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Isolation of Prodigiosin Producing Bacteria from Marine Ecosystem and Exploration of its Fabric Dyeing Potential

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Abstract: Mahim creek a salt-water body located in Mumbai district Maharashtra was explored for isolation of Prodigiosin producing bacteria. A total of 38 pigmented isolates were obtained of which 15.78% were red pigmented. Two isolates namely MK 12 and MK 23 successfully qualified for prodigiosin production. Acetone extraction was more effective with a yield of 23.6ug/10gm and 47.30ug/ml for MK12 and MK23, respectively. Based on isolates morphological and biochemical characteristics they were identified as Serratia rubidaea and Serratia marcescens. MIC for antibacterial activity against standard pathogens for MK 12 and MK 23 was in the range of 2ug/ml to 3ugml and 2ug/ml to 4ug/ml. While for both the extracts MIC for antifungal activity was 4ug/ml. Both the extracts were effective in dyeing cotton fabric in presence and absence of mordants ($FeSO_4$ and $Al_2(SO_4)_3$). Simultaneous mordanting with $FeSO_4$ was the more effective method for dyeing cotton fabrics for both the extracts. Stability studies showed percent loss in the range from 3% to 12%, with dye being most stable under alkaline conditions. Dyeing was most effective for wool and nylon fabrics as detected on the multifibre strip.

Key words: Antibacterial, Antifungal, Dyeing, Mordant, Prodigiosin

1. INTRODUCTION:

The textile industry is an exponentially growing market, driven solely on consumer appreciation and demand. The industry already has an annual turnover of 1 trillion dollars, with key drivers like China, the European Union, the United States, and India. India is the third-largest textile manufacturing industry and is responsible for more than 6% of the total textile production, globally. An indispensable ally associated with textiles is the dye industry. India with its lower production costs is the second largest producer of dyes (Mathur *et al.*, 2006; Ghally *et al.*, 2014).

A major concern with this blooming textile and dye industry is the extensive environmental damage, due to release of untreated dye effluents in water habitats Most of the textile effluents have very strong and persistent color which deteriorate the aesthetics of water bodies. Apart from noticeable aesthetic changes they hamper light penetration leading to impairment of photosynthesis in aquatic flora. This has a direct impact on aquatic fauna and the human food chain. (Lellis *et al.*, 2019; Wang *et al.*, 2007). Many of the dyes employed by the industry are not only toxic but also may be mutagenic and carcinogenic. Such water if gains contact with

humans by means of agricultural irrigation or drinking purpose can lead to long term after-effects. To add on majority of the dyes are produced from coal tar and petrochemical by products, which raises the toxicity concern **(**Kumar *et al.,* 2021). Currently environmentalists are raising their concerns using effective means, which has led the industry to approach ecofriendly alternatives (McDonagh *et al.,* 1995).

Natural dyes also called as biochromes are a promising alternative for developing green, and safe textile dyeing process. Biochromes are dyes derived from plants, insects, and microorganisms. They exhibit long endurance, charm and beauty as perceived in nature. Biochromes are completely renewable and do not release toxic secondary by-products. In fact, use of biochromes in textile industry can be configured to ancient civilizations of Harappan and Mohenjo-Daro (Saharan and Rani 2005; Gupta, 2019). Currently microbes are considered as an apt option for biochrome production as they grow steadily and consistently on cheap substrates, produce diverse biochromes, and are independent from environmental changes as they can be cultivated in fermenters (Hibbing *et al.,* 2010).

