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Purification and Characterization of Red-Pigment from *Serratia nematodiphila* Isolated from Soil and Evaluation of Its Antifungal Efficacy

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ABSTRACT
Microbial pigments are increasingly valued for their biological activities and eco-friendly nature. Among them, *Serratia* species are known producers of bioactive compounds, including prodigiosin. Prodigiosin is a secondary metabolite of *Serratia nematodiphila*. This study aimed to purify and characterize the red pigment produced by *Serratia nematodiphila* and evaluate its antimicrobial potential. Taxonomic identification was confirmed by 16S rRNA sequencing. Pigment identity was validated using UV–Vis, FTIR, and ^{1}H NMR spectroscopy, supported by chromatographic purification. The red-coloured eluent is collected in six fractions. Thin-layer chromatography (TLC) is used to monitor the fractions and confirm the presence and purity of the prodigiosin. The third fraction absorption maximum of prodigiosin was found to be 535nm in UV- VIS study, and FTIR analysis revealed that the functional group of prodigiosin is well matched with prior studies N–H stretch (3431 cm^{-1}), C–H stretch (2923 cm^{-1}), C=O stretch (1734 cm^{-1}), C–N stretch (1026 cm^{-1}) functional groups. The LC-MS analysis confirmed that prodigiosin was present, eluted with a molecular ion peak at m/z 324.2 ([M+H]+). Prodigiosin exhibited notable antifungal activity against selected pathogenic fungi when evaluated by the agar well diffusion method. At a concentration of 1 mg/mL, the pigment produced an inhibition zone of 11 mm against *Aspergillus flavus* (NFCCI 384) and a comparatively higher inhibition zone of 21.5 mm against *Fusarium oxysporum* (NFCCI 708). Although the standard antifungal agent nystatin showed stronger inhibitory effects, prodigiosin pigment demonstrated appreciable antifungal potential. These results indicate that prodigiosin pigment produced by *Serratia nematodiphila* possesses promising antifungal properties and may serve as a natural bioactive metabolite with potential applications in the agriculture

1. INTRODUCTION:

1.1 Microbial Pigments: Significance and Applications

Microbial pigments are a diverse group of secondary metabolites produced by bacteria, fungi, and algae, serving as alternatives to synthetic colorants in various industries. The growing concern over the environmental and health hazards posed by synthetic dyes—such as toxicity, carcinogenicity, and ecological persistence—has driven the search for natural pigments with safer profiles (Hamada & Mohamed, 2024). Microbial pigments offer several advantages over plant-derived colorants, including higher yields, ease of cultivation, independence from seasonal variations,

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and the ability to tailor production through metabolic engineering (Anuradha Pendse, 2020). These pigments are not only valued for their vibrant colors but also for their bioactive properties, including antibacterial, antifungal, antioxidant, and anticancer activities, making them attractive for applications in food, pharmaceuticals, cosmetics, textiles, and agriculture (Metwally *et al*., 2017). Microbial pigments possess significant potential for applications across diverse sectors, particularly in health-related fields, due to their notable biological activities. Advances in microbial biotechnology have greatly enhanced cultivation strategies, enabling optimized pigment production. These developments include the use of cost-effective substrates, such as agro-industrial waste materials, to support high-yield growth of pigment-producing microorganisms (Paillіè-Jiménez *et al*., 2020).

1.2 Prodigiosin: Structure, Biosynthesis, and Biological Activities

Prodigiosin is a linear tripyrrole red pigment belonging to the prodiginine family, characterized by a unique pyrrolylpyrromethene skeleton and a molecular formula of $C_{20}H_{25}N_3O$ (molecular weight ~323.43 Da) (de Araújo *et al*., 2010, Paul *et al*., 2024; Zhao *et al*., 2021). It is produced by several bacterial genera, most notably *Serratia marcescens*, but also by *Streptomyces*, *Pseudomonas*, *Vibrio*, and *Hahella* species (Hamada & Mohamed, 2024). The biosynthesis of prodigiosin in *Serratia* involves a well-characterized gene cluster (pigA–N), regulated by quorum sensing and environmental factors such as carbon/nitrogen source, pH, and temperature (Hamada & Mohamed, 2024).

