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Characterization of *Micrococcus alovera* pigment

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Abstract: Non-toxicity and biodegradability of natural pigments give them an attractive property for human use. Pigmented bacteria excrete pigments as secondary metabolites when exposed to stress. The bacterial pigments have many features giving them as natural pigments many industrial uses over the synthetic pigments such as simple techniques of culturing, extraction and scaling up make them preferable in pharmaceutical, textile, cosmetics and food industries. Also they have antimicrobial, anticancer and antioxidant properties. In the present study, yellow pigmented bacteria were isolated from soil sample collected from the farm of Mansoura university campus on L.B media. The biochemical and molecular data exposed the identification of this isolate as *Micrococcus alovera*. The yellow pigment secreted in the medium was extracted in methanol, and then characterized using Fourier Transform Infrared (FTIR) spectroscopic techniques. The spectroscopic profile presented the characteristic peaks of carotenoid pigment. The isolated carotenoid pigment of *Micrococcus alovera* showed a significant antioxidant activity (IC50 of 0.355 mg/ml). In conclusion, *Micrococcus alovera* is considered as a good source of yellow carotene pigment that needs further analysis for finding more applications **.**

keywords: Micrococcus alovera, Pigments, Carotene, Antioxidant.

1.Introduction

Micrococci are Gram-positive tetrads, obligate aerobe, non-spore forming and non-motile bacteria[1]. These characters enable them to survive in a wide variety of environmental conditions. They belong to genus *Micrococcus* (class Actinobacteria, family Micrococcaceae)[2]. *Micrococcus* could be found in soil, water, air, plant and human body. The wide spread of these microorganisms in different environmental conditions occurred through their bioactive and radio-protective abilities. *Micrococcus* sp. have application such as bioremediation, heavy metal resistance, producing enzymes[3]. Colors give a catchy look to marketable products such as food products, textiles, and pharmaceutical products [4].Pigments as vital compounds to many industries, having many colors that are water-soluble or water in-soluble[5]. Taxonomists used the pigments as a mean for the classification and characterization of bacteria [2]. Pigments diversity is determined by their chemical structures and occurrence of certain chromospheres. Synthetic pigments are widely used in foods, dyes, cosmetics products and drugs making processes, comprising various fatal effects. Many restrictions of synthetic pigments are due to carcinogenic harms, not being ecofriendly and degradable[6]. There are many sources of natural pigments or bio-pigments such as fruit, roots, vegetables and microorganisms. There are many bio-pigments such as carotene, prodigision, flavones, monascins, quinines, and melanin. Bio-pigment production is a faster, cheaper and more safe than synthetic pigments[7]. Pigmented microorganisms like bacteria exhibit several special features, due to their short life cycle, resistance to environmental conditions, ability of production of pigments with wide range of colors [6]. Pigment has wide variety of applications which include a- being indictors to indicate pollution in drinking water and contamination sites with heavy metals, b-in food and nutrition by using it as a precursor of vitamin A and food colorant and c- controlling diseases using its bioactive properties like antimicrobial and antifungal activities[6].

Carotenoids are important group of hydrophobic natural pigments that give yellow to red color. The carotenoid pigments are known as isoprenoid polyenes, which are extracted from plant and microbial sources[8]. Pigment production is affected by many parameters such as temperature, pH, minerals, salts source, carbon source, nitrogen source, moisture content, light condition and incubation time. This study aimed to isolate and identify pigmented bacteria. Also the extraction and characterization of pigments applied.

MATERIALS AND METHODS

Isolation of pigmented bacteria from soil:

Soil samples were collected from the garden of Faculty of Science - Mansoura University. Using sterilized spatula the soil samples were collected and then transported to the laboratory in sterile plastic bags. Serial dilutions of the soil samples have been prepared and then inoculated on LB agar media. Anti-fungal Mycostatin was added to the LB media to inhibit fungal growth. The inoculated petri dishes were incubated at 37^{o}C for 24 hours.

After bacterial growth formation, the pigmented colonies were selected and sub-cultured on LB agar media to obtain pure single colonies of the pigmented bacteria and stored in 50% glycerol stock solution [9].

Morphology of pigmented bacteria:

Cultural properties

The pigmented bacteria were cultured on solid and liquid LB media to determine its morphological characteristics included cultural properties such as color, elevation, shape and margin of colonies. Using serial dilution technique, inoculation of bacteria on solid media then incubated at 37^{o}cfor 24 hour.

