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Screening and Partial Purification of Hydroxamate Type Siderophore from *Pseudomonas sp.*

Kuppuswamy Kavitha[1], Arumugam Suganya[1], Kuppusamy Umaa Rani[2] and Balasubramaniyan Ramesh[2*]

[1]Department of Microbiology, Sri Sankara Arts and Science College, Enathur, Kanchipuram – 631561, Tamil Nadu, India
[2]Department of Biotechnology, Sri Sankara Arts and Science College, Enathur, Kanchipuram – 631561, Tamil Nadu, India
**Corresponding author*

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A B S T R A C T

The present study document the isolation and enumeration of siderophore producing microbes from iron contaminated soil. Identification of potential strains and optimization studies for the production of siderophore was carried out. The Iron contaminated soil samples were collected from welding shops, factories in and around Kanchipuram and Thiruvallur. Ten morphologically different strains (AS1-AS10) were selected for further screening for the production of siderophores by Ferric Perchlorate Assay. Strain AS-6 showed a maximum OD value of 0.82 and was further identified as *Pseudomonas sp* and optimization studies were carried out. Siderophore production was maximum when using Glucose medium, 72 hours of incubation; pH 5.5, 30^0C; Ferric Chloride as additive. The partially purified siderophore showed good zone of inhibition against water borne pathogens such as *Salmonella, Vibrio, Shigella, and E.coli.*

Introduction

Micro-organisms growing in aerobic conditions need iron for a variety of functions including reduction of oxygen for the synthesis of ATP, for formation of heme and other essential purposes (Neilands, 1995). Siderophore dissolve these ions by chelating as Fe^{3+} soluble complexes that can be taken by active transport mechanisms. Many siderophore are non-ribosomal peptides (Julie, 1999). Siderophores are defined as relatively low molecular weight, ferric ion specific chelating agents synthesized by bacteria and fungi growing under low ionic stress. Strategies other than siderophores to enhance iron solubility and uptake are the acidification of the surrounding or the extra cellular reduction of $Fe3^+$ into the more soluble $Fe2^+$ ions. Examples of siderophore produced by various bacteria and fungi are Ferrochrome (*Ustilago sphaerogena*), Enterobactin (*Escherichia coli),* Mycobactin (*Mycobacterium* species), Enterobactin and Bacillibactin (*Bacillus subtilis*), Ferrioxamine B (*Streptomyces pilosus*), Fusaninine C

(*Fusarium roseum*), Yersiniobactin (*Yersinia pestis*); Pseudobactin *(Pseudomonas aeruginosa*) *etc.*

Siderophore producing algae are also reported. These are *Anabena* species which facilitate iron uptake (Balagurunathan and Radha Krishnan 2007). Carboxylates earlier called complex ones are produced by bacteria (*Rhizobium* and *Staphylococcus* strains) and Fungi (Dave and Dube, 2000). Carboxylate nature of siderophore are detected by Vogel's chemicals test (Vogel, 1987) and further confirmation by spectrometric assay (Shenker *et al.,* 1992).

Hydroxamate siderophores are present in various soils and they are also produced in aquatic environments. Burton *et al.,* (1954) had shown the importance of siderophores in maintaining the balance in mixed microbial population in the soil and that some microbes synthesize siderophores, while others use them without synthesizing any. Desferrioxamine has also been used to remove vanadium. In rats desferrioxamine reduced the vanadium content in kidney by 20% in lungs by 25 % and in liver by 26% when administered at 100µ mol kg^{-1} following a dose of 5µ mole kg^{-1} of $Na_{48}VO_3$. Both urinary and fecal excretion increased at this dose (Hansen *et al.,* 1984). Desferrioxamine has proved successful in the treatment of dialysis encephalopathy, which is a major complication of long term dialysis. It is caused by the accumulation of aluminum in the brain from dialysis water supply. Siderophores from *Klebsiella pneumoniae* has been used as an antimalarial agent (Gysin *et al.,* 1991) and in cosmetics as deodorants (Johnson *et al.,* 2003).