Prodigiosin has attracted significant attention due to its multifaceted biological activities. It exhibits potent antibacterial effects against both Gram-negative and Gram-positive bacteria, antifungal activity against a range of pathogenic fungi, anticancer properties through induction of apoptosis in malignant cells, immunosuppressive effects, and antiviral activities (Zhao *et al*., 2021; Lu *et al*., 2024). Mechanistically, prodigiosin disrupts bacterial membranes, induces reactive oxygen species (ROS), interferes with DNA and protein synthesis, and modulates pH homeostasis (Lu *et al*., 2024).

1.3 Rationale for Isolating Prodigiosin from Soil Bacteria

Soil is a rich reservoir of microbial diversity, harboring bacteria capable of producing novel bioactive compounds. The isolation of prodigiosin from soil bacteria, particularly *Serratia nematodiphila*, is of interest due to the potential for discovering strains with unique pigment yields, stability, and bioactivity profiles (Hamada & Mohamed, 2024; Paul *et al.*, 2024). *Serratia nematodiphila* is a Gram-negative, facultative anaerobe, previously described as a symbiont of nematodes but also found in soil environments. Its ability to produce prodigiosin expands the repertoire of prodiginine-producing bacteria and offers opportunities for sustainable pigment production using low-cost substrates and optimized fermentation processes (de Araújo *et al*., 2010; Paul *et al*., 2024).

The present study aims to purify and characterize prodigiosin from *Serratia nematodiphila* obtained from soil samples of Hanamkonda, Telangana, and to evaluate its antifungal activity against Phytopathogenic fungi. This work addresses the need for natural, multifunctional pigments and contributes to the understanding of soil-derived prodigiosin properties and applications.

2. MATERIALS AND METHODS:

2.1 Preparation of Bacterial culture

The bacterial strain used in the present study was previously isolated from soil during a preliminary investigation aimed at screening pigment-producing bacteria. Isolation was carried out using the serial dilution technique on nutrient agar medium supplemented with 1% glycerol, followed by purification through repeated streaking to obtain a stable pure culture. The isolate was maintained under laboratory conditions and preserved at 4 °C for subsequent use. Initial characterization included assessment of colony morphology, Gram staining, and biochemical traits, while molecular identification was performed using 16S rRNA gene sequencing.

For the present investigation, the preserved strain was revived by streaking onto freshly prepared nutrient agar plates supplemented with 1% glycerol and incubated at 28 ± 2 °C to obtain actively growing colonies. A single well-isolated colony was further subcultured to ensure purity, and the freshly revived culture was used for further studies.

2.2 Pigment production and extraction

The selected bacterial isolate was found to produce extracellular pigment. For inoculum preparation, a loopful of a 24-h actively growing pure culture was inoculated into 10 mL of nutrient broth supplemented with 1% glycerol and incubated at 28 ± 2 °C for 48–72 hours (de Araújo *et al.,* 2010, Paul *et al.,* 2024, Zhao *et al*., 2021). The developed inoculum was subsequently transferred into 250 mL of fresh broth and incubated on a rotary shaker at 120 rpm for 5 days at 28 °C to facilitate pigment production. After Incubation, the cells were

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harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was resuspended in methanol to extract pigment (Hamada & Mohamed., 2024; de Araújo *et al*., 2010; Paul *et al*., 2024). The collected biomass was transferred into sterile microcentrifuge tubes containing 5–10 mL of methanol, a commonly used solvent for pigment extraction due to its high solubility and ability to disrupt bacterial cell structures (Williamson *et al*., 2006). The supernatant also contained visible pigmentation. From the supernatant the pigment was extracted by ethyl acetate, using liquid-liquid separation method. The pigment was collected and concentrated using a rotary evaporator under reduced pressure. The concentrated pigment extract was stored for further analysis. The extraction of the pigment from the culture has been carried out according to the method described by Yip *et al.,*2019.

2.2.1 Purification by Silica Gel Column Chromatography

The crude pigment extract was loaded onto a silica gel column (60–120 mesh) as the stationary phase equilibrated with chloroform: methanol: acetone (4:3:3 v/v) as the mobile phase. Elution was performed at a flow rate of 1 mL/min, and red-coloured fractions were collected. Purity was assessed by thin-layer chromatography (TLC) using chloroform: methanol (9:1) as the mobile phase; Rf values were compared to prodigiosin standards (Jinxiang Ge e*t al*.,2019)

