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STUDIES ON OPTIMIZATION OF SIDEROPHORE PRODUCTION BY *PSEUDOMONAS AERUGINOSA* AZAR 11 ISOLATED FROM AQUATIC SOIL AND ITS ANTIBACTERIAL ACTIVITY

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ABSTRACT

***Background**: Siderophores are low molecular weight iron-chelating molecules produced by micro-organisms to carry out multiple metabolic processes, where iron serves as a cofactor. **Objectives:** The objectives of the current study were to optimize and characterize siderophore production by bacterial isolate and determine its antibacterial activity. **Method:** In the present study, thirty-five bacterial cultures were isolated from five soil samples collected from aquatic environment and checked for siderophore production using CAS-agar assay method. Optimization of various physicochemical parameters was carried out for siderophore production by the most promising isolate which was identified by 16s rRNA gene sequencing analysis. Finally, the antibacterial activity of the siderophores was determined against the laboratory cultures as well as β-lactamase producing uropathogens. **Results:** Maximum siderophore production (59.18%) was obtained in king's B medium (pH7) by Pseudomonas aeruginosa azar 11, in 72h at 37°C using a 5% inoculum size of 0.6O. D_{600nm} under shaker conditions (120rpm). It was further observed that supplementation of 0.45% glycine (59.18%) in Kings B medium did not affect siderophore production, whereas 0.05% maltose (57.35%) slightly inhibited the same. However, other nutrient sources like ammonium nitrate (47.27%SU), proline (45.00%) and citric acid (33.89%SU) showed a considerable reduction in siderophore production. Among metal ions, cobalt sulfate (4.91%SU) significantly inhibited siderophore production. The cell-free extract of test culture also showed antibacterial activity against few of the test isolates. **Conclusion:** All these results collectively suggest appropriate industrial application of P. aeruginosa azar 11 for siderophore production after further optimization studies.*

KEY WORDS

16s rRNA, Antibacterial, CAS-agar assay, Siderophore

INTRODUCTION

Iron is among the most vital elements required to carry out several physiological processes in all living things. From unicellular micro-organisms to multicellular complex life forms, every being may face significant impairment of necessary life functions under iron-deficient conditions leading to severe malformation or even death. The major roles of iron in plants and animals include the biosynthesis of chlorophyll, redox reactions in ATP and ribonucleotide synthesis, formation of heme, cell cycle regulation and detoxification [1]. Although optimum levels of iron is a requisite for biologically processes, high concentrations are extremely toxic to living cells due to its involvement in Fenton reaction i.e., production of free radicals. Hence, the availability of free iron is tightly regulated in cells where it is found in the bound state to proteins such as hemoglobin, transferrin, lactoferrin and ferritin [2].

In order to overcome this limitation, microorganisms synthesize and secrete low molecular weight organic

ferric ion- specific chelating agents called siderophores. It helps in sequestering, and extra-cellular solubilization of iron from minerals or organic compounds present in the environment [3, 4]. In nature, more than 500 types of siderophores are studied, of which 270 have been structurally characterized [5]. Various species of bacteria belonging to genus Escherichia, Salmonella, Klebsiella, Vibrio, Aeromonas, Aerobacter, Enterobacter, Yersinia and Mycobacterium are known to produce siderophores. Bacterial siderophores are basically classified by the ligands used to chelate the ferric iron. It is of four types' viz., hydroxamate, catecholate, salicylate and carboxylate [6]. Besides bacteria, several common species of fungi e.g., Penicillium, Mucor, Rhizopus, Saccharomyces; actinomycetes e.g., Nocardia, Streptomyces; and algae e.g., Anabaena are also known to produce siderophores [6]. Arguably, the genus Pseudomonas contributes to the most diverse and ecologically significant group of bacteria [7]. The ability of this species to produce siderophores, thus enable us to anticipate abundant siderophore production and its probable applications in various fields in near future.

Plants face iron-deficiency due to the presence of insoluble ferric oxides in alkaline or porous soil that cannot be absorbed by the roots [8]. It is only under acidic conditions that iron is freed from the ferric oxides and becomes more available for uptake by roots [9]. Hence, plants tend to produce phytosiderophores to meet their iron requirement; however, it is often inefficient to meet the demands of a growing plant. Studies have shown that the bacterial siderophore treatments significantly increase plant yield, chlorophyll and iron content. Thus, it indicates that the siderophores are effective in providing iron to the plants in presence as well as the absence of bacterial isolates producing them [10]. The symbiosis between the bacteria and its host plant is exhibited by the formation of root nodules. Within the nodules, the bacteria fix atmospheric nitrogen into biologically useful compounds that are shared with the host. Proper formation of these nodules is dependent upon sufficient iron acquisition by the bacteria. Additionally, due to the competition for iron, siderophore production has been identified as one of the important mechanisms for the suppression of phytopathogens [11].

Although siderophores contribute to plant and animal nutrition, its functions are not limited to the same. They also find extensive applications in biogeochemical cycling of iron in oceans and soil mineral weathering. The biotechnological applications include bioremediation of environmental pollutants like metals, petroleum hydrocarbons, nuclear fuel reprocessing, biobleaching of pulp etc. [12, 13]. The obligate nutritional requirement for iron by pathogens is also exploited to control infections in fish and plants by selective drug delivery using a trojan horse strategy i.e., use of siderophore-antibiotic conjugates known as Sideromycins [14]. Other major clinical applications of siderophores include treatment of diseases like hemochromatosis, thalassemia, and dialysis encephalopathy [15].

The current study emphasizes the isolation of indigenous bacteria from an aquatic environment having the ability to produce siderophores. The characterization of these siderophores was also carried out by analytical techniques. Further attempts were made to understand how metabolic and physicochemical alterations affect siderophore production by bacteria. In addition, the antibacterial activity of the siderophore produced by the test isolate was also studied against laboratory cultures as well as β-lactamase producing uropathogens.

MATERIALS AND METHODS

Iron Decontamination

All glassware used in our study were soaked overnight in 6M hydrochloric acid (HCl) and rinsed with distilled water several times to remove any traces of iron [16].

Collection of soil sample and enrichment of bacterial isolates

Five pond soil samples were collected from different sites in Mumbai i.e kemps corner, Baan Ganga, Borivali, Bandra, and Vikhroli. The soil suspension was prepared by adding 1g of soil-sample to 10mL sterile distilled water, and 1mL of this suspension was inoculated in sterile Nutrient Broth (NB) and incubated at 37°C for 24h in order to propagate the possibly stressed organisms. After the incubation period, the cultures were streaked on to Nutrient Agar (NA) plates and further incubated at 37°C for 24h. Later, well-isolated colonies were grown on NA slants and maintained at 4°C to carry out further screening of siderophore producers [17].

Preparation of Chrome Azurol S medium for screening and isolation of siderophores producers

The Chrome Azurol S (CAS) medium used in our study was the same as suggested by Schwyn and Neilands, 1987; except that all nutrients were removed [18]. It was prepared by mixing 4 solutions given below.

Solution 1 (Fe-CAS indicator solution): It was prepared by mixing 10mL of 1mM $FeC1_3.6H_2O$ (prepared in 10mM HCl) with 50mL aqueous solution of CAS (1.21mg/mL). The resulting dark purple mixture was added slowly, with constant stirring, to the 40mL aqueous solution of Hexadecyl trimethyl ammonium bromide (HDTMA) (1.82mg/mL) yielding a dark blue solution.

Solution 2 (Buffer solution): It was prepared by dissolving piperazine-N, N'-bis(2-ethane sulfonic acid (PIPES) in 750mL of a salt solution containing 0.3g KH_2PO_4, 0.5g NaCl, and 1g NH_4Cl. The pH was adjusted to 6.8 with 50% KOH, and water was added to bring the volume to 800mL. The solution was autoclaved after adding 15g of agar.

Solution 3 (Mineral solution): It was prepared by adding sugars and minerals in 70mL distilled water. It included 2g glucose, 2g mannitol, 493mg $MgSO_4.7H_2O$, 11mg $CaCl_2$, 1.17mg $MnSO_4.H_2O$, 1.4mg H_3BO_3, 0.04mg $CuSO_4.5H_2O$, 1.2mg $ZnSO_4.7H_2O$ and 1mg $Na_2MoO_4.2H_2O$.

Solution 4 (amino acid solution): It was prepared by filter sterilizing 30mL solution of 10% casamino acids.

All solutions were sterilized separately, cooled to 50°C and mixed together, with constant stirring taking precaution to avoid the formation of bubbles [19].

The Modified CAS agar plate was prepared by mixing two solutions given below.

Solution 1: It was prepared by dissolving 60.5mg CAS in 50mL de-ionized water and mixing it with a 10mL iron solution (containing 1mM $FeCl.6H_2O$ and 10mM HCl).

Solution 2: It was prepared by dissolving 72.9mg of HDTMA in 40mL de-ionized water resulting in a dark blue solution.

Both solutions were autoclaved separately and mixed slowly. The final mixture of 100mL volume was added to 900mL of sterile Luria Bertani agar (pH6.8) [20].

Qualitative screening of siderophore producers

The qualitative screening of siderophore producers was carried out by spot inoculating 24h old test isolates on modified CAS agar plate. The change in the blue color of the medium to orange, or the presence of yellow to light orange halo surrounding the test colony after 24-72h incubation indicated the production of siderophores [19].

