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Characterization and optimization of bacterium isolated from soil samples for the production of siderophores☆

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ABSTRACT

Siderophores are small molecules that can easily bind to ferric iron. As a chelating agent, they transport iron molecules inside the bacterial cell for various biochemical reactions. Due to its various applications in medicinal, industrial and environmental related aspects, this paper deals with characterization and optimization of few siderophores producing bacteria from the soil samples, collected from Chikkamagaluru district, Karnataka. The siderophores production was assayed qualitatively and quantitatively through Chrome Azurol S and the results showed positive for the strains VITVK5 and VITVK6 that grown in succinate medium. Further characterization and optimization results revealed that both the bacterium has the ability to yield siderophores (~60–80% units) in the optimum condition of pH 8, at 37 °C with glucose and sucrose as a carbon source and NaNO3 as a nitrogen source. Thus, the study concludes that strains VITVK5 and VITVK6 can be promising candidates for the siderophores production which can play major applications in medicinal and industrial aspects.

1. Introduction

Most of the microorganisms are highly dependent on the requirement of iron, except some *Lactobacilli sp.* Under aerobic conditions, free metal iron Fe (III) forms insoluble hydroxides and oxyhydroxides that leads to the reduction of iron availability to the microbes. In such cases, bacteria has a strategy of solubilizing the metal form of iron for their uptake. The common strategy is the synthesis of low molecular weight chelators that shows high association constants for complexing iron [1,2]. These chelators have the ability to form stable complexes with other metal atoms such as Al, Cd, Cu, Ga, In, Pb, Zn [3,4]. Around 500 biomolecules were classified under siderophores where many genes and regulators are involved in their synthesis, transport, and re-import into the cells [5,6]. These siderophores are structurally classified as hydroxamate, catecholate or mixed hydroxyl carboxylic ligand groups. Previous literatures has reported that the gram negative and gram positive bacteria synthesizes siderophore beneath iron deprived conditions for complex formation with the iron from different habitats [7–9].

The mechanism of siderophore is to first bind with a ferric form of iron and form a complex of siderophore-iron that enters the cells through specific siderophore receptors present in the cell membrane. For gram-positive bacteria, transport of the siderophore-iron complex is carried out by the involvement of siderophore irrevocable proteins, permeases, and ATPases. Whereas, in the gram-negative bacteria the transport mechanism is quite different due to their complex membrane structure . Here, they transfer the siderophore-iron complex through a periplasmic binding protein and a cytoplasmic membrane protein corresponding to ATP-binding cassette transporter (ABC-transporter) [10].

As soon as the complex enters the cytosol, the ferric iron gets reduced to a ferrous form which becomes free from the siderophore chelator complex. The released ferrous iron form is further utilized for their metabolic processes. The free form of siderophore is either besmirched or reprocessed by excretion through efflux pump system [11].

Though, the primary application of siderophore is to provide soluble iron to microbes for its growth. They also play various roles in fields such as agriculture, bioremediation, biosensor, and medicine. Hence, our study is focused on the isolation of siderophore-producing bacteria from iron-enriched soil collected from Chikkamagaluru district; Karnataka, South India. This study enumerates the siderophore production and optimized culture condition in which the isolates produced a higher concentration of siderophores.

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2. Materials and methods

2.1. Isolation and identification of the isolate

Iron- enriched soil sample from which we had isolated our bacterial strains VITVK5 and VITVK6 were collected from Chikkamagaluru district; Karnataka, South India. Previous studies have reported the isolation of bacteria producing from rhizospheric soil [12] and Chikkamagaluru district has rhizospheric soil itself. So, we produced siderophore from Chikkamagaluru soil sample. The samples were serially diluted and inoculated to grow in nutrient agar medium for 24 h at 37 °C. The colonies were distinguished and pure cultured in separate plates [13].

