

 <p>ISSN NO. 2320-5407</p>	<p>Journal Homepage: - www.journalijar.com</p> <h2 style="text-align: center;">INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)</h2> <p style="text-align: center;">Article DOI: 10.21474/IJAR01/6230 DOI URL: http://dx.doi.org/10.21474/IJAR01/6230</p>	
---	--	---

RESEARCH ARTICLE

TO COMPARE THE ANTIMICROBIAL POTENTIAL OF THE PROTONATED AND ZWITTERIONIC STATE OF BIOACTIVE PYOCYANIN ISOLATED FROM *Pseudomonas aeruginosa*.

Payel Sutradhar¹, Ayan Majumder², Sudipa Chakravarty³ and Amit Chakravarty⁴.

1. Department of Microbiology, Institute of Genetic Engineering. 30A Thakurhat Road. Kolkata- 700128, WestBengal, India.
2. MSc Microbiology, 1st year , Jiwaji University ,Gwalior,Madhya Pradesh -474001,India
3. Department of Genetics, Institute of Genetic Engineering. 30A Thakurhat Road. Kolkata- 700128, West Bengal, India.
4. Department of Biotechnology, Institute of Genetic Engineering. 30A Thakurhat Road. Kolkata- 700128, West Bengal, India.

Manuscript Info

Manuscript History

Received: 05 November 2017
Final Accepted: 07 December 2017
Published: January 2018

Key words:-

Pseudomonas aeruginosa, pyocyanin ,
virulence factor, chloroform,
zwitterionic (blue) state, protonated (red)
state, antimicrobial.

Abstract

Though *Pseudomonas aeruginosa* is medically important as an infectious agent due to its ubiquity , the emergence of this organism in the field of biotechnology is commercially valuable to date . The increasing scopes and interests in order to exploit the metabolites from this organism is a boon to the biotechnologists of this era. *Pseudomonas aeruginosa* is an opportunistic , Gram-negative bacterium responsible for severe nosocomial infections. The bluish secondary metabolite known as pyocyanin secreted by this bacteria is an important virulence factor. It has been reported that the redox active tricyclic pyocyanin is able to readily cross biological membranes, possessing antimicrobial activity ^(1, 2) and can exist in different states in various pH ranges. In our present study we have focussed on the comparison between the antimicrobial effects of the protonated and the zwitterionic state of this bioactive pigment pyocyanin against the Gram negative bacteria (*E.coli* , *Vibrio cholerae*) and the Gram positive bacteria (*Bacillus subtilis*). Pyocyanin was extracted from the broth culture by Chloroform /HCl technique and agar well diffusion method was followed thereafter to assay the antagonistic effects . The protonated (red) state in acidic environment was found to be more potent antimicrobial agent as compared to the zwitterionic (blue) state in neutral or slightly alkaline environment. The maximum zone of inhibition was noted for the acidic (red) state of pyocyanin against Gram positive *Bacillus subtilis* .Further high quality in vivo studies are needed to fully assess the physiological manifestations of pyocyanin exposure on the various cells including eukaryotic systems.

Copy Right, IJAR, 2018,. All rights reserved.

Introduction:-

Pseudomonas aeruginosa is a ubiquitous Gram negative; citrate, catalase, and oxidase positive bacterium that can cause disease in plant and animals, including humans. It is medically important as a multidrug resistant pathogen

Corresponding Author:- Payel Sutradhar.

Address:- Asst. Prof. Department of Microbiology, Institute of Genetic Engineering. 30A Thakurhat Road. Kolkata- 700128. WestBengal. India.

and the agent for nosocomial infections. The organism is considered opportunistic because it produces a battery of secretory virulence factors causing infections often during existing diseases or conditions notably cystic fibrosis and burn cases.

The major group of these secretory virulent factors are known as phenazines⁽³⁾. One of them is called pyocyanin which has been widely emerged as an bioactive compound having antimicrobial properties. These pigments are also involved in iron acquisition (siderophores) and quorum sensing^(4,5).