Qualitative estimation of siderophore production

For quantitative estimation of siderophore production, the test isolates were inoculated in sterile NB and incubated at 37°C for 24h. After incubation, the fermented broth was centrifuged at 10,000rpm for 10mins under refrigerated conditions. A small aliquot of the cell-free supernatant (0.5mL) was then mixed with 0.5mL CAS solution. The color intensity was determined using the spectrophotometer at absorbance 630nm after 20mins of incubation. The reference solution used in our study was prepared by mixing 0.5mL CAS solution with 0.5mL un-inoculated NB media.

The percentage of siderophore type iron-binding compounds was calculated by using the formula

$$\% \text{ siderophore units} = \frac{(Ar).(As)}{(As)} \text{ X } 100$$

Where "Ar" is the absorbance of reference and "As" is the absorbance of the test sample [4, 21].

Identification of isolates

Primary identification of the isolate was done on the basis of morphological, cultural and biochemical tests. The strain was confirmed by 16s rRNA gene sequence analysis. PCR based 16S rRNA gene amplification and sequencing of the isolated bacterium was carried out using universal primers at Sai Biosystems Private Limited, Nagpur India.

Optimization of physicochemical parameters for siderophore production

The biological production of siderophores is affected by several environmental factors like growth medium, pH, temperature, oxygen, incubation time, NaCl concentration, inoculum size etc. In our study, the optimization experiments were initiated by determining the optimum nutrient medium for siderophore production. The 8 different iron-deficient nutrient media tested in the current study and its composition (in g/L) are given below.

Nutrient broth: [NB containing peptone (5), beef extract (3), NaCl (5); pH 7.0] [22];

Glycerol medium: [Glycerol (10); $(NH_4)_2SO_4$ (1), $MgSO_4.7H_2O$ (1), K_2HPO_4 (4)] [7];

King's B: [King's B medium containing proteose peptone (20), K_2HPO_4 (1.5), $MgSO_4.7H_2O$ (1.5)] [7];

Aspargine medium: [Asparagine (5), $MgSO_4$ (0.1), K_2HPO_4 (0.5)] [7];

Malt medium: [Malt extract agar containing malt extract (2.0), agar (2.2); pH 5.6] [4];

Sodium succinate medium: [Sodium succinate medium containing KH_2PO_4 (6), K_2HPO_4 (3), $(NH_4)_2SO_4$ (1), $MgSO_4.7H_2O$ (0.2), sodium succinate (4)] [23];

Succinate medium: [Succinate medium containing K_2HPO_4 (6), KH_2PO_4 (3), $(NH_4)_2SO_4$ (1), $MgSO_4.7H_2O$ (0.2), succinic acid (4)] [16].

In our study, the siderophore production was monitored by using 50mL medium inoculated with 0.25mL of 24h old culture and incubating it at 37°C under shaker conditions (120rpm) for 24h. The optimization of other physicochemical parameters for production of siderophores was investigated by varying one parameter at a time while keeping the others constant. These varying parameters included temperature (28°C, 37°C and 45°C), aeration i.e., static or shaking (120rpm), incubation time (24h, 48h, 72h, 96h, 120h), inoculum size (1-10%), optical density of test isolate ($O.D_{600nm}$ 0.2, 0.4, 0.6, 0.8, 1.0), pH (5-10), concentration of NaCl (1-10%) and iron (10-100µM) [24, 25, 26].

In addition the effect of 0.1% solution of different carbon sources (glucose, sucrose maltose, galactose, fructose, lactose, sorbitol, xylose and mannitol), organic nitrogen (peptone, yeast extract, soya, glycine and tryptone) and inorganic nitrogen sources (urea, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate, ammonium oxalate, tri-ammonium citrate and sodium nitrate), amino acids (arginine, leucine, pyruvate, asparagine, valine, methionine, alanine and proline), organic acids (oxalic acid, malic acid, lactic acid, palmitic acid and citric acid) and 100µM metal ions (aluminium potassium sulfate, manganese sulphate, cadmium chloride, lead acetate, cobalt sulphate, zinc chloride, cupric sulphate and silver nitrate) was also studied on siderophore production. The concentration of optimized nutrient sources was further determined by studying the siderophore production in the respective nutrient range of 0.05-5% [12, 16, 26-33].

Antimicrobial activity of siderophores

The antimicrobial assay of siderophores was performed using agar cup method. For this purpose, 20mL sterilized and molten NA butt was mixed with a 0.5mL suspension of 4h old test cultures adjusted to 0.1O. D_{530nm} along with 2% trizolium tetrachloride (TTC) and poured in sterile petri plates. Wells of 8mm diameter were made in plates with the help of a cork borer. Siderophore extract was prepared by growing the test isolate in Kings B media and centrifuged to obtain a cell-free extract. This extract was added to the wells and the plates were incubated for pre-diffusion at 4°C for 3h, followed by 37°C for 24h. Control wells containing sterile media were also set up [34]. The antimicrobial activity was checked against 10 laboratory cultures including *Staphylococcus aureus, S. aureus* 6538p, *Streptococcus pyogens*, *Salmonella typhi*, *Salmonella paratyphi* A, *Salmonella paratyphi* B, Shigella sp., *Vibrio cholerae, Klebsiella pneumoniae* and *Escherichia coli*. In addition 20 β-lactamase producing isolates including 17 *E. coli* and 3 *K. pneumoniae* previously identified in our study were also tested [35].

Characterization of siderophores

Detection of Hydroxamate nature

Two different tests were carried out to determine the Hydroxamate nature of siderophores.

1. Neilands' spectrophotometric assay: 1mL of cell-free supernatant was mixed with 1-5mL of freshly prepared 2% aqueous $FeCl_3$ solution, and absorbance was measured between 400-600nm. A peak between 420-450nm indicated the hydroxamate nature of the siderophores [36].
2. Tetrazolium salt test: A pinch of tetrazolium salt and 1-2 drops of 2N NaOH was added to 0.1mL of the test culture supernatant. Instant appearance of a red to deep-red color indicated the presence of hydroxamate siderophores [36].

Detection of Catecholate nature

1. Neilands' spectrophotometric assay: 1mL of cell-free supernatant was mixed with 1-5mL of freshly prepared 2% aqueous $FeCl_3$ solution, and absorbance was measured between 400-600nm. The formation of wine-colored complex and the occurrence of a peak at 495nm indicated the catecholate nature of the siderophores [36].
2. Arnow's test: 1mL of cell-free supernatant was mixed with a solution containing 1mL 0.5N HCl, 1mL nitrite molybdate reagent and 1mL 1N NaOH. The volume was made up to 5mL and absorbance was measured at 500nm using 2, 3 dihydroxybenzoic acid as standard [36].

Detection of Carboxylate nature

1. Vogel's chemical test: A test reagent was prepared by adding 3drops of 2N NaOH to 1 drop of phenolphthalein. Distilled water was added to this reagent until the development of pink color. The disappearance of pink color on the addition of test

sample indicated carboxylate nature of siderophores [36].

2. Spectrophotometric assay: 1mL of cell-free supernatant was mixed with a solution containing 1mL 11M $CuSO_4$ and 2mL acetate buffer (pH4). The formation of the copper complex was observed by scanning for absorption maximum between the entire range of 190-280nm, since there is no specific wavelength for absorption of copper complexes [36].

Detection of siderophores by Thin Layer Chromatography (TLC)

The culture supernatant of siderophore producer was spotted on 10×20mm silica gel plates and allowed to dry. The plates were run in an n-butanol: acetic acid: distilled water (12:3:5) solvent system until the solvent front reached the top. It was then dried and sprayed with 0.1M $FeCl_3$ prepared in 0.1N HCl. The formation of a wine-colored spot indicated a hydroxamate-type siderophore, while a dark gray spot indicated production of a catechol-type siderophore. Siderophores were separated on the basis of hydrophobicity using these plates [37].

RESULTS AND DISCUSSION

Sample collection, enrichment, isolation and screening of siderophore producers

Thirty-five isolates were obtained from 5 pond samples collected in our study. Out of these, 22 isolates showed siderophore production on CAS agar plates, characterized by a yellow/orange zone around the growth as shown in Fig. 1. Quantitative estimation of siderophores produced by these isolates revealed maximum activity in 6 isolates viz., SID10, SID16, SID29, SID33, SID34, and SID35. The zone sizes produced by these isolates and percentage siderophore production are represented in Table 1. Among these 6 isolates, SID10 showed maximum siderophore production ie.45.65%SU, and therefore was used for further optimization studies.

