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Effect of iron, temperature and incubation periods on biofilm formation by multi-drug resistant *Pseudomonas aeruginosa* PS31

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ABSTRACT : **Most microorganisms form biofilm as protection mechanism that provides cells advantages as infectivity, antibiotic resistance and survivability of a variety of infections in humans. *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen cause of infections in cystic fibrosis patients such as pneumonia and chronic lung infections. Although biofilm control is an important in medicine, clinically, there is not available effective inhibitors of biofilm formation but some environmental factors could influence on biofilm formation. This research investigated the effect of incubation periods, temperature and iron concentration on the formation of biofilm which is critical for virulence by multi-drug resistant *P. aeruginosa* taken from the sputum of pulmonary infection patient. It was found that moderate biofilm was observed on day two and day one was low in formation of biofilm but on day three was the optimum period for *P. aeruginosa* to switch into a strong biofilm. We investigated the effect of temperature and found that biofilm showed the highest level at temp 37°C. After using different concentrations of iron, it was showed that biofilm production was higher at the addition of $FeSO_4$ at 25 mM incubated at 37°C for 3 days. It concluded that some factors can affect and control the biofilm formation as a significant part of virulence system plays a critical role in antibiotic resistance in *P. aeruginosa* infection.**

Keywords : **Multidrug resistant; *Pseudomonas aeruginosa*; Biofilm; Iron concentration; Temperature; Incubation periods**

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I. INTRODUCTION

Pseudomonas aeruginosa is an important clinically opportunistic pathogen responsible for nosocomial infections in immunocompromised patients. It has been found in lung infections in patients with cystic fibrosis. This pathogen has several factors to aid in its pathogenicity and virulence such as biofilm formation that is the factor of protection of bacteria against the attack of the host immune defenses and antimicrobial medication **(Grabski *et al.*, 2017)**.

Because biofilms are more resistant to antibiotics and biocides, they are one of the sources of issues in the medical field. Also, it can reduce the host immune responses. So, the biofilms of bacteria have been the subject of several studies to control and manage biofilm formation **(Grabski *et al.*, 2017)**.

The biofilms of microorganisms are known to possess increased resistance to stress factors **(Cochis *et al.*, 2016)**. Temperature and osmolality are the abiotic environmental elements that have been investigated the most in relation to how bacteria create biofilms **(Yang *et al.*, 2016)**. Gram-positive bacteria have much more specific (30–37°C) than that of Gram-negative bacteria (4–50°C) in the range of optimal temperature from comparative data on the temperature optima for biofilm formation by various microbes **(Ponomareva *et al.*, 2018)**.

There are some of factors that affect biofilm formation such as the availability of nutrients, material for adhesion, light, N_2O, H_2S, NO^{3-}, pH and metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}) **(Powell *et al.*, 2016)**. Iron is an important cofactor for metabolism, growth and survival of bacteria and for induction of infection by pathogenic bacteria in host including *P. aeruginosa*. Various iron binding proteins produced by mammalian systems such as

ferritin that decrease free iron bioavailability for progress of pathogens infection and growth, thus ferritin is acting as an innate immunity molecule against of bacteria. Under iron limitation conditions, siderophores are produced to acquire iron from the host **(Gomila *et al.*, 2018)**.

The characterization of *P. aeruginosa* PS31 (LC619328.2) strain on the basis of the capability of biofilm formation under different conditions and the detection of the effect of time incubation, temperature and iron concentration on biofilm formation were the aim of this study. Therefore, this research aimed to detect the optimum factors for *P. aeruginosa* to form biofilm, to control and inhibit the biofilm virulence factor.

. II.Materials and Methods

2.1. Bacterial strain: *Pseudomonas aeruginosa* PS31 LC619328.2 was obtained from sputum of pulmonary infection patient at Zagazig University Hospital, Sharkia, Egypt **(Hamza *et al.*, 2022)**.

2.2. Effect of incubation period: *P. aeruginosa* PS31 was cultivated in Brain heart infusion broth medium with pH 7 and incubated at 37°C for different incubation periods (1, 2, 3, 4 and 5 days). After incubation, the assay of biofilm was measured O.D (570 nm) as described by **Bose *et al.*, (2009)**.

