



## BIO PROSPECTING OF *PSEUDOMONAS AERUGINOSA* FOR THEIR POTENTIAL TO PRODUCE SIDEROPHORE, PROCESS OPTIMIZATION AND EVALUATION OF ITS BIOACTIVITY

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**Abstract:** Siderophores produced by *Pseudomonas spp.* play a vital role in controlling several plant diseases. In this study, two siderophore producing bacterial strains were isolated from waste water sample and labeled them as RM1 and RM2. Among these two isolates, RM1 strain was found to produce more siderophore and therefore selected for further study. 16s rRNA analysis and biochemical characterization as described in the Bergey's manual of determinative bacteriology, identified the RM1 strain as *Pseudomonas aeruginosa* 6A (bc4). Siderophore production was determined by more sensitive and reliable Chrome azurol-S (CAS) method. Chemical and spectrophotometric assays showed that *P. aeruginosa* 6A strain produced 99% siderophore units. Two siderophores, hydroxamate (Pyoverdine) and phenol catechololate (pyochelin) were mainly found to be produced by this strain. Different parameters such as temperature, pH, carbon and nitrogen sources were optimized for maximum production of siderophores. Maximum production (95.65%) was observed on succinate medium, pH 7.0 and at 40°C temperature. Several amino acids were used as carbon and nitrogen sources. Among them serine resulted in a maximum production of siderophore (99.80% unit). The *Pseudomonas spp.* were also tested as seed inoculants and found to be very effective in seed germination and plant growth promotion of *Glycine max* JS-335 plant under pot culture conditions. It also exhibits antifungal activity against *Aspergillus spp.* In summary, the present study has identified a novel siderophore producing bacterial strain, which can also be used as an antifungal agent.

**Key Words:** *Pseudomonas aeruginosa*, Siderophore, Process optimization, Chrome azurol-S, Antimicrobial activity of Siderophore

### INTRODUCTION

Siderophores are low molecular weight iron chelating compounds secreted under iron stress conditions (Suryalala D. et al., 2004). Chelators acquire useable iron by forming multiple bonds with the metal ions. The main function of siderophores is to scavenge iron from the environment and make them available to the living cells for their various metabolic processes. In many soils, the presence of micro quantity of iron limits the efficient uptake and utilization of iron by plants. Under such condition, siderophores produced by rhizosphere bacteria like *Pseudomonas fluorescence*, *Pseudomonas putida* and *Methylobacterium spp.* (Bholay et al., 2012, Lacava et al., 2008), increase the bioavailability of iron near the root and thus supports the plant growth. Iron is essential to plants because it is required in many anabolic activities such as for synthesis of metallo enzymes, for the synthesis of chlorophylls which basically required for photosynthesis.

Bacteria require iron for survival and growth (Andrews et al., 1999 and Conrad et al., 2000, Gao et al., 2006). Prokaryotes have evolved a variety of mechanisms to scavenge the insoluble form of iron Fe<sup>3+</sup>. One such mechanism is the production of siderophores, which chelate iron with a high affinity (Guerinot, 1994). Siderophores are secreted into the

extracellular environment where it binds to and transport iron through interactions with specific receptors. More than 500 siderophores have been identified in different plants and bacteria (Ratledge & Dover, 2000; Wandersman & Delepelaire, 2004). Acquisition of iron from siderophores produced by many other bacteria has already been described like *Escherichia coli*, *Salmonella typhimurium* (Martinez et al., 1990) *Actinobacillus pleuropneumoniae* (Diarra et al., 1996) *Streptomyces spp* (Imbert et al., 1995) *Arthrobacter flavescence* (Winkelmann, 1991). Siderophores are thought to facilitate biocontrol by sequestering iron from pathogens, thus limiting their growth (Champomier et al., 1999, Chiriam et al., Sullivan et al., 1992).

Interestingly, most of the siderophores exhibit quite conserved structure, consisting a functional unit that ligate with iron molecules (transferrins and lactoferrins) and a peptide backbone interacting with a receptor present on the bacterial outer membrane. An important property of siderophores is their specificity. The siderophore receptor of a bacterium is usually recognized by the peptidic chain of a single siderophore (Hohnadel & Meyer, 1988, Cornelis et al., 1989, de Chial et al., 2003, Spencer et al., 2003), although in exceptional cases some strains can

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recognize multiple siderophore types by expressing different receptors (Barelmann et al., 2002, Ghysels et al., 2004).

