Bioscience Discovery, 8(3): 388-396, July - 2017

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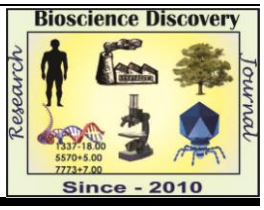
Print & Online, Open Access, Research Journal Available on http://jbsd.in
ISSN: 2229-3469 (Print); ISSN: 2231-024X (Online)

Research Article



Isolation, Identification and Optimization Study of Prodigiosin from *Serratia marcesces*

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Article Info

Received: 05-03-2017,
Revised: 16-04-2017,
Accepted: 12-06-2017

Keywords:
Prodigiosin , *Serratia marcescens*, Optimization, biopigments.

Abstract

Investigations were made in the present study to screen new strain designated as *S. marcescens* was isolated. The parameter viz., temperature, pH, oil substrate, amino acid, carbon source, nitrogen sources and time were optimized to increase the production of prodigiosin. It was observed maximum amount of prodigiosin was produced at temperature 30°C and pH 7.0. Among the different sugar substrates tested, 0.4% mannose when amended in the medium yielded maximum Prodigiosin 400 mg/mL. Oil substrate plays a vital role in prodigiosin production. Among the various oil substrates used for the production of prodigiosin was maximum 500 mg/mL, when the medium was amended with peanut oil 0.1%. Amino acids like combination of 0.01%Methionine and 0.003% Cysteine was found effective, 0.1% Ammonium chloride supported maximum Prodigiosin production. Time required for maximum pigment production was also observed, it was found that 3 days time period required for maximum pigment production.

INTRODUCTION

A natural product represents one of the critical sources of chemical diversity and potential medicinal use. Pigments produced by organisms as reminiscence of its secondary metabolism are commonly referred as biopigments. These biopigments have wide synthetic and commercial application (Shirata *et a*l., 2000). Prodigiosin is a red pigment produced by many strains of the bacterium like *Serratia marcescens* and some other unrelated microbial st rains, such as *Vibrio psychroerythrus, Sreptomycin griseoviridis* and *Hahella chejuensis* was found to exhibit antibacterial, antimycotic, immunomodulating, anti-tumor and anti-malarial properties (Frustner,2003) Prodigiosin {5[3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl]-2-methyl-3-pentyl-1H-pyrrole}is an alkaloid secondary metabolite with a unique tripyrrol chemical structure. First characterized from *S. marcescens* a Gram negative bacterium which is an opportunistic pathogen with nonchromogenic biotypes posing a public health threat (Kalesperis *et a*l., 1975; Gargallo *et al*., 1987; Gargello, 1989). Chromogenic biotypes from the natural environment have rarely implicated in infections and the function of this red pigment remains unclear because clinical isolates are rarely pigmented (Hejazi and Falkiner, 1997). Interestingly, the water insoluble red pigment produced by *S. marcescens* has been reported to have antibiotic activity (Tsuji *et al.*, 1990; Kataoka *et al*., 1992; Tsuji *et al.*, 1992; Songia *et a*l., 1997). Some strains of *S. marcescens* also produce a water-soluble, redish-violet pigment with supoeroxidase mimentic activity (Hardjito *et al*., 2002).

Kobayashi and lchikawa (1991) and Matsuyama *et a*l. (1986) reported that Prodigiosin is associated in extracellular vesicles or present in intracellular granules. Most pigments absorb light at some defined wavelength, and pigment expression may be easily monitored spectrophotometrically (Cerdeno *et al*., 2001). Prodigiosin can exist in two

district forms, depending upon the hydrogen ion concentration of the solution. In an acid medium the pigment is red and exhibits a sharps spectral peak at 535 nm. In alkaline medium the pigment is colored orange-yellow and posses a broader spectral curve centered at 470nm (Williams *et al.*, 1955)

The present study focus isolation and identification of Prodigiosin producing organism from soil and attempt was made to optimize the physio-chemical parameters for the production of pigment in *Serratia marcescens*.

