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Isolation and application of siderophore producing bacteria

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Abstract
Siderophores are low molecular weight metal chelating agents which are produced by plants and microorganisms in Fe- limiting conditions. In this study bacteria were isolated from different soil sample and were characterized for PGPR traits. Biochemical characters were studied and the isolates were identified. Influence of different environmental factors, such as carbon source, nitrogen source, pH, temperature and salt stress, on siderophore production was studied. Isolates were further used to increase the fertility of the soil to help the plantlets grow well and increase the Fe content of the plantlets. It was found that different siderophore producing bacteria produced siderophore at different time and with different percentage. At different environmental condition the amount of siderophore produced was different. They also increased the iron concentration in plantlets grown in different soil samples.

Keywords: Siderophore, Chrome Azurol S, Hydroxamate, Catecholate

Introduction
Iron is an essential element for the growth of all the living microorganisms as it acts as a catalyst in enzymatic process, oxygen metabolism, electron transfer, and DNA and RNA synthesis (Aguado- Santacruz *et. al.,* 2012) [1]. Iron is also important in biofilm formation as it regulates surface motility and stabilizes the polysaccharide matrix (Weinberg, 2004; Chhibber *et. al.,* 2013) [25, 10]. In an environment deficient of iron, the microbial surface hydrophobicity decreases which alter the surface protein composition and thus limit the biofilm formation (Simões *et al*., 2007) [22]. Thus, the microorganisms have developed a special strategy such as the production of siderophores. Siderophores are low molecular weight (200-2000 Da) metal chelating agents which are produced by plants and microorganisms in Fe- limiting conditions (Schwyn and Neilands, 1987) [21]. Siderophore forms complex with free iron and transport it into the cell by membrane receptor molecules, these molecules are encoded by five genes in operon which is turned off when sufficient iron has been taken into the cell (Lewin, 1984) [13]. Marine organisms such as phytoplankton (Trick *et al*., 1983) [24] and cyanobacteria (Armstrong and Van Baalen, 1979) [4] can also produce siderophores. The primary role of siderophore is to scavenge Fe, but they can also form complexes with other essential elements (e.g., Mo, Mn, Co and Ni) in the environment and makes them available for the microbial cells (Bellenger *et al*., 2008; Braud *et al,* 2009 a, b) [6, 7, 8]. Depending upon the characteristics functional groups siderophores are divided into three main families, i.e., catecholates, hydroxamates and carboxylates. The formation of Fe (III)-siderophore complexes is affected by the change in the pH because of competition for the free siderophore ligands between free photon and Fe (Albrecht- Gary and Crumbliss, 1998) [3]. Siderophores not only contributes to provide nutrition to plant and microorganism but also in other environmental applications such assoil mineral weathering, biogeochemical cycling of Fe in oceans, and biotechnological applications such as enhancing growth and pathogen biocontrol of plants, biocontrol of fish pathogens, microbial ecology and taxonomy, bioremediation of environmental pollutants, petroleum hydrocarbons, nuclear fuel reprocessing, optical biosensor, bio- bleaching of pulp (Ahmed and Holmström, 2014) [2].
This work deals with the isolation of bacterial isolates from various sources having the ability to produce certain PGR's and siderophores. These isolates were further used to increase the fertility of the soil to help the plantlets grow well and increase the Fe content of the plantlets.

Materials and methods

Isolation and characterization of bacterial isolates

The samples were collected from five sites: garden soil, soil near petrol pump, soil near garage, ground soil of Birla College, Kalyan and soil near trees of Birla College, Kalyan. Soil suspension was prepared by adding 1g of soil in 10 ml sterile distilled water. This was streaked on to nutrient agar plates. The plates were incubated at 28 ^{0}C ± 2 ^{0}C for three days. Well isolated colonies were selected, purified and maintained on nutrient agar slants (Harley and Prescott, 2002) [12].

