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Original Article

Optimization of Prodigiosin Production in *Serratia marcescens*: Effects of Growth Parameters on Antimicrobial and Cytotoxic Activities

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Abstract

In this study, we isolated a red-colonial strain from marine soil collected in Colachel, Kanyakumari, Tamil Nadu, and identified it as *Serratia marcescens* via 16S rRNA sequencing. Optimization of various growth parameters was performed to increase pigment prodigiosin production. Mannitol was found to be an ideal carbon source with an optical density (OD) value of 1.99. A pH level (OD 1.92) and temperature of 27°C (OD 1.95) also promoted the favorable synthesis of pigment. Moreover, low salt concentration (0.5% NaCl) and a prolonged incubation period (120 h) significantly increased prodigiosin yield. It was found that higher prodigiosin production was achieved under shaking conditions (OD 2.25) than under static conditions (OD 1.12), and the pigment was maintained in methanol. The prodigiosin exhibited a strong antimicrobial effect against *Staphylococcus aureus,* with a zone of inhibition (ZOI) of 38 mm. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the produced prodigiosin were 62.50 µg/mL. The index of 0.50 of the fractional inhibitory concentration (FIC) was an indication of the strong synergistic effect between prodigiosin and streptomycin. The pigment neutralized DPPH radicals and exhibited strong anticancer activity against MCF-7 breast cancer cells. This thorough study not only explains why optimizing the various growth parameters is important for maximizing prodigiosin synthesis but also examines their utility with respect to potency in both therapeutic and textile applications.

Keywords: *Anticancer; Antioxidant; Antimicrobial; Pigments; Prodigiosin; Serratia marcescens*

Introduction

Pigments are natural substances that provide color to a broad range of organisms and play a primary role in the environment and industries. Natural pigments have intrinsic qualities that make them a viable alternative to synthetic dyes. They can be utilized as food additives, color enhancers, and preservation agents. Numerous natural pigments are recognized as intriguing bioactive substances with possible health advantages in addition to serving as colorants (Pailliè-Jiménez *et al*., 2020). Although all viable organisms can produce some pigments, the main cell factories that produce large quantities of pigments and food colorants for industrial use are plants and microorganisms (Paul *et al*., 2020). Plants and some microorganisms can produce pigments, which are crucial for their survival in ecological conditions (Swami *et al*., 2020). In addition, the pigments protect the plants from photodamage and also enhance their overall growth and longevity (Sajjad *et al*., 2020). Natural plant-derived pigments have several limitations. Moreover, it is not readily soluble in polar solvents and is not readily accessible year-round. Conversely, pigments extracted from microorganisms exhibit notable persistence at elevated pH or temperature and high solubility in a variety of solvents. Moreover, fermentation processes enable easy growth of microorganisms at industrial and laboratory scales, and downstream processing is relatively straightforward (Sen *et al*., 2019). The bacterial and fungal pigments, prodigiosin, melanin, and pyocyanin, can be used as antimicrobials and as shields

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against UV radiation and oxidative stress (Agarwal *et al*., 2023). Bacterial pigments are among the most fascinating chemical compounds, with a wide range of therapeutic actions. These substances have raised concern among scientists due to their potential in biomedicine. Prodigiosin, synthesized by other bacterial species, possesses unique properties that make it the most appropriate for various therapeutic purposes (Guryanov & Naumenko, 2024). Microbial pigments may vary in response to factors such as light, temperature, pH, and nutrients. Understanding how these pigments are produced and regulated in cells can be used to apply them in medicine, cosmetics, food, and agriculture (Groeneveld *et al*., 2023). The appeal of natural pigments lies in their safety, biodegradability, and as an alternative to synthetic dyes (Yadav *et al*., 2023).

Prodigiosin is one such bright red pigment produced by several bacteria, particularly Serratia species. The most commonly studied producer is Serratia marcescens. Prodigiosin is irregular with a dipyrrole core and a long-chain fatty acid, which makes it colorful and chemically active (Mnif *et al*., 2022). Prodigiosin can kill microbes and protozoa, making it of interest to biotech and pharmaceutical researchers (Darshan & Manonmani, 2015). Prodigiosin is a red coloring substance that is formed by some bacterial species, and it is not only coloured by it. It is widely distributed as a secondary metabolite in the membranes and granules of Gram-positive and Gram-negative bacteria. The prodigiosin and its bacterial derivatives showed protective effects against tumor cells (Paul *et al*., 2020). The antimicrobial activity of the pigment prodigiosin is another, and it is produced in response to ambient conditions that demand higher parameters to allow microbial competitiveness within their microbiome (Jaber *et al*., 2023).

In addition to imparting color, prodigiosin also exhibits antimicrobial activity against various microorganisms. The property is applied in textiles, where it is inappropriate to allow microbial growth, e.g., in medical fabrics, sports apparel, and sourcing materials (Choi *et al*., 2021). The fact that prodigiosin can be used in both functional and aesthetic applications makes it developable in the field of textile technology. To sustain intensive applications, scientists have sought to increase prodigiosin production by optimizing fermentation and genetic modification of the strains (Kumar & Kumar, 2015). Pigment yield has been reported to be enhanced by approaches such as pathway modification and co-culture techniques. Altogether, the biosynthesis of prodigiosin by microorganisms is a more sustainable and cost-effective alternative to artificial dyes and should be considered, given the growing use of natural pigments and biologically active compounds (Paul *et al*., 2020). The current study was devoted to the isolation and characterization of a bacterial strain producing prodigiosin isolated from marine sediments. The experiment was also set out to identify the appropriate circumstances under which pigments could be produced with a high level of success. Moreover, the antimicrobial, antioxidant, and cytotoxic properties of prodigiosin were analyzed using cancer cell lines to determine its therapeutic and textile-industry applications.

Materials and Methods

Isolation and characterization of the prodigiosin-synthesizing strain

The soil samples were collected from the Colachel area in Kanyakumari, Tamil Nadu, India. It was diluted in series and plated on sterile nutrient agar plates. The red-colony-producing strains were isolated from agar plates after incubation and further purified. The biochemical tests were performed, and the isolated strain was identified using 16S rRNA gene sequencing. An accession number was obtained by submitting the acquired gene sequence to GenBank. BLAST analysis was performed, and the Mega 11 software was used to construct the phylogenetic tree.

Optimization of growth parameters for increased prodigiosin production

A set of experiments was conducted to improve prodigiosin production by S. marcescens TPA03 through systematic modifications to fermentation conditions. Carbon sources, including sucrose, glucose, fructose, maltose, lactose, and mannitol, were tested at a 1% concentration. The effects of temperature on growth and pigment production were examined at seven different temperatures: 0°C,

7°C, 17°C, 27°C, 37°C, 45°C, and 65°C. The pH of the growth medium was adjusted over a wide range (1–14) to identify the optimal pH for cellular activity. The sodium chloride concentration was varied from 0% to 5% in increments of 0.5% to examine how salinity influences growth and prodigiosin synthesis. The incubation periods were set at 0, 24, 48, 72, 96, 120, and 144 hours to explore the influence of time on production yield. Experiments were conducted under agitated and stationary conditions to investigate the impact of aeration on fermentation. After optimal growth conditions were established, prodigiosin was extracted via a range of solvents, including distilled water, acetone, DMSO, ethyl acetate, methanol, ethanol, chloroform, and petroleum ether, to identify the most effective solvent for pigment recovery (Gondil *et al*., 2017; Srimathi *et al*., 2017).

Characterization of prodigiosin

Preliminary test for prodigiosin detection

A preliminary examination of the pigment prodigiosin was conducted according to the protocol outlined by Balasubramaniam *et al*. (2019). Initially, bacterial cultures producing prodigiosin were grown under optimal conditions, and the clarified supernatant was obtained by centrifugation at 10,000 rpm for 10 min. Following centrifugation, the supernatant was carefully acidified by adding concentrated hydrochloric acid (HCl) dropwise until the pH reached approximately 3. The resulting mixture was then observed for any color change, indicative of the presence of the pigment prodigiosin. The acidified pigment extract was subsequently alkalinized by gradually adding concentrated ammonia solution until a noticeable pH shift was observed. Characteristic color changes in both the acidified and the alkalinized samples were meticulously recorded. These color changes were analyzed qualitatively, allowing the identification of pigments based on the expected responses of prodigiosin under acidic and alkaline conditions.

Ultraviolet-visible spectroscopic assessment

In order to understand the light absorption by the pigment, we used UV light spectrophotometry. The solvent containing the pigment was extracted and diluted in an appropriate solvent to provide a clear reading. An equal amount of prodigiosin solution was made similarly and compared. The absorbance spectrum of the pigment was noted down, and the wavelength at which the peak absorbance was shown was recorded as the maximum absorption (en=max). The spectra of the extracted pigment, when compared with the standard, were quite similar, indicating that prodigiosin was extracted (Mandal *et al*., 2017).

Fourier transform infrared (FTIR) spectroscopic analysis.

