Isolation and Identification of pigment producing marine isolate and testing of Antioxidant activity and Cytotoxicity effect of pigment

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ABSTRACT *As the marine is a good source of microorganisms. The aim of the present study is to isolate biotechnologically potential bacteria from the marine environment. Antimicrobial compounds, antioxidant compounds producers are very essential at industrial level as they comprise the essential values in cosmetic, food and pharmaceutical industries. In the present study, out of 19 pigmented bacteria, isolate 10(Y) was characterized as Planococcus maritimus and produce pigment in Zobell marine broth. Methanol optimized as best for extraction of pigment. The maxima absorbance was obtained at 463.2 nm, which indicates the carotenoid nature of the pigment. Rf value of TLC was 0.66 which also indicates the carotenoid pigment by using optimized Ethyl acetate: Benzene (2:1) solvent system. The pigment showed the antimicrobial activity against S. aureus, followed by E.oli and B,subtilis. In addition, the DPPH and ABTS activity of the pigment was 18% and 22% of inhibition, respectively. There was no effect on lymphocytes while treated with pigment in cytotoxicity test.*

Keywords: *Marine bacteria, pigment production and extraction, characterization, antibacterial and antioxidant properties.*

I. Introduction

Color plays an important role in our life. In market, synthetic colors are available but they are highly toxic. Hence, it is essential to produce such colored pigments from natural resources. Pigments are produced by plants and microorganisms like fungi, bacteria, yeasts, actinomycetes, etc. natural pigments can be extract not only from vegetables, fruits, roots but also form microorganisms. Pigments are secondary metabolites in microorganisms. The carotenoid pigments produced by pigmented bacteria are beneficial for various industries like food, dyes, pharmaceutical and cosmetics. In addition, the marine bacteria are more potential to have bioactive compounds than terrestrial microorganisms. Nowadays there is a demand and interest for color from natural sources has been increased rather than synthetic color as people get aware of the toxicity of the synthetic food color/dyes on health. Microorganisms, vegetables, fruits are sources of bio color as they are safe and convent to use for humans and other life because of its biological origin. Carotenoids are the most important pigment group of yellow-orange- red variants, which are ubiquitous nature with proven anti-carcinogenic and immune-modulation properties (Browning *et al.,* 2003).

The aim of the present study is to evaluate the antibacterial and antioxidant properties of carotenoid pigment isolated from marine bacteria. The pigment extract of *P. maritimus* isolated from marine soil samples and its pigment was identified as carotenoid based on the spectral analysis. The orange pigment extract was characterized by using thin layer chromatography. Antioxidant activity and Antibacterial activity was examined. Cytotoxicity effect of pigment on lymphocytes was also checked

II. Materials and Methods

Sample collection

Marine water and soil samples were collected aseptically in a clean and sterilized container and polythene bags, respectively. Samples were collected from four different sites (Site 1- Tithal (Valsad), Site 2- Dandi (Navsari), Site 3- Umbharat (Surat), and Dumas (Surat)) of South Gujarat Coastal regions. These collected samples were immediately sent to the laboratory for further study. Two samples of each sites were taken for the bacterial examination. Each samples were stored at 4°C prior to examination.

Isolation of marine bacteria

The samples were labeled as per their location sites. The soil and water samples were firstly enriched into artificial seawater. After enrichment, each sample was serially diluted from 10^{-1} to 10^{-6} in normal saline water. Then, each sample was spread on to the specific media Zobell marine agar media procured from Hi-Media ltd, Mumbai and incubated for 24 to 48 hrs at 28 C in incubator. The well-isolated pigmented colonies

of marine bacteria were selected and studied for the morphological and cultural characterization. The studied isolates were restreaked on the Marine agar plates and incubated for 24 hrs to checked for the purity and gram staining was checked as well. The selected bacteria were stored on Marine agar slants and was over layered by sterile paraffin for preservation and kept at 4°C for further use.

