Isolation, screening, characterization, and optimization of bacterium isolated from calcareous soils for siderophore production

REDDY KIRAN KALYAN V S (✉ vskirankalyan@gmail.com)

Tamil Nadu Agricultural University <https://orcid.org/0000-0001-9390-4970>

Meena S

TNAU: Tamil Nadu Agricultural University

Karthikeyan S

Tamil Nadu Agricultural University

Jawahar D

Tamil Nadu Agricultural University

Research Article

Keywords: Arachis hypogea, calcareous soils, iron, phytopathogenic and siderophores

Posted Date: February 28th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1365991/v1>

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Abstract

The most effective agricultural practice to prevent iron deficiency in calcareous soils is fertilizing with synthetic chelates. These compounds are non-biodegradable, and persistent in the environment and hence there is a risk of leaching metals into the soil horizon. To tackle iron deficiency-induced chlorosis (IDC) in crops grown under calcareous soils, an environmentally friendly effective solutions are needed rather than chemical application as it affects the soil health further. Hence the present work aimed at isolating and screening calcareous soil-specific bacteria capable of producing the iron-chelating siderophores. Siderophore producing bacteria (SPB) was isolated from the rhizosphere of calcareous soil-grown groundnut (*Arachis hypogea* L.), of which seventeen bacterial isolates were positive for siderophore production assayed by Chrome Azurol Sulphonate. The performance of SPB isolates was compared for siderophore kinetics, level of siderophore production, type of siderophore produced (using Arnou and Csaky's tests), and iron-chelating capacity under 15mM KHCO_3 . Four best performing bacterial isolates were screened, with average siderophores yield ranging ~60–80% under pH 8, with sucrose as a carbon source and NH_2SO_4 as a nitrogen source at 37°C. The four efficient SPB were molecularly identified as *Bacillus licheniformis*, *Bacillus subtilis*, and *Ochrobactrum grignonense* based on 16S rDNA sequencing. The strains also showed a strong antagonistic effect against the phytopathogenic fungal strains viz., *Sclerotium rolfsi* and *Macrophomina phaseolina* *in vitro*. Our results indicate that the optimized conditions enhanced siderophores chelation and by suppressing the stem and root rot fungi which could help in cost effective and environmentally friendly manner.

1. Introduction

Even though Fe is one of the most predominant elements in the Earth's crust (Manthey et al., 1994) its availability to plants and microorganism is insufficient. A transition metal can be found in two oxidation states, Fe (III) and Fe (II). It is a crucial limiting factor for plants and microorganisms as it occurs as Fe^{3+} (ferric form) which is not soluble at physiological pH (Bou-Abdallah, 2010) which is insoluble in the form of iron (Zuo and Zhang, 2011; Saha *et al.*, 2016). Iron is an essential element for plant growth and development (Barker and Pilbeam, 2015). Though Fe is not a chlorophyll component, it is vital for photosynthesis and the functioning of the photosynthetic apparatus (Broadley et al., 2012; Briat et al., 2015; Rangani et al., 2018). It also controls the biosynthesis of antibiotics, porphyrins, pigments, toxins, siderophores, cytochromes, and aromatic compounds (Messenger and Barclay, 1983). The iron deficiency of plants results in chlorosis, characterized by the yellow young leaves due to lack of iron for efficient chlorophyll production (Lucena, 2000; Barhoumi et al., 2021). These effects of chlorosis in plants result in lower biomass and yield, decreased flowers and fruits, and in critical cases total crop fatigue (Tripathi et al., 2015; Kabir et al., 2016).

Because of iron chemical composition, low solubility, low bioavailability, and dissolubility kinetics makes problematic in absorbing iron on calcareous soils. IDC is widespread in plants grown in alkaline and calcareous soils as low Fe (Fe^{2+}) levels. Calcareous soils are estimated to cover about 30% of the world's cultivated soils (Chen and Barak, 1982; Di Gregorio, 2005; Barker and Pilbeam, 2015) which underscores the importance of iron deficiency as a major global agricultural challenge. This significantly affects crop yields and hinders low bioavailability in soil (Martins et al., 2017). Synthetic chelating agents viz., *o,o*-ethylenediamine - di (*o*-hydroxyphenylacetate)-Fe(III) and *o,o*-EDDHA/Fe(III) chelate (García-Marco et al., 2006; Bin et al., 2016) have been commonly used to overcome chlorosis in calcareous soils, but their non-biodegradable nature poses a

hazard to soil health. Since these are powerful chelating agents, the pursuit of environmental protection and the proper chelating of iron fertilizers has therefore become a major challenge.

Microbes have developed many strategies to scrounge iron from their atmosphere in response to iron limitation. One mechanism of microorganisms and plants is to produce low molecular (500–1000 Daltons) iron chelators termed siderophores (Greek *sideros* meaning iron and *phores* meaning bearer) to enhance the acquisition of iron from the soil environment, particularly under the Fe-limited circumstances, which selectively complex iron (III) with very high affinity (Sandy and Butler, 2009; Lis *et al.*, 2015). They exhibit strong interaction constants for iron complexation (Neilands, 1995). Siderophores are usually classified into three major classes, such as hydroxamates, catecholates, and carboxylates based on their structural units (Hider and Kong, 2010; Rungin *et al.*, 2012). Some bacteria possess more than one form of siderophores and multiple absorption pathways for iron acquisition. The main feature of the siderophores is the chelating of Fe (III) and accumulation of complex heavy metals such as Fe^{3+} , Cu^{2+} , Zn^{2+} , Ni^{2+} and Cd^{2+} and the production and retention of toxic metals such as Cu^{2+} , Zn^{2+} , and Pb^{2+} affecting metal mobility (Braud *et al.*, 2009; Dimkpa *et al.*, 2009).