MATERIALS AND METHODS

Isolation Screening and Identification

Soil samples were collected from different parts of Akola region and screened for *Serratia marcescens*. The collected soil samples were serially diluted and plated on nutrient agar plates after incubation and preserved at 4°C. The isolated colonies were identified based on Bergey's classification of determinative bacteriology.

A loopful of culture was inoculated in pre-sterilized 100ml nutrient broth. The broth flask was kept in a shaker at 120 rpm for 16-18h at 30°C. The culture broth was centrifuged at 6,000 rpm 30 min. Cell suspension was prepared using sterile distilled water and adjust to 0.2 OD using UV Visible spectrophotometer. One percent of the above under UV-visible spectrophotometer was used as inoculums for the production of Prodigiosin. The bacterial isolate was subcutured in 100 ml of nutrient broth and incubated in a rotary shaker for 48hrs at 37°C, and then extracted Prodigiosin from production broth or medium (Slater *et al*., 2003).

Pigment Extraction

The organisms were harvested by centrifugation at 6,000 rpm for 30 minutes. The supernatant was discarded and the pellet was resuspended in acidified ethanol (4% 1M HCL in 96 ml ethanol) The mixture was vortexed and the suspension was transferred to a fresh vial and observed under UV- visible spectrophotometer at 534nm. The prodigiosin produced was quantified using known concentration of prodigiosin.

Determination of absorption spectra

Spectral analysis was made on dried pigment by dissolving in 10ml of absolute ethanol. Acidic condition for spectral analysis were obtained by adding 1ml of 1N Hydrochloric acid to 10ml of the ethanol extract (Williams *et al*., 1955). Spectral analysis was made on a UV-Visible Spectrometer.

a) Effect of temperature on Prodigiosin production

Equal volume of the bacterial isolates was inoculated Nutrient broth and incubated at different temperature viz. 25, 30, 37, 45 and 55°C for 48h. The prodigiosin production was estimated after incubation. The temperature at which maximum production of prodigiosin was observed was chosen and maintained in the following studies.

b) Effect of pH on Prodigiosin production

Equal volume of the bacterial isolate was inoculated in nutrient broth with various intial pH viz. 3, 5, 7, 11, and 12. The flasks were incubated at 37°C for 48h. The prodigiosin production was estimated after incubation. The initial pH at which maximum production of prodigiosin was observed was chosen maintained in the following studies.

***c*) Effect of different oil substrate on Prodigiosin production.**

The bacterial isolates was cultivated in medium amended with peanut, sesame, coconut soya and neem oil. It was observed that maximum amount of prodigiosin resulted in the medium and supported source of oil chosen for further study.

d) Effect of amino acids on Prodigiosin production

The bacterial isolates cultivated in medium containing Cystein, Methionine, Tryptophane, Tyrosin and combitation of various concentration with increase Cystein & Methionine, was studied on Prodigiosin production.

e) Effect of different carbon sources on Prodigiosin production

To study the effect of different carbon sources on Prodigiosin production 0.5% of different sugars concentration of Glucose, Sucrose, Fructose, Mannose and Maltose used for maximum Prodigiosin production.

f) Effect of different nitrogen sources on Prodigiosin production

The effect of different nitrogen sources on Prodigiosin production was studied by replacing 0.1% ammonium chloride of different organic and inorganic nitrogen Nitrogen source. such as Ammonium chloride, Ammonium nitrate, Ammonium oxalate, Ammonium citrate and urea. The nitrogen source at which maximum production of Prodogiodin was observed and chosen in following study.

h) Effect of different time on Prodigiosin production

The effect of time on Prodigiosin production was performed by inoculating culture on media and incubated at various time period viz., 1, 2, 3, 4, 5, 6 and 7 days.

RESULTS AND DISCUSSION

Twenty samples were collected from various region of Akola and screened for Prodigiosin production. Among the 20 soil sample, 6 soil samples were positive for Red pigmented colonies.

Isolation of the pigment producing organisms from different samples resulted in six isolates showing orange to maroon coloration. The promising isolate which showed Prodigiosin production on the basis of presemptive test was selected for present study, and was maintained on nutrient agar slant at 4°C. The cultural, morphological and biochemical test were used to identify this isolate as *Serratia marcescens*.

