Turkish Journal of Health Science and Life

Effect of Different Culture Media on *Pseudomonas aeruginosa* Biofilm Formation

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ARTICLE INFO

RESEARCH ARTICLE

Article history:
Received: 4 November 2023
Accepted: 22 December 2023
Available : 31 December 2023

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Turkish Journal of Health Science and Life
2023, Vol.6, No.3, 133-140.
DOI: https://doi.org/10.56150/tjhsl.1386213

ABSTRACT

The opportunistic pathogen *Pseudomonas aeruginosa* (PA) causes nosocomial infections, and it is the most common pathogen that can form biofilm. PA biofilm formation is important as an environmental bacterium in hospital wastewater, in vivo, in the environment, and in infection control. Besides many antibiotic resistance mechanisms, biofilms may play an important role as in PA forming biofilms that have a minimum inhibitory concentration (MIC) for antibiotics up to 1,000-fold higher than that of planktonic bacteria. Multiple biofilm-specific mechanisms contribute to the high levels of antibiotic resistance. Therefore, PA biofilm-associated infections lead to important clinical outcomes. The aim was to investigate the efficacy of four different culture media used in two biofilm formation protocols on the assessment of biofilm production by 11 PA isolated from hospital wastewater. The crystal violet microtiter plate-based method was used to evaluate the quantification of the biofilm formation capacity of PA. Results of culture media used in the formation of biofilm capacity were; TSB with %1 glucose no-biofilm, 63.6%, and 36.4%; BHI 18.2%, 36.4%, and 45.5%; LBB 9.1%, 27.3%, and 63.6% of isolates were strong, moderate, and weak biofilm producers, respectively. However, in MHB, 27.3%, 63.6%, and 9.1% of isolates were moderate, weak, and non-biofilm producers, respectively. The biofilm levels in protocol one were higher than the other protocol used (OD570). PA biofilm formation and quantification in these media used may help to search for antibiofilm agents in laboratories to prevent the spread of antimicrobial resistance, develop effective precautions, and prevent PA infections in hospitals.

Keywords:
Biofilm formation, crystal violet, culture media, *Pseudomonas aeruginosa*

1. INTRODUCTION

Pseudomonas aeruginosa (PA), an aerobic, motile, nutritionally versatile, gram-negative, opportunistic pathogen, is the most persistent cause of infection in non-fermentative bacteria, affecting immunosuppressed patients. PA presents an important challenge to clinicians, both in the community and in hospitals, because of their increasing resistance leading to long therapy and excess mortality. Biofilm formation is critical in PA being an important nosocomial pathogen (1-7). Biofilm formation as an important virulence factor of PA infections allows for adherence on surfaces, and provides protection from harsh environmental conditions, from the immune system, and from antibiotics in vivo aiding in the formation of Multidrug -Resistant (MDR) strains. The minimum inhibitory concentrations (MICs) of bacteria inside the biofilm may be 10–10,000 times higher, compared to planktonic cells, and antimicrobials even in high doses may not kill bacteria inside a biofilm. PA also can tolerate antiseptics and disinfectants and prevents the elimination of PA from hospital environments (1,2). PA is mostly isolated from

infections, environmental sources, food, and plants (8). They can live in many habitats because of being non-fastidious and highly adaptive to various environmental conditions (9). This is due to its genomic flexibility and quorum sensing (QS) regulatory network of pathways for metabolic activities and virulence factors (10-12). Biofilm production is one of the distinctive features of their existence in vivo and in extreme environmental circumstances thus, biofilm-eradication is important in treatment and infection control aspects (13,14).

Biofilm is formed when bacteria change from a free-swimming phase to a surface-attached phase (15) and its steps are attachment to a surface, colony formation, maturation, and detachment (16). Biofilm is a network of microorganisms in an extracellular matrix adhering to surfaces of substances (on medical instruments, hospital surfaces, and tissue), protecting the bacteria against extreme conditions, toxic compounds, and lack of nutrients, and causing problems in antibiotic treatments (15-21,24). Biofilm which is made of exopolysaccharides, nucleic acids, and adhesins, plays a role in antibiotic defense and prevents phagocytosis in the immune system (18,19,22,23). In PA biofilms, as multidrug resistance increases, treatment of PA becomes complicated (21 -23). PA causes infections, especially in old people, patients at the hospital, immunosuppressed patients, and cystic fibrosis (24-26,28).

