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ORIGINAL ARTICLE

# Response Surface Optimization of Prodigiosin Production by Mutagen-Treated *Serratia marcescens* in Different Growth Media

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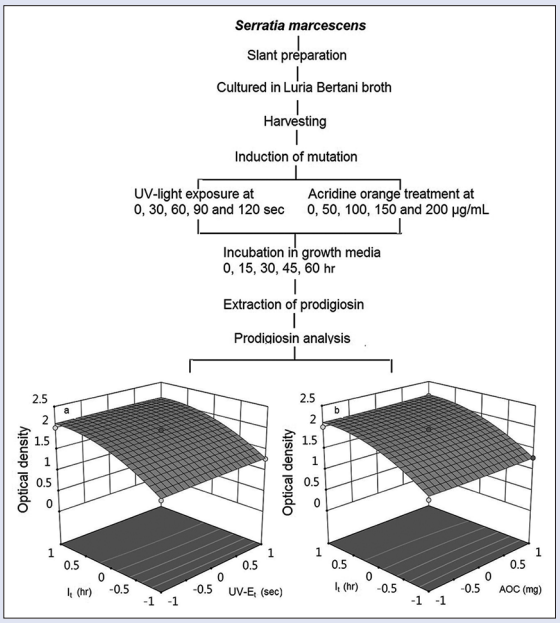
## ABSTRACT

**Background:** Prodigiosin is a bioactive bacterial pigment produced by *Serratia marcescens* and possesses anticancer, antibiotic, antifungal, antibacterial, and immunosuppressive activities without any toxic effects on human cells. **Objectives:** The study was planned to optimize the effect of ultravoilet (UV) light and acridine orange (AO), as mutagens and incubation time on prodigiosin production by *S. marcescens* in nutrient agar broth and peptone glycerol broth. **Materials and Methods:** The optimization was done by constructuing a quadratic polynomial response surface model using a bifactorial central composite design. The *S. marcescens* starian was treated with UV light (0, 30, 60, 90, and 120 s) and various concentrations of AO (0, 50, 100, 150, and 200 µg/mL) and incubated in the selected growth media for different times (0, 15, 30, 45, and 60 h). The prodigiosin production was measured in terms of change in pH and optical density (OD) of the ethanolic extracts of culture media. **Results:** The prodigiosin production was found to be a significant linear and quadratic function of incubation time in both of the culture media. However, a statistically non-significant increased prodigiosin production, in terms of pH and OD of the extracts, was observed under the influence of both mutagens. The optimum values of incubation time to achieve optimal production of prodigiosin from UV light-exposed and AO-treated cultures were found to be in the range of 37–60 h, respectively. **Conclusion:** The pigment production was increased significantly with an increase in the incubation time. The study provides the optimum conditions for the production of prodigiosin in research and commercial field.

**Key words:** Acridine orange, prodigiosin production, response surface optimization, *Serratia marcescens,* ultravoilet light

## SUMMARY

- Prodigiosin is a bioactive bacterial pigment produced by *Serratia marcescens*. In the present study, the effect of ultravoilet (UV) light, acridine orange, and incubation time on prodigiosin production by *S. marcescens* was optimized by constructing quadratic polynomial response surface models. The prodigiosin production was found to be a significant linear and quadratic function of incubation time. However, UV light and acridine orange treatment showed statistically non-significant increase in prodigiosin production. The optimum values of incubation time to achieve optimal production of prodigiosin were found to be in the range of 37–60 h.




**Abbreviations used:** AP: Adequate precision; AO: Acridine orange; AOC: Acridine orange concentration; AOLs: Actual optimum levels; CCD: Central composite design; CE: Coefficient estimate; COL: Coded optimum levels; CV: Coefficient of variation; $I_t$: Incubation time; LBB: Luria Bertani Broth; NAS: Nutrient agar slant; NB: Nutrient broth; OD: Optical density; PGB: Peptone glycerol broth; $R^2$: Regression coefficient; SD: Standard deviation; UV: Ultravoilet; UV-$E_t$: UV exposure time.

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## INTRODUCTION

*Serratia marcescens*, a Gram-negative, facultative anaerobic human pathogen, produces prodigiosin, a red-colored pigment as a secondary metabolite.[1,2] Prodigiosin performs a protective role for bacterial cell under stress. It protects the bacterial cell by minimizing the production of reactive oxygen species in aerobic growth conditions through an energy spilling mechanism.[3-5] Prodigiosin is a biologically active compound possessing anticancer, antibiotic, antifungal, antibacterial, and immunosuppressive activities with no



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toxicity toward human cells. It has been found to cause apoptotic effect against cancerous cell lines, particularly the multidrug resistant cells, through activation of tumor necrosis factors which leads to cell shrinkage, chromatin condensation, and finally, the DNA fragmentation.[6-8] Prodigiosin shows antifungal activity along with some chitinolytic enzymes and other substances leading to the cell wall rupture and finally the fungal cell lysis.[9] It has been also found to show antibacterial activity against certain species of bacteria by pH modulation, enzymatic cleavage of bacterial DNA, and inhibition of cell cycle.[4] Prodigiosin is also considered as an alternative to synthetic pigments and gained importance as a dye in the paper, rubber latex, and polymethyl methacrylate preparation.[7,9]

The production of prodigiosin by *S. marcescens* may be affected by exposure to physical, chemical and viral mutagens and culture conditions such as pH and nutritional composition of the growth medium, incubation time, and temperature.[10,11] Ultraviolet (UV) light has been reported to be a physical mutagen for *S. marcescens* which cause distortion of the DNA structure leading to lethal mutation at 254 nm. Although *S. marcescens* continues its survival after UV exposure for a short period of time, a prolonged UV exposure results in a full stop on its prodigiosin-producing ability.[3,12-15] Acridine orange is a dye which is used for staining of bacterial strains. It shows mutagenic activity against a variety of bacteria including *S. marcescens* and interfere with their metabolic activities. The prodigiosin producing ability of *S. marcescens* is inversely correlated with acridine orange concentration (AOC) in the culture medium. High concentrations of AOC results in a full stop on the survival frequency of *S. marcescens* strains and a decrease in the prodigiosin production.[16,17] Prodigiosin is aerobically produced by *S. marcescens* at optimal conditions of pH (5–9) and temperature (30°C–32°C). The composition of the culture medium also affects the growth curve and finally the production of metabolites including prodigiosin.[1]

