IDENTIFICATION AND CHARACTERIZATION OF BIOACTIVE PIGMENTS FROM MARINE BACTERIA ISOLATED FROM COASTAL REGION OF KELWE-MAHIM, PALGHAR

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Abstract: Marine bacteria though rich in pigments largely remain unexplored for its bioactive components. Synthetic pigments used in various industries pose various harmful effects, to overcome these problems it is important to explore varied natural sources for natural pigments.
Pigmented bacterial isolates were screened for sediment sample of the coastal region of kelwe beach,palghar. 20 different bacterial isolates were obtained on st.zobells agar. From these 20 bacterial isolates, 2 visually pigmented bacteria orange and yellow pigment producing bacteria were further studied.
The MIC of both microbial isolates were found to be at 8% NaCl and hence was moderate halotolerant in nature. Optimum growth of both pigment producing bacteria was found to be at pH 9. Pigment was extracted from bacteria using different solvents such as methanol, ethanol, acetone and chloroform, the maximum absorbance of the pigment was found to be 430nm and methanol was found to be the best solvent for maximum pigment extraction for both the isolates.
The identification of pigment was done by chromatography technique and the Rf value was 0.48 and 0.58 for orange and yellow pigment, probably may contain zeaxanthin and lutein respectively. Antioxidant activity of pigment was checked by DPPH method, the percentage of scavenging activity of orange and yellow pigment was found to be 18.841% and 33.334% respectively.
Antimicrobial activity of the extracted pigment was studied against *E.coli*, *S.aureus* and *S.typhi* by agar cup method and both pigment show maximum antimicrobial activity against only *S.aureus*.

Keywords: *Marine pigments, thin layer chromatography, Antioxidant, DPPH assay, Antimicrobial activity.*

Introduction:

Marine environment covers 71% of the earth surface area which remains largely unexplored for its novel compounds. Marine covers almost 80% of life on earth. These contains different types of species from which many are still unknown and these species produces different types of metabolites. Microorganisms produces different unique metabolites because of the complexity and dynamic system in oceans. Marine microorganisms live in extreme conditions such as pressure, salinity and temperature. Marine microorganisms can be divided on the basis of habitat into:-Psychrophilic- living at low temperatures, Halophiles- living at high salinity, Barophilic- living under high pressure.Some marine microbes' offers production of secondary metabolites like pigments. Marine microorganisms are very beneficial as compare to terrestrial microorganisms because marine microorganisms and their products have unique properties under extreme marine environmental conditions. Bioactive compounds:-A compound that has an effect on a living organism, tissue /cell. A bioactive compound is any compound present in the foods of humans, animals, or plants that has an effect on the organism consuming it (example-Pigment).

Different types of colors or dyes are used in industries but these are synthetic and may be harmful for living organisms, so as avoid its harmful effects it is necessary to replace these harmful synthetic colorants with natural colorants. Pigments can be classified by their origin as natural & synthetic. Natural pigments are produced by living organisms such as plants, animals, fungi, and microorganisms. Synthetic pigments are synthesized in laboratories.

Nature produces various substances that have colors and are produces from biological materials hence called as bio-colors or pigments. Nowadays, use of synthetic pigments decreases because of its harmful effects so the industries move towards natural resources and they try to obtain novel pigments. In nature pigments are produce by plants as well as by microbes but it is easy to grow microbes in its optimum condition and produce large amount of desire pigment as compared to plants. Pigments are used

in various industries such as pharmaceuticals, food industry, and cloth industry, because of its unique characteristics most of pigments are used for medicine preparations as antibacterial or antiviral compounds. Some pigments have antioxidant like properties which are beneficial in medicinal industries.

Production and application of microbial pigments as natural colorants increases and it is necessary to study there unique properties because of this colorants have been studied by various researchers and is one of the emerging fields of research. Most of the pigments are still under study so as to discover some other unique properties

Materials and Methods:

A) Screening and isolation of pigment producing bacteria.

Isolation of bacteria

The soil samples were collected from kelwe(Palghar) coastal region of west coast [19.643959°N 72.713201°E] in clean, sanitized and autoclaved bumper tube by adopting 'Scoop method'. *(EPA-600/8-78-017. U.S. Environmental Protection Agency, 1978, pp: 337.)* . Samples collected from kelwe beach,Palghar were serially diluted in triplicates and spread on zobell's marine agar by spread plate technique and incubated at 28ºC for 48hrs.

Screening of pigment producing bacteria

After incubation the isolated pigment producing colonies were subcultured on zobell"s agar plate with the help of streak plate technique and plates were incubated at 28ºC for 48 hrs. After incubation at 48 hrs the individual pigment producing colonies were further enriched in St. Zobell"s medium, the enriched flasks were incubated at 28ºC for 7 days.

B) Morphological and Biochemical Identification of pigment producing bacteria.