Absorption spectrum

In order to determine the wavelength at which maximum absorption occurs, Spectrum scan was done using UV-visible spectrometer. The spectrum scan results showed that the Prodigiosin molecule in acidic conditions showed an absorption maximum at 534nm (Fig 1).

Effect of temperature on Prodigiosin production

The effect of different temperature 25, 30, 37, 45 and 55°C was observed and the Optimum Temperature for Prodigiosin production by *Serratia marcescens* was found 30°C. (Fig. 2)

Effect of pH on Prodigiosin production

The effect of different pH like 3, 5, 7, 9 and 11 like was observed and the Optimum pH for Prodigiosin by *Serratia marcescens* was found 7.0 (Fig3)

Effect of different oil substrate on Prodigiosin production.

The effect of different oils supported with medium like peanut, sesame, coconut, soya and neem oil. It was observed that maximum amount of prodigiosin resulted in the medium supplemented with peanut oil followed by sesame oil, coconut oil, soya oil and neem oil.(Fig4)

Effect of amino acids on Prodigiosin production

Effect of different amino acid was studied on Prodigiosin production, it was found that there was no production of Prodigiosin when medium amended with Tryptophan and Tyrosin, Initially different ranges of Cystein (0.01-0.1%) was studied on Prodigiosin production of which 0.01% supported maximum production of Prodigiosin and showed a diminution in production of Prodigiosin with increase in concentration of Cystein (Fig 5.a) and therefore the range was expanded, from 0.001-0.010% and it was found to be 0.006% (Fig 5.a) Optimized Prodigiosin production was at 0.02% Methionine as shown in Figure. Combination of Methionine (0.01%)and Cystein (0.003%) exhibited maximum production of Prodigiosin (Fig 5.b)

Effect of different carbon sources on Prodigiosin production

Effect of different 0.4% carbon sources like Maltose, Glucose, Mannose, Sucrose and Fructose was observed, after 24 hrs. 0.4% Mannose resulted in maximum Prodigiosin yield as compared to other C sources. However, after 72hrs there was no increase in Prodigiosin yield in the medium containing mannose or any other sugar (Fig 6).

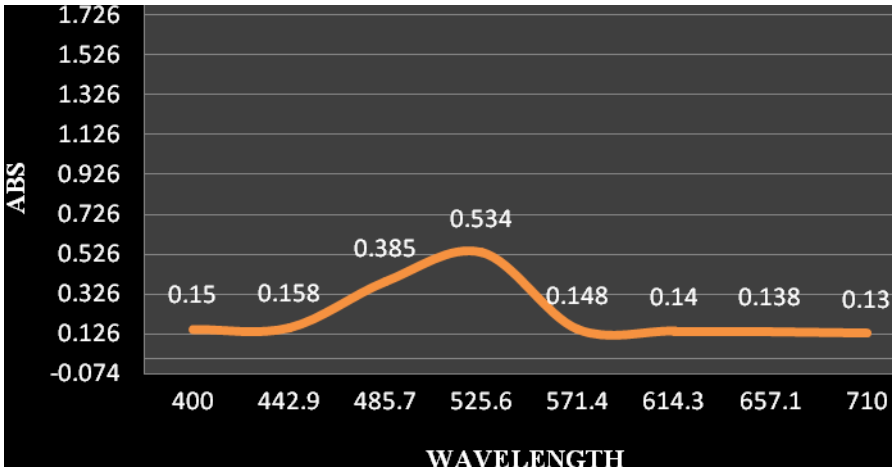

Fig 1. Spectrum scan of Prodigiosion produced by *Serratia Marcescens*

Effect of different nitrogen sources on Prodigiosin production

Effect of different Nitrogen sources like Ammonium chloride, Ammonium nitrate, Ammonium oxalate and Urea was observed. In presence of Ammonium nitrate and Ammonium citrate good pigmentation was observed, however, in presence of Ammonium oxalate less Prodigiosin was produced as compared to that with Ammonium nitrate and citrate, while urea showed very less amount of prodigiosin production. Among all the N sources. 0.1% Ammonium chloride supported maximum Prodigiosin production (Fig7)

