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Rhizosphere Soils of Warangal District as a Source of Pigment-Producing Bacteria: Isolation and Molecular Identification

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ABSTRACT
Microbial pigments have gained significant attention due to their diverse biological activities and eco-friendly nature. Microbial pigments serve as eco-friendly alternatives to synthetic dyes, which often pose toxicity, allergenicity, and environmental hazards. The present study aimed to isolate and characterize a red-pigment-producing bacterium from soil samples collected from different regions of Hanamkonda, Telangana. A highly intense red-pigmented isolate was recovered and identified as a *Serratia marcescens* based on its colony morphology and biochemical characteristics. The pigment exhibited characteristic pH-dependent color changes, turning pink under acidic conditions and yellow under alkaline conditions, confirming its presumptive identity as prodigiosin. Molecular identification using 16S rRNA sequencing further supported the taxonomic assignment of the isolate.

1. INTRODUCTION:

Microbial pigments serve as eco-friendly alternatives to synthetic dyes, which often pose toxicity, allergenicity, and environmental hazards (Venil *et al.*, 2014). Natural pigments produced by microorganisms have gained considerable scientific attention due to their ecological functions, structural diversity, and potential applications in pharmaceuticals, food systems, textiles, and biomedical industries. Among microbial pigments, bacterial pigments are particularly valuable due to their high yield, stability, and broad biological activities, including antioxidant, anticancer, immunosuppressive, and antimicrobial properties (Darshan & Manonmani, 2015).

Soil is recognized as a rich reservoir of diverse pigmented bacteria, and systematic screening helps to isolate strains capable of producing industrially significant biomolecules. One of the most widely studied bacterial pigments is prodigiosin, a bright red tripyrrole pigment primarily produced by *Serratia marcescens* and related species. Prodigiosin exhibits potent antimicrobial, antifungal, anticancer, and immunomodulatory activities due to its unique ability to induce membrane disruption, DNA cleavage, and apoptosis in target organisms (Williamson *et al.*, 2006; Lapenda *et al.*, 2015). The increasing emergence of multidrug-resistant pathogens has further intensified interest in prodigiosin as a natural antimicrobial compound.

Although *Serratia* species are extensively studied, local soil environments often harbor unique pigment-producing strains with distinctive biochemical and antimicrobial properties. Identifying such strains contributes to both biodiversity documentation and potential biotechnological exploitation. However, limited studies have documented pigment-producing bacteria from the soils of Hanamkonda, Telangana. Thus, the present study aimed to isolate pigment-producing bacteria from soil samples collected from Hanamkonda, identify the selected red-pigmented isolate through morphological, biochemical, and 16S rRNA sequencing methods, extract and characterize the pigment.

2.0 MATERIAL AND METHODS:

2.1 Sample Collection:

Soil samples were collected aseptically from five locations in Hanamkonda (Hasanparthy, Unikicherlla, Madikonda, Subbayyapalli) (Table

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1.1) and the Regional Agriculture Institute (Mulugu Road) at a depth of 5–10 cm to ensure the inclusion of active microbial populations (Alexander, 2019). The samples were labeled according to their place of collection (Table 1.1).

2.2 Serial Dilution Technique:

Approximately 1 g of soil was suspended in 9 mL sterile distilled water to obtain a 10^{-1} dilution. Serial dilution was performed up to 10^{-8} by transferring 1 mL from each tube into the next containing sterile diluent. Aliquots (0.1 mL) from dilutions 10^{-4} and 10^{-7} were spread onto yeast extract malt extract agar (YEMA) plates using a sterile L-rod and incubated at 28 ± 2°C for 24–48 hours (Cappuccino and Sherman, 2014).

2.3 Morphological Identification:

2.3.1 Colony Characteristics:

The selected isolate was examined for macroscopic features, including colony shape, pigmentation, surface texture, elevation, opacity, and margin. Pigmented bacteria often show consistent macroscopic features that assist in preliminary identification (Madigan *et al.,* 2018).

2.3.2 Microscopic Identification:

Microscopic observation was carried out using Gram staining. A thin smear of the isolate was prepared, heat fixed, and subjected to staining with Crystal violet, Iodine, Decolorizer, and Saffranin.

2.3.3 Biochemical Characterization:

Biochemical assays were performed using standard protocols described in Bergey's Manual of Systematic Bacteriology (Whitman *et al.,* 2012). The isolate was tested for the following biochemical tests, including Indole, Methyl red (MR), Voges–Proskauer (VP), Citrate utilization, Catalase, Oxidase, Nitrate reduction, Urease variable, and Gelatin hydrolysis.

