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RESEARCH ARTICLE

BIOCONTROL ACTIVITY OF SIDEROPHORES PRODUCING *BACILLUS LICHENIFORMIS* DS3 AGAINST SEVERAL PATHOGENIC FUNGI IN BLACK GRAM [*VIGNA MUNGO* (L.) HEPPER]

Silpa, D., Brahmaji Rao, P. and *Kranthi Kumar, G.

Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur, India

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ABSTRACT

Plant growth promoting *Bacillus licheniformis* DS3 strain isolated from Agriculture field soils of Guntur, Andhra Pradesh, India. The identification of bacteria was done by 16 S rRNA sequencing analysis. Siderophore production by microorganisms in solid medium, Chrome Azurol' S (CAS) Agar plate assay was used. Siderophore produced by the strain *Bacillus licheniformis* DS3 was identified as hydroxamate type. Maximum siderophore production was achieved from 96 h of incubation period with 14.4µg/ml. Carbon and nitrogen sources on the production medium also greatly influenced the siderophore production. Maximum siderophore production 18.2 µg/ml was obtained with glucose as the carbon source and ammonium sulphate 14.6 µg/ml used as nitrogen source. This strain showed significant fungal activity against *Aspergillus niger, Alternaria solani, Fusarium oxysporium and Fusarium solani.* Therefore our results elucidate that the *Bacillus licheniformis* DS3 strain shows potential activity and biological agent for the control of several fungal pathogens of black gram plant.

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INTRODUCTION

Siderophores are low molecular weight compounds that can chelate ferric iron from many insoluble compounds in the environment, ranging in size from 500-1500 Daltons, which are synthesized by many microbes when growing under low iron conditions. PGPR produces a range of siderophores which have a very high affinity for iron. Therefore the low availability of iron in the environment would suppress the growth of pathogenic organisms. Siderophores, the iron chelating agents produced by microorganisms, have been classified into three main types: hydroxamates, catecholate and carboxamate types depending on the iron ligation groups (Wandersman and Delepelaire 2004). However siderophore producing microorganisms which improves the soil fertility for sustainable agriculture and ecofriendly. These microorganisms may improve the plant growth. Many reports related to the plant growth promoting ability to produce siderophores. There are few reports on siderophores producing by bacteria (Arora, 2001, Schwyn and Neilands, 1987). 'Siderophores' (derived from the Greek meaning "iron carriers") are low molecular weight ferric-ion-specific chelating agents produced by bacteria and fungi to combat low iron stress (Ngamau *et al.,* 2014). Further extensive studies on Siderophores are iron chelating agents in agriculture.

Corresponding author:* **Kranthi Kumar, G.,
Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur, India.
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Plant growth promoting activity has been reported in bacterial strains belonging to several genera, such as *Acetobacter* sp, *Azospirillum* sp, *Azotobacter* sp, *Bacillus* sp, *Burkholderia, Pseudomonas* sp. and *Rhizobia* (Lugtenberg and Kamilova, 2009). Strains with Plant Growth Promoting activity, belonging to genera *Bacillus licheniformis* also have been reported by (Hurek and Reinhold-Hurek 2003). The species of *Bacillus* showed a number of PGP were extensively studied. Bacterial species produce different types of siderophores like hydroxamate-type, carboxylates and catechol-type (Neilands and Nakamura, 1991). These Siderophore produced by bacteria's have the ability to employ as biocontrol agents against some soil-borne plant pathogens (Arora, 2001). Plant growth-promoting strains act as the suitable alternative to chemicals, to facilitate eco-friendly biological control of several soil and seed borne pathogens. Much work was done by the siderophore production by bacteria and fungi. There were limited studies on siderophore production by bacteria and type of siderophores. Our study mainly focussed on the present bacteria shows which type of siderophores, optimization studies and antagonistic activities are present in *B. licheniformis* DS3 isolated from agriculture field soils.

MATERIALS AND METHODS

Microorganisms: Microorganisms *Bacillus licheniformis* DS3 used in the present study was isolated from the banana rhizospheric soil sample collected from Guntur region and

maintained in our laboratory. Isolation and media composition of bacterial strains was followed by Silpa *et al.,* (2018). The preliminary characterization and identification of the bacterial isolate was made following Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Partial 16 S rRNA sequence analysis of the isolate DS3 exhibited 99% homology with *Bacillus licheniformis* and the sequence was deposited in Gen Bank with accession number MG870112. Seven fungal strains, *Aspergillus niger, Aspergillus flavus, Cercospora sp, Helmenthosporium sp. Alternaria solani, Fusarium oxysporium and Fusarium solani* isolated from Black gram plants. These are showing the symptoms of powdery mildew, leaf spot, wilt and root rot. After retrieving fungal and bacterial strains they were maintained on Potato Dextrose Agar (PDA) and Nutrient Agar (NA) media for further studies.

