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ISOLATION AND CHARACTERIZATION OF PIGMENT PRODUCING BACTERIA ISOLATED FROM FRESH & MARINE WATER HABITATS IN THANE DISTRICT, M.S, INDIA

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ABSTRACT

In the decade of stringent guidelines put forth by FDA in manufacture of consumer goods, microbial pigments, owing to its nature of colour, nutritive value & medicinal Properties; very aptly has provided an alternative over its chemical counterparts. Natural Habitats viz soil, water will always remain a never-ending source of micro-organisms; with huge potential in many sectors. In the present study, isolation of pigment providing bacterial isolates from fresh & marine water habitats was carried out. Identification was carried out as mentioned in Bergey's manual of Determinative Bacteriology, 9th Edition. Extraction and Optimization studies of growth conditions for maximum pigment production are carried out.

KEYWORDS: FDA, Microbial pigments, Natural Habitats, Bergey's manual.

INTRODUCTION

A pigment is a material that changes the colour of reflected or transmitted light as a result of wavelength – selective absorption. Materials that humans choose and develop for use as pigments usually have special properties that make them ideal for colouring other materials. Biological pigments are substances produced by living organisms that have a colour resulting from selective colour absorption. Both natural pigments and synthetic dyes have been extensively used in various fields of everyday life such as foods, feeds, textile, paper, printing inks, cosmetics, pharmaceuticals etc. (Tiber 2007).

As rightly said, "We inevitably eat with our eyes". (Stitch et al 2002). Colour is an important attribute that determines the consumer's acceptance of foods, colour additives are essential in food industry. As a result, various synthetic food colours have been manufactured but many of them comprise of various hazardous effects (Faber et al 1993). There is an increasing demand for natural colours from industries as well as consumers. However, the range of natural colour – shades is still limited compared to synthetic dyes.

Natural colours used in food industry are largely plant extracts having several disadvantages such as instability against light, heat or adverse pH, low water solubility and non- availability throughout the year. Microbial pigments are a promising alternative source for natural food grade pigments & have a great potential for varied applications due to their natural colour, safety to use, medicinal properties, nutrients like vitamins, production being independent of season & geographical conditions with controllable & predictable yield (Francis et al 2000; Johnson & Schroeder, 1996).

Microbial pigment production is now one of the emerging fields of research. Pigment producing micro- organisms (bacteria, fungi, yeast, protozoa) (Duffose, 2009) offer considerable scope for commercial production of biological pigments like carotenoids, anthraquinone, cholorophyll, melanin, flavins, quinones, violacein etc.(Kenini & Gupta 2011).

The present study was aimed at isolation of bacterial isolates from fresh & marine water habitats, capable of producing pigments of commercial importance. Isolates were identified through morphological & biochemical characteristics.

MATERIALS AND METHODS

1. Chemicals: Analytical grade chemicals & solvents were obtained from SRL, E.Merck India. Gram staining kit and reagents required for biochemical tests were obtained from E.Merck, India. Bacteriological media were obtained from Himedia Pvt. Ltd.

2. Sample Collection: Water samples were collected from fresh & marine water sources from different areas in Kalyan-Badlapur region of Thane district & Alibaug region of Raigad District, M.S, India. Distance of the water source from ground and depth were taken into consideration during sample collection. Four water samples were collected from individual source. Fresh water sources include samples from Kalyan, Ambarnath & Badlapur while

marine water sources include samples from Kalyan & Alibaug. All samples were refrigerated at 4^0C until use.

3. Isolation of Pigment producing micro-organisms: For isolation of Pigment producing micro-organisms, collected water samples were plated on Nutrient Agar and incubated at room temperature for 24 hrs. Following incubation, different pigment producing colonies viz; Orange, red, yellow, green, violet were selected and propagated on the same medium until pure cultures were obtained.

4. Taxonomic identification of the microorganisms: Taxonomic identification of the bacterial isolates under study was done by following the Bergey's Manual of Determinative Bacteriology, 9th edition. (Bhat & Khan)

a. Morphological characterization: The isolates producing higher intensity of colour pigmentation were selected and identified using Gram Staining, morphology & biochemical tests. The pure culture of isolates was maintained on Nutrient agar slants for further investigation.

b. Biochemical Identification: In order to determine the biochemical characterization of the bacterial isolates under study, a series of biochemical tests were performed.

5. Preparation of Inoculums: Pure culture of test isolates from NA slant was transferred in pre-sterilized 100ml nutrient broth with 1% mannitol and incubated under static conditions for 48 hrs at RT. One percent of the above cell suspension was used as inoculum.

