EVALUATION OF BIOSURFACTANT PRODUCING AND ANTIMICROBIAL RESISTANCE *PSEUDOMONAS* FOR HEAVY METALS TOLERANCE

Al-Sajad M. S. **H. A. A. Alsalim**
Researcher **Prof.**
Dept. of Biotech, Coll. Sci, University of Baghdad, Baghdad - Iraq
alsajad.sami1206@sc.uobaghdad.edu.iq **hutaf.alsalim@sc.uobaghdad.edu.iq**

ABSTRACT

This study aimed to evaluate biosurfactant production and antibiotic resistance in *Pseudomonas* bacteria isolated from some agricultural fields to detect the relationship of these isolates traits with some heavy metals resistance. Bacterial isolates were screened for biosurfactant production through blood hemolysis, oil spreading, emulsification activity, and surface tension. The antibiotic sensitivity was determined using disk diffusion method. Then, identification of the selected isolates and subjected to gradient concentrations of heavy metals to determine the minimum inhibitory concentration (MIC). Biosurfactant production was found in 74.29% of these isolates. The isolates resistance to Ticarcillin-clavulanate, Aztreonam, Piperacillin, and Imipenem were 92.86%, 31.43%, 2.86% and 1.43%, respectively. The eight selected isolates were identified by biochemical tests and VITEK 2 system as *P. aeruginosa*. The resistance of these isolates to heavy metals differed significantly. The isolate B49 recorded the highest resistance to Cu (MIC=3200 µg/ml) and Zn (MIC=2600 µg/ml), while the isolate B66 recorded the highest resistance to Cd (MIC=1000 µg/ml) and isolate B25 had higher resistance to Hg (MIC=80 µg/ml), and Pb (MIC=2800 µg/ml). The correlation coefficient between emulsification (E_{24}%) and $CdCl_2$ (r=0.27) and Pb (r=0.38) was significant positive, while E_{24}% had a significant negative correlation with Zn (r= -0.63) and non-significant correlation to copper (r=0.02) and mercury (r=0.19) resistance.

Keywords: *Pseudomonas* spp., emulsification, antibiotics, pollution, rhizosphere.

تقييم قابلية بكتريا الزائفة الزنجارية المنتجة للمستحلب الحيوي والمقاومة للمضادات الحيوية في تحمل المعادن الثقيلة

السجاد ماجد سامي **هتاف عبد الملك احمد السالم**
باحث **أستاذ**
قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد

المستخلص

هدفت الدراسة الحالية الى تقييم إنتاج المستحلب الحياتي ومقاومة المضادات الحيوية لبكتريا الـ *Pseudomonas* المعزولة من بعض الحقول الزراعية للكشف عن علاقة صفات هذه العزلات ومقاومتها لبعض المعادن الثقيلة. تم غربلة العزلات البكتيرية لإنتاج المستحلب الحياتي من خلال تحلل الدم، وانتشار الزيت، ونشاط الاستحلاب، والشد السطحي. وتم تحديد الحساسية للمضادت الحيوية باستخدام طريقة انتشار الاقراص. ثم شخصت العزلات المنتخبة وعرضت لتراكيز متدرجة للمعادن الثقيلة لتحديد أدنى تركيز مثبط (MIC). وجد ان 74.29٪ من هذه العزلات منتجة للمستحلبات الحياتية. وكانت العزلات مقاومة لـ Ticarcillin–clavulanate و Aztreonam وPiperacillin وImipenem بنسبة 92.86٪ و 31.43٪ و 2.86٪ و 1.43٪ بالترتيب. كما شخصت العزلات المنتخبة الثمانية بواسطة الاختبارات الكيموحيوية واختبار الفايتك بأنها *P. aeruginosa*. اختلفت مقاومة هذه العزلات للمعادن الثقيلة معنوياً. وسجلت العزلة B49 أعلى مقاومة للنحاس (MIC= 3200 مايكروغرام/مل) والزنك (MIC= 2600 مايكروغرام/مل)، بينما سجلت العزلة B66 مقاومة أعلى للكادميوم (MIC = 1000 مايكروغرام/مل)، و B25 مقاومة أعلى للزئبق (MIC= 80 مايكروغرام/مل) والرصاص (MIC= 2800 مايكروغرام/مل). وكان الارتباط بين الاستحلاب ومع كل من الكادميوم (r = 0.27) و الرصاص (r = 0.38) ارتباطًا إيجابيًا معنويًا ، في حين كان ارتباطًا سلبيًا عالي المعنوية مع الزنك (r= −0.63) وارتباط غير معنوي مع ومقاومة النحاس (r = 0.02) والزئبق (r = 0.19).

الكلمات المفتاحية: بكتيريا الزائفة الزنجارية، المستحلبات الحياتية، المضادات الحياتية، التلوث، مناطق الجذور.

Received: 28/3/2022, Accepted:8/6/2022

INTRODUCTION

Biosurfactants are amphiphilic substances with hydrophobic and hydrophilic parts (1,21). Surface-active molecules are synthesized commonly by bacteria, yeast, and fungi (5,13). The biological surfactants are safe, biodegradable, have good surface activity, high specificity, and are effective in stressful environments, making them eco-friendly (23, 43). *Pseudomonas* spp. are well known to produce biosurfactants such as rhamnolipids and lipopeptides biosurfactants. Also, biosurfactants can encapsulate *Pseudomonas* by micelles formation to protect them from heavy metals and harmful compounds (12, 26). Soil pollution with heavy metals is an environmental concern issue worldwide. Most endogenous soil microorganisms cannot decompose the heavy metals, and their long-term presence in soil poses a significant threat to human health (14). The biosurfactants, produced by rhizobacteria, have possible roles in mobilizing heavy metals and increasing hydrocarbons degradation in agricultural soil (39). Heavy metals in microbial environments have continuously increased, so microbes have evolved a variety of mechanisms to survive in the presence of these metals. (19,26,32). *Pseudomonas* genera are largely present in the soil, water, sediments, rhizosphere, and most genus is known to have antibiotic resistance, specifically to a class of β-lactam antibiotic (7). They are known as a degrading agent of environmental contaminants and play a role in bioremediation programs, biological control, and other applications. Multiresistant and pathogenic bacteria presence reported in soils containing organic fertilizer or soils rich in mineral nutrients and humus (4). Once infected, these pathogens can spread across the ecosystem, infecting humans, animals, and plants (32). Organic fertilizers and sewage run-off have been identified as selective pressures that may aid in the development, uptake, and spread of antibiotic resistance (16). Furthermore, using inoculums that carry antibiotic resistance can enhance the risk of their spread and be passed on to animals and humans by entering the food chain through consuming fresh vegetables or fruits (14). The presence of both heavy metals and antibiotic tolerance in soil microbes is a potential risk to human health and the environmental system (33). Hence, the environmental application that uses *Pseudomonas* isolated from polluted and agricultural fields as inoculums may be hiding a hazard of antibiotic resistance emergence. This study was amid to detect the correlation between heavy metals resistance and biosurfactants production and antibiotic sensitivity of *Pseudomonas* isolates collected from agricultural field.