Previous work done on *Pseudomonas* and other bacteria found to produce siderophore units in the range of 60 to 80 percent that is either a hydroxymate or catecholate type. So search for such a novel strain that can give maximum siderophore units (more than 90%) and able to produce both the types of siderophores has become essential.

*Pseudomonas* sp. is ubiquitously present in agricultural soils and it has many desired traits that make them well suited as plant growth promoting rhizobacteria (PGPR). *Pseudomonas* spp. can grow very fast and shows antagonistic effect against phytopathogen. Recently Pandey et al., (2013) reported the implications of *Pseudomonas* strains as plant growth promoting endorhizospheric bacteria. Considerable research is underway globally to exploit the potential of one group of bacteria belonging to *Pseudomonas* spp. as PGPR. So far the most effective PGPR strain of *Pseudomonas* has been found is fluorescent *Pseudomonas* spp. This species plays vital role in stimulating plant growth and in controlling several plant diseases (Lemanceau P. et al., 1993). They function as a biocontrol agent by depriving the pathogen from iron nutrition, thus resulting in increased yield of crop. Growth and siderophore production by PGPR is attributed to organic acids, sugars, amino acids, minerals, enzymes and several other components of root exudates (Deelip C. et al., 1998, Nehl DB. et al., 1996).

*Pseudomonas* spp produces several types of siderophores, the main class of which is called pyoverdines (Cornelis & Matthijs, 2002). Of these there exist three distinct structural types: Type 1, 2 and 3. Each strain of *Pseudomonas* can produce only one of these types and possess the corresponding type-specific pyoverdine receptor (Cornelis et al., 1989). Moreover, considerable variation has been reported within each structural type (Smith et al., 2005, Bodilis et al., 2009).

Smith et al., (2005) has proposed a model on how selectivity can cause diversity at the pyoverdine receptor locus. The authors argued that siderophore diversity could be a defense against exploitation by non-siderophore-producing bacteria. If there are many non-siderophore producers, it would be beneficial for a mutant to produce a siderophore that is distinct in structure and then incompatible with the dominant population, which would create diversifying selection on the pyoverdine gene (Smith et al., 2005, Tummler & Cornelis, 2005). However this explanation assumes that

non-siderophore-producing strains are sufficiently abundant to drive this selection.

Chrome azurol S (CAS) is a method used for the detection of the mobilization of iron. Previously several studies have used CAS assay to demonstrate the siderophore production by various microorganisms (Alexzander et al., 1991, Neilands JB. 1995, Milagres et al., 1999). Moreover, in an ecological study CAS was used to detect the mobilization of iron by testing the presence of siderophore in soil samples collected from the study site. Thus, CAS can be used for the determination of micro quantity of siderophores present in ecological as well as medical samples.

The RM 1 Strain is found to be more advantageous than the others because it can produce highest percentage of siderophore as well as it promotes the plant growth very fast and can be used as a biocontrol agent.

The major findings of this study are maximum production (99%) of siderophore was observed on succinate medium, pH 7.0 and 40°C temperature. Several amino acids were used as carbon and nitrogen sources. Among them serine resulted in maximum production of siderophore (99.80% unit). The *Pseudomonas* spp. were also tested as seed inoculants and found to be very effective in seed germination and plant growth promotion of *Glycine max* JS-335 plant under pot culture conditions and also exhibits antifungal activity against *Aspergillus* spp. In summary, the present study has identified a novel siderophore producing bacterial strain, which can also be used as antifungal agent.

## MATERIALS AND METHODS

### Sample collection

For the isolation of organism, 5 different sewage samples were collected in plastic bottles, and were stored in a refrigerator until further use.

### Enrichment of bacterial culture

For the enrichment of microorganism, one ml of each of five samples were inoculated in nutrient broth and incubated at room temperature for 6 days.

### Isolation of microorganisms

After incubation, the enriched culture was serially diluted from  $10^{-1}$  to  $10^{-6}$  with sterile saline 0.1ml culture from  $10^{-5}$  dilution was spread on nutrient agar plates and incubated at room temperature for 24 h.

