



Optimization of prodigiosin production by *Serratia marcescens* using crude glycerol and enhancing production using gamma radiation



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ABSTRACT

Prodigiosin is a red pigment produced by *Serratia marcescens*. Prodigiosin is regarded as a promising drug owing to its reported characteristics of possessing anti-microbial, anti-cancer, and immunosuppressive activity. A factorial design was applied to generate a set of 32 experimental combinations to study the optimal conditions for pigment production using crude glycerol obtained from local biodiesel facility as carbon source for the growth of *Serratia marcescens*. The maximum production (870 unit/cell) was achieved at 22 °C, at pH 9 with the addition of 1% (w/v) peptone and 10⁹ cell/ml inoculum size after 6 days of incubation. Gamma radiation at dose 200 Gy was capable of doubling the production of the pigment using the optimized conditions and manipulating production temperature. Our results indicate that we have designed an economic medium supporting enhanced *Serratia marcescens* MN5 prodigiosin production giving an added value for crude glycerol obtained from biodiesel industry.

1. Introduction

The massive demand for fuels to support the industrial needs, accompanied with retreating crude oil reserves and environmental concerns, have increased the need to put more effort on developing renewable energy [1]. Biodiesel is one of the promising alternative renewable fuels. Although there has been a growing effort to develop its production capacity there has been a major limitation which is the generation of about 10% (w/w) crude glycerol as the main byproduct [1]. Utilization of the produced crude glycerol in industrial microbiology by offering an economic feasible medium to support growth of different microorganisms is one of the potential options for lowering the production cost and promoting biodiesel production on a large industrial scale by solving its disposal problem [2].

Serratia marcescens is gram-negative bacilli belonging to the family Enterobacteriaceae, which are opportunistic to human, plant and insect. *Serratia marcescens* has been isolated from soil, water, plants and air. There are two types of *Serratia marcescens*; pigmented (red) and non pigmented (white) strains [3]. Prodigiosin is a red pigment produced as a secondary metabolite by *Serratia marcescens*, characterized with unique tripyrrole structure which is regarded as responsible for its reported pharmacological characteristics as anti-cancer, anti-microbial, anti-oxidant and immunosuppressant [4] and its unique application as a natural based dye for olefins and textiles [5].

Pigmented *Serratia marcescens* strains have been shown to cause infections in much less frequency than non-pigmented strains, thus reducing the risk of infection during mass production of pigment [6].

The expansion of industrial biotechnology considerably increased the need to seek overproducing microbial strains for the improvement of the production and obtaining better yield [7]. The successful examples of strain improvement in biotechnology are mostly attributed to the application of mutation and selection technique that results in choosing the best producer strain for the desired product [8].

Gamma radiations are short wave highly energetic electromagnetic radiations emitted from certain radioactive isotopes such as Cobalt 60. Exposure of microbes to gamma radiation may result in wide effects. Certain mutations to the genes may occur due to single or double strand breakage of DNA, oxidation of bases, structural change of DNA cross linking proteins [9]. The reaction of gamma rays with water inside the cell is the main contributor of those effects due to the resulting reactive oxygen species (ROS) and molecular products like OH⁻ and H₂O₂ [10]. Mutagenic effectiveness of gamma rays has been reported to be higher than the chemical like ethyl methane sulfonate (EMS) due to higher penetration of gamma rays into cells [11].

In our study we seek to utilize crude glycerol obtained from a local biodiesel facility to optimize the growth conditions required to support *Serratia marcescens* pigment production and to get a hyperproducing strain through gamma irradiation.

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2. Materials and methods

2.1. Screening of *Serratia marcescens* strains for prodigiosin

Twelve locally isolated *Serratia marcescens* strains from various hospitals in Cairo identified using (BioMérieux's API[®] kit, Marcy-l'Étoile, France) were kindly provided by Prof. Dr. Magdy Amin, from (Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University). The strains were screened for prodigiosin production on modified Luria Bertani (LB) agar plates where the tryptone was replaced with peptone at 25 °C [3]. The pigment producing strains were detected by appearance of pink red growth.

