

# Isolation and Characterization of Pigments from Marine Soil Microorganisms

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## ABSTRACT

Bacterial pigments have many applications in the current day to day life. The pigments produced by chromobacteria can be used for various applications like dairy, pharmaceutical, and food etc. In this study, three types of pigments were isolated i.e. yellow from *Xanthomonas* sp., pinkish red from *Rhodotorula* sp., and orange from *Sarcina* sp. Pigmented bacterial isolates were obtained from the soil samples and used for the pigment extraction study. We studied that the pigment producing bacteria and identified the color producing pigments. Soil samples from Pondicherry, Cuddalore, Chennai, and Andhra sea coast were collected and used for isolation of microbes producing pigments. Purification of extracting pigments was done by column chromatography, whereas identification and characterization of purified pigment done by UV-Visible spectrophotometry and GC/MS analysis etc. The pigment isolated from bacterial sp. were used for the antimicrobial activity, antioxidant, and anticancer & transformation studies. The bacterial extracts of carotenoid pigment extracted and used as natural colorants for food products and dyeing of cloth.

**Key-words:** Carotenoid, GC/MS analysis, Pigment extraction, Soil samples, UV-Visible spectrophotometry

## INTRODUCTION

Carotenoids are a class of compounds that have coloring power and have been widely used in food industry, leading its market to full development. Carotenoids occur widely in nature and, in general, all fruits and vegetables of color are good sources of these compounds <sup>[1]</sup>. Microorganisms are the most versatile tools in biotechnology to produce variety of molecules including enzymes, antibiotics, organic acids and pigments. Recent studies have shown that microorganisms are a promising source for natural colors. The presence of pigments has been reported among the entire microbial world including bacteria, fungi, yeast, algae and protozoa.

Industrial production of natural food colorants by microbial fermentation has several advantages such as cheaper production, easier extraction, higher yields through strain improvement, no lack of raw materials and no seasonal variations <sup>[2]</sup>. Pigments are compounds with characteristics of importance to many industries. In the food industry they are used as additives, color intensifiers, antioxidants, etc. Pigments come in various colors, some of which are water soluble <sup>[3]</sup>. Microorganisms are the most powerful creatures in existence and determine the life and death on this planet.

## MATERIALS AND METHODS

**Sample Collection-** Soil samples were collected from different marine sources of India such as (Pondicherry, Cuddalore, Chennai, Tuticorin, and Andhra sea coast). After the collection of soil samples from different coastal areas, further study was done at the Department of Biotechnology Women's Christian College, Chennai, India. The collected soil samples were stored at 4°C for further study.

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**Isolation of pigment producing bacteria from Soil samples-** Pondicherry, Cuddalore, Chennai, Tuticorin, and Andhra sea coast.

#### Identification of pigment producing bacterial species

**Cultural characteristic-** The isolated pure culture was maintained in nutrient agar slant for further experimental use.

**Morphological characteristic-** The bacterial species were subjected to Gram staining for morphological identification.

**Biochemical characteristic-** The isolated bacterial sp. were subjected to the following biochemical tests i.e. Indole test, methyl red and voges proskauer test, citrate test, oxidase test, catalase test, triple sugar iron agar test, urease test, and carbohydrate fermentation test.

**Test for carotenoids in bacteria-** The bacterial cell isolates were grown in Luria Bertini broth and the pigments were extracted from the organisms. Carotenoid pigments were identified the using of UV-Visible spectroscopy ranging from 450 nm to 600 nm <sup>[4]</sup>.

**Thin layer chromatography (TLC)-** Silica gel TLC plates are cut as per need. The bacterial pigment extracts, the carotenoid yellow, orange, pink red pigment spots observed were marked and  $R_f$  value determined <sup>[5]</sup>.

**Isolation of carotenoid pigments by column chromatography-** The bacterial pigments were purified by column chromatography whereas, the fractions collected were evaporated and the thickly concentrated carotene fractions are used for TLC. The stationary phase of silica gel (100–200  $\mu$ m) and mobile phase of chloroform: methanol 95: 5 <sup>[6]</sup> was used.

**Determination of the antimicrobial activity of the bacterial pigments-** The antimicrobial activity was checked by 2 different methods (phosphomolybdenum method and  $H_2O_2$  scavenging assay) <sup>[7]</sup> given below-

**Total antioxidant activity by phosphomolybdenum method-** The phosphomolybdenum assay <sup>[8]</sup> used for determining the antioxidant capacity was based on the reduction of Mo (VI) - Mo (V) by the antioxidants and subsequent formation of a green phosphate/Mo (V) complex at acidic pH.

