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Screening and Characterization of Several siderophore Producing Bacteria as Plant Growth-Promoters and Biocontrolling agents

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Abstract

86 bacterial strains belonging to *Pseudomonas*, *Enterobacter*, *Stenotrophomonas* and *Bacillus* from sunflower roots and associated regions were tested for the Siderophore production. The Siderophore producing ability of rhizobacteria belonging to the genus *Pseudomonas*, *Azotobacter* and *Bacillus* were detected in culture supernatants in MM9 and modified King's Medium by the universal CAS assay. *P. aeruginosa*, *P. flourescens*, *Stenotrophomonas*isolated from sunflower rhizosphere were able to produce Siderophores was well documented by the CAS assays. Methods like CAS assay, $FeCl_3$ test, Arnows test, TTC test, were used. In the CAS test, *Pseudomonas spp.* and *Enterobacter spp.* showed orange zone of decolourization whereas others produced light yellow colourization and were positive for siderophores. Several of the bacteria were possessing Hydroxamate, Catecholate and Carboxylate type siderophores. The role of these rhizobacteria against several of the plant pathogenic fungi was also investigated. These bacteria also inhibited the growth of such plant pathogenic fungi. These rhizobacteria were also able to increase seedling emergence and also promoted plant growth during early stages.

Key-Words: Siderophore, Biocontrol, rhizobacteria, CAS assay, Hydroxamate

Introduction

Iron is an essential element and the second most abundant metal after Aluminium in the earth's crust.At low iron concentration many aerobic and facultatively anaerobic bacteria are known to produce iron chelators, Siderophores for ensuring their iron availability. Siderophores are non-porphyrin, non-protein compounds that bind iron and their synthesis is repressed when this element is abundant Neilands (1995).Although iron accounts for about 4% of the total content of minerals in the earth crust, under aerobic conditions or in alkaline or neutral environment it occurs in the form that is refractory to solubilisation which makes it little available to microorganisms. Mainly, Siderophores belong to the category of Hydroxamates or Catechols.

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General methods for Siderophore determination include chemical or microbiological. Hydroxamate and Catecholate types of Siderophores can be measured by chemical methods. In general most assays for Siderophore detection are qualitative. Most common is the universal assay of Schwyn and Neilands. The assay is based on competitive exchange of iron (III) from an indicator dye, chrome azurol S (CAS). The affinity of CAS for iron (III) seems to be slightly lower than that of most Siderophores and hence the metal ion is quantitatively released to a competing ligand.

Over a past few decades producers became dependent on agro chemicals as a reliable method of crop production. Increasing use of such chemical inputs cause severe negative effects. Biological control thus is being considered as an alternative or supplementary way of reducing the use of chemicals in agriculture in a developing country like India. Plant associated bacteria act as agents for stimulating plant growth. *Pseudomonas* species have been known for their Siderophore production for many years with many reports been published. Pathogenic microorganisms affecting plant health are a major and chronic threat to

food production and eco system. Several fluorescent Siderophores have also been used for biological control in various experiments related to the soil system. The well studied are the Plant Growth-Promoting Rhizobacteria (PGPR) closely associated with the root surface and the adhering soil. Some PGPR also enter root and establish endophytic populations.

The aim of this study was to detect the Siderophores in the rhizosphere bacteria, their ability to inhibit several plant pathogenic fungi and thus their use for plant growth promotion as a biocontrol agent.

Material and Methods

Sample collection

Samples were collected and studies were carried out during year 2009-2011 from sunflower field situated in Mankana village in KamrejTaluka, Dist. Surat in the southern part of Gujarat (Latitude 21°10'and Longitude 72°55'). In the present study the samples considered were Non-Rhizosphere soil (Bulk Soil) that detaches from the root when the plant is shaken, Rhizospheric soil (fraction of soil that remains attached to the root) and along with these, rhizoplane and endorhizosphere region samples associated with *Helianthus annuus* plant roots were also collected and processed. They were then analyzed by plating them on various media.

Characterisation of isolates

The bacteria obtained from these samples were first identified on the basis of their morphological, colonial and biochemical reactions. Later those promoting plant growth and possessing biocontrolling ability were confirmed by molecular characters.

Detection of microbial Siderophores

Several qualitative and quantitative methods were used. Iron decontamination was carried by soaking all glasswares overnight in HCl (6M) and rinsed with distilled water several times to remove traces of iron.

