ORIGINAL RESEARCH PAPER

Agricultural Science

ISOLATION OF MICROBIAL SIDEROPHORES FROM THE RHIZOPHERE SOIL AND ITS GROWTH PROMOTION IN PLANTS

KEY WORDS: Siderophore, blue agar medium, biocontrolling

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ABSTRACT

Iron is essential to the majority of microorganisms, it is an important cofactor in many cellular processes and enzymes. Many bacteria have the ability to produce siderophores, low molecular weight compounds that have a high affinity for Fe^{3+}. The objective of this study was to isolate microbial siderophores from the soil sample and its growth promotion of plants. From the rhizophere soil, siderophore producing bacteria were isolated. The isolates were screened using blue agar medium and estimation of siderophores were done by using iron percolate assay. The isolate which has shown high siderophore activity was selected and characterized by using 16Sr RNA sequencing method. The result of this experiment were confirmed using pot experiment, *Bacillus cereus* showed high amount of plant growth rate like shoot length (7.57), root length(4.92) and also protein content. This results indicates that this if we continue the large scale biocontrolling agents against fungal infection in plants.

Introduction

Agriculture is the main stay of nutrition, food security and livelihood of the country. Availability of micronutrient to human being can be primarily determined by output of the food produced from agricultural systems. Deficiency of any micronutrient leads to poor health, disease, morbidity, mortality, infertility and even to death. It also leads to lower micronutrient content in edible plant parts and thereby causing a health problem in animal and human beings. So, it is very important to maintain healthy micronutrient profile of the plants because major source of dietary micronutrients are from soil *via* plants (Beneduzi *et al.,* 2012).

In the recent years iron deficiency is known to occur in many regions of the world, the reason is low solubility of $Fe^{2+}$and Fe^{3+} under toxic conditions. Fe is involved in chlorophyll biosynthesis, thylakoid synthesis and chloroplast development. Therefore, Fe deficiency impairs chlorophyll biosynthesis and chloroplast development in both dicotyledonous and monocotyledonous species (Jin *et al*., 2014). Consequences of iron deficiency in human are IDA, lower resistance to infection, reduced learning abilities, stunted growth, fatigue and reduced productivity.

In 2017 WHO estimates that globally two billion peoples are affected by iron deficiency. In order to survive under such iron-depleted environment, microorganisms produce certain organic compounds with low molecular masses called siderophore (Ahmed and Holmstrom, 2014). They are the metal- chelating agents that primarily function to capture the insoluble ferric iron from different habitats (Nagoba and Vedpathak. 2011). They are low molecular weight organic molecules which can compete for ferric iron in ferric hydroxide complexes. It can be classified into three main categories, namely, hydroxamate, catecholate, and carboxylates.

Mechanism of siderophores:

Siderophore first binds with iron (Fe^{+3}) tightly and then the siderophore- iron complex moves into the cell through the cell membrane using the specific siderophore receptors. The membrane network of gram- negative bacteria is markedly different from that of gram- positive bacteria. In case of gram-positive bacteria, siderophore- binding proteins, *permeases*, and *ATPases* are involved in the transport of siderophore iron (Fe^{+3}) complex in the cell membrane (Ahmed and Holmstrom. 2014). Once siderophores bound to ferric iron moves to cytosol, the ferric iron gets reduced to ferrous form and the ferrous form of iron becomes free from the siderophores. After release of iron, siderophores either get degraded or recycled by excretion through efflux pump system. Plant is able to produce siderophores which assimilate Fe from environment for their own purpose or to be used by the plants sliving system and involve in symbiosis. It is especially essential in the time of environment stress resulting from the inadequate amount of iron in the soil, which main lead to the inhibition of plant growth as well as disturbing their functions.

The siderophores are applied in both in agricultural and in medical field. The applications of siderophores in biotechnological aspects are enhancing the plant growth and for bio-control of pest, bio-bleaching of pulp, bio-control of fish pathogens, bioremediation of heavy metals, alters microbial community, used as biosensors, and it is also used as an antibiotic in medical field. To isolate microorganism from the soil this is responsible for producing siderophore activity. Objective to screen and characterize the isolated microbial samples for siderophore production and to check the plant growth promotion activity (*in vitro)*.

Methodology

Collection of soil sample and its isolation of bacteria

Soil samples were collected from Coimbatore area. The sample was collected and sealed in fresh polythene bag, to avoid contamination and it was brought to laboratory. The collected sample was used for isolation. The nutrient agar medium was prepared and autoclaved at 121°C for 15 mins. The plates were prepared. One gram of soil sample was weighed and suspended in 100ml distilled water and were kept in rotatory shaker for about 30 mins. Then the sample was serially diluted in order to determine the bacterial and fungal colonies present in the soil.