**Hydrogen peroxide ( $H_2O_2$ ) scavenging assay-** The ability of the extracts to scavenge hydrogen peroxide was determined and calculated <sup>[9]</sup> by given below formula:

**% scavenged ( $H_2O_2$ ) =**

$$(A \text{ of control} - A \text{ of test} / A \text{ of control}) \times 100$$

whereas, A= Absorbance

**Reducing power assay-** A spectrophotometric method reducing power assay <sup>[10]</sup> was used for the measurement of the reducing power of the sample.

**GC-MS analysis-** The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m  $\times$  0.25 mm ID  $\times$  250  $\mu$ m df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. Total 1  $\mu$ L of extract sample injected into the instrument the oven temperature as follows: 60°C (for 2 min); followed by 300°C at the rate of 10°C min<sup>-1</sup>, where it was held for 6 min. Mass detector conditions were transferred line temperature 240°C; ion source temperature 240°C and ionization mode electron impact at 70 eV, scan time 0.2 sec and scan interval of 0.1 sec. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

**Confirmation test for carotenoids-** The bacterial cell isolates were grown in LB broth in a rotary shaker at 120 rpm and 37°C temperature. After 3 days, the cells were subjected to centrifuge at 8000 RPM for 10 minutes at 4°C. Discard the supernatant and the pellet collected. Collect pellet with distilled water and spin at 4000 RPM for 15 minutes. Collected pellet with 5 ml methanol and incubated in water bath 60°C for 15 minutes. Again centrifuge at 4000 RPM for 15 minutes and collected the supernatant and filter through Whatman filter paper and collected the bacterial extracts of yellow, orange, and pink red. To the extracts of carotenoid pigment added 1 ml of sulphuric acid in 9 ml of water. The appearances of blue color confirm the presence of the carotenoids <sup>[11]</sup>.

**Transformation study-** A single colony was picked from a freshly grown plate of *E. coli* DH5 $\alpha$  and inoculated in the 100 ml of LB broth and kept for overnight incubation

at 37°C with vigorous shaking for approximately 3 hours. Cell density was monitored by determining OD<sub>600</sub> nm and it should be less than 10<sup>8</sup> cells/ml (log phase of growth-the healthiest in bacteria). The culture was subjected to centrifugation at 6000 rpm at room temperature for 5 minutes and the pellet was re-suspended with 0.1 M ice cold calcium chloride. The aliquot was taken in pre-chilled vial along with plasmid DNA and gently tapped and incubated in ice for 20 minutes. The cells were subjected to heat shock for uptake the plasmid DNA by placing in 42°C water bath for 20 minutes, then returned to ice to chill immediately for 5 minutes <sup>[12]</sup>.

### Anticancer activity of the extracts

**MTT assay-** Cancer cell lines were purchased from Cancer Institute, Chennai. The cells were grown in the 96 well plate in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum and antibiotics (Penicillin-G). About 200 µl of the cell suspension was seeded in each well and incubated at 37°C 48 hours with 5% CO<sub>2</sub> for the formation of monolayer of cells. The monolayers of cells in the plate were exposed to various concentrations of the bacterial carotenoid pigment and were incubated for 24 hours. Cytotoxicity was measured using MTT (5 mg/ml). After

incubation at 37°C in a CO<sub>2</sub> incubator for four hours, the medium was discarded and 200 µl of DMSO was added to dissolve the formazan crystals. The absorbance was read in a micro-plate reader at 570 nm <sup>[13]</sup>.

**Test for carotenoids-** The bacterial carotenoid pigment (2 ml) extract from the purified compound from column chromatography was mixed with alum potassium aluminium sulphate (6%). The cotton fabric and thread was kept immersed in the solution for about 5 minutes and kept for drying <sup>[14]</sup>.

**Washing performance-** Dried cotton fabrics were soaked in the detergent solution for 20 minutes and then washed using tap water and dried for 30 minutes. The bacterial pigment purification carotenoid pigments were applied as food colorants.

### RESULTS

The microorganisms were identified on the basis of Gram staining and Biochemical characteristics. The Table 1 shows the all the microorganisms were Gram negative but each one giving different color pigments.

