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Isolation and characterisation of phenazines producing *Pseudomonas* spp.

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ABSTRACT: Bacterial strain was isolated from rhizosphere soil and identified as *Pseudomonas aeruginosa* PI21 and *Pseudomonas putida* PI33 by 16s rRNA gene sequence analysis. The bacterial gene sequences were deposited in the GenBank. The desired isolates were screened for the production of pyocyanin, pyorubin and pyoverdine on pseudomonas isolation agar. The extracted pyocyanin was analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and n-butanol - acetic acid -water (13:4:7) as the mobile phase and where as the pigments pyorubin and pyoverdine was analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and Methanol- Dichloromethane (1:1) as the mobile phase. The study concluded, the maximum yield of pyocyanin and pyorubin by strain PI21, where as the strain PI33 produced maximum yield of pyoverdine at alkaline conditions. Further, different parameters such as pH, temperature, carbon sources, Nitrogen sources, Amino acids, Metal ions and Agro-wastes will be used to improve the production of phenazines by *Pseudomonas aeruginosa* PI21 and *Pseudomonas putida* PI33.

Keywords: *Pseudomonas aeruginosa* PI21, *Pseudomonas putida* PI33, pyocyanin, pyoverdine, pyorubin, optimization.

I. INTRODUCTION

[1]. Bacteria are unicellular organisms which grow very rapidly in the presence of moisture and moderate temperature. Bacteria reproduce by cell division, which can occur every 20 minutes under optimum growing conditions, turning in bacterium into 8 million bacteria in a short time as 8 hours. [2], [3]. Further, sub divisions in the bacteria family are Gram positive (e.g. *Staphylococcus aureus*), Gram negative (e.g. *E. coli*), spore bearing or non spore bearing type. Some specific types of bacteria are pathogenic and cause disease.

[4]. Microbial pigments are of great structural diversity. They may be derivatives of the material classes of carotenoids, phenazine dyes, pyrrole dyes, azaquinones etc. Moreover, the yield of pigment may yet be improved by optimizing the parameters at the various method steps (dwell time, solvent ratios etc.), in particular during cultivation of the bacteria (nutrients, shaking frequency, oxygen charge, pH value, salt content, temperature etc.). Overall speaking, at moderately process control and in relatively short time, results of great economic advantages, especially in respect to large scale technical production can be achieved.

[5]. Pigmentation is one of the most striking cultural characteristics. In some pigment producing bacterial species, the pigment is retained within the cell and the mass of bacterial cells is coloured, whereas for others the pigment is excreted and colours the medium. The intensity of the pigment is influenced by the composition of the medium and the conditions of incubation. Pigment production best observed from growth on solid media. Bacteria are known to produce iron-chelating compounds i.e. percent siderophores, which are often colour pigments.

The genus *Pseudomonas* consists of a very diverse assemblage of bacteria in which some are pathogen to most of the plants and some are non-pathogenic. The non-pathogenic Pseudomonadsare divided into different groups, amongst which most of the species have the ability of plant growth promotion and biological control of plant pathogens.

[6], [7], [8]. *Pseudomonas* spp. are well known for producing several pigments such as pyocyanin, pyorubin and pyoverdines. All three pigments have various applicational properties such as antimicrobial, [9]. biocontrol, [10]. textile dyesand [11]. siderophore activity. [12]. Pyocyanin is a blue coloured water-soluble pigment, synthesized by 90–95% *Pseudomonas aeruginosa* under the control of quorum sensing coordination. Pyroverdine is yellow fluorescent pigment which can observed under Ultraviolet rays, where as Pyorubin is reddish brown, water soluble pigment.

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II. MATERIALS AND METHODS

Isolation and screening : The samples from lake water, rhizosphere soil sample and raw milk were collected under aseptically. The samples were serially diluted and plated on the pseudomonas isolation agar. The inoculated agar plates were then incubated at 37^0C for 48 hours. Only 35 bacterial colonies were isolated. Among 35 colonies, 14 isolates were further selected based on the pigments produced by the colonies on the pseudomonas isolation agar. Both pyocyanin and pyorubin, were screened based on distinct bluish-green colour pigment and brownish red colour pigment on the agar medium. [13]. The pyoverdine producing isolates were identified by exposing the culture plates to the UV light by using Ultraviolet transilluminator at 312 nm.

Identification of selected isolates : the two isolates were studied to have different morphological and cultural characteristics. These were identified by 16s rRNA gene sequence analysis. The bacterial gene sequences were deposited in the GenBank.

