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Antibiofilm activity of pyocin against local isolates of *Pseudomonas aeruginosa*

Zainab Z. Khalaf [1*] , Laith A. Hussein[2]
[1] Biology Department, College of Science, University of Baghdad, Baghdad, Iraq.
[2] Ministry of Education, Baghdad, Iraq.
*Corresponding email: zainab.alnaji@sc.uobaghdad.edu.iq

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ABSTRACT

Background: Pyocins are narrow-spectrum bacteriocins that kill bacteria closely related to the producing strain and play a key role in colonization and competition in bacterial communities. This study aims to investigate the antibacterial and antibiofilm activity of pyocin. **Methodology**: Twenty bacterial isolates of *Pseudomonas aeruginosa* were isolated from wounds and burns; the bacteriocin-producing isolates were detected using the cup assay method. Then, the bacteriocin was extracted from a highly producing strain, and this bacteriocin was tested against bacterial isolates other than the producing strain in both planktonic and biofilm form. The ability to form biofilm was tested using the microtiter plate method to isolate isolates that form strong biofilms. The antibiofilm activity of bacteriocin was also studied in this study using the same procedure as that used for testing biofilm. **Results**: The results showed that all bacterial isolates possess the ability to produce bacteriocin against each other, but not against their own bacteriocin, because each isolate contains an immunity gene against its bacteriocin. The results of the biofilm study showed that six isolates produced a strong biofilm, while others produced a moderate biofilm. Other findings showed that bacteriocin exhibited high activity against biofilms formed by potent isolates, with the percentage of inhibition ranging from 48 to 67%. **Conclusion**: The bacteriocin can be used to inhibit biofilm-producing isolates of the same species, making it a potential alternative to antibiotics.

Key words: Bacteriocin, pyocin, biofilm, *Pseudomonas aeruginosa*.
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INTRODUCTION

Pseudomonas aeruginosa is a common, Gram-negative, opportunistic bacterium that can infect individuals with weakened immune systems, leading to life-threatening lung illnesses (1). It is one of the primary pathogens associated with hospital infections. It can interfere with host defenses by using a wide range of pathogenicity factors. *P. aeruginosa* pathogenesis targets the extracellular matrix, modifies or disrupts host cell pathways, and enhances adhesion (2). It's also the most researched microbe in terms of quorum sensing (QS), which is regarded as a model organism for research on biofilm development (3). Biofilms are compact collections of cells that generate extracellular matrix constituents, which serve to stabilize the community, facilitating the interchange of genetic material. The biofilm mode of growth shields cells against a range of chemical and environmental stressors, including phagocyte engulfment (4). In biofilm states, *P. aeruginosa* may endure in extremely severe settings, such as hypoxic atmospheres. Furthermore, because *P. aeruginosa* rapidly adapts to acquire antibiotic resistance, treating infections caused by it can be quite challenging (5). Biofilms are less susceptible to host inflammatory and immune responses and have higher antibiotic tolerance than free-living planktonic cells. Developing treatments against biofilms requires an understanding of bacterial biofilm-specific physiological traits (6). Bacteriocins are antimicrobial peptides produced by some bacteria. Pyocins are bacteriocins produced by more than 90% of *P. aeruginosa* strains, many of which produce several pyocins. Pyocin production is dependent on increased expression of the recA gene, which is

known to be activated in response to ultraviolet light. It was also found that environmental stress and mutagenic agents may induce bacteriocin production (7). Due to the limited studies on the antibiofilm activity of bacteriocins and the multidrug resistance of biofilms, this study aimed to investigate bacteriocin-producing isolates and examine the antibacterial and antibiofilm effects of bacteriocin crude extracts against other *Pseudomonas* isolates.

