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 natural mixes with low atomic masses that are created by microorganisms. Under the iron-limited condition, numerous microscopic organisms create press chealating siderophores. Siderophore chealate iron and supply to the bacterial cell by external layer receptors. An incredible variety is found in the siderophore structure created by numerous microscopic organisms. Bacterial strains additionally create fluorescence as the one like *Pseudomonas fluorescence*. They are pervasive in fertilizer soil condition. They have gotten much consideration as of late on account of their potential jobs and application in different zones of ecological research. Their criticalness is a result of their capacity to eliminate bacterial and contagious pathogens. They go about as an anti-microbial and they have an extensive variety of substance structures and particular properties. Despite the fact that siderophores have been accounted for from an assortment of living beings occupying various situations. The investigation of marine siderophores is in its earliest stages when contrasted with their earthbound partners. Subsequently, the present examination was completed to identify and portray siderophores of interesting *Pseudomonas spp*. secluded from dregs gathered from the marine condition. Fish squander is one of a noteworthy wellspring of marine microorganisms like *Pseudomonas*, *Vibrio* and *Streptococcus Sp*. The secluded strains were affirmed by biochemical portrayal. The biosynthesis of a yellow-green, fluorescent, water-solvent color by *P.fluorescens* happened just when the microscopic organisms were press lacking and was not straightforwardly impacted by the idea of the natural carbon source. The color shaped an exceptionally steady Fe3+ complex and was purged in this frame. *P.fluorescens* delivered just a single atomic sort of fluorescent shade; be that as it may, its lability under mellow basic conditions prompted the arrangement of a few pigmented disintegration items. The two its biosynthesis and its substance properties (arrangement of a stable Fe3+ complex) recommend that the fluorescent shade is a desferrisiderophore. The strain was developed in King`s B fluid for fluorescent color generation and from that point forward, it was removed with CH3)2CO. Siderophore and their subordinates have an expansive application in agribusiness as it expands soil ripeness and is a biocontrol specialist for the contagious and bacterial pathogen. The present examination manages the disengagement of the fluorescent separates of *P.fluorescens* having an assortment of promising properties which improve them organisms. Twelve *P.fluorescens* disengages were

segregated on King's B and Pseudomonas separation agar medium and their creation of fluorescence under an UV transilluminator were surveyed. The point of the present examination is to diagram and talk about the vital jobs and type of siderophores in fish squander compost soil and to stress their critical jobs that these little natural atoms could assume an essential job in biocontrol forms. A straightforward encounter test for recognizing potential enemies was created. Fluorescent *Pseudomonas* segregates PS6, PS7, PS8, and PS10 were observed to be opposing against both Bacterial and Fungal Pathogens.

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

1. INTRODUCTION:

Iron (Fe) is a fundamental component for the development of every living microorganism, since, it goes about as an impetus in enzymatic procedures, oxygen digestion, electron exchange and DNA and RNA combinations (Aguado-Santacruz Received 18 December 2013). It is a basic necessity for the development and multiplication of microorganisms, and the productivity of its procurement in microscopic organisms is for the most part accepted to be connected to their pathogenicity (Grffths 1999).

Siderophores are metabolites delivered by microorganisms. These mixes tie ferric iron, advance the rate of Fe3+ transport, and subsequently ease the issue of iron inaccessibility. Press is the fourth most normal component in the world's outside layer. It is available in the dirt, with uncommon special cases, as oxide hydrates, which have little separation constants. Complexation by peptides can likewise make press inaccessible (Budzikiewicz, 1997). These actualities clarify why press isn't promptly accessible to microorganisms. Marine living beings, for example, phytoplankton (Trick et al., 1983) and cyanobacteria (Armstrong and Van Baalen, 1979) can likewise create siderophores. The job of siderophores is principally to rummage Fe, however they likewise shape buildings with other basic components (i.e. Mo, Mn, Co, and Ni) in the earth and make them accessible for microbial cells (Bellenger et al., 2008; Braud et al., 2009).

The biosynthesis of siderophores is one of the regular techniques that numerous organisms utilize to acquire press from their condition (Neilands 1995; Byers and Arceneaux 1998). These high-proclivity press chelates are biosynthesized by the cell and discharged to solubilize and sequester Fe (III) and are then perceived by the host as an iron-siderophore conjugate by particular receptors on the cell surface (FaraldoGomez and Sansom 2003).

