ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Antagonistic Activity of Siderophore Producing *Pseudomonas Aeruginosa* against *Aspergillus Spp.* and *Candida Albicans*

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen and a leading causes of hospital-aquired pneumonia. In present study 50 *Pseudomonas* strains was isolated from patients and hospital environment and were identified *Pseudomonas aeruginosa*. All isolates were studied for siderophore production on different medium at different pH and iron concentration also they were studied for antifungal activity. The result indicated that all 50 isolates showed siderophore production on CAS agar plate. Among the 50 isolates PAUTI4 strain showed maximum siderophore production on succinate medium (61mg/L) without addition of iron at optimum pH 7.0. The cell free supernatant of PAUT14 showed growth inhibition of *Aspergillus flavus, A. fumigates, A.niger* and *Candida albicans.* Maximum growth inhibition was found with *Aspergillus flavus* (67%). Hence *Pseudomonas aeruginosa* can be found efficient for siderophore production and biocontrol for fungal pathogen.

Key words: Siderophore, *Pseudomonas aeruginosa, Aspergillus spp*

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ISSN: 0975-8585

INTRODUCTION

Pseudomonas aeruginosa is ubiquitous in the nature and is one of the most commonly found microorganisms with versatile metabolic activities; it can be used to produce various industrial and pharmaceutical products. *Pseudomonas spp*. has been known for their siderophore production for many years and therefore many reports on the isolation and characterization of their siderophore have been published [9]. Siderophore produced by *Pseudomonas spp*. have been employed efficiently as biocontrol agents and present time there are some commercial product in the market [23].The applications of purified siderophore, as bacteriostatic or fugistatic agents in combination with other antibacterial factors will certainly raise a great interest [17]. Administration of desferrioxamine which is form of siderophore lowers the level of aluminum in the body and relives the symptoms of the disease [2]. *P. aeruginosa* produced two types of siderophore, phychelin [7] and pyoverdine [8] which has been widely studied as biological agents in the control of phytopathogenic fungi and bacteria [5]. Recently it has been reported that *Candida albicans* is the commonest species of Candida which causing infection in immune-compromised and HIV patients [6]. Other fungal infections seen in HIV-infected individuals include aspergillosis due to *Aspergillus flavus*, *A. fumigatus* and *A.niger* [15]. A rapid emergence of resistance to various antifungal drug, it is important to exist a novel antifungal remedies. The aim of present investigation to evaluate siderophore production of *Pseudomonas aeruginosa* and their antagonistic activity against pathogenic fungi.

MATERIALS AND METHODS

Experimental materials

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*

Fifty *Pseudomonas spp*, were isolated from different patients out of which 15 from urinary tract infection, 12 from burn skin, 12 from wound, 11 from hospital environment and reference strain of *Pseudomonas aeuroginosa* NCIM-2200 obtained from NCL Pune (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 sample 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 and characterization.

ISSN: 0975-8585

Identification and characterization of *Pseudomonas spp*.

The clinical specimen obtained from different patients was cultured on citramide agar plates and incubated at a temperature of 37°C for 24 hours. All isolates of *Pseudomonas* were examined for colony morphology, fluorescence on King B medium and gram reaction as per the standard procedures given by Anonymous [1] and Bartholomew and Mittewer [3].

Detection of siderophore production

Siderophore production by different strain of *Pseudomonas aeruginosa* and reference was tested by chromo azural S (CAS) assay [21]. The strains were spread over citramide agar and incubated for 48h at 30°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°C formation of yellow orange zone around the colonies indicates siderophore production.

Estimation of siderophore production

Siderophore production was studied by inoculating loopful culture of each isolate and reference strain in 250ml of succinate medium [16], 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. It was incubated for 24-30hrs 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 then mixed with 0.5 ml CAS solution and 10 L shuttling solution (Sulfosalicyclic acid). The color obtained was determined using the spectrophotometer at Absorbance 630 nm after 20 min of incubation with blank (Succinate medium). The percentage of siderophore units was estimated as the proportion of CAS blue color shifted to pink colur showed in using the formula $[(A_r - A_s)/A_r] \times 100$, where A_r is the $A630_{nm}$ of reference sample (medium + CAS assay solution + shuttle solution) and A_s is the $A630_{nm}$ of the sample (supernatant + CAS assay solution + shuttle solution).

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

To evaluate siderophore production the pH of succinate medium was adjusted at 5, 6,7,8,9 and 10 pH before inoculation with 1 N HCL or 1N NaoH by keeping all other condition at their optimum level.

ISSN: 0975-8585

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. 1-100□M. The flask was incubated for 24-30h at 28^{o}C with constant shaking at 120 rpm on rotator shaking incubator.

