International Journal of Molecular and Clinical Microbiology 7(1) (2017) 809-815

International Journal of
Molecular and Clinical Microbiology

# Antimicrobial and Antioxidant Activity of Carotenoid Pigment Produced by Native *Rhodococcus* spp. Isolated from Soil

**Mahdieh Sadat Haddad[1] and Soheil Aghaei[*1] , Mohsen Zargar[1]**

*1. Department of Microbiology, Qom Branch, Islamic Azad University, Qom, Iran*

ARTICLE INFO

*Article history:*
Received 5 April 2017
Accepted 28 May 2017
Available online 1 June 2017

*Keywords:*
*Rhodococcus rhodochrous*
*carotenid pigment*
*antimicrobial activity*
*antioxidant property*
*GC-MS*

ABSTRACT

In recent years, scientists and researchers from all over the world pay much attention to carotenoid pigments as a substitute for antibiotics and synthetic chemical compounds with antioxidant property and the plants, various microorganisms including bacteria and algae are also capable of producing carotenoid pigments. In this research, the production of antimicrobial and antioxidant carotenoid pigment in native strain of *Rhodococcus* bacterium was studied and evaluated for the first time in Iran. The activation and growth of the native *Rhodococcus rhodochrous* isolate was done using cultivation on different culture media including Triptic soy agar, BHI agar, ISP5, Bennett's Agar and LB Agar at 30°C. Pigment extraction was carried out with different organic solvents and carotenoid pigment components were analysed using GC-MS method. Antimicrobial activity of the pigment was examined using Agar well diffusion method and its antioxidant property by DPPH(2,2-diphenyl-1-Picrilhydrazil) method was examined and analysed by comparing to antioxidant BHT. The best media for growth and pigment production were determined TSA and TSB. The antimicrobial activity of carotenoid pigment was obtained against to *Staphylococcus aureus* and *Candida albicans* with 21mm and 35mm in diameter of inhibition zone respectively. Antioxidant activity of pigment was determined at the concentration of 8 mg/ml (75/59181 %). GC-MS analyses of extracted pigment showed compounds with the highest peak area. The results of this research indicated that native *Rhodococcus rhodochrous* strain as a non-pathogenic soil bacterium can be candidate biological source with antimicrobial and antioxidant activity for various applications in farmaucidal and biotechnological products.

## 1. Introduction

According to Netzer, Stafsnes *et al.* (2010), carotenoids are natural pigments that are synthesized by bacteria, fungus, algae and plants (Netzer *et al*., 2010). Carotenoids are intracellular products and usually located in the membranes of mitochondria, chloroplasts or the endoplasmic reticulum and are generally hydrophobic compounds. Also they are in connection with such membranes as hydrophobic structure. Noviendri, Hasrini *et al.* (2011), declare that Animals cannot synthesize and produce carotenoids and they must obtain carotenoids from nutrients.

Sandmann (2003) indicated that Carotenoids are considered as important nutriceutical and lipophilic antioxidants compounds. Potential antioxidant carotenoids depend on chemical properties, such as the number of conjugated

*Corresponding author: Soheil Aghaei
E-mail address:soheilaghaee@Yahoo.com

double bonds, structure of the final groups and oxygenated alternatives. Based on epidemiological and clinical studies conducted by Linnewiel-Hermoni *et al.*, (2015), the direct effect of carotenoids can be used as lowering factors of chronic diseases. (Linnewiel-Hermoni *et al*., 2015).

One of the most important sources for searching the antimicrobial agents and important molecules in biotechnology field is microbial pigments that can be used as natural colors as well as antimicrobial agents instead of antibiotics (Rashid *et al*., 2014). One of the carotenoid producing bacteria, is *Rhodococcus*. The genus *Rhodococcus* of *Actinobacteria*, was first explained in 1891 by Zopf (Bell *et al.*, 1998).