The variety *Pseudomonas*, initially depicted by Migula in1894 is described as straight or somewhat twisted Gram-negative bars with at least one polar flagellae, not shaping spores (Fuchs et al., 2001). Its digestion is chemoorganotrophic and entirely vigorous with a respiratory kind in which oxygen is utilized (Fuchs et al.,2001). *Pseudomonas* "sensu stricto" group I is the biggest of the groups and incorporates both fluorescent and non-fluorescent ones. A few types of rRNA group I *pseudomonads* can deliver and discharge, under the state

of iron impediment, solvent yellow-green colors that fluorescent under UV light (Bultreys et al., 2003) named pyoverdines (PVDs) or pseudobactins, which go about as siderophores for these microscopic organisms (Meyer, 2000). These atoms are believed to be related with bio control of contagious pathogens in the biosphere (Fuchs et al., 2001). Fluorescent pseudomonads have been considered as an essential bio inoculant because of their natural potential to deliver plant development advancing hormones (Latour et al., 2003) and antimicrobial optional metabolites (Costa et al., 2006; Dong and Zhang, 2005). The portrayal of the Pseudomonas variety is looked with troubles dependent on their hereditary heterogeneity. As of late, the advancement of atomic procedures has yielded inventive elective devices for showing the components hidden bio control properties (Massart and Jijakli, 2007) and understanding the job of these microscopic organisms in bioremediation, plant decay and pathogenicity (Ravi Charan et al., 2011). *Pseudomonas spp.* create a weapons store of antimicrobials (counting hydrogen cyanide (HCN), pyoluteorin, phenazines, pyrrolnitrin, siderophores, cyclic lipopeptides and 2,4-diacetylphloroglucinol (DAPG) (Thomashow and Weller 1996; Weller 2007). They likewise can advance plant development and incite fundamental obstruction (ISR) in plants (Raaijmakers et al. 2009; Glick 2014). *Pseudomonas spp*. have been utilized proficiently as business biocontrol specialists (Loper and Lindow 1987; Walsh et al. 2001).

Pseudomonas spp. have been utilized effectively as business biocontrol operators (Loper and Lindow 1987; Walsh et.al). Certain Pseudomonas species may likewise deliver extra colors, for example, quinolobactin (yellow, dim green in nearness of iron, a siderophore) a rosy shade called pyorubrin and pyomelanin (dark colored shade).

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 2800C 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 fluorescents*

States demonstrating yellow pigmentation on King's B medium were gotten. In view of the shade arrangement and fluorescence under UV light, the confines were isolated and they were

then described based on biochemical tests according to the methodology sketched out in the Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986). Segregated provinces of fluorescent *Pseudomonads* were additionally streaked onto KB agar plates to get unadulterated societies. Every one of the trials were performed in triplicates.

2.5 Assay for Siderophore Production

Siderophore generation was tried by developing *Pseudomonas fluorescens* secludes on the lord's B medium at 28oc for 48 hours. The plates were presented to UV light for few moments and the states showing fluorescence (Ramyasmruthi et al., 2012). The separation and distinguishing proof of siderophores of the fluorescent *pseudomonas* disconnects are introduced in table 2.

2.6. Biochemical characterization of *Pseudomonas fluorescens*

After segregation, the life forms were subjected to biochemical tests for affirming them. Tests like Indole generation, MR, VP, citrate, TSI, urease were completed.

Phenotypic and class level portrayal of the secludes were completed by subjecting them to social (oxygen necessity), morphological (settlement morphology), minute (Gram recoloring) and biochemical tests (usage of various carbon sources and catalyst movement) following standard techniques according to Holt et al. (1994). All media were gotten from Hi-Media. Biochemical tests for recognizable proof of microbes were given in table-3.