Qualitative detection of Siderophores by plate assay

The chrome azurolsulfonate (CAS) assay – [universal assay - Schwyn&Neilands] was used since it is comprehensive, exceptionally responsive, and most convenient. The chrome azurolsulfonate assay agar was used. For the qualitative assay cultures were spot inoculated onto the blue agar and incubated at 28°C for 3-5 days The results were interpreted based on the colour change due to transfer of the ferric ion from its intense blue complex to the Siderophore. The sizes of yellow-orange haloes around the growth indicated total Siderophore activity. The result was scored either negative or positive.

Quantitative assay

For the quantitative estimation of Siderophores, the cultures were inoculated onto Trypticase soy broth (TSB), Succinic medium (SA). The tubes with the cultures were then incubated at 28°C for 24-72 h with constant shaking at 120 rpm. After incubation, the culture broths were centrifuged in a centrifuge at 10,000 rpm for 10 min. The cell free supernatants were subjected to quantitative estimation of Siderophores by CAS-shuttle assay (Payne 1994). In this assay, 0.5 ml of CAS reagent and 0.5 ml of supernatant were taken against uninoculated broth as reference. Spectrometric readings were taken and percentdecolorisation was calculated by the following formula (Rane et al. 2005).

% decolorization = Ar-As/Ar x 100 where, Ar = absorbance of reference at 264 nm (CAS reagent) and As = absorbance of sample at 264 nm.

Further, chemical nature of Siderophores produced was confirmed by TTC test (Snow 1954) for hydroxamate nature, Arnow's test for catecholate nature and for carboxylate naturePhenolphthalien test.(Vogel's chemical test, 1992) was performed.

Deection of hydroxamate Siderophores

$FeCl_3$ test- The formation of orange coloured ferric hydroxamates, showing maxima at 420-450 nm indicated presence of hydroxamatesiderophores.

Tetrazolium test- This test is based on the capacity of hydroxamic acids to reduce tetrazolium salt by hydrolysis of hydroxamate group in presence of strong alkali. Instant appearance of a deep red colour on addition of tetrazolium salt and NaOH to the test sample indicated presence of a hydroxamate Siderophore.

Detection of catecholate Siderophores

Arnow's test- For detection of catecholates, 1ml culture supernatant was mixed after each orderly addition, 1ml HCl followed by 1ml of nitrite-molybdate (catechols produced yellow colour) & then 1ml NaOH was added. (pinkchromogen forms) that absorbs maximally at 515nm. According to a study catechol-type of Siderophores produce purple or blue colour whereas hydroxamate-type of Siderophore produces orange colour on CAS media. (Milagres et al1999, Arnows 1937.)

Detecton of Carboxylate Siderophores

Carboxylate nature was determined by thedisappearance of pink colour on the addition ofphenolphthalein to Siderophore sample.

Evaluation of Antifungal activity of the bacteria

To assess the ability of the isolates to inhibit fungi, each isolate was tested against four different fungi with the circle method (Da Luz, 1990). The bacterial isolates were seeded in a 5 cm diameter circle on a PDA plate. After 24 h at room temperature, a 5 mm plug of each fungus was placed on the plates. And the plates were incubated at 28°C for 3-6 days. Fungal

growth inhibition was assessed by measuring the mycelial radial growth (Cattelan, 1999).

Effect of bacterization on seed germination and plant growth:Seed germination in plates

In the experiments, seeds of sunflower were wetted in 20 M ethanol for 5 seconds and surface sterilized in 0.21 M NaOCl for 5 min. The seeds were washed once in sterile distilled water and soaked for 10 minutes in 0.01 M HCl (to remove traces of NaOCl) and washed 5 times in sterile distilled water to remove traces of HCl. All the bacterial isolates were grown on 0.1 x TSB for 24 to 48 hr, agitated on a rotary shaker (120 rpm, 24°C) and before harvest, centrifuged. The pellets were collected and suspensions prepared in 0.1 M $MgSO_4$ to give an absorbance of 0.1 at 620 nm. These suspensions were used for the bacterization. The sunflower seeds were bacterized with the selected test strains by soaking 10 seeds in the above prepared bacterial suspensions for 30 minutes. Control seeds were treated with 0.1 M $MgSO_4$ only. After treatment the seeds were placed, 10 seeds per plate in 9 cm diameter glass petri plates lined with 2 filter papers, moistened with sterile de-ionized water (SDW) to test for germination. Petri plates were covered and incubated in the dark at ambient temperature. SDW was added to the plates to provide moisture for germination when necessary. Germination was recorded at every 24 hr (Wocoma 2008; Egamberdiyeva 2007).

