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ORIGINAL ARTICLE

Study of cultural parameters for enhanced Production of prodigiosin from *Serratia marcescens*

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ABSTRACT
Among natural pigments, pigments from microbial sources are potentially good alternative to synthetic pigments. Prodigiosin is a classical secondary metabolite, produced in late-log to stationary phases of growth. It was first characterized from Serratia marcescens. It possess anti-bacterial, anti-fungal, anti-protozoal and immunosuppressive properties. In this study different cultural parameters were studied to determine the optimum conditions for prodigiosin production by Serratia marcescens. These factors include media, temperature, pH, incubation time, effect of aeration, effect of enhancers and inhibitors. The antibacterial potency of the pigment was also studied by agar cup diffusion method. It was observed that maximum amount of prodigiosin was produced in peanut powder broth at temperature 28°C and pH 9.0. The higher levels of prodigiosin are found in the media kept in shaker conditions. It was also found that maximum prodigiosin was at 48h incubation. MgSO4 and SLS (sodium lauryl sulphate) enhanced the pigment production and CuSO4 and FeSO4 showed inhibition of pigment production. The extracted pigment showed antibacterial action against E.coli and S.aureus. The findings of the study proved that, the red pigmented prodigiosin is an alternative to chemical antibacterial agent.
Keywords: Prodigiosin, Serratia marcescens

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INTRODUCTION

Pigments are chemical compounds that absorb light in the visible range of the electromagnetic spectrum [1]. There are natural and synthetic pigments. Synthetic colours are found technically more suitable but many are carcinogenic and teratogenic in nature. With the increasing concern for health, people avoid using synthetic colours. Artificial dyes like azodye, nitrodye, nitrosodye etc. are toxic, recalcitrant and cause environmental pollution [2].

So, Pigments produced from natural sources are of worldwide interest and is gaining significance. Therefore, it is essential to explore various natural sources of pigments and their potentials. Natural pigments not only have the capacity to increase the marketability of products, but they also display advantageous biological activities as antioxidants, antimicrobial, anti-proliferative and anticancer agents. They are nontoxic and biodegradable too [3]. In spite of the availability of variety of pigments from fruits and vegetables, there is an ever growing interest in microbial pigments [4]. The advantages of pigment production from microorganisms comprise easy and fast growth in the cheap culture medium, independence from weather conditions and colours of different shades [5]. 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 [6, 7]. Prodigiosin compound was first isolated from *Serratia marcescens* in pure form in but a wide variety of bacterial taxa, including *Serratia rubidaea, Vibrio gazogenes, Alteromonas rubra, Rugamonas rubra, Streptoverticillium rubrireticuli,* etc.produces prodigiosin and/or derivatives of this molecule [8].

It has been discovered that prodigiosin possesses antifungal, antibacterial, anticancer, immunosuppressive, anti-tumour, anti-neoplastic, antiproliferative and antioxidant activity [9–13]. In light of its potential commercial 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 such as media, temperature, pH, incubation time, effect of agitation, effect of enhancers and inhibitors to achieve the enhanced production of prodigiosin from *S. marcescens* followed by exploration of the antibacterial potency of the pigment.

MATERIALS AND METHODS

Microbial Samples

The bacterial cultures used were *Serratia marcescens, Staphylococcus aureus*, and *Escherichia coli.*

Optimization of Prodigiosin Production

Effect of Different Media on Pigment Production

Fresh pigmented culture suspension (5%) was inoculated in 50ml of St. Nutrient broth, 50 ml of St. Peptone glycerol broth, 50ml of St.2% powdered peanut broth [Peanut seeds were finely powdered and 1g was added to 50 ml distilled water in a 250 ml Erlenmeyer flask] [14] and 50ml of St. Milk broth .The pH of all the media were maintained at 7.0. The various media were autoclaved at 120°C for 20 minutes and incubated after inoculation.

Extraction of pigment

Acetone extraction: The pigment was extracted by adding 4 volumes of acetone to the cell suspension and then it was shaken for 3 hrs at room temperature, and then centrifuged. Then sedimented cell debris was suspended in 50 ml of acetone. Solution was filtered. Pigment was extracted from small portions of the filtrate by mixing thoroughly 1 volume of the acetone solution with 2 volumes of petroleum ether in a separatory funnel. The separating funnel was shaken vigorously for 10 - 15 min. The pigment was extracted in the petroleum ether phase. This petroleum ether layer was poured in a small beaker and kept at 40 – 50°C in order to evaporate the solvent completely. 2 ml of acidified methanol was added and the pigment was scraped off from the beaker and stored in a screw capped tube [15].

