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Production and Optimization of Siderophore from Plant Growth Promoting Rhizobacteria

Meenu Saraf[1*], Sarita Sharma[2] and Aarti Thakkar[3]

[1] Department of Microbiology, University School of Science, Gujarat University, Ahmedabad.
[2, 3] Asst. Professor, President Science College, Affiliated to Gujarat University, Ahmedabad.
E-mail: sarafmeenu@gmail.com
*Corresponding Author

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Abstract

The aim of this study was to determine the effect of siderophore producing bacteria on plant growth promotion in *Glycine max* and iron chelation from rhizospheric soil. Bacterial strains were isolated from rhizosphere on the basis of siderophore production by using chrome azurolsulphonate assay. Maximum siderophore production was observed with strains S-6 and S-26on standard succinic acid medium.Siderophore production was found to be influenced by different carbon, nitrogen and amino acid sources. Optimization of succinic acid medium enhances siderophore production. The pot culture experiment clearly demonstrate the beneficial effect of siderophore producing strains S-6 and S-26 with significance increase in biometric parameters of *Glycine max*. Effect of rhizospheric bacterial isolates (S-6, S-26) was estimated by detecting the iron in soil as well as plant by using atomic absorption spectrophotometer. The iron concentration of soil was decreased after treatment from 38.32 ppm to 26.66 ppm and the iron concentration of plant was increased from 10.18 ppm to 36.05 ppm after treatment with S-6 which indicate the iron chelating capacity of bacterial isolates.

Keywords: Siderophore, Optimization, PGPR (Plant growth promoting rhizobacteria)

Introduction

Iron plays a key role in electron transport, oxidation–reduction reactions, and detoxification of oxygen radicals, synthesis of DNA precursors and in many other biochemical processes [1]. Being a transition element, iron gets rapidly oxidized from soluble ferrous (Fe2+) to insoluble ferric (Fe3+) state. In order to facilitate iron(III) acquisition, plants and microorganisms, such as fungi

and bacteria, produce and excrete strong iron(III) chelators, i.e., siderophores[2,3,4,5]. Siderophores (Greek: "iron carrier") are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses [6]. Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe3+ complexes that can be taken up by active transport mechanisms. Many siderophores are non-ribosomal peptides [6], although several are biosynthesized independently heterologous siderophores or its producer organism may bring about various responses in other target bacterial species that are present within the same niche. Growth of some species may be inhibited and this has been attributed to be one of the mechanisms by which biocontrol agents' act in inhibiting the growth of pathogens in the rhizosphere[7]. PGPR produces extracellular siderophores (microbial iron transport agents) which efficiently complex environmental iron, making it less available to certain native microflora. Siderophore production by PGPR is influenced by source of C, N and minerals found. Plant growth benefits resulting from PGPR application include increases in germination rate, root and shoot weight, lateral root growth, leaf surface area, chlorophyll content, nitrogen content, and yield. In general, yield can be enhanced up to 10% for cereal crops and 15 to 50% for different vegetable crops with PGPR applications [8].Ability to produce siderophores by an organism under iron limiting conditions can promote plant growth by directly supplying iron for plant utilization and by removing iron from the environment for the growth of phyto pathogens thereby reducing their competitiveness[9].The objectives of this research paper are to evaluate the potency of natural isolates for siderophore production *In vitro*as well as plant growth promotion *In vivo* using *Glycine max.*

Materials and Methods

Thirty isolates were isolated from rhizosphere soil of agricultural fields located in semi-arid regions of India. Selected isolates were identified based on the biochemical analyses. Further 16S rRNA gene sequencing was carried out for identification of bacterial isolates. Amplification of the 16S rRNA gene was attempted by PCR using 16S rRNA gene sequence of the isolate was submitted to NCBI and compared with related gene sequences. Selected sequences were aligned in Bio-Edit. Phylogeny was examined by neighbour-joining dendrogramusing MEGA software.

Screening for siderophore production

For siderophore production, isolates were screened on iron depletedsuccinic acid medium. After incubation, the cell free supernatant (10,000 rpm for 15 min) was examined for siderophore production by $FeCl_3$ test and CAS agar plate method. Nature of siderophore produced by the isolates was ascertained by Arnow's [10], Csaky's [11] and Shenker's [12] assay. The amount of siderophore in the culture supernatant was quantified by Chrome azurolsulphonate(CAS) shuttle assay. Various physico-chemicals parameters were optimized for siderophore production[13].