2.3. Effect of incubation temperature: *P. aeruginosa* PS31 was cultivated in Brain heart infusion broth medium with pH 7 at different incubation temperatures 25, 30, 35, 37, 40, 45 and 50°C. After incubation for 3 days, the assay of biofilm at each temperature was measured O.D (570 nm) as mentioned previously.

2.4. Effect of iron concentration: *P. aeruginosa* PS31 was cultivated in Brain heart infusion broth medium with different iron concentration of $FeSO_4$ (5, 10, 20 and 25 mM) with pH 7 at 37°C for 3 days. After incubation for 3 days, the assay of biofilm at each iron concentration was measured O.D (570 nm) as mentioned previously.

2.5. Statistical analysis: The obtained information was analyzed using the one-way ANOVA test. IBM SPSS Corp was used for conducting the statistical analysis. The significance was calculated (p-value < 0.05). The standard deviation of the mean of 3 replicates is represented in each value of the collected findings.

III.RESLUTS

3.1. Effect of incubation periods

After incubation of *P. aeruginosa* PS31, biofilm production was determined after one, two, three, four and five days were (0.484), (0.506), (0.870), (0.595) and (0.539), respectively but biofilm formation was the strongest adherent on day three (0.87) (Table 1).

Table (1): Incubation periods effect on biofilm assay produced by *P. aeruginosa* PS31 LC619328.2.

Incubation periods (Day)	OD (570nm)
1	0.484 ± 0.001^{e}
2	0.506 ± 0.004^{d}
3	$\mathbf{0.870\pm.005^{a}}$
4	0.595 ± 0.004^{b}
5	0.539 ± 0.005^{c}

3.2. Effect of incubation temperatures

The biofilm production of *P. aeruginosa* PS31 incubated for 3 days at different temperatures of 25, 30, 35, 37, 40, 45 and 50C°were (0.45), (0.59), (0.66), (0.87), (0.26), (0.18) and (0.01) OD, respectively. Also, these results showed that biofilm production was higher at 37°C (Table 2).

Table (2): Incubation temperatures effect on biofilm assay produced by *P. aeruginosa* PS31 LC619328.2.

Temperature (°C)	OD (570nm)
25	0.451±0.003[d]
30	0.597±0.003[c]
35	0.663±0.006[b]
37	**0.870±0.006[a]**
40	0.266±0.005[e]
45	0.185±0.003[f]
50	0.016±0.003[g]

3.3. Effect of iron concentration

The influence of iron on biofilm production of *P. aeruginosa* PS31 was showed that biofilm production was higher at the addition of FeSO4 at 25 mM incubated at 37°C for 3 days. The results revealed that the biofilm formation increased with increasing iron concentration up to 25 mM (Table 3).

Table (3): Iron concentration effect on biofilm assay produced by *P. aeruginosa* PS31 LC619328.2.

Iron Conc. of $FeSO_4$ (mM)	OD (570nm)
0	0.205±0.004[b]
5	0.172±0.005[d]
10	0.195±0.002[c]
20	0.137±0.003[e]
25	**0.288±0.002[a]**

IV.Discussion

In this study, after 5 days of incubation of *P. aeruginosa* PS31, biofilm production was determined after one, two, three, four and five days were (0.484), (0.506), (0.870), (0.595) and (0.539), respectively but biofilm formation was the strongest adherent after three days (0.87).

This is similar to study that showed biofilms by *P. aeruginosa* and *Streptococcus pyogenes* on day three (1.0) and (0.96), respectively **(Al-Kafaween *et al.*, 2019)**.

In other study, in microtiter plate resulting strong biofilm formation occurred at an optimum number of bacteria after three days (Jama *et al.*, 2017). Also, producing strong biofilm by *Proteus mirabilis* in another study was after three days **(Emineke *et al.*, 2017)**.

On other hand, other study on *Escherichia coli* showed that biofilm produced on day six **(Culotti and Packman 2014)**. Another study showed that the optimum day was after five days for *Leptospires* to produce high biofilm **(Apun *et al.*, 2018)**.