FT-IR analysis was used to identify the functional groups present in the extracted pigment, prodigiosin. It was analyzed using an FT-IR spectrophotometer. The recording was made within the 4000-400 cm -1 spectrum. The analysis was performed in ATR mode, and a ZnSe crystal was used for the measurement. The FI-IR profile of the extracted pigment was compared with the FT-IR profile of a known pigment, prodigiosin, purchased from Cayman Chemical, USA, to obtain the spectrum. This comparison was used to establish the characteristic functional groups of prodigiosin, which included hydroxyl and carbonyl groups, as indicated by the observed absorption bands. (Dos Santos *et al*., 2021).

Gas chromatography-mass spectrometry (GC–MS)

The GC 7890A system with the MS 5975C detector was used for GC-MS analysis. The separation of the compounds was performed on a DB5MS capillary column, 30 m long. The samples were properly prepared using appropriate extraction techniques, ensuring they could be easily identified during analysis. The instrument was also calibrated using standard compounds before the samples were run. A sample of low volume (1-2µl) was injected in spitless mode. The constant carrier gas rate was 1 mL/min, using helium. The oven was set to 60°C for 2 minutes, then slowly ramped to 250°C at 10°C/min, and held at the final temperature for 5 minutes. As the mass analysis method, electron ionization was applied, and mass spectra were obtained in the 50-500 m/z range. The spectra

obtained were matched against the NIST library to identify the compounds present in the sample (Renukadevi *et al*., 2017).

Antimicrobial activity of prodigiosin

The antimicrobial activity of prodigiosin was tested using the well-diffusion method according to standard NCCLS guidelines. Different disease-causing bacterial strains, such as *Bacillus subtilis*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus sp*., *Salmonella typhi*, *Shigella sp*., *Staphylococcus aureus*, and *Streptococcus pyogenes,* were obtained from PSG Institute of Medical Science and Research (PSG-IMSR), Coimbatore. Each bacterial strain was grown in Mueller-Hinton Broth and incubated until the turbidity matched the 0.5 McFarland standard. Mueller Hinton Agar plates were prepared, and wells of 6 mm diameter were made using a sterile borer. The agar surface was evenly swabbed with the respective bacterial cultures. Approximately 100 µL of prodigiosin (10 µg/mL, prepared in methanol) was added to each well. The plates were incubated at 37°C for 24 hours. After this period, the inhibition zones around the wells were measured in mm to evaluate the antimicrobial effectiveness of prodigiosin against the tested pathogens (Sruthy *et al*., 2014).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of prodigiosin

The MIC and MBC of prodigiosin were determined using the microbroth dilution method in sterile 96-well microplates, following standard protocols. In each well, 100 µL of double-strength Mueller-Hinton Broth was added. Prodigiosin stock solutions were prepared in methanol and diluted to achieve final concentrations ranging from 1 to 50 µg/mL. An overnight culture was converted into a bacterial inoculum, which was then adjusted to a 0.5 McFarland standard. There were also control wells to test whether it was sterile and whether it grew as usual. The plates were incubated at 37 °C for 24 hours after all components were added. MIC was defined as the minimal concentration at which bacterial growth was not observed. During MBC establishment, well samples that did not show growth were placed on Mueller-Hinton agar plates and incubated again at 37 °C for 24 hours. MBC was defined as the lowest concentration at which no bacterial colonies formed on agar plates (Ramesh *et al*., 2020).

Synergistic action of prodigiosin with the antibiotic streptomycin

The study employed a checkerboard titration assay to evaluate the enhanced antimicrobial activity of prodigiosin in combination with a selection of antibiotics to combat pathogenic bacteria, in accordance with ASM standards. Various concentrations of prodigiosin and antibiotics were combined in a 2D layout in a sterile 96-well flat-bottom microtiter plate, with each well corresponding to a distinct combination of the two agents. The inoculum for the bacterial strains was standardized as previously described. Control wells were included to ensure the validity of the experiments. After adding the combinations, the plates were incubated at 37°C for 24 hours. The MBC for each combination was determined, and the fractional bactericidal concentration (FBC) index was calculated by summing the FBC values for prodigiosin and antibiotics (∑ FBC index = FBC_prodigiosin + FBC_antibiotic). The FBC was defined as the MBC of the combined drugs divided by the MBC of each drug used alone. The interaction between prodigiosin and the antibiotics was identified as synergistic if the FBC index was ≤ 0.5, additive if it was > 0.5 to ≤ 1.0, indifferent if it was > 1.0 to ≤ 2.0, and antagonistic if it was > 2.0 (Ji & Kim, 2019).
where,

$$\text{FICA} = \text{MIC in combination}\frac{\text{Prodigiosin} + \text{Streptomycin}}{\text{MIC of Streptomycin}}$$

$$\text{FICB} = \text{MIC in combination}\frac{\text{Prodigiosin} + \text{Streptomycin}}{\text{MIC of Prodigiosin}}$$

The FICA was calculated as the MIC of the prodigiosin-streptomycin combination relative to the MIC of streptomycin. In contrast, FICB is determined by dividing the MIC of the combination by the MIC of

prodigiosin. An interaction was deemed synergistic when the FIC index was ≤ 0.5, additive when >0.5 to ≤1.0, indifferent when >1.0 to ≤2.0, and antagonistic when it was greater than 2.0.

Antioxidant activity

The radical scavenging activity of prodigiosin extracted from *S. marcescens* TPA03 was evaluated via the DPPH method. A stock solution of DPPH was prepared by dissolving 24 mg in 100 mL of methanol to obtain a concentration of 100 µM, and the solution was stored in the dark to prevent degradation. Prodigiosin was extracted and diluted to concentrations of 50, 150, 250, 350, 450, and 550 µg/mL. In the assay, 1 mL of each prodigiosin solution was mixed with 3 mL of DPPH solution in separate test tubes. A control was established using 1 mL of methanol instead of a prodigiosin solution. The mixture was gently vortexed and incubated in the dark at room temperature for 30 min to enhance the reaction. After incubation, the absorbance of the resulting solution was measured at 517 nm (Formagio *et al*., 2014).

Statistical analysis

ANOVA, the Kruskal-Wallis chi-square test, and diagrammatic representation were performed in R (version 4.0.2).

Anticancer activity

The cytotoxicity of prodigiosin extracted from *S. marcescens* TPA03 was assessed using the MTT assay in the human breast cancer cell line MCF-7. MCF-7 cells were cultured in complete growth medium and incubated under standard conditions (37°C, 5% CO_2) until 70–80% confluence was reached. The cells were harvested and seeded into 96-well plates at a density of 1 × 10^4 cells per well and allowed to attach for 24 hours. Purified prodigiosin was dissolved in dimethyl sulfoxide (DMSO) to prepare a concentrated stock solution, which was subsequently diluted into complete culture medium to obtain various prodigiosin concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg/mL). The final DMSO concentration in all wells was maintained below 0.1% (v/v) to avoid solvent-induced cytotoxicity. These concentrations were selected based on preliminary range-finding experiments and previously reported cytotoxic ranges. After treatment with prodigiosin for 24 hours, 20 µL of MTT solution was added to each well, and the plates were incubated for an additional 4 hours to allow viable cells to reduce MTT to insoluble formazan crystals. The formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm using a microplate reader (Van de Loosdrecht *et al*., 1994).

$$\text{Percentage of viability (\%V)} = 100(At/Ac)$$

$$\text{Percentage of inhibition (\%I)} = 100\,[1-(At/Ac)]$$

where At – is the absorbance of the treated cells, and Ac – is the absorbance of the control cells

Results

Isolation and identification of the prodigiosin-producing strain

A marine sediment sample was collected from Colachel, Kanyakumari District, Tamil Nadu, India. After serial dilution of the sediment, it was spread onto nutrient agar plates, resulting in the isolation of a red colony-forming strain after incubation. Table 1 presents the biochemical characteristics of strain TPA03. The indole test yielded a negative result, indicating the absence of indole production, whereas the methyl red test was negative, suggesting a lack of stable acid production from glucose fermentation. In contrast, the Voges-Proskauer test result was positive, confirming acetoin production. The citrate utilization test results were positive, indicating that strain TPA03 can utilize citrate as its sole carbon source. The urease and oxidase tests were negative, indicating that this strain did not hydrolyze urea and lacked cytochrome oxidase. Conversely, the catalase test was positive, indicating catalase activity. The starch hydrolysis test was negative, suggesting that strain TPA03 did not hydrolyze starch, whereas the lactose hydrolysis test was positive, indicating its ability to ferment

lactose. Finally, the hydrogen sulfide test returned negative results, confirming that strain TPA03 did not produce hydrogen sulfide.

Table 1: *Biochemical characteristics of strain TPA03*

S. No.	Biochemical test	Result
1	Indole test	Negative
2	Methyl red test	Negative
3	Voges Proskauer	Positive
4	Citrate utilization test	Positive
5	Urease	Negative
6	Oxidase	Negative
7	Catalase	Positive
8	Starch hydrolysis	Negative
9	Lactose hydrolysis	Positive
10	Hydrogen sulfide test	Negative

This strain was purified and identified via 16S rRNA sequencing. The sequence obtained was submitted to the GenBank database under accession number OM475689.1. A subsequent BLAST analysis revealed that the isolate exhibited 100% similarity to *S. marcescens*. A phylogenetic tree was constructed using MEGA 11, further confirming the close evolutionary relationship between the isolate and *S. marcescens* (Figure 1 and Table 2).