Borisova (2011) tested 16 different species of *Rhodococcus* and showed 11 types of carotenoid pigments in them. 4 species produce beta - carotene and 11 species produce a gamma-carotene-like substance. Some species can also produce derivatives of beta and gamma - carotene, such as mixoxanthophyl-like, zeaxanthin-like and B-citraurin-like (Borisova, 2011).

The aim of this study was to assess the evaluation of carotenoids pigments production in native *Rhodococucs* strain and determining its antimicrobial and antioxidant activity in vitro conditions.

## 2. Materials and Methods

### *2.1. Preparation of bacterial strains*

The native bacterial strain used in this research was isolated by Aghaei *et.al* in 2015 from manganese mine soils in Qom city. (Aghaei *et al.*, 2015). Pathogenic strains for antimicrobial assay were prepared from accredited academic research centers such as the Iranian Research Organization for Science and Technology.

The isolates were cultivated in different agar media such as: ISP5, TSA, BHI Agar, Bennett's Agar (Zhang *et al.*, 2005) and LB Medium (Indra Arulselvi *et al*., 2014) purpose for good growth and primary screening of the pigment production.

### *2.2. The macroscopic and microscopic properties of native bacterial strain*

Morphologically characteristics of the native isolate in terms of shape and appearance of colonies was examined by gram stain and through optical microscope. Biochemical and metabolic properties of the isolate were tested for assurance (McNeil and Brown, 1994).

### *2.3. Determining the growth curve of the isolate*

At this step, 20 number of 250-ml flask containing 50 ml of sterile Tryptic soy Broth (TSB) and 1 ml of suspension with 0.5 McFarland opacity of native *Rhodococcus rhodochrous* isolate was inoculated to these media and they were placed at 30°C and 180 rpm, in the shaking incubator. The absorption spectrum growth rate was recorded every 10 hours at 600 nm wavelength by spectrophotometry device with 3 repetitions and the growth curve was designed based on the obtained absorption spectrum (Hall *et al.*, 2014).

### *2.4. Determining of the pigment production curve*

10 number of 250-ml containing 50 ml of sterile Tryptic soy Broth (TSB) and 1 ml of suspension with 0.5 McFarland opacity of *Rhodococcus rhodochrous* was inoculated to these media and were incubated at 30°C and 180 rpm, in the incubator shaker. Also the absorption spectrum growth rate of *Rhodococcus rhodochrous* isolate was recorded every 10 hours at 400-600 nm wavelength with a gap of 10 nm by spectrophotometry device with 3 repetitions and the growth curve of this bacterium was designed based on the obtained absorption spectrum.

The absorption spectrum of pigment production with different resources and conditions was recorded every 24 hours at 460 nm wavelength by spectrophotometry device with 3 repetitions and pigment production curves was designed based on the obtained absorption spectrum (Ichiyama *et al*., 1989).

### *2.5. Pigment extraction from R. rhodococcus*

Several 250-ml erlenes containing 100 ml of sterile Trypticase soy Broth (TSB) and 2 ml of suspension with 0.5 McFarland opacity of *Rhodococcus rhodochrous* isolate was inoculated to these liquid culture media and

incubated at 30°C and 200 rpm, in the incubator shaker. After that, in order to extraction of the pigment, 100 ml Tryptic soy broth containing pigment production was centrifuged at 12000 rpm for 10 minutes. The liquid phase (supernatant) was discarded. Then, methanol – stone cold solvents (1:1) was added to the precipitated cells. After a complete mixture of pellet with solvent, they were stored in the fridge for 1 to 6 hours. These mixture were centrifuged for 10 minutes at 12000 rpm and after collecting the extracts, the absorption spectrum of the sample was recorded by spectrophotometer at a maximum wavelength of 460 nm pigment production(Oren and Rodríguez-Valera 2001).