Methanol extraction: Methanol extraction method was also carried out. Methanol was added to the cell suspension in the ratio 2:1. Then this methanolic extract was filtered to remove residual biomass. After that, in a separating funnel chloroform: water: concentrated extract was added in the ratio 1:1:1 to remove hydrophilic impurities. The organic phase is collected and the hydrophilic phase was re extracted with chloroform to remove residual pigment. The pooled pigment was dried at 50°C and then redissolved separately in methanol [16].

Presumptive test for prodigiosin

The culture broth was centrifuged at 4500 rpm for 15 mins. 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. The tubes were observed for colour change to red or pink colour in the acidified solution and a yellow or tan colour in the alkaline solution. This gives a positive presumptive test for prodigiosin [17].

Spectral analysis of Prodigiosin

Spectral analysis was made on pigment extracted by the above methods by using a UV-Visible spectrophotometer and the methanolic extract was scanned in the range of 400 - 700 nm to find out the maximum absorption spectra. Methanol was used as a blank**.**

Isolated prodigiosin was estimated using the following formula**,** [18]

Prodigiosin unit/cell = [OD534 – (1.381 x OD 620)] x 1000/OD620

Where, OD - Optical density; OD534 – Pigment absorbance; OD620 – Bacterial cell absorbance; 1.381 – Constant

Effect of temperature on Pigment Production

Equal volume of the bacterial isolate was inoculated in St. 2 % powdered peanut broth and incubated at 28°C and 37°C. The temperature at which *Serratia marcescens* gave maximum pigment production was maintained for further studies.

Effect of pH on Pigment Production

Equal volume of the bacterial isolate was inoculated in St. 2 % powdered peanut broth with various pH such as 4, 7 and 9. The flasks were incubated at 28°C. The pH at which *Serratia marcescens* gave maximum pigment production was maintained for further studies.

Effect of Aeration on Pigment Production

To study the influence of aeration on pigment production, the 24 h grown culture of bacteria was inoculated in St. 2 % powdered peanut broth and the flasks were incubated on static and shaker conditions at 28°C .

Effect of incubation on pigment production

To study the effect of incubation time on pigment production the prodigiosin concentration was measured at different incubation time viz., 8, 16, 24, 32, 40, 48, 56 h.

Study of enhancers

The enhancement of prodigiosin formation by sodium lauryl sulfate (SLS) and enhancement by addition by certain salts such as MgSO4 was determined. Effect of SLS was studied using agar cup method. 1ml of *Serratia marcescens* 0.05 OD culture at 530nm was added to sterile molten powdered peanut agar butts. Concentrations of SLS used include 10mg/ml, 20 mg/ml and 30mg/ml. After 48 hrs of incubation zone size were measured [19].

For studying effect of MgSO4, various concentrations such as 0.04%, 0.06%, 0.08% and 1.0% were added in the peanut powder broth.

Study of inhibitors

The inhibitors used for the study was ferrous sulphate and copper sulphate. The concentrations of ferrous sulphate and copper sulphate such as 0.1%, 0.2%, 0.3% were added in the peanut powder broth. The effect of copper sulphate was also studied by agar cup method. After incubation time, the pigment production was determined.

Study of Antimicrobial activity of prodigiosin

The agar plate surface was inoculated by spreading a volume of the bacterial inoculum over the entire St. Nutrient agar surface. Then, a hole with a diameter of 6 to 8 mm was punched aseptically with a sterile cork borer and a volume 100 µL of the methanolic extract of prodigiosin was introduced into the well. Then, agar plates were incubated at 37°C.

Results

Optimization of Prodigiosin Production

Effect of Different Media on Pigment Production

Out of 4 different medias used, Serratia *marcescens* gave more pigmentation on peanut powder broth. Figure 1 shows the biopigment accumulation by *S. marcescens* in St. Nutrient broth medium, St. Peptone glycerol broth, St. Milk broth and in St.peanut powder broth.