MATERIALS AND METHODS

Samples collection

The soil samples were collected from many plants' rhizosphere using sterilized plastic bags until transferred to the laboratory. Different agricultural fields were targeted, including those in Karbala, Hilla, Baghdad-Abu-Ghraib, and College of Agricultural Engineering Science\University of Baghdad.

Isolation and identification of bacterial isolate: One gram of rhizospheric soil was added to 9 ml of normal saline to form the first stock test tube, followed by successive serial dilutions and the three last dilutions were used. Plates containing Cetrimide agar, King's B, and *Pseudomonas* isolation agar (PIA) were covered with 0.1 ml from the dilutions 10^{-4}, 10^{-5}, and 10^{-6}, then incubated for 48 h at 30°C. 0.1% of Nystatin, as an antifungal, has been added in the culture medium to prevent fungal growth. The suspected colonies were re-culture in the same medium and incubated for 48 h at 30°C, then cultured on MacConkey agar to detect the lactose non-ferment Gram-negative bacteria. The isolates were maintained in nutrient agar slants at 4°C for further use. *Pseudomonas* spp. were identified depending on the shape, size, texture of colonies, and pigment production. As well as, biochemical tests were performed including oxidase, catalase, Simon citrate, methyl red, voges proskauer, starch hydrolysis, indole tests, and motility.

Screening the isolates for biosurfactant production on agar medium

Hemolysis activity (HA): Hemolysis method was used to detect biosurfactant production since biosurfactants can cause erythrocyte lysis. This test was performed by streaking the bacterial isolates on nutrient agar plates supplemented with 5% (v/v) blood. After 48 hours of incubation at 30°C, the formation of a

clear area around the colonies indicated the presence of biosurfactant synthesis microorganisms (1).

Biosurfactant production in nutrient broth media: Biosurfactant production was determined in 50 ml of nutrient broth medium supplemented with 1% olive oil in Erlenmeyer flasks (250 ml). The pH of the medium was adjusted to 7 and autoclaved at 121°C, then inoculated with 2% of bacterial growth (O.D = 0.5 on McFarland; 1×10^8 CFU. ml^{-1}), the non-inoculated flask considered as a control. The flasks were incubated at 30±2°C for 96 hours in an incubator shaker set at 120 rpm. Then the broth culture was centrifuged for 15 minutes at 10,000 rpm. Biosurfactant was estimated by subjecting the supernatant to the following tests: oil spreading test (OST), emulsification activity (E_{24}%), and surface tension (ST) (18).

Measurement of oil spreading test (OST): In this test, petrol oil (1 ml) was added to the surface of a Petri dish (15 cm in diameter) containing 50 ml of distilled water to form a thin layer. Then 20 µl cell-free supernatant was carefully dropped into the center oil layer, and oil displacement occurred within 30 seconds. The zone formed has been measured to confirm the presence of the biosurfactant (36).

Measurement of the emulsification index (E_{24}%): Emulsification activity for culture supernatants was estimated by mixing 2 ml of toluene with 2 ml of culture supernatants and vortexed for 2 minutes, then incubated at 30°C for 24 hours. The emulsification index was determined using the formula below (35).

Emulsification index (E_{24}%) = (The height of the emulsion layer/The total height of the mixture) ×100

Measurement of surface tension (ST)

The surface tension of cell free supernatant was determined by a plate of Wilhelmy platinum QBZY-2 Tensiometer after device calibration by weight. A glass beaker (50 ml) was filled with 20 ml supernatant and placed on the tensiometer platform. The test value for tension appears after dipping the plate in a liquid sample at 25°C. According to the procedures described in the device guide, the platinum plate had been washed thoroughly with alcohol and burned between measurements to ensure pollutants removal. For more accurate results, the average of three values was read. The surface tension of DW, ethanol (72 mN/m), and (22 mN/m), respectively, were used for this study as a standard range (20).

Antibiotic susceptibility testing

This test was conducted using four antibiotic discs, Aztreonam (30 µg), Piperacillin (75 µg), Imipenem (30 µg), and Ticarcillin-clavulanate (85 µg). The disk diffusion method was performed following Clinical Laboratory-Standards Institute guidelines (9). The used antibiotic discs were obtained from Liofilchem (Italy). The bacterial colony regrown and then suspended in normal saline (O.D = 0.5 on McFarland; 1×10^8CFU.ml^{-1}) and spread using a cotton swab on the surface of Muller Hinton agar (Himedia, India). Finally, the antibiotic disc was put on plates agar, then incubated at 30ºC for 18 h.

Bacterial identification

Vitek 2 compact system was used to identify the selected bacterial isolates using a gram-negative (GN) card. The isolates were activated on MacConkey ager at 30°C for 24 hours then, the bacterial suspension was prepared by transferring to 3 ml of sterile saline using a disposable plastic loop. The turbidity was fixed at 0.5 OD using the McFarland standard. The kit (GN card) was added for each tube and incubated for 18 hours in the apparatus.

The minimum inhibitory concentration (MIC): The MIC refers to the lowest heavy metal concentration that inhibits bacterial growth. The bacterial isolates were exposed to the gradient concentration of $CuSO_4$, $CdCl_2$, $ZnCl_2$, $HgCl_2$, and Pb $(NO_3)_2$ on a Mueller Hinton agar plate. The concentration of heavy metals was initiated with 100 µg/ml and raised within 50 µg/ml each time, while increased within 10 µg/ml for $HgCl_2$. After 48 h of incubation at 30°C, the MIC was determined by the absence of growth (2).