**Table 1:** Gram staining characterization of selected bacterial species

Pigment	Bacteria name	Examination
Yellow	<i>Xanthomonas</i> sp.	Gram –ve, rod shape
Orange	<i>Sarcina</i> sp.	Gram – ve, rod shape
Pink red	<i>Rhodotorula</i> sp.	Gram – ve, rod shape

**Table 2:** Biochemical analysis of selected bacterial species

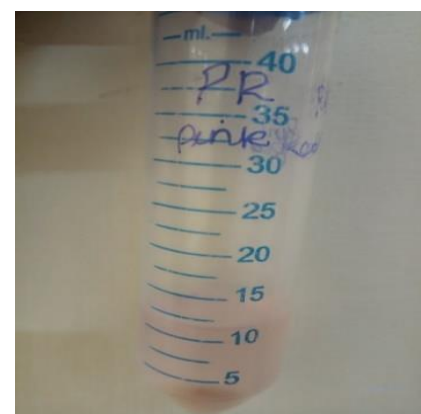
Pigments	Bacteria name	Indole	MR	VP	TSI	Urease	Simons citrate	Sugar fermentation			Catalase
								Glucose	Sucrose	Lactose	
Yellow	<i>Xanthomonas</i> sp.	+	+	–	–	–	+	+	+	+	+
Orange	<i>Sarcina</i> sp.	–	+	–	–	–	+	+	+	+	+
Pinkish red	<i>Rhodotorula</i> sp.	–	+	–	–	–	+	+	+	+	+



a. Yellow



b. Orange

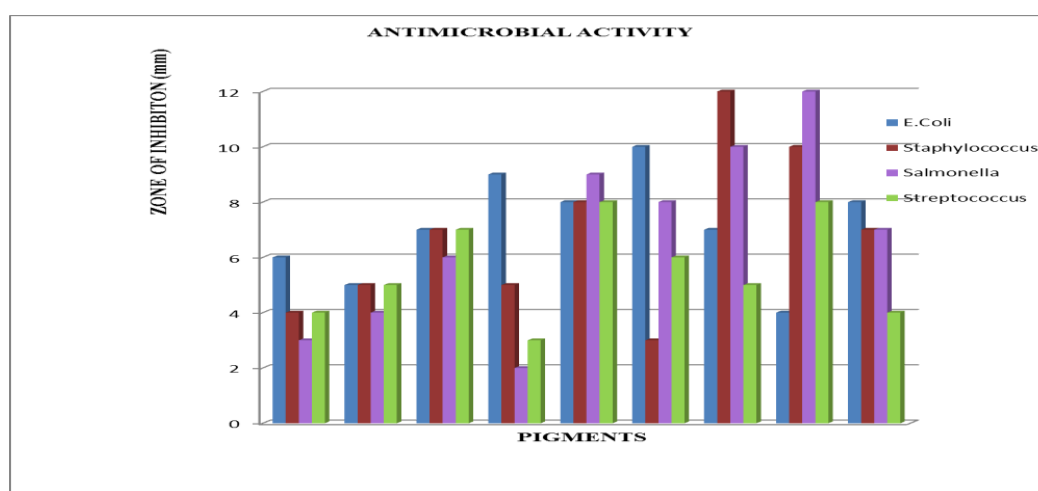
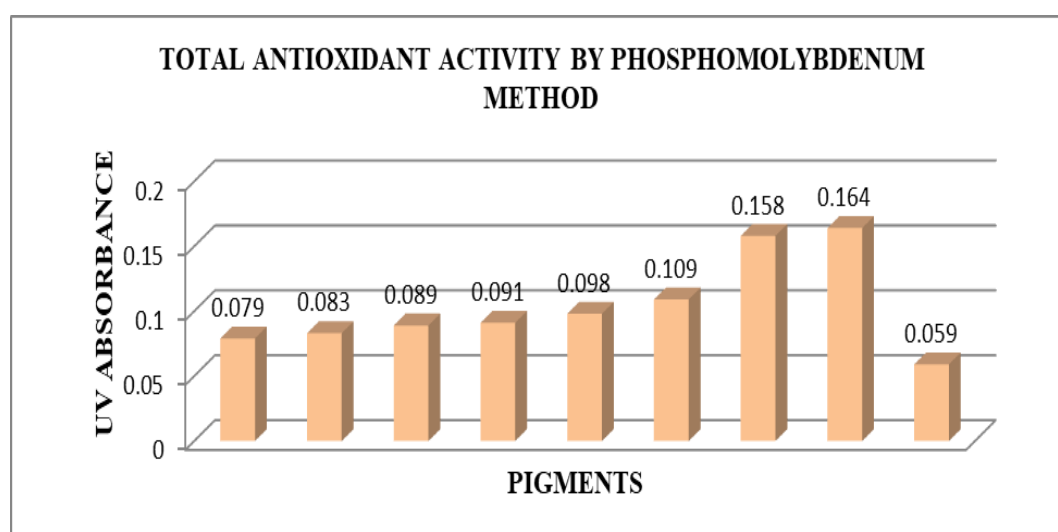


