Growth promoting and antagonistic activity of pyoverdine producing Pseudomonas isolated from marine source of Alibagh, Maharashtra

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***Abstract*—Fourty two bacterial isolates were obtained from marine water sample using Zobell marine agar. These isolates were further evaluated for the production of pyoverdine in succinate media and estimated both qualitatively and quantitatively using CAS plate and tube assays. 87 % siderophore units were recovered. The promising isolate was identified as *Pseudomonas aeruginosa* by morphological, biochemical tests and 16S rRNA sequencing. The pyoverdine producing *Pseudomonas* strain was evaluated for growth promoting and antifungal activity. Excellent growth promoting results were obtained in gram seed germination experiments. The isolate was also found to suppress plant deleterious fungal pathogens viz *Pythium* and *Fusarium* causing crown and root rot, pink rot on fruits wilts in vegetables respectively. These in vitro antagonistic actions of marine *Pseudomonas* against phytopathogens and growth promoting results from seed germination experiment suggest that this pyoverdine producing marine *Pseudomonas* can be used simultaneously as growth promoter as well as to serve as a bio-control agent.**

***Index Terms*—Pyoverdine, Marine *Pseudomonas*, CAS, Phytopathogens**

I. INTRODUCTION

Marine organisms are of great scientific interest because they often possess unique structures, metabolic pathways, reproductive systems, and sensory and defence mechanisms. The marine environment, with its physicochemical properties, is a unique habitat for a variety of living forms. Microbes, which adopt this diverse environment and produce a variety of metabolites, play a significant role in fields like Agriculture, pharmaceuticals, etc (Armstrong et al., 2001). Currently, microbes from terrestrial sources are employed as bio inoculants and bio-control agents for agricultural use, but the potential for synthesis of several novel secondary metabolites by marine microorganisms has been recognized (Jaytilake, et al., 1996).

Microbial secondary metabolites represent a rich source for drug discovery, plant protective agents, and biotechnologically relevant compounds. Among them are siderophores, iron-chelating molecules that show a great influence on bacterial community assembly and the potential to control pathogen invasions. One of such a siderophore is pyoverdine that is produced by fluorescent *Pseudomonas* members and consists of different peptide chains specific to each bacterial species.

Plant diseases are caused by variety of pathogens from bacteria, viruses and fungi. Control of many plant diseases has remained a challenge to mankind. Conventional strategies of disease control were replaced with the use of chemical pesticides. However, these pesticides affected soil fertility and the ecosystem (Bayer1986). To overcome this problem, bio-control agents have been well exploited (Kloepper et al., 1980; Chincholkar, et al., 2000).

Fluorescent *Pseudomonads* are known to produce a variety of secondary metabolites that have a dual effect toward plants: plant growth promotion and suppression of phytopathogens (Johri, 1997). Fluorescent *Pseudomonads* synthesize the peptide-derived green-yellow diffusible florescent molecule pyoverdine as their primary siderophore, together with secondary siderophores that have lower affinity for iron (Meyer and Abdallah, 1978; Cornelis et al., 2002.)

The present study attempted to identify and characterize a marine *Pseudomonas*, to optimize its pyoverdine production, its purification, in vitro antagonistic action against fungal plant pathogens and growth promoting abilities.

II. MATERIAL AND METHODS

Isolation of marine *Pseudomonas*:

Marine water sample was collected in sterile bottles from Alibagh beach, located in District Raigad ($18^0$38' 29'' N $72^0$52'' 20' E; temperature 29.7 ºC), Maharashtra (south India), and brought to the lab within 24 hours. Marine bacterial isolates were obtained using the standard tenfold dilution method, and by cultivating microbes on sterile Zobell marine agar (peptone 5 g, sodium chloride 19.45 g, yeast extract 1, magnesium chloride 8.8 g, ferric citrate 0.1 g, sodium sulphate 3.24 g, calcium chloride 1.8 g, potassium chloride 0.55g,sodium bicarbonate 0.16 g, potassium bromide 0.08 g, strontium chloride 0.16 g, disodium phosphate 0.008 g, boric acid 0.022 g, sodium silicate 0.004 g, sodium fluorate 0.0016 g, distilled water 1000ml, agar 15g.,pH 7.6) plates after incubation at 28 °C for 72 hrs. After incubation, isolated colonies were purified by sub culturing and maintained on slants at 4 ºC. Isolated colonies were then screened for *Pseudomonas* using Cetrimide agar.