The well isolated colonies of pigments producing bacteria were studied for their Morphological characteristics both cultural characteristics and gram staining was performed.
Biochemical characteristics of the pigments producing bacteria were studied by performing various tests like sugar fermentation , catalase test , coagulase test , nitrate reduction test , salt tolerance etc.

C) Optimization of pigment production by the isolates.

The maximum pigment production was determined by inoculating pure isolates in 100 ml flask containing st. zobell's broth with different pH (i.e. 2, 4, 7, 9 and 11) and incubated at 27° C for 48 hrs. This helps to check the impact of different pH on pigment production.

D) Extraction of pigment using solvent.

The extraction of the pigments was carried out by centrifugation method. Different solvents were used for pigment extraction such as Methanol, Ethanol, Acetone and Chloroform.

E) Antioxidant activity of pigment by using DPPH.

Antioxidant property of pigments were determined by DPPH assay method. DPPH reagent was used and the absorbance was determined colorimetrically at 520nm. The percentage scavenging activity was calculated by using formula

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

Ao= Absorption of control

As= Absorption of tested compound

F) Identification of pigments by Thin layer chromatography

For identification of pigments thin layer chromatography was performed. Silica gel was used as stationary phase and mixture of methanol: chloroform (95:5 v/v) used as mobile phase. Crude pigments were mixed with solvent (95% methanol) and then spotted on TLC plate. Retention factor (Rf) was determined by formula

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

G) Antibacterial activity of purified pigments.

Purified pigments were further checked for its antimicrobial activity on st. Mueller hinton agar by using agar cup method against different test cultures such as *Staphylococcus aureus*, *Salmonella typhi* & *Escherichia coli.*

RESULT AND DISCUSSION:

A) Screening and isolation of pigment producing bacteria.

After incubation at 27°C for 48 hrs by using spread plate method. 20 different bacterial isolates were obtained on st. zobells agar from kelwe beach,Palghar. From these 20 bacterial isolates, 2 visually pigmented bacteria orange and yellow pigment producing bacteria were further studied.

Fig.1:- Yellow and Orange pigment producing bacterial colonies on st. zobells agar media plate.

B) Morphological and Biochemical Identification of pigment producing bacteria.

Morphological and biochemical characteristics were studied and it was found that Yellow colony producing was found to be gram positive I nature and morphology was bacillus and orange pigment producing bacteria was found to be gram positive rods. Both pigments producing microbes showed only acid production in all the sugar tests while coagulase and catalase test were positive and the nitrate test was negative. From vasai coast total 227 bacteria's were found from that morphological study said that gram negative bacteria were dominant over gram positive bacteria (Mukesh.R.Pimpliskar* and Rahul.N.Jadhav ,et al,2014).orange pigment producing microbes were isolated from loar salt sarovar (Mali Devyani,et al, 2017).

C) Optimization of pigment production by the isolates.

Bacteria show increase in growth rate at a particular pH, this is nothing but the optimum pH of the bacteria. This increase growth of bacteria helps to increase the pigment production. The maximum pigment production at different pH was done in flask containing zobell's agar media obtained from himedia with different pH (i.e. 2, 4, 7, 9, and 11). The maximum pigment was obtained in the flask which was set at pH 9. Maximum growth of pigment producing bacteria was found to be at pH 9. From one of the study it was observed that the isolated strain produce extracellular red pigment on nutrient agar medium at 30 ℃ – 37 ℃ at pH 7 after 7 days (Bhaswati Goswami, et al, 2014.).

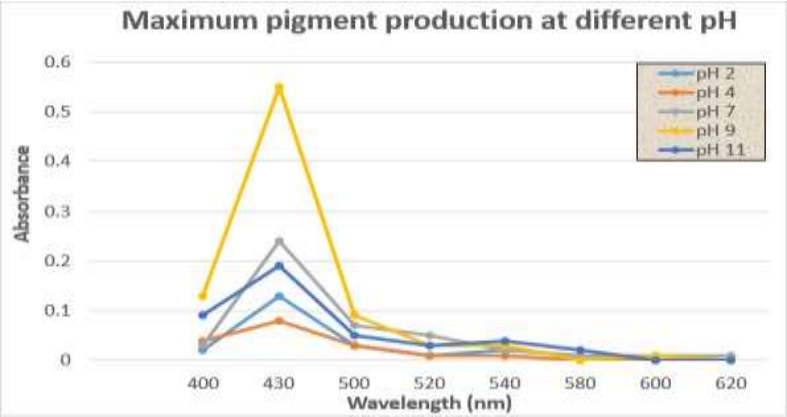

Fig 2 Maximum pH production at different pH by Yellow pigment producing bacteria.