2.2. Quantitative and qualitative estimation of siderophores

All the bacterial isolates were grown in iron-deficient succinate medium and incubated for 48 h with constant shaking at 120 rpm. All the isolates were screened for siderophore manufacturing via a spectrophotometric means which was further confirmed by CAS agar test.The production of siderophore by the isolate was quantitatively determined using Chrome Azurol sulphonate (CAS) assay as described by Schwan and Neiland. To set up 100 ml of CAS solution, 60.5 mg of CAS was diffused in 50 ml of deionized water to which 10 ml of $FeCl_3.6H_2O$ solution was added. 72.9 mg HDTMA (Hexa-decyl Trimethyl Ammonium bromide) dissolved in 40 ml of deionized water was added to CAS to make the volume to 100 ml. 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. The percent of siderophore was intended in terms of % of siderophore units by means of the following formula:

$$\%\ \textbf{of siderophoreunits} = \frac{Ar - As}{Ar} * 100$$

where, Ar = absorbance of reference (CAS reagent); As = absorbance of the sample at 630 nm.

Further, this was confirmed qualitatively by performing CAS agar test. In this, the CAS solution prepared were added to King's B medium and inoculated with the bacterial isolate and incubated at 28 °C under the dark condition for two weeks. The appearance of orange zones confirms siderophore production. All the assays were carried out in triplicates [14,15].

2.3. Characterization of efficient siderophore-producing isolate

Bacterial isolate showing efficient siderophore production was further characterized based on morphological, biochemical and molecular level. Isolates were Gram stained to understand the cell shape, size, arrangement and gram nature. The purified isolates were further analyzed to the biochemical characterization of detection of organisms up to genus level. Further, the molecular characterization was carried out by forward and reverse DNA sequencing reaction of PCR amplicon with 27F/1492R primers using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA).The consensus sequence of approximately 1400 bp 16S rDNA gene was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). Then the sequences were subjected to homology search using BLAST program of the National Centre for Biotechnology Information (NCBI) [16].

2.4. Optimization of siderophore production

The bacterial isolates were allowed to grow in different fermentation conditions, such as pH, temperature, nitrogen source, carbon source, iron concentration and organic acid were investigated in order to allow the utmost production of siderophores. The isolates were grown in succinate medium for 48 h with the providence of different fermentation conditions. 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 [17,18].

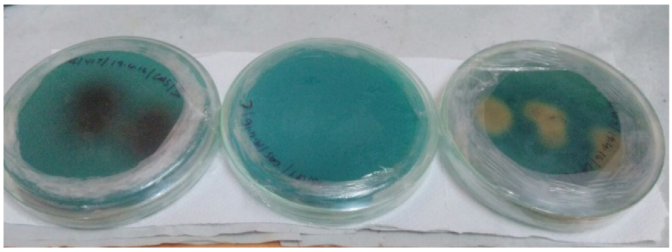

Fig. 1. The appearance of orange color and zone formation indicating siderophore production in CAS agar plate assay.

3. Results and discussion

3.1. Isolation and screening of siderophores producing bacteria

Siderophores are low molecular weight chelating agents highly synthesized by microorganism for their competence of ferric iron in ferric hydroxide complex. They have great applications in plant growth promotions, biocontrol activity, and several other ecological factors. They also show advantages in the field of medicine as a potential drug for the iron deficient diseases and acts as antimicrobial agents [19]. Some of the commonly known siderophores are schizokinen from *Rhizobium leguminosarum* IARI 917 [20], pyoverdine by *Pseudomonas fluorescence* [21], protochelin by *Azotobacter vinelandii* [22]; Rhizobactin by *Rhizobium meliloti* [15] and much more. In this study, the siderophore-producing bacterium was isolated from the soil samples and analyzed for their optimum fermentation condition to understand the culture medium paving high concentration of siderophore production.