Effect of Incubation time on siderophore production

The selected isolates showing high siderophore production were inoculated in this SM (Succinic acid medium) broth and the flask was then incubated on shaker at 150 rpm production of siderophore was estimated at regular time interval [14].

Effect of pH on siderophore production

The effect of pH 4.0 to 10.0 on siderophore productions was studied in succinic acid medium by adjusting the pH before inoculating the strain with 1N HCl and 1N NaOH and keeping all other condition constant. Sample were harvested at 24 h, each set was subjected to siderophore quantification [15].

Effect of inoculum size on siderophore production

To study the effect of inoculum size on siderophore production was studied in succinic acid medium by inoculating the strain S-6, S-26 with 0.5 %, 1.0 %, 1.5 %, 2 %. The production flasks were then incubated on shaker at 150 rpm, and maximum siderophore production was checked by harvesting the sample at 24 h [16].

Effect of different sugars on siderophore production

To study the effect of different sugar on siderophore production was studied in succinic acid medium which was individually supplement with different sugar such as glucose (1gm/l), glycerol (1gm/l), sucrose (1gm/l), dextrose (1gm/l), mannitol (1gm/l), and keeping all other condition constant. Sample were harvested at 24 h, each set was subjected to siderophore quantification [17].

Effect of different organic acids on siderophore production

To study the effect of different organic acids on siderophore production was studied in succinic acid medium which was individually supplement with different organic acid such as succinic acid (4 gm/l), oxalic acid (4 gm/l), malic acid (4 gm/l), citric acid (4 gm/l). Each set was separately inoculated with strain S-6 and S-26, incubated on shaker at 150 rpm for 24 h at room temperature. After incubation each set was subjected to siderophore production [18].

Effect of different amino acids on siderophore production

To study the effect of different amino acid on siderophore production the succinic acid medium was individually supplemented with 0.05 gm per 50 ml of cystein, lysine, threonine, tyrosine, and serine. Each set was separately inoculated with strain S-6 and S-26 and incubated. After incubation of 24 h each set was subjected to siderophore quantification [15].

Effect of nitrogen source on siderophore production

To study the effect of different nitrogen source on siderophore production the succinic acid medium was individually supplemented with 1 gm/l of ammonium sulphate, urea. Each set was separately inoculated with strain S-6 and S-26 and incubated. After incubation of 24 h each set was subjected to siderophore quantification [16].

Influence of iron on siderophore production

In order to determined threshold level of iron at which siderophore biosynthesis repressed in organisms under study. Both the cultures were grown in the medium supplemented with 0-100 uM of iron. Reports showed increase in growth of *Pseudomonas* with increase in $FeCl_3$ concentration revealing that presence of FeCl3 is vital for its growth [9].

Pot trials and measurement of biometric parameters of *Glycine max* L

Two isolates were selected on basis of their high siderophore producing activity for pot study.The plant chosen was *Glycine max* L and cultures designated as S-6 and S-26 were used. Soybean seeds were soaked in 0.02% sodium hypochlorite for 2 min and washed five times with sterilized distilled water. Seeds were coated with 1% CMCas adhesive. Then seeds were treated with bacterial strain for 30 min. seeds were sown in each earthen pot filled with sterile sandy loam soil and watered regularly. For each treatment, three such pots were maintained.Uninoculated seeds were sown in pot served as control. After 30 days of plant growth, plant were carefully uprooted from sand. Intact root system was carefully uprooted to prevent breakage. The plant growth promoting parameters such as root length, shoot length, fresh weight, dry weight, number of leaves, number of lateral root and chlorophyll content were recorded[13].

Detection of iron in soil as well as in plant by using AAS (Atomic absorption spectroscopy)

Atomic absorption spectrophotometer with following accessories; HVG (Hydride vapor generator) & GFA (Graphic furnace Atomizer) was used to determine the concentration of iron in soil and plants [19].

Statistical analysis

Statistical analysis was done using ANOVA software for individual parameters for triplicate samples. All hypotheses were tested at the 1% confidence level.

