To Study The Antimicrobial Potential of Pyocyanin Extracted From Pseudomonas aeruginosa.

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Abstract **- A total of 10 Isolates of Pseudomonas aruginosa were obtained from15rhizospheric soil sample of Different plant roots. The isolated colonies were identified based on their morphology and Biochemical Characteristics . Antimicrobial compounds produced by Pseudomonas aeruginosa are the secondary metabolites .These compounds are used therapeutically and some times prophylactically in the control of infectious disease .Among the different compound produced Phenazine are nitrogen containing compound produced by Pseudomonas aeruginosa . These compound have broad spectrum antibacterial activity against pathogenic bacteria like Staphylococcus aureus, Preteus vulgaries, Escherichia coli, Salmonella typhi by in vitro technique. The result indicates that strains of Pseudomonas aeruginosa showed a significant role in controlling against above pathogenic organisms**

keywords **- Rhizospheric soil , Pseudomonas , phenazine**

INTRODUCTION

Rhizosphere is the zone around the root of plant is also called as Rhizospheric region. Rhizosphere is a narrow region of soil that is directly influenced by root secretions and associated soil microorganisms. Rhizosphere contains many bacteria and other microorganisms that feed on sludge off plant cells several studies have indicates that bacteria are most numerous inhabitant of rhizosphere. Most efficient root colonizers belong to the genus*Pseudomonas, Anthrabacter, Azatobacter, Rhizobium species, Alcaligence, Micrococcus, Bacillus* etc. Nature has been a source of medicinal agent for thousands of years.

An impressive number of modern drugs have been isolated from microorganism, mainly based on their use in traditional medicine. However, an increasing role has been played bymicroorganisms in the production of antibiotics and other drugs (Fenical,1993). The importance of bacteria and fungi as sources of valuable bioactive metabolites is very well established for more than half a century. As a result, over 120 of the most important medicines (Penicillins, Cyclosporine A, Adriamycine, etc.) in use today are obtained from microorganisms (Alanis, 2005).

Microorganism have been the study of importance in recent years because of the production of novel metabolites, which exhibits antibacterial, antiviral, anti tumour as well as anticoagulant properties. Most of the current antimicrobial drugs are the derivatives of the earlier generation and microbial resistance against them, further intensify the need for new drug discovery. Acceptable options available are the metabolites of plants or animal origin, which are biocompatible, biodegradable and non-toxic in nature. These metabolites are widely studied and are produced by various groups of microorganisms like *Pseudomonas* (Chain and Mellows, 1997) and *Streptomyces* (Shanshoury*et al*., 1996) which are studied for their secondary metabolites.

*Pseudomonas aeruginosa*is gram-negative rod shaped, asporogenous, and monoflagellated bacterium. It is about 1-5µm long and 0.5 - 1.0 µm wide. *P. aeruginosa*is obligate bacteria that respire aerobically as its optimal metabolism However it can also respire anaerobically on nitrate or other alternative electron acceptors. Therefore this makes *P. aeruginosa*very invasive microorganisms and has been found in environments such as soil, water, humans, animals, plants, sewage, and hospitals (Leaderbery, J., 2000). One of the applications of *P. aeruginosa*in biotechnology is its ability to degrade aromatic hydrocarbon such as methyl benzenes, which are the by-products of petroleum industries and used as solvents for enamels and paints as well as in production of drugs and chemicals.

Methyl benzenes are considered as environmental contaminants that ubieties in atmosphere, underground and soil, and in surface water . *P. aeruginosa*can also degrade toluene, the simplest form of methyl benzene by the oxidation of methyl group to aldehyde, alcohol, acid, and convert it to catechol. Therefore it can be used in pollution control (Johnson G. R. *et al.,* 1997). Phenazines are redox-action pigments produced by these bacteria. These pigments are involved in virulence and iron acquisition (Dietrich. E. *et al.,* 2006). Pyocyanin is a water-soluble blue green, phenazine pigment produced by active cultures of *Pseudomonas aeruginosa*. Pyocyanin also has antibiotic activity toward different microorganisms, phenazine are bioactive compounds have antimicrobial activity and *P. aeruginosa* frequently produce such compounds.

Among the recognized phenazine pigments, pyocyanin, 1-hydroxy phenazine, phenazine-1-carboxamide and phenazine carboxylic acid are notable. It has been found that phenazine compounds are associated with biofilm development, such as Phenazine-1-Carboxylic acid, which is secreted by some pseudomonads, promote bacterial biofilm formation via acquisition of ferrous iron.