2.2.2 Spectroscopic Characterization

- **UV–Vis Spectroscopy:** The purified pigment was dissolved in ethanol and scanned from 200 to 800 nm. It exhibits a maximum absorbance at ~535 nm in acidic ethanol, shifting to ~470 nm in alkaline conditions (Hamada., & Mohamed., 2024, de Araújo *et al*., 2010, Miglani *et al*., 2023).
- **FTIR Spectroscopy:** Functional groups were identified using FTIR (4000–400 cm^{-1}, KBr pellet). Characteristic peaks include N–H stretch (~3431 cm^{-1}), C–H stretch (~2923 cm^{-1}), C=O stretch (~1734 cm^{-1}), and C–N stretch (~1026 cm^{-1}) (Paul *et al*., 2024).
- **^{1}H-NMR Spectroscopy:** Samples dissolved in $CDCl_3$ were analyzed at 500 MHz. Chemical shifts corresponding to aromatic protons (6.3–7.1 ppm), methoxy (3.2–3.7 ppm), methyl (2.0–2.3 ppm), and aliphatic chains (0.8–1.3 ppm) were recorded, confirming the tripyrrole structure (de Araújo *et al*., 2010).

2.3.3 Analytical Confirmation: Mass Spectrometry

- **Mass Spectrometry (LC-MS/GC-MS):** The pigment was analysed by ESI-MS, revealing a molecular ion peak at m/z 324.2 ([M+H]+), and was consistent with pigment (Hamada & Mohamed, 2024; de Araújo *et al*., 2010, Paul *et al*., 2024).

2.4 Antifungal Assay

Antifungal activity of the pigment was assessed using the agar well diffusion method against *Aspergillus flavus* NFCCI 384 and *Fusarium oxysporum* NFCCI 708. (El-Batal *et al*., 2018). These Fungal cultures were procured from the National Fungal Culture Collection of India, Pune.

Pure Pigment concentration of 1mg/ml and Fungal suspensions were prepared at 10^5 CFU/mL, inoculated, and spread on potato dextrose agar plates. 6mm wells were punched using a cork borer, and each well was loaded with positive control, negative control, and purified pigment and incubated at 28–30°C for 48-72 h. Positive control includes nystatin, and negative control includes methanol. The zone of inhibition was recorded in millimeters using standard scale.

2.5 Data Analysis and Statistical Methods

All assays were performed in triplicate. Data were analysed using one-way ANOVA, and significance was determined at $p < 0.05$.

3. RESULTS”

3.1 Preparation of Bacterial Culture

3.1.1 Colony Morphology and Microscopy

The bacterial culture on nutrient agar supplemented with 1% glycerol was observed to successfully produce well-defined red-pigmented colonies developed within 48–72 h of incubation at 28 ± 2 °C. The obtained isolate consistently produced pigmented colonies, indicating stable pigment production. Colonies were circular, smooth, convex, and exhibited a deep red coloration characteristic of prodigiosin-producing *Serratia* species.

Microscopic examination following Gram staining revealed Gram-negative, short rod-shaped cells, confirming the morphological features typical of the genus *Serratia (*Grimont & Grimont,2006*)*. These observations verified the purity and phenotypic stability of the revived culture used for further analysis.

3.1.2 Molecular identification

The molecular identity of the revived red-pigmented isolate was reconfirmed through 16S rRNA gene sequencing. PCR amplification using universal primers 27F and 1492R yielded an amplicon of approximately 1500 bp. Sequence analysis using BLAST showed greater than 99% similarity with *Serratia nematodiphila*.

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Phylogenetic analysis further clustered the isolate within the *Serratia* clade, closely aligning with other reported prodigiosin-producing strains, thereby confirming its taxonomic identity.

3.2 Pigment Extraction, Purification, and Characterization

3.2.1 Pigment Extraction

Pigment was efficiently extracted through sequential extraction from both the bacterial biomass and the culture supernatant. From the pellet and supernatant, the pigment was extracted using methanol and ethyl acetate. The extracted crude pigment (**Figure 1**) was concentrated using a rotary evaporator and stored for further purification and characterization.

Figure 1: Pigment extract

3.2.2 Purification

Silica gel column chromatography using chloroform: methanol: acetone (4:3:3 v/v) as the mobile phase yielded six fractions (**Figures 2 & 3**). TLC analysis was performed for all six fractions using a chloroform: methanol (9:1). Among the six fractions, the third fraction showed a single red band with an Rf value of 0.81, matching the prodigiosin standards **(Figure 4).**

Figure 2: Partial purification of pigment using Colum chromatography.