More than five bacterial consortiums were isolated and pure cultured from the iron-enriched soil sample. The distinct siderophore- producing bacterial isolates were screened out by performing CAS assay (both qualitatively and quantitatively). The cultures were grown in succinate broth medium for 48 h and the supernatant was separated and spectrophotometric analyzed for CAS assay test. Out of five culture, two bacterial isolates showed turbidity in the succinate medium and CAS test positive. The detection of siderophores was further confirmed by plating that two bacterial isolates in the CAS agar plate method. It was found that the bacterial isolates were showing distinct zone with the appearance of orange color (Fig. 1) indicating the production of siderophore and then, those two strains were taken for further studies. Orange zone appearance clearly demonstrates siderophore production. Similar results were reported by Ghosh et al. where they used fungal strains *Trichoderma viride*-1, *T. harzianum*-1, *Candida famata*-1 and three bacterial strains *Bacillus subtilis*-1, *B. megatericus* 1, *Pseudomonas aeroginosa*1for siderophore production [23].

The CAS or HDTMA forms a tight complex with the ferric ion to create a blue color in the medium, and when the iron chelators like the siderophores are added to the medium, it removes the iron from the dye complex and the color eventually changes from blue to orange [24].

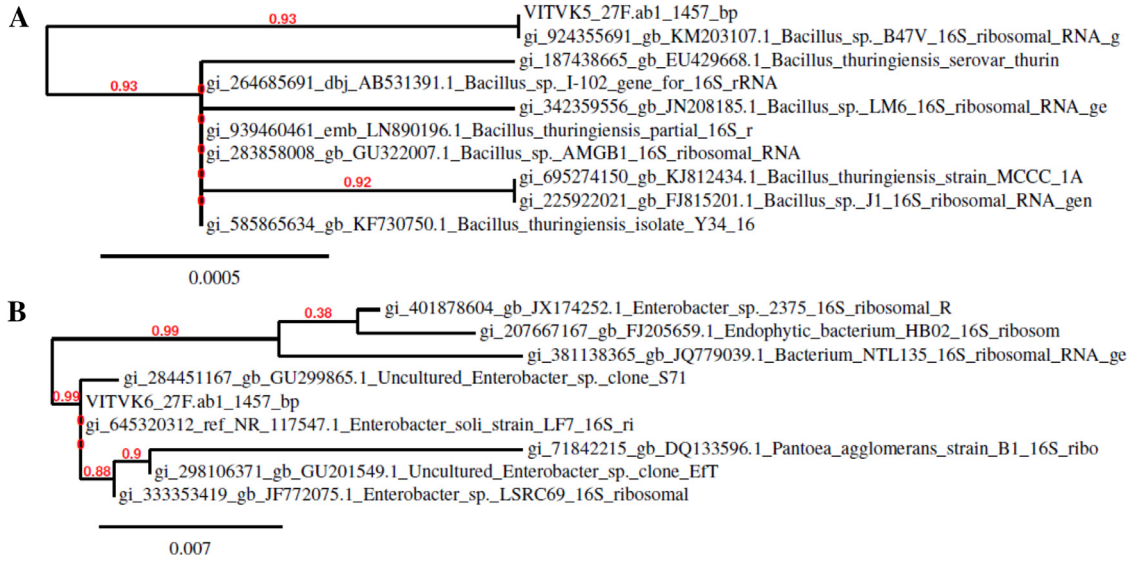

Fig. 2. Evolutionary relationships A) bacterial isolate VITVK5 showing a close relationship with *Bacillus sp.* B) bacterial isolate VITVK6 showing a close relationship with *Enterobacter sp.*

Table 1
Characterization of siderophore- producing bacterial isolates.

Morphological characteristics		
	VITVK5	VITVK6
Size	Long	Long
Shape	Rod	Straight rods
Arrangement	Chain	Single
Gram reactivity	Gram-positive	Gram-negative
Biochemical characteristics		
Test		
Citrate	Positive	Positive
MR (methylene red)	Positive	Negative
Indole	Negative	Negative
Catalase	Negative	Postive
VP (Voges-Proskauer)	Positive	Positive
Oxidase	Positive	Negative
Urease	Positive	Negative
Motility (Hanging drop technique)	Negative	Positive
TSI	Negative	Negative
Glucose fermentation (Acid/Gas)	Positive/-	Positive/Positive

