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Studies on siderophore production by microbial isolates obtained from aquatic environment

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ABSTRACT

The paper investigates and reports siderophores production from bacteria isolated from aquatic environment. In the experimental findings, out of 125 bacterial isolates only twelve morphologically different bacterial cultures were isolated and identified as Escherichia coli(6 isolates, strains CH1-CH6), Pseudomonas aeruginosa(6 isolates, strains CH7-CH12) all of which showed siderophores production by Chrome Azurol Sulphonate(CAS) assay. E.coli Strain CH6showed maximum siderophores production (46%) and Pseudomonas aeruginosa strain CH8 showed the lowest production of siderophore (10%). Siderophores produced by the E.coli strain (strains CH1-CH6) were hydroxymate type and those produced by P. aeruginosa(strains CH7-CH12) were catecholate type. E.coli strain CH6 was chosen for further examination as it showed maximum siderophore production on succinate medium (75%) with10mM Fe at optimum pH 7.0. The prime interest of this experimentation is to further explore and re-examine the biological application in the current Indian context of Agricultural development.

Key words: Siderophore, CAS, *E. coli, Pseudomonas aeruginosa*, Hydroxmate, Catecholate

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INTRODUCTION

Microorganisms have evolved a wide range of strategies enabling them to acquire iron from the environment, including reduction of ferric to ferrous ions, direct acquisition of iron from host iron binding proteins by pathogens and the uptake of iron in the form of ferric siderophores (microbial iron chelates). They express a variety of low molecular weight, high affinity chelating agents that solubilize ferric iron in the environment and transport it into the cell and are known generically as siderophores[1].Siderophores are important for the survival and growth of bacteria in the soil and in aqueous environments [2]. Siderophores are commonly produced by aerobic and facultative anaerobic bacteria and fungi under iron limiting conditions. Research on siderophore-mediated iron transport has mainly focused on Gram-negative bacteria, although Gram-positive bacteria such as *Bacillus*, *Staphylococcus* and *Streptomyces* also possess siderophore-mediated iron transport systems [2]. To date nearly 500 siderophores are reported from selected microorganisms. In general, siderophores are classified as hydroxamates, catecholates, salicylates and carboxylates and more recently with new group polycarboxylates[3, 4]. Variations are seen in siderophores structure from one species to another[5]. Siderophores and their substituted derivatives have varied

applications in agricultural, environmental and medical sciences [6].The present investigation describes the isolation of siderophore from water samples.

MATERIALS AND METHODS

Collection of Water Samples and Isolation of Bacteria

Water samples were collected from Adyar River, Chennai, Tamilnadu during the month of November 2012. Water samples for microbiological examination were collected using the standard procedure [7], at a depth of few centimeters using 10 ml sterile bottles for each sample. Samples were placed in ice and transported to the laboratory and processed within 2 hours of collection. The water samples were serially diluted up to 8 fold in sterile water. 100µl of the 10^{-2} to 10^{-8} diluted water sample were evenly spread plated method on Nutrient agar (Hi-media Mumbai, India) and incubated at 37°C overnight. Following incubation, colony-forming units (CFU) were counted and the results were recorded. Different bacterial isolates were picked from the plates based on morphological appearance and sub-cultured. Pure cultures were stored in 30% glycerol (stock) at – 40°C for further studies.

Identification of the Isolates of Bacteria

The bacterial isolates obtained were Gram stained, tested for their biochemical characteristics and identified using Bergey's manual of Determinative Bacteriology [8].

Isolation of Siderophore Producing Bacteria

Qualitative Assay[9]

60.5 mg CAS was dissolved in 50 ml glass distilled water and mixed with 10 ml of iron (III) solution (1mM $FeCl_3.6H_20$ in 10mM HCl). This was added to 72.9 mg of hexadecyltrimethylammoniumbromide (HDTMA) in 40 ml of distilled water. The dark blue colored CAS reagent was then autoclaved for 15 minutes. This reagent was added to PIPES agar medium(30.24 g of PIPES buffer dissolved in 750ml of Distilled water +15g of agar, whose pH was adjusted to 6.8 using 1.06g of NaOH pellets). The solidified CAS agar plates were punched with 2.5 to 5mm diameter holes by using a gel puncher. Each hole was filled with 25 µl of the bacterial culture supernatant and kept for incubation in dark at 28^{o} C for 7days.