c. Pinkish red

**Fig. 1:** Extracted carotenoid pigments

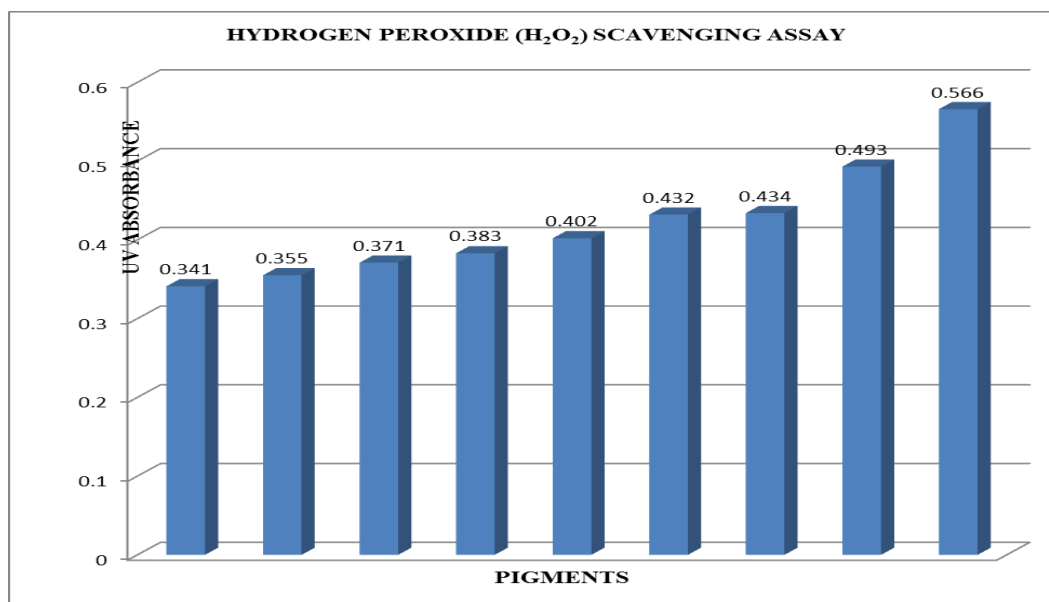
The three pigments i.e. yellow, orange and pink red was obtained from the bacterial sp. The extracted pigment was purified using the column chromatography and  $R_f$  value was determined using the TLC. The pigments were

checked for their absorption maxima using a spectrophotometer in the wavelength ranging from 450 to 600 nm.

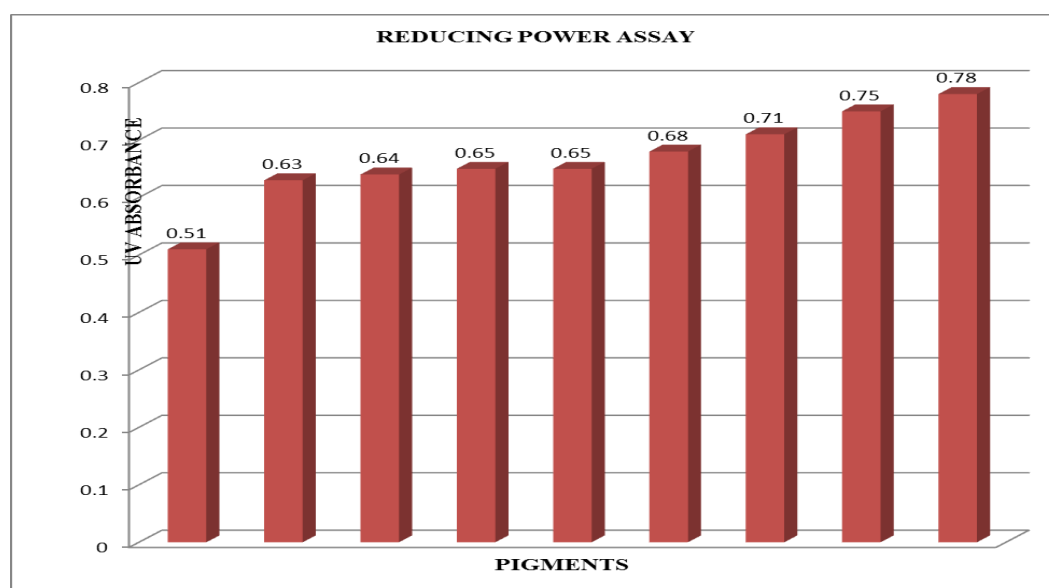
**Fig. 2:** Zone of inhibition against bacterial test species**Fig. 3:** Total antioxidant activity by Phosphomolybdenum method of pinkish red pigment