Calculations of concentration of the pigments:

[14]. Quantification of pyocyanin and pyrubin from *Pseudomonas aeruginosa* strain PI211.

Pyocyanin concentration = Luminar density of pyocyanin suspension at 690 nm / Absorption coefficient

Pyorubin concentration = Luminar density of pyorubin suspension at 320 nm / Absorption coefficient

Quantification of pyoverdine from *Pseudomonas putida* strain PI33.

Pyoverdine concentration = Luminar density of pyoverdine suspension at 400 nm / Absorption coefficient

2.1. Production of phenazines

Pseudomonas spp. produces certain phenazines which are nitrogen containing heterocyclic redox agents. Phenazines (pyocyanin, pyorubin and pyoverdines) were produced by strains PI21 and PI33 in nutrient broth under shaking conditions incubated at 25°C, 150 rpm for 24 hours.

[15].These strains were streaked on nutrient agar plates and incubated at room temperature (25°C) for 24 hours. A single colony were picked up, inoculated into 500 ML conical flask containing 100 ML nutrient broth and incubated at 25°C, 150 rpm for 48 hours.

2.2. Extraction of phenazines and separation by TLC:

The bacterial cells were removed from the growth medium by centrifugation at 7000 rpm for 10 minutes. The supernatant was further used to extract the pigments. The cell-free supernatant was extracted twice with equal volume of chloroform in a separating funnel. The pyocyanin pigment in chloroform layer were separated and re-extracted using 0.1 N HCl. The acidic fraction was separated, neutralized using 0.1 N NaOH, and again re-extracted using chloroform, where as pyorubin and pyoverdine were extracted from the aqueous fraction. [15]. The obtained pyocyanin, pyorubin and pyoverdine pigments were dissolved in methanol, and absorbance was recorded on a UV–vis spectrophotometer at 690 nm, 320 nm and 400 nm respectively.

The extracted phenazines was analyzed by thin-layer chromatography (TLC) with silica gel as the static phase. For PYC, n-butanol - acetic acid – water (13:4:7) was used as mobile phase where as, for pyorubin and pyoverdine Methanol- Dichloromethane (1:1) was used as the mobile phase (Jayapriya et al., 2012).

III. RESULTS

Screening of Isolates:

Among the samples collected, only 35 pigment producing *Pseudomonas* spp. were isolated from the lake water and rhizosphere soil using pseudomonas isolation agar. Where as, none of the isolates obtained from raw milk sample produced any pigments on pseudomonas isolation agar.

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The pyocyanin producing isolates were identified by the distinct bluish-green colour pigment production on the pseudomonas isolation agar and the isolates producing pyorubin pigment were identified by the reddish colour pigment on the agar medium . [13]. Where as the proverdine pigment producing *Pseudomonas* spp. were screened by exposing culture plates to UV light by usingUltraviolet transilluminator at 312 nm. Total 10 isolates were positive for both pyocyanin and pyoverdine production, 2 isolates were positive for both pyocyanin and pyorubin, 2 isolates were positive for only pyoverdine production , where as 1 isolate was positive for only pyocyanin production. The selected pigment producing isolates for the pyocyanin, pyorubin and pyoverdine pigments are tabulated as shown in Table.1.

The desired 14 isolates where further grown in nutrient broth under desired conditions. Among the 14 colonies, strain PI21 was selected for the maximum yield production of pyocyanin and pyorubin, where as the strain PI33 for pyoverdine based on UV–vis spectrophotometeric estimation.

Table.1. Screening of Isolates producing pyocyanin, pyorubin and pyoverdine

Screening of Isolates producing pyocyanin, pyorubin and pyoverdine											
Isolates	Pyocyanin	Pyorubin	Pyoverdine	**Isolates**	Pyocyanin	Pyorubin	Pyoverdine	**Isolates**	Pyocyanin	Pyorubin	Pyoverdine
PI1	_	_	_	**PI13**	+	_	+	**PI25**	+	_	+
PI2	_	_	_	**PI14**	+	_	+	**PI26**	+	+	+
PI3	_	_	_	**PI15**	_	_	_	**PI27**	_	_	_
PI4	_	_	_	**PI16**	_	_	_	**PI28**	_	_	+
PI5	_	_	_	**PI17**	_	_	_	**PI29**	_	_	_
PI6	_	_	_	**PI18**	+	_	+	**PI30**	_	_	_
PI7	_	_	_	**PI19**	_	_	_	**PI31**	_	_	_
PI8	_	_	_	**PI20**	_	_	_	**PI32**	_	_	+
PI9	+	_	+	**PI21**	+	+	+	**PI33**	_	_	+
PI10	_	_	_	**PI22**	+	_	_	**PI34**	_	_	_
PI11	_	_	_	**PI23**	+	_	+	**PI35**	_	_	_
PI12	+	_	+	**PI24**	+	_	+				