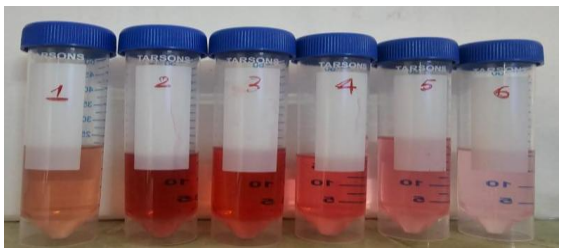

Figure 3: Six fractions were eluted from the column.

Figure 4: Third fraction showing a single red band on the TLC

3.2.3 Spectroscopic Characterization

- **UV–VIS:** The pigment exhibited a maximum absorbance at 535 nm in acidic ethanol, shifting to 470 nm in alkaline conditions. This spectral behaviour is specific to prodigiosin.
- **FTIR:** Key peaks included N–H stretch (3431 cm^{-1}), C–H stretch (2923 cm^{-1}), C=O stretch (1734 cm^{-1}), C–N stretch (1026 cm^{-1}), consistent with prodigiosin's functional groups.

UV-VIS, IR, and ^{1}HNMR and ^{13}CNMR Spectroscopic and chromatographic analyses were used to characterize the pigment (**Figure 5.1, 5.2, & 5.3**). These analyses collectively validated the pigment as prodigiosin, aligning with earlier characterizations reported for *Serratia marcescens* (Lapenda *et al*., 2015). The chemical properties of the characterized pigment are shown below **(Table 1).** The structure of the prodigiosin is shown in **Figure 5 .4.**

Table 1: Chemical Identity and Properties of Prodigiosin

Parameter	**Details**
Common Name	Prodigiosin
IUPAC Name	(5-[(3-methoxy-5-methyl-2-pyrrolyl)(pyrrol-2-yl)methylidene]pyrrol-2-yl)pentane
Chemical Class	Tripyrrole red pigment
Molecular Formula	**$C_{20}H_{25}N_3O$**
Molecular Weight	**323.44 g/mol**
Appearance	Deep red crystalline pigment
Odor	Odorless
State at Room Temperature	Solid
Solubility	Soluble in methanol, ethanol, chloroform, acetone; poorly soluble in water
Melting Point	**~95–97 °C**
Boiling Point	Not well defined (decomposes before boiling)
pH Sensitivity	Acidic → pink/red; Alkaline → yellow
Stability	Stable under acidic conditions; moderately unstable under strong alkaline conditions
UV–Visible Absorption (λmax)	**~535 nm (methanol)**
IR Functional Groups	–NH (pyrrole), C=C (aromatic), C–O (methoxy), C–N

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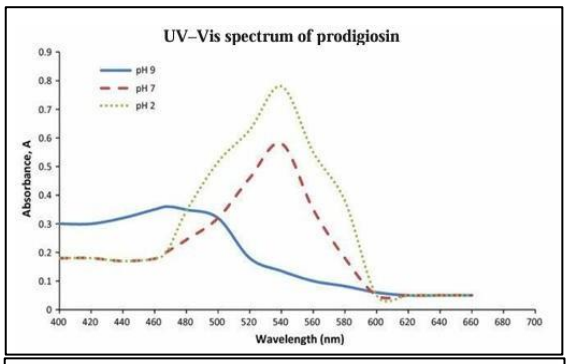
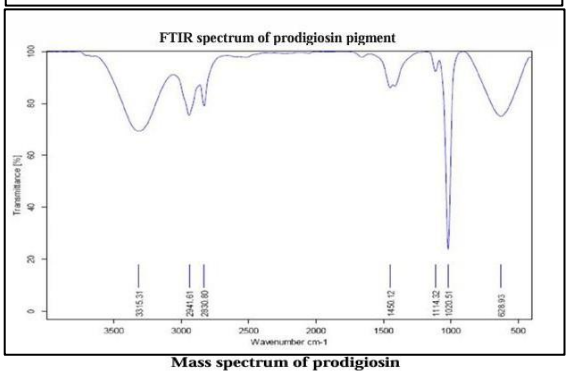
Major Functional Groups	Pyrrole rings, methoxy group, alkyl side chain
Density	~1.1 g/cm³ (estimated)
Ionic Nature	Neutral
Polarity	Moderately non-polar
Photostability	Sensitive to prolonged light exposure
Thermal Stability	Stable





