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Siderophore Production by *Pseudomonas aeruginosa* Isolated from the Paddy Fields of Kuttanad, Kerala

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Abstract: ***Siderophores are low-molecular-weight chelating agents excreted under iron-limited conditions by microorganisms which strongly and specifically bind, solubilise and deliver iron to microbial cells via specific cell surface receptors. In natural habitats, certain bacteria can acquire iron by uptake of exogenous siderophores through receptors showing that they can utilize siderophores produced by other microorganisms as an iron source. This property can be made use of in increasing the nutritional value of agricultural areas. Kuttanad is well noted for paddy cultivation below sea level in Kerala. Considering its agricultural importance, this area was selected for the isolation of siderophore producing bacteria. One of the isolates, KSB3, which was later identified as Pseudomonas aeruginosa, was screened for the ability to produce siderophore. Siderophore production by submerged fermentation and the parameters influencing siderophore production were optimized. The chemical nature of the siderophore produced by KSB3 was also determined.***

Keywords: Iron, siderophore, bacteria, *Pseudomonas aeruginosa,* submerged fermentation

1. Introduction

Iron is the fourth most abundant element in the earth's crust. However, at biological pH and under aerobic conditions, iron is oxidized to insoluble oxy-hydroxides polymers, which are unavailable to a microorganism. To overcome this limitation, microbial systems produce compounds called Siderophores, which play an important role in sensing and uptake of iron (Rachid & Ahmed, 2005)[1]. The term Siderophore was coined in 1973 and was described as low molecular weight molecules that bind ferric iron with an extremely high affinity (Lankford, 1973)[2]. They are generally synthesized and secreted by bacteria, fungi and some monocotyledonous plants in response to iron unavailability. Siderophores scavenge iron from mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by energy dependent membrane transport mechanism and thus bind it and transport it to plants or bacterial cells. Their molecular weight ranges from nearly 600 – 1500 Daltons (Neilands, 1995; Leong, 1986)[3,4]. Siderophores are classified into hydroxamate, catecholate, carboxylate and mixed type on the basis of the chemical functional groups they use to chelate iron. The present study reports siderophore production by *Pseudomonas aeruginosa* isolated from Kuttanad agrarian ecosystem of Kerala, which has been assigned as a *Globally Important Agriculture Heritage Site*, by FAO. Process parameters influencing growth and siderophore production by the bacteria under submerged fermentation were also optimized and the siderophore was characterized.

2. Materials and Methods

Isolation of siderophore producing bacteria

For the present study, soil sample from Kuttanad was collected aseptically and serially diluted up to 10^{-7} as per standard microbiological methods and spread plated on King's B agar. After 24 hours of incubation, well isolated and morphologically distinct bacterial colonies were selected to screen for siderophore producers.

Screening for siderophore producers

Siderophore producers were identified by the fluorescence they emit when they were grown on KB agar and viewed under UV light (King *et al,* 1954)[5].

A selective medium namely modified Chrome Azurol Sulphonate (CAS) agar was used to identify pure siderophore producing bacteria (Alexander and Zuberer, 1991)[6]. Siderophore producers were identified by the presence of orange halos surrounding the bacterial colonies when incubated on blue CAS agar for 24 hrs at 30^0C. Out of five strains, one bacterial isolate named as KSB 3 was selected for further studies.

Siderophore production under Submerged Fermentation (SmF)

All the fermentation experiments were carried out in 250 ml Erlenmeyer flasks as batch cultures and in duplicate. Constant aeration was provided to the cultures by incubating the cultures in a shaking incubator at a constant speed of 120 r p m. Appropriate controls were maintained.

Quantification of siderophore produced

The quantity of siderophore produced was determined using CAS liquid assay (Schwyn & Neilands, 1987)[7]. This assay is based on a competition for iron between the ferric complex of the dye Chrome Azurol Sulphonate (CAS) and the siderophore. 0.5 ml centrifuged supernatants were used for assay along with 0.5 ml CAS reagent. The change in colour of the blue dye in the CAS assay solution to purple-orange indicates the presence of siderophore. Siderophore production was calculated as percentage siderophore units (PSU or % SU)

$$PSU = [(A_r - A_s)/A_r] \, 100$$

where,

A_r = Absorbance of reference at 630 nm.
A_s = Absorbance of Sample at 630 nm.

Optimization of process parameters influencing siderophore production under Submerged Fermentation (SmF)

Seven important process parameters affecting siderophore production were selected for the optimization studies. The strategy followed was to optimize one parameter at a time and include it in the optimized level in the next experiment.

Effect of various media on siderophore production

KSB 3 was inoculated to various media to study the effect of media on siderophore production. Submerged fermentation was carried out using succinate medium, King's B broth and nutrient broth. After 24 hours of incubation, the cell free culture supernatant was used for the quantification of siderophore as per the method mentioned above.

Effect of temperature of incubation

The effect of temperature on siderophore production was determined by incubating the flasks inoculated with KSB 3 at the following temperatures: 25°C, 30°C, 35°C, 40°C and 45°C.