Production of pigment

Marine isolate 10(Y) was further selected for pigment production. 10(Y) isolate was inoculated into 100 ml of Marine broth in 250 ml Erlyn flask. The broth was incubated at room temperature (28°C) for 4 -5 days at 120 rpm. As the color developed from yellow to orange, pigment extraction was carried out.

Extraction of pigment

10 ml of culture broth was centrifuged at 10,000 rpm for 15 min. and colored pallet was obtained. The supernatant was discarded and the colored palette was washed with sterile distilled water for three times. This could remove the remaining media components. Now, the colored pallete was dissolved with different organic solvents for pigment extraction. The extracted pigment was filtered through Whatman filter paper to remove the impurities from the extract and preceded for the characterization of pigment by spectroscopic analysis. The pigment extract was concentrated by evaporating the solvent and partially purified by using chloroform/petroleum ether to remove the lipid content of the culture broth. It was evaporated and dried pigment was obtained.

Characterization of pigment extract

1.) **Spectral analysis**
The pigment extract was examined for spectroscopic analysis in the UV range of 400-800nm on a UV-visible spectrophotometer. This was to check the characteristic of carotenoid of the pigment.

2.) **TLC of pigment**
The partially purified pigment was characterization by thin layer chromatography (TLC) using silica gel plates developed by using different solvent system for the better separation of carotenoids. Methanol: ethyl acetate, methanol, petroleum ether: methanol were used for separation.

Molecular identification of marine isolate

The molecular identification of the isolate 10(Y) was carried out by 16S rRNA sequencing method. It was done at Saffron Life sciences, Bilimora, Navsari, India.

Antibacterial activity of pigment extract

The antibacterial potentiality of pigment extract was evaluated by Well diffusion assay method by using Muller Hinton agar media (Hi-Media). Antibacterial activities were tested against test organisms (*Escherichia coli, Staphylococcus aureus, Enterobacter sp., Bacillus subtilis, Shigella, Pseudomonas aeruginosa, Proteus vulgaris,* and *Klebsiella pneumoniae*). Agar plates were spread plated with individual test organism on separate plates. The wells were punctured with sterile borer. The pigment extract (100µl) was added to the each well while the control plate was maintained with methanol solvent. All the plates were kept in the refrigerator for 10 min for diffusion. The plates were incubated in upright position at 37°C for 24hrs. Next day, observe and measure the zone of inhibition in comparison with control plate having solvent.

Antioxidant activity of pigment extract

1.) **DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay**
The DPPH radical scavenging activity of the pigment extract was determined according to the method reported by Chang et al., (2001) with Ascorbic acid and Trolox were used as a standard antioxidants. The inhibition of DPPH free radicals by the sample extract calculated as per the following equation,
DPPH (%) = [1-(Absorbance of the sample/Absorbance of the control)] × 100

2.) **ABTS (2,20-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) assay**
The ABTS assay was employed to evaluate the total antioxidant capacity of the pigment extract. The absorbance was measured at 734 nm and the antioxidant activity was expressed in percent inhibition. Ascorbic acid and Trolox used as a standard antioxidants.

Cytotoxicity effect of pigment

The human lymphocytes were treated with the different concentration of pigment. The first test was performed in control culture in which no pigment was added. In second test, the lymphocytes were tested with 0.1 ml of sample having 10 µg of pigment yield, and the in last, 1.0 ml of sample having 100 µg of pigment.

Results and Discussion

Isolation of marine bacteria

By performing serial dilution of soil and water samples, seventy-four various types of colonies were obtained. All the different colonies were further purified by four-flame method on Zobell marine plates and gram reaction was performed. 72% of bacteria were gram positive and 28% of isolates were gram negative. In the present study, gram-positive bacteria were dominating than gram-negative bacteria. Out of 74 isolates, 19 bacterial strains were pigmented bacteria and only six isolates having different pigmentation were chosen for the further study.