Antifungal activity of *Pseudomonas aeruginosa*

The test fungal organisms used for study are *Aspergillus flavus* NCIM-532, *A. fumigates* NCIM-902*, A.niger* NCIM-621*, Candida albicans* NCIM-3471 were obtained from National Chemical Laboratory (NCL) Pune, Maharashtra, India. Inoculum of fungi was maintained on sabouraud's dextrose agar during experiment. The obtained fungal pathogens were grown on sabouraud's dextrose agar media and incubated for 8 days to get profuse growth of selected fungi, thereafter 10mm diameter dish of each fungus was obtained by cork borer which was placed at the center of plate containing 0.2ml spread supernatant of PAUTI4 strain and incubated for 96h at 32^{0}C temperature. The inhibition of growth of fungal pathogen were recorded after 96 hrs of incubation and compared with the PDA plate inoculated with only pathogens as a control.

The radial growth of mycelium was measured and per cent inhibition (PI) was calculated.

$$PI = \frac{A - T}{A} X\ 100$$

Where, A is the growth of test pathogen (cm) in the absence of the antagonistic strain, T is the growth of test pathogen (cm) in the presence of the antagonistic strain.

RESULTS

All 50 isolates of *Pseudomonas* were examined for their morphological and biochemical characters and showed similar characteristic to *P. aeruginosa*. All the isolates were gram negative rod shaped produced light green coloured colnies with high fluorescence on King B medium. Colonies of the most of isolates were creamy round, white irregular or circular. Also isolates showed good growth on medium containing glucose, moderate growth on arginine and alanine and poor growth on valine and meso-inosital. They were also capable to grow at 41^{o}C but not at 4^{o}C. All isolates were negative for starch hydrolysis and positive for gelatin liquefaction, casein hydrolysis, lipid hydrolysis and denitrification.

Siderophore production by different *P. aeruginosa* and reference strain were confirmed by growing them individually on citramide agar, after spreading layer of CAS reagent and incubation each colony has developed yellow to orange coloured zone on CAS agar plate indicating siderophore production (Fig.1). 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 blue color shifted to pink color showed in fig.2 using the formula $[(A_r - A_s)/A_r] \times 100$. It's found that amount of siderophore production varies

ISSN: 0975-8585

from 32 to 61mg/L in *Pseudomonas spp* showed in table 1. The maximum percent of siderophore production were showed by strain PAUTI4 isolated from urinary tract infection (61mg/L), followed by wound, burn skin, and hospital environment strain which indicate that the amount of siderophore produced by *P. aeruginosa* is depend upon availability of free iron present in human host [14]. While environmental strain has shown low amount of siderophore production which revealed that expression of siderophore producing gene is very important to initiate siderophore production. Similar finding of maximum siderophore production among the *Pseudomonas* sp. have been reported by Bashan, [4] and Seuk *et al*.,[22]. The reference strain NCIM 2200 showed 58mg/L of siderophore production.

Fig.1 Orange halo Zone of siderophore produced by selected *Pseudomonas aeruginosa*

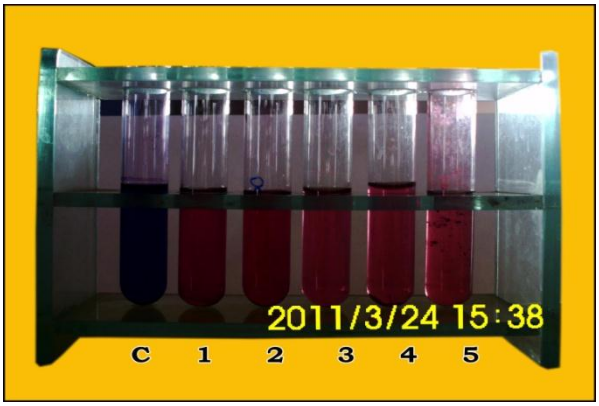

Fig.2 Siderophore production by selected *Pseudomonas aeruginosa* on CAS liquid assay

Table: 1 Isolation of *Pseudomonas aeruginosa* and amount of siderophore production

Sl. No	Source of isolates	Isolates code	Siderophore production mg/L	Sl. No	Source of isolates	Isolates code	Siderophore production mg/L
1	Urinary Tract infection	PAUTI1	53	26	Burn Skin	PABS11	54
2		PAUTI2	54	27		PABS12	56
3		PAUTI3	60	28	Wounds	PAW1	54
4		PAUTI4	61	29		PAW2	57
5		PAUTI5	58	30		PAW3	56
6		PAUTI6	58	31		PAW4	53
7		PAUTI7	56	32		PAW5	50
8		PAUTI8	58	33		PAW6	48
9		PAUTI9	60	34		PAW7	56
10		PAUTI10	59	35		PAW8	57
11		PAUTI11	54	36		PAW9	59
12		PAUTI12	58	37		PAW10	50
13		PAUTI13	60	38		PAW11	56
14		PAUTI14	54	39		PAW12	45
15		PAUTI15	58	40		PAHE1	43
16		PABS1	43	41		PAHE2	23
17		PABS2	56	42		PAHE3	57
18		PABS3	48	43		PAHE4	48
19		PABS4	59	44		PAHE5	32

ISSN: 0975-8585

20	Burn Skin	PABS5	57	45	Hospital Environment (Air)	PAHE6	48
21		PABS6	58	46		PAHE7	56
22		PABS7	57	47		PAHE8	53
23		PABS8	32	48		PAHE9	51
24		PABS9	34	49		PAHE10	50
25		PABS10	56	50		PAHE11	43
				51	Reference Strain		58