2.2. 16S rDNA sequencing

Sequencing of 16S rDNA was done for genetic identification of the pigmented strains. Extraction of DNA from pigmented strains and analysis of the sequence of its 16S rDNA was carried out using the PrepMan[®] ultra sample preparation reagent protocol method for DNA extraction; (Applied Biosystems, USA), identification was done using Microseq[®] 500 kits protocol (Microseq[®] system; Applied Biosystems, USA). A 527-bp fragment of the 16S rDNA gene of the bacterial strains was amplified in a reaction volume of 25 µl containing: 12.5 µl of MicroSeq 500 PCR master mix, 1 µl of each forward and reverse primers (Microseq[®] 500 Forward or Reverse sequencing primers), 9.5 µl of sterile distilled water, and 1 µl of the bacterial DNA extract, using the 9700 Thermal Cycler (ThermoFisher Scientific, USA). The 16S rDNA sequence of the strains of *Serratia marcescens* was done using 3500 Genetic Analyzer (ThermoFisher Scientific, USA) and was compared to available sequences using the BLAST program from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) and submitted for revision and getting an I.D accession number.

2.3. Screening for the highest prodigiosin producing strain

The pigmented strains were further screened on 100 ml Luria Bertani broth in 250 Erlenmeyer flasks for three days and the three strains with the highest pigment production were selected for further screening on different carbon sources to choose the strain giving maximum pigment production to be used throughout of the study.

2.4. Screening of carbon and nitrogen sources

The three strains with the highest production were screened for pigment production using four carbon sources: crude glycerol 1% (v/v) from local biodiesel facility, cotton seed cake 1% (w/v), soybean cake 1% (w/v) and black seed cake 1% (w/v) (all brought from local oil refinery facility and were ground to 30 mesh powders screen using electric grinder). The effect of the five nitrogen sources; peptone, tryptone, urea, ammonium sulphate and ammonium nitrate, all in 1% (w/v) concentration was identified to obtain a suitable nitrogen source to be used with the selected carbon sources on the highest strain. The used amounts were added to 100 ml distilled water and incubated at 25 °C.

2.5. Extraction and quantification of prodigiosin

At the end of the incubation period, 20 ml of acidified ethanol were added to 10 ml of the flask content and put in a shaker at 200 rpm (LAB-Line R Orbit Environ, U.S.A) for 15 min and then centrifuged in cooling centrifuge (Hettich Universal 16 R, Germany) for 15 min at 6 °C and centrifuged at 2415 × g to remove pigment free pellet [12].

Prodigiosin quantification using relative prodigiosin concentration was expressed per cell ($A_{535} \text{ ml}^{-1} \text{ OD}_{600} \text{ unit}$) by measuring the absorbance spectrophotometrically (JASCO V/560 UV/Vis, Japan). Prodigiosin in acidified ethanol displays a characteristic wavelength

Table 1

The screening of seven *Serratia marcescens* strains (MN1-MN7) on modified LB broth.

<i>Serratia marcescens</i> strain	Pigment production (unit/cell)
MN1	470
MN2	400
MN3	250
MN4	300
MN5	500
MN6	440
MN7	380

of 535 nm [3]. Estimation of prodigiosin was expressed as unit/cell after measuring the absorbance at 600 nm at the end of incubation [13–15].

2.6. Factorial design

The effect of the five variables in two level forms was assessed to study the optimal conditions for pigment production (Table 1). A total of 32 possible experimental combinations (done in triplicates) were carried out where the choice of the variables was based on the fact that production of prodigiosin by *Serratia marcescens* is highly regulated by environmental factors which includes; media composition, temperature and pH [16]. Factors included nitrogen source, pH (adjusted using 0.1N HCl to adjust the medium at the lower level of pH 6), inoculum size was determined by measuring the absorbance of an overnight broth of *Serratia marcescens* at 600 nm using spectrophotometer (JASCO V/560 UV/Vis, Japan). Designing the experiment and analysis of experimental data was done using the statistical software package (Minitab 16, U.S.A). The adequacy of the model was tested and the parameters with statistically significant effects were identified using Fisher's test for the analysis of variance (ANOVA). The main effects were estimated by subtracting the mean responses of variables at their lower levels from their corresponding higher levels and divided by the total number of experimental runs. Regression analysis of the experimental data and plotting the relationship between variables were done.

