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Inhibition of Catheter Biofilm-Acquired Urinary Tract Infections by Purified Pyoverdine from Pseudomonas aeruginosa Isolated from Agricultural Soils

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Abstract

One of the most prevalent healthcare-acquired diseases is catheter-acquired urinary tract infection because of use of catheters can result in the introduction of bacteria into the bladder. Pyoverdine production was assessed qualitatively and quantitatively in all *Pseudomonas* spp. isolates and in comparison to *P. fluorescens, P. aeruginosa* isolates produced the most pyoverdine, ranging from 1.45 to 2.23 %. Pyoverdine was purified on a Sephadex G-150 column and produced three peaks with pyoverdine levels of 2.13 to 4.54|%. The purified pyoverdine led to inhibition of biofilm formation with 62-85 % after 24 hours and increased to 69-89 % after 48 hours. The highest percentage of inhibition was found in *Escherichia coli* isolates, followed by 77 % in *Enterococcus faecalis*, while lesser inhibition was found in *Proteus mirabilis* at 62 %

Keywords: Agricultural Soils ; pyoverdine, biofilm formation;

INTRODUCTION

Pseudomonas aeruginosa is a multidrug-resistant opportunistic bacterium that poses a serious hazard to hospitalized patients, particularly those in intensive care units (1). Patients are increasingly becoming exposed to multi- or pan-drug-resistant bacteria, affecting chronic disease therapy and resulting in acute, life-threatening infections. Antimicrobial resistance infections are linked to significant increases in morbidity and mortality, as well as significantly higher healthcare costs (2). P*seudomonas aeruginosa* is a common nosocomial bacterial pathogen that causes a wide range of infections, especially in people with immune system abnormalities, cystic fibrosis, and extensive burn scars (1, 3).

Siderophores are ferric ion-specific chelating agents with a low molecular weight (200–2000 Da) released by bacteria and fungi to get iron essential for growth(4--7). As a result, such critters produce specific precious parts that can be put to great use for the acquisition of irons as well as human improvement. These compounds are typically produced by creatures for their protection from hunters or natural selection.

One of the most prevalent healthcare-acquired diseases is catheter-acquired urinary tract infection (4,5) with 70–80 percent of these infections linked to the use of an indwelling

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urethral catheter. Biofilms act as a barrier to antimicrobial treatment and immune cell access, making treatment and clearance of nosocomial *Pseudomonas aeruginosa* infections extremely difficult(8). As a result, the goal of this study was to look into *Pseudomonas aeroginosa*'s pyoverdine production and purification, as well as its efficacy as an antibiofilm agent for treating urinary tract infections in catheterized patients.

MATERIALS AND METHODS

Contaminated soil samples collection

Twenty samples of agricultural soils were gathered and placed in sterile plastic bags from general parks in Baghdad. The bacteriological tests were completed within 4 hours of the samples being collected.

Isolation and identification of *Pseudomonas* spp.

One gram of each soil sample was combined with 99 mL of distilled water, agitated for 30 minutes, and then allowed to settle for 30 minutes. From 1 ml of soil dilution and 9 mL of distilled water, serial dilutions ranging from 10-2 to 10-6 were created. One loop full of each sample was inoculated onto MacConkey agar, Cetrimide agar, and Baird Parker agar plates, which were then incubated at 30°C for 18-24 hours. Physiological and biochemical testing was performed on the growing colonies (9). These isolates are also protected by the Vitek 2 system.

Screening for pyoverdine producers

Qualitative Assay

The ability to secrete pyoverdine was tested in all fluorescent *Pseudomonas* isolates. The detection of pyoverdine was carried out using chrome azurol S CAS agar plates, where the detection is based on the high affinity of pyoverdine for chelated iron, causing the medium's color to shift from greenish blue to orange in the presence of pyoverdine. *Pseudomonas* isolates were cultivated for 24 hours at 200 rpm and 30 °C in a succinic medium containing 4 g succinic acid, 6 g KH2PO4, 4 g K2HPO4, 0.2 g MgSO47H2O, 1 g (NH4)2SO4, and dH2O up to 1000 ml (10,11). The supernatant was then filtered through a 0.2 syringe filter after 10 mL of each culture was centrifuged at 10,000 rpm for 10 minutes. Each CAS agar plate had five wells, each of which was filled with 80 liters of culture filtrate. Plates were then incubated at 30°C for 24 hours, and pyoverdine was visually recognized.

