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Assessment of pyocyanin activity produced by *Pseudomonas aeruginosa* against *Rhizoctonia solani* and *Macrophomina phaseolina*

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ABSTRACT

Samples were taken from different environments such as soil (195) and clinical specimens (30), burns and wounds. One hundred and eighty isolates were found belong to the genus *Pseudomonas*, sixty eight of them were identified as *Pseudomonas aeruginosa*. *P. aeruginosa* isolates were screened for pyocyanin production. The isolate B4 (from burn) was the higher pyocyanin producers reached to 7.172µg/ml. Pyocyanin extracted from production enhancing media, glycerol alanine minimal medium, and purified partially by silica gel technique. Different concentrations of pyocyanin (50, 100, 150µg/ml) were examined for their effect against plant pathogenic fungi, *Rhizoctonia solani* and *Macrophomina phaseolina*. The results showed that crude and purified pyocyanin act as antifungal activity agent against *Rhizoctonia solani* and *Macrophomina phaseolina*, *Rhizoctonia solani* suffered more inhibition. The inhibition of 150µg/ml of pyocyanin was more than other concentrations.

Keywords: *Pseudomonas aeruginosa*, pyocyanin, antifungal activity, *Rhizoctonia solani*, *Macrophomina phaseolina*.

1. INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative rod which can cause serious infections that lead to morbidity and mortality. It has adaptable capacity to survive and persist under a broad range of environmental conditions [1]. It can found in soil, marshes and coastal marine habitats, as well as in hospitals, clinical instruments, cosmetics and medical products [2].

P. aeruginosa produces many types of soluble pigments such as pyocyanin (PCN) and pyoverdin are the most common, other pigments produced are pyorubin (red), pyomelanin (brown) and pyoverdin (yellow/green) [3]. Pyocyanin was the first phenazine compound discovered in nature from *P. aeroginosa* in 1890. This pigment has various pharmacological effects on prokaryotic and eukaryotic cells, its biological activity related to similarity in the chemical structure to isoalloxazine, flavoproteins, flavin mononucleotide and flavin adenine dinucleotide compounds [4]. Thus it is used to control phytopathogens [5] and also bioprocess and downstream processing of pyocyanin for aquaculture applications [6].

The objectives of this study were: (1) Isolation and identification of *P. aeruginosa*. (2) Screening of the efficient pyocyanin producer isolate of *P. aeruginosa* via optical method. (3) Extraction and partial purification of pyocyanin pigment. (4) Investigate the activity of pyocyanin against two plant pathogenic fungi *Rhizoctonia solani* and *Macrophomina phaseolina*.

2. MATERIALS AND METHODS

2.1 Isolation of Pseudomonas *spp*.

A total of (30) clinical sample of burns and wounds were collected from patients from teaching laboratories in Baghdad. Collected samples were cultured onto blood agar media and incubated overnight at 37°C.

Soil samples (195) were collected from different soils such as garden soil, parks, farm soil, hydrocarbon contaminated soil, and poultry soil. Series of dilutions were made, 0.1ml of each dilution was cultured on cetrimide agar at 37°C for 24 hr.

2.2 Identification of *P. aeruginosa*

All clinical and soil isolates were cultured on cetrimide agar at 37°C overnight to detect growth. Isolates ability to produce pyocyanin pigment was detected on King A and King B agar. Growing colonies and pigmented colonies were re-cultured on MacConkey and blood agar at 37°C for 24 hr to detect lactose fermentation and red blood cells lysis ability [7].

Standard biochemical tests including; oxidase, catalase, gelatin liquefaction, citrate utilization as well as their capability to grow at 4 and 42°C were performed [8]. Isolates were identified depending on cultural, morphological, and biochemical characteristics [9, 10]. The Api 20E system test was performed for further detection.

2.3 The efficient pyocyanin producer

Pseudomonas aeroginosa isolates were cultured on King A and King B medium, and incubated at 37°C for 48 hr. The selected isolates, have darker blue-green pigment, were cultured in 250 ml conical flasks containing 50ml of glycerol supplemented nutrient broth medium (GSNB) and incubated at 37°C for four days [11]. After incubation, the tubes centrifuged at 10000 rpm for 10 min. The O.D measurement was performed by adding 3ml of chloroform and then re-extracted by adding 1ml of 0.2M HCl to give pink to red solution in the upper layer, the top layer was removed, and the absorbance of this solution was measured at 520nm. Concentration was determined by multiplying the optical density at 520nm by 17.072 according to the following equation [12].

Concentration of pyocyanin (μg/ml) = $O.D_{520} \times 17.072$

2.4 Extraction of pyocyanin pigment

The isolate that produced high concentration of pyocyanin was cultured on glycerol-alanine minimal media to enhance the production of pyocyanin pigment and incubated at 37°C for 72hr. The change in color from white to blue was observed, after that the plates were exposed to light source at room temperature for 24hr, this makes bacteria increase the production of its pigment [13].

