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ORIGINAL ARTICLE

Screening, characterization and Evaluation of Antibacterial Activity of Pigment-Producing Bacteria Isolated from Soil

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ABSTRACT
Pigments are colored compounds that play important roles in various biological systems, such as photosynthesis, vision, and signalling. Pigment-producing bacteria are a diverse group of microorganisms that can produce a wide range of pigments with different colours and properties. The present study aims to isolate the pigment-producing bacteria from the soil with the antibacterial activity of the pigment. Soil samples were collected from Kakatiya University, Warangal, Telangana. The isolated organisms were characterised with the help of Bergey's Manual of Determinative Bacteriology. In this investigation, isolation and screening of pigment-producing bacteria resulted in the identification of 7 coloured colonies from 17 soil samples. Of the seven pigmented colonies, 5 exhibited red colouration, whereas 2 had yellow colouration due to the resemblance in growth patterns and the extreme intensity of pigmentation. One isolate was chosen for further research. The isolated red colony was labelled as JS 05. The crude pigment extract showed significant antibacterial activity against both Gram-negative and Gram-positive bacteria. The crude pigment extract of Serratia nematodiphila exhibited greater inhibitory efficacy against Gram-positive bacteria compared to Gram-negative bacteria.
Keywords: *Pigment, Soil bacteria, Extraction, Antimicrobial activity.*

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INTRODUCTION

For humans, colour serves as a source of allure. It illustrates the diversity of geographical, cultural, sociological, and economic conditions within the population. Colour impacts human lives in various aspects of daily existence. Historical records indicate that pigments have served as colouring agents since antiquity. [1]

A pigment is a substance that alters the colour of reflected or transmitted light due to wavelength-selective absorption. This physical process is distinct from fluorescence, phosphorescence, and other luminescent phenomena, in which a substance emits light. [2] .

Natural pigments are derived from ores, insects, plants, animals, and bacteria. Bacteria that synthesise pigment are referred to as chromogenic bacteria [3]. The creation of pigments in bacteria is crucial for protection against UV radiation, oxidative stress, severe temperatures, and desiccation. Pigments are recognised for their role as bacterial shields against some natural antibacterial chemicals generated by other microbes [4] . Certain bacteria synthesise pigments as a component of their standard metabolic processes, resulting in various shades of colour. Bacteria are recognised for synthesising a range of pigments, including carotenoids and their derivatives (β-carotene, astaxanthin, canthaxanthin,

zeaxanthin), prodigiosin, pyocyanin, melanin, and violacein, among others [5,6]. Microbial pigments are distinctive attributes of certain bacteria, which can aid in identification, as pigmented bacteria produce cultures that display specific colours. The production of pigments may occur extracellularly or intracellularly, contingent upon the microorganisms involved.

The investigation of soil-derived pigment-producing bacteria and the subsequent analysis of their pigments for antimicrobial properties is driven by the increasing demand for innovative antimicrobial agents due to escalating antibiotic resistance. Soil, a dynamic and diverse environment, is a prolific source of microorganisms that generate bioactive chemicals with potential therapeutic applications. [7]

Pigment-producing bacteria occupy several niches within soil ecosystems, such as the rhizosphere, bulk soil, and decomposing organic materials [8] These bacteria utilise colours as secondary metabolites to acclimatise to environmental conditions and compete with other microbes. Many bacterial taxa, including Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes, possess the capability to synthesise colours. The antibacterial efficacy of bacterial pigments has gained significant recognition in recent years [9]. Research has documented the antibacterial efficacy of pigments against several diseases, encompassing bacteria, fungi, and protozoa. The processes underlying the antimicrobial activity of pigments are complex and may include disruption of cell membranes, inhibition of vital enzymes, and production of reactive oxygen species.

The production of microbial pigments by fermentation technology is more efficient and economical than the extraction of pigments from plants and animals.[10]. These pigments are biodegradable substances having prospective commercial applications as colourants. [11,12 13] Fungal and bacterial microbes provide a viable alternative supply of naturally generated colours. Compared to fungi, bacteria exhibit superior pigment production due to their shorter life cycle and greater ease of genetic modification.