Statistical Analysis

The Statistical Analysis System- SAS (37) was used to detect the differences between the studied parameters. Chi-square and T-test were used to compare percentages and means (0.05 and 0.01 probability) by LSD values. The Correlation coefficient (r) between

biosurfactant production (E_{24}%) and Heavy metals tolerance was performed by using Pearson's correlation.

RESULTS AND DISCUSSION

Isolation of *Pseudomonas* spp.

Seventy bacterial isolates were isolated from agricultural fields soil samples (Table 1) and characterized depending on the morphological, cultural, and biochemical tests. The results of all isolates were positive for oxidase, catalase, Simon citrate, and motile while negative for Gram stain, indole, Voges-Proskauer, methyl red, starch hydrolysis and lactose fermentation. Depending on Holt *et al*. (17), all the isolates belonged to *Pseudomonas* spp.

Table 1. Source and site of the bacterial isolates

Source of the sample (Plant rhizosphere)	No. of the samples	No. of the isolates	Site of the sample
Banana (*Musa* spp.)	N = 1	N = 4	Karbala (Tuwairej)
Bean (*Phaseolus vulgaris*)	N = 4	N = 9	Karbala (Tuwairej)
Alfalfa (*Medicago sativa*)	N = 2	N = 6	Karbala (Tuwairej)
Okra (*Abelmoschus esculentus*)	N = 1	N = 1	College of Agriculture
Conocarpus (*Conocarpus* spp.)	N = 2	N = 1	College of Agriculture
Mint (*Mentha* spp.)	N = 4	N = 6	Baghdad (House garden)
Alfalfa (*Medicago sativa*)	N = 2	N = 5	Hilla City
Corn (*Zea mays*)	N = 2	N = 5	Hilla City
Barley (*Hordeum vulgare*)	N = 3	N = 6	Hilla City
Soil (sewage polluted)	N = 2	N = 6	Baghdad (Abu-Ghraib)
Wheat (*Triticum aestivum*)	N = 3	N = 5	Baghdad (Abu-Ghraib)
Sunflower (*Helianthus annuus*)	N = 2	N = 6	Baghdad (Abu-Ghraib)
Jerusalem artichoke (*Helianthus tuberosus*)	N = 5	N=10	Baghdad (Abu-Ghraib)
Total		N=70	

Biosurfactant production

The isolates' ability to produce biosurfactants evaluated, by hemolysis activity, oil spreading, emulsification activity, and surface tension tests to help select higher, medium, and lower biosurfactant producer. Then find the correlation with different heavy metals resistances.

Hemolysis activity (HA)

This method was used to detect the biosurfactant producer strain as a primary assay. *Pseudomonas* spp. isolates (70) were screened on blood agar plates, 35 isolates (50%) showed β-hemolytic activity (Table 2). *Pseudomonas* is a well-known biosurfactant-producing bacteria (34). Verma *et al*. (40) proved that *P. aeruginosa* culture that exhibited β-hemolysis was able to produce biosurfactants. Another study showed that bacterial isolates that produced biosurfactants (by the E_{24}% test) had different types of hemolysis on blood agar including β-hemolysis, α-hemolysis, and γ-hemolysis with 37.5%, 50%, and 12.5%, respectively. Also, two *P. aeruginosa* isolates (PS2 and PS4) produced 60.68±1.47% and 60.77±0.79% respectively in the E24% test, were showed different hemolysis activity, β-hemolysis, and γ-hemolysis respectively (1).

Oil spreading test (OST): The oil spreading experiment was started by adding a few drops of a bacterial culture supernatant to a petroleum oil-containing plate. The displaced oil formed a clear zone in middle of the plate, 52 (74.29%) isolates were showed distinct zones, indicating a biosurfactant production. Isolates' zones ranged in diameter were between 1.2±0.20 to 9.4±0.11cm. B7 isolate revealed a great surface activity by displacing 9.4±0.11cm of oil (Figure 1-B) and (Table 2), which differs significantly from the rest isolates, followed by B56 (7.6±0.57 cm), with no significant difference from B7. Oil spreading test has been used for biosurfactant screening. It is an easy-to-do, rapid method and needs simple equipment with a small

volume of samples (1). Persson *et al*. (31) revealed that the clear zone of oil spreading is proportional to the amount of the biosurfactant produced. The strain belonging to *P. aeruginosa* SA3 isolated from wheat rhizosphere by Oluwaseun *et al*, (28) showed that better oil displacement result was occurred after 24 h of incubation and it was 4.2 cm. Another study showed that *P. aeruginosa* L10 strain isolated from reed plants gave eight cm clear zone, and can promote plant growth, degrade petroleum oil, and enable phytoremediation of the polluted site (40). Sun and his team (37) obtained similar results, where they found that *P. aeruginosa* S5 has the highest oil spread (8.83 cm).

Emulsification index (E_{24}%)

The results illustrated in Table (2) revealed that from 70 *Pseudomonas* spp. isolates, 52 (74.29%) isolates showed emulsification activity, ranging between 12.88±0.62% and 96.20±0.92%. The isolate B7 exhibited maximum emulsification activity (96.20±0.92 %) (Figure 1-B), which did not differ significantly from B56 (85.08±1.35%) but did from all the other isolates, while the isolate B62 exhibited the minimum activity (12.88±0.62%). The emulsification index is an often criterion for estimating biosurfactant production. A value ≥30% indicates significant emulsification activity (1). Sometimes biosurfactant biosynthesis is suppressed due to the production of by-products that can interface with emulsion formation and the adsorption of surfactant molecules at the oil-water interface (20). The strain CGA1 of *P. aeruginosa*, which was isolated from engine oil polluted soil by Anaukwu *et al*. (5), showed a powerful positive biosurfactant in the displacement test and the highest emulsification activity (93.3%). Zouari *et al*. (42) noticed three strains of *P. putida* out of eleven *Pseudomonas* spp. isolates had the highest emulsifying activity. They showed higher oil displacement and had an emulsion index ranging from 79.0%, for E39 and E313 strains, to 78.0% for E311 strain. A study mentioned that *P. fluorescens* showed a high emulsification index in the culture supernatant which was 90.38% (29). In another study, they showed that out of 168 screened bacterial isolates, *P. aeruginosa* (A3) showed a higher emulsification index, 52% (13).

