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Isolation of Pseudomonas fluorescence species from fish waste, assessment of siderophore production and their antibiotic activity by dual culture plate techniques

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ABSTRACT

Siderophores are organic compounds with low molecular masses that are produced by microorganisms. Under the iron-restricted condition, many bacteria produce iron chealating siderophores. Siderophore chealate iron and supply to the bacterial cell by outer membrane receptors. A great variation is seen in the siderophore structure produced by many bacteria. Bacterial strains also produce fluorescence as the one like Pseudomonas fluorescence. They are prevalent in compost soil environment. They have received much attention in recent years because of their potential roles and application in various areas of environmental research. Their significance is because of their ability to kill bacterial and fungal pathogens. They act as an antibiotic and they have a wide range of chemical structures and specific properties. Even though siderophores have been reported from a variety of organisms inhabiting diverse environments. The study of marine siderophores is in its infancy as compared to their terrestrial counterparts. Therefore, the present investigation was carried out to detect and characterize siderophores of unique Pseudomonas spp. isolated from sediments collected from the marine environment. Fish waste is one of a major source of marine bacteria like Pseudomonas, Vibrio and Streptococcus Sp. The isolated strains were confirmed by biochemical characterization. The biosynthesis of a yellow-green, fluorescent, water-soluble pigment by p.fluorescens occurred only when the bacteria were iron-deficient and was not directly influenced by the nature of the organic carbon source. The pigment formed a very stable Fe3+ complex and was purified in this form. P.fluorescens produced only one molecular type of fluorescent pigment; however, its lability under mild alkaline conditions led to the formation of several pigmented decomposition products. Both its biosynthesis and its chemical properties (formation of a stable Fe3+ complex) suggest that the fluorescent pigment is a desferrisiderophore. The strain was grown in King`s B liquid for fluorescent pigment production and after that, it was extracted with acetone. Siderophore and their derivatives have a large application in agriculture as it increases soil fertility and is a biocontrol agent for the fungal and bacterial pathogen. The present study deals with the isolation of the fluorescent isolates of P.fluorescens possessing a variety of promising properties which make them better microbes. Twelve P.fluorescens isolates were isolated on King's B and Pseudomonas isolation agar medium and their production of fluorescence under a UV transilluminator were assessed. The aim of the present study is to outline and discuss the important roles and form of siderophores in fish waste compost soil and to emphasize their significant roles that these small organic molecules could play an important role in biocontrol processes. A simple confrontation assay for identifying potential antagonists was developed. Fluorescent Pseudomonas isolates PS6, PS7, PS8, and PS10 were found to be antagonistic against both Bacterial and Fungal Pathogens.

Keywords— *Fluorescent pseudomonads, Yellow-green fluorescent pigment, Siderophore*

1. INTRODUCTION

Iron (Fe) is an essential element for the growth of almost all living microorganisms, because, it acts as a catalyst in enzymatic processes, oxygen metabolism, electron transfer and DNA and RNA syntheses (Aguado-Santacruz Received 18 December 2013). It is an essential requirement for the growth and proliferation of microbes, and the efficiency of its acquisition in bacteria is generally believed to be linked to their pathogenicity (Grffths 1999).

Siderophores are metabolites produced by microorganisms. These compounds bind ferric iron, promote the rate of Fe3+ transport, and thus alleviate the problem of iron unavailability. Iron is the fourth most common element in the earth's crust. It is present in the soil, with rare exceptions, in the form of oxide hydrates, which have small dissociation constants. Complexation by peptides can also make iron unavailable (Budzikiewicz, 1997). These facts explain why iron is not readily available to microorganisms. Marine organisms such as phytoplankton (Trick et al., 1983) and cyanobacteria (Armstrong and Van Baalen, 1979) can also produce siderophores. The role of siderophores is primarily to scavenge Fe, but they also form complexes with other essential

elements (i.e. Mo, Mn, Co, and Ni) in the environment and make them available for microbial cells (Bellenger et al., 2008; Braud et al., 2009).