Prodigiosin, is a pink to red biochrome produced as secondary metabolite by several bacteria such as *Serratia marcescens, Pseudomonas magneslorubra, Vibrio psychroerythrous, Serratia rubidaea, Vibrio gazogenes, Alteromonas rubra, Rugamonas rubra* and certain Actinomycetes (Kobayashi and Ichikawa, 1991). Structurally it is pyrrolyl dipyrromethene skeleton containing 4-methoxy, 2-2 bipyyrole ring system. Bacterial prodiginines have been classified as cyclic and linear derivatives. The linear derivatives include prodigiosin and undecylprodigiosin while cyclic derivatives include streptrorubin B, cycloprodigiosin and cyclononylprodigiosin. (Furstner *et al.,* 2001; Williamson *et al.,* 2006; Mo *et al.,* 2008). Apart from being environmentally safe prodigiosin has functional character like antibacterial, anticancer, and antiprotozoal nature. Over the past decade immunomodulatory activity of prodigiosin has also surfaced. It has shown to reduce polyclonal proliferation of T cells and can be harnessed for treatment of diseases. (Venil *et al.,* 2009).

Currently, textile industry globally employs pink Rhodamines chemically known as 9-(2-Carboxyphenyl)-3,6-bis(diethylamino) xanthylium chloride for brilliant pink coloration. It has been categorized under GHS classification (OSHA HCS) as chronically toxic to aquatic organisms, causing severe eye damage and lethal if swallowed. (Sigma Aldrich, 2014). Once it enters the food chains concerns like liver dysfunction, cancer, acute poisoning, and oxidative damage arise. (Mahdi and Pratama, 2019; Sulistina *et al.,* 2020).

Prodigiosin thus with the same color range can be used to replace pink Rhodamines at a small scale and can be expected to have good acceptance in baby apparels and clothing.

2. MATERIALS AND METHODS:

All chemicals, solvents and bacteriological media used in our study were of analytical and microbiological grade. They were procured from Himedia and Sigma Aldrich, Mumbai. For preparation of media and chemicals distilled water was used. All borosilicate acid and alkali resistant glassware were used during the study.

2.1 Sample Collection:

Mahim creek (19.0467° N, 72.8330° E) acts as a separation boundary for Mumbai city and its suburbs. Subsurface water samples (100ml) were collected from 10 different selected sites of Mahim creek in alcohol sterilized containers. Each site was approximately 1 to 2 meters

distinct from each other. The samples were processed for isolation within 4 hours of collection. Physical parameters like pH, color, odor, and turbidity of samples were recorded. (Sapkale *et al.,* 2019; Singare *et al.,* 2014).

2.2 Isolation

From each sample isolation was done on sterile Nutrient Agar plates (Himedia) using T-streak method. Incubation was done at room temperature for 48hrs to 72hrs. All pigmented colonies were selected and propagated on the same medium to obtain pure cultures. Their morphological appearance (as detected by Gram staining) was recorded. Pure cultures were maintained on Nutrient agar slants and stored at 4°C until used.

2.3 Confirmation of Prodigiosin:

Isolates producing pink to red pigmentation were selected for the test. Each isolate was cultivated on Sterile Nutrient Agar plates. Biomass: acetone at a ratio of 1g:25ml was kept on shaker for 4 hours for pigment extraction. On centrifugation, 2ml of supernatant was dispensed in tubes to which addition of 0.1ml of (0.1N) hydrochloric acid or 0.1ml of (0.1N) sodium hydroxide was done to attain pH in the range of 4 to 9. Retention of pink color under acidic conditions and change to yellow color under alkaline condition is a positive presumptive test for Prodigiosin. (Hariyali & Manisha, 2019).

2.4 Extraction of Prodigiosin:

Isolates with positive presumptive tests were considered for extraction studies. For extract preparation, presumptive isolates were plated on sterile Nutrient Agar plates followed by incubation for 2 days at room temperature (28^0C). Two methods were used for extraction.

For acetone extraction method, prodigiosin was extracted from biomass at ratio of 1:4, followed by solvent-solvent extraction using acetone: petroleum ether in the ratio of 1:3. The petroleum ether layer was collected, dried over water bath and yield was recorded. (Park *et al.,* 2012)

For methanol extraction method, prodigiosin was extracted from biomass at ratio of 2:1 followed by solvent-solvent extraction using, methanol: chloroform: water at ratio of 1:1:1. The chloroform layer was collected, dried over water bath and yield was recorded. (Alihosseini et.al, 2008).

