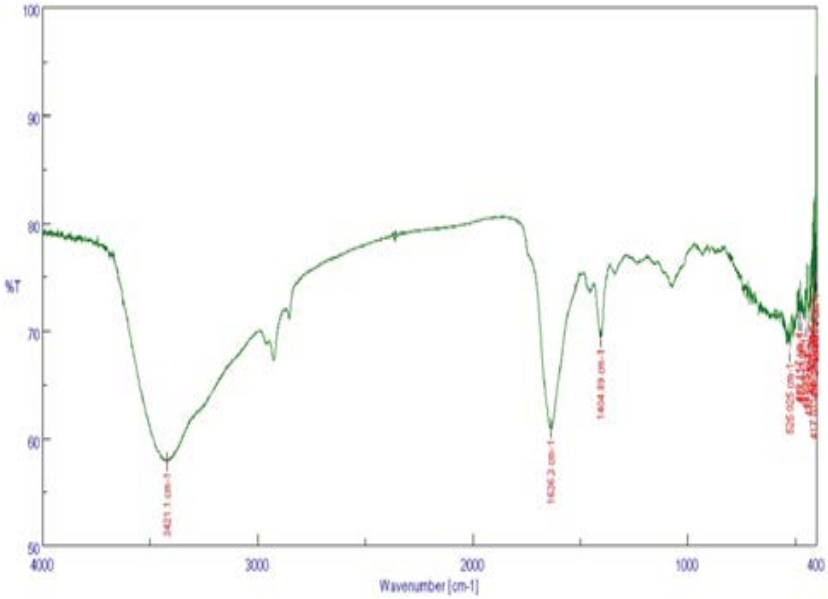

Figure 8. FT-IR profile of purified pigment.

4. Conclusion

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 BAA2, 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 d in a shaker at 28ºC and 150 rpm, and showed the maximum growth, biomass and pigment production at pH 7. Moreover, the structural elucidation of the molecule needs to be carried out further by NMR. Efficacy of the pigment needs to be confined by antioxidant, cell line, and bioinformatics aided computational docking studies on various cellular targets.

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