Gram staining:

A bacterial smear prepared on clean dry slides by spreading of bacterial suspension and fixation using gentle heating. Drops of crystal violet were added on the smear for 30 seconds, the excess of stain was washed by distilled water. After that, iodine solution was applied for 30 seconds. De-colorization step was made using 95% ethyl alcohol after that the slides were washed by distilled water. Secondary stain safranin, was applied for 30 seconds; the slides were washed by distilled water and then dried. For microscopic examination under oil immersion lens, a drop of cedar oil was added [10].

Phase contrast microscopy:

A smear of the bacteria prepared from pure bacterial suspension and then examined by Phase Contrast Microscope Leica DM 500 with ICC50W camera.

Molecular identification of pigmented isolate:

The yellow pigmented isolate was identified via DNA sequencing of 16S rRNA gene. The gDNA was used as a template for PCR amplification. Forward and reverse primers were used. The PCR program was: 95°C for 10 min, 95°C for 30 s, 60°C for 30 s, 72°C for 45 s and 72°C for 10 min (30 cycles). The sequencing reaction was performed in the 9700 thermal cycler at a total volume of 20 µl (7 µl of the purified PCR product and 13 µl of the Sequencing Module) by adjusting the thermal cycling conditions to 96°C for 10 s, 50°C for 5 s and 60°C for 4 s (25 cycles). The amplicon was purified and then sequenced. The generated sequence was analyzed by Finch TV software, phylogenetic tree was generated by Seaview software comparing to sequences of the closest published type strains[11].

Extraction of pigments

For pigment extraction, 250 ml Erlenmeyer flask containing 50 ml of LB liquid media were inoculated with single colony of the pigmented bacteria and then incubated using shaker incubator at 37^{o}C and 120 rpm for 4 days. The pellet was collected by centrifugation of liquid media at 6000 rpm for 5 minutes. A group of several organic and non-organic solvents; water, hexane, acetone, methanol, ethanol, ethyl acetate, and chloroform and petroleum ether were tested for selecting the best solvent for extraction of pigment. To obtain dry pigment powder, the pigment extract was lyophilized *[12]*.

FT-IR spectroscopy: The yellow pigment was identified by FT-IR spectroscopy. The concentrated methanolic extract of the yellow pigment was precipitated with potassium bromide (KBr) and analyzed using FTIR (Nicolet is10). The relative intensity of the transmitted light was calculated against the wavelength of absorption in the range 400-4000 cm^{-1}[1]

Antioxidant test

DPPH radical scavenging assay was used to determine the antioxidant activities of the pigment. The pigment was dissolved in the best solvent, which is methanol, in different concentrations like (10:25 mg/ml). Freshly prepared DPPH 40 g dissolved in 1

ml methanol, incubated in dark for 30 min. The control was methanol whereas ascorbic acid was used as standard. For spectrophotometry, 2ml of pigment in methanol and DPPH in methanol were measured at 512 nm using UV-VIS spectrophotometer [13]. The percentage of scavenging activity of compound on DPPH radical was calculated as percentage of inhibition of DPPH (I %) using the following formula:

$$I\% = 100 \times \frac{\mathrm{Ao} - \mathrm{As}}{\mathrm{Ao}}$$

Where Ao is the absorption reading of control and As is the absorption reading of the pigment.

Antimicrobial test:

Using disc diffusion method, the lyophilized pigment powder was dissolved in methanol after that sterile filter paper discs were immersed in it for 3 hours. LB agar media were poured into petri dishes and then inoculated with several pathogenic bacteria; *Escherichia coli, Salmonella typhi, Pseudomonas, Staphylococcus aureus, Candida, Klebsiella and Bacillus cereus*. Saturated discs were applied into the surface of the media and incubated at 37°C for 24 hours. Saturated methanol discs were used as control [14][15].[3, 12].

RESULTS

Isolation and characterization of pigmented bacteria from soil:

Yellow Pigmented bacteria were isolated from soil sample on LB agar media. The bacterial colonies appeared yellow, circular, smooth, opaque, convex with entire margin (Figure 1 A and B). The isolated bacteria was Gram-positive tetra-coccid shaped structure (Figure 2 A and B). The tetrad coccoid structure formation leads to identification of the isolated bacteria as *Micrococcus* sp.

