Fibers and Polymers 2015, Vol.16, No.4, 802-808
DOI 10.1007/s12221-015-0802-6

ISSN 1229-9197 (print version)
ISSN 1875-0052 (electronic version)

Optimization of Prodigiosin-type Biochrome Production and Effect of Mordants on Textile Dyeing to Improve Dye Fastness

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(Received October 15, 2013; Revised November 12, 2014; Accepted December 28, 2014)

Abstract: The environmental toxicity concerns raised by the synthetic origin pigments have lead to escalating interest towards natural pigments. In the present study, prodigiosin type biochrome- a member of prodiginine dye family was obtained from indegenously isolated bacterial strain *Serratia* sp. KH-1. Cultural and physiological parameters for production of pigment along with its application on dyeing fabrics have been studied. The identity of prodigiosin type biochrome was confirmed by fourier transform infrared (FT-IR) spectroscopy, mass spectroscopy and nuclear magnetic resonance (NMR). The detailed dyeing ability of this biochrome was evaluated using cotton and wool fabrics. The fabric showed the maximum dye uptake (*K/S*) at of 50 °C for 50 min duration, and 4.3 g/*l* dye concentration. Pre-mordanting by sodium chloride proved to be effective on increasing *K/S* values of the dyed fabrics under the optimum dyeing conditions. Fastness ratings to washing and light showed acceptable fastness for both cotton and wool fabrics. Hence, the present study represents the application and detailed investigation of prodigiosin type biochrome for textile dyeing using mordants. This study will thus, be helpful for designing the dyeing protocol for cotton and wool fabrics using eco-friendly pigment.

Keywords: *Serratia* sp., Prodigiosin, Biochrome, Dyeing, Mordanting

Introduction

In the present scenario, management of the water pollution problem is one of the major concerns of scientific proceedings. Textile industrial effluents contribute significantly to water pollution due to the unchecked release of these effluents in the open water bodies. Majority of the dyes used in textile coloration technology comprises of a wide range of synthetic dyes which are recalcitrant in nature. Moreover, due to inefficiency of the dyeing process, about 10 % of the dyes are lost in the environment [1]. Removal of these recalcitrant dyes from the effluent is of the essence owing to their mutagenic property, carcinogenicity and intense coloration [2]. Owing to these limitations and hazards of synthetic dyes, the use of natural dyes in textile coloration technology has gained research momentum due to their higher bio-degradability, ecological safety and high compatibility with the environment [3-7].

Among the microbially produced pigment, the chrimson biochrome, prodigiosin has gained scientists' attention due to its ease of production and extraction as well as its wider scopes of applications [8]. Prodigiosin is a linear tripyrrole and a typical secondary metabolite, synthesized in the later stages of bacterial growth [9]. Moreover, prodigiosin is of great interest due to its tremendous medicinal properties [10]. While much work has been done to assess the effect of antifungal, anti-cancer, immunosuppressive and anti-proliferative activity of prodigiosins and prodigiosin like pigments [11,12] reports are lacking on detailed studies of dyeing of fabrics using such type of pigments [13-16].

Synthetic dyes used in textile industries pose a considerable health and environmental hazard. This necessitates the need for amendment in the textile coloration technology by using natural origin pigments to replace the existing toxic, mutagenic and teratogenic dyes. Prodigiosin can be used for textile dyeing but study of improved dyeing with prodigiosin is still unfinished.

Therefore the present work is focused on the optimization of nutritional parameters for production of chrimson biochrome from *Serratia* sp. KH-1 along with its structural analysis and detailed evaluation of its dyeing properties using mordants as dyeing enhancers on cotton and wool fabrics.