The prodigiosin production may be enhanced by optimizing these factors in the region of its optimal yield. Previously, optimization studies have reported the optimum levels of pH, temperature and nutritional composition of the medium to enhance the yield of prodigiosin from *S. marcescens*.[1,18,19] Recently, another study has reported the optimized levels of crude glycerol as a carbon source and gamma radiation dose to enhance the production of prodigiosin by *S. marcescens*.[20] However, no literature was found on the optimization of prodigiosin production by *S. marcescens* under the influence of UV light and acridine orange (AO) as mutagens and incubation time.

The present study was, therefore, designed to optimize the effect of the physical as well as chemical mutagens and incubation time on the prodigiosin production capacity of *S. marcescens* in two different growth media. A bifactorial response surface central composite design (CCD) was developed which determined the relationship between UV light as a physical mutagen, acridine orange as a chemical mutagens, incubation time and prodigiosin production in nutrient broth (NB), and peptone glycerol broth (PGB) as culture media. The study would be a valuable contribution to the literature and a useful guideline for the microbiologists regarding the production of prodigiosin from *S. marcescens* for therapeutic purpose.

# MATERIALS AND METHODS

## Experimental design

The study was planned to optimize the effect of UV light a physical mutagen and acridine orange as chemical mutagen and the duration of incubation on the production of prodigiosin by *S. marcescens* in two different growth media. A bifactorial response surface CCD with 4 factorials, 4 axial, and 5 central points was constructed to optimize the effect of two variables: $X_1$: UV-exposure time (UV-$E_t$) or AOC and $X_2$: Incubation time ($I_t$) on prodigiosin production by *S. marcescens* in different growth media. The coded levels of the selected factors were selected on a scale of −2–2 including 0 as the center point. The actual five levels of the selected factors were set as UV-$E_t$: 0, 30, 60, 90, 120 s, AOC: 0, 50, 100, 150, 200 µg/mL and $I_t$: 0, 15, 30, 45, 60 h. The coded values at specific levels of the selected input variables were calculated using the following generalized equation (Eq. 1).[21]

$$X_i = \left[\frac{v_i - c_{vi}}{d_i}\right] \quad i = 1, 2, 3 \ldots k \tag{1}$$

Where $X_i$ is the coded value of a particular level of the selected input variable, $v_i$ is the specific level of the input variable, $c_{vi}$ is the central level of the input variable, and $d_i$ is the interval between two levels of the input variable.

The coded levels of UV-$E_t$, AOC, and $I_t$ at specific levels were calculated from Eq. (2–4):

$$X_1 = \left[\frac{UV - Exposure\ time\,(s) - 60}{30}\right] \tag{2}$$

$$X_1 = \left[\frac{Acridine\ orange\ concentration\,(\mu g/mL) - 100}{50}\right] \tag{3}$$

$$X_2 = \left[\frac{Incubation\ time\,(s) - 30}{15}\right] \tag{4}$$

The coded values of the selected input variables as calculated from the above equations along with the actual values are presented in the design matrix [Table 1]. The CCD suggested 13 experiments for each of the UV-$E_t$ and AOC with 6-factorial, 4-axial, and 5-center points.

The wild strain of *S. marcescens* was cultured in Luria Bertani Broth (LBB) and treated with UV light at selected levels of exposure time as a physical mutagen. The wild strain was also treated with different concentrations of acridine orange as a chemical mutagen as selected by CCD. The wild and mutant strains were incubated for selected levels of $I_t$ in two different media including NB and PGB for growth and production of prodigiosin. The cultures were extracted in ethanol and the production of prodigiosin was measured in terms of change in pH and optical density (OD) ($\lambda_{max}$ = 560 nm) of the extracts. The design matrices containing the coded and actual levels of input variables and their random combinations as selected by CCD for both the physical and chemical mutagenesis are presented in Table 1.

## Preparation of culture media

### *Nutrient agar slant preparation*

The nutrient agar slant (NAS) was prepared by dissolving nutrient agar (2.8 g) in distilled water (100 ml) at pH 7.0. The solution was distributed into test tubes (5 mL each) and sterilized in an autoclave at 121°C at 15 lb pressure for 15 min. The test tubes were placed in a slanting position until the medium could solidify.

### *Luria Bertani Broth*

The LBB was prepared by mixing 1% bactotrytone, 0.5% bactoyeast extract, and 1% NaCl at pH 7.0 and then dissolving the mixture (1.5 g) in distilled water (100 mL) at pH 7.0 in Erlenmeyer flask (250 mL) followed by sterilization in an autoclave at 121°C for 30 min.

**Table 1:** Experimental values of pH and optical density of the extracts obtained from two different cultures of the control and mutant strains of *Serratia marcescens* treated at various levels of mutagenic factors and incubation time as selected by central composite design