In this study, the biofilm production of *P. aeruginosa* PS31 incubated for 3 days at different temperatures of 25, 30, 35, 37, 40, 45 and 50 °C were (0.45), (0.59), (0.66), (0.87), (0.26), (0.18) and (0.01) OD, respectively. Also, these results showed that biofilm production was higher at 37°C.

Also in other study, biofilm formation of *P. aeruginosa* incubated at temperatures 28, 33, 37 and 42 °C were (1.25), (2.20), (2.80) and (2.30) OD, respectively. The results showed that biofilm formation at 37°C would have a higher mechanical stability than biofilm formation at 28, 33 and 42°C **(Kannan and Gautam 2015)**.

On other hand, there was study on clinical and environmental strains of *Vibrio vulnificus*, showed that biofilms were produced at 37°C but higher biofilm at 24°C was 2 to 3 times greater than at 30 and 37°C for all strains **(Cam and Brinkmeyer 2020)**.

The influence of iron in this study on biofilm production of *P. aeruginosa* PS31 was tested by the addition of different concentration of $FeSO_4$ (5, 10, 20 and 25 mM) to the culture medium and incubated at 37°C for 3 days. The results showed that the formation of biofilm was higher at the addition of $FeSO_4$ at 25 mM, and revealed that biofilm production increased with increasing iron concentration.

Similar to the present results, with increasing iron concentration up to 200 mM, *Staphylococcus aureus* biofilm was increased **(Lin *et al.*, 2012)**. Other study on *Vibrio vulnificus*, the effect of iron concentrations 18, 30, 50, 100 and 200 mM showed there is a direct correlation between iron concentration in medium of growth and biofilm production **(Cam and Brinkmeyer, 2020)**.

According to some authors, an increase in the concentration of Fe^{2+} stimulated formation of biofilms by *E. coli*, *P. aeruginosa*, *Acidithiobacillus ferrooxidans*, *Geobacter sulfurreducen*, *A. ferrivorans*, *Pinnularia aljustrelica, and P. putida* **(Rinaudi *et al.*, 2006; Liu *et al.*, 2014; Ebrahimi and Hosseini, 2015)**. The increase of the stability of mixed river biofilms to an acidic environment was shown by Fe^{2+} **(Luís *et al.*, 2014)**.

From other studies on *Streptococcus mutans* and *Legionella pneumophilia* have shown that iron limitation that induced biofilm production, however, under low iron concentrations in growth medium of *Vibrio cholerae* and *Escherichia coli*, biofilm formation was repressed. So, iron dependent regulation of biofilm production can differ between bacterial species **(Wu and Outten, 2009)**.

Bacterial biofilm formation and maturation starts with the introduction of bacteria to a surface during a process originated by Brownian, gravitational and hydrodynamic forces **(Beloin *et al.*, 2008)**. Environmental factors such as pH and temperature as well as nutrient levels are reported to be influential on the strength of forces involved in adherence of bacteria on surfaces **(Donlan, 2002)**.

The next stage after the adherence of the bacteria to the surface is the formation of extracellular polymeric substance (EPS) and maturation of the biofilm **(Di Martino, 2018)**. **Harmsen *et al.* (2010)** have reported the important role of environmental conditions such as pH and temperature on the composition of extracellular matrix. In the third stage of biofilm growth the dispersion mechanisms start. Biofilm is a community of microorganisms that can actively change the structure of the biofilm and maintain the living environment **(Kostakiot *et al.*, 2013)**.

3 During the dispersion stage of the biofilm growth, some mature parts of EPS are detached from the structure and the cells within the part are released to the medium, in a process known as sloughing **(Kaplan, 2010)**.

Biofilms, are formed by *P. aeruginosa*, are cell populations that resulting in the resistance of bacteria to the antibiotics medication alongside the host immune defenses **(Pestrak *et al.*, 2019)** as this cell populations are encased as the matrix of self-secreted extracellular that defends the cells from antimicrobial and the host immune responses **(Cassin and Tseng, 2019)**.