6. Production, Extraction & Analysis of Pigment: The inoculums of the isolated strain were grown in 250 ml Erlenmeyer flasks containing 100 ml of nutrient broth. Fermentation was carried out under static conditions for 48 hrs at RT. Extraction of the pigment from the fermentation broth was done by solvent extraction method. The organism was harvested by centrifuging at 6,000rpm for 10 mins. Supernatant was discarded and the pellet was resuspended in distilled water for cell lyses to occur. The pigment was then extracted with methanol by repeated centrifugation until the cell debris turned colorless. These supernatants containing the diffused pigment were filtered through Millipore membrane filter (pore diameter 0.22µm).The filtrates were then collected in sterilized screw-cap tubes. Visible absorption spectrum of the separated pigments in nutrient broth was analysed with UV-Spectrophotometer (SPECTRO 2080) between the wavelength of 350-750nm.Maximum absorbance of the pigments was recorded.

RESULTS AND DISCUSSIONS

1. **Sample Collection:** Distance of the water source from ground and depth for Fresh water source was reported as follows:

Table 1: Distance of water source & its depth for Fresh water environment.

Fresh water Source: Nandivali Pond, Kalyan, Thane District		
Sample Code	Distance from ground to source (in feets)	Depth of water (in feets)
A	2.5	1.5
B	4.5	3.5
C	6.0	4.0
D	7.5	6.0

Fresh water Source: Lotus Pond, Ambarnath, Thane District		
Sample Code	Distance from ground to source (in feets)	Depth of water (in feets)
A	4.5	4.0
B	6.5	4.5
C	8.0	7.0
D	10.0	8.5

Fresh water Source: Pond, Badlapur, Thane District		
Sample Code	Distance from ground to source (in feets)	Depth of water (in feets)
A	2.0	1.0
B	3.0	2.0
C	4.5	3.5
D	6.0	4.0

The distance of the water source from the ground and depth for Marine environment was reported as follows:

Table 2: Distance of water source & its depth for Marine environment.

Marine water Source: Durgadi Creek, Kalyan, Thane District		
Sample Code	Distance from ground to source (in feets)	Depth of water (in feets)
A	6.0	4.5
B	8.0	7.0
C	10.0	9.0
D	12.0	10.0

Marine water Source: Alibaug, Raigad District		
Sample Code	Distance from ground to source (in feets)	Depth of water (in feets)
A	4.5	3.5
B	6.5	5.0
C	8.0	6.5
D	10.0	8.0

2. Isolation of pigment producing organisms

A total of 57 isolates were obtained on NA plates. For the present study of isolating pigment producing organisms, 12 water samples from 3 fresh water sources and 8 water samples from 2 marine water sources were procured. The samples obtained were plated on Nutrient agar plates and incubated at room temperature. 33 bacterial isolates were obtained from fresh water and 24 bacterial isolates were obtained from marine water sources. Among them 5 Pigment producing bacterial isolates were obtained from fresh water and 3 pigment producing bacterial isolates were obtained from marine water sources. Isolates which showed different morphological characteristics were selected for further screening.

Table 3: Sampling & Isolation Details – Aquatic habitats.

Sampling area	No. of samples collected	No. of isolates obtained	No. of Pigment producing isolates
Fresh water habitat			
Nandivali Pond, Kalyan, Thane District	04	16	02
Lotus Pond, Ambarnath, Thane District	04	12	01
Pond, Badlapur, Thane District	04	5	02
Marine water habitat			
Durgadi Creek, Kalyan, Thane District	04	10	02
Alibaug, Raigad District	04	14	01

3. Identification of isolates

Taxonomic identification of the test isolates under study was reported by performing Gram's test, studying cultural characteristics of the isolates and biochemical tests as recommended in the Bergey's Manual of Determinative Bacteriology. Morphological characteristics of the isolates are mentioned in Table 4.

Table 4. Morphological characteristics of the isolates.

Colony Characteristics			
	ISO-1	**ISO-2**	**ISO-3**
Colony shape	Circular	Circular	Irregular
Colony size	Small	Small	Pin-point
Margin	Entire	Entire	Irregular

Surface	Smooth	Smooth	Smooth
Colour	Red	Purple	Bluish green
Cell morphology			
Cell shape	Rods	Rods	Short rods
Motility	Motile	Non-motile	Motile
Spore(s)	-	-	-
Gram staining	Gram negative	Gram negative	Gram negative

By recording pigment colour and morphological characteristics of test isolates it was confirmed that ISO-1 belonged to *Serratia spp*, ISO-2 belonged to *Chromobacterium spp* and ISO-3 belonged to *Pseudomonas spp.*

Identification Scheme mentioned in Bergey's manual of Determinative Bacteriology, 9th edition for Gram positive and Gram negative bacteria is followed to identify isolate up to species level. Key biochemicals and Special tests are performed to avoid confusion.

4. Absorption characteristics of pigments from different strains

Absorption spectra of pigments from three isolates under investigation were studied in the visible range between the wavelengths of 350-750nm and spectrophotometric analysis at the respective wavelengths at which maximum absorbance (λ max) were observed were determined.

Table 5: Absorption maxima of the pigments.

