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Characterization of siderophore produced by *Pseudomonas* sp. MT and its antagonist activity against *Fusarium oxysporum* f. sp. *cubense* and *F. oxysporium* f. sp. *ciceris*

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Abstract

Siderophores are low molecular weight iron scavengers produced by bacteria to combat iron stress and also suppress deleterious rhizobacteria. In the present study, microbes were isolated from wheat and tobacco farm in Changa village, Anand district, India, and were screened for their siderophore production. Out of 11 isolates, 6 were siderophore producers as they produced orange halos on CAS agar. Isolated bacteria were examined for their hydroxamate, catechol, and carboxylate type of siderophore, and it revealed that all produced hydroxamate siderophore. Among all the isolates, a potential bacterium was selected for further studies and identified by the biochemical test as *Pseudomonas* sp. MT. Temporal effect on growth and siderophore production revealed that both were higher at 24 hrs of incubation and remained active up to 8 days and then after the decline. Siderophore was partially purified and chemically characterized by FTIR. A particle size analyzer measured the particle size of the siderophore and showed 91.36 nm in size. The siderophore producer and non-producer were examined for their ability to uptake iron by providing external siderophores, which gave positive results. The isolated bacterium was tested for its antagonistic activity against *Fusarium oxysporum* f. sp. *cubense* and *F. oxysporium* f. sp. *ciceris*, resulting in inhibition of both the species. Hence, *Pseudomonas* sp. MT can be effectively used to control *Fusarium* spp.

Keywords: biocontrol; *Fusarium oxysporum; Pseudomonas;* siderophore; PGPR

Introduction

Banana (*Musa* spp.) is the fourth most important global food commodity in gross value production after rice, wheat, and maize (Ref). Banana is grown in more than 120 countries in the tropical and subtropical regions, and more than 400 million people use it as their staple food (Molina and Valmayor, 1999). However, wilt is the most common disease found in bananas, spread throughout the world, and ranked as one of the six important plant diseases (Ploetz and Pegg, 1997). *Fusarium oxysporum* f. sp. *cubense* (Foc) is a causative agent found throughout the world except for some islands in the South Pacific, the Mediterranean, Melanesia, and

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Somalia. Gujarat is the second largest banana production in India after Tamil Nadu (National Horticulture Board).

Generally, the root of banana plants is infected and colonized by fungus, and affects the vascular system of rhizomes and pseudostem. Symptoms are usually found after 5 - 6 months of planting (Wardlaw, 1961; Stover, 1962). When there is an infection, it inhibits bunch formation, and if produced, only a few fingers develop and are smaller in size. Moreover, the ripening process is also irregular, and the flesh is pithy and acidic. The fungus survives in soil for up to 30 years as chlamydospores in infested plant material or alternative hosts' roots (Ploetz, 2015). In the present study, bacteria were isolated from the rhizosphere for their siderophore production. A potential siderophore producer was identified, and the type of siderophore was also characterized. Their efficacy to inhibit *Fusarium oxysporum* f. sp. *cubense* and *F. oxysporum* f. sp. *ciceris* pathogens was evaluated.

Materials and Methods

Sample collection and isolation

To isolate siderophore-producing bacteria, rhizospheric soil samples were collected from wheat and tobacco plant rhizosphere. Bacteria were isolated using CAS overlay plate assay (Parez-Miranda *et al.,* 2007). The change in color of overlaid CAS medium from blue to yellow/orange indicated a positive result. Isolates that showed a color change were further screened for their siderophore production by CAS agar plate (Schwyn and Neilands, 1987) and CAS diffusion plate assay (Shin *et al.,* 2001). All the glassware was treated with 6M HCL overnight and rinsed thoroughly with glass distilled water in all the experiments to minimize iron contamination.

Quantification of siderophore

Deferoxamine mesylate and rhizoferrin (kindly supplied by Gunther Winkelmann, Germany) were used to prepare a standard curve for hydroxamate and carboxylate, respectively. The quantity of siderophore produced was extrapolated from the standard curve using CAS assay solution, and the absorbance was measured at 630 nm after 1 hr incubation (Fekete *et al*., 1989; Trivier *et al*., 1995) and denoted as µg/ml.