Pyocyanin also has significance as quorum sensing (QS) signaling molecule as well as a virulence factor for *P. aeruginosa.* Although pseudomonads are frequently reported for their pathogenicity, the ability of this microorganism to produce antimicrobial pigment opened the door of using this as biological control agent. Researchers invented an appreciable number of new antimicrobial agents in the last three decades, bacterial resistance to antimicrobial agent has also increased simultaneously common antimicrobial agents are not working properly against those infectious organisms.

The present study was designed to isolate some high pyocynin yielding *P. aeruginosa* isolate to extract antimicrobial pigment from its culture,purify the pigment, augment it production and test as antimicrobial activity against *Salmonella typhimurium* (Laine *et al.*, 1996), *Bacillus substilis*, *Proteus valgaris* and *Candida albicance* (Trugillo*et al.*, 2007).
Pseudomonas aeruginosa. Nearly 90–95% of all isolate of *P. aeruginosa* produces pyocyanin pigment, which is referred to us" blue pus". A variety of redoxactive phenazine compound are produced by strain of *P. aeruginosa*, including pyocyanin, phenazine 1- carboxylic acid and phenazine 1- carboxamide (Budzikiewicz, 1993). Pyocyanin production is abundant in medium with low iron content and playsan important role in iron metabolism. The presence of pyocyanin is easy to detect due to its blue color that turns stationary phase cultures of *P. aeruginosa*into green color. It has various pharmacologic effects on prokaryotic cell and also used to control phytopathogen (Sudhakar*et al*., 2013).
The aim of this study is to produce pyocyanin pigment from different *P. aeruginosa* strains isolated from different soil and to determine the pyocyanin production.The purpose of this study was to examine the antimicrobial activity of *Pseudomonas aeruginosa*against some bacterial pathogens of health significance such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Proteus vulgaries*, *Escherichia coli and Salmonellatyphi*by *in vitro* techniques.

MATERIAL AND METHODS

1) Sample collection :

A total of 10 soil samples were collected from different area of Akola city. About 50 gm of soil sample were collected in sterile plastic (zipper) polythene bags from different sites around the Rhizospheric soil of some inhabitant plants.

2) Isolation of *Pseudomonas aeruginosa* from rhizospheric soil :

1gm of R hizosphere soil sample was suspended in 99 ml of sterile distilled water. Samples were serially diluted and 0.1 ml of sample was spreaded on nutrient agar plates and incubated at 37°C for 24 hrs. Colonies were counted and the results were expressed as CFU/g.

3) Isolation of pathogenic bacteria:

The various target bacterial pathogens of health significance were obtained from different clinical samples on differential as well as selective media. andwere maintained by growing in Nutrient agar medium and stored as Glycerol stock at 4° C. Strains were propagated twice before use in experiments.

4. Antimicrobial Activity of Culture Supernatant against Clinical Pathogens

Screening of Antibacterial Activity of *Pseudomonas aeruginosa*isolated from rhizosphere soil by Agar well diffusion method. Antibacterial activity of *Pseudomonasaeruginosa* isolated from rhizosphere soil was tested against target bacterial pathogens of health significance like *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*,, *Salmonella typhi* and *Proteus vulgaris* by *in vitro* techniques using Muller Hinton agar plates (MHA) at 37 °C for 24 hrs.

A fresh colony of potential antibacterial *Pseudomonas aeruginosa* isolated from rhizosphere soil was inoculated in nutrient broth and incubated at 37 °C for 24 hrs. After swabbing the target pathogenic bacteria of health significance on the sterile Muller Hinton agar plates, wells of 6mm were punched for agar well diffusion assay method. Different concentration of overnight culture of potential antibacterial *Pseudomonas aeruginosa*was added to the wells of Muller Hinton agar plates. Then the plates were incubated at 37°C for 24 hrs, where upon inhibitory activity was observed as a zone of clearing around thewells. The diameter of the clearing zones was measured in mm. The experiment was done in triplicate for each pathogenic bacterium (V. Rekha, *et al.*, 2010).

Production of pyocynin:

Selected single colonies from Nutrient Agar, wereinoculated into king's B Broth (KB) (KB: Peptone 20g; Glycerol 10g; $MgSO_4$ 1.5 g; K_2PO_4 1.5 g; D.W1000 ml) and incubated for overnight at 37°C on 120 rpm rotary shaker for 24 -48 hours and were observed for color change. The Pigment was extracted using chloroform system (Mayur Gahlout, *et al.*, 2017).