Surface tension (ST): The surface tension was determined using a tensiometer, and the results were ranged between 63.6±0.98 to 27.86±0.30 mN/m. The isolate B7 reduced the surface tension to 27.86±0.30 mN/m (Figure 1-C), and the isolate B37 reduced it to 31.1±0.55 mN/m, while the highest surface tension was obtained by B40 (63.6±0.98 mN/m) after 96 h of incubation (Table 2). The isolate B7 showed a significant difference from that gave surface tension values more than 35.88 mN/m. The biosurfactant production varied between isolates and may be affected by the type of carbon source used. The lowest surface tension was observed in the B7 isolate when using a nutrient broth and olive oil. The highest biosurfactant production in nutrient broth may be explained by the presence of its high protein content. The criteria used to classify isolates as biosurfactant producers are the ability to decrease the surface tension of nutrient broth below 40 mN/m (18). The molecular weight of the biosurfactant plays a role in reducing the surface tension lower than 40 mN/m, and a high molecular weight can stabilize emulsions without remarkable surface tension reduction (20). Das and Kumar (10) showed that surfactant produced by *Pseudomonas* spp. AJ15 strain had lowered the surface tension as far as 30.5 mN/m. Other studies by Sun *et al*. (38) found that seven bacterial isolates isolated from wastewater had surface tension ranging between 64.7 ± 0.1 and 28.4 ± 0.2, whereas *P. aeruginosa* S5 reduced surface tension to 28.4 mN/m. While *P. aeruginosa* PG1strain isolated by Patowary *et al.* (30) decreased the surface tension to 26.7 ± 0.30 mN/m during 48 h of incubation, they noted slowly increased in surface tension during 48-120 hours. They attributed that to biosurfactant degradation during the incubation period.

Table 2. Biosurfactant production using hemolysis activity (HA), oil spreading test (OST), emulsification index (E24%), and surface tension (ST)

No. of Isolate	HA	OST (cm)	E_{24} (%)	ST (mN/m)	No. of Isolate	HA	OST (cm)	E_{24} (%)	ST (mN/m)
B1	-	-	-	-	B36	+	4.6±0.50	52.30±1.47	37.3±0.30
B2	-	-	-	-	B37	+	7.1±0.28	75.71±0.97	31.1±0.55
B3	-	-	-	-	B38	+	5.8±0.28	72.96±0.51	34.3±0.15
B4	-	-	-	-	B39	-	-	-	-
B5	-	3.2±0.26	47.91±0.61	40.1±0.62	B40	-	1.2±0.20	14.02±0.98	63.6±0.98
B6	-	-	-	-	B41	-	5.7±0.30	56.60±0.75	34.9±0.77
B7	+	9.4±0.11	96.20±0.92	27.86±0.30	B42	-	-	-	-
B8	-	3.8±0.23	45.43±0.84	39.1±0.60	B43	+	6.5±0.12	66.05±1.75	32.8±0.30
B9	+	7.1±0.17	66.62±0.08	34.4±0.32	B44	+	7.3±0.29	78.18±0.89	34.3±0.20
B10	-	2.7±0.28	38.61±1.29	42.8±0.57	B45	-	-	-	-
B11	-	-	-	-	B46	+	1.4±0.50	27.34±1.66	48.8±0.45
B12	-	-	-	-	B47	+	4.1±0.15	52.57±1.87	38.5±0.35
B13	-	7.3±0.28	78.19±0.80	33.7±0.20	B48	+	7.3±0.57	73.82±0.57	33.1±0.30
B14	-	-	-	-	B49	+	6.5±0.5	58.84±1.66	34.5±0.17
B15	-	1.2±0.25	20.31±0.08	57.6±0.2	B50	+	7.1±0.29	62.33±1.73	35.8±0.64
B16	-	1.3±0.20	33.32±1.35	55.7±0.51	B51	+	2.6±0.57	38.026±0.62	43.7±1.58
B17	-	-	-	-	B52	+	4.3±0.15	46.01±0.63	40.8±0.770
B18	-	5.3±0.28	42.35±1.4	40.1±0.35	B53	+	5.5±0.86	68.83±1.43	35.5±0.15
B19	-	-	-	-	B54	-	-	-	-
B20	-	4.6±0.57	50.42±0.06	36.4±0.12	B55	+	5.2±0.28	50.23±0.43	39.4±0.30
B21	+	4.2±0.34	45.86±0.24	38.5±0.85	B56	+	7.6±0.57	85.08±1.35	33.8±1.35
B22	-	7.1±0.18	60.98±0.83	35.1±0.30	B57	-	2.0±0.50	31.98±1.16	45.7±0.40
B23	+	5.5±0.05	53.15±0.46	35.7±1.17	B58	+	3.8±0.35	36.94±0.39	43.4±0.20
B24	+	5.7±0.26	47.69±0.69	39.4±0.15	B59	+	5.5±0.5	52.41±1.71	38.7±0.15
B25	+	6.2±0.43	60.14±0.37	34.7±0.20	B60	-	-	-	-
B26	+	5.4±0.05	53.59±0.23	38.2±0.87	B61	+	6.3±0.28	51.69±0.93	39.1±0.40
B27	-	3.9 ±0.11	47.76±1.41	39.1±0.28	B62	-	1.2±0.20	12.88±0.62	62.8±0.40
B28	-	2.3±0.57	30.39±0.14	46.7±0.58	B63	+	6.2±0.20	54.06±1.29	38.3±0.30
B29	-	2.16±0.28	30.91±0.45	43.8±0.92	B64	-	2.3±0.58	27.77±0.57	54.6±1.15
B30	-	-	-	-	B65	+	3.4±0.11	24.50±1.07	53.5±0.49
B31	-	-	-	-	B66	+	6.5±0.30	50.95±0.48	36.9±0.70
B32	+	7.3±0.35	64.47±1.72	36.2±0.55	B67	+	4.3±0.2	53.77±0.49	39.5±0.15
B33	-	-	-	-	B68	+	2.1±0.1	32.33±0.78	45.4±0.20
B34	+	5.1±0.28	55.46±1.51	37.1±0.55	B69	+	5.5±0.15	49.73±0.88	39.5±0.23
B35	+	6.2±0.20	60.42±0.12	34.7±0.72	B70	+	7.3±0.2	67.63±0.92	34.6±0.40
		LSD value** (P≤0.01)					1.952 **	13.637 **	8.027 **

