Production of catechol type of Siderophores by *Bacillus* Altitudinis and *Paenibacillus* species isolated from root nodules of *Vigna Trilobata* (L.) Verdc. Cultivars

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Abstract
In the present study, Bacillus and Paenibacillus strains were isolated from root nodules of Vigna trilobata (L-Verdc) plants raised in soils collected from geographically different areas in Andhra Pradesh, India. The identification of strains was done by biochemical tests and using 16 S rRNA sequencing analysis. The sequences were deposited in GEN bank (NCBI). For the Production of siderophores Chrome azurol'S (CAS) Agar media was used.

The two strains Bacillus altitudinus MRR 122 and Paenibacillus sp. MRR 124 showed the catechol type of siderophores. Siderophore production was started initially at 72 h of incubation and it reached maximum with 144 h of incubation. Bacillus altitudinus MRR 122 showed the maximum colony size and clear zone diameter of 8 mm and 18 mm respectively. Sucrose and sodium nitrate supported that the maximum siderophore production was used as carbon and nitrogen sources.

Keywords: Siderophore, catechol, *Bacillus*, *Paenibacillus*.

Introduction

Many bacteria and fungi are capable of producing more than one type of siderophore or have more than one iron-uptake system to take up multiple siderophores.[10] *Rhizobia* are also known to produce a wide variety of siderophores, only a few of which have been characterized. These include anthranilate, citrate, rhizobactin and other carboxylates, rhizobactin 1021, vicibactin as well as other unidentified catechols and hydroxamates.[4] Both *R. leguminosarum* and *B. japonicum* have been identified as producing catechol-type siderophores, but the siderophores have not yet been characterized.[9,11]

The most commonly studied of these siderophores are the dihydroxamate rhizobactin 1021 and trihydroxamate vicibactin, both of which have known structure. Many other strains of rhizobia have not been examined for siderophore production, have been labelled as CAS positive or negative, or are identified as producing catechol or hydroxamate-type siderophores.

Rhizobia have been characterized for hydroxamate type siderophore production as being species specific. In general, *Sinorhizobium* produce dihydroxamates, the other fast-growing rhizobia trihydroxamates, and bradyrhizobia produces neither of these.[3]

Vigna trilobata is a wild species belonging to the subgenus Ceratotropis in the genus *Vigna*. *Vigna trilobata*, commonly called as 'Pillipesara', was mainly cultivated as short term Pasteur and green manure crop in India, Pakistan, Sudan and Indonesia. So far, the symbiont in the root nodules was reported as *Rhizobial* strains but not characterized completely.

Therefore, the present study involved the production of catechol type of siderophores from root nodules of *Vigna trilobata*. There were no reports on the production of catechol type of siderophores from *Rhizobial* species isolated from *Vigna trilobata*.

Materials and Methods

Isolation: *Rhizobial* strains were isolated from the root nodules of *Vigna trilobata* plants raised in earthen pots filled with soils collected from various districts of Andhra Pradesh and maintained properly in the botanical garden of our university. Nodulation studies were done by the method followed by Somasegaran and Hoben.[15] Procedure for isolation of rhizobia was previously mentioned.[8]

Siderophore production

Siderophore assays: For the detection of siderophore production, each rhizobacterial isolate 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 and Arnow's assay.

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 stirring, resulting in dark blue solution (100 ml total volume) which was then autoclaved.

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, 2 ml of the 50% glucose solution was added to the basal agar

medium with constant stir followed by 10 ml of the CAS indicator solution which was added slowly along the walls of the flask with constant stirring. Once mixed thoroughly, the resulting solution (100 ml) was poured into sterile plates.

Chrome Azurol S (CAS) Agar medium: For the detection of siderophore, each rhizobacterial isolate was grown in synthetic media 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.[13] 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.

Optimization studies for Siderophore production: Optimization studies including effect of iron concentration, effect of carbon and nitrogen sources influence the siderophore production.

Effect of iron concentration on catechol type of Siderophore production: In order to study the effect of different concentrations (20, 40, 60, 80 and 100 µM) of $Fecl_3$ was determined by growing the rhizobacteria in the basal media upto 144 h of incubation for siderophore production.

Effect of carbon and nitrogen sources on catechol type of Siderophore production: Instead of mannitol in original YEMA (Yeast extract mannitol Agar), media different carbon sources (1%) were tested for siderophore production. The rhizobacteria were inoculated in the basal medium for 144 h of incubation and estimated the siderophore production. Different nitrogen sources (Ammonium sulphate, Sodium nitrate, Urea, Glutamine and Glycine) were introduced into the medium.

Statistical Analysis: Three replicates were maintained for each treatment. ANOVA two way and Duncan's multiple tests were carried out and the results are considered to be significant at $P<0.05$.