3.1. Identification:

The morphological and cultural characteristics of the two isolates were studied to be different and 16s rRNA gene sequence analysis was carried out for the two isolates. The bacterial gene sequences were deposited in the GenBank. The maximum pyocyanin and pyorubin pigments yielded strain PI21 was identified as *Pseudomonas aeruginosa* by using 16s r RNA sequencing method. The accession number is MK966380.

The maximum pyoverdine pigment yielded strain PI 33 was identified as *Pseudomonas putida* by using 16s r RNA sequencing method.The accession number is MK966379.

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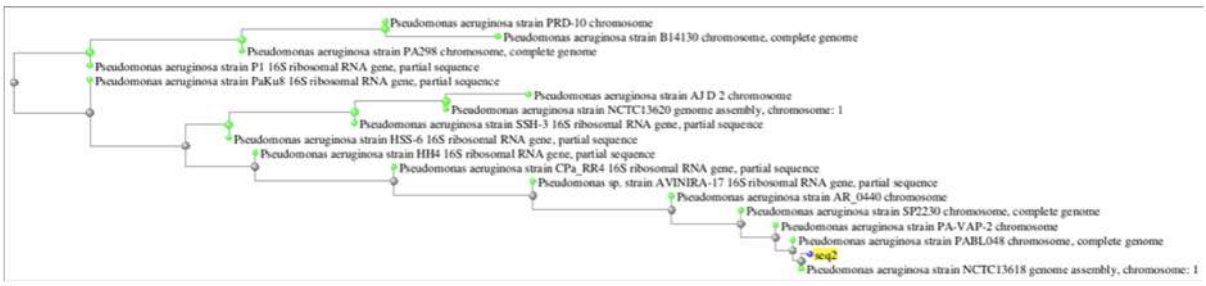

Fig. 1. Evolutionary relationship of *Pseudomonas* strain PI21(GenBank (NCBI) accession no. MK966380) with its relative inferred using Neighbor Joining method

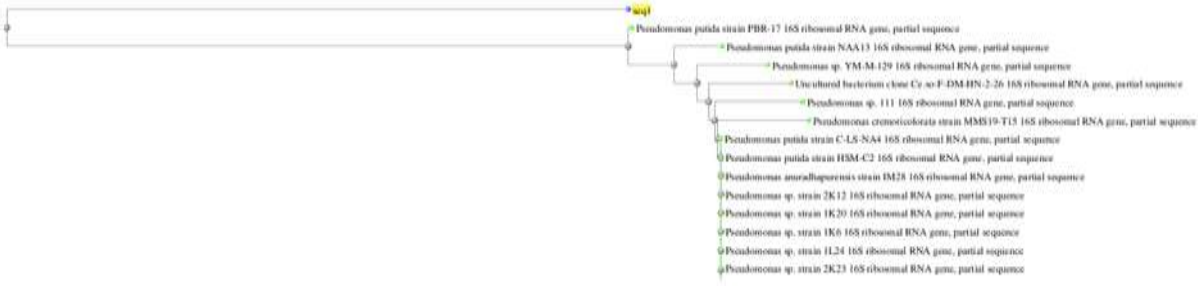

Fig. 2. Evolutionary relationship of *Pseudomonas* strain PI33 (GenBank (NCBI) accession no. MK966379) with its relative inferred using Neighbor Joining method

3.2. Extraction of pigments

According to Saosoong et al (2009), the crude phenazine were purified by SPE method using a Chromabond SiOH cartridge. The study suggested that 3 bands isolated appeared on the cartridge as yellow, yellow-green and blue band when eluted with dichloromethane, 5% methanol: dichloromethane and follow by 10% methanol: dichloromethane. As per the TLC assessment, purified phenazines obtained TLC plate with R_f 0.77and R_f 0.53, respectively.

The extracted pyocyanin was analyzed by thin-layer chromatography (TLC) with silica gel as the staticphase and n-butanol - acetic acid –water (13:4:7) as the mobile phase and R_f values was match with R_f value of pyocyanin reported by (Saosoong et al. 2009; Jayapriya et al. 2012).