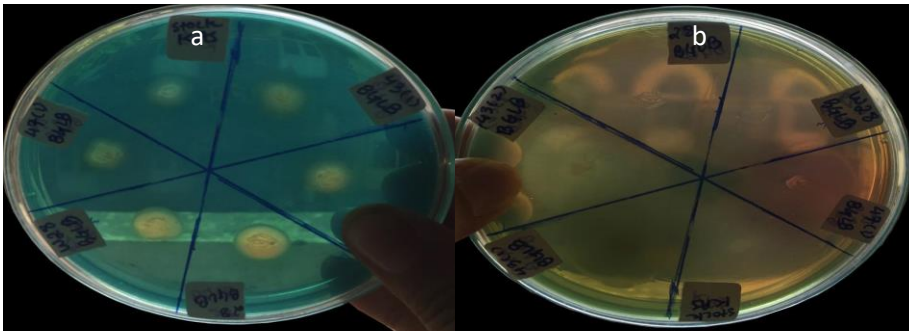

Fig.1: Qualitative screening of siderophore production by using CAS agar plates. Light orange yellow hallos observed on day 1 (a) and day 5 (b)

Table 1: Qualitative and quantitative estimation of siderophore production by test isolates

Culture	*Zone size of halos observed*					*%Siderophore Production*
	Day1	*Day2*	*Day3*	*Day4*	*Day5*	
SID10	12	21.33	32	60.66	66.66	45.65
SID14	11	16	27.33	28.83	55.33	30
SID 29	11.33	17.16	25.33	48.33	62.66	26.53
SID33	15	21.83	27.33	48.66	51	20
SID34	12	19.5	21.83	46.33	57.66	37.77
SID35	12.5	21	29.66	58	64	34.37

Identification of the isolate

The cultural, morphological and biochemical tests identified the promising isolate (i.e., SID10) as *Pseudomonas aeruginosa* and 16s rRNA analysis also confirmed the same. The nucleotide sequence analysis of the isolate was done at BlastN site on NCBI server (http://www.ncbi.nlm.nih.gov/ BLAST) and corresponding sequences were downloaded. The

identified isolate was deposited as *Pseudomonas aeruginosa* azar 11 at National Centre for Biotechnology Information (NCBI) with an accession number LC333996.

Many gram-negative bacteria are known to secrete siderophores under iron-limiting conditions, either in the environment or in an animal host, in the case of pathogens. However, most of the research has been carried out using the iron transport systems of *E. coli* [16]. Over the years many studies have reported production of siderophores in Rhizobium species viz., *R. ciceri*, *R. meliloti* and *R. radiobacter*; Azotobactor species viz., *A. vinelandii* and *A. chroococcum*, and Bacillus species like *B. shackletonii*, *B.subtilis* and *B. cereus* [38-42]. Among the genus Pseudomonas, *P. ultimum*, *P. aeruginosa*, *P. syringae*, *P, cepacia* are reported to be siderophore producers [39, 43-45]. According to a recent study, Pseudomonas are key models to assess beneficial plant-bacteria interactions, because they display a wide range of properties which is beneficial to plants like disease suppression in soil [46].

Optimization of physicochemical parameters for siderophore production

The results for the optimization of physicochemical parameters for siderophore production are represented in Fig. 2-20. The optimized culture condition for siderophore production by *P. aeruginosa* azar 11 was achieved when it was grown in King's B medium (pH7) and incubated at 37°C for 72h under shaker conditions using a 5% inoculum size of 0.6O.D_{600nm}.