Table 2: *Sequences producing significant alignments*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Serratia marcescens subsp. sakuensis strain KRED	2730	2730	99%	0	98.87%	NR_036886.1
Serratia marcescens strain NBRC 102204	2682	2682	98%	0	99.73%	NR_114043.1
Serratia marcescens subsp. marcescens ATCC 13880 strain DSM 30121	2682	2682	98%	0	99.59%	NR_041980.1
Serratia marcescens subsp. marcescens ATCC 13880 strain JCM 1239	2680	2680	98%	0	99.66%	NR_113236.1
Serratia surfactantfaciens strain YD25	2673	2673	98%	0	99.59%	NR_169468.1
Serratia ureilytica strain NiVa 51	2575	2575	98%	0	98.56%	NR_042356.1
Serratia entomophila strain DSM 12358	2567	2567	99%	0	98.10%	NR_025338.1
Enterobacter soli ATCC BAA-2102 strain LF7	2556	2556	99%	0	97.71%	NR_117547.1
Serratia ficaria strain DSM 4569	2553	2553	98%	0	98.09%	NR_041979.1
Serratia odorifera strain PADG 1073	2549	2549	98%	0	97.96%	NR_037110.1

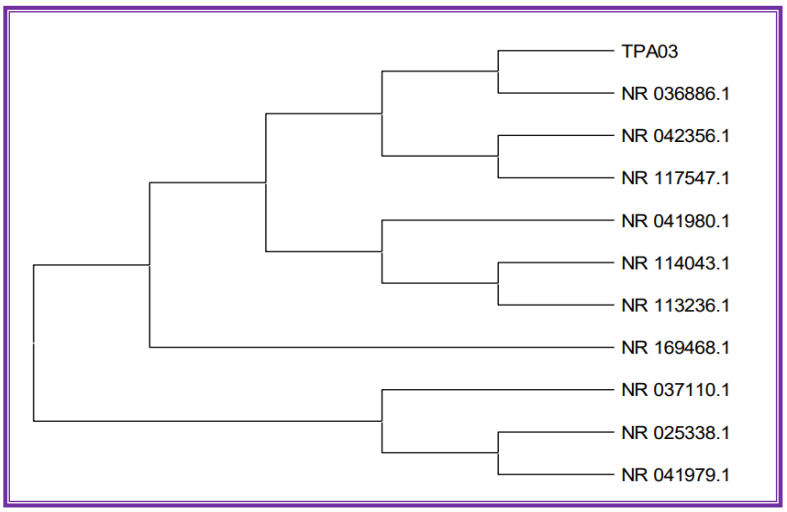

Figure 1: *Phylogenetic analysis*

Optimization of growth parameters for increased prodigiosin production

Effect of the carbon source on prodigiosin production:

This study assessed the impact of different carbon sources on prodigiosin pigment production by measuring the OD at 530 nm. The results revealed significant differences in pigment production across the tested carbon sources. Mannitol yielded the highest OD value (1.99), indicating it was the most effective carbon source for promoting prodigiosin synthesis. Sucrose had excellent performance, with an OD of 1.81, while maltose had an OD of 1.78. Lactose achieved a moderate OD of 1.53, whereas fructose and glucose were less effective, with OD values of 1.12 and 1.08, respectively. These findings suggest that the choice of carbon source plays a critical role in optimizing prodigiosin production, with mannitol and sucrose being the most conducive for enhancing pigment synthesis in *S. marcescens* (Figure 2). $p < 0.001$ indicates a highly significant effect of the carbon source on prodigiosin production.

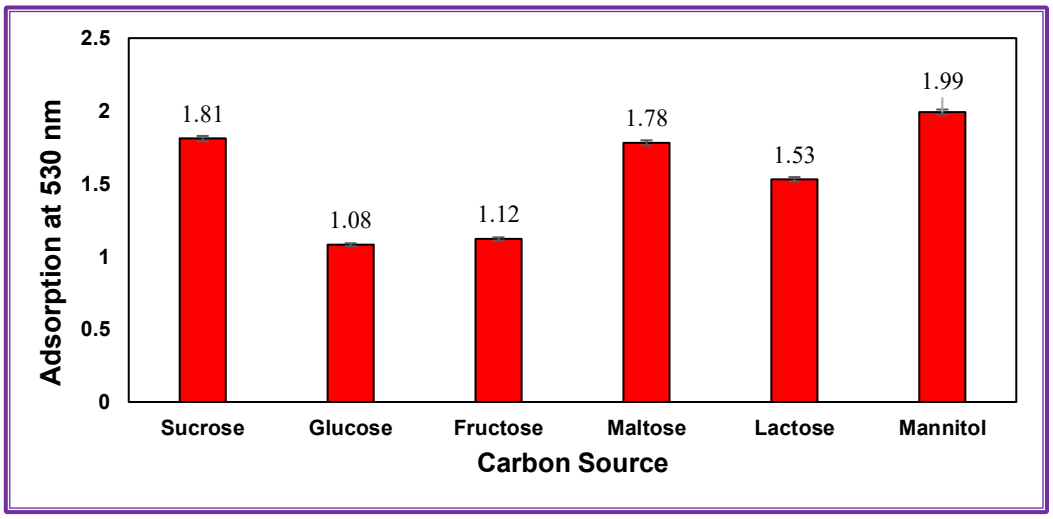

Figure 2: *Effect of the carbon source on prodigiosin pigment production*

Effect of pH on prodigiosin production

The impact of pH on prodigiosin pigment production was evaluated by recording the OD at 530 nm over a range of pH values. The results indicated that pigment production varied significantly with pH. The highest OD value of 1.92 was observed at pH 7, suggesting optimal conditions for prodigiosin synthesis at neutral pH. Additionally, pH 6 also supported notable production with an OD of 1.45. Other pH levels, including 8 (OD 1.76) and 9 (OD 1.64), resulted in moderate pigment production,

whereas more acidic conditions (pH 1-5) and highly alkaline conditions (pH 11-14) resulted in little to no prodigiosin production, with OD values of 0. These findings indicate that a neutral to slightly alkaline pH maximizes prodigiosin synthesis in S. marcescens, highlighting the importance of pH in optimizing production conditions for this pigment (Figure 3). $P < 0.05$ (0.005519) indicates that there is a statistically significant difference in prodigiosin production across different pH levels.

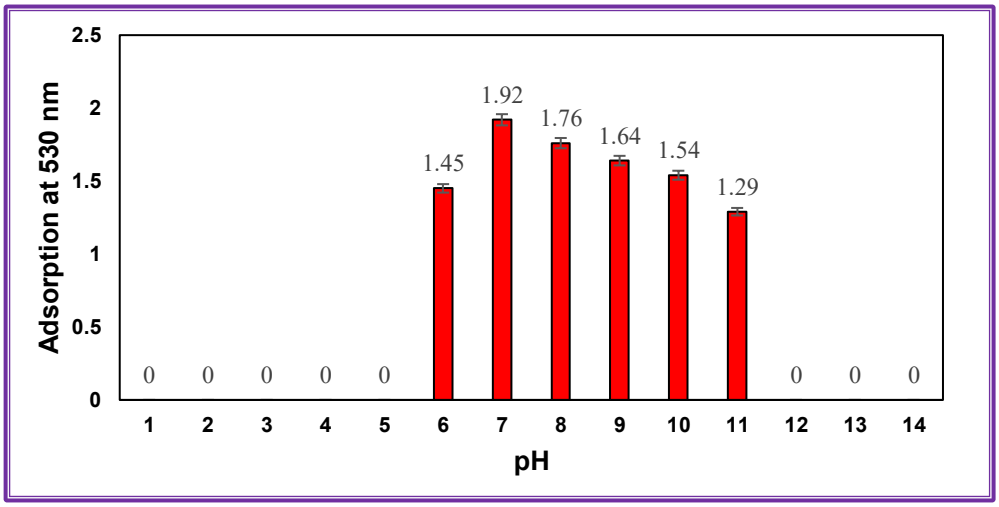

Figure 3: *Effect of pH on prodigiosin pigment production*

Effect of temperature on prodigiosin pigment production:

The effect of temperature on prodigiosin pigment production was evaluated by measuring the OD at 530 nm across various temperatures (Figure 4). These results indicated a clear temperature dependency in pigment synthesis. Notably, the highest OD value of 1.95 was recorded at 27°C, demonstrating optimal conditions for prodigiosin production. In contrast, temperatures below 17°C and above 37°C resulted in minimal to no pigment production, with OD values of 0.11 at 17°C and 0.1 at 37°C, whereas extreme temperatures of 0°C, 7°C, 45°C, and 65°C yielded OD values of 0. These findings suggest that prodigiosin production in *S. marcescens* is significantly increased at moderate temperatures, particularly around 27°C, whereas both low and high temperatures adversely affect pigment synthesis. $p < 0.05$ indicates a statistically significant difference in prodigiosin production across different temperature levels.