Figure 1. β-hemolytic on blood agar (A) Oil displacement in petroleum oil (B), and Emulsification index in toluene (C) for B7

Antibiotic susceptibility of *Pseudomonas* spp. : The antibiotics' effect on the bacterial isolates showed high significant differences. As well, the response of bacterial isolates revealed a highly significant difference (Table 3). 65 (92%) isolates exhibited ticarcillin-clavulanate resistance, and 22 (31%) isolates aztreonam resistance. Only 1 (1.43%) and 2 (2.86%) isolates were resistant to imipenem and piperacillin, respectively. On the other hand, 69(98.57%) isolates were sensitive to imipenem and 60(85.71%) to piperacillin. Most studies on antibiotic resistance focus on clinical isolates, although environmental microorganisms have a critical parameter for susceptibility to antibiotics, whereas heavy metals (e.g., copper and zinc) pollutants have selective pressure for antimicrobial resistance (33). Camiade *et al*. (7) isolated 316 *Pseudomonas* spp. from faecal wastes and

exposed them to 25 antibiotics, less than 3% were resistant to piperacillin and imipenem, 80% were resistant to ticarcillin-clavulanate, and 90% to aztreonam. However, Luczkiewicz *et al.* (25) noted that *Pseudomonas* strains isolated from wastewater showed 54.8%, and 40.4% resistance against aztreonam and ticarcillin-clavulanate, respectively, as well as only one isolate resistant to piperacillin. Another study found only 27.7 % of the date palm *Rhizopseudomonas* isolates exhibited resistance to ticarcillin. However, the 36 isolates showed no resistance to imipenem and piperacillin (14). Estepa *et al.* (11) mentioned that twenty isolates of *Pseudomonas* spp. were exhibited high resistance to ticarcillin (85%) and less to aztreonam (30%), and imipenem (10%). Meanwhile, Khdair *et al*. (22) indicated the high resistance in fifty *Pseudomonas aeruginosa* isolated from sewage and tap water in Baghdad to ticarcillin (92%, 95%) and less to aztreonam (4%, 35%), respectively. Finally, Pitondo- Silva *et al.* (32) isolated 46 isolates of *P. aeruginosa* from soil samples and subjected them to 16 antimicrobial agents. Their results showed that 42 (91%) isolates exhibited resistance to aztreonam and 32 (70%) to ticarcillin. This *Pseudomonas* associated with rhizospheric soils showed resistance to beta-lactam antibiotics, especially ticarcillin-clavulanate and Aztreonam. This could be due to the presence of intrinsic resistance and beta-lactamase or having novel resistance in some rhizobacteria, as suggested by Ferjani *et al.* (14). Other study reported that the presence of heavy metals in the environment acts as selective pressure and leads to development resistance to both antibiotics (34).

Table 3. Antibiotic susceptibility test of *Pseudomonas* spp. isolates

The antibiotics	No. of isolates (ratio%)			P-value
	Resistance	Intermediate	Sensitive	
PC	2 (2.86%)	8(11.43%)	60(85.71%)	0.0001 **
TIM	65(92.86%)	0(0.00%)	5(7.14%)	0.0001 **
IPE	1(1.43%)	0(0.00%)	69(98.57%)	0.0001 **
ATM	22(31.43%)	17(24.29%)	31(44.29%)	0.0001 **
P-value	0.0001 **	0.0094 ** (P≤0.01)	0.0001 **	------

PC, Piperacillin; TIM, Ticarcillin-clavulanate; IPE, Imipenem; and ATM, Aztreonam

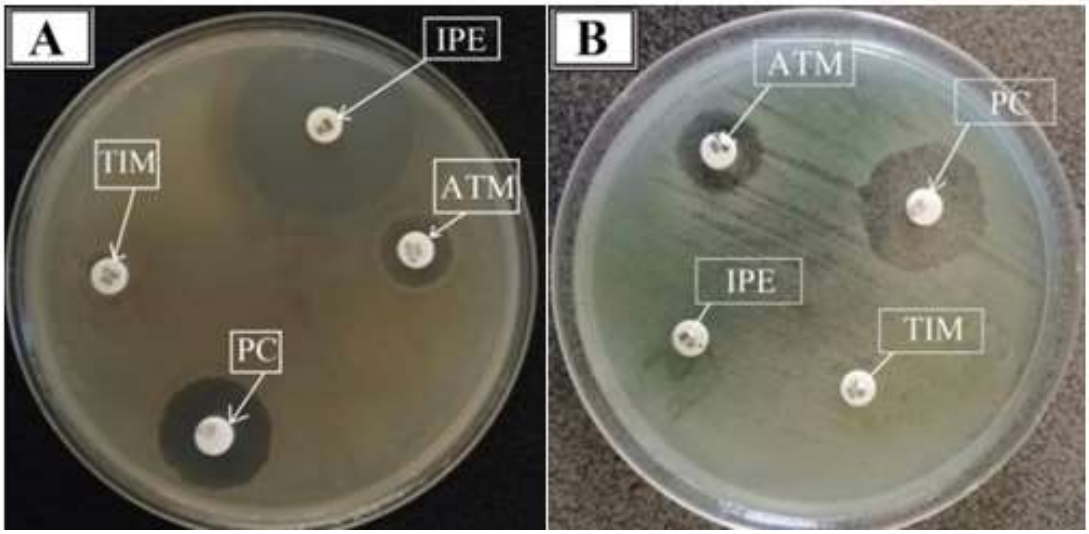

Figure 2. Antibiotic test of some isolates

Identification of bacterial isolates

Eight isolates were selected based on biosurfactant production, high, medium, and low (Table 2). B7, B25, B40, B46, B49, B50, B66, and B69 isolates were identified by Vitek 2 compact system instrument. The results showed that they belong to *Pseudomonas aeruginosa* with a probability of 97, 97, 97, 95, 98, 97, 99, and 97 %, respectively.

