"ISOLATION OF PRODIGIOSIN PRODUCERS FROM AQUATIC ENVIRONMENT AND STUDY OF IT'S APPLICATIONS."

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Abstract: Harmful effects of synthetic dyes and pigments used in various industries have forced us to look for alternative preparation of dyes from natural sources. Prodigiosin, natural red pigment characterized by a common pyrrolylpyrromethene skeleton is produced by various bacteria like *V. psychroerythrus, S. marcescens*, *P. Magnesiorubra, N. pelletieri* etc. *Serratia marcescens* is one of the suitable candidate for production of prodigiosin. Prodigiosin is emerging as a valuable molecule because of it's large scale applications in pharmaceuticals to textile industry. Isolation of prodigiosin producing organisms was done from different aquatic sources; screening was done for selection of orange to red pigment producing organisms. Pigmented strains were confirmed by morphological and biochemical studies. The water insoluble red pigment was extracted using methanol and further purified. Antioxidant activity of prodigiosin was determined by using standard antioxidant 2,2 Diphenyl-1-picrylhyadrazyl (DPPH). Pigment also showed inhibitory effect on bacterial pathogens. The pigment was evaluated for application as a dye in the textile industry. Prodigiosin may further have broad applications in cancer treatment and other industries such as food, plastic, etc.

Keywords: Prodigiosin, Serratia marcescens.

Introduction: Pigments are one of the classes of secondary metabolites [1]. These bio pigments can be obtained from two major sources, plant and micro-organisms. Bio pigments from the micro-organisms have been preferred over those from plants because of their stability and their cultivation technology throughout the year. On the other hand, bio pigments from plants have numerous drawbacks like instability towards light, heat or adverse pH, low water solubility and often variation in concentration and availability throughout the year.

One of the studied biopigments of microbial origin is the prodigiosin. Prodigiosins are a family of natural red pigments, characterized by a common pyrrolylpyrromethene skeleton having low molecular weight (323.4 Daltons) [2], appearing only in stationary phase of bacterial growth. Prodigiosin $C_{30}H_{26}N_8O$ is produced by many strains of the bacterium *Serratia marcescens* and other unrelated strains such as *Nocardia madurae, Pseudomonas magnesiorubra*, *Vibrio psychroerythrus, Nocardia pelletieri* and *Streptomyces longisporusruber* [3], in the bacteria it has been shown to be associated with extracellular vesicles or present in intracellular granules [4]**.** It has also been discovered that prodigiosin possesses antibacterial, antifungal, antiprotozoal, anticancer [5], antimalarial, antidiabetic, nonsteroidal anti-inflammatory properties. Inspite of potential commercial values, cost effective bioprocesses for prodigiosin production has still to be developed.The present study focuses on exploration of antioxidant, antimicrobial and effectiveness of prodigiosin as a dyeing agent.

Material and Methods:

Sample Collection: Water samples were collected from aquatic environment (fresh and

marine) sources of different areas in Maharashtra. Fresh water samples were collected from Hiraghat (Ulhasnagar), Karjat, Badlapur, Thane, Satara, Kalu and Ulhas river, while marine water samples were collected from Kalyan (reti bunder), Thane creek and Mahim creek [6]. Distance of the water source from ground and depth were taken into consideration during sample collection and further refrigerated at 4°C until use.

Isolation and Identification:

Prodigiosin producing organisms were isolated on Nutrient Agar plates from each water samples. Plates were incubated at RT for 24 hours. Following incubation, orange to red coloured colonies were selected and propagated on the same medium until pure cultures were obtained. Gram staining and biochemical characterization was done using Bergey's Manual of Determinative Bacteriology, 8th edition, 1974, for confirming the isolate as *Serratia marcescens*. Pure cultures was maintained on Nutrient agar slants and stored at 4°C until use.