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

3.2. Characterization of efficient siderophore-producing isolate

Further, the isolates VITVK5 and VITVK6 was taken for morphological, molecular and biochemical characterization. The gram stain results of VITVK5 showed rod shape that was connected continuously as chains with violet color giving an idea gram-positive *Bacillus sp.* Similarly, results of isolate VITVK6 showed gram negative, rod shaped, and motile *Enterobacter sp.* Also, the biochemical characterization of the bacterial isolate VITVK5 and VITVK6 has supported the results of gram staining. The results of biochemical characterization are given in Table 1. The molecular identification was confirmed by 16S rDNA sequencing of bacterial isolate VITVK5 and VITVK6 were showing similarity to *Bacillus sp.* more close to *Bacillus thuringiensis* and *Enterobacter sp.* more close to *Enterobacter soli.* The evolutionary relationship of the identified 16S rDNA sequencing was shown in Fig. 2. Solanki et al. also reported efficient siderophore production using *Enterococcus sp.* and its activity against plant pathogen *Rhizoctonia solani* [25].

3.3. Quantitative estimation of siderophore produced from VITVK5 and VITVK6 strains

For quantitative estimation, Chrome Azurol sulphonate (CAS) assay as described by Schwyn and Neiland was employed and OD value was measured at 630 nm. The percentage of siderophore was calculated according to the above- mentioned formulae at room temperature and neutral pH. 60.06% of siderophore production was estimated by VITVK5 and 61.79% by VITVK6 which confirmed the siderophore production quantitatively after which various optimization parameters were selected.

3.4. Different culture conditions for optimum production of siderophores

In order to understand the significant effect of various culture conditions that relates the bacterial growth and siderophore production, optimization was performed. Though iron is the major factor involved in siderophore production, other culture conditions also play some significant role in siderophore productions. Taken into considerations, conditions such as different pH, temperature, carbon source, nitrogen source, organic acids, iron concentration, cell biomass concentration etc. Table 2 depicts the comparative study of siderophore production under optimized conditions of VITVK5 and VITVK6 with previously reported literatures.

3.4.1. Influence of pH

Effect of pH on the microbial growth plays a significant role. The iron solubility and the availability to the developing microorganisms depend upon the pH of the medium. Tailor and Joshi have reported the maximum production of S-11 siderophore at pH 7, considering the fact that iron is insoluble at neutral pH and bacteria can grow optimally in the physiological environment [16]. Our study reports the maximum production of the iron chelators is initiated when the pH is at 8. Agro services international reported that the insolubility of iron increases at high pH value which supports our findings. At pH 8, iron becomes more insoluble in the soil solution and it might have stimulated the production of siderophore. It was observed from the Fig. 3, that both the bacterial isolate is showing a higher concentration of siderophore production at pH 8 which is comparatively higher than the siderophore-

Table 2
Comparison between siderophore producing organisms.