| Effect of UV-$E_t$ (s) and $I_t$ (h) on prodigiosin production | | | | | | | |
|---|---|---|---|---|---|---|---|
| Standard | Run | $X_1$ (UV-$E_t$) | $X_2$ ($I_t$) | Prodigiosin concentration | | | |
| | | | | pH | | OD | |
| | | | | NB | PGB | NB | PGB |
| 1 | 3 | −1 (30)* | −1 (15) | 7.83 | 6.73 | 1.21 | 0.57 |
| 2 | 2 | 1 (90) | −1 (15) | 7.92 | 6.66 | 1.28 | 0.52 |
| 3 | 13 | −1 (30) | 1 (45) | 9.2 | 7.87 | 2.01 | 1.40 |
| 4 | 1 | 1 (90) | 1 (45) | 9.18 | 7.98 | 2.04 | 1.36 |
| 5 | 9 | −2 (0) | 0 (30) | 8.89 | 7.42 | 1.91 | 1.08 |
| 6 | 4 | 2 (120) | 0 (30) | 8.98 | 7.75 | 1.95 | 1.23 |
| 7 | 10 | 0 (60) | −2 (0) | 7.19 | 6.91 | 0.16 | 0.05 |
| 8 | 8 | 0 (60) | 2 (60) | 9.36 | 8.00 | 1.89 | 1.78 |
| 9 | 5 | 0 (60) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.26 |
| 10 | 7 | 0 (60) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.26 |
| 11 | 6 | 0 (60) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.26 |
| 12 | 12 | 0 (60) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.26 |
| 13 | 11 | 0 (60) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.26 |
| Mean±SD | | | | 8.73±0.18 | 7.49±0.27 | 1.71±0.06 | 1.10±0.12 |
| **Effect of AOC (µg/mL) and $I_t$ (h) on prodigiosin production** | | | | | | | |
| Standard | Run | $X_t$ (AOC) | $X_2$ (It) | Prodigiosin production | | | |
| | | | | pH | | OD | |
| | | | | NB | PGB | NB | PGB |
| 1 | 2 | −1 (50) | −1 (15) | 7.91 | 6.66 | 1.20 | 0.52 |
| 2 | 7 | 1 (150) | −1 (15) | 7.84 | 6.74 | 1.29 | 0.57 |
| 3 | 3 | −1 (50) | 1 (45) | 9.20 | 7.87 | 2.01 | 1.39 |
| 4 | 6 | 1 (150) | 1 (45) | 9.22 | 7.97 | 2.14 | 1.49 |
| 5 | 13 | −2 (0) | 0 (30) | 8.93 | 7.67 | 2.02 | 1.14 |
| 6 | 10 | 2 (200) | 0 (30) | 8.95 | 7.65 | 1.99 | 1.29 |
| 7 | 12 | 0 (100) | −2 (0) | 7.20 | 6.91 | 0.16 | 0.05 |
| 8 | 4 | 0 (100) | 2 (60) | 9.37 | 8.00 | 1.89 | 1.78 |
| 9 | 8 | 0 (100) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.27 |
| 10 | 1 | 0 (100) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.27 |
| 11 | 11 | 0 (100) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.27 |
| 12 | 9 | 0 (100) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.27 |
| 13 | 5 | 0 (100) | 0 (30) | 8.98 | 7.62 | 1.95 | 1.27 |
| Mean±SD | | | | 8.73±0.18 | 7.50±0.27 | 1.71±0.06 | 1.12±0.11 |

*The values in parentheses present the actual values of the input factors at the respective codes as selected by CCD. UV-$E_t$: Ultraviolet-Exposure time; $I_t$: Incubation time; OD: Optical density; NB: Nutrient broth; PGB: Peptone glycerol broth; AOC: Acridine orange concentration; SD: Standard deviation; CCD: Central composite design

## Preparation of prodigiosin producing media

### *Nutrient broth*

Peptone (10 g), sodium chloride (5 g), and yeast extracts (3 g) were dissolved in distilled water (100 mL) at pH 7.0. The medium was dispensed into erlenmeyer flask (250 mL) and autoclaved for 30 min at 121°C.

### *Peptone glycerol broth*

Bacto-peptone (5 g) and glycerol (10 mL) were dissolved in distilled water (100 mL) at pH (7.0). The medium was added into Erlenmeyer flask (250 mL) and autoclaved for 30 min at 121°C.

### *Peptone glycerol agar*

Bacto peptone (5 g), bacto agar (15 g), and glycerol (10 mL) were dissolved in distilled water (100 mL) at pH (7.0). The medium was added into Erlenmeyer flask (250 mL) and autoclaved for 30 min at 121°C.

## Bacterial strain and culture condition

The wild-type *S. marcescens* strain (BDCS-N-S1) was acquired from Biodiversity Collection Section (BDCS), Nuclear Institute of Agriculture and Biology (NIAB) Faisalabad, Pakistan. The wild-type *S. marcescens* was isolated from the rhizoplane of rice and was maintained on NAS by subculturing. The solidified NAS was cultured with freshly purified wild-type *S. marcescens* strain in a microbial safety cabinet and incubated at 28°C for 24 hr for growth using VWR scientific incubator.

## Induction of mutation

Random mutations in bacterial strains were induced using UV light, a physical mutagen and acridine orange, a chemical mutagen. UV light induces mutation in the DNA structure by formation of pyrimidine dimmers in adjacent bases while the acridine orange works by intercalating in the DNA structure between the base pairs that push the nucleotides away or by introducing an additional nucleotide to the developing chain of DNA during replication.

### *Induction of mutation with ultravoilet-exposure*

The wild-type *S. marcescens* strain from the slant was grown on LBB followed by overnight incubation at 28°C with continuous shaking at 150 rpm in a shaker incubator (lab-line shaker-incubator). The culture was diluted in LBB (1:50) to achieve a cell density of

approximately 2 × $10^8$ cells measured in terms of OD at 600 nm (0.4) using a spectrophotometer (CECIL CE 2041). The diluted culture was reincubated at 28°C in a water bath for 2 hr, placed on a rotor for further 2 hr and chilled in ice to prevent further growth. The bacterial culture was harvested by centrifugation at 5000 × *g* for 5 min, suspended in 0.1 M $MgSO_4$ solution (1:1 w/v), placed on ice for 10 min and subjected to UV light exposure for mutagenesis.

*S. marcescens* culture was exposed to UV light following the previously reported method of Miller (1992) as described by Arshad *et al.*[22] The bacterial culture was exposed to UV light at 254 nm in a Petri plate with overlaid cell suspension under a UV lamp at 30 cm sample to source distance for different levels of exposure time (30, 60, 90, and 120 s) as selected by CCD. The control (UV-$E_t$ = 0) and UV light-exposed cultures (0.1 mL each) were incubated at 37°C for 24 hr.

