

MARINE BACTERIA AS SOURCE OF PIGMENT FOR APPLICATION AS DYE IN TEXTILE INDUSTRY

Jissa G. Krishna, Soorej M. Basheer, P. S. Beena & M. Chandrasekaran

Microbial Technology Laboratory, Department of Biotechnology,
Cochin University of Science and Technology, Cochin 682 022.

E-mail: chansek10@gmail.com

ABSTRACT : The textile industry is one amongst the rapidly growing industries world wide, which utilizes enormous amounts of synthetic dyes. Consequently, the effluent from these textile industries poses serious threat to the environment which is often very difficult to treat and dispose. This has become a very grave problem in environment conservation and hence natural pigments have drawn the attention of industry as safe alternative. In this context, in the present study an attempt was made to bioprospect marine bacteria towards isolation of a suitable and ideal pigment that could be used as a natural dye. A marine *Serratia* sp. BTWJ8 was recognized to synthesize enormous amounts of a prodigiosin-like pigment. The pigment was isolated and characterized for various properties. The pigment was evaluated for application as a dye in the textile industry. Results of the studies indicated that this pigment could be used as a natural dye for imparting red-yellow colour to various grades of textile materials. The colour was observed to be stable after wash performance studies.

Key words: Textile dyes, *Serratia* sp. BTWJ8, Prodigiosin.

INTRODUCTION

The textile industry is one among the rapidly growing industries world wide. In India it accounts for 14 % of the total industrial production, and contributes to nearly 30 % of the total exports. Textile industry utilizes enormous amounts of synthetic dyes and consequently the textile effluent, which is often very difficult to treat and dispose, poses serious threat to the environment and has become a very grave problem in environment conservation. Hence natural pigments have drawn the attention of industry as safe alternative. Among the natural sources of colourants, microorganisms offer great scope and hope. The ease of cultivation, extraction, the genetic diversity in microbes and sophistication of technology has made their choice more feasible (Juailova *et al.*, 1997). Among the different organisms bacteria, yeast, algae, fungi, and actinomycetes appear more efficient and attractive sources of biocolourants. The microorganisms such as *Monascus*, *Rhodotorula*, *Bacillus*, *Achromobacter*, *Yarrowia* and *Phaffia* produce a large number of pigments. Commonly found microbial pigments are carotenoids and astaxanthin. Carotenoids are yellow, orange and red pigments, which are widely distributed in nature. They are utilized as food or feed supplements and as antioxidants in pharmaceutical formulations (Miura *et al.*, 1998).

Prodigiosin is a tripyrrole first characterized from *Serratia marcescens*, which forms beautiful pillar box red colonies. Its name is derived from "prodigious" - something marvellous. The prodigiosin tripyrrole was shown to be localized in extracellular and cell-associated vesicles and in intracellular granules (Kobayashi and Ichikawa, 1991). Secondary metabolites related to prodigiosins are produced by a wide variety of bacteria including *Serratia marcescens*, *Serratia rubidaea*, *Vibrio gazogenes*, *Vibrio psychroerythrus*, *Pseudomonas magnesorubra*, *Alteromonas rubra*, *Rugamonas rubra*, *Streptomyces longisporus*, *Streptomyces spectabilis* and *Streptoverticillium rubrireticuli* (Variyar *et al.*, 2002). Species of *Serratia marcescens* are the major producers of prodigiosin (Furstner, 2003). Secondary metabolites may be located in the cell envelope of microorganisms, as is true of prodigiosin (Purkayastha and Williams, 1960), although prodigiosin is not released into the medium, as is characteristic of many secondary metabolites.

Prodigiosin has several biological activities such as immunomodulatory, antibacterial, antimycotic and antimalarial activities and so on (Lazaro *et al.*, 2002; Pandey *et al.*, 2003). It has been reported that prodigiosin could induce apoptosis in various kinds of cancer cells, such as haematopoietic, colorectal and gastric cancer cells. (Diaz-Ruiz *et al.*, 2001; Montaner and Perez-Tomas, 2001). In the present study an attempt was made to isolate and identify a red pigment-producing bacterium from the water and sediment samples of Kerala coast and also to check the dyeing property of the same to different grade of textile materials.



