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# Assessment of process parameters influencing the enhanced production of prodigiosin from *Serratia marcescens* and evaluation of its antimicrobial, antioxidant and dyeing potentials

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## ABSTRACT

**Aims:** Prodigiosin is a bright red pigment produced by certain strains of *Serratia marcescens*, characterized by a common pyrrolylpyrromethane skeleton. This pigment is found to possess antibacterial, antifungal, immunosuppressive and antiproliferative activity. The present study aimed at designing process parameters for the enhanced production of this pigment.
**Methodology and Results:** Peptone glycerol broth was selected as the best synthetic medium. The effects of various media components and process parameters like carbon and nitrogen sources, temperature, pH, incubation period and other supplements were investigated. Maximal amount of prodigiosin was produced at temperature 25 °C, pH 7.0 and incubation period of 48 h. Supplementation of media with maltose and peptone yielded maximal amount of prodigiosin. Incorporation of minimal amount of supplements like silica gel, iron salts, inorganic phosphate also showed promising results. Chromatographic separations suggested that prodigiosin is made up of three different fractions (purple, orange and red). Further investigation of antimicrobial properties of prodigiosin revealed that it is a potent inhibitor against gram positive bacteria like *Staphylococcus aureus* and *Bacillus cereus* and fungal pathogens like *Candida albicans, C. parapsilosis* and *Cryptococcus* sp. This antimicrobial potency remained stable under a wide range of temperature and pH. The antioxidant capacity of prodigiosin was found to be 22.05 µg ascorbic acid equivalents/ml of extract. When applied to textiles, prodigiosin resisted the action of acid, alkali and detergent.
**Conclusion, Significance and Impact of study:** Besides combating gram positive bacterial pathogens and some pathogenic yeasts, prodigiosin with strong dyeing and antioxidant activity may find broad applications in textile and therapeutic industries.

*Keywords:* *Serratia marcescens,* antimicrobial, antioxidant, biopigment, prodigiosin

## INTRODUCTION

Natural products either synthesized or secreted by organisms represent one of the critical sources of potential medicinal use. One of these natural products is the small molecular weight compounds secreted by organisms having no demonstrable function in the cells and known as secondary metabolite. They have a major effect on the health, nutrition and economics of our society. There are several organisms which can produce pigments, which are one of the important classes of these secondary metabolites and are often referred to as biopigments. These biopigments can be obtained from two major sources, plants (Papageorgiou *et al*., 1979) and microorganisms (Cho *et al*., 2002). Biopigments from the micro-organisms have been preferred over those from plants because of their stability (Raisainen *et al*., 2002) and the availability of their cultivation technology (Kim *et al*., 1999; Parekh *et al*., 2000) throughout the year. On the other hand, biopigments from plants have numerous drawbacks like instability against light, heat or adverse pH, low water solubility and often non-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 pyrrolylpyrromethane skeleton, having low molecular weight (323.4 Dalton), appearing only in the late stages of bacterial growth. Prodigiosin ($C_{20}H_{25}N_3O$) is produced by many strains of the bacterium *Serratia marcescens*, and other gram negative bacteria and some other unrelated microbial strains, such as *Vibrio psychroerythrus*, *Streptomyces griseoviridis,* and *Hahella chejuensis* (Hubbard and Rimington, 1950), where it has been shown to be associated with extracellular vesicles or present in intracellular granules (Kobayashi and Ichikawa, 1991; Matsuyama *et al*., 1986). It has been discovered that prodigiosin possesses antibacterial, antifungal, antiprotozoal (Croft *et al*., 2002), cytotoxic (Nakashima *et al*., 2005) antitumor (Perez-Tomas *et al*., 2003), antimalarial, antidiabetic, nonsteroidal and anti-inflammatory properties. In light of its potential commercial

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values, there is a demand to develop high throughput and cost effective bioprocesses for prodigiosin production.

The present investigation focuses on the optimization of cultural parameters to achieve the enhanced production of prodigiosin from *S. marcescens*, followed by its chromatographic analysis. Exploration of the antimicrobial, antioxidant and dyeing properties of prodigiosin were also important criteria of the study. To our knowledge this is the first report on the effect of various temperatures and pH on the antimicrobial potency of the pigment.