Heavy metals resistance

P. aeruginosa isolates were tested on Mueller-Hinton agar for resistance to $CuSO_4$, $CdCl_2$, $ZnCl_2$, $HgCl_2$, and Pb $(NO_3)_2$ at different concentration. The isolates' resistance to all the tested heavy metals differed significantly

(Table 4). The MIC for Copper ranged from 800 to 3200 µg/ml, and the isolate B49 recorded a maximum MIC (3200 µg/ml), which differs significantly high from other strains, followed by the B46 (2800 µg/ml). Cadmium MIC values ranged between 600 to 1000µg/ml, B66 showed the maximum followed by B7 (950 µg/ml) and B49 (900 µg/ml). The isolate B66 showed higher significant resistance to $CdCl_2$ compared with other strains, except B7. The MIC values for Zinc were ranged from 1050 to 2600 µg/ml, the isolate B49 (2600 µg/ml) showed a highly significant increase in resistance. Mercury MIC values ranged from 10 to 80 µg/ml, with a clear superiority for the isolate B25, which exhibited a highly significant difference from all isolates in its resistance. Finally, lead MIC values ranged from 1200–2800 µg/ml, and isolate B25 demonstrated the highest resistance that differs significantly high from other isolates. Metals resistances were appeared to be heterogeneous among *P. aeruginosa* isolates. The Cd, Hg, and Pb consider as a most toxic heavy metals which explain the lower MIC were obtain. Bacteria have evolved a variety of mechanisms to survive in the presence of these metals, active transport which can be encoded by chromosomal or plasmid. Some examples of plasmid encoding genes, mer operon for Hg^{2+} resistance in *P. stutzeri* and *czc* operons for Cd^{2+} resistance in *P. aeruginosa* to release heavy metals from the cytoplasm (19). Lal *et al.* (24) mentioned that biosurfactants could participate in heavy metal resistance. Imron *et al*. (19) reported that *Pseudomonas aeruginosa* collected from sanitary landfills had a minimum inhibitory concentration (MIC) of more than 20 mg/l for Zn, Mg, Cd, and Pb, using the disk diffusion method. Ferjani *et al*. (14) isolated 36 strains of *Pseudomonas* spp. from date palm roots and mentioned that only one isolate had resistance to $CuSO_4$ in 1800 µg/ml, while no isolates showed resistance up to 100 µg/ml for CdCl2 and $ZnCl_2$. Nath *et al*. (24) isolated eight strains of *Pseudomonas aeruginosa* from polluted field and found that lead and cadmium MIC were ranged from 400-1800 µg/ml and 80-170 µg/ml, respectively. According to Chakraborty and Das (8), the MIC of mercury, lead, and cadmium for Pseudomonas aeruginosa (strain JP-11) were 10, 400, and 1250 ppm (µg/ml), respectively. Another study (15) isolated four strains of *Pseudomonas aeruginosa* from Al-Dora farms, the isolates exhibited MIC values for Cadmium ranged between 600 and 900 mg/L. Furthermore, a study performed in Hilla by Al-Charrakh and Al-Enzi (3) isolated 43 strains of Pseudomonas aeruginosa found that their MIC for $CuSO_4$ was between 400 and 3200 µg/ml, the majority of the isolates showed 1600 µg/ml, while most of the isolates had 86.4 µg/ml and 3200 µg/ml MIC value for HgCl2, $PbNO_3$, respectively. The presence of heavy metals in the environment acts as selective pressure and leads to resistance mechanisms development to heavy metals (34).

Table 4. Heavy metal resistance (MIC) and antibiotic resistance in eight selected isolates

No.	*P. aeruginosa* isolate	$CuSO_4$ µg/ml	$CdCl_2$ µg/ml	$ZnCl_2$ µg/ml	$HgCl_2$ µg/ml	$Pb(NO_3)_2$ µg/ml
1	*B7*	1800	950	1050	20	2400
2	*B25*	2600	600	1800	80	2800
3	*B40*	1600	850	2400	10	2100
4	*B46*	2800	700	2250	40	1500
5	*B49*	3200	900	2600	30	2000
6	*B50*	2200	850	1250	60	1850
7	*B66*	2700	1000	1850	20	1200
8	*B69*	800	600	1100	20	1600
	LSD value	271.48 **	84.52 **	163.87 **	7.42 **	175.53 **

** (P≤0.01).

Correlation between emulsification index (E24%) and heavy metals resistance

Pseudomonas spp. are well known to produce biosurfactants such as rhamnolipids and lipopeptides biosurfactants. E_{24}% is one of the production indicators. This study estimated the relation between bacterial ability to produce biosurfactants represented as emulsification index (E_{24}%) and resist heavy metals (Cu, Cd, Zn, Hg, and Pb). The data (Table 5) indicated that E_{24}% has a statistically significant positive correlation with lead (r = +0.38) and cadmium (r = +0.27) resistance, which may be due to the biosurfactants' ability to encapsulate

Pseudomonas bacteria in micelles formation to protect them from heavy metals (41). E_{24}% has a non-significant correlation with copper (r = 0.02) and mercury (r = 0.19) resistance. Meanwhile, a statistically significant negative correlation was observed between E_{24}% and zinc (r = -0.63) resistance. The biosurfactant has a role in heavy metal resistance by metal complexation or sorption mechanisms (24). The negative correlation with zinc may occur by expressing unique systems, such as efflux pumps. Rizzo *et al*. (31) reported that biosurfactant production in bacteria is an adaptive strategy of cells to defend against the stress condition resulting from heavy metals. Bendaha *et al*. (6) reported that using rhamnolipids produced by *P. aeruginosa* S7PS5 is efficient in the remediation of Cd and Zn ions from the soil and mainly depends on the soil structure, texture, clay content, and cation exchange capacity.

Table 5. Correlation coefficient-r between E24% and heavy metals

The heavy metals	Correlation coefficient-r with E24 %	P-value
$CuSO_4$	0.02 NS	0.957
$CdCl_2$	0.27 *	0.0452
$ZnCl_2$	-0.63 **	0.0026
$HgCl_2$	0.19 NS	0.643
$Pb(NO_3)_2$	0.38 *	0.0309

* (P≤0.05), ** (P≤0.01).