2.3.4 Molecular Identification Using 16S rRNA Sequencing:

Genomic DNA was extracted, and the 16S rRNA gene was amplified using universal primers 27F and 1492R. PCR products were purified and sequenced. BLAST analysis of the obtained sequence revealed >99% similarity to the *Serratia* genus, confirming that the isolated pigment-producing bacterium belonged to Serratia spp., consistent with previously reported prodigiosin-producing strains (Khanafari *et al.,* 2006).

2.4. Production and Extraction of Pigment:

The selected isolate was observed to produce extracellular pigment. A loopful of 24 hours active culture was transferred to the 10ml of Yeast extract malt extract broth (YEMA) for the development of inoculum and incubated it for 48 hours at 28 ± 2°C. The inoculum was then transferred to the 250ml of broth for the pigment production and incubated in a shaker at 120 rpm for 5 days at 28°C. After incubation, the broth was centrifuged at 10,000 rpm for 10 min at 4°C to obtain the bacterial pellet. The supernatant was collected, and the pigment was extracted using the solvent extraction method. Different solvents like ethyl acetate, petroleum ether, and n-hexane were used for the extraction of pigment from the Supernatant. The obtained pellet was washed twice with sterile distilled water. For pigment extraction, the cell pellet was subjected to three freeze-thaw cycles to facilitate cell lysis. The lysed cells were then extracted using different solvents like methanol, ethanol, and acetone by centrifugation.

2.4.1 Pigment Identification by Presumptive Test:

The presumptive identification of the extracted pigment was carried out based on its characteristic acid–base sensitivity. Briefly, 1 mL of the pigment extract was transferred into two separate, clean test tubes. To the first tube, 2–3 drops of concentrated hydrochloric acid (HCl) were added and gently mixed, and the development of a stable pink to deep red coloration was immediately observed. To the second tube, 2–3 drops of aqueous ammonia solution (NH_4) were added, resulting in a distinct color change from red to yellow. These reversible color transitions under acidic and alkaline conditions are attributed to the protonation and deprotonation of the tripyrrolic chromophore of the pigment and are considered a reliable presumptive indication of its presence. This qualitative assay is widely employed as a rapid screening method for red pigment prodigiosin before confirmatory spectroscopic analyses (Williamson *et al.,* 2006; Song *et al.,* 2006).

3.0 RESULTS:

3.1 Morphological characterization:

The serial dilution and plating of soil samples collected from five regions of Hanamkonda resulted in the recovery of multiple distinct colouerd colonies, among which a highly intense red-pigmented colony was consistently observed across several plates. The isolate that was exhibiting intense and bright pigmentation was selected, and it was observed as circular, smooth, convex colonies with deep red pigmentation. Gram staining revealed the isolate as a Gram-negative rod. The results are represented in Table 1.2

Table 1.1 Soil sample collection from the different locations of Hanamkonda.

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Region	Region Code	Sample Labels	Latitude (°N)	Longitude (°E)
Hasanparthy	HP	HP-1, HP-2	18.0688	79.5244
Unikicherla	UK	UK-1, UK-2	18.0198	79.4731
Madikonda	MK	MK-1, MK-2, MK-3	17.9900	79.5400
Subbayyapalli	SB	SB-1, SB-2	18.0700	79.5200
Regional Agriculture Institute (Mulugu Road)	RAI	RAI-1, RAI-2	18.0072	79.5583

Table 1.2 Morphological characterization of the selected bacteria isolated from the soil samples collected from different regions of Hanamkonda

Parameter	Observation	Interpretation
Colony Colour	Deep red pigment	Presumptive prodigiosin producer
Colony Shape	Circular	Typical of *Serratia* spp.
Colony Margin	Entire (smooth)	Indicates pure culture
Elevation	Convex	Consistent with *Serratia* colonies
Surface Texture	Smooth, glistening	Healthy active growth
Opacity	Opaque	Mature colony
Colony Size	2–4 mm (24–48 h)	Normal for *Serratia*
Gram Reaction	Gram-negative	Matches *Serratia* genus
Cell Shape	Rod-shaped	Bacillary morphology
Motility	Motile	Common in *Serratia* spp.
Pigmentation	Stable red pigment	Prodigiosin confirmed

3.2 Biochemical characterization:

Biochemical characterization further supported the identity of the isolate, showing a VP-positive, citrate-positive, oxidase-negative, and catalase-positive profile. Such biochemical characteristics are typical for *Serratia* species, supporting preliminary identification (Hejazi & Falkiner, 1997). The results are represented in Table 1.3

Table 1.3 Biochemical characterization of the bacteria isolated from the soil samples collected from different regions of Hanamkonda

Test	Result
Indole Test	Negative
Methyl Red (MR)	Negative
Voges-Proskauer (VP)	Positive
Citrate Utilization	Positive
Catalase Test	Positive
Oxidase Test	Negative
Urease Test	Variable
Gelatin Hydrolysis	Positive
Nitrate Reduction	Positive
Carbohydrate Fermentation	Positive

3.3 Molecular Identification Using 16S rRNA Sequencing

Genomic DNA of the selected red-pigmented bacterial isolate was extracted and subjected to 16S rRNA gene amplification using universal primers 27F and 1492R. The amplified PCR product was purified and sequenced. The obtained partial 16S rRNA gene sequence was analyzed using the BLASTn program against the NCBI GenBank database. The sequence showed **>99% similarity with *Serratia marcescens* strain JS1**, confirming the taxonomic identity of the isolate as *Serratia marcescens* (Table 1.4). This molecular identification further validated the morphological and biochemical characterization results of the isolate.