The zone of inhibition against various pathogens were measured using Kirby Bauer's method. The ability to scavenge radicals was studied using phosphomolybdenum method, hydrogen peroxide assay, and reducing power assay using the three pigments.

All the pigment showed good results however the Fig. 3, to Fig. 5 shows the results of pinkish red pigment. Antioxidant properties were checked using two procedures.



**Fig. 4:** Hydrogen peroxide scavenging assay of pinkish red pigment



**Fig. 5:** Reducing power assay of pinkish red pigment

The transformation of *E. coli* DH5 $\alpha$  was done with the plasmids isolated from pigment producing microorganisms. The transformation was observed but pigment formation was not found in the *E. coli* DH5 $\alpha$ .

The colored pigment was used to dye the fabrics. The colors were taken from the fabric and lasted even after washing with detergents.



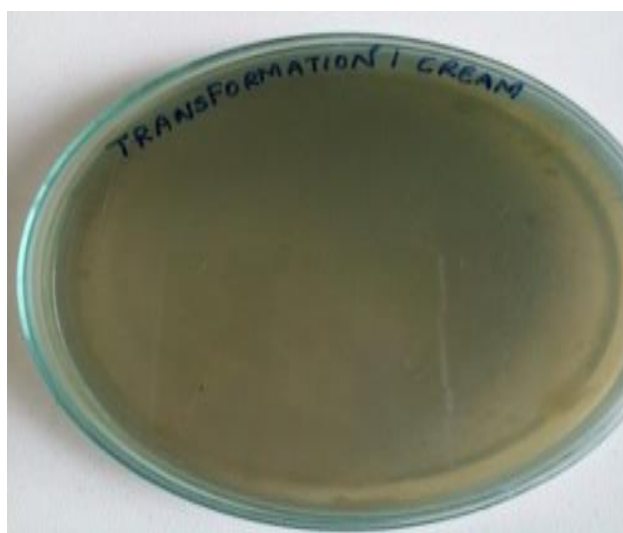


Fig. 6: Transformation colonies



Fig. 7: Colorization done in cotton cloth by different pigments

**Anticancer activity-** The orange color pigment isolated from *Sarcina* sp., pink from *Rhodotorula* sp. and yellow from *Xanthomonas* sp. were tried for anticancer properties. Fig. 8 shows the titre plate with MTT, which was incubated for 4 hours, while after Fig. 9 shows the

plate with DMSO added to stop the reaction and take the reading on the ELISA reader. The wells with different concentration of pigment inhibited the Lymphoma cells and then the readings were taken to determine the amount of inhibition.

