Journal of Scientific Research and Reports

Volume 30, Issue 8, Page 313-326, 2024; Article no.JSRR.120236
ISSN: 2320-0227

Optimizing Siderophore Production in *Bacillus subtilis* to Enhance Seed Germination and Biocontrol Efficacy against *Alternaria triticina* and *Bipolaris sorokiniana*

Neha Saini [a], Vindhya Bundela [a], Shivani Singh [a], Manvika Sahgal [a] and Ajay Veer Singh [a*]

[a] Department of Microbiology, G.B. Pant University of Agriculture & Technology, Pantnagar-263145, Uttarakhand, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author NS conceptualized the study, performed the methodology, did experimental preparation, analysis of data, and prepared the final draft of the manuscript. Authors VB and SS reviewed and edited the manuscript. Author MS supervised the study, did data validation and reviewed the manuscript. Author AVS reviewed the final draft. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/jsrr/2024/v30i82251

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/120236

Original Research Article

Received: 22/05/2024
Accepted: 25/07/2024
Published: 27/07/2024

ABSTRACT

Introduction: *Bacillus subtilis* (UP11) is a gram-positive, plant growth-promoting rhizosphere bacterium (PGPR) isolated from the wheat rhizosphere. This bacterium produces siderophore, are low-molecular-weight, high-affinity molecules produced under iron-limiting conditions. This study

**Corresponding author: E-mail: ajaygbpuat@gmail.com;*

Cite as: *Saini, Neha, Vindhya Bundela, Shivani Singh, Manvika Sahgal, and Ajay Veer Singh. 2024. "Optimizing Siderophore Production in Bacillus Subtilis to Enhance Seed Germination and Biocontrol Efficacy Against Alternaria Triticina and Bipolaris Sorokiniana". Journal of Scientific Research and Reports 30 (8):313-26. https://doi.org/10.9734/jsrr/2024/v30i82251.*

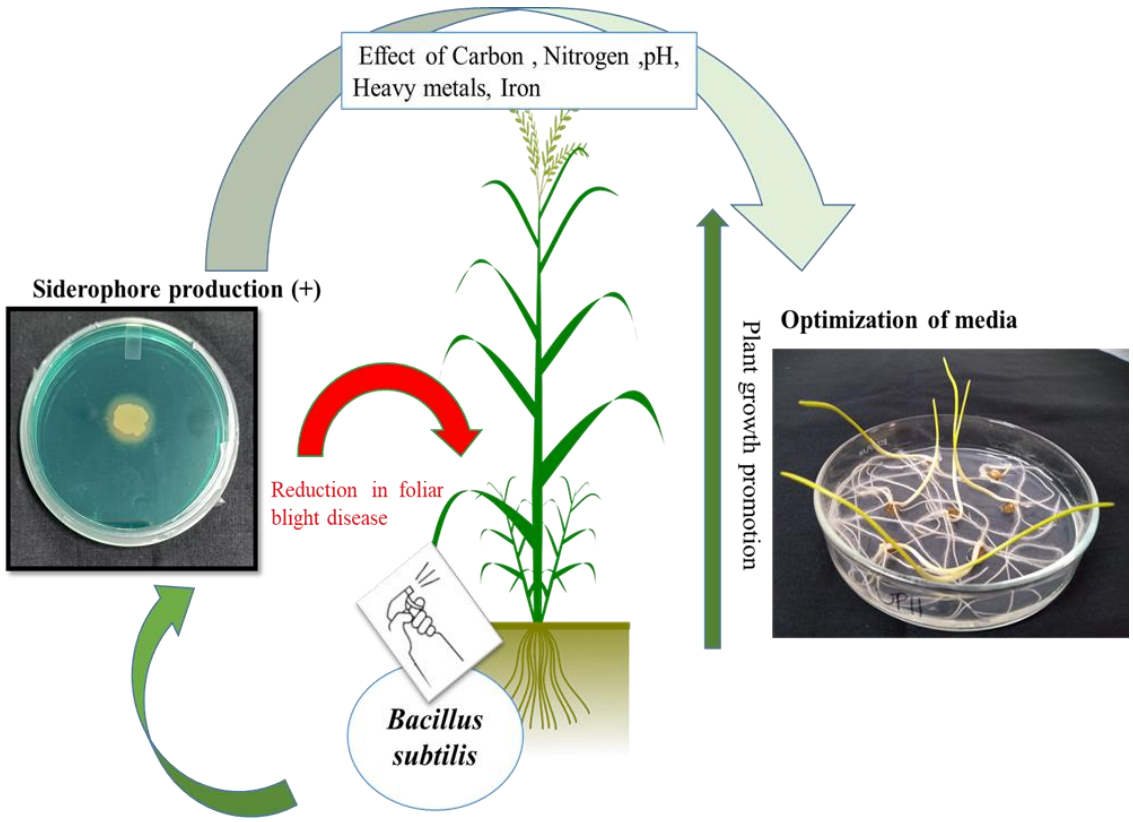
aimed to optimize culture conditions for siderophore production and investigate the antagonistic activity against foliar blight pathogens.

Work: The present study optimized the culture conditions for maximum siderophore production by evaluating various factors. The optimum pH for siderophore production was pH 7, yielding a maximum siderophore unit of 62.95%. Mannitol was identified as the best carbon source (59.14%), and ammonium nitrate as the best nitrogen source (63.04%). The presence of heavy metal $Pb(NO_3)_2$ in the succinate broth led to the highest yield (61.36%). The optimal iron concentration for siderophore production was determined to be 50 µM. The one-factor-at-a-time (OFAT) approach was used to investigate these parameters and their effects.

Result: The optimized siderophore was partially purified using ethyl acetate extraction. This extract exhibited significant mycelial fungal inhibition, with 59.64% and 50.88% inhibition against *Alternaria triticina* and *Bipolaris sorokiniana*, respectively. The filtrate was also tested for its germination potential, demonstrating its dual role as a biocontrol and plant growth-promoter. The filtrate enhanced the percentage of germination, root and shoot length.

Conclusion: Our findings indicate that *Bacillus subtilis* (UP11) can produce substantial quantities of siderophores, which exhibit potent antagonistic activity against foliar blight pathogens and promote plant growth. This study holds promise for advancing the development of novel biological control agents, offering the dual benefits of disease control and plant growth promotion.

GRAPHICAL ABSTRACT



Siderophore producing bacteria (*Bacillus subtilis*) shows both antifungal and plant growth promoting activity.

Keywords: Siderophore unit; Bacillus subtilis; antagonistic; antifungal.

1. INTRODUCTION

"Rhizosphere microbiota benefit plants through direct and indirect mechanisms. Direct benefits include the production of phytohormones, siderophores, antioxidants, cell wall-degrading enzymes, and volatile organic compounds (VOCs). Additionally, they enhance plant health by solubilizing minerals such as phosphorus, potassium, and zinc, and by fixing biological

nitrogen. Indirect benefits are conferred through antibiosis, induced systemic resistance, and the formation of biofilms" [1]. "Microorganisms produce various secondary metabolites for growth and survival. During iron stress, they produce secondary metabolites termed siderophores. Siderophore-producing plant growth-promoting rhizobacteria (PGPRs) play a significant role in pathogen biocontrol by competing for iron (Fe3+)" [2].

