*IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)*
*ISSN: 2455-264X, Volume 2, Issue 5 (Jul. – Aug. 2016), PP 58-61*
*www.iosrjournals.org*

# Study of Biological Activities and Characterization of the Crude Orange Pigment isolated from A Distinct *Salinicoccus Sp*. MKJ997975

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***Abstract:*** *In the present study crude orange pigment isolated from a distinct Salinicoccus sp. MKJ997975 was investigated for antibacterial and antioxidant activity. Elucidation of the structural characteristic of the pigment was performed using the techniques of FTIR and NMR. Antibacterial activity using disc diffusion method for two gram negative and two gram positive bacteria concluded that the orange pigments did not show any toxicity. Radical scavenging activity by DPPH test for analysing antioxidant activity showed a good antioxidant property. The FTIR results suggested that the pigments can be xanthophylls – Peridinin and Pyrrhoxanthin with the supporting results of TLC and UV spectroscopy. The NMR results exhibited more of background noise and thus no satisfactory results were obtained. Characterization can be improved by further purification of the crude pigments and then comparing with the standards.*

***Keywords:*** *Salinicoccus sp MKJ997975, Antibacterial, Antioxidant, orange, pigment, Peridinin, Pyrrhoxanthin.*

## I. Introduction

Pigment is one of the alluring features on the Earth. In ancient time's painting was one of the interesting arts in which the paints were made by naturally available pigments. The traditional paintings of Indian arts of Kerela included 'mural paintings' in which colours extracted from soots of oil lamps and from leaves extract of *Neelamari* and *Eravikkara*[1]. Replacing synthetic pigments with natural pigments isolated from various sources have led to a step forward in the field of industrial waste management. Pigments extracted from different microorganisms are found to have some biological activities. Various applications of naturally derived have been found in textile industries for dyeing purpose. *Roseomonas fauriae*, a gram negative bacteria has application of dyeing the cotton fabrics[2]. Food colours bixin, anthraquinone and actinorhodin from *Bixa orellana*(shrub), *Penicillium oxalicum*(fungus) and *Streptomyces coelicolor*(bacteria) respectively is used as natural colouring agents[3,4,5]. Xanthophyll from the plant of *Tagetes erecta*-L can also be used for coloration purpose if the levels of the lutein content are increased[6]. The scrutiny and negative assessment of synthetic food dyes by the modern consumer have given rise to a strong interest in natural colouring alternatives. This study is an attempt to extract, purify and characterize an orange coloured pigment from a novel bacterial species and evaluate its antibacterial and antioxidant potential.

## II. Materials and Methods

### 2.1 Isolation and extraction of the pigment

The organism used in the study was isolated from forest soil of Tungareshwar, Vasai, Maharashtra. The organism was identified as *Salinicoccus* sp. M KJ997975 by 16S rRNA sequencing method[7]. The protocol for extraction of the pigment was modified[8,9,10]. The bacterial culture was inoculated in the nutrient broth and cultivated at room temperature for 72 hrs in shaking conditions. After 3 days the culture was centrifuged at 10000rpm at 4°C for 20 minutes. Different solvents were used for analysing the best extraction of pigment. The pellet was further treated with methanol and Acetone: Methanol (1:1) in the water bath for 1 hr. The extracted orange supernatant was filtered by Whatman no 1 filter paper and analyzed in Shimadzu UV spectrophotometer between wavelength 400-600nm. The extracts were concentrated under reduced pressure in rotary evaporator at 50°C.

### 2.2 Stability test of the pigment

Stability test of crude pigment extract was carried out at 4°C, RT and 37°C in light and dark conditions for 24hrs. The extract was then analyzed at 472nm using UV-VIS spectrophotometer (UV-1700 Shimadzu)[9].

### 2.3 Antibacterial activity test

The protocol was modified for antibacterial activity testing[11]. Antibacterial activity of pigment was determined by well diffusion method. Four 8mm wells were made in each plate with the sterilized cork borer.

Various dilutions were made and Acetone : Methanol (1:1) was used as a control.100µl bacterial suspensions of *Bacillus subtilus, Proteus vulgaris, Sarcina lutea* and *Pseudomonas aeruginosa* was uniformly swabbed on nutrient broth plates. These plates were incubated at 30±2°C for 24hrs.

**2.4 Antioxidant activity test**

The protocol was modified for antioxidant testing[12]. The free radical scavenging activity of the crude extract was tested by the DPPH method with some modifications. DPPH (1,1-diphenyl-2-picrylhydrazyl) of 0.3mM(5.91mg in 50ml of methanol) was prepared in methanol. Different dilutions of pigment extract were prepared ranging from 50µl-450µl and ascorbic acid was used as a standard (0.4mg/ml) in methanol. The reaction mixture was prepared and vortexed for 10s following with incubation at room temperature for 30min in dark conditions. The absorbance was measured using UV-VIS spectrophotometer (UV-1700 Shimadzu).The assay was performed in duplicates.