Experimental

Culture Media and Micro-organism

All chemicals used in the study were of analytical grade. Bushnell and Haas Broth, agar agar powder and yeast extract were purchased from Hi Media (Mumbai, India). All chemicals and enzymes used in molecular biology were procured from New England Bioloabs (NEB) GmbH, Germany. For mordanting studies, aluminum ammonium sulfate, cupric sulfate, ferric sulfate, tannic acid and sodium chloride were procured from Sisco Research Laboratory (SRL, Mumbai, India). The red pigmented organism designated as KH-1 was isolated from an oil contaminated site near Vidyanagar city, Gujarat by serial dilution technique on Bushnell and Haas Medium supplemented with groundnut oil (0.5 % v/v) and yeast extract (0.5 % w/v). The isolate KH-1 was routinely grown at 30 °C in Bushnell and Haas broth with groundnut

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oil (0.5 % v/v) and yeast extract (0.5% w/v) for 24 h at 150 rpm. Ten % of this was used as inoculum unless specified.

Identification of Strain KH-1 by 16S rRNA Gene Sequencing

The isolate KH-1 was grown in liquid culture, centrifuged at 6000 g for 15 min and the pellet obtained was subjected to genomic DNA extraction using the phenol-chloroform method as described by Ausubel *et al.* [17]. This genomic DNA was used as template in PCR reaction and purified PCR products were sequenced using internal overlapping primers [18]. Sequence was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov/) using BLAST (blastn) tool and corresponding sequences were downloaded. Similarly matrix was prepared using Dnadist program in PHYLIP analysis package using Jukes Cantor corrections. Phylogenetic tree was constructed by neighbour-joining method using the MEGA package. The gene sequence was submitted to NCBI with accession number JQ966939.

Production, Extraction and Characterization of Crimson Biochrome

Ten percent (v/v) of the grown culture of the isolate KH-1 was inoculated in 100 m*l* of BHM broth supplemented with 0.5 % (v/v) groundnut oil and yeast extract (0.5 % w/v) and incubated at 30 °C for 24 hours. The cell mass was harvested by centrifugation at 6000 g for 10 minutes. The collected cell mass was suspended into 40 m*l* of methanol and subsequently filtered. The cell mass was washed repeatedly till methanol extract became colourless. The coloured filtrates were then combined and the extracted biochrome was dried over anhydrous Na_2SO_4.

The extracted biochrome was characterized based on its color, absorption maxima, fourier transform infrared spectroscopy (NICOLET 6700, Thermo Scientific, USA), mass spectroscopy (MS) and nuclear magnetic resonance (Bruker, USA). FTIR analysis was done in the mid IR region of 400-4000 per cm.

Evaluation of Effect of Concentration of Media Components (oil and yeast extract) and Inoculum Size on Biochrome Production Through Response Surface Methodology

Response Surface Methodology (RSM) was used to evaluate the individual as well as the combined effect of media components like groundnut oil concentration, yeast extract concentration and inoculum size on pigment production using the central composite design (CCD). According to this design the total number of experimental combinations was 2^k+2k+n_0, where k is the number of independent variables and n_0 is number of repetition of experiments at the center point. The 'Design-Expert' version 8.0, State-Ease Inc., Minneapolis, USA was used for experimental design, regression and graphical analysis of the data obtained [19]. Groundnut oil concentration, yeast extract concentration and inoculum size were studied at five different concentrations.

A set of 20 experiments was performed. The minimum and maximum ranges of variables were used and the full experiment design with respect to their values was generated. The data obtained from RSM on biochrome production was subjected to the analysis of variance (ANOVA) and the results of RSM were used to fit a second order polynomial equation (1).

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_1\beta_2AB+\beta_1\beta_3AC+\beta_2\beta_3BC+\beta_1\beta_1A^2+\beta_2\beta_2B^2+\beta_3\beta_3C^2 \quad (1)$$

where Y is response variable (dependent variable), β_0 is intercept (constant), β_1, β_2, β_3 are linear coefficients, $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_2\beta_3$ are interaction coefficients, $\beta_1\beta_1$, $\beta_2\beta_2$, $\beta_3\beta_3$ are squared coefficients and A, B, C, AB, AC, BC, A^2, B^2 and C^2 are concentrations of independent variables.

