Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2025.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

Comparison between the biofilm formation components by pathogenic and the soil rhizosphere zone Pseudomonas aeruginosa isolates

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Received: 19/01/2025, Accepted: 11/02/2025, Published: 30/06/2025

Abstract

The study aims to compare biofilm formation quantitatively and compositionally between two selected isolates of *Pseudomonas aeruginosa* from soil rhizosphere zone and infection cases. Fifteen isolates were isolated out of 50 samples from the root rhizosphere zone of maize plants from the fields of the College of Agriculture / University of Baghdad during November, 2023, Ten isolates from burn injuries, and 10 isolates from 25 identified strains obtained from the isolate bank / College of Biotechnology, Al-Nahrain University. Isolates from soil and infections were identified by biochemical tests and compared with identified strains. The isolates were subjected to a qualitative biofilm formation test, and 15 isolates were selected from the soil and 15 from the infections and standard strains (10 from the specimens and 5 from the pure strains (P11-P15). Statistical analysis of qualitative biofilm formation indicated to significant differences between these selected isolates. It was found also the significant differences at P-value (0.0017) among pathogenic isolates and 0.0001 among soil isolates. So, ten soil isolates and 10 pathogenic isolates were selected for quantitative test of the biofilm using the Microtiter plat method. The results of quantitative measurement of biofilm formation showed that all selected pathogenic and the soil isolates were of the strong biofilm formation category and to the significant differences at P- value (≤0.05) among pathogenic isolates and also among soil isolates, but in comparison between soil and pathogenic isolates, the most of soil isolates have significance P- values at (≤0.05) over pathogenic isolates. Soil isolate (S2) and pathogenic isolate (P10) were selected for their high concentration of biofilm formation, but soil isolate has more biofilm concentration compared with P10. Antibiotic sensitivity test using the antibiotic disc diffusion method showed that 71% of the soil isolates were resistant to all 10 antibiotics from 8 groups of antibiotics, and 62% of the pathogenic isolates. As for the selected pathogenic isolates p10 and S2 from the soil, they were resistant to 80 % of antibiotics. The results of FTIR analysis showed that the components of the biofilm of the pathogenic isolate are more complex than those of the soil isolate and that the soil isolate contains more lipids and mixed materials. The results of HPLC analysis of polysaccharides showed to the significant differences for GIcL and GaIA in soil isolate at (P ≤0.05) and (P≤0.01), respectively compared with pathogenic isolate and the absence of D-mannuronic acid ɤ-lactone (ManL) in biofilm of pathogenic isolate.

Keywords: Biofilm; *P. aeruginosa*; Soil; Infection; FTIR; HPLC

Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2025.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

Introduction

Pseudomonas aeruginosa bacterium is one among important biofilm producing opportunistic clinical and environmental bacteria[1]. It is a G-ve bacterium that can grow in a broad environment such as a host-associated and niches ecosystem because of its high ability to biofabricate of extra- multicellular mass known as biofilms. Secreted polysaccharides are an essential component *of P. aeruginosa* biofilms, and this bacterium is capable of producing three types of polysaccharides; alginate, the polysaccharide synthesis locus (*PSL*) and polysaccharide encoding locus (*PEL*)[2]. Strains of *P. aeruginosa* isolated clinically from cystic fibrosis patients have been found to overproduce each of these polysaccharides, meanwhile the environmental isolates produced primary the *PEL* and/or *PSL* in biofilm matrix formation[2,3,4]. *P. aeruginosa* explores the biofilm formation as a major strategy for survive, and the regarded as one of the major barrier in antibiotic resistance a problem for patient therapies. It was found that less than 10% of the dry mass corresponds to microorganisms biomass, whereas the remaining percent (90%) retained to the biofilm matrix [5]. In the 3D of biofilm structure, the exo-polymeric substances (EPS) function as a diffusion barrier for chemical compounds and antimicrobial agents and act as defensive mechanism protecting bacteria from unsuitable environmental conditions[6]. The biofilm also protects organisms against UV- radiation, drying, oxidizing agents, some antibiotics and biocides, metallic cations, and host immune defenses, among others[5].