Siderophore first bonds to the ferric form of iron and then forms a siderophore-iron complex, which enters the cells through unique cell membrane siderophore receptors. Many siderophores produced by microorganisms are attributable to the low atmospheric abundance of Fe and are more selective to Fe (III) than divalent metals. Approximately 500 biomolecules have been listed under siderophores where many genes and regulators are involved in their synthesis, distribution, and re-import into cells (Challis, 2005; Visca *et al.*, 2007; Chu *et al.*, 2010). Previous studies reported the siderophore beneath iron-deprived conditions forms complex with iron (Boukhalfa and Crumbliss, 2002; Tian *et al.*, 2009; Prathap *et al.*, 2015).

Several siderophore-producing rhizobacteria (SPR) have been assessed and reported as biocontrol agents against plant pathogens (Sulochana *et al.*, 2014; Sasirekha *et al.*, 2016), including the species of *Pseudomonas* (Weller, 2007; Ghazy and El-Nahrawy, 2021), *Bacillus* (Crosa *et al.*, 2004; Peralta-Yahya *et al.*, 2012; miao Sheng *et al.*, 2020; Ghazy and El-Nahrawy, 2021), and *Enterobacter* (Costa and Loper, 1994; Grobelak and Hiller, 2017; Sinha *et al.*, 2020). These strains develop siderophores under iron-restricting conditions that chelate the available iron and deprive the respective phytopathogens of iron nutrients (Lemanceau *et al.*, 1993; Ahmed and Holmström, 2015; Santoyo *et al.*, 2016), thus limiting the dissemination of phytopathogens and root colonization. SPR is also known to impart induced systemic resistance (ISR) to plants (Pieterse *et al.*, 2000; Aznar and Dellagi, 2015) and has been documented in biocontrol of *M. Phaseolina*, *Rhizoctonia solani*, *Phytophthora nicotianae* var. *Parasitic*, *Pythium spp.*, and *Fusarium spp.* (Blumer and Haas, 2000; Haas and Défago, 2005; Singh *et al.*, 2009; Ali *et al.*, 2018; El-Shabrawy and Shehata, 2018). With this context, our research focuses on the isolation and characterization of calcareous soil siderophore-producing bacteria and the culture conditions for higher siderophore production have also been optimized.

2. Materials And Methods

2.1. Micro-organisms and culture conditions

Soils were sampled from both rhizosphere and bulk soils of groundnut, grown under calcareous soils in June 2019 near the villages of Thondamuthur block of Coimbatore, Tamil Nadu, India (10°99'N, 76°79'E). These sites were located about at an altitude of 473 m above sea level. The annual average precipitation is 618 mm and an

annual average temperature of 28.9°C. The bedrock in the area is charnockite and gneiss. The soil samples for this study were taken from the rhizosphere soil and kept cold (4°C) until further analysis.

2.2. Isolation and identification of siderophore producing bacteria from soil

The aerobic and facultative anaerobic siderophore-producing microorganisms were isolated from the soil solution. The samples were serially diluted (to 10^{-5}) and inoculated in nutrient agar (NA) medium and incubated for 24 h at room temperature. The colonies were distinguished, sub-cultured, and purified (Gaonkar and Bhosle, 2013). The purified colonies were further tested for the production of siderophore using CAS (Schwyn and Neilands, 1987). The positive colonies were purified by three times subculture method on CAS Agar plates and stored in 20% (v/v) glycerol at -20°C.

2.2.1. Identification of strains

Bacterial isolate showing efficient siderophore production was further characterized based on the morphological, biochemical, and molecular levels. Isolates were gram stained to understand the cell shape, size, arrangement, and gram nature. The purified isolates were subjected to biochemical characterization (Aneja, 2007) for the detection of organisms up at the genus level. Further, the molecular characterization was carried out using fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3') primers. The reaction mixture in each tube consisted of 20 µl with DNA template 50 ng, 1x Taq buffer, 0.2 mM of each dNTP mixture, 1 µM of each primer, 1.5 mM MgCl₂, and 2U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed and products were separated by electrophoresis on 1% agarose gel (Sambrook et al., 1989). The 16S rRNA gene was sequenced in both directions and the obtained sequences were compared to sequences in the NCBI GenBank database using the BlastN (Altschul *et al.*, 1997). The recovered sequences, as well as the closest identified relatives, were aligned in Molecular Evolutionary Genetics Analysis (MEGA) Software ver. 10.0. All the sequences were submitted to the GenBank and accession numbers were acquired.