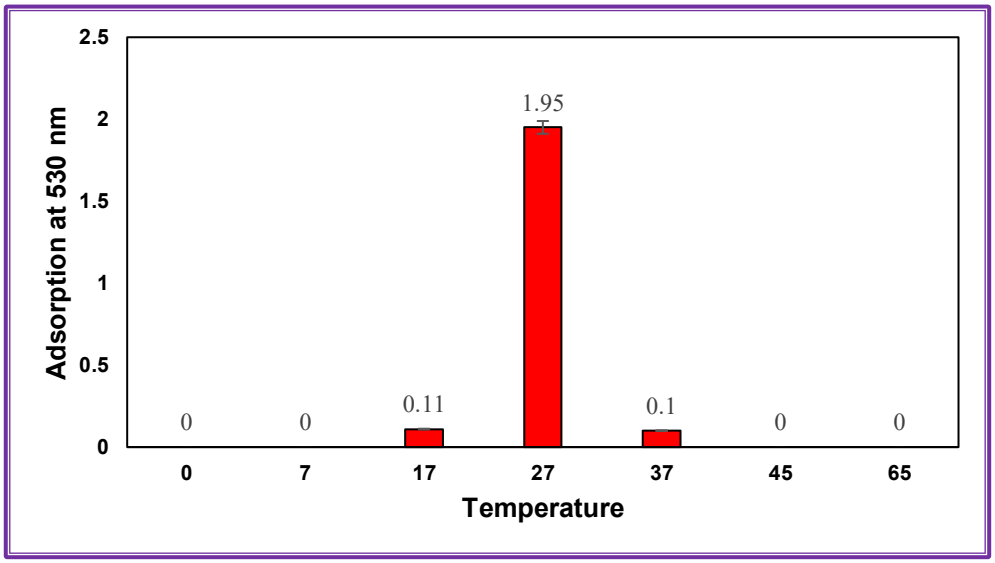

Figure 4: *Effect of temperature on prodigiosin pigment production*

Effect of salt concentration on prodigiosin pigment production

The impact of salt concentration on prodigiosin pigment production was investigated by measuring the OD at 530 nm across a range of salt concentrations (Figure 5). The results revealed that salt presence significantly influenced pigment synthesis. The highest OD value of 1.97 was observed at 0.5% salt, indicating that low salt levels enhance prodigiosin production. As the salt concentration increased to 1% and 1.5%, the OD values were 1.73 and 1.43, respectively, indicating a gradual decline in pigment production. Further increases in salt concentration led to a marked decrease in OD, with values decreasing to 1.26 at 2%, 0.97 at 2.5%, and 0.21 at 3%. Concentrations above 3% resulted in negligible production, with OD values of 0.05 at 3.5% and 0 at 4% and above. These findings suggest that while low salt concentrations can promote prodigiosin synthesis in *S. marcescens*, high salt concentrations are detrimental to pigment production. $p < 0.001$ indicates a highly significant effect of salt concentration on prodigiosin production.

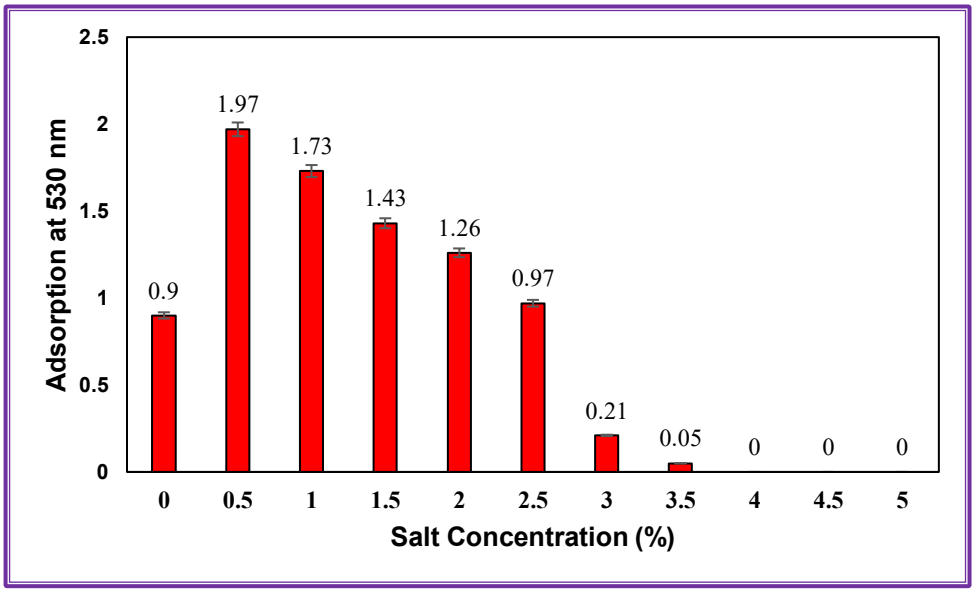

Figure 5: *Effect of salt concentration on prodigiosin pigment production*

Effect of incubation period on prodigiosin pigment production

The effect of the incubation period on prodigiosin pigment production was assessed by measuring OD at 530 nm at multiple time points. The results indicated a clear trend of increasing pigment synthesis over time, with the OD value starting at 0 at 0 hours and gradually increasing. At 24 hours, the OD was 0.32, and it increased to 0.51 at 48 hours. Notably, a significant increase in prodigiosin production was observed at 72 hours, with an OD of 0.9. The highest OD value of 1.92 was recorded at 120 hours, indicating peak pigment production at this time point. Although there was a slight decrease to 1.8 at 144 hours, the overall trend demonstrated that extended incubation promoted prodigiosin synthesis in *S. marcescens*. These findings suggest that optimal pigment production occurs between 120 and 144 hours of incubation (Figure 6). $p < 0.001$ indicates a highly significant effect of incubation period on prodigiosin production.

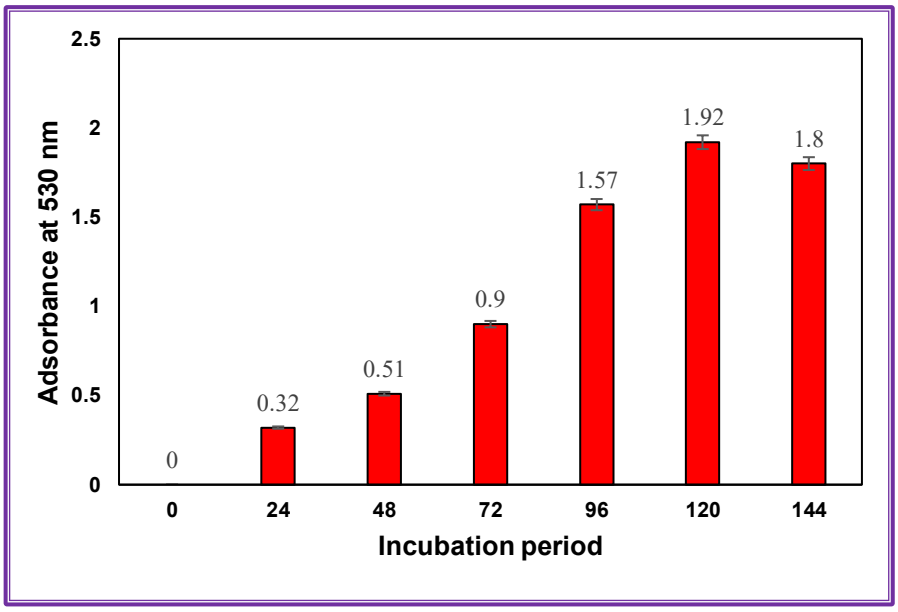

Figure 6: *Effect of incubation period on prodigiosin pigment production*

Effect of shaking/static conditions on prodigiosin pigment production

The effects of shaking and static conditions on prodigiosin pigment production were evaluated by measuring the OD at 530 nm (Figure 7). The results indicated a substantial difference in pigment synthesis under the two conditions. Shaking conditions yielded a significantly higher OD of 2.25, suggesting that agitation enhances prodigiosin production in *S. marcescens*. In contrast, under static conditions, an OD of 1.12 was observed, indicating reduced pigment synthesis. These findings demonstrate that shaking, which likely facilitates better oxygen transfer and nutrient availability, is more conducive to maximizing prodigiosin production than static cultivation. $p < 0.001$ indicates a highly significant effect of shaking *vs* static conditions on prodigiosin production.