Identification of bacteria by 16 S rRNA sequencing

DNA extraction and PCR amplification: Genomic DNA was isolated according to the following procedure. 50 ml LB broth was inoculated with a single bacterial colony and grown to an absorbance at 600 nm of 0.5–1.0 and cells were collected by centrifugation at 5000 rpm, at 4° C, for 10 minutes. The genomic DNA was isolated from the *B. licheniformis* DS3. Amplification of the 16s rRNA gene sequence was performed by using the universal primers. Amplification of the 16s rRNA gene was performed using the universal primers.

Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3'
Reverse Primer: 5'-ACGGCTACCTTGTTACGACTT-3'

Bioinformatics analysis: Sequences were compared to the non-redundant NCBI database by using BLAST, with the default settings used to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignments with the default settings. The multiple alignment file was then used to create phylogram using MEGA 5 software.

Siderophore production

i. **Siderophore assays:** For the detection of siderophore production, *B. licheniformis* DS3 was grown on the medium containing 0.5 µM of iron, and incubated for 24 h on rotary shaker at 200 rpm at room temperature. The assays used to detect siderophore were the Chrome Azurol S assay, Atkin's assay (Hydroxamate type of siderophore) and Arnow's assay **(**Catechol type of siderophore).

ii. **Chrome Azurol S (CAS) Agar medium:** For the detection of siderophore, *B. licheniformis* DS3 was grown in synthetic medium, containing 0.5 µM of iron and incubated for 24 h on a rotary shaker at 200 at room temperature. Chrome Azurol S (CAS) assay is used to detect the siderophore. Culture supernatant was added to the wells made on the CAS agar plates and incubated at room temperature for 24 h. Formation of yellow to orange coloured zone around the well indicates siderophore production (Schwyn and Neiland's, 1987). All glassware used to store stock solution of the medium were treated with concentrated HNO_3 and left to overnight. After 24 h, the acid was removed and the glassware was rinsed thoroughly with double distilled water.

iii. **Preparation of CAS indicator solution:** Initially 60.5 mg of chrome Azurol S dissolved in 50 ml of double distilled water 10 ml of Fe III solution (27 mg $FeCl_3.6H_2O$ and 83.3 µl concentrated HCl in 100 ml double distilled water) was added along with 72.9 mg hexadecyl trimethyl ammonium bromide (HDTMA) dissolved in 40 ml double distilled water. The HDTMA solution was added slowly while stirring, resulting in dark blue solution (100 ml total volume) which was then autoclaved.

iv. **Preparation of CAS agar plates:** The autoclaved basal agar medium was cooled to 50°C in a water bath. The CAS indicator solution was also cooled to 50°C, along with a 50% solution of glucose. Once cooled, to 2 ml of the 50% glucose solution was added to the basal agar medium with constant stirring, followed by 10 ml of the CAS indicator solution, which was added carefully and slowly along the walls of the flask with constant stirring. Once mixed thoroughly the resulting solution (100 ml) was poured into sterile plates. The strain *B. licheniformis* DS3 was grown

If siderophore are present an orange halo is visible. A halo was formed the supernatant of cultures grown in iron-restricted media and cultures grown under high iron conditions did not create any colour change. In addition to using supernatant from culture grown in high iron medium as a control, uninoculated medium is also added to a separate well to ensure the medium alone does not cause a colour change.

Atkin's assay for estimation of hydroxamate- type of siderophores: The culture supernatant was further using Atkin's assay for the estimation of hydroxamate type of siderophores (Atkin *et al.,* 1970). For hydroxamate type of siderophores production, Fiss-glucose mineral medium (K_2HPO_4 -5.0 g; L-Asperagine -5.0 g; glucose - 5.0 g; $ZnCl_2$ - 0.05; $MnSO_4.7H_2O$ - 4.0 g/l) was used. Atkin's assay is also a colorimetric assay, and the reaction was allowed to incubate at room temperature for approximately 5 minutes for orange colour development. Once the colour developed the absorbance of the solution is measured at 480 nm using uninoculated medium with reagents as a blank. The control assay was colour less to slightly yellow in colour and a positive reaction is orange colour.

Antifungal activity of siderophores: Antifungal activity of partially purified siderophores was determined by the Slightly modified method as described by Schorth and Hancock (1981). Test organisms used in this study include *Aspergillus niger, Aspergillus flavus, Cercospora species, helmenthosporium sp. Alternaria solani, Fusarium oxysporium and Fusarium solani* by well diffusion method. Zone of inhibition was measured after 72 hours of incubation at 28° C.