Screening of marine *Pseudomonas* for pyoverdine production:

Marine *Pseudomonas* isolates were screened for the production of Pyoverdine by inoculating 1% inoculum in flasks containing sterilized iron-deficient succinate medium (succinate 4.0 g, KH_2PO_4, 3.0 g, K_2HPO_4 100 mg, $(NH_4)_2SO_4$ 1.0g, $MgS0_4.7H_2O$-20 mg, pH 7; Meyer *et al.*,1978) Growth medium was extracted with 1.5% 8 hydroxyquinoline and chloroform to remove trace of irons (Tailor and Joshi, 2012). Flasks were incubated at 28$\pm2^O$C for 48 h till the visible yellow green fluorescent diffusible pigment appeared. After every 12 h of incubation, the culture was centrifuged at 10,000g for 15 min at 4°C, and the cell-free supernatant was subjected to qualitative and quantitative determination/confirmation of siderophores**.**

Qualitative analysis of Pyoverdine:

The absorption maxima of cell-free supernatant at 404 nm and instant appearance of golden yellow colour after reacting with Chrome Azurol S (CAS) reagent confirmed production of the pyoverdine type of siderophore. Pyoverdine production was also confirmed by development of yellow - orange zones around the growth on Chrome azurol sulphonate agar medium plate (Schwyn and Neilands, 1987).

Quantitative analysis of Pyoverdine:

Pyoverdine production was quantitatively analysed by CAS solution tube assay (Schwyn and Neilands, 1987). Isolated *Pseudomonas aeruginosa* strain was grown in minimal medium, under non-saline and saline conditions (100mM and 200mM salt concentrations) for 24 h at 30 °C with constant shaking at 120 rpm. Following the incubation, cells were pelleted at 10,000 g for 15 min to separate cell-free supernatant. 0.5ml culture supernatant and 0.5ml CAS reagent were mixed along with 10 µL of shuttle solution (4mM 5-sulfosalicylic acid) and incubated for 5 minutes at room temperature. Reduction of the blue colour of the solution was observed in 6 h, and measured at 630 nm absorbance. The minimal medium was used as a blank, and the minimal medium plus CAS assay solution plus shuttle was used as a reference (r). Siderophore units are defined as

Siderophore units (%) ¼ [(Ar - As) /Ar] _ l00

Extraction and Purification of Siderophores:

Siderophores were extracted from culture grown in iron-free succinate medium according to Stintzi and Meyer (Stintzi and Meyer, 1995). Siderophore containing supernatant was purified by subjecting the acidified (pH 6.0) culture-filtrate through an XAD-2 column (Aldrich) and eluting with 50% methanol. The brown fraction was concentrated and dried under vacuum on a rotary evaporator and checked for CAS.

Detection of pyoverdine production by HPLC:

The liquid fractions from 3 days grown culture was transferred to individual tube, and 40 µl of an FeCl3 solution (1 M) was added to tube. Culture was shaken for 20 min, and the bacteria were removed by centrifugation (22 min, 10,000 g) and filtration through a 0.2 µm pore size membrane filter. The HPLC analyses were carried out by using the filtered culture media adjusted to pH 5.0 to 5.3. Pyoverdine production was estimated by measuring the absorbance at 403 nm in order to determine the injection volume for each strain and the retention times (RT) of peaks with comparable heights were recorded.

Growth promoting activity of *Pseudomonas* strain:
Growth promoting activity of *Pseudomonas* strain was checked by seed germination studies.20 healthy Gram seeds from same batch were surface sterilized by 0.1 % $HgCl_2$ followed by ethanol and then washing with sterile distilled water. These seeds were then soaked in succinate broth for 24 h and then placed in a glass dish on moistened tissue paper and incubated till the germination and appearance of rootlets and leaflets. Simultaneously gram seeds were also placed in a tube containing soil extract agar as nutrient medium and incubated till the development of rootlets and leaflets. Control using sterile saline was also run simultaneously. Observations were continued intermittently and progress in the growth of rootlets and leaflets were recorded.