Biofilms in soil consist of diverse microorganisms species engulfed in EPS, immobilizing to soil particles or roots rhizosphere and fungal hyphae and decomposing organic material[7,8]. EPS can provide numerous beneficial functions in soils such as protecting microorganisms against biotic and abiotic stresses [9]. It was found that EPS can store about 15 - 20 times its own weight in water, this led to the significance increasing in water retention of soils [10]. EPS also confers stability of soil agglomerates in which the area of the effect is mostly dependent on the features of EPS and soil physicochemical properties [11]. Finally, the *Psl* is composed of a repeating pentasaccharide of D-mannose, D-glucose, and L-rhamnose [12]. *P.aeruginosa* strain type PAO1 is mainly produces *Psl* associated to the biofilm matrix stability but it can also generate *Pel*[13]. Extracellular proteins are also regarded to be important components of biofilm, including the flagella, type IV fimbriae, cytoadhesions, and lectins. It was found that these components mainly play an assistant role as adhesion factors and structural support in the process of *P. aeruginosa* biofilm formation [14].

Materials and Methods

Collection the samples:

Number of 50 samples of soil rhizosphere zone of maize was collected from college of Agriculture, Baghdad University during November, 2023. The samples were collected in sterile screw –cupped container of 100 g capacity and transferred to laboratory. Also 25 specimens were collected from burnes of patients in Al-Yarmouk hospital during the same period in addition to 25 pathogenic strains of bacterium obtained from isolates bank, College of Biotechnology, Al-Nahrain University. The specimens were collected by swabs in ready transmitted medium in cooling box and transferred directly to the laboratory.

Isolation and identification of *P. aeruginosa*:

Decimal dilutions (10^{-1} – 10^{-7}) of soil samples were prepared in sterile saline. Volume of 0.1 ml of dilutions 10^{-4} to10^{-7} was spread on the nutrient agar medium. The plates were incubated at 30 °C for 24 h.

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Small pale colonies were selected and examined by Gram's stain. Selected colonies were streaked on MacConkey Agar (MA) and incubated under the same conditions.[15] All pathogenic specimens swabs and the isolated supplied by isolates bank were streaked directly on MA and incubated at 37 ℃ for 24 h. Selected colonies form from soil rhizosphere and pathogenic samples were selected from MA agar and inoculated on King A agar, Blood agar base and cetrimide agar. Oxidase, catalase, motility tests, and growth at 42 °C were performed. The pathogenic strains supplied by isolates bank were used as indicator for isolation and identification. All these tests were done according to Health protection agency UK [16].

Qualitative and quantitative biofilm formation:

Qualitative measurement of biofilm formation by *P .aeruginosa* isolates was conducted using Congo red agar (CRA) assay [17].

The Congo red agar test was used for qualitative measuring the capacity of biofilm formation and ability of isolates to adhesion. From each active pure isolates colonies, one loopfull was inoculated on CRA plate, then incubate at 37 °C for pathogenic isolates and 30 ℃ for soil isolates for 24–48 h. A positive result indicate either strong produce of crude black color or light black zone denote to moderate biofilm formation, whereas the negative result was denoted with developing a red or dark pink colonies.

The quantitative test for biofilm formation using Microtiter plate (MTP) assay:

Quantitative determination of biofilm formation was achieved using the microtiter plate (MTP) assay of 96 wells flat bottom array and accordance to the method of Banerjee (2014) [18]. The experiment was replicated three times accordance to the criteria in which optical density (OD) of biofilm was obtained by using the absorbance microplate (ELISA) auto-reader at wavelength 570nm [19].

Antibiotic Susceptibility Test of Selected Isolates:

Disc diffusion agar technique according to procedure CLSI (2020)[20] was used for determination the sensitivity of *P. aeruginosa* isolates toward antibiotics. Ten types of antibiotics from 8 groups were used., these are Erythromycin (Em), 10mg; Caftazidime (Cef), 30mg; Cefotaxime (Ctx), 30mg; Aztreonam (Azt), 30mg; Meropenem (Mem), 10mg; Tetracycline (TC), 10mg; Amoxicillin (Am), 10mg; Vancomycin (Va), 30mg; Levofloxacin (Lev), 5mg; Gentamicin (Gen), 10mg.

Biofilm Components Analysis

Extraction and partially purification of biofilm: The method of Auhim and Hassan (2013)[21] was applied for extraction and partially purification of biofilm. Volume of 0.1 ml from suspension of active colonies prepared at 0.5 McFarland (1.5 X 10^8 cell/ ml) in normal saline was spreading on brain heart infusion agar (BHIA) for each selected isolate and incubated for 48 h at suitable temperature. The colonies were crushed for each plate and transfer to 20 ml of deionized distilled water (ddH_2O) and mixed well. One milliliter of 0.5 M EDTA–sodium salt and 0.5 ml of 5M NaCl was added to a 20-ml of suspended cells. After being mixed for 5 min, the sample was centrifuged at 12000 rpm at 4 °C for 30 min to precipitate the cells. The cells were washed twice with distilled water, re-centrifuged and then dried at 80 °C for 24 h. The supernatant was cooled, and biofilm was then precipitated by the addition of 3 volumes of ethanol absolute,

Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2025.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

which was then recovered by centrifugation at 12000 at 4 °C for 30 min. The precipitate was dissolved in water, precipitated again, centrifuged, and then finally dried at 80°C for 24 h and weight. The dry samples were used for analysis by FTIR and HPLC instruments.