The biosynthesis of siderophores is one of the common strategies that many microbes employ to obtain iron from their environment (Neilands 1995; Byers & Arceneaux 1998). These high-affinity iron chelates are biosynthesized by the cell and released to solubilize and sequester Fe (III) and are then recognized by the host as an iron-siderophore conjugate by specific receptors on the cell surface (FaraldoGomez & Sansom 2003).

The genus *Pseudomonas*, firstly described by Migula in1894 is characterized as straight or slightly bent Gram-negative rods with one or more polar flagellae, not forming spores (Fuchs et al., 2001). Its metabolism is chemoorganotrophic and strictly aerobic with a respiratory type in which oxygen is used (Fuchs et al.,2001). *Pseudomonas* "sensu stricto" group I is the largest of the groups and includes both fluorescent and non fluorescent ones. Several species of rRNA group I pseudomonads have the ability to produce and excrete, under the condition of iron limitation, soluble yellow-green pigments that fluorescent under UV light (Bultreys et al., 2003) named pyoverdines (PVDs) or pseudobactins, which act as siderophores for these bacteria (Meyer, 2000). These molecules are thought to be associated with biocontrol of fungal pathogens in the biosphere (Fuchs et al., 2001). Fluorescent pseudomonads have been considered as an important bioinoculant due to their innate potential to produce plant growth promoting hormones (Latour et al., 2003) and antimicrobial secondary metabolites (Costa et al., 2006; Dong and Zhang, 2005). The characterization of the *Pseudomonas* genus is faced with difficulties based on their genetic heterogeneity. Recently, the development of molecular techniques has yielded innovative alternative tools for demonstrating the mechanisms underlying biocontrol properties (Massart and Jijakli, 2007) and understanding the role of these bacteria in bioremediation, plant spoilage and pathogenicity (Ravi Charan et al., 2011). *Pseudomonas* spp. produce an arsenal of antimicrobials (including hydrogen cyanide (HCN), pyoluteorin, phenazines, pyrrolnitrin, siderophores, cyclic lipopeptides and 2,4-diacetylphloroglucinol (DAPG) (Thomashow and Weller 1996; Weller 2007). They also are able to promote plant growth and induce systemic resistance (ISR) in plants (Raaijmakers et al. 2009; Glick 2014). *Pseudomonas* spp. have been employed efficiently as commercial biocontrol agents (Loper and Lindow 1987; Walsh et al. 2001).

Pseudomonas spp. have been employed efficiently as commercial biocontrol agents (Loper and Lindow 1987; Walsh et.al). Certain *Pseudomonas* species may also produce additional pigments such as quinolobactin (yellow, dark green in presence of iron, a siderophore) a reddish pigment called pyorubrin and pyomelanin (brown pigment).

2. MATERIALS AND METHODS

2.1 General methods

All chemical reagents were purchased from Aldrich, LOBA Chemicals. All glassware was rinsed in distilled water and ethanol prior to use and used without further purification.

2.2 Sample Collection

The fish waste procured from a local farmer from Pazhayar and was transported immediately to the laboratory.

2.3 Bacterial isolation

The collected sample was serially diluted up to 10^{-6} dilution in aseptic conditions. The 10 g of fish waste compost was transferred to 250ml of the conical flask containing 100 ml of blank sterile distilled water and was kept in a shaker for about 24hrs. Then, 1ml of this suspension was made into serial dilution up to 10^{-6} and about 0.1ml of that was placed on to King's B medium to isolate the colonies. The plates were incubated at 280^0C for 48 hrs. The total viable count (TVC) of the colonies were finally noted. The summary of the total colonies of *Pseudomonas* spp is presented in table 1.

2.4 Isolation of *Pseudomonas fluorescens*

Colonies showing yellow pigmentation on King's B medium were picked up. Based on the pigment formation and fluorescence under UV light, the isolates were separated and they were then characterized on the basis of biochemical tests as per the procedures outlined in the Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986). Isolated colonies of fluorescent Pseudomonads were further streaked onto KB agar plates to obtain pure cultures. All the experiments were performed in triplicates.

