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ENHANCE PRODIGIOSIN PRODUCTION UNDER OPTIMAL CONDITION IN MUTANT STRAIN OF *Serratia marcescens* QBN

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ABSTRACT

Prodigiosin, a red tripyrrole pigment primarily synthesized by *Serratia marcescens*, has attracted significant scientific interest due to its wide ranging bioactivities, notably its pro-apoptotic effects on cancer cells. In addition to its anticancer potential, prodigiosin demonstrates antibacterial, antifungal, antibiotic, and immunosuppressive properties, highlighting its relevance for pharmaceutical development. This study aimed to enhance prodigiosin biosynthesis through UV-induced mutagenesis of a wild-type *S. marcescens* QBN strain, followed by phenotypic selection. A mutant strain, *S. marcescens* UV1, was obtained through UV irradiation of the wild-type strain and selected based on enhanced pigment production. The results demonstrated a significant improvement in prodigiosin yield from the UV1 mutant compared to the wild-type strain. Specifically, UV1 produced approximately 700 mg/mL of prodigiosin, representing a 1.84-fold increase relative to the wild-type strain's yield of 380 mg/mL under the same conditions. This enhancement suggests that UV-induced mutagenesis conferred improved biosynthetic efficiency and potentially upregulated the metabolic pathway involved in prodigiosin synthesis. Consequently, *S. marcescens* UV1 represents a promising candidate for industrial-scale production of prodigiosin in biotechnological and therapeutic contexts.

Keywords: Prodigiosin, red pigment, *Serratia marcescens*, mutant, stress condition.

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INTRODUCTION

Prodigiosin is a secondary metabolite soluble in solvent, insolvent in water, chemical formula $C_{20}H_{25}N_{30}$ and is red at pH 7 and absorption at 530–540 nm. Prodigiosin has been demonstrated to induce apoptosis in hematopoietic cancer cells, colon cancer cells, B-cell, chronic lymphocytic leukemia cells, but hasn't any toxicity in normal cell lines (Kavitha et al., 2010; Perez-Tomas & Montaner, 2003; Prabhu et al., 2016). Other important biological activity of prodigiosin is antimicrobial thought machines including autolysin, inhibition biofilm or stimulation of the production of reactive oxygen species (Danevcic et al., 2016; Kimyon et al., 2016). Besides, with characteristic natural pigment, nontoxic, prodigiosin is a potential food color, color intensifier and preservative. Prodigiosin was found in various bacteria such as *Pseudoalteromonas rubra*, *Vibrio* sp., *Janthino bacterium*, *Pseudomonas putida*, *Streptomyces lividans*, *Hahella chejuensi*, *Pseudovibrio denitrificans*, *Vibrio psychroerythreus*, *Serratia marcescens*, *Zooshikella rubidu*s (Hu et al., 2016; Vitale et al., 2020). Among these *Serratia marcescens* is the best known due to its high prodigiosin production ability (de Araujo et al., 2010).

Its biosynthesis is regulated by a gene cluster known as *pig* (pigA–N), which plays a crucial role in gene expression and pigment formation (Harris et al., 2004). Recent studies have revealed that regulatory proteins, such as PtrA, positively modulate the *pig* cluster and affect bacterial motility, biofilm formation, and stress tolerance (Lin et al., 2023). However, prodigiosin production is highly sensitive to cultivation conditions. Temperature is a key factor; when the incubation temperature is increased from 30 to 37°C, prodigiosin synthesis significantly decreases, and the cell and culture color turns pale pink or colorless (Romanowski et al., 2019). In addition, environmental factors like light exposure can also reduce pigment stability, as prodigiosin is light-sensitive (Lu et al., 2024).