Results

Isolation and Screening

Thirty isolates were isolated from rhizospheric soil of agricultural fields located in semi-arid regions of India from Rajasthan and Gujarat and screened on iron depleted succinic acid medium for the siderophore production.CAS assay based on the color change (colored halo) around the microbial colonies from blue to orange after chelation of the bound iron by siderophoresproduced by isolates [20]. 16 out of 30 isolates were positive for the siderophore production. The positive isolates were S-1, S-2, S-3, S-4, S-5, S-6, S-7, S-17, S-21, S-23, S-24, S-25, S-26, S-28, S-29, and S-30. The zone diameter was measure around positive isolates on CAS agar plates (Table 1).

Culture No.	CAS Agar plate Zone diameter (in mm)		Mean (in mm)
S-1	20	18	20
S-2	25	21	23

S-3	24	25	24.5
S-4	26	28	27
S-5	20	23	21.5
S-6	32	36	34
S-7	16	18	17
S-17	20	21	20.5
S-21	18	16	17
S-23	17	13	15
S-24	25	28	26.5
S-25	30	33	31.5
S-26	35	30	32.5
S-28	20	23	21.5
S-29	9	10	9.5
S-30	22	20	21

Table 1 Diameter around colonies of siderophore producers

Quantitative determination of bacterial siderophore

All siderophore producing bacterial isolates produced moderate reaction with the hydroxamate assay [10] while S-6 and S-26which showed higher siderophore production (36.5 ug/ml and 33ug/ml respectively) as compare with other bacterial strain (Figure1). However, isolates does not show catecholate [11] and carboxylate [12] type of siderophore.

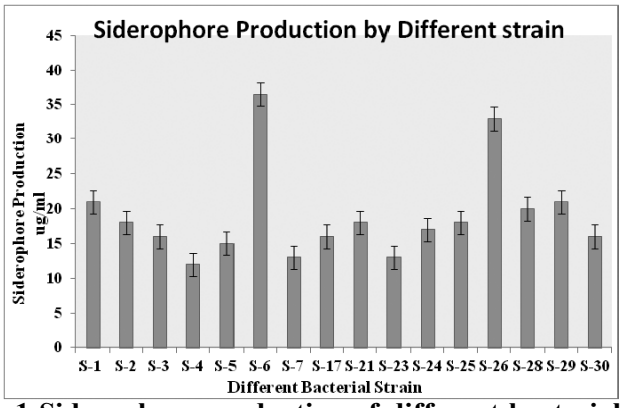

Figure 1 Siderophore production of different bacterial strain

Identification

The two isolates showing maximum siderophore production- S-6 and S-26 were identified basedon 16S rRNA assay. They were *Rhizobium* and *Enterobacter* respectively. The sequence was submitted to NCBI and compared with related gene sequences under the accession number KF984469 and KF984470 respectively. Selected sequences were aligned in Bio-Edit.Phylogeny was examined by neighbour-joining dendrogramusing MEGA software.

Optimization of the conditions for maximum siderophore production

Optimization of various parameters and development of media are the most important criteria for enhance production of siderophore. Various physical and chemical factors have been known to affect the production of siderophore such as incubation time, pH, inoculum size, different sugars, different organic acids, different amino acid, different nitrogen sources, different concentration of iron. Interactions of this parameter were reported to have a significant influence on the production of the siderophore. Hence several cultural parameters were studied to optimize the siderophore production from S-6 and S-26.

Effect of different incubation time on siderophore production

For the optimization of incubation time for maximum siderophore production sample was harvested at interval of every 24 h, 28 h, 48 h, and 52 h and centrifuged at 10,000 rpm for 10 mins. The results obtained were shown in the graph for S-6, S-26 isolate (Figure 2). From the graph it was concluded that maximum siderophore production was observed at the end of 24 h and declined thereafter.