Pyocyanin is a redox active nitrogenous heterocyclic compound belonging to the tricyclic phenazine class of compounds secreted extracellularly as secondary metabolites. Due to its redox-active properties, pyocyanin generates reactive oxygen species. The presence of the phenol group in the zwitterionic state gives a weak acidic character (pKa of 4.9). Pyocyanin can exist as protonated or red form in acidic environment. The pigment is wine-red at acid condition due to the basic property of one of the N atoms (Friedheim et al 1931). At physiological pH (7.4) or neutral or slightly alkaline condition, pyocyanin exist in blue state or zwitterionic state (**Fig 1**). The low molecular weight and zwitterionic properties of pyocyanin are believed to allow the toxin to easily penetrate the cell membranes⁽³⁾. It has been already reported that pyocyanin also possess antimicrobial activity^(2,6). Due to the great solubility of pyocyanin in chloroform, it can be easily isolated chemically.

Thus, our initial work involved the extraction of pyocyanin(blue) secreted by *P.aeruginosa* chemically by chloroform and its conversion into the red protonated state by acidification. The next phase of the work emphasized on the comparison of the antimicrobial activities of red(acidic) and blue(zwitterion) states separately against few test bacterial cultures (*B.subtilis*, *V.cholerae*, *E.coli*) by agar well diffusion method.

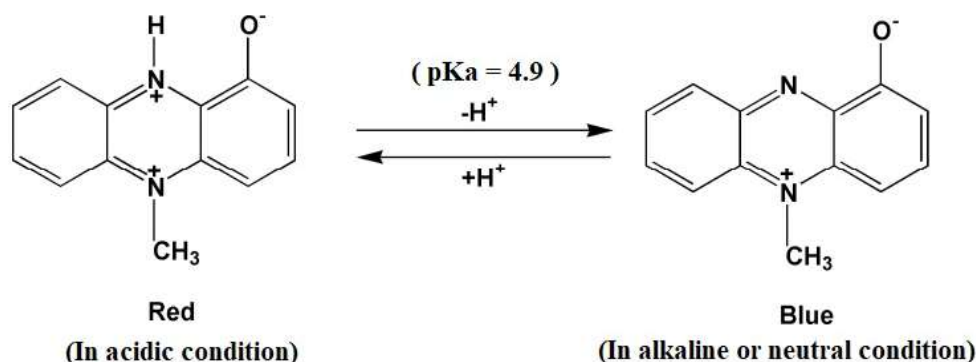


Fig 1:- Chemical structure of pyocyanin in acidic and alkaline conditions.

Materials and Method:-

Preparation of microbial test cultures:-

Pure cultures of *E.coli*, *V.cholerae*, *B.subtilis* were grown in nutrient media for 18hours at 37°C from the stock of the laboratory (IGE).

Isolation of *P.aeruginosa*:-

The laboratory (IGE) stock sample of *P.aeruginosa* was analyzed to investigate its presence by subculturing in Pseudomonas Agar (King et al) and incubated for 48 hours at 37°C. UV illuminator was used to detect the presence of the organism by fluorescence. The colony morphology was studied, screened morphologically by Gram stain and biochemical tests for further confirmation.

Biosynthesis and isolation of crude pyocyanin:-

The suspected colonies were subcultured onto Kings A medium^(7,8) and incubated overnight at 37 °C; then the incubation was continued for 6 days in room temperature. Centrifugation at 10000 rpm for 10 min was done to remove the bacterial cells and media components (**Fig 2**). The supernatant was separated for Pyocyanin extraction. 3 ml of chloroform (1:2 ratio) was added to the supernatant (**Fig 3**). The distinct bottom organic layer (blue)⁽⁸⁾ was separated (**Fig 4**) and divided into two parts. The top greenish layer was also assayed for antimicrobial activity.