## MATERIALS AND METHODS

### Isolation and identification of *S. marcescens*

For isolation of *S. marcescens*, swab sample was collected from a toilet water pipe, serially diluted and plated on nutrient agar and incubated at 37 °C for 24 h. Following incubation, red coloured colonies were selected and propagated on the same medium until pure cultures were obtained. Pigmentation of the colonies and Gram's staining results followed by standard biochemical characterization confirmed that the colonies were of *S. marcescens*. Pure culture was maintained on nutrient agar slants in duplicates and stored at 4 °C until used.

### Selection of the media for the maximal production of prodigiosin by *S. marcescens*

In order to determine the media supporting the maximal production of prodigiosin, *S. marcescens* was grown in different media (nutrient broth, peptone glycerol broth, gelatin broth, LB broth, tryptone yeast extract broth, yeast extract malt extract broth, glycerol beef extract broth and tryptone soya broth) at 37 °C for 24 h.

### Confirmation and quantification of prodigiosin

Bacterial cell absorbance in the broths was measured at 620 nm, following which the broth suspensions were subjected to centrifugation at 5000 x g for 15 min to collect the cell pellet. 10 mL of 95% methanol was added to the cell pellet and centrifuged under the same condition. Debris was removed and the 2mL of the supernatant was taken in two test tubes. The content of one of the test tube was acidified with a drop of concentrated HCl and the other alkalinized with a drop of concentrated ammonia solution. A red or pink colour in the acidified solution and a yellow or tan colour in the alkaline solution indicated a positive, presumptive test for prodigiosin (Gerber and Lechevalier, 1976). 5 mL of the supernatant was subjected to spectrum scanning in the range of 300 to 700 nm using a SANYO Gallenkamp UV-VIS spectrophotometer (UK). 95% methanol was used as a blank. Methanolic extract of prodigiosin showed characteristic maxima at 499 nm. Extracted prodigiosin was estimated using the following equation (Mekhael and Yousif, 2009).

Prodigiosin unit/cell = $([OD_{499} – (1.381 \times OD_{620})]) \times 1000/OD_{620}$

Where, $OD_{499}$ – pigment absorbance
$OD_{620}$ – bacterial cell absorbance
1.381 – constant

### Optimization of the parameters for maximal production of prodigiosin

Media optimization was done to standardize the conditions favouring the maximal production of prodigiosin by *S. marcescens*. The selected media which yielded the maximum amount of prodigiosin was varied with different carbon sources (1% w/v: sucrose, fructose, lactose, mannitol, glucose and maltose), nitrogen sources (1% w/v: tryptone, ammonium chloride, ammonium sulphate, urea, ammonium oxalate, ammonium nitrate, ammonium acetate, yeast extract and peptone).

Effect of different concentrations of inorganic salts like NaCl (0.25, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 %), $FeCl_3$ (1, 2, 3, 4, and 5 µg/mL of media) and $K_2HPO_4$ (0.1, 0.3, 0.5, 1 and 10 mM) were examined. The effect of silica gel was being studied by amending its various concentrations into the media (2, 4, 6, 8 and 10 mg silica gel/mL of media).

In order to determine the effect of physical parameters on the maximal production of prodigiosin, *S. marcescens* was grown at various pH (5, 6, 7, 8, 9 and 10), temperature (20, 25, 30, 35 and 40 °C).

### Separation of prodigiosin fractions by Thin Layer Chromatography

The CFS was put in a separating funnel and double the volume of petroleum ether was added. The separating funnel was shaken vigorously for 10-15 min allowing the two liquids to separate. Prodigiosin was extracted in the petroleum ether layer which was removed carefully from the separating funnel. This petroleum ether layer was poured in a petri dish and kept at 30 °C to 40 °C in order to evaporate the solvent completely, following which 2 mL of 95% methanol was added. The methanolic extract of prodigiosin was stored in a sterile container.

Methanolic extract of prodigiosin was separated by the solvent system containing methanol, ethyl acetate and chloroform in the ratio of 6: 3: 1 (v/v). 10 µL of methanolic extract of prodigiosin was loaded on to the silica gel slides and run against the solvent till the solvent front reaches $2/3^{rd}$ of the slide. After the development of the chromatograms, slides were removed and dried. The retardation factor (Rf) values of the chromatogram were calculated (Lynch *et al*., 1968).