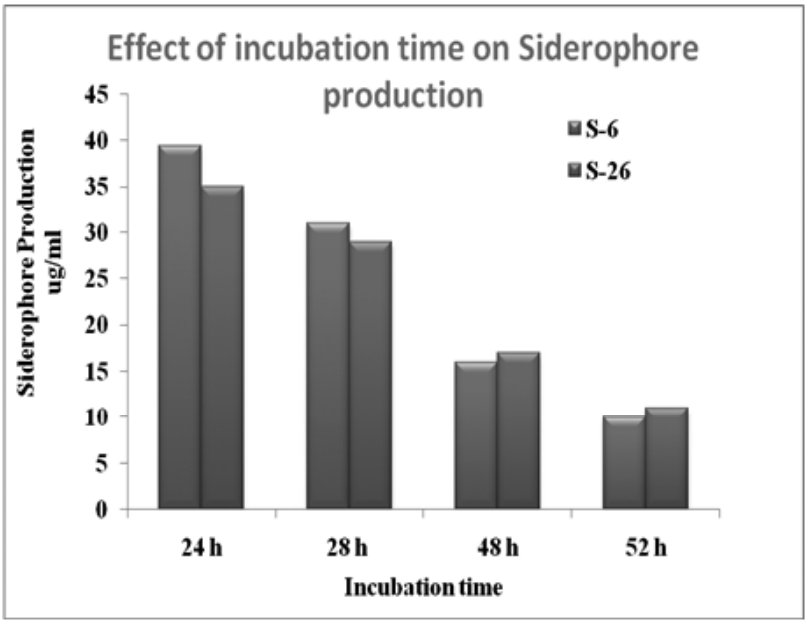

Figure 2 Effect of different incubation time on siderophore production

Effect of different pH on siderophore production

PH plays an important role in the solubility of iron and thereby availability to the growing organism in the medium. In order to check the effect of pH on siderophore production by S-6 and S-26 pH of the medium was set from 4 to 10. Analysis of results indicate that S-6 and S-26 showed maximum siderophore production i.e. 43.5 ug/ml, 39 ug/ml respectively at pH 7 (Figure 3).

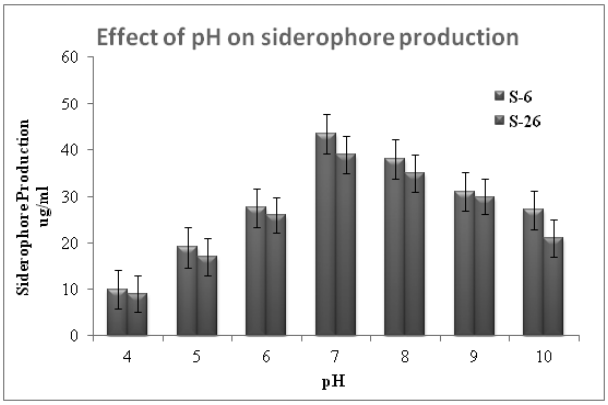

Figure 3 Effect of different pH on siderophore production

Effect of different inoculum size on siderophore production

Effect of inoculum size for maximum siderophore production was checked. Different inoculum size such as 0.2%, 0.5%, 1.0 %, 1.5%, 2.0% were inoculated in production medium. It was concluded thatinoculum size of 0.5% is optimum for maximum siderophore production byS-6 and S-26was 65ug/ml, 52ug/ml respectively (Figure 4).

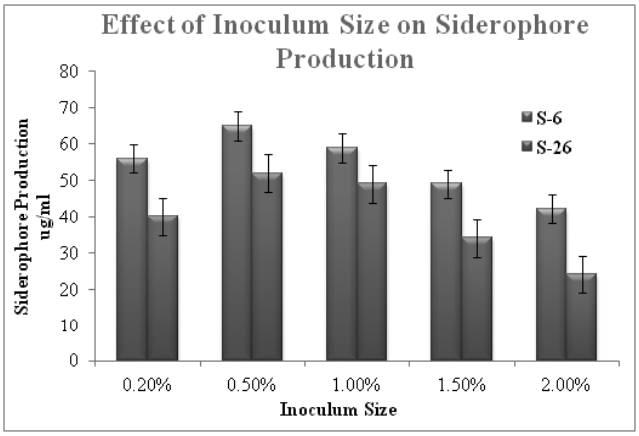

Figure 4 Effect of different inoculum size on siderophore production

Effect of different sugars on siderophore production

Effect of Sugar for maximum siderophore production was checked. Different sugar such as glucose, glycerol, sucrose, dextrose, and mannitol were used in production medium for both the

strains (S-6, S-26). From the graph it was observed that glucose did not show maximum siderophore production. This study shows that both the strain (S-6, S-26) showed the best siderophore production when grown on glycerol (39.6 ug/ml, 34 ug/ml respectively) as compare with other sugars (Figure 5).