*2.6. Gas Chromatography - Mass Spectrometry analyses of Extracted Pigment*

Chromatograph device with model 6890 coupled with mass spectroscopy model N - 5973 made by the Agilent Company with capillary column of HP- 5MS and 5% methyl phenyl of the stagnant phase (30 m length, internal diameter of 0.25 mm, the thickness of stagnant layer 0.25 µm) and the ionization energy of 70 electron volt was analysed to qualify the components. At first thermal planning for analyses started at the temperature of the oven - that is 60°C. Injection and detector temperature was 250°C, injectable sample volume was 1 µl and with 1.50 split and helium gas with the flow rate of 1.5 ml / min. To prepare the sample for mass spectrometric - gas chromatography, after sample extraction (which in here is *Rhodococcus rhodochrous*) using the solvent, through a specific device at 37°C and 120 rpm, we condensed the sample as much as possible, and what remains is extracted from the pigment which is almost non-solvent (Elumalai *et al*., 2014).

*2.7. Antioxidant activity of the extracted pigment*

In this stage of our work, 1 ml of serial diluted pigment and 1 ml of 2-diphenyl-1-Picril hydrazil were mixed in the glassial tube. Then, 1 ml of the standard series suspension, 1 ml of standard suspension of Butylated hydroxytoluene (BHT) and 1 ml of suspension 2-diphenyl-1-Picril hydrazil were mixed in the experiments tube and they were put in darkness for 30 minutes. Then the absorbance of mixtures were recorded at 517 nm with 3 repetitions. The amount of free radical DPPH (2,2-diphenyl-1-picrilhydrazil) by *Rhodococus rhodochrous* pigment is calculated from the following formula (Mohana, Thippeswamy *et al*., 2013).

$$I\% = \frac{(A_{Control} - A_{Sample})}{A_{Control}} \times 100$$

Sample : Pigment *Rhodococcus rhodochrous*

Control : DPPH

A : Absorbance

*2.8. Antimicrobial activity of the extracted pigment*

Antimicrobial activity of the extracted pigment was evaluated by agar well diffusion method. To do this, 100 µl from the methanolic suspension of *Rhodococcus rhodochrous* extracted pigment was against some pathogenic strains: *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Shigella dysenteriae* (PTCC 1188), *Bacillus subtilis* (PTCC 1715), *Enterococcus faecalis* (ATCC 51299), *Listeria monocytogenes* (PTCC 1295), *Candida albicans* (ATCC 10231). Plates were incubated at 37°c for 24 hours. (Rashid *et al*. 2014).

## 3. Results

*3.1. The morphology of Rhodococcus rhodochrous*

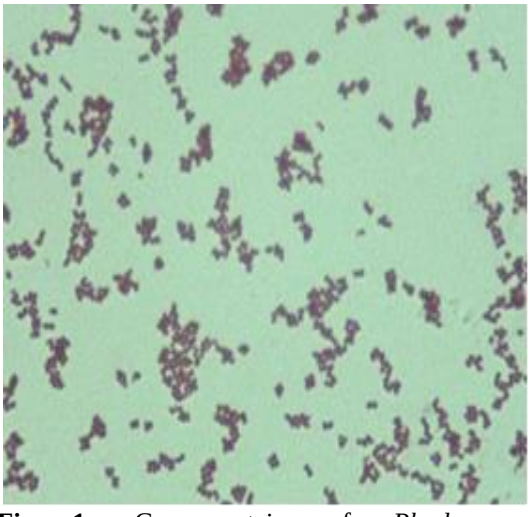


**Figure1.** Gram stain of *Rhodococcus rhodochrous* isolate cultivated on Tryptic Soy agar medium after 72h.

**Figure 2.** Mucoid orange colonies of *Rhodococcus rhodochrous* isolate on Tryptic Soy agar after 72h.

### *3.2. Rhodococcus rhodochrous growth curve*

The absorption spectrum growth of *Rhodococcus rhodochrous* isolate was recorded every 10 hours at 600 nm wavelength by spectrophotometry device with 3 repetitions. The growth curve of this bacterium was obtained based on the optical spectrometry method and Statistical analysis were performed using SPSS v 11/5 (Diagram 1).