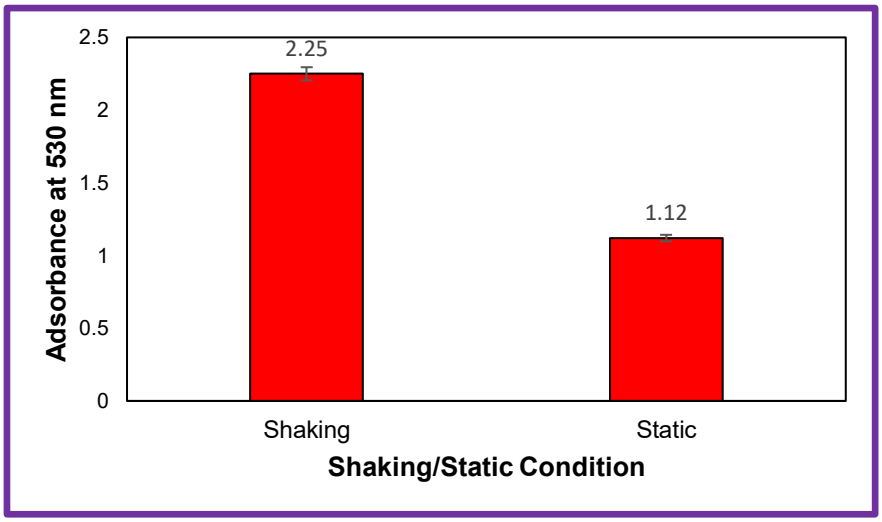

Figure 7: *Effect of shaking/static conditions on prodigiosin pigment production*

Selection of a suitable solvent for maximal prodigiosin extraction

The effects of different solvents on prodigiosin pigment production were assessed by measuring the OD at 530 nm (Figure 8). The results indicated significant variability in pigment extraction efficiency among the solvents tested. Methanol emerged as the most effective solvent, yielding the highest OD value of 1.53, suggesting that it is optimal for extracting prodigiosin from *S. marcescens*. DMSO had an OD of 0.77, indicating good solvent properties for pigment extraction. Ethyl acetate also showed

moderate efficacy with an OD of 0.51. In contrast, acetone, ethanol, chloroform, and petroleum ether yielded OD values of 0.4, 0.55, 0.2, and 0.33, respectively, whereas distilled water showed no detectable pigment production. $p < 0.001$ indicates highly significant differences in prodigiosin extraction efficiency across solvents.

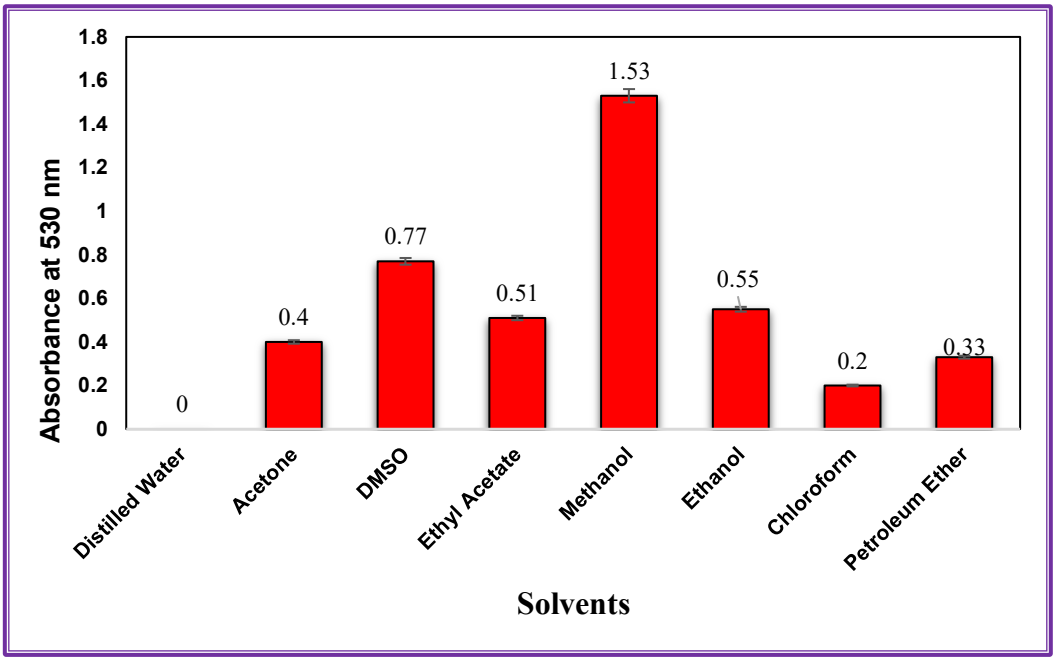

Figure 8: *Effect of solvents on prodigiosin pigment production*

Characterization of prodigiosin

Preliminary test for prodigiosin detection

A significant color change was observed during the experiment: the tube acidified with concentrated hydrochloric acid (HCl) turned pink, whereas the one treated with concentrated ammonia solution turned yellow. These distinct color changes indicate the presence of prodigiosin: the pink color usually represents the protonated form of the pigment, whereas the yellow color indicates its deprotonated state under alkaline conditions (Figure 9).

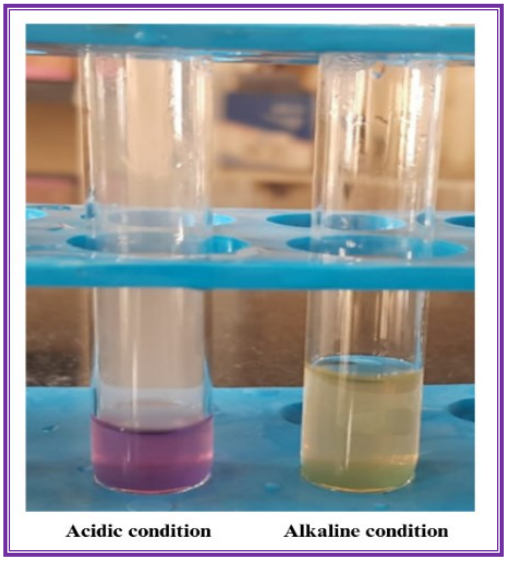

Figure 9: *Presumptive test for prodigiosin pigment*

Ultraviolet-visible spectroscopic assessment

The maximum absorbance of prodigiosin was determined to be 534 nm for the sample and 534.50 nm for the standard. Notably, under acidic conditions, prodigiosin appeared red, with a maximum absorption at 535 nm. These results confirm the characteristic absorption properties of prodigiosin and demonstrate its stability in acidic environments (Figure 10).

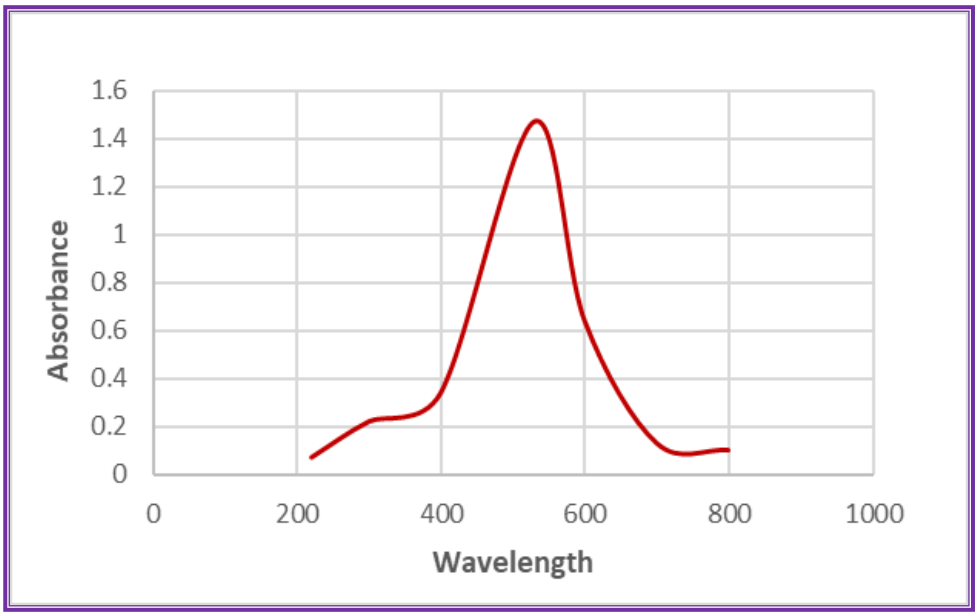

Figure 10: *UV analysis of the prodigiosin pigment isolated from S. marcescens TPA03*

Fourier transform-infrared (FTIR) spectroscopic analysis.

FT-IR analysis of prodigiosin revealed a range of significant absorption peaks that provide insights into its functional groups and molecular structure (Figure 11). Notable peaks were observed at 432.05 cm^{-1}, 501.49 cm^{-1}, and 555.50 cm^{-1}, which may correspond to the bending vibrations of various functional groups. The prominent peak at 601.79 cm^{-1} suggests the presence of out-of-plane bending vibrations, which may indicate aromatic structures—the peaks at 671.23 cm^{-1} and 817.82 cm^{-1} further support the aromatic character of the molecule. The spectrum also displayed peaks at 879.54 cm^{-1} and 1049.28 cm^{-1}, which are likely associated with C–H bending and C–O stretching vibrations, respectively. The additional peaks at 1249.87 cm^{-1} and 1396.46 cm^{-1} indicate the presence of –C–N and –C–H bending modes, reflecting the nitrogen functionality within the prodigiosin structure. The peak at 1543.05 cm^{-1} corresponds to C=C stretching vibrations, whereas the peak at 1627.92 cm^{-1} suggests the presence of carbonyl (C=O) groups. Furthermore, significant peaks at 2306.86 cm^{-1}, 2978.09 cm^{-1}, and 3286.70 cm^{-1} indicate the presence of C–H stretching and O–H stretching vibrations, which are consistent with the alcohol or phenolic functional groups in prodigiosin. The peak at 3718.76 cm^{-1} indicates the presence of hydroxyl (–OH) groups, reinforcing the molecule's potential bioactive properties.