### *Induction of mutation with acridine orange*

The mutant strain of *S. marcescens* was also prepared by treating the wild strain with AO following the previously described method.[17,22] The NB was inoculated with a fresh culture of *S. marcescens* strain maintained on NAS and the culture was incubated at 28°C with shaking at 150 rpm for 18 hr using Lab-line shaker-incubator. The bacterial culture grown in NB (2 mL) was treated with different concentrations of filter-sterilized AO (0, 50, 100, 150, and 200 µg/mL) as selected by experimental design. The AO treated culture was incubated at 37°C for 18 hr and centrifuged at 3000 × *g*. The AO was removed by repeated washings with distilled water, the bacterial cells were suspended in phosphate buffer (pH 7.5), and the absorbance of the media was recorded at 600 nm to access the growth.

## Prodigiosin production and analysis

The production of prodigiosin by the wild and mutant strains at various incubation times in NB and PGB growth media was analyzed in terms of the change in pH and OD of the media following the previously described protocols with some modification.[23] For the production of prodigiosin, the wild and mutant strains were grown in NB as well as PGB. The fresh inoculums from wild and mutant strains (0.5% v/v) were added to each of the prodigiosin producing medium (100 mL) in 250 mL Erlenmeyer flask and incubated at 28°C in PGB and 37°C in NB for selected levels of incubation time (0, 15, 30, 45, and 60 h) with continuous shaking at 150 rpm. The pigmented cultures were harvested by centrifugation at 5000 × *g* for 15 min and the cells were suspended in 95% ethanol. The cell suspension was incubated overnight at 28°C followed by the separation of clear pigment solution from the debris by centrifugation at 5000 × *g* for 15 min. The pigment solution (2 mL) was subjected to the measurement of pH and OD at 600 nm (CECIL CE 2041) as a response for prodigiosin production. The 95% ethanol was used as a blank.

## Statistical analysis

Response surface models were created for the prediction of changes in the response and optimization of independent variables. The significance, adequacy and reliability of the suggested model were determined by analysis of variance (ANOVA). The trends of variation in the response in relation to the independent variables were studied by developing the second order polynomial regression equations based on coefficient of estimates as shown by the following generalized expression (Eq. 5).

$$Yi = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (5)$$

Where *Yi* is the predicted response, $\beta_0$ is a constant, $\beta_1$ and $\beta_2$ are the regression coefficients for linear effects, $\beta_{11}$ and $\beta_{22}$ for quadratic effects, and $\beta_{12}$ for the interaction effect of input variables on the response.

The significance of variation in the response was determined by calculating the lack of fit (*F*-value) and the probability (*P* value) at 95% confidence level. The adequacy, reliability, and validity of the response surface model were determined by calculating the adequate precision (AP), the coefficient of variation (CV), and regression coefficient ($R^2$), respectively. The development of experimental design, data analysis, and optimization procedures were performed using the statistical software Design Expert 8.0.4.1 (Stat-Ease, Inc., Minneapolis, USA).

# RESULTS

## Production of prodigiosin

The concentration of prodigiosin was measured in terms of pH and OD of the medium. The actual values of pH and OD of the ethanolic extract of the culture media obtained at selected levels of UV-$E_t$ and $I_t$ are given in Table 1. The pH of the extracts obtained from NB growth medium at various combinations of UV-$E_t$ and $I_t$ ranged from 7.1952 to 9.3625 with a mean value 8.73 ± 0.18 while that of the extracts obtained from PGB medium at the similar combinations of input variables was found to be in range of 6.66–8.0025 with mean value 7.49 ± 0.27. The OD of the extracts obtained from NB and PGB growth media at various combinations of UV-$E_t$ and $I_t$ ranged from 0.1605 to 2.035 and 0.051 to 1.7755 with mean values 1.71 ± 0.06 and 1.10 ± 0.123, respectively.

The actual values of pH and OD of the ethanolic extract of the culture media obtained at selected levels of AOC and $I_t$ are also given in Table 1. The pH and OD of the extracts obtained from NB growth media at various combinations of AOC and $I_t$ ranged from 7.195 to 9.3625 and 0.1605 to 2.135 with mean values 8.73 ± 0.18 and 7.50 ± 0.27, respectively. The pH and OD of the extracts obtained from PGB medium at the similar combinations of input variables were found to be in the range of 6.6575 to 8.0025 and 0.051 to 1.7755, with mean values 1.71 ± 0.06 and 1.12 ± 0.11, respectively. The wild and the mutant strains cultured in NB as growth medium showed higher values of pH and OD as compared to those cultured in PGB.

## Response surface analysis and optimization of results

The variation in the experimental results at various combinations of the input factors was analyzed statistically by one-way ANOVA applying response surface methodology. The experimental data were found to be fit in a response surface polynomial quadratic model to locate the region of optimal response under the influence of the selected factors. The response surface models yielded the following quadratic polynomial regression equations (Eq. 6–13) showing an empirical relationship between the UV-$E_t$, $I_t$, and prodigiosin production in different media.

$$pH\ in\ NB = 8.91 + 0.0206X_1 + 0.5806X_2 - 0.0170X_1^2 - 0.1798X_2^2 - 0.0269X_1X_2 \quad (6)$$

$$pH\ in\ PGB = 7.54 + 0.0569X_1 + 0.3865X_2 - 0.0126X_1^2 - 0.0441X_2^2 + 0.0444X_1X_2 \quad (7)$$

$$OD\ in\ NB = 1.93 + 0.0140X_1 + 0.4186X_2 - 0.0075X_1^2 - 0.2329X_2^2 - 0.0123X_1X_2 \quad (8)$$

$$OD\ in\ PGB = 1.21 + 0.0164X_1 + 0.4267X_2 - 0.0293X_1^2 - 0.0903X_2^2 + 0.0052X_1X_2 \quad (9)$$