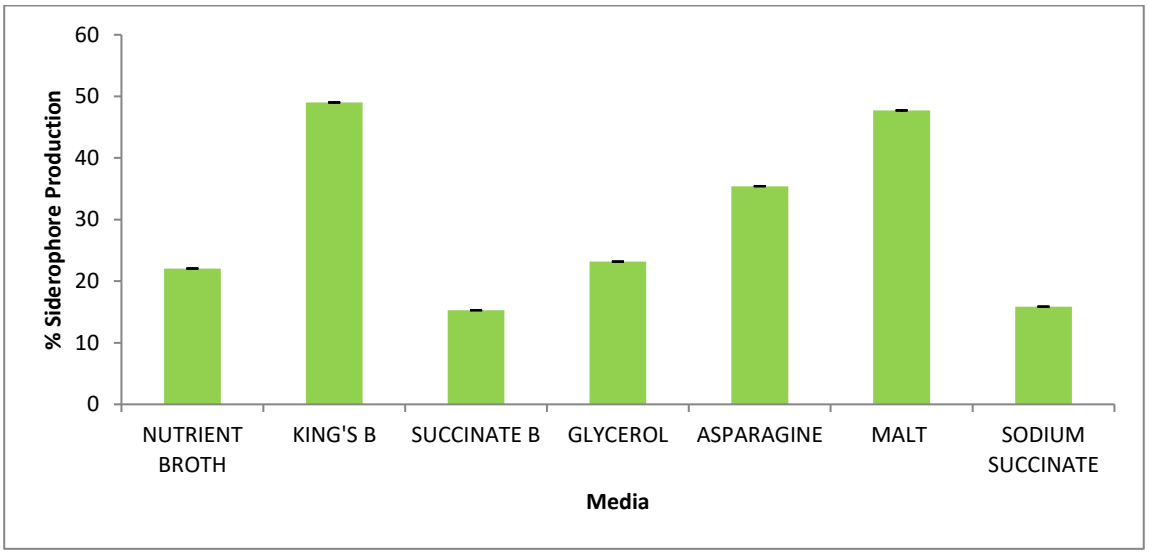

Fig.2: Effect of different media on siderophore production

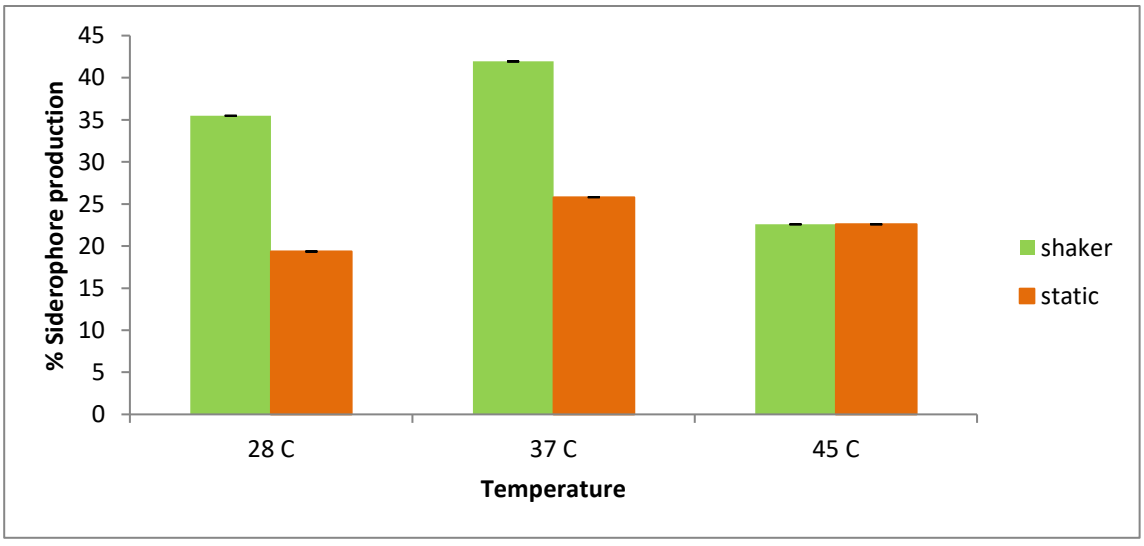

Fig.3: Effect of temperature and aeration on siderophore production

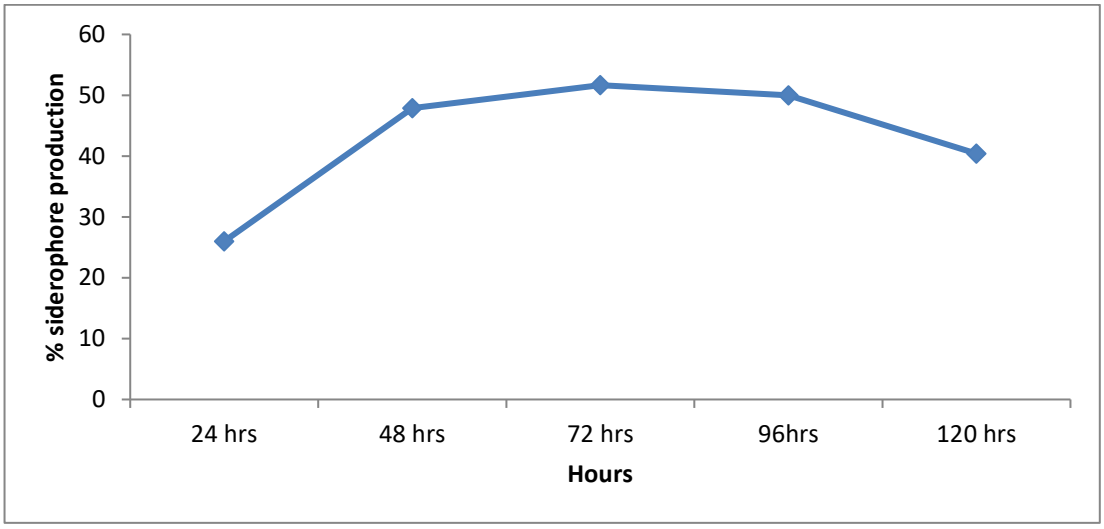

Fig.4: Effect of incubation time on siderophore production

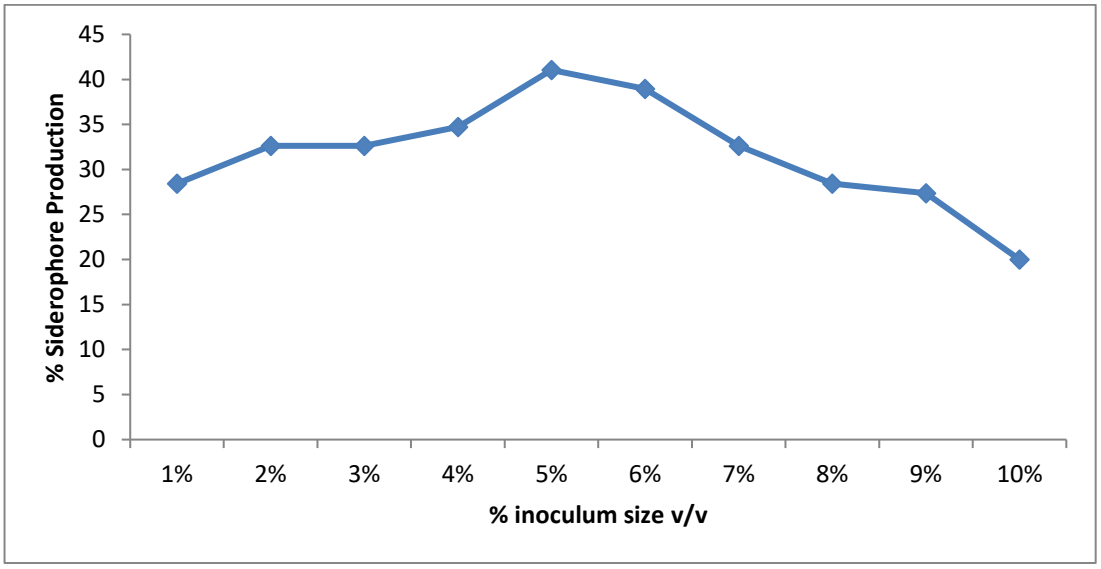

Fig. 5: Effect of inoculum size on siderophore production

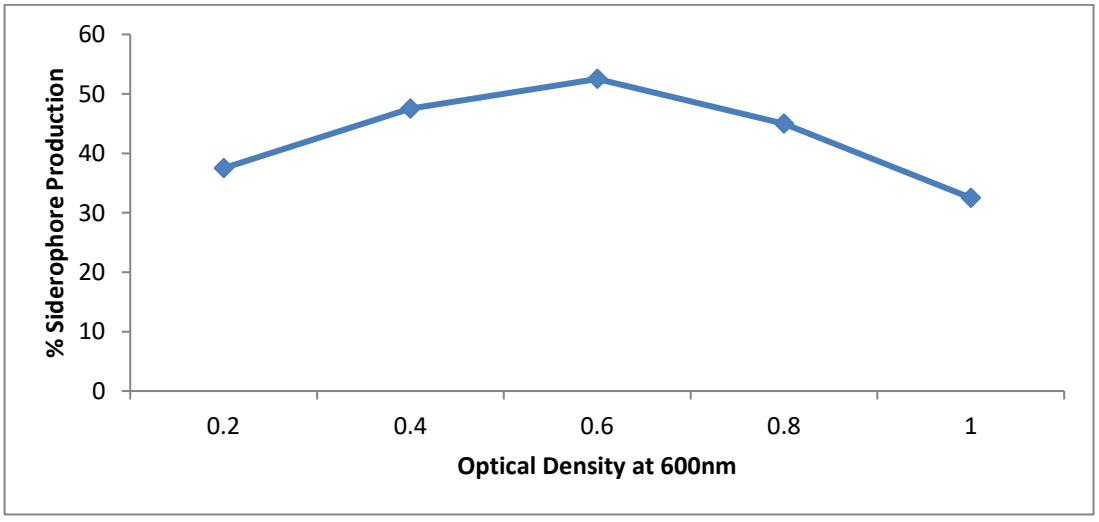

Fig.6: Effect of optical density on siderophore production

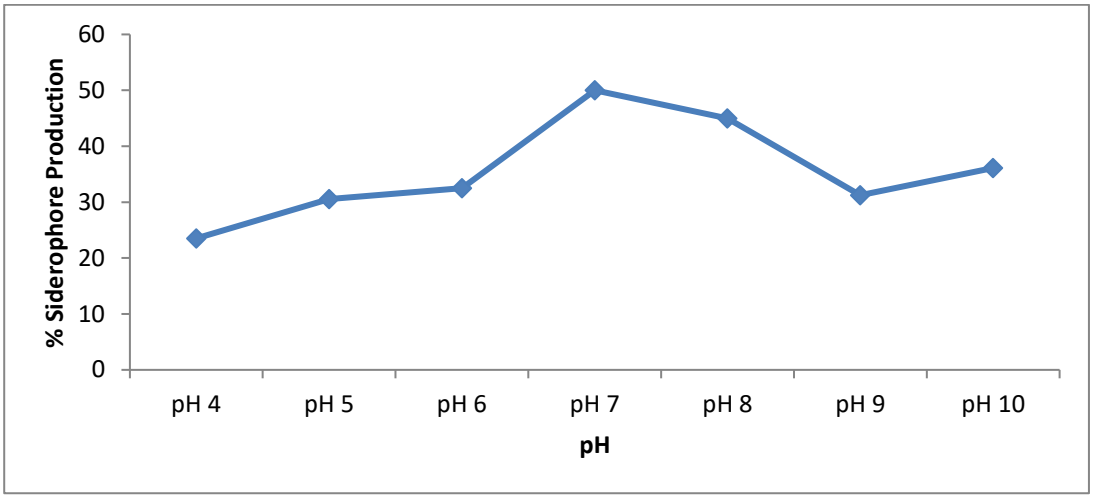

Fig. 7: Effect of pH on siderophore production

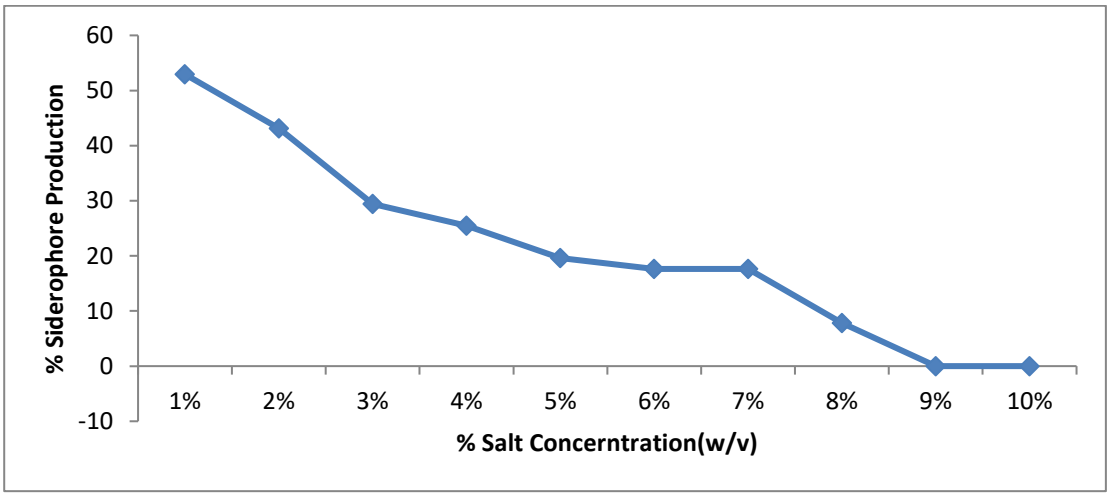