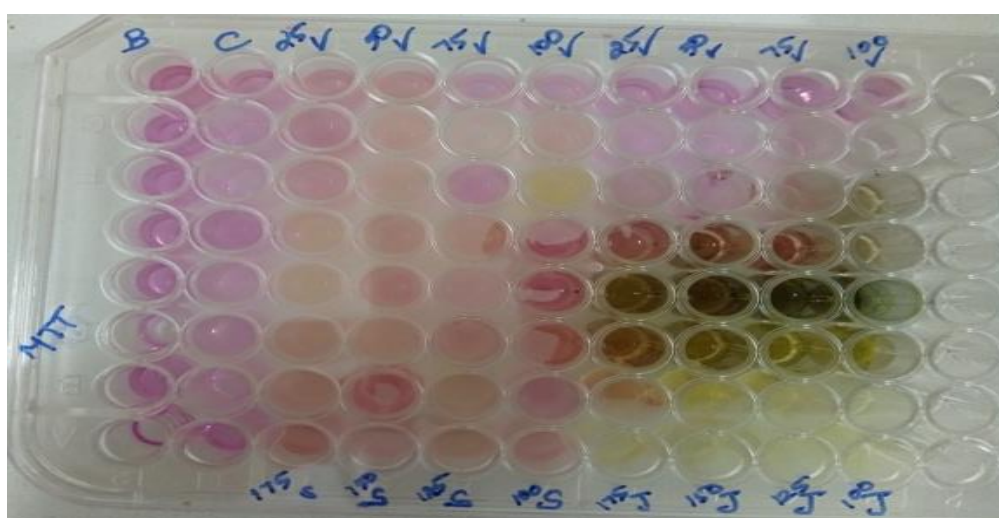
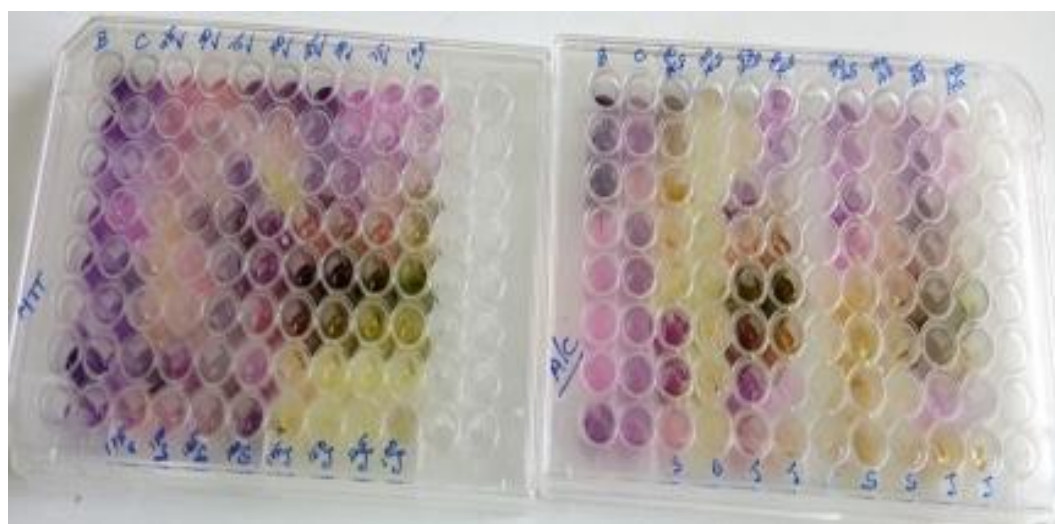


Fig. 8: After addition of MTT

a. Orange (*Sarcina* sp.), b. Pink (*Rhodotorula* sp.) c. Yellow (*Xanthomonas* sp.)



**Fig. 9:** After addition of DMSO

**a.** Orange (*Sarcina* sp.), **b.** Pink (*Rhodotorula* sp.) **c.** Yellow (*Xanthomonas* sp.)

## DISCUSSION

Nutrient agar media was prepared and soil samples subjected to serial dilution were spread on the plate containing the nutrient agar media then the plates were incubated at 37°C for 24 hours. Results of the gram staining and biochemical tests were used for the identification of the microorganisms i.e. *Xanthomonas* sp., *Sarcina* sp. and *Rhodotorula* sp. (Table 1).

## Extraction of pigments

***Xanthomonas* sp.-** The bacterial carotenoid yellow pigment was extracted with methanol and it has been confirmed with UV-Visible spectrophotometry to get single peaks of the pigment.

***Sarcina* sp.-** The bacterial carotenoid orange pigment was extracted with methanol and it has been confirmed using spectrophotometry and GC-MS.

***Rhodotorula* sp.-** The bacterial carotenoid pinkish red pigment was extracted with methanol and it had been confirmed using spectrophotometry and GC-MS.

**Thin layer chromatography** <sup>[15]</sup>- The average  $R_f$  value obtained from bacterial carotenoid pigment was found to be 0.99, which was comparable with the standard pigment  $R_f$  value, which observed as 0.97.  $R_f$  value of carotenoids were in the range of 0.99 to 0.97, which were matched with the standard so yellow, orange, pink red are carotenoids. The results were comparable with the reported results.