Biofilm analysis

Analysis using FTIR was done using Shimadzu launched the AIM-9000 FTIR microscope at Department of Chemistry, Science Collage, Al- Nahrain University, Iraq. Dry grinding samples of extracted biofilm from selected isolates cultures were mixed with KBr powder and made the pellets ground in a mortar and pressed. The spectral IR scanning started from 200 and 4000 cm1 [20]. HPLC analysis was conducted by Water and Environment Directorate, Ministry of Sciences and Technology, Baghdad. HPLC instrument (Sykam Company, German) was used. Mobile phase consists of Acetonitrile: Methanol (15: 85%); temp., 15- 85 ℃; flow rate, 0.3- 1.2 ml/ min. UV- detector at 254nm was used. Standard of 10 ppm of polysaccharide was used as a reference. The concentrations of sample contents were calculated as follow:

Conc. of sample (C), ppm = A sample/A St. X C St

Where A: area; St: Standard concentration (10 ppm).

A values of standard components are as follow: D-mannuronic acid ɤ - lactone (ManL), 2.000; D-Glucuronic acid ɤ - lactone (GlcL), 2.672; D- mannuronic acid (ManA), 3.232; D- glucuronic acid (GlcA), 3.563; D- mannuronic acid- D- galctronic acid (ManA- GalA), 3.810; D- galactronic acid (GalA), 5.450

Results and Discussion

Isolation and identification of *P. aeruginosa*

From 50 soil samples from maize rhizosphere root zone, 15 isolates were selected. Ten isolates from burned infection injuries were selected in addition to 10 strains from 25 strains supplied by isolates bank. These isolates were identified as *P. aeruginosa* accordingto the tests mentionedin methodology. The identified isolates and strains are non-lactose fermenting, production pyocyanin on King Agar and certrimide agar (at 42 ℃), catalase and oxidase positive, G-ve, motile and growth at 42 ℃**.** The strains were used as indicator for identification.

Qualitative and quantitative biofilm formation

The qualitative biofilm formation by selected *P. aeruginosa* isolates from soil rhizosphere samples and pathogenic isolates (specimens and strains). Biofilm formation was carried out in congo red agar. Number of 15 isolate from soil, 10 from wound injuries and 5 pure strains were selected (P11-P15) by formation the large deep black zone. Ten soil isolates (S1-S10) and ten pathogenic isolates (P1-P10) were selected for quantitative biofilm determination accordance to the statistical analysis which indicated to the no significant differences between these selected isolates comparing with remaining excluded isolates. It was found also the significant differences at P-value (0.0017) among pathogenic isolates and 0.0001 among soil isolates. The current study is corresponding to the results mentioned El-Sayed *et al*., (2020)[22] on the qualitative biofilm formation who indicated that among 187 isolates of *P. aeruginosa*, 135 (92%) were strong and moderate biofilm formation [23,24].

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Published Online First: 30/ June /2025.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

Table (2) shows the results of quantitative determination of biofilm formation using microtiter plate. Soil isolates generally produce higher biofilm concentration than pathogenic isolates. All soil and pathogenic isolates fall into the strong biofilm formation category (> 0.240) [17]. Statistical analysis in table (2) shows the significant differences at P- value (≤0.05) among pathogenic isolates and also among soil isolates, but in comparison between soil and pathogenic isolates, the most of soil isolates have significance P- values at (≤0.05) over pathogenic isolates. No significant difference between soils isolate (S2) and pathogenic isolate (P10), but soil isolate has more biofilm concentration rather than P10. There are many reasons for these differences return to the components and feature of EPS, environmental factors, source and location, the type of infection, soil composition, the period of bacterial colonization and the efficiency of host immunity[25]. Another study support and agree with the present study was mentioned, that found 96% of the clinical isolates of P. *aeruginosa* were biofilm formation[26].