2.7 Anti-Microbial Activity on Fungal pathogen

Fluorescent Pseudomonas disconnects were increased on King's B soup and hatched for 2 days at 28 ^{o}C till the fluorescent color showed up in the stock. Petri-plates containing pre-disinfected potato dextrose agar (PDA) medium were vaccinated with plant pathogenic growths, *Rhizoctonia solani* and *Aspergillus* (in the inside) and hatched at 252oC for 3 days till the organism totally secured the whole plate. Bipartite associations were performed following a straightforward showdown test which was created over the span of the examination. To distinguish imminent bio-operator, the edge of a glass pipe was sent for bio-specialist inoculum testimony encompassing pre-vaccinated contagious pathogen. It was then cleaned by dunking in liquor pursued by flaring. Juices containing youthful developing cells (3-day-old) of fluorescent *Pseudomonas* was apportioned in a sterile petri dish and picked from the edge of the channel by plunging. Care was taken to expel the abundance inoculum by delicately shaking the pipe. Vaccination was finished by delicately contacting the edge of the channel (containing fluorescent *Pseudomonas*) which encompassed the pre-immunized plant pathogenic organisms on agar plug equidistantly. Restraint zone was estimated after 72 h of hatching at 28oC. 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):

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

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	-

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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 75.92		63.77	
Max. 21.73		18.91	
Min. 15.38		12.12	
C.V 3.745		2.985	

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.

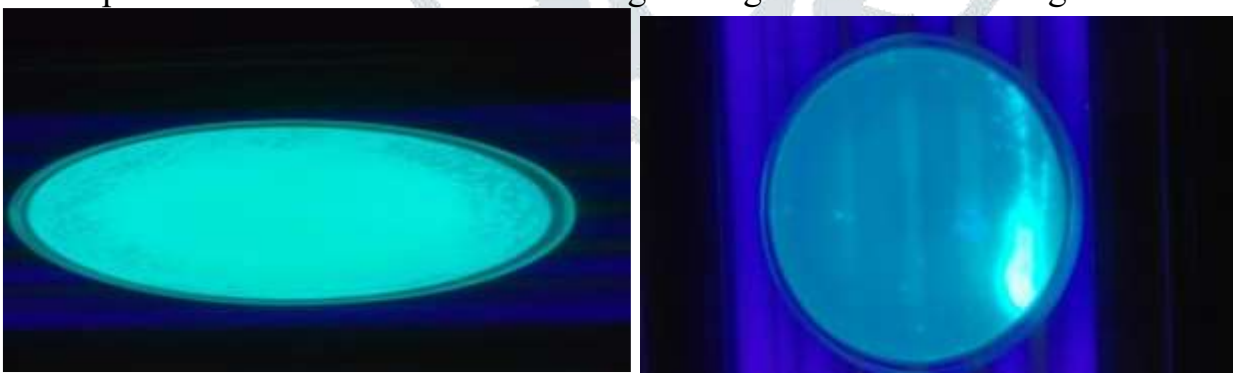

Fig. 1: Siderophore production by *Pseudomonas fluorescens* isolates on King's B Agar plates when observed Under UV light

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 creation by strains of *Pseudomonas spp*., for bio control, is of extraordinary intrigue in light of its potential outcomes in the substitution of synthetic pesticides. Also, microbial cyanogenesis has been exhibited in a couple of bacterial animal types (having a place with the genera Pseudomonas, Chromo bacterium, Rhizobium and a few cyanobacteria (Blumer and Haas 2000). In the present investigation, we have thought about the capacity of a few fluorescent Pseudomonads to create siderophores, cyanogenesis, and hostility in plate examine. Our examination uncovered that the confines fluctuate in their component and capacity to restrain pathogens. Amid the examination, a straightforward showdown measure procedure was produced which was profitable when contrasted with before revealed systems (Dennis and Webster 1971; Fokkema 1978; Santoyo et al. 2010), wherein, bipartite cooperation were performed on media plates by streaking bacterial bio-specialists (framing quadrant) and putting mycelia attachment of … mm in the inside. Our consolidated in vitro and double plates methods of measurable information demonstrated the capability of detaches PS2, PS7 was potential and they can be utilized as a business bio agent for the control of Fungal Pathogens.

The proposed strategy has the accompanying preferences: (1) uniform inoculum testimony amid all mixes of bipartite collaborations. (2) Replica-plating should be possible of the inoculum singled out the edge of the pipe. (3) Ability to assess the opposing capability of a sporulating bio-inoculant (e.g. *Trichoderma spp*, etc...). The main impediment with this system is that, for each bio-inoculant in the stock, a different plate is required to apportion the inoculums.

5. ACKNOWLEDGMENT

The authors thank the SERB New Delhi for financially supporting the present research work. They also thank the Annamalai University for providing the facilities required to do the work successfully.

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