2.5 Assay for Siderophore Production

Siderophore production was tested by growing *Pseudomonas fluorescens* isolates on the king's B medium at 28°c for 48 hours. The plates were exposed to UV light for few seconds and the colonies exhibiting fluorescence (Ramyasmruthi et al., 2012). The isolation and identification of siderophores of the fluorescent *pseudomonas* isolates are presented in table 2.

2.6 Biochemical characterization of *Pseudomonas fluorescens*

After isolation, the organisms were subjected to biochemical tests for confirming them. Tests like Indole production, MR, VP, citrate, TSI, urease were carried out.

Phenotypic and genus-level characterization of the isolates were carried out by subjecting them to cultural (oxygen requirement), morphological (colony morphology), microscopic (Gram staining) and biochemical tests (utilization of different carbon sources and enzyme activity) following standard procedures as per Holt et al. (1994). All media were obtained from Hi-Media. Biochemical tests for identification of bacteria were given in table-3.

2.7 Anti-Microbial Activity on Fungal pathogen

Fluorescent *Pseudomonas* isolates were multiplied on King's B broth and incubated for 2 days at 28 ºC till the fluorescent pigment appeared in the broth. Petri-plates containing pre-sterilized potato dextrose agar (PDA) medium were inoculated with plant pathogenic fungi, *Rhizoctonia solani and Aspergillus* (in the centre) and incubated at 252ºC for 3 days till the fungus completely covered the entire plate. Bipartite interactions were performed following a simple confrontation assay which was developed during the course of the investigation. To identify prospective bio-agent, the edge of a glass funnel was deployed for bio-agent inoculum deposition surrounding pre-inoculated fungal pathogen. It was then sterilized by dipping in alcohol followed by flaming. Broth containing young growing cells (3-day-old) of fluorescent *Pseudomonas* was dispensed in a sterile petri dish and picked from the edge of the funnel by dipping. Care was taken to remove the excess inoculum by gently shaking the funnel. Inoculation was done by gently touching the edge of the funnel (containing fluorescent *Pseudomonas*) which encircled the pre-inoculated plant pathogenic fungi on agar plug equidistantly. Inhibition zone was measured after 72 h of incubation at 28ºC. Percent inhibition of pathogens by *Pseudomonas* isolates over control was calculated using the formula (Vincent 1947):

$$Inhibition = \frac{Radial\ growth\ in\ control - Radial\ growth\ in\ treatment}{Radial\ growth\ in\ control} \times 100\ mm\ \%$$

3. RESULTS

A total of 12 isolates of fluorescent *pseudomonads* were collected from a local farmer from Pazhayar. All the isolates were gram-negative, rod shaped, while nine of the isolates produced pigment in King's B medium and showed fluorescence under UV light.

Table 1: Total viable counts of bacterial colonies at different dilutions

S. no	Dilution	CFU/g
1	10^{-1}	55×10^{-1}
2	10^{-2}	25×10^{-2}
3	10^{-3}	13×10^{-3}
4	10^{-4}	11×10^{-4}
5	10^{-5}	5×10^{-5}

The identity of the fluorescent *Pseudomonas* isolates which produce siderophores producer under UV light is presented in table 2.

Table 2: Characterization and identification of fluorescent *Pseudomonas* isolates

S. No	Isolates Designation	Fluorescent/Non-Flurescent	Siderophore production	Identification
1	PS-1	Fluorescent	+ve	*Pseudomonas fluorescens*
2	PS-2	Non-Fluorescent	-ve	*Pseudomonas spp.*
3	PS-3	Fluorescent	+ve	*Pseudomonasfluorescens*
4	PS-4	Non-Fluorescent	-ve	*Pseudomonas spp.*
5	PS-5	Fluorescent	+ve	*Pseudomonasfluorescens*
6	PS-6	Fluorescent	+ve	*Pseudomonasfluorescens*
7	PS-7	Fluorescent	+ve	*Pseudomonasfluorescens*
8	PS-8	Fluorescent	+ve	*Pseudomonasfluorescens*
9	PS-9	Non-Fluorescent	-ve	*Pseudomonas spp.*
10	PS-10	Fluorescent	+ve	*Pseudomonasfluorescens*
11	PS-11	Fluorescent	+ve	*Pseudomonasfluorescens*
12	PS12	Fluorescent	+ve	*Pseudomonasfluorescens*

Table 3: Morphological, physiological and biochemical characteristics of identified bacterial strains.