Table (1): Quantitative determination of biofilm formation by *Pseudomonas aeruginosa* isolates

Isolate source	Biofilm formation at 570 nm										
	No. of isolate										
	1	2	3	4	5	6	7	8	9	10	P-value
Pathogens	0.330	0.396	0.400	0.390	0.388	0.378	0.398	0.356	0.400	0.410	0.035 *
Soil	0.434	0.498	0.440	0.330	0.200	0.336	0.332	0.376	0.376	0.318	0.0229 *
P-value	0.047 *	0.041 *	0.502 NS	0.126 NS	0.025 *	0.607 NS	0.122 NS	0.792 NS	0.,527 N	0.042 *	---
* (P≤0.05), ** (P≤0.01).											

(1): MTP/OD:< 0.120 Non-biofilm producer; 0.120-0.240 Moderate biofilm producer; >0.240 Strong biofilm producer[17].

Susceptibility test of selected isolates

Tables 3 and 4 show the results of antibiotic discs susceptibility test of soil and pathogenic isolates against 8 groups of antibiotics. The results showed that soil isolates were more resistant to antibiotics, and that 71% were resistant to all antibiotics belonging to some groups of antibiotics, while 62% of the pathogenic isolates were resistant to these antibiotics. The soil isolate S2 and the pathogenic isolate P10 were selected as the most resistant to antibiotics, with a percent of 80% for both isolates.[27] Referring to biofilm production, the two isolates were the most biofilm producing isolates (Table 1) and this confirms the existence of a direct relationship between biofilm formation and resistance to antibiotics. Statistically analysis in tables (3, 4) show a significant differences at P-value (P≤0.01). This relation was confirmed by Sousa and Pereira (2014)[28].

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Table (3): Antibiotic disc diffusion test against *Pseudomonas aeruginosa* pathogenic isolates

Antibiotic disc	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
R: (%)	60%	40%	50%	50%	70%	50%	50%	50%	60%	80%
S: (%)	30%	60%	40%	50%	30%	40%	40%	50%	30%	20%
I: (%)	10%	0%	10%	0%	0%	10%	10%	0%	10%	0%
P-value	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **
** (P≤0.01).										

1: CLSI (2020).

Table (4): Antibiotic disc diffusion test against *Pseudomonas aeruginosa* soil isolates

Antibiotic disc	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
R: (%)	60%	80%	60%	30%	70%	50%	70%	60%	60%	60%
S: (%)	30%	20%	40%	60%	30%	40%	20%	20%	20%	10%
I: (%)	10%	0%	0%	10%	0%	10%	10%	20%	20%	20%
P-value	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **	0.0001 **
** (P≤0.01).										

Biofilm Components Analysis

Fourier-transform infrared spectroscopy (FTIR)

Figures 3A and 3B show the results of FTIR analysis of the biofilm of the pathogenic isolate and the soil isolate, respectively. The results show the complexity of biofilm formation for pathogenic isolate P10 in comparison with soil isolate S2 in spite of high concentration of biofilm in S2. In the figures A and B, four major components were detected, lipids, polysaccharides, protein and extracellular DNA in addition to the mixed region of protein and polysaccharides. The IR spectrum for both biofilm samples refers to presence of lipids and mixed compounds in high concentrations in the S2 compared to the P10. Polysaccharides especially alginate is found in high quantity in P10 than in S2. The results indicate that alginate sample contain bending acetyl group in 1620.09 1/cm, stretching OH group in 3417.63 1/cm , and C-O-C bending group in 1072.35 and 1026.06 1/cm[29]. The IR spectrum for carboxyl group (OH) stretch band will appear as a broadband at approximately 3738-33669 cm^{-1} in both isolates and in P10, the