### Evaluation of in vitro antimicrobial activity of prodigiosin

The antimicrobial activities of prodigiosin were studied on Mueller Hinton agar by the disc diffusion technique against clinical isolates of *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa,*

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*Candida albicans*, *C. parapsilosis* and *Cryptococcus* sp. maintained in the Department of Microbiology, Genohelix Biolabs, Bangalore.

Sterile filter paper discs (6 mm) were individually impregnated with 50 µL of methanolic extract of prodigiosin. 95 % methanol was taken as control. All the discs were dried and placed on the surface of the test bacterial and fungal lawn. Following 18 to 24 h of incubation at 37 °C the plates were examined for the zones of inhibition.

**Effect of temperature treatment on inhibitory activity of prodigiosin**

Methanolic extracts of prodigiosin were exposed to different temperatures (30, 50 and 80 ºC for 30 min and at 121 ºC for 15 min, 15 lbs pressure). A control was maintained by incubating methanolic extract at 37 ºC. Antimicrobial activity was performed as mentioned earlier.

**Effect of pH on inhibitory activity of prodigiosin**

The sensitivity of prodigiosin to different pH was estimated by adjusting the pH of the methanolic extracts for 30 min to pH 2, 4, 6, 8, 10, and 12 by using 1 *N* HCl or 1 N NaOH. A control was maintained by adjusting the methanolic extract of prodigiosin to neutral pH. Antimicrobial activity was performed.

**Total antioxidant capacity**

The total antioxidant capacity of the methanolic extract was evaluated by phosphomolybdenum method (Prieto *et al*., 1999). 0.1 mL of the extract solution was mixed with 1 mL reagent solution (6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min. The absorbance of the solution was spectrophotometrically measured at 695 nm using a blank. The antioxidant capacity of the extract was evaluated as equivalents ascorbic of acid (µg AE/mL extract).

**Effectiveness as dyeing agent**

1 $cm^2$ of each fabric (wool, silk, nylon, cotton and polyester) was soaked in 2 mL methanolic extract of prodigiosin taken in different test tubes and incubated for 48 h at room temperature, following which each fabric was dried and cut into 5 smaller pieces. These smaller pieces were then treated with acid, alkali, cold water, cold water and detergent and hot water and detergent for 1 h in respective test tubes.

## RESULTS AND DISCUSSION

Biopigments produced by bacteria possess enormous efficiency as medicinally important products. Prodigiosin, a red pigment synthesized by *S. marcescens*, belongs to the family of tripyrrole and exhibits antimicrobial, immunomodulating and anti-tumor properties. The present investigation focused on formulation of a production medium for effective prodigiosin production and separation of the pigment followed by its antimicrobial activity evaluation.

**Selection of synthetic media**

Peptone glycerol broth was found to be suitable medium for prodigiosin production (763.55 prodigiosin unit/cell) by *S. marcescens* (Figure 1). This result is in perfect agreement with the previous results where enhanced pigment production was observed in the glycerol broth at 30 °C over nutrient broth at 28 °C (Giri *et al.,* 2004). Hejazi and Falkiner, 1997 also reported the highest increase in biomass and maximal pigmentation in cultures grown on glycerol medium.

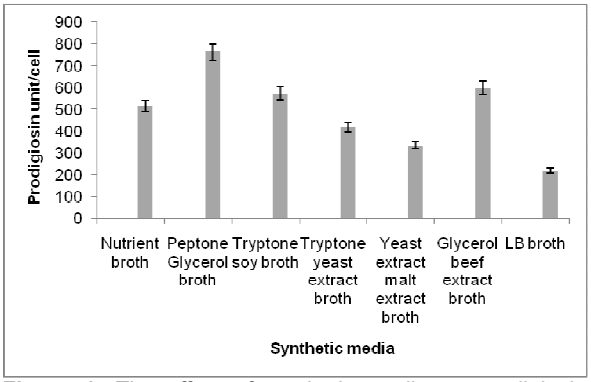


**Figure 1:** The effect of synthetic media on prodigiosin production

**Effect of carbon sources**

Different sugars when added to the peptone glycerol medium, the maximum amount of pigment production was observed in the presence of maltose (784.15 prodigiosin unit/cell), followed by moderate level of pigment production in medium amended with lactose, fructose and sucrose and the least being in the medium amended with mannitol (Figure 2). A similar result was observed, wherein a new strain designated as *S. marcescens* NY1 yielded 425±40 mg/L of prodigiosin which is maximum of all sugars tested, when maltose was amended in the media. Moderate level of pigment production was observed in medium amended with lactose and sucrose (Sundaramoorthy *et al.,* 2009).The role of glucose in pigment production is critical. Our study revealed that glucose when incorporated in the media resulted in decreasing the pigment production which is in accordance the previous investigations (Oller, 2005; Clements-Jewery, 1976).