2.7. Radiation experiment

2.7.1. Testing of hyper producing strain

Gamma irradiation was used as a mutagenic agent to produce a hyper producing strain [7]. Irradiation process was carried out using Co-60 Gamma Chamber (4000-A-India) at a dose rate 1.915 kGy/h at the time of experiment. Overnight grown slant of the highest pigmented strain was irradiated at different doses of gamma irradiation (50, 100, 200, 400, 600, 800, 1000, 1500 and 2000 Gy).

Serial dilution was done accompanied with colony count to plot the survival curve of *Serratia marcescens*. A dose response curve was constructed to determine the (D10) radiation dose (Gy) required for reducing the number of *Serratia marcescens* by 10-fold (one log cycle) or the radiation dose required to kill 90% of the total number of microorganisms. D10 value varies for different microbes, irradiation conditions, and purposes [17]. The lethal dose (LD) was determined, where 100% of irradiated cells died to test the effect of gamma irradiation that can kill *Serratia marcescens* (for sterilization purposes) [17], then screening for a hyper producing strain was done and the strain isolated was cultivated at optimized conditions for prodigiosin production. Non-irradiated strain was used as control.

2.7.2. Testing for prodigiosin production by *Serratia marcescens* above 30 °C

At temperatures over ≥ 30 °C, *Serratia marcescens* stops producing pigment and colonies appear in a creamy white color, above that temperature, the activity of one or more enzymes contributing in prodigiosin synthesis pathway may be lost [4]. The gamma irradiated

Serratia marcescens was tested for pigment production at temperatures (32, 34, 36, 38 and 40 °C).

2.8. Radiation of the non pigmented strains

To test the ability of gamma radiation to induce pigmentation, radiation of overnight grown slants of non-pigmented strains was done at the same radiation dose used to obtain hyperproducing strains of *Serratia marcescens*.

3. Results

3.1. Screening of *Serratia marcescens* strains for prodigiosin

The screening of twelve *Serratia marcescens* strains revealed that seven strains demonstrated red growth (pigmentation) while the remaining five strains showed creamy white growth (no pigmentation). The seven pigmented strains were named *Serratia marcescens* (MN1-MN7).

3.2. 16S rDNA gene sequencing

The 16S rDNA gene sequences of the seven pigmented strains were submitted for review and were given accession numbers in the Gene bank. *Serratia marcescens* MN1: KX601161, *Serratia marcescens* MN2: KX601268, *Serratia marcescens* MN3: KX601278, *Serratia marcescens* MN4: KX601721, *Serratia marcescens* MN5: KX601170, *Serratia marcescens* MN6: KX602154 and *Serratia marcescens* MN7: KX592863.

3.3. Screening for the highest prodigiosin producing strain

The screening of the seven pigmented strains revealed that *Serratia marcescens* MN 1, *Serratia marcescens* MN 5 and *Serratia marcescens* MN 6 showed the highest prodigiosin production (Table 2).

3.4. Screening of carbon and nitrogen sources

Serratia marcescens MN 5 demonstrated the highest pigment production (560 unit/cell) among the three examined strains with crude glycerol as carbon source. It was elected to carry out the whole study due to relatively high production and consistent reproducibility as shown in (Table 3). Among the four tested carbon sources, crude glycerol displayed easy prodigiosin recovery due to its liquid state which did not absorb part of the produced pigment. The addition of the five tested nitrogen sources to crude glycerol revealed that peptone improved prodigiosin production to 610 unit/cell.

3.5. Factorial design

The factorial design results showed that the maximum production (870 unit/cell) was obtained at run number 16 with the conditions: 22 °C as incubation temperature, pH 9, 1% (w/v) peptone as nitrogen

Table 2
The five studied variables and their levels.

Levels	Variables				
	Nitrogen Source (% v/v)	pH	Inoculum size (cell/ml)	Incubation period (Days)	Temperature (°C)
High Level (+1)	1	9	~10 ⁹	6	28
Low Level (-1)	0	6	~10 ⁶	3	22

Table 3
Effect of carbon source on the highest three strains *Serratia marcescens* MN1, MN5 & MN6.