METHODOLOGY

Bacterial Isolation and Identification

Different specimens were collected from patients with wound and burn infections in period from November 2018 to January 2019, using the streak plate method (8), and all samples were first cultivated in the nutrient broth at 37 °C for 18–24 hours. Afterward, they were subcultured onto MacConkey and Cetrimide agar to investigate the colony morphology (shape, size, surface texture, edge and elevation, color, opacity, etc.). Other biochemical tests performed include the Indole, Methyl Red, Voges-Proskauer, and Citrate utilization tests (IMVIC tests), as well as Triple Sugar Iron Agar (TSI) test, Motility Test, and urease test (9,10). For further identification, the Vitek 2 compact system was also used.

Detection of Bacteriocinic isolates by the cup assay

Cup assay was done according (11) as the following:Each producer isolate's loopful is vigorously streaked on Brain Heart Infusion Agar and incubated at 37˚C for eighteen hours after being cultured in Luria bertani (LB) broth overnight. Eight millimeter cork porer was used to create the discs. For each isolate plate (duplicate), the indicator isolate suspension was applied to the Muller Hinton agar surface and allowed to dry at 37°C for ten minutes. After removing the discs from the Brain Heart Infusion Agar medium, the sensitive isolate of was spread out over the Muller Hinton Agar surface, and the mixture was incubated at 37˚ C for a whole night. The zone of inhibition was evaluated in order to identify producing isolates.

Extraction of bacteriocin crude extract

Bacteriocin extraction was carried out according to (12). One hundred milliliters of sterile LB broth supplemented with 5% glycerol was inoculated in a shaker incubator with 2.5 milliliters of an overnight culture of *P. aeruginosa* isolates in LB broth. After reaching a late logarithmic phase (approximately 14 hours of incubation) at a cell density of about 3 × 10^8 cells/mL, 2 µg/mL of mitomycin C was added to the mixture, and the mixture was shaken for an additional three hours. The culture was spun for 30 minutes at 5000 rpm in a chilled centrifuge. The supernatant was extracted for protein analysis and the pyocin activity test. To ensure that any cells remaining in the supernatant were destroyed, chloroform was added. To ensure that no *P. aeruginosa* cells were present, all supernatants were cultured on Brain Heart Infusion Agar. They were then stored at 4°C until the assay.

Detection of bacteriocin activity by well assay

The method of well diffusion was used to test the antimicrobial activity according to (13). Using the streak plate method, Mueller-Hinton agar was inoculated with various selected bacterial strains. Using a sterile micropipette, 100 µL of the bacteriocin crude extract was pipetted into the wells created on the agar surface using a 6 mm cork borer. The antibacterial efficiency of the extracts was quantitatively evaluated based on the inhibition zone following a 24-hour incubation period at 37°C. Tests for antibacterial activity were conducted using a variety of isolates, including *P. aeruginosa* (except for the one producing one).

Biofilm detection

Biofilm detection of *P. aeruginosa* was carried out according to the Deka (14) method, in brief. Trypticase soy broth (TSB) was inoculated with an overnight culture of the isolate obtained from nutrient agar plates, and the results were compared to the 0.5 MacFarland standard. Subsequently, the primary inocula were injected in triplicate for each isolate into 96-well flat-bottom microtiter polystyrene plates using 1% glucose in TSB. After covering and incubating the plates for 24 hours at 37°C under aerobic conditions, the wells were decanted and then cleaned three times with regular saline. The wells were then decanted and dyed for 15 minutes using 0.1% crystal violet. Once

more, the wells were decanted and cleaned using distilled water. Ultimately, 10 minutes of 100% ethanol extraction were added to the wells to extract the stain. The optical density of each well was determined at 490 nm using an ELISA microplate reader. The degree of biofilm was calculated as follows:

Cut off OD_{490}=Negative control.