Results and Discussion

The bacteria were characterized by morphological, physiological, biochemical reactions. Later several of the bacteria possessing plant growth promoting abilities were analysed by 16S rRNA gene analysis.

Analysis of sequencing result:

Comparative analysis of the sequences with already available database showed that the species were close members of the genera *Pseudomonas, Enterobacter, Stenotrophomonas, Microbacterium*and*Bacillus*spp. The obtained sequences were subjected to BLAST (Basic Local Alignment Research tool) analysis with online tool with the preexisting sequences available in NCBI/Genbank to confirm the sequences. The obtained 16S ribosomal RNA sequences of strains were aligned with various rhizosphere bacterial species obtained from GenBank, NCBI. Widely, the biochemical test of identification is found to be preliminary identification in which the genus will be identified and species can be identified to a certain extent. Whereas the molecular identifications through 16S ribosomal RNA coding sequence analysis is widely accepted. BLAST hit results showed that strain M1R2 gave 99% similarity with *Pseudomonas flourescens.* Bacterial isolates were identified based on percentage of sequence similarity with that of a prototype strain sequence in the GenBank.

The partial 16S rDNA sequences were submitted in the Gen Bank under accession numbers KF 561998 - 562005.Several bacteria possessing some other growth promoting activities were then analysed further for Siderophore producing abilities.

Siderophores by CAS plate assay showed that 37 % of bacteria were positive and mainly belonged to Enterobacter, Pseudomonas and Bacillus.Table 1.The results of orange halo around the colonies are shown in.Figure1.Broth assay showed 73% of the strains were positive.Figure:2 shows the decolourization in broths. *Pseudomonas* spp. (M1R2), *Enterobacter* spp. (M6S3),*Microbacterium*(M7ER1, M7ER2) spp. gave more than 50% decolorization of the supernatants in the broth CAS assay when grown in Tryptic soya broth. Table 2. The bacteria grown in Succinic medium resulted in slow decolourization of supernatant and only 30% were positive in the medium. According to a study catechol-type of Siderophores produce purple or blue colour whereas hydroxamate-type of Siderophore produces orange colour on CAS media. (Milagres et al 1999).

Hydroxamate Siderophores were produced by 43% of the strains. Among them bacteria belonging to *Bacillus*, *Stenotrophomonas* and *Enterobacter* recorded the presence of Hydroxamate Siderophores. Whereas Catecholate and Carboxylate nature of Siderophores were shown by 27% of bacteria. Bacillus species produced Catecholate type Siderophores whereas Stenotrophomonas and Pseudomonas were showing Carboxylate type and Enterobacter species produced both types. Table: 3

All the isolates had antagonistic activity against the tested phytopathogenic fungi.*Pseudomonas fluorescens*,*Pseudomonas aeruginosa*, *Enterobactersakazakii*and *Bacillus* speciesgave good antifungal activity against the tested fungi *Fusarium*, *Aspergillus*, *Curvularia*and*Helminthosporium* species. Table:4 shows the selected bacterial isolates and their antifungal nature.

Seed germination observed in plates showed that Vigorindex(VI) was the highest in *Pseudomonas fluorescens* isolated from rhizosphere i.e. 1480. Secondly *S. maltophilia* gave a VI of 1420, followed by *Microbacterium* species of 1292, *Bacillus* species was 1120, *Enterobactersakazakii* gave an index of 840 and *Pseudomonas aeruginosa* at 582. PGPR in addition to stimulating plant growth and nutrient uptake; also enhanced the number and length of root hairs was also shown in our study (Bertrand et al., 2000) Table

5.Results of seed bacterization on growth parameters are as shown in figure3.

The present investigation confirms the earlier studies. It revealed that under *in vitro* conditions, seed treatment with PGPR strains improved seed germination, seedling vigor, seedling emergence over the control (Nezarat and Gholami, 2009). Similar improvement of seed germination parameters by rhizobacteria has also been reported in other cereals such as sorghum and pearl millet (Niranjan et al., 2004). Our study also is in synergy with the above researchers where all the isolates showed an increase in root length as compared to control.