Effect of different time on Prodigiosin production

Effect of different time was observed on Prodigiosin production. It was found that the maximum prodigiosin produced at 72hrs. As comapared 24, 48hrs. While at 4day(96hr) to 5day(120hr) it remains constant.(Fig 8)

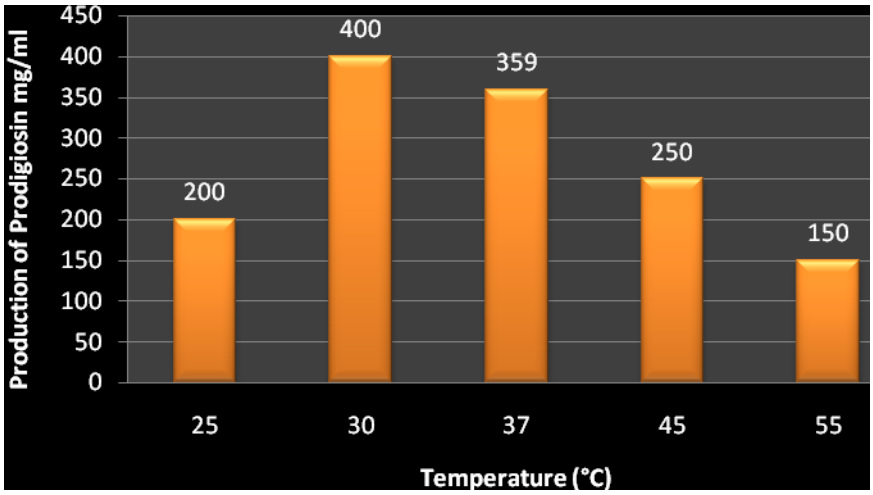

Fig. 2 : Effect of temperature on Prodigiosin production

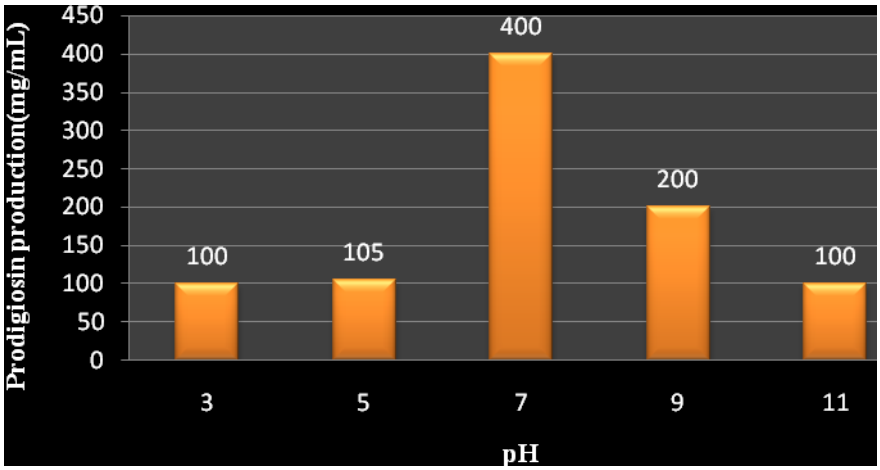

Fig. 3: Effect of pH on Prodigiosin production by *Serratia marcescens*