"Iron is crucial for the growth and survival of most microorganisms. At acidic pH (pH < 7), Fe^{2+} is prevalent in anaerobic conditions and more soluble in aerobic conditions but easily oxidized to Fe^{3+}, which precipitates. At physiological pH (7.0–7.4), Fe^{3+} (10^{-18} M at pH 7.0) predominates but is not readily accessible without a chelating agent, making it a primary growth-limiting factor for most organisms" [3]. "Microbes have developed unique strategies to absorb Fe, including producing and secreting Fe^{+3}-chelating molecules called siderophores [4]. Siderophores are small, low molecular weight secondary metabolites (400–2,000 Da) with a strong affinity for Fe^{3+}. They are produced and secreted by microorganisms to sequester iron from their surroundings as needed" [5]. "In aerobic environments, where Fe^{2+} is scarce, bacteria must actively uptake Fe^{3+} to maintain cellular iron homeostasis. Based on their iron-binding moieties, siderophores can be classified into three basic types: catecholate, hydroxymate, and carboxylate and mixed types" [6]. "In iron-deficient environments, microorganisms produce siderophores to acquire iron for growth. Some plant growth-promoting rhizobacteria (PGPR) synthesize siderophores, enabling them to compete with pathogens for iron and inhibit pathogen growth" [7]. **"**We previously reported that *Bacillus subtilis* used biocontrol bacterium against foliar blight pathogens" [8,9]. The primary objective of this study was to identify and maximize siderophores produced by *Bacillus subtilis.*

"Various factors, including carbon source, nitrogen source, pH, temperature, and metal ions, influence siderophore synthesis" [10]. "Additionally, carbon and nitrogen sources play vital roles; for instance, maltose as a carbon source and NH_4NO_3 as a nitrogen source significantly enhance siderophore production in endophytic fungi from *Cymbidium aloifolium"* [11]. Optimal conditions for maximum siderophore production vary between strains. For example, Bendale found that *Streptomyces fulvissimus* achieves peak siderophore production with sucrose as the carbon source [12], whereas Santos identified glycerol as optimal for *Bacillus megatherium* growth [13]. "*Bacillus* sp. PZ-1 showed highest siderophore production with a glucose concentration of 21.84 g/L, B. *brevis* sucrose as carbon source and asparagine as nitrogen" [14,15]. "pH is critical; plays a sensitive role, neutral pH generally supports maximum siderophore production in fluorescent pseudomonads" [16,17]. "Iron availability crucially impacts organism growth by influencing siderophore production. Furthermore, the presence of metal ions in the medium, such as cadmium, influences siderophore production in *Streptomyces tendae*, $MnCl_2$ in *P. monteilii* B8" [18,10].

"Bacteria that produce siderophores promote plant growth, protect against fungal infections, and degrade pesticides" [19]. "Siderophores enhance iron bioavailability and benefit plant roots, helping soil microbes combat iron deficiency in tough environments. The exact mechanism is unclear, but two possible methods include plant transport systems receiving Fe^{+2} from microbial siderophores and microbial siderophores chelating Fe from soils to exchange ligands with phytosiderophores" [20]. "Siderophore-producing bacteria are crucial for enhancing crop nutrition and suppressing phytopathogens" [21], (Sayyed et al., 2013). Ghazy and El-Nahrawy [22] studied "*Bacillus subtilis* MF497446 and *Pseudomonas koreensis* MG209738, showing their efficacy against *Cephalosporium maydis* in maize". Additionally, Wang et al. [23] demonstrated that "siderophore-producing *Pseudomonas putida* controls *Fusarium* wilt in cucumber, radish, and flax, highlighting their role as effective biocontrol agents (PGPB)". Siderophore-producing bacteria offer a sustainable alternative to chemical pesticides in agriculture. They help immobilize toxic metals in soil [24,25] and assist in bioremediation efforts [26]. "Commercializing these bacteria as bio-inoculants can effectively control fungal diseases by limiting iron availability to pathogens [27,28], promoting sustainable agriculture" [29]. Our study reported that *Bacillus subtilis* produce hydroxymate-catechol mixed type siderophores and has a strong antagonistic effect on wheat foliar blight fungal pathogens. However, in this study also included the factor that influence the production of siderophore production and their effect on antifungal and seed germination were also evaluated.

2. MATERIALS AND METHODS

2.1 Antagonistic Strains and Medium

Antagonistic wheat rhizobacteria strains (UP11) *Bacillus subtilis* accession no MN099431.1 previously isolated and stored at the Rhizosphere Biology Laboratory, Department of Microbiology, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand were cultured and maintained on Nutrient Agar (NA).

2.2 Chrome Azurol S (CAS) Medium Assay

The CAS agar plate method [30] was used to qualitatively assay siderophore production. Strains were grown in Nutrient Broth (NB) for 48 hours at 30°C and 120 rpm, then 0.05 ml of a suspension (2 × 10^8 cells/ml) was spotted onto CAS-agar plates and incubated for 5 days at 30°C. Clear halo around colonies indicated siderophore production.

2.2.1 Quantitative assay

To quantify siderophore activity in culture supernatant extracts, the CAS solution method [30] was used. Tested strains were grown in Nutrient Broth for 48 hours on a rotary shaker at 30°C and 120 rpm, then centrifuged for 15 minutes at 3000 rpm. A mixture of 0.5 ml of supernatant (2 × 10^8 cells/ml), 0.5 ml CAS solution, and 10 µl shuttle solution (sulfosalicylic acid) was incubated at room temperature for 2 hours. Absorbance was measured at 630 nm using a UV/Vis spectrophotometer. Reference samples (blanks) were prepared with Nutrient Broth medium, CAS solution, and shuttle solution. Siderophore units (SU) were calculated using the formula SU = [(Ar - As) / Ar] × 100%, where Ar represents the reference absorbance and As represents the sample absorbance.

2.3 Characterization of Siderophores

2.3.1 Hydroxamate

Tetrazolium salt test: A small amount of tetrazolium salt and 1-2 drops of 2N NaOH were added to 0.1 mL of the test culture supernatant. The immediate appearance of a red to deep-red color indicated the presence of hydroxamate siderophores [31].

2.3.2 Catecholate

Arnow's test: 1 mL of the culture supernatant, 0.1 mL of 0.5 M HCl is added, followed by 1 mL of nitrate moly date reagent and 1 mL of NaOH. The presence of catecholate in the supernatant is indicated by a color change from yellow to bright orange-red after 5 minutes of incubation [32].