S.No.	Name of organism	Gram nature	pH	Temperature	Carbon sources	Nitrogen sources	Iron sources	Organic acids	Yield	References
1.	*Pseudomonas fluorescens NCIM 5096 and Pseudomonas putida NCIM 2847*	–	7	–	Glucose	Urea	30 µM	Succinic acid	87 83	[26]
2.	*E. coli ST2*	Negative	2.5 or 8.5	Room temperature	–	–	–	–	70 mg/L	[34]
3.	*P. aeruginosa FP6*	–	–	–	Sucrose and mannitol	Yeast extract and urea	5 µM	–	104.8 mM (yeast extract) and 92.9 mM(urea)	[35]
4.	*Escherichia coli, Bacillus spp. ST13,* and *Streptomyces pilosus*	–	6	55	Glucose Or sucrose	–	–	–	48 µg/ml 31 µg/ml 32 µg/ml	[17]
5.	*Anabaena oryzae*	–	–	–	–	–	6.55 ± 0.045 mg kg^{-1}	–	79.36	[36]
6.	*Rhodotorula strain*	–	8	–	–	Urea	–	–	60	[10]
7.	*Pseudomonas fluorescens*	Negative	7	29°C	Tyrosine	Ammonium sulphate, urea	20 µM	Succinic acid	96	[16]
8.	*Pseudomonas aeruginosa*	Negative	7.08	27.80°C	–	–	–	–	68.41%	[28]
9.	*Pseudomonas spp.-PB19*	Negative	7	29°C	Glucose (1)	Urea (0.6)	20 µM	Citric acid (4)	78%	[32]
10.	*Bacillus subtilis* (PS – 8) and *Pseudomonas fluorescens* (PS- 4)	Negative	–	–	–	–	–	–	(PS – 8) 8.30 µg/ml PS- 4 (6.10 µg/ml).	[29]
11.	*Bacillus sp.* (VITVK5) *Enterobacter sp.* (VITVK6)	Negative	8	37 °C	Sucrose and glucose	Sodium nitrate	2 g/l	Citric acid	~60–80%	In our present study

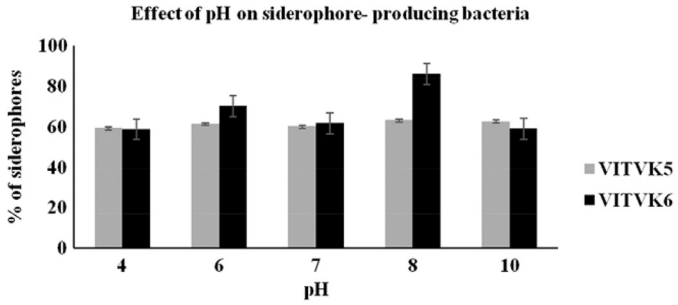

Fig. 3. Effect of pH on the production of siderophore by bacterial isolateVITVK5 and VITVK6.

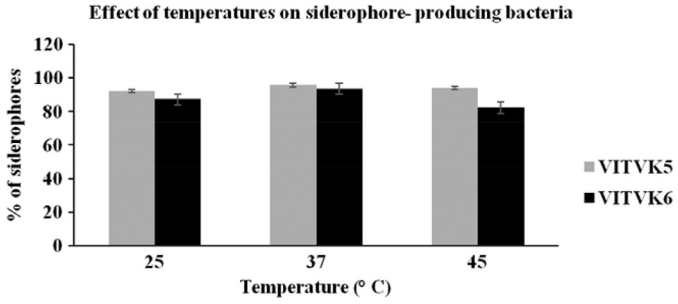

Fig. 4. Effect of temperature on the production of siderophore by bacterial isolate VITVK5 and VITVK6.

producing bacterium *Pseudomonas* and *Rhizobacteria* that are involved in plant growth promotion and stress tolerance activity [24,26]. Calvente et al. also reported similar results [10].

3.4.2. Influence of different temperatures

Further, the culture conditions were changed with constant pH and different temperature. Previous studies on siderophore production and optimization using Plackett–Burman method reported that the bacterial isolate *Pseudomonas aeruginosa* showed higher siderophore concentration at 27 °C [27]. Comparing the results of the previous study, bacterial isolates VITVK5 and VITVK6 showed production of siderophore in high concentration in all the temperature ~90% of siderophores (Fig. 4). Room temperature might be the optimum temperature for the growth of microorganism so; 35 °C shows optimum siderophore production for both the strains.

3.4.3. Influence of nitrogen sources

The optimization was carried out with the different source of nitrogen such as ammonium sulfate, sodium nitrate, and Urea. Both the bacterial isolate has produced siderophore in equal concentration in all the nitrogen source of ~60%, as shown in the Fig. 5. The results were similar to the siderophore production under different nitrogen source by the isolates *Rhodotorula sp.* [10]. *Pseudomonas fluorescence* and *Pseudomonas putida* showed maximum productivity in the presence of urea [16,24].