Weak biofilm= 0 cut off OD_{490} up to 2 cut off OD_{490} value

Moderate =2 cut off OD_{490} up to 4 cut off OD_{490} value

Strong biofilm with more than four cut-off OD_{490} values

Inhibitory effect of crude extract of bacteriocin on pre-formed biofilm

For the inhibition of biofilm the procedure by (15,16) was followed, the isolates of *P.aeruginosa* that form strong biofilm were selected to be assayed according to the inhibition activity of bacteriocin against planktonic cells of it on agar plates. A bacterial suspension, after incubation in tryptic soy broth, was added to a microtiter plate in a volume of 100 µL per well. After 30 minutes, 100 µl of bacteriocin extract was added to each well. The plate was incubated for 24 hours at 37 ºC.

Following incubation, D.W. was used to wash each well in order to remove any detached cells. Subsequently, each well received 200µl of 0.1% crystal violet, and the plates were shaken three times to ensure the colorant reached the bottom of each well. Following a 15-minute rest period at room temperature, 200 µl D.W. was added to each well. There were three iterations of this process. After binding to the biofilm, the crystal violet was extracted using 200µl of ethyl alcohol, and the absorbance was measured using an ELISA reader at 490 nm. Crystal violet binding was used as a control, applied only to the bacterial culture medium in the wells. The following equation was used to calculate biofilm inhibition percentage (17):

Biofilm inhibition (%) = (Control OD- Test OD / Control OD) ×100

RESULTS

Isolation and Identification of *P.aeruginosa*

Out of the samples of burns and wounds, twenty isolates were obtained. *P. aeruginosa* organisms exhibiting distinctive colony morphology were repeatedly subcultured onto Cetramide agar until a pure culture of homogeneous colonies was achieved. The colonies of *P.aeruginosa* on MacConkey agar appeared as pale or transparent colonies (Lactose non-fermenter), whereas on Cetramide agar appeared as pigmented blue, blue-green, or non-pigmented. Cetrimide increases the synthesis of pyocyanin and fluorescein pigments while inhibiting the growth of all bacteria, except *P. aeruginosa.*(18).

Screening of bacteriocin-producing isolates

To select effective isolates for bacteriocin synthesis, locally obtained *P. aeruginosa* isolates were examined. After culturing the isolates on Brain Heart Infusion Agar at 37 °C, their ability to produce bacteriocin was assessed. Muller-Hinton Agar containing the indicator isolates was then placed in wells created on the agar. To measure the antagonistic effect against the sensitive strain, the cup assay method was employed. The results, as indicated in Table (1), showed that all bacterial isolates (100%) were pyocin producers, as evidenced by the inhibition zones against the indicator isolates (P. aeruginosa, except for one isolate), as shown in Figure (1).

Table (1): Screening of bacteriocin production by *P.aeruginosa*

Indicator Isolates	Producing Isolates																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	-	-	-	-	12 +	-	-	-	-	-	12	10	15	-	12	10	15	12	8	10
2	-	-	-	-	-	-	-	-	-	-	30	-	22	-	-	-	-	-	10	12
3	-	-	-	-	-	-	-	-	-	-	10	-	-	10	-	-	-	-	8	15
4	-	-	-	-	-	-	-	-	-	10 +	-	-	-	-	-	-	-	-	-	10
5	-	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-	10
6	10	8	10	9	20	10	13	12	12	12	-	-	-	13	10	12	12		10	10
7	10	10	12	11	-	-	-	-	-	-	-	-	-	-	-	-	11	10	-	-
8	11		15	10	10	8	-	-	10	-	8	-	8	-	-	10	-	10	10	10
9	12	10	10	8	17	12	15	10		14	10	14	10	11	-	10	8	10	8	8
10	12	12	12	12	12	10	13	11	12	-	8	11	8	11	10	10	10	10	10	10
11	25	20	-	-	-	-	-	-	-	-	-	-	-	-	10		30	20	10	10
12	-	-	-	12	-	-	-	15	13	-	-	-	-	-	25	20	-	-	12	10
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	10
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	–	-	10
15	-	-	-	10	12	-	-	-	-	-	-	-	-	-	-	12	-	12	10	10
16	-	-	8	-	-	-	-	-	-	-	-	-	-	13	-	-	-	8	-	10
17	-	-	15	-	12	-	-	-	-	-	10	-	-	-	-	-	-	20	-	-
18	-	-	8	-	-	-	-	-	13	-	-	-	20	25	-	12	-	-	13	-
19	20	-	8	-	-	-	-	30	12	-	-	-	25	-	25	20	30	20	-	-
20	-	-	-	-	-	-	-	-	30	-	28	-	-	-	30	20	20	-	10	-