Antagonistic ability of pyoverdine producing *Pseudomonas*: -
Isolated and purified pyoverdine producing *Pseudomonas* was checked for the antifungal property on potato dextrose agar (potato infusion 200 g, dextrose 20 g and agar 15 g/L) against two common fungal plant pathogens such as *Pythium* and *Fusarium* which causes the diseases in fruits and vegetable, fruits and also causes seed rot, wilts disease in plant. Pure *Pseudomonas* culture was spot inoculated on PDA and then spread covered by test fungal cultures separately. Plates were incubated at 28°C ± 2°C for 2 to 4 days. After incubation the plates were observed for inhibition of fungal growth.

II. RESULTS AND DISCUSSION

Isolation of marine *Pseudomonas*:
Fourty two bacterial isolates were obtained from the sea water samples after inoculation on Zobell marine agar and incubation at 28 °C for 72 hrs. (photo1). As results of screening of these isolates using cetrimide agar only eighteen isolates were reported as *Pseudomonas* based on morphological and cultural characteristics as per Bergey's manual.
Previously Raut et al., (2008) obtained siderophore producing organisms from screening of rhizospheric soils of Ground nut and Soybean.

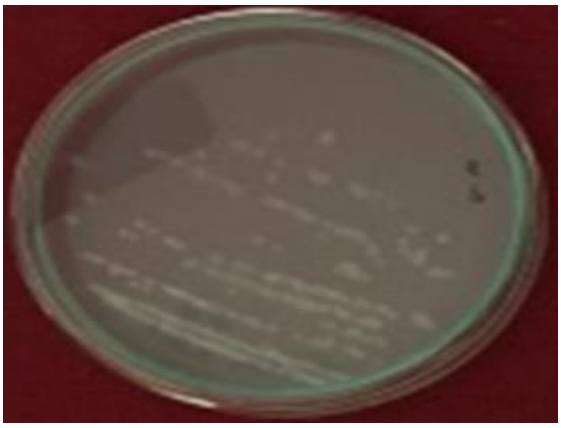

Photo1: Isolated marine bacteria on Zobell agar

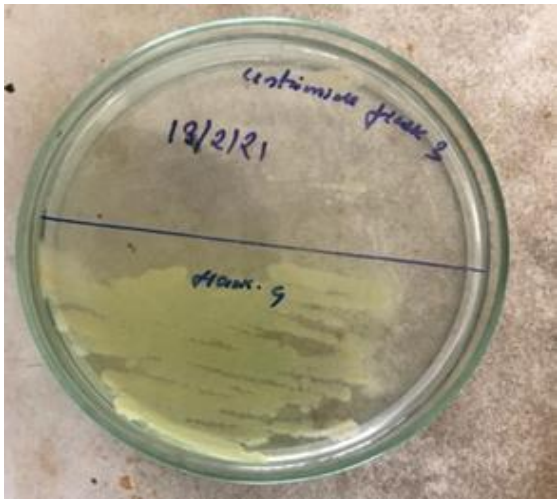

Photo 2: *Pseudomonas* on Cetrimide agar

Out of these 18 isolates, only four isolates were obtained after confirmation of appearance of dark fluorescent coloured growth on Cetrimide agar by ultra violet exposure (photo 2). Most intensively fluorescent selected single isolates showed pyoverdine production in liquid succinate medium (photo 3).
Previously many researchers obtained siderophore production from *Pseudomonas* using succinate media. Among them were Sujatha and Stella (2009) who obtained siderophore from *Pseudomonas* and *Azosprillum*, Syed and Vidhale (2011) obtained maximum siderophore on succinate medium at pH 7.0 and Sujatha and Ammani (2013) obtained siderophore from fluorescent *Pseudomonads.*

Photo 3: Pyoverdine production using Succinate media

Identification of promising isolate:
Morphological, biochemical and molecular tests were considered for identification of isolated *Pseudomonas* strain. Isolated *Pseudomonas* strain was reported as gram negative rod shaped motile, non-capsulated, non-sporing, with citrate, catalase and oxidase tests positive while urease, methyl red and Voges proskauer test negative. Along with these tests the promising isolate was identified as *Pseudomonas aeruginosa* on the basis of 99.95 % similarity by 16 r RNA gene sequencing.

