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Cytotoxic potential of a pigment from *Serratia marcescens* against HepG2 and Jurkat cell lines and optimization of culture conditions for enhanced pigment production

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Abstract
Background: Pigment from micro-organism is one of the emerging areas of research and many pigments have been extensively studied for their therapeutic potential. **Methodology**: Our aim was to analyze the anticancer property of a red pigment extracted from *Serratia marcescens* JGI 27against HepG2 and Jurkat cell lines. The cell viability was evaluated by MTT assay. As the red pigment extracted from *S. marcescens* exhibited high cytotoxicity to the tested cancer cell lines, the pigment production was optimized under various culture conditions like, temperature shock, incubation time, carbon sources, nitrogen sources and metal ions. Pigment yield was analyzed spectrophotometrically at 487 nm. **Results:** The IC_{50} value of the red pigment was calculated around < 20µg/mL concentration. The percentage viability of lymphocytes and CHO was found to be negligible at all the selected concentration even after 72 hrs of incubation. When the bacterial culture was kept at 37°C for 48 hrs and then 50°C for 48 hrs, the yield of the pigment was found to be highest. Supplimentation of sucrose (1%), beef extract (1%) and $Fecl_30$. 01% resulted in enhanced pigment production from *S. marcescens* JGI 27. Supplementation with the metal ions with at concentration resulted in highest pigment production. **Conclusion:** It is concluded that red pigment from *S. marcescens* JGI 27 was found to have a promising cyotoxic effect against HepG2 and Jurkat cell lines and it is non-toxic to normal peripheral human lymphocytes and CHO cell lines. At optimum physical and cultural parameters the pigment production was found to be highest.
***Keywords**:* Cytotoxicity, HepG2 cells, Jurkat cells, CHO cells, *Serretia marcescens.*

1. Introduction

Colors are vital to the sensing of the environment and have evolved in higher living organisms to guide their interactions with others[1]. A large number of bacteria, moulds, yeasts and algae produce pigments like carotenoids, melanins, flavins, quinones, prodigiosins and more specifically monascins, violacein or indigo[2]. The pigments produced by microbes received the attention in this context. Natural pigments can be obtained from two major sources, plants[3] and microorganisms[4]. Many pigment from microbes have been extensively studied for their therapeutic potential. Prodigiosin a reddish-pink linear trypyrrole multifaceted secondary metabolite produced by many strains of the bacterium like *Serratia marcescens*, *Vibrio psychroerythrus*, *Streptomycin griseoviridis* and *Hahellachejuensis*and was found to exhibit antibacterial, antimycotic, immunomodulating, anti-tumor and anti-malarial properties and in many cytotoxic and anticancerous activities prodigiosin play an active role[5,6]. Our aim was to analyze the anticancer property of a red pigment extracted from *Serratia marcescens* JGI 27, isolated from soil sample of Bangalore, against the liver cancer cell line HepG2 and leukemia cell line Jurkat and yield of the cytotoxic pigment production. The pigment was also checked for its safety on humans by treating on to normal human peripheral lymphocytes and normal

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ovarian cell line CHO.

2. Materials and methods

2.1 Isolation and identification of pigment producing bacteria

Pigment-producing bacteria were isolated from soil samples collected from different parts of Bangalore, serially diluted in sterile distilled water, plated on to nutrient agar plates and incubated at 37°C. Red pigmented colonies of bacteria were isolated from the plates after incubation and propagated on the same medium by cross streaking method to maintain the pure culture and stored at 4°C. The isolated red colonies were identified based on Bergey's classification of determinative bacteriology by studying morphological, microscopical and biochemical characters of the organism.

2.2 Isolation of lymphocytes

Lymphocytes were isolated from blood of healthy individuals. HiSep medium (HI-MEDIA Laboratories, Mumbai, India) was used for the isolation of lymphocytes and were suspended in RPMI-1640 medium supplemented with 10% FBS, 5g/ml phytohemagglutinin and maintained in a CO_2 humidified incubator at 37°C.