Characterization of the microbes

The isolated bacterial samples were identified based on their morphology, colony characteristics and standard biochemical reactions using bergey's manual 1962. Isolated samples were confirmed by using blue agar medium (selective media). The different isolates were streaked in to the plates and incubated at 37°C for 24 hrs. After incubation, the isolate which produce cream color colonies were confirmed as siderophore producing organisms. Those organisms were isolated and used for mass cultivation. The confirmed siderophore producing isolate was selected and sequenced.

Mass cultivation of siderophore producing microorganism

Kings B broth (selective media) was used for the siderophore production. In the autoclaved broth, 1% of the inoculum was added and kept in rotatory shaker at 700 rpm and maintained at 37°C for 48 hrs for the siderophore production. Characterization of catecholate using Ferric Chloride Test (Neilands 1981) and estimation of siderophore concentration (Atkin *et al*., 1970).

Pot culture experiment

Pot culture method was performed. The soil was sterilized, cooled and transferred into plastic cups. *Fenugreek* seeds were surface sterilized with 0.2% mercuric chloride for 30 secs and then washed with sterile distilled water. Seeds were then soaked into

the siderophore producing microbes for different time interval of 10, 20, 30 and 40 mins, before inoculation. The un-inoculated seeds were soaked as a control. The cups were watered regularly, and maintained in open shade at 27°C- 30°C for the germination of seed and development of the root, and shoot. All datas were recorded. The amount of protein content present in the leaf samples was estimated by *Lowry's* method.

Results and discussion

The nine bacterial isolates were isolated and checked colony morphology and were characterized using biochemically test and confirmed in the blue agar medium (Table 1).

Table 1: Morphological and Biochemical test of the bacterial isolates

Isolates	Gram's reaction	Morphology	MR	VP	Indole	Citrate Utilization	Gelatin Test	Starch Hydrolysis Test	Blue agar Test
1	-ve	Cocci	-	-	-	+	-	+	+/-
2	+ve	Rod	-	-	-	-	+	+	+
3	-ve	Cocci	-	-	-	-	+	+	+
4	+ve	Rod	-	-	-	+	-	-	+
5	-ve	Cocci	-	-	-	+	-	+	+
6	-ve	Cocci	+	-	-	+	-	-	+
7	-ve	Cocci	+	-	+	-	+	-	+
8	+ve	Rod	-	-	-	-	-	+	+
9	-ve	Cocci	-	-	-	-	-	-	-

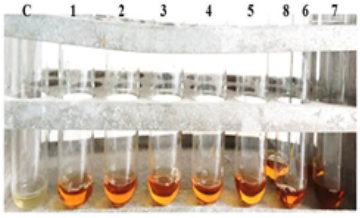
Confirmation of Catecholate type of siderophore by Ferric chloride Test

1ml of 2% aqueous FeCl3 solution was added to 1ml of culture filtrate, and examined for the appearance of orange or reddish brown color which indicates positive test for siderophore production (fig 1)

Determination of Catecholate type of siderophore by Iron percolate assay

The siderophore produced by all isolates were of the Catecholate or Phenolate type (yellow color) table 2. Unni et al., 2014 reported the presence of Catecholate type (pyovirdine) of siderophore in P.aeruginosa BUP2 isolated from the Malabari Goat.

Fig. 1: Confirmation of Siderophore by Ferric chloride Test



C-Control; Isolates 1, 2, 3, 4, 5, 6, 7, 8, 9

Table 2: Determination of siderophores by iron percolate assay

S. no	Isolates	Optical Density @480nm
1	Blank	0.00
2	1	0.18
3	2	0.22
4	3	0.25
5	4	0.28
6	5	0.23
7	6	0.25
8	7	0.24
9	8	0.26
10	9	0.23

Molecular identification and sequencing

Isolate 4 was shown high siderophore activity @ 480nm and further sequenced with 16Sr RNA sequence. The 480bp sequence obtained, submitted in the NCBI database (accession noMF136788) and phylogenetic tree was generated with other *Bacillus cereus* (Eden *et al*., 1991). It showed 91% similarity with the exciting *Bacillus* sp in the NCBI database (fig 2 & 3).