2.3. Partial purification of siderophore

The cultures were grown in Fiss-glucose minimal media (Vellore, 2001) for 24 hours at 28°C on a rotary shaker. After incubation, the culture supernatant was collected by centrifuging at 7,000 rpm for 30 minutes. The supernatant was then acidified to pH 2.0 with 6 M HCl to make the siderophore less soluble in water and passed the acidified supernatant through a 30 x 5 cm column packed with Amberlite XAD-2 (Sigma, USA), which binds cyclic compounds were dissolved in distilled water and kept overnight for soaking. The loaded column was pre-washed twice with water, methanol, and water. The aqueous supernatant was allowed to slowly pass through the column at the rate of 5 mL min⁻¹. Loading of supernatant was continued until the saturation of the column, which was indicated by the browning of the column. The column was then washed with five to ten-bed volumes of distilled water to remove all unbound components of the medium. The column was then equilibrated by changing the solvent to 50% methanol to facilitate the extraction of siderophores (Budzikiewicz, 1993). Different fractions were separately collected; filtrate, water wash, and eluted fractions were checked for CAS test. Fractions positive for siderophore were combined in a 100 ml boiling flask and the pH was adjusted to 3.0 with H₂SO₄ and 50 percent ammonium sulfate solution was added to deproteinize and evaporated to dryness using a rotary evaporator. This aqueous phase was concentrated in a lyophilizer (Lark) and set aside in cold to

crystallize. The filtrate was neutralized, reduced to dryness, extracted in dry hot methanol and were then separated on Whatman filter paper no. 44. The obtained purified siderophore crystals obtained were then subjected to Fourier transform infrared spectroscopy (FTIR)-ATR analysis (JASCO FT/IR-6800) for determination of the functional groups. The spectrum was recorded in the range from 400 to 4000 cm⁻¹. The infrared spectrum wavelengths were determined based on their functional groups (Tank et al., 2012).

2.4. Quantitative and qualitative estimation of siderophores

The purified bacterial isolates were grown in an iron-deficient succinate medium and incubated for 48 h with constant shaking at 120 rpm. All the isolates were screened for siderophore production *viz* spectrophotometric means at A630 nm (Grimm and Allen, 1954) which was further confirmed by the Chrome Azurol sulphonate (CAS) agar test. The production of siderophore by the isolate was quantitatively determined using (CAS) as described by (Schwyn and Neilands, 1987). From the prepared CAS solution, 0.5 ml was taken to which 0.5 ml of culture supernatant was added and incubated for 5 min. Then the mixture was measured at 630 nm and calculated for the siderophore production (Maindad et al., 2014; Fazary et al., 2016). The percent of siderophore was intended in terms of % of siderophore units by the following formula:

$$\% \text{ of siderophore units} = [Ar - As)/Ar] * 100$$

Where Ar = absorbance of reference (CAS reagent); As = absorbance of the sample at 630 nm. Further, the qualitative confirmation was performed by CAS agar test, where the bacterial isolates streaked on the CAS plates show orange zone.

2.5. Screening of bacteria for calcareousness tolerance

The succinate medium (SM) medium was to screen calcareous tolerant bacteria. The succinate medium concentration per liter: KH₂PO₄, 3.0 g; K₂HPO₄, 6.0 g; MgSO₄.7H₂O, 0.2 g; (NH₄)₂SO₄, 1.0 g; succinic acid, 4.0 g; peptone, 1.0 g; final pH 6.5. This medium was enriched with KHCO₃ (calcareous source) to mimic calcareous soil. The KHCO₃ were added at different concentrations *viz.*, 10, 15, 20, and 25 µM at room temperature. The production of siderophore by the isolates under calcareous stress was quantitatively determined using CAS (Schwyn and Neilands, 1987).

Succinate medium (SM)	KH ₂ PO ₄ 3.0 g per litre; K ₂ HPO ₄ 6.0 g per litre; MgSO ₄ .7H ₂ O 0.2 g per litre; (NH ₄) ₂ SO ₄ 1.0 g per litre; succinic acid 4.0 g per litre; peptone 1.0 g per litre; distilled water 1000 ml; final pH 6.5
Treatment 1	SM + 10 mM KHCO ₃
Treatment 2	SM + 15 mM KHCO ₃
Treatment 3	SM + 20 mM KHCO ₃
Treatment 4	SM + 25 mM KHCO ₃

2.6. Chemical Characterization of Siderophores

The type of siderophore was determined by specific tests using culture supernatants prepared as described above. Hydroxamate type of siderophore was detected by tetrazolium test (Snow, 1970), catecholate type of siderophores was detected by Arnow's test (Arnow, 1937); carboxylate type was detected using Vogel (1992).

2.7. Optimization for maximum siderophore production

The bacterial isolates were allowed to grow in succinate broth under different fermentation conditions, such as pH (5, 6, 7, 8 and 10), temperature (27, 37 and 45⁰C), nitrogen sources (urea, ammonium sulphate (NH₂SO₄) and sodium nitrate (NaNO₃), carbon sources (sucrose, glucose, fructose and lactose), and iron concentration (1, 2 and 3 ppm) for 48h to investigate the maximum level of siderophore production. For siderophores analysis, the supernatant was centrifuged at 5000 rpm for 10 min and cell-free supernatant was analyzed using CAS assay test. The production of siderophore was measured at 630 nm and calculated (Maindad et al., 2014; Fazary et al., 2016).