REFERENCES

1. Adejumo, S. A., A. N. Oli, E. I. Okoye, C. D. Nwakile, C. M. Ojiako, U. M. Okezie, I. J. Okeke, C. M. Ofomata, A. A. Attama, J. N. Okoyeh and C. O. Esimone. 2021. Biosurfactant production using mutant strains of *Pseudomonas aeruginosa* and *Bacillus subtilis* from agro-industrial wastes. Advanced Pharmaceutical Bulletin, 11(3): 543-556.

2. Adekanmbi, A. O., O. O. Adelowo, Okoh, A. I., and O. E. Fagade. 2019. Metal-resistance encoding gene-fingerprints in some bacteria isolated from wastewaters of selected printeries in Ibadan, South-western Nigeria. Journal of Taibah University for Science, 13(1): 266-273

3. Al-Charrakh, A. H., and R. M. Al-Enzi. 2014. Resistance of *Pseudomonas aeruginosa* From Clinical and Environmental Sources to Heavy Metals in Hilla City, Iraq. Science Journal of University of Zakho, 2(1): 107–115.

4. Al-Salmany, S. W. K., and I. A. Ibrahim. 2021. Phytoextraction of cadmium and lead from a contaminated soil using Eucalyptus seedings. Iraqi Journal of Agricultural Sciences, 52(4): 810–827. https://doi.org/10.36103/ijas.v52i4.1390

5. Anaukwu, C. G., C. M. Ogbukagu and I. A. Ekwealor. 2020. Optimized Biosurfactant Production by *Pseudomonas aeruginosa* Strain CGA1 Using Agro-Industrial Waste as Sole Carbon Source. Advances in Microbiology, 10(10): 543–562

6. Bendaha, M. A., B. Meddah, H. Belaouni, A., M. Mokhtar and A.Tirtouil. 2016. Removal of zinc and cadmium ions from contaminated soils with rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* S7ps5. Journal of Fundamental and Applied Sciences, 8(3): 1146-1165

7. Camiade, M., J. Bodilis, N. Chaftar, W. Riah-Anglet, J. Gardères, S. Buquet, A. F. Ribeiro and B. Pawlak. 2020. Antibiotic resistance patterns of *Pseudomonas* spp. isolated from faecal wastes in the environment and contaminated surface water. FEMS Microbiology Ecology, 96(2).

8. Chakraborty, J. and S. Das. 2014. Characterization and cadmium-resistant gene expression of biofilm-forming marine bacterium *Pseudomonas aeruginosa* JP-11. Environmental Science and Pollution Research, 21(24): 14188-14201

9. Clinical and Laboratory Standards Institute. 2020. Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI supplement M100

10. Das, A. J. and R. Kumar. 2016. Bioremediation of petroleum contaminated soil to combat toxicity on Withania somnifera through seed priming with biosurfactant producing plant growth promoting rhizobacteria. Journal of Environmental Management, 174, 79-86

11. Estepa, V., B. Rojo-Bezares, C. Torres, and Y. Sáenz. 2015. Genetic lineages and antimicrobial resistance in *Pseudomonas* spp.

isolates recovered from food samples. Foodborne Pathogens and Disease, 12(6): 486-491

12. Faqri, A. M. A., N. H. Hayder and A. J. Hashim. 2019. Induction of rhamnolipid production by *Pseudomonas aeruginosa* A3 using chemical and physical mutagenic factors. Iraqi Journal of Agricultural Sciences, 50(4): 1174-1185. https://doi.org/10.36103/ijas.v50i4.766

13. Faqri, A. A. and N. H. Hayder A. J. 2019 Lab-Scale production of Rhamnolipid by *Pseudomonas aeruginosa* A3 and study its synergistic effect with certain antibiotic againts some pathogenic bacteria , Iraqi Journal of Agricultural Sciences, 50(5) :1290-1301. https://doi.org/10.36103/ijas.v50i5.794

14. Ferjani, R., H. Gharsa, V. Estepa-Pérez, E. Gómez-Sanz, M. Cherni, M. Mahjoubi, A. Boudabous, C. Torres, and H.I. Ouzari, 2019. Plant growth-promoting *Rhizopseudomonas*: expanded biotechnological purposes and antimicrobial resistance concern. Annals of Microbiology, 69(1): 51-59.

15. Ghaima, K. K., A. I. Mohamed, W. Y. Al Meshhdany and A. A. Abdulhassan. 2017. Resistance and bioadsorption of cadmium by *Pseudomonas aeruginosa* isolated from agricultural soil. International Journal of Applied Environmental Sciences, 12(9): 1649-1660

16. Guo, Y., T. Qiu, M. Gao, Y. Sun, S. Cheng, H. Gao and X. Wang. 2021. Diversity and abundance of antibiotic resistance genes in rhizosphere soil and endophytes of leafy vegetables: Focusing on the effect of the vegetable species. Journal of Hazardous Materials, 415-125595.

17. Holt, J.C. ; N.R. Krieg, 1984 .Bergeys' Manual of Systemic Bacteriology .4th (ed). William and Willkins, Baltimor. London. 9: 40-97

18. Hentati, D., A. Chebbi, F. Hadrich, I. Frikha, F. Rabanal, S. Sayadi, A. Manresa, and M. Chamkha. 2019. Production, characterization and biotechnological potential of lipopeptide biosurfactants from a novel marine *Bacillus stratosphericus* strain FLU5. Ecotoxicology and Environmental Safety, 167: 441-449.

19. Imron, M. F., S. B. Kurniawan and S. R. S. Abdullah. 2021. Resistance of bacteria isolated from leachate to heavy metals and the removal of Hg by *Pseudomonas aeruginosa* strain FZ-2 at different salinity levels in a batch biosorption system. Sustainable Environment Research, 31(1): 1-13

20. Jameel, A. A., and N. H. Haider. 2021. Determination of the optimum condition for biosurfactant production by local isolate of *Lactobacillus plantarum* and evaluate its antibacterial activity. Iraqi Journal of Agricultural Sciences, 52(1): 170-188. https://doi.org/10.36103/ijas.v52i1.1249

21. Jameel, A. A., and N. H. Haider. 2021. Study the antimicrobial and antiadhesive activity of purified biosurfactant produced from from *Lactobacillus plantarum* against pathogenic bacteria. Iraqi Journal of Agricultural Sciences, 52(5):1194–1206. https://doi.org/10.36103/ijas.v52i5.1457