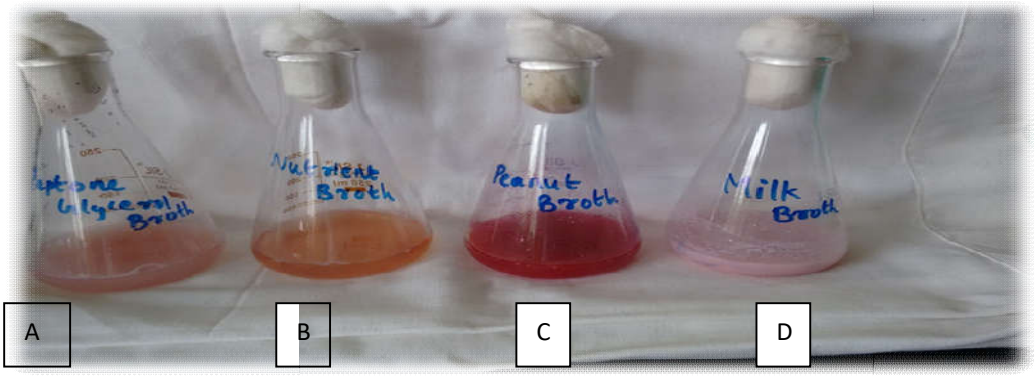

Figure 1. Effect of different medias used for pigmentation. A- Peptone glycerol broth, B- nutrient broth, C- peanut powder broth, D- milk broth. Here peanut powder broth is showing higher pigmentation.

Presumptive test for prodigiosin

On addition of 1drop of concentrated HCl to the methanol extract of pigment and to the cell free supernatant of the broth culture, the colour changed to deep red and pink respectively. When concentrated ammonia solution was added to methanol extract and cell free supernatant, there was a colour change to yellow and tan respectively. This showed a positive presumptive test for prodigiosin.

Spectral analysis of pigment

In order to confirm that the extracted pigment is prodigiosin, spectral analysis of the pigment was carried out using UV-Vis spectrophotometer. The pigment obtained by acetone extraction showed a characteristic peak at 540 nm and 560nm for extract obtained by methanol extraction (figure 2).

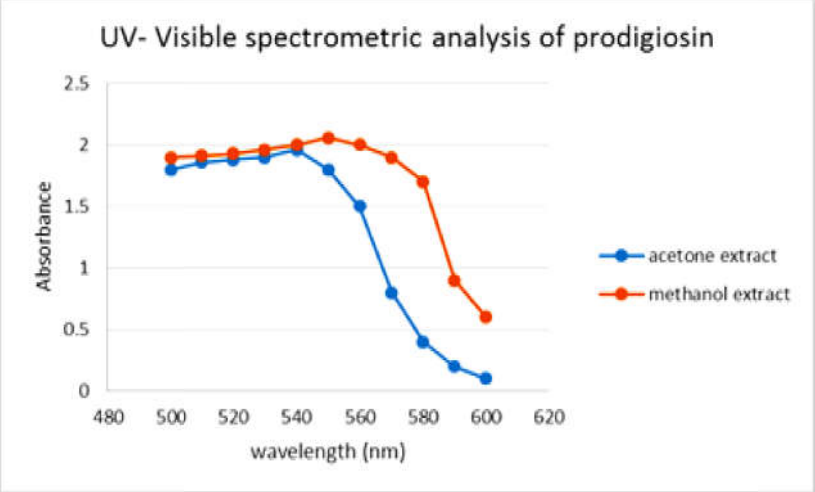

Figure 2: UV Vis spectrophotometric analysis of prodigiosin showing peak at 540 nm for extract obtained by acetone extraction and 560nm for extract obtained by methanol extraction.

Effect of temperature on Pigment Production

In order to determine the optimum incubation temperature for prodigiosin production by *S. marcescens*, incubation temperatures 28ºC and 37 ºC were used for this purpose. It was found that the maximum production of prodigiosin was obtained when the temperature of medium was 28°C and there was only negligible amount of prodigiosin was obtained at 37°C (Figure 3). This might be due to the fact that the terminal step in prodigiosin biosynthesis i.e., condensing of mono and bipyrrole moieties is temperature sensitive [20].