Pigment production

Out of six pigmented bacteria, isolate 10(Y) was selected for pigment production as it has intense pigmentation. As color of marine broth has been changed from yellow to orange, after 4-5 days of incubation, the optical density was taken and pigment extraction was carried out (Fig 1 & 2)

Extraction of pigment

Various organic solvents were used for the maximum pigment extraction of isolate 10(Y). Among all, methanol has given the best yield of pigment (Fig 3). The methanolic extract was further partially purified by using petroleum ether and chloroform. The yield of dried pigment was calculated.

Characterization of Pigment extract

Spectroscopical analysis

UV-Visible absorbance spectra of carotenoid are very importane as they help in determining the structure of carotenoid (Medicharla *et al*., 1991)The methanolic exract was analysed for the determination of nature of carotenoid. It was analyzed in the UV and visible range 400-800 nm by UV visible spectrophotometer (Systronics, Germany) for obtaining the maximum absorbance (λ_{max}). It observed that the λ_{max} was 463.2 nm which indicates the nature of carotenoid (Fig. 4). The polyene chromophore of carotenoids absorb light in the 400 to 550 nm range that provides the characteristic of yellow-to-red colors and their ability to quench singlet oxygen (Umeno *et al*., 2005).

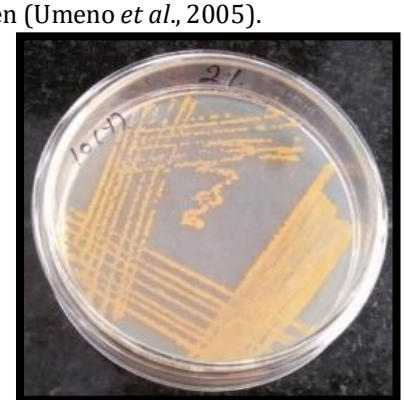

Fig. 1 Isolation of marine pigmented bacteria

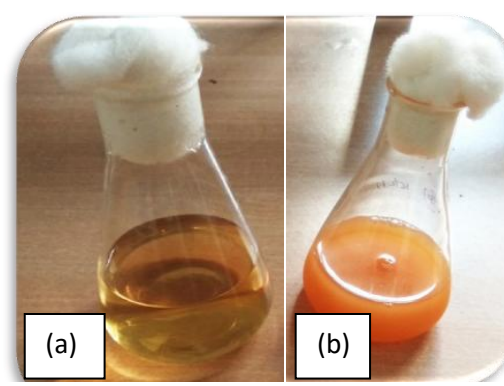

Fig. 2 Production of pigment in Zobell marine broth (a) Uninoculated brith (b) 5th day of inoculation