MATERIALS AND METHODS

Isolation of pigment producing bacteria: Bacterial strains, capable of pigment production were isolated from the water and sediment samples from the Coastal areas of Kerala and the mangrove environments of Cochin using Zobell Marine Broth 2216 (HiMedia). From among 60 chromogenic cultures the isolate **BTWJ8** which recorded considerable amount of red pigmentation on the agar as well as in the broth was selected as potential strain. and identified based on the morphological and biochemical characteristics, as outlined in Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974). In addition molecular ribotyping was also performed in order to confirm the identity. Genomic DNA was isolated according to the method of Sambrook *et al.* (2000). Ribotyping was performed using universal primer pair for 16S rDNA. A portion of the 16S rRNA gene (1.5 kb) was amplified with a PCR from the genomic DNA (Reddy *et al.*, 2002a; 2002b; Shivaji *et al.*, 2000). The sequences of forward (16SF) and reverse (16SR) primers used for amplifying 16S rDNA were as follows:

Sequence	Reference
16SF 5' AGTTTGATCCTGGCTCA 3'	(Shivaji <i>et al.</i> , 2000)
16SR 5' ACGGCTACCTTGT TACGACTT 3'	(Reddy <i>et al.</i> , 2002a; 2002b)

Nucleotide sequences of the PCR amplicon was determined by using the ABI Prism 310 genetic analyzer, using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. The identity of the sequences determined was established by a comparison with the sequences obtained with the gene sequences available in the database using BLAST software (Altschul *et al.*, 1980). Phylogenetic tree was constructed using the neighbour joining method implemented in CLUSTALW. Tree was constructed using nucleotide evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. Tree was visualized using the CLUSTALW N-J tree.

Standardization of procedure for extraction of pigment: Initially the solvent that could support maximal yield of pigment on extraction of culture broth was standardized, using different solvents *viz*; ethanol, acetone, methanol, petroleum ether, ethyl acetate, chloroform, hexane, diethyl ether and distilled water. A preculture was developed in 5 ml of Zobell Marine broth by inoculating the broth with one loopfull of selected bacterial strain and incubating at room temperature for overnight. Fifty ml of freshly prepared Zobell Marine broth taken in 250 ml Erlenmeyer flasks was inoculated with the preculture (Optical density 1.0) as inoculum at 1 % (v/v) level and incubated for 24 h on a rotary shaker at 150 rpm, at room temperature ($28 \pm 2^\circ\text{C}$). Extraction of the pigment was done according to Slater *et al.*, (2003) with slight modification. The standardized extraction procedure is as follows. (i) One ml of the fermentation broth was taken in a microfuge tube and centrifuged at 10,000 rpm for 10 min. at 4°C . (ii) The colourless supernatant was discarded. The coloured cell pellet was resuspended in one ml of solvent. (iii) The microfuge tubes with the suspended cell pellet was then kept in a water bath, at 60°C , for 20 min. and again centrifuged for 10 min. at 4°C at 10,000 rpm (Sigma-laboratory Centrifuge, Germany). The coloured supernatant was then analyzed by scanning in a UV-Visible spectrophotometer (Shimadzu, Japan) for detecting the λ_{max} . The scanning range selected was 400-600 nm. Absorbance at the λ_{max} was measured.

Purification of the pigment: Pigment produced by the bacterium was purified according to Song *et al.* (2006), with some modification. Equal volume of petroleum ether was added to the methanol extract taken in a separatory funnel and mixed well. Equal volume of distilled water and concentrated solution of sodium chloride was then added to the separatory funnel in order to enhance the phase separation. Slowly the pigment got transferred to the epiphase (petroleum ether phase). The hypophase with methanol and water soluble impurities was removed. The petroleum ether phase is washed 4 or 5 times with distilled water to remove residual methanol. The pigment collected from the hypophase was treated with 1 N HCl (9:1; v/v) and concentrated by evaporating the solvent in a 40°C water bath. Pigment was separated by silica gel column chromatography and was eluted with a solvent mixture of n-hexane and ethyl acetate (2:1; v/v) at a flow rate of 1 ml/min. The eluted fraction was then analyzed by scanning in UV-Visible spectrophotometer. The selected scanning range was 400-600 nm.