2.3 Cell lines

HepG2 (liver cancer cell line), Jurkat (leukemia cell line) and CHO (normal ovarian cell line) cell lines were procured from National Center for Cell Sciences (NCCS), Pune. For maintaining the cell lines, MEM medium i. e Modified Eagle Medium (HIMEDIA Laboratories Pvt. Ltd) is used for HepG2 cell line, RPMI -1640 (HIMEDIA Laboratories Pvt. Ltd) for Jurkat cell line and DMEM medium i. e Dulbecco's Modified Eagle's Medium (HIMEDIA Laboratories Pvt. Ltd) supplemented with 10% fetal bovine serum (HIMEDIA Laboratories Pvt. Ltd), 100 U/ml of penicillin and 100 µg/ml of streptomycin. Cells were incubated at 37°C in a humidified incubator with 95% air and 5% CO_2. All cells were used during the linear phase of growth. Subculturing is required to maintain the cell lines and is done by trypsination and replacing the old media with the fresh one.

2.4 Extraction of the pigment and quantification

S. marcescens JGI 27 cells grown in nutrient agar plates were subjected to centrifugation at 6000 rpm for 15 min with methanol for extraction of red pigment. Cell debris was removed and supernatant (containing pigment with methanol) is collected and subjected to spectrum scanning in the range of 200 to 700 nm using a UV-VIS spectrophotometer (Schimadzu 1800, Japan) with methanol as a blank. The methanol extract was evaporated to dryness and the amount of pigment obtained on a dry weight basis was calculated.

2.5 Cell viability assay (MTT-Assay)

Cell viability was determined by MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide)] colorimetric assay (Mosmann, 1983). HepG2, Jurkat and CHO cells were incubated in 96-well microtitre cell culture plates in for 24 hrs and then again cells were incubated in the absence (control cells) or in the presence of 10µg/ml to 40 µg/ml of crude extract in triplicates for 24 hrs at 37°C in a humidified 5% CO_2 incubator. After 24 hours incubation, 20 µl of MTT (5mg/ml,diluted in PBS) was added to each well for an additional three hours in a dark chamber. The blue MTT formazan crystals was dissolved in 100 µl of DMSO and the absorbance at 540 nm was recorded using ELISA plate reader. The percentage viability of the cells were calculated by Viability (%) = $OD_s/OD \times 100$**,** where OD_s = Optical density of the sample and OD = Optical density of the control.

2.6 Inoculum Preparation

Bacterial cultures were transferred from stock to 100-mL sterile distilled water, and the optical density (OD) was read at 660 nm. Accordingly, suspension with an OD_{660} of 0. 8 was selected as the standard inoculum size which contains 5. 5 x 10^7cells/mL.

2.7 Composition and preparation of the media for optimization of the pigment production

Different carbon source viz. , dextrose, fructose, lactose, maltose and sucrose and different nitrogen source viz. ,

urea, casein, peptone, beef extract, yeast extract, tyrosine, $NaNO_3$, NH_4Cl and $(NH_4)_2SO_4$ were supplemented in a nutrient agar spread plates at 1% concentration. The plates were then incubated at 37°C for 72 hours to analyse the influence of various carbon source for enhancing the yield of the pigment. After incubation, the growth of *Serratia marcescens* JGI 27 and the yield of the pigment produced was estimated. Growth was estimated by cell dry weight of the bacteria and pigment production by *Serratia marcescens* JGI 27 was estimated as absorbance at 499 nm, and after getting a straight line graph based on Beer lambert's law, absorbance was converted into the yield of pigment in mg/ml. Minimal amount of mineral component including $FeCl_3$, $CaCl_2$, $MgSO_4$, $MnCl_2$, $ZnSO_4$ and KH_2PO_4 at three different concentration (0. 01%, 0. 1% and 1%) in a nutrient agar spread plate was incorporated to evaluate the effect on yield of the pigment and bacterial cell dry weight. Influence of temperature shock condition on pigment production was also studied. The culture was kept at two different incubation time in combination with different temperature to study the effect of temperature shock conditions on pigment production and growth of the bacteria.