Evaluation of Dyeing Efficiency of Prodigiosin on Cotton and Wool Fabrics

In order to evaluate use of extracted biochrome in dyeing efficiency, two different fabric materials were used for dyeing purpose. The cotton and wool fabrics were dyed under optimized conditions of biochrome concentration (4 g/*l*), dyeing temperature (50 °C) & duration (50 min) to achieve maximum dye uptake (Figure 2(a), (b) supplementary material). The liquor ratio was set fixed to 1:100. Mordanting was done in 3 % (o.w.f.) aqueous solution of each mordant (aluminium ammonium sulfate, cupric sulfate, ferrous sulfate, tannic acid and sodium chloride) with a liquor ratio of 1:200 at 40 °C for 20 min via both the pre-mordanting and post mordanting methods. After pre-mordanting, excess liquor was removed from the fabric and then transferred into the dyeing bath. A total of three pieces of cotton and wool fabric were dyed under each dyeing condition as well as mordanting and their mean value of *K/S* for each condition was obtained to optimize dyeing condition. The fabric was rinsed with cold water after dyeing and air-dried at ambient temperature.

Dye Uptake

Dye absorbed on the fiber was estimated by measuring the light reflectance of the dyed fabric using a spectrophotometer. The *K/S* value as used for color strength was calculated to find out the dye uptake of each dyed fabric at a maximum absorption wavelength of 535 nm from the reflectance values using the Kubelka- Munk equation as follows;

$$K/S = (1 - R_{\lambda max})^2/2R_{\lambda max}$$

where K is the coefficient of absorption; S is the coefficient of scattering; $R_{\lambda max}$ is the reflectance value of the fabric at peak wavelength.

Fastness Properties

The dyed samples were tested for their fastness according to the ISO Standard and AATCC methods. The specific tests

includes color fastness to washing, ISO 105-CO2 (1989) and color fastness to light (AATCC 117).

Results and Discussion

Identification of KH-1 Culture

The DNA sequencing and BLAST analysis of 16S rRNA gene sequence and phylogenetic analysis of the isolate KH-1 showed maximum sequence identity (99 %) with the complete sequence of *Serratia marcesens* 16S rRNA gene (Figure 1), and hence in the present study it is referred as *Serratia* sp. KH-1.

Structural Analysis of Biochrome Produced by *Serratia* sp. KH-1

UV Visible spectra of the extracted biochrome (Figure 2(a))

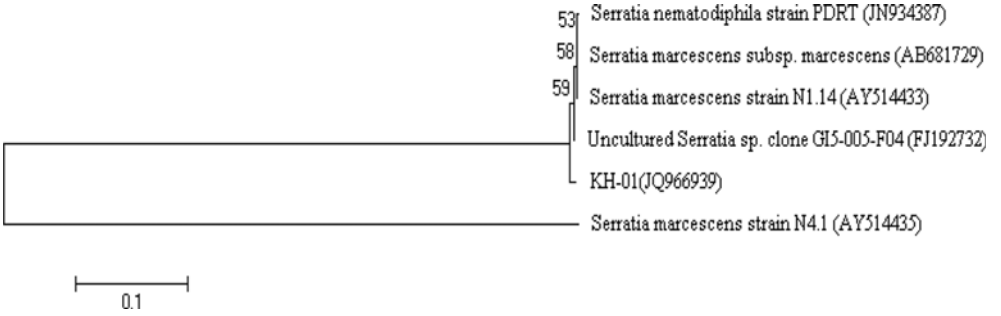

Figure 1. Phylogenetic tree derived from 16S rRNA gene sequence of isolate KH-1. and sequences of closest phylogenetic neighbors obtained by NCBI BLAST (n) analysis. The NJ-tree was constructed using neighbor joining algorithm with Kimura 2 parameter distances in MEGA 5.0 software. The numbers in the parentheses indicate the NCBI accession numbers and the nodal numbers indicate the bootstrap value.