2.3.3 Carboxylate

Vogel's chemical test: Prepare a test reagent by adding 3 drops of 2N NaOH to 1 drop of phenolphthalein. Add distilled water until the solution turns pink. Add the test sample to this solution. If the pink color disappears, it indicates the presence of carboxylate siderophores.

2.4 Effect of Different Medium on Siderophore Production

Bacillus subtilis was separately inoculated into nutrient broth, Luria-Bertani (LB) medium, *Bacillus* medium broth and succinate medium. Each medium was inoculated with a loopful of bacteria and incubated at 37°C for 48-72 hours in a rotary shaker set at 120 rpm. After incubation, the cultures were centrifuged to obtain the culture filtrate. 1 ml of cell filtrate was mixed with 1 ml of CAS (Chrome Azurol S) solution. The mixture was thoroughly mixed, and the absorbance was measured at 630 nm using a spectrophotometer. The percentage of siderophore units was then calculated based on the absorbance readings.

2.5 Optimization of the Cultural Conditions for Siderophore Production

One factor at a time (OFAT) was conducted to investigate the impact of various physicochemical parameters—including pH, carbon source, nitrogen source, and heavy metal, iron concentration on siderophore production of the UP11 strain.

2.5.1 Effect of carbon and nitrogen sources

The effects of different carbon sources including sucrose, glucose, maltose, sucrose, fructose, lactose, and mannitol as well as different nitrogen sources such as sodium nitrate, ammonium sulfate, soybean meal powder, yeast extract, ammonium nitrate, peptone, and potassium nitrate were investigated in succinate

broth (100 ml) by supplemented with 1 g/l of these different carbon and nitrogen sources separately. Then flasks were incubated at 37°C for 48–72 hours at 120 rpm. After incubation, 1 ml of the culture filtrate was mixed with 1 ml of CAS solution, and the absorbance was measured at 630 nm to calculate the percentage of siderophore units [33,34].

2.5.2 Effect of pH and heavy metals

The pH of 100 ml of succinate broth was adjusted to 3.0, 5.0, 7.0, 9.0, and 11.0. To investigate the effect of metal ions, the succinate broth was supplemented with 10 µM of $HgCl_2$, $ZnSO_4$, $MnCl_2$, $CdCl_2$, $MnSO_4$, and $NiCl_2$ incubated at 37°C for 48–72 hours. The percentage of siderophore units was measured at 630 nm [35].

2.5.3 Effect of iron concentration

Log-phase bacterial cultures were separately inoculated into succinate broth containing varying concentrations of $FeCl_3 \cdot 6H_2O$ (0, 25, 50, 100, and 150 µM) and incubated at 37°C for 48–72 hours at 120 rpm to identify threshold level that inhibits siderophore production. Siderophore production was calculated as a percentage of siderophore units [36].

2.6 Preliminary Purification of Siderophore

After a 48-hour incubation at 30°C and 200 rpm, the liquid culture was centrifuged at 1,000 ×g for 10 minutes to collect the cell-free supernatant. It was tested for siderophores using the CAS assay. This supernatant was further purified by extraction with ethyl acetate. The pH of the supernatant was adjusted to 2 - 2.5 using strong acids before being quickly subjected to ethyl acetate extraction. The organic fractions were collected and dried by evaporation. The resulting dried siderophore crystals were suspended in sterile double-distilled water. The purified siderophore solutions were subsequently used in antifungal experiments.

2.7 Antifungal Activity of Purified Siderophore

The purified siderophore was tested for antifungal activity against foliar blight fungal plant pathogens, *Alternaria triticina* and *Bipolaris sorokiniana* on potato dextrose agar. The fungal mycelium (5 mm) were placed onto center then 5 mm wells were prepared. These wells were then filled with the purified siderophore and incubated at 28°C ± 2°C for 6 – 7 days. After the incubation, fungal growth inhibition was observed.

2.8 Seed Germination

To evaluate the effect of bacterial siderophores on seed germination, wheat seeds were collected from a Norman E. Borlaug Crop Research Centre U.S. Nagar, Uttarkhand. Healthy seeds of similar sizes and shapes were selected for the experiments. The seeds were surface-sterilized by soaking in 75% ethanol, repeatedly washing with sterile deionized water to remove any chemicals, and drying with tissue paper. Fifteen seeds were soaked in the cell-free supernatant of the siderophore production medium in a Petri dish and incubated at 25°C for 1-2 hours. Seedlings were monitored and considered germinated when the root protruded at least 2 mm from the seed coat. Seedlings per plate were randomly selected to measure root and shoot lengths. The germination percentage was calculated using Equation [37].

Germination percentage (% GP) = (Seeds germinated/Total seeds) × 100

2.9 Statistical Analysis

All experiments were conducted in triplicates. Statistical analysis of the data was done using Statistical Package for Social Studies (SPSS), version 21.0. software. All data are expressed as means ± SE.

3. RESULTS AND DISCUSSION

3.1 Qualitative and Quantitative Estimation of Siderophore

Clear halo zone was observed on chrome-azurol S-agar medium (CAS) agar medium shows the strain UP11 was siderophore positive. CAS–Fe^{+3}–HDTMA complex was broke by siderophores and formed Fe^{+3}–siderophore complex. As dye released, resulted in clear zone formation [8,38]. The quantitative estimations were found to produce siderophores in the range of 53.26 ± 0.72 siderophore units (Fig. 1).

3.2 Identification of Type of Siderophore Production

The immediate appearance of a red to deep-red color confirmed the presence of hydroxamate

siderophores. *B. subtilis* had a strong reaction to tetrazolium tests and Arnow's, which indicate the presence of a hydroxamate- catecholate type siderophore in the supernatant of the culture. this indicating the occurrence of a mixed-type siderophore (both catecholate and hydroxamate). The no disappearance of the pink color upon adding the test sample to the prepared reagent confirmed the absence of carboxylate siderophores (Table 1, Fig. 2).

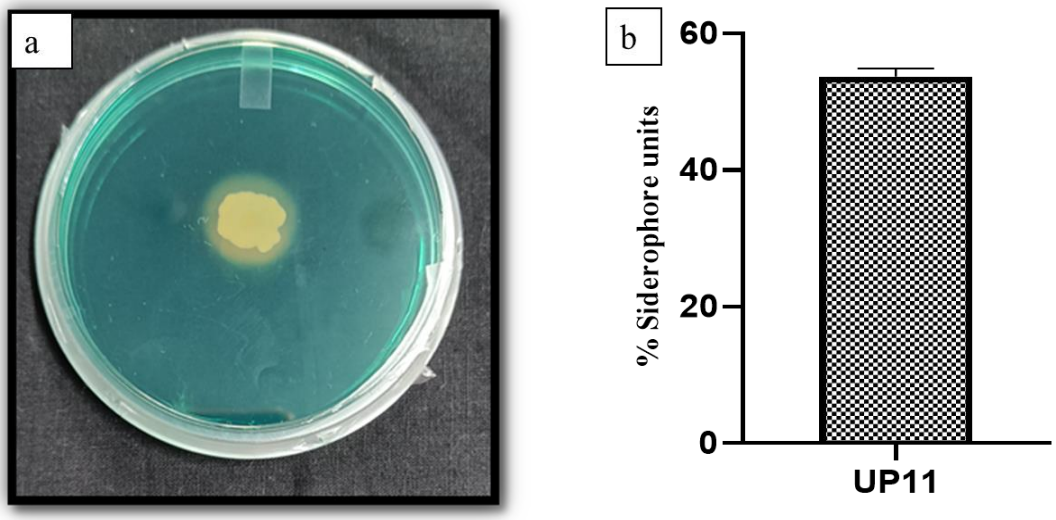


Fig. 1. Qualitative and Quantitative estimation of siderophore of *Bacillus subtilis* (a) Chrome-azurol S-agar medium plate assay for siderophore production (b) % Siderophore unit of UP11