3. Results and Discussion

The bacteria was identified as *Serratia marcescens* JGI 27 based on Bergey's classification of determinative bacteriology and characteristic maxima (λ-max) of methanolic extract of red pigment was found at 487 nm and the yield of the pigment was found to be 40 mg/L.

Screening of cytotoxic potential of the crude extract was carried out by MTT Assay. We found that when HepG2 and Jurkat cells were treated with red pigment of different concentration (1, 5, 10 and 20µg/mL) the percentage viability of the HepG2 cell and Jurkat cells decreased from 64. 4% to 56. 4 % and 74. 4% to 54. 4 % respectively after 24 h of incubation and it further decreased to 55. 9 % and 53. 2 % after 72 h as the concentration increases from 2 µg/mL to 10 µg/mL (**Figure I**). The cytotoxic potential of the red pigment was found to be most effective at 20 µg/mL. The IC_{50} value was calculated around < 20µg/mL concentration, indicated that red pigment extract have significant cytotoxic potential against human liver cancer and leukemia cell line. When lymphocytes and CHO cells were treated with pigment of four different concentration (1, 5, 10, and 20 µg/mL) the percentage viability of lymphocytes and CHO was found to be 100% at 1 µg/ mL of concentration after 48 hrs, and lowest at 20 µg/mL after 72 hrs of incubation, indicating that the effect of pigment on lymphocytes was negligible at all the selected concentration even after 72 hrs of incubation

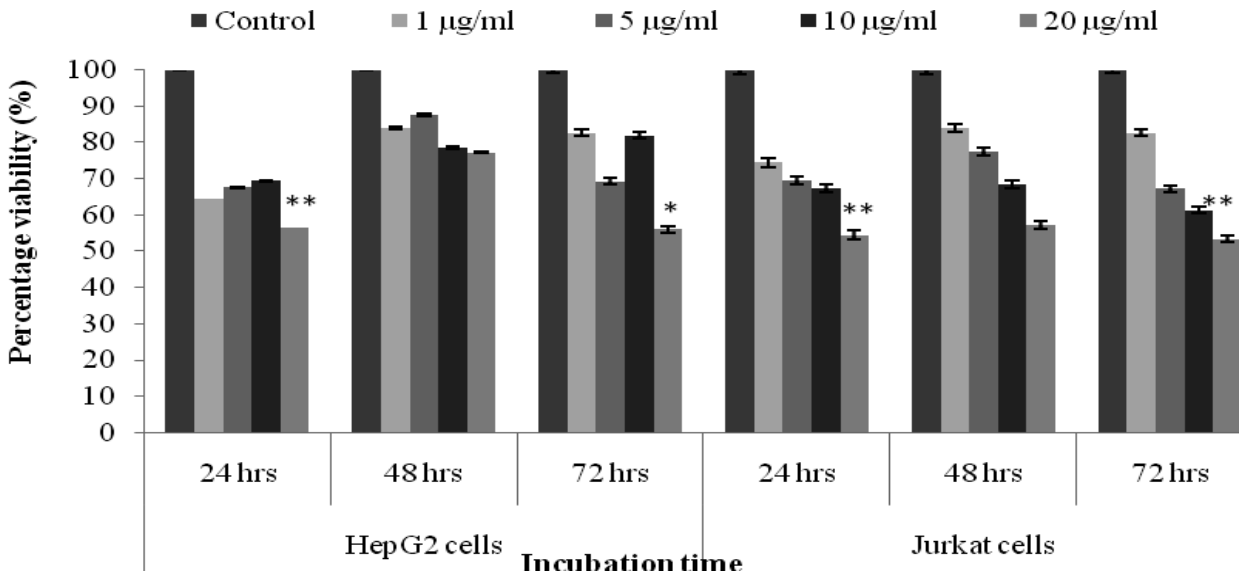

Figure I. Effect of different concentrations of *S. marcescens* crude extract on the percent viability of HepG2 and Jurkat cells when treated for 24, 48, and 72h. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0. 05 and ** indicates p< 0. 001 as compared to the control