2.8. Antagonism tests in vitro

2.8.1. Growth of bacterial strain and fungal mycelium in dual culture

The antimicrobial activity of every bacterial strain was surveyed by the dual culture method (Campanile et al., 2007) to screen antagonism. Petri dishes (9 cm) containing 20 ml of sterile PDA were inoculated with a loop of potential antagonists of bacterial strains and the 0.5 cm sterile plug of a 3 day-old pure culture pathogens *S. rolfsi* and *M. phaseolina*, which were collected from the department of plant pathology, Tamil Nadu Agricultural University, Coimbatore. The distance between the pathogen and antagonist was 4 cm. Each combination of pathogen/antagonist was repeated 3 times and as negative controls. All Petri dishes were incubated at 25⁰C in the dark and randomly distributed. The experiment was replicated three times. Radial growth was recorded by measuring colony diameter at 1-day intervals for the time required to reach the margin of the dish in controls. The Antagonism Index (AI) was assessed according to the following formula: $AI = (RM - rm) / RM * 100$, where *rm* = ray of the colony towards the antagonist and *RM* = average of the three rays of the colony in the other directions. The zone of inhibition around the spots was recorded as a positive result.

2.8.2. Interactions between bacterial strains and antagonistic fungi in dual culture

The antagonistic ability of the selected bacterial cultures against *S. rolfsi* and *M. phaseolina* was tested using the dual-culture method described by (Badalyan et al., 2002; Badalyan et al., 2004). A loop of bacterial cultures and a plug (0.5 cm diameter) of antagonistic fungus was cut from the margin of a 3 day-old culture and placed respectively on opposite sides (4 cm from the margin) of Petri dishes containing PDA. Each combination of pathogen/antagonist was repeated 3 times and plates were randomly incubated at 25⁰C for 3 days. As negative controls, 3 Petri dishes were inoculated with selected bacterial cultures and a water agar plug. Interactions were examined daily. Antagonism towards bacterial cultures was scored using the Badalyan et al. (2002) rating scale into 3 types (A, B, and C) and 4 subtypes (CA1, CA2, CB1, and CB2), where: A = deadlock with mycelial contact, B

= deadlock at a distance, C = replacement, overgrowth without initial deadlock; CA1 and CA2 = partial and complete replacement after initial deadlock with mycelial contact, CB1 and CB2 = partial and complete replacement after an initial deadlock at a distance. The following scores were assigned to each type or subtype of reaction: A = 1.0; B = 2.0; C = 3.0; CA1 = 3.5; CB1 = 4.0; CA2 = 4.5; and CB2 = 5.0. The AI was calculated for each species using the formula: $AI = SN \times I$, where N = number (frequency) of each type or subtype of reaction and I = the corresponding score.

2.9. Statistical analysis

All the data were analyzed using SPSS software 23.0 version (SPSS IBM). The significant differences in the means were analyzed based on Tukey's multiple comparison test ($p < 0.05$).

3. Results

3.1. Isolation and screening of siderophores producing bacteria

A total of 33 bacterial isolates were obtained, purified, and cultured. The siderophore-producing bacterial isolates were screened using a CAS assay (both qualitatively and quantitatively). The cultures were grown in a succinate medium and incubated for 48 hours before being tested for CAS in a spectrophotometer. 17 of the thirty-three isolates tested positive for turbidity in the SA medium and turbidity in the CAS test. The presence of siderophores was confirmed on CAS agar plates by the presence of a distinctively orange-colored zone (Fig. 1) indicating siderophore production.

3.1.1. Quantitative Screening of Siderophore Producing Bacteria

For quantitative estimation, CAS assay was employed and their cell concentration was measured at A630 nm. Out of thirty-three isolates, 17 were found to be positive for siderophore and selected for quantitative estimation of siderophore production. Thirteen isolates produced siderophore units in the range of 8.4–54.9%, while 4 isolates SID 13, SID 25, SID 30, and SID 33 produced significantly higher quantities of siderophores (< 65% siderophore units (SU) (Fig. 2).

3.1.2. Screening of bacteria for calcareousness tolerance

Potassium bicarbonate ($KHCO_3$) is used in the culture medium to simulate calcareousness artificially and their influence was studied in terms of bacterial population and siderophore production. All the isolates followed similar trend, siderophore production increased with increasing the concentration of sodium bicarbonate. Among the four isolates, *Bacillus licheniformis* showed marked increase in bacterial colony forming units (cfu) and siderophore production with rising concentration of bicarbonate. At higher concentrations *i.e.* at 25 mM $KHCO_3$, the siderophore production percentage ranges from 65.9 to 74.1% irrespective of the bacterial strains (Table.1).

3.2. Characterization of efficient siderophore-producing isolate

Further, the isolates SID 13, SID 25, SID 30, and SID 33 were taken for morphological, molecular, and biochemical characterization. The results of biochemical characterization are given in Table.2. The molecular

identification was confirmed by 16S rDNA sequencing of bacterial isolate SID 13 was showing similarity to *B. licheniformis* (98%); SID 25 was showing similarity to *B. subtilis* (100%); SID 30 was showing similarity to *B. licheniformis* (99.9%) and SID 33 was more close to *O. grignonense* with the percent identity of 93%. The evolutionary relationship of the identified 16S rDNA sequencing and the acquired accession numbers from NCBI were shown in Fig. 3.