The extraction of novel antimicrobial drugs from natural resources is deemed a crucial endeavour, especially in developing nations where the risk of microbial drug resistance is heightened. Consequently, bacterial pigments, as secondary metabolites, are regarded as a promising agent in this context. This study focused on the isolation and identification of pigment-producing bacteria from natural habitats such as dirt. The pigment derived from the isolates undergoes processing for synthesis, extraction, and assessment of antibacterial activity.

MATERIAL AND METHODS

Collection of soil samples

Soil samples were collected from Kakatiya University (18.0275° N, 79.5545° E). The soil sample was serially diluted in six test tubes. Subsequently, 1 gram of soil sample was added into 9 millilitres of distilled water. Dilution was performed up to 10^-6. The NA plates were subsequently inoculated with the dilutions. Following the distribution of the plates, they were incubated at 37°C for 24 hours. Various colonies of microorganisms were acquired. We extracted the red colony from the plate. The red-colored colony was inoculated onto the petri plates using the streaking method. The sample was subsequently incubated at 25°C for 72 hours, after which growth was assessed. Subsequently, the Red colonies were acquired.

Grams Staining

Bacterial cultures grown overnight were smeared onto a sterile glass slide, heat-fixed, and then incubated with crystal violet for 30 seconds. The slides were washed with deionised water for 5 seconds and immersed in Gram's iodine for 1 minute. The slides were cleaned and decolourised with 95% ethanol till the complete removal of the detached crystal violet for 30 seconds. The glass slides were washed with dH2O for 5 seconds, and safranin was employed for counterstaining for 60 seconds. The slides were subsequently washed to eliminate excess staining, blotted, dried, and viewed under a light microscope at 100X magnification using oil immersion.

Identification of pigmented isolates

Bacterial isolates were identified according to the procedures outlined in Bergey's Manual of Systematic Bacteriology, which includes colony characterisation, cellular morphology, and biochemical assays.

Enrichment for pigment production

A pre-culture was set up in 5 ml of NB medium by inoculating the broth with one loopful of selected bacteria and kept at room temperature overnight. The pre-culture was subsequently utilised to inoculate 1 ml of the nutrient broth production medium. The cell concentration was estimated using the McFarland standard graph. The pigment was produced in 50 ml of nutrient broth within a 100 ml conical flask and incubated at 37 °C for 48 hours.

Extraction of pigment

To extract the pigment, bacterial cells were cultivated for 48 hours in nutrient broth, followed by centrifugation at 3000 rpm for 20 minutes. The pigment-containing layer (pellet/supernatant) was collected and combined with 10 ml of 95% (v/v) methanol for extraction [14]. The coloured methanolic supernatant extract was obtained and filtered using Whatman No. 1 filter paper. The filtrate was transferred to an evaporating dish and incubated at 50 °C in a hot air oven for 30 minutes to yield a dry pigment extract devoid of solvent. The bulk was preserved at 4 °C till subsequent utilisation.

Spectrophotometric analysis

The pigment extract was further examined by measuring the absorbance in the wavelength range of 200–700 nm using a UV-Vis spectrophotometer, which facilitates the determination of the maximum absorption spectra (λmax) with 95% methanol as a blank.

Molecular Characterization of the Bacterial Isolate

Based on the data collected, the most important isolate, JS 05, was chosen and sent to Barcode Biosciences in Bengaluru, Karnataka, for molecular characterisation using 16S rRNA gene sequencing. Bacterial genomic DNA was extracted and analysed by electrophoresis on a 1% agarose gel. Segments of the 16S rRNA gene were amplified using Polymerase Chain Reaction (PCR). The 16S rRNA F and 16S rRNA R primers served as forward and reverse primers, respectively, employing the Bacillus Direct Testing (BDT) v3.1 Cycle sequencing kit on the ABI 3730xl Genetic Analyser. The consensus sequences obtained from the sequencing data are aligned using alignment techniques and examined with the Basic Local Alignment Search Tool (BLAST) to identify potential matches. Ten sequences were selected based on the highest identity scores, followed by a multiple sequence alignment of 70 conducted using CLUSTAL W. The phylogenetic tree was built using Molecular Evolutionary Genetics Analysis (MEGA) 10 software.

Test organisms

The test organisms employed for evaluating the antibacterial activity of soil isolates were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh. Bacteria were cultivated on nutritional agar at 37°C for 24 to 48 hours.