3.4.4. Influence of different carbon sources

The bacterial isolate was analyzed for their optimum conditions of carbon and nitrogen source in which they produce the siderophore. Supplementing the growth media with carbon sources increases the growth capacity of bacteria and the siderophore production capability. Hence we have taken major three carbon sources glucose, fructose, and sucrose. Of which both the bacterial isolate showed a higher concentration of siderophore production when they are influenced b the carbon source as sucrose shown in Fig. 6. Still, when we compared the bacterial isolate themselves with each other. The production of siderophore was found

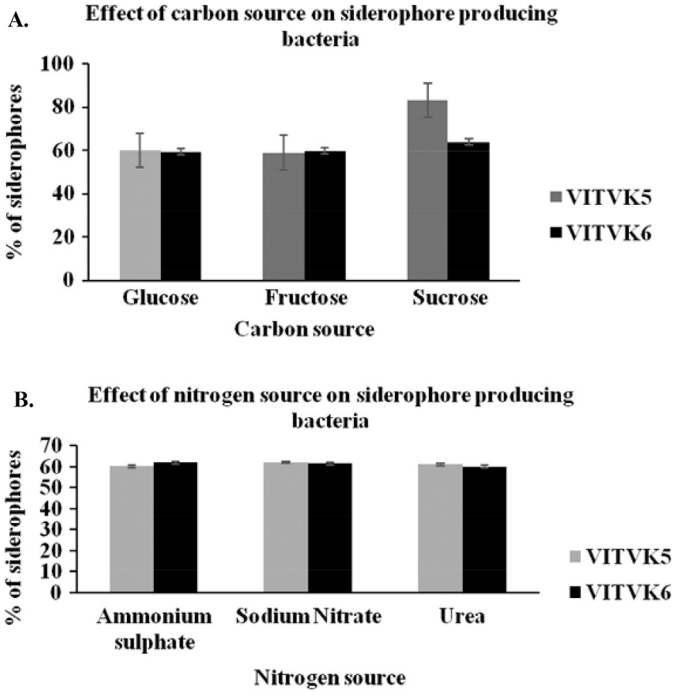

Fig. 5. Production of siderophore by bacterial isolate VITVK5 and VITVK6 under the influence of A) carbon source and B) nitrogen source.

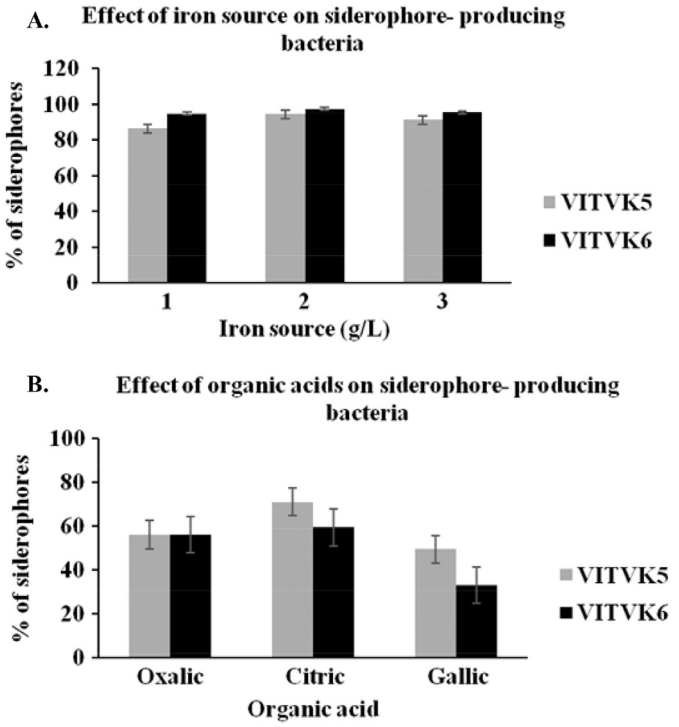

Fig. 6. Production of siderophore by bacterial isolate VITVK5 and VITVK6 under the influence of A) iron concentration and B) organic acid.