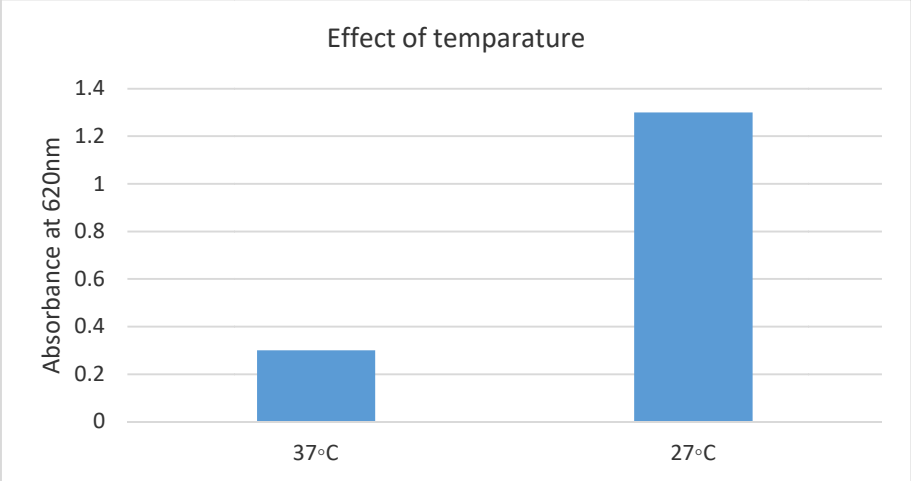

Figure 3. The effect of temperature on prodigiosin production.

Effect of pH on Pigment Production

From the graph (Figure 4) it can be seen that the production of prodigiosin increase is more at pH 9.0 and it progressively decreases with decrease in pH. This shows that the colour production is more efficient at alkaline pH than at acidic pH.

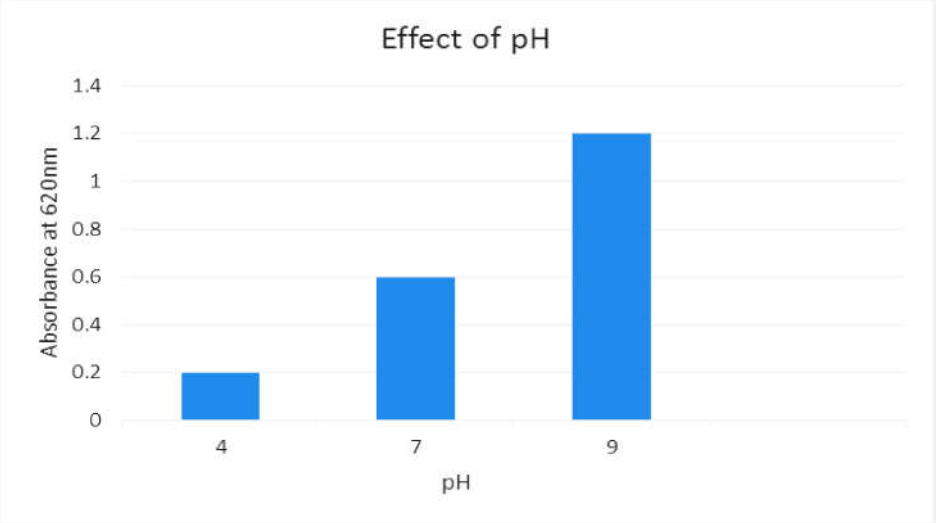

Figure 4: The effect of media pH on prodigiosin production

Effect of Aeration on Pigment Production

Pigment production was found more in shaker conditions (figure 5). Agitation allows the oxygen to dissolve in the medium hence, influence the metabolism of cells which increase the requirement of amino acids and hence cause more production of pigment.

