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A Study of the Relationship Between Pigment Production and Biofilm Formation in Pseudomonas aeruginosa

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Abstract

Eighty eight isolates of *Pseudomonas aeruginosa* were obtained from Central Child Teaching Hospital, Imamin Al-Kadhimin Medical City, Baghdad Teaching Hospital, National Center for Educational Laboratories, Imam Ali Hospital, Ibn Al-Baladi Hospital, as well as (38) clinical isolation obtained from previous studies and (22) environmental isolation obtained from previous studies and it was studied the effect of the type of cultural medium, temperature and different pH numbers in the production of pigments. The results showed that the isolates of *Pseudomonas* clinical (Pc18) and (Pc40) had the ability to form green pigments after 24 hours of incubation in the nutrient broth and after 72 hours in the heart infusion broth and Luria bertani broth after 72 hours, and they were yellowish-green in color. It was also noted that the isolate Pc18 showed the green pigment at a temperature of 25, 37, and 41°C after an incubation period of 72, 24, and 120 hours respectively, while the isolate Pc40 showed the yellowish green pigment at a temperature of 25°C after an incubation period of 72 hours, and it had a green pigment at a temperature of 37 and 41°C at a 24 and 120 hours incubation period. The results also showed the formation of a yellowish-green color after 72 hours of incubation of Pc18 isolate, while Pc40 isolate showed its ability to form green after 48 hours in the alkaline medium. In the acidic medium, the ability of *Pseudomonas aeruginosa* bacteria to form color during the incubation period of 120 hours was not observed. In the neutral medium, the results showed that the pigment for the two isolates was absorbed after 24 hours. The results of morphological detection showed biofilm formation by 11.11% was poorly formed, 88.89% was medium formation, and no strong isolates were observed. There was no effect of pigment formation on the ability of *Pseudomonas aeruginosa* to form biofilm.

Keywords: *Pseudomonas aeruginosa*, Luria bertani, Pigment, Biofilm**.**

1. Introduction

Pseudomonas aeruginosais is gram-negative, and it is in the form of bacilli with a length ranging between 1 and 5 micrometers and a width of 0.5 and 1.0 micrometers. It is facultative aerobic and

characterized by growth through aerobic respiration and anaerobic respiration with nitrates as a peripheral receptor of the electron.

It can grow anaerobic using arginine and has the ability to grow on a medium of small salts with a single source of carbon and energy [1].

They are catalase and oxidase positive [2], and *P. aeruginosa* is non-spore-forming and motile by polar flagellum [3]. *P. aeruginosa* prefers to grow in a wide range of pH ranging from 5.6 to 8.0, but the optimum pH value is 6.6–7.0 [4], and it is characterized by their grape-like smell due to their production of 2-aminoacetophenone [5]. It is considered one of the most opportunistic pathogens that cause morbidity and mortality [6], and it is characterized by its production of four types of pigment, which include pyocyanin, pyoverdin, pyorubin, and pyomelanin. The most common is the soluble blue-green pigment phenazina called pyocyanin [7,8]. The pigments pyocyanin and pyoverubin are toxic to human cells, *P. aeruginosa's* ability to inhibit lymphocyte proliferation [9]. Pyocyanin is responsible for producing hydrogen peroxide and superoxide from host cells [10]. Strains unable to produce pyocyanin are less pathogenic and more susceptible to the immune system response [11,12] and the toxic effect of pyocyanin through the secretion of cellular catalase [13]. *P. aeruginosa* causes a serious infection involving mainly all human organs, including pneumonia, lung infection, soft tissue infection in burns and open wounds, urinary tract infection, keratitis, and diabetic foot ulcers [14], may cause multiple infections such as meningitis and bacterial infection in hospitals [15], and produces many virulence factors that can cause extensive tissue damage, bloodstream invasion and distribution [16], and possess exotoxin A with a toxic effect and necrosis at the site of infection [17]. Exoenzyme U is excreted, which has a role in producing high levels of cytotoxicity [18]. They also have *phzm* and *phzs* genes that express enzymes that convert phenazine-1-carboxylic acid into pyocyaninphenazine [19]. In addition to having biofilms, which is one of the patterns of growth of self-protective bacteria, biofilm cells have a barrier that prevents or reduces the effect of environmental pressures and the host's immune system [20]. Additionally, the biofilm plays a role in the adhesion of bacteria to living and non-living surfaces [21], as well as its role in encapsulating bacteria together on surfaces, thus hindering phagocytosis and preventing colonization and its long-term survival [22]. The aim of the study was to determine the effect of media type, temperature,temperature and pH on the production of pigment and the ability of pigment production by *P. aeruginosa* on biofilm formation.