Identification of bacteria

The bacterial isolates were characterized using biochemical test: motility test, MR-VP test, TSI test, nitrate test, indole test, sugar fermentation test (Glucose, Sucrose, Lactose, Mannitol and Maltose), oxidase test, catalase test, sodium thioglycolate broth test. The results obtained were compared with Bergey's manual of bacterial identification. The growth curves for all bacterial isolates were studied. 2ml of 24 hrs old grown bacterial suspension was inoculated in 25ml of nutrient broth in side arm flask. Absorbance was measured at 540 nm using colorimeter [CL 157 (ELICO)] at an interval of 30 mins. A graph of time in mins. versus absorbance at 540 was plotted and the growth rate was calculated for each bacterial isolate (Harley and Prescott, 2002) [12].

Characterization of the isolated bacteria for PGPR traits

The selected isolates were screened for different PGPR traits like:

Phosphate solubilization: The organisms were spot inoculated onto Pikovskaya's Agar medium (Nautiyal, 1999) [16] Plates were incubated for three days at 28 °C ± 2 °C. Phosphate solubilization index (SI) was calculated by measuring the diameter of halo zone and bacterial colony using the formula (Edi- Premono *et al*., 1996) [11]:
SI (Solubility Index) = colony diameter + halo zone diameter/colony diameter

Organic acid production: The organisms were spot inoculated on MM9 agar medium with methyl red as pH indicator dye. Plates were incubated for three days at 28 °C ± 2 °C (Sambrook and Russell, 2001) [19].

Production of auxin (IAA): IAA production was carried out on a medium containing 1% peptone water, 1% tryptophan and the isolates were inoculated in the medium and incubated at 28 ^{0}C $\pm$ 2 ^{0}C for 24 hours. After incubation the cells were separated by centrifugation and the supernatant was mixed with two drops ortho-phosphoric acid and 4 ml Salkowaski's reagent (50 ml of 35% perchloric acid, 1ml 0.5 N FeCl3 solution) (Brick *et al*., 1991) [9]. Optical density was measured at 540 nm. Concentration of IAA was estimated by using the standard IAA (0.1mg/ml).

HCN production: Nutrient agar was amended with glycine (4.4 g/L) and isolates were spot inoculated on the modified agar plate. A Whatman filter paper No. 1 was soaked in 2% sodium carbonate in 0.5% picric acid solution and placed on the top of the plate. Plates were sealed with parafilm and incubated at 28 °C ± 2 °C for 4 days (Lock, 1948) [14].

Siderophore production: Isolates were screened for siderophore production on Chrome Azurol S (CAS) plates. The organisms were spot inoculated and incubated at 37 °C for 24- 48 hours (Milagres *et al.*, 1999) [15].

Influence of environmental factors on growth and Siderophore production

Freshly grown cultures were subjected to different conditions to study the effect of various environmental factors, media components and physical parameters. All culture flasks were incubated for 24- 48 h at 28 °C ± 2 °C. In each case the quantification of siderophore was done by using universal chemical assay as per Schwyn and Neilands (1987) [21]. Quantification was done by CAS- shuttle assay (Payne, 1994) [17]. In this method, 0.5ml of aliquot of culture filtrate was taken in a test tube and then 0.5ml of CAS reagent was added and in reference/ blank, 0.5 ml of uninoculated medium and 0.5ml of CAS reagent was added. Colour change was observed and measured colorimetrically and then percentage siderophore units was measured by the formula

$$\% \text{ siderophore units (SU)} = \left[\frac{(Ar - As)}{As}\right] \times 100$$

$Ar -$ Absorbance of reference/ blank at 600 nm
$As -$ Absorbance of sample at 600 nm

The effect of environmental factors on Siderophore production was determined by various parameters. The effect of carbon source was checked using different carbon sources such as citric acid, lactate, glucose, sucrose, glycerol, maltose, sodium acetate and lactosein nutrient broth at concentration of 2 g/ml (2%) resp. The organism was inoculated in the medium and incubated at 37 ^{0}C for 24 hours. The Siderophore production are known to be varied with pH of the medium. The organisms were grown in the succinate medium with a pH of 3, 5, 7, 9 and 11. The effect of temperature was determined by inoculating growing cells in succinate medium and incubating at various temperatures viz., 10 °C, 28 °C, 37 °C and 55 °C. The influence of different nitrogen sources viz. ammonium sulphate, urea, ammonium chloride, and sodium nitrate was studied at the concentration of 0.1% w/v. To check the effect of osmotic pressure on growth and siderophore production, sodium chloride was added at a final concentration of 2%, 4%, 6%, 8% and 10% within the medium. Siderophore production was quantified in the culture by adding CAS reagent in the medium and was measured colorimetrically at 600 nm (Sayyed *et al.*, 2005) [20].