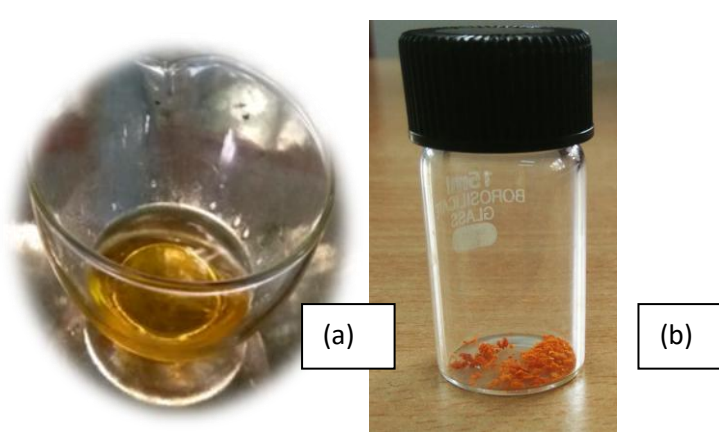

Fig. 3 (a) Extraction of pigment with Methanol and (b) Dried pigment yield

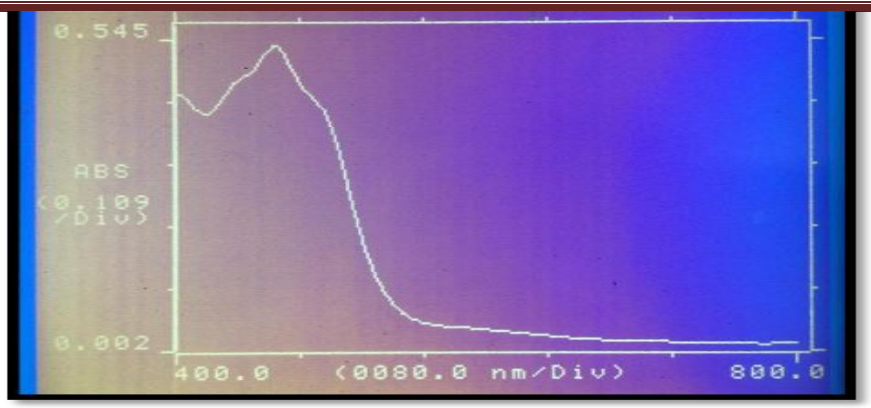

Fig. 4 Spectroscopic analysis of pigment extract

TLC of pigment

Different solvent systems were used to get the better separation. Hence, optimized solvent system was Benzene: ethyl acetate (2:1). Fig. 5 clearly showed that Rf value was 0.66 which indicate the nature of carotenoid pigment.

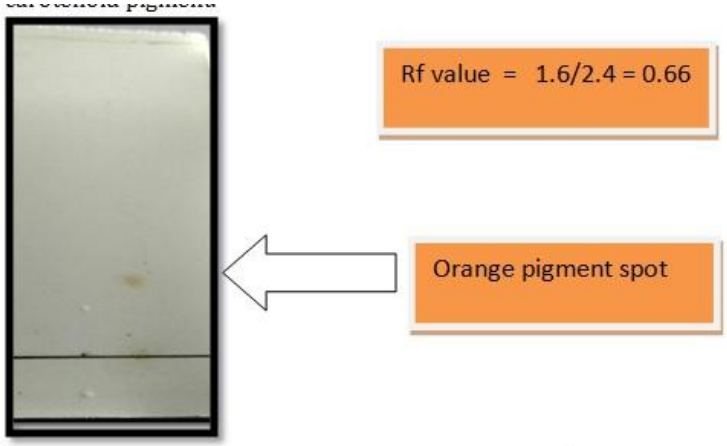

Fig. 5 TLC of pigment extract

Molecular identification of marine isolate 10(Y)

By performing sequencing method, the isolate was identified as *Planococcus maritimus* SP034 with 99% of similarity (Fig.6). The Accession number (KR028440.1) was submitted in GenBank.

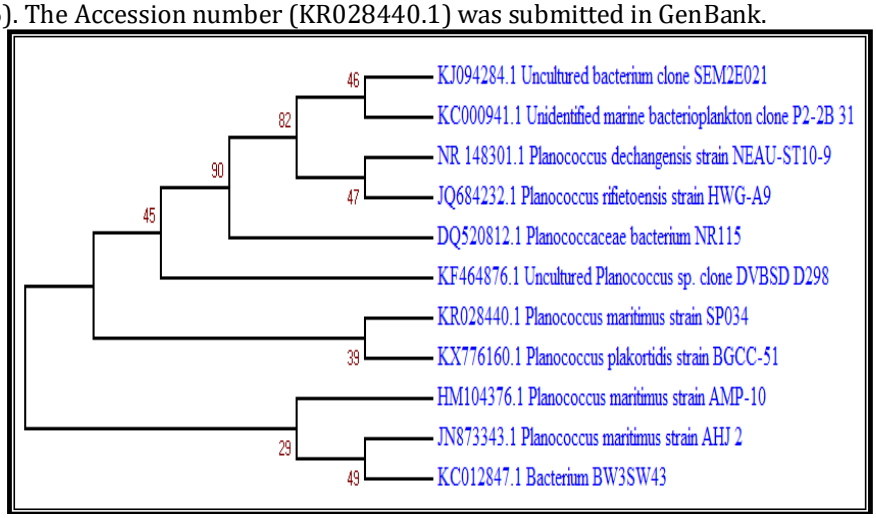

Fig. 6 Phylogenetic tree of potential marine isolate *Planococcus maritimus* SP034