Structural identification of the pigment: Pigment produced by the *Serratia* sp. was characterized according to Song

et al.(2006), with some modification. The was separated and purified, The chemical structure of the purified pigment was determined by Thin-Layer Chromatography (TLC), Liquid chromatography-Mass spectrometry (LC-MS), Nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infra red spectroscopy (FT-IR), and Fourier transform-Raman spectroscopy (FT-Raman) (Jissa PhD Thesis, 2008).

Application Studies: In the present study, scope for probable application of the bacterial pigment was evaluated for different grades of textile materials commercially available in the market which included 'Cotton', 'Chiffon', 'Poplene', '2 by 2', 'Pure silk', 'Century cotton', 'Dupoil silk', '2 by 1', 'Organdi', 'Polyester', 'Terry cotton' and 'Nylon.' Each material was cut into equal size of 2 cm². Bacterial pigment in methanol (40 µg/L) was used as the stock solution. From this stock solution 100 µl (0.004 µg; w/v), 200 µl (0.008 µg; w/v) and 300 µl (0.012 µg; w/v) was applied to the cloth material in a warm surface and was allowed to dry at room temperature for about 1 h. One set of experiment was done with the application of thiourea as a mordant (Shirata et al., 2000). The dyed cloth materials were dipped in thiourea solution (1 %; w/v) for 30 min. at 70°C. For all the experiments white cloth material were taken as a control.

Wash performance of the textile materials: All the dyed textile materials were washed with soap solution (sunlight 0.7 %; w/v) for 30 min. at room temperature as well as at 40°C. After 30 min. (random selection) the cloths were washed with tap water and allowed to dry at room temperature (28 ± 2°C). Absorbance of the soap solution after washing was measured at 535 nm in a UV-Visible spectrophotometer. Appropriate blank was also used for the experiment. The same procedure was repeated for the dyed textile material treated with thiourea.

RESULTS

Screening and selection of potential strain: The isolate BTWJ8 obtained from Puthuvypin, Kochi showed considerable amount of red pigment production both on the agar medium and in the liquid medium after 6 h of incubation in Zobell Marine medium compared to other isolates which produced red pigment only after 24 h besides recording rapid growth. Hence, the strain **BTWJ8** was selected as the potential strain for pigment production in the present study. The selected strain BTWJ8 was identified as *Serratia* sp. according to the morphological and biochemical characteristics. The partial sequence of the 16S rRNA gene (Fig 1) obtained for *Serratia* sp. BTWJ8 was submitted to Genbank (Accession number EU239958) through BankIt programme, at NCBI site (<http://www.ncbi.nlm.nih.gov/BankIt>). The identity of the *Serratia* sp. BTWJ8 could be confirmed by comparing the sequences with Genbank entries, by BLAST programme (Altschul et al., 1980). The partial gene sequence of 16S rRNA of *Serratia* sp. BTWJ8 showed only 75 % similarity with the other reported 16S rRNA gene sequences of *Serratia* sp. With the sequence data generated for the partial gene sequence of 16S rRNA of *Serratia* sp. BTWJ8, a phylogram was constructed using CLUSTALW N-J programme (Fig. 2).

Standardization of protocol for isolation of pigment: It was noted that this pigment could be assayed at 535 nm where the pigment shows maximum absorption λ_{max} . It was inferred from the results (data not shown) obtained that methanol is an ideal solvent for extracting the maximum of the water insoluble membrane bound pigment.