However, the response surface quadratic polynomial regression equations showing the relationship between the AOC, $I_t$ and production of prodigiosin in different media were obtained as follows:

$$pH\ in\ NB = 8.91 - 0.0015X_1 + 0.5840X_2 - 0.0144X_1^2 - 0.1797X_2^2 + 0.0244X_1X_2 \quad (10)$$

$$pH\ in\ PGB = 7.54 + 0.0123X_1 + 0.3860X_2 + 0.0058X_1^2 - 0.0445X_2^2 + 0.0056X_1X_2 \quad (11)$$

$$OD\ in\ NB = 1.93 + 0.0127X_1 + 0.4269X_2 + 0.0112X_1^2 - 0.2328X_2^2 + 0.0098X_1X_2 \quad (12)$$

$$OD\ in\ PGB = 1.21 + 0.0367X_1 + 0.4374X_2 - 0.0146X_1^2 - 0.0900X_2^2 + 0.0112X_1X_2 \quad (13)$$

These polynomial regression equations included the main, interaction and quadratic effects, and coefficient for intercept. The influence of each of the selected factor on the responses is shown by the sign and magnitude of the main effect.

The optimum values of the input variables to achieve the optimal response were obtained from numerical optimization of the data. The coded optimum levels (COLs) of the selected factors to achieve a maximal response of prodigiosin production in NB and PGB is presented in Table 2. The actual optimum levels (AOLs) of the factors were calculated from the coded values in the design using the following generalized equation (Eq. 14).

$$AOL = COL \times I_{AL} + CP_{AL} \quad (14)$$

Where AOL is the specific AOL of a particular variable whereas COL is the COL of the factors, $I_{AL}$ is the interval between two actual levels, and $CP_{AL}$ is the central point of actual levels of that variable.

The calculated values of AOL along with their respective COL and prediction of the response are given in Table 2. The optimum levels of UV-$E_t$ and $I_t$ to achieve optimal production of prodigiosin in terms of pH (9.377, 8.104) in the extracts obtained from NB and PGB were found to be 49.25 and 76.46 s and 57.86 and 53.43 hr, respectively. The optimum levels of UV-$E_t$ to achieve an optimal value of OD (2.113, 1.709) in the extracts obtained from NB and PGB were found to be 92.26 and 73.73 s and 43.54 and 60 hr, respectively. On the other hand, the optimum levels of AOC to achieve an optimal value of pH (9.374, 8.154) in the extracts obtained from NB and PGB were found to be 99.94 and 167.19 µg/mL and 50.65 and 58.56 hr, respectively, while those to achieve an optimal value of OD (2.151, 1.778) in the extracts obtained from NB and PGB were found to be 190 and 174.56 mg/mL and 37.50 and 58.90 hr, respectively.

## DISCUSSION

The present study was designed to optimize the effect of physical and chemical mutagens on the production of prodigiosin by *S. marcescens* in two different culture media. Prodigiosin is a medicinally important bacterial pigment which can be extracted in alcohols and effectively used for the treatment of various diseases.[6,8] The production of prodigiosin by the bacteria has been reported to be affected by various factors including the mutagens and culture conditions including the incubation time, temperature, pH, and the nutritional composition of the growth medium.[13,18,19,24] The optimization of these factors would be helpful for the researchers and the manufacturers in enhancing the production of prodigiosin on experimental as well as industrial level.

The response surface optimization of the data showed significant main effect ($P < 0.05$) of UV-$E_t$ and $I_t$ on the pH and OD of the extracts

**Table 2:** Coded and actual optimum levels of input variables at optimal response

| Response | Culture medium | X1: UV-$E_t$ (s) | | X1: AOC (µg/mL) | | X2: $I_t$ (h) | | Prediction |
|---|---|---|---|---|---|---|---|---|
| | | COL | AOL | COL | AOL | COL | AOL | |
| pH | NB | −0.358 | 49.25 | | | 1.857 | 57.86 | 9.377 |
| | PGB | 0.549 | 76.46 | | | 1.562 | 53.43 | 8.105 |
| | NB | | | −0.0013 | 99.94 | 1.376 | 50.65 | 9.374 |
| | PGB | | | 1.340 | 167.19 | 1.904 | 58.56 | 8.154 |
| OD | NB | 1.076 | 92.26 | | | 0.903 | 43.54 | 2.113 |
| | PGB | 0.458 | 73.73 | | | 2.00 | 60.00 | 1.709 |
| | NB | | | 1.800 | 190.00 | 0.50 | 37.50 | 2.151 |
| | PGB | | | 1.490 | 174.56 | 1.927 | 58.91 | 1.778 |

UV-$E_t$: Ultraviolet-Exposure time; AOC: Acridine orange concentration; $I_t$: Incubation time; COL: Coded optimum level; AOL: Actual optimum level; NB: Nutrient broth; PGB: Peptone glycerol broth; OD: Optical density

**Table 3:** Analysis of variance of the data based on the effect of ultraviolet-exposure time and incubation time on prodigiosin production

| Source | NB | | | | | | PGB | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH | | | OD | | | pH | | | OD | | |
| | CE | *F* | *P* | CE | *F* | *P* | CE | *F* | *P* | CE | *F* | *P* |
| Model | 8.91 | 30.27 | 0.0001 | 1.93 | 198.78 | <0.0001 | 7.54 | 5.21 | 0.0259 | 1.21 | 31.39 | 0.0001 |
| A: UV-$E_t$ (s) | 0.0206 | 0.1602 | 0.7009 | 0.0140 | 0.6831 | 0.4358 | 0.0569 | 0.5371 | 0.4874 | 0.0164 | 0.2143 | 0.6574 |
| B: $I_t$ (h) | 0.5806 | 126.96 | <0.0001 | 0.4186 | 608.38 | <0.0001 | 0.3865 | 24.80 | 0.0016 | 0.4267 | 144.35 | <0.0001 |
| AB | −0.0269 | 0.0907 | 0.7721 | −0.0123 | 0.1758 | 0.6875 | 0.0444 | 0.1090 | 0.7510 | 0.0052 | 0.0071 | 0.9353 |
| $A^2$ | −0.0170 | 0.2075 | 0.6625 | −0.0075 | 0.3702 | 0.5621 | −0.0126 | 0.0501 | 0.8293 | −0.0293 | 1.30 | 0.2913 |
| $B^2$ | −0.1798 | 23.25 | 0.0019 | −0.2329 | 359.82 | <0.0001 | −0.0441 | 0.6175 | 0.4577 | −0.0903 | 12.34 | 0.0098 |
| $R^2$ | 0.9558 | | | 0.9930 | | | 0.7883 | | | 0.9573 | | |
| Adjusted $R^2$ | 0.9242 | | | 0.9880 | | | 0.6371 | | | 0.9268 | | |
| Predicted $R^2$ | 0.6746 | | | 0.9467 | | | −0.8974 | | | 0.6949 | | |
| CV (%) | 2.05 | | | 3.44 | | | 3.59 | | | 11.19 | | |
| AP | 19.1512 | | | 48.7969 | | | 8.4641 | | | 20.4208 | | |