2. Materials and Methods

2.1.Identification of isolates

Eighty-eight isolates belonging to *P. aeruginosa* were obtained, and the clinical isolates were 28 obtained from different hospitals in Baghdad, 38 clinical isolates from previous studies, and 22 environmental isolates from previous studies from soil near oil fields. The isolates were cultured on MacConkey agar and Cetrimide agar, and all isolates were diagnosed by microscopic examination, biochemical tests, and the Vitek-2 device.

2.2. Study Pigment Formation

The isolates were cultured on *Pseudomonas* agar and nutrient agar, and all isolates were characterized by their ability to form pigments on *Pseudomonas* agar and nutrient agar.

2.3. Study some physical factors in pigment formation in *Pseudomonas aeruginosa*

2.3.1. Growth in different media

The test was performed by inoculating tubes containing nutrient broth, Luria broth, and brain heart infusion broth with *P. aeruginosa* and incubating at 37 °C for 24, 48, and 72 hours with the observation of changes in the color of the medium [23].

2.3.2.Growing at different temperatures

The test was carried out by inoculating tubes containing a nutrient broth with *P.aeruginosa* and incubating them at a temperature of 30, 37, and 41 °C for 24, 48, and 72 hours with the observation of changes in the color of the medium [23].

2.3.3. Growth in different pH media

The test was performed by inoculating tubes containing a nutrient broth, adjusted to pH (5, 7, and 8), and incubated at a temperature of 37 °C for 24, 48, and 72 hours with the observation of changes in the color of the medium [23].

2.4. Detection of biofilm formation

The ability of *P. aeruginosa* to biofilm formation was detected using the Microtiter plate method (MTP), according to the method used by Gajdács et al. (2021) [24]. Some isolates of *P. aeruginosa* were selected in the study because of their ability to form biofilm. They included clinical and environmental isolates and had the ability to form different pigments (green, yellowish green and transparent).

3. Results and Discussion

Pseudomonas aeruginosa isolates were diagnosed based on microscopic characteristics and biochemical tests and their growth on Cetrimide agar medium and Pseudomonas agar, and the diagnosis was confirmed using a VITEC device. It included (7) burn isolates, two wound isolates, one otitis media isolate, one lung isolate, one dialysis isolate, two blood isolates, (7) UTI isolates, and (6) sputum isolates, as well as (38) clinical isolates obtained from previous isolated studies of burns and wounds and (22) environmental isolates obtained from previous studies (isolated from the soil near the oil fields), as shown in **Table 1**.

Table 1. Source and number of *Pseudomonas aeruginosa* isolates.

Source of isolate	No.
Burns	7
Wounds	2
Otitis Media	1
Lung	1
Dialysis	1
Blood	2
UTI	7
Stool	1
Sputum	6
Previous studies (Burns and wonds)	38
Previous studies (Soil near oil fields)	22
Total Number	88

3.1. Growth of *Pseudomonas aeruginosa* on the Pseudomonas agar

All isolates obtained from hospitals were planted on Pseudomonas agar and appeared in dark green (37.87%), light green (16.66%), yellowish green (31.81%), and colorless colonies (13.63%). As for the environmental isolates obtained from previous studies, colonies were formed in dark green (36.36%), light green (9.09%), yellowish green (18.18%), and colorless colonies were also formed (36.36%). This is pointed out by [25] that (40%) of the isolates had the ability to grow on the Pseudomonas agar and formed a fruit-like smell, and all of them belonged to *P. aeruginosa*, and [26] indicated that (32.5%) of the isolates only grew on the Pseudomonas agar, which produced a bluish pyocyanin pigment that spread in the medium and belonged to *P. aeruginosa* **Table 2**.