Chemical assays

Hydroxamates

$FeCl_3$ test (Neilands, 1981)

1 ml of culture filtrate was mixed with 1–5 ml of 2% ferric chloride solution. The formation of red or purple color indicates the presence of siderophores. A peak between 420 and 450 nm of ferrated siderophores showed its hydroxamate nature. The peak was noted on a UV–visible spectrophotometer (Shimadzu 160A).

Tetrazolium test (Snow, 1954)

To a pinch of tetrazolium salt, 1-2 drops of 2N NaOH and 1 ml of the test sample were mixed. If it develops, the immediate deep red color indicates hydroxamate siderophores.

Catecholates

Arnow's test (Arnow, 1937)

To 1 ml of culture filtrate, 0.1 ml of 0.5N HCl was added. To this, 0.5 ml of reagent containing 10 g each of $NaNO_2$ and $Na_2MoO_4.2H_2O$ in 50 ml water was subsequently added. After the formation of yellow color at this point, 0.1 ml of 10N NaOH (a red color resulted) and enough distilled water were added to make the volume 5 ml. Absorbance was noted at 515 nm.

$FeCl_3$ test (Neilands, 1981)

1 ml of culture filtrate was added to 1 ml of 2% $FeCl_3$. A peak at 495 nm of ferrated siderophores indicated the presence of catecholate siderophores.

Carboxylates

Spectrophotometric test (Shenker *et al*., 1992).

To 1 ml of test culture filtrate, were added 1 ml of 250 AM $CuSO_4$ and 2 ml of acetate buffer at pH 4. The copper complex formed was observed for absorption maximum between 190 and 280 nm. There is no specific wavelength at which the copper complex was absorbed. The entire wavelength of 190-280 nm was scanned to observe siderophores' peak absorption.

Identification of siderophore producing bacteria

A potential siderophore-producing organism was identified using Bergey's Manual of Determinative Bacteriology and Bergey's Manual of Systematic Bacteriology.

Temporal effect on siderophore production

The temporal effect on siderophore production was monitored for up to 8 days. Growth was measured at 600 nm, while siderophore production was estimated as mentioned above.

Partial purification of siderophore

Cell-free supernatant was used to partially purify siderophore as described by Jadhav and Desai (1992). Briefly, the supernatant was acidified to pH-2 (with concentrated HCl) and extracted with an equal volume of ethyl acetate. Ethyl acetate was evaporated to dryness, and the residue was used further for analysis.

FT-IR analysis

For characterization, dried partially purified siderophore and standard desferal was ground with KBr separately and pressed to obtain pellets. Infrared absorption spectra were recorded on an FT-IR in the 4000-500 cm-1 range (Nicolet IR200 FT-IR Spectrometer). KBr pellet was used as the background reference.

Size analysis

To analyze the size of the siderophore, the partially purified sample was dissolved in water, and size was measured with a particle size analyzer (Malvern Zetasizer Nano S90).

Cross utilization assay

Iron-free nutrient agar plates containing minimum inhibitory EDTA concentrations were surface spread with the test organism (Joshi *et al*., 2006). Wells of 8 mm diameter were made using a cup borer in plates and the supernatant containing siderophores (Isolate M10) was added to the wells. $FeSO_4$ (100 mg/ml) and saline water (pH 8.0) were added as positive and negative controls, respectively. Desferrioxamine B (100 mg/ml) and ferric citrate (100 mg/ml) were added as standard hydroxamate and citrate-type siderophores. Plates were incubated at 30 °C and growth around wells was monitored for up to 48 h.

Antagonistic activity

Antagonistic activity of isolate was examined dual culture method. Briefly, both the fungus i.e., *Fusarium oxysporum* f. sp. *cubense* and *F. oxysporum* f. sp. *ciceris* were spot inoculated on the four corners while the isolate was spot inoculated in the center. Plates were incubated at 30 °C for 48-72 hrs, and the zone of inhibition was recorded.

Results

Isolation

Morphologically different 11 bacterial isolates which showed orange to yellow-colored halos near colony on CAS overlay plates were selected and purified (Figure 1). All the isolates were designated as M1-M11 and stored on nutrient agar slant at 4 °C until the use.