To improve prodigiosin yield, both classical mutagenesis and molecular biology techniques have been employed. Due to the complexity of genetic engineering, classical mutagenesis using chemical agents such as NTG (N-methyl-N'-nitro-N-nitrosoguanidine), EMS (ethyl methanesulfonate), ethidium bromide, and physical mutagens like UV radiation or gamma rays has proven effective in enhancing pigment production (Dikshit & Tallapragada, 2018; Elkenawy et al., 2017; Fakorede et al., 2019). Recent approaches have also focused on optimizing fermentation conditions using alternative substrates such as agro-industrial waste to increase cost-efficiency and yield (Pereira & de Carvalho, 2024). In our previous studies, the *S. marcescens* QBN strain exhibited high prodigiosin production at 28–30 ^{o}C, but the yield dropped drastically at 37 ^{o}C, which is often the ambient temperature during summer. Similarly, the addition of antibiotics to the fermentation medium commonly used to prevent microbial contamination also affects prodigiosin production. Although *S. marcescens* shows resistance to various antibiotics due to the presence of R-factors carrying resistance genes (Carbonell et al., 2000), increasing antibiotic concentrations leads to a reduction or complete inhibition of prodigiosin synthesis. Therefore, in this study, we employed mutagenic agents to generate mutant strains and screened for variants with resistance to heat and antibiotic stress. This strategy aims to enhance the efficiency and robustness of prodigiosin production under industrial fermentation conditions.

MATERIALS AND METHODS

Bacteria strain and medium

The strain of *Serratia marcescens* strain QBN VTCC 910026 (GenBank: KX674054.1) were provided by Enzyme Biotechnology Laboratory, Institute of Biology, Vietnam Academy of Science and Technology. The basic medium used for culture *S. marcescens* was Lysogeny Broth (LB) (1% peptone, 0.5% yeast extract, 1%

NaCl, pH 7) and Nutrient broth (NB) (0.5% peptone, 0.3% yeast extract, 0.5% NaCl). Standard prodigiosin, methanol, ethyl acetate, chloroform, acetone, toluene was procured from Merck (Darmstadt, Germany). Peptone, yeast extract was from Bio Basic Inc (New York, USA) and others from China.

Mutagenesis

The wild strain of *S. marcescen* QBN was used for mutation. The wild type *S. marcescen* QBN was cultured in 5 ml LB at 28 ^{o}C with shaking at 150 rpm overnight. The 1% of overnight culture were transferred into 25 mL LB at 28 ^{o}C with continuous shaking at 150 rpm for 3 h to achieve OD_{600} = 0.6–0.8 then were used to treat by mutant agent. Mutagen by UV: The culture was diluted in LB (1:10) and was spread on LB agar with 300 µg/mL ampicillin plates and was exposed under a UV lamp at 30 cm sample to source distance with exposure time 10, 20, 30, 60 s. The control and UV light exposed plates were incubated in a dark place at 37 ^{o}C for 24 h.

Mutagen by EMS, NTG and Ethidium bromide: The culture was treated with 1% EMS/NTG/Ethidium bromide and the chemical mutagent treated was incubated at 37 ºC for 2 h, 4 h and 6h at 28 ºC. With EMS, the reaction with EMS was terminated by adding 200 µL of 5% $Na_2S_3O_2$ centrifuged at 5,000 rpm in 5 min to harvest the cell. The reaction with NTG and Ethidium bromide was also centrifuged at 5000 rpm in 5 min to harvest the cell. The chemical mutagent treated cell was washed with H_2O to remove EMS/NTG/Ethidium bromide the bacterial cells were suspended in LB and spread on LB with 300 µg/mL ampicillin plate and was incubated at 37 ^{o}C in 24 hours to select strain resistance thermal and chemical stress.

The high prodigiosin production mutants were screened according to colony color. The colonies with deep red color were selected to be examined further. The mutant and wild colonies were cultured in 5 mL LB with 300 µg/mL ampicillin, shaken at 150 rpm, at 37 ^{o}C for 24 hours. The pigment was extracted by adding 95% ethanol (ratio 1:1). The extract solution containing prodigiosin was determined by a spectrophotometer at 534 nm. The absorbance values are converted to prodigiosin concentration using the standard curve. A standard curve of prodigiosin was established using various concentrations of purified prodigiosin (10–30 mg/L).

Solid stated fermentation

The mutant strain was precultured in LB medium, shaken at 200 rpm at 28 ^{o}C for 24 h, the overnight culture was spread on agar plates containing solid fermentation medium including 15 g/L ground peanut powder, 15 g/L agar containing 50 µg/mL ampicillin. The plates were continuously incubated at 37 ^{o}C for 72 hours. The layer of cells growing on the surface of the soil was harvested to extract prodigiosin with a ratio of 1 g wet cell: 10 mL ethyl acetate.