Preparation of the different states of pyocyanin:-

One part of the blue organic layer is treated with 0.2M HCl to give a deep wine-red colour solution in the upper layer⁽⁹⁾ (**Fig 5**). This upper red layer was separated and divided into two parts. Addition of 500 mM NaOH to one part of red layer reconverts it into blue zwitterion (**Fig 6**). Both the red (protonated/acidic) state and the blue (slightly alkaline) state were subjected further for antimicrobial assay.

Antimicrobial assay/antagonistic activity:-

The crude forms of the pyocyanin extractives which were obtained in the present work were labelled for our successive work of antimicrobial susceptibility assay. The forms were labelled as follows: (**Fig 7**)

1. G → Top aqueous greenish layer (after chloroform addition)
2. B1 → Blue (bottom organic layer after chloroform addition)
3. R → Red (top layer after acidification of B1 by HCl)
4. B2 → Blue form (NaOH treated top red layer R)

All the above mentioned crude extractives were evaluated for the zone of inhibitions separately against the test microbial cultures (**Table 1,2,3**). Nutrient agar plates were spreaded aseptically with 20 µl of 18 hours grown test cultures (*E.coli*, *V. cholerae* and *B.subtilis*). Wells were bored over these agar plates in proper spacing and filled with 100 µl of the above mentioned crude extractives separately. The plates were incubated for 24 hours at 37°C. The solvent of extraction (CHCl₃, HCl and NaOH) were also assayed for antimicrobial activity against the same said test microbial samples considering as Control.

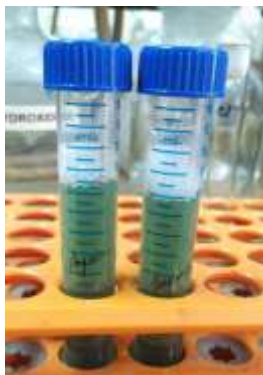


Fig 2:- Greenish supernatant after centrifugation



Fig 3:- Blue pyocyanin dissolves in chloroform layer(bottom)



Fig 4:- Separation of chloroform layer containing crude pyocyanin(blue)



Fig 5:- Crude protonated pyocyanin in acidic condition on treatment with 0.2M HCl



Fig 6:- Crude zwitterionic pyocyanin in alkaline condition Of 500 mM NaOH

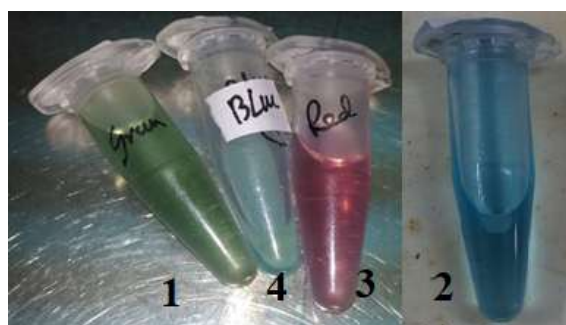


Fig 7:- Different states of crude pyocyanin prepared in order to assay the antimicrobial efficacy.

Results:-

The different forms of crude pyocyanin extracted in our present study were evaluated for the antimicrobial activity against each of the test microbial samples; *V.cholerae* , *E.coli* , *B.subtilis* (Fig 8a,b,c).The diameter of the clear zone of inhibitions were measured in cm and tabulated.(Table 1,2,3).

TABLE 1

Test microbial sample	Crude Pyocyanin isolates	Diameter of Clear Zone of inhibitions (in cm)
<i>V.cholerae</i> (Gram negative)	G(green top aqueous layer)	1.6
	B1(Blue bottom chloroform layer)	2.5
	R(red protonated,acidic layer)	4.4
	B2(blue alkaline isolate)	1.2

(#Solvent of extractions taken as control showed no zone of inhibitions)

TABLE 2

Test microbial sample	Crude Pyocyanin isolates	Diameter of Clear Zone of inhibitions (in cm)
<i>E.coli</i> (Gram negative)	G(green top aqueous layer)	1.9
	B1(Blue bottom chloroform layer)	2.4
	R(red protonated,acidic layer)	4.7
	B2(blue alkaline isolate)	1.5