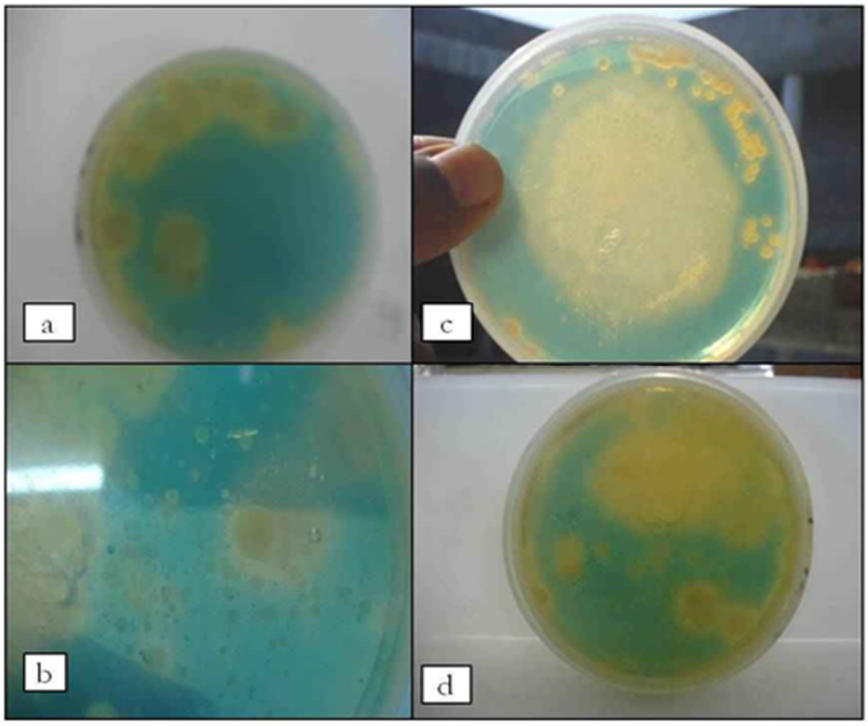

Figure 1. CAS overlay method. Figure a-d shows zone in CAS overlayer by different soil sample

All these isolates were further screened for siderophore production by CAS plate and CAS diffusion assays. However, only 7 isolates have shown a yellow to orange color zone on CAS agar (Table 1). On the CAS diffusion agar plate, six isolates showed a zone near well (Table 1). Among all isolates, M10 showed a maximum zone of 5 mm diameter, followed by M11 with 3 mm diameter. The rest of the isolates showed a 2 mm diameter.

Table 1. Isolates showed positive for siderophore on CAS agar and their response to the $FeCl_3$ test

Isolates	CAS Overlay	CAS plate	CAS diffusion (mm)	Color with $FeCl_3$	$FeCl_3$ test
M1	+ Ve	- Ve	-	Yellow	NA
M2	+ Ve	+ Ve	2	Orange	451
M3	+ Ve	- Ve	-	Yellow	NA
M4	+ Ve	+ Ve	2	Orange	450
M5	+ Ve	- Ve	-	Yellow	NA
M6	+ Ve	+ Ve	-	Orange	451.4
M7	+ Ve	+ Ve	2	Orange	451.6
M8	+ Ve	+ Ve	2	Orange	434.8
M9	+ Ve	- Ve	-	Yellow	NA
M10	+ Ve	+ Ve	5	Red wine	449
M11	+ Ve	+ Ve	3	Red wine	449.2

Identification

Isolate M10 was a potential siderophore producer as it showed a 5 mm diameter on CAS diffusion and red wine color with the $FeCl_3$ test (typical of siderophore). Isolate M10 was identified by various physiological and biochemical tests. Morphological examination by Gram's staining, spore staining, motility, and flagella staining revealed that the isolate is Gram-negative, non-spore former, motile, and flagellated. Results for various biochemical tests were: oxidase-positive, nitrogenase negative, growth on 12-15% NaCl negative, the requirement of cysteine and iron salts for growth negative, arginine dihydrolase positive, gelatin liquefication positive, citrate utilization positive, O-F test positive, intracellular polyhydroxybutyrate accumulation positive, growth on cetrimide agar positive. These results identify the isolate as belonging to the family Pseudomonadaceae and the genus *Pseudomonas*.