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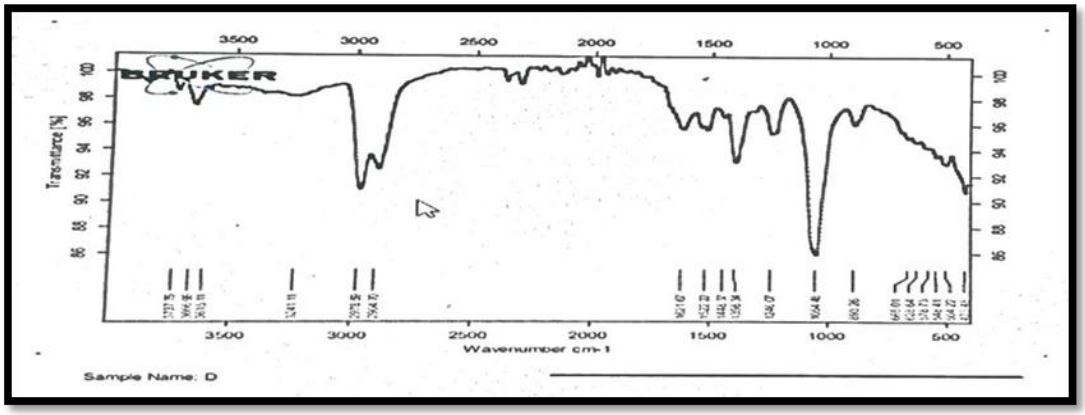
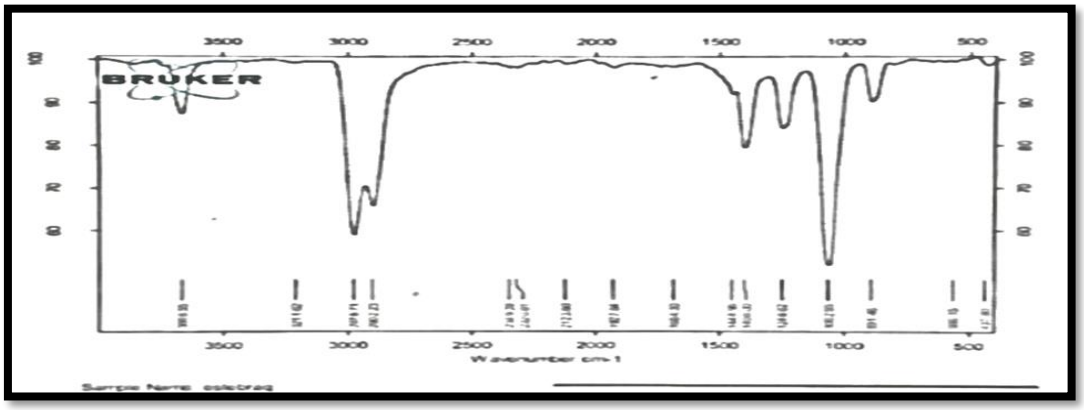
p-ISSN: 3007-5491 / e-ISSN:3007-5505

concentration is higher refers to the polysaccharides especially alginate . Amide I (C=O) stretching found in P10 and absence in S2. Amide II (N–H) bending and (C-N) stretching were observed in peaks at 1650 cm^{-1} to 1400 cm^{-1}, respectively as shown in figures and these results were agreement with the results of Arango *et al*.(2019)[30] in terms of IR analysis.



A:P10



B:S2

Figure (3): FTIR analysis of purified biofilm formation by; A: Pathogenic *Pseudomonas aeruginosa*; B: Soil rhizosphere *Pseudomonas aeruginosa.*

The separation process using a high liquid performance chromatograph (HPLC) depends on the molecular weight. The results of the HPLC analysis are shown in Table (5) that the soil isolate had high concentrations of 6 types of polysaccharide compared to the pathogenic isolate that have not contains the D-mannuronic acid ɤ-lactone (ManL) in their biofilm. Statistically analysis shows the significant differences for GIcL and GaIA in soil isolate at ($P \leq 0.05$) and ($P \leq 0.01$), respectively compared with pathogenic isolate. It was concluded from the results of the quantitative analysis and the components of the biofilm, that the soil isolation is more efficient than the pathogenic isolate, whether in producing the biofilm or its components. This may be attributed to the soil environment and the intense competition between

Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2025.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

groups of microorganisms, whether pathogenic or beneficial. This leads to the occurrence of genetic adaptation for the purpose of the bacteria continuing to survive in this environment.

Table (5): HPLC analysis of polysaccharides content of biofilm in soil and pathogenic *Pseudomonas aeruginosa* bacterium

Compound	Conc. (ppm) / Isolate		P-value
	P10	S2	
GIcL	1.345	2.350	0.0361 *
ManA	3.100	3.450	0.084 NS
GIcA	3.100	3.500	0.077 NS
ManA- GaLA	1.100	1.100	0.00 NS
GaIA	14.500	21.500	0.0052 **
ManL	-	5.610	--
* (P≤0.05), ** (P≤0.01).			

Conclusions

The study of difference between soil and pathogenic bacteria samples for biofilm production, which is one of the factors that resistance diseases and pests, It has been shown that soil samples are the most productive for biofilm, as well as the compound (ManL), which is not found in the pathogenic samples.

Acknowledgments

The authors are grateful to their respective College of Biotechnology for their support.

Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

Istabrq H. Mohammed: Contributed to the conception and design of the study, conducted some experiments, data rearrangement and drafted the initial manuscript.

Ali S. Ahmed: conducted some experiments, collection a part of literature review andconducted some characteristics of the products.

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p-ISSN: 3007-5491 / e-ISSN:3007-5505

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