Antibacterial Activity

The antimicrobial efficacy of a crude pigment extract derived from methanol was evaluated against the pathogens Bacillus subtilis (NCIM 2010), Escherichia coli (NCIM 2065), Staphylococcus aureus (NCIM 2079), Pseudomonas aeruginosa (NCIM 2200). The antibacterial activity of the crude pigment extract was assessed using the agar well diffusion method. Approximately 100 µl of standardised inoculum (0.5 MacFarland) of each test bacterium was inoculated into Petri dishes containing nutrient agar medium under aseptic circumstances. A standard cork borer with a diameter of 6 mm was utilised to create consistent wells, into which 60µl of a 10 mg/ml crude pigment extract dissolved in methanol was introduced. Methanol was employed solely as the negative control. The plates were subsequently incubated at 37°C for 24 hours. Following a 24-hour incubation at 37°C, the zone of inhibition was quantified using a standard scale (HiMedia).

Results and Discussion:

Soil samples were collected at Kakatiya University, located at latitudes (17.957273, 79.614976), in Warangal, Telangana, India. The samples were labelled JS01-JS17 based on their collecting sites. The soil samples underwent serial dilution and were subsequently spread-plated onto a nutrient agar medium, leading to the isolation of 17 morphologically different pigmented bacterial colonies labelled JS01 to JS17. The morphological differences among the isolates were confirmed after an extensive examination of colony morphology. The red-pigmented bacterial strain was isolated onto NA plates, and then quadrant streaking was performed to acquire colonies of the pigmented strain. The quadrant streaking technique has been employed to isolate distinct bacterial colonies.

In this investigation, isolation and screening of pigment-producing bacteria resulted in the identification of 7 coloured colonies from 17 soil samples. Of the seven pigmented colonies, 5 exhibited red colouration, whereas 2 had yellow colouration due to the resemblance in growth patterns and the extreme intensity of pigmentation. One isolate was chosen for further research. The isolated red colony was labelled as JS 05. (Figure 1).

Fig 1: The isolated red colony (pigment-producing bacteria)

The isolate JS 05 showed robust growth on the nutrient agar medium. The bacterial isolate exhibited colony morphology and cellular characteristics on Nutrient agar, resulting in the formation of dense red colonies measuring 0.5-2 mm in diameter with a smooth texture within 3 to 5 days of incubation. [15]. The morphology of the isolates and their staining properties were determined using Gram stain, while the biochemical parameters were assessed according to the Bergey Manual of Determinative Bacteriology (Table 1). The microscopic examination of the isolate revealed comparable biochemical findings as reported by researchers Fatima and Anuradha, Prakash et al., and Arulselvi et al. in their studies [16-18]

Table.1. Displaying morphological, physiological, and biochemical properties of *Serratia nematidophila*

Characteristics	**Nature**
Colony morphology	Red, pigmented, smooth
Cell shape	Rod
Gram staining	Gram-Negative
Indole	Negative
Methyl red	Negative
Voges Proskauer	Positive
Citrate test	Positive
Gelatinase	Positive
Catalase	Positive
Nitrate test	Negative

Molecular characterisation of the isolates. Subsequent to the comprehensive biochemical characterisation, the bacterial isolate JS 05 was subjected to molecular characterisation procedures. The sequencing of the 16S rRNA gene revealed that the isolate JS 05 was a pigmented bacterium. The sequences were analysed, validated, and submitted to the GenBank database. Phylogenetic trees were constructed utilising Clustal W for sequence alignment and MEGA 6 software for the phylogenetic analysis of the strains. (Figure 2)