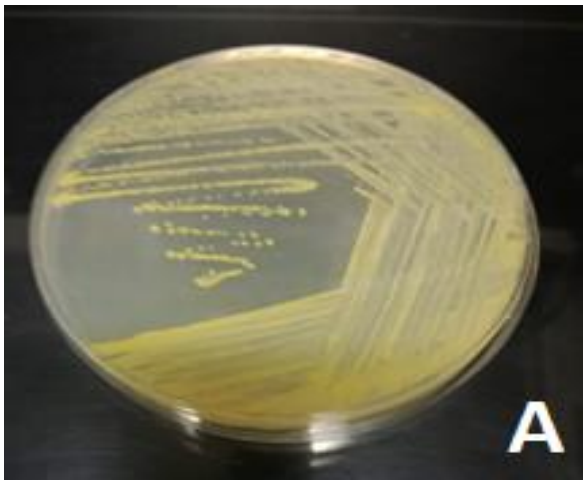

Figure 1: *Micrococcus* bacteria on LB agar medium (A) and magnified view of its colonies (B).

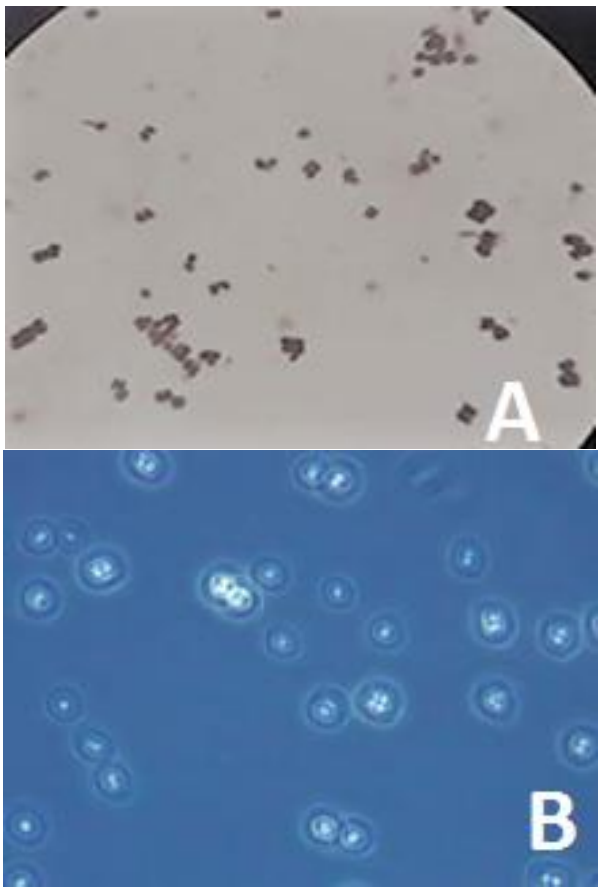

Figure 2: Gram staining of*Micrococcus* (A) and its view under phase contrast microscope (B).

Identification of yellow pigmented isolate:

The yellow pigmented isolate was identified via analysis of the 16S rRNA gene sequences and comparing to the closest type strains obtained from the database. The analysis showed that isolate could be identified as *Micrococcus alovera* species exposing high sequence similarity (99.89%) to Type Strain *Micrococcus aloeverae* strain AE-6 (Figure 3)**.**

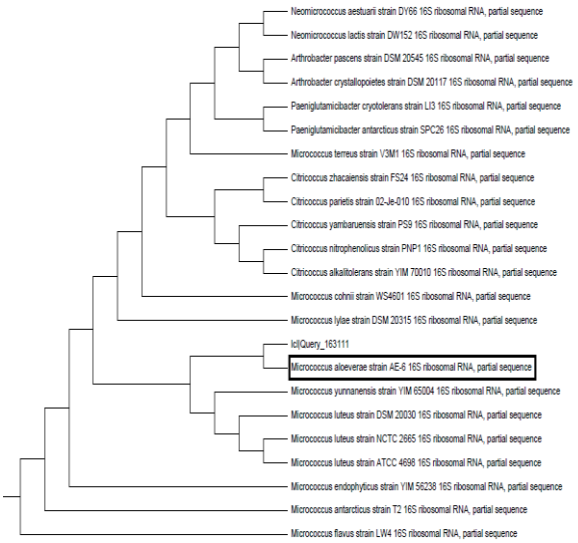

Figure 3: Phylogenetic tree of *Micrococcus aloverae*

Extraction of the pigment:

After using several solvents such as acetone, ethyl acetate, chloroform, hexane, diethyl ether, ethanol, butanol, propanol and methanol in different concentrations, methanol was the best solvent in extraction of the yellow pigment of *Micrococcus* sp. It was noted that the dry pigment powder was not soluble in water (Figure 4).