Table 1.4 BLAST Similarity Analysis of 16S rRNA Gene Sequence

Accession Number	Organism Name	Sequence Identity (%)	Query Coverage (%)	E-value
PV342635.1	*Serratia marcescens* strain JS1 (Present isolate)	**100**	**100**	**0.0**
MN691639.1	*Serratia marcescens*	99.8	100	0.0
MN691629.1	*Serratia marcescens*	99.7	100	0.0
MN691654.1	*Serratia marcescens*	99.6	99	0.0
MN691685.1	*Serratia marcescens*	99.6	99	0.0
MN691682.1	*Serratia marcescens*	99.5	99	0.0
MN691680.1	*Serratia marcescens*	99.5	99	0.0
MN691684.1	*Serratia marcescens*	99.4	99	0.0
MN691683.1	*Serratia marcescens*	99.4	99	0.0
MN691380.1	*Serratia marcescens*	99.3	99	0.0
KC790285.1	*Serratia marcescens*	99.2	98	0.0
MN691674.1	*Serratia marcescens*	99.2	98	0.0
MN691386.1	*Serratia marcescens*	99.1	98	0.0
MN691371.1	*Serratia marcescens*	99.0	98	0.0
KT223626.1	*Serratia marcescens*	98.9	97	0.0
MN691644.1	*Serratia marcescens*	98.8	97	0.0
MW742369.1	*Serratia entomophila*	97.6	96	0.0
MN691632.1	*Serratia marcescens*	99.0	98	0.0
MN691398.1	*Serratia marcescens*	98.9	97	0.0
KX456108.1	*Staphylococcus aureus* (outgroup)	89.2	85	3e-120
MN691620.1	*Serratia marcescens*	98.7	97	0.0

3.3.1 Phylogenetic Tree Description:

A phylogenetic tree was constructed using the

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neighbor-joining method based on aligned 16S rRNA gene sequences of the isolated strain and closely related reference sequences retrieved from the GenBank database. The evolutionary distances were calculated using the Kimura two-parameter model, and the robustness of tree topology was evaluated by bootstrap analysis with 1000 replications. The isolate clustered closely with *Serratia marcescens* reference strains, confirming its phylogenetic placement within the genus *Serratia*. The phylogenetic tree is shown in **Figure 1**

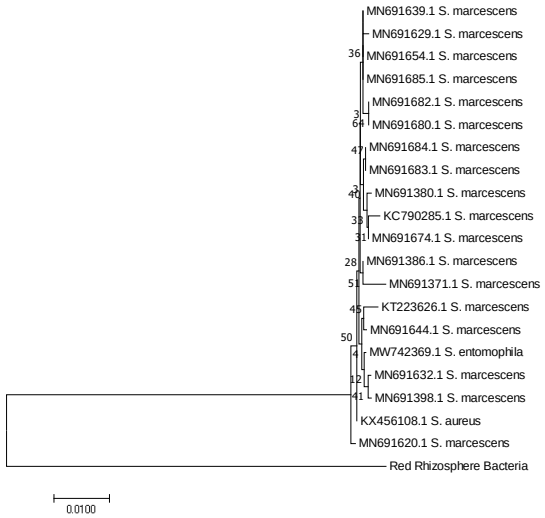

Figure 1: Phylogenetic tree showing the evolutionary relationships of the isolated bacterium

3.4 Production and Extraction of Pigment:

Among the various solvents tested for pigment extraction, ethyl acetate yielded a higher amount of pigment from the supernatant, whereas methanol extracted a greater quantity of pigment from the pellet. The methanol extract and ethyl acetate extract were concentrated using a rotary evaporator under reduced pressure. The concentrated pigment extract was stored for further analysis.

3.4.1 Pigment Identification by Presumptive Acid–Base Test:

The extracted pigment exhibited characteristic pH-dependent color changes that are indicative of prodigiosin (Figure 2 D). Upon the addition of concentrated hydrochloric acid, the pigment solution immediately changed from red to a stable pinkish-red coloration, confirming its acid sensitivity (Figure 2D). Conversely, treatment of the pigment extract with aqueous ammonia solution resulted in a rapid transition from red to yellow, indicating an alkaline response. These distinct and reversible color changes under acidic and basic conditions confirm the protonation–deprotonation behavior of the tripyrrolic chromophore, which is a diagnostic property of prodigiosin. The observed acid–base response strongly supports the presumptive identification of the extracted pigment as prodigiosin (Figure 2D).