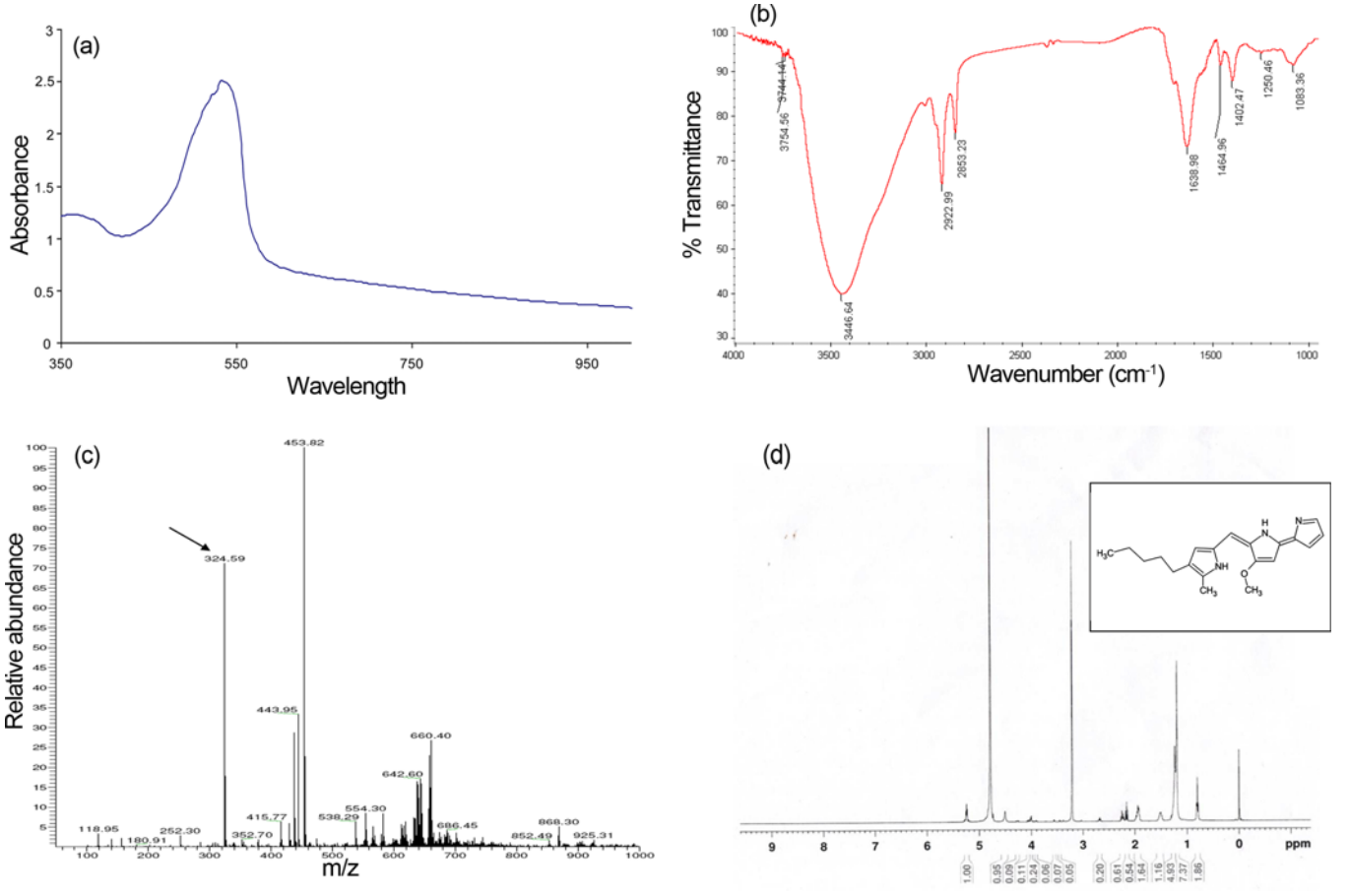

Figure 2. (a) UV-visible scan of the biochrome, (b) fourier Transform InfraRed spectrum of chrimson biochrome, (c) mass Spectrum of chrimson biochrome, and (d) nuclear magnetic resonance spectrum of chrimson biochrome.

showed a sharp peak at 535 nm. Based on the color and absorption maxima, the biochrome was found to belong to tripyrrol class of biochromes [20].