**Effect of nitrogen sources**

Study regarding the effect of nitrogen source on the prodigiosin production, revealed that *Serratia marcescens* proliferated and produced maximum pigment (793.48

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prodigiosin unit/cell) in the presence of peptone (Figure 3), whereas, pigmentation was delayed in media amended with urea and ammonium oxalate. The organism failed to grow in the media supplemented with ammonium chloride, ammonium sulphate, ammonium nitrate and ammonium acetate, possibly indicating the toxicity of ammonium salts towards the organism. Previously, *S. marcescens* grown on mineral media did not produce pigment when the carbon source was glucose or the nitrogen source was ammonium chloride (Hejazi and Falkiner, 1997).

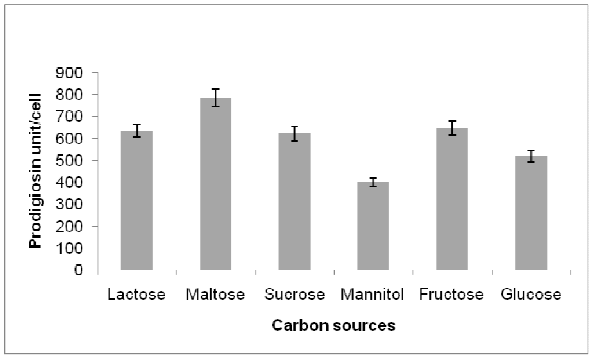


**Figure 2:** The effect of carbon sources on prodigiosin production

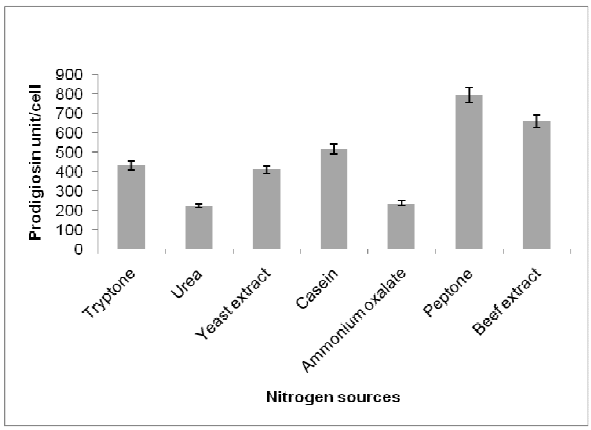


**Figure 3:** The effect of nitrogen sources on prodigiosin production

**Effect of inorganic salts**

0.25 % of NaCl supported the maximal pigment production and there was a decline in the production with the increase in the % of NaCl (being the minimal at 4.0% of NaCl). This result was at par with that obtained by Silverman and Munoz, 1973, who studied the effect of NaCl in two strains of *S. marcescens*- wild-type 264 and wild-type Nima and observed that both strains showed decrease in the prodigiosin synthesis per unit of cell mass at higher NaCl concentrations, often reaching complete inhibition at salt concentrations varying between 0.5 to 3.0 % NaCl in strain 264 and 2.5 to 3.0 % NaCl in strain Nima. Moreover, in the present study, high % of NaCl (4 %) favoured the growth of the white mutants of the bacteria, inhibiting the production of prodigiosin completely.

Iron salt, inorganic phosphate and silica gel enhanced the pigment production when amended in the medium in minimal amount. This result is in contradiction with an earlier study, where it was found that 8 mg/mL of silica gel when amended in the liquid peptone glycerol media yielded the maximal amount of prodigiosin (Yamashita *et al.*, 2001). A high elevation of pigment formation was obtained in the media amended with 0.1mM of inorganic phosphate. As the concentration of inorganic phosphate increases, the ability of *S. marcescens* to produce the prodigiosin decreases. A high elevation in pigment formation at less than or equal to 0.3 mM Pi by *S. marcescens* supports our study (Witney *et al.*, 1977).