Siderophores plays a main role in the biodegradation of petroleum hydrocarbon in marine ecosystems by facilitating microbial acquisition of iron. Barbean *et al.,* (2000) isolated oil degrading marine bacterium *Marinobacter hydrocarbonclasticus*. It produces a siderophores called Petrobactin that readily undergoes a light mediated decarboxylated reaction. Petrobactin is the first structurally characterized siderophores produced by the known hydrocarbon degrading marine bacterium. *Pseudomonas fluorescens AH2* on inhibition of pathogenic bacteria in fish was studied under iron rich and iron limited condition (Gram *et al.,* 1999). Neilands (1995) reported that desferrioxamine B produced by *Streptomyces pilosus* is advocated for the removal of excess iron resulting from the supportive therapy for thalassemia.

The present study aims at isolation and enumeration of siderophore producing microbes from iron contaminated soil. Attempts were also made in identification of potential strains and optimization studies for the production of siderophore.

Materials and Methods

Sample collection

Iron contaminated soils samples were collected from welding workshops, factories in and around Kanchipuram and Thiruvallur. Soil samples were collected by using sterile spatula and polythene cover and transported to the laboratory (Braud *et al.,* 2009).

Isolation of bacteria from soil samples

One gram of soil sample was weighed and mixed with 99ml distilled water. Then the sample was serially diluted up to 10^{-8} dilution about 0.1ml of aliquot from 10^{-2} to 10^{-8} dilutions were taken and platted in triplicate on sterile nutrient agar plate and one plate was kept as control. All the plates were incubated at 28^{o}C for 48 hours. After incubation, plates were observed for presence of colonies, which were purified and streaked on nutrient agar plates (Cappuccino, 1999).

Screening for siderophore producing microorganisms

Ten morphologically different strains were subjected to screening for the production of siderophores by ferric perchlorate assay as recommended by Payne, 1994. The maximum optical density to the culture supernatant indicated the level of siderophores.

All the 10 strains were subjected to ferric perchloric acid assay consisted in mixing 0.5ml of culture supernatant or a suitable dilution there with 2.5ml of 5mM ferric chloride was dissolved in 0.1M perchloric acid in a cuvette followed by using sterile medium as the blank then the cuvette was subjected to calorimetric analysis.

Identification of potential strain

The potential strain AS6 was identified up to genus level by studying phenotypic characters like gram staining, motility and biochemical characteristics like oxidase, catalase, IMVIC, urease, nitrate reduction and sugar fermentation tests. The methods described by Microbiology: A laboratory Manual by (Cappuccino 2013) was followed for all the procedures. All these results were compared with Bergey's manual of determinative bacteriology to determine the genus.

Based upon the expressed phenotypic characters, the potential strain AS6 was identified as *Pseudomonas sp.*

Optimization studies

Optimization studies were carried out by changing various parameters for siderophore production. Different parameters studied were incubation period, pH, media namely Glucose medium, King's B medium, Glutamic medium and Succinate medium (Patel *et al.*, 2009). The presence of siderophores was confirmed by using TLC. Culture supernatant was spotted on silica gel plates and spots were allowed to dry. The plates were then developed using Butanol: Acetic acid: Water (12:3:5) solvent system until the solvent front reaches the top of the plate. Plates were then dried and sprayed with 0.1M $FeCl_3$ in 0.1N HCl (Patel *et al.*, 2009). The protein fraction from culture broth was purified using SG 25 gel column.

The column was equilibrated with 50mM potassium phosphate buffer (pH 2.0). The sample was loaded and the unbound fractions were washed off with phosphoric acid. The bound sample was eluted using 0.1M phosphate buffer (pH 7.0). The fractions were monitored by taking absorbance at 380nm on UV Spectrophotometer (Patel *et al.*, 2009).