to be comparatively higher of ~83% in the bacterial isolate VITVK5 (showing an evolutionary relationship with the *Bacillus sp.*). Previous studies of siderophore-producing bacillus species have also supported the results that most of the siderophore-producing bacteria are falling under the *Bacilli sp.* [28,29]. *Pseudomonas fluorescence* showed a diminished rate of siderophore production when supplemented with sucrose, glucose, mannitol, lactose and xylose because it might have used all the available carbon source for its growth alone and not siderophore production [16]. A previous study of siderophore production by *Rhodotorula sp.* reported by Calvente et al. also shows similar results of enhanced siderophore production when supplemented with sucrose [10].

3.4.5. Influence of iron concentrations

Siderophore production under different culture conditions was further analyzed by the influence of different organic acid and iron concentrations. The results obtained shown in Fig. 6. Observation depicts that the increase in iron concentration increases the siderophore production of ~ 96%.The increase in iron concentration up to the limit of 2 g/L. might have induced an enhanced rate of siderophore production in order to bind with the available iron and provide it to the cell but as the concentration increased beyond a threshold level, siderophore production started decreasing with the increasing concentration of iron. This might be have happened because of negative transcriptional regulation by fur protein were Fe^{+2} acts as a co-repressor [16,24,30]. Tailor and Joshi also reported similar results where the concentration of siderophore produced from *Pseudomonas fluorescence* decreased after the concentration of 1 µM due to negative transcription control of iron- regulated gene [16].

3.4.6. Influence of organic acids

The production of siderophores was found to be higher in the influence of citric acid. The concentration of siderophore production in the influence of organic acid is shown in Fig. 6. The results obtained were in contrary to the results obtained by Sayeed et al. they reported that citric acid was not suitable for the production of siderophore by *P.fluorescence* and *P.putida* [26]. Sharma et al. also reported similar results of enhanced siderophore production when supplemented with citric acid [27,31–33]. This might be because our strains were best capable of assimilating citric acid out of all the organic acids supplemented.

4. Conclusion

As a vital element, iron is needed by all the living organisms from unicellular to multicellular for their numerous cellular processes. Microorganisms under iron-deficient conditions produce siderophores, low molecular weight chelators that trap iron molecules from the atmosphere, host etc., for their survival. The characterization of siderophore production by CAS assay test showed positive which was confirmed by qualitative CAS agar plate test. The appearance orange color and halo zone formation confirmed that the bacterial isolate VITVK5 and VITVK6 had the ability to producing siderophores. The morphological and molecular characterization of siderophore-producing bacteria depicts that the bacterial isolates VITVK5 and VITVK6 were showing a close resemblance to the bacterial species of *Bacillus* and *Enterobacter sp.* Further, these preliminary results paved an idea on proceeding with optimization parameter analysis where the bacterial isolates were allowed to grow in different culture conditions such as pH, temperature, carbon source, nitrogen source, organic acid and iron concentration. Results of the influence of different culture conditions showed that these bacterial isolate had the efficiency of producing siderophore in higher concentration at pH 8 (approximately equivalent to 63.2% by VITVK5 strain and 86% by VITVK6 strain), at 37 °C (95.75% by VITVK5 strain and 93.71% by VITVK6 strain), with glucose (60.06% by VITVK5 strain and 59.43 by VITVK6 strain) and sucrose (83.17% by VITVK5 strain and 63.83% by VITVK6 strain) as carbon source, $NaNO_3$(61.94% by VITVK5 strain and 61.32% by VITVK6 strain) as nitrogen source and at a cell biomass concentration of 200 µl (58.8% by VITVK5 strain and 58.33% by VITVK6

strain). The increase in iron concentration increased the production of siderophore but after a certain concentration, an increase in iron concentration inhibited the siderophore production which might be due to the negative transcriptional regulation of genes involved in siderophore production. However, further research is needed to be elucidating in detail for the production and purification of siderophore from the bacterial isolate and the application in the various fields.

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