**2.5 Characterization of the crude pigment**

The characterization of the crude pigment was done using FTIR spectroscopy (3000 Hyperion Microscope with Vertex 80 FTIR System, Bruker, Germany) at SAIF, IIT, Bombay. The method for FTIR spectroscopy of pigment was modified[13]. The dried sample was mixed with the KBr powder. The range was set to mid-IR range from 4000cm$^{-1}$ to 400 cm$^{-1}$. A spectral resolution of 2 cm$^{-1}$ was set.

## III. Results and Discussion

**3.1 Isolation and extraction of the pigment**

The organism used in the study was isolated from forest soil of Tungareshwar, Vasai, Maharashtra. The organism was identified as *Salinicoccus* sp. M KJ997975 by 16S rRNA sequencing method. Various solvents were used on bacterial pellet to check the best solvent for extraction of the pigment. At 472nm the best extraction was observed in Acetone:Methanol(1:1)> Methanol> Acetone: Methanol(7:3) > Propanol> Ethanol> Acetone: Methanol(3:7)> Acetone> Ethyl Acetate> Acetonitrile/Acetic acid> Benzene> Hexane. The absorption spectrum of Acetone: Methanol extract of the cell pellet from the bacterial isolates showed maximum absorption at 472nm which suggested for presence of carotenoids.

**3.2 Stability test of the pigment**

The stability assessment of the pigment proved that the different temperatures did not change the optical densities of the pigment at the greater extent but when the methanolic extract was kept for more 5 days it was found that as methanol is volatile in nature and thus at high temperatures at both room and 37°C the amount in eppendoff tube was decreased. The effect of light on pigment colour was tested and it was seen that light affected its colour brightness indicating the pigment was light sensitive. Stability at varied temperatures and different solvents till long time can be checked. Different vegetable oils like sunflower oil, groundnut oil, coconut oil, sesame oil and palm oil at different temperatures in presence of light and dark conditions for 30 days[13].

**3.3 Antibacterial activity test**

The antimicrobial activity of the pigment was checked against four pathogens *Pseudomonas aeruginosa*, *Sarcina lutea*, *Bacillus subtilus* and *Proteus vulgaris* depicted no zone of inhibition. Carotenoids exhibit excellent antimicrobial activity but only some xanthophylls have shown good antimicrobial activity. The xanthophylls extracted from the plants of *Acacia catechu* (L.), *Pterocarpus marsupium, Toddalia asiatica* (L.) *and Ventilago denticulate* has shown excellent antibacterial activity with zone of inhibition ranging from 14 to 16mm[14]. The pigment needs to be concentrated in order to analyze antibacterial activity at the higher concentration.

**3.4 Antioxidant activity test**

DPPH test is an easy and fast method based on the stable free radical which fades purple colour and turns to yellow in the presence of antioxidants. Antioxidant activity of crude pigment extracted in methanol and ascorbic acid was measured. It was found that from 3µg/ml to 9µg/ml the antioxidant activity of pigment was higher than that of ascorbic acid and decrease in the activity of pigment was observed at higher concentrations when compared to ascorbic acid. A major number of xanthophylls such as violoxanthin, neoxanthin, zeaxanthin and lutein have shown good antioxidant activity[15]. It can be concluded that the power of antioxidant activity of extracted pigment is excellent.

**3.5 Characterization of the crude pigment**

The comparison of the FTIR peaks was accessed by the online database –Lipid Bank[16]. The TLC screened for pigment initially suggested that the pigment is a xanthophyll and secondly UV spectra showed for

the maximum absorption near 472nm in methanol[17]. The FTIR resulted in five major peaks and two minor peaks. The FTIR reports were screened according to UV absorption and TLC. The FTIR results suggested that the major peaks obtained were matched with four compounds. Fucoxanthin, Fucoxanthinol, Peridinin and Pyrrohxanthin. But the fucoxanthin and Fucoxanthinol was brownish in colour and the lambda max was less likely near 470nm in methanol. Thus it can be concluded that the extracted pigment can be Peridinin or Pyrrhoxanthin or the derivatives. These two compounds revealed many other small peaks which suggests for the further purification. Some peaks of samples were exhibited by pigments such as Lycopene, Fritschiellaxanthin, Zeaxanthin, Canthaxanthin, Astaxanthin and (3R,3'R)-Astaxanthin showed some peaks nearby as pigment peaks but not all the peaks matched the sample pigment. This suggests that the pigment can be combination of two known pigments or a new pigment or the pigments needs to be compared with supporting results of high end techniques like NMR, HPLC, Raman spectroscopy etc.

