Microbicidal Response of Natural Pigment Pyocyanin Isolated from *Pseudomonas aeruginosa*

Dr. Ratna Trivedi[1] * Mita .J. Vakilwala[2]

[1]*Department of Environment science,*
Shri Ramkrishna Institute of Computer Education and Applied Sciences, Surat
[2]*Department of Medical Technology*
KBS Commerce and Nataraj Sciences College, Vapi

***Abstract*: *Pseudomonas aeruginosa* produces various phenazine pigments like pyrroles, quinolines and others. These pigments possess antibacterial antifungal and antiviral activities. In the present study *Pseudomonas aeruginosa* was studied for pigment production. The maximum production of pigment was obtained at a temperature of 37ºC after 48-72 hr of incubation. The pigment was extracted by chloroform extraction method forming a green to deep blue colour, conforming for pyocyanin. Further the pigment was partially purified by using chromatographic technique and analyzed by UV-Vis spectrophotometer. The maximum absorption peak was observed at 278nm characteristic of pyocyanin. The pigment was tested for its antibacterial and antifungal activity towards the test pathogens, like *Staphylococcus aureus, Escherichia coli, Aspergillus niger* and *Candida albicans.* The antibacterial and antifungal activities were evaluated by *in vitro* disk diffusion and MIC methods. Different concentrations of pigment (1, 2, 4, 8, 16, 32, 64 and 128µg/ml) were tested for antibacterial and antifungal activity. The MIC of pyocyanin was found at 32µg/ml and 64µg/ml against *E. coli* and *S. aureus,* respectively.**

***Key words*: *Pseudomonas aeruginosa,* pyocyanin, antibacterial and antifungal activity.**

I. INTRODUCTION

Pseudomonas aeruginosa produces large quantities of water soluble blue-green phenazine pigment, pyocyanin. The blue green chloroform soluble phenazine pigment extracted from *Pseudomonas aeruginosa* has antimicrobial (Preetha *et al*;2010) and antifungal (Kerr *et al*;1999) activity. It inhibits bacterial and fungal growth both in vivo and in vitro condition. It is a redox active secondary metabolite soluble in chloroform. The antagonistic action of pyocyanin is due to its unique redox potential (Sheeba Jayaseelan *et al*:2014). The phenazines are heterocyclic nitrogen containing natural products synthesized by fluorescent *Pseudomonas spp.*, members of a few other bacterial genera, and thousands of chemically synthesized derivatives (Blankenfeldt *et al*., 2004). Nearly 90–95 % of all isolates of *P. aeruginosa* producing pyocyanin pigment, is referred to as ‘‘blue pus’’ (from pyocyaneus) (Ran HM;2000).

II. MATERIALS AND METHODS

Isolation and Identification of pigments producing strains

Different clinically suspected samples infected with *Pseudomonas spp* were collected from different places in and around the Valsad district. Out of 5 strains of *Pseudomonas spp* one pigment producing strain from the clinical sample was isolated on King’s B medium at 37°C for 24 hours. The pure isolate was maintained on King’s B medium and slants at 4 °C. The biochemical analysis was done according to Bergey’s Manual of Systematic Bacteriology, to characterize *Pseudomonas aeruginosa* (Saha.S;2008).

Pathogenic microbes used in this study

Four tested pathogens, used in this study (Table 1), were obtained from the Microbial type culture collection (MTCC; Chandigarh). The stock cultures of tested pathogens were maintained on nutrient agar slants at 4 ˚C with monthly transfer, whereas, fungal mycelia were maintained on Sabouroud's agar slants.

Table 1: Pathogenic Tested Microorganisms

Type	Microorganisms
Gram +ve	*Staphylococcus aureus* MTCC-47077
Gram -ve	*E. Coli* MTCC- 25922
Fungi and Yeast	*Aspergillus niger* MTCC- 16888
	Candida albicans MTCC- 10231

Production of pyocyanin pigment

Pseudomonas aeruginosa 1 strain produces soluble blue green pigment named pyocyanin. Pyocyanin production was carried out using King’s B liquid medium as described by King *et al*. *Pseudomonas aeruginosa* was inoculated into King’s B liquid

medium (pH 7) and incubated under shaking condition (120 rpm) at 37 °C for 3 days. Coloured supernatant free bacterial cells were removed by centrifugation at (10,000 rpm x 10 min) and filterd through 0.45 µm filter for further analysis.