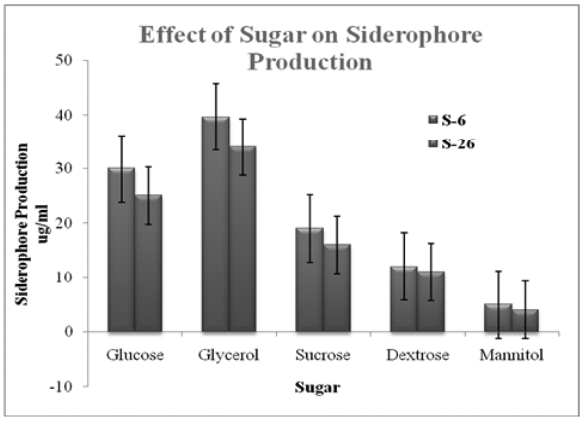

Figure 5 Effect of different Sugar on siderophore production

Effect of different organic acids on siderophore production

Different organic acids such as succinic acid, oxalic acid, malic acid, citric acid were used in production medium for both the strains (S-6, S-26). It was observed (Figure 6) that succinic acid was act as the most efficient carbon source with maximum siderophore production for both the strains (74.6ug/ml, 64.3ug/ml respectively).

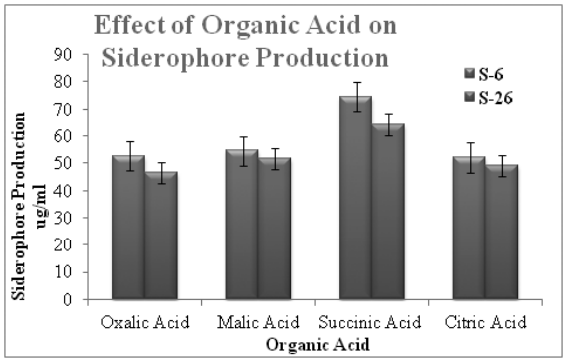

Figure 6 Effect of different organic acids on siderophore production

Effect of different amino acids on siderophore production

The succinic acid medium was individually supplemented with 0.1% of cystein, lysine, threonine, tyrosine,serine was used in production medium for both the strains (S-6, S-26). Threonine showed the maximum siderophore production (Figure 7) in both the strains S-6, S-26 (78.9ug/ml, 71ug/ml respectively).

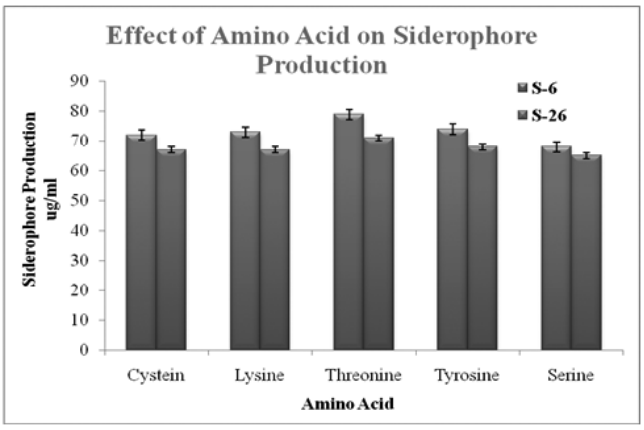

Figure 7 Effect of different amino acids on siderophore production

Effect of different nitrogen source on siderophore production

Effect of 0.1% ammonium sulphate, and urea was studied forsiderophore production by both the strains (S-6, S-26).From the graph it was observed that ammonium sulphate showed the most efficient nitrogen source with maximum siderophore production (80.6ug/ml, 75.9ug/ml

Respectively and it was observed that strain S-6 gave higher siderophore as compare with strain S-26.