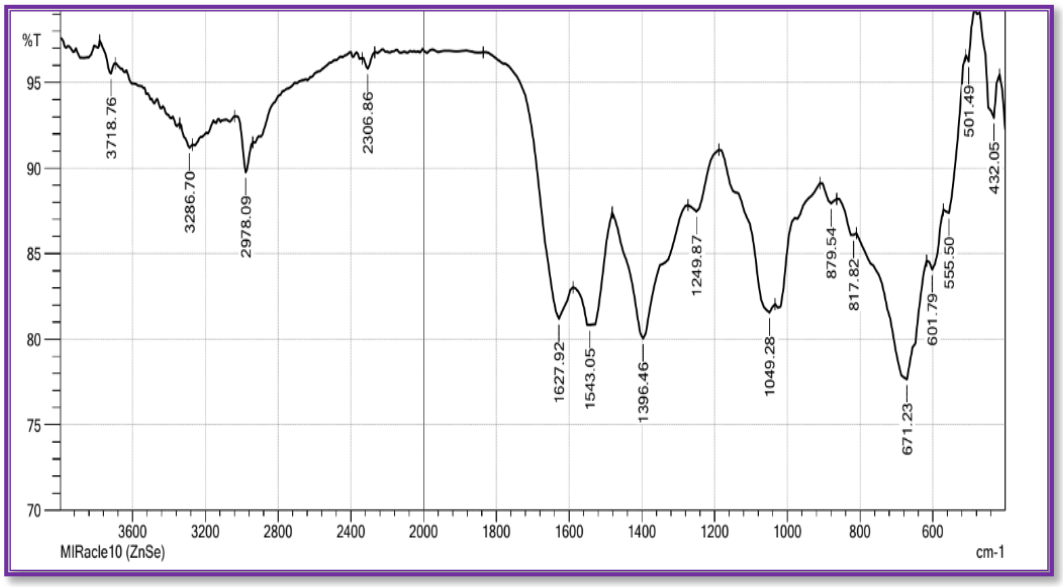

Figure 11: *FTIR analysis of the prodigiosin pigment isolated from S. marcescens TPA03*

Gas chromatography-mass spectrometry (GC–MS)

The GC-MS analysis of prodigiosin revealed a diverse range of compounds, characterized by varying retention times and area percentages (Table 3). Notably, the analysis revealed several significant peaks, with 1,3-benzodioxole, 5-(1-propenyl)- and eugenol exhibiting the highest area percentages of 8.51% and 7.63%, respectively, at retention times of 7.898 and 8.464 min. Other prominent compounds included 2-cyclohexene-1,4-diol, which accounted for 13.46% at 14.619 min, suggesting its potential relevance to the sample's composition. Additionally, 1,2,5-oxadiazol-3-carboxamide, tridecanoic acid, and 12-methyl-, methyl ester were also detected, contributing to the overall profile, with area percentages of 1.18% and 8.01%, respectively. The complexity of the resulting chromatogram suggests a rich chemical composition that may contribute to the biological activity of prodigiosin (Figure 12).

Table 3: GC-MS analysis of prodigiosin

S. No.	Compound Name	Retention Time	Area Percentage
1.	Tungsten, dicarbonyl-	4.487	1.10
2.	Benzene, 1,2,3-trimethyl-	4.842	2.58
3.	Pyrazine	5.142	4.81
4.	Pyrrylmethyl(triethyl)stannane	5.209	3.81
5.	5-Oxazolecarbonitrile, 4-methyl-	5.365	1.48
6.	1,2,5-Oxadiazol-3-carboxamide, -azobis-, 2,2'-dioxide	5.509	1.18
7.	Oxane-2,4-dione, 6-(4-bromophenyl) -5-ethyl-3,3-dimethyl-, trans	5.898	1.11
8.	Pentane, 3-ethyl-2-methyl-	5.953	4.88
9.	1,2,3,6-Tetrahydropyridine	6.964	2.94
10.	1,3-Benzodioxole, 5-(1-propenyl)-	7.898	8.51
11.	Eugenol	8.464	7.63
12.	1,3,6-Octatriene, 3,7-dimethyl-	9.120	2.77
13.	2,6-Dimethyl-3-amino benzoquinone	9.497	2.07
14.	alpha.-(N,N-Dimethylamino)-3'-hydroxy-4'-methoxy acetophenone	9.731	3.05
15.	Benzoic acid, 4-hydroxy-	9.908	2.66
16.	Spiro(1,3-dioxolane)-2,3'-pregn-5' -en-20'-ol, 11'-acetoxy-18'-(methyl amino)-	10.342	1.20
17.	1-Methyl-1,2-cis-cyclopropane dicarboxylic acid	11.253	1.49
18.	1-(4-Amino-furazan-3-yl)-5-dimethylaminomethyl-1H-[1,2,3]triazole-4-carboxylic acid ethyl ester	11.286	1.76
19.	Methyl 10-methyl-undecanoate	11.864	1.88
20.	Tridecanoic acid, 12-methyl-, methyl ester	11.919	8.01

21.	Hexadecanoic acid, methyl ester	12.575	2.85
22.	Methyl 11-cyclopentyl undecanoate	12.819	1.86
23.	Phthalic acid, 2-methyl allyl octyl ester	13.075	1.21
24.	Vanadium, (eta.7-cycloheptatrienylium) (.eta.5-2,4-cyclopentadien-1- yl)	14.375	2.68
25.	+/-.-trans-2-Cyclohexene-1,4-diol	14.619	13.46
26.	N-Methyl-1-adamantaneacetamide	15.452	2.41
27.	Cyclotrisiloxane, hexamethyl-	15.852	1.49
28.	Silane, 1,4-phenylenebis [trimethyl	16.796	3.82
29.	Cyclotrisiloxane, hexamethyl-	17.463	1.84
30.	Cyclotrisiloxane, hexamethyl-	17.741	3.48

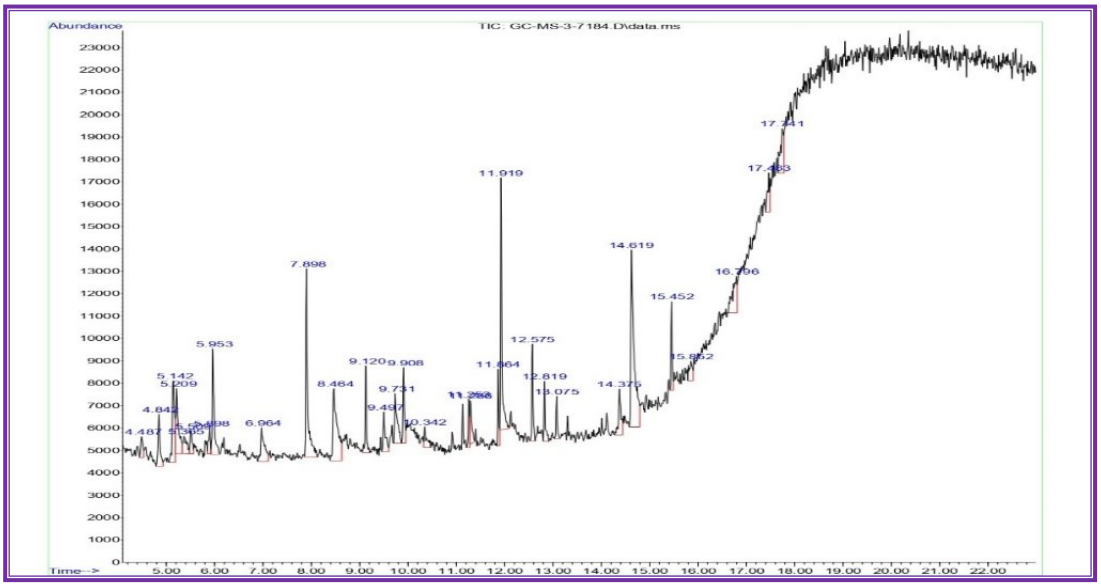

Figure 12: *Chromatogram of prodigiosin*

Antimicrobial activity of prodigiosin

The antimicrobial activity of prodigiosin (10 µg/mL) was assessed by measuring the zones of inhibition against various pathogens (Table 4), with the results indicating notable efficacy. *Staphylococcus aureus* exhibited the largest zone of inhibition (38 mm), indicating strong susceptibility to prodigiosin. Other gram-positive bacteria, such as *Streptococcus pyogenes* and *Bacillus subtilis*, presented zones of 28 mm and 10 mm, respectively. Among the gram-negative bacteria, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* displayed zones of 20 mm and 28 mm, respectively, indicating moderate sensitivity. *Escherichia coli* and *Salmonella enteritidis* showed inhibition zones of 16 mm and 17 mm, respectively, whereas Proteus sp. and Salmonella sp. showed inhibition zones of 29 mm and 22 mm, respectively. Finally, *Shigella* sp. displayed a zone of 24 mm. Notably, prodigiosin demonstrated broad-spectrum antimicrobial activity, inhibiting both gram-positive and gram-negative bacteria. Such dual efficacy is uncommon among natural products, highlighting the potential of prodigiosin as a versatile antimicrobial agent for further therapeutic applications.