After incubation the plates were observed for the presence of greenish yellow colored colonies. Isolated colonies were restreaked on the same media

to get pure culture. The obtained isolates were labeled as RM 1 and RM 2. Morphological characteristics of each isolates were noted.

#### Biochemical characterization

The morphological and biochemical characterization of isolates were performed by using following tests

- I. **Gram Staining:** The Gram nature of the isolated organisms was determined by a standard Gram staining procedure.
- II. **Catalase Test:** 1ml of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution was taken in a test tube and the colony was immersed in to it and observed for effervescence.
- III. **Oxidase Test:** Filter paper strip dipped in NNNN'-tetramethyl paraphenylene diamine dihydrochloride (TMPD or DMPD) reagent was smeared with single colony and observed for color change. Formation of blue color within 10 seconds indicates positive test.
- IV. **Sugar Utilization test:** The isolates were checked for their ability to utilize various carbon sources by growing them in minimal medium containing 0.1% carbon source at room temperature for 24 h.
- V. **Citrate Utilization Test:** The isolates were checked for their ability to convert citrate into oxaloacetate. For this, isolates were inoculated in Simmon's citrate media and incubated at room temperature for 24 h. Change in colouration from green to bright blue indicates positive test.
- VI. **Casein Hydrolysis Test:** This test was conducted on milk agar, which is a complex media containing casein, peptone and beef extract. The isolates were streaked on milk agar plates, incubated at room temperature for 24 h and observed for zone of casein hydrolysis

#### Siderophore detection

Detection of siderophore was carried out by following two methods Siderophore production by the isolated strains of *Pseudomonas* were determined by Chromazurol S assay. The strains were spread over cetrimide agar and incubated for 48 h at 30°C. After incubation, a thin layer of CAS reagent in 0.7% agar was overlaid on the bacterial growth and plates were again incubated for 24 h at 30°C. Formation of yellow orange zone around the colonies indicate siderophore production.

#### Siderophore Characterization

##### Hydroxamate type of siderophore

**Tetrazolium test:** This test is based on the capacity of hydroxamate to reduce tetrazolium salt by hydrolysis of hydroxamate group using a strong alkali. The reduction and release of alkali show red color. To a pinch of tetrazolium salt, 1-2 drops of NaOH and 0.1 ml

of the test sample were added. Instant appearance of a deep red color indicated the presence of hydroxamate siderophore.

**Neilands Spectrophotometric Assay:** The hydroxamate nature of siderophore was detected by Neilands spectrophotometric assay, where a peak between 420-450nm on addition of 2% aqueous solution of FeCl<sub>3</sub> to 1ml of cell free supernatant indicated the presence of ferrate hydroxamate.

##### Catecholate type of siderophore

**Arnow's Test:** To 1 ml of cell free supernatant 1 ml of 0.5 N HCL and 1 ml of nitrite molybdate reagent were added. After the production of yellow color, 1 ml NaOH solution was added immediately, which resulted in the production of red color and the absorbance was measured at 510 nm using UV-Vis spectrophotometer.

##### Spectrophotometric Assay

Catecholate siderophore production was detected by spectrophotometric assay. The appearance of a peak at 495nm on addition of 2% aqueous solution of FeCl<sub>3</sub> to a 1ml of cell free-supernatant indicate the presence of catecholate siderophore.

##### Siderophore quantification

The quantitative estimation of siderophore produced by *Pseudomonas* was done by CAS-shuttle assay. In this assay the strains were grown on succinate medium containing of gm/l of K<sub>2</sub>HPO<sub>4</sub> (6.0), KH<sub>2</sub>PO<sub>4</sub> (3.0), MgSO<sub>4</sub> (0.2), (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> (1.0) and succinic acid (4.0), pH 7.0 and incubated for 24-30 h at 28°C at 120 rpm on a rotary shaking incubator. After incubation the fermented broth was centrifuged at 10,000 rpm at 4°C for 10 minutes. The cell free supernatant was mixed with 0.5 ml of CAS solution and incubated for 20min. The intensity of the color produced was determined by measuring the absorbance at 630nm using the spectrophotometer. The sample without cell culture supernatant was used as control. The percentage of siderophore units produced was estimated as the measure of proportion of CAS color shifted using the formula  $[(Ar-As)/Ar] \times 100$ , where Ar is absorbance of reference at 630 nm (CAS assay solution + uninoculated medium) and As is the absorbance of Sample at 630nm (CAS assay solution + cell supernatant).