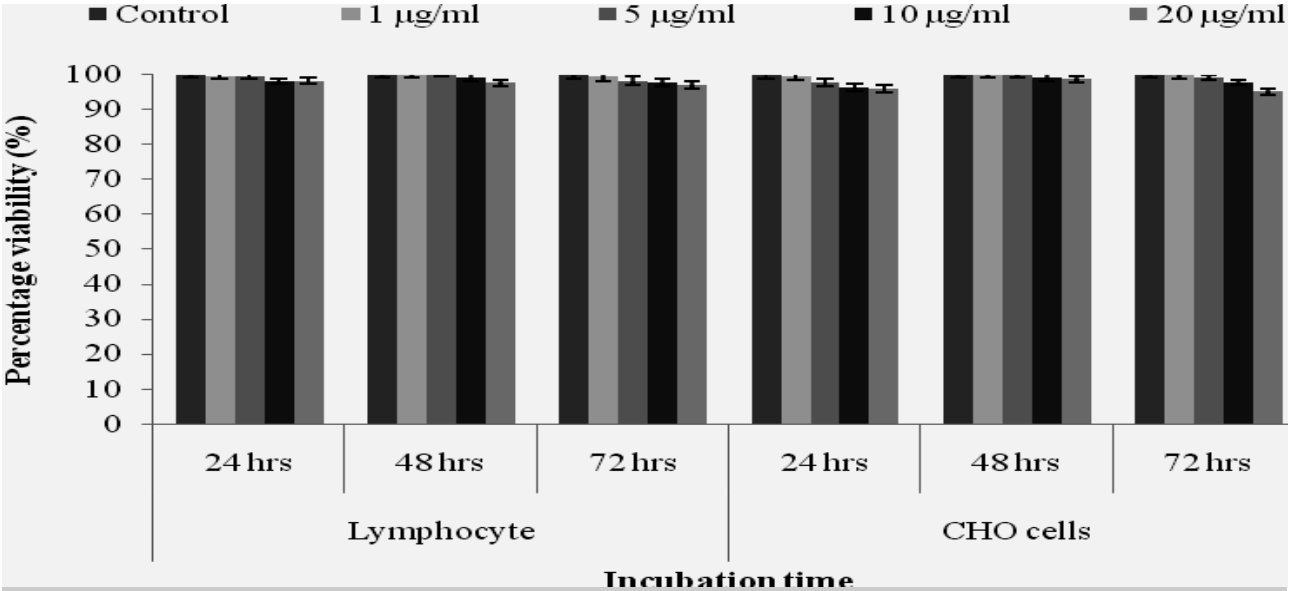

Figure II. Effect of different concentrations of *S. marcescens* crude extract on the percent viability of Lymphocytes and CHO cells when treated for 24, 48, and 72h. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0. 05 and ** indicates p< 0. 001 as compared to the control