3.3. Siderophore Chemotyping

The type of siderophore present in each medium filtrate was subjected to Arnow's, Snow's, and Vogel's tests. Both *B. licheniformis* strains and *O. grignonense* were positive for all three tests, indicating the presence of catechol, carboxylate, and hydroxamate siderophores in the filtrate of the culture medium. *B. subtilis* filtrate was positive only for hydroxamate. (Table 3; Fig. 4.).

3.4. FTIR analysis

The infrared spectrum analyses of the partially purified SPB cultures extract pellet showed a broad peak at 3221, 3242, 3264 and 3265 cm^{-1} indicating the presence of aromatic OH moiety of siderophores. The peak at 3170 cm^{-1} is attributed to NH stretching. The appearance of a peak at 2950 cm^{-1} showed the presence of saturated alkanes. The spectrum also provides evidence for the presence of amide linkage in the structure. The intense peak at 1640 cm^{-1} typically indicated an amide C = O stretching suggesting a secondary amide functionality. A conjugation and intramolecular H bonding may have caused the lowering of the C = O stretch peak. The peak observed at 1451 and 1452 cm^{-1} revealing the presence of one -C-H bending with functional group -CH₂ and one -N-O structure, which shows that similarity towards hydroxamate functional groups. Furthermore, a peak at around 1200 cm^{-1} indicated a -C-O-C- bond of the ether linkage. Thus, the spectrum indicated the presence of catechol siderophores (Fig. 5.).

3.5. Different culture conditions for optimum production of siderophores

To understand the significant effect of various culture conditions that relates the bacterial growth and siderophore production, the cultures were grown under different growth conditions such as pH, temperature, carbon source, nitrogen source, and iron concentration. Hence we tried to optimize conditions for the maximum production of siderophore by the SPB strains.

3.5.1. Influence of pH

The optimum siderophore production in all the four SPB strains was achieved at pH 8. The maximum amount of siderophore produced (84.8% SU) was found with SID 30 strain at pH 8. Irrespective of the strains the lowest siderophore yield was found at pH 10 in SID 13 with the value of 52.8% SU. This might be due to at alkaline pH of the medium decreases the solubility of iron thus making it unavailable to the growing bacteria creating an iron-depleted environment suitable for siderophore production (Fig. 6.).

3.5.2. Influence of temperature

Further, the culture conditions were changed with constant pH and different temperatures. Our study also showed higher production of siderophore accounting for 70% at 25°C in all the strains. Upon increasing the

temperature levels the siderophore production showed a sheer decline to 37.2% at 45⁰ C in SID 25 (Fig. 6.).

3.5.3. Influence of nitrogen sources

All the four, selected strains from this study were able to synthesize siderophore > 67% irrespective of applied nitrogen source. Different nitrogen sources influenced siderophore production significantly. The highest % SU was detected with ammonium sulfate (80.6%) as the nitrogen source in SID 30, whereas, the lowest siderophore production was registered with SID 25 (67.2%) with urea as a nitrogen source as shown in Fig. 6.

3.5.4. Influence of carbon sources

The SPB strains were assessed for their optimal requirement of carbon source for maximal production of the iron-chelating siderophores. Sucrose was found to be a favourable carbon source for all the four tested SPB (Fig. 6). The production of siderophore was comparatively higher 80.4% in *B. licheniformis* (showing an evolutionary relationship with the *Bacillus sp.*).

3.5.5. Influence of iron concentrations

The addition of FeSO₄ in the medium significantly affected the siderophore production. The addition of 2 PPM of FeSO₄ in the medium resulted in higher siderophore production up to 77% SU in SID 13. This might have induced an enhanced rate of siderophore production to bind with the available iron and provide it to the cell. Upon increasing iron concentration, there was a steep decline in the % SU to 61.3. This could be because once iron concentration in the medium reached above the threshold value required for siderophore production, it negatively regulates iron acquisition genes (Fig. 6).

3.6. Antagonistic potential of SPB in dual culture

Varying degrees of mycelial growth inhibition of *S. rolfsi* and *M. phaseolina* were observed with antagonistic bacterial and fungal isolates. *Trichoderma viride* had the maximum inhibitory effect on mycelial growth of both *M. phaseolina* and *S. rolfsi* with a reduction of 69.3 and 65.1 respectively compared to control. The isolates *B. licheniformis*, *B. subtilis*, *B. licheniformis*, and *O. grignonense* expressed significantly lower mycelial growth inhibition with reductions in the range of 37.3–57.5% for *M. phaseolina* and 48.3–52.6% for *S. rolfsi* (P < 0.05, compared to controls and other antagonists) (Table.4.).

The antagonistic index in the dual-culture method, based on the AI values for both the *M. phaseolina* and *S.rolfsi* in the presence of antagonistic isolates were divided into three groups: active (*T. viride*), moderate (*B. licheniformis* and *B. subtilis*) and weakly active (*O. grignonense*) only for *S. rolfsi*. The most important parameter determining antagonistic activity was the inhibiting speed of colony growth (Table.5 & Fig. 7).