The FTIR spectral analysis of the pink biochrome (Figure 2(b)) was dominated by broad peak at 3446.64 cm^{-1} indicated the presence of -NH of primary amines. Very strong bands at 2922.99 cm^{-1} indicated the presence of methyl groups (aromatic -CH_3) in the molecule and at 1638.98 cm^{-1} indicated the presence of C=N of pyrolenine group. The fingerprint region for the red biochrome was characterized by medium intensity bands at 1464, 1379 and 1083 indicating the presence of aromatic C=C, C-O group of ester bond and -C-H bond of aliphatic amine. The above information is consistent with the published literatures of prodigiosin structure indicating the red pigment to be of prodigiosin type [21].

The molecular mass of the chrimson pigment was 324 Da as m/z 324.59 (Figure 2(c)) which is in line with the one reported for prodigiosin pigment ($C_{20}H_{25}N_3O$). An indication of the structure of the pigment was attempted through use of NMR spectroscopy (Figure 2(d)). ^1H NMR (400 MHz, MeOD-d6) depicted peak shifts at the following δppm : - 0.83 (s, 3H, terminal methyl), 0.85 (s, 3H, terminal methyl), 1.31 (m, 2H, H9"), 1.59 (m, 2H, H8"), 2.44 (t, 2H, H7"), 2.58 (s, 3H, H6"), 3.51 (s, 3H, -oCH_3), 4.04 (s, 3H, -oCH_3), 5.1 (d, 2H, olefinic protons) implying the pigment to be of prodigiosin type (Figure 2(d)-inset). However, a detailed ^{13}C NMR is required to elucidate exact structure of the extracted pigment.

Optimization of Biochrome Production by Various Parameters

The effect of different concentrations of groundnut oil, yeast extract and initial inoculum size on biochrome production was evaluated using response surface method. From analysis of variance (ANOVA) (Table 1) it was established that only inoculum size was significant (p= 0.0034) for pigment production. Analysis of variance (ANOVA) shows significance of experiment with *F*-test= 3.62 (ρ=0.0417).

The contour plot in Figure 3(a) exhibits the behavior of pigment production (g/*l*) with respect to changes in the initial inoculum size and oil concentration in the selected range, while yeast extract concentration was constant at 0.1 %. Higher pigment production was achieved at higher inoculum size where as increasing in oil concentration has no effect of production. The contour plot in figure 3b describes pigment production (g/*l*) with respect to changes in the initial inoculum size and yeast extract concentration in the selected range, while oil concentration was kept constant (0.1 %). Concentration of yeast extract was ineffective for pigment production whereas increased in inoculum size drastically increased pigment production. Effect of the initial oil and yeast extract concentration on pigment production (g/*l*) is displayed in Figure 3(c), with inoculum size kept constant at 20 %. Both are equally effective for pigment production at lower concentrations.

Dyeing Performance

Chemically synthesized dyes have been proved to be potentially toxic and also carcinogenic to humans. Therefore an attempt was made to dye cotton and wool fabrics with microbial pigment. The microbial pigment was found capable of dyeing these fabrics though to a different extent (Figure 4) with respect to k/s value. This value was high for cotton and wool. Alihosseini *et al.* [13] reported k/s value for wool fabric only 3.43. According to the Kubelka-Munk theory, *K/S* is directly proportional to the color strength (content) of a solid surface. Thus prodiogiosin produced in present study

Table 1. ANOVA for response surface quadratic model

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Block	6.166667	2	3.083333			
Model	68.93641	9	7.659601	3.626507	0.0417	significant
A-Inoculum size	35.73309	1	35.73309	16.91815	0.0034	
B-Yeast extract	1.863961	1	1.863961	0.882509	0.3750	
C-Oil concentration	10.54416	1	10.54416	4.992225	0.0559	
AB	8	1	8	3.787672	0.0875	
AC	8	1	8	3.787672	0.0875	
BC	0	1	0	0	1.0000	
A^2	1.478539	1	1.478539	0.700028	0.4271	
B^2	3.55991	1	3.55991	1.685471	0.2304	
C^2	0.015798	1	0.015798	0.00748	0.9332	
Residual	16.89692	8	2.112116			
Lack of fit	11.39692	5	2.279385	1.243301	0.4571	not significant