Our results are in accordance with Manwar et al. (2004) who demonstrated siderophore based maximum inhibition by *Pseudomonas* species against *Aspergillusniger*. Similarly Jagadeesh et al. (2001) reported the role of fluorescent Siderophores in the biological control of plant pathogens. Reports suggest that *Pseudomonas fluorescens* and *Bacillus subtilis* and their siderophore complex help in controlling Alternaria disease and Sclerotinia infection (Sadi and Masoud 2012) in *Helianthus annuus*. The Siderophore mediated antagonism of rhizoplane isolates of *Pseudomonas* from chilli, cotton, groundnut and soybean against species of *Aspergillus, Fusarium, Mucor, Penicillium, Pythiumaphanidermatum, Rhizopusoryzae, Rhizoctoniasolani, Sclerotiumrolfsii*and *Sclerosporagraminicola*has been reported by Yeole, Dave and Dube (2001).

Two different strains of *Bacillus* species and *Pseudomonasaeruginosa* species have shown to effectively reduce sunflower necrosis virus disease more than the other treatments. Besides, they also effectively increased the seed germination, seedling vigor and other growth parameters in sunflower plant (Srinivasana, Krishnaraj and Mathivanan, 2009). Seed bacterization would be an optimal method of inoculation for immediate root colonization by the PGPR strains. Seed bacterization by biocontrol strains would provide a barrier against infection by soil-borne phytopathogens.

Reports by Zahir et al. (2008) showed that *Pseudomonas fluorescens* was the most effective in increasing root length; shoot length, fresh weight of pea plants. *Enterobacter* species is known to be good colonizers of plants and have been used to promote plant growth (Gyaneshwar et al., 1999). Ambroisini et al. (2012) reported the species of *Enterobacter* associated with sunflower roots and rhizosphere capable of producing phytohormone and siderophores which promoted plant growth. *Enterobactersakazakii* was also recently reported by Fernando et al. (2012) which has phytohormone, phosphate solubilizing and siderophore producing abilities and thus can promote growth of sunflower plant. Seed bacterization with *Pseudomonas fluorescens* UTPF61 as reported by Sadi and Masoud (2012) showed that the strain can be used for sunflower plant growth promotion as well as biocontrolling agent for soilborne pathogens. Sadaghiani (2008) studied the effect of Siderophore producing Pseudomonads on the growth of wheat and reported the positive effects of seed bacterization by *P. aeruginosa*. This positive effect may be attributed to their plant growth-promoting properties like IAA, Siderophore and phosphate solubilization which is in accordance with our studies. The occurrence of *Bacillus* species in the rhizosphere soil has been reported by various workers (Wipat and Harwood, 1999; Garbeva et al., 2003; Chakraborty et al., 2005*Stenotrophomonasmaltophilia* isolated from rhizospheres of wheat, oat, cucumber, maize, oilseed rape and potato showed plant growth promoting activity and antagonistic properties (Berg et al. 1994; Debette and Blondeau 1980; Hauer 1999; Lambert and Joos 1989). *Microbacterium* species when used as inoculants in apple plants by various PGP mechanisms like nitrogen fixation and phosphate solubilization helped in plant growth promotion was reported by Karlidag et al. (2007). Our study also shows the plant growth promotion using such organisms. *Microbacterium* species possessing Siderophores and nitrogen fixing abilities associated with rice rhizospheres can also be exploited as Biofertilizers as suggested by Shahi et al. (2011). Cavalca et al. (2010) reported the bioremediation and Siderophore producing potentials of *Microbacterium* species associated with wild thistle (*Cirsiumarvense* L.). Ali et al. (2012); Abou-Shanab et al. (2006) and Waranusantigul et al. (2011) reported the bioremediation abilities of *Microbacterium* species associated with peas, yellowtuft (*Alyssum murale*) and eucalyptus respectively.

Conclusion:

The bacteria belonging to the rhizosphere and associated regions of *Helianthus annuus*, *Pseudomonas* and *Bacillus* species. possessing the different plant growth promoting mechanisms showed effective results in plate experiments along with Siderophore producing abilities. The *Bacillus* species possessed the highest antagonistic activity against the tested phytopathogenic fungi. The *Microbacterium*spp. *a*nd*Stenotrophomonasmaltophilia*spp. were also effective in increasing growth parameters and had good vigor index so could be used for plant growth promotion. A consortium of these organisms with

various formulations can be prepared to be used as biocontrolling agents in agriculture. As these bacteria are adapted to and associated with the rhizosphere of the sunflower they may provide effective plant growth promotion and can be used as adjuncts to agricultural and horticultural practices. The application of such organismsalong with other disease control strategies will augment the benefits to the plant in integrated disease management systems also.