4. Discussion

Siderophores are low molecular weight compounds (between 500 and 1500 dalton), of high affinity and selectiveness to bind and complex Fe (III). As a part of a strategy to extract iron from the atmosphere because of the poor bioavailability of iron, these siderophores are produced both from plants and microbes (Hider and Kong, 2010; Ahmed and Holmström, 2014). In the last few years, several researchers have drawn attention to this particular function. They have wider applications in plant growth, biocontrol activity, and several other ecological factors. In this present study, the siderophore-producing bacteria were isolated from the soil samples

and the optimal fermentation condition was configured to understand the culture medium capable of a high level of siderophore production. Similar results were reported by Ferreira et al. (2019) under alkaline conditions where they evaluated five bacterial strains for siderophore kinetics, percent siderophore production, type of siderophore produced, and iron-chelating capacity at pH 9.0. Ghosh *et al.* (2015) used both fungal strains (*T. viride*-1, *T. harzianum*-1, *Candida famata*-1) and bacterial strains (*B. subtilis*-1, *B. megatericus*, *P. aeruginosa*) for siderophore production. The CAS or HDTMA forms a strong complex with a ferric ion to produce a blue color to the medium, when iron chelators like siderophores are added to the medium, the iron is separated from the dye complex and the colour gradually changes from blue to orange (Louden et al., 2011).

Out of 17, 4 isolates (SID 13, SID 25, SID 30, and SID 33) produced higher siderophore units (SU) above 65% in CAS-liquid assay. When these siderophore producing isolates subjected to calcareous stress showed positive results even under 25 mM K HCO₃ concentration, without any decline in % siderophore units. Earlier results showed that *E. coli*, *P. aeruginosa*, *S. aureus*, *S. agalactiae*, *E. faecalis* and *H. influenza* growth significantly inhibited when subjected to NaHCO₃ (100 mmol l⁻¹) and suggesting that HCO₃⁻ can suppress bacterial growth in general (Dobay et al., 2018). As several researchers have also confirmed that high bicarbonate concentration directly caused iron deficiency chlorosis (Coulombe et al., 1984; Pearce et al., 1999), our strain proved to withstand at all the four levels of bicarbonate concentration showing its ability of tolerance against calcareousness along with higher siderophore production.

Microbial siderophores are usually grouped as catecholates, hydroxamates, carboxylates, and mixed type since these compounds shows the highest affinity towards iron. Evolutionary relationship of our bacterial strains showed maximum relative percentage towards *Bacillus sp.* Earlier researchers also found that most of the siderophore producing bacteria are subsiding under the *Bacilli sp* (Sivasakthi et al., 2013; Shaikh et al., 2016). Most of our isolates were tested positive for more than one type of siderophore. Earlier reports showed that several soil bacteria could produce more than 65 percent of SU and multiple types of siderophore (Hider and Kong, 2010; Ahmed and Holmström, 2015; Ferreira et al., 2019).

The partial purified siderophores using Amberlite XAD-2 shows similar trends of Sayyed and Chincholkar (2006) who purified siderophores of *Alcaligenes faecalis* on Amberlite XAD-400 resins. Further, the FTIR spectrum of LSBS2 showed the adsorption bands at 3445, 2951, 1652, 1455, and 1143 cm⁻¹, respectively, which indicates the presence of (-OH), aromatic (-CH), (-C = O), (-CH₂) and (C-O-C) linkage. These functional groups are present in the catecholate type of siderophore so that the purification of siderophore production in FTIR analysis confirmed the presence of a catecholate type of siderophore that is specific to 2,3 dihydroxybenzoic acid (Nithyapriya et al., 2021).

To optimize the bacterial strains capable of siderophores potential ability, the performance of the bacteria was optimized with the following characteristics: culture media composition (carbon and nitrogen sources), iron complexation capacity, pH range, and under different temperatures conditions. Though iron is the major factor involved in siderophore production, other cultural conditions also play a significant role in siderophore production. Tailor and Joshi (2012) reported that bacteria can grow optimally in the physiological environment. Storey et al. (2005) indicated that under normal conditions with Fiss-glucose medium, the hydroxamate siderophore was lower and, this can further be optimized to higher siderophore production with certain modifications in the growth medium.

Because its solubility and availability are affected by the pH of the medium, pH plays an important role in microbial proliferation. We detected that the increased siderophore concentration was recorded when the pH was lower than 8. Given that Fe^{2+} is soluble and Fe^{3+} is insoluble at physiological pH (7.35–7.40) (Bou-Abdallah, 2010) alkaline pH decreases iron solubility, rendering it unavailable to bacteria, resulting in an iron-depleted environment ideal for siderophore synthesis. Gérard (2016) discovered that the insolubility of iron increases at high pH values, which supports our observations. At pH 8, iron becomes more insoluble in soil solution, which may have accelerated siderophore production. The present finding agrees with Calvente et al. (2001) who reported similar results as pH near 8 stimulates higher siderophore production. In some cases, it was reported that the highest iron-chelating capacity, at pH 9.0, was obtained by *B. megaterium* followed by *B. subtilis* and *A. vinelandii* (Ferreira et al., 2019)

The percentage of siderophore production varied with incubation temperature, being optimum at 27°C, lesser at 35°C, and least at 45°C. The incubation temperature considerably influenced siderophore production; these findings are in accordance with Dave and Dube (2000) who have reported that maximum siderophore production occurred at 30 °C. Percent siderophore units were recorded maximum at 30°C i.e. 83.9% by *A. oryzae* (Singh and Mishra, 2015). The *P. fluorescens* strain was also found to produce maximum siderophore at 29°C (Tailor and Joshi, 2012). Otherness Kumar et al. (2017) reported that 35°C (room temperature) showed optimum bacterial siderophore production in VITVK5 and VITVK6 strains.