Sr.no.	Isolate code	Colour of pigment	Absorption maxima (λ max)nm
1.	ISO-1	Red	420nm
2.	ISO-2	Violet	547nm
3.	ISO-3	Bluish green	520nm

Table 6: Biochemical characterization of the isolates.

Tests	Standard result *Serratia spp*	Observed result ISO-1	Standard result *Chromobacterium spp*	Observed result ISO-2	Standard result *Pseudomonas spp*	Observed result ISO-3
Glucose Aerobic Anaerobic	 + -	 + -	 + +	 + +	 + -	 + -
Sucrose	+	+	-	-	-	-
Lactose	-	-	-	-	-	-
Maltose	+	+	-	-	-	-
Mannose	+	+	-	-	-	-
Xylose	-	-	-	-	-	-
Glycerol	+	+	+	+	-	-

With Arginine	-	-	+	+	+	+
Without Arginine	-	-	+	+	+	+
With Lysine	+	+	-	-	-	-
Without Lysine	+	+	-	-	-	-
M.R test	-	-	+	+	-	-
V.P.Test	+	+	-	-	-	-
Indole test	-	-	-	-	+	+
Nitrate	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Urease	D	+	-	-	-	-
TSI Slant	Y	Y	Y	Y	P	P
Butt	Y	Y	Y	Y	P	P
H_2S	-	-	-	-	-	-
Gas	-	-	-	-	-	-
Pigment	Orange-Red	Red	Violet	Violet	Blue/Green/Bluish green	Bluish green

Key: D: Variable, Y: Yellow, +: Positive, - : Negative

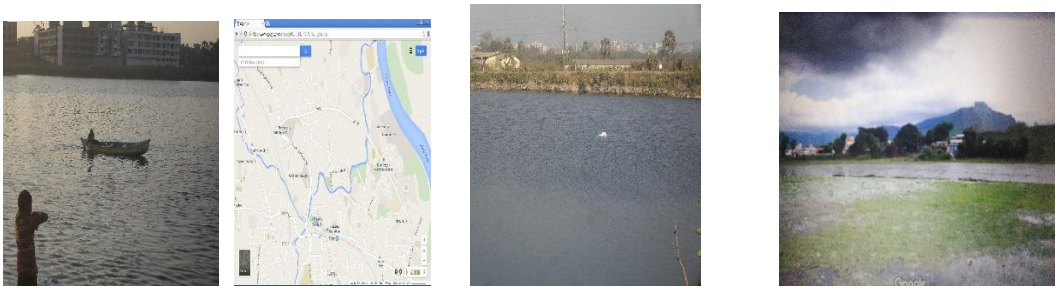
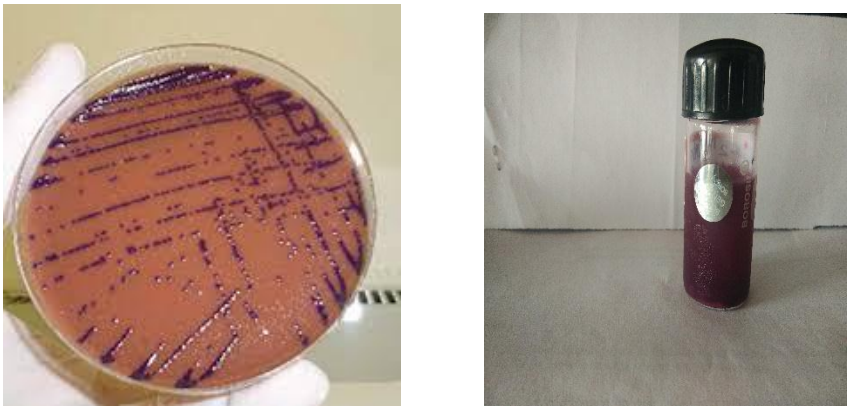


Fig 1: Sampling locations: KDC site, BLP site, KNP site, Lotus Pond (left to right)



ISO-2 *Chromobacterium spp* & *extracted pigment*

ISO-3 Pseudomonas spp

ISO-1 Serratia spp

CONCLUSION

The study was intended to isolate pigment producing micro-organisms in fresh & marine water samples. Three pigment producing bacterial isolates, viz; *Serratia marcescens, Chromobacterium spp* & *Pseudomonas aeruginosa* were screened from above mentioned sampling sites. Natural colours or biocolours have numerous applications in textile as well as in food industries as colouring agents. The use of synthetic dyes in textiles pose a major threat to the environment and the toxic dyes released in waste water were highly toxic to the aquatic as well as for human life. Moreover, the synthetic food colouring agents used in food industries cause numerous human health related issues due to its toxic nature. In this regard, this study is an initiative approach towards the use of biocolours which find its applications in numerous sectors as an alternative for synthetic chemicals. Further, the natural pigments obtained in this study would be tested for its applications in diverse fields.

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Conflict of Interests: The authors declare that there is no conflict of interest regarding the publication of this paper.

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