Extraction and purification of pyocyanin:

King's B broth medium was used for the extraction of pigment where the organism was inoculated and incubated for 2-3 days at 35°C. The change in color of the pigment to bluish green indicated the pigment production. The color change the pigment was extracted from culture supernatants and measured based on the absorbance of pyocyanin in acidic solution at 520 nm (Baron, 1981). The broth culture was centrifuged at 5000 rpm for 10 minutes. The culture supernatants were transferred into new test tubes and extracted with chloroform (1:2) and theaqueous phase was removed. The bottom layer was reextractedwith 1 ml of 0.2 N HCl until red color change was observed. Following this, the absorbance of the pigment solution was measured using spectrophotometer at 520 nm (Mayur Gahlout, *et al.*, 2017).

Determination of antimicrobial activity of the purified pigment:

The antibacterial activity of the purified pigmentproduced by the selected isolates against different bacterial strains - *S. aureus*, *S. typhi*,*E. coli* and *P. vulgaris* – were investigated following agar well diffusion method. Mueller-Hinton Agar was used as agar medium. First the prepared media was seeded with the test organism (OD equivalent to 0.5 McFarland), poured in the petriplate, solidified and wells were made in the agar medium. Then 100 µL of each prepared pigment solutions were poured in different wells of the agar plate and incubated for 24 hours at 37ºC. After incubation the diameter of zone of growth inhibition was measured to determine the antimicrobial activity of the test agent. Each experiment was replicated trice.

RESULTS AND DISCUSSION

Observation Tables :

Table1: Isolation of Pathogens from clinical samples

Sr. No.	Clinical Samples	Isolated Strains

1	Urine	*P. vulgaris*
2	Blood	*S. typhi, E. coli*
3	Pus Samples	*S. aureus*
4	Faeces	*E. coli*

Table2 :Antimicrobial Activity of Culture supernatant of P5 isolate against clinical pathogens

Sr. No.	Clinical Isolates	Zone of inhibition in mm
1	*S. aureus*	6.2
2	*S. typhi,*	5.2
3	*E. coli*	7.7
4	*P. vulgaris*	6.0

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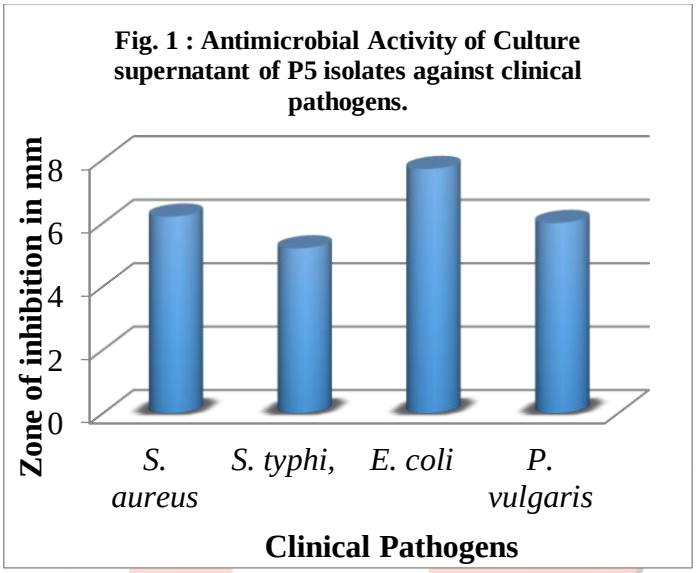


Table-3:Pyocyanin production yield by isolates

Sr. No.	Isolates	Pyocyanin Yield (µg/ml)	
		After 48 hrs	After 72 hrs
1	P1	1.20	2.80
2	P2	2.60	5.30
3	P3	1.30	2.60
4	P4	1.0	3.60
5	P5	5.80	10.90
6	P6	0.5	2.10
7	P7	1.50	4.2
8	P8	0.76	3.01
9	P9	3.25	5.0
10	P10	0.94	4.06