The extracted phenazines (pyorubin and pyoverdine) were analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and Methanol- Dichloromethane (1:1) as the mobile phase and R_f values was match with R_f value of pyorubin, where as the extracted pyoverdine was analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and Methanol- Dichloromethane (1:1) as the mobile phase and R_f values were recorded as given in table 2.

Table.2. Rf values of pyocyanin, pyorubin and pyoverdine after thin-layer chromatography

Phenazines	Solvent system	Sample R_f value
Pyocyanin	n-Butanol-Acetic acid- Water (13:4:7)	0.87

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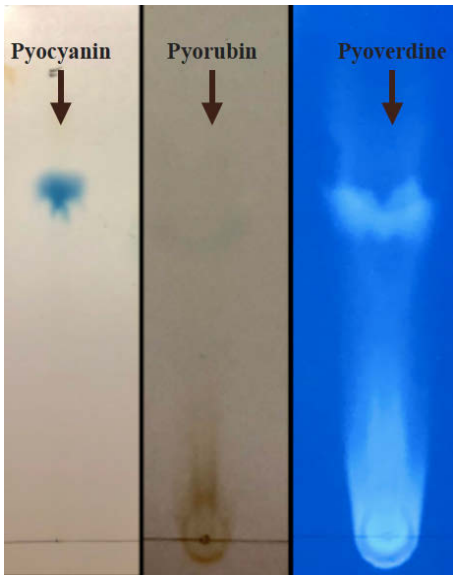
Phenazines	Solvent system	Sample R_f value
Pyorubin	Methanol- Dichloromethane (1:1)	0.74
Pyoverdine	Methanol- Dichloromethane (1:1)	0.88

Fig. 3. Thin-layer chromatography of pyocyanin, pyorubin and pyoverdine



IV. DISCUSSION

PYC is one of the most common marker phenazine derivative produced by *P. aeruginosa* compared to PYR and PYV. According to Abdul et al (2016) chromatogram of colored spot on TLC plate showed an RF value of 0.83 for pyocyanin. The result was comparable with previous studies reported by Genevive et al (2006). Baron and John obtained RF value of pyocyanin (0.71) by eluting with chloroform solvent. The phenazines were analysed by thin-layer chromatography (TLC) using silica gel as the stationary phase and methanol and dichloromethane (1:1) as the mobile phase and found to match the R_f values reported by Saosoong et al. (2009).

In the present study The extracted pyocyanin was analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and n-butanol - acetic acid –water (13:4:7) as the mobile phase and R_f values was match with R_f value of pyocyanin reported by (Saosoong et al. 2009; Jayapriya et al. 2012).

The extracted phenazines (pyorubin and pyoverdine) were analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and Methanol- Dichloromethane (1:1) as the mobile phase and R_f values was match with R_f value of pyorubin, where as the extracted pyoverdine was analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and Methanol- Dichloromethane (1:1) as the mobile phase and R_f values were recorded.

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V. CONCLUSION

In conclusion, the strain PI21 produced maximum yield of pyocyanin and pyorubin, where as the strain PI33 produced pyoverdine at alkaline conditions using basal nutrient growth medium at 25^0C for 24 hours which were in consistency with reports. 16s rRNA gene sequence analysis was carried out for the two isolates. The maximum pyocyanin and pyorubin pigments yielded strain PI21 was identified as *Pseudomonas aeruginosa* and the maximum pyoverdine pigment yielded strain PI 33 was identified as *Pseudomonas putida* by using 16s r RNA sequencing method. The extracted pigment pyocyanin was analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and n-butanol - acetic acid –water (13:4:7) as the mobile phase and R_f values was match with R_f value of pyocyanin, where as pyorubin and pyoverdine were analyzed by thin-layer chromatography (TLC) with silica gel as the static phase and Methanol-Dichloromethane (1:1) as the mobile phase and R_f values match with R_f value of pyorubin. Further, different parameters and economical wastes such as pH, temperature, carbon sources, Nitrogen sources, Amino acids, Metal ions and Agro-wastes will be used to improve the production of phenazines by *Pseudomonas aeruginosa* PI21 and *Pseudomonas putida* PI33.