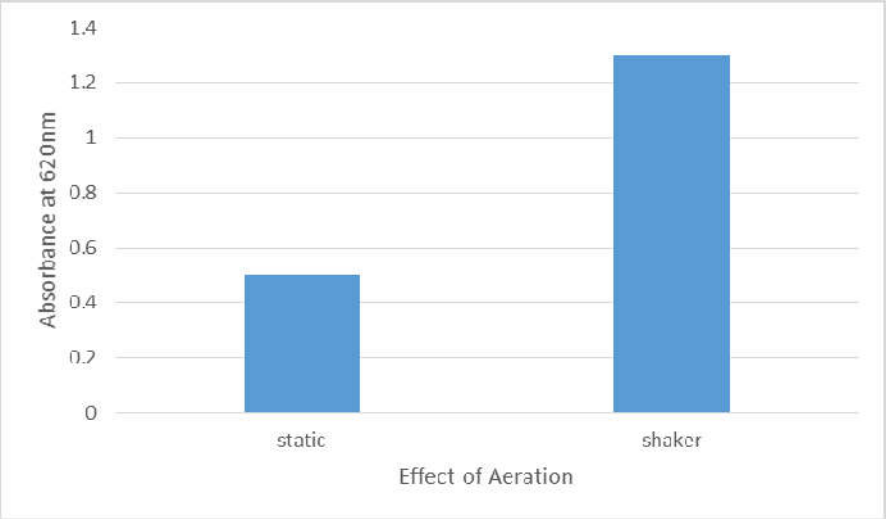

Figure 5: Effect of aeration on pigment production

Effect of incubation on pigment production

The pigment production of *S. marcescens* was observed at different time intervals. The pigment production was started after 16 h of incubation and increased pigmentation was seen till 48 h of incubation. After 48 h incubation there was no increase in pigmentation. From the figure (figure 5), it may be examined that the stationary phase begins from 16 h. So the optimum pigment production is observed during the stationary phase. This results indicates that prodigiosin is produced by the organism as a secondary metabolite.

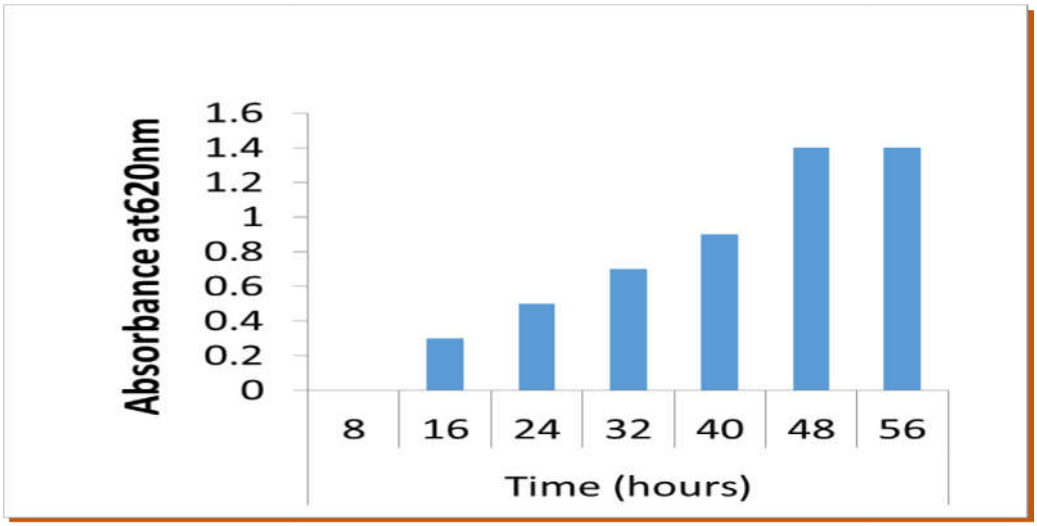

Figure 5: The effect of incubation time on prodigiosin production

Study of enhancers

MgSO4 and SLS showed enhancement of pigment production (figure 6 and 7). The possible mechanism of the SLS enhancement effect could be explained by an increase in negative binding sites by the association of SLS with a cell envelope component(s). These binding sites may be required for prodigiosin synthesis **[19]**.

Figure 7: MgSO4 is showing enhanced pigment production

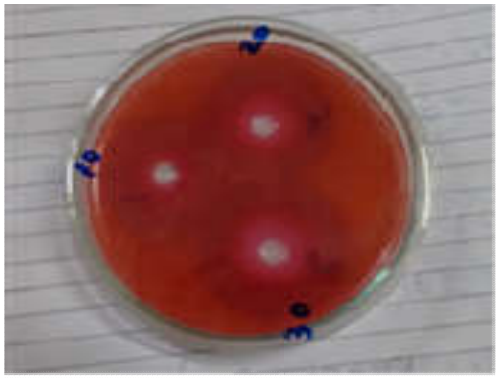

Figure 6: SDS is showing enhanced pigment production

Study of inhibitors

Ferrous sulphate and copper sulphate showed complete inhibition of pigment production (Figure 8 and 9). The inhibitory effect of these compounds might be due to inhibition of enzymatic activity.