Fig. 8: Effect of NaCl on siderophore production

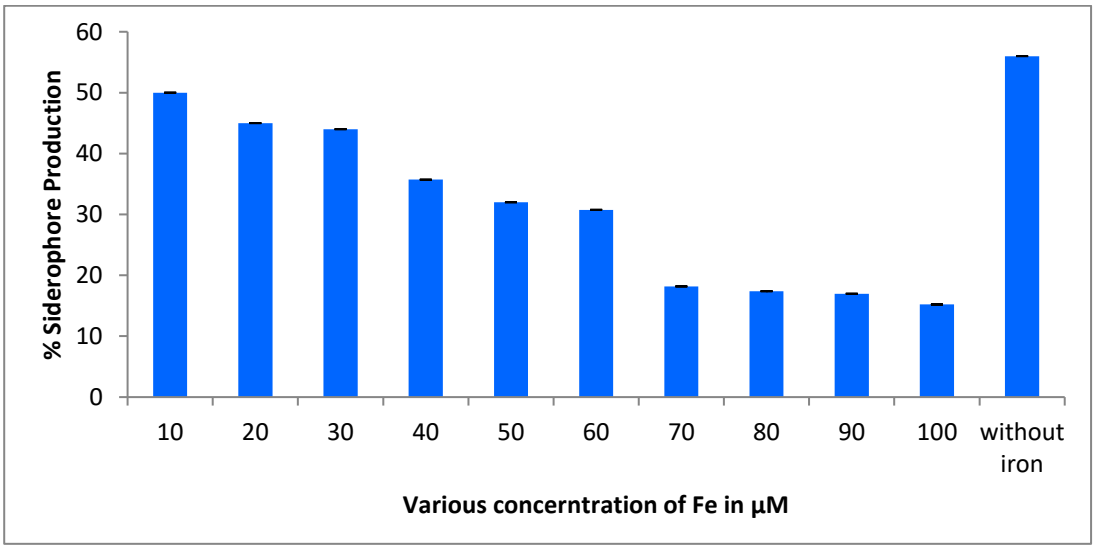

Fig.9: Effect of Different Iron concentration on siderophore production

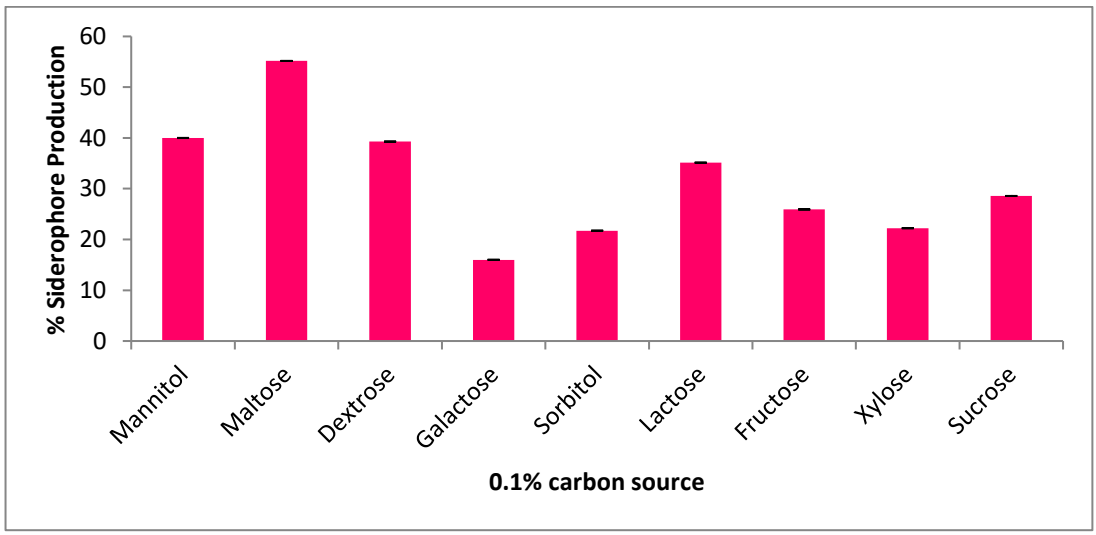

Fig.10: Effect of different carbon sources on siderophore production

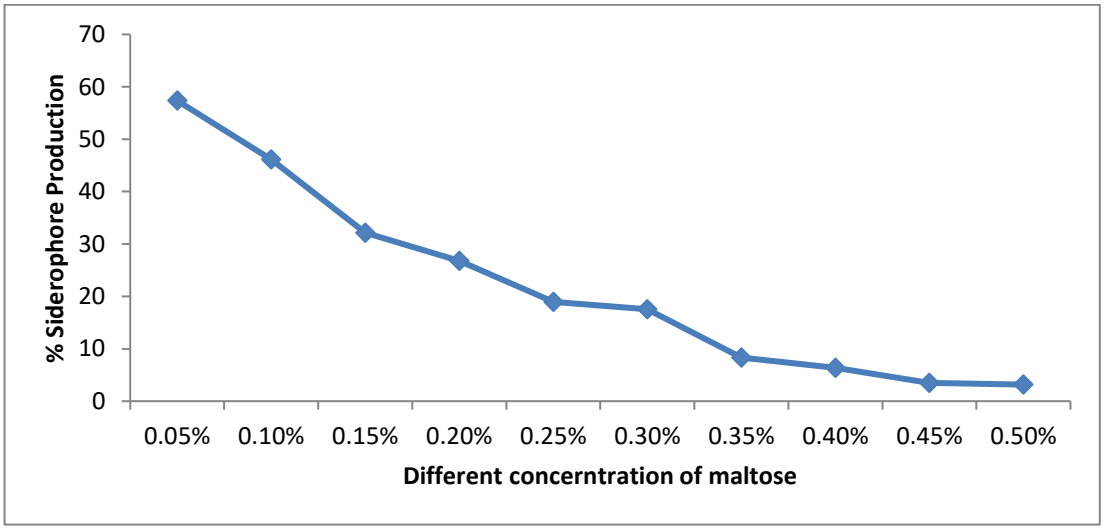

Fig. 11: Effect of various concentrations of maltose on siderophore production

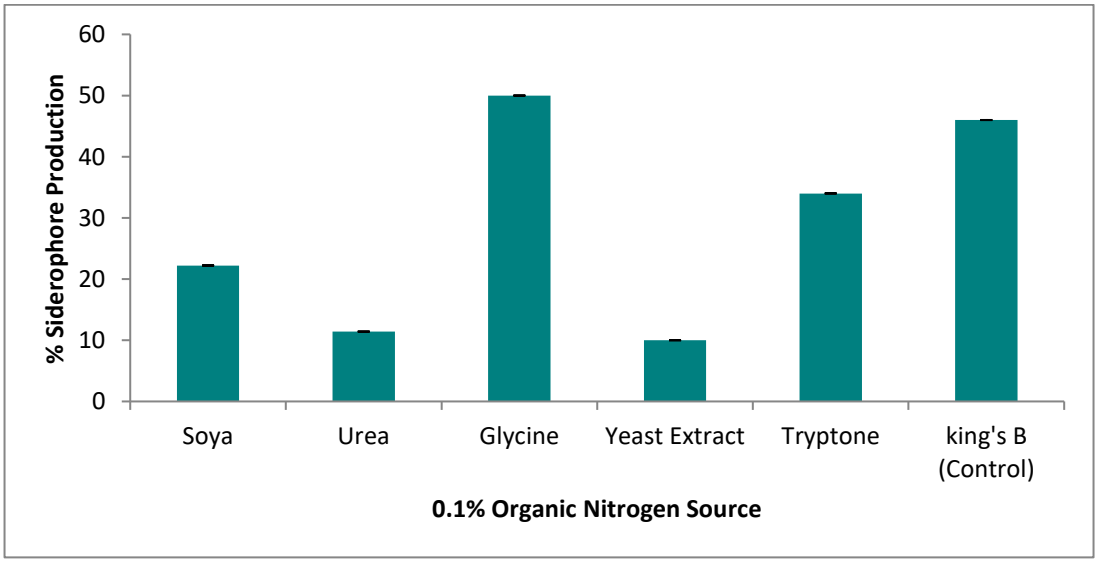

Fig.12: Effect different Organic Nitrogen Source on siderophore production

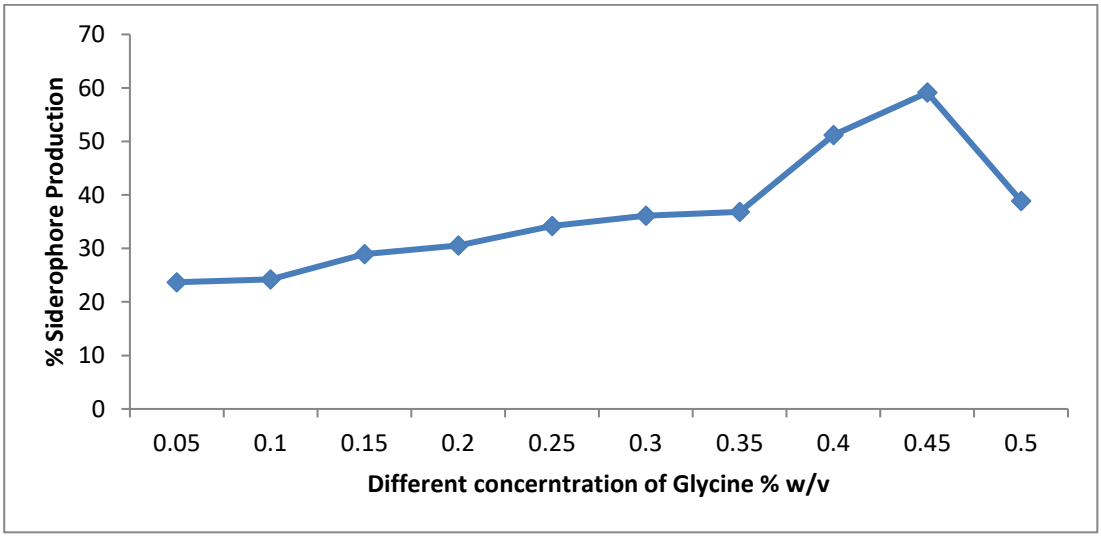

Fig.13: Effect of various concerntration of glycine in King's B medium on siderophore production