All 20 isolates were tested against each other using the cup assay method, and the results showed that all isolatesproduced bacteriocin (pyocin). However, the isolates that produced the highest amounts were P19 and P20.

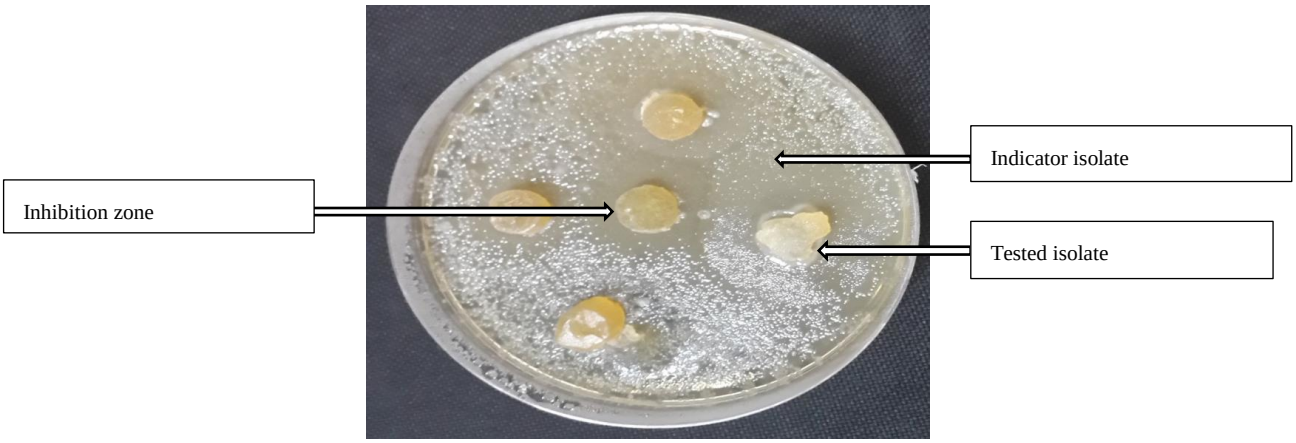

Figure (1): Cup assay for bacteriocin detection

Extraction of bacteriocin from highly producing isolate:

The highly productive isolate was achieved based on the results of the cup assay; the isolate (P20) was selected, and crude bacteriocin was extracted from it. The concentrations were then detected using the Lowry method (19). After that, the extracted bacteriocin (pyocin) was tested against other isolates by the well assay method. The results revealed that bacteriocin exhibited activity against different isolates of *P. aeruginosa* (excluding the producing isolate), as shown in Figure (2).

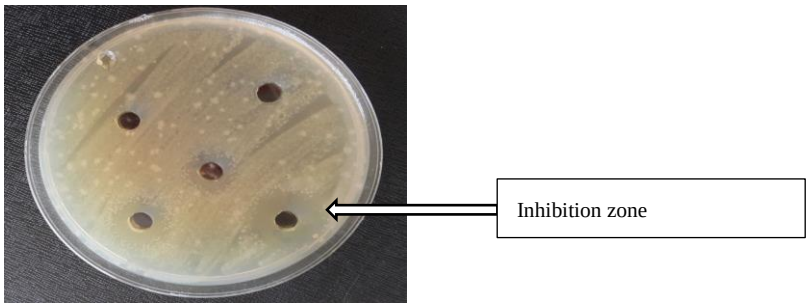

Figure (2): Well assay method for detection of pyocin activity

Biofilm formation

All *P. aeruginosa* isolates were tested for their ability to produce biofilm using the microtiter plate method Figure (3). The results showed that all bacterial isolates can form biofilms, with this ability ranging from strong to moderate, as indicated in Table (2).