PAUTI –*P. aeruginosa* of urinary tract infection
PABS- *P. aeruginosa* of burn skin
PAW- *P. aeruginosa* of wound
PAHE- *P. aeruginosa* of hospital environmental

As *Pseudomonas* strain PAUTI4 showed maximum siderophore production further evaluation of the siderophore production studied continued with same strain. The results of effect of medium on siderophore production reveled that a highest siderophore production was observed in succinate medium i.e. 6l mg/l, while low concentration in Asparagin medium. Glucose, King B, Cas-amino acid medium showed intermediate amount of siderophore production (Table-2).

Table:2 Effect of medium on siderophore production

Sl. No	Medium	Siderophore production in mg/L
1	Succinate	61
2	Kind B	58
3	Cas-amino acid	50
4	Glucose	48
5	Asparagin	54

The maximum siderophore production was found on succinate medium is due to pyoverdine, in which the 3-aminomoiety of the chromophore is substituted with various groups derived from siccinate, malate, a-ketoglutarate. [13, 11]. Siderophore production was also studied at different pH in succinate medium. The maximum siderophore production was found at neutral pH 7.0 in which bacteria grow better and iron is present in insoluble form at neutral pH and therefore is not available to the bacteria [20] (Fig. 3). Siderophore are iron-specific compounds which are secreted under low iron stress conditions. The optimal iron concentration for maximum siderophore production was studied at 10□M in succinate medium. While production of siderophore repressed when iron concentration in increased but in our finding maximum siderophore production were found without addition of $Fecl_3$ (Fig. 4). Similar result was obtained by Raaska *et al*.,[17]. Who examined detection of siderophore in growing cultures of *Pseudomonas spp*.

ISSN: 0975-8585

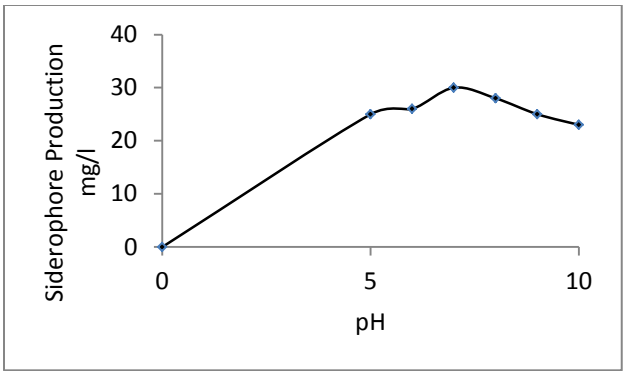

Fig .3 Effect of pH on siderophore production

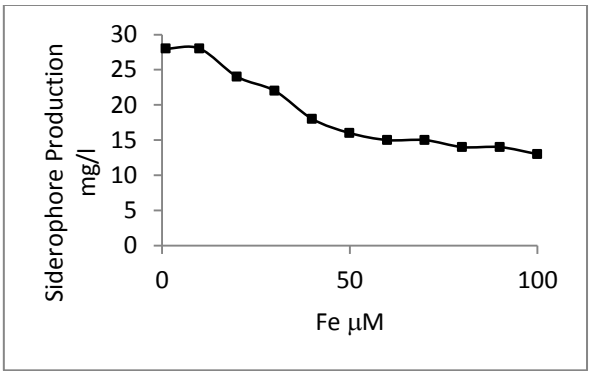

Fig .4 Effect of iron concentration on siderophore production

The cell free supernatant of PAUTI4 strain showed fungal growth inhibition in range 38 to 67%. Maximum inhibition was found with *Aspergillus flavus* (67%) (Table.3). Followed by *Aspergillus fumigates, Aspergillus niger and Candida albicans.* The results of fungal inhibition are in accordance with Sayyed et al., [19] and Kaleli et al., [12]. Singh et.al., [23] reported that *Pseudomonas* shows fungal growth inhibition by mechanism like antibiosis, site competition, HCN production, fluorescent pigments, antifungal volatiles metabolites and siderophore production.

Table -3 Antifungal activity of siderophore

Sr. No	Test Fungi	Percent Inhibition
1	*Aspergillus flavus*	67%
2	*Aspergillus fumigatus*	66%
3	*Aspergillus niger*	62%
4	*Candida albicans*	38%

P. aeruginosa is ubiquitous in nature and easily cultivated on different medium and it is efficient siderophore producer. Siderophore produced by *P. aeruginosa* has wide application in medicine, biotechnology and agriculture. In present study *P. aeruginosa* PAUTI4 showed maximum siderophore production on succinate medium without addition of iron at neutral pH. Hence can employed for large scale siderophore production purpose. Also it showed antifungal activity against various fungi which indicated that *P. aeruginosa* can be used as biocontrol for various phytopathogenic fungi and its metabolite such as siderophore can be used as growth inhibitor or in combination with some antimicrobial compound for human fungal pathogen.

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