Optimization studies for Siderophore production: Various factors like effect of incubation period, effect of carbon and nitrogen sources influence the siderophore production. Quantitative estimation of siderophore production was done by using spectrophotometer at 480 nm.

Effect of incubation period on Hydroxamate type of Siderophore production: In order to study the siderophore production, different incubation periods (24, 48, 72, 96,120 and 144 h) was determined by growing the bacterial strain in the basal medium upto 144 h of incubation for siderophore production.

Effect of carbon sources on type Hydroxamate type of Siderophore production: To study the siderophore production by using different carbon sources (Starch, glucose, sucrose, Succinate, and citrate) were replaced with 1% mannitol. The bacterial strain was inoculated in the basal medium for 144 h of incubation and estimated the siderophore production.

Effect of nitrogen sources on type Hydroxamate type of Siderophore production: To study the siderophore production by using different nitrogen sources (ammonium sulphate, sodium nitrate, L-Aspargine, glutamine and glycine) were replaced with 0.1% yeast extract. The rhizobacteria were inoculated in the basal medium for 144 h of incubation and estimated the siderophore production.

Statistical analysis

Triplicates were maintained for each treatment. Statistical data are recorded on biomass of the strain and enzyme production by using One-way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

The bacterial strain *B. licheniformis* DS3 was isolated from agriculture field soils and this strain was showed maximum clearance zone on starch hydrolysis test. Molecular identification and classification on the basis of 16 S rRNA sequencing analysis is important for perfect identification of microbial species then the morphological, physiological and biochemical characterization (Aneja, 2012). The results of PCR sequences were compared with other sequenced bacteria in National Center for Biotechnology Information (NCBI) in Gene Bank. Phylogenetic tree was conducted by taking the sequences obtained in blast search. Sequence obtained from BLASTN (nucleotide blast) was obtained in FASTA format and relation between each sequence could be known by multiple sequence alignment using a software CLUSTAL algorithm. The tree was generated using neighbour joining (NJ) a distance-based algorithm of phylogenetic analysis. Bacterial isolate (ANU-MCB-DS3) was clustered. Based on 16S rRNA gene analysis, isolate ANU-MCB-DS3 was grouped into genus *Bacillus*. The sequence of DS3 was most closely related to *B. licheniformis* with similarity of 99% (Figure-1). Bacterial strain was tested for the siderophore production on CAS agar medium. Solubilization (zone of clearance) was observed after 48 h of incubation period.

Effect of incubation period on siderophore production: Siderophore production was started with initial incubation period from 24 hours of incubation period with 2.40 µg/ml. Maximum siderophore production 14.4 µg/ml was observed at 96 hours of incubation period (Table-1). Production was increased with increasing incubation period upto 96 h. After increasing the incubation period siderophore production was decreased. From the studies on 24-48 hours of incubation period was optimum for hydroxamate type of siderophores by *Bacillus* sp. SD12 (Radhakrishnan *et al.,* 2014).

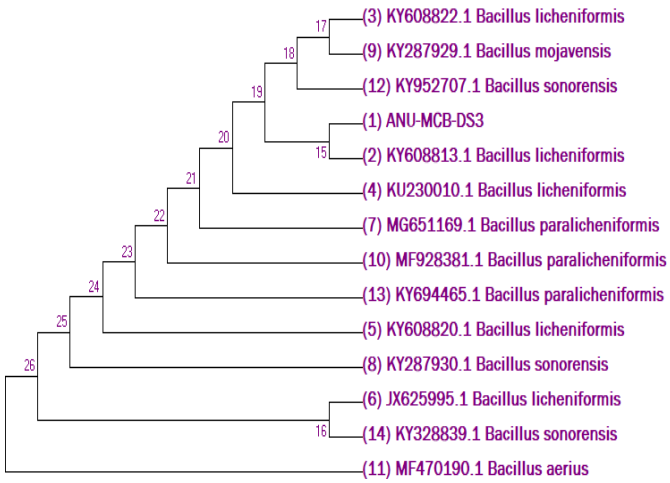

Figure 1. Phylogenetic tree constructed based on 16S rRNA gene sequence analysis from the isolates obtained from agriculture field soils Guntur district of Andhra Pradesh, India, with reference sequences available at NCBI through BLAST analysis. Phylogenetic tree based on 16S rRNA sequences of the genus Bacillus obtained from BLAST search showing the position of isolate (ANU-MCB-DS3- *Bacillus licheniformis* DS3) and related strains