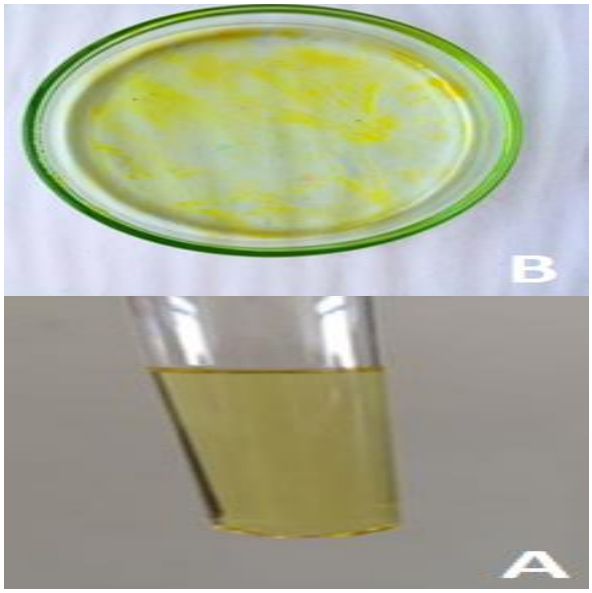

Figure 4: Methanolic extract of *Micrococcus* yellow pigment (A) and a petri dish containing the pigment after lyophiliztion (B).

FT-IR absorption of the yellow pigment gave strong and broad peak at 3447 cm^{-1} as (Figure 5). The spectrum obtained after analysis also showed medium peaks at 1635 and at 1404 cm^{-1}. The peaks at 3447, 1635 and 1404 cm^{-1} correspond to different functional groups[1].

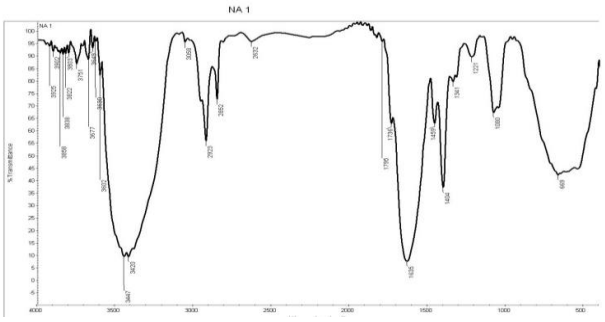

Figure (5): FTIR profile of the yellow pigment

FTIR spectrophotometry

Antioxidant activity (DPPH radical scavenging activity)

The free radical-scavenging activity of the *Micrococcus* yellow pigment was analyzed. It gave inhibition percentage of 80.08% with IC50 of 0.355 mg/ml.

Antimicrobial activity of methanolic pigment

From the antimicrobial activity of the methanolic pigment extract of *Micrococcus* against *Escherichia coli, Salmonella typhi, Pseudomonas, Staphylococcus aureus, Candida, Klebsiella and Bacillus cereus*, no clear zones were observed. This means that *Micrococcus* pigment has no antimicrobial activity against the previously mentioned microorganisms.

Discussion

The need for natural pigments is increasing day by day, because of its environmental safety as well as beneficial effects on human health[1]. Natural pigments possess anticancer activity, contain pro-vitamin A and have some desirable properties like stability to light, heat and pH[16].

Micrococcus sp. which showed up as yellowish colonies was identified as Gram-positive coccoid bacteria[17]. In this study, one yellow pigmented isolate was obtained from the soil samples for their ability of pigment production. The isolate was phylogenetically identified as *Micrococcus alovera.*

FT-IR helps to define the functional groups in the sample. Different functional groups absorb certain frequencies of IR radiations in different way. FT-IR absorption of the yellow pigment gave strong and broad peak at 3447 cm^{-1}. The spectrum obtained after analysis also showed medium peaks at 1635 and at 1404 cm^{-1}. The peaks at 3447, 1635 and 1404 cm^{-1}

correspond to different functional groups[1]. Spectroscopic profile showed the characteristic peaks of carotenoid pigment. Carotenoid is one of the pigments of natural source play an important role in human diet because of their properties e.g., action as antioxidant, pro-vitamin or possibly their role in tumor inhibition[18].

The yellow pigment produced by *M. luteus* was a dihydroxy C50 carotenoid; while α or β carotene derivative with canthaxanthin as the main pigment was produced by *M. roseus* [1].

In antioxidant activity assay, the results were compared with ascorbic acid. The free radical-scavenging activity of the *Micrococcus alovera* yellow pigment was analyzed. It gave inhibition percentage of 80.08% with IC50 of 0.355 mg/ml, which means that the yellow pigment has antioxidant activity [19].

The antimicrobial test gave negative results against pathogenic microorganisms mentioned above unlike searches which prove that the pigment have antimicrobial activity, which may be affected by the used technique in each search [19].

From all the previous work it can be concluded that the yellow pigment produced by *Micrococcus alovera* has promising applications that need further analysis.

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