(#Solvent of extractions taken as control showed no zone of inhibitions)

TABLE 3

Test microbial sample	Crude Pyocyanin isolates	Diameter of Clear Zone of inhibitions (in cm)
<i>B.subtilis</i> (Gram positive)	G(green top aqueous layer)	1.5
	B1(Blue bottom chloroform layer)	1.3
	R(red protonated,acidic layer)	5.2
	B2(blue alkaline isolate)	1.7

(#Solvent of extractions taken as control showed no zone of inhibitions)

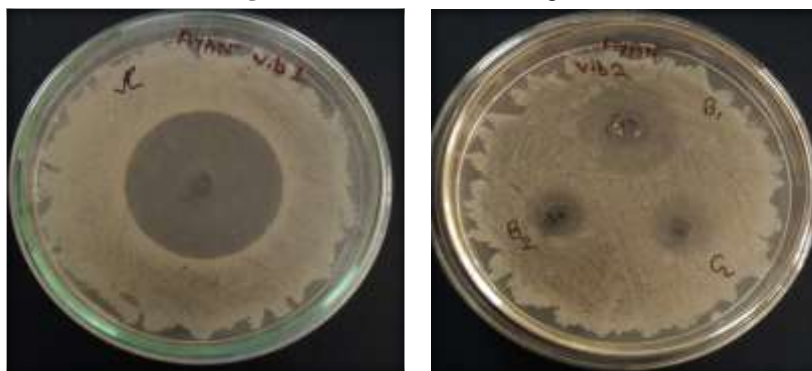
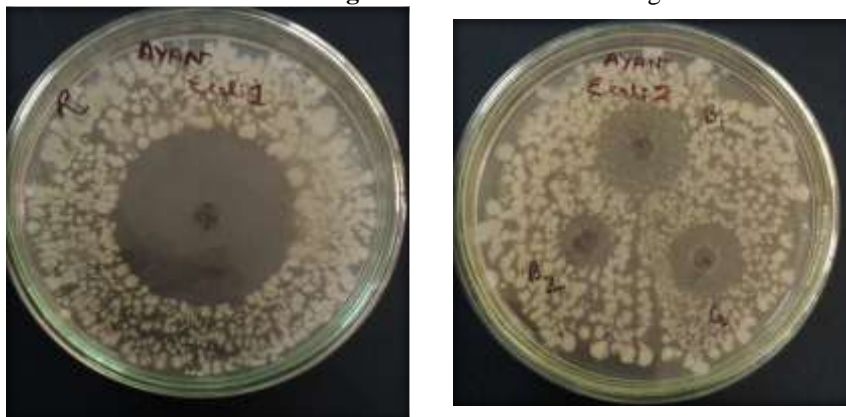
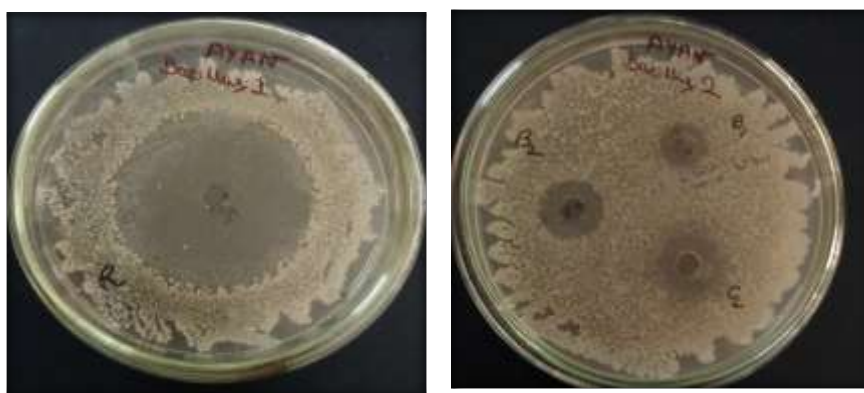
Fig 8 a:- Zone of inhibitions against *V.cholerae*