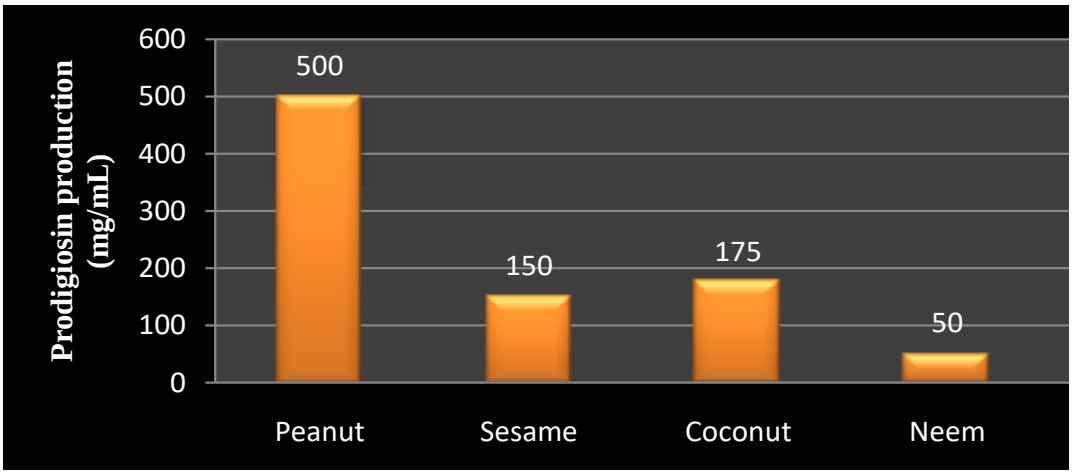

Fig. 4 : Effect of Oil substrate on Prodigiosin production by *Serratia marcescens*

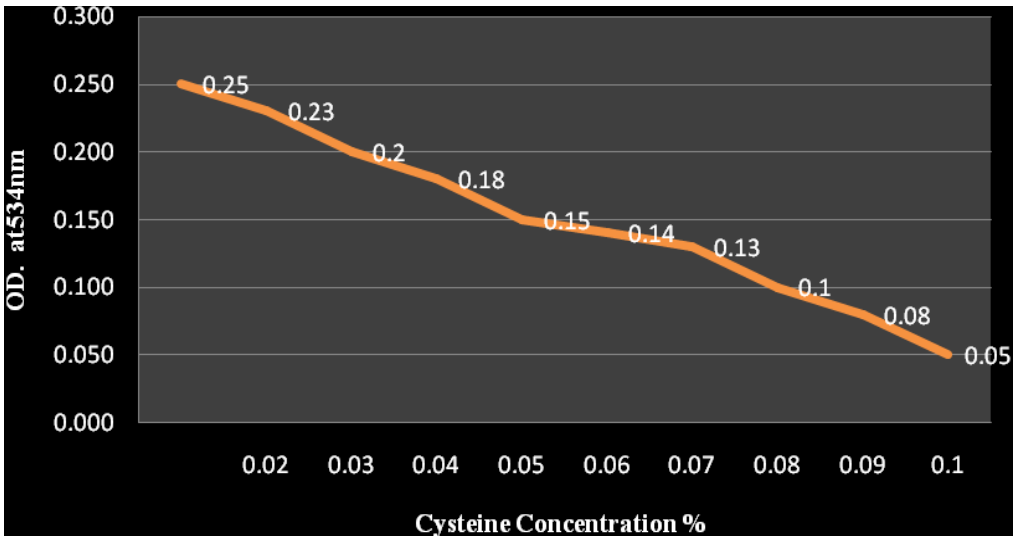

Fig 5.a Effect of various concentration of Cysteine on Prodigiosion production by *S. marcescens*

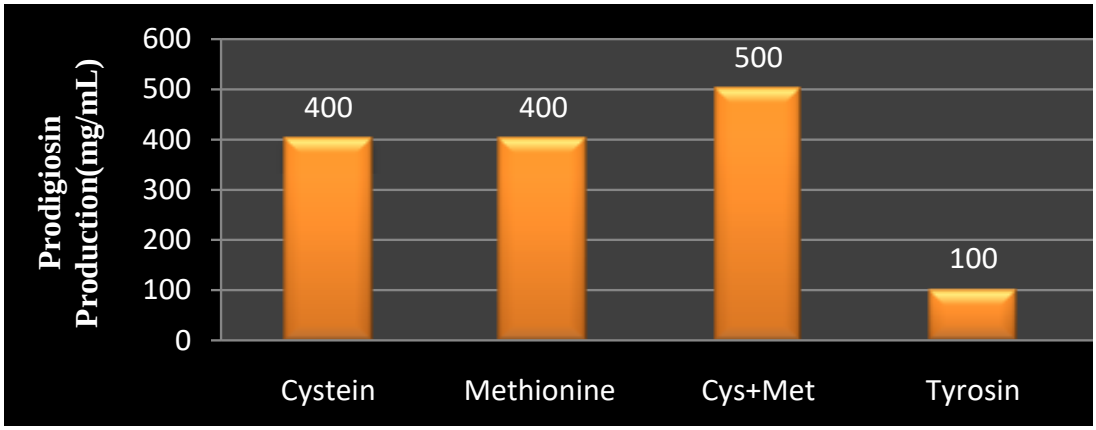

Fig. 5.b : Effect of various concentration of aminon acid on Prodigiosin production by *Serratia marcescens*