Extraction, purification and identification prodigiosin

The prodigiosin was extracted and purified from cell harvest using solvent, then purified prodigiosin was identified using HPLC. This process was described in our previous study (Nguyen et al., 2022). The concentration of prodigiosin was calculated by using the standard curve of pure standard prodigiosin (Merck).

Effects of culture conditions on Prodigiosin production by mutant *Serratia marcescens* UV1

To investigate the effect of cultivation time on prodigiosin (PGs) production by mutant and wild-type *S. marcescens*, 20 mL of *S. marcescens* UV1 and *S. marcescens* QBN were inoculated into 100 mL shake flasks containing 20 mL of LB medium. The cultures were incubated at 28 ^{o}C, pH 7.0, with shaking at 200 rpm for various durations (24, 48, 72, 96, and 120 hours). After incubation, the cultures were analyzed for prodigiosin concentration. To assess the effect of medium pH on PGs production, 20 mL of *S. marcescens* UV1 and *S. marcescens* QBN were inoculated into 100 mL shake flasks containing 20 mL of LB medium adjusted to

different pH levels (6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0). Cultures were incubated at 28 ^{o}C with shaking at 200 rpm for 72 hours, followed by determination of prodigiosin concentration. To evaluate the effect of natural substrates on PGs production, *S. marcescens* UV1 and *S. marcescens* QBN were cultured in 100 mL shake flasks containing 20 mL of nutrient broth-based media supplemented with various natural substrates: Mt 1: NB + 1% black sesame oil; Mt 2: 2% black sesame oil + distilled water; Mt 3: 2% coconut oil + distilled water; Mt 4: NB + 1% coconut oil; Mt 5: 2% peanut powder + distilled water, substrate concentrations were optimized in previous studies (Nguyen et al., 2022). All cultures were incubated at 28 ^{o}C with shaking at 200 rpm for 72 hours. Prodigiosin concentration was then measured.

RESULTS

Screening of mutants resistant to high temperature and antibiotic stress

Following mutagenesis using chemical agents including EMS, NTG, and ethidium bromide, subsequent screening at 37 ^{o}C in the presence of ampicillin revealed a slight decline in survival rate with increasing exposure time; however, the overall survival remained relatively high. Despite this, all resulting colonies exhibited white or light pink pigmentation, indicating minimal or no prodigiosin production.

In contrast, UV-induced mutagenesis resulted in a time-dependent decrease in colony survival, with very few colonies remaining after 60 seconds of UV exposure. Among the surviving colonies screened under the same stress conditions (37 ^{o}C with ampicillin), several displayed a distinct red pigmentation (Fig. 1), suggesting continued or enhanced prodigiosin biosynthesis.

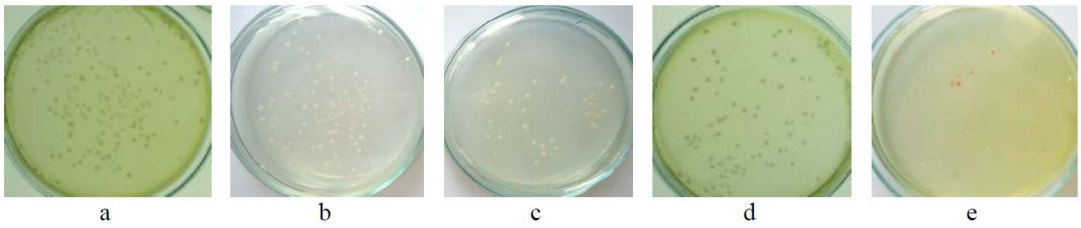


Figure 1. Colonies of *Serratia marcescent* wild type (a: *Serratia masescen* QBN) and after treatment by mutagens (b: Treated mutagen EMS; c: Treated mutagen NTG; d: Treated mutagen Ethidium bromide; e: Treated mutagen UV) on nutrient agar with antibiotic supplement at 37 ^{o}C