Table 1. Characterization of siderophore type in endophytic strain UP11 (*Bacillus subtilis*)

Strain	Peak at 425 nm (Hydroxamate)	Peak at 495 nm (catecholate)	Hydroxamate (Tetrazolium test)	Catecholate (Arnow's test)	Carboxylate (Vogel's test)
UP11	+	+	+	+	-

Catecholate, carboxylate and hydroxamate-type siderophores were identified using Arnow´, Vogel's and Tetrazolium test tests, respectively. + positive result; − negative result

Fig. 2. Positive results of different types of siderophore produced by *Bacillus subtilis*

3.3 Influence of Different Media on Siderophore Production

Bacillus subtilis shows the maximum % siderophore unit in succinate medium (67.20%) followed upto on *Bacillus* broth (55.06%), LB (50.37%), NB (47.82%). Before optimizing physicochemical parameters, it is crucial to optimize the nutrient media to ensure maximum growth of the test culture and, consequently, maximum siderophore production. Among the four different nutrient media tested, succinate medium yielded the highest siderophore production at 630 nm (Fig. 3). Similarly, a study reported that succinate B medium to be ideal for siderophore production by P. aeruginosa KSB, *P. aeruginosa* PAUTI4, *P. aeruginosa* PB19, *B. amyloliquefaciens, Bacillus subtilis* MF497446, and *Pseudomonas koreensis* [39,10,22]. So, succinate media is further used for optimization culture condition for siderophore production.

3.4 Optimization of Culture Condition for Siderophore Production

The literature indicates that several factors, such as initial pH, carbon source, nitrogen source, iron concentration and metal ions, influence siderophore synthesis and secretion [10]. Additionally, these conditions can vary significantly depending on the bacterial species and strain used to optimize siderophore production [15]. All these parameters and their effects were investigated using the one-factor-at-a-time (OFAT) approach (Fig. 4).

Therefore, the optimum pH of 7 was found to yield the maximum siderophore unit (62. 95%). pH significantly affects siderophore production and iron solubility [40]. Generally, a neutral pH (~7) is favorable for maximum siderophore production in most bacteria and fungi [41]. This study observed peak production at pH 7, consistent with similar findings in *Bacillus subtilis* [42,4,43] and *B. brevis* GZDF3, as well as fluorescent pseudomonads [15,44], where neutral pH supports optimal growth and siderophore production.

The carbon and nitrogen sources in the culture medium significantly influence microbial metabolism and siderophore production [40,15]. Different carbon sources lead to varying rates of siderophore production, affecting the quality of the medium and bacterial metabolism [45]. Selecting a suitable carbon source is crucial for optimal siderophore production [46]. For instance, glucose, sucrose, and dextrose produce intermediate amounts of siderophores in *P. fluorescens* [13], while *P. aeruginosa* FP6 shows increased production with sucrose and mannitol [47]. Similarly, *Bacillus licheniformis*, *Bacillus subtilis*, and *Ochrobactrum grignonense* exhibit the highest siderophore production with sucrose, and glycerol enhances production in *Bacillus megaterium*, *Bacillus spp*., and *Escherichia coli* more effectively than galactose, lactose, or maltose [48,14,13]. The nitrogen source is a key factor influencing both bacterial growth and siderophore production, with its impact varying depending on whether the nitrogen is inorganic (e.g., sodium nitrate, ammonium nitrate) or organic (e.g., urea, peptone, yeast extract). For example, *B. subtilis* DR2 exhibited maximum siderophore production using $NaNO_3$ [49]. Additionally, yeast extract and urea enhanced siderophore production in *P. aeruginosa* [47], and sodium nitrate was effective for *Pseudomonas* strain JAS-25 [10]. *Bacillus* species utilize glucose, sucrose, and sodium nitrate [45], while *Bacillus* sp. PZ-1 uses asparagine as a nitrogen source [14]. In our study it was found that *Bacillus subtilis* uses mannitol as carbon source (59.14%), ammonium nitrate as nitrogen source (63.04%) to maximize the production of siderophore unit.

Metals can either stimulate or inhibit siderophore production and cell growth, depending on their type and concentration. Besides Fe^{+3}, trace amounts of metals like Mg^{+2}, Cu^{+2}, Mn^{+2}, Mo^{+4}, Ni^{+2}, and Zn^{+2} are essential as micronutrients, acting as co-factors in various microbial enzymes [47]. Notably, Pb has the highest affinity for siderophores and can gradually enhance their production by participating directly in siderophore biosynthesis or by competing with Fe-siderophore complexes, leading to Fe deficiency and increased siderophore synthesis [50]. *Bacillus cereus* has been shown to produce siderophores at different lead concentrations [51] Additionally, *Bacillus taeanensis* SMI_1, *Enterobacter* sp. AABM_9, and *Pseudomonas mendocina* demonstrated growth and siderophore production in the presence of metals like Fe^{+3}, Cu^{+2}, Mn^{+2}, and Zn^{+2} [52]. In present study it was found that the heavy metal $Pb(NO_3)_2$ shows (61.36%) highest yield of siderophore in the succinate broth.

In bacteria, the FUR (Ferric Uptake Regulator) system plays a key role in transporting the siderophore-Fe^{3+} complex. A high-affinity receptor outside the membrane transfers the complex into the cell, aided by siderophore

irrevocable proteins, permeases, and ATPases. In this study, iron concentration increased siderophore production by 56.96% in *Bacillus subtilis*, respectively. However, beyond a certain iron concentration, siderophore production decreased due to negative transcriptional regulation by the FUR protein. This decrease occurs because Fe^{2+} acts as a corepressor. Previous research has shown similar results, with siderophore production by *Pseudomonas fluorescence* decreasing at 1 µM iron concentration due to this regulation. For *P. monteilii* strain MN75947, 50 µM is ideal, while a minimum of 30 µM is required for *P. fluorescens* NCIM 5096 and *P. putida* NCIM 2847 [16,53]. Lower iron levels induce higher siderophore production to bind Fe3+ ions, but production sharply decreases beyond a threshold due to negative transcriptional regulation by FUR proteins, with Fe^{2+} acting as a cation [17]. *Bacillus cereus* and *B. subtilis* also show increased siderophore production with rising iron concentrations from 10 nM to 50 µM but decreases when the concentration of iron is enhanced above 50 µM [54,55].