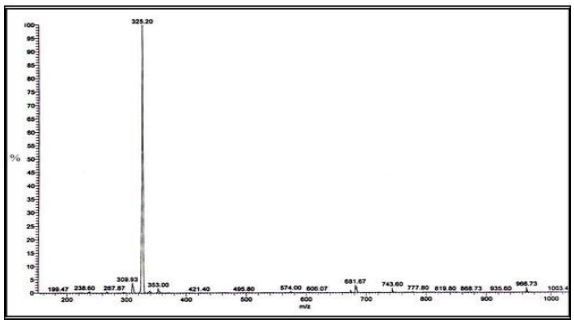


Figure 5.1: UV, FTIR, MASS characterization of Prodigiosin

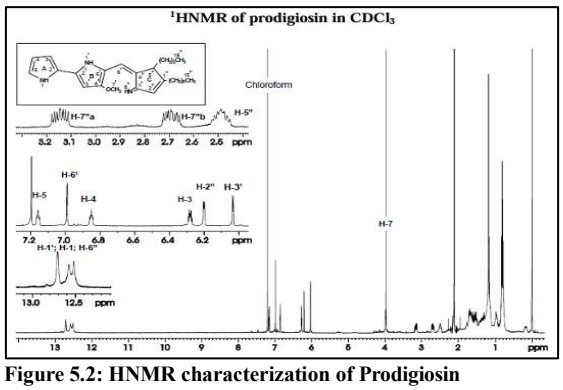


Figure 5.2: HNMR characterization of Prodigiosin

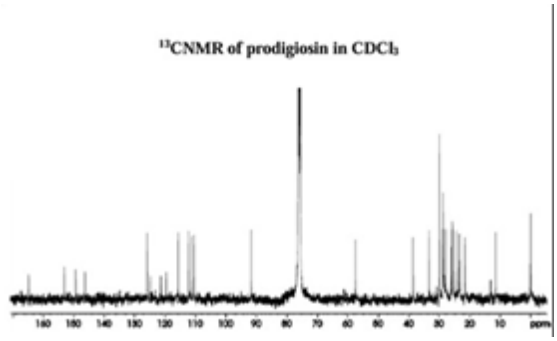


Figure 5.3: 13C characterization of Prodigiosin

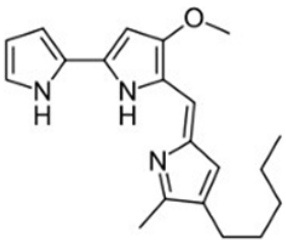


Figure 5.4: Structure of the prodigiosin isolated from the *Serratia nematodiphila*

3.2.4 Analytical Confirmation

- **Mass Spectrometry:** LC-MS revealed a molecular ion peak at m/z 324.2 ([M+H]+), confirming prodigiosin identity **Table 2**.

Table 2: Pigment Characterization

Technique	Key Findings
UV–Vis	λmax = 535 nm (acidic), 470 nm (alkaline)
FTIR	N–H, C–H, C=O, C–N functional groups
¹H-NMR	Tripyrrole backbone, OCH_3, CH_3, CH_2
Mass Spectrometry	m/z = 324.2 ([M+H]+)

3.3 Antifungal Activity

The maximum antifungal activity of prodigiosin was observed at a concentration of 1 mg/mL. At this concentration, prodigiosin produced an inhibition zone of 11 mm against *Aspergillus flavus* NFFCI 384 and 21.5 mm against *Fusarium oxysporum* NFCCI 708. In comparison, the standard antifungal drug nystatin (1mg/mL) exhibited a significantly larger inhibition zone of 18mm and 25 mm **(Figure 6A & B)**. Although prodigiosin showed comparatively lower activity than the standard, it demonstrated moderate and notable antifungal efficacy. Among the tested fungal pathogens, *Fusarium oxysporum* was found to be more susceptible to prodigiosin than *Aspergillus flavus*. These results confirm the antifungal potential of prodigiosin and highlight its promising role as a natural antifungal agent as shown in **Table 3**.