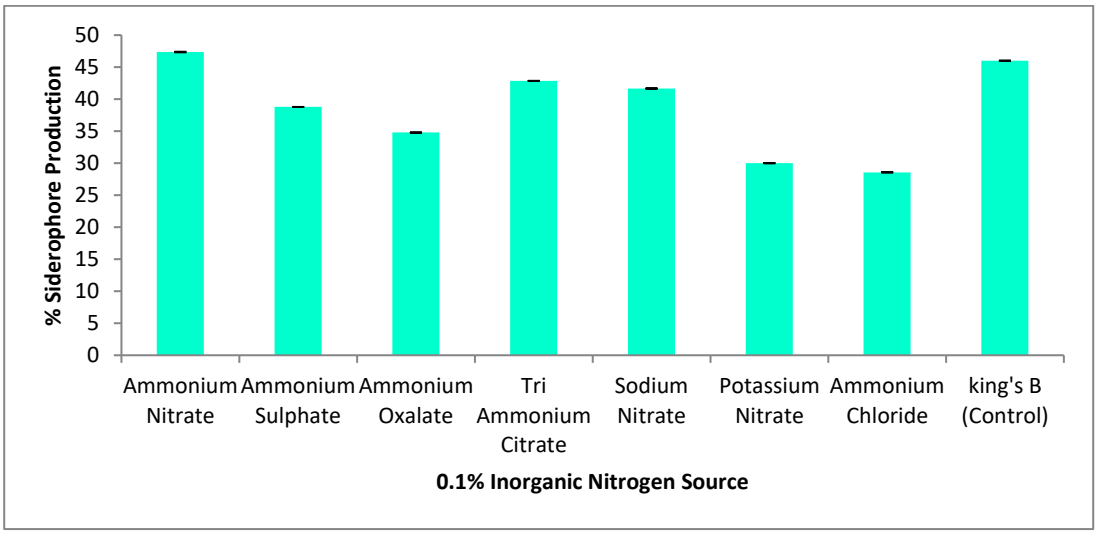

Fig. 14: Effect of different Inorganic Nitrogen Source on siderophore production

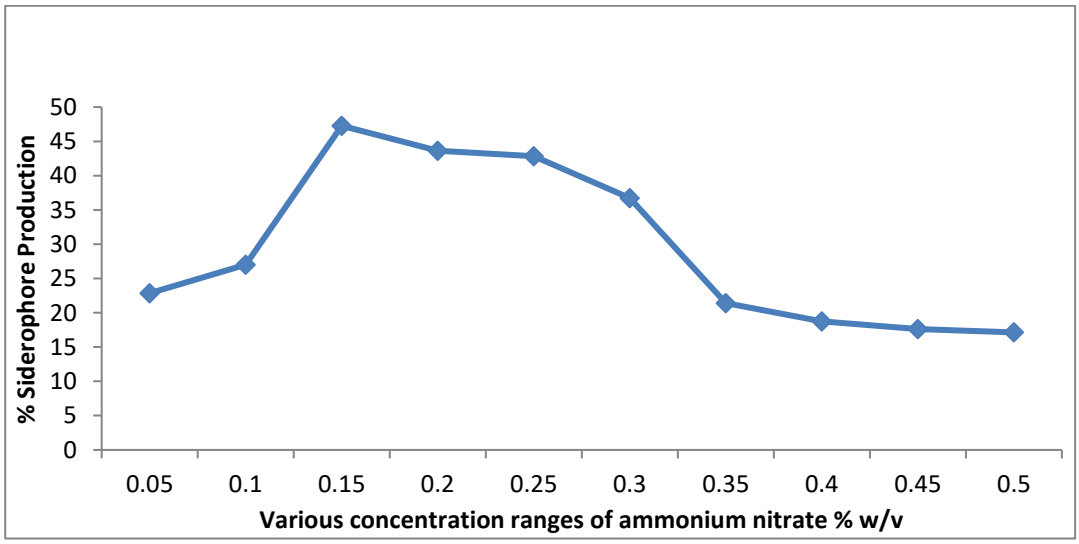

Fig.15: Effect of Different concentration range of Ammonium Nitrate on siderophore production

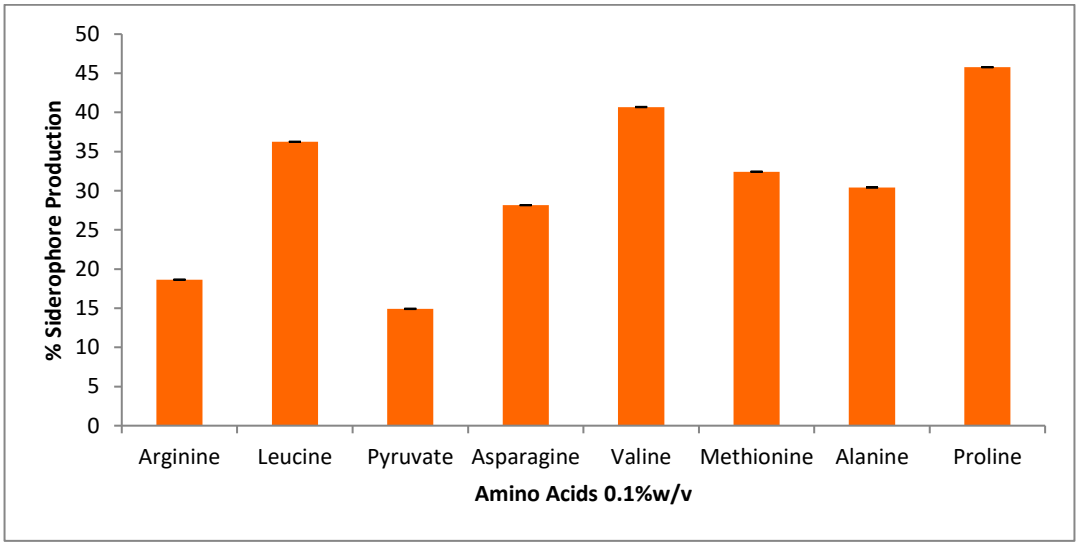

Fig.16: Effect of different amino acids on siderophore production

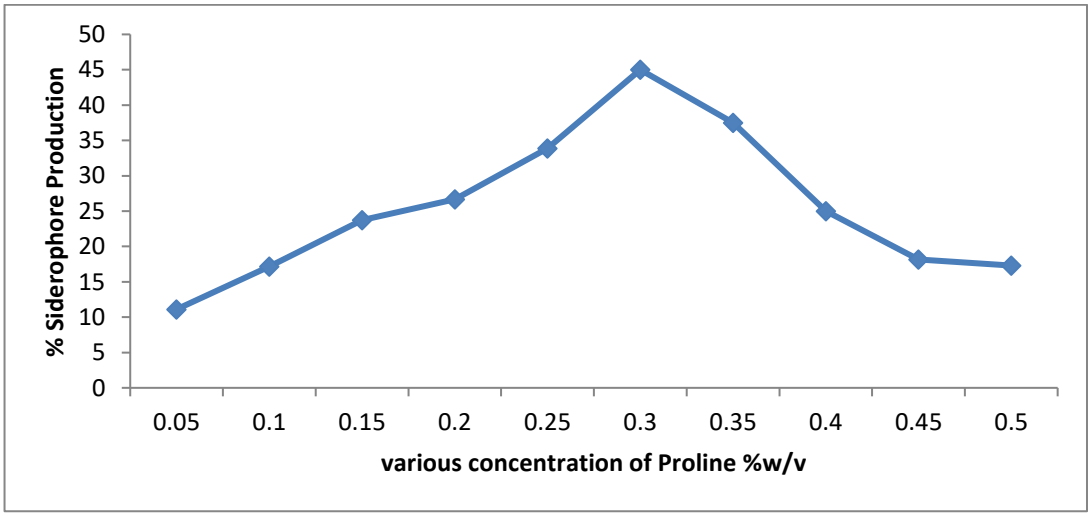

Fig.17: Effect of different concentration range of proline on siderophore production