Quantitative Assay

Pseudomonas isolates were grown for 24 hours at 200 rpm and 30°C in a succinic medium. Each culture was centrifuged for 10 minutes at 10,000 rpm in a 1.5 mL volume. The relative level of pyoverdine was determined using the CAS assay method described by (10, 12) in a set volume of supernatant (10ml). Following that, a 0.5 ml CAS assay solution was added to 10 ml of culture supernatant and thoroughly mixed, followed by 10 ml of Shuttle solution, which was thoroughly mixed and kept at room temperature for a few minutes. The existence of pyoverdine is linked to the elimination of the blue color. Using the media as a

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blank, the absorbance was measured at 630 nm. The following formula was used to compute the relative level of pyoverdine: Relative level of pyoverdine % = Ar − As/Ar × 100

Ar denotes CAS solution plus medium-plus shuttle solution absorption, whereas As denotes CAS solution plus culture supernatant plus shuttle solution absorption.

Extraction and purification

The chosen bacterial isolate was cultured in a succinic medium (production broth) at 30°C for 24 hours, after which it was centrifuged at 8000 rpm for 20 minutes. The supernatant was employed as a crude extract, and purification was done in a different way for (11). The cell-free solution of crude pyoverdines was obtained by membrane filtering the supernatant. The absorbency at 400 of the supernatant was used to estimate the amount of pyoverdine generated. At room temperature, all purification operations were carried out. 1M phosphate buffer, pH 7, was added to the cell-free supernatant. The solution was then prepared for Sephadex G-150 and eluted using the same buffer as before. The absorbency at 400 of each fraction was measured after collecting 3 ml of fractions. Furthermore. The pyoverdine-containing fractions were combined, lyophilized, and kept until needed.

Isolation and identification of bacteria from catheterized patients

Total of 24 swabs from catheterized patients in the hospital were obtained and cultured on selective media. Morphological and biochemical tests were used to diagnose the grown colonies, as well as two types of cards (GN-cards and GP-cards) in the VITEK 2 system to confirm the diagnosis.

Biofilm formation assay

According to (13) provides a detailed procedure. In a 6-well plate (2 ml/well), overnight bacterial cultures established in Luria Broth (LB) were diluted 20-fold into M9 medium. Bacteria were cultivated statically at 30°C for 24 hours. Crystal violet 0.1 % (w/v) in 20 |% (v/v) ethanol/water was used to stain biofilms. Biofilm stain was evaluated using an Elisa reader after dissolving crystal violet in a 30 % (v/v) acetic acid solution and detecting absorbance at 450 nm. The average optical density of the control well containing sterile brain heart infusion broth was used as a cut-off value. Samples with absorbance greater than the cutoff value are regarded as positive, whereas those with absorbance less than the cutoff value are considered negative.

Pyoverdine as biofilm inhibitor

To assess pyoverdine activity against biofilm formation, 125 µl of pure pyoverdine in 50 mM sodium phosphate buffer, pH 7.0, was mixed with 125 µl of selected cells suspension in a microtiter plate method. The biofilm experiment was carried out as mentioned above after a 24- and 48-hour incubation period at 30°C. The biofilm activity was replicated, as previously indicated. The percentage of biofilm inhibition was calculated using (14): **Percentage of biofilm inhibition (%) = [O.D control-O.D treatment] / O.D control x100**

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RESULTS AND DISCUSSION

Isolation and identification of *Pseudomonas* spp. from agricultural soils

The findings of culturing agricultural soils on selective media and identifying the growing colonies revealed that 7 isolates of *Pseudomonas* were found out of 20 collected samples. *Pseudomonas aeruginosa* was found in four of the isolates (57%) while *Pseudomonas fluorescens* was found in three of the isolates (43%) (figure 1).