(a) Crude glycerol	
Strain	Prodigiosin (Unit/cell)
MN1	500
MN5	560
MN6	485
(b) Soya been cake	
Strain	Prodigiosin (Unit/cell)
MN1	410
MN5	450
MN6	370
(c) Cotton seed cake	
Strain	Prodigiosin (Unit/cell)
MN1	325
MN5	350
MN6	260
(d) Black seed cake	
Strain	Prodigiosin (Unit/cell)
MN1	150
MN5	200
MN6	110

source and ~10⁹ (cfu/ ml) inoculum size after 6 days of incubation.

The model determination coefficient (**R = 0.95**) with **R-Squared (R²) = 95%** (**R-Squared-adjusted**) = **94.1%** suggests that this model could explain 95% of the total variation, which indicates a satisfactory representation of the process by the model. The coefficient of determination (R-value) lies between 0 and 1. The closer the value of R is to 1, the stronger the model and the better it predicts the response. The analysis of variance for the selected factorial model showed that the model constant was significant (P ≤ 0.05) as shown in (Table 4).

The effect of the five variables was tested for significance using one way (ANOVA). The P-value was used to assess the significance of factors studied as shown in (Table 4). The results shown proved that incubation period, temperature and pH all had a significant effect (P ≤ 0.05).

The mathematical expression for the relationship between the variables for prodigiosin production is given below in the regression equation of the model shown: **Pigment = 0.356 + 0.200 pH – 0.00000 Inoculum conc. + 0.00625 Nitrogen source + 0.0250 Incubation period – 0.0750 Temperature**

The equation allows the prediction of the pigment production response (Y) in future experiments where a relationship mathematical formula is originally: [Y = a + bx], where Y is the enzyme activity, a is the constant (slope of the line) and x is the concentration of the variable (five variables were tested). The values before every factor are

Table 4
The significance of five tested variables.

Predictor	Effect (*P ≤ 0.05)
Constant	0.000*
pH	0.000*
Inoculum conc.	1.000
Nitrogen source	0.523
Incubation period	0.016*
Temperature	0.000*

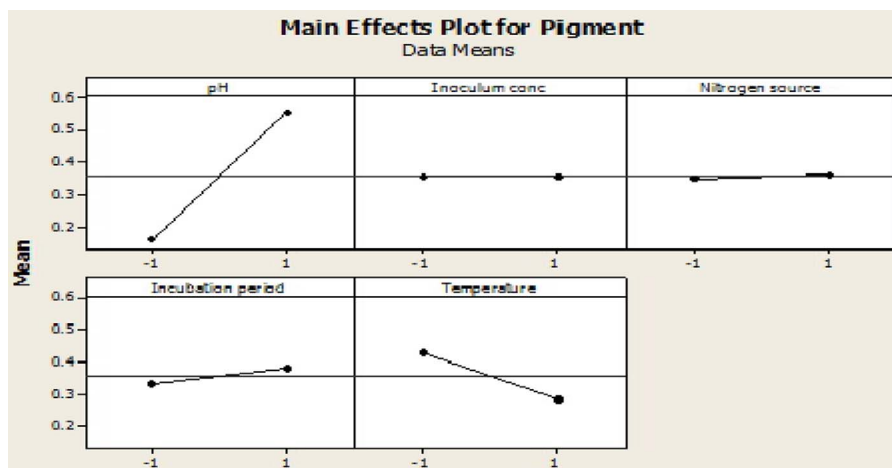


Fig. 1. Main effects plot for pigment production.

constants generated by the program based upon our results.

The Main effects plot of variables (Fig. 1) demonstrated the significant positive effect of pH and temperature and the significant negative effect of incubation period where the pigment production was better after 3 days, while the insignificant effect of nitrogen source and inoculum concentration was observed.

3.6. Radiation experiment

(a) Radiation of the highest producing strain

The survival curve of *Serratia marcescens* in response to different doses of gamma radiation was done, where D_{10} and lethal dose (LD) were calculated. A D_{10} of 100 Gy was able to decrease the count of *Serratia marcescens* by 1 log cycle and the LD causing complete death was 1700 Gy (Fig. 2). Gamma radiation at dose 200 Gy produced an intense red colony that was examined to produce the pigment using the optimized conditions (Fig. 3). The gamma irradiated strain (named *Serratia marcescens* MN200) was able to double the production of the pigment compared to the control (*Serratia marcescens* MN5) (Fig. 4). *Serratia marcescens* MN200 was cultured several times to ensure that gamma radiation caused permanent effect.