The optimization was carried out with the different sources of nitrogen such as ammonium sulfate, sodium nitrate, and urea. All the above mentioned isolates have produced siderophore more than 65% with all the nitrogen sources and the maximum was registered with ammonium sulfate, while *Ochrobactrum* sp. influenced to produce higher siderophore with as N source as shown in our results (Louden et al., 2011; Tailor and Joshi, 2012). The results were similar with Tailor and Joshi (2012) who also confirmed maximum siderophore yield up to 96% in *P. fluorescence* and *P. putida* in the presence of ammonium sulfate and urea.

Based on previous reports we have chosen to evaluate four major carbon sources namely glucose, fructose, lactose, and sucrose. Among these, sucrose had a stimulating effect on the production of siderophore followed by glucose. The present findings prove that the presence of sugars such as sucrose in the growth media increased the growth of *Nocardia levis* MK-VL_113 for the elaboration of bioactive metabolites (Kavitha and Vijayalakshmi, 2009). Sucrose concentration at 2% roused the growth and siderophore production in *Rhizobium* strains (Sridevi and Mallaiah, 2008). A previous study of siderophore production by *Rhodotorula* sp. reported by Calvente et al. (2001) also shows similar results of enhanced siderophore production upon sucrose supplementation. Supplementing the growth media with carbon sources increases the growth and metabolism of bacteria and the siderophore production capability. The siderophore production by an organism depends on the availability of organic and inorganic nutrients in the medium (Neilands, 1981; Abd-Alla, 1998).

Among various abiotic factors, the pH of the medium plays a major role in Fe availability. Notably with increasing concentration beyond a threshold level suppresses the siderophore production. Our findings also showed that the percentage siderophore enhanced with increasing pH and Fe concentration up to 8 and 2 PPM respectively beyond that siderophore production started to decline, which might be due to native availability of iron. Tailor and Joshi (2012) showed similar results, where the concentration of siderophore produced from *P. fluorescence* decreased after 1µM. Muthuselvan et al.(2013) who also reported that at low concentration (1–

3μM) of iron enhanced the siderophore production of *Azotobacter* sp. up to 82%. Excess iron concentration harmed siderophore production along with the morphology alterations and growth (Singh and Mishra, 2015).

Identification and selection of effective antagonistic organisms is the first and foremost step in biological control (Kamakannan *et al.*, 2004). Our findings witness the significant antagonistic activities of *B. licheniformis*, *B. subtilis*, *B. licheniformis*, and *O. grignonense* against *M. phaseolina* and *S. rolfsi* in *in-vitro* dual cultures. Siderophores are also thought to facilitate biocontrol by sequestering iron from pathogens, thus limiting their growth (Chiarini *et al.*, 1993; Champomier-Vergès *et al.*, 1996). Siderophore positive *P. fluorescens* is known to control bacterial pathogens including bacterial soft rot of potato, the bacterial canker, and bacterial wilt of tomato (David *et al.*, 2018). These results are also in agreement with Solanki *et al.* (2014) who also reported efficient siderophore production using *Enterococcus* sp. and its activity against plant pathogen *R. solani*. These beneficial effects of siderophore include both plant growth enhancement and biological control of phytopathogenic fungi (Ghazy and El-Nahrawy, 2021). Secondary metabolite like siderophore production is a key for plant growth-promoting bacteria by fulfilling the plants' iron requirements and protecting against plant pathogens. Nursery and field trials in cardamom with siderophore producing bacteria, by *P. putida* TAUC10, had higher ligand formation constants than those of the soil pathogens (Panchami *et al.*, 2020).

In the present study, 17 bacterial isolates were found to be positive for qualitative CAS agar assay. Upon qualitative and quantitative analysis, 4 isolates (*B. licheniformis*, *B. subtilis*, *B. licheniformis*, and *O. grignonense*) were found to produce above 65% siderophore units having higher potential use for iron-induced chlorosis amendment in calcareous soils. The morphological and molecular characterization of siderophore-producing bacteria depicts close resemblance to the bacterial species of *Bacillus* sp. and *Ochrobactrum* sp. Results of the influence of different culture conditions showed that the bacterial isolate had the efficiency of producing siderophore in higher concentration at pH 8, 37 °C with sucrose as carbon source and Na₂SO₄ as nitrogen source. The increase in iron concentration increased the production of siderophore, which saturated after a threshold limit. As these strains are capable of producing higher siderophore units even upto 25 mM calcareous level, it can be used a tool for overcoming iron related stress under calcareous soils (> 30% of world area), where iron deficiency is a major constrain. The present investigation also revealed that these bacterial isolates exhibited other plant growth-promoting traits *viz.* citrate utilization, catalase activity, IAA production and subdues the root and stem rot fungi in groundnut *viz.*, *S. rolfsi* and *M. phaseolina* has added advantage. However, further research is needed to elucidate the tolerance limits for calcareousness and optimisation of siderophore under field conditions, which can lower the impact of iron-induced chlorosis.

Declarations

ACKNOWLEDGEMENT

The financial help by Science and Engineering Research Board (SERB), Ministry of Science and Technology, Govt. of India, New Delhi, India, Grant/Award number: E28 ACC for the research is gratefully acknowledged.