Conclusion

There are many factors that affect bacterial growth and biofilm formation by several human pathogens. These could regulate the virulence factors produced by most of the clinically important opportunistic pathogens such as *P. aeruginosa*. This study came to the conclusion that the formation of biofilms is significantly influenced by a number of parameters, including iron, temperature, and incubation periods by *P. aeruginosa* PS31 (**LC619328.2**) and showed biofilms are formed at various environmental factors and that important to survival in human host. Such studies could be beneficial for providing new insights to control biofilms, for antibiofilm experiments and determining the amount and structure of biofilms.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests to the work reported in this paper.

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REFERENCES

Al-kafaween, M.A.; Hilmi, A.M.; Jaffar, N.; Al-Jamal, H.A.N. and Zahri, M.K. (2019): Determination of optimum incubation time for formation of *Pseudomonas aeruginosa* and *Streptococcus pyogenes* biofilms in microtiter plate. Bulletin of the National Research Centre 43:100.

Apun, K.; Jalan, J.; Pui, C.F.; Bilung, L.M.; Hashim, H.F. and Aina, A. (2018): Biofilm forming ability of intermediate and saprophytic Leptospira on abiotic and biotic surfaces. Malays J Microbiol 14(4):313–319.

Beloin, C.; Roux, A. and Ghigo, J.M. (2008): *Escherichia coli* biofilms. *Curr Top Microbiol Immunol* 322: 249–289.

Bose, S.; Khodke, M.; Basak, S. and Mallick, S. (2009): Detection of biofilm producing staphylococci: Need of the hour. Journal of clinical and diagnostic research.

Çam, S. and Brinkmeyer, R. (2020): The effects of temperature, pH, and iron on biofilm formation by clinical versus environmental strains of *Vibrio vulnificus*. Folia Microbiologica, 65:557–566.

Cassin, E.K. and Tseng, B.S. (2019): Pushing beyond the envelope: the potential roles of OprF in *Pseudomonas aeruginosa* biofilm formation and pathogenicity. DOI: 10.1128/JB.00050-19.

Cochis, A.; Azzimonti, B.; Valle, D.C.; De Giglio, E.; Bloise, N.; Visai, L.; Cometa, S.; Rimondini, L., and Chiesa, R. (2016): The effect of silver or gallium doped titanium against the multidrug resistant *Acinetobacter baumannii*, Biomaterials, vol. 80, pp. 80–95.

Culotti, A. and Packman, A.I. (2014): *Pseudomonas aeruginosa* promotes *Escherichia coli* biofilm formation in nutrient-limited medium. PLoS One 9(9): e107186.

Di Martino, P. (2018): Extracellular polymeric substances, a key element in understanding biofilm phenotype[J]. AIMS Microbiology,4(2): 274-288.

Donlan, R.M. (2002): Biofilms: Microbial life on surfaces. *Emerg Infect Dis* 8: 881–890.

Ebrahimi, S. and Hosseini, M., (2015): Model-based evaluation of ferrous iron oxidation by acidophilic bacteria in chemostat and biofilm airlift reactors, J. Industr. Microbiol. Biotechnol., vol. 42, no. 10, pp. 1363–1368.

Emineke, S.; Cooper, A.J.; Fouch, S.; Birch, B.R. and Lwaleed, B.A. (2017): Diluted honey inhibits biofilm formation: potential application in urinary catheter management. J Clin Pathol 70(2):140–144.

Gomila, A.; Carratalà, J.; Eliakim-Raz, N.; Shaw, E.; Wiegand, I.; Vallejo-Torres, L.; Gorostiza, A.; Vigo, J.; Morris, S. and Stoddart, M. (2018): Risk factors and prognosis of complicated urinary tract infections caused by *Pseudomonas aeruginosa* in hospitalized patients: A retrospective multicenter cohort study. Infection and drug resistance.

Grabski, H.; Hunanyan, L.; Tiratsuyan, S. and Vardapetyan, H. (2017): Interaction of N-3-oxododecanoyl homoserine lactone with LasR protein of *Pseudomonas aeruginosa*: insights from molecular docking and dynamics simulations. bioRxiv.:121681.

Hamza, E.H.; El-Shawadfy, A.M.; Allam, A.A. and Hassanein, W.A. (2022): Study on pyoverdine and biofilm production with detection of LasR gene in MDR *Pseudomonas aeruginosa*, Saudi Journal of Biological Sciences,103492.