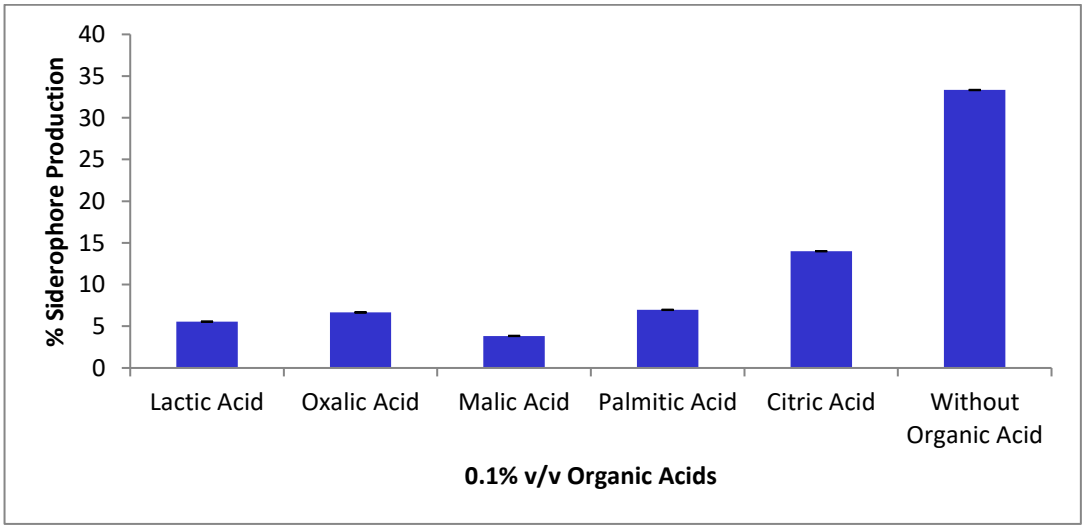

Fig.18: Effect of various Organic acids on siderophore production

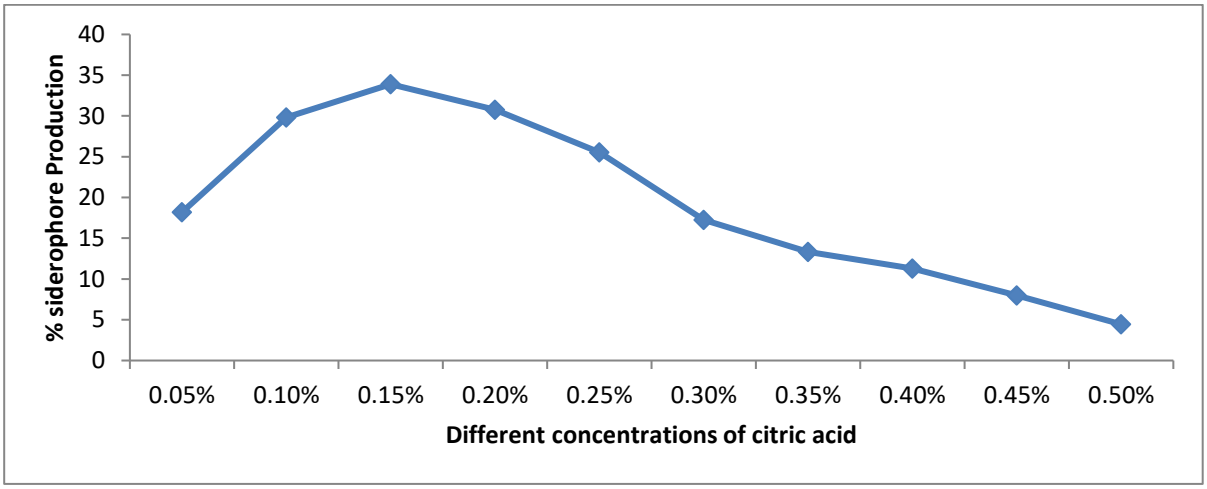

Fig.19: Effect of Different concentration of citric acid on siderophore production

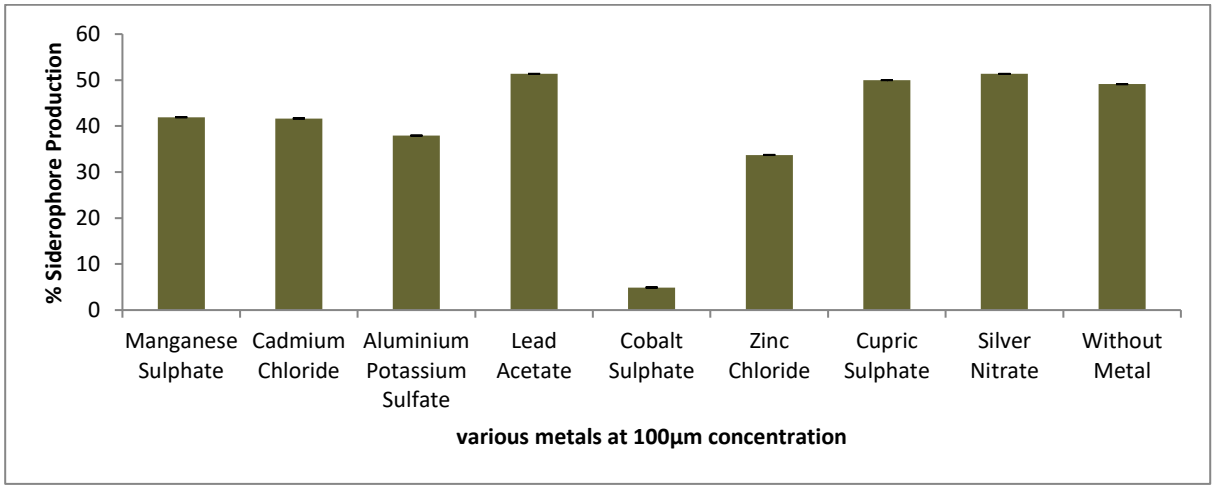

Fig.20: Effect of different metals on Siderophore Production

Optimization of media

Prior to optimization of physicochemical parameters, it is essential to optimize the nutrient media in order to ensure maximum growth of test culture and hence maximum siderophore production. Among the 8 different nutrient media tested, maximum siderophore production was observed in King's B medium (49.01%SU) as represented in Fig. 2. A similar study has reported maximum siderophore production in King's B media by *P. aeruginosa* PAO1 [47]. In contrast to our study, succinate B media was found to be ideal for siderophore production by *P. aeruginosa* KSB, *P. aeruginosa* PAUTI4 and *P. aeruginosa* PB19 [48-50]. In another study, succinic acid medium showed optimum siderophore production by *Alcaligenes sp.* [33]. Apart from King's B and succinate medium, efficient siderophore production was observed in Grimm-Allen iron-limited medium by *P. chyrsogenum* NCIM 707, Derx's medium by *Azotobacter sp.,* and NB by *B. amyloliquefaciens* NAR38.1 [30, 51, 52].

Optimization of temperature and aeration

Fig. 3 represents the siderophore production at various temperatures under static and shaker conditions. It was observed that the maximum production of siderophores was achieved at 37°C under the shaker condition at 120rpm (41.93%SU).

Previous studies have reported 30ºC and 27°C as an optimum temperature for siderophore production by *P. aeruginosa* strains [25]. Optimum production of siderophores are also reported over a wide range of temperature i.e., 25°C-45°C by *Bacillus* VITVK5, *Bacillus* VITVK6 *and Enterobacter sp.* [12, 21, 53]. Elevated temperatures (i.e., above 30°C) are shown to inhibit siderophore production in a *P. aeruginosa* and *C. albicans* strains [22, 54]. However, similar to our findings, most of the cited literature report the temperatures of 28°C to 37°C as optimum for siderophore production in *Enterobacter sp, Erwinia sp, Citrobacter sp, Bacillus sp.* and *Micrococcus sp.* [12, 55]. Also, aeration was found to be important for

Pseudomonas stutzeri CCUG 36651 and *P. stutzeri* RC-7 to produce siderophores which were grown under shaker conditions of 220 and 200rpm respectively [56, 57]. In contrast, another *P. stutzeri* strain showed no siderophore production under aerobic conditions [58]. Another study has reported optimum siderophore production in both static as well as shaker conditions at 37°C within 24h by a clinical *E. coli* isolate [3].

Optimization of incubation time

Fig. 4 represents the siderophore production at various time intervals and was found to be optimum after 72h incubation (51.66%SU). The *P. aeruginosa* azar11 isolate showed a gradual increase in siderophore production till 72h to 96h, after which the production declined up to 120h of incubation as noted in our study.

Other studies have reported an increase in siderophore production after 3-12h of inoculation and an optimum production was achieved after 30h for *P. aeruginosa* PB19 and Azotobacter sp., and 12h for an unidentified strain of *P. aeruginosa* [30, 49, 59].

Optimization of inoculum size and optical density

Fig. 5 and 6 represents the effect of inoculum size and density of *P. aeruginosa* azar 11 on siderophore production respectively. It was observed that *P. aeruginosa* azar 11 was most effective in producing siderophores when a 5% inoculum size (41.05% SU) of 0.6O. D_{600nm} (52.50% SU) was used.

A recent study reported a 0.5% inoculum size to be optimum for siderophore production in Rhizobacteria sp. S-6 and S-29 [60]. Similarly, the culture density adjusted to $6x10^6$cells/mL and $2\text{-}4x10^6$cells/mL was reported to be optimum for siderophore production in *Alcaligenes sp.* and *C. albicans* respectively [33, 54]. Other studies reported 1.0 O. D_{600nm} to be ideal for *P. syringae* [61]. Another published study reported similar siderophore production among three Aspergillus species when monosporic inoculum was used. However, the experiments with *A. flavus* using blocks as inocula produced the highest CAS-reaction rate [4].

Optimization of pH and NaCl concentration

Fig. 7 and 8 represent the effect of pH and NaCl concentration on siderophore production by *P. aeruginosa* azar 11. A neutral pH (48.71%SU) and a low salt concentration of 1% (52.94%SU) were found to be optimum for siderophore production in our study. This observation may be attributed to the general characteristic of bacteria that prefer neutral pH and a low salt concentration for its growth and survival. Similar to our findings, *Alcaligenes sp.* and *Azotobacter sp.* were reported to produce siderophores under neutral pH conditions [30, 33]. The decrease in siderophore production under acidic pH could be attributed to the solubilization of iron at a lower pH resulting in iron availability [52]. In contrast, a study reported siderophore production by an unidentified strain of *P. aeruginosa* over a broad range of pH values between 5.0-11.0, with an optimum pH of 9.0 [25]. Similarly, *P. stutzeri* AS22 and *P. oxalicum* showed an optimal pH of 8.0 and 4.5 respectively for siderophore production [26, 62]. Among fungal cultures, *A. niger* was found to be unaffected by different pH conditions, but other fungi viz., *A. tamarii* and *A. flavus* showed a lower rate of siderophore production at pH5.5 [4].