Competing interests

Authors have declared that no competing interests exist

Authors' Contributions

'V.S. Reddy Kiran Kalyan' performed the laboratory experiment, analysis, statistical analysis, wrote the protocol, and drafted the manuscript. 'S.Karthikeyan, S.Meena, D.Jawahar' designed the study, managed the analyses of the study, literature searches, and manuscript.

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Tables

Table 1

Bacterial population and siderophore production (%) on different calcareousness levels

Isolates code	Bacterial population ($\log_{10}$ cfu)				Siderophore production (%)			
	10mM KHCO ₃	15mM KHCO ₃	20mM KHCO ₃	25mM KHCO ₃	10mM	15mM	20mM	25mM
<i>B licheniformis</i> (MW279241)	5.80	6.11	6.52	7.21	62.3	69.5	70.1	70.9
<i>B subtilis</i> (MW279240)	6.98	6.52	6.53	6.62	79.8	76.4	74.7	74.1
<i>B licheniformis</i> (MW279255)	6.47	6.06	6.20	6.91	59.1	61.7	63.9	65.9
<i>O grignonense</i> (MW279256)	6.68	6.63	6.72	6.61	70.4	69.9	72.2	72.9

Table 2

Characterization of siderophore producing bacterial isolates

Morphological parameters

	<i>Bacillus licheniformis</i>	<i>Bacillus subtilis</i>	<i>Bacillus licheniformis</i>	<i>Ochrobactrum grignonense</i>
Gram reactivity	+	+	+	+
Shape	Rod	Rod	Rod	Rod
Colony color	white	white	white	White
Elevation	Raised	Raised	Raised	Raised
Opacity	Translucent	Translucent	Translucent	Translucent

Biochemical characteristics test

Citrate utilization test	-	+	+	+
Catalase	+	+	+	+
MR (methylene red)	+	-	+	-
VP (Voges-Proskauer)	+	+	+	-
Indole acetic acid	-	+	+	-

Note: Positive and Negative show the results of biochemical results.

Table 3

Chemotyping of the siderophore-type produced by the bacteria

Bacteria	Arnow ^a	Snow ^b	Vogel ^c
<i>Bacillus licheniformis</i>	+	++	+
<i>Bacillus subtilis</i>	-	++	-
<i>Bacillus licheniformis</i>	++	++	++
<i>Ochrobactrum grignonense</i>	++	++	+

^aCatechol, ^bhydroxamate, and ^ccarboxylate type siderophores were identified using Arnow's, Snow's, and Vogel's tests, respectively. ++ Strong positive result; + positive result; - negative result

Table 4

Effect of antagonistic isolates on the growth of MP and SR

Antagonistic isolate	Mycelial growth of pathogens		(% reduction over control)*
	MP	SR	
<i>Bacillus licheniformis</i>	57.5 ^b	52.6 ^c	
<i>Bacillus subtilis</i>	48.2 ^c	51.0 ^c	
<i>Bacillus licheniformis</i>	54.1 ^b	76.3 ^a	
<i>Ochrobactrum grignonense</i>	37.3 ^d	48.3 ^c	
<i>T. Viride</i>	69.3 ^a	65.1 ^b	
Control**	0.0 ^e	0.0 ^d	

* Mean of three replicates

**Growth of *Macrophomina phaseolina* (MP) and *Sclerotium rolfsi* (SR) in the absence of antagonistic fungal isolates

Table 5

Antagonism Index (AI) and type/subtype of interaction between antagonistic isolates and SR, MF scored on Badalyan's scale

Antagonistic isolate	AI*		Type/Subtype*	
	MP	SR	MP	SR
<i>Bacillus licheniformis</i>	10	10	B	B
<i>Bacillus subtilis</i>	10	10	B	B
<i>Bacillus licheniformis</i>	10	10	B	B
<i>Ochrobactrum grignonense</i>	5	20	A	CA1
<i>T. Viride</i>	5	5	A	A

* Mean of three replicates

A = deadlock with mycelial contact; B = deadlock at a distance; CA1 = partial replacement after initial deadlock with mycelial contact

Figures

Figure 1

The appearance of orange color and zone formation indicating siderophore production in CAS agar plate assay.

Figure 2

Siderophore production by the bacteria isolates

Figure 3

Phylogenetic tree based on the 16S rRNA gene sequence of siderophore producing rhizobacteria along with NCBI accession code

Figure 4

Results of hydroxamate-type and catechol-type siderophore activity, using cell-free culture supernatants

Figure 5

FTIR spectrum of siderophore positive bacterial extract (A – *Bacillus licheniformis* (SID 13); B – *Bacillus subtilis* (SID 25); C – *Bacillus licheniformis* (SID 30) and D – *Ochrobactrum grignonense* (SID 33))

Figure 6

Effect of different a) pH, b) temperature, c) nitrogen source, d) carbon source and e) iron concentration on the production of bacterial siderophores

Figure 7

a & b, Complete replacement with *T.viride* mycelium on *M.phaseolina* and *S.rolfsi* after 7 and 6 days of inoculation in dual culture respectively; c, partial replacement after initial deadlock at a distance between *B. licheniformis* isolate against *M.phaseolina*; d shows deadlock at a distance between *B. licheniformis* isolate against *S.rolfsi* in dual culture method.