Insecticidal properties of water diffusible prodigiosin produced by Serratia nematodiphila 213C

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Abstract

Water diffusible pigment producing rhizospheric bacteria was isolated and identified as Serratia nematodiphila 213C through phenotypic characters, FAME and 16S rDNA analysis. In glycerol - peptone media at 28 °C, 72 h and 180 rpm; dark red colored pigment was biosynthesized, which showed UV – Visible absorbance at λ 535 and Rf of 0.9 on TLC (10 M ethyl acetate; methanol- 6:4), suggesting close similarity toward prodigiosin type of pigment. Insecticidal performance of isolated pigment on Helicoverpa armigera and Spodoptera litura showed 70 and 100 % larval mortality at 20 and 30 mgml⁻¹ concentrations.

Keywords: Serratia, insecticide, prodigiosin, Helicoverpa armigera

Introduction

The extensive use of chemical pesticides has resulted in (i) environmental degradation, (ii) adverse effects on human health, (iii) effect on other organisms, (iv) eradication of beneficial insects and (v) resistance developed by insect pest with broad host range e.g. Helicoverpa armigera and Spodoptera litura. Thus, failures of chemical pesticide and demand of organic food has led to search for new avenue for insect pest control (11). In this regard, biopesticide demonstrated a sustainable approach over chemical pesticide because of its (i) target specific, (ii) easy biodegradable, (iii) less shelf-life and (iv) user friendly for sustainable agriculture (6, 30). Nature had maintained host parasite relations; hence entomopathogenic bacterium and their nematode association can be considering trail for this challenges.

Serratia genus is omnipresent in the environment and found in different geoclimatic conditions as well as in close association with other microbes, insects (nematode), mammals and plants (12). Root association of Serratia sp. and corn root worm (Coleoptera: Chrysomelidae) was explained earlier (24). Similarly biocontrol potential of Serratia sp., like S. marcescens, S. ficaria, S. rubidaea etc. has been explained previously (18, 23, 27, 28, 32). In view of the broad action of Serratia sp. the biochemical mechanism shows production of potentially active molecules (secondary metabolite as well as proteins) like pyrrolnitrin, oocydin A, carbapenem, prodigiosin, surfactant serrawettin as well cell-wall/membrane degrading enzymes like chitinase (1, 15, 19, 21, 35).

Serratia produces both water insoluble as well as water soluble pigments, like in Serratia marcescens red water insoluble pigment has antibiotic activity (16, 34, 38, 39) whereas water soluble reddish-violet pigment with superoxidase dismutase mimetic activity (14, 36). Amongst all the pigments, prodigiosin is well studied because its abundance in Serratia sp.; consisting of alkaloid with a tripyrole group i.e. 5[(3-methoxy-5-pyrrol-2-yldene-pyrrol-2-yldene)-methyl]-2-methyl-3-pentyl-1H pyrole (C₂₀H₂₅N₃O) covering three rings and forming a pyrroly/pyrromethane
skeleton with a C-4 methoxy group of average molecular weight of 323.44 D (33, 41). It showed its bioactive potential as antibacterial, antimycotic, immunomodulating, antitumor and anti-malarial agent (2, 8).

In order to elucidate natural biocontrol mechanism in insect pest infested sorghum rhizosphere, current study relates the pigment producing bacteria from rhizosphere and insecticidal activity of pigment with the objectives (i) isolation of pigment producing bacteria and its identification by phenotypic, FAME and 16S rDNA analysis, (ii) isolation and identification of pigment and (iii) determination of activity of pigment against the common insect pest i. e. *H. armigera* and *S. litura*.

**Materials and Methods**

**Isolation and identification of organism:** Sorghum rhizospheric soil sample was serially diluted up to 10⁻⁷ dilution on suspending in sterile phosphate buffer saline (pH – 7.0) in proportion of 1% and mixed it carefully. A 0.1 ml sample was spread on nutrient agar and incubated at room temperature for 24 hrs. Colonies with diffusible pigment was observed and isolated colony was sub cultured and stored at 4°C. The isolated bacteria was further characterized for identification by phenotypic characters i.e. morphological (staining and motility), cultural (nutrient agar, glycerol-peptone medium), biochemical tests (IMVIC test, catalase test, oxidase tests) (3).