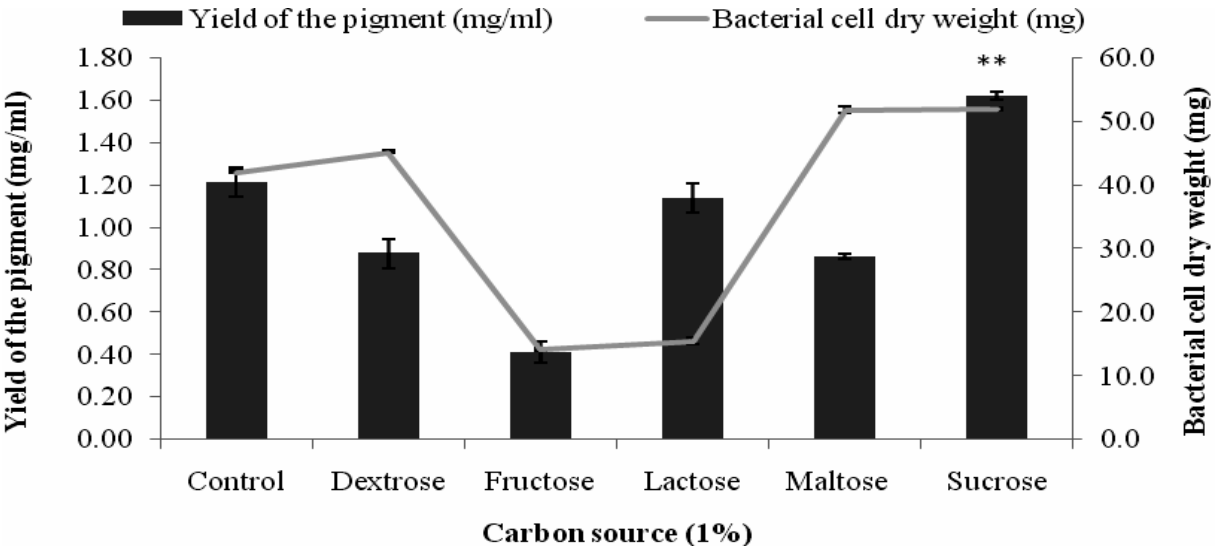

Figure III. Effect of various carbon source on pigment production and bacterial cell dry weight. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0. 05 and ** indicates p< 0. 001 as compared to the control.

(**Figure II**).

When *S. marcescens* were allowed to grow in a media supplemented with different carbon source (1%), maximum pigment production and dry weight of the bacterial cell were observed with treatment of Sucrose and yield of the pigment was found to be 1. 62 mg/ml (**Figure III**). Supplimentation of media with 1% dextrose, fructose, lactose and maltose did not influence the production of pigment . There is around 2 fold decrease in pigment production in media added with fructose as compared with control. Sundaramoorthy *et al*[7] found that, media supplemented with maltose highly influence the production of prodigiosin by *Serratia marcescens*. Chang *et al*[8] was reported the maximum prodigiosin production in a media supplemented with dextrose and it was found to be 3 mg/ml. Palanichamy *et al*[9] reported the highest biomass production of *Streptomyces* spp. in glycerol supplemented medium whereas highest pigment intensity was observed in both glycerol and mannose .

The effect of various organic and inorganic nitrogen source on pigment production and bacterial cell biomass

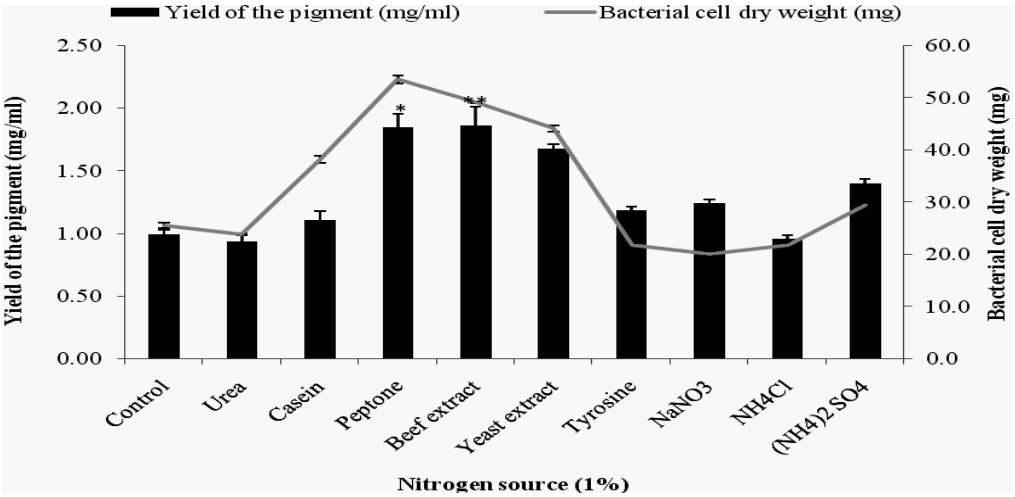

Figure IV. Effect of various nitrogen source on pigment production and bacterial cell dry weight. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0. 05 ** indicates p< 0. 001 as compared to the control