Detection of functional group

The 24 hr old culture's supernatant was subjected to various tests for the detection of the iron- chelating function group. The various iron chelating functional groups were assessed by performing the following tests:

Tetrazolium test (Snow, 1954) [23] For determining hydroxamate type of siderophore, a pinch of tetrazolium salt was added in a test tube to which 1-2 drops of 2 N NaOH was added and then 1ml of test sample was added.
Arnow's test (Arnow, 1937) [5] For catecolate type of siderophore, 1 ml of culture supernatant was added in a test tube to which 1 ml of 0.5N HCl was added then 1 ml of nitrite molybdate reagent was added. After formation of yellow colour 1ml of 1N NaOH was added.

Application of bacterial siderophore

Bacterial isolates were grown in nutrient broth at 37 ^{0}C till their OD reached 0.2 extinction units. The culture was centrifuged and the pellet was washed free of any medium. The pellet was resuspended in sterile Saline (0.8% w/v). The 10 ml of the culture was added to 20 gms of sterile cocopeat and garden soil respectively. Moong seeds were sterilized using 1% bavistin and sowed in the inoculated cocopeat and garden soil respectively. The seeds were kept at room temperatures and watered daily. After 7 days, the grown plantlets were removed, washed to remove the traces of soil. The Fe content of the plantlets was checked using ICP-AES (SAIF, IIT Bombay) analysis. Untreated cocopeat and garden soil was used as blank for growing moong seeds

Results and Discussion

11 morphologically distinct bacterial isolates were isolated from the various sources and were studied for their colony characters. After performing the biochemical test and comparing with Bergey's manual, it was observed that the above bacterias may belong to *Enterobacter spp., Erwinia spp., Citrobacter spp., Bacillus spp., Micrococcus Spp.* or *Microbacterium spp.* The optimum temperature for growth was found to lie between 28 to 37 ° C at a pH range of 7 to 9. All the 11 isolates were screened for the PGPR (Plant growth promoting *rhizobacteria*) traits. All the isolates produced IAA at varying concentrations. The concentration of IAA produced by each strain was estimated by comparing with the standard (Fig 1).

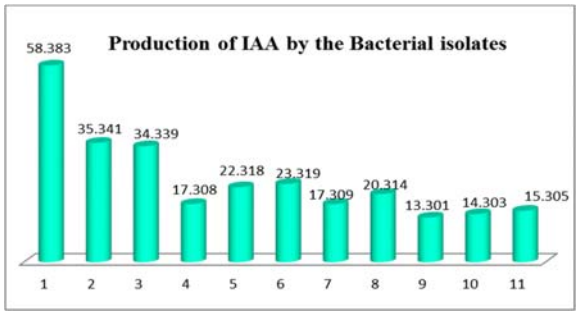

Fig 1: Production of IAA by the bacterial isolates

The isolates showed negative results for phosphate solubilisation and HCN production. Four organisms, *i.e*., one organism from the soil near the garage (Garage 1) and three organisms from the soil near the tree (NT1, NT2, NT3), turned the blue colour of the agar to pink colour indicating utilization of Fe and thus indicate the production of siderophore. NT3 and Garage 1 showed the production in 24 hours whereas NT 1 and NT2 showed the production in 48hours (Fig 2). These four isolates were further studied for their siderophore production.

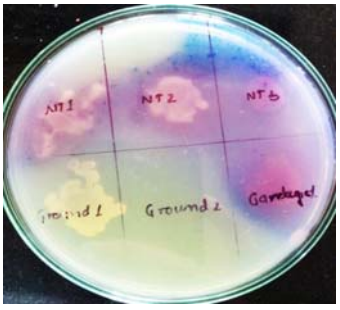

Fig 2: Siderophore production by the bacteria isolates

Influence of environmental factors on growth and siderophore production

All the organisms producing siderophore were subjected to different conditions to study the effect of various environmental factors, media components and physical parameters. The concentration of siderophore produced was estimated and calculated (Schwyn and Neilands, 1987) [21].