Table 2. Growth of *Pseudomonas aeruginosa* isolates on Pseudomonas agar.

Isolates	Total	Dark green		Light green		Yellowish green		Colorless	
	No.	No.	%	No.	%	No.	%	No.	%
Total hospital isolates	66	25	37.87	11	16.66	21	31.81	9	13.63
Environmental isolates from previous studies	22	8	36.36	2	9.09	4	18.18	8	36.36

3.2. Study the effect of some physical factors in the production of pigments

3.2.1. Effect of pH on pigments production

Two isolates of dark green *Pseudomonas* isolated from a clinical source (Pseudomonas clinical PC) they are Pc18 and Pc40, were selected to know the effect of the pH of the culture medium on the formation of pigments. **Table (3A)**, as it was observed that the yellowish-green color was formed after 72 hours of incubation of the Pc18 isolate, while the isolation Pc40 showed its ability to form green after 48 hours in the base medium, **Table (3B)**. In the acidic medium, the ability of *P. aeruginosa* bacteria to form color during the incubation period of 120 hours was not observed. In the neutral medium, see **Table 3C**. showed that the pigment of the two isolates after 24 hours was formed. [27] indicated that there was no difference in pyocyanin production when growing in the nutrient broth medium at pH (7) and pH (8) for the p12 strain; on the contrary, a significant decrease in pyocyanin production was observed when the pa14 isolate grew at pH (8) in the nutrient broth compared to pyocyanin yield at pH 7. [28] indicated that the neutral pH was suitable for the growth of the organism and that the maximum production of pyocyanin was at pH (7). It was noted in the results that the acidic pH did not help the formation of pigments, while the neutral and alkaline mediums were similar in terms of their effect on the formation of pigments and that the ability of *P. aeruginosa* bacteria to form pigments varies according to isolation or strain.

Table 3. The Ability of *P.aeruginosa* to form pigments in different pH.

A: Alkaline pH (8).

No. Isolate	Source of isolate	Period of incubation/ hour				
		24	48	72	96	120
Pc18	Clinical	/	/	Yellowish green	Yellowish green	Yellowish green
Pc40	Clinical	/	Green	Green	Green	Dark green

B: Acidic pH (5)

No. Isolate	Source of isolate	Period of incubation/ hour				
		24	48	72	96	120
Pc18	Clinical	/	/	/	/	/

Pc40	Clinical	/	/	/	/	/

C: Neutral pH (7)

No. Isolate	Source of isolate	Period of incubation/ hour				
		24	48	72	96	120
Pc18	Clinical	Green	Green	Green	Green	Green
Pc40	Clinical	Green	Green	Green	Green	Green

Pc: *Pseudomonas* clinical

3.2.2. Effect of temperature on pigment production

The ability of *P. aeruginosa* to form pigments at different temperatures is noted in **Table 4**. The results show that the isolate Pc18 showed the green pigment at a temperature of 25 °C after an incubation period of 72 hours, the pigment was green at a temperature of 37 °C after an incubation period of 24 hours, and the dye showed at a temperature of 41 °C after an incubation period of 120 hours. The results showed that a temperature of 37 °C was the best temperature for the formation of the pigment, followed by a temperature of 25 °C, while atemperature of 41 °C led to a longer time for the formation of the pigment. This is indicated by [29] that pyocyanin production at 32 °C is the minimum pigment production in the luria broth and the nutrient broth, while pyocyanin production was significantly higher in cultured media at 37 °C. A temperature of 42 °C showed a decrease in pyocyanin production compared to 37 °C and showed that the production of pyocyanin by *P. aerugionsa* can be achieved in the nutrient broth at a pH of 7.2 and a temperature of 37 °C. While [30] and [29] indicated an increase in the production of pigment in the nutrient broth and the incubation period due to the initiation of phenazine formation after the exponential phase of microbial growth as well as the biosynthesis of aromatic amino acids that contribute to the formation of pigments, While [27] indicated that pyocyanin production at a temperature of 37 °C significantly decreased at the incubation of bacterial strains at 30 °C, [31] indicated that the temperature of 37 °C had an effect on the gene expression of the genes *phzs and phzm* responsible for the synthesis of pyocyanin.