REFERENCES:

1. Clemo, B. 2003. Applying Ultra-fresh. Colourage. 50(10): 59-64.
2. Shanmugasundaram, O. L. 2007. Antimicrobial finish in textiles. The Indian Textile Journal 117(11): 53-58.
3. Sivaramakrishanan, C. N. 2007. Antimicrobial finishes. Colourage 54(9): 50-54.
4. Meyer, D. & Montforts, F.P. (2005) Transfer of Technology: Violacein (Black Beauty) http://www.awi-bremerhaven.de/TT/viola/index-e.html
5. Pelczar, M. J.; Reid, R. D. and Chan, E. C. S. 1982. Microbilogy. Tata mcgraw hill publishing company ltd, New Delhi. p.936.
6. Ozyurek SB, Gur SD, Bilkay IS. Production of pyocyanin pigment from *Pseudomonas aeruginosa* strains and investigation of the antimicrobial effect of pyocyanin on other microorganisms. *Current Opinion in Biotechnology*. 2011;22:S113. doi:https://doi.org/10.1016/j.copbio.2011.05. 363
7. Priyaja P, Jayesh P, Correya NS, Sreelakshmi B, Sudheer NS, Philip R, Singh ISB. Antagonistic effect of *Pseudomonas aeruginosa* isolates from various ecological niches on Vibrio species pathogenic to crustaceans. *Journal of Coastal Life Medicine*. 2014;2(1):76-84.
8. Laxmi M, Bhat SG. Characterization of pyocyanin with radical scavenging and antibiofilm properties isolated from *Pseudomonas aeruginosa* strain BTRY1. 3 Biotech. 2016;6(1). doi:https://doi.org/10.1007/s13205-015-0350-1
9. P. Priyaja, Pyocyanin (5-methyl-1-hydroxyphenazine) Produced by *Pseudomonas Aeruginosa* as Antagonist to Vibrios in Aquaculture: over Expression, Downstream Process and Toxicity, Cochin University of Science and Technology, India, 2012 Ph.D. Thesis.
10. Mishra, A. "Natural Dyes from Pseudomonas fluorescens: Extraction, Characterization and Optimization of Dyeing Process for Textiles."GB Pant University of Agriculture and Technology, Pantnagar-263145 (Uttarakhand) (2007).
11. Chaudhari, B. L., Patil, S. N., Paradeshi, J. S., Chaudhari, M. A., & Chaudhari, C. S. (2017). Premier Biocontrol Traits of Pseudomonads: Siderophores, Phenazines or What Else?. In Microorganisms for Green Revolution (pp. 351-390). *Springer*, Singapore.
12. M.Z. El-Fouly, A.M. Sharaf, A.A.M. Shahin, H.A. El-Bialy, A.M.A. Omara, Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*, *J. Radiat. Res. Appl. Sci.* 8 (1) (2015) 36–48.
13. Dietrich LEP, Price-Whelan A, Petersen A, Whiteley M, Newman DK. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Molecular Microbiology*. 2006;61(5):1308-1321. doi:https://doi.org/10.1111/j.1365-2958.2006.05306.x
14. Chapter 4 Isolation , purification and partial characterization of pyocyanin and rhamnolipids from *Pseudomonas aeruginosa* BTRY 1 2, (2016).
15. Patil S, Nikam M, Patil H, Anokhina T, Kochetkov V, Chaudhari A. "Bioactive pigment production by *Pseudomonas* spp. MCC

3145: Statistical media optimization, biochemical characterization, fungicidal and DNA intercalation-based cytostatic activity."*Process Biochemistry.* 2017;58:298-305.

16. Saosoong K, Wongphathanakul W, Poasiri C, Ruangviriyachai C. Isolation and analysis of antibacterial substance produced from P. aeruginosa TISTR 781. *Kkv. Sci. J.* 2009;37(20):163-172.
17. Saranya R, Jayapriya J, Tamilselvi A. Dyeing of silk fabric with phenazine fromPseudomonasspecies. Coloration Technology. 2012;128(6):440-445. doi:https://doi.org/10.1111/j.1478-4408.2012.00397.x
18. Abdul-Hussein, Z. R., & Atia, S. S. (2016). Antimicrobial effect of pyocyanin extracted from *Pseudomonas aeroginosa*. *European Journal of Experimental Biology*, 6(3), 1-4.
19. Genevive G, Sharief B, Sebastein R, Tjeerd V, Ben J, et al. (2006) Pip, a novel activator of phenazine biosynthesis in *pseudomonas chlororaphis* PCL1391. *J Bacteriol* 188: 8283-8293.
20. Baron SS, Rowe JJ (1981) Antibiotic action of pyocyanin. *Antimicrob Agents Chemotherapy,* 20:814-820.