NB: Nutrient broth; PGB: Peptone glycerol broth; OD: Optical density; CE: Coefficient estimate; UV-$E_t$: Ultraviolet-exposure time; $I_t$: Incubation time; $R^2$: Regression coefficient; CV: Coefficient of variation; AP: Adequate precision

obtained from NB and PGB [Table 3]. However, the UV-$E_t$ showed a non-significant linear positive effect and non-significant quadratic negative effect on the pH and OD of the extracts obtained from the cultures in NB and PGB respectively. However, the $I_t$ showed significant linear and quadratic positive effects on the pH and OD of the extracts obtained from each culture. The interaction effect of both variables was also found to be non-significant on prodigiosin production in each media.

The AOC and $I_t$ also showed a significant main effect ($P < 0.05$) on the pH and OD of the extracts obtained from NB and PGB [Table 4]. The AOC showed non-significant linear positive and quadratic negative effects on both the pH and OD of the extracts obtained from each culture. The $I_t$ showed a significant positive linear and quadratic effect on pH and OD of extracts each obtained from each of the culture media. However, the interaction effect of AOC and $I_t$ on pH and OD in extracts from each culture was found to be non-significant.

The measurement of regression coefficient indicated that the model is significant with high degree of fitness of the data points on the regression line. The values of the adjusted regression coefficient also advocated the significance of the model. The values of CV for pH in NB, pH in PGB, OD in NB and OD in PGB was found to be 2.04, 3.78, 3.30, and 10.84, respectively. A relatively low value of CV indicates the better precision

**Table 4:** Analysis of variance of the data based on the effect of AOC and it on prodigiosin production

| Source | NB | | | | | | PGB | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | pH | | | OD | | | pH | | | OD | | |
| | CE | *F* | *P* | CE | *F* | *P* | CE | *F* | *P* | CE | *F* | *P* |
| Model | 8.91 | 31.00 | 0.0001 | 1.93 | 193.37 | <0.0001 | 7.54 | 4.92 | 0.0300 | 1.21 | 44.10 | <0.0001 |
| A: AOC (μg/mL) | −0.0015 | 0.0008 | 0.9781 | 0.0127 | 0.5247 | 0.4923 | 0.0123 | 0.0242 | 0.8808 | 0.0367 | 1.42 | 0.2715 |
| B: $I_t$ (h) | 0.5840 | 130.31 | <0.0001 | 0.4269 | 590.54 | <0.0001 | 0.3860 | 23.84 | 0.0018 | 0.4374 | 202.43 | <0.0001 |
| AB | 0.0244 | 0.0757 | 0.7912 | 0.0098 | 0.1043 | 0.7562 | 0.0056 | 0.0017 | 0.9684 | 0.0112 | 0.0441 | 0.8397 |
| $A^2$ | −0.0144 | 0.1504 | 0.7096 | 0.0112 | 0.7764 | 0.4075 | 0.0058 | 0.0104 | 0.9216 | −0.0146 | 0.4304 | 0.5328 |
| $B^2$ | −0.1797 | 23.55 | 0.0018 | −0.2328 | 335.45 | <0.0001 | −0.0445 | 0.6043 | 0.4624 | −0.0900 | 16.36 | 0.0049 |
| $R^2$ | 0.9568 | | | 0.9928 | | | 0.7784 | | | 0.9692 | | |
| Adjusted $R^2$ | 0.9259 | | | 0.9877 | | | 0.6201 | | | 0.9473 | | |
| Predicted $R^2$ | 0.6767 | | | 0.9414 | | | −0.9541 | | | 0.7890 | | |
| CV (%) | 2.03 | | | 3.52 | | | 3.65 | | | 9.52 | | |
| AP | 19.40 | | | 48.69 | | | 8.30 | | | 24.18 | | |

NB: Nutrient broth; PGB: Peptone glycerol broth; OD: Optical density; CE: Coefficient estimate; AOC: Acridine orange concentration; $I_t$: Incubation time; $R^2$: Regression coefficient; CV: Coefficient of variation; AP: Adequate precision