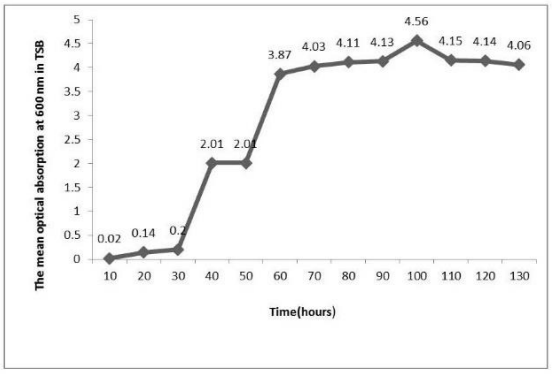



**Diagram 1.** Growth curve of *Rhodococcus rhodochrous*

### *3.3. Pigment production curve* of *Rhodococcus rhodochrous*

The absorption spectrum of *Rhodococcus rhodochrous* pigment production was recorded every 10 hours at 400-600 nm wavelength with a gap of 10 nm by spectrophotometry with 3 repetitions and pigment production curve was designed according to the obtained absorption spectrum. Statistical analysis were performed with SPSS v. 11/5 and The maximum of pigment production at 460 nm wavelength has been mentioned (Diagram 2).

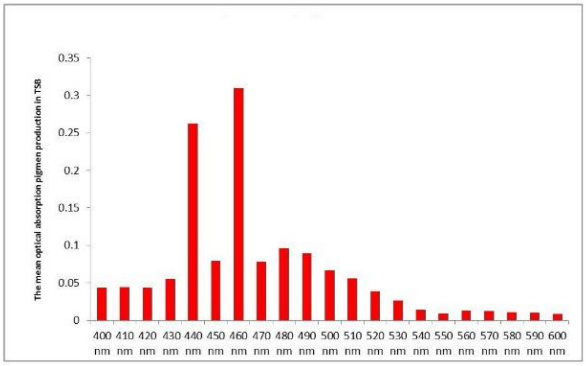



**Diagram 2.** *Rhodococcus rhodochrous* pigment production curve at 10th hour on Tryptic Soy Broth at 30°C and 180 rpm.

### *3.4. Gas Chromatography–Mass Spectrometry*

In this method, special chromatogram At Different time (diagram 3-10) And mass spectrum has shown the existence of compounds in the related peaks including Ethyle Acetate, Bis (2-ethylhexyl) phthalate, Hexadecanoic acid, 1,2 BenzenedeCarboxylic acids, Octa decanoic acid (table 1).

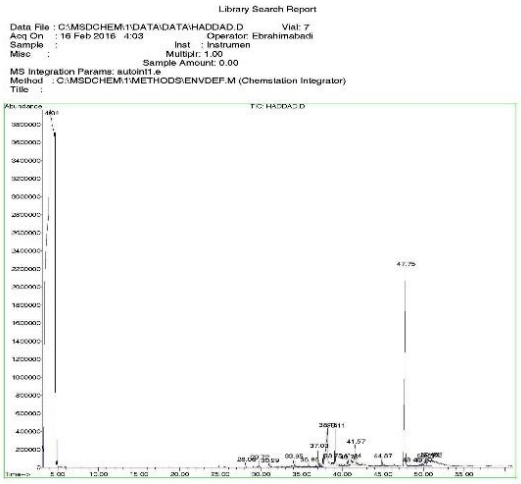



**Figure 3.** The extracted pigment from isolated *Rhodococcus rhodochrous* using GC-Mass method.

### *3.5. Antioxidant activity of the extracted Pigment*

According to the results, antioxidant activity of extracted pigment of *Rhodococcus rhodochrous* isolate was depended to the density of methanol extract of pigment. The free radical scavenging rate and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was observed at the density of 8 mg per liter (75/59181%) of methanol extract of pigment (Fig 3).