Further confirmation was done with (i) whole-cell fatty acids derivatized to methyl esters (FAME) were analyzed using Gas Chromatography (Agilent 6850 Series II) with the help of MIDI Sherlock software for FAME (MIDI, Newark, DE, USA) as per Rajan et al. (2011) at Royal Life Sciences Pvt. Ltd, Secunderabad and (ii) nucleotide sequence of 16 S rRNA gene. The genomic DNA of strain 213C was isolated as per Rajan et al. (2011) at Royal Life Sciences Pvt. Ltd, Secunderabad where, 16S rDNA subjected to amplification (1.5 kb) using universal primer 16S F (5' AGA GTT TGA TGG CTC AG 3') and 16S R (5' ACG GCT ACC TTG TTA CCA CTG 3'). The PCR amplification conditions were (i) 25 repeat cycles of 95 °C denaturation for 1 min, (ii) annealing at 55 °C for 1 min and extension of annealing at 72 °C for 1.30 min (Gene AMP PCR System 9700) in 0.2 mL PCR tubes. The PCR products were purified using Qiagen PCR product purification kit. Purified PCR products were taken for cycle sequencing with ABI Big Dye Terminator chemistry using either forward primer or reverse primer in Gene AMP PCR System 9700. The cycle sequencing products were purified according to Applied Biosystems protocol. Purified products were sequenced in ABI 3730XL Sequencer. The 16S rRNA nucleotide sequences were aligned using BLAST analysis at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). For comparison currently available sequences at NCBI were used and multiple sequence alignment performed by using Bioedit 7.0. The evolutionary dendrogram was inferred using the Neighbor-Joining method (29). The bootstrap consensus tree inferred from 1000 replicates (5) was taken to represent the evolutionary history of the taxa analyzed (5). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (5). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (17) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (37). The nucleotide sequence of 16 S rRNA was deposited to Gene Bank database under accession number JN 166084.

**Fermentation condition:** A pre-culture was developed in 50 ml of nutrient broth in Erlenmeyer flask by inoculating a single colony of selected bacterial strain (213 C) and incubating at room temperature, 180 rpm for 24 h. A 50 ml of freshly prepared fermentation medium (gL⁻¹): glycerol, Patil et al
0.2; peptone, 0.5 (10) of pH 7.0 in 250 ml Erlenmeyer flasks was inoculated with the pre-culture of 1.0 absorbance at 600 nm at 1% (v/v) concentration and incubated at 28 °C, 180 rpm for 72 h.

**Isolation of Pigment:** The pigment naturally concealed into the glycerol - peptone broth medium after 72 h incubation was separated by either filtration in glass wool tied funnel or by centrifugation at 10,000 rpm for 15 min and subjected to concentrated in rotary vacuum evaporator (BÜCHI Rotavapor, R -124) at 50 °C under vacuum (10⁻⁵ torr).

**Determination of chromatographic and spectral properties:** Thin layer chromatography - The concentrated pigment was checked for its electrophoretic mobility as well separation using TLC. The silica gel TLC plate (20 x 20 cm) were prepared and developed as per standard protocol (22, 31). A mobile phase (10 M ethyl acetate: methanol – 6: 4) was run to separate the pigment components as well as to determine its retardation factor (Rf) (20).

**UV-Visible spectral trait:** The concentrated pigment (10 mgL⁻¹) was dissolved in de-ionized Millipore water and scanned for its absorbance on UV – Visible spectrophotometer (UV-1800, Shimadzu, Japan) from 700 – 400 nm.

**Insect bioassay:** The insect bioassay was carried out on second instar larvae of *H. armigera* (pod borer) and *S. litura* (cotton leaf eating caterpillar) in 12 or 24 well flat bottom plates using pigment as a diet feed in triplicate plate was sealed and placed in a humidified growth chamber at 28 °C. 24 - 30 larvae were placed in each of the petri plates containing different concentrations of pigment (10, 20, 30, 40, 50 mgmL⁻¹) sprayed on okra pods as diet, with a control kept without pigment spray. The larvicidal effect of pigment was determined by counting the number of dead larvae after 72 h on observing failed motility after probing with needle.

**Statistical analysis:** The dose–response data was subjected to probit regression analysis to determine the statistical significance of performed experiment.

**Results and Discussion**

Different *Serratia* sp. has been studied for their physiological and pigment production (9, 13). Sorghum is one of the contamination sites for different insects like *H. armigera*, *S. litura*. Very few reports are available for isolation of *Serratia* sp. from Sorghum rhizosphere. Present study is related with nematode contaminate sorghum field and its natural control by pigment, producing microbes as biocontrol agent. In view of this, nine different pigment producing bacteria were isolated on nutrient agar after incubation for 24 h at room temperature. Among 09 isolates, one red pigmented mucoid bacterial colony (designated as 213 C) showing water diffusible pigment production was selected for further study. Colonies were small, low convex, smooth, bright red colored, non spore forming, Gram negative, motile, rods. The biochemical performance showed -, -, +, + reaction trait for IMViC test and catalase (+) and oxidase (-) test suggest relevance of *Serratia* genus. The FAME analysis showed similarity index of 0.654 with *Serratia marcescens*-GC-subgroup with 14 reference peaks (data not shown). The partial 16S rRNA gene sequence was compared with sequences available from databases. Phylogenetic tree was constructed from available 16S rRNA of *Serratia* sp. and selected *B. subtilis* using molecular evolutionary analysis tool i. e. Clustal X and MEGA software (Fig. 1). Based

![Fig. 1. Phylogenetic position of strain 213C among genus Serratia in a neighbour-joining tree based on analysis of 16S rRNA gene sequence.](image-url)
on 16 S rRNA homology, the isolate 213 C suggest (99%) with a *Serratia nematodiphila* HQ123425. Thus the phenotypic, FAME and 16S r DNA reveals the similarity of isolate 213 is a *Serratia nematodiphila* (Gene bank accession no. JN166084). The results obtained are similar to Zhang *et al.* (2009).