Effect of carbon sources: Different carbon sources were introduced in to the synthetic medium for siderophore production. Maximum siderophore production 18.2 µg/ml was observed in glucose containing the medium (Table-2) Maximum siderophore production was also observed in Sucrose 16.8 µg/ml and Succinate 14.0 µg/ml respectively. Yu *et al.,* (2017) reported that the siderophore production varied with carbon source, where the maximum siderophore production (80.68% SU) was obtained with glucose as the carbon source. According to Mahmoud and Abd-Alla, (2001) glucose proved to be the most suitable source for *Pseudomonas aeruginosa* (16.9), *Aspergillus nidulans* (11.4) and *Pseudomonas chrysogenum* (13.7). The siderophore production by an organism depends on the availability of organic and inorganic nutrients in the medium (Neilands 1981 and Abd-Alla, 1998).

Effect of nitrogen sources: Different nitrogen sources were incorporated into the synthetic medium for siderophore production. Maximum siderophore production was observed in nitrate source Ammonium sulphate 14.6 µg/ml followed by sodium nitrate 14.0 µg/ml and glutamine 12.0 µg/ml. Lowest production 8.4 µg/ml was observed in L-Aspargine containing the medium (Table-3). Kumar *et al.,* 2017 reported that the bacterial strains showed siderophore production was maximum in presence of ammonium sulphate and sodium nitrate was used as nitrogen sources. Sasirekha and Srividya (2016) reported that the Siderophore production by *Pseudomonas aeruginosa* in the presence of Yeast extract proved to be the most suitable nitrogen source. Utilization of urea by the isolate in the current study also suggested its possible exploitation for bioremediation of alkaline soils by reducing the excess amount of urea present in the soil (Sayyed *et al.,* 2010).

Anti phytopathogenic activity

Fungicidal activity of bacterial strain B. licheniformis DS3 inhibited the growth of the pathogenic fungi. From this study 7 fungal strains were tested for antagonistic activity (Table-4). Among them 4 strains fungal mycelia did not cover the surface

Table 1: Effect of incubation period on hydroxamate type of siderophores by *Bacillus licheniformis* DS3

Incubation periods (hours)	Siderophore production (µg/ml)
24	2.40
48	5.20
72	11.8
96	14.4
120	8.04
144	5.08

Table 2: Effect of carbon sources on hydroxamate type of siderophores by *Bacillus licheniformis* DS3

Carbon sources (1%)	Siderophore production (µg/ml)
Control	1.20
Starch	11.4
Glucose	18.2
Sucrose	16.8
Succinate	14.0
Citrate	8.20

Table 3: Effect of nitrogen sources on hydroxamate type of siderophores by *Bacillus licheniformis* DS3

Nitrogen sources (0.5%)	Siderophore production (µg/ml)
Control	3.0
Ammonium sulphate	14.6
Sodium nitrate	14.0
L-Aspargine	8.4
Glutamine	12.0
Glycine	13.2

Table 4. Anti phytopathogenic activity of the isolate *B. licheniformis* DS3

S. No	Test strain	Diameter of inhibition zone (mm)
1	*Aspergillus niger*	14.00
2	*Fusarium solani*	16.00
3	*Cercospora sp*	0.00
4	*Helmenthosporium sp.*	0.00
5	*Alternaria solani*	12.00
6	*Fusarium oxysporium*	15.00
7	*Aspergillus flavus*	0.00

*Zone diameter is not include the diameter of the agar well (10 mm)

of the inhibition ring it indicates the antagonism was done. This test reveals the growth of the bacteria inhibited the development of fungal pathogens. Siderophore-producing bacteria have been used as biocontrol agents to combat plant pathogens reported by Gram, (1996). Hu *et al.,* (2008), also reported that the siderophore producing *B. subtilis* QM3, a spore-forming bacterium commonly used in commercial and research bio-control products to control a variety of plant pathogens.

Conclusion

Presence of orange color and halo zone formation confirmed that the bacterial isolate *B. licheniformis* DS3 had the ability to producing siderophores. Atkins assay proved to be *B. licheniformis* DS3 showed the hydroxamate type of siderophores. For the first time we are reporting the optimization studies and hydroxamate type of siderophores in *B. licheniformis* DS3 isolated from agriculture field soils. Maximum siderophore production was recorded with glucose as the carbon source and ammonium sulphate was used as nitrogen source. This strain shows significant fungal activity against *Aspergillus niger, Alternaria solani, Fusarium oxysporium and Fusarium solani.* This is also constitutes the first report. Siderophores produced by *Bacillus licheniformis* DS3 has got the potentials to control plant diseases in Black gram.

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