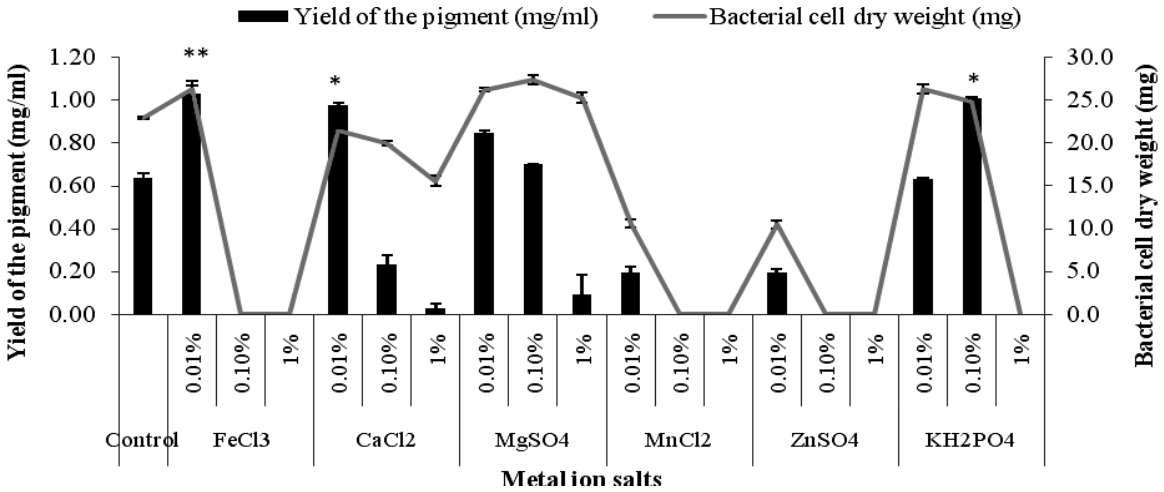

Figure V. Effect of various metal ion salts on pigment production and bacterial cell dry weight. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0. 05 ** indicates p< 0. 001 as compared to the control

was studied. When the bacteria was cultured in a media supplemented with different nitrogen source (1%), beef extract was found to be the best nitrogen source for maximum production of pigment (1. 87 mg/ml) (**Figure IV**). Yield of the pigment in a media incorporated with peptone was found to be 1. 85 mg/ml. Yeast extract, tyrosine, $NaNO_3$, and $(NH_4)_2SO_4$ also contributed in the pigment production. Pigment production was reduced in a media added with 1% urea, NH_4Cl and yeast extract, as compared to control. Hardjito *et al*[10] reported that yeast extract and beef extract are suitable substrate for growth of *Serretia marcescence*. Pradeep *et al*[11] reported the maximum prodigiosin pigment production from strain *S. marcescens* MBB05 in a media supplemented with dried yeast extract Chung *et al*[12] reported that among all the nitrogen sources tested monosodium glutamic acid (MSG) gave the highest specific productivity of red pigments.

Incorporation of 0. 01%, 0. 10% and 1% of metal ion salts including $FeCl_3$, $CaCl_2$, $MgSO_4$, $MnCl_2$, $ZnSO_4$ and KH_2PO_4 on the pigment yield was studied. Among all the metal ion salts, media incorporated with 0. 01% $FeCl_3$ showed the highest pigment production and it was found to be 1. 03 mg/ml. Media supplemented with 1% and 0. 1% of $FeCl_3$, $MnCl_2$, $ZnSO_4$ and 1% KH_2PO_4 resulted in no pigment and bacterial biomass production (**Figure V**). Chung *et al*[12] reported that out of three metals, Zn2+, Mn2+, and Fe2+, Fe2+ showed the strongest stimulatory

