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EVALUATION OF SIDEROPHORE PRODUCED BY DIFFERENT CLINICAL ISOLATE *PSEUDOMONAS AERUGINOSA*

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Abstract- A total thirteen strains of Pseudomonas spp. were isolated from patients and hospital environment and were identified as a *Pseudomonas aeruginosa*. All isolated strains were shown siderophore production on chromo azural S agar plate. The maximum siderophore production was found in strain PAUTI4 i.e. 67%. Evaluation study of siderophore shown that maximum siderophore were obtained on succinate medium at 7.0 pH with iron concentration 10μM.

Key words – Evaluation**,** siderophore, clinical, *Pseudomonas aeruginosa,*

INTRODUCTION

Siderophore are low molecular weight (< 10 KD) iron chelating compounds synthesized by microbes in large quantity under iron limited conditions.[1,2] Pseudomonas spp. have been known for their siderohore production for many years and therefore many reports on the isolation and characterization of their siderohores have been published [3,4]. The importance of siderophore extends their application in biotechnology and medicine. Siderophore produced by Pseudomonas spp. have been employed efficiently as biocontrol agents against certain soil-borne plant pathogens. Also siderophore use in medicine for iron and aluminum overload therapy and antibiotic for better targeting [5]. One potentially powerful application of siderophore is to use the iron transport abilities of siderophore to carry drugs into cell by preparation of conjugates between siderophore and antimicrobial agents. Because microbes recognize and utilize only certain siderophore such conjugates are anticipated to have selective antimicrobial activity [6]. *P. aeruginosa* produced two types of siderophore, phyochelin [7] and pyoverdine [8]. Pyoverdine have high affinity for iron but phyochelin shows lesser affinity. To make available siderophore for biotechnological and medicinal application is important to increase siderohore production by evaluating process parameter. The aim of present investigation to evaluate siderophore production of clinical isolates pseudomonas aeruginosa.

MATERIALS AND METHODS

Experimental Material

All the ingredients and media used in these experiments were procured from Hi-media Laboratories Pvt. Ltd (India) and S.D. Fine chemicals. Pvt. Ltd (India).The glassware used in experiment was periorly washed with 6N HCl to remove residual iron and rinsed with pure water.

Isolation of *Pseudomonas aeruginosa*

Thirteen Pseudomonas spp. were isolated from different patients. Out of which 04 from urinary tract infection, 03 from burn skin, 03 from wound and 03 from hospital environment (Table-1). Urine sample were collected from patients aseptically with the help of sterile wide mouthed screw capped plastic containers and processed by pour plate method using citramide agar. While sample of burn skin and wound were collected by sterile cotton swabs and directly inoculated into citramide agar. Hospital environment samples were collected by direct exposure of citramide agar plate. The plates where incubated at 28^{o}C for 24 hrs a well isolated colony were selected for identification.

Identification of Pseudomonas spp.

The clinical specimen obtained from different patients were cultured on blood agar and MacConkey agar plates and incubated at a temperature of 37^{o}C for 24 hours. The culture plates were processed using standard microbiological procedures, characterization and identification of *P. aeruginosa* was carried out using a combination of colonial morphology, Gram stain characteristics, motility tests, pigmentation, oxidation-fermentation tests, catalase and oxidizer activity tests and pyocyanin production.

Detection of Siderophore Production

Siderophore production by different strain of *Pseudomonas aeruginosa* was tested by chromo azural S (CAS) assay [9]. The strains were spread over citramide agar and incubated for 48h at 30^{o}C. After incubation a thin layer of CAS reagent in 0.7% agar was spread on the bacterial growth and plates were again incubated for 24h at 30^{o}C formation of yellow orange zone around the colonies indicates siderophore production.

Estimation of Siderophore Production

The quantitative estimation of siderophore produced by clinical isolated *Pseudomonas aeruginosa* was done by CAS-shuttle assay [10]. In which the strain grown on succinate medium [11]. Containing of gm/l K_2HPO_4 6.0, KH_2PO_4 3.0, $MgSO_4$ 0.2, $(NH_4)_2SO_4$ 1.0 and succinic acid 4.0, pH 7.0 and incubated for 24-30h at 28°C with constant shaking at 120 rpm on rotator shaking incubator. After incubation the fermented broth were centrifuge at 10,000 rpm in cooling centrifuge at 4°C for 10 minute 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 min of incubation with reference containing 0.5ml uninoculated succinate medium and 0.5 ml CAS solution. The percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula $[(A_r - A_s)/A_r] \times 100$, where A_r is the $A630_{nm}$ of reference (CAS assay solution+ uninoculated media) and A_s is the $A630_{nm}$ of the sample (CAS assay solution+ supernatant).

