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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].

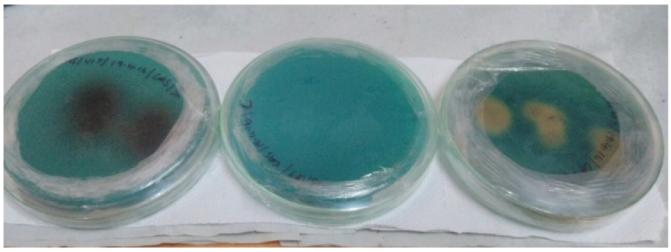

Fig. 1. The appearance of orange color and zone formation indicating siderophore production in CAS agar plate assay.

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].

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