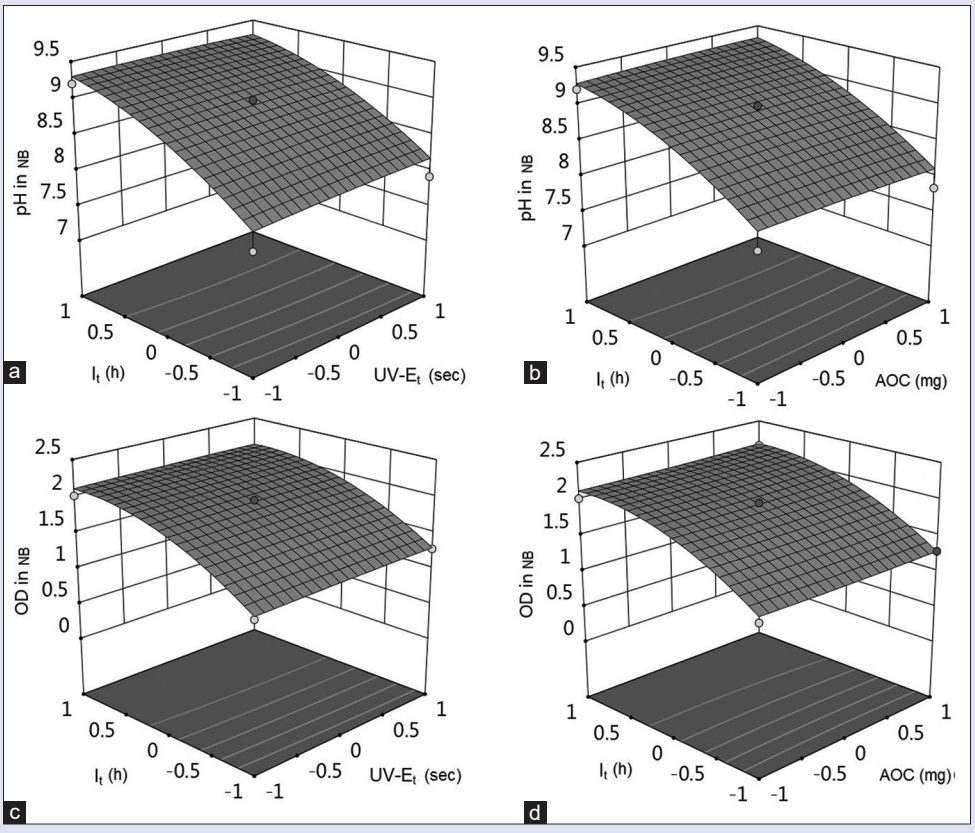


**Figure 1:** Three-dimensional plots of pH (a and b) and optical density (c and d) of the extracts obtained from nutrient broth culture at various levels of the selected factors. *OD: Optical density, AOC: Acridine orange concentration, UV-$E_t$: UV exposure time, $I_t$: Incubation time, NB: Nutrient broth

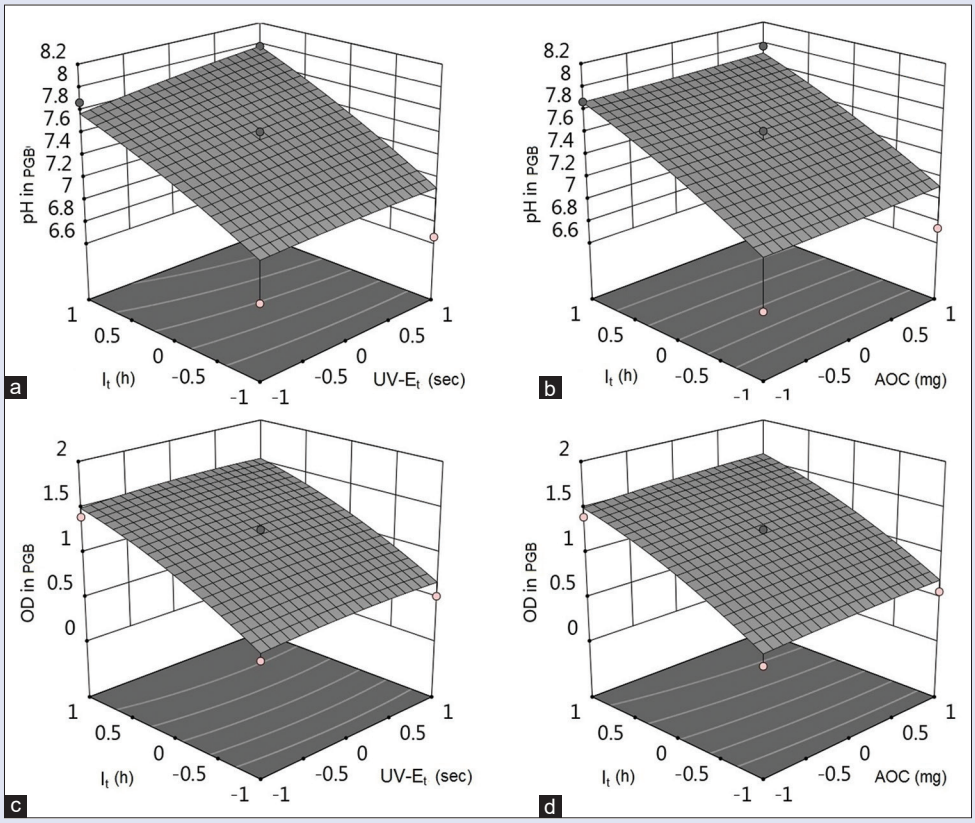


**Figure 2:** Three-dimensional plots of pH (a and b) and optical density (c and d) of the extracts obtained from peptone glycerol broth culture at various levels of the selected factors. *OD: Optical density, AOC: Acridine orange concentration, UV-$E_t$: UV exposure time, $I_t$: Incubation time, PGB: Peptone glycerol broth

of experiments carried out in the study. The values of AP for pH in NB, pH in PGB, OD in NB, and OD in PGB were calculated as 19.2494, 8.8601, 49.5630, and 20.3626, respectively. The observed values of AP suggest that the precision, reliability and suitability of the proposed model to navigate the design space.

Three-dimensional response surface plots were drawn to show the main and interaction effects of the input factors on the pH and OD of the extracts obtained from NB [Figure 1a-d] and PGB [Figure 2a-d]. The significance and adequacy of the model were measured in terms of *F*-value and *P* value at 5% significance level. The variation in corresponding variables with relatively higher *F*-values and smaller *P* values were considered more significant. The measurement of *F* and *P* values for main effects indicated that $I_t$ has significant effect on pH and OD in NB.

The applicability of the suggested model was also tested by plotting the experimental values against predicted values calculated from the polynomial regression equations [Figure 3a-h]. A good agreement between the experimental and predicted values with high values of $R^2$ is observed. Value of $R^2$ for pH in NB is 0.9568, for pH in PGB $R^2$ = 0.8047, for OD in NB $R^2$ is 0.9927, and for PGB $R^2$ is 0.9597. The measurement of regression coefficient indicates the significance of the model. The values of adjusted coefficient of the responses also advocated the significance of the model.