The four isolates were able to grown and produce siderophore in all the carbon sources except in lactate and citric acid. It was found that maximum amount of siderophore production was observed in media containing glycerol. NT 1 produced maximum amount of siderophore in glycerol whereas NT 2 produced minimum amount of siderophore in glycerol (Fig 3).

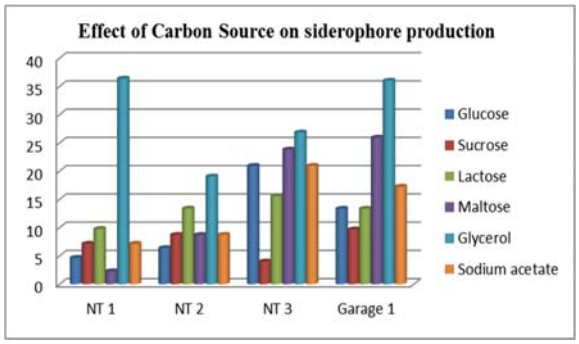

Fig 3: Effect of Carbon Source on siderophore production

pH plays an important role in the solubility of iron and thereby its availability to the growing organism in the medium. It was found that maximum amount of siderophore production was observed at pH 9, in all the bacterial isolates (Fig 4).

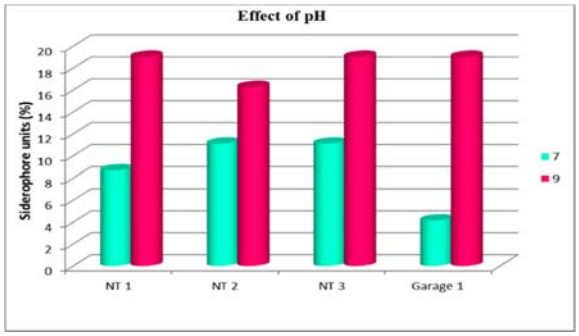

Fig 4: Effect of pH on siderophore production

It was observed that NT 1 produced maximum amount of siderophore at 37 °C, whereas, NT 2, NT 3 and Garage 1 produced maximum amount of siderophore at 28 °C (Fig 5).

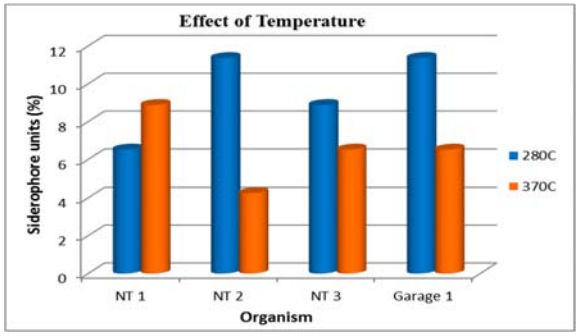

Fig 5: Effect of temperature siderophore production

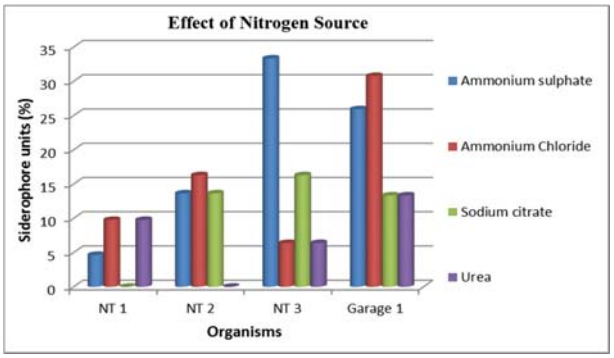

Fig 6: Effect of nitrogen on siderophore production

It was observed (fig 6) that NT 1 produced maximum amount of siderophore in a media containing ammonium sulphate and urea and least amount of siderophore production was observed in sodium citrate. NT 2 produced maximum amount of siderophore in a media containing ammonium chloride and minimum amount of siderophore production was observed in urea. NT 3 produced maximum amount of siderophore in a media containing ammonium sulphate and minimum amount of siderophore production was observed in ammonium chloride and urea. Garage 1 produced maximum amount of siderophore in a media containing ammonium chloride and minimum amount of siderophore production was observed in sodium citrate and urea.