**Table 1.** Identified compounds in gas chromatography–mass spectrometry from *Rhodococcus rhodochrous* pigment.

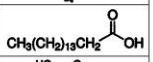
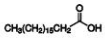
| Metabolite profile | Molecular Formula | Molecular Weight | Area% | Chemical Structure |
|---|---|---|---|---|
| Ethyl Acetate | C4H8O2 | 88 /11 g/mol | 85/49 |  |
| Bis(2-ethylhexyl) phthalate | $C_{24}H_{38}O_4$ | 390/56 g/mol | 6/22 |  |
| Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256/42408 g/mol | 2/54 |  |
| 1,2-Benzenedicarboxylic acid | $C_8H_6O_4$ | 166/14 g/mol | 1/07 |  |
| Octadecanoic acid | $C_{18}H_{36}O_2$ | 284/4772 g/mol | 0/77 |  |

**Figure 4.** The free radical scavenging rate and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was observed at the density of 8 mg/liter (75/59181%) of methanol extract of pigment *Rhodococcus rhodochrous.*

### *3.6. Antimicrobial activity of the extracted pigment*

Antimicrobial activity of extracted pigment was observed with inhibition zones of the growth against to *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC 10231) (Figure 5-6).


**Figure 5.** Inhibition zone of *Staphylococcus aureus* (ATCC 25923) with 21 mm in diameter on the Muller Hinton agar medium against pigment of *Rhodococcus rhodochrous.*

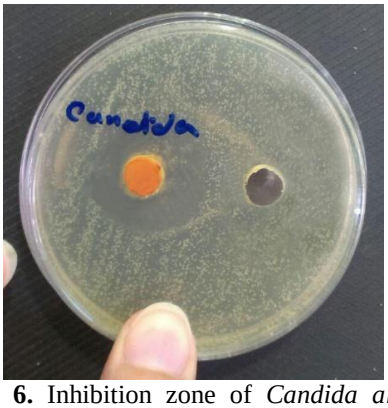



**Figure 6.** Inhibition zone of *Candida albicans* growth (ATCC 10231) with 35 mm in diameter on the Muller Hinton agar medium against pigment *Rhodococcus rhodochrous*.

## 4. Discussion

Native strain of *Rhodococcus* in this study was isolated by Aghaei and Nima Bahador in 2014 from manganese mine soils in Qom (Aghaei *et al*., 2015). In this study the best medium for optimum growth of native *Rhodococcus rhodochrous* Tryptic soy agar (TSA) and Brain Heart Infusion Agar (BHI Agar) at 30°C. Zhang, Li *et al.*, in 2005 by separating strain from *Rhodococcus rhodochrous*, observed a proper growth from *Rhdococcus* genus on tryptic soy agar and ISP5 agar media at 28°c (Zhang *et al.*, 2005).

In our research, in terms of pigment production, Tryptic Soy agar medium was chosen as the best. In the studies of Satoshi *et al.* in 1998 concerning the proof of pigment production carotenoid in the *Rhodococcus* genus, they used souton agar medium at 28°C for *Rhodococcus rhodnii* and 37°C for other *Rhodococcus* genus (Ichiyama *et al*., 1989). In 2012, Wang *et al*., in a study about the optimization of beta-carotene production in the *Serratia marcescens* strain used the nutrient medium agar and broth nutrient at 30°C in beta-carotene production (Wang *et al*., 2012). The maximum growth rate of *Rhodococcus rhodochrous* was at 10 hour (fifth day after inoculation) and from the fifth day onward, the average optical absorption was almost constant, so *Rhodococcus rhodochrous* entered a Stationary phase in terms of growth. In 2014, Hamidi *et al*., in a study about the optimization of carotenoid in the *Halorubrum* Sp. TBZ126, Growth rate increases up to a maximum of 9 days and then enters the Stationary phase (Hamidi *et al*., 2014).