Extraction of Prodigiosin:

Culture was spread on Nutrient Agar and incubated at RT for 24hrs. Harvested cells were mixed in methanol, sonicated for 20 min. at 20 KHz, 120 watts and centrifuged at 12,000 rpm for 10 min. at 2°C. Pellet obtained was discarded while supernatant was used as pigment. Evaporation till dryness was done to get residue of prodigiosin which was redissolved in methanol to get pure extract

Confirmation of prodigiosin:

2ml of concentrated pigment was taken in two test tubes. The content of one of the test tube was acidified with a drop of concentrated hydrochloric acid and the other alkalinized with a drop of concentrated ammonia solution. A red or pink colour in the acidified solution and yellow or tan colour in the alkaline solution indicated a positive, presumptive test for prodigiosin [3].

Detection of λ max:

The methanolic extract of prodigiosin was analyzed by using a colorimeter for detecting the λ_{max}. The absorption range selected was 400 – 600nm.

Quantification of prodigiosin [4]: Bacterial cell absorbance in the broth was measured at 620nm, methanolic extract of prodigiosin showed maxima at 499 nm .

$$\textbf{\textit{Prodigiosin unit per cell}} = \frac{[\textbf{\textit{O.D.}}499-(1.381\ x\ \textbf{\textit{O.D.}}620)]x\ 1000}{\textbf{\textit{O.D.}}620}$$

Where, O.D. 499 = pigment absorbance, O.D. 620 = bacterial cell absorbance, 1.381 = constant

Evaluation of in vitro antimicrobial activity of Prodigiosin:

The antimicrobial activity of prodigiosin was studied on Mueller Hinton Agar against laboratory isolates of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium.*

0.1ml and 0.5ml of the methanolic extract of the pigment was added in sterile Mueller Hinton Agar butts, poured and solidified. 95% methanol was used as control in same volumes. Laboratory isolates of 0.1 O.D. were further streaked on the solidified plates. Following 24 hours incubation at 37°C, plates were examined for growth of the isolates.

Total antioxidant capacity:

The total antioxidant capacity of the methanolic extract was evaluated by DPPH method. 0.4ml of the methanolic extract was mixed in 3.6ml of 25mg% DPPH reagent solution in methanol. Incubation was done at RT in dark for 30 minutes. The absorbance of the solution was calorimetrically measured at 530nm, using methanol as blank.

The % scavenging capacity for prodigiosin was

calculated using the following formula:

$$\% \text{ Scavenging} = \frac{\text{(Abs of control – Abs of test)}}{\text{Ab of control x 100}}$$

Effectiveness as dyeing agent: Six pieces of 2cm of each fabric (Satin, cotton, polyester and rubia 2 x 2) was soaked in 4ml methanolic extract of prodigiosin taken in different petri dishes and incubated for 48 hours at room temperature for absorption of the pigment. Following this each fabric was dried at room temperature. All the dried six pieces of each fabric were treated with acid, alkali, cold water, hot water, cold water and detergent and hot water and detergent for study of retention of pigment.

Results and discussion:

Prodigiosin, a secondary metabolite has varied application in pharmaceuticals, food, textile and other industries too. The present study focuses on isolation of prodigiosin producers from aquatic environment and study of its applications.

Screening and identification of bacterial isolates:

Pigmented colonies from orange to red colour were obtained from water samples collected at Satara (fresh water) and Mahim (marine water), colonies were selected from these sources and identified depending on morphological and biochemical characteristics.

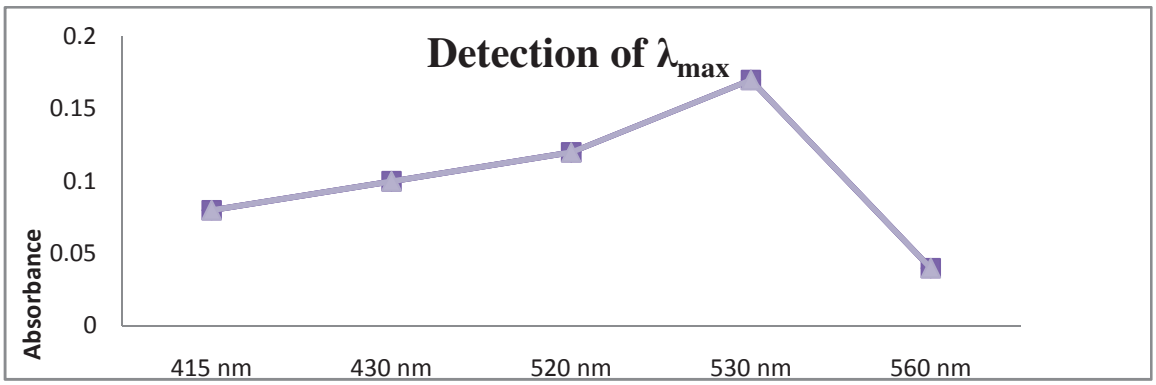
Table 1: Morphological and biochemical characteristics of *Serratia marcescens*.