3.5 Antifungal Activity of Crude Siderophore Filtrate

Siderophore produced by UP11 has a stronger antifungal effect shows fungal mycelial inhibition (59.64%, 50.88%) against *Alternaria triticina* and *Bipolaris sorokiniana* (Fig. 5). These results indicated that siderophore is a crucial component produced by UP11 and plays a significant role in antifungal activity. This effect is due to siderophores sequestering iron, depriving fungi of this essential nutrient and inhibiting their growth. Additionally, siderophores may directly interfere with fungal iron acquisition systems and enhance plant defenses, making plants more resistant to fungal infections [22]. A similar study by Khan et al. [56] reported that catecholate siderophore produced by *Escherichia coli* exhibited antifungal activity against *Aspergillus nidulans*. Numerous reports have suggested that siderophores produced by *P. fluorescens* MPF47, *P. putida*, *Alcaligenes faecalis* and *Pseudomonas syringae* BAF.1 demonstrate inhibitory activity against many fungal pathogens [57,14,58,59]. Additionally, siderophore-producing *Pseudomonas fluorescens* RB5 inhibited the phytopathogens *R. cerealis,* which causes wheat sheath blight [60].

This indicates a significant dependence of the organism on siderophores for iron acquisition. It was speculated that the iron stress induced by the bacterial catecholate- hydroxymate mixed type siderophore could not be countered by the fungus due to the lack of an alternative iron acquisition mechanism.

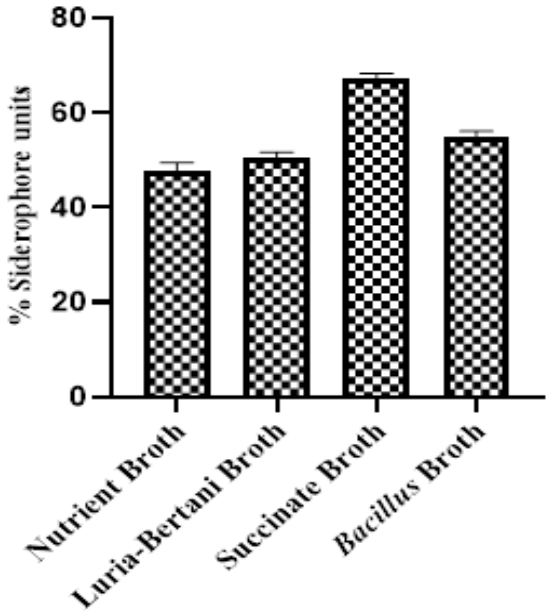


Fig. 3. Effect of different media on % Siderophore units of *Bacillus subtilis* strain. Values are presented as mean ± SE (n = 3)

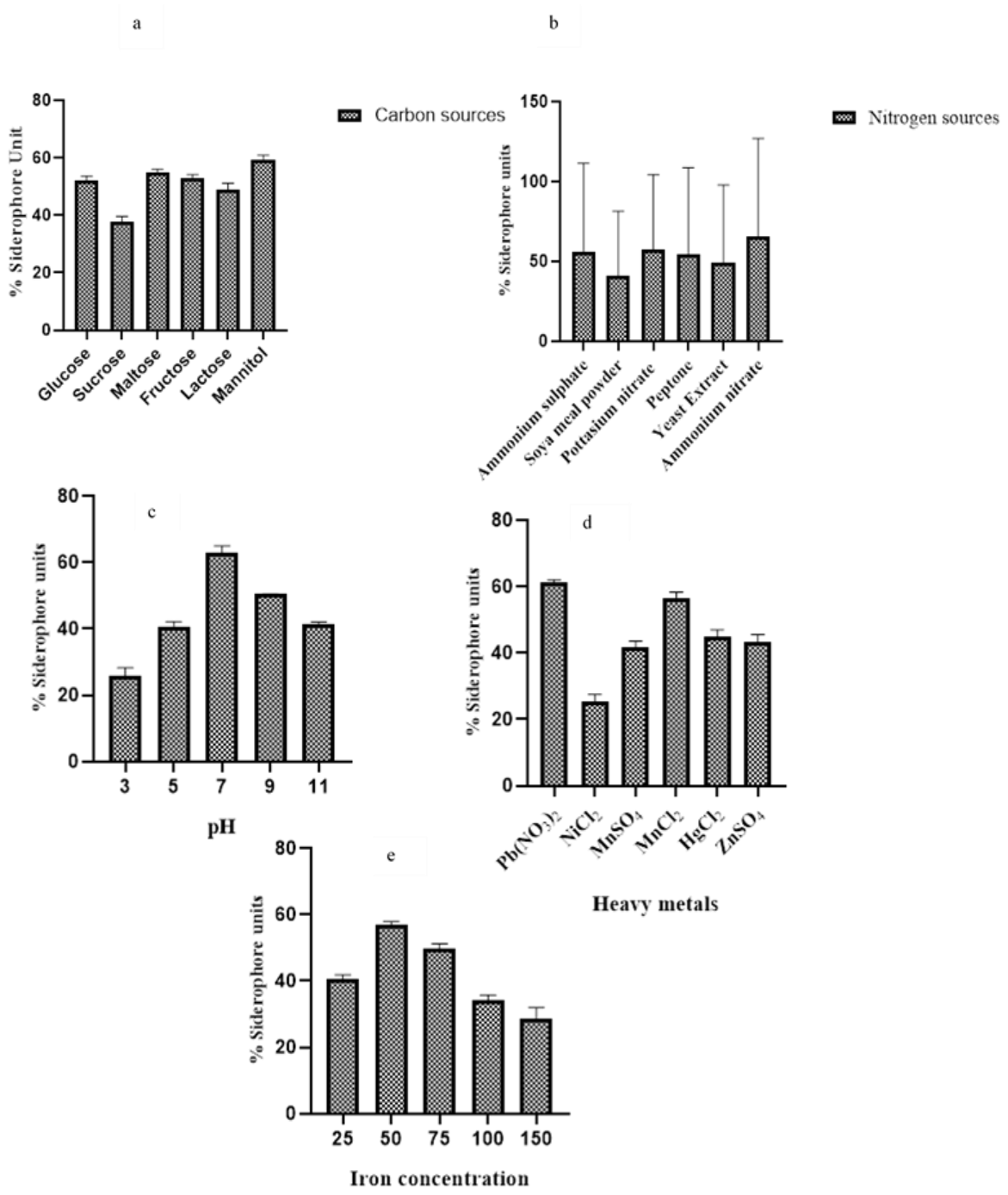


Fig. 4. Effect of physicochemical parameters (a) carbon (b) nitrogen sources, (c) initial pH (d) heavy metals (e) iron concentration on siderophore production of *Bacillus subtilis*