In this study, the maximum absorption of *Rhodococcus rhodochrous* pigment production was recorded at a wavelength of 460 nm. In a study by Satoshi *et.al* about the carotenoid pigment in the genus of *Rhodococcus* the maximum absorption of pigment production in nanometers was (430-425), (440-435), 449, 450, 460, 470, 474, 475, 477, 489, 500 and 503 (Ichiyama *et al*., 1989). Indra *et al*., in 2014 isolated gram positive bacterium strain YCD3b, with the highest production of carotenoid pigments at a maximum wavelength of 450 nm (Indra *et al*., 2014). In 2013, Mohana *et al*. based on a study about the Carotenoid pigment of *Micrococcus* species reported that the maximum absorption for *Micrococcus roseus* and *Micrococcus luteus* pigment were at the wavelength of 476.31 and 437.16 respectively (Mohana *et al*., 2013).In this investigation carotenoid pigment extraction was done using a solvent method in methanol to acetone ratio 1: 1 and centrifugation at 12000 rpm for 10 minutes. According to studies of Aharon Oren *et al*., 2001 on holographic bacteria with red pigment, they used the same method to extract pigments (Oren and Rodríguez-Valera, 2001). In the present study, using gas chromatography, mass spectrometry for the chromatograph device model 6890 coupled with mass spectrometer model 5973N made by Agilent company has a capillary column HP-5MS with methylene phenylsiloxane stagnant phase 5% (30 m length, internal diameter 0.25 mm, thickness of the stagnant layer 0.25 micrometer) and were analysed for qualitative identification of compounds such as ethyl acetate (85/49%), bis (2-ethylhexyl) phthalate (6/22%), hexadecanoic acid (2/54%), 1,2 benzene dicarboxylic acid (1/07%) and octadecanoic acid (0/77%).

In 2014, Alumley *et al*., in a study on the extraction of carotenoids in a new strain of *haematococcus filial* using SHIMADZU device with capillary column VF-5ms (30 m length, internal diameter 0.25 mm, thickness of the stagnant layer 0.25 micrometer) analysed ionization energy of 70 electron volts for qualitative identification of compounds such as hexadecanoic acid, ethyl ester (17.75%), ethyl oleate (11.65%), 2- hexadecanol (78.7%), 5 - Octadecanol (6.98%), Cyclopentane, 1-phenyl-3-(-phenyl-1-trimethylsilymethylene) (10/92%) (Elumalai *et al*., 2014). In this study, the antimicrobial activity of *Rhodococcus rhodochrous* pigment by a well-agar method and antimicrobial activity of *Rhodococcus rhodochrous* pigment against *Staphylococcus aureus* was with 21 mm inhibition zone and *candida albicans* was with 35 mm inhibition zone. In 2013, Mohana *et al*., reported antimicrobial activity of the carotenoid pigment of *Micrococcus roseus* and *Micrococcus luteus* using a disk diffusion method against *Streptococcus* with inhibition zone of 6.5 – 15 mm diameter (Mohana *et al*., 2013). In the present study, the antioxidant property of *Rhodococcus rhodochrous* pigment was evaluated and examined by 2 , 2 d-phenyl-1-picyrilhydrazil (DPPH) method with comparing to antioxidant butyllithium hydroxytoluene (BHT) at 517 nm wavelengths. The maximum antioxidant activity of *Rhodococcus rhodochrous* pigment in this study was 8 mg/ml (75/59181%).

In a study by Mohana *et al*., in 2013, carotenoid pigment antioxidant properties of *Micrococcus roseus* and *Micrococcus luteus* were conducted in the same way and antioxidant property of *Micrococcus roseus* was reported at a density of 3.5 mg / ml and *Micrococcus luteus* a with a density of 4.5 mg / ml (Mohana *et al*., 2013).

## Conclusion

The results of this research for the first time showed that native *Rhodococcus rhodochrous* strain isolated in Iran, is one of the most important carotenoid pigment producing microorganism as a non-pathogenic soil bacterium and also its antimicrobial activity against to *staphylococcus aureus* and *candida albicans* can be used in the field of pharmaceutical biotechnology, food, cosmetics and sanitation industry.

## Refereces

Aghaei, S., Bahador, N., Baserisalehi, M. (2015). Isolation and identification of bioactive compound producing *Rhodococcus* spp. isolated from soil samples. International Journal of Molecular and Clinical Microbiology 5(1): 463-468.