Characterization of siderophore

Siderophores were characterized based on the group which binds to the Fe+3 and form a ferric siderophore complex. With the addition of $FeCl_3$, the siderophore formed a complex and gave orange to red wine colour, indicating the presence of siderophores (Figure 2). If the siderophore is absent in the medium, it will not form a ferri-siderophore complex, and no colour change was observed. Out of the 11 isolated bacteria, M7, M6, M2, M8, and M4 form orange colour with $FeCl_3$ and shows λmax between 400-450 nm indicating the hydroxamate type siderophores (Table 1). MG10 and MT11 gave red wine colour. The rest of the organisms didn't form the ferri-siderophore complex; hence, no color change was observed. Moreover, the λmax of the rest of the isolates was not in the range of 400-450 nm indicating the absence of siderophore.

The tetrazolium test further confirmed the hydroxamate nature. The isolate showed a positive $FeCl_3$ test that gave instant red colour on the addition of tetrazolium salt (Figure 2). Here, isolates confirm their hydroxamate nature by providing instant red colour with tetrazolium salt. Spectrophotometric assay for catechol type of siderophore gave negative results and didn't show λmax at 495 nm, indicating the absence of catechol siderophore. The absence of catechol nature was further confirmed by Arnow's test, which showed a negative result.

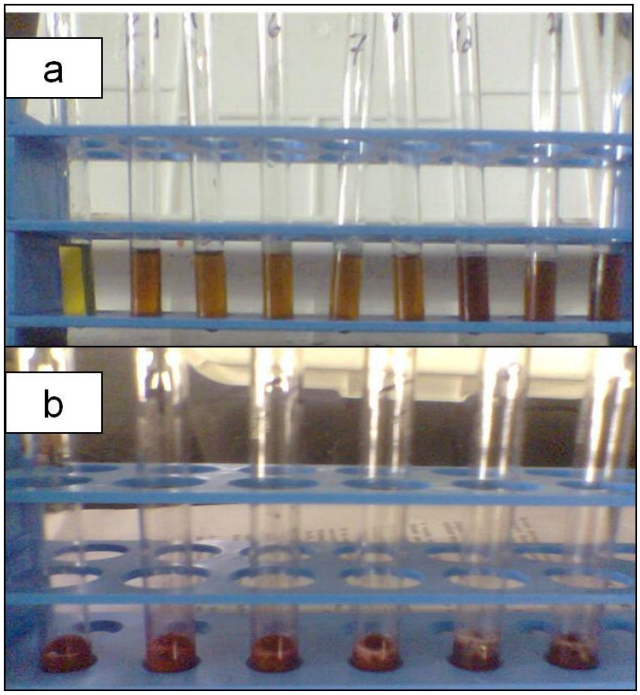

Figure 2. Colour change by the siderophore producing isolates in $FeCl_3$ and tetrazolium test

The temporal sequence of siderophores production

Siderophore production and growth were monitored for up to eight days at 24 hrs intervals. Maximum growth was achieved after 24 hrs of incubation, decreasing and again increasing on the 3rd day (Figure 3).

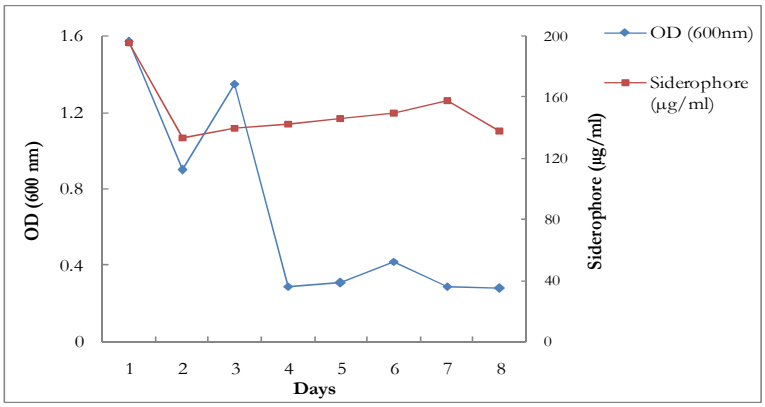

Figure 3. Temporal effect on growth and siderophore production

Siderophore production was also maximum after 24 hrs of incubation and decreased after that. Thus, growth and siderophore production both go hand in hand and decrease after 48 hrs of incubation.