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Table 1: Siderophore detection in selected bacterial cultures by CAS broth and plate assay

Test isolate	TSB	SA medium	CAS Plate Assay
M1R1	++	+	-
M1R2	++	+	+
M1ER1	+	+	-
M1ER2	-	-	
M1ER32	+	-	-
M2R3	-	-	-
M5S2	+	-	-
M5R1	+	+	-
M6S3	+	-	++
M6ER1	-	-	-
M7S1	++	+	++
M7S2	++	-	-
M7R1	++	++	+
M7R2	++	++	+
M7ER1	+	-	+
M7ER2	+	-	+
M8R3	+	+	+
M8ER3	+	-	+
M9R2	+	-	-
M9ER2	-	-	-
M10S1	++	-	+
M10S3	++	+	-
M10ER1	++	-	-
M10ER2	+	-	+
M11R2	-	-	+
M12R1	+	-	
M12R3	-	-	-
M12ER3	+	-	-

Figure 1: Observation of orange coloured zones around the bacterial colonies on CAS agar

Table 2: Percentage decolourization of supernatants by selected bacterial isolates in CAS assay

Test isolate	OD 264nm	Percentage
M1R1	2.9	-
M1R2	1.326	50
M6S3	1.278	52
M7S1	1.27	35
M7R1	1.448	45
M7ER1	1.306	51
M7ER2	1.257	53
M11R2	2.483	7
M12R1	2.961	-

Figure 2: Decolourization of culture supernatants in broth assay

Table 3: Detection of chemical nature of Siderophores

Test isolates	$FeCl_3$ test	TTC test	Arnows test	Phenolphthalein test
M1R1	-	+	-	-
M1R2	+	-	++	-
M1ER1	-	-	-	-
M1ER2	-	-	-	-
M1ER32	-	+	-	-
M2R3	-	-	-	-
M5S2	+	+	-	++
M5R1	+	+	-	+
M6S3	+	++	++	+
M6ER1	+	+	-	-
M7S1	+	+	+	+
M7S2	+	-	-	+
M7R1	-	+	++	+
M7R2	-	+	++	+
M7ER1	+	+	-	+
M7ER2	+	+	-	-
M8R3	+	+	+	+
M8ER3	+	-	-	-
M9R2	-	-	-	-
M9ER2	-	+	+	-
M10S1	+	-	-	-
M10S3	-	-	-	-
M10ER1	+	-	-	-
M10ER2	+	-	+	-
M11R2	+	-	+	-
M12R1	+	+	+	-
M12R3	+	-	-	-
M12ER3	-	-	-	-

Table 4: Antifungal activity of the selected bacterial isolates against plant pathogenic fungi (Radial diameter in mm)

Test isolates	control	*Fus.spp.*	control	*Asp.spp.*	Control	*Curv.spp.*	Control	*Hel.spp.*
Pseudomonas spp.	15	6	25	16	18	15	16	10
Pseudomonas spp.	15	5	25	10	18	5	16	5
Enterobacter spp.	15	5	25	5	18	5	16	5
Stenotrophomonas spp.	22	15	20	15	18	10	20	10
*Pseudomonas*spp.	22	8	20	5	18	5	20	5
*Microbacterium*spp.	22	8	20	10	18	8	20	12
*Microbacterium*spp.	22	8	20	20	18	10	20	15
Bacillus spp.	12	5	40	10	25	7	25	5
Bacillus spp.	12	6	40	12	25	6	25	6

Table 5: Influence of bacterial isolate inoculation on sunflower germination and growth in plate experiments

No. given	Bacterial species	Root length	Shoot length	Vigor index	Germination%
C	control	5.6	3.1	609	70%
M1R2	*Pseudomonas* spp.	9.6	5.2	1480	100%
M7R1	*Pseudomonas* spp.	4.2	5.5	582	60%
M6S3	*Enterobacter* spp.	5.4	6.6	840	70%
M7S1	*Stenotrophomonas* spp.	9	5.2	1420	100%
M7ER1	*Microbacterium* spp.	9.9	5.3	1292	85%
M7ER2	*Microbacterium* spp.	6	3.1	728	80%
M11R2	*Bacillus* species	7.8	6.2	1120	80%
M12R1	*Bacillus* spp.	10.6	3.5	930.6	66%

Figure 3: Results of bacterization experiments and observation of various growth parameters

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