Antibacterial activity by Well diffusion method

The pigment was screened for the antibacterial activity against different test organisms. Fig, 7 showed that the pigment was highly effective against Staphylococcus aureus, followed by Escherichia coli and Bacillus subtilis. The analysis result found potential antimicrobial compound of pigment. The result also showed

that the gram positive bacteria were more sensitive against the pigment than gram negative bacteria. *Pseudomonas aeruginosa, Proteus vulgaris* were resistant against pigment. (Table 1).

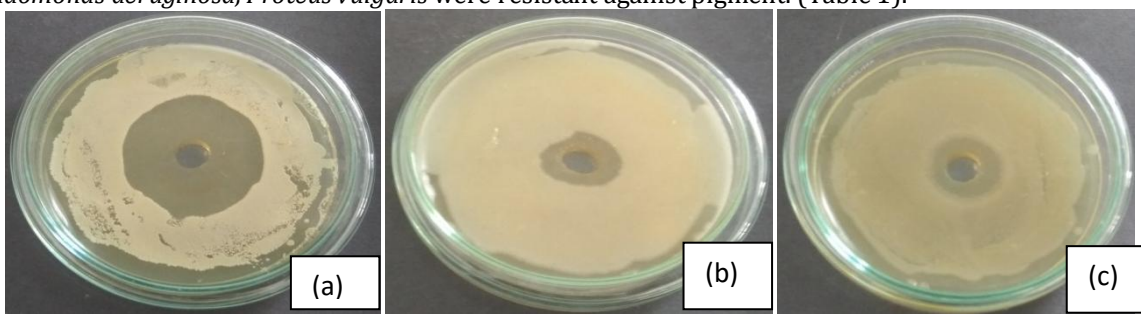

Fig. 7 Antibacterial activity of pigmnet against (a) *S.aureus* (b) *E.coli* (c) *B. subtilis*

Antioxidant activity

1.) DPPH scavenging activity

Carotenoid pigment has the ability to act as antioxidants and hence they can protect cells against photooxidation. As antioxidant, the carotenoids have the ability to quench singlet oxygen with

Table 1. Antimicrobial activity of pigment against test organisms

	Test organism	Inhibition of Zone (mm)
Pigment extract of *P.maritimus* SP034	*Escherichia coli*	18
	Enterobacter sp.	13
	Klebsiella pneumoniae	12
	Pseudomonas aeruginosa	-
	Proteus vulgaris	-
	Salmonella typhi	-
	Shigella sp.	14
	Bacillus subtilis	15
	Staphylococcus aureus	34

radical species (Edge *et al*., 1997). If carotenoids are taken as dietary carotenoids, it can inhibit the onset of many diseases such as cataract, age related muscular degeneration, and mainly cancer (Bhosale, 2004). Michalowska and Stachowiak (2010) reported that pigment produced from *Phaffia rhodozyma* has highest percentage of DPPH scavenging properties (94.58%) at 0.05% of carotenoid extract. In the present study, the scavenging activity of DPPH exerted by pigment of P.maritimus SP034 at the 50 µg ml -1 exhibited 20% of inhibition (Fig 8 a).

2.) ABTS activity

Likewise, to evaluate the capacity of antiradical characteristics, ABTS assay was performed. Fig 3 showed 22% of inhibition of the pigment extract, while the standard Trolox showed 98% of inhibition (Fig.8 b). Form the graph it was observed that the pigment of *P.maritimus* also showed antioxidant activity as well.