**Pigment isolation and its preliminary characterization:** After 72 h of incubation at 28 °C, yellow white colored glycerol peptone media of pH 7.0 turns in lust red. Pigment isolated after centrifugation at 10,000 rpm for 10 min and concentrated in rotary vacuum evaporator and finally showed dark red colored powder.

The TLC scan (Fig. 2) showed single spot after evaporation of solvent at room temperature. The Rf value at 0.9 is similar to prodigiosin type of pigments i.e. 0.9 – 0.95 as reported earlier (22, 31). Similarly the UV-Visible spectrum (Fig. 3) from 700 – 400 nm gives absorption maxima at 536 nm which is analogous to prodigiosin type of pigment as reported by Patil *et al.* (2012). The electrophoretic and spectral evidence reveals the biosynthesis of extracellular water diffusible sole prodigiosin type of pigment by an isolate *Serratia nematodiphila* 213C in glycerol peptone media. Prodigiosin is high value pigment as described earlier and hence sole synthesis of pigment in fermentation media will be helpful in down streaming of large scale pigment production.

**Insecticidal performance of pigment:** In earlier reports (23, 24) *Serratia marcescens* and other *Serratia* sp. have been shown its biocontrol potential. Although, Patil and coworker have shown role of prodigiosin in mosquito control, till...
Fig. 4. Effect of crude prodigiosin on second instar *H. armigera* larvae with increase in dose (10 – 50 mg/ml) and incubation time (24 – 72 h) with 5 % SD

Fig. 5. Effect of crude pigment on second instar *S. litura* larvae with increase in dose (10 – 50 mg/ml) and incubation time (24 – 72 h) with 5 % SD

*Insecticidal water diffusible pigment*
Supportive information - Preliminary phenotypic and biochemical characteristics of bacterial isolate 213C

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony on nutrient agar</td>
<td>Mucoid, sticky, convex, entire surrounded with red pigment</td>
</tr>
<tr>
<td>Gram character</td>
<td>Gram negative, rod, single, motile, non</td>
</tr>
<tr>
<td>Microscopy</td>
<td>-</td>
</tr>
<tr>
<td>spore forming</td>
<td>-</td>
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<tr>
<td>Indole production</td>
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<tr>
<td>Methyl red</td>
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<td>Voges-proskauer</td>
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<tr>
<td>Citrate utilization</td>
<td>+</td>
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<tr>
<td>Catalase</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
</tr>
<tr>
<td>Gelatinase</td>
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<tr>
<td>Amylase</td>
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</tr>
<tr>
<td>H₂S production</td>
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</table>

Statistical analysis for insecticidal performance

1. H. armigera

<table>
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<th>Parameters for correlation</th>
<th>24h</th>
<th>46h</th>
<th>72h</th>
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<tbody>
<tr>
<td>Number of XY Pairs</td>
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<td>5</td>
<td>5</td>
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<tr>
<td>Pearson r</td>
<td>0.9776</td>
<td>0.9902</td>
<td>0.8796</td>
</tr>
<tr>
<td>95% confidence interval</td>
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<td>0.8543 to 0.9994</td>
<td>-0.01228 to 0.9920</td>
</tr>
<tr>
<td>P value (two-tailed)</td>
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<td>0.0012</td>
<td>0.0492</td>
</tr>
<tr>
<td>P value summary</td>
<td>**</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>Is the correlation significant? (alpha=0.05)</td>
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<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>R square</td>
<td>0.9556</td>
<td>0.9805</td>
<td>0.7737</td>
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</table>

2. S. litura

<table>
<thead>
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<th>Parameters for correlation</th>
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<th>46h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of XY Pairs</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Pearson r</td>
<td>0.9887</td>
<td>0.9824</td>
<td>0.8796</td>
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<td>95% confidence interval</td>
<td>0.8338 to 0.9993</td>
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<td>R square</td>
<td>0.9776</td>
<td>0.9651</td>
<td>0.7737</td>
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nematodiphila 213C, proved to have insecticidal effect on Lepidopteran pests i.e. H. armigera and S. litura. The statistical analysis recommends the data in correlation is significant with \( p < 0.05 \).

**Conclusion**

Present investigations showed insecticidal capability of prodigiosin at laboratory conditions on H. armigera and S. litura. To our knowledge, this is the first report of S. nematodiphila isolated from sorghum rhizosphere as well as having water diffusible pigment production capability. Sole prodigiosin synthesis, suggest efficacy of bacterium for biocolor production. At crude concentrate level, prodigiosin showed insecticidal activity implies its econo - value. However, further purification, cytotoxicity testing and field trial will be carried out in future for its commercial use.

**Acknowledgement**

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**References**


28. Roberts, D. P., McKenna, L. F., Lakshman, D. K., Meyerc, S. L. F., Konga,