Similarly, significant decrease is noted in siderophore production with an increase in salt concentration among rhizobium strains [52]. However, *Macrotyloma uniflorum* was found to support siderophore production up to 8-9% of salt concentration [63]. A study reported maximum siderophore production in different Rhizobium isolates at varying salt concentrations ranging from 400mM-1000mM. In their study, the amount of siderophore production gradually increased with increase in salt concentration up to 600mM. However, the growth of the isolate decreased gradually with further increase in salt concentration except for strain HGR23, which showed maximum siderophore production at 1000mM salt concentration [64].

Effect of iron on siderophore production

Fig. 9 represents the effect of iron on siderophore production by *P. aeruginosa* azar 11. As expected maximum siderophore production of 56%SU was observed when media was devoid of iron. This may be because of negative transcriptional regulation by 'Fur' protein where Fe^{+2} acts as a co-repressor. Siderophores being iron-specific compounds are secreted under iron stress conditions and several studies have proved the inverse proportionality of iron to improve siderophore production [16, 53, 65, 66]. Few studies, however, have reported tolerance to moderate concentrations of iron where *P. fluorescens* NCIM 5096 and *Pseudomonas putida* NCIM 2847 showed siderophore production up to 100mM iron concentration [67]. The cell growth of *P. fluorescens* P5-18 reached a maximal value with 200µg/L iron but siderophore production was found to decrease significantly. It was further reported that the optimal iron concentration for high siderophore

production was observed below 50µM concentration of iron [65]. In another study, the mean production of siderophores, under iron-limited conditions, increased by 100% for clinical isolates and 50% for the environmental isolates of *Aeromonas hydrophila* when compared to normal iron-rich culture conditions. The clinical isolates produced 73% more siderophores under iron-limiting conditions than environmental isolates and possessed 44% more siderophore activity than the environmental isolates [54].

Optimization of nutrient sources

Fig. 10 -19 represents the effect of various nutrient sources and optimized concentrations of the same on siderophore production by *P. aeruginosa* azar 11. In our study, supplementation of 0.45% glycine (59.18%) in optimized kings B medium did not affect siderophore production, whereas 0.05% maltose (57.35%) slightly inhibited the same. However, the addition of nutrients like ammonium nitrate (47.27%SU), proline (45.00%) and citric acid (33.89%SU) showed a considerable reduction in siderophore production. Even optimization of the most effective nutrient source concentration did not result in increased siderophore production, hence confirming inhibitory activity of these compounds.

Previous studies have reported glucose to be the most suitable carbon source and malate to be a poor substrate for siderophore production in *P. aeruginosa, A. nidulans* and *P. chrysogenum* [27]. In a similar study, 0.5% glycerol, 2% glycine and 2% peptone was ideal for siderophore production in *Pseudomonas* sp. KSB3, whereas it was found to be repressed in presence of urea and sodium acetate [22]. In contrast to our study, *Pseudomonas* sp. P1, P2, P3 showed maximum siderophore production in SM (succinate medium) when supplemented with $1gL^{-1}$ urea, whereas supplementation of sugars resulted in decreased siderophore production [7]. Urea was also found to be effective for siderophore production in *Pseudomonas* sp. PB19 at $0.6gL^{-1}$ concentration [50]. In another study, mannitol and urea proved effective for siderophore production in *Alcaligenes* sp. [33].

In other reports of Pseudomonas, all tested amino acids positively affected siderophore production in isolate P2. However, histidine resulted in maximum siderophore production [7]. Similar observations were reported in the case of *Pseudomonas sp*. PB19 [50]. Other studies have reported optimum production of siderophores in presence of L-asparagine for *Pseudomonas sp.* KSB3 and tyrosine for Alcaligenes sp. [33, 48].

Effect of metal ions on siderophore production

Fig. 20 represents the effect of metal ions on siderophore production by *P. aeruginosa* azar 11. The maximum siderophore production was obtained in silver nitrate and lead acetate (51.35%SU) solutions. Significant inhibition of siderophore production was observed in presence of cobalt sulfate (4.91%SU). Another study has reported enhanced siderophore production in presence of 10µM mercury and inhibitory effects of magnesium, cobalt and molybdenum on *Pseudomonas* sp. [7]. Two other studies carried out on siderophore production in *Pseudomonas fluorescens* and *Pseudomonas putida* suggested an increase in production of siderophores in presence of lead and reduced production in presence of manganese, mercury and cobalt [65, 68]. However, *A. vinelandii* showed complete inhibition of siderophore production in presence of all tested metal ions [30].

Antimicrobial activity of siderophores

Table 2 represents the antimicrobial activity of siderophores on test cultures. It showed antibacterial activity against few laboratory cultures and drug-resistant β-lactamase producing uropathogens. Maximum inhibition was observed against *S. aureus* and *S. paratyphi* B in our study.

Table 2: Antimicrobial activity of siderophore against β-Lactamase producing uropathogens and laboratory cultures

Sr.No.	Cultures	Zone of inhibition in mm
Laboratory cultures		
1.	*S. aureus*	21
2.	*S. pyogens*	0
3.	*S. paratyphi A*	13
4.	*S. paratyphi B*	21
5.	*Shigella sp.*	12
6.	*S. typhi*	0
7.	*E. coli*	14

Sr.No.	Cultures	Zone of inhibition in mm
8.	*K. pneumoniae*	0
9.	*S. aureus 6538p*	14
10.	*V. Cholerae*	0
β-Lactamase producers		
11.	*E. coli* 1	12
12.	*E. coli* 2	0
13.	*E. coli* 3	0
14.	*E. coli* 4	12
15.	*E. coli* 5	0
16.	*E. coli* 6	0
17.	*E. coli* 7	0
18.	*E. coli* 8	0
19.	*E. coli* 9	11
20.	*E. coli* 10	0
21.	*E. coli* 11	0
22.	*E. coli* 12	0
23.	*E. coli* 13	0
24.	*E. coli* 14	0
25.	*E. coli* 15	11
26.	*E. coli* 16	0
27.	*E. coli* 17	0
28.	*K. pneumoniae* 1	0
29.	*K. pneumoniae* 2	0
30.	*K. pneumoniae* 3	10

A similar study reported fungal inhibition by cell-free supernatant of *Pseudomonas PAUTI4* strain. Maximum inhibition was observed against *A. flavus* followed by *A. fumigates, A. niger* and *C. albicans* in their study [49]. In another study, inhibition of *R. solani* was observed in absence of $FeCl_3$ by *P. aeruginosa* suggesting siderophore-mediated antagonism. In addition, *C. gloeosporioides* culture was inhibited in the presence as well as the absence of $FeCl_3$ by *P. aeruginosa*, which may be due to the production of other antifungal metabolites by the test strain [16]. A study carried out to determine the antifungal activity of *Azotobacter* sp. against plant root pathogens reported excellent activity, as all the test pathogens viz., *Fusariurm sp., Alternaria sp., Phytophthora sp., Rhizoctonia sp., Colletotrichum sp.* and *Curvularia sp.* were completely inhibited [30]. The exposure to diverse biotic stress has helped plants in developing various defense mechanisms. Siderophores are well known for bio-control of phytopathogens [69]. Apart from pre-formed physical and chemical barriers, plants can detect pathogen attacks and activate complex signaling networks, leading to induced defenses that confer a more tolerant state. Induced innate immune processes include phosphorylation events, accumulation of reactive oxygen species, cell wall rigidification, callose deposition, defense hormone signaling, and expression of genes encoding pathogenesis-related proteins [70]. Plants are equipped with sentry systems consisting of proteins defenses against potential microbial pathogens, devoted to the recognition of pathogen-derived elicitors. In the rhizosphere, several bacterial species can be beneficial to plants and protect them against pathogens. These kinds of bacteria are called plant growth-promoting rhizobacteria (PGPR) and can induce systemic resistance by producing Siderophores and increasing plant immunity [71].

Characterization of siderophores

The chemical nature of the siderophores could not be detected by the analytical tests carried out in our study. It may be due to the presence of mixed ligands of siderophore containing 2 functional groups. In such cases, the characterization of siderophores becomes difficult by using conventional protocols.

Other studies have also suggested the inability to characterize the siderophores. The siderophore produced by *A. salmonicida* could not be extracted from the culture supernatant into any of the organic solvents examined; therefore, the chemical nature of siderophore could not be detected [72]. In contrast, the spectrophotometric analysis reported hydroxamate nature of siderophore in *P. aeruginosa FP6, P.*

fluorescens and *Mesorhizobium loti* [73-75]. Arnow's method revealed hydroxamate nature of siderophores in *P. chrysogenum* NCIM707 [51]. A similar study revealed an exceptional observation in a fungal isolate i.e., HF-8 which produced both hydroxamate and catecholate siderophores as evidenced by the positive reaction to both hydroxamates and catecholates [76].

CONCLUSION

The results presented in our study clearly indicate the possible applications of the isolate *P. aeruginosa* azar 11 in siderophore production. Siderophore as chelating compounds, not only find application in agriculture but also in medicinal industries for generating genetically modified drugs for life-threatening diseases like tuberculosis and thalassemia. Moreover, minimum optimization of culture conditions resulted in a 10.17% increase in siderophore production in our study, using a common laboratory set-up. Hence *P. aeruginosa* azar 11 can be further improved by genetic engineering to obtain a high yielding siderophore strain.

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