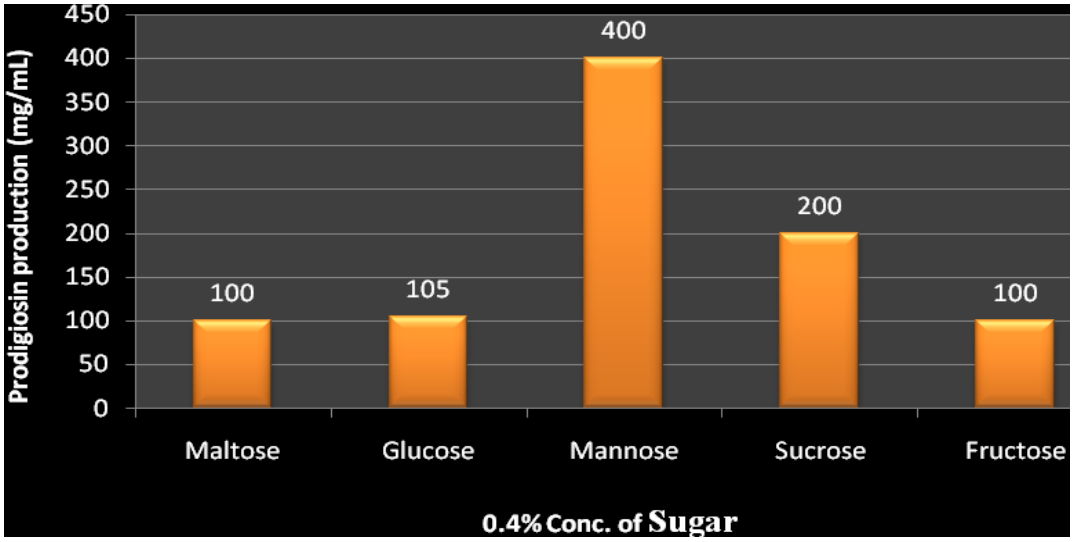

Fig. 6 : Effect of Carbon source on Prodigiosin production by *Serratia marcescens*

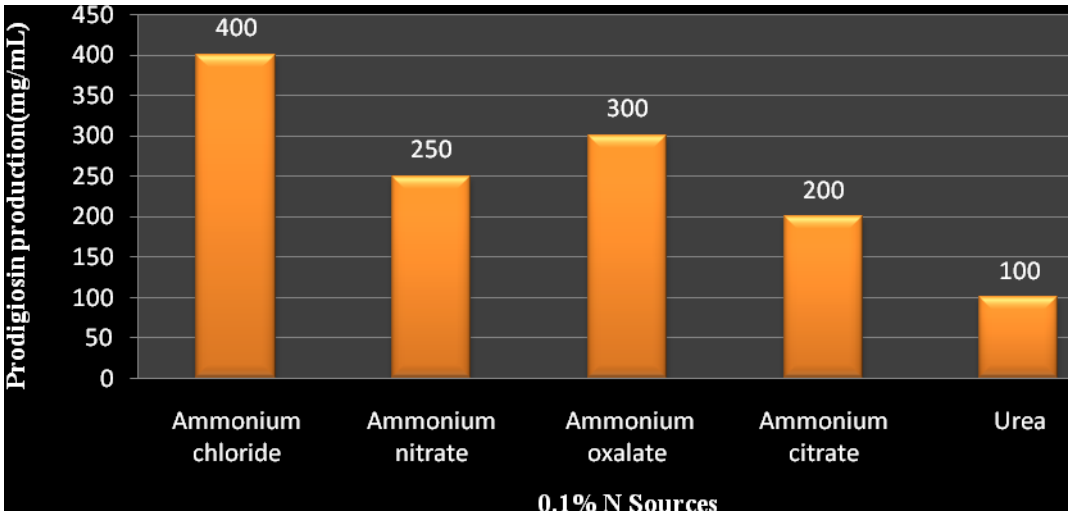

Fig. 7 : Effect of Nitrogen source on Prodigiosin production by *Serratia marcescens*