Characterization of Siderophore

Hydroxamate type of siderophore was determined by hydrolyzing 1ml supernatant of overnight grown culture with 1ml of 6 N H_2SO_4 in a boiling water bath for 6h or 130°C for 30 min. Further this hydrolyzed sample was buffered by adding 3ml of sodium acetate solution. To this 0.5ml iodine was added and allowed to react for 3-5 min. After completion of reaction the excess iodine was destroyed with 1 ml of sodium arsenate solution. Finally 1 ml α – Naphthylamine solution was added as allowed to develop the colour. Wine red colour formation indicates production of hydroxamate type of siderophore [12]. While caecholate type of siderophore was determined by taking 1 ml of supernatant in a screw capped tube. To this 1 ml of nitrite-molybdate reagent with 1 ml NaoH solution was added. Finally 1 ml of 0.5 N HCl was added and allowed to develop colour. Yellow colour formation indicates production of catecholate type siderophore [13].

Effect of culture media on siderophore production

The culture was grown on different medium such as Succinate, King B, Cas-amino acid, Glucose and Asparagin medium. King B medium containing gm/l Glycerin 1.0, Protease-Peptone 20, $MgSO_4$1.5, Cas-amino acid medium containing gm/l Cas-amino acid 5.0, K_2HPO_4 1.180, and $MgSO_4$. $7H_2O$ 0.25, Glucose medium containing gm/l K_2HPO_4 0.56, Glucose 10, urea 0.85, Asparagin medium containing gm/l Asparagin 5.0, $MgSO_4$ 0.1 and K_2HPO_4 0.5. Each medium was separately inoculated and incubated at 28°C on rotatory shaking incubator at 120 rpm.

Effect of pH on siderophore production

The effect of pH on siderophore production were studied on succinate medium by adjusting pH at 5, 6,7,8,9 and 10 before inoculating the strain with 1 N HCL or 1N NaoH by keeping all other condition constant.

Effect of iron concentration on Siderophore production

To determine the effect of iron concentration the *Pseudomonas* strain were grown in succinate medium containing $FeCl_3$ in increasing amount i.e. 1-100μM. The flask was incubated for 24-30h at 28°C with constant shaking at 120 rpm on rotator shaking incubator.

RESULTS AND DISCUSSION

Pseudomonas aeruginosa was used for experimental study were isolated from urinary tract infection, wound, burn skin of patients and hospital environment and identified as a Pseudomonas *aeruginosa* on the basis of morphology and biochemical tests (Table 2). Siderophore production by different *P. aeruginosa* were confirmed by growing them individually on citramide agar, after spreading layer of CAS reagent and incubation each colony has developed yellow to orange colored zone on CAS agar plate indicating siderophore production. The color change from blue to orange resulting from siderophoral removal of Fe from the dye. Similar finding have been reported by Wilhelmina M. Huston., 2000 [14].

In order to estimate the amount of siderophore produced by different isolates, a CAS liquid assay has performed. Percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula $[(A_r - A_s)/A_r] \times 100$. It's found that amount of siderophore production varies in clinical and environmental isolates. In clinical isolates maximum percentage of siderophore were found in PAUTI4 strain which isolated from urinary tract infection, secondly in wound strain and thirdly in burn skin strain (Fig 1). This indicates that the amount of siderophore produced by *Pseudomonas aeruginosa* is depend upon availability of free iron present in human host [15]. While environmental strain has shown low amount of siderohore production which revealed that expression of siderohore producing gene is very important to initiate siderophore production. Further the type of siderophore was determined by Csaky and Arnow assay, where all isolate has shown both type of siderophore production i.e. wine red colour formation in supernatant indicated production of hydroxamate type (pyoverdine) while yellow colour formation in supernatant showed presence of catecholate or phenolate type (pyochelin) siderophore [16]. The maximum siderophore production were found on succinate medium as compare to other media (Table 3). This is due to pyoverdine, in which the 3-aminomoiety of the chromophore is substituted with various groups derived from siccinate, malate, α-ketoglutarate [17, 18]. The optimum pH for siderophore production was found 7.0 (Fig 2) in which bacteria grow better and iron is present in insoluble form at neutral pH and therefore is not available to the bacteria [19]. Siderophore are iron-specific compounds which are secreted under low iron stress conditions. The optimal iron concentration for maximum siderophore production was studied at 10μM in succinate medium (Fig 3), while production of siderophore repressed when iron concentration is increased but in our finding maximum siderophore production were found without addition of

$FeCl_3$. Similar result was obtained by Raaska., 1993 [20]. Who examined detection of siderophore in growing cultures of *Pseudomonas spp*. Hence the clinical isolated strain of *Pseudomonas aeruginosa* can be employed for large scale siderophore production by optimizing the above process parameter.