Pyocyanin was extracted by adding of chloroform (95%) for two hours, blue chloroform was collected and the pyocyanin was extracted by adding (0.1M HCl). The red layer was collected and neutralized to 7 by 1M Tris base. Then addition chloroform was added to extract most of pyocyanin, pyocyanin was removed from chloroform by adding 0.05M HCl and then the pH was adjusted to 7.5 by 1M of NaOH. The final solution was derided in oven at 45°C and kept in the refrigerator [14, 15].

2.5 Purification of pyocanin pigment

The crude pyocyanin pigment previously stored in sterile containers at 4°C was re-dissolved in 5ml of D.W. and absorbed onto small quantity of silica gel (mesh size 200-500). Silica gel absorbed crude pigment was loaded on column (30 cm length × 5 cm diameter) that had been equilibrated with 1% methanol. Purified pyocyanin was eluted with 15%. The eluted fractions were examined by scanning UV-Vis spectrophotometer; fractions have the same ϫ-max were collected together and dried on a rotary evaporator at 37°C. The purified pyocyanin was subjected to spectroscopic analysis. Ultraviolet and visible absorption spectra of purified pyocyanin dissolved was recorded at 520nm.

2.6 Antifungal activity of pyocyanin

Different concentrations of purified and crude pyocyanin pigment (50,100 and 150μg/ml) were prepared with D.W.

Risoctonia solani and *Macrophomina phaseolina* fungal cells were activated on Sabouraud Dextrose Agar plates (SDA). Pyocyanin concentrations were added separately to cultured fungi then the plates were incubated at 28°C for 4-7 days.

3. RESULTS AND DISCUSSION

3.1 Isolation and characterization of *Pseudomonas aeruginosa*

One hundred and eighty isolates of *Pseudomonas* spp. where obtained from 225 soil and clinical samples. Depending on microscopic, cultural and biochemical characteristics, 68 isolates were characterized as *Pseudomonas aeruginosa,* (43) from soils and (25) from patients.

Pseudomonas aeruginosa cells appeared as gram negative small single rods, non-spore formation, without capsule and arranged in single or short chain [9, 10].

The isolates were non-lactose fermentation for their ability to grow on MacConkey agar medium with bale colonies and grape-like odor, as well they showed positive hemolysis activity on blood agar medium [7]. All 68 isolates have positive results for oxidase, catalase, gelatinase and Simmon Citrate tests, and able to grow at42°C [16, 8]. Characterization results are confirmed by Api 20E system.

3.2 Production and purification of pyocyanin pigment

Forty isolates of *P. aeruginosa* can produce blue-green pigment (pyocyanin) on King A medium, 20 from soils and 20 from clinical specimen.

Approximately 59% of the isolates were able to produce pyocyanin when they growing on King A medium, due to its sufficient concentrations of salts (Potassium and Magnesium) which accelerate pyocyanin production through enhancing the genes

encoding this pigment, and suppress production of fluorescein (pyoverdin), while king B medium contains less of these salts and it contains phosphate that inhibit pyocyanin production [17].

All isolates that have the ability to produce pyocyanin were evaluated by determination the absorbance at 520nm on Glycerol Supplemented Nutrient Broth (GSNB) [14].

Absorbance result show large amount of pyocyanin produced by B4, G1, W5, P3 and B5 isolates which gave the concentrations 7.172, 5.941, 5.326, 4.814 and 4.336µg/ml respectively (Table 1). The variation in pyocyanin production among different strains could be attributed to regulator mechanism. Liang *et al*., (2011) identified a novel regulator of the quorum sensing system in *P. aeruginosa* and called it QteE [18].

The isolate B4 from burn gave highest pyocyanin concentration and this may due to O_2 abundance in the environment of this isolate. Pyocyanin is secondary metabolite produced by *P. aeruginosa,* has aerobically metabolism, in stationary phase [19]. Moreover, O_2 induces genes that responsible for encoding pyocyanin.

Table 1: Pyocyanin concentration determined by absorbance density at 520 nm

Symbols of *P. aeruginosa* strains	Source of isolation	O.D 520 nm	Concentration µg/ml
B4	Clinical specimen (burn)	0.420	7.172 µg/ml
B5	Clinical specimen (burn)	0.254	4.336 µg/ml
W5	Clinical specimen (wounds)	0.312	5.326 µg/ml
G1	Agricultural soil from garden soil	0.348	5.941 µg/ml
P3	Agricultural soil from park soil	0.282	4.814 µg/ml

The crude pigment of selected *P. aeruginosa* isolate (B4) was purified using silica gel, so the pyocyanin fractions appeared in blue color. The blue fractions were collected, the measurement of O.D was performed at 520nm. The highest absorption was occurred at fraction number 51 (0.532nm), which was dried and stored at -20°C for antifungal application.