Harmsen, M.; Yang, L.; Pamp, S.J. and Tolker-Nielsen, T. (2010): An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. *FEMS Immunol Med Microbiol* 59: 253–268.

Jama, C.; Abdallah, M.; Boukherroub, R.; Faille, C. and Chihib, N.E. (2017): Effect of incubation duration, growth temperature, and abiotic surface type on cell surface properties, adhesion and pathogenicity of biofilm-detached *Staphylococcus aureus* cells. AMB Express 7(1):191.

Kannan, A.A. and Gautam, P., (2015): Quantitative study on the formation of *Pseudomonas aeruginosa* biofilm, SpringerPlus, vol. 4, no. 1, p. 3.

Kaplan, J.B. (2010): Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses. Journal of Dental Research.89(3):205-218.

Kostakioti, M.; Hadjifrangiskou, M. and Hultgren, S.J. (2013): Bacterial biofilms: development, dispersal,

and therapeutic strategies in the dawn of the postantibiotic era. Cold Spring Harb Perspect Med.3(4): a010306.

Lin, M.H.; Shu, J.C.; Huang, H.Y. and Cheng, Y.C. (2012): Involvement of iron in biofilm formation by *Staphylococcus aureus*. PLoS One 7: e34388.

Liu, X.; Tremblay, P.L.; Malvankar, N.S.; Ward, J.E.; Nevin, K.P.; Woodard, T.L.; Smith, J.A.; SnoeyenbosWest, O.L.; Franks, A.E.; Tuominen, M.T. and Lovley, D.R. (2014): *Geobacter sulfurreducens* strain expressing *Pseudomonas aeruginosa* type IV pili localizes OmcS on pili but is deficient in Fe (III) oxide reduction and current production, Appl. Environ. Microbiol., vol. 80, no. 3, pp. 1219–1224.

Luis, A.T.; Bonet, B.; Corcoll, N.; Almeida, S.F.P.; da Silva, E.F.; Figueira, E. and Guasch, H. (2014): Experimental evaluation of the contribution of acidic pH and Fe concentration to the structure, function and tolerance to metals (Cu and Zn) exposure in fluvial biofilms, Ecotoxicology, vol. 23, no. 7, pp. 1270–1282.

Pestrak, M.J.; Baker, P.; Dellos Nolan, S.; Hill, P.J.; da Silva, D.; Silver, H.; Lacdao, I.; Raju, D.; Parsek, M.R.; Wozniak, D.J. and Howell, P.L. (2019): Treatment with the *Pseudomonas aeruginosa* glycoside hydrolase PslG combats wound infection by improving antibiotic efficacy and host innate immune activity. Antimicrobial agents and chemotherapy, 63(6), pp. e 00234- 19.

Ponomareva, A.L.; Buzolevaa, L.S. and Bogatyrenkoc, E.A. (2018): Abiotic Environmental Factors Affecting the Formation of Microbial Biofilms. Biology Bulletin, Vol. 45, No. 5, pp. 490–496.

Powell, L.C.; Khan, S.; Chinga-Carrasco, G.; Wright, C.J.; Hill, K.E. and Thomas, D.W. (2016): An investigation of *Pseudomonas aeruginosa* biofilm growth on novel nanocellulose fibre dressings, Carbohydr. Polym., vol. 137, pp. 191197.

Rinaudi, L.; Fuji Shige, N.A.; Hirsch, A.M.; Banchio, E.; Zorreguieta, A., and Giordano, W. (2006): Effects of nutritional and environmental conditions on *Sinorhizobium meliloti* biofilm formation, Res. Microbiol., vol. 157, pp. 867– 875

Wu, Y. and Outten, F.W. (2009): IscR controls iron-dependent biofilm formation in *Escherichia coli* by regulating type I fimbria expression. J Bacteriol 191:1248–1257.

Yang, Y.; Mikš-Krajnik, M.; Zheng, Q.; Lee, S.B.; Lee, S.C. and Yuk, H.G. (2016): Biofilm formation of *Salmonella enteritidis* under food-related environmental stress conditions and its subsequent resistance to chlorine treatment, Food Microbiol., vol. 54, pp. 98–105.