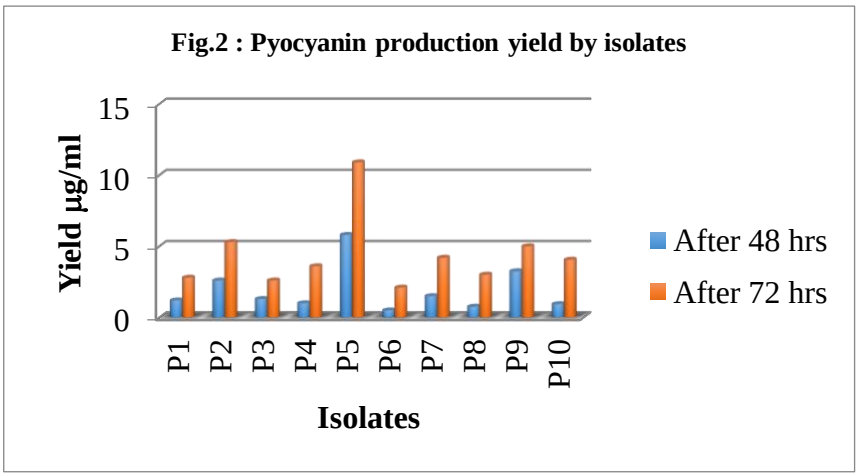
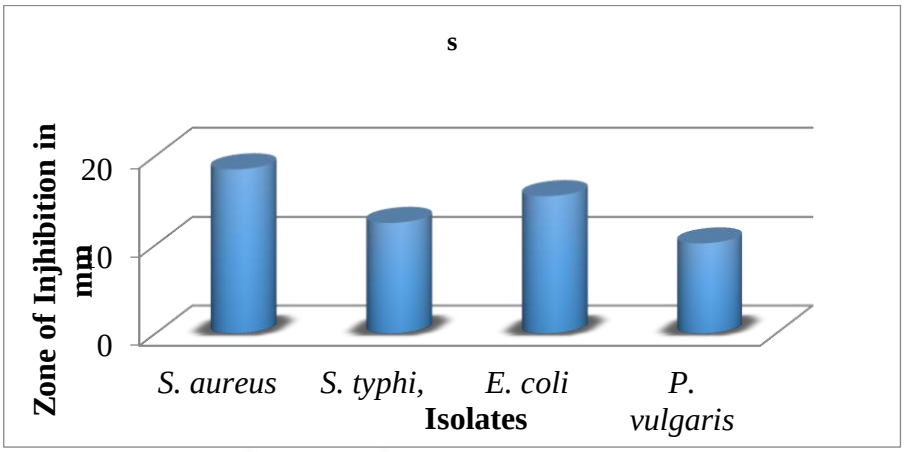


Table 7 : Antimicrobial Activity of Extracted Pyocyanin obtained from P5 isolates

Sr. No.	Clinical Isolates	Zone of inhibition in mm
1	*S. aureus*	18.5
2	*S. typhi,*	12.5
3	*E. coli*	15.5
4	*Proteus vulgaris*	10.2



Photoplate 1

Antimicrobial Activity of Culture Supernatant of P5 Isolate against Clinical Pathogens

Antimicrobial activity against *E. coli*

Antimicrobial activity against *S. aureus*

Results and Discussion

As microorganisms grow in unique and extreme habitats, they may have the capability to produce unique and unusual metabolites. So,Rhizospheric soil gives an excellent option as a source for search of some new alternative medicines.The rhizosphere of plants is heavily populated by differentmicroorganisms that also include fluorescent Pseudomonads (Weller, 1988). These have been isolated from the rhizospheres of many plants.

Characterization of fluorescent *Pseudomonads.*

In our study, we focused on the isolation of bacteria from the rhizosphere of different plants of Akola city and 10 bacterial strains were recovered, were fluorescent Pseudomonads as shown by their fluorescent characteristics on KB medium and their biochemical properties. Rhizosphere-competent bacteria are potential biocontrol agents as they may suppress pathogens and reduce disease incidence by competition, antibiosis or parasitism (Velusamy and Gnanamanickam, 2008; Chet *et al*., 1990). Fluorescent Pseudomonads that protect plants from soil-borne fungal pathogens are thought to act, in part, through the secretion of compounds with antifungal (James and Gutterson 1986; Smirnov andKiprianova, 1990) and antibacterial activity (Smirnov and Kiprianova, 1990; Veselova*et al.*, 2008).

Total ten isolates from the rhizospheric soil were characterized as fluorescent Pseudomonads on the basis of cultural, morphological and biochemical characteristics, as described in Bergey's Manual of Determinative Bacteriology (Garrity *et al.*, 2004). All were Gram negative isolates. They were positive for catalase and cytochrome oxidase, could use glucose but not lactose as a carbon source and produced pyoverdines. Three types of colony morphology was observed on the selective medium such as Cetrimide Agar, colonies with vigorous yellow-green pigmentation,with scanty pigmentation and no pigmentation. The selective media contain a quaternary ammonium compound, cetrimide, which has broad spectrum bactericidal activity against a wide range of Gram-positive and some Gram-negative microorganisms. A number of water soluble iron chelators, like the yellow-green or yellow-brown fluorescent pyoverdin are also produced by *P. aeruginosa*. The characteristic bright green colour of *P. aeruginosa*broth culture is developed when water-soluble blue pyocyanin combines with pyoverdin.