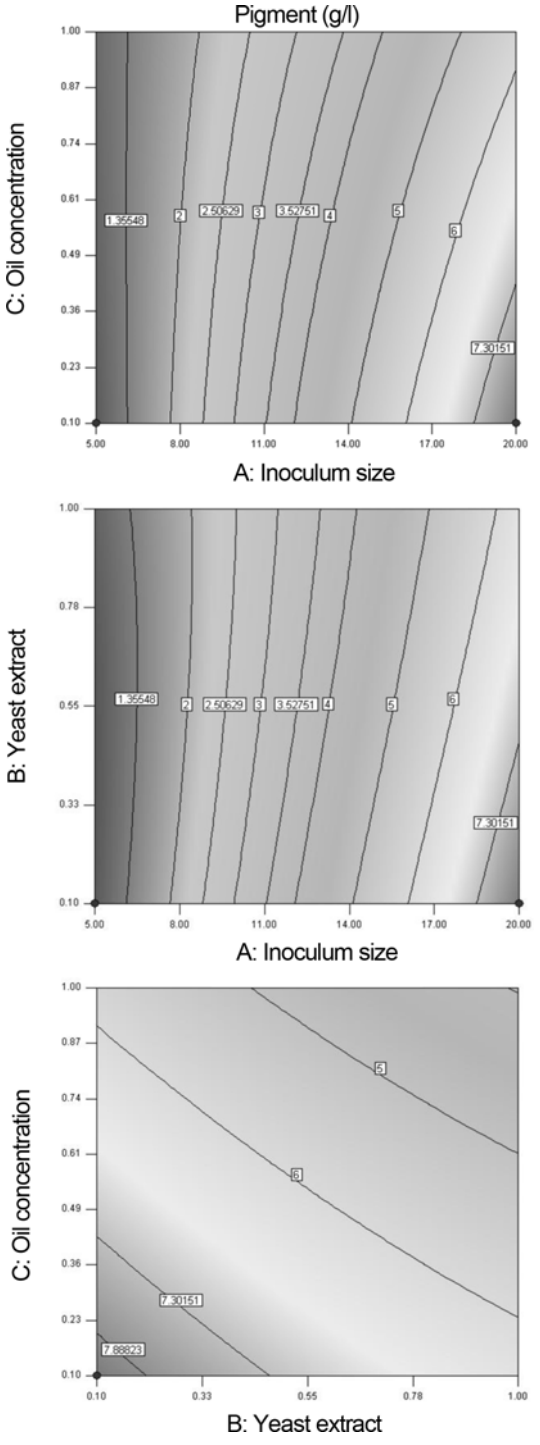

Figure 3. Countour plot showing interaction effect of (a) inoculum size and oil, (b) inoculum size and yeast extract, and (c) oil and yeast extract.

binds more strongly as compared to the reported prodiogiosin by Alihosseini *et al.* [13]. Uniform and deep dyeing was obtained for only wool fabrics. The fabrics were dyed pinkish by the microbial biochrome (Figure 2-supplementary reading).

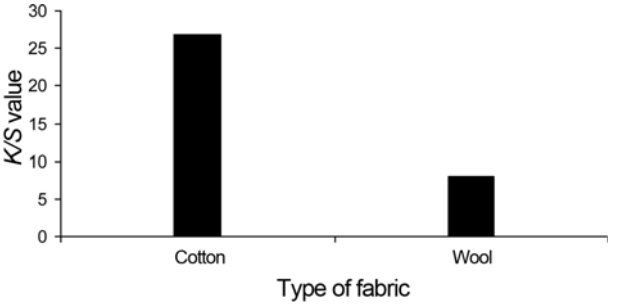

Figure 4. Dyeing performance of biochrome on cotton and wool fabrics.

A similar finding is reported by Vaidyanathan *et al.* [16] and Alihosseini *et al.* [13].