##### Optimization of siderophore production

To optimize the production of siderophore, the effect of incubation time, pH and temperature were checked by incubating the bacteria in succinate medium. The percentage of siderophore produced was determined by the proportion of CAS color shifted using the formula mentioned.

To observe the effect of different amino acids on siderophore production, the bacteria were grown in succinate medium supplemented with alanine, arginine, lysine, serine and tyrosine at 40°C and 120 rpm. Sample is taken after 12 hours of incubation and the percentage of siderophore produced was estimated by CAS-shuttle assay as described.

#### Antimicrobial activity

**Antifungal activity of *Pseudomonas*:** The antifungal activity of *Pseudomonas* strains was tested against *Aspergillus flavus* NCIM-532, *Aspergillus fumigatus* NCIM-902, *Aspergillus niger* NCIM-621, *Candida albicans* NCIM-3471 obtained from National Chemical Laboratory (NCL) Pune, Maharashtra, India. Fungal cultures were maintained routinely on Sabouraud's dextrose agar. Fungal cultures were spread on the Potato Dextrose Agar (PDA) plates using sterile glass spreader. A standard cork borer of 8 mm diameter was used to make the wells on the PDA plates. 20 µl of bacterial suspension was then added in to each well. The plates were then incubated in refrigerator for 30 minutes for pre-diffusion of the bacterial culture followed by incubation at room temperature for 24 h. After incubation the plates were observed for the zone of inhibition.

#### Pot culture

**Preparation of soil samples:** The soil samples were collected from Baramati of Pune district, Maharashtra. The collected samples were air dried under sunlight. Visible roots and debris were removed from the soil and discarded. After air-drying, the soil aggregates were broken down by gently crushing them using a wooden hammer. Grounded samples were then pass through 2mm stainless still sieve. The sieved samples were mixed thoroughly for making the composite sample and preserved in plastic bags.

#### Pot preparation

Sterilized and air dried 5mm sieved soil samples amounting 2 kg were added in to each of the pot.

#### Sowing of seeds

Certified seeds of *Glycine max* were collected from the Baramati Agricultural Research Institute (BARI). 8 seeds of *Glycine max* were sown in each of the pots and allowed to germinate. After germination five seedlings were kept in each pot and allowed to grow. All the pots were arranged in the net-house in a completely randomized design.

#### Collection and processing of plant samples

The pots were watered twice daily. Positions of the pots were allowing equal exposure to Sunlight. Three weeks after germination the plant were

separated from the pot and dried over night at 105°C before determining the dry weight of whole plant. The plants were harvested manually by uprooting them carefully from the pots. The roots of the harvested plants were washed with tap water and then again with distilled water three times to remove ions from the root free space and observed for shoot length, root length and seed germination with respect to the control after each 20 days. Plants were grown in pots for three months under open natural condition at temperature 28°C to 30°C during the day and 20°C to 24°C during the night in summer.

## RESULTS AND DISCUSSIONS

#### Isolation of Microorganisms

The growth was observed on all five plates of nutrient agar. However only the greenish yellow colored colonies, which are peculiar characteristics of *Pseudomonas spp.* were selected as potential isolates. Two greenish yellow colored isolates were obtained from two different sewage samples. These isolates were labeled as RM1 and RM2.

#### Morphological and Biochemical Characteristics of Isolates

After growth on nutrient agar both isolates RM1 and RM2 formed greenish yellow colored colonies by producing a diffusible pigment. The biochemical characters were performed by using standard methods described in Bergey's Manual of Determinative Bacteriology. According to King EO. et al., (1954) *Pseudomonas aeruginosa* colonies appear green to bluish-green due to production of pyocyanin pigments. The results (Table 1) obtained with morphological and biochemical characteristics for RM1 and RM2 were compared with the characters of reference *Pseudomonas aeruginosa* (Bergey's Manual of Determinative Bacteriology) and it was found that RM1 exhibits more similarity with the *Pseudomonas aeruginosa*.