**Effect of pH on pigment production**

For the selected isolate of *Serratia,* the maximum prodigiosin yield (811.88 prodigiosin unit/cell) was observed at pH of 7.0 (Figure 4). pH of the media plays a very crucial role in the synthesis of secondary metabolites and therefore affects the biosynthesis of prodigiosin. Maximum amount of prodigiosin by a new strain designated as *S. marcescens* NY1 was produced at pH 7.0 (Sundaramoorthy *et al.*, 2009). Moreover, studies revealed that the inhibitory effect of carbon sources on prodigiosin production may be due to a lowering of the pH of the medium (Sole *et al.*, 1997). This suggests the importance of pH in the media since its altered value can either increase or decrease the amount of prodigiosin.

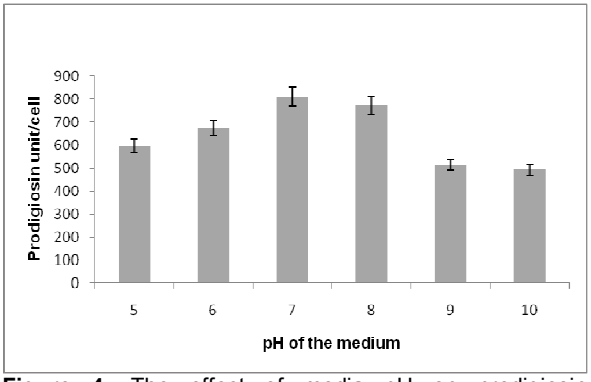


**Figure 4:** The effect of media pH on prodigiosin production

**Effect of temperature on pigment production**

In a synthetic media comprising of maltose, peptone, NaCl and glycerol, the maximal prodigiosin (709.29 prodigiosin unit/cell) was obtained at a temperature of 25 °C (Figure 5). A survey on the role of the temperature and incubation time on the pigment synthesis implies that these are important physical factors which decide the prodigiosin production depending on the type of media. Biosynthesis

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of prodigiosenes (prodigiosin and prodigiosin like pigments) by *S. marcescens* occurred over a relatively narrow range of temperatures with maximal production being between 24 and 28 ºC, although the bacteria grow over a broad range of temperature (Williams, 1973). However, peptone glycerol broth gave the maximum amount of prodigiosin at 30 ºC (Giri *et al*., 2004).

In the present study, a complete block in the prodigiosin production was observed when *S. marcescens* was incubated at 35 °C or above characterized by the growth of white mutants of *Serratia* which did not synthesize prodigiosin. Reduction in the pigment production at elevated temperatures is well documented (Sundaramoorthy *et al*., 2009; Pryce and Terry, 2000).

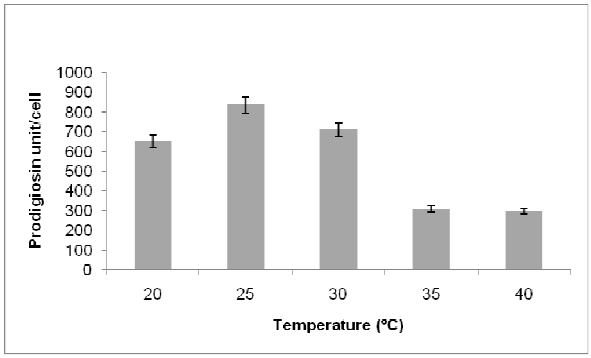


**Figure 5:** The effect of temperature on prodigiosin production

**Thin Layer Chromatography**

Three different fractions, characterized by purple, red and orange bands were obtained with Rf of 0.27, 0.64 and 0.82 respectively, when the methanolic extract of prodigiosin was run against a mixture of methanol: ethyl acetate: chloroform in the ratio of 6:3:1 (v/v), as indicated in Table 1. It was observed that purple fraction was the first to be separated followed by orange and red fractions. The orange component was found to be unstable and got merged with the red component very rapidly soon after the separation as suggested in previous report (Bunting, 1940; Weiss, 1949).