Fig 8 b:- Zone of inhibitions against *E.coli***Fig 8 c:-** Zone of inhibitions against *B.subtilis***Discussion:-**

The present work demonstrated the successful extraction of the crude natural bioactive pigment pyocyanin from *Pseudomonas aeruginosa* and showed a significant antimicrobial property against few test microbes. In our present work, it has been evaluated that among all the different forms of the pyocyanin, the red protonated state showed a maximum clear zone of inhibitions with a diameter of 4.4 cm, 4.7 cm and 5.2 cm against *Vibrio cholerae*, *E.coli*, and *Bacillus subtilis* respectively. The Gram positive bacteria (*B.subtilis*) was found to be relatively more susceptible to the antibiotic action of red (protonated) and blue (zwitterion) form of pyocyanin than Gram negative bacteria.

Conclusion:-

The study showed that the red protonated state of pyocyanin is a potential antimicrobial agent as compared to the alkaline blue zwitterionic form. This observation may offer a new insight to put forward a hypothesis that the difference in the cell wall structures between Gram positive and Gram negative bacteria might play an important role in the penetration of the Pyocyanin molecule. The thick LPS layer of Gram negative bacteria might behave as a relative barrier for the entry of the toxin at low concentration. At the same concentration the red protonated form was proved to be more potent as an antimicrobial. This wine-red state at acidic condition possessing a basic property due to one of the N atoms may explain the easy attraction of this toxin towards the negatively charged cell wall components of the Gram positive bacteria.

Funding statement:-

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors. All the research work done by the affiliated institution funding.

Competing Interests Statement:-

The authors declare that they have no competing interests.

Data Sharing Statement:-

We cannot share any unpublished data with other laboratory or person.

Acknowledgement:-

Author acknowledges Institute of Genetic Engineering for funding and affiliation. We would like to express our sincere thanks and appreciation to Mr Anirban Roy Chowdhury for his contribution in all ways. We are also thankful to other laboratory members and associated persons of IGE for their enthusiastic participation.

References:-

1. Hall S et.al , “Cellular Effects of Pyocyanin, a Secreted Virulence Factor of *Pseudomonas aeruginosa*”, 2016 Aug 9;8(8). pii: E236. PMID: 27517959 , doi: 10.3390/toxins8080236.
2. Abdul-Hussein ZR, Atia SS. Antimicrobial Effect of Pyocyanin Extracted from *Pseudomonas aeruginosa*. Eur J Exp Bio. 2016, 6:6.
3. Alexa Mari (2009) Physiology and Mechanisms of Pyocyanin Reduction in *Pseudomonas aeruginosa*. Dissertation (Ph.D.), California Institute of Technology.
4. Dietrich LE, PriceWhelan A, Petersen A, Whiteley M, Newman DK (2006) .The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. Mol Microbiol 61: 1308-1321.
5. Jasmine Lee and Lianhui Zhang .The hierarchy quorum sensing network in *Pseudomonas aeruginosa* . PMCID: PMC4286720, 2014 Sep 25. doi: 10.1007/s13238-014-0100-x
6. Baron SS, Rowe JJ (1981) Antibiotic action of pyocyanin. Antimicrob Agents Chemother 1981 Dec; 20(6): 814–820. PMCID: PMC181804
7. 1.King E. O., Ward M. K. and Raney D. E., 1954, J. Lab and Clin. Med., 44:301-307.
8. Sanaa R Oleiwi , Study the Effect of Pyocyanin Extracted from *Pseudomonas aeruginosa* on DNA Fragmentation of Human Lymphocytes Cells, Iraqi Journal of Science, 2015, Vol 56, No.2B, pp: 1366-1371.
9. Rajkumar Cheluvappa . Standardized chemical synthesis of *Pseudomonas aeruginosa*
10. Pyocyanin. PMCID: PMC4472851. 2014 Jul 8. doi: 10.1016/j.mex.2014.07.001.