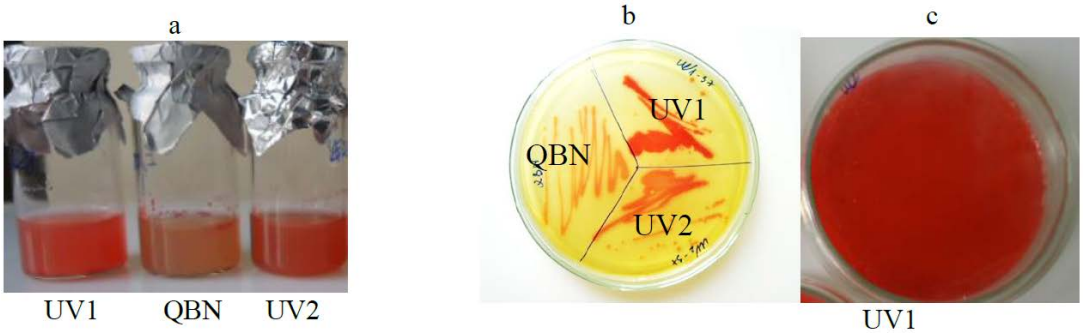


Figure 2. Culture wild type strain (QBN) and mutant strains (UV1, UV2) in stress condition (a, b). Solid stated fermentation mutant strain UV1 on peanut agar added antibiotic at 37 ^{o}C (c)

These red-pigmented colonies were further analyzed for prodigiosin content. Two mutants with the highest pigment production were selected and designated as UV1 and

UV2. Across successive generations, both UV1 and UV2 consistently produced approximately double the amount of prodigiosin compared to the wild-type strain when cultivated under identical conditions (Fig. 2).

Prodigiosin production via solid state fermentation using *Serratia marcescens* UV1

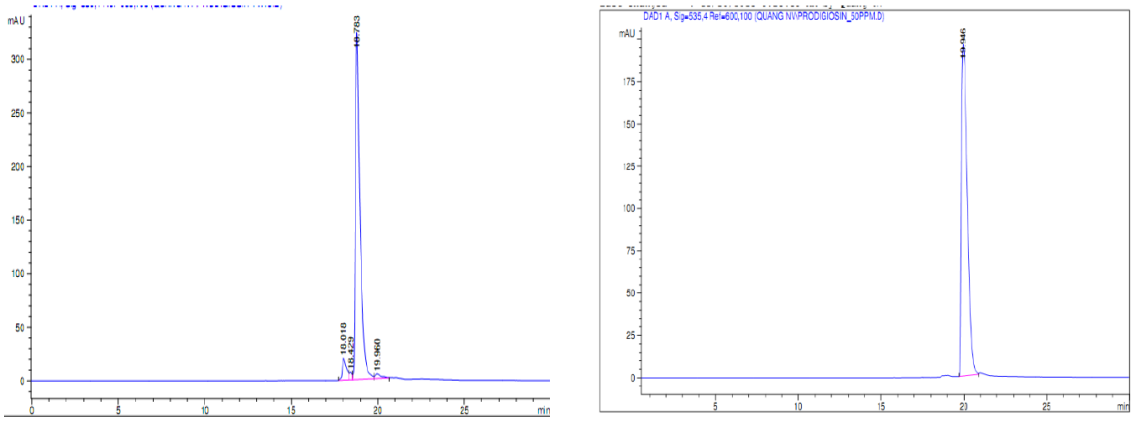
Prodigiosin production was carried out using the UV1 mutant strain of *S. marcescens* under solid-state fermentation (SSF) conditions, as illustrated in Figure 2. The fermentation process was conducted under stress conditions, specifically at an elevated temperature of 37 ^{o}C and in the presence of antibiotics, to mimic selective pressure environments that enhance metabolite synthesis.

The results demonstrated a significant improvement in prodigiosin yield from the UV1 mutant compared to the wild-type strain. Specifically, the UV1 mutant produced approximately 700 mg/mL of prodigiosin, which corresponds to a 1.84 fold increase relative to the wild-type strain, which yielded 380 mg/mL under the same conditions. This enhancement in pigment production indicates that UV-induced mutagenesis not only conferred stress resistance but also substantially upregulated the biosynthetic pathway responsible for prodigiosin synthesis.

These findings suggest that the UV1 mutant strain holds considerable promise for industrial-scale prodigiosin production, particularly under challenging fermentation environments.