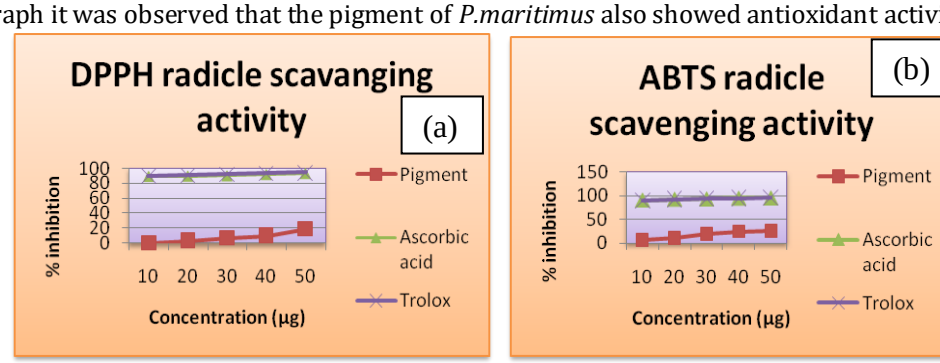

Fig.8 Antioxidant activity of pigment (a) DPPH activity (b) ABTS activity

Cytotoxicity effect of the pigment

A significant effect of pigment on lymphocytes was not observed. The effect of pigment on lymphocyte has been checked by setting up lymphocyte culture. It was observed that there is no significant change in the cell

morphology. Lymphocyte size and shape found in control slide was more or less same as that of in 0.1ml (10µg), and 1ml (100µg) pigment containing slide (Fig. 9 a, b, and c).

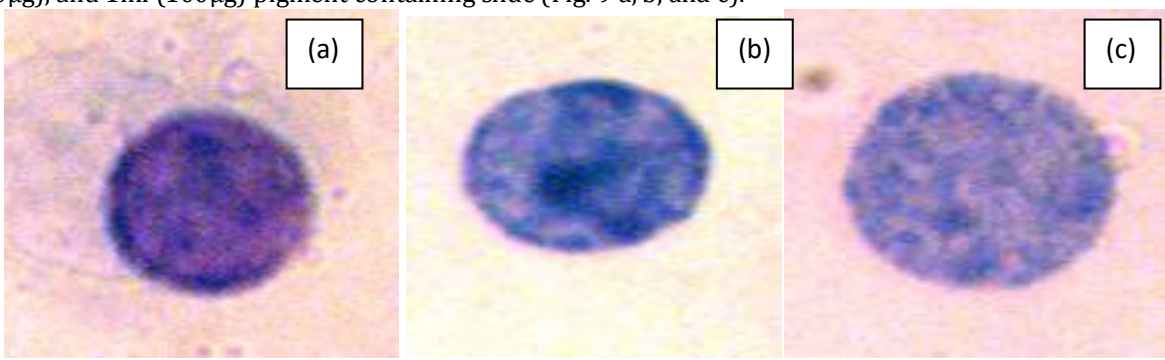

Fig. 9 Lymphocytes treated with (a) control (b) 10µg , and (c) 100 µg of pigmnet

IV Conclusion

On the basis of results obtained from two ntioxidant capacity assays, the pigmnet extarct of P.maritimus has shown a significant total antioxidant activity. Antibacterial activity was also determined by well difussion method, in which it was showed that the S.aureus was highly sensitive aginst the pigment extract. It was followed by *E.coli*, *B.subtilis*, *Shigella*. Hence, P.maritimus has capability to produce the antimicrobial compound. As the pigment has such capabilities to be used in food industry, cosmetic industry, pharnaceutical industries, the cytotoxicity effect of the pigment was checked. There was no significant effect observed on lymphocytes with pigment exract.

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