Results

The rhizobacterial strains *Bacillus altitudinus* MRR 122 and *Paenibacillus* sp. MRR 124 were isolated from the root nodules of *Vigna trilobata*. The two strains were identified by using 16S rRNA sequencing analysis. Siderophore production by the isolate was tested by chrome azurol S agar medium. The two strains were inoculated on CAS agar medium and the plates were incubated for 144 h at 35^0C. Formation of yellow orange halo around the colonies indicates the siderophore production. Siderophore production started after 72h of incubation and it reached maximum at 144 h (Table 1).

Bacillus altitudinus MRR 122 showed the maximum colony size and clear zone diameter 8 mm and 14 mm respectively. The other strain *Paenibacillus* sp. MRR 124 showed colony size 4 mm and clear zone of 6 mm after 144h of incubation. To determine the type of siderophore, culture supernatant of the synthetic medium were used. The presence of catechol type of siderophores was tested according to Arnow's assay.

Catechol type of siderophore production decreases with increasing iron concentration up to 100 µM (Table 2). Maximum siderophore production were observed in 20 µM by *Bacillus altitudinus* MRR 122 (18.8 µg/ml^{-1}) and *Paenibacillus* sp. MRR 124 (18.6 µg/ml^{-1}).

Table 1
Effect of incubation time on siderophore production

Strain name	Incubation periods (Hours)							
	72		96		120		144	
	Cs	Cz	Cs	Cz	Cs	Cz	Cs	Cz
Bacillus altitudinus MRR 122	3	6	8	12	8	14	8	14
Paenibacillus sp. MRR 124	4	6	4	6	4	6	4	6

CS= Colony size (mm): CZ= Clear zone (mm)

Table 2
Effect of iron concentration Catechol type on Siderophore production

Iron concentrations (µM)	Catechol type of siderophores (µg/ml)	
	***Bacillus altitudinus* MRR 122**	***Paenibacillus* sp. MRR 124**
100	8.00	8.40
80	10.6	10.3
60	11.4	11.8
40	16.8	16.4
20	18.8	18.6

* The overall model is significant with $p<0.05$

Table 3
Effect of carbon sources on Catechol type Siderophore production

Carbon sources (%)	Catechol type of siderophores (µg/ml)	
	***Bacillus altitudinus* MRR 122**	***Paenibacillus* sp. MRR 124**
Control	3.0	4.0
Mannitol	11.0	12.4
Glucose	14.4	16.0
Sucrose	19.2	18.0
Succinate	8.9	11.2
Citrate	6.0	8.40

* The overall model is significant with $p<0.05$

Table 4
Effect of nitrogen sources on hydroxamate type Siderophore production

Nitrogen sources	Catechol type of siderophores (µg/ml)	
	***Bacillus altitudinus* MRR 122**	***Paenibacillus* sp. MRR 124**
Control	2.0	3.2
Ammonium sulphate	10.2	10.0
Sodium nitrate	18.6	16.2
Urea	11.3	11.2
Glutamine	12.8	10.6
Glycine	12.0	11.8

* The overall model is significant with $p<0.05$

Among the 5 carbon sources tested for catechol type of siderophores sucrose containing the medium (Table 3), the strain *Bacillus altitudinus* MRR 122 (19.2 µg/ml^{-1}) and *Paenibacillus* MRR 124 (18.0 µg/ml^{-1}) showed the maximum siderophore production when compared to control. These two strains showed the fewer amounts of siderophores in Succinate used as carbon source.

Siderophore production by the tested microorganisms was affected by different nitrogen sources (Table 4). According to this, sodium nitrate proved to be the most suitable nitrogen source. Maximum siderophore production was in *Bacillus altitudinus* MRR 122 and *Paenibacillus* sp. MRR 124 (18.0 µg/ml^{-1}) and (16.2µg/ml^{-1}) respectively. Among the nitrogen sources supplemented, the synthetic medium, ammonium sulphate showed the less amount of siderophores.

Discussion

The present two strains, *Bacillus altitudinus* MRR 122 and *Paenibacillus* sp. MRR 124 showed the catechol type of siderophore production. Siderophore production by the isolates was tested by Chrome Azurol'S (CAS) assay.[13] A clear orange hallow zone was formed on CAS plates. Formation of yellow-orange halo around the colonies indicates siderophore production.[4]

The rhizobacterial strains *Bacillus altitudinus* MRR 122 and *Paenibacillus* sp. MRR 124 strongly reacted with catechol. Previous reports on *Bacillus subtilis* QM3, a potential plant growth promoting bacteria showed the orange halos around the colonies indicates siderophore excretion and act as biocontrol agent as reported by Hu et al.[6] Waheed et al[16] reported that out of seven *Rhizobium* strains only 4 were siderophores producers.