Identify Prodigiosin by HPLC

The determination of an effective solvent system for Pg extraction from *S. marcescens* culture was previously established (Nguyen et al., 2022). In this investigation, a solvent mixture of ethyl acetate and acetone (1:1) was employed for extracting prodigiosin from *S. marcescens*.



a b

Figure 3. Detection of purified prodigiosin from mutant strain UV1 with HPLC. (a) Detection of standard prodigiosin samples; (b) Detection of purified prodigiosin samples

The resultant cell-free extract underwent chromatographic separation on a silica gel column. The prodigiosin-enriched fractions were selectively collected and subjected to a second purification step through another silica gel column. Two fractions were obtained, and subsequent analysis revealed a singular band representing the putative compound. To test purified pigment from mutant clones, the purified pigment was analyzed by HPLC, and as expected, the peak detected at 532 nm matched purified stand prodigiosin (Fig. 3).

Effects of culture conditions on prodigiosin production by *Serratia marcescens* UV1 mutant

Effect of growth kinetics

Under optimal conditions for *S. marcescens* growth (28 °C, no antibiotics), the UV1 mutant strain demonstrated superior prodigiosin production compared to the wild-type strain during the 48−72 hour period. However, beyond 72 hours, the prodigiosin concentration in the mutant strain declined and fell below that of the wild-type at 120 hours. Both strains achieved peak pigment production on the third day of culture.

For the UV1 mutant, prodigiosin concentration increased from 208 mg/L at 24 hours to 487 mg/L at 48 hours, reaching a maximum of 713 mg/L at 72 hours. Subsequently, pigment levels decreased to 557 mg/L at 96 hours and 455 mg/L at 120 hours (Fig. 4). This pattern is consistent with bacterial growth kinetics, in which pigment production typically peaks during the late exponential phase before declining in the stationary or death phases.

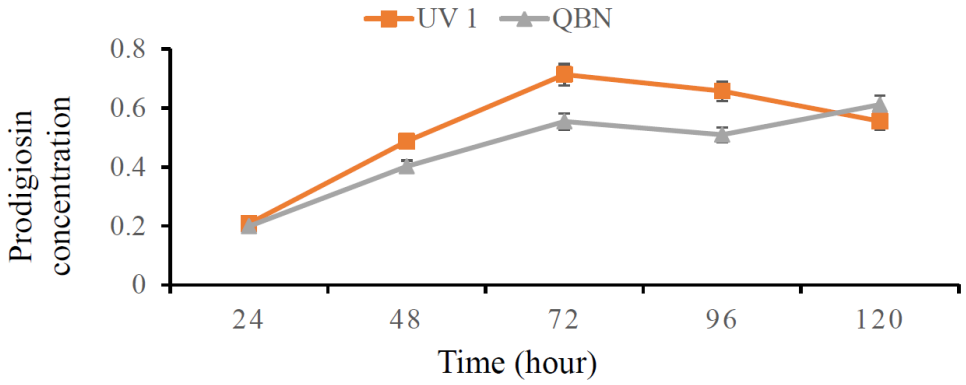


Figure 4. Growth kinetics of mutant strains and concentration of PG was produced. UV 1: the PGs concentration of *Serratia marcescens* UV1; QBN: the PGs concentration of *Serratia marcescens* QBN

Effect of pH

In addition to temperature, environmental pH plays a crucial role in regulating prodigiosin biosynthesis. The ability of the UV1 mutant and wild-type strains to synthesize prodigiosin under varying pH values (6.0–9.0) was assessed. Across all tested pH values, the UV1 mutant consistently produced higher pigment levels than the wild-type strain.

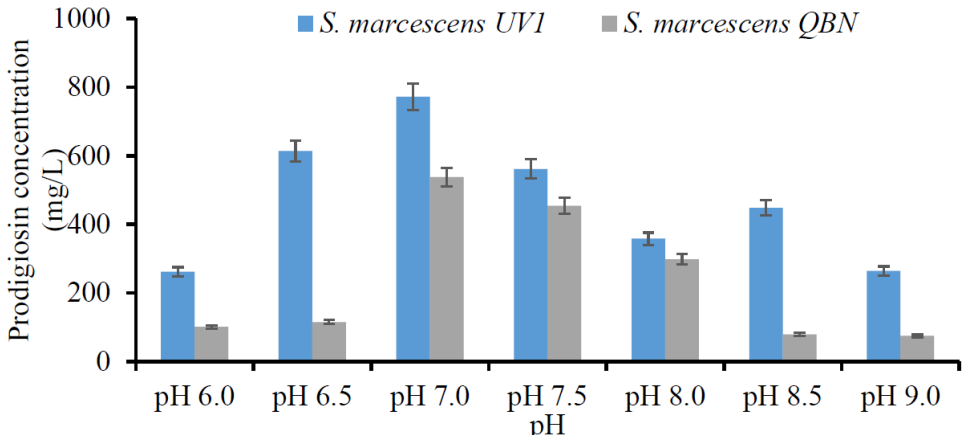


Figure 5. Prodigiosin concentration of mutant and wild strain in pH effect experiments