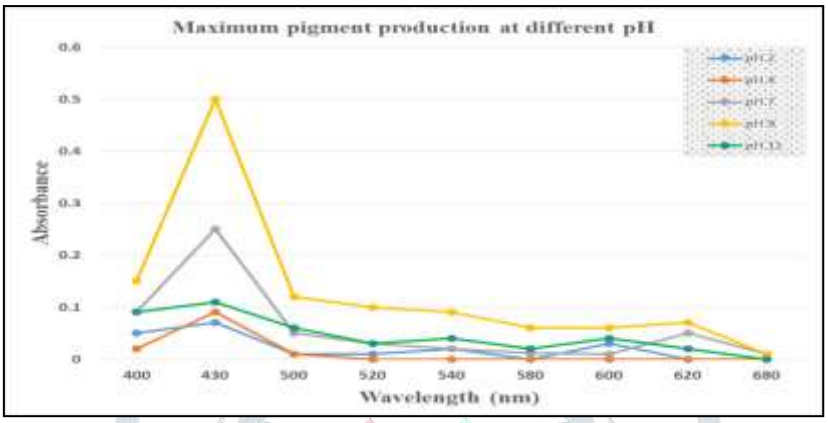

Fig 3 Maximum pH production at different pH by Orange pigment producing bacteria.

D) Extraction of pigment using solvent.

Pure culture was inoculated in the st. 100 ml Zobell's broth and was kept at 27°C for 7 days to obtain dense growth of organisms which leads to more production of pigments. After 7 days of incubation, the culture was sent for pigment extraction by centrifugation method using 95% methanol. Both the pigments were extracted and dried to get crude pigment. Different solvents like ethyl acetate, methanol, acetone, hexane was used to check for the maximum solubility of pigments and the pigments were isolated using liquid-liquid extraction method (Minal R. Dave, et al, 2018).

Table 1 Maximum absorbance of pigments extracted by using methanol as solvent.

Wavelength	Yellow pigment	Orange pigment
400	0.07	0.11
430	0.08	0.12
500	0.06	0.08
520	0.04	0.05
540	0.05	0.05
580	0.03	0.04
600	0.04	0.03
620	0.01	0.02
680	0.02	0.01

E) Antioxidant activity of pigment by using DPPH.

The anti-oxidant property was determined by DPPH assay of the pigments. The 0.05mM DPPH reagent was used and the absorbance was determined colorimetrically at 520nm and the percentage of scavenging activity of Yellow and

Orange pigment were found to be **33.334** and **8.841%** respectively. Scavenging activity of the fungal pigment crude was found to be 1.72mg/ml (V. manon Mani, et al, 2015). Scavenging DPPH free radicals radical determines the free radical scavenging activity which helps to decrease various injuries against the biological system (Lee et al., 2001).

Table 2 DPPH assay of both pigments.

	Absorbance at 520nm	Percentage Scavenging activity
DPPH	0.69	-
Yellow pigment	0.46	33.334%
Orange pigment	0.56	18.841%

F) Identification of pigments by Thin layer chromatography

For identification of pigments Thin layer chromatography was use and by comparing the standard Rf value it concludes that Yellow and Orange pigment contains lutein and zeaxanthin respectively. Photosynthetic pigments such as chlorophylls, xanthophyll's and β-carotene are soluble in organic solvents (Quach et,al…2004).

Fig 4 Thin layer chromatography of both pigments.

G) Antibacterial activity of purified pigments.

Bacterial pigments are reported to have antibacterial activity against both Gram-positive and Gram-negative bacteria. August et al. (2000). The antimicrobial activity of the pigment dissolved in methanol was tested by agar well diffusion method. Methanol were used as control. The plates were incubated at 37° C for 24h after which activity was evidenced by the presence of zone of inhibition surrounding the well. Marine Actinomycetes and pigmented organisms were isolated from marine water sample. The extracted bacterial pigments had antibacterial activity (Amrita M. bhatial, et, al). The antimicrobial activities exhibited by Bacillus sp. which indicates that the red pigment extracted from the isolated Bacillus sp. were able to inhibit the growth of all the test microorganisms such as *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas spp.* (Bhaswanti Goswami, et, al 2014)

Table 3 Antibacterial activity of both pigments against test organisms.

Name of Pathogens	Antibacterial activity-inhibition zone (mm)		
	Negative Control (Methanol)	Yellow Pigment (in Methanol)	Orange Pigment (in Methanol)
Salmonella typhi	0	0	0
Escherichia coli	0	0	0
Staphylococcus aureus	10	18	13

Fig 5 Plates showing antibacterial activity.

Conclusion:

The results of this study showed that there are potential pigment producing bacteria present in the soil of kelwe-beach. 20 different isolates were found of among which only 2 were pigmented. The Cultural and biochemical characteristics of the orange and yellow isolate on comparison with bergy's manual was found to be *Bacillus spps. & Micrococcus spps.* Methanol was found to be the best solvent for maximum pigments extraction. R_f value for yellow and orange pigment was found to be 0.48 and 0.58 respectively, which indicates pigments may contains lutein and zeaxanthin. As this study showed that the both yellow and orange pigments obtained from the marine isolate possess both anti-oxidant property and anti-bacterial properties.

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