(b) Testing for prodigiosin production above 30 °C

Serratia marcescens MN200 showed pigment production at temperatures 32, 34 and 36 °C (Fig. 5). At 38 and 40 °C no growth was detected. *Serratia marcescens* MN200 demonstrated gradual loss of pigmentation upon repeated sub culturing.

(c) Radiation of the non pigmented strains

The five non pigmented strains gave no evidence of pigmentation where the colonies remained creamy white after exposure to doses 100 and 200 Gy (Fig. 6).

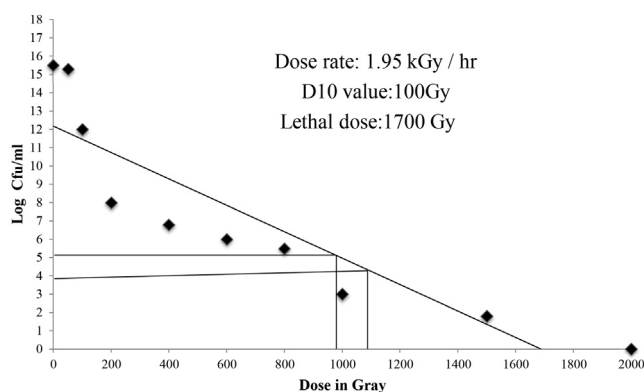


Fig. 2. Dose response curve for *Serratia marcescens* MN5.

4. Discussion

With the strong demand to search for novel compounds with multiple pharmaceutical applications, prodigiosin is a bacterial secondary metabolite that owns various medical applications serving as anticancer and immunosuppressant with reported positive effect on the increased chances of survival in mice undergoing heart transplantation [18].

Utilization of industrial wastes, optimization of media components, culture parameters and strain improvement are essential tools to improve the performance of the bacterial system which helps to increase the yield of its products in economic manner and also solves an environmental disposal problem.

Crude glycerol, a waste obtained from biodiesel industry was used to support growth of *Serratia marcescens* MN5, giving the highest prodigiosin production among the tested carbon sources due to its unique composition where it contains glycerol, methanol, soap, water and trace amounts of ions (mainly Na^+ and K^+) in variable percentages depending upon the method of production. Our results were in accordance with Tao et al. [19] who used pure glycerol to produce prodigiosin, as well as Cang et al. [20] who used ethanol as medium for *Serratia marcescens*. Among the four tested carbon sources, crude glycerol also showed the easiest prodigiosin recovery due to its liquid state not absorbing part of the produced pigment like the other carbon sources.

Peptone was used as nitrogen source when used with glycerol and gave the highest production of prodigiosin which was similarly observed with Gulani et al. [5], other inorganic nitrogen sources didn't support prodigiosin production and that was in accordance with Sumathi et al. [21] where sodium nitrate, ammonium sulfate, and potassium nitrate medium didn't support production of prodigiosin. Our results were in agreement with Giri et al. [22] and Gulani et al. [5], where peptone glycerol medium was their selected medium for production.

Conventional practice in medium optimization of components and parameters, which allows testing one variable at a time is time consuming and does not allow evaluation of the combined effects of all the variables in the involved in the fermentation process. Factorial design was used to identify the main effect of five variables simultaneously affecting the pigment production from *Serratia marcescens*.

The main effects plot revealed that pH was a significant factor affecting the pigment production, pH 9 supported better pigment production than pH 5 as it was proved that pH of the media possess a crucial role in the synthesis of secondary metabolites and it was proved that the decrease in pH caused negative influence on pigment production [29], also *Serratia marcescens* belongs to family Enterobacteriaceae

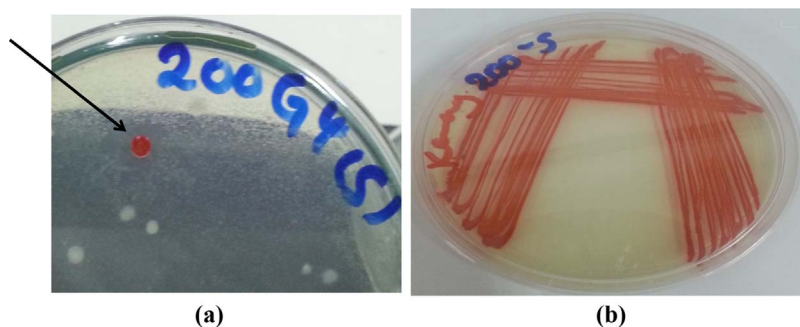


Fig. 3. (a) The intense red colony obtained at 200 Gy. (b) Sub culturing of the colony on LB agar.

which grows better at higher pH [3].