Extraction and Purification of pyocyanin

Pyocyanin was separated from supernatant by the addition of chloroform solvent system. Extractable chloroform layer was further mixed with 1 ml of 0.2 N HCl which converted pyocyanin to the acidic (red) form. Red colour obtained pigment was separated and subjected to UV spectrophotometric analysis and scanned at range of 200-800 nm. Absorption was measured at 520 nm (Essar DW *et al*;1954).

Analysis of pigment by UV-visible spectrophotometer

The extracted pigment of the strain *Pseudomonas aeruginosa* 1 was subjected to U-V visible spectrophotometer colour analysis by APHA standard and maximum absorption was recorded by UV spectrophotometer.

Antimicrobial activity of pyocyanin

The antimicrobial activity of pyocyanin was tested by disc diffusion technique to test the antimicrobial activity of bacterial isolates. Agar plates of Müller Hinton for bacteria and potato dextrose for yeasts and fungi were prepared. The plates were inoculated with 0.1 ml containing 106 cfu/ml of fresh bacterial cultures and spore suspensions of pathogenic strain. Sterile discs of 5 mm diameter were loaded by 10 µl of pyocyanin dissolved in chloroform. Negative control disc was applied for chloroform. Plates were left for two hour in the refrigerator to allow the diffusion. The plates were incubated for 24 h at 30 °C for bacteria and incubated for 72 h at 30 °C for fungi and examined for inhibition zone. All the assays were performed in duplicate (Noura E;2014).

Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) of purified pyocyanin fractions against the pathogenic bacteria was determined by micro dilution method (M. Jennifer;2001). One mille litre of the nutrient broth containing pyocyanin fractions (P1 and P2) at various concentrations was distributed in eight sterile test tubes (1µl to 128 µl) besides keeping a positive and negative control (Table I). The concentration of the pigments was maintained in each tube at geometric progression such as 1, 2, 4, 8, 16, 32, 64 and 128µg/ml. Bacterial suspension or fungal suspension was prepared by dissolving in 5 ml equivalent to a Mc Farland 0.5 standard (M. Jennifer;2001). 0.1 ml of bacterial suspension or fungal suspension was added to 9.9ml of saline with a fresh pipette. From which 0.1 ml of diluted bacterial or fungal suspension transferred to broth tubes containing the compound pyocyanin with varying concentration, numbered 1 through 8 and also to the positive control tube. 0.1ml sterile saline was added to the negative control. Contents in all the tubes were mixed well and incubated for 24hrs. Test tube containing lowest concentration of the compound with no bacterial growth was considered as minimum inhibitory concentration.

Table-2

MICRO-DILUTION TUBES CONTAINING PYOCYANIN CONCENTRATION IN DESCENDING ORDER

Tube No	1	2	3	4	5	6	7	8	9	10
Conc. of pyocyanin µg/ml	128	64	32	16	8	4	2	1	Positive control	Negative control

III. RESULTS AND DISCUSSION

ISOLATION AND IDENTIFICATION OF *Pseudomonas spp*

Out of various clinical samples, 5 strains were identified as *Pseudomonas aeruginosa* based on Gram's staining, motility, cultural characteristic, pigment production by various media and by various biochemical reactions. The *Pseudomonas aeruginosa* strain 1 produced maximum pigment on King's B media. This study revealed that, the higher pyocyanin pigment producer *P. aeruginosa* strain 1 was selected.

Fig-1 *Pseudomonas aeruginosa* on King's B medium

Production and Extraction of Pyocyanin

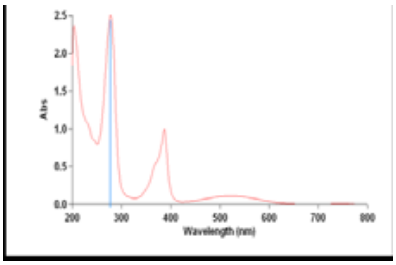
The maximum pigment production was achieved at optimum temperature and pH of 37°C and 7 respectively after 48-72 hours of incubation at shaking condition of 120rpm. Pyocyanin compound was produced and extracted. The results of the study are in accordance to the work (Chandran Masi;2014). A blue-green shade colour of the solution was obtained, extracted by adding chloroform which separated a blue colour compound. It was then confirmed by adding 0.2 N HCl and a pinkish red colour compound was obtained which indicated the presence of pyocyanin pigment (Ra'oof W M;2010). The work correlated with the study of (Sudhakar.T;2015)

Purification of pyocyanin pigment

The pigment was separated by column chromatography and yielded one single fraction of light blue color. It was then eluted with Chloroform and methanol. It correlated with the study of (Sudhakar.T; 2015).