Structural identification of the pigment: The purified pigment was analysed for its structural identification. A single band with an R_f value of 0.42 was obtained (Fig. 3) after thin-layer chromatography with chloroform: methanol (95:5; v/v). The molecular mass of the sample pigment was 324.2 Da, as shown in Fig. 4, which corresponds to that of prodigiosin (C₂₀H₂₅N₃O). FT-IR absorption in KBr for the red pigment (Fig. 5) was dominated by strong bands at 2924.78 cm⁻¹ and 2853.67 cm⁻¹ (aromatic CH), 1736.21 cm⁻¹ and 1710.44 cm⁻¹ (C=O), 1611.15 cm⁻¹ (aromatic C=C), 1548.24 cm⁻¹ (N-H), 1459.18 cm⁻¹ (C-H), 1264.91 cm⁻¹ (C-N). The spectrum obtained after the FT-Raman analysis (Fig. 6). showed strong bands at 2924.79 cm⁻¹ and 2834.96 cm⁻¹ very similar to that of FT-IR spectrum which indicates the presence of aromatic CH bonds in the pigment molecule. NMR spectral analysis presented a distinct spectrum, which indicates the position of each proton in the sample molecule (Fig. 7). In the spectrum, a chemical shift of the methoxy group in the molecule exhibited δ 4.012 ppm as a single peak. In addition, the chemical shift (in CDCl₃) of NH protons in pyrrole ring was δ 12.72 ppm. All the results were very similar to that of prodigiosin pigment. It is therefore concluded that the identity of the pigment isolated from *Serratia* sp. BTWJ8 is prodigiosin-like pigment.

Application studies: Results presented in Fig. 8 clearly evidence that the pigment produced by *Serratia* sp. BTWJ8 can be effectively used to dye all the textile materials studied. During the wash performance studies with the textile



materials treated with pigment, it was found that the pigment is lost from the cloth after washing in soap solution at room temperature; $28 \pm 2^\circ\text{C}$ (Fig. 9) and also at 40°C (Fig. 10). Whereas, the loss of pigment from the same textile materials treated with mordant was found to be less. So it is inferred that thiourea is an effective mordant for treating the dyed textile materials and it can withstand at hot wash conditions.

DISCUSSION

Serratia sp. occurs in water, soil, plants, insects and vertebrates, and it has various characteristics including the pigment prodigiosin (Hejazi and Falkiner, 1997). *Serratia* sp. BTWJ8, isolated from seawater was identified as a potential strain that produced bright red pigment. The identity of the bacterium was further confirmed by ribotyping using partial 16S rRNA gene. From the phylogram it was inferred that this bacterium is closely related to *S. marcescens* (76%), *S. rubidea* (75%), *S. fonticola* (75%), *S. proteamaculens* (75%).

The pigment produced by *Serratia* sp. BTWJ8 is water insoluble and methanol was found to be an ideal solvent for the maximal extraction of the pigment among the different solvents studied. The results obtained for spectrophotometric and chromatographic analysis indicate that the pigment produced by the strain is prodigiosin or a close derivative. It has been reported that certain strains belonging to genus *Serratia* as well as other genera of marine bacteria produce prodigiosin, red antibiotic pigment (Cang *et al.*, 2000) which is insoluble and accumulates in the cells (Allen *et al.*, 1983).

Serratia sp. is reported to produce cell associated red colour pigment prodigiosin (Carbonell *et al.*, 2000; Singleton and Sainsbury, 2001). Microscopic observation of *S. marcescens* colonies showed that prodigiosin pigment was localized in vesicles (extracellular and cell associated) or as intracellular granules (Matsuyama *et al.*, 1986).

In the present study, the pigment could be completely transferred to the solvent after incubation at 60°C in a water bath testifying that the pigment produced by *Serratia* sp. BTWJ8 is membrane bound. Prodigiosin was reported to display a characteristic absorption spectrum in ethanol, with a maximum at 534 nm (Slater *et al.*, 2003) and single peak absorbance at 535 nm (Giri *et al.*, 2004, Song *et al.*, 2006). Montaner *et al.* (2000), extracted prodigiosin by shaking the *S. marcescens* 2170 cells with a mixture of methanol/1N HCl in the ratio 24:1. Pigment produced by *Serratia* sp. BTWJ8 recorded maximum absorption at 535 nm suggesting that this pigment is prodigiosin.

Purification and characterization of the pigment: Pigments from *S. marcescens* were purified by extraction with acetone followed by transfer to petroleum ether and the petroleum ether extract was evaporated *in vacuo* at 30 to 40°C in order to obtain dry pigment. A single red coloured band was obtained after column chromatography using silica gel column and the same was eluted with n-hexane and ethyl acetate (2:1; v/v) and concentrated by evaporation (Williams *et al.*, 1960). In the present study, the red pigment from the *Serratia* sp. BTWJ8 was purified by extraction with methanol followed by transfer to petroleum ether and dry pigment was obtained by evaporation of the solvent at 40°C . A single band with an R_f value of 0.42 was obtained after thin-layer chromatography with chloroform: methanol (95:5; v/v) solvent system. Song *et al.*, (2006) reported single red prodigiosin band with R_f value 0.43. The molecular mass of the pigment produced by *Serratia* sp. BTWJ8 was 324.2 Da, which corresponds to that of the molecular mass of prodigiosin ($\text{C}_{20}\text{H}_{25}\text{N}_3\text{O}$). Similar result was reported earlier for *Serratia* sp. (Giri *et al.*, 2004; Song *et al.*, 2006).