Table 4. The ability of *Pseudomonas aeruginosa* bacteria to form pigments at different temperatures.
A: 25°C temperature

No. Isolate	Period of incubation/ hour				
	24	48	72	96	120
Pc18	/	/	Green	Green	Green
Pc40	/	/	Yellowish green	Yellowish green	Yellowish green

B: 37°C temperature

No. Isolate	Period of incubation/ hour				
	24	48	72	96	120
Pc18	Green	Green	Green	Green	Green
Pc40	Green	Green	Green	Green	Dark green

C: 41°C temperature

No. Isolate	Period of incubation/ hour				
	24	48	72	96	120
Pc18	/	/	/	/	Green
Pc40	/	/	/	/	Green

Pc: *Pseudomonas* clinical

3.2.3. Effect of media type on pigment formation of *Pseudomonas aeruginosa*

The results of **Table 5** showed that the isolates Pc18 and Pc40 had the ability to form green pigments after 24 hours of incubation in the nutrient broth and after 72 hours in the brain-heart infusion broth and Luria bertani broth, and they were yellowish-green in color. It was noted from the results that nutrient broth was the best medium for the formation of pigments compared to the Brain Heart Infusion broth and Luria bertani broth. This is indicated by [29] that the highest pyocyanin production is in the nutrient broth, followed by luria broth, and also [27] pointed out that the King's broth medium is the best medium used for pyocyanin production compared to luria broth and nutrient broth, and this result can be changed by the large effect of carbon and nitrogen sources on the amount of pyocyanin production as indicated [33] and [34]. In contrast to low-K_2SO_4 energy-source media such as nutrient broth, these differences in media composition largely affect bacterial growth and, thus, pyocyanin production. [35] pointed out that luria broth had an effect on the enzymatic activity of the enzyme protease in stimulating the *opra*gene contributing to pyocyanin synthesis. [28] noted that among the various media, it was observed that nutrient broth showed an increase in the amount of pyocyanin production and showed an additional growth rate, so nutrient broth can be preferred to pyocyanin production after 3–4 days of incubation, and then the color of the medium changed to green.

Table 5 . ability of *P. aeruginosa* to form pigments in different media**.**

A: Nutrient broth

No. Isolate	Period of incubation/ hour				
	24	**48**	**72**	**96**	**120**
Pc18	Green	Green	Green	Green	Green
Pc40	Green	Green	Green	Green	Green

B: Brain heart infusion broth

No. Isolate	Period of incubation/ hour				
	24	**48**	**72**	**96**	**120**
Pc18	/	/	Yellowish green	Yellowish green	Yellowish green
Pc40	/	/	Yellowish green	Yellowish green	Yellowish green

C. Luria broth

No. Isolate	Period of incubation/ hour				
	24	**48**	**72**	**96**	**120**
Pc18	/	/	Yellowish green	Yellowish green	Yellowish green
Pc40	/	/	Yellowish green	Yellowish green	Green