After the dyeing, majority of the prodigiosin-dyed natural or synthetic fabrics have low durability. Alihosseini *et al.* [13] practiced a good dyeing of various fabrics but failed to report their fastness. Gulani *et al.* [14] reported instability of dyed textile in a simple washing with hot, cold water and with detergent. Various attempts to unravel these problems and to form the stable complex between the dye and the fiber have focused on the use of metallic salts as mordants. Therefore, in this study pre-mordanting and post-mordanting during the dyeing process was carried out and evaluated with *K/S* value.

All of fabrics with pre mordant and post mordant treatments were of original pinkish shade which was of the same hue to that of control and untreated ones. Fabrics treated with cupric sulfate and ferrous sulphate were rendered discoloured and of different shade. This means that mordanting by aluminum, tannic acid and sodium chloride had no effect hue of the prodigiosin-dyed cotton and wool fabric (data not shown). Moreover, none of the fabric treatment with mordant, except with tannic acid and sodium chloride, showed a significant improvement in the dye uptake by the wool fabric (Figure 5(a), (b)) or by the cotton fabric (Figure 5(c), (d)). Pre-mordanting with sodium chloride gave significant increase in the dye uptake by both cotton and wool fabrics. Therefore, the present dyeing technique eliminates the use of harmful metallic salts as mordants in a normal textile dyeing process.

Evaluation of Fastness Properties

Dye fastness is related to the washing and light for prodigiosin dyed wool and cotton fabric without mordanting are depicted in Figure 6(a) and (b) respectively. As for washing fastness, color change rates were very good (*K/S* 18.1 for cotton and 5.9 for wool). For light fastness, color change rate was 13.4 *K/S* for cotton and 4.4 for wool. So both cotton and wool fabrics failed to get good rates as for its shade changes by light. All studies reported till date reports fading of colour with exposure to light [15,22]. Thus, considering that natural materials usually display very low