3.6 Seed Germination

This study investigated the effect of siderophores on the germination of wheat seeds. Seeds were incubated in siderophore-positive supernatant, with water-incubated seeds as controls. The siderophore presence was confirmed via the CAS assay, only siderophore-positive

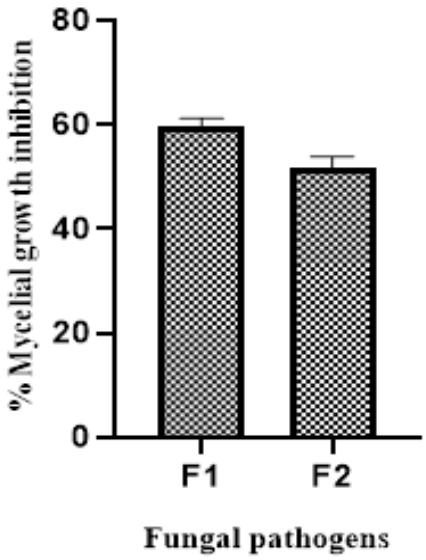


Fig. 5. Antagonistic activity of purified siderophore extract of UP11 against *Alternaria triticina* (F1) and *Bipolaris sorokiniana* (F2)

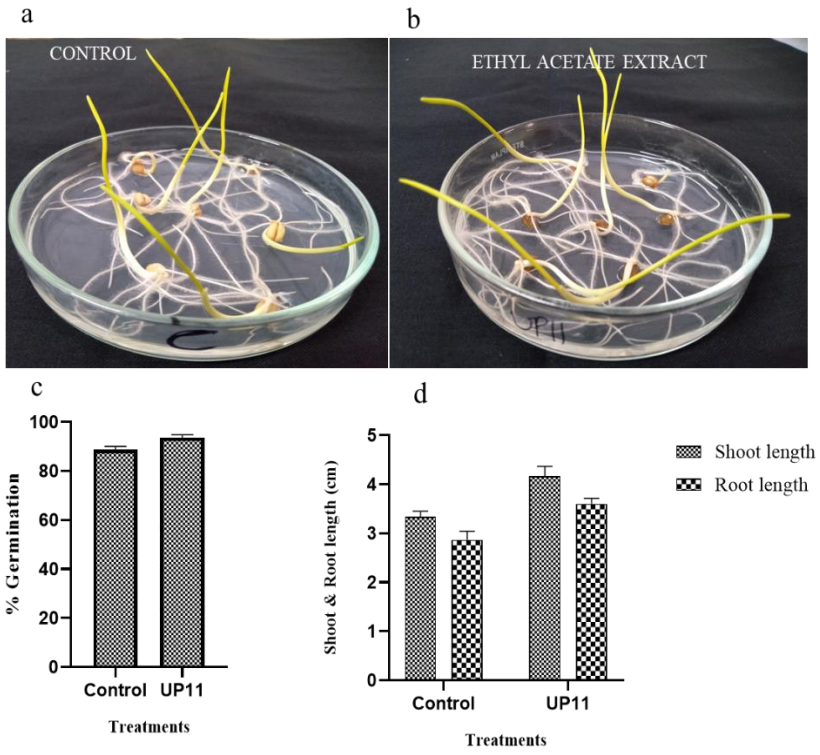


Fig. 6. *In vitro* seed bioassay to check the effect of partial purified siderophore on seeds (a) control (water) (b) Ethyl acetate extract of siderophore (c) percent germination (d) Shoot and root length. Values are presented as mean ± SE (n = 3)

supernatant was used. Germination percentage was assessed after 36 hours, with sprouting monitored and root length measured every 12 hours. *Bacillus subtilis* enhanced seedling growth i.e., root, shoot length and germination percentage for each seed with the respective

supernatant are shown in the Fig. 6. The siderophore positive *Bacillus subtilis* ethyl acetate filtrate shows the maximum germination root (3.60 cm) and shoot length (4.17 cm). The similar study was also reported by Yue et al., [61] under Fe limitation, siderophore-producing microorganisms, such as *Bacillus* sp. WR12, can be used as bio-fertilizers to enhance wheat growth by increasing Fe acquisition and alleviating growth. Similar study was also found in, *Enterobacter* AABM_9's siderophores significantly improved peanut seedling length compared to other legumes, with the effect varying based on seed-inoculant symbiosis [52,62]. Thus, siderophore-producing bacteria can serve as bio-fertilizers to boost plant growth and as biocontrol agents against plant pathogens.

4. CONCLUSIONS

Iron is an essential and abundant metal on Earth's surface, but it is unavailable to living organisms at physiological pH. Under iron-limiting conditions, siderophore-producing microorganisms can act as bio-fertilizers to enhance wheat growth. Iron deficiency hampers growth and impairs photosynthesis. *Bacillus* sp. WR12 has been shown to improve iron acquisition and mitigate these adverse effects [61]. Our study found that *Bacillus subtilis* as an effective siderophore producing bacterium and it produces mixed siderophore types that enhance the antifungal as well as elevated the plant growth of wheat. So, it induces the disease resistance of foliar blight disease in wheat plant and enhancement of growth promotion and yield. Further research is needed to explore gene-level interactions related to siderophore production in plant growth-promoting rhizobacteria (PGPR), pathogenic fungi, and host plants.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

DATA AVAILABILITY STATEMENT

All the data are presented in the paper.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Singh P, Chauhan PK, Upadhyay SK, Singh RK, Dwivedi P, Wang J, et al. Mechanistic insights and potential use of siderophores producing microbes in rhizosphere for mitigation of stress in plants grown in degraded land. Frontiers in Microbiology. 2022;13:898979.
2. Murthy KN, Soumya K, Udayashankar AC, Srinivas C, Jogaiah S. Biocontrol potential of plant growth-promoting rhizobacteria (PGPR) against *Ralstonia solanacearum*: Current and future prospects. In Biocontrol Agents and Secondary Metabolites. Woodhead Publishing. 2021;153-180.
3. Kalyan VRK, Meena S, Karthikeyan S, Jawahar D. Isolation, screening, characterization, and optimization of bacteria isolated from calcareous soils for siderophore production. Archives of Microbiology. 2022;204(12),
4. Nithyapriya S, Lalitha S, Sayyed RZ, Reddy MS, Dailin DJ, El Enshasy HA, et al. Production, purification, and characterization of bacillibactin siderophore of Bacillus subtilis and its application for improvement in plant growth and oil content in sesame. Sustainability. 2021;13(10):5394.
5. Albelda-Berenguer M, Monachon M, Joseph E. Siderophores: From natural roles to potential applications. Advances in Applied Microbiology. 2019;106:193-225.
6. Khan A, Gupta A, Singh P, Mishra AK, Ranjan RK, Srivastava A. Siderophore-assisted cadmium hyperaccumulation in *Bacillus subtilis*. International Microbiology. 2020;23:277-286.
7. Leventhal GE, Ackermann M, Schiessl KT. Why microbes secrete molecules to modify their environment: The case of iron-chelating siderophores. Journal of the Royal Society Interface. 2019;16(150): 20180674.
8. Saini N, Sahgal M, Singh AV. Mechanisms of cell wall degrading enzymes from *Bacillus methylotrophicus* and *Bacillus subtilis* in suppressing foliar blight pathogens. Journal of Advances in Biology & Biotechnology. 2024;27(8):239-249.
9. Jaggi V, Joshi S, Dasila H, Pareek N, Sahgal M. Functional and molecular characterization of wheat rhizosphere bacteria and their antagonistic activity