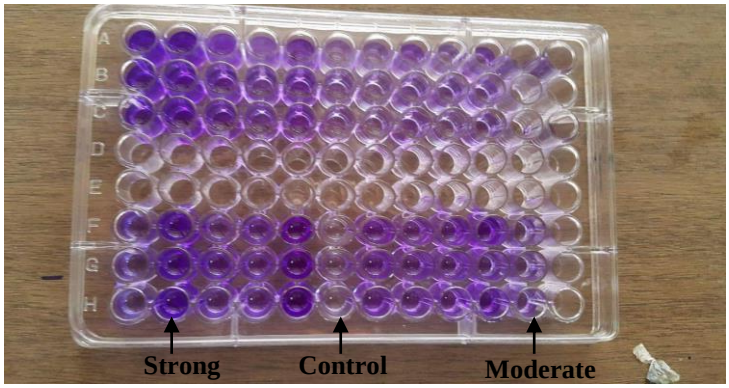

Figure (3): Detection of biofilm activity by the microtiter plate method

Table (2): Biofilm produced by *P.aeruginosa* before treatment, using microtiter plate assay

Isolates	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
O.D	0.185	0.299	0.162	0.442	0.211	0.531	0.312	0.391	0.189	0.326
Biofilm type	M	M	M	S	M	S	M	S	M	S
Isolates	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20
O.D	0.264	0.377	0.184	0.299	0.220	0.197	0.243	0.308	0.223	0.180
Biofilm producing	M	S	M	M	M	M	M	S	M	M

Antibiofilm activity of bacteriocin

The antibiofilm activity was achieved using the same method employed for detecting biofilm activity. The crude extract of bacteriocin was used as an inhibitor for the strong biofilm formed by six isolates from the twenty P. aeruginosa isolates. The results showed that bacteriocin can inhibit the biofilm formed by the six isolates (strong biofilm), albeit to varying degrees, as shown in Table (3). The percentage of inhibition ranged from 48 to 67.7%. The highly affected isolate was P8, while the least affected one was P18.

Table (3): The antibiofilm activity of bacteriocin produced by *P.aeruginosa* against other isolates of the same species

Isolates	P4	P6	P8	P10	P12	P18
O.D of Biofilm before treatment	0.442	0.531	0.391	0.326	0.377	0.308
Biofilm type	S	S	S	S	S	S
O.D of Biofilm after treatment	0.186	0.172	0.126	0.128	0.129	0.159
Biofilm type	M	M	W	W	W	M
The inhibition percentage%	57.9%	67.6%	67.7%	60.7%	65.7%	48%

DISSCUSION

Findings by Charkhian *et al.* (20) indicate that *P. aeruginosa* isolates obtained from clinical sources can produce antibacterial agents. Their bactericidal activity is attributed to the secretion of bacteriocin compounds. Doshi *et al*. (21) reported the onset of pyocin secretion after three hours of culturing. Similarly, Gholizadeh *et al*. (22) and Shokri *et al*. (23) also noted that the secretion of pyocin became detectable after 5 and 6 hours, respectively. Mitomycin C, as an inducer of pyocin production, significantly enhanced antibacterial peptide production by an average of 67.78% (2). A previous study by Saleh and Khalaf (24) reported the same result: all isolates of P. aeruginosa had the ability to form biofilms, but in different degrees, ranging from strong to moderate to weak biofilms. Microbial pathogenicity and resistance are frequently linked to the development of biofilms (25). Some investigations have shown that *P. aeruginosa* isolates from cystic fibrosis cases possess a high capacity for biofilm generation, although the underlying mechanism remains unclear.