The colony morphology and cultural characteristics of the isolated organisms was identified as *Pseudomonas aeruginosa.* Gram staining and motility showed gram negative rods with actively motile organism. Pigment production was accomplished after overnight incubation. Soluble pigments namely pyocyanin production were indicated by color change in the solid media. In case of liquid media, pyocyanin production was demonstrated in shades of green color. The change in color of

the pigment to deep pink observed upon addition of chloroform and 0.2N HCl that confirmed the presence of pyocyanin. The absorbance of this solution was maximum at 520 nm. A total of 10 different of pyocyanin producing *pseudomonas* strain was isolated in pure form and characterized for their morphological characteristics(Table-1) upon screening in liquid media maximum pyocyanin production was shown by isolate P5.

Pseudomonas aeruginosa identification was confirmed by cultural and morphological observation and biochemical tests as noted in Table-2 and it was also found that all the isolates had the same characteristics. The organisms have diverse metabolic and physiological ability like catalase activity, sugar fermentation, etc.

Various clinical samples such as urine, blood, pus sample, faeces were collected for the isolation of clinical pathogens of health significance. From urine sample *P. vulgaris*, from blood sample two different pathogens namely *S. typhi* and *E. coli* were isolated. Similarly *S. aureus*from pus and *E. coli* from faeces sample were isolated.

Primary screening for the Isolates having Antimicrobial Activity :

The effect of bacterial secondary metabolites on bacterial and fungal pathogens of rice. In :Karlovsky P. (ed.). Secondary Metabholities in Soil Ecology, Soil Biology, pp 93 – 106. Springer-Verlag synthesizes various bioactive substances, among these pyocyanin is the best known for its antimicrobial activity against *S. aureus, S. typhi, P. vulgaris*, and *E. coli.* Primary screening for antimicrobial activity of *P. aeruginosa* was carried out against different test organism following Agar well diffusion method. Results revealed that *E. coli* (7.7 mm) and *S. aureus*(6.2 mm) showed visually maximum sensitivity as shown in Table 5 and Fig. 1. Whereas, *P. vulgaris* showed intermediate sensitivity and *S. typhi*was weakly sensitive. *P. aeruginosa* showed antagonistic activity against *S. aureus, E. coli, S. typhi* and *P. vulgaries* and is graphically represented in Fig. 1. Similar findings were obtained by Machan *et al.,* (1990)for the anti-staphylococcal activity of *P. aerugvinosa* with cross steak test.

PyocyninProduction :-

The pyocyanin produced from *P. aeruginoas*was variable from one another, the sample that gives the higher productivity of pyocyanin was selected. In Table 6 the results showed that the higher concentration of pigment found in isolate P5 and the optical density was determined by spectrophotometric analysis at 520 nm by 17.072 (Essar*et al.,*1990) and conc. of pyocyanin expressed as microorganisms of pyocyanin produced per milliliter of culture supernatant. Sneha*et al.,* (2008) reported that pyocyanin pigment showed a steady increase in concentration throughout the culture period of 72 h. Onbasli and Belma, (2008) showed that the highest pyocyanin production occurred after 72 h of incubation.

Antimicrobial Activity of Extracted Pyocyanin :

Previously in the primary screening process, we have found that *P. aeruginosa* have good antimicrobial activity against Gram +ve and Gram –ve bacteria. The subsequent antimicrobial activities of purified pigment shows that the bactericidal effect pyocyanin was dependent on concentration in all cases. Among the test organisms *S. aureus*was the most sensitive to pyocyanin producing 18.5 mm of inhibition zone, *E. coli* 15.5 mm, *S. typhi* 12.5mm and *P. vulgaris* showed 12.2 mm zone of inhibition respectively. From the results it is clear that Gram –ve bacteria were less sensitive than Gram positive bacteria. It was reported earlier that purified pyocyanin showed good antimicrobial properties against *S. aureus, E. coli, Klebsiells sp., S. typhi, Shigella sp., C. albicans* (Sudhakar T. *et al.,* 2013).

In future different soil ecosystem must be explored for the discovery of new strains, development of effective antimicrobial drug with novel mechanisms of action and bioactive metabolites to overcome possibilities of spreading of drug resistant and new pathogenic strain.

CONCLUSION

In the present study pyocyanin pigment was produced by P*seudomonas* P5.The pyocyanin pigment was extracted using chloroform system which produce blue colour and turn to red on addition of 0.2NHCl.sThe *Pseudomones* strain isolated during the study seemed to be highly potential in controlling bacterial pathogens of health significance.

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