**Column chromatography-** From the column chromatography, the compounds were separated based on the differences in partitioning between mobile and stationary phases. The pigments obtained were purified as yellow, orange and pink red <sup>[16]</sup>.

**Antimicrobial activity** <sup>[17]</sup>- The comparison of antimicrobial efficacy in terms of zone of inhibition of pigment against gram positive and gram negative organism *E. coli*, *Staphylococcus* sp., *Salmonella* sp., and *Streptococcus* sp. The pigments were found to exhibit maximum zone of inhibition i.e. 13.5 mm against *Staphylococcus* sp. and 12.5 mm against *E. coli* were exhibited the carotenoid pigments (Fig. 2).

**Conformation test for carotenoid pigment** <sup>[18]</sup>- The nature of the extracted carotenoid pigment sample were tested. The appearance of blue color in addition of sulphuric acid indicated the presence of carotenoids.

**Gas chromatography-mass spectrometry** <sup>[19]</sup>- GC-MS Chromatogram of the methanolic extracted pigment showed different peaks, the highest peak was observed and identified. The compound names are given below-

**Yellow (*Xanthomonas* sp.)-** 2-piperidinon, n-[4-bromo-n-butyl]

**Orange (*Sarcina* sp.)-** Triarachine

**Pink red (*Rhodotorula* sp.)-** Octacosane

**Total antioxidant activity by phosphor-molybdenum method-** In total antioxidant activity by phospho

molybdenum method had maximum in pinkish red pigment<sup>[15]</sup> (Fig. 3).

**Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging assay-** Total antioxidant activity of hydrogen peroxide scavenging assay for carotenoid pigment was found to be good in pinkish red pigment.<sup>[16]</sup> (Fig. 4).

**Reducing power assay-** Reducing power assay<sup>[17]</sup> carried on the pigments was found to be positive shown in (Fig. 5).

**Transformation colonies**<sup>[20]</sup>- Several experiments were carried out. Transformed colonies were obtained in these experiments. A similar set of experiment was carried out with carotenoids. In this case, the transformation was observed. This probably resulted in more efficient transformation. The blue white colonies were observed, indicating transformed colonies (Fig. 6).

**Application of pigment to cotton cloth**<sup>[21]</sup>- The isolated bacterial purified pigment was applied to dye cotton cloth. The dye was applied to cotton fixed in potassium aluminium sulphate (alum) solution and kept for drying. The fabric retained the respective yellow, pink red color. These pigments can be utilized in the textile industries replacing synthetic dyes, hence being more eco friendly (Fig. 7).

**Anticancer activity**<sup>[22]</sup>- In 50 µl, 125 µl, 150 µl, and 170 µl orange (*Sarcina* sp.), pink (*Rhodotorula* sp.) have high OD value indicates that the pigment inhibits normal cells. Therefore cannot be used by human beings. The cytotoxicity in cell other than cancer cells is an indicator that they are harmful. Yellow (*Xanthomonas* sp.) have low OD values in comparison to orange and pink indicating that these yellow pigment can be used for anticancer activity and thereby can be used to benefit human beings. Tested compound of bacterial carotenoid pigments showed the weak anticancer activity against cancer cell lines as detected by the MTT assay. The results showed the lowest IC<sub>50</sub> (the highest anticancer activity) against lymphoma cells by the pigments, however there cytotoxicity against non-cancerous cell lines indicate the limited application of the pigment as anticancer agent (Fig. 8 & Fig. 9). Anticancer compounds from marine microorganisms inhibit cell growth in various cells through bacterial pigments has already been reported.

## CONCLUSIONS

The microorganisms were isolated and characterized by the Gram staining and Biochemical tests. Pigment producing organisms isolated from different sea-shore's soil were selected for pigment extraction. Natural color pigments were extracted from bacteria i.e. Yellow (*Xanthomonas* sp.), Orange (*Sarcina* sp.), and Pink red (*Rhodotorula* sp). Extracted pigments were purified by the column chromatography and identified by the TLC. Pigments were characterized by spectrophotometry and GC/MS analysis. Pigments were tested for antimicrobial activity, antioxidant, and anticancer activity against test isolates. The isolated organisms were used for transformation study. The extracted bacterial pigment was used for dyeing cotton cloth, fabric thread and food samples. Application as an anticancer agent was limited to the pinkish red.

## CONTRIBUTION OF AUTHORS

All authors equally contributed in this article.