3.3 Antifungal Activity of Pyocyanin Pigment

Pyocyanin pigment showed antifungal activity against *Rhizoctonia solani* and *Macrophomina phaseolina*. This effect increased with increasing of its concentration (Fig. 1 and 2). Maximum inhibition was at 150µg/ml of pyocyanin concentration. Stephan, (1981) demonstrated that pyocyanin activity have concentration dependence which means that the biological activity increased when the concentration of pyocyanin increasing [20].

Pyocyanin pigment was found to be more effective against *Rhizoctonia solani* than *Macrophomina phaseolina*. The inhibition effect of purified pigment was less than crude pigment against *Rhizoctonia solani*, while the average of its effect was more than crude pyocyanin against *Macrophomina phaseolina*.

The antifungal effect of pyocyanin may relate to its ability to disrupt the electron transport chain of fungi [21]. Accordingly, Karpagam *et al*., demonstrated that pyocyanin has high antifungal effect on *C. krusei*, *C. glabrata*, *C. tropicalis*, *Cryptococcus neoformans* [22].

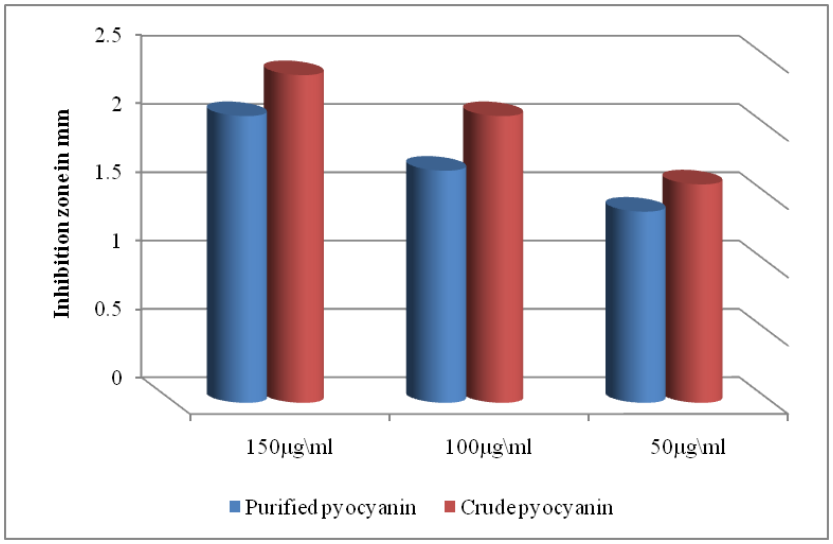

Figure 1: Antifungal activity of purify and crude pyocyanin extracted from isolate B4 against *R. solani* at different concentrations (150, 100 and 50µg/ml)

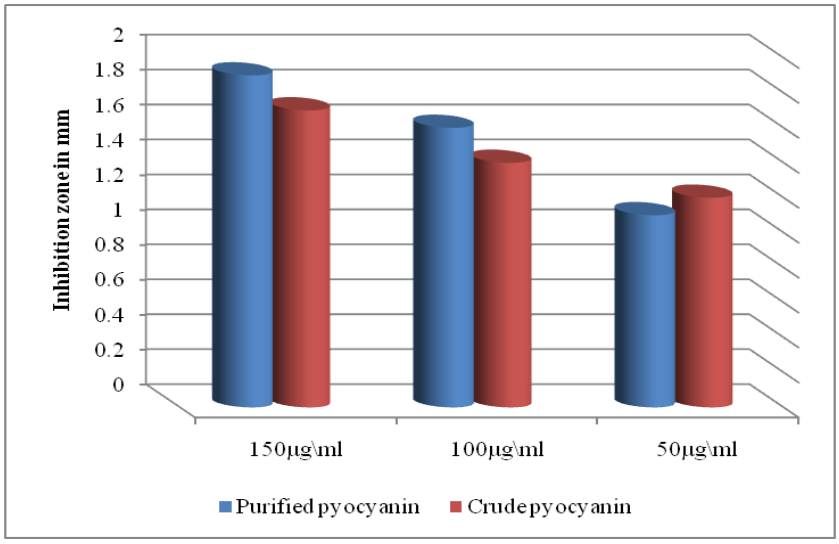

Figure 2: Antifungal activity of purify and crude pyocyanin extracted from isolate B4 against *M. phaseolina* at different concentrations (150, 100 and 50µg/ml)