Quantitative Assay (CAS- Shuttle Assay)[9]

The quantitative estimation of siderophore produced by the bacterial isolates was done by CAS- shuttle assay**(**Schwyn and Neilands, 1987). The strain was grown in succinate medium and incubated for 24 to 30 hrs at 28˚C with constant shaking at 120 rpm on rotator shaking incubator. After incubation the fermented broth was taken and centrifuged at 10,000 rpm in a cooling centrifuge at 4˚C for 10 minutes and cell- free supernatant was mixed with 0.5 ml CAS solution. The color obtained was determined using the Spectrophotometer at absorbance 630 nm after 20 minutes of incubation with reference containing 0.5 ml CAS solution with 0.5ml uninoculated succinate medium. The percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula

$[(A_r - A_s) / A_r] * 100$

Where A_r is the Absorbance of reference (CAS assay solution + uninoculated media) and A_s is the Absorbance of the sample (CAS assay solution + cell-free supernatant).

Determination of the Type of Siderophore

Iron PercholateAssay[10]

The presence of hydroxymate type strain was detected by the Iron percholate assay. To 0.5ml of culture supernatant, 2.5ml of Iron percholate solution (5mM $Fe(ClO_4)3$ in 0.1M $HClO_4$) was added and allowed to incubate for 5min to develop the orange red colour solution and the absorbance was measured at 480nm. Desferal was used as standard.

Arnow's Assay [11]

For detection of catechols, 1ml of culture supernatant was mixed with 1ml of HCl, 1ml of nitrite molybdate (catechols produce yellow color),1ml of NaOH(color changes to red) followed by distilled water to make up the volume to 5ml. Absorbance was measured at 500 nm using 2,3 dihydroxybenzoic acid as the standard in UV-Visible spectrophotometer.

Effect of Culture Media on Siderophore Production [12]
The culture was grown on different media such as Succinate, Glucose and King B to check the effect of culture media on siderophore production. Each medium was separately inoculated with bacterial cultures and incubated at 28 ˚C on shaking incubator at 120rpm. After incubation the fermented broth was taken and centrifuged at 10,000 rpm in cooling centrifuge at 4˚C for 10 minutes and cell-free supernatant was mixed with 0.5 ml CAS solution. The color obtained was determined using the Spectrophotometer at absorbance 630 nm after 20 minutes of incubation. The reference contained 0.5 ml CAS solution with 0.5ml uninoculated medium. The percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula

$[(A_r - A_s) / A_r] *100$

Where A_r is the Absorbance of reference (uninoculated media) and A_s is the Absorbance of the sample (supernatant).

Effect of pH on Siderophores Production [12]
The effect of pH on siderophore production was studied on succinate medium by adjusting the pH to 5, 6, 7, 8,9and 10 using 1N HCl or 1N NaOH, keeping all other conditions constant. The bacterial isolates were inoculated in the succinate medium. After incubation the fermented broth was taken and centrifuged at 10,000 rpm in a cooling centrifuge at 4˚C for 10 minutes and the cell-free supernatant was mixed with 0.5 ml CAS solution. The color obtained was determined using the Spectrophotometer at absorbance 630 nm after 20 minutes of incubation with reference containing 0.5 ml CAS solution with 0.5ml uninoculated succinate medium. The percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula

$[(A_r - A_s) / A_r] *100$

Where A_r is the Absorbance of reference (uninoculated media) and A_s is the Absorbance of the sample (supernatant).

Effect of Iron Concentration on Siderophore Production[12]
To determine the effect of iron concentration the bacterial strains was grown in succinate medium containing $FeCl_3$ in increasing amount(10-50mM).The flask was inoculated with the bacterial isolate and incubated for 24-30 hours at 28˚C with constant shaking at 120rpm in rotator shaking incubator. After incubation the fermented broth was taken and centrifuged at 10,000 rpm in cooling centrifuge at 4˚C for 10 minutes and cell free supernatant was mixed with 0.5 ml CAS solution. The color obtained was determined using the Spectrophotometer at absorbance 630 nm after 20 minutes of incubation with reference containing 0.5 ml CAS solution with 0.5ml uninoculated succinate medium. The percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula

$[(A_r - A_s) / A_r] *100$

Where A_r is the Absorbance of reference (uninoculated media) and A_s is the Absorbance of the sample (cell-free supernatant).

RESULTS AND DISCUSSION

Isolation of Bacteria from Water Samples
A total of 125 bacterial isolates were isolated from the Nutrient agar plates based on colony morphology.