against wheat foliar blight pathogens; 2020.
10. Srivastava P, Sahgal M, Sharma K, Enshasy HAE, Gafur A, Alfarraj S, et al. Optimization and identification of siderophores produced by Pseudomonas monteilii strain MN759447 and its antagonism toward fungi associated with mortality in Dalbergia sissoo plantation forests. Frontiers in Plant Science. 2022; 13:984522.
11. Chowdappa S, Jagannath S, Konappa N, Udayashankar AC, Jogaiah S. Detection and characterization of antibacterial siderophores secreted by endophytic fungi from *Cymbidium aloifolium*. Biomolecules. 2020;10(10):1412.
12. Bendale MS, Chaudhari BL, Chincholkar SB. Influence of environmental factors on siderophore production by *Streptomyces fulvissimus* ATCC 27431. Current Trends in Biotechnology and Pharmacy. 2009; 3(4):362-371.
13. Santos S, Neto IF, Machado MD, Soares HM, Soares EV. Siderophore production by Bacillus megaterium: Effect of growth phase and cultural conditions. Applied Biochemistry and Biotechnology. 2014;172:549-560.
14. Yu S, Teng C, Liang J, Song T, Dong L, Bai X, et al. Characterization of siderophore produced by *Pseudomonas syringae* BAF. 1 and its inhibitory effects on spore germination and mycelium morphology of *Fusarium oxysporum*. Journal of Microbiology. 2017;55:877-884.
15. Sheng M, Jia H, Zhang G, Zeng L, Zhang T, Long Y, et al. Siderophore production by rhizosphere biological control bacteria Brevibacillus brevis GZDF3 of *Pinellia ternata* and its antifungal effects on *Candida albicans*. Journal of Microbiology and Biotechnology. 2020;30(5):689.
16. Sayyed RZ, Badgujar MD, Sonawane HM, Mhaske MM, Chincholkar SB. Production of microbial iron chelators (siderophores) by fluorescent Pseudomonads; 2005.
17. Tailor AJ, Joshi BH. Characterization and optimization of siderophore production from *Pseudomonas fluorescens* strain isolated from sugarcane rhizosphere. Journal of Environmental Research and Development. 2012;6(3A):688-694.
18. Sayyed RZ, Chincholkar SB, Reddy MS, Gangurde NS, Patel PR. Siderophore producing PGPR for crop nutrition and phytopathogen suppression. In Bacteria in agrobiology: Disease management. Berlin, Heidelberg: Springer Berlin Heidelberg. 2012;449-471.
19. Yadav R, Singh AV, Kumar M, Yadav S. Phytochemical analysis and plant growth promoting properties of endophytic fungi isolated from Tulsi and Aloe vera. Int J Agric Stat Sci. 2016;12(1):239-248.
20. Sun Y, Wu J, Shang X, Xue L, Ji G, Chang S, et al. Screening of siderophore-producing bacteria and their effects on promoting the growth of plants. Current Microbiology. 2022;79(5):150.
21. Yasmin S, Alcazar-Fuoli L, Gründlinger M, Puempel T, Cairns T, Blatzer M, et al. Mevalonate governs interdependency of ergosterol and siderophore biosyntheses in the fungal pathogen *Aspergillus fumigatus*. Proceedings of the National Academy of Sciences. 2012;109(8):E497-E504.
22. Ghazy N, El-Nahrawy S. Siderophore production by Bacillus subtilis MF497446 and Pseudomonas koreensis MG209738 and their efficacy in controlling *Cephalosporium maydis* in maize plant. Archives of microbiology. 2021;203(3): 1195-1209.
23. Wang Y, Huang W, Li Y, Yu F, Penttinen P. Isolation, characterization, and evaluation of a high-siderophore-yielding bacterium from heavy metal–contaminated soil. Environmental Science and Pollution Research. 2022;29:3888-3899.
24. Trindade IB, Hernandez G, Lebègue E, Barrière F, Cordeiro T, Piccioli M, Louro RO. Conjuring up a ghost: Structural and functional characterization of FhuF, a ferric siderophore reductase from *E. coli*. JBIC Journal of Biological Inorganic Chemistry. 2021;26:313-326.
25. Handore AV, Khandelwal SR, Karmakar R, Handore DV. Exploration of bacterial siderophores for sustainable future. In Climate Change and Microbial Diversity. Apple Academic Press. 2022; 163-189.
26. Rani R, Kumar V, Gupta P. Plant growth-promoting rhizobacteria-assisted bioremediation of toxic contaminant: Recent advancements and applications. In Microbial Biodegradation and Bioremediation. Elsevier. 2022;327-341.
27. Basu A, Prasad P, Das SN, Kalam S, Sayyed RZ, Reddy MS, El Enshasy H. Plant growth promoting rhizobacteria (PGPR) as green bioinoculants: Recent

