Optimization and production of siderophore

Production and optimization of siderophore from plant growth promoting rhizobacteria

Abstract

The aim of this study was to optimize the production of siderophores by bacterial strains isolated from rhizosphere soil. Chrome azurol sulphonate assay confirms siderophore production by all

30 bacterial isolates. Maximum siderophore production was observed with strains S-6 and S-26 on standard succinic acid medium. Siderophore production was found to be influenced by different carbon, nitrogen and amino acid sources. Optimization of SM medium nutrient composition enhances siderophore production. The pot culture experiment clearly demonstrate the beneficial effect of strain S-6 and S-26 with significance increase in biometric parameters of soybean. Effect of rhizospheric bacterial isolates (S-6, S-26) on siderophore production 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.

Key words: Siderophore, Optimization, PGPR

Introduction

Iron plays a key role in electron transport, oxidation–reduction reactions, 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]. Siderophores (Greek: “ iron carrier”) are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses [3]. 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 [3], although several are biosynthesised 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 [4]. 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 [5]. 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 phytopathogens thereby reducing their competitiveness [6].

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 dendrogram using MEGA software.

Screening for siderophore production

For siderophore production, isolates were screened on iron depleted succinic acid medium. After incubation, the cell free supernatant (10, 000 rpm for 15 min) was examined for siderophore production by FeCl3 test and CAS agar plate method. Nature of siderophore produced by the isolates was ascertained by Arnow’s [7], Csaky’s [8] and Shenker’s [9] assay. The amount of siderophore in the culture supernatant was quantified by Chrome azurol sulphonate (CAS) shuttle assay. Various physico-chemicals parameters were optimized for siderophore production [10].

Effect of Incubation time on siderophore production

The selected isolates showing high siderophore production were inoculated in this SM broth and the flask was then incubated on shaker at 150 rpm Production of siderophore was estimated at regular time interval [11].

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 [12].

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 [13].

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 [14].

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 [15].

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 [12].

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 [13].

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 FeCl3 concentration revealing that presence of FeCl3 is vital for its growth [6].

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% CMC as 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 [10].

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 [16].

Results and Discussion

Isolation and Screening

Thirty isolates were isolated from rhizosphere soil of agricultural fields located in semi arid regions of India from Rajasthan and Gujarat and screened on iron depleted succinic acid medium. CAS assay based on the

color change (colored halo) around the microbial colonies from blue to orange after chelation of the bound iron by siderophores produced by isolates [17].

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).

Quantitative determination of bacterial siderophore

All Positive rhizospheric bacterial isolates produced moderate reaction with the hydroxamate assay [7] while S-6 and S-26 which showed higher siderophore production (36. 5 ug/ml. 33 ug/ml respectively) as compare with other bacterial strain (Figure 1). However, any isolate did not show catecholate [8] and carboxylate [9] type of siderophore.

Identification

The two isolates showing maximum siderophore production- S-6 and S-26 were identified based on 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 dendrogram using MEGA softwar.

Optimization of the conditions for maximum siderophore production

Optimization of various parameters and development of media are the most important criteria for the overproduction 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. Supernatant used for the siderophore production. 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. However, overall trend of siderophore production level during time course study are similar in both the isolates. On the other hand in pseudomonas fluorescens the siderophore synthesis started after

12 h of incubation, which increased up to 28 h and declined thereafter [15]. 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 [18].

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. For that production media was set at different initial pH 4 to10 pH in order to check the effect of pH on siderophore production by culture S-6 and S-26. The result was shown in graph (Figure 3).