Qualitative and Quantitative analysis of pyoverdine:
This promising isolate was then used for pyoverdine production. The formation of the yellow orange zone on CAS agar gives clear evidence of siderophore production (photo 4) and fluorescent coloured cell free supernatant under U.V illumination supported the presence of pyoverdine. (Photo5). By quantitative tube analysis (photo 6) 87 % units of siderophore were recovered.

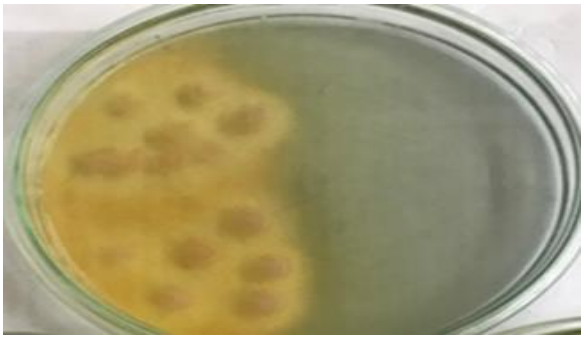

Photo 4: CAS assay: Appearance of Orange coloured zone indicate siderophore production.

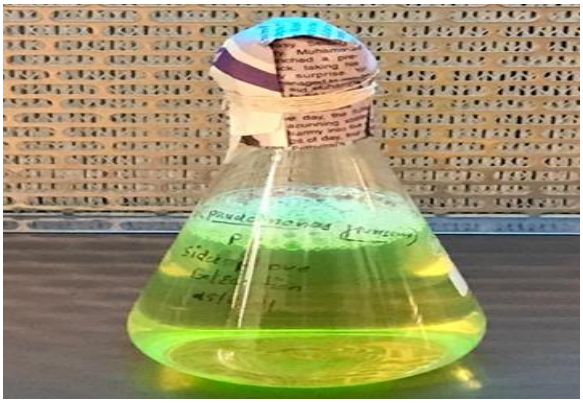

Photo 5: Fluorescent coloured Cell-free supernatant under U.V indicating pyoverdine

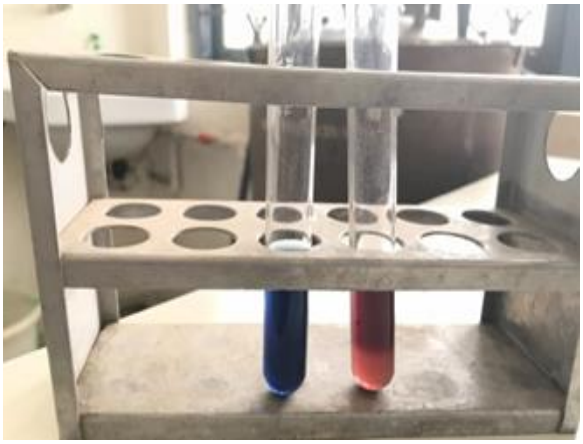

Photo 6: Pyoverdine detection by tube assay.

Similar results were obtained by Ghosh et al., (2015) while studying fungal species *Trichoderma*, *Bacillus species*, and *Pseudomonas aeruginosa* for siderophore production.

Pyoverdine produced by *Pseudomonas aeruginosa* strain was extracted by XAD 2 Column extraction method (Photo 7) and cell free supernatant from succinate broth was analyzed spectrophotometric for CAS assay test and appearance of fluorescent color under U.V light indicated presence of pyoverdine Spectrophotometric analysis of the siderophore sample showed a sharp peak at 404 nm (Fig 1) which is characteristic of the pyoverdine type of siderophore (Meyer, et al., 1978). Srivastava et al., (2022) also used XAD 2 column chromatography.