Similar results as that of the present study were obtained by Williams *et al.* (1956), who developed the circular chromatogram in which acetone-extracted prodigiosin was separated into at least four bands- blue, red, red and orange at Rf 0.18, 0.48, 0.70 and 0.89. The orange band at Rf 0.89 has only an evanescent orange colour which rapidly turns red when exposed to air. A blue band at Rf 0.18 is consistently present. The presence of this blue component was suggested in previous reports (Bunting, 1940; Weiss, 1949) since they had observed a slow-moving purple band in their chromatograms of prodigiosin. The purple band was probably the same blue component reported here, but admixed with red fractions.

**Table 1:** Rf values of different fraction of methanolic extract of prodigiosin

| Fractions of methanolic extract of prodigiosin | Rf value |
|---|---|
| Purple | 0.217 |
| Red | 0.64 |
| Orange | 0.82 |

**In vitro antimicrobial potency**

By the use of disc-agar diffusion technique, it was observed that the prodigiosin was able to inhibit majority of the test bacteria. As indicated in Table 2, the inhibitory zones for bacteria varied between 10.5±0.47 mm and 17.5±0.47 mm, whereas, fungicidal activity was evident from the clear zones of inhibition observed against *C. parapsilosis* and *Cryptococcus* sp. Fungistatic activity was observed against *C. albicans*. Prodigiosin possesses antibacterial activity against gram positive bacteria like *Staphylococcus* species and not against gram negative bacteria like *E.coli* and *Pseudomonas* species. The fungal pathogens being sensitive to this potent antimicrobial agent were the *C. parapsilosis*, *Cryptococcus* sp. and to a lesser extent *C. albicans* (Khanafari *et al*., 2006).

**Table 2:** Antimicrobial activity of prodigiosin

| Pathogens | Zone of inhibition (mm) |
|---|---|
| *S. aureus* | 17.5±0.47 |
| *B. cereus* | 10.5±0.47 |
| *E. coli* | – |
| *P. aeruginosa* | – |
| *Cryptococcus* sp. | 11.3±0.47 |
| *C. parapsilosis* | 15.0±0.81 |

**Effect of temperature and pH on antimicrobial activity of prodigiosin**

The biocidal activities of prodigiosin have been already identified *in vitro* conditions; however, there is no report illustrating the stability of the antimicrobial potency of prodigiosin when exposed to different temperature and pH variations. The antimicrobial property of the methanolic extract of prodigiosin was relatively stable during heat treatments at 30, 50, 80 and 121 °C for a given time period. Treatment of the culture supernatants at these temperatures did not show significant difference from the control. Similar to the effect of temperature, the antimicrobial activity of the culture supernatants after treatment at different tested pH was not significantly affected. Prodigiosin showed better antimicrobial activity at the acidic pH than the basic pH. The highest zone of inhibition was at pH 6 (16.0±0.31 mm) and the lowest (9.0±0.24 mm) at pH 2 and pH 12. To the best of our knowledge this study provides the first scientific record regarding the relation between prodigiosin and the stability of its antimicrobial property under different temperature and pH range.

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**Total antioxidant capacity**

Total antioxidant capacity was reported as ascorbic acid equivalents. The total antioxidant capacity of the methanolic extract was 22.05 µg AE/mL. There is a little information about total antioxidant activity of prodigiosin by phosphomolybdate method which employs cost-effective reagents. It is based on the reduction of Mo (VI) to Mo (V) in presence of antioxidant compound and subsequent formation of a green phosphate/Mo (V) complex at acidic pH and at higher temperature.

**Prodigiosin as textile colourant**

It was observed that when the different fabrics (wool, silk, nylon, cotton and polyester) were subjected to the treatment with acid, alkali, cold water, hot water, cold water and detergent and hot water and detergent for 1 h, the colour of the dye for all the fabrics was completely retained in cases of acid and cold water treatments whereas a small amount of discolouration resulted when treated with alkali, cold water and detergent and hot water and detergent.

## CONCLUSION

This study demonstrated a successful optimization of the cultural parameters that facilitated the enhanced production of the prodigiosin. The antimicrobial and antioxidant potential of the pigment may aim at the possible future usage of prodigiosin as a therapeutic. Prodigiosin being resistant to acid, alkali and detergents may be explored further as a colourant in the textile industry.

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