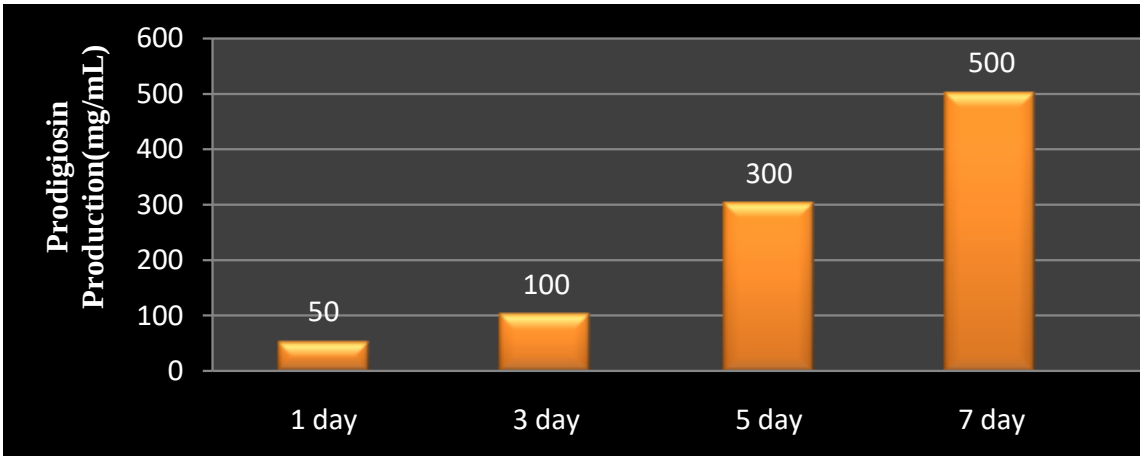

Fig. 8: Effect of Time on Prodigiosin production by *Serratia marcescens*

Fig. 10 : Production of Prodigiosin by *Serratia marcescens*

Fig. 9 : *Serratia marcescens*

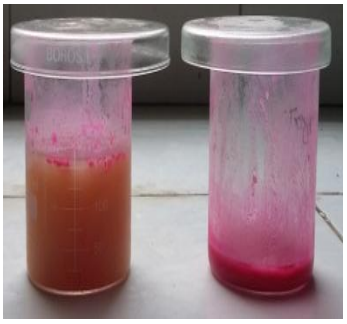

Fig. 10 : Prodigiosin extracted by *Serratia marcescens*

DISCUSSION

Biopigment produced by bacteria possess enormous efficieny as medicinally important products. Prodigiosin, a red pigment, belongs to the family of tripyrrole was found to anti-tumor and anti-malarial properties. These pigments were found associated with cell wall vesicle of the bacteria. The present investigation centered on isolation of prodigiosin producing organism from soil and formulating optimized condition for effective production of Prodigiosin.

The bacterial isolate elaborated the pigment at 30^0C. and the rate was reduced as the temperature increases. Williams & Hussain Quadri (1970) Reported that the no prodiodin was observed when the temperature was sahifted to 27^0C. A complete block in prodigiosin was observed in most of the basically used media tested at 37^0C was similar to the result observed by Pryce & Terry (2000).

While considering the basic role of carbon source in augmenting the pigment production, the addition of maltose or sucrose or lactose was shown to enhance the prodigiosin yield and addition of glucose was found to be ineffective. Anna R Oller (2005) reported that glucose and sorbitol had a repressive effect on prodigiosin production synthesis. Chang *et al.* (2000) has reported 3mg/ml of prodigiosin when dextrose was used in the medium. Jungdon *et al.*(2001) reported that in a bioreactor study with an internal absorbent for prodigiosin, the final yield was 13mg/ml in which dextrose was used in culture broth.