**Table 1:** Morphological and Biochemical characterization of isolate RM1 and isolate RM2

Characters	Observations	
	RM1	RM2
<b>Morphological</b>		
Greenish yellow pigment	+	+
Cell Shape	Rod	Rod
Gram staining	Gram negative	Gram negative
Motility	Motile	Motile

**Table 2:** Biochemical Details of isolate RM1 (As per the API Test)

Sr. No.	Biochemical Test	Mnemonic	Result	Sr No.	Biochemical Test	Mnemonic	Result
1	Ala-Phe-Pro-Arylamidase	APPA	-	24	D-Trehalose	dTRE	+
2	H <sub>2</sub> S production	H <sub>2</sub> S	-	25	Succinate Alkalinization	SUCT	+
3	Bit-a-glucosidase	BGLU	-	26	Lycine D-carboxylase	LDC	-
4	L-Proline- Arylamidase	ProA	+	27	L-Arabitol	IARL	-
5	Saccharose/Sucrose	SAC	-	28	D-Glucose	dGLU	+
6	L-Lactate alkalinization	ILATk	+	29	D-Manose	dMNE	+
7	Glycine- Arylamidase	GlyA	-	30	Tyrosine Arylamidase	TyrA	+
8	O129R resistance	O129R	+	31	Citrate (Sodium)	CIT	+
9	Adonitol	ADO	-	32	Bit-a-N Acetyl Galactosamidase	NAGA	-
10	Bit-a-N-acytil-Glucosaminidase	BNAG	-	33	L-Histidine assimilation	IHISa	-
11	D-Maltose	dMAL	-	34	Ellaman	ELLM	-
12	Lipase	LIP	-	35	D Celobiose	dCEL	-
13	D-Tagatose	dTAG	-	36	Gama-Glutamyl Transferase	GGT	+
14	Alfa-Glucosidase	AGLU	-	37	Beta Xylosidase	BXYL	-
15	Ornithine D-Carboxylase	ODC	-	38	Urease	URE	-
16	Glu-Gly-Arg-Arylamidase	GGAA	-	39	Malonet	MNT	+
17	L-Pyrrolydonyl Arylamidase	PyrA	-	40	Alfa Galactosidase	AGAL	-
18	Glutiamyl Arylamidase pNA	AGLTp	+	41	Coumarate	CMT	+
19	D-Manitol	dMAN	-	42	L-Lactate Assmilaton	ILATa	+
20	Palatinose	PLE	-	43	Beta-Galactosidase	BGAL	-
21	Fermentation/Glucose	OFF	-	44	5 Keto-D- Gluconate	5KG	-
22	Beta Alanine Aralamidase pNA	BALap	+	45		BHOS	+
23	D-Sorbitol	dSOR	-	46	Beta-Glucunoridase	BGUR	-

The 16s rDNA gene sequence of RM1 isolate was used to BLAST with the nt database of NCBI genbank. Based on maximum identity score first ten sequence were selected and aligned using multiple alignment software program, clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4. The sequencing and phylogentic analysis identified the RM1 as *Pseudomonas aeruginosa* strain 6A (bc4) (Genbank Accession Number JX661716.2).

### Siderophore detection

Siderophore production by isolated strains of *Pseudomonas aeruginosa* (RM1 strain) was tested by a Chromeazurol assay as described in the methods. Yellow orange zones, an indicator of siderophore production (Schwyn B and Neilands JB 1987) were observed around the colonies.

**Figure 1:** Chromazurol S assay in Liquid Medium

### Siderophore characterization

Characterization of Siderophore production was performed by Tetrazolium and Neilands

Spectrophotometric Assay (for Hydroxamate type of siderophore) and Arnow's and Spectrophotometric Assay (for Catechol type of siderophore). It was found that *Pseudomonas* produced both hydroxamate and catechol type of siderophores.

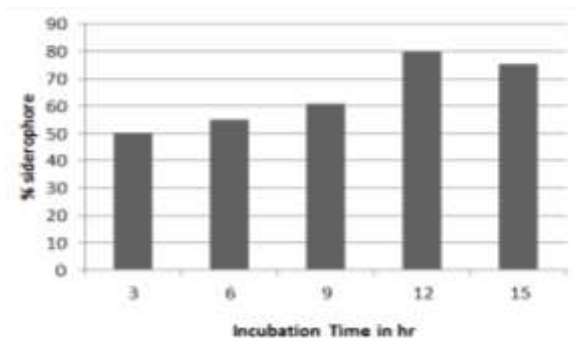
**Figure 2:** Chromazurol S assay on Solid Medium