The results clearly indicate that the production of prodigiosin in both media was significantly increased by increasing the incubation time which may be correlated with the time-dependent increase in the bacterial count in nutrient-rich media. However, a prolonged incubation time beyond to the selected range may result in a decreased production of prodigiosin due to the fast consumption of the nutrients leading to the death phase of the microbial growth curve.

Although both the physical and chemical mutagens resulted in a non-significant increase in prodigiosin production by *S. marcescens*, it may be correlated with the mutation in the genes coding for or regulating the expression of prodigiosin. The results support the previous findings that prodigiosin production by *S. marcescens* can be enhanced by protecting from UV light induced mutations in its DNA.[12,15] The exposure to UV light at 260 nm wavelength has been found to result in the distortion of the DNA structure leading to lethal changes in base pair sequence.[12,16] These mutations further lead to the alterations in the production of various metabolites and enzymes by the microbes. The increase in prodigiosin production by AO treated mutant strain may also be correlated with AO induced DNA damage.[17]

## CONCLUSION

It is concluded that the selected physical and chemical mutagens and the incubation time showed a positive effect on the production of prodigiosin from *S. marcescens* in each of the selected growth media. The prodigiosin production, measured in terms of pH and OD of the extracts obtained from each of the culture medium, was found to be the significant linear and quadratic function of incubation time which may be correlated with the time-dependent increase in bacterial count resulting in increased production of prodigiosin. However, the increase in pH and OD with respect to an increase in UV-$E_t$ and AOC was found to be non-significant, which indicates non-significant induction of mutations in the bacterial strains. NB was found to be a preferable culture medium for the production of prodigiosin by the wild as well as the mutant strains of *S. marcescens*. The data would be a significant contribution to the literature and a valuable guideline regarding the production of prodigiosin, a naturally occurring bioactive pigment, from *S. marcescens*.

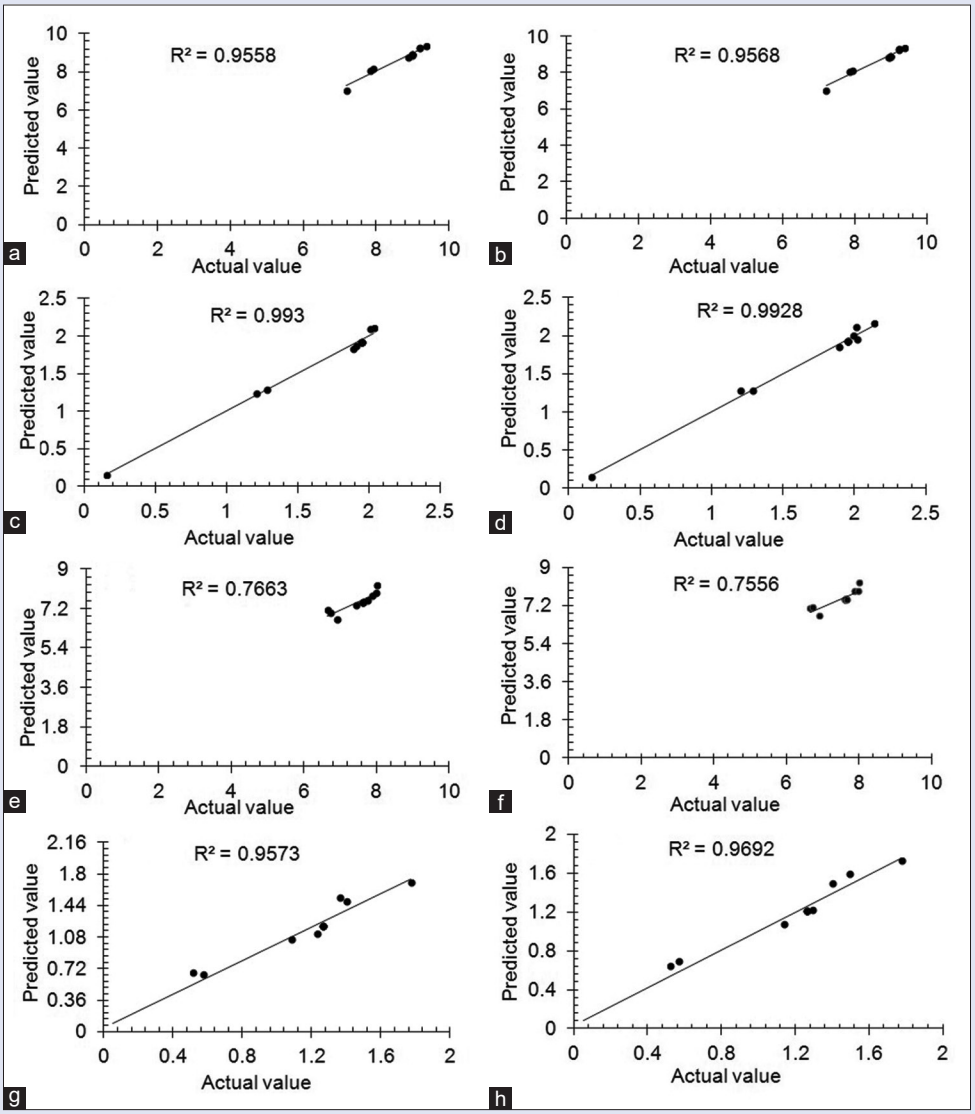


**Figure 3:** Correlation between the experimental and predicted values of the response at various levels of the selected factors. (a: pH and b) Optical density at selected levels of UV-$E_t$ and $I_t$ in NB. (c: pH and d) Optical density at selected levels of AOC and $I_t$ in NB. (e: pH and f) Optical density at selected levels of UV-$E_t$ and $I_t$ in PGB. (g: pH and h) Optical density at selected levels of AOC and $I_t$ in PGB. *OD: Optical density, AOC: Acridine orange concentration, UV-$E_t$: UV exposure time, $I_t$: Incubation time, NB: Nutrient broth, PGB: Peptone glycerol broth

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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