2.5 Determination of Absorption maxima:

Absorption maxima of extracts by both the methods was recorded using a spectrophotometer scan from 400nm to 650nm.

2.6 Biochemical identification of promising isolates:

The promising isolates were subjected to several biochemical tests (table 2) and by referring to Bergey's Manual of Determinative Bacteriology identification of the promising isolates was done.

2.7 Antibacterial activity of Prodigiosin extracts:

Prodigiosin was tested against standard pathogens namely *Pseudomonas aeruginosa* 1688, *Escherichia coli* 1885, and *Staphylococcus aureus* 3160 procured from IMTECH, Chandigarh. To 19ml of molten Mueller and Hinton agar butts, 1ml of different concentrations of extracts prepared in DMSO were added to attain the final concentrations in the range of 0.5ug/ml to 5.0ug/ml at an interval of 0.5ug/ml. The above pathogenic isolates were then streaked across to record signs of growth. DMSO control processed in similar manner was kept as control.

2.8 Antifungal activity of Prodigiosin Extracts:

Prodigiosin was tested against *Aspergillus niger* (laboratory isolated) by media dilution method as described for antibacterial activity. (Sabouraud's agar butts were used instead of Mueller and Hinton's agar butts).

2.9 Prodigiosin as a dyeing agent:

a. Fabric Scouring:

Scouring helps remove the finishing coat, which interferes with dye and fabric interaction. Cotton fabric (1 meter) purchased from local shop was soaked in distilled water overnight followed by treatment with sodium carbonate and liquid soap for 60mins at 95°C. The fabric was then washed under tap water multiple times, dried and then used for dyeing. (Poorniammal *et al.,* 2013)

b. Dyeing (without mordant):

In a stainless-steel container, fabric and pigment extract (acetone) at ratio of 1g:100ml were added. The container was then heated in a water bath at 80^0C for 60 mins. The fabric was removed from the extract, rinsed under cold water and dried. (Ren *et al.,* 2017).

c. Effectiveness of mordants in Fabric dyeing:

Mordants are metal salts which aid in dye fabric interaction and are commonly employed for natural dyes. They can be applied at three different stages, namely, Pre-mordanting: Mordant treatment before dyeing; Simultaneous-Mordanting: Mordant treatment along with dyeing; Post-mordanting: Mordants treatment after the dyeing process. $Al_2(SO_4)_3$ (5%) and $FeSO_4$ (3%) were prepared with distilled water were the mordants used. Conditions employed for mordanting were 60^0C for 30mins (pre and post mordanting). (Yi Ding *et. al.,* 2017; Morales-Oyervides *et al.,* 2017; Chauhan *et al.,* 2015).

Calculation:

Percent Absorbance of dye was determined using the formulae:

$$\frac{\textbf{Absorbance of Test (WM/PM, SM, POM) – Absorbance of control}}{\textbf{Absorbance of Test}} \textbf{ X 100}$$

Where WM stands for without mordant; PM stands for pre mordanting, SM stands for simultaneous mordanting, POM stands for post mordanting.

2.10 Stability Studies

The dyed and mordanted fabrics were evaluated for color fastness to washing and color fastness to acid/alkali and light using ISO 105-C10:2006 and ISO 105-E04 guidelines respectively. (Jahan and Datta, 2015).

2.11 Multi-fabric strip Dyeing:

A multi-fabric strip (MFS) consists of 6 different fabrics namely acetate, cotton, nylon, polyester, acrylic, and wool arranged consecutively. Dye binding capacity varies from one fabric to another thus, using MFS gives us an easy estimate towards six fabrics simultaneously. The most effective dyeing process as determined by preliminary assessment was employed for multi-fabric strip dyeing.

3. RESULTS AND DISCUSSION:

Biochromes produced by bacteria possess enormous efficiency as a dyeing agent in textile industry. Prodigiosin, a pink red tripyrrole pigment synthesized by bacteria, exhibits multifaceted functional properties. The present study focuses on isolation of prodigiosin producing bacteria from marine environment and study its fabric dyeing potential.