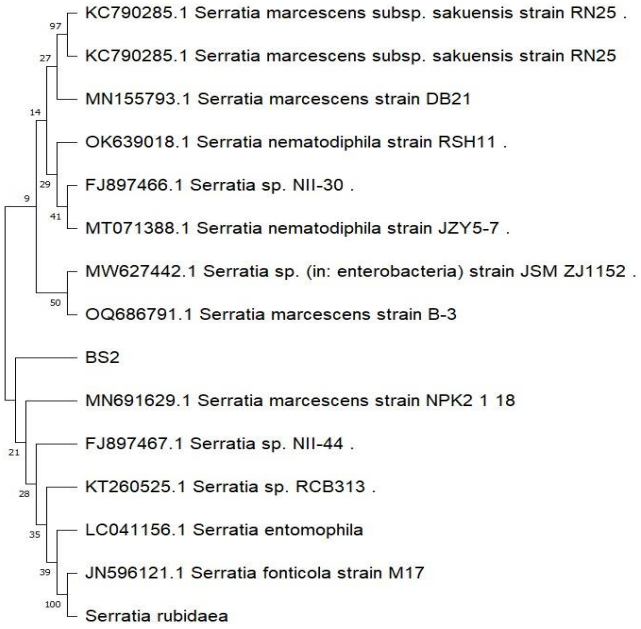

Figure 2. Phylogenetic tree of *Serratia nematodiphila* JS 05

The pigment extracted with ethanol from *Serratia nematodiphila* JS 05 exhibited characteristic peaks at 530 nm, indicating that the ethanol-extracted pigment was prodigiosin. Prodigiosin isolated with ethanol from S. *nematodiphila* exhibited characteristic peaks at 535 and 470 nm in acidic and alkaline solutions, respectively. (Figure 3) [19,20].

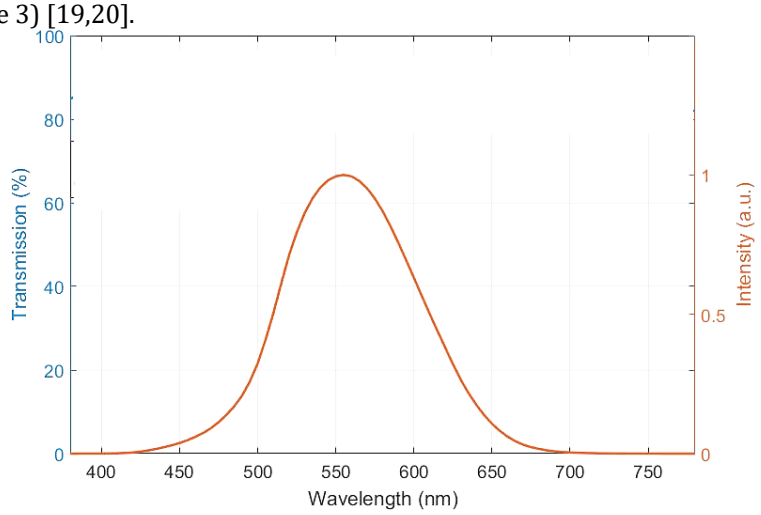

Figure 3 UV -Vis spectrophotometric analysis of pigment extract showing maximum absorbance (λmax)

The crude pigment extract from *Serratia nematodiphila* was evaluated for antibacterial activity using the agar well diffusion method against typical pathogens, including *Bacillus subtilis* (NCIM 2010), *Escherichia coli (NCIM 2065), Staphylococcus aureus (NCIM 2079),* and *Pseudomonas aeruginosa (NCIM 2200).* The crude metabolites have remarkable antibacterial efficacy against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa.* The crude pigment extract showed significant antibacterial activity against both Gram-negative and Gram-positive bacteria. The crude pigment extract of *Serratia nematodiphila* exhibited greater inhibitory efficacy against Gram-positive bacteria compared to Gram-negative bacteria. as shown in Table 2.

Sno	Test organism	Diameter of zone of inhibition in mm		
		Pigment	Methanol	Standard
1	*Bacillus subtilis*	24	5	30
2	*Escherichia coli*	17	6	32
3	*Staphylococcus aureus*	25	5	30
4	*Pseudomonas aeruginosa*	18	5	34

Table 2 Antibacterial activity of the pigment extract against gram positive and gram-negative bacteria

CONCLUSION

This study highlights the significant potential of soil-derived pigment-producing bacteria as sources of innovative antibacterial compounds. We identified pigment-producing bacteria by isolating them from soil. Soil serves as a substantial reservoir of pigment-producing microorganisms with considerable antibacterial capabilities. The ongoing investigation and advancement of these natural compounds may yield revolutionary solutions in healthcare, agriculture, and environmental management, facilitating the identification of novel antimicrobial agents and sustainable practices

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