Visual observations (S. 1A, 1B) revealed that *S. marcescens* QBN produced red-pigmented cultures that ranged from light to dark red at most pH levels, except at pH 8.5, where a cream-white color appeared. In contrast, the UV1 mutant showed red pigmentation across all pH conditions, with color intensity varying by pH. Quantitative analysis showed that the UV1 strain produced the lowest prodigiosin concentration (261.6 mg/L) at pH 6.0 and the highest at pH 7.0 (772 mg/L). A moderate increase in pigment production was observed at pH 8.5 (from 358 mg/L to 448 mg/L), followed by a sharp decline to 264 mg/L at pH 9.0. These results suggest that while both strains can produce prodigiosin under mildly acidic conditions, alkaline conditions (pH 8.0–9.0) lead to variable and generally reduced pigment synthesis (Fig. 5).

Effect of natural substrates

The influence of different natural substrates on prodigiosin biosynthesis was evaluated using coconut oil, peanut powder, and black sesame oil. As shown in S. 2A and 2B, culture color varied significantly depending on the substrate, ranging from light milky white to deep pink and purple.

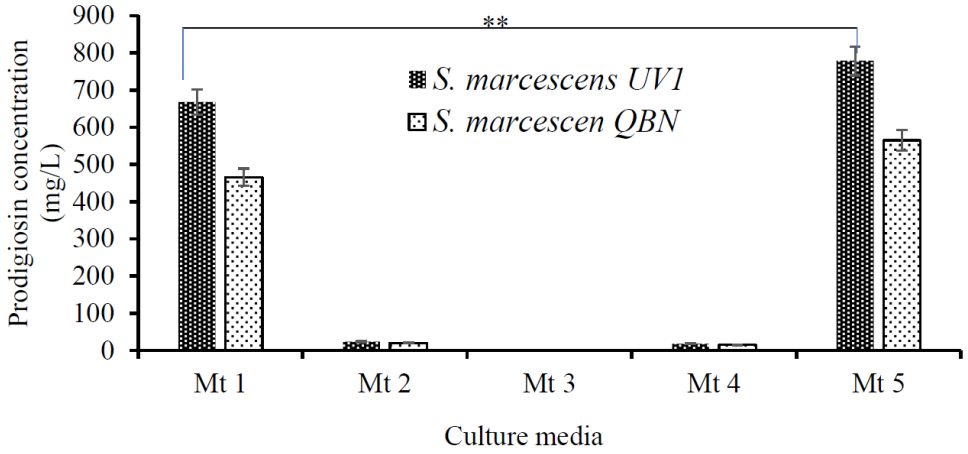


Figure 6. PGs concentration of mutant and wild strain in different natural substrates. ** P < 0.05. *Serratia marcescens* UV1: PGs concentration of *Serratia marcescens* UV1*; Serratia marcescens* QBN*:* PGs concentration of *Serratia marcescens* QBN*;* Mt 1: NB + 1% black sesame oil; Mt 3: 2% coconut oil + H_2O; Mt 2: 2% black sesame oil + H_2O; Mt 4: NB + 1% coconut oil; Mt 5: 2% peanut powder + H_2O

For the UV1 mutant, the highest prodigiosin yield was obtained using peanut powder (Mt5), reaching 778.8 mg/L, compared to 565 mg/L in the wild-type strain (Fig. 6). No pigment production was observed with coconut oil, likely due to its antimicrobial fatty acid content. Pigment yields in media containing peanut powder and black sesame oil were approximately 40 times higher than in nutrient broth containing coconut oil. Among the substrates tested, prodigiosin yield ranked in the following order for the UV1 strain: peanut powder (Mt5) > black sesame oil (Mt1, 668 mg/L) > others (Mt2 and Mt4, with minimal production). Coconut oil (Mt3) did not support pigment biosynthesis, while sesame oil, being poorly soluble in water, dispersed only partially under agitation, limiting its effectiveness as a substrate.