S. no	Isolates	Morphological Characteristics			Biochemical-test					
		Gram Staining	Motility	Colony Morphology	Indole	MR	VP	Citrate	TSI	Urease
1	PS1	G (-)Rod	Motile	Small, round, greenish colonies.	-	-	-	+	A/A H2S	-
2	PS3	G (-)Rod	Motile	Small, round, greenish colonies.	-	-	-	+	A/A H2S	-
3	PS5	G (-)Rod	Motile	Small, round, greenish colonies.	-	-	-	+	A/A H2S	-
4	PS6	G (-)Rod	Motile	Small, round, greenish colonies.	-	-	-	+	A/A H2S	-
5	PS7	G (-)Rod	Motile	Small, round, greenish colonies	-	-	-	+	A/A H2S	-
6	PS8	G (-)Rod	Motile	Small, round, greenish colonies	-	-	-	+	A/A H2S	-
7	PS10	G (-)Rod	Motile	Small, round, greenish colonies	-	-	-	+	A/A H2S	-
8	PS11	G (-)Rod	Motile	Small, round, greenish colonies	-	-	-	+	A/A H2S	-
9	PS12	G (-)Rod	Motile	Small, round, greenish colonies	-	-	-	+	A/A H2S	-

Efficacy of two (PS-2 and PS-7) potential isolates of *Pseudomonas fluorescens* were evaluated against a range of predominant pathogens such as *Fusarium, Rhizoctonia solani, Macrophomina and Aspergillus* through dual culture technique. The results of the dual plate are presented in table 3. Among them, PS7 (21.73%) produced the maximum amount of antibiotic property of siderophore, which is followed by (Anand et al., 2010). So PS7 has shown maximum percent inhibition of mycelial growth of all pathogens compared to PS2 (12.12 %).

Table 3: Inhibition of mycelial growth of spectrum of plant pathogens by *Pseudomonas fluorescens* under dual culture

S. No	Pathogens	% of Mycelial growth inhibition over control	
		PS2	PS7
1	***Fusarium***	17.85 %	21.73 %
2	***Rhizoctonia solani***	12.12 %	17.39 %
3	***Macrophomina***	18.91 %	21.42 %
4	***Aspergillus***	14.89 %	15.38 %
Mean		63.77	75.92
Max.		18.91	21.73
Min.		12.12	15.38
C.V		2.985	3.745

CV- coefficient of variance

Assay for siderophore Production

Production of Siderophore was detected by all the isolates of *Pseudomonas fluorescens* and colonies were exhibiting yellowish green pigment production on King's B Agar plates as shown in figure 1 and figure 2, without using UV light, *Pseudomonas fluorescens* isolates developed white colonies on streaked King's B Agar as indicated in figure 3.