Effect of pH of succinate medium

pH of the succinate medium was varied from acidic to alkaline nature by treating with 1N NaOH and 1N HCl as required. Succinate medium of varying pH was inoculated with the bacteria and then incubated to find out the effect of pH on siderophore production, in the range of 5 to 9.

Effect of hours of incubation

The effect of the time period of incubation on siderophore production was monitored by assaying the culture supernatant for quantifying the siderophore produced after 24, 48, 72 and 96 hours of incubation.

Effect of glucose as a carbon source

The effect of glucose on siderophore production was studied by treating the succinate medium with increasing concentrations of glucose. Glucose was added to the medium at the concentrations of 0.5%, 1%, 1.5%, 2%, 2.5% and 3% w/v. Succinate medium devoid of glucose served as the control.

Effect of sodium nitrate as a nitrogen source

Sodium nitrate as a nitrogen source was added to the succinate medium at the concentrations of 0.5%, 1%, 1.5%, 2%, 2.5% and 3% w/v prior to sterilization. After inoculation and incubation, the cell free supernatant was assayed to determine the quantity of siderophore produced.

Effect of asparagine as an amino acid source

The effect of asparagine on siderophore production was found out by supplementing the succinate medium with the same at 0.5%, 1%, 1.5%, 2%, 2.5% and 3% w/v concentrations. Asparagine was filter-sterilized through a 0.4µ pore sized syringe driven filter and then added separately to the culture flask after autoclaving. This was done in order to prevent the denaturation of the amino acid while autoclaving.

Chemical characterization of the siderophore

Various tests were carried out to determine the chemical nature of the iron binding group of the siderophore produced by the selected bacterial strain. All tests were carried out using the cell free supernatant of the culture. Hydroxamate nature of the siderophore was confirmed by Csaky assay (1948)[8] and Tetrazolium salt test (Snow, 1954)[9] whereas Arnow's assay (1937)[10] was used to determine the catecholate nature whereas Vogel's test (1987)[11] was the basis for the determination of carboxylate siderophores.

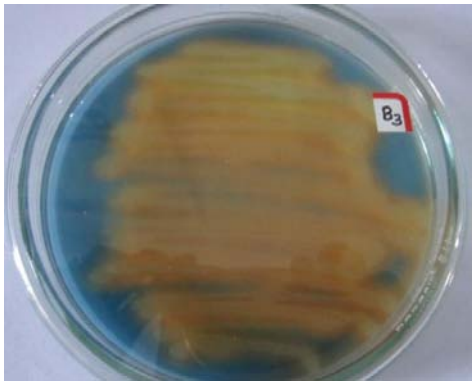
3. Results and Discussion

As a part of primary screening, the Petri plates containing microbial growth were visualized under UV light to detect fluorescence producing colonies. A well isolated and pigmented colony which showed fluorescence was selected for the present study. The colony was designated as KSB3. Slant grown culture of KSB3 was again inoculated to 50ml of King's B broth in a 250ml Erlenmeyer flask to clearly visualize the fluorescence under UV light, after 24 hours of incubation (Photograph – 1).

After studying the colony characteristics it was streaked on to slants of KB agar to maintain pure culture.

Photograph 1: KB broth grown KSB3 showing fluorescence under UV light

24 hour old slant grown culture of KSB3 was later streaked on Chrome Azurol Sulphonate agar as a method of secondary screening. The colony produced orange halos around them confirming that they produce siderophore (Photograph - 2). It was identified as *Pseudomonas aeruginosa* at MTCC, Chandigarh.



Photograph 2: KSB3 showing orange halo in CAS agar.

Optimization Studies

Selection of medium for siderophore production

Chrome Azurol Sulphonate liquid assay showed that *Pseudomonas aeruginosa* produced maximum siderophores in succinate medium than in King's B broth. Though nutrient broth supported the bacterial growth it did not stimulate siderophore production. Siderophore production by *Pseudomonas aeruginosa* in succinate medium was evidenced by the formation of yellow-green pigment whereas un-inoculated succinate medium remained colourless (Photograph - 3).

Photograph 3: Siderophore production by *P. aeruginosa* in succinate medium

Effect of temperature on siderophore production

Temperature of incubation had a profound effect on siderophore production by *P. aeruginosa*. It preferred lower temperatures for siderophore production where a maximum production of 53.88 percentage siderophore units (PSU) was obtained at 30°C. 25°C also supported siderophore production whereas temperatures above 30°C did not favour siderophore production.

Effect of pH and hours of incubation on siderophore production

P. aeruginosa preferred an alkaline pH for siderophore production giving a maximum of 35.36 PSU at pH 8. As acidity increased the siderophore production decreased. *P. aeruginosa* produced 41.40 PSU after 24 hours of incubation. The yield increased to 45.13 PSU when the culture was incubated for another 24 hours. On further incubation it was found that the siderophore yield decreased.