In the present study, the data obtained for the spectroscopic analyses of the red pigment with LC-MS, FT-IR, FT-Raman and $^1\text{H-NMR}$ very clearly testify that the pigment produced by *Serratia* sp. BTWJ8 is prodigiosin-like pigment.

Application studies: Synthetic dyes are extensively used as a dye in textile, rubber, plastic and paper industries. Now-a-days people are concerned about harmful effect of using synthetic dye and going for natural dye in spite of synthetic dye. The effluent released from the dyeing of the synthetic dyes are toxic and cause environmental pollution and harmful to health. The discharge of these waste residues into the environment eventually poison, damage or affect one or more species in the environment, with resultant changes in the ecological balance. There are several attempts being made to evolve ideal processes for safe and effective disposal of dye effluent from industries that use dyestuff. The harmful effects of synthetic dye and chemicals used at the time of dyeing have forced us to concern about the alternative preparation of dye using natural sources. With concern for environment protection becoming so important, there is a challenge to evolve environment friendly technologies and yet be competitive on a global level.

So in this study an attempt was made to explore the probable use of natural pigment produced by *Serratia* sp. BTWJ8 for dyeing purpose in textiles, rubber, paper and plastic.

Results obtained in the present study strongly evidence that the pigment produced by *Serratia* sp. BTWJ8 has the dyeing property and could be used to dye different grades of textile materials. Further, the wash performance studies with the textile materials treated with pigment and thiourea, which is generally considered as a safe and effective mordant, suggest that there is ample scope for using this pigment as a dye in textile industry. In an earlier study the blue pigment from *Janthinobacterium lividum* was used to dye natural fibers and the colour shade depending on the material. Dyeing was performed by a simple procedure consisting of either dipping in the pigment extract or boiling with the bacterial cells. It was found that when the dyed material was subjected to post-treatment with thiourea solution, the fading of the bluish-purple colour to light was considerably reduced (Shirata *et al.*, 2000).

Whereas, this is the first time, to the best of our knowledge that the pigment was evaluated as a dyeing agent for use in textile industry and could record satisfactory performance. Being a natural pigment it is definitely harmless and would be ecofriendly.

**“TATCCGACTCTCCCTTGCCCTGCGGTGCTCGCCATGATGGGATCGACGCTCATA
CGGTGCGATGGCTATGGGTAACAAGGGTGCCTTCGGGTGTCCCGCTTCTCGATATGG
GCATGATGATTTGCTAGCTGCTGACGTTGCGCGGTTTATGAGAGGCTGGCTACCTGT
CTTACCCACATGATCTGCTTCAGTGGAACTTTAAGTGTTC AATAGTGCCTAGGGGAG
GACGC AGCGTTGAATATTGCACCCTGGCAACACCCTGATGCACCCGCCTCAGAGCTC
CCAAACGTCAGGGCAATCTCATCCGTTTTACAGAAACGCAAAAAATAAGTTTGGCATA
TTGCTGTTTGAATATAAATAGTACCGCAGGGGGCCTCCCCCCTTTTTGTTTGAGTGGAGC
TAGAACGTAATCGGACGGGAGGACAAAAACAATGTGGCGGCGCG”**

Fig. 1: The partial nucleotide sequence (452 bp) of the amplicon of 16S rRNA gene obtained from *Serratia* sp. BTWJ8 (GenBank Accession No: EU239958)