Isolation of SiderophoreProducing Bacteria
Qualitative Assay and Quantitative Assay (CAS- Shuttle Assay)
All the 125 bacterial isolates isolated were screened for production of siderophore on CAS agar plates. Only bacterial twelve isolates were siderophore positive with varying intensity of orange zones. The 12 bacterial isolates were further characterized by biochemical tests and identified as *E.coli* (6 isolates and designated as strains CH1, CH2, CH3, CH4, CH5, CH6) and *P.aeroginosa* (6 isolates and designated as strains CH7, CH8, CH9, CH10, CH11, CH12). The percentage of siderophore produced varied from 10%(*P.aeruginosa* strain CH8) to46% (*E.coli*strain CH6) (Table 1). The CAS Assay is the universal assay for siderophore detection and is based on a siderophore's high affinity for ferric iron. Chrome azurol S (CAS) dye complexes with ferric iron and the CAS assay plates are

blue in color. Iron bound to CAS is easily chelated by siderophores to produce a color change from blue to orange.*E. coli* strain CH6was chosen for further studies as it produced the highest percentage (46%) of siderophore.

Table 1 Percentage of Siderophore Production by *E.coli* and *P.aeruginosa* strains

Bacterial strains	% of siderophore production	Bacterial strains	% of siderophore production
E.coli		*P.aeruginosa*	
CH1	13	CH7	18
CH2	22	CH8	10
CH3	19	CH9	35
CH4	36	CH10	27
CH5	45	CH11	24
CH6	46	CH12	42

Determination of the Type of Siderophore

Iron Percholate Assay and Arnow's Assay

The type of siderophore produced by *E. coli*(CH1,CH2,CH3,CH4,CH5,CH6) were of the Hydroxmate type (wine red color) whereas the *P.aeruginosa* isolates CH7, CH8, CH9, CH10, CH11, CH12 were of the Catecholate or Phenolate type (yellow color) Table 2.[13]reported the presence of Catecholate type (pyovirdine) of siderophore in *P.aeruginosa* BUP2 isolated from the Malabari Goat. [14]has reported Hydroxmate type siderophore from the gut flora of *E. coli*.

Table 2 Determination of the Type of Siderophore: Iron Percholate Assay and Arnow's Assay

Sample	Iron Percholate Assay- OD value at 480nm	Sample	Arnow's Assay - OD value at 500nm
Blank	000	Blank	000
Standard	0.301	Standard	0.091
CH1	0.222	CH7	0.087
CH2	0.132	CH8	0.086
CH3	0.207	CH9	0.045
CH4	0.121	CH10	0.048
CH5	0.142	CH11	0.054
CH6	0.152	CH12	0.032

Effect of Culture Media on Siderophore Production

The maximum siderophore production (75%) was found on succinate medium as compared to other media (Table. 3). The maximum siderophore production found in succinate medium is due to pyoverdine, in which the 3-aminomoiety of the chromophore is substituted with various groups derived from succinate, malate, a-ketoglutarate [15, 16]. Similar results were obtained by [17].

Table 3 Effect of Culture Media on Siderophore Production

Medium	%Siderophore Production
Succinate	**75**
Kings B	62
Glucose	41

Effect of pH on Siderophore Production

The maximum siderophore production was found at neutral pH 7.0 (Table.4). This may be because bacteria grow better and iron is present in insoluble form at neutral pH and therefore is not available to the bacteria. Thus siderophore are produced under low stress condition similar to the results of [18].

Table 4 Effect of pH on Siderophore Production

pH	%Siderophore Production
5	15
6	36
7	**77**
8	52
9	17
10	10

Effect of Iron Concentration on Siderophore Production

The optimal iron concentration was found at 10mM in succinate medium (Table. 5), while production of siderophore repressed when the iron concentration increased in concordance with the results obtained by [19]. Similar reports were recorded by [20] in 20 species of Fungi and three rhizobacterial Pseudomonas.

Table 5 Effect of iron concentration on siderophores production

Fe mM	%Siderophore Production
10	**78**
20	67
30	40
40	31
50	16

CONCLUSION

The findings of the present study which foregrounds *E. coli* strain CH6 is a potential candidate for the production of hydroxamate siderophores. Further purification and characterization of siderophores from strain CH6 may pave the way for its utilization in the field of Medicine and Agriculture.

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