Table 4: Antimicrobial activity of prodigiosin

Pathogen	**Zone of Inhibition (mm)**
Staphylococcus aureus	38
Streptococcus pyogenes	28
Bacillus subtilis	10
Pseudomonas aeruginosa	28
Klebsiella pneumoniae	20
Proteus sp.	29
Salmonella sp.	22
Escherichia coli	16
Salmonella enteritidis	17
Shigella sp.	24

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of prodigiosin

The antimicrobial activity of prodigiosin was evaluated through MIC and MBC assays (Table 5). The results indicated that no growth inhibition was observed at concentrations of 1000, 750, 500, and 250 µg/mL. However, at a concentration of 125 µg/mL, growth was present, suggesting a lack of significant antibacterial activity. In contrast, concentrations of 62.50 µg/mL and below demonstrated both inhibitory and bactericidal effects, as indicated by positive results for growth inhibition and bactericidal activity at 62.50, 31.25, 15.62, 7.81, 3.90, 1.95, and 0.97 µg/mL. These findings suggest that prodigiosin exhibits potent antimicrobial properties at relatively low concentrations, effectively inhibiting and killing bacteria as the concentration decreases.

***Table 5*:** MIC and MBC of prodigiosin

Concentration (µg/mL)	MIC of prodigiosin	MBC of prodigiosin
1000	-	-
750	-	-
500	-	-
250	-	-
125	-	+
62.50	+	+
31.25	+	+
15.62	+	+
7.81	+	+
3.90	+	+
1.95	+	+
0.97	+	+

Synergistic activity of prodigiosin with the antibiotic streptomycin

The synergistic activity of prodigiosin in combination with the antibiotic streptomycin was evaluated through minimum inhibitory concentration (MIC) testing (Table 6). The results indicated that prodigiosin exhibited significant antimicrobial activity at concentrations of 125 µg/mL or lower, while streptomycin also showed activity at 125 µg/mL. Notably, at a concentration of 62.50 µg/mL, both prodigiosin and streptomycin effectively inhibited the bacteria, with MIC values indicating that the combined treatment increased the overall antimicrobial activity. The calculations revealed that the FIC for prodigiosin (FICA) was 0.50 µg/mL, and the FICB value of 1.00 indicates that, while the combination maintains the effectiveness of both agents, it does not exhibit synergy to the same extent as prodigiosin.

These FIC values indicate a synergistic interaction between prodigiosin and streptomycin, as an FIC index of ≤ 0.5 indicates a combined effect. The combination treatment retains individual efficacy and enhances overall antimicrobial action, suggesting that prodigiosin, alongside streptomycin, could be a promising strategy for combating resistant bacterial infections.

***Table 6:** Synergistic activities of prodigiosin with streptomycin antibiotic*

Concentration (µg/mL)	MIC of prodigiosin	MIC of streptomycin	MIC of prodigiosin + streptomycin
1000	-	-	-
750	-	-	-
500	-	-	-
250	-	-	-
125	-	+	-
62.50	+	+	+
31.25	+	+	+
15.62	+	+	+
7.81	+	+	+
3.90	+	+	+
1.95	+	+	+
0.97	+	+	+

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Antioxidant activity

The results of the DPPH radical scavenging assay showed that prodigiosin extracted from Serratia marcescens TPA03 exhibited significant antioxidant activity at concentrations of 50, 150, 250, 350, 450, and 550 µg/mL (Figure 13). As the concentration of prodigiosin increased, the percentage of DPPH radical scavenging also increased, indicating a dose-dependent response. At the lowest concentration of 50 µg/mL, a modest scavenging effect was observed. In contrast, at higher concentrations, particularly at 550 µg/mL, the scavenging activity reached its peak, effectively neutralizing a substantial proportion of the DPPH radicals.

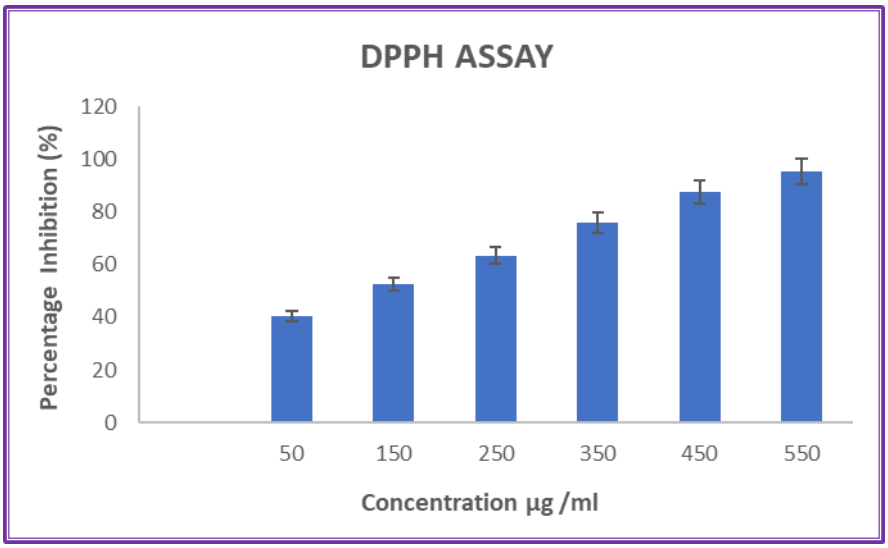

Figure 13: *DPPH scavenging activity of prodigiosin*

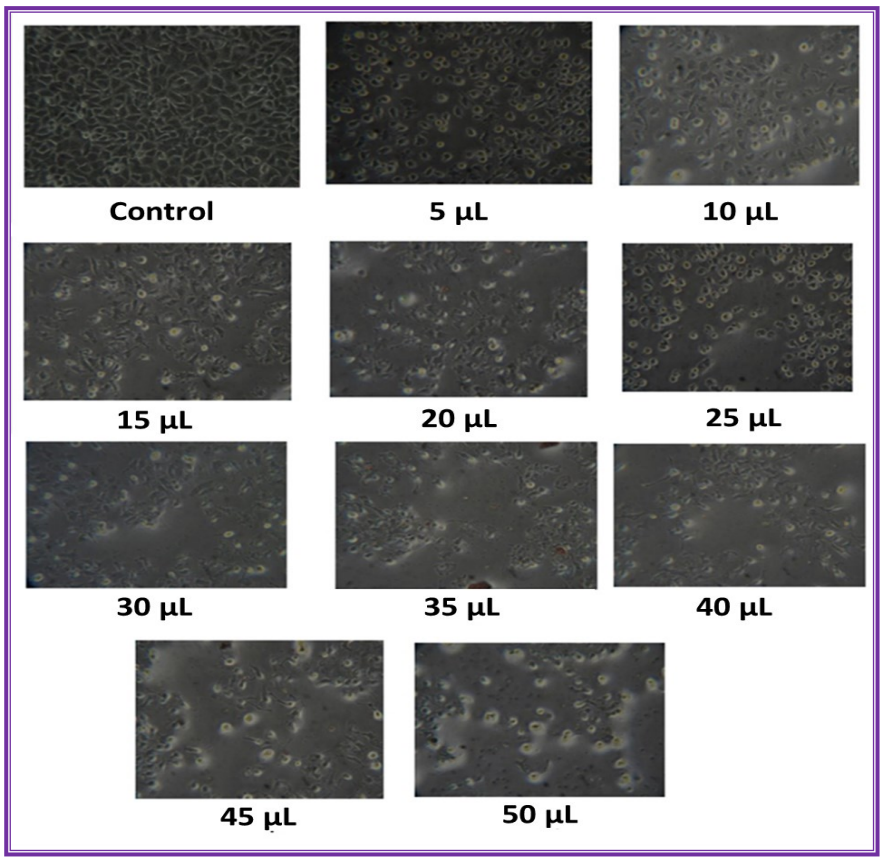

Figure 14a: *Anticancer activity of prodigiosin pigment isolated from S. marcescens TPA03.*