22. Khdair, S. R., Hassan, A. M., Wahab, H. M. A. and N. N. Mahmood. 2017. Detection of blaCTX-M gene among *Pseudomonas aeruginosa* isolated from water samples in Baghdad. Al-Mustansiriyah Journal of Science, 28(1): 35-40

23.Kadhum, M. KH., and N. H. Haydar. 2020. Production and characterization of biosurfactant (Glycolipid) from *Lactobacillus helviticus* M5 and evaluateits antimicrobial and antiadhesive acvtivity. Iraqi Journal of Agricultural Sciences, 51(6):1543–1558. https://doi.org/10.36103/ijas.v51i6.1182

24. Lal, S., S. Ratna, O. B. Said and R. Kumar (2018). Biosurfactant and exopolysaccharide-assisted rhizobacterial technique for the remediation of heavy metal contaminated soil: an advancement in metal phytoremediation technology. Environmental Technology & Innovation, 10: 243-263

25. Luczkiewicz, A., E. Kotlarska, W. Artichowicz, K. Tarasewicz and S. Fudala-Ksiazek. 2015. Antimicrobial resistance of *Pseudomonas spp*. isolated from wastewater and wastewater-impacted marine coastal zone. Environmental Science and Pollution Research, 22(24): 19823-19834

26. Lopushniak, V. I. and H. M. Hrytsuliak. 2021. The Intensity of the Heavy Metals by Topinambur in the Conditions of the Oil-Polluted Areas. Iraqi Journal of Agricultural

Sciences, 52(6): 1334–1345. doi: 10.36103/ijas.v52i6.1473. https://doi.org/10.36103/ijas.v52i6.1473

27. Nath, S., B. Deb, I. Sharma and P. Pandey. 2014. Role of cadmium and lead tolerant *Pseudomonas aeruginosa* in seedling germination of rice (*Oryza sativa* L.). Journal of Environmental and Analytical Toxicology, 4(4): 1

28. Oluwaseun, A. C., O. J. Kola, P. Mishra, Singh, J. R., A. K. Singh, S. S. Cameotra, and B. O. Micheal. 2017. Characterization and optimization of a rhamnolipid from *Pseudomonas aeruginosa* C1501 with novel biosurfactant activities. Sustainable Chemistry and Pharmacy, 6: 26-36

29. Panjiar, N., S. G. Sachan, and A. Sachan. 2017. Biosurfactant Production by *Pseudomonas fluorescens* NCIM 2100 Forming Stable Oil-in-Water Emulsions. In Applications of Biotechnology for Sustainable Development pp: 97-107

30. Patowary, K., M. Das, R. Patowary, M. C. Kalita, and S. Deka. 2019. Recycling of bakery waste as an alternative carbon source for rhamnolipid biosurfactant production. Journal of Surfactants and Detergents, 22(2): 373-384

31. Persson, A., E. Oesterberg and M. Dostalek. 1988. Biosurfactant production by *Pseudomonas fluorescens* 378: growth and product characteristics. Applied Microbiology and Biotechnology, 29(1):1-4

32. Pitondo- Silva, A., G. B. Gonçalves and E. G. Stehling. 2016. Heavy metal resistance and virulence profile in *Pseudomonas aeruginosa* isolated from Brazilian soils. Apmis, 124(8): 681-688

33. Poole, K. 2017. At the nexus of antibiotics and metals: the impact of Cu and Zn on antibiotic activity and resistance. Trends in Microbiology, 25(10): 820-832

34. Rizzo, C., L. Michaud, M. Graziano, E. De Domenico, C. Syldatk, R. Hausmann and A. Lo Giudice. 2015. Biosurfactant activity, heavy metal tolerance and characterization of *Joostella* strain A8 from the Mediterranean polychaete Megalomma claparedei (Gravier, 1906). Ecotoxicology, 24(6): 1294-1304

35. Rasheed, H. G., and N. H. Haydar. 2023. Purification, characterization and evaluation of biological activity of Mannoprotein produced from *Saccharomyces cerevisiae* BY. Iraqi Journal of Agricultural Sciences, 54(2): 347-359. https://doi.org/10.36103/ijas.v54i2.1709 doi:10.36103/ijas.v54i2.1709.

36. Sanjivkumar, M., M. Deivakumari, and G. Immanuel. 2021. Investigation on spectral and biomedical characterization of rhamnolipid from a marine associated bacterium *Pseudomonas aeruginosa* (DKB1). Archives of Microbiology, 1-18

37.SAS. 2012. Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA

38. Sun, S., Y. Wang, T. Zang, J. Wei, H. Wu, C. Wei, G. Qiu, and F. Li. 2019. A biosurfactant-producing *Pseudomonas aeruginosa* S5 isolated from coking wastewater and its application for bioremediation of polycyclic aromatic hydrocarbons. Bioresource Technology, 281: 421-428

39. Sun, W., B. Zhu, F. Yang, M. Dai, S. Sehar, C. Peng, I. Ali and I. Naz. 2021. Optimization of biosurfactant production from *Pseudomonas* sp. CQ2 and its application for remediation of heavy metal contaminated soil. In Chemosphere, 265, 129090.

40. Verma, S., R. Prasanna, J. Saxena, V. Sharma, and L. Nain. 2012. Deciphering the metabolic capabilities of a lipase producing *Pseudomonas aeruginosa* SL-72 strain. Folia Microbiologica, 57(6): 525-531

41. Wu, T., J. Xu, W. Xie, Z. Yao, H. Yang, C. Sun, and X. Li. 2018. *Pseudomonas aeruginosa* L10: a hydrocarbon-degrading, biosurfactant-producing, and plant-growth-promoting endophytic bacterium isolated from a reed (*Phragmites australis*). Frontiers in Microbiology, 9, 1087

42. Zouari, I., F. Masmoudi, K. Medhioub, S. Tounsi, and M. Trigui. 2020. Biocontrol and plant growth-promoting potentiality of bacteria isolated from compost extract. Antonie Van Leeuwenhoek, 113(12): 2107-2122

43. Zouari, O., D. Lecouturier, A. Rochex, G. Chataigne, P. Dhulster, P. Jacques and Ghribi, D. 2019. Bio-emulsifying and biodegradation activities of syringafactin producing *Pseudomonas* spp. strains isolated from oil contaminated soils. Biodegradation, 30(4): 259-272.