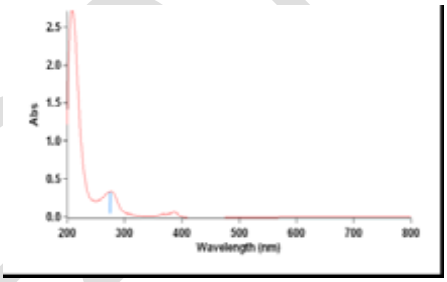
Characterisation of pigment by U.V visible spectrophotometer

Fig-2



P1-278 λmax

Fig-3



P2- 276 λmax

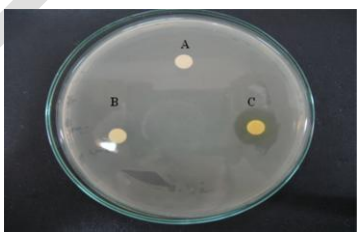
The partially purified compound was subjected to UV-spectrophotometer and the absorbance of this solution was maximum at 278nm. This peak indicates the presence of pyocyanin compound. The results of the study correlated with the work of (Sudhakar.T; 2013)

Antimicrobial activity of pyocyanin pigment

Table-3

Sr.No	Bacterial strain	Control(mm) (chloroform)	P1 (µg/ml)				P2(µg/ml)			
			5	10	15	20	5	10	15	20
1	*S.aureus*	-------	0	0	5	10	0	0	5	5
2	*E.coli*	-------	0	0	5	10	0	0	0	10
3	*Candida albicans*	-------	0	0	0	5	0	0	0	0
4	*Aspergillus niger*	--------	0	0	0	5	0	0	0	5

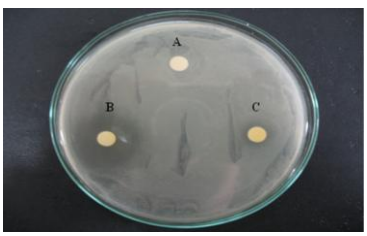
Fig-4 *E.coli*



A-Control

B- P1 (15µg/ml) C-P1 (15µg/ml)

Fig-5 *S.aureus*



A- Control

B- P1(20 µg/ml) C-P2 (20µg/ml)

The pigment produced by *Pseudomonas aeruginosa* strain 1was subjected to antibacterial and antifungal activity using *E. coli, S.aureus, Candida albicans and Aspergillus niger* as shown in (Table 3). It was found that antibacterial and antifungal activity of pyocyanin was against *S.aureus* and *E.coli*. Diameter of zone of inhibitions was 5mm and 10 mm for *E.coli* and *S.aureus* respectively. Also antifungal activity of pyocyanin was found against *Candida albicans* and *Aspergillus niger*. The zone of inhibitions was 5mm respectively. In this connection, the antifungal activity of fluorescent pigment produced by *Pseudomonas* spp. was detected by Cook *et al*.,1995 Chythanya *et al*;2002 and Weller; 2008.

Minimum Inhibitory concentration (Micro-tube dilution method)

Table-4

Sr.No	Concentration of pyocyanin pigment(P1&P2µg/ml)	MIC of pyocyanin pigment(P1 andP2) for *E.coli*	*S.aureus*
1	128	++++	+++
2	64	++++	+++
3	32	No visible growth	No visible growth
4	16	No visible growth	No visible growth
5	8	++++	++++
6	4	++++	+++
7	2	++++	++++
8	1	++++	+++

The MIC's of pyocyanin P1 and P2 for *E.coli* and *S. aureus* is shown in the above table-4.There is no visible growth at conc. 32 and 16 µg/ml for *E.coli*. and *S.aureus*. The results varied according to Hassanein, W.A.,*et al*;2009.