Bell, K., Philp, JC., Aw, DWJ., Christofi, N. (1998). The genus *Rhodococcus*. Journal of Applied Microbiology 85(2): 195-210.

Elumalai, S., Santhose, B., Kanna, Rajesh G. (2014). Extraction of carotenoid and thin layer chromatography (TLC), GC-MS, FT-IR and HPLC analysis of pharmaceutically important pigment astaxanthin from a new strain of *Haematococcus pluvialis.* Weekly Science Research Journal 2(8): 2321-7871.

Hall, B. G., Acar, H., Nandipati, A., Barlow, M . (2014). Growth rates made easy. Molecular biology and evolution 31(1): 232-238.

Hamidi, M., Abdin, M.Z., Nazemyieh, H., Hejazi, M.A., Hejazi, M.S. (2014). Optimization of Total Carotenoid Production by *Halorubrum* sp. TBZ126 using response surface methodology. Journal of Microbial & Biochemical Technology. 6(5): 286-294.

Ichiyama, S., Shimokata, K., Tsukamura, M. (1989). Carotenoid pigments of genus *Rhodococcus*. Microbiology and immunology 33(6): 503-508.

Indra Arulselvi, P., Umamaheswari, S., Ranandkumar Sharma, G., Karthik, C., Jayakrishna, C. (2014). Screening of Yellow Pigment Producing Bacterial Isolates from Various Eco-climatic Areas and Analysis of the Carotenoid Produced by the Isolate. J Food Process Technol 5(292):1-4.

Linnewiel-Hermoni, K., Khanin, M., Danilenko, M., Zango, G., Amosi, Y., Levy, J., Sharoni, Y. (2015). The anti-cancer effects of carotenoids and other phytonutrients resides in their combined activity. Archives of biochemistry and biophysics 572: 28-35.

McNeil, M.M. and Brown, J.M. (1994). The medically important aerobic actinomycetes: epidemiology and microbiology. Clinical microbiology reviews 7(3): 357-417.

Mohana, D.C., Thippeswamy, S., Abhishek, R.U. (2013). Antioxidant, antibacterial, and ultraviolet-protective properties of carotenoids isolated from *Micrococcus* spp." Radiation Protection and Environment 36(4): 168-174.

Netzer, R., Stafsnes, Marit H., Andreassen, T., Goksøyr, A., Bruheim, P., Brautaset, T., (2010). Biosynthetic pathway for γ-cyclic sarcinaxanthin in *Micrococcus luteus*: Heterologous expression and evidence for diverse and multiple catalytic functions of C50 carotenoid cyclases. Journal of bacteriology 192(21): 5688-5699.

Noviendri, D., Hasrini, R., Octavianti, F. (2011). Carotenoids: Sources, medicinal properties and their application in food and nutraceutical industry. Journal of Medicinal Plants Research 5(33): 7119-7131.

Oren, A., and Rodríguez-Valera, F. (2001). The contribution of halophilic Bacteria to the red coloration of saltern crystallizer ponds. FEMS Microbiology Ecology 36(2-3): 123-130.

Rashid, M. M., Fakruddin, Md., Mazumdar, R. M., Kaniz, F., Chowdhury, Md. A. (2014). Anti-Bacterial Activity of Pigments Isolated From Pigment-Forming Soil Bacteria. British Journal of Pharmaceutical Research 4(8): 880-894.

Wang, B., Lin, L., Lu, L., Chen, W. (2012). Optimization of β-carotene production by a newly isolated *Serratia marcescens* strain. Electronic Journal of Biotechnology 15(6): 3-3.

Zhang, Y.Q., Li, W.J., Kroppenstedt, R.M., Kim, C. J., Chen, G.Z., Park, D.J., Xu, L.H. Jiang, C.L, .(2005). *Rhodococcus yunnanensis* sp. nov., a mesophilic actinobacterium isolated from forest soil. International Journal of Systematic and Evolutionary Microbiology 55(3): 1133-1137.