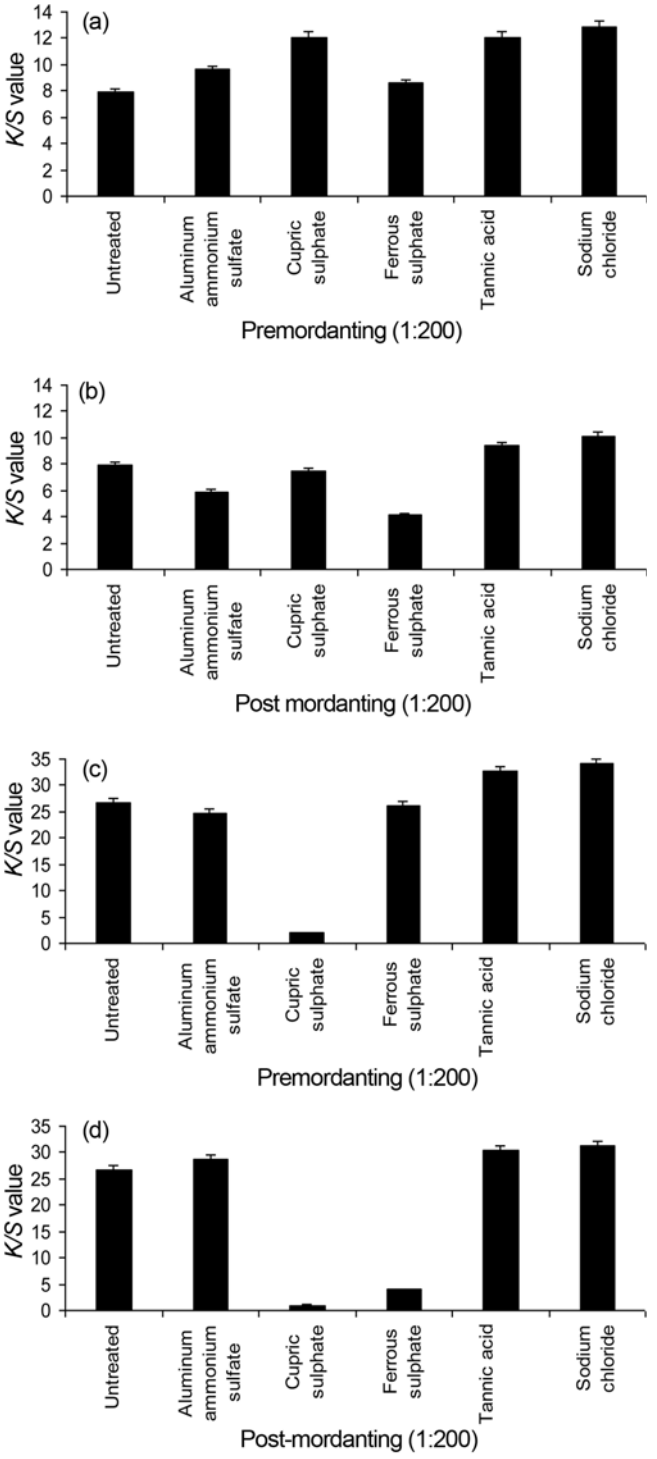

Figure 5. (a) Pre-mordanting on wool fabrics, (b) post-mordanting on wool fabrics, (c) pre-mordanting on cotton fabrics, and (d) post-mordanting on cotton fabrics.

substantively towards fibers, it can be thought that prodigiosin type pigment provide acceptable fastness to washing and light when it was dyed on cotton and wool fabrics. However,

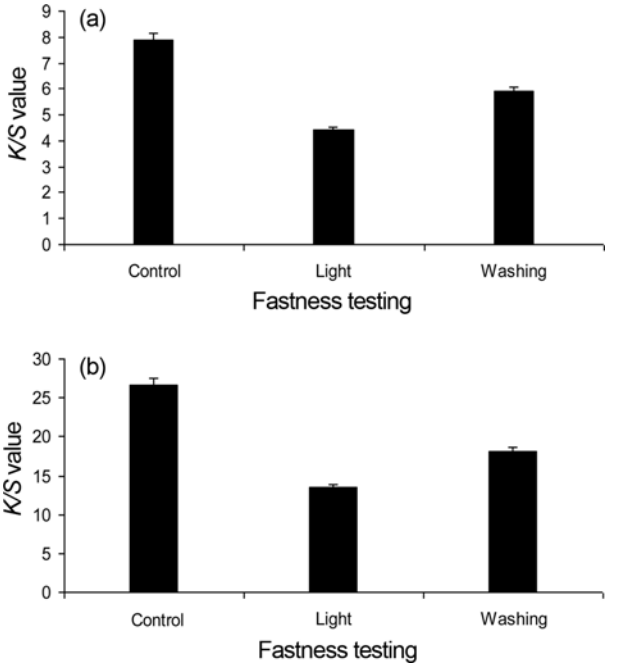

Figure 6. (a) Fastness testing of dyed wool fabric and (b) fastness testing of dyed cotton fabric.

efforts are required to improve dye fastness to light of the prodigiosin dyed fabrics.

In conclusion, through the present study we report the production of prodigiosin type pigment by newly isolated *Serratia* sp. KH-1. The biochrome was subjected to structural analysis and was identified to be of the type of prodigiosin pigment. Further, the pigment was applied to cotton and wool fabrics for dyeing purpose. The dyeing conditions were optimized to obtain maximum dye uptake by the fabric. In the case of the mordant-dyed fabrics, either by pre- or post mordanting, all of dyed fabrics showed lower *K/S* values than that of un-mordanted one, except for sodium chloride, which implies mordanting with metallic salts on the chrimson biochrome dyed fabrics was not effective on improving affinity of the dyes with cotton or wool fibers. This eliminates the use of harmful metallic salts and introduces more eco-friendly compound – sodium chloride for improving the dyeing efficiency of the process. Fastness ratings to washing were very good for both cotton and wool fabrics. However, light fastness needed to be improved in the future. Owing to acceptable dye uptake of cotton and wool fabrics as well as good fastness properties suggests that this pigment can prove a promising candidate for textile dyeing technology in near future.

Electronic Supplementary Material (ESM) The online version of this article (doi: 10.1007/s12221-015-0802-6) contains supplementary material, which is available to authorized users.

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