Effect of carbon, nitrogen and amino acid sources on siderophore production

Glucose as a carbon source stimulated siderophore production by *P. aeruginosa* at concentrations above 1% w/v. The production was consistent for glucose concentrations ranging from 1.5% to 2.5%. A maximum of 43.90 PSU was obtained when the medium was supplemented with 2.50% glucose. Sodium nitrate on the other hand suppressed siderophore production in *P. aeruginosa* showing that it is not a preferred nitrogen source for siderophore production by *P. aeruginosa.* Maximum siderophore yield was obtained when the medium was devoid of sodium nitrate. The amino acid asparagine also stimulated siderophore production by *P. aeruginosa.* 89.76 PSU was the maximum yield of siderophore obtained when the succinate medium was supplemented with 2.5% asparagine. Asparagine stimulated *P. aeruginosa* to produce siderophores at all concentrations ranging from 0.5% to 3% w/v (Figure 1).

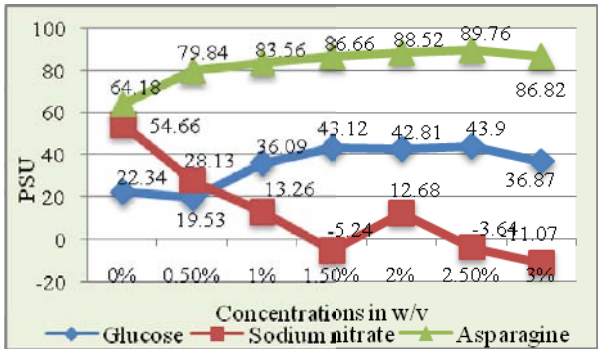
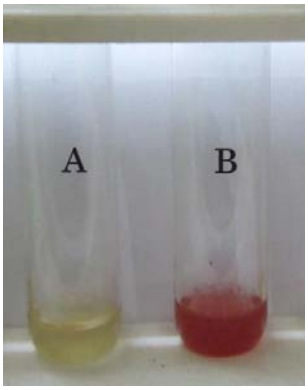


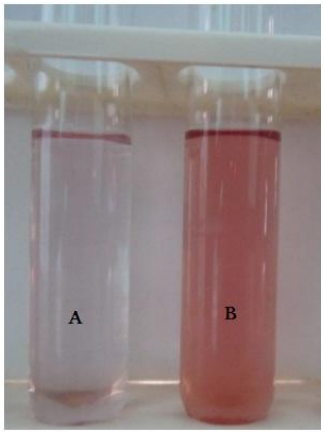
Figure 1: Effect of glucose, sodium nitrate and asparagine on siderophore production by *P. aeruginosa*

Chemical characterization of the *P. aeruginosa* siderophore

The cell free supernatant of *P. aeruginosa* culture was assayed to determine the chemical nature of the siderophore and it was found to be of hydroxamate type siderophore. Positive results were obtained for both tetrazolium salt test (Photograph 4) and Csaky assay (Photograph 5).



Photograph 4: Tetrazolium salt test
A – Control, B - Sample



Photograph 5:- Csaky assay
A – Control, B – Sample

Hydroxamate nature of the siderophore produced by *P. aeruginosa* was further confirmed when negative results were obtained for Arnow's assay and Vogel's test ruling out the possibility of catecholate and carboxylate type siderophore respectively.

Anubrata Paul and Rajendra Dubey (2015)[12] had reported that siderophore production by bacteria was at its maximum at an incubation temperature of 30ºC-35ºC. Bendale *et al* (2009)[13] reported that maximum siderophore production by *Streptomyces fulvissimus* was at an alkaline pH of 8, which clearly support our result. Studies carried out by Sharma *et al* (2016)[14] also found that glucose stimulated siderophore production in *Pseudomonas* which yielded a maximum of 60.05 PSU. According to Bendale (2009)[13] the highest siderophore yields were obtained by *Streptomyces fulvissimus* when the culture media was supplemented with sodium nitrate as the nitrogen source.

Siderophore producing *Pseudomonads* are very important in relation to plant growth and yield. They increase the availability of absorbable iron in the vicinity of plants thus making it available to them. This can reduce the input of chemical pesticides to an extent (Schenk *et al* 2012)[15]. They act as bio-control agents by blocking the iron source to other pathogenic bacteria (Prema & Selvarani, 2013)[16]. In recent years *Pseudomonads* has gained popularity because of the fact that the siderophores produced by them can be used as bio pesticides (Wilson, 1997)[17].

4. Conclusion

Although reports of siderophore production using bacteria isolated from soil and water samples from different parts of India and elsewhere are available, this is a pioneering study on the isolation and characterisation of siderophore producing bacteria from Kuttanad region of Alleppey district in Kerala, where a vast area of the land is used for paddy cultivation (Bindu & Nagendra Prabhu, 2012, 2016) [18, 19].

5. Future Scope

The use of siderophore produced by *P. aeruginosa* as bio-control agent against selected plant pathogens and field studies as an environmentally friendly alternative to hazardous pesticides are in progress.

6. Acknowledgement

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