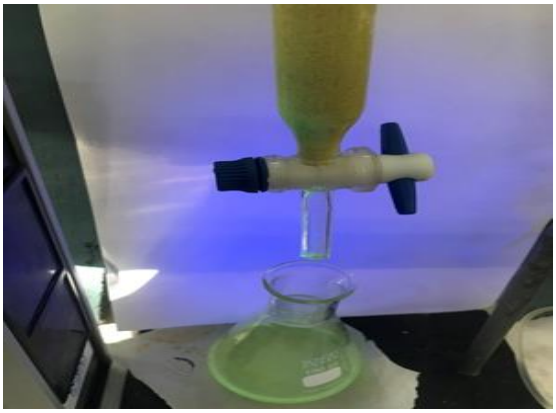

Photo 7: XED 2 Column extraction of Pyoverdine

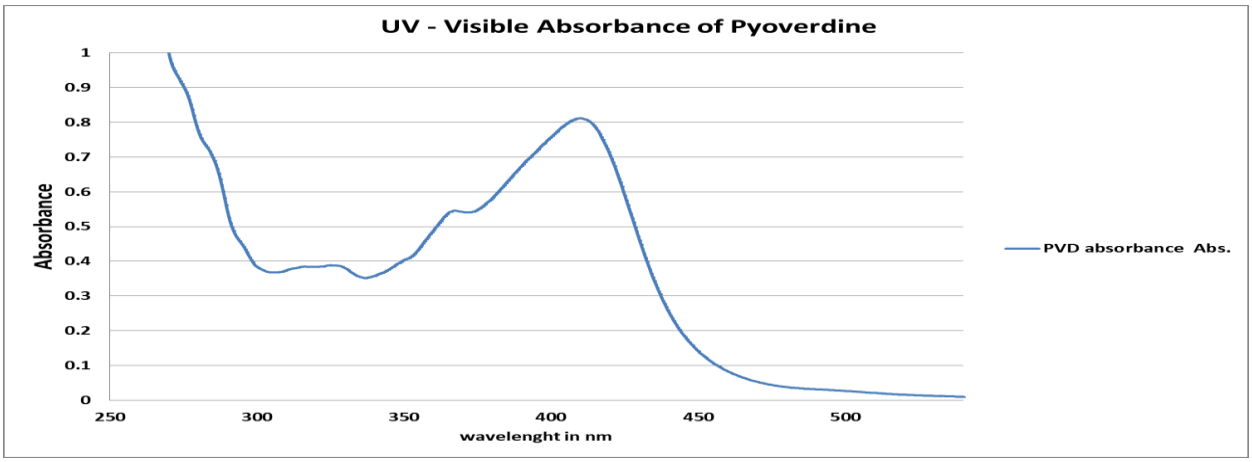

Fig 1: Spectrophotometric analysis showing peak at 404nm

Detection of pyoverdine production by HPLC:

The visual and spectrophotometric analyses of the liquid culture media were generally informative enough to draw conclusions about the types of pyoverdine produced. As the pH of the HPLC buffer used was pH 5.3, the spectral characteristics of the molecules obtained in line allowed the types of pyoverdine produced to be determined. The RT data allowed discrimination between pyoverdine with different peptide chains produced by different species. In the present analysis the graph shows the results of a HPLC analysis (detection at 404 nm) with spectral characteristics, analysed in line. Present strain of *Pseudomonas* was found to produce a typical pyoverdine with an RT of 6.104 min. (Fig 2)