Cross utilization

Both siderophore producers and non-producers were examined for iron uptake by external siderophores. $FeCl_3$ was served as a positive control, and normal saline was administered as a negative control. All the organisms grow near $FeSO_4$, dessferral, and siderophore extracted from *Pseudomonas*, suggesting using external siderophore for their growth. Non-siderophore producers were also grown using external siderophore provided in media, indicating their ability for cross-utilization.

Antifungal activity

Fusarium oxysporum f. sp. *cubense* and *F. oxysporum* f. sp. *ciceris* both were inhibited by the siderophore. *F. oxysporum* f. sp. *ciceris* produced wilt in chickpea, while *F. oxysporum* f. sp. *cubense* produced wilt in the banana plant. The growth of both the fungi was inhibited by the bacterial cell (Figure 4).

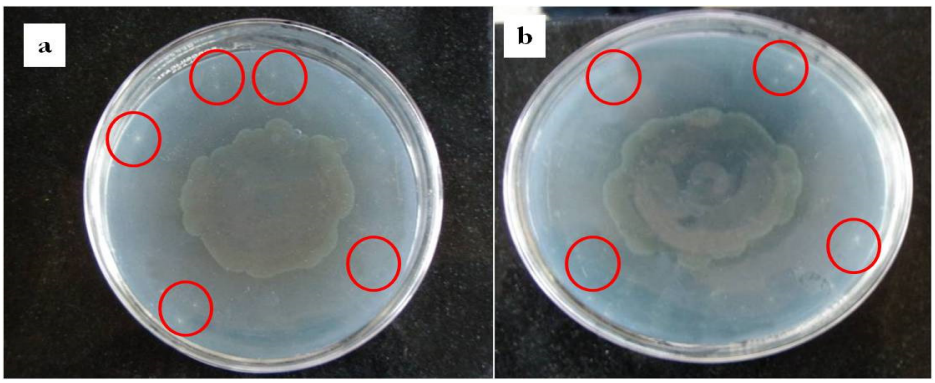

Figure 4. Antagonistic activity against *Fusarium oxysporum* f. sp. *cubense* (a) and *F. oxysporum* f. sp. *ciceris* (b)

FT-IR analysis

FT-IR analysis of the siderophore extracted from isolate and standard desferal was done using KBr as a reference blank. Hydroxamate nature was confirmed by N-H, C-H, C=O, and O-H stretching. Amine stretch was observed at 164 and 1568, C=O at 1760-1670, N-O bond between 1660-1500, and 1390-1260. C-H stretching was observed at 1340-1020. The presence of the above-mentioned group confirms the hydroxamate nature of siderophore (Figure 5).