The suitable fatty acid for pigment production was optimized by cultivating the bacterial isolate in the presence of peanut, sesame, coconut soya and neem oil. In case of fatty acid alternatives, peanut oil broth gave a maximum yield (500mg/mL) and this result was compared with the existing litreture that uses different carbon source. The observed enhancement in the yield of prodigiosin in peanut oil has been attributed to its higher saturated fatty acid content. On the other hand, sesame and soya oil broth contains higher level of unsaturated fatty acid (approx 4%) than the rest. From these arguments it was speculated that the bonded fatty acid are of limited carbon source and less accessible by *S.marcescens*. Giri *et al.,(*2004) reported peanut seed powder increased the production of prodigiosin and they have concluded that the saturated from of fatty acid plays a role in enhanced cell growth and prodigisin production. *Serratia marcescens strain* exhibited pigmentation in presence of amino acid Cystein and Methionine individually , However with this isolate, pigmentation was found to be absenr with Tyrosin and Tryptophan which have pyrrole structures and are known to enhanced pigmentation(Wei and Chen 2005). In another study with orange mutant of *Serratia marcescens*, it was indicated that the methyl group on C6 of Prodigiosin comes from Methionine and hence an important role of methionine in biosynthesis of Prodigiosin is methylation of the pigment (Qadri and Williams, 1973). In addition, isotope from Cystein and Methionine, was found to be incorporated to a small extend in Prodigiosin (Qadri *et al.*, 1974). The significance of the result in the fact that pigmentation is seen in presence of Cystein and Methionine individually than with Tyrosin and Tryptophan, At the higher concentration of Cystein and Methionine results in less yield or none of Prodigiosin by *Serratia marcescens*. Also Methionine (0.01%) and Cystein (0.003%) in combination enhances Prodigiosin production showing to have a syngerstic effect.

Prodigiosin is typical secondary metabolite is appearing only in later stages of bacterial growth (Harris *et al*., 2004). The production of Prodigiosin has been shown to be influenced by numerous environmental factors including media composition and pH, (Weinberg, 1970; Williamsons *et al*., 2005). Furthermore, the formation of Prodigiosin by *Serratia marcescens* in buffered medium with a pH of 7.0 was 10 times greater than the formation in an unbuffered medium.

0.1% of Ammonium chloride as a N source supported maximum Prodigiosin production, In presence of Ammonium oxalate good pigmentation was observed, however in presence Ammonium nitrate and Ammonium citrate only little Prodigiosin was produced as compared to Ammonium oxalate (Silverman and Munoz, 1973). Prodigiosin production increases from 12 to 72 hrs, maximum production was observed at 72hrs. Further increase in incubation does not show effect on Prodigiosin production. Davaraj N. et al.,(2009) has reported that, significant pigment production was observed on fifth and seventh day of incubation whereas the Prodigiosin completely reduced after seventh day of incubation.

Samrot, *et al.* (2011) reported that, more Prodigiosin was produced after incubation of 72 hrs. After 72hrs, the rate of Prodigiosin production was reduced.

A Prodigiosin producing bacterium was isolated from soil and identified as *S. marcescens*. It was found that optimum condition for Production of Prodigiosin temperature 30°C and pH 7.0 , the addition of mannose of 0.4% produced 400mg/mL pigment, Pea nut oil 0.2%, 0.01% methionine and 0,003% Cystein and 0.1% Ammonium chloride , Incubation period 3 days under shaker conditions (120rpm) produced 500 mg/mL pigment . Methionine and Cystein together resulted in better Prodigiosin production and found to exhibit a synergistic effect. Mannose and Peanut oil almost yielded same amount of Prodigiosin but since mannose induced pigmentation within 24hrs. So it was selected as a better C source, for Prodigiosin production.

Acknowledgement

The auther thankful to Prof. and Head of Dept Dr. Archana S. s Pethe and Principle Dr. Shubhas Bhadange Shri Shivaji College of Arts, Commerce and Science Akola For providing infrastructure facility and their constant support for this study.

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How to Cite this Article:

Monika T Rokade and Archana S Pethe, 2017 Isolation, Identification and Optimization Study of Prodigiosin from *Serratia marcesces*. *Bioscience Discovery*, **8**(3):388-396.