These findings highlight the importance of substrate composition and solubility in influencing prodigiosin production by *S. marcescens* and suggest that peanut powder is a highly effective and economical substrate for large-scale pigment production.

DISCUSSION

Our findings revealed a marked difference in colony pigmentation following mutagenesis treatments. Specifically, after chemical mutagenesis, most of the *S. marcescens* colonies exhibited a non-pigmented phenotype, whereas UV mutagenesis yielded a greater number of red-pigmented colonies. This observation can be attributed to the distinct mechanisms by which these mutagens affect cellular function. While chemical agents primarily act as stress inducers, UV radiation not only induces mutations but also serves as an environmental signal that stimulates prodigiosin biosynthesis, likely as a protective response. Prodigiosin has been reported to protect bacterial cells against UV-induced damage, with pigmented cells exhibiting enhanced survival under UV exposure (Boric et al., 2011; Cediel Becerra et al., 2022). Consequently, UV irradiation functions both as a mutagen and as a selection pressure, facilitating the isolation of mutant strains with enhanced prodigiosin production and increased stress tolerance. Numerous studies have reported successful enhancement of prodigiosin yield through mutagenesis. For instance, El-Bialy & El-Nour (2015) applied both UV and EMS mutagenesis to *S. marcescens*, generating strains with prodigiosin production levels 2- and 8-fold higher than the wild type, respectively. Notably, these mutant strains were also capable of synthesizing prodigiosin at 38 ^{o}C, a condition under which the wild-type strain failed to produce pigment. Similarly, Fakorede et al. (2019) reported a fivefold increase in prodigiosin production using ethidium bromide-induced mutants.

In our previous research, the wild-type *S. marcescens* QBN strain successfully produced prodigiosin using solid-state fermentation (SSF). This method requires significantly less solvent for pigment extraction compared to submerged fermentation due to the higher concentration of pigment within the biomass. Therefore, in this study, we continued to employ SSF for the cultivation of the mutant strain. The results demonstrated that biomass collected from the surface of the solid substrate facilitated efficient extraction with reduced solvent use. Visual observation revealed that by the first day of fermentation, colonies of the UV1 mutant strain exhibited strong red pigmentation, whereas the wild-type QBN strain appeared colorless or faint pink. By the third day, pigmentation in the QBN strain intensified; however, the total prodigiosin yield from the UV1 strain remained approximately twice as high. This disparity may be attributed to gradual antibiotic degradation over time and the wild strain's delayed adaptation to stress conditions.

Under optimal temperature (28 ^{o}C) and antibiotic-free conditions, prodigiosin production in both the mutant UV1 and wild QBN strains increased steadily from 24 hours to 72 hours, reaching peak levels at 72 hours before declining at 96 hours. This trend corresponds with the bacterial growth curve. During the lag phase, the expression of genes related to fundamental cellular processes is upregulated (Brejning et al., 2005), resulting in slow pigment accumulation. During the exponential (log) phase, rapid cell division promotes elevated metabolic activity, including prodigiosin synthesis. However, as cells enter stationary and death phases, pigment production diminishes due to declining cell viability (Rolfe et al., 2012).

The pH of the fermentation medium was found to significantly influence pigment yield. As shown in Figure 5, deviations from the optimal pH led to substantial reductions in prodigiosin production. This may be due to pH-dependent modulation of the expression or activity of biosynthetic enzymes, as previously reported by Sole et al. (1994). Moreover, pH affects nutrient solubility, transport, and pigment stability (Ibrahim, 2009). In this study, the optimal pH for prodigiosin production in mutant strains was determined to be 7.0, aligning with findings by Hardjito et al. (2002), though other studies reported peak yields at pH 8.0–8.5 (Sole et al., 1994).

Regarding substrate selection, both UV1 and QBN strains exhibited the highest prodigiosin yields when cultured on peanut

powder (Mt5), while no pigment was detected when coconut oil (Mt3) was used. This observation is consistent with the work of Pradeep et al. (2012), who reported enhanced cell growth and pigment production using peanut-based substrates. While oils such as sesame and coconut oil are rich in unsaturated fatty acids, the fatty acids in coconut oil particularly lauric (~50%) and capric (~7%) acids have known antimicrobial properties that likely inhibited bacterial growth. In contrast, peanut powder may provide not only accessible carbon sources but also essential nitrogen and micronutrients that support prodigiosin biosynthesis (Giri et al., 2004).