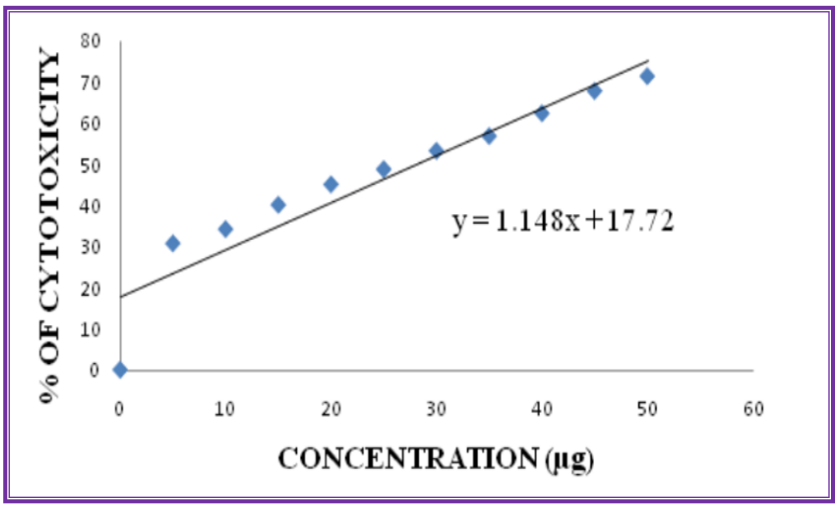

Figure 14b: *Anticancer activity of the prodigiosin pigment isolated from S. marcescens TPA03*

Anticancer activity

The results of the MTT assay showed that the extracted pigment from Serratia marcescens TPA03 exhibited significant cytotoxicity against the MCF-7 human breast cancer cell line (Figure 14a and b). Following 24 hours of exposure to varying pigment concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg), a dose-dependent inhibition of cell growth was observed. The lowest concentration of 5 µL had minimal effect on cell viability, whereas concentrations of 30 µL or higher significantly reduced cell viability, indicating a strong cytotoxic effect. At the highest concentration of 50 µL, the viability of the MCF-7 cells was markedly diminished, demonstrating the potential of the pigments as an effective anticancer agent.

Discussion

Natural microbial products are important secondary metabolites with therapeutic uses. Various types of microbes produce secondary metabolites along with pigment synthesis. The lower-molecular-weight pigments are biopigments derived from both microbes and plants (Chauhan *et al*., 2017). One of the secondary metabolites is the prodigiosin pigment, which has various applications that include medicine, agriculture, and food technology, and these applications are extensive (Srilekha *et al*., 2024). In this study, the pigment prodigiosin-producing strain was isolated from marine sediment, and its parameters were examined to increase pigment production. The antimicrobial, antioxidant, and cytotoxicity activities of the prodigiosin pigment against cancer cells were also tested, highlighting its potency in the medical and textile sectors. To identify the strain, 16S rRNA gene sequencing was performed. The role of the bacterial pigment has not changed over the years, and the gene spans approximately 1500 base pairs and is used to identify bacterial species and genera (Garcha *et al*., 2016). Based on BLAST analysis of the 16S rRNA gene, a red-colored bacterium, NS-17, isolated from Nanchang soil, was identified as Serratia marcescens, to which it is 99% related (Fu *et al*., 2019). Similarly, a red-colored bacterium from marine water was also identified as Serratia nematodiphila DZ0503SBS1, with a 99.59% BLAST match (Manas *et al*., 2020).

Serratia is a Gram-negative bacterium commonly found in diverse environments. It is distinguished by its production of the red pigment prodigiosin, a tripyrrole compound known for its immunosuppressive, antioxidant, anticancer, and antimicrobial properties (Elkenawy *et al*., 2017). In this research paper, the red strain was obtained as a production of *S. marcescens* (Pereira *et al*., 2024). The production of prodigiosin pigment from *S. marcescens* basically depends on several growth factors. The factors include temperature, pH, medium composition, natural components, and phosphate availability. Pigment yield can be increased by changing the medium, pH, or temperature (Paul *et al*., 2024). The carbon sources had a significant effect on pigment production. In which the sucrose yields 1147.32 mg/L, glycerol gave 1138.71 mg/L, and the mannitol shows 994.38 mg/L (Sujitha & Rajanarayanan,

2020). Carbon sources play a major role in pigment synthesis. Previous studies reported high pigment yields with sucrose, glycerol, and mannitol, whereas glucose was less effective (Sundararajan & Ramasamy, 2024). UV-visible spectroscopy showed the pigment had maximum absorption at 535 nm when grown on shrimp heads (Nguyen *et al*., 2021). Other reports found it to be 532 nm (Sajjad *et al*., 2020).

FTIR analysis revealed a characteristic functional group at 3435.22 cm^{-1}, suggesting N–H groups. Methylene vibrations appeared at 2926.01 cm^{-1} and 2856.58 cm^{-1}. Symmetric and asymmetric stretching gave a peak at 2960.17 cm^{-1}. Peaks at 1718.97 cm^{-1} and 1445 cm^{-1} were from N–H and methyl groups. C–O groups caused a peak at 1381.26 cm^{-1} (Maurya *et al*., 2020). In the fingerprint region, 1707.36 cm^{-1} showed C=O, and 3288.96 cm^{-1} showed NH stretching (Dos Santos *et al*., 2021).

The pigmented *S. marcescens* strains are known to cause infections much less often than the non-pigmented bacterial strains. This ensures safer large-scale pigment production (Elkenawy *et al*., 2017). Prodigiosin has demonstrated strong antimicrobial activity, particularly against Gram-positive bacteria such as *S. aureus* and *Bacillus species* (Gugu *et al*., 2020). The purified prodigiosin pigment inhibited all tested bacteria, with MIC values ranging from 32 to 64 µg/mL (Ji & Kim, 2019). The MBC values were higher than the MIC values, and the lowest MBC was for *L. monocytogenes*. For clinical isolates of P. aeruginosa, MICs ranged from 8 to 64 µg/mL. The MIC and MBC of strain PAO1 were 32 µg/mL and 128 µg/mL, respectively (Ma *et al*., 2024). When Hazarika *et al*. (2021) tested prodigiosin against these bacterial strains, *S. aureus*, *B. subtilis*, and *B. cereus*, the MIC values resulted in 3, 5, and 4 µg/mL, respectively. It had a final MBC of 50 0 ug/mL with B. subtilis, and 200 ug/mL with S. aureus and B. cereus. The Prodigiosin pigment was tested against streptomycin, which has an FIC value of 4.50. This suggests that the prodigiosin pigment treatment could be sufficient for staphylococcal infection (Sundararajan & Ramasamy, 2024). The pigment prodigiosin was active in other tests and, in combination with various antibiotics, showed a synergistic effect. None of the combinations had any antagonistic or neutral effect (Gohil *et al*., 2020).

The prodigiosin pigment has the capacity to neutralize free radicals, thereby inhibiting damage to lipids, proteins, and cellular DNA and preventing preliminary health conditions. (Nguyen *et al*., 2020) reported that the prodigiosin pigment by *S. marcescens* has a high ability to quench DPPH radicals of between 86 and 99 percent. The Prodigiosin pigment was synthesized by the Egyptian strain of *S. marcescens*, which showed a higher DPPH inhibition percentage of 92.63 (Othman *et al*., 2019). Similarly, *S. marcescens* produced prodigiosin pigment in large amounts, and it was studied to determine whether the pigment has the capacity to scavenge DPPH. The results showed that free radicals are fully eliminated by prodigiosin (10 mg/L) (Arivizhivendhan *et al*., 2018). Serratia marcescens is commonly associated with cell proliferation and acts as a potent anticancer agent with immunosuppressive effects. The hydrochloride of the prodigiosin pigment has shown promising anticancer activity due to its cell-cycle inhibitory and apoptotic effects (Srimathi *et al*., 2017). In vitro anticancer effects on different cell lines were determined on the purified prodigiosin pigment. It shows a strong suppressive effect across all cancer cell lines, and tumor size is reduced by 36.82% over 28 days, indicating that the isolated prodigiosin pigment is effective in reducing tumor growth (Nguyen *et al*., 2022). Treatment of the human breast cancer cell line MDA-MB-231 with purified prodigiosin showed inhibitory effects at all tested concentrations throughout the treatment periods (Muslim *et al*., 2024). These findings further support the strong anticancer potential of prodigiosin against different breast cancer cell models.

Limitations:

While the study focused on optimized laboratory conditions using a selected bacterial strain, it establishes a strong foundation for broader applications. Further studies under diverse conditions can enhance the general applicability of the findings.

Future Scope:

Future studies can focus on in vivo evaluation and elucidation of molecular mechanisms underlying prodigiosin's bioactivities. Scale-up production and formulation development may further enhance its potential for pharmaceutical and industrial applications.

Conclusions

In summary, this study successfully isolated and identified a *S. marcescens* strain producing prodigiosin from marine sediment in Tamil Nadu, India. The biochemical characterization and phylogenetic analysis of this strain confirm its classification and underscore its importance and significance in marine microbiology. Optimizing growth parameters revealed that mannitol, neutral pH, moderate temperatures, and low salt concentrations are critical for maximizing prodigiosin production. These findings indicate that shaking substantially enhances pigment synthesis compared with static cultivation, underscoring the importance of aeration for microbial growth. Moreover, the extracted prodigiosin exhibited potent antimicrobial properties, particularly against *S. aureus*, and showed significant antioxidant and cytotoxic activities against the MCF-7 breast cancer cell line. These properties suggest that prodigiosin has considerable potential for therapeutic applications, particularly as an antimicrobial agent and a candidate for anticancer therapies.

Conflict of interests

The authors declare no competing interests.

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