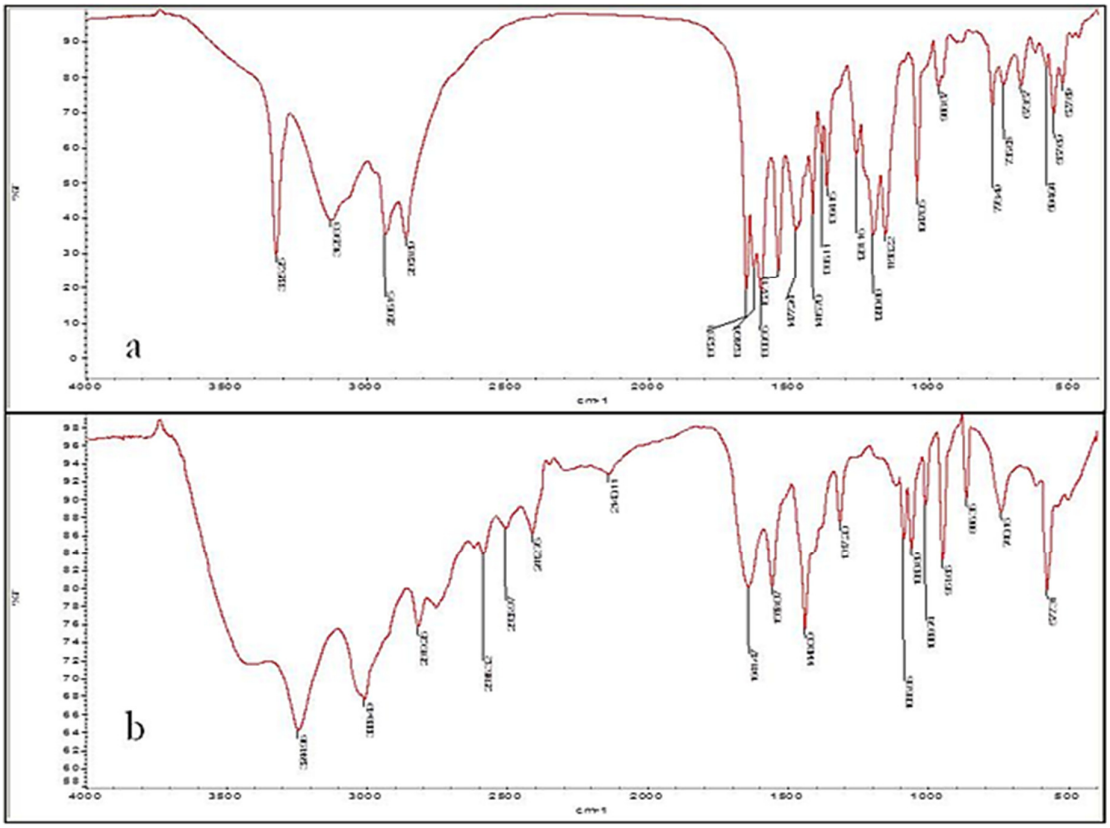

Figure 5. FTIR spectra of standard desferal (a) and siderophore extracted from *Pseudomonas* sp. MT

Particle size analysis

The particle size of the siderophore was analysed by a particle size analyser, indicating that the siderophore produced by *Pseudomonas* has 91.36 nm while the standard desferal has 77.55 nm at 25 °C temperature. Thus, it shows that siderophore produced by Pseudomonas sp. MT was larger compared to standard desferal (Figure 6).

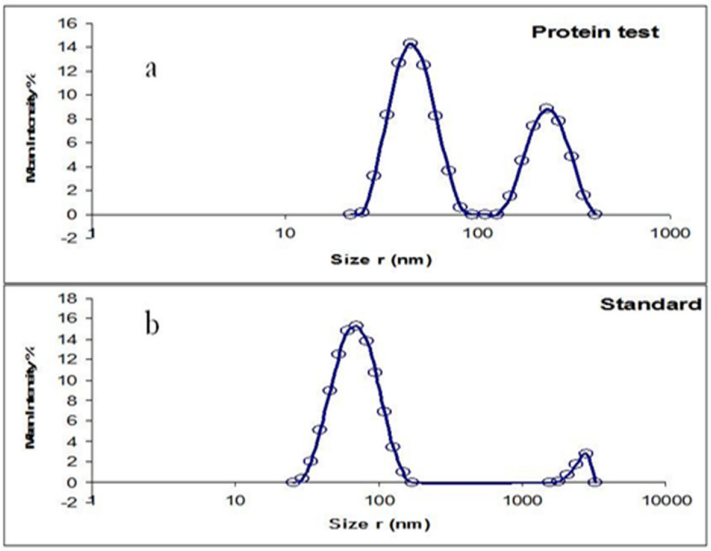

Figure 6. The particle size of standard desferal (a) and extracted siderophore from *Pseudomonas* sp MT

Discussion

Instead of chemical practice, people prefer a biological approach as fertilizer, and controlling insect/pests in the agricultural field to improve yields and quality of agricultural produce is extremely important for developing countries (Cohen *et al*., 1998). There has been an increasing interest in applying biocontrol agents instead of chemical pesticides. Microorganisms produce siderophores, a biomolecule that suppresses phytopathogenic microbes in the soil. Iron sequestration by siderophore producing microbes indirectly limits the growth of pathogens (Champomier-Veges *et al*., 1996; Chiriani *et al*., 1993). Hence, many fluorescent and non-fluorescent pseudomonas that produces siderophore can be used to substitute chemical pesticides as they reduce phytopathogens (Buysens *et al*., 1996). In the present study, bacterium isolate was identified as a species belonging to the Pseudomonadaceae family, and it showed maximum similarity with Pseudomonas sp.