Determination of antimicrobial activity of siderophore

Muller Hinton agar plates were prepared and the water borne pathogens such as *Salmonella sp, Shigella sp, E. coli sp, Klebsiella sp, Vibrio sp* were swabbed on the plate by using sterile cotton swabs. Then wells were cut through the gel and the purified iron binding protein was inoculated into wells. The plates were incubated at 28°C for 24hrs for formation of zone of inhibition (Patel *et al.*, 2009).

Results and Discussion

Isolation of bacteria from soil samples

Bacterial population in nutrient agar plates was estimated as $136x10^5$CFU/gram, $83x10^5$CFU/gram, $46x10^5$CFU/gram for soil sample I, II and III respectively. From the plates 10 morphologically different strains (AS1-AS10). All the 10 strains were subjected to screening for the production of siderophores by Ferric perchlorate assay as recommended by Payne, 1994. The maximum optical density to the culture supernatant

indicated the presence of siderophores. Strain AS-6 showed maximum OD value of 0.82 and was selected for further identification and optimization studies.

Identification of potential strain

The potential strain AS6 was identified based upon the expressed phenotypic characters, the potential strain AS7 was tentatively identified as *Pseudomonas sp* (Fig. 1).

Optimization studies

The siderophore production was maximum when using Glucose medium (OD: 0.85). The effect of incubation period on siderophore production was maximum at 72 hours with absorbance of 0.98 and the maximum at pH and temperature 5.5 (OD 1.20); 30^0C (OD: 1.19) respectively.

Finally, maximum siderophore production was observed when using Ferric Chloride as additive (OD: 1.40).

Detection of siderophore by thin layer chromatography

Siderophores were confirmed by using TLC (Fig. 2).