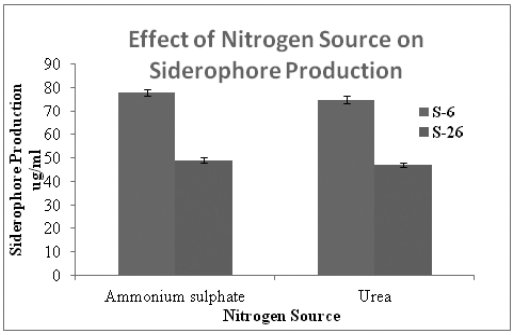

Figure 8 Effect of different Nitrogen source on siderophore production

Effect of different iron concentration on siderophore production

To determine the effect of iron concentration on the siderophore production, the succinic acid medium supplemented with 1 uM, 3 uM, 5 uM, 10 uM, 15 uM, 20 uM, 40 uM, 60 uM, 80 uM, 100 uM for both the strains (S-6, S-26) (Figure 9).It has been reviewed that iron-stressedconditions lead to production of strong iron-chelating agents such as siderophores. Hence studies were done to evaluate the influence of iron concentration on siderophoresynthesis. As it is depicted from the observations, the isolate S-6, S-26 showed gradual decrease in siderophore production and was found to be completely repressed at higher concentrations.

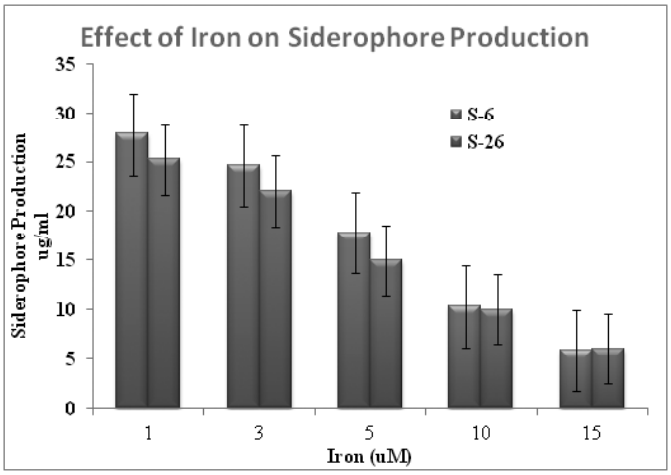

Figure 9 Effect of different iron concentration on siderophore production

Pot trials

The pot culture experiment clearly demonstrate the beneficial effect of strain S-6 and S-26 with significance increase in the plant growth which was shown in Table 2. The result of pot study showed that inoculation of soybean seeds with bacterial strain showed a positive effect on root and shoot length. Both the strain increases the root length and shoot length as compare with

Treatments	Totallength =Shoot length + Root length (cm)	Fresh Weight (gm)	Dry Weight (gm)	Shoot width (mm)	No. of lateral roots	No. of leaves	Chlorophyll a(mg/gm)	Chlorophyll b(mg/gm)
Treatment 1	44	1.428	0.3699	2.4	526	8	1.21	0.98
Treatment 2	65.5	2.768	0.9307	2.5	810	17	2.21	1.44
Treatment 3	61.5	1.811	0.6364	2.5	725	13	1.62	1.12

Table 2 Effect of inoculation of rhizospheric bacterial isolates on different parameters of soybean plant after 30 day of experiment

number of leaves as compare with control.The chlorophyll content in plants treated with S-6 (2.21 mg/g), S-26 (1.62 mg/g), which was higher than the chlorophyll content of control plant (1.21 mg/g).

Detection of iron in soil as well as in plant by using AAS (Atomic absorption spectrophotometry)

Effect of rhizospheric bacterial isolates (S-6, S-26) on siderophore production was estimated by detecting the iron in soil as well as plant. Sample of soil was collected at 0 day from treatment 1 and estimated iron concentration in soil and plants. The iron concentration in soil was decreased from 35.18 ppm to 26.66 ppm with S-6 and 28.31 ppm with S-26. The iron concentration in plants was increased from 10.18 ppm to 36.05 ppm when treated with S-6 and 25.13 ppm in plants treated with S-26 (Table.3).

Table 3 Detection of iron in plants and soil by AAS

Treatment	Iron concentration (ppm) At 0 DAS	
	Soil	Plant
Treatment 1	35.18	10.18
Treatment 2	26.66	36.05
Treatment 3	28.31	25.13

Discussion

However, overall trend of siderophore production level during time course study are similar in both the isolates.On the other hand in *pseudomonasfluorescens* the siderophore synthesis started after 12 h of incubation, which increased up to 28 h and declined thereafter [18]. Moreover, in case of strain MR-AI and WR-W2 highest accumulation of siderophore level was observed after 94 h of growth but both the strain demonstrate a significant decline in siderophore production level after 120 h of growth [21].