	Standard results for *Serratia marcescens*.	Observed results of the bacterial isolates.
Colony colour	Red	Red
Cell shape	Rod	Rod
Gram Nature	Gram negative	Gram negative
Glucose	+	+
Sucrose	-	-
Lysine Decarboxylase	+	+
Nitrate reduction	+	+
Urease	-	-
Motility	+	+

Extraction and study of Pigment:

Extraction was done in methanol till the residual cells were colourless. After drying the pooled methanolic extracts, the residual powder was redissolved in methanol to get purified extract. Confirmation of pigment was done using acid and base method. Under acidic condition pigment appears red and in basic condition appears yellow in colour, this shift in colour due to change in pH is confirmatory test for prodigiosin.[3]. λ_{max} was measured and found to be at 530nm.

Graph 1: Detection of λ_{max} for methanolic extract of prodigiosin



Quantification of prodigiosin: Absorbance of the 1:100 diluted methanolic extract at 530nm was found to be 0.17 O.D. By using absorbance value in the formulae the concentration of 1:100 diluted methanolic extract of prodigiosin was found to be 0.24µg/ml. Hence the concentration of neat extract is 24 µg/ml

Antibacterial activity of prodigiosin: As indicated in table 2, when 0.1ml of pigment was used it did not inhibit any of the bacterial cultures used. When 0.5ml of pigment was used all cultures except salmonella typhimurium were inhibited. The methanol control in both the cases did not show inhibition of any culture used. The antibacterial activity of prodigiosin is the result of their ability to pass through the outer membrane and inhibiting target enzymes such as DNA gyrase and Topoisomerase IV, which inhibits the cell growth [6].

Table 2 : Antibacterial activity of prodigiosin

	0.1 ml		**0.5 ml**	
Laboratory isolates	pigment	Methanol (control)	Pigment	Methanol (control)
Escherichia coli	+	+	-	+
Pseudomonas aeruginosa	+	+	-	+
Salmonella typhimurium	+	+	+	+
Staphylococcus aureus	+	+	-	+

Key : + : Growth., - : No growth

Total antioxidant property: DPPH is a purple coloured compound which in reduced state gets decolourised to yellow colour. An antioxidant reacts with DPPH (purple), by donating hydrogen to it, which results in conversion of DPPH to reduced state (yellow). The change in colour from deep violet to light yellow was read calorimetrically at 530nm, using methanol as blank. From table 3 the % scavenging capacity of the prodigiosin pigment was determined to be

60% by using the formulae as mentioned above.

Table 3: Antioxidant capacity of prodigiosin		
		Absorbance at 530nm
TEST	0.4ml of methanolic extract + 3.6ml of DPPH reagent	0.20
CONTROL	0.4ml of methanol + 3.6ml of DPPH reagent	0.50

Prodigiosin as textile colourant: Prodigiosin can be used to dye many fibres including wool, nylon, acrylics and silk [7]. The red pigment prodigiosin got absorbed to the fabrics after incubation. The fabrics retained the pigment after acid, alkali, cold water, cold water and detergent, hot water and hot water and detergent. Thus the fabrics were successfully coloured.

Fig 1 : Fabric staining

Conclusion: The study demonstrated a successful isolation of prodigiosin producing organisms from aquatic environment. The antibacterial and antioxidant nature of the pigment may aim at the possible future usage of prodigiosin as a therapeutic agent[8]. Prodigiosin can also be explored in textile industry as its shows stability to various treatment post dyeing.

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