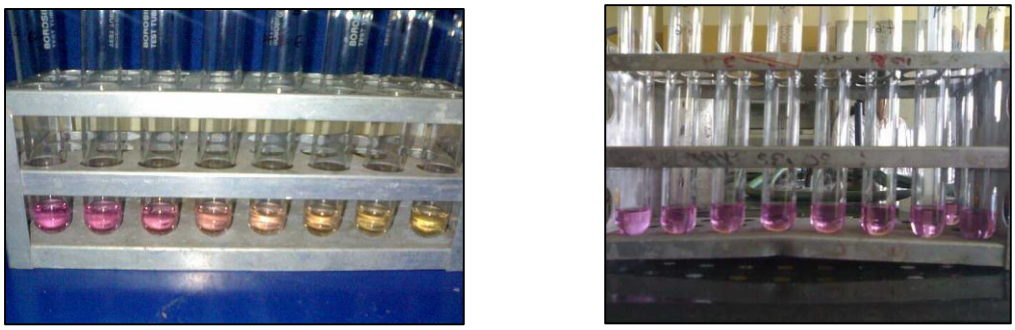
3.1 Isolation for Prodigiosin producing bacteria:

Physical analysis from different sites of Mahim creek led to similar outcomes. The pH was in the range from 7.5 to 8.5. the water samples had blackish colour, foul odour and high turbidity. A large amount of effluent and domestic waste released by adjoining slums could be the

probable reason for undesirable changes in the water body. A total of 38 pigmented isolates were obtained which were labelled from MK1 to MK38. Bacterial isolates namely MK-2, MK-7, MK-12, MK-23, MK-25 and MK-27 produced pink to red pigmentation while the remining were yellow, orange, green and buff colored. Morphologically 47.36% were Gram positive cocci, 31.57% were Gram negative rods, and 21.05% were Gram positive rods.

3.2 Chemical confirmatory test for Prodigiosin:

All the six suspected isolates were processed for chemical confirmatory test for which two isolates namely MK 12 and MK 23 were positive. MK 2, MK 25, and MK 27 did not show colour change in presence of alkali, while MK 7 showed reduced pigmentation on subculturing. Thus, MK12 and MK23 were considered for further studies.



(a) (b)

Figure 1: (a) Results for MK 23 and (b) for MK2

3.3 Extraction of Prodigiosin:

MK12 and MK23 biomass was collected for prodigiosin extraction using acetone and methanol method. The pigment yield obtained were as follows:

Table 1: Pigment yield using Acetone and Methanol extraction methods

Isolate No	Acetone Extraction	Methanol Extraction
MK 12	30.2ug/10gm biomass	23.6 ug/10gm biomass
MK 23	50.4 ug/10mg biomass	47.3 ug/10gm biomass

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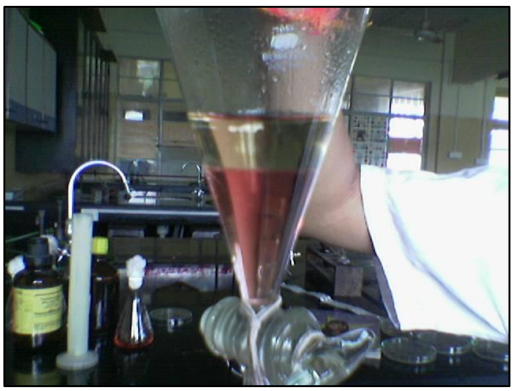


Figure 2: Extraction of Prodigiosin

As seen in table 1, prodigiosin yield for both the isolates by acetone extraction was marginally higher. Park *et al.* (2013) reported acetone as an efficient solvent for prodigiosin extraction. Shahitha & Poornima, (2012) used acetone extraction procedure to extract prodigiosin from *S. marcescens* using different substrates.