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Table- 1-List of Sample collected

Sr. No	Isolates	Origin	Number
1	PAUTI1	Urinary Tract infection	04
2	PAUTI2		
3	PAUTI3		
4	PAUTI4		
5	PABS1	Burn Skin	03
6	PABS2		
7	PABS3		
8	PAW1	Wound	03
9	PAW2		
10	PAW3		
11	PAHE1	Hospital Environment (Air)	03
12	PAHE2		
13	PAHE3		

Table-2 -Colony Morphology and biochemical Characteristic of isolated Pseudomonas aeruginosa

Sr. No	Morphology, Physiology & Biochemical Characters	Isolated *Pseudomonas aeruginosa*												
		PAUTI1	PAUTI2	PAUTI3	PAUTI4	PABS1	PABS2	PABS3	PAW1	PAW2	PAW3	PAHE1	PAHE2	PAHE3
1	Gram staining	Gr -	Gr -	Gr -	Gr -	Gr -	Gr -	Gr -	Gr -	Gr -	Gr -	Gr -	Gr -	Gr -
2	Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Colony on Cetrimide agar	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C	B.L.C
4	Growth at different temperature													
	4^0C	-	-	-	-	-	-	-	-	-	-	-	-	-
	42^0C	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Growth at different pH													
	5.7	+	+	+	+	+	+	+	+	+	+	+	+	+
	6.8	+	+	+	+	+	+	+	+	+	+	+	+	+
	8.0	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Growth on Nacl(25%)	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
9	Simmon's citrate medium	+	+	+	+	+	+	+	+	+	+	+	+	+
10	Urease	-	-	-	-	-	-	-	-	-	-	-	-	-
11	Indole	-	-	-	-	-	-	-	-	-	-	-	-	-
12	Methyl red	-	-	-	-	-	-	-	-	-	-	-	-	-
13	Vogues Prosker	-	-	-	-	-	-	-	-	-	-	-	-	-
14	Nitrate Reductase	+	-	+	+	+	+	+	+	+	+	+	+	-
15	Gelatin Hydrolysis	-	+	+	+	+	+	+	+	+	+	+	+	+
16	Glucose	+	+	+	+	+	+	-	+	+	+	+	+	-
17	Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
18	Manitol	+	+	+	+	+	+	+	+	+	+	+	+	+
19	Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-	-
20	Starch hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-

+ Posative, - Negative, Gr- Gram negative, B.L.C Blue color colony.

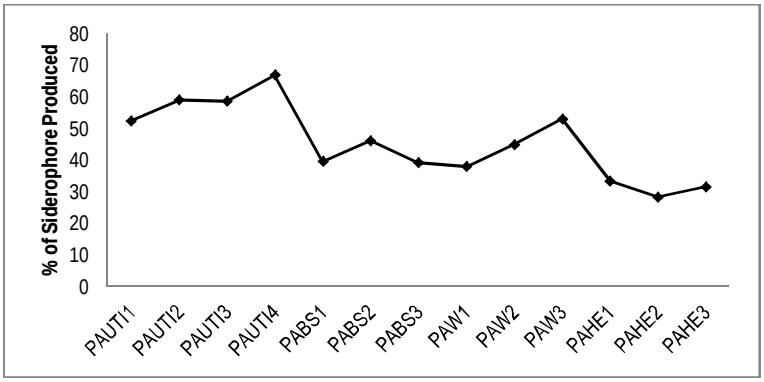

Fig.1- Percentage of Siderophore produced by isolates Pseudomonas aeruginosa

Table -3 -Effect of medium on Siderophore production.

Sr. No	Medium	Siderophore production in %
1	Succinate	68%
2	Kind B	62%
3	Cas-amino acid	56%
4	Glucose	52%
5	Asparagin	58%

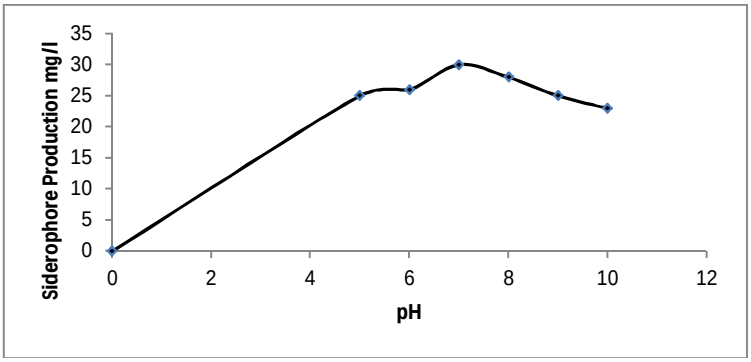

Fig. 2- Effect of pH on Siderophore production

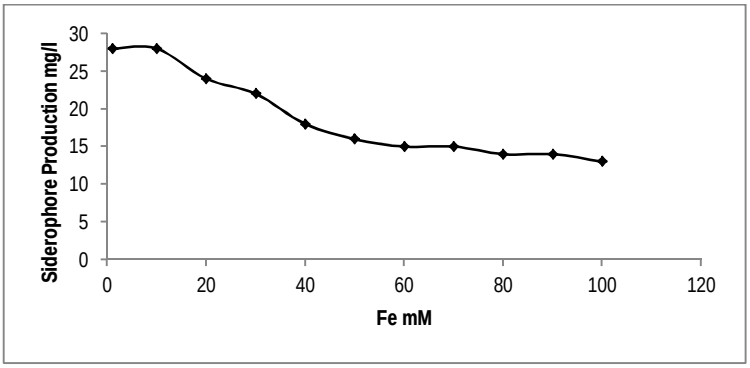

Fig.3- Effect of iron Concentration on siderophore Production