The analysis of significance for temperature as a variable (22 °C and 28 °C) revealed that temperature was a very significant factor where maximum prodigiosin production by *Serratia marcescens* MN 5 was observed at 22 °C, this observation was in agreement with Giri et al. [22] as higher production was obtained at the lower temperature on the peptone glycerol medium.

An incubation period of 6 days indicated significant effect as after such time interval, the strain expressed better pigment production which would be due to prodigiosin being a secondary metabolite produced at stationary phase.

The insignificant effect of nitrogen source would indicate that crude glycerol was a sufficient medium for production and consequently no added expenses is required for an additional nitrogen source. The inoculum concentration didn't implicate a significant effect on the production although previous studies stated that pigment production was regulated by quorum sensing where higher population of bacteria would lead to successful pigmentation due to increased gene expression [23].

Strain improvement has been the hallmark of all industrial fermentation processes which can reduce the cost of the production process by escalating productivity and possessing specialized desirable characteristics [24]. Gamma radiation was applied at different doses to improve the production of *Serratia marcescens* MN5 where intensely red colony was isolated at dose 200 Gy. The increased production might indicate mutation in limited region sequence which is expressed phenotypically and detected by selection as Hori et al. [25], who detected the mutant *Echerichia coli* strains depending on their rifampicin resistance on rifampicin selection plate.

The 100% increase in production of prodigiosin by *Serratia marcescens* MN200 compared to the parent strain *Serratia marcescens* MN5 was

similarly detected with Li et al. [26] where an increase of 81% in lipase activity has also been reported by *Penicillium expansum* by gamma irradiation and with Iftikhar et al. [7] who also detected 114% higher lipase activity than the parent strain at 60 Gy.

Fermentation industries widely use gamma irradiation to produce thermo tolerant microbe mutants, especially in sugar fermentation where high temperature (35–45 °C) and high ethanol concentration (over 20%) are used, *Saccharomyces cerevisiae* mutant was produced through gamma radiation that is highly tolerant to ethanol concentrations and temperatures (up to 42 °C) [27].

Gamma radiation was able to manipulate the temperature at which the pigmentation can still occur where *Serratia marcescens* MN200 could produce prodigiosin up to 36 °C. A thermotolerant *Phialocephale humicola* mutant was also generated by gamma radiation yielding thermostable α -amylase [9]. Prodigiosin production in temperatures above 30 °C would be an economic aspect in case of batch production, where any technical problems concerning temperature won't affect the production.

The effect of gamma radiation on bacteria is due to the high amount of free radicals and reactive oxygen species produced resulting in random mutation that affects pigment production pathway and temperature sensitivity mechanism. However, the identification of the exact mutation location and manner would require comparative genomic sequencing of the parent strain and the gamma irradiated one (mutant).

The tendency of reversion of the gamma radiation effect on irradiated strain *Serratia marcescens* MN200 is a common occurring event with low dose irradiated strains as in low dose microwave irradiation. Reports demonstrated reversible nature of microwave effect in over producing mutants of *Xanthomonas campestris*, were shown to revert back to the parent phenotype and in low dose microwave