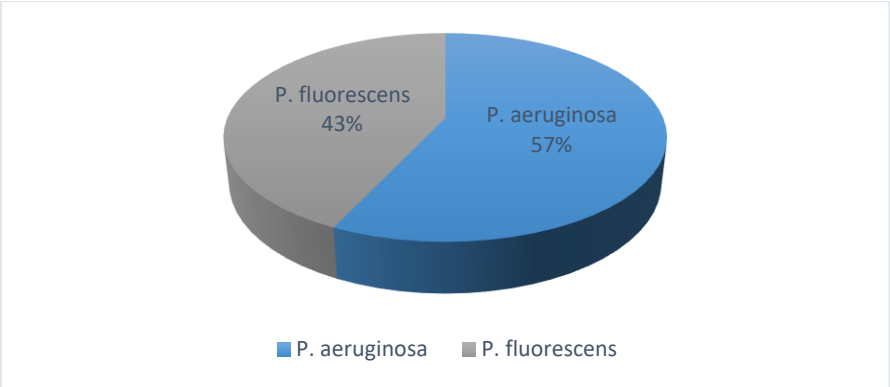

Figure 1: Percentage of *Pseudomonas* spp in agriculture soils

In Baird Parker agar, these isolates generated black colonies with no halo, while in cetrimide agar, they developed yellow-green pigment. These traits appeared to match those of *Pseudomonas aeroginosa* as described by (15). Soil, in particular, contains an incredible variety of microbes and is a productive, easily accessible, and typically safe resource for bacterial isolation. Although certain Pseudomonas species have long been recognized as plant pathogens (16), others are emerging as plant-associated growth promoters with potential biocontrol roles (17). This genus' nutritional plasticity makes it common isolation in bioremediation studies (18), and it can also be used as an enrichment approach. A typical carbon and nitrogen sources in a minimal enrichment medium exploit the degradative potential of *Pseudomonas* species and boost their abundance for easy isolation (19).

Screening for pyoverdine producers

Pyoverdine production was assessed qualitatively and quantitatively in all bacterial isolates. In the first stage, CAS agar plates were used to do a qualitative screening. Pyoverdine production was positive in all isolates that formed a yellow to an orange zone surrounding the pure colony. Furthermore, when cell-free supernatant from each culture was applied to the wells of CAS agar plates, a yellow to orange halo was noticed around the wells, indicating the formation of pyoverdine in the agar well diffusion method. Pyoverdine generated by all bacterial isolates was quantitatively quantified in liquid cultures to confirm previously observed results. In comparison to *P. fluorescens*, *P. aeruginosa* isolates produced the most pyoverdine, ranging from 1.45 to 2.23% (figure 2). *Pseudomonas* species isolated from soil were found to be capable of producing pyoverdine, which was extracted and filtered from each stock and critter. Pyoverdine was discovered in the hydroxymate group (20).

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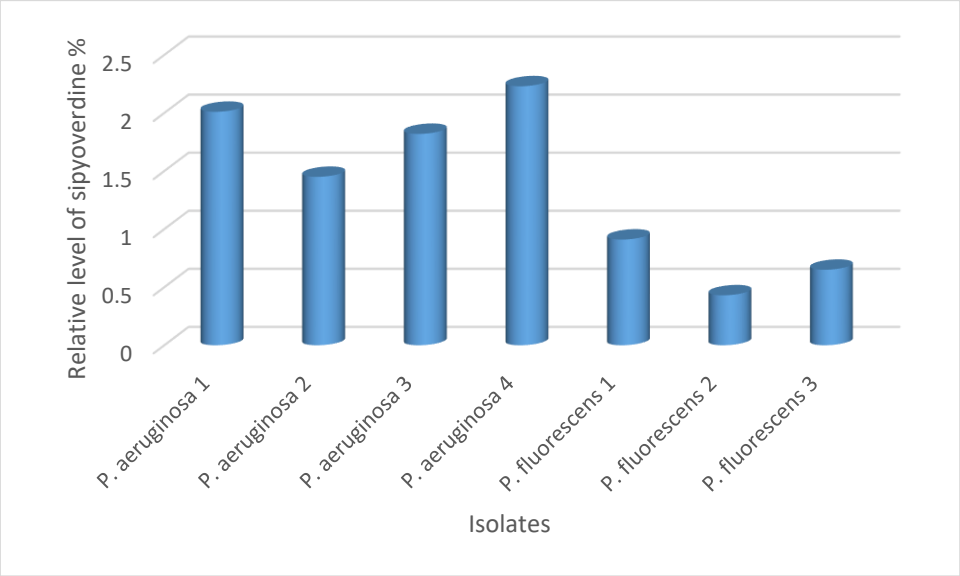

Figure 2: Screening pyoverdine production by *Pseudomonas* spp.

Extraction and purification

The chromatographic separation of *P. aeruginosa*4 pyoverdine on a sephadex G-150 column produced three peaks, as illustrated in figure(3). The relative level of pyoverdine content infractions that yielded readings at 400 nm ranged from 2.13 to 4.54 |%. The fractions were combined and lyophilized, yielding 2.15 mg of pyoverdine. Three pyoverdines, Pf-A, Pf-B, and Pf-C, were isolated from *Pseudomonas fluorescens* 2-79 using copper-chelate Sepharose and Sephadex G-15 columns, with yields of 2.8, 21.6, and 3.2 mg per 100 ml of culture supernatant, respectively (2).