Siderophore production started after 72 h of incubation and it reached maximum at 144 h of incubation. Jadav and Desai[7] reported that the *Rhizobium* sp. isolated from cowpea produced catechol type of siderophores after 22-24 h of incubation. Arnow's assay formation of pink colour confirms the catechol type of siderophores. The detection of both a hydroxamate-type siderophore and a catechol-type siderophore produced by a strain of *R. leguminosarum* was interesting because catechol-type siderophores are much more uncommon in rhizobial species than hydroxamate-type siderophores.[3]

Siderophore production by the tested *Bacillus altitudinus* MRR 122 and *Paenibacillus* sp. MRR 124 was affected by iron concentration in the synthetic medium. Generally, siderophore production was observed in iron restricted medium.

Among the 5 carbon sources tested, maximum siderophore production was observed in 1% sucrose containing medium. Sridevi and Mallaiah[14] reported that the *rhizobium* sp. isolated from stem nodules showed the catechol type of siderophores with for mannitol 2% containing the medium.

Conclusion

From this study it concludes that catechol type of siderophore production was more common in *Bacillus altitudinus* MRR 122 and *Paenibacillus* sp. MRR 124. Siderophores play an important role for the sustainable

farming. Therefore, these two strains were proved to be best isolates with best PGPR characters for better yielding.

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References
1. Arnow L.E., Colorimetric determination of the components of 3, 4-di hydroxy phenyl alanine-tyrosine mixtures, *Journal of Biological Chemistry*, **118(2)**, 531-537 **(1937)**

2. Atkin C.L., Neilands J.B. and Phaff H.J., Rhodotorulic acid from species of *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces* and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*, *Journal of Bacteriology*, **103(3)**, 722-733 **(1970)**

3. Carson K.C., Holliday S., Glenn A.R. and Dilworth M.J., Siderophore and organic acid production in root nodule bacteria, *Archives of Microbiology*, **157(3)**, 264-271 **(1992)**

4. Carson K.C., Meyer J.M. and Dilworth M.J., Hydroxamate siderophores of root nodule bacteria, *Soil Biol Biochem*, **32**, 11-21 **(2000)**

5. Dilworth M.J., Carson K.C., Giles R.G., Byrne L.T. and Glenn A.R., *Rhizobium leguminosarum bv. viciae* produces a novel cyclic trihydroxamate siderophore, vicibactin, *Microbiology*, **144(3)**, 781-791 **(1998)**

6. Hu Q.P. and Xu J.G., A simple double-layered chrome azurol S agar (SD-CASA) plate assay to optimize the production of siderophores by a potential biocontrol agent Bacillus, *African Journal of Microbiology Research*, **5(25)**, 4321-4327 **(2011)**

7. Jadhav R.S. and Desai A.J., Isolation and characterization of siderophore from cowpea Rhizobium (peanut isolate), *Current Microbiology*, **24(3)**, 137-141 **(1992)**

8. Kranthi Kumar G. and Raghu Ram M., Effect of carbon and nitrogen sources on exopolysaccharide production by *rhizobial* isolates from root nodules of *Vigna trilobata*, *African Journal of Microbiology Research*, **8(22)**, 2255-2260 **(2014)**

9. Nambiar P.T.C. and Sivaramakrishnan S., Detection and assay of siderophores in cowpea *rhizobia* (*Bradyrhizobium*) using radioactive Fe (59Fe), *Letters in Applied Microbiology*, **4(2)**, 37-40 **(1987)**

10. Neilands J.B., Microbial iron compounds, *Annual Review of Biochemistry*, **50(1)**, 715-731 **(1981)**

11. Patel H.N., Chakraborty R.N. and Desai S.B., Isolation and partial characterization of phenolate siderophore from *Rhizobium leguminosarum* IARI 102, *FEMS Microbiology Letters*, **56(2)**, 131-134 **(1988)**

12. Persmark M., Pittman P., Buyer J.S., Schwyn B., Gill Jr. P.R. and Neilands J.B., Isolation and structure of rhizobactin 1021, a siderophore from the alfalfa symbiont *Rhizobium meliloti* 1021, *Journal of the American Chemical Society*, **115(10)**, 3950-3956 **(1993)**

13. Schwyn B. and Neilands J.B., Universal chemical assay for the detection and determination of siderophores, *Analytical Biochemistry*, **160(1)**, 47-56 **(1987)**

14. Sridevi M. and Mallaiah K.V., Production of catechol-type of siderophores by Rhizobium strains from Sesbania sesban (L.) Merr, *Asian Journal of Bio Science*, **3(1)**, 187-194 **(2008)**

15. Somasegaran P. and Hoben H.J., Methods in legume – *Rhizobium* technology NIFTAL, University of Hawalii, Paia **(1985)**

16. Waheed A., Afzal A., Sultan T., Ijaz F., Manan S., Zia M.A., Khan K. and Shah A., Isolation and biochemical characterization of *rhizobium* from pea crop at Swabi, *International Journal of Biosciences*, **4(8)**, 231-240 **(2014)**.

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