IV. CONCLUSION

Pseudomonas aeruginosa isolated from various clinically suspected samples was studied for pigment production. The culture conditions for the isolate were optimized to produce the activity of the pigment. The pigment was characterised by U.V visible spectrophotometer and maximum absorbance was found at 278 nm. The peak indicated presence of pyocyanin. Antimicrobial activity and antifungal activity of the pigment pyocyanin was done with the indicator bacteria like *E.coli, Staphylococcus aureus Candida albicans* and *Aspergillus niger*. Minimum antimicrobial activity of the pigment was found. MICs of pyocyanin pigment showed no visible growth at higher concentrations. The pigment can be further analysed and characterised to elucidate its structure successively.

REFERENCES

[1]. Blankenfeldt W., Kuzin A., Skarina T., Korniyenko Y., Tong L. And Bayer P. 2004. Structure and function of the phenazine biosynthetic protein *phz* F from *P. fluorescens*. PNAS. 101(47): 1631-16436.

[2]. Chythanya, R., Indrani Karunasagar and Iddya Karunasagar, 2002. Inhibition of shrimp pathogenic vibrio by a marine *Pseudomonas* 1-2 strain. Aquaculture, 208: 1-10.

[3]. Cook, R.J., L.S. Thomashow, D.M. Weller, D. Fujimoto, M. Mazzola, G. Bangera and D. Kim, 1995. Molecular mechanisms of defense by rhizobacteria against root disease. Proceedings of the National a cademy of Sciences of the United States of America, 92: 4197- 4201.

[4]. Essar DW, Eberly L, Hadero A, Crawford IP. J Bacteriol 1990;172: 884–900.

[5]. Hassanein, W.A., Awny, N.M., El-Mougith, A.A. and Salah El-Dien, S.H. The Antagonistic Activities of Some Metabolites produced by *Pseudomonas aeruginosa* Sha8 Journal of Applied Sciences Research, 5(4): 404-414, 2009, INSInet Publication

[6]. Kerr, J.R; G.W. Taylor, A. Rutman, N.Hoiby, P.J. Cole and R. Wilson.1999.Pseudomonas aeruginosa pyocyanin and 1-hydroxyphenazine inhibit fungal growth. Journal of Clinical Pathology, 52:385-387.

[7]. King EO. J Lab Clin Med 1954;44(2): 301-30. 7.

[8]. M. Jennifer, Andrews, "Determination of minimum inhibitory concentrations," *Journal of Antimicrobial chemistry*, vol. 48, pp. 5-16, July 2001.

[9]. Noura E, Nayera AM and Darwesh. J Microbiol. Biotechnol 2014; 24(4): 453–464.

[10]. Preetha,R; S.Jose, S.Prathapan, K.K, Vijayan, N.S. Jayaprakash, R.Philip and I.S. Bright Singh.2010. An inhibitory compound produced by Pseudomonas with effectiveness on Vibriyo harveyi. Aquaculture Research, 41: 1452-1461.

[11]. Ran HM, Hassett DJ, Lau GW. Proc Natl Acad Sci USA 2003;100: 14315-14320.

[12]. Ra'oof W M and Latif. European J Scient Res 2010; 47(3):405–421

[13]. Saha S, Thavasi R, Jayalakshmi. Res J Microbiol 2008;3 (3): 122-128.

[14]. Sheeba Jayaseelan, D.RamaSamy, S.Ethiraj, S.Dharmaraj. Production of Pyocyanin and its antagonistic activity against selected Fungal rice pathogens .IJCRBS 1(3) 2014: 26-32 ISSN:2393-8560

[15]. T. Sudhakar, S. Karpagam and Sabapathy Shiyama. Analysis Of Pyocyanin Compound And Its Antagonistic Activity Against Phytopathogens International Journal of ChemTech Research CODEN(USA): IJCRGG ISSN : 0974-4290 Vol.5, No.3, pp 1101-1106, April-June 2013

[16]. Weller, D.V., 2007. *Pseudomonas* Biocontrol agents of soilborne pathogens: Looking back over 30years. *Phytopathol.*, 97: 250-256. 414.