Fig. 2: 16S r RNA sequences of *Bacillus cereus*

>AAGAATTTGCCCCGGGTGTTTTTCTGGAATATGAATTTACACG
TTTGGTTTCTTGAAGGCGAGTGGCAGCCTCCAATCCGAACTGA
GAACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTGCAGCT
CTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAA
GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTG
TCACCGGCAGTCACCTTAGAGTGCCCAACTTAATGATGGCAA
CTAAGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
CTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACT
CTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTTCAGAGG
ATTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACC
ACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAG
TTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGC
GTTAACTTCGGCACTAAAGGGCGGAAACCCTCTAACACTTAG
CACTCATCGTTTACGGCGTGGAC

Fig. 3: Phylogentic tree for *Baccillus cereus*

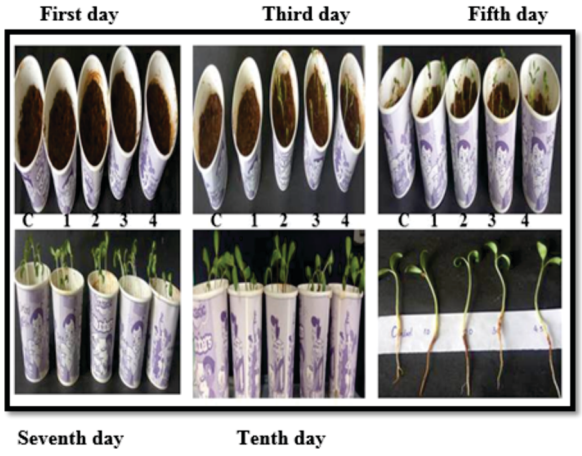
Plant growth promotions by *Bacillus cereus*

Siderophore producing plant growth promoting rhizobacteria have shown to play a vital role in iron nutrition of the plant and therefore in plant growth promotion leading to healthy plants (Ravindran and Menon 2015). It was therefore checked in the present study whether the plant is capable of utilizing microbial siderophores for iron nutrition. For this Fenugreek plants like *Trigonella foenum-graecum* was grown under iron limiting conditions and under iron limiting conditions with siderophore supplements. Plants growth under Iron supplemented conditions was used as control (Table 3). T4(40mins) showed better growth in terms of increase in root length, shoot length and number of leaves was observed in plant grown under iron limiting conditions with siderophore supplements as when compare to the plants grown under iron limiting conditions (Figure 4 & 5). Tilak and Reddy 2006 reported that strains of *Bacillus circulans* and *B. cereus* increased growth in corn, wheat, and pigeon pea, but the highest response to the bacterial treatments was found in corn. *Bacillus* are increasingly used in agriculture to promote plant growth and to protect against plant pathogens (Qiao *et al*., 2017).

Table 3: Plant growth promotion by *Bacillus cereus* on the *Fenugreek* seeds (different time intervals)

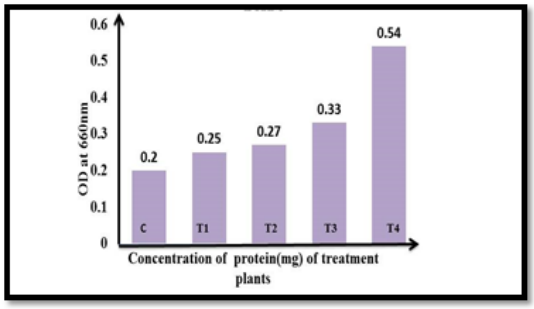
S. no	Seeds (inoculated)	Soaking time	Germination (Days)	Leaves	Shoot length (cm)	Root length (cm)	Protein Content
1	C	-	4	3	5.35	4.07	0.20
2	T1	10	2	4	5.95	4.05	0.25
3	T2	20	2	6	6.15	4.40	0.27
4	T3	30	1	8	7.07	4.65	0.33
5	T4	40	1	8	7.57	4.92	0.54

Fig. 4: Plant growth promotions by *Bacillus cereus*



C-control; 1, 2, 3, 4 (seeds treated siderophore culture for10, 20, 30, 40 mins)

Fig. 5: Estimation of protein from leaves after 15 Days



Conclusion

Thus in the present study *Bacillus cereus* was able to overcome the major problem related to the adverse effects of chemical fertilizers on plant growth and productivity. Thus a biological platform was built to combat this problem. *Bacillus cereus* produce extracellular water soluble yellow green Siderophore which was proved to be *Fenugreek* when grown under iron limiting conditions with siderophore supplements. Thus siderophore can be used in combination with other biofertilizers to increase crop productivity

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