.Pc: *Pseudomonas* clinical

3.4.Ability of *P.aeruginosa* in biofilm formation

Some isolates of *P. aeruginosa* were selected in the study because of their ability to form biofilm. They included clinical and environmental isolates and had the ability to form different pigments (green, yellowish green, and transparent). The results of the phenotypic detection showed that the composition of the biofilm by the microtiter plate method was 11.11% weak composition and 88.89% medium composition, and no isolates were observed that had the ability to form the biofilm strongly. It was noted from the results that *P. aeruginosa* has the ability to *form* pigments and non-pigments to procedure the biofilm in an amedium way. [36] also noted that the ability of *P. aeruginosa* to form biofilm was 19.87% weak, 20.86% moderate, and 59.27% strong. While

[37] indicated that the number of isolates producing strong, medium, and weak biofilms is equal to 34, 52, and 14, respectively, [24] indicated that 9.87%, 20.86%, and 59.27% were weak, medium, and strong producers of biofilms, respectively. [38] also stated that 33 isolates out of 35 isolates that were components of biofilms included: 15 isolates that formed a strong biofilm, 8 isolates that formed a biofilm medium, 10 that formed a weak biofilm, and 2 that did not produce biofilms, while [39] indicated that *P. aeruginosa* bacteria can use various exogenous polysaccharides such as *pel*, *psl*, and alginate to synthesize their biofilm matrix and that the CdrA protein binds to *psl*, which promotes the formation of biofilms and overall stability.

While [40] indicated that the low nutritional level is better in the formation of membranes than the high nutrient level in the Luria broth and that the isolates grown on the Luria broth were found to be weak and medium biofilms, [41] pointed out that the percentage of biofilm formation was 75.9%, as 42.3% were poorly formed, 15.4% were moderately formed, 42.3% were strong, and 24% were non-formation, and this study showed a significant correlation between the presence of genes (*PSID*, *PeIF*, *algD*) and biofilm formation. [42] showed that *FIeQ* has an important role in biosynthesis through the bioregulation of exosaccharides in *P. aeruginosa*, while [5] in his study indicated that (13%) of *P. aeruginosa* isolates formed a biofilm while (8.6%) were unable to form a biofilm. The difference between isolates in biofilm formation may be due to several reasons, including differences in the ability of isolates to form a biofilm or perhaps differences in the initial number of cells that it succeeded in adhesion and differences in the presence and quantity of particles of quorum sensing signals (automatic inducers) that result from each isolate that play important roles. As well as the different types of medium used in the study of biofilm formation. There was no difference between clinical and environmental isolates in the formation of the biofilm, and also, the ability of bacteria to form pigments did not have an effect on the formation of the biofilm, and this may be due to the presence of many factors that affect the ability of bacteria to form the biofilm.

Table 6. Ability of *P.aeruginosa* in biofilm formation.

No. isolate	Source of isolate	Ability of color formation	Non-formation	Poor	Moderate	Strong
Pc18	Clinical	Green	-	-	+	-
Pc40	Clinical	Green	-	-	+	-
Pe26	Environmental	Green	-	-	+	-
Pc23	Clinical	Yellowish green	-	+	-	-
Pc29	Clinical	Yellowish green	-	-	+	-
Pe27	Environmental	Yellowish green	-	-	+	-
Pc10	Clinical	Transparent	-	-	+	-
Pc31	Clinical	Transparent	-	-	+	-
Pe33	Environmental	Transparent	-	-	+	-

Pc: *Pseudomonas* clinical

Pc: *Pseudomonas* environment

4. Conclusion

Pseudomonas aeruginosa showed that the highest formation of pigments was in nutrient broth at a temperature of 37 °C and a pH of 7. It also showed the ability of *P. aeruginosa* to form a medium biofilm, and no difference in the ability of pigment- and non-pigment-forming isolates in biofilm formation was noted.

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Conflict of Interest

Estabraq A. Mahmoud declares that she is a member of IHJPAS editorial board at the time of submitting the manuscript. The editor-in-chief of IHJPAS confirms that (Estabraq A. Mahmoud) was excluded from any decisions made regarding this paper.

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Ethical Clearance

The samples were gained according to local research Ethics committee approval in the Iraqi Ministry of Health No. 26416 on 29/6/2022.

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