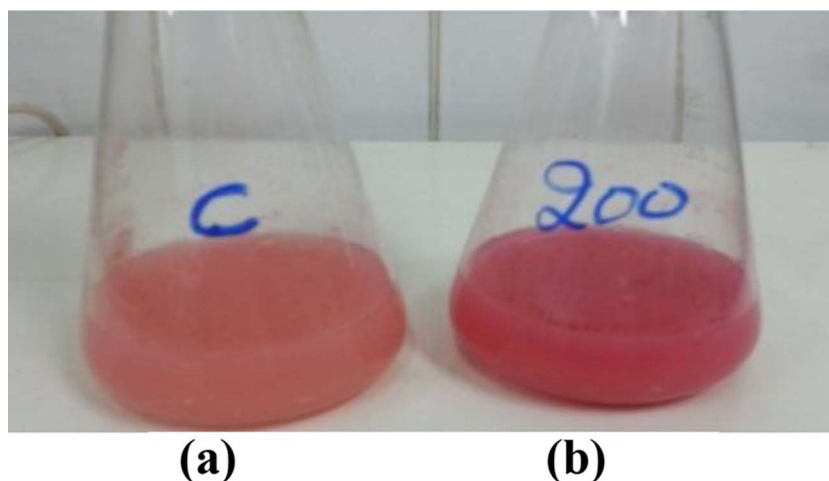


Fig. 4. (a) The non-irradiated strain *Serratia marcescens* MN5 (Control) grown under optimized conditions. (b) The gamma irradiated strain at 200 Gy *Serratia marcescens* MN200 grown under same optimized conditions.

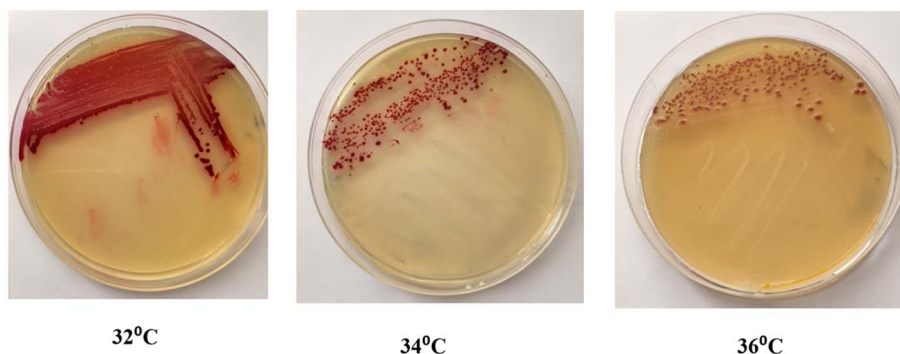


Fig. 5. Prodigiosin production at 32, 34 and 36 °C by *Serratia marcescens* MN200.

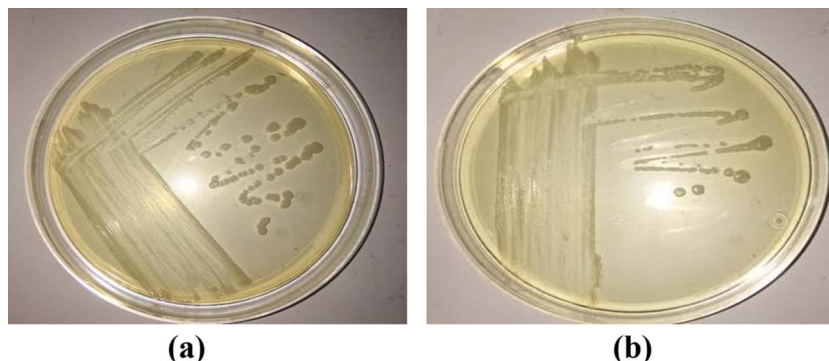


Fig. 6. The non-pigmented strain (a) before radiation (b) after radiation at dose 200 Gy.

induced effects in *Drosophila melanogaster* which disappeared after few generations. The reversion of mutation was due to restoration of microbial repair system to its efficiency after few subcultures [28].

Above 36 °C *Serratia marcescens* MN200 was not capable of pigment formation which might be due to denaturation of some heat sensitive enzymes involved in the biosynthesis pathway. Radiation of the non-pigmented strains didn't induce prodigiosin formation which might be due to permanent damage in the biosynthesis mechanism that didn't respond to low dose of gamma radiation or requiring higher dose to induce mutation.

Our results indicate that we have successfully formulated a new economically feasible medium and identified the set of conditions for optimally supporting *Serratia marcescens* MN5 production of prodigiosin giving an added value for crude glycerol resulting from biodiesel industry. In addition, gamma radiation was able to produce a hyper producing strain that can produce the pigment up to 36 °C.

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