developments, constraints, and prospects. Sustainability. 2021;13(3):1140.
28. Malgioglio G, Rizzo GF, Nigro S, Lefebvre du Prey V, Herforth-Rahmé J, Catara V, Branca F. Plant-microbe interaction in sustainable agriculture: The factors that may influence the efficacy of PGPM application. Sustainability, 2022;14(4): 2253.
29. Khan A, Singh AV, Kumar R, Kukreti B, Bundela V, Upadhayay VK. Relative impact of PGPR inoculation on biofortification and yield of wheat under field conditions and their performance assessment through statistical tools. Pharma Innovation. 2022;11(8):490-495.
30. Schwyn B, Neilands J. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry. 1987;160(1):47-56.
31. Rashmi V, Shylaja Naciyar M, Rajalakshmi R, D'Souza SF, Prabaharan D, Uma L. Siderophore mediated uranium sequestration by marine cyanobacterium *Synechococcus elongatus* BDU 130911. Bioresource Technology. 2013;130:204-210.
32. Arnow BYLE. Hydrochloric acid and enough distilled water to make a volume of 1 Liter. Preserve under Toluene. 531. Comp. A J. Comp. Educ. 1937;531-537.
33. Waday YA, Girma Aklilu E, Bultum MS, Ramayya Ancha V, Beyene D. Isolation and characterization of plant growth-promoting rhizobacteria from coffee plantation soils and its Influence on Maize Growth. Applied and Environmental Soil Science. 2022;2022(1):5115875.
34. Cornu JY, Gutierrez M, Randriamamonjy S, Gaudin P, Ouedraogo F, Sourzac M, et al. Contrasting effects of siderophores pyoverdine and desferrioxamine B on the mobility of iron, aluminum, and copper in Cu-contaminated soils. Geoderma. 2022; 420:115897.
35. Wang H, Liu R, You MP, Barbetti MJ, Chen Y. Pathogen biocontrol using plant growth-promoting bacteria (PGPR): Role of bacterial diversity. Microorganisms. 2021; 9(9):1988.
36. Patel N. Siderophores. Practical Handbook on Agricultural Microbiology. 2022;351-359.
37. Kumar P, Thakur S, Dhingra GK, Singh A, Pal MK, Harshvardhan K, et al. Inoculation of siderophore producing rhizobacteria and their consortium for growth enhancement of wheat plant. Biocatalysis and Agricultural Biotechnology. 2018;15:264-269.
38. Murakami C, Tanaka AR, Sato Y, Kimura Y, Morimoto K. Easy detection of siderophore production in diluted growth media using an improved CAS reagent. Journal of Microbiological Methods. 2021; 189:106310.
39. Bindu P, Nagendra PG. Siderophore production by *Pseudomonas aeruginosa* isolated from the paddy fields of Kuttanad, Kerala. Int J Sci Res. 2016;5:1577-1581.
40. Singh P, Khan A, Kumar R, Kumar R, Singh VK, Srivastava A. Recent developments in siderotyping: Procedure and application. World Journal of Microbiology and Biotechnology. 2020; 36:1-15.
41. Soares EV. Perspective on the biotechnological production of bacterial siderophores and their use. Applied Microbiology and Biotechnology. 2022;106(11):3985-4004.
42. Miethke M, Marahiel MA. Siderophore-based iron acquisition and pathogen control. Microbiology and Molecular Biology Reviews. 2007;71(3):413-451.
43. Kumari S, Kumar P, Kiran S, Kumari S, Singh A. Optimization of siderophore production by *Bacillus subtilis* DR2 and its effect on growth promotion of Coriandrum sativum. Russian Agricultural Sciences. 2022;48(6):467-475.
44. Le VVH, Olivera C, Spagnuolo J, Davies IG, Rakonjac J. *In vitro* synergy between sodium deoxycholate and furazolidone against enterobacteria. BMC Microbiology. 2020;20:1-11.
45. Kumar V, Menon S, Agarwal H, Gopalakrishnan D. Characterization and optimization of bacterium isolated from soil samples for the production of siderophores. Resource-Efficient Technologies. 2017;3(4):434-439.
46. Vindeirinho JM, Soares HM, Soares EV. Modulation of siderophore production by *Pseudomonas fluorescens* through the manipulation of the culture medium composition. Applied Biochemistry and Biotechnology. 2021;193:607-618.
47. Sasirekha B, Srividya S. Siderophore production by Pseudomonas aeruginosa FP6, a biocontrol strain for *Rhizoctonia*

solani and *Colletotrichum gloeosporioides* causing diseases in chilli. Agriculture and Natural Resources. 2016;50(4):250-256.
48. Valdebenito M, Crumbliss AL, Winkelmann G, Hantke K. Environmental factors influence the production of enterobactin, salmochelin, aerobactin, and yersiniabactin in *Escherichia coli* strain Nissle 1917. International Journal of Medical Microbiology. 2006;296(8):513-520.
49. Singh A, Mishra AK. Influence of various levels of iron and other abiotic factors on siderophorogenesis in paddy field cyanobacterium *Anabaena oryzae*. Applied biochemistry and biotechnology. 2015; 176:372-386.
50. Naik MM, Dubey SK. Lead-enhanced siderophore production and alteration in cell morphology in a Pb-resistant *Pseudomonas aeruginosa* strain 4EA. Current Microbiology. 2011;62:409-414.
51. Perez-Cordero A, Montes-Vergara D, Aguas-Mendoza Y. *In-vitro* evaluation of siderophore production by bacteria in the presence of heavy metal. Webology. 2022;19(5).
52. Sarvepalli M, Velidandi A, Korrapati N. Optimization of siderophore production in three marine bacterial isolates along with their heavy-metal chelation and seed germination potential determination. Microorganisms. 2023;11(12):2873.
53. Sayyed RZ, Seifi S, Patel PR, Shaikh SS, Jadhav HP, Enshasy HE. Siderophore production in groundnut rhizosphere isolate, *Achromobacter sp.* RZS2 influenced by physicochemical factors and metal ions. Environmental Sustainability. 2019;2(2):117-124.
54. Sinha AK, Parli Venkateswaran B, Tripathy SC, Sarkar A, Prabhakaran S. Effects of growth conditions on siderophore producing bacteria and siderophore production from Indian Ocean sector of Southern Ocean. Journal of Basic Microbiology. 2019;59(4):412-424.
55. Rizzi A, Roy S, Bellenger JP, Beauregard PB. Iron homeostasis in *Bacillus subtilis* requires siderophore production and biofilm formation. Applied and Environmental Microbiology. 2019;85(3): e02439-18.
56. Khan A, Singh P, Kumar R, Das S, Singh RK, Mina U, et al. Antifungal activity of siderophore isolated from *Escherichia coli* against *Aspergillus nidulans* via iron-mediated oxidative stress. Frontiers in Microbiology. 2021;12:729032.
57. Solanki MK, Singh RK, Srivastava S, Kumar S, Kashyap PL, Srivastava AK, Arora DK. Isolation and characterization of siderophore producing antagonistic rhizobacteria against *Rhizoctonia solani*. Journal of Basic Microbiology. 2014; 54(6):585-597.
58. Yang X, Yousef AE. Antimicrobial peptides produced by *Brevibacillus spp*.: Structure, classification and bioactivity: A mini review. World Journal of Microbiology and Biotechnology. 2018;34:1-10.
59. Sayyed RZ, Chincholkar SB. Siderophore-producing *Alcaligenes feacalis* exhibited more biocontrol potential vis-à-vis chemical fungicide. Current microbiology. 2009; 58(1):47-51.
60. Yi Y, Hou Z, Shi Y, Zhang C, Zhu L, Sun X, et al. Pseudomonas fluorescens RB5 as a biocontrol strain for controlling wheat sheath blight caused by Rhizoctonia Cerealis. Agronomy. 2023; 13(8):1986.
61. Yue Z, Chen Y, Hao Y, Wang C, Zhang Z, Chen C, et al. *Bacillus sp*. WR12 alleviates iron deficiency in wheat via enhancing siderophore-and phenol-mediated iron acquisition in roots. Plant and Soil. 2022;471(1):247-260.
62. Khan A, Singh AV, Gautam SS, Agarwal A, Punetha A, Upadhayay VK, et al. Microbial bioformulation: A microbial assisted biostimulating fertilization technique for sustainable agriculture. Frontiers in Plant Science. 2023;14:1270039.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/120236