## REFERENCES

- [1] Bhat S, Tawheed A. Isolation and Characterization of Pigment Producing Bacteria from Various foods for their possible use as biocolors. Int. J. Sci. Res., 2013; 4(10): 1605-09.
- [2] Goswami G, Chaudhuri S, Dutta D. Effect of pH and temperature on pigment production from an isolated bacterium. Chem. Eng. Trans., 2010; 543(42): 127-32.
- [3] Duffose L. Microbial production of food grade pigments. J. Food Techn. Biotechnol., 2006; 44(3): 313-21.
- [4] Balraj J, Pannerselvam K, Jayaraman A. Isolation of pigmented marine bacteria *Exiguobacterium* sp. from peninsular region of India and a study on biological activity of purified pigment. Int. J. Sci. Technol. Res., 2014; 654(78): 375-85.
- [5] Bhat SV, Khan SS, Amin T. Isolation and characterization of pigments producing bacteria from various foods for their possible use as biocolors, Int. J. Recent Sci. Res., 2013; 127(54): 1605-09.
- [6] Edge R, Mcgarvey DJ, Truscott TG. The carotenoids as antioxidants, A review. Journal of Photochemistry and Photobiol., 1997; 41(87): 189-200.
- [7] Fabre CE, Goma G, Blanc PJ. Production and Food Applications of the red pigments of *Monascus ruber*. J. Food Sci., 1993; 58(5): 1099-102.



- [8] Williamson NR, Fineran PC, Gristwood T, Chawrai SR, Leeper FJ, et al. Anticancer and Immunosuppressive properties of bacterial prodigionines. *Future Microbial.*, 2007; 283(289): 605-18.
- [9] Indra, AP, Umamaheswari S, et al. 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.*, 2014; 5(1): 01-04.
- [10] Khanna SK, Singh GB. Toxicity of commonly used food colors, A review *J. Sci. Indian Res.*, 1975; 34: 631-35.
- [11] Jiang, Y, Chen, F, Hyde KD. Production potential of water-soluble *Monascus* red pigment by a newly isolated *Penicillium* sp. *J. Agr. Techn.*, 2005; 1(1): 113-126.
- [12] Lee PC, Schmidt-Dannert C. Metabolic engineering towards biotechnological production of carotenoids in microorganisms, *Appl. Microbiol. Biotechnol.*, 2007; 60: 01-11.
- [13] Manimala MR, Murugesan R. In vitro antioxidant and antimicrobial activity of carotenoid pigment extracted from *Sporobolomyces* sp. isolated from natural source. *J. Appl. Nat. Sci.*, 2014; 6: 649-53.
- [14] Mukherjee S, Mitra AK. Identification and Characterization of a green pigment producing bacteria isolated from Bakreshwar Hot spring, West Bengal, India. *Int. J. Environ. Sci. Res.*, 2012; 2(1): 126-29.
- [15] Pattnaik P, Roy U, Jain P. 1997. Biocolors, new Generation. Additives For Food, Indian Food Industry, 16(5): 21-32.
- [16] Sudhakar T, Karpagam S, Premkumar J. Biosynthesis, antibacterial activity of pyocyanin pigment produced by *Pseudomonas aeruginosa* SU1. *J. Chem. Pharm. Res.*, 2015; 321(71): 921-24.
- [17] Prasad MP. Optimization of media parameters for pigment production in bacteria from effluent water samples. *Bioline*, 2015; 3(2): 428-33. doi: 10.17812/blj2015.32.9.
- [18] Raj DN, Dhanasekaran D, Thajuddin N, Panneerselvam A. Production of Prodigiosin from *Serratia marcescens* and its Cytotoxicity Activity. *J. Pharma. Res.*, 2009; 284(73): 590-93.
- [19] Nakamura Y, Asada C, Sawada T. Production of antibacterial violet pigment by psychrotropic bacterium RT102 strain. *Biotechnol Bioprocess Eng.*, 2003; 432(32): 37-40.
- [20] Sandmann G. Carotenoid biosynthesis in microorganisms and plants. *European J. Biochem.*, 1994; 223: 07-24. doi: 10.1111/j.1432-1033.1994.tb18961.x.
- [21] Tokatl M, Ozcelik F. Food colorants production from microbial sources, Turkey 10. *Food Congr.*, 2008; 21-23.
- [22] Tibor C. Liquid Chromatography of natural pigments and synthetic dyes. *J. Chromatographic Lib.*, 2007; 71: 01-91.

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