### Siderophore quantification

The quantitative estimation of Siderophore produced by *Pseudomonas* was determined by CAS-Shuttle assay. The isolated strain of *Pseudomonas* spp. Showed 71% siderophore production

### Siderophore optimization

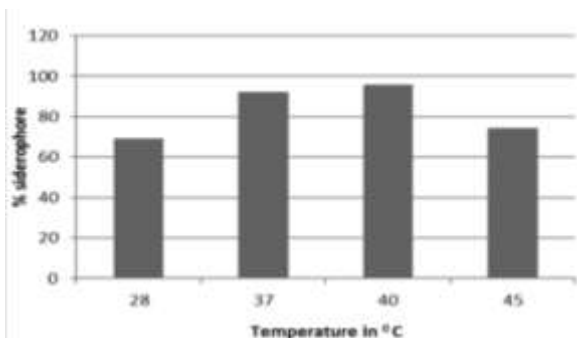
**Effect of incubation Time on Siderophore Production:** To determine the effect of incubation time the bacteria were grown at 28°C at 120 rpm and the samples were removed at different time intervals. As shown in Fig. 3 maximum production of siderophore was observed after 12 h of incubation. Further incubation did not increase the siderophore yield.



**Figure 3:** Effect of Incubation Time on siderophore production

**Effect of incubation Temperature on Siderophore Production**

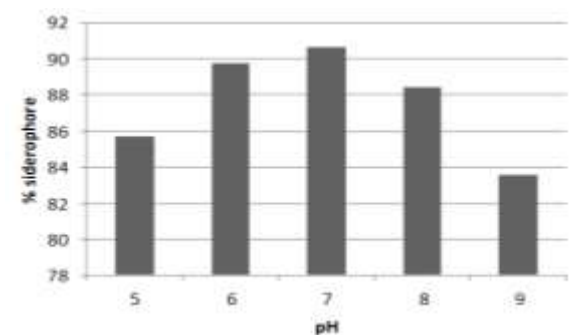
To determine the optimum temperature bacteria were grown at different temperatures (28°C, 37°C, 40°C, 45°C) for 12 h at 120rpm. As shown in Figure. 4 Maximum siderophore production was observed at 40 °C.



**Figure 4:** Effect of Incubation Temperature on siderophore production

**Effect of pH on Siderophore Production**

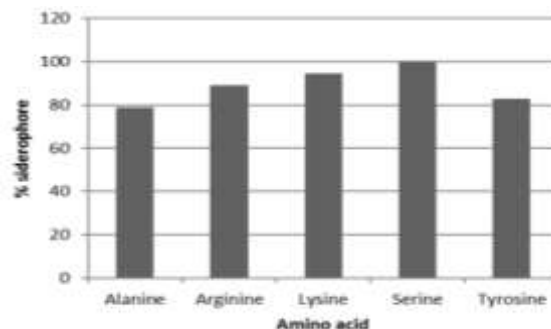
To check the effect of pH on Siderophore production, the bacteria were grown in Succinate medium at different pH (5.0, 6.0, 7.0, 8.0, and 9.0) at 40°C with constant shaking at 120 rpm for 12 h. As shown in Fig. 5 maximum siderophore production was observed at pH 7.0



**Figure 5:** Effect of pH on siderophore production

**Effect of amino acid on Siderophore Production**

To determine the effect of different amino acids on siderophore production, the succinate medium was supplemented with 0.1% of alanine, arginine, lysine, serine and tyrosine. After incubation of 12 h at 40°C with pH 7 and constant shaking at 120 rpm on rotary shaker, it was found that maximum production of siderophore was observed in serine containing medium (Fig.6).



**Figure 6:** Effect of Amino acid on siderophore production

**Antimicrobial activity**

**Antifungal activity of Pseudomonas:** Antifungal activity of *Pseudomonas* was tested against *Aspergillus niger* (NCIM 621), *Aspergillus flavus* (NCIM 532) *Aspergillus fumigatus* (NCIM-902) and *Candida albicans* (NCIM3471). using agar well diffusion assay. After 24h of incubation the plates were observed for zone of inhibition. It was found that the *Pseudomonas* isolate exhibits inhibitory activity against both *Aspergillus niger* and *Aspergillus flavus* (Fig. 7 and 8).