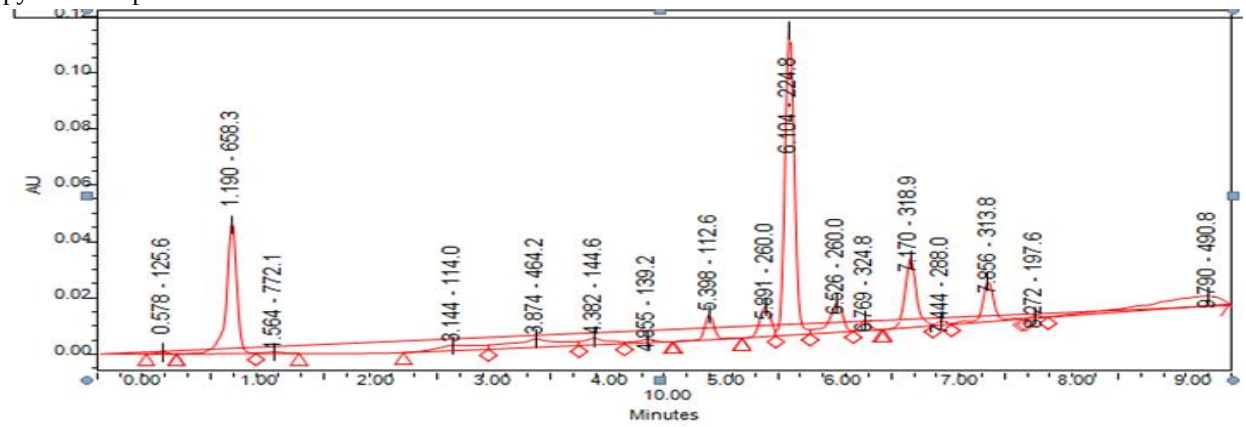

Fig 2: HPLC analysis showing RT of pyoverdine.

Karoline Rehm et al., (2022) used Ultra-high-performance liquid chromatography high-resolution tandem mass spectrometry (UHPLC-HR-MS/MS) for separation of highly polar pyoverdines and their derivatives produced by fluorescent *Pseudomonas.*

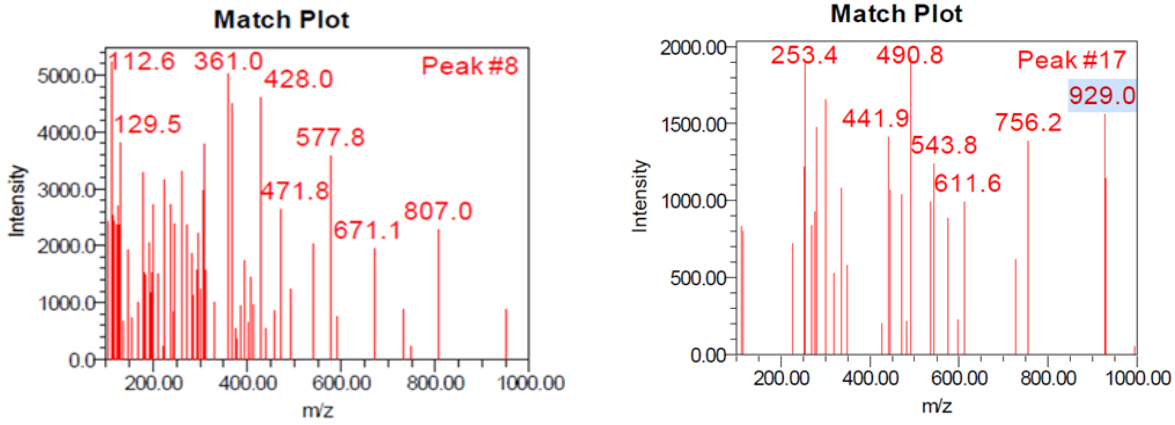

Fig. 3 & 4: Theoretical and measured exact monoisotopic masses and compositions

Match plot Fig 3 & 4 shows the comparisons of the theoretical and measured exact masses achieved with high mass accuracy detection of the ions, within 4.4 ppm or lower which revealed the presence of pyoverdine as per the theoretical and exact mass of molecule reported as 428.1570 suggesting molecular formula $C_{20}H_{22}N_5O_6$ (peak 8 match plot: Fig 3) and 929.3753 suggesting molecular formula $C_{39}H_{53}N_{12}O_{15}$ (peak 17 match plot: Fig 4)

Hua Wei and Ludmilla Aristilde (2015) also characterized the pyoverdine and determined the formula using same type of theoretical and measured exact mono-isotopic masses and compositions by referring the match plots of various peaks. So, our results are supported by these workers.