3.4 Absorption maxima Studies: The absorption maxima for both the extracts was 530nm.

3.5 Biochemical Identification:

Promising isolates had the following morphological and biochemical characters.

Table 2: Morphological and biochemical characteristics of *Serratia marcescens* and *Serratia rubidaea*

	Standard results for *Serratia rubidaea*	Observed results of the bacterial isolates (MK 12)	Standard results for *Serratia marcescens.*	Observed results of the bacterial isolate (MK 23)
Colony colour	Mild pink	Mild pink	Red	Red
Cell shape	Rod	Rod	Rod	Rod
Gram Nature	Gram negative	Gram negative	Gram negative	Gram negative
Catalase	+	+	+	+
Oxidase	-	-	-	-
Glucose	+	+	+	+
Sucrose	+	+	-	-
Xylose	+	+	-	-
Lysine Decarboxylase	-	-	+	+
Nitrate reduction	+	+	+	+
Urease	-	-	-	-
Indole	-	-	+	+
Methyl red	-	-	-	-
Vogues Proskauer	-	-	-	-
Citrate	+	+	+	+

3.6 Antibacterial Activity

Table 3: Antibacterial activity against standard pathogens

Sr. No.	**Culture**	**MIC for MK 12**	**MIC for MK 23**
1.	*Pseudomonas aeruginosa* 1688	**3.0 ug/ml**	**3.0ug/ml**
2.	*Escherichia coli* 1885	**4.0ug/ml**	**3.0ug/ml**
3.	*Staphylococcus aureus* 3160	**2.0ug/ml**	**2.0ug/ml**

As seen in the table 3, antimicrobial activity of prodigiosin extracts from MK 23 and MK12 did not differ significantly. Prodigiosin from MK 23 was slightly more effective against *Escherichia coli* 1885, as compared to MK 12. Ji and Kim *et al.,* (2019) reported antibacterial activity of prodigiosin in the range of 3ug/ml to 30mg/ml against several intestinal tract pathogens. Suryawanshi *et al.,* (2017) reported prodigiosin treated bacterial cells show 30% higher membrane leakage than normal cells. This leakage leads to excessive loss of solutes, metabolites and ions eventually leading to bacterial cell death.

3.7 Antifungal Activity

Fig 3: Antifungal activity of prodigiosin

Prodigiosin extract from MK 23 and MK 12 both demonstrated antifungal activity against *Aspergillus niger* with an MIC of 4ug/ml. Parani and Saha, (2008) reported antifungal activity of prodigiosin against phytopathogens. Someya *et al.,* (2001) reported synergistic effect of prodigiosin with chitinolytic enzymes against fungal phytopathogens.

3.8: Prodigiosin as textile colourant

Biosynthesis of colorants for textile applications has attracted increased interest in recent years. Nature produces many biochromes from various resources like plants, animals, and micro-organisms, which are possible alternatives to synthetic dyes and pigments. (Shahitha S, *et al.*, 2012). In the present study cotton fabrics were dyed using pigment extracts from isolates MK12 and MK 23, in presence and absence of mordants.

Figure 4: Fabric dyeing capability of prodigiosin

3.9: Mordants in dyeing:

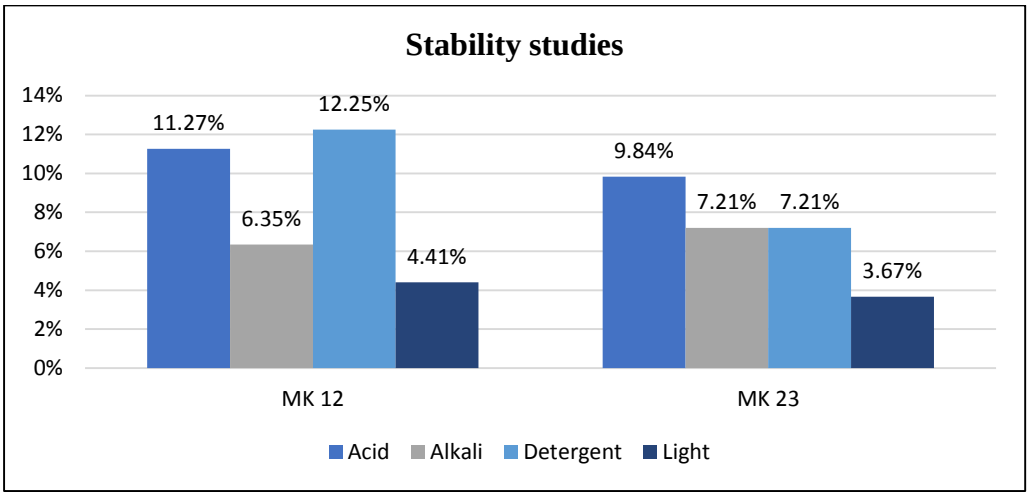
Table 4: Percent absorption in presence and absence of mordants

3% $FeSO_4$				
Extract	Without Mordant	Pre-Mordanting	Simultaneous Mordanting	Post-Mordanting
MK 12	40.33±0.01	52.31±0.93	56.36±0.69	49.93±1.07
MK 23	58.63±0.83	66.53±1.73	72.41±0.83	58.05±0.77
5% $Al_2(SO_4)_3$				
Extract	Without Mordant	Pre-Mordanting	Simult	Post
MK 12	40.33±0.01	42.74±1.08	50.96±1.07	43.27±1.47
MK 23	58.63±0.83	61.40±1.76	63.63±0.70	58.43±0.76

As seen in table (4) the dyeing capacity of MK 23 prodigiosin is approximately 10% higher than that of MK 12, for both the mordants used and across different stages of mordanting. Mordanting has contributed effectively by increasing the interaction between the dye and the fabric resulting in an approximate percent increase of 5% to 30%. The order for effective stage of mordanting is Simultaneous>Pre mordanting> Post Mordanting > Without mordant. $FeSO_4$ (3%) is more effective mordant than 5% $Al_2(SO_4)_3$ this may be due to transitional properties of Fe. As a transition metal Fe has an empty orbital which can participate in effective back-bonding enabling and enhancing the photostability of dyes (Raisanen *et al.*, 2001).

3.10 Stability studies:

For stability studies, cotton fabric was dyed using $FeSO_4$ (3%) as mordant and simultaneous mordanting method. It was subjected to treatment with acid, alkali, detergent, and light. The results obtained for each treatment are shown in the graph below.



Graph 1: Stability studies of dyed fabrics

As seen in graph 1, the highest loss in dye was recorded on exposure to detergent in case of MK 12 and to acid in case of MK 23. For most of the treatments the losses were in the range of 3% to 12% which can be considered as non-substantial when compared to percent color absorbed during the dyeing process.

3.11 Multi-fabric strip Dyeing:

Figure 5 : Fabric dyeing

The sequence of fabrics, beginning from extreme top is wool, acrylic, polyester, nylon, cotton, and acetate. As seen from the figure, good binding capacity of prodigiosin can be seen to most of the fabrics with highest for wool and nylon. Alihosseini *et al.,* (2008) reported use of prodigiosin from *Vibrio* spp. in dyeing multifabric strip. They observed prodigiosin was more effective in dyeing nylon, wool, and acrylic. Wool and nylon have amide groups which can interact with dyes to enhance dyeing potential.

4. CONCLUSION:

The study demonstrated a successful isolation of prodigiosin producing organisms from marine environment. The prodigiosin producers were identified as *Serratia rubidaea* and *Serratia marcescens*. Extracts from both the isolates demonstrated antibacterial and antifungal activity. Simultaneous dyeing with mordant $FeSO_4$ was found to be most effective dyeing process. The presence of anti-bacterial and anti-fungal, functional character multiples the aesthetic value of prodigiosin as an alternative to pink rhodamines. Thus natural dyes like prodigiosin are environmentally friendly, biodegradable and give a soft soothing hue compared to toxic counterparts.

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