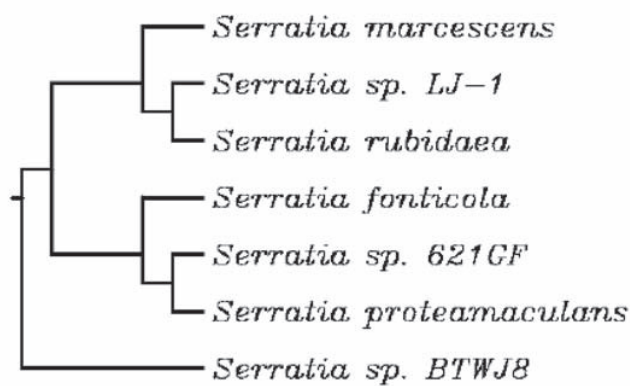


Fig. 2: Phylogram of *Serratia* sp. BTWJ8

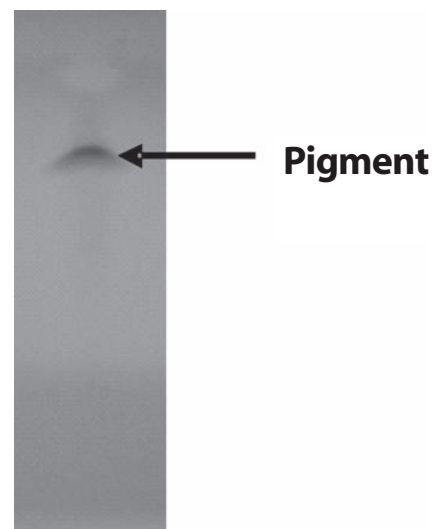


Fig. 3: Thin layer chromatogram of the pigment isolated from *Serratia* sp. BTWJ8

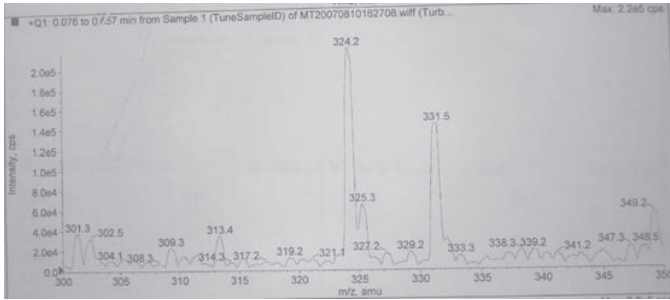


Fig. 4: Liquid chromatography-Mass spectrum of the red pigment isolated from *Serratia sp. BTWJ8*