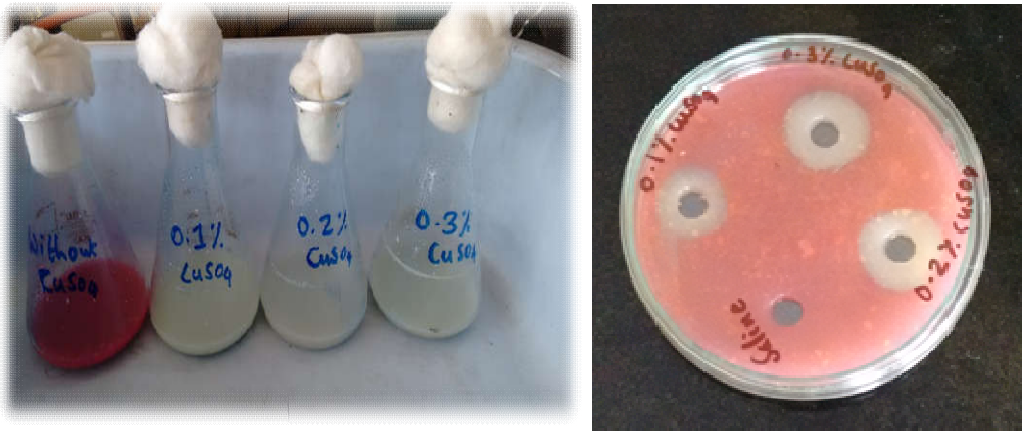

Figure 8: Inhibition of pigment production by CuSO4

Figure 9: Inhibition of pigment production by FeSO4

Antibacterial activity of pigment extract

Prodigiosin showed inhibitory activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* bacteria with zone of inhibition of 11 and 6 mm in diameter respectively at 100 µg ml−1 concentration.

Organisms used	Zone of inhibition (mm)
Staphylococcus aureus	11
Escherichia coli	6

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 [21]. The present investigation focused on selection of a production medium, effect of temperature, pH, incubation time and aeration for effective prodigiosin production. Effect of enhancers and inhibitors of the pigment was also studied followed by its antimicrobial activity evaluation.

Powdered peanut broth was found to be suitable medium for prodigiosin production by *S. marcescens.* This result is in perfect agreement with the previous results where enhanced pigment production was observed in the powdered peanut broth over other medias. That is because the fatty acid form of carbon source has play in enhanced cell growth and prodigiosin production [14]. Temperature and pH are the common environmental factors that play a crucial role in cell growth and prodigiosin production of *Serratia* strains [8]. No prodigiosin was produced when cultures were incubated at 38°C; however pigment production was observed when the temperature was shifted to 27°C. Prodigiosin is previously reported to be produced maximally at pH 7 [22].But we here report highest yield of prodigiosin at pH 9 in powdered peanut broth. In the present study both static and agitated conditions were examined. Increase in pigment production was observed with increased aeration suggesting a key role of shaking speed in pigment production. Aerobic conditions influence the metabolism of cells which increase the requirement of amino acids and hence cause more production of pigment [8]. In the present work, 48 hrs of incubation resulted in the maximum production of prodigiosin.

Magnesium sulfate, and SLS, has a role in physiology and growth of organism. However, inhibition of prodigiosin production was found with different concentrations of copper sulphate and ferrous sulphate.

The inhibitory effect of prodigiosin on Gram positive and Gram negative bacteria was studied and it was observed that it has a more inhibitory effect on Gram positive bacteria. Antibacterial effect against *E. coli*, and *S. aureus* was in comparison with previous reports [17]. The antibacterial activity of prodigiosin was the result of the ability of prodigiosin to pass through the outer membrane and inhibiting target enzymes such as DNA gyrase and topoisomerase IV, which inhibited the cell growth [9].

CONCLUSION

This study demonstrated a successful optimization of the cultural parameters that facilitated the enhanced production of the prodigiosin. The antimicrobial potential of the pigment may aim at the possible future usage of prodigiosin as an alternative to chemical antibacterial agent.

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