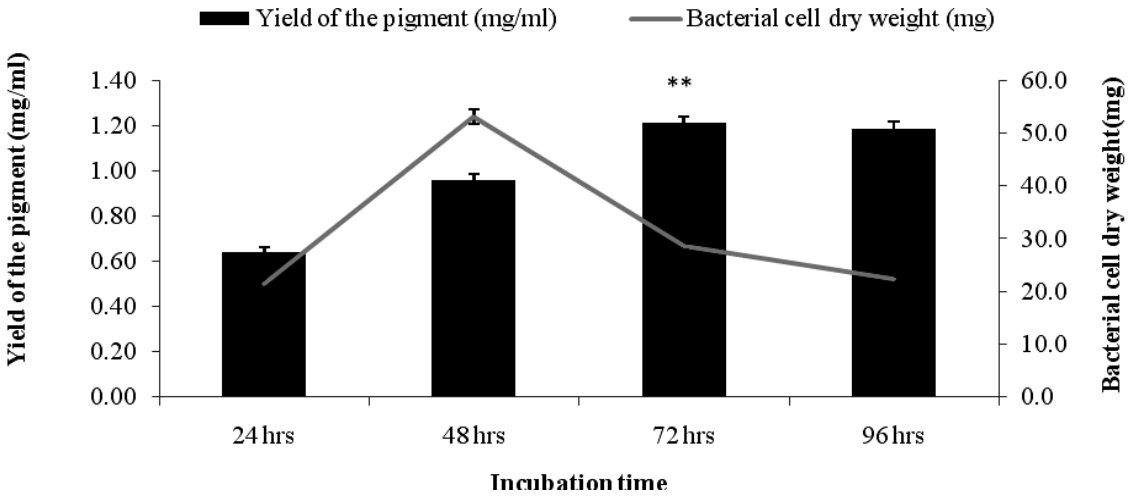

Figure VI. Effect of incubation time on pigment production and bacterial cell dry weight. Vertical bars indicate SE of the mean for n = 3, *indicates significance with p< 0. 05 ** indicates p< 0. 001 as compared to the control