Fig.1 Colony morphology of *Pseudomonas species* on Nutrient Agar plates

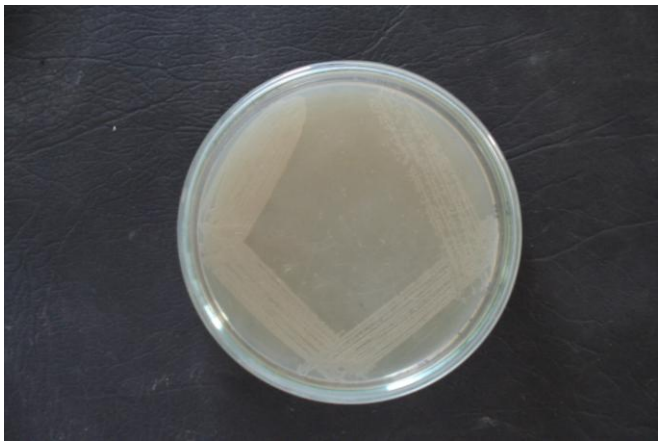


Fig.2 Detection of siderophore by thin layer chromatography

Fig.3 Elution profile of siderophore separation by column chromatography

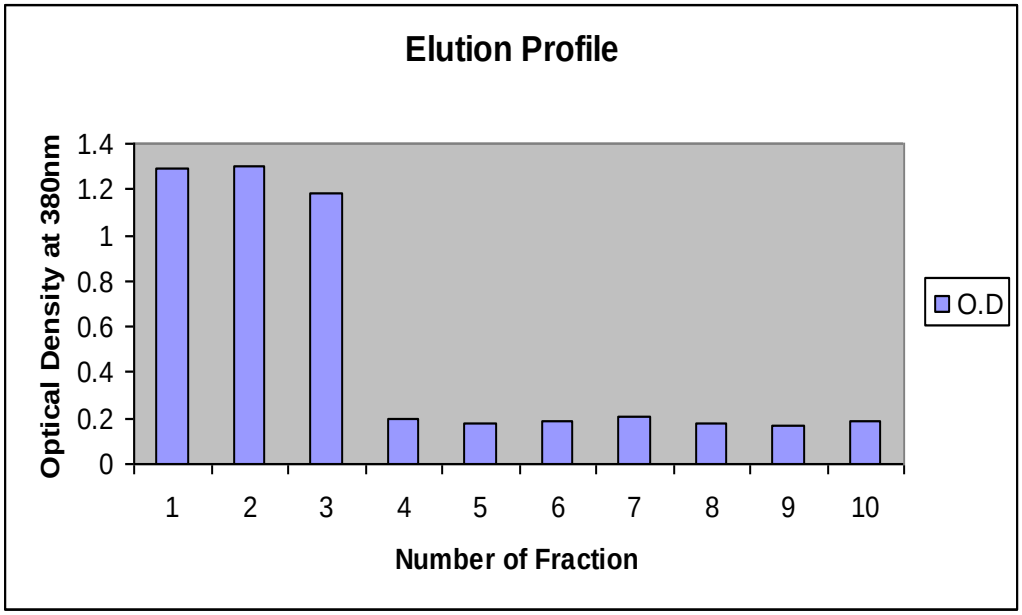
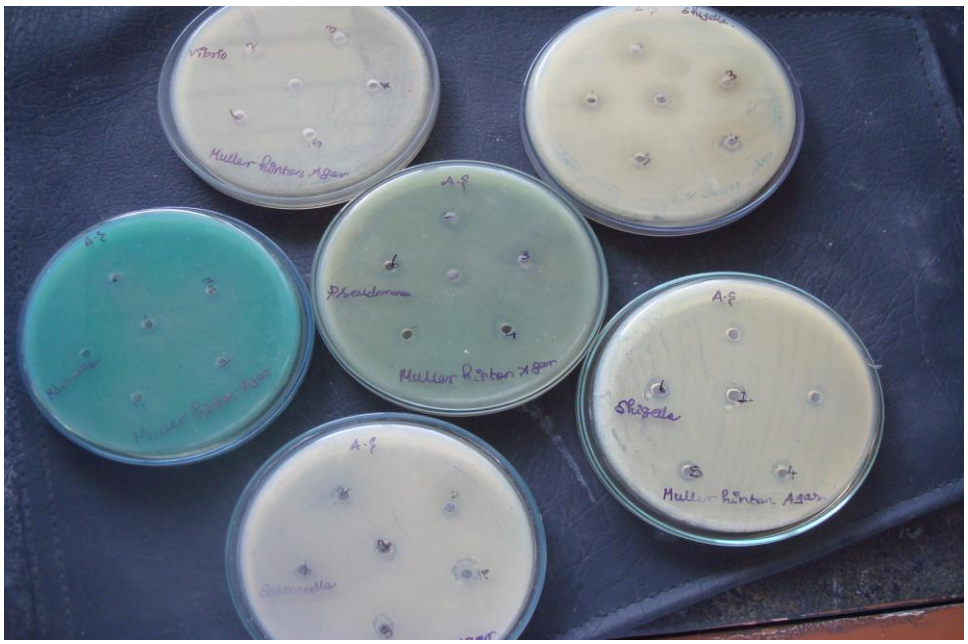


Fig.4 Determination of antimicrobial activity of siderophore



Purification of protein fraction by column chromatography

Chromatography was performed using Sephadex G25 gel column (Patel *et al.*, 2009). The results clearly indicate that the Fractions 1, 2 and 3 showed high optical density at 380 nm and were pooled and used as single active fraction for further antimicrobial activity studies (Fig. 3).

Determination of Antimicrobial activity of siderophore

Antimicrobial activity of active fraction was assed against water borne pathogens such as *Salmonella sp, Shigella sp, E. coli, Klebsiella sp, Vibrio sp*. (Fig. 4). The active fraction showed good zone of inhibition against water borne pathogens such as *Salmonella, Vibrio, Shigella* and *E. coli* in Muller Hinton agar plates.

The study reports the isolation and enumeration of siderophore producing microbes from iron contaminated soil. It is concluded that the iron contaminated soil samples are good source for the isolation of siderophore producing microbes and also indicates that these siderophores can be used against pathogenic bacteria.

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