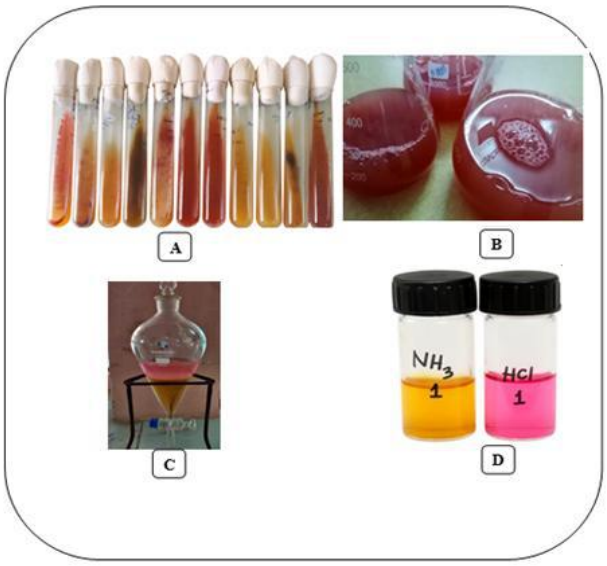

Figure 2. A. Screening of Pigmented Bacteria. **B**. Production of Pigment. **C**. Extraction of Pigment from culture broth. **D**. Presumptive Test for Prodigiosin.

4.0 DISCUSSION:

The present investigation successfully reports the isolation of a red pigment–producing bacterium from the rhizosphere soil samples collected from Hanamkonda in the Warangal region. Based on a comprehensive evaluation of its morphological, biochemical, and molecular characteristics, the isolate was identified as *Serratia marcescens* (Hejazi & Falkiner, 1997*;* Garg *et al.*, 2016). The colony exhibited distinct red pigmentation, smooth texture, and a characteristic mucoid appearance, features that are widely recognized in *Serratia marcescens* isolates. These traits, together with the biochemical profile—including carbohydrate utilization patterns and enzyme activity—support its classification within the genus *Serratia*.

The production of a bright red pigment was a prominent feature of the isolate, consistent with the biosynthesis of prodigiosin, a tripyrrole-based pigment known for its distinctive coloration and biological significance. Prodigiosin has been historically described as exhibiting pH-dependent chromatic variations, a characteristic also observed in the present study (Williams, 1973). These color changes arise from protonation states of the pigment structure, reflecting its sensitivity to environmental factors such as pH, temperature, and nutrient availability. The expression of such pigments is typically regulated by quorum sensing and secondary metabolic pathways within *Serratia* species, indicating the physiological adaptability of the isolate.

The recovery of a potent pigment-producing

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bacterial strain from the rhizosphere soils of Warangal highlights the ecological richness of this region. Rhizosphere ecosystems are well known as microbially dense environments filled with metabolically diverse and functionally important bacteria. These soils, affected by plant root exudates and organic matter, often contain microorganisms with enhanced biosynthetic abilities, including pigment production (Venil *et al.,* 2014). The discovery of S. marcescens in this context emphasizes the potential of local soil microbiota as untapped bioresource pools capable of producing novel biopigments and bioactive compounds with pharmaceutical or industrial applications and, prodigiosin, a characteristic red tripyrrole pigment predominantly produced by *Serratia marcescens*, serves as a reliable phenotypic marker during the isolation and preliminary identification of the species, while molecular confirmation using 16S rRNA gene sequencing ensures accurate taxonomic placement and validates its biosynthetic potential for diverse bioactive applications (Srilekha *et al.,* 2024).

Additionally, the molecular identification of the isolate using 16S rRNA gene sequencing confirmed its precise taxonomic classification, supporting the phenotypic observations. The use of molecular markers, especially 16S rRNA, continues to be a gold standard for accurate bacterial identification because of its high phylogenetic resolution and evolutionary stability (Hejazi & Falkiner, 1997). This study thus emphasizes the importance of combining traditional microbiological methods with molecular techniques to ensure reliable identification and characterization of microbial isolates.

5.0 CONCLUSION:

The present study successfully isolated and characterized a prodigiosin-producing *Serratia* species from soil samples collected in Hanamkonda, highlighting the region as a valuable reservoir of bioactive pigment-producing microorganisms. The isolate exhibited characteristic morphological, biochemical, and molecular features consistent with *Serratia marcescens* and the pigment was conclusively identified as prodigiosin based on presumptive acid–base testing.

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CONFLICT OF INTEREST

Authors disclose no conflict of interest

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