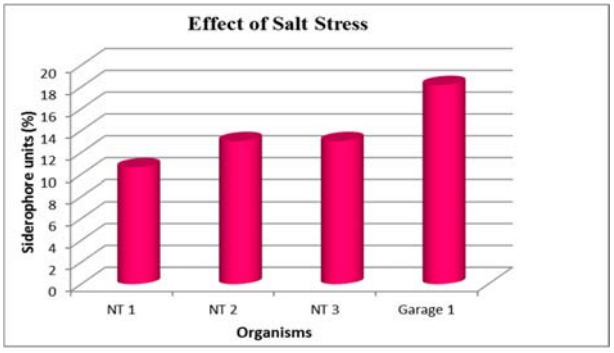

Fig 7: Effect of salt stress on growth and siderophore production

It was found that Garage 1 produced maximum amount of siderophore whereas, NT 1 produced minimum amount of siderophore at 2% salt stress (Fig 7).

Detection of functional group

All the four siderophore producing bacteria's were subjected to various tests for identifying the iron- chelating functional group. All the four bacteria's showed the development of deep red colour, thus, indicating the presence of hydroxamate type of siderophore (Snow, 1954) [23]. Powell *et al*. (1980) [18] have shown that hydroxamate siderophores are present in various soils and they are also produced in aquatic environments.

Application of bacterial siderophore

Two organisms were selected on the basis of production of siderophore by the bacteria. The selected organisms were NT 3 and Garage 1 as they produced more amount of siderophore as compared to the other two i.e., NT 1 and NT 3, on the CAS media. The Fe content of the plantlets was checked using ICP-AES (SAIF, IIT Bombay) analysis.

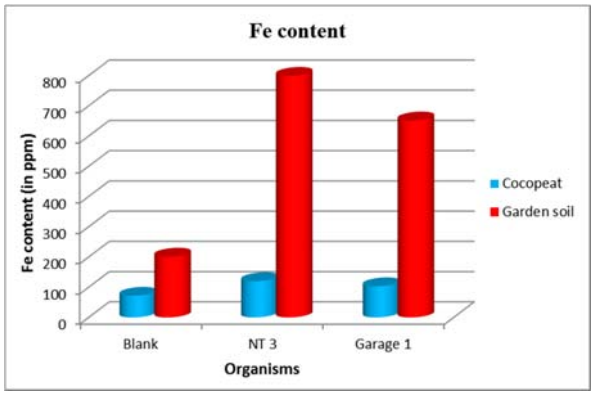

Fig 9: Fe content in plantlets

From the graph (Fig 9), it was observed that NT 3 and Garage 1 increased the Fe content in the plantlet when the organisms were added to the soil, i.e., cocopeat and garden soil. NT 3 is more suitable for the growth of the plantlet as it increases the Fe content in the plantlet more than that of Garage 1. NT 3 was found to be more suitable for the growth of the plantlet as it increases the Fe content 120.077 (cocopeat), 799.375 (garden soil) in the plantlet, which was more than that of Garage 1 103.291 (cocopeat) and 651.801 (garden soil).

Conclusion

The 11 bacterial isolates were screened for different PGPR traits. These isolates were able to produce IAA and Siderophores. Maximum IAA production was estimated in NT- 1 (58.383) and minimum IAA production was estimated in P- 1 (13.301). Siderophore production was estimated in four isolates, i.e., Garage 1, NT 1, NT 2 and NT 3. Garage 1 and NT 3 produced siderophore in 24 hours whereas other two produced in 48 hours. Different environmental factors influence the growth and siderophore production. All the siderophore producing bacteria produced maximum siderophore at pH 9, in a medium containing glycerol and with 2% salt concentration. Siderophores are small molecules with large biotechnological potential. With the advent of new molecular methods and technologies further research and development is required to harness the beneficial aspect of these molecules in the field of both medical and environmental microbiology.

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