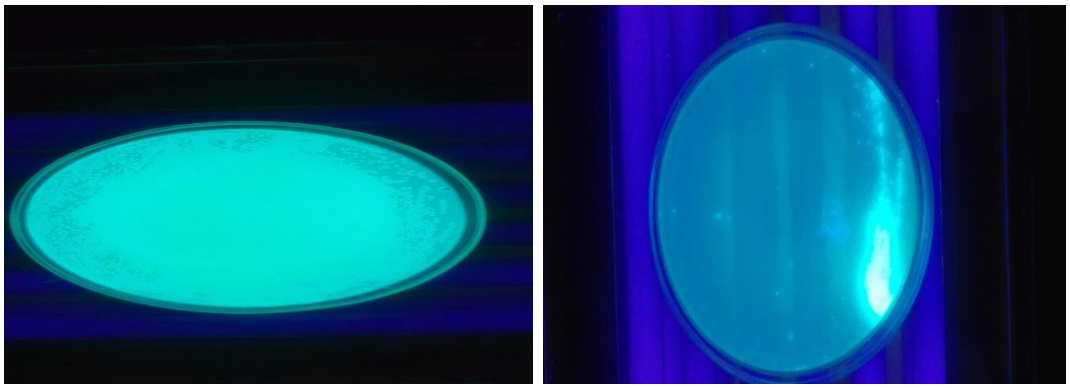

Fig. 1: Siderophore production by *Pseudomonas fluorescens* isolates on King's B Agar plates when observed Under UV light

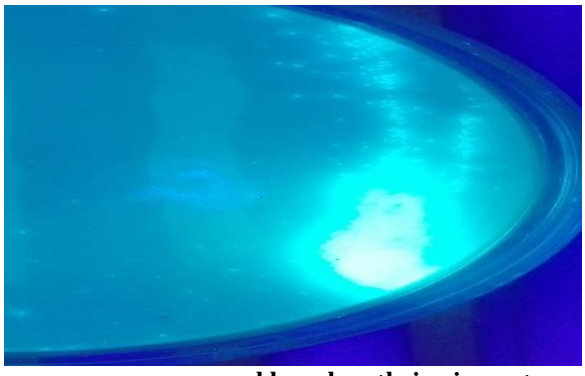

Fig. 2: Pseudomonas fluorescens was screened based on their pigment production under UV light

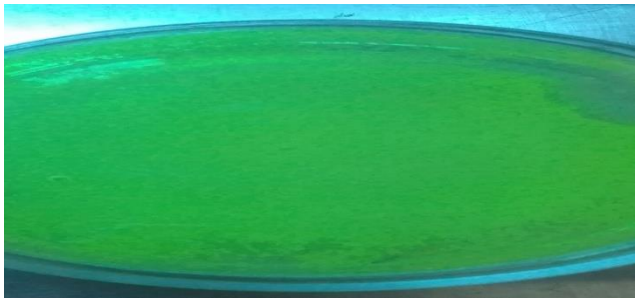

Fig. 3: Siderophore production by *Pseudomonas fluorescens* isolates on King's B Agar plates without using UV light

In this study, a qualitative estimation of siderophores produced by *Pseudomonas fluorescens* isolates was made which showed that they were powerful producers of siderophore under limited iron on King's B medium. The production of siderophores by *Pseudomonas fluorescens* isolates indicated that they can be used as biocontrol agents against soil-borne phytopathogens.

4. DISCUSSION

Siderophore production by strains of *Pseudomonas* spp., for biocontrol, is of great interest because of its possibilities in the substitution of chemical pesticides. Similarly, microbial cyanogenesis has been demonstrated in a few bacterial species (belonging to the genera *Pseudomonas, Chromobacterium, Rhizobium* and several cyanobacteria (Blumer and Haas 2000). In the present study, we have compared the ability of several fluorescent Pseudomonads to produce siderophores, cyanogenesis, and antagonism in plate assay. Our study revealed that the isolates vary in their mechanism and ability to inhibit pathogens. During the study, a simple confrontation assay technique was developed which was advantageous as compared to earlier reported techniques (Dennis and Webster 1971; Fokkema 1978; Santoyo et al. 2010), wherein, bipartite interactions were performed on media plates by streaking bacterial bio-agents (forming quadrant) and placing mycelial plug of …mm in the centre. Our combined in vitro and dual plates techniques of statistical data showed the potential of isolates PS2, PS7 was potential and they can be used as a commercial bioagent for the control of Fungal Pathogens.

The proposed technique has the following advantages: (1) uniform inoculum deposition during all combinations of bipartite interactions. (2) Replica-plating can be done of the inoculum picked on the edge of the funnel. (3) Ability to evaluate the antagonistic potential of a sporulating bio-inoculant (e.g. *Trichoderma* spp, etc...). The only disadvantage with this technique is that, for each bio-inoculant in the broth, a separate plate is required to dispense the inoculums.

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