Both the organism shows maximum sidrophore production was observed at pH 7. This may be because bacteria grow better at physiological pH and iron is present in insoluble form at neutral pH. Siderophore production was found to be induced under iron stress conditions [18]. However, in the case of the *Pseudomonas sp.*, the optimum pH for siderophoregenesis was found to be 7.0, but *P. fluorescens*was more stable to pH variations as compared to *P. aeruginosa*. Moreover both the organisms had retained at least 50% of siderophoregenesis activity at all the pH levels tested [15]. Observations reported that alkaline pH (8.5) favoured growth while acidic pH (6.5) supported siderophoregenesis on *Alcaligenesfeacalis*in succinic acid medium [16].

With increase in inoculum size decrease in siderophore production was observed. Optimum siderophore production with 1 % inoculum level in *P. fluorescens* NCIM 5096 and further increase does not support growth and siderophoregenesis [16].

This may be due to utilization of sugar for growth and not siderophore production. However, glucose proved to be the most suitable source for *Ps.Aeruginosa, A. nidulan* and *P. chrysogenum* whereas *B. japonicum* gave its higher siderophore yield when grown on mannitol [17].

It was reported that disaccharides (fructose, xylose, maltose and lactose) and monosaccharide (glucose) support growth, while polysaccharides (starch) supports both growth and siderophore yield with *Alcaligenesfeacalis* [16].

Both the organism shows maximum sidrophore production was observed by using succinic acid. But in case of strain MR-AI and WR-W2 malate and acetate were the best sole carbon source for siderophore production [21]. The lactic acid supported growth while succinic acid supported both growth and siderophore yield with *Alcaligenesfeacalis*[16].

Threonine showed the maximum siderophore production in both the strains. Reports suggested that supplement of cystein, histidine boosted the growth while metheonine, isoleucine, tyrosine and proline enhanced siderophore production with *Alcaligenesfeacalis* [16].

It was observed that ammonium sulphate showed the most efficient nitrogen source with maximum siderophore production. In the case with *Pseudomonas fluorescence* it was found that urea was the best nitrogen source for siderophore production [18]. Reports found that NH_4SO_4 supported good growth and maximum siderophore yield with *Alcaligenesfeacalis* [16].

Maximum siderophore production was obtained at 1 uM concentration (27.8ug/ml, 25.2ug/ml respectively). It was found that concentration of iron >10 uM had a negative effect on siderophore production. It was reported that siderophore production occurred at an iron concentration of >50 uM [22]. The siderophore production was inversely proportional to the iron concentration while no siderophoregenesis was reported at iron concentration above 25 µM by *P. fluorescens*and *P. aeruginosa*[15].

Azospirillum sp., on the growth of some agriculturally important plants and observed a significant increase in the dry weight of both the root system and aerial parts of the PGPR inoculated plants, resulting in better development and flowering. Total root length, surfacearea and volume in tomato and cucumber roots increased after inoculation with *Pseudomonas fluorescens* 92rk and P190r [23].

Conclusion

The current study highlights the efficiency of bacterial isolates S-6 and S-26 as PGPR of *Glycine max*plant in addition to their siderophore production attributeunder *in vitro*conditions. Siderophore production was optimized and highest amount of siderophore was produced by S-6 after 24 hours at pH 7 by the addition of 0.5% of inoculum with the addition of succinic acid, threonine, and 1 µM of iron concentration in the medium by using ammonium sulphate as nitrogen and glycerol as a corbon source.The experimental results also showed that siderophore producing microbes are involved in plant growth promotion in *Glycine max*. The bacterial suspension of S-6 showed best performance in yield attributes, physiological and biochemical parameters indicating its plant growth promoting potential. The results of atomic absorption spectrophotometer were showed that the decrease in iron concentration in soil after inoculation with bacterial isolates S-6 and increased in plants as compare with untreated soil (control). The results are promising fordesign of potentially active Siderophore producing S-6 strain based formulation whichwould be beneficial for *Glycine max* cropimprovement and crop protection. The potential lof this strain could be investigated in detail andfield application shall be studied for its plant growth promoting and biocontrol potential.

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