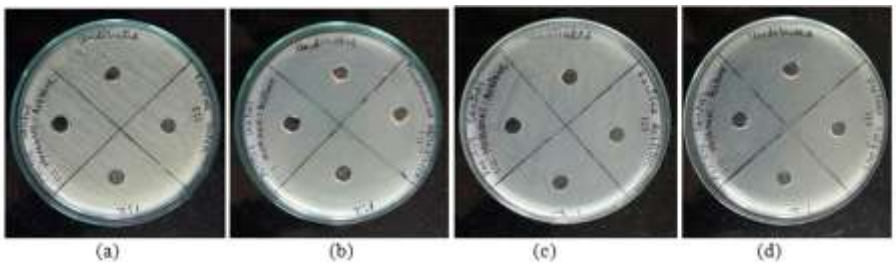


**Fig. 3.1.** Depicts the results of antibacterial activity on pathogens- a) *Sarcina lutea*, b) *Pseudomonas aeruginosa*, c) *Bacillus subtilus d*) *Proteus vulgaris*

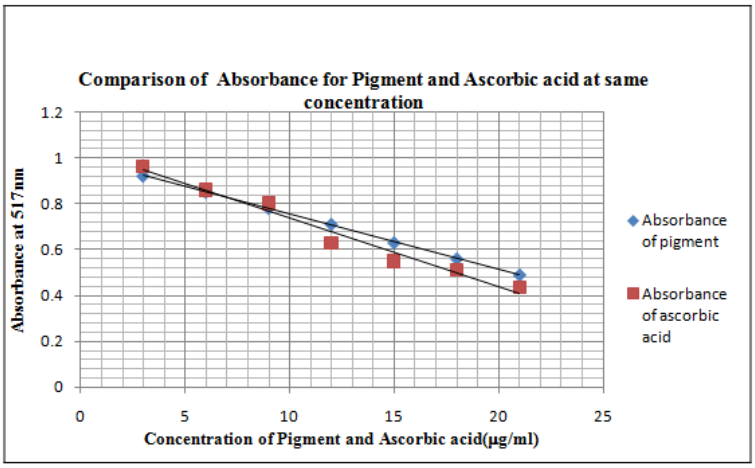


**Fig. 3.2.** Comparison of absorbance for pigment and ascorbic acid at same concentrations.

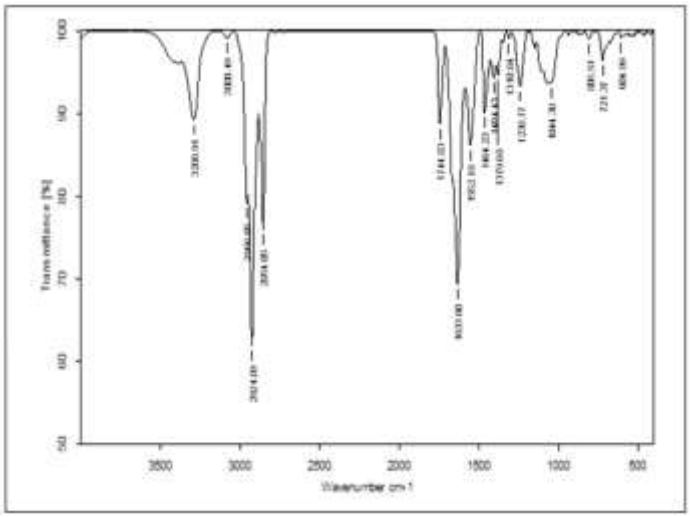

**Fig. 3.3.** FTIR analysis of the crude pigment

| Peak No | Peaks of major wavelength ($cm^{-1}$) | Description of functional groups | |
|---|---|---|---|
| I) | 2924.84 | i) | Includes C-H bond, Alkanes |
| | | ii) | Includes O-H bond, Carboxylic acids. |
| II) | 2864.06 | i) | Includes C-H bond, Alkanes |
| | | ii) | Includes O-H bond, Carboxylic acids. |
| III) | 1744.83 | i) | Includes saturated aliphatic esters |
| | | ii) | Includes amides with four atom ring |
| | | iii) | Includes unsaturated anhydrides |
| | | iv) | Includes C=O, Carbonyl group |
| IV) | 1633.60 | N-H bend, Saturated amides or primary amines | |
| V) | 1552.18 | N-H asymmetric bend, Nitro compounds | |

**Table. 3.1.** Bands designated for the following peak

## IV. Conclusion

The biochrome isolated from a novel species *Salinicoccus sp*. MKJ997975 was best extracted with the combination of methanol and acetone solvent. The photosensitivity of the pigment revealed the characteristic of a carotenoid. The extracted pigment did not show any antibacterial activity with species *Bacillus subtilus, Proteus vulgaris Sarcina lutea* and *Pseudomonas aeruginosa*; however the pigment showed good antioxidant activity. The characterization of the crude pigment sample was found closely related to the pigments like Perdinin or Pyrrhoxanthin. The structural purification of the crude pigment needs to be repeated by further purification.

## Acknowledgements

We would like to thank the director, school of Biotechnology & Bioinformatics, D. Y. Patil University Navi Mumbai, for support and providing necessary facilities to pursue the research work. We also thank SAIF, IIT Bombay for the analytical service of FTIR.

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