Verma *et al*. (2019) reported that in *Pseudomonas aeruginosa*, siderophore production was increased with time in the presence of cadmium for up to 6 days; after that, siderophore production was reduced. Their finding suggests that isolate can produce siderophore for a more extended incubation period. In the present study, the isolate was identified by the biochemical test.

Parveen and Latha (2019) also identified isolated bacteria morphologically and biochemically, and their results revealed that the isolate was Pseudomonas sp. Pseudomonas sp. produces hydroxamate and carboxylate type of siderophore, as reported by Parveen and Latha (2019). They have also reported other plant growth-promoting activities by isolate and found that isolate has the potential for plant growth promotion.

In the present study, FTIR analysis was carried out to confirm the chemical nature of siderophore. Various peaks confirm the hydroxamate nature. A similar study was carried out by Verma and a co-worker (2019) to characterize siderophores using FTIR analysis. Their spectrum peak revealed that siderophore possessed a hydroxamate functional group. Their data corroborate with our data and confirm hydroxamate type siderophore.

Cross utilization

Both siderophore producers and non-producers were examined for the uptake of iron by external siderophores. $FeCl_3$ was served as positive control and normal saline was served as a negative control. All the organisms grow near $FeSO_4$, dessferral, and siderophore extracted from *Pseudomonas* indicating the use of

external siderophore for their growth. Siderophore non-producers were also grown using external siderophore provided in media.

Antagonistic activity

Microbial interactions play a vital role in the ecological niche and are predominantly cooperative or competitive (Schiessl *et al*., 2017). Siderophore is one such molecule secreted by microbes for chelating iron and competing for its availability for other microbes. Thus, indirectly siderophore producers suppress the growth of deleterious rhizobacteria in the soil rhizosphere and thus protect plants. Vadnerker *et al*. (2018) have isolated *Pseudomonas aeruginosa* AP from Dandi, Gujarat, examining their plant growth-promoting activities and siderophore production. They have reported 84.6% siderophore production after three days of incubation.

Lurthy and co-workers (2020) have examined the impact of three ferropyoverdines in two pea cultivars for ion and ionome for their tolerance to iron deficiency chlorosis. They have reported that pyoverdine-producing pseudomonads possibly contributed to tolerance to iron deficiency chlorosis between pea cultivars

Ghazy and El-Nahrawy (2021) reported that six isolates viz., *Bacillus subtilis, B. circulance, B. coagulanse, B. licheniformis, Pseudomonas fluroscence* and *P. koreensis* produce siderophore. Isolates showed antagonistic activity against *Cephalosporium maydis*. Thus, their results support present findings that siderophore has the potential to inhibit specific plant pathogens.

In present study, particle size of standard siderophore and siderophore extracted from isolated bacterium was measured. Data revealed that siderophore produced by isolated bacterium is little bit larger (91.36 nm) than the standard desferal (77.55 nm). However, size of the siderophore produced by isolated bacterium fall under nanoparticles size making it more easily accessible for transport by plant roots.

Conclusions

Eleven siderophore-producing bacteria were isolated from different tobacco, wheat, and garden soil rhizosphere soil samples. Among them, a potential siderophore producer was selected and identified as Pseudomonas. Siderophore typing revealed that isolate produced hydroxamate type siderophore, which was further confirmed by the presence of N-H, C-H, C=O, and O-H stretching in FT-IR analysis. Antagonistic activity against plant pathogens, i.e., *Fusarium oxysporum* f. sp. *cubense* and *F. oxysporum* f. sp. *ciceris*, inhibited plant pathogenic fungi. The particle size of crude siderophore produced by Pseudomonas was 91.36 nm as analysed by a particle size analyzer. Thus, present study revealed that siderophore produced by Pseudomonas could be effectively used to control the wilt of banana and plant disease. However, field research is required before its application as an antagonistic agent.

Authors' Contributions

Conceptualization and design of experiments (TKV); Methodology (MA); data collection, analysis, and interpretation (MA); Supervision (TKV); Writing - original draft (TKV); Writing – review, and editing (TKV). Both authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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