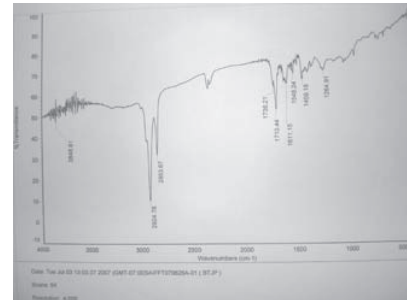


Fig. 5: FT-IR spectrum of the red pigment isolated from *Serratia sp. BTWJ8*

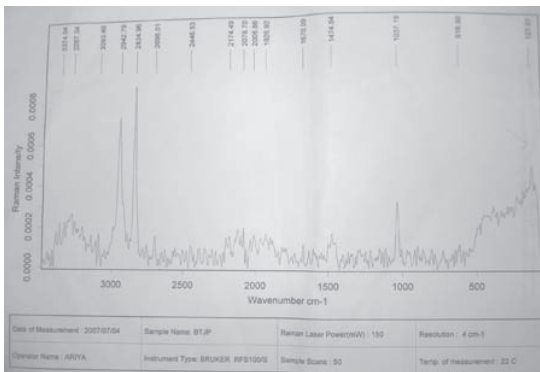


Fig. 6: FT-Raman spectrum of the red pigment isolated from *Serratia sp. BTWJ8*

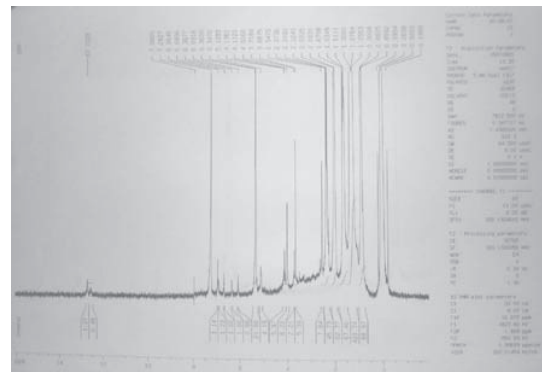


Fig. 7: NMR spectrum of the red pigment isolated from *Serratia sp. BTWJ8*

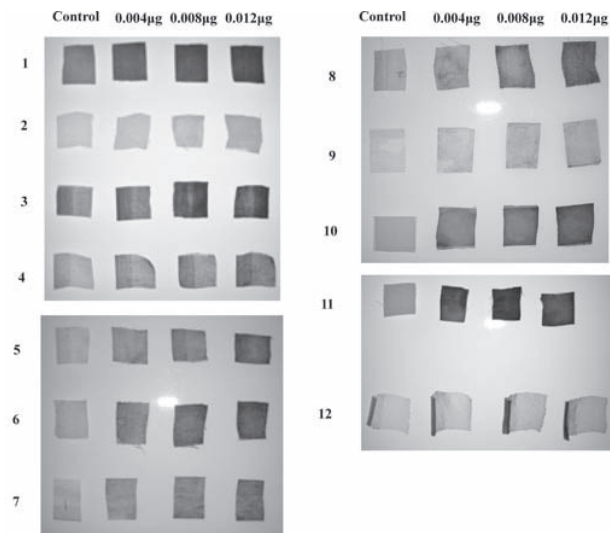


Fig. 8: Cloth materials dyed with prodigiosin pigment: 100 μ l (0.004 μ g), 200 μ l (0.008 μ g) and 300 μ l (0.012 μ g) of pigment were applied in a warm surface to the cloth material from a stock solution of 40 μ g/L. The cloth material was allowed to dry at room temperature for about 1 h.

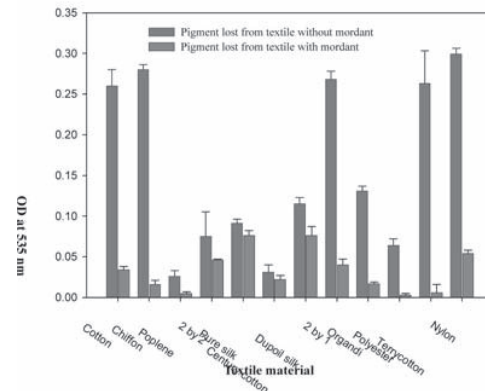


Fig. 9: Wash performance of textile materials in soap solution (room temperature; $28 \pm 2^\circ\text{C}$): Dyed textile materials were washed with soap solution (sunlight 0.7 %; w/v) for 30 min. Absorbance of the soap solution after washing was measured at 535 nm.

1- Cotton	5- Pure silk	9- Organdi
2- Chiffon	6- Century cotton	10- Polyester
3- Poplene	7- Dupoil silk	11- Terrycotton
4- 2 by 2	8- 2 by 1	12- Nylon

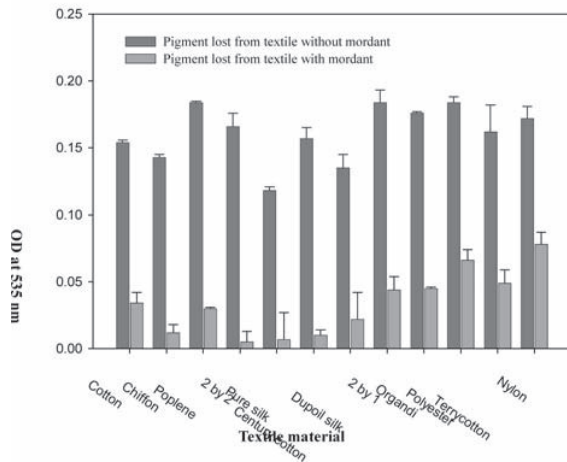


Fig. 10: Wash performance of textile materials in soap solution (at 40°C): Dyed textile materials were washed with soap solution (sunlight 0.7 %; w/v) for 30 min. Absorbance of the soap solution after washing was measured at 535 nm

Acknowledgement: One of the authors, Jissa G. Krishna is grateful to Kerala State Council for Science Technology and Environment (KSCSTE) for the fellowship.

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