**Figure 7:** Antifungal activity of Isolates against *Aspergillus flavus* (NCIM 532) using agar well diffusion method



**Figure 8:** Antifungal activity of Isolates against *Aspergillus niger* (NCIM 621) using agar well diffusion method.

**Table 3:** Effect of *Pseudomonas* on Seed Germination, Root length, Shoot length and on Weight of Glycine max (JS-335)

Treatment	Germination Time	Root length (cm) (after 20 days)	Shoot length (cm) (after 20 days)	Weight	
				Initial	After drying
Control (uninoculated)	2 days	6.5	17.5	0.83	0.14
Test (Inoculated)	1 day	18.05	18.05	1.67	0.29

**Pot culture assay**

Influence of *Pseudomonas* spp. on seed germination and plant growth promotion of Glycine max (JS-335) Plant was studied by pot culture technique. Interestingly *Pseudomonas* strain significantly improved the seed germination and growth of Glycine max plant. Addition of *Pseudomonas* culture showed 50% decrease in the time of germination, 30% increase in the root length and 20 % increase in the shoot length of Glycine max (JS-335) seeds as compared to control.

Cornelis and Matthijs, 2002). Pyoverdine (PVD), the fluorescent Siderophore produced by the rRNA group I species of Genus *Pseudomonas* constitutes a large family of iron chelators (Wahyudi et al., 2011). Moreover, microorganisms able to produce siderophores can protect themselves by binding toxic metals (Al, Pb, Cd,) (Mureseanu et al., 2003, Olmo et al., 2003). Although essential metals have important biological role, at high levels they can damage cell membranes, alter enzyme specificity, disrupt cellular functions, damage the DNA structure (Bruins et.al.2000; Canovas et al., 2003, Teitzel et al., 2006) and can reduce crop yields and soil fertility (Stuczynski et al., 2003).



**Figure 8:** Effect of *Pseudomonas* on Root Length



**Figure 9:** Effect of *Pseudomonas* on plant height

**DISCUSSION**

The Fe (III) Siderophore complex is transported into bacterial cell via cognate-specific receptor to enzymatic reduction (Meyer et al., 2000,



**Figure 10:** Stem Width of Control plant (Uninoculated)



**Figure 11:** Stem Width of Test plant (Inoculated)

Sr. No	Control (uninoculated)	Test (Inoculated)
1	Diameter 0.23	0.27

In present work, two *Pseudomonas* strains RM1 and RM2 were isolated and RM1 was identified as *Pseudomonas aurogenosa* on the basis of 16s rDNA, morphological and biochemical characterization. Optimization of different parameters showed that maximum siderophores were produced in succinate medium supplemented with 150µ/ml Fe<sup>3+</sup> at pH 7, temperature 40°C, time 12h and in the presence of amino acid serine. These results are in agreement with a previous result obtained by Raaska et al., Maximum Siderophore production was 99 units for RM1. Minimum production of siderophore was found in a Kings B medium. We also found that the production of siderophore decreased after increasing the concentrations of iron in the medium. At pH 7.0, maximum (90.66%) siderophore yield was obtained. This may be due to the fact that alkaline pH helps in excess solubilization of iron, which increases the iron content of the medium. (Schwyn and Neilands, 1987 and Olsen et al., 1981). Among the different amino acids tested, maximum production (99.80% SU) was observed when Serine was supplied as carbon and nitrogen source. The present work indicates that in order to satisfy their need for iron, microorganisms start to excrete large amounts of specific Fe<sup>3+</sup> scavenging molecules (Siderophores), when cells are grown under iron deficiency.

**Figure 12:** Effect of *Pseudomonas* on Growth of plant



**Figure 13:** Effect of *Pseudomonas* on Seed Size and weight.



The selected isolated bacteria are able to increase the growth and nutrient uptake of Soybean

in loamy soil. Increased nutrient uptake by plant inoculated with effective bacteria was attributed to the production of plant growth regulator by the bacteria at the root interface, which stimulated root development and resulted in better absorption of water, nutrients and iron from the soil (Höflich et al., 1996). The positive effect of *Pseudomonas aurogenosa* in these experiments indicated that bacterial production of plant growth promoting substance might be responsible for the observed effect.

In summary, the final result of plant growth promotion in our experiment showed that growth promoting siderophore-producing bacteria can play an essential role in helping the plant establishment and growth.

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