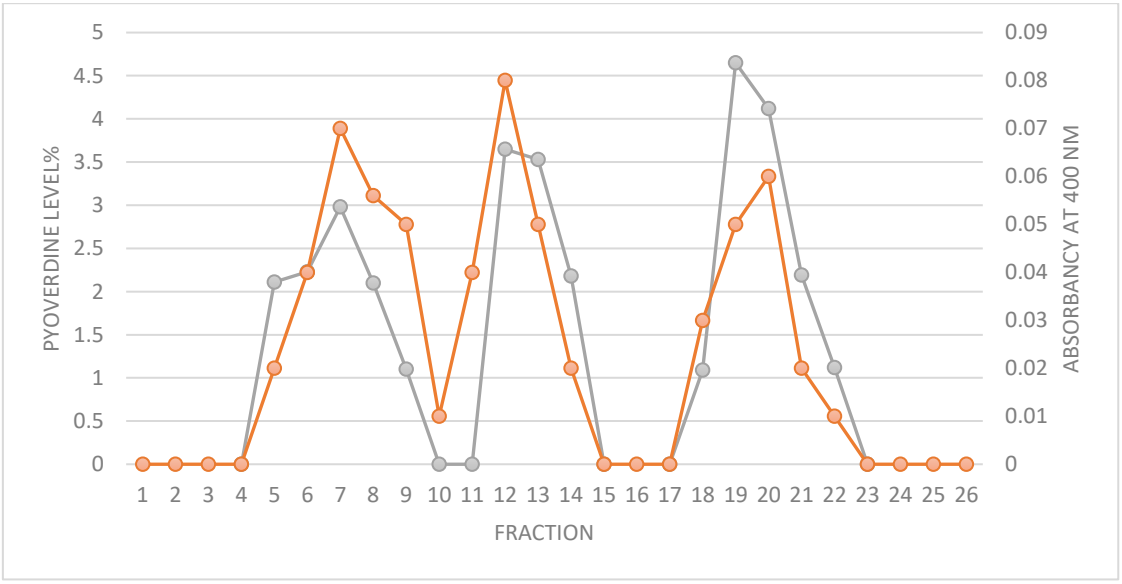

Figure 3: Purification of pyoverdine from *P. aeruginosa*4 on a sephadex G-150 column

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Isolation and identification of bacteria from catheterized patients

Three isolates of *Escherichia coli* and two isolates of *Proteus mirabilis*, one isolate of *Enterococcus faecalis*, and five *Staphylococcus epidermidis* isolates were found out of 24 cotton swab samples obtained from catheterized patients, One of the most common diseases acquired by patients in health care institutions is urinary tract infection linked to the use of an indwelling urine catheter (3). Catheterized patients' urinary tracts are particularly prone to infection. Changes in the microbiological and antibiotic sensitivity patterns of the organisms detected are recurrent concerns with these nosocomially acquired catheter-related urinary tract infections (21).

Biofilm formation assay

Only seven bacterial isolates were found to be stronger biofilm makers, with the remaining isolates classed as weak biofilm formers (table 1).

Organisms growing in biofilms are in an environment that is relatively immune to antimicrobials and host defenses. Following the implantation of an indwelling catheter, the first episode of bacteriuria is usually recognized as a single species. Polymicrobial bacteriuria becomes the norm if the catheter is left in place and a mature biofilm forms. About 3–5 organisms are commonly identified in patients with long-term indwelling catheters (22).

Pyoverdine as biofilm inhibitor

Pyoverdine activity against biofilm growth was investigated using the strongest producers. Pyoverdine isolated from *P. aeruginosa* was discovered to have antibiofilm activity against several genera. The proportion of biofilm inhibition was 62-85 % after 24 hours and increased to 69-89 %after 48 hours, according to figure (3). The highest percentage of inhibition was found in *Escherichia coli* isolates, followed by 77 percent in *Enterococcus faecalis*, while lesser inhibition was found in *Proteus mirabilis* at 62 % (table-1). Pyoverdine's main inhibitory mechanism was iron chelation from the environment, which influenced biofilm growth (23).

Table 1: Biofilm formation and its inhibition by purified pyoverdine

Isolate	Absorbency of biofilm formation	Biofilm inhibition (%) after 24h	Biofilm inhibition (%) after 48 h
E. coli 1	2.34	85	89
E. coli 2	2.12	78	83
E. coli 3	2.51	81	86
Pro. Mirabilis1	1.99	62	69
Pro. Mirabilis2	1.62	74	79
Enter. faecalis	1.11	77	82
Staphy. epidermidis 1	2.26	72	84
Staphy. epidermidis 2	1.87		
Staphy. epidermidis 3	2.78		
Staphy. epidermidis 4	1.76		
Staphy. epidermidis 5	2.02		

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CONCLUSION

The purified pyoverdine led to inhibition of biofilm formation with 62-85 % after 24 hours and increased to 69-89 % after 48 hours. The highest percentage of inhibition was found in *Escherichia coli* isolates, followed by 77 % in *Enterococcus faecalis*, while lesser inhibition was found in *Proteus mirabilis* at 62 %

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