Growth promoting activity of *Pseudomonas:*

It is evident from the results of gram seed germination in plate (photo 8) that present pseudomonas strain had played a significant role in growth as revealed from the excellent root length and leaflet formation

Photo 8: Effective seed germination with root length and rootlet formation

Excellent seedling development of Gram in tube experiment (photo 9) also proved the significant role of isolated *Pseudomonas aeruginosa* strain and pointed out its potential as growth promoter to be used in agriculture sector.

Photo 9: Growth promoting activity of *Pseudomonas* on test gram growth in comparison to control

Wani et al., (2007) reported plant growth promoting activity by number of bacteria like *Pseudomonas, Bacillus, Enterobacter* and pointed out that these bacteria participate in many important biological activities such as biological control of plant pathogens. So, our results of growth promoting potential of isolated *Pseudomonas* strain are in accordance to and supported by Wani et al., (2007)

Antagonistic action against phytopathogenic fungi:

The isolated *Pseudomonas aeruginosa* showed antifungal activity against plant deleterious fungi viz *Fusarium* (photo10) and *Pythium* (photo 11)

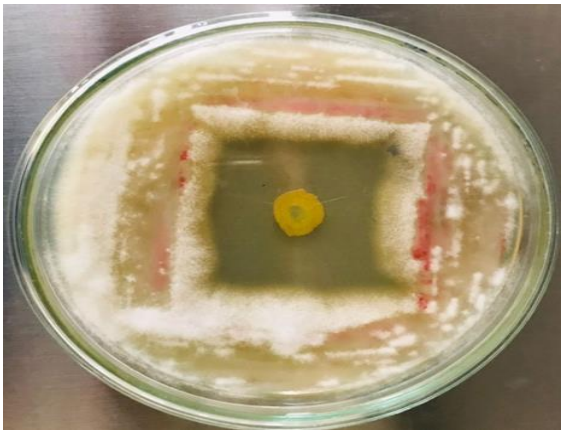

Photo 10: Antifungal activity against *Fusarium* fungus

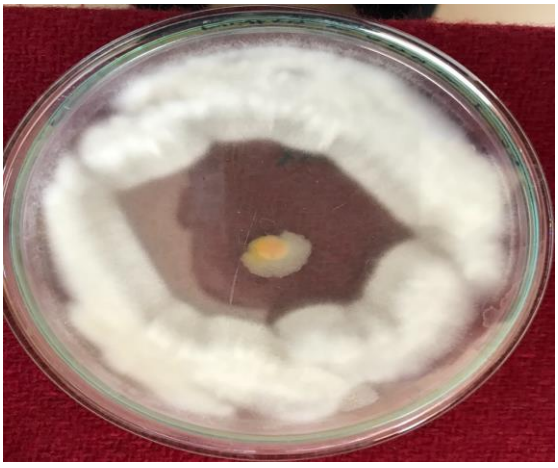

Photo 11: Antifungal activity against *Phythium* fungus

Commercialization of siderophore-producing bacteria as bio-inoculants will thus enable the control of several fungal plant diseases by depriving iron-scavenging pathogens (Basu et al., 2021; Malgioglio et al., 2022) and holds the promise of achieving sustainable agriculture goals.

According to Jagadeesh et al., (2001) fluorescent *Pseudomonads* was documented as bio-control agents against some soil borne plant pathogen requiring iron for their growth and pathogenesis. According to Qessaoui et al., (2022) plant growth-promoting bacteria possessing biofungicide potential are residents of the rhizosphere. Srivastava et. al., (2022) reported antagonistic activity of *P. monteilii* against *F. oxysporum*. In the present investigation, antifungal activity of isolated marine *Pseudomonas* against *Fusarium* and *Pythium* fungal pathogens was reported. Thus, our results of antifungal activity are in accordance with these previous researchers.

III. CONCLUSION

From the observations and results, it was concluded that the pyoverdine producing *P. aeruginosa* of marine origin has specific growth promoting as well as bio-control abilities against phytopathogens viz *Fusarium* and *Pythium* and is eco-friendly hence having much scope in agriculture sector.

IV. ACKNOWLEDGEMENT

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