The mutant UV1 strain exhibited enhanced resilience and productivity under various culture conditions compared to the wild-type QBN strain. Despite sharing the same optimal pH, culture time, and substrate preferences, the UV1 mutant consistently produced higher prodigiosin yields, highlighting its potential for industrial-scale production under solid-state fermentation.

CONCLUSION

In summary, Serratia marcescens QBN was successfully mutated into S. marcescens UV1 using ultraviolet (UV) mutagenesis with an exposure time of 10 seconds. The resulting mutant strain exhibited a 1.84-fold increase in prodigiosin production compared to the wild-type strain under stress conditions involving elevated temperature and chemical exposure. Furthermore, under optimal temperature and antibiotic-free conditions, the highest prodigiosin yield from the UV1 strain was achieved when cultured in a medium containing 2% peanut powder at pH 7 for 72 hours. Under these conditions, the prodigiosin levels produced by the UV1 strain consistently surpassed those of the wild-type QBN strain.

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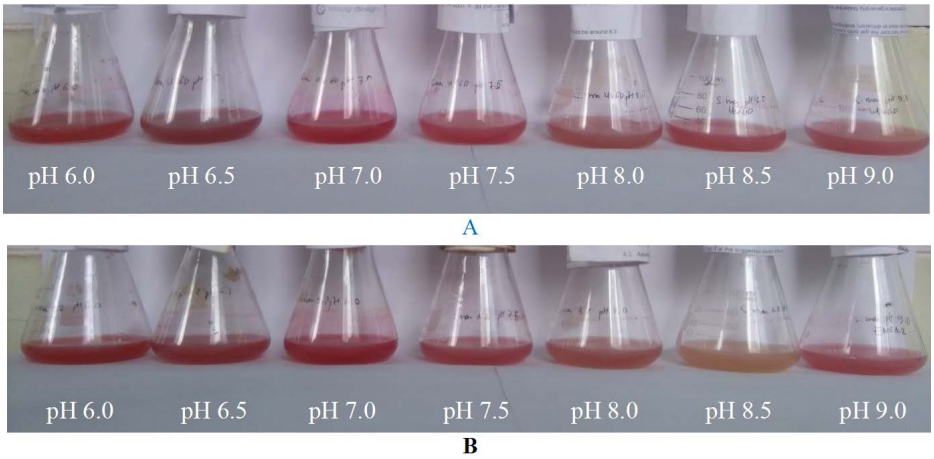
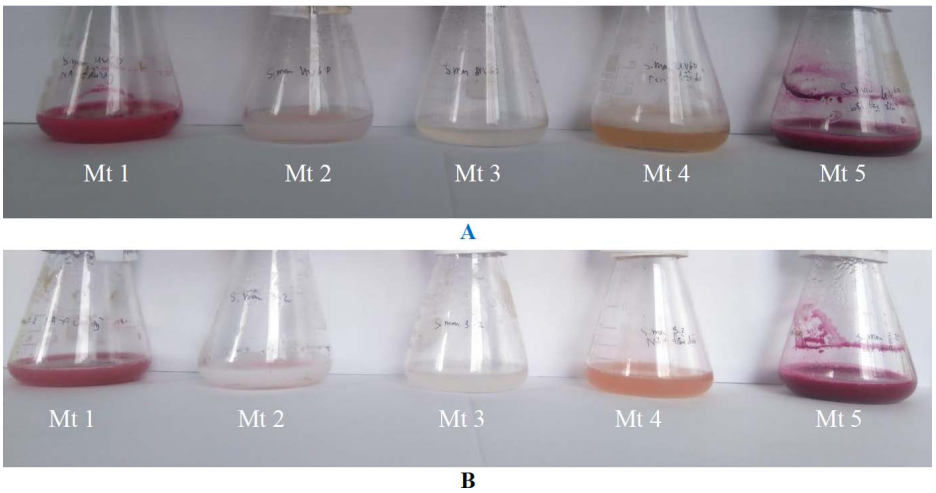


Supplementary 1. (A) Effect of pH on *Serratia marcescens* UV; (B) Effect of pH on *Serratia marcescens* QBN



Supplementary 2. (A) *Serratia marcescens* UV cultured in different natural substrates; (B) *S. marcescens* QBN cultured in different natural substrates