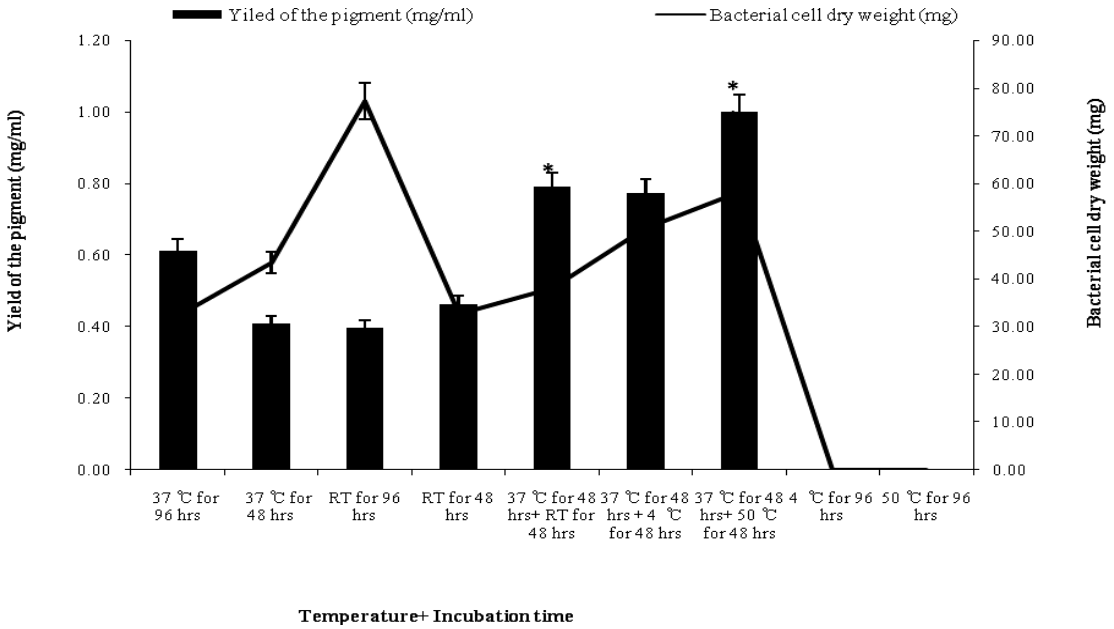

Figure VII. Effect of temperature shock conditions on pigment production and bacterial cell dry weight. Vertical bars indicate SE of the mean for n = 3. *indicates significance with p< 0. 05 ** indicates p< 0. 001 as compared to the control

effect on the red pigment production.

The production of pigment and bacterial cell dry weight was found to be highest after 72 hrs of incubation at 37° C. From 24 hrs to 72 hrs duration, both pigment production and bacterial cell dry weight increased (**Figure VI**) and yield of the pigment ranged between 0. 64 mg/ml to 1. 21 mg/ml. After 72 hrs the pigment production rate again decreased and it was found to be 1. 19 mg/ml. Pradeep *et al*[11] reported that during 60, 72, 84 and 96 h of incubation prodigiosin production ranged between 32. 2 and 116. 1 mg/mL, and whereas at 12, 24, 36 and 48 h it ranges between 110 to 177 mg/mL.

Influence of temperature shock on pigment production was also studied and maximum pigment production was found when the culture was kept at 37°C for 48 hrs then shifted to 50°C again for 48 hrs of incubation and yield was found to be 1 mg/ml which is more than2 fold increase as compared with the yield found when culture was kept at 37°C for 48 hrs which was found to be 0. 41 mg/ml (**Figure VII**). Out of nine different combination (37°C for 96 hrs, RT[24±2] for 96 hrs, RT[24±2] for 48 hrs, 37°C for 48 hrs+ RT[24±2] for 48 hrs, 37°C for 48

hrs+ 4°C for 48 hrs, 37°C for 48 hrs+ 50°C for 48 hrs, 4° C for 96 hrs and 50°C for 96 hrs, pigment production was found to be enhanced as compared with that of the control i. e 37°C for 48 hrs. Our isolated *Serratia marcescens* was very sensitive to temperature shock and this stress was probably responsible for the higher pigment production when incubated at 50 and 4°C after growth for 48hrs at 37°C. Cardona *et al*[13], reported that the bacteria *V. indigofera,* optimal temperature to produce the pigment was at 25°C, that for optimal growth was at 32°C but at that temperature there was no pigment production. They also reported that the bacteria may be sensitive to thermal shock, causing the bacteria to lose the pigment at temperatures higher or lower than 25 °C. Hejazi and Falkiner[14] reported that *Serratia marcescens* produce the red pigmentation only when grown at 25°C.

4. Conclusion

Serratia marcescens JGI 27 was isolated from soil and identified. Red pigment from *S. marcescens* JGI 27 was found to have a promising cyotoxic effect against HepG2 and Jurkat cell lines and IC_{50} value was < 20µg/mL and it is non-toxic to normal peripheral human lymphocytes and CHO cell lines. Production of the red pigment from *S. marcescens* was highly dependent on various parameters. Optimization of various physical and cultural parameters that effect the production of pigment was demonstrated. Based on the experiment conducted it was found that sucrose was the most suitable carbon source and beef extract was the best nitrogen source for enhanced pigment production and yield was found to be 1. 62 mg/ml and 1. 87 mg/ml respectively which was found to be an efficient enhancement. Among all the metal salts selected media incorporated with 0. 01% $FeCl_3$ showed the enhanced pigment production and it was found to be 1. 03 mg/ml. The optimum incubation time for pigment production was found to be 72 hrs. Influence of temperature shock on pigment production was also studied and maximum pigment production was found when the culture was kept at 37°C for 48 hrs then shifted to 50 °C for 48 hrs. At optimum physical and culture parameters the pigment production was found to be highest.

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Conflict of interest

The author's declares none.

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