4. CONCLUSION

Most of clinical (80%) and about half of soil (46.5%) *P. aeruginosa* isolates have the ability to produce pyocyanin pigment. The pyocyanin pigment has antifungal activity against some plant pathogenic fungi such as *Rhizoctonia solani* and *Macrophomina phaseolina* and the more efficient concentration of the pigment was 150µg/ml. Purified and crude pyocyanin was active against *Rhizoctonia solani* more than *Macrophomina phaseolina.*

5. REFERENCES

1. Todar K (2008). Online Textbook of Bacteriology 330 Lecture Topics: *Pseudomonas aeruginosa*. Annual Reports of Wisconsin University.
2. Franzetti L and Scarpellini M (2007). Characterization of *Pseudomonas* spp. isolated from foods. Annals of Microbiology. 57: 39-47.
3. Krieg, NR and Holt JG (2001**)**. *Bergey's Manual of Systematic Bacteriology*. Vol. 2. Williams and Wilkins Publishers, Baltimore.
4. Ohfuji K, Sato N, Hamada-Sato N et al. (2004). Construction of a glucose sensor based on a screen-printed electrode and a novel mediator pyocyanin from *Pseudomonas aeruginosa*. *Biosensors and Bioelectronics*. 19: 1237-1244.
5. Sudhakar T, Karpagam S and Shiyama S (2013). Analysis of pyocyanin compound and its antagonistic activity against phytopathogens. *International Journal of ChemTech Research*. 5: 1101-1106.
6. Priyaja P, Jayesh P, Correya N et al. (2014). Antagonistic effect of *Pseudomonas aeruginosa* isolates from various ecological niches on Vibrio species pathogenic to crustaceans. *Journal of Coastal Life Medicine*. 2: 76-84.
7. Baron ET and Finegold S (1990). Diagnostic Microbiology, 8th ed. Bailey & Scott's the C.V.Mosby, Co., USA. Pp.825.
8. Vandepitte J, Verhaegen J, Engbaek K et al. (2003). Basic laboratory procedures in clinical bacteriology (2nd Ed.). Geneva: World Health organization.
9. Collee JG Miles RS and Watt B. (1996)**.** Test for the identification of bacteria. Mackie and Macartney. Practical Medical Microbiology. 14th Edition. Churchill Livingstone, New York. pp. 413-424.
10. MacFaddin, JF (2000). Biochemical Tests for Identification of Medical Bacteria, 3rd edition. Lippicott Williams and Wilkins. USA.
11. Rahman P, Pasirayi G, Auger V et al. (2009). Development of a simple and low cost microbioreactor for high-throughput bioprocessing. *Biotechnology Letters*. 31: 209-214.
12. Essar DW, Eberly L, Hadero A et al. (1990). Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. Journal of Bacteriology. 172: 884-900.
13. Ulmer AJ, Pryjma J, Tarnok Z et al. (1990). Inhibitory and stimulatory effects of *Pseudomonas aeruginosa* pyocyanine on human T and B lymphocytes and human monocytes. *Infect. Immun.* 808-815.
14. Ingledew WM. and Campbell JR (1969). A new re-suspension medium for pyocyanin production. *Canadian Journal of Microbiology*. 15: 595-598.
15. Al-Azawi SH (2006). Cytotoxic effect of pyocyanin extracted from *Pseudomonas aeruginosa* on some human and animal cancer and normal cell lines. Ph.D. Thesis. College of Science. University of Baghdad. (In Arabic).
16. Atlas RM and Snyder JW (2006). Handbook of Media for Clinical Microbiology. 2ed. Taylor and Francis group. *CRC press*. USA.
17. Ramalho R. Cunha J. Teixeira P et al. (2002). Modified *Pseudomonsa* agar: new differential medium for the detection/ enumeration of *Pseudomonas aeruginosa* in mineral water. *Microbiological Methods*.49: 69-74.
18. Liang H, Duan J, Sibley CD et al. (2011). Identification of mutants with altered phenazine production in Pseudomonas aeruginosa. *Joumal of Medical Microbiology*. 60: 22-34.
19. Mavrodi DV, Bonsall RF, Delaney SM et al (2001). Functional Analysis of Genes for Biosynthesis of Pyocyanin and Phenazine-1 Carboxamide from Pseudomonas aeruginosa PAO1. J.Bacteriol. 183(21):6454-6465.
20. Stephen S, Baron and John J (1981). "Antibiotic action of pyocyanine" Antimicrobi. Agent Chemoth. 20(5): 814-820.
21. Wilson R, Pitt T, Taylor G et al. (1986). Pyocyanin and 1-Hydrophenazine produced by Pseudomonas aeruginosa inhibit the beating of human respiratory cilia in vitro, J. Clin. Invest. 79: 221-22

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