Table 3: Antifungal activity of prodigiosin pigment

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Test organism	Zone of Inhibition(mm) for pigment	Zone of Inhibition(mm) for standard
Aspergillus flavus	11mm	18mm
Fusarium oxysporum	21.5mm	25mm

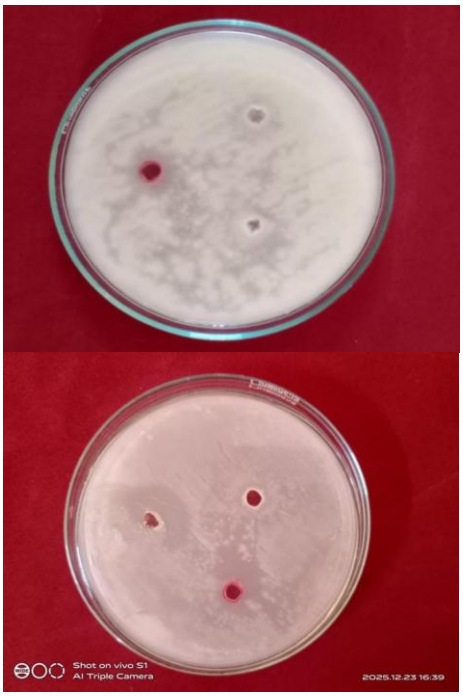

Figure 6 (A, B): prodigiosin showed antifungal activity for *Aspergillus flavus* 11 mm inhibition zone at 1mg/mL, and *Fusarium oxysporum* 21.5 mm inhibition zone at 1mg/mL. When compared with the standard antifungal drug nystatin (1mg/mL).

3.4 Statistical Analysis

All antifungal assays were statistically significant ($p < 0.05$), confirming the reproducibility and potency of prodigiosin.

4. DISCUSSION:

4.1 Comparison with Existing Literature:

The isolation of prodigiosin from *Serratia nematodiphila* in Hanamkonda, Telangana, adds to the growing body of evidence that soil bacteria are valuable sources of bioactive pigments (1,5). The yield of 7.8 mg/L is comparable to other *Serratia* species, with *S. marcescens* often cited as the highest producer under optimized conditions (Paul *et al*., 2024). The pigment's spectral properties (λmax = 535 nm, m/z = 324.2) and functional group profile confirm its identity as prodigiosin, consistent with previous reports (Hamada & Mohamed 2024, de Araújo *et al*., 2010; Paul *et al*., 2024).

The antifungal activity supports prodigiosin's role as a natural antifungal agent, with potential applications in food preservation and wound healing (Hamada & Mohamed, 2024; Zhao *et al*., 2021).

4.2 Novelty and Potential Applications:

This study is among the first to systematically characterize prodigiosin from *Serratia nematodiphila* isolated from Indian soils, expanding the known diversity of prodiginine producers. The use of silica gel column chromatography, combined with advanced spectroscopic and chromatographic techniques, ensured high purity and accurate identification of the pigment.

Potential applications of prodigiosin include:

- **Pharmaceuticals:** As an antimicrobial and anticancer agent, prodigiosin could be developed into novel therapeutics, especially for multidrug-resistant infections and cancer (Lu *et al*., 2024).
- **Food Industry:** Its antibacterial and antioxidant properties make it suitable as a natural preservative and colorant (Hamada Mohamed, 2024, K *et al*., 2023).
- **Cosmetics and Textiles:** The pigment's stability and vibrant color support its use in eco-friendly dyes (Zhao *et al*., 2021; Pooja Rane and Anuradha Pendse, 2025).
- **Agriculture:** Prodigiosin's antifungal and insecticidal activities could be harnessed for crop protection and biocontrol (Hamada & Mohamed, 2024, K. *et al*., 2023).

4.3 Limitations and Future Directions:

While prodigiosin demonstrated promising antifungal activity, further studies are needed to elucidate its mechanism of action, toxicity profile, and pharmacokinetics. Scaling up production using low-cost substrates and optimizing fermentation conditions could enhance yield and reduce costs. Genetic engineering of *Serratia nematodiphila* or heterologous expression in safe hosts may further improve prodigiosin production. Preclinical and clinical studies are required to validate its therapeutic potential.

5. CONCLUSION:

This study successfully purified and characterized prodigiosin pigment from soil-derived *Serratia nematodiphila*. The pigment exhibited a characteristic red color, tripyrrole structure, and strong absorbance at 535 nm. Advanced spectroscopic and chromatographic analyses confirmed its identity and purity. Prodigiosin demonstrated significant antifungal effect, with additional biofilm inhibition properties.

The findings underscore the value of soil bacteria as sources of multifunctional pigments and support the development of prodigiosin as a natural antifungal agent. Future research should focus on optimizing production, exploring synergistic effects with existing antibiotics, and assessing safety for

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pharmaceutical and industrial applications. Unlocking the potential of prodigiosin and its biosynthetic gene clusters could have a profound impact on combating antimicrobial resistance and advancing sustainable bioproducts.

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