Preventing the initiation of biofilm formation is the most crucial step for combating biofilm-associated pathogens, as the ability of pathogens to resist antibiotics is enhanced 10 to 1000 times once biofilms are formed (26). In a study by Khalaf and Flayyih (27), it was found that the bacteriocin produced by *Escherichia coli* affected the biofilm of *Escherichia coli* and other bacterial species. Another study by Hussein et al. (28) reported that

bacteriocin produced by Proteus mirabilis had antibiofilm activity against biofilm. Khalaf and Hussein (29) showed the antibiofilm activity of bacteriocin produced by *Klebsiella pneumonia*e. On the other hand, findings by Hussein and Khalaf (30) proved that klebocin (bacteriocin of Klebsiella) had antibacterial activity against planktonic cells of Staphylococcus aureus, in addition to antibiofilm activity against both premature and mature biofilms of MRSA. These previous studies were in agreement with the present study.

CONCLUSION

The current study concluded that all *P. aeruginosa* isolates possess the ability to produce bacteriocins. On the other hand, these isolates were biofilm producers, ranging from strong to moderate. This study also suggested that Bacteriocin extract can affect biofilm formation in the initial stage.

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التأثير المضاد للغشاء الحيوي للبايوسينات ضد العزلات المحلية لبكتريا الزوائف الزنجارية

زينب زامل خلف ، ليث علي حسين

قسم علوم الحياة / كلية العلوم/ جامعة بغداد / بغداد/ العراق
وزارة التربية / بغداد/ العراق

الخلاصة

خلفية عن الموضوع: البايوسينات هي بكتيريوسينات ذات طيف ضيق تقتل البكتيريا المرتبطة ارتباطًا وثيقًا بالسلالة المنتجة وتلعب دورًا رئيسيًا في الاستعمار والتنافس في المجتمعات البكتيرية. **الهدف من الدراسة:** دراسة الفعالية الضد بكتيرية وضد الغشاء الحياتي للبايوسين. **طرق ومواد العمل:** تم عزل عشرين عزلة بكتيرية من الزائفة الزنجارية من الجروح والحروق؛ تم الكشف عن العزلات المنتجة للبكتيريوسين بطريقة اختبار الكأس. بعد ذلك، تم استخراج البكتيريا من عزلة عالية الإنتاج واختبار هذه البكتيريا ضد عزلات بكتيرية أخرى غير منتجة في كل من شكل العوالق والأغشية الحيوية. تم اختبار القدرة على تكوين الأغشية الحيوية بطريقة لوحة الميكروتيتر من أجل الحصول على العزلات التي تشكل أغشية حيوية قوية. كما تم إجراء النشاط المضاد للأغشية الحيوية للباكتيريوسين في هذه الدراسة بنفس الإجراء المستخدم لاختبار الأغشية الحيوية. **النتائج :** أظهرت النتائج أن جميع العزلات البكتيرية لديها القدرة على إنتاج البكتريوسين ضد بعضها البعض ولكن ليس ضد نفسها لأن كل عزلة تحتوي على جين مناعة ضد البكتريوسين الخاص بها. أظهرت نتائج الأغشية الحيوية أن 6 عزلات لديها القدرة على إنتاج أغشية حيوية قوية بينما أنتجت عزلات أخرى أغشية حيوية معتدلة. أظهرت نتائج أخرى أن البكتريوسين أعطى نشاطًا عاليًا ضد الأغشية الحيوية التي تشكلها العزلات القوية وتراوحت نسبة التثبيط من (48 إلى 67٪). **الاستنتاجات:** يمكن استخدام البكتريوسين لتثبيط إنتاج الأغشية الحيوية لعزلات من نفس النوع حتى نتمكن من استخدامه كعامل مضاد للميكروبات بديلاً عن المضادات الحيوية.

الكلمات المفتاحية: بكتريسين، بايوسين، الغشاء الحياتي، الزوائف الزنجارية.