PA is one of the model organisms to study biofilms (7). Among the biofilm formation models, the 96-well microtiter plate assay determines the adherence of bacteria to the surface of the plate using established criteria such as temperature and media. (15). The information given in the study by O'Toole et al. on biofilm formation is widely used in biofilm experiments (15,31,33). The aim of our study was to investigate the efficacy of four different culture media used in two biofilm formation protocols on the enhancement and the assessment of biofilm production capacity by eleven PA isolated from our hospital wastewater.

2. MATERIALS AND METHODS

2.1. Collection of samples

Hospital wastewater was collected from the Dokuz Eylul University Research and Application Hospital in Izmir Turkey in April 2023. The hospital wastewater samples were collected from two points four times during the day at different time intervals (10.00 am, 14.00 pm, 17.30 pm, 19.30 pm) and transferred into sterile 1-liter bottles without sodium thiosulfate, (MOS LAB, Turkey) as composite samples and were transported to the laboratory in an ice box and kept at 4 °C.

In addition to this, each hospital wastewater sample from two points for the above given hours at different time intervals was also separately studied for each time period as eight hospital wastewater samples besides total of two composite hospital wastewater samples of the two points.

2.2. Isolation and Identification

Hospital wastewater samples were cultured for isolation by standard methods. 10 mL of hospital wastewater samples were diluted from 10^{-1} to 10^{-8} in buffered peptone water [(10 gr Gibco Bacto-Peptone, 5 gr NaCl, 9 gr/L Na2 HPO4 X 12 H2O, 1.5 g/L K2HPO4) 25.5 g/L.] and filtered by the membrane filtration method using a filtration manifold system and 45 µm pore-sized membrane filters (Merck Millipore, Germany). After the membrane filtration, each membrane filter was carefully placed on each specific selective culture media in plates and incubated at 37 °C for 24 hours. Colonies from the plates were chosen according to the morphology of the colony. Sub-cultures were made on their selective media for identification. Then biochemical tests were applied to identify the pathogens. Identification of PA isolates was accomplished by microbiological and biochemical methods [pigment production, oxidase test, catalase tests, triple sugar iron (TSI) agar reactions, and motility test]. Above mentioned tests were done for two hospital wastewater samples in April to find out the differences in strains and quantity between two collection points of the hospital wastewater coming

from different buildings and for eight hospital wastewater samples to find out the differences in bacterial strains and quantity during the different times of the day.

All isolated bacteria were transfered into stock media and stock cultures were stored at −80 °C.

A total of eleven PA were isolated and included in this study.

PA ATCC 27853 was used as a standard strain, which was obtained from the Dokuz Eylul University Research and Application Hospital, Bacteriology Laboratory.

2.3. Biofilm Production

The biofilm formation capacity of all PA isolates was assessed and quantified in our study. The media used were Tryptic Soy Broth (TSB) (LAB M UK) with 1% glucose (AppliChem, Germany), Brain-Heart Infusion Broth (BHI) (Oxoid Ltd. England), Luria-Bertani Broth (LBB) (CONDA pronadisa, Spain), Mueller-Hinton Broth (MHB) (Oxoid Ltd. England).

Protocol I:

Biofilm formation capacity in hospital wastewater PA was quantified using a sterile flat-bottomed microtiter -plate technique described by Kamali et al. and Stepanović et al. (31,32). The strain was transferred from the stock culture onto blood agar (BD, Germany) and incubated overnight aerobically at 35 °C–37 °C. After verifying the purity of the strain, three to four well-isolated identical colonies were suspended in 5 ml of TSB, and the other broth medium and incubated without shaking for 18 h ± 30 min. An overnight broth media cultures of PA was adjusted to the turbidity of 1 McFarland standard. Bacterial suspensions were diluted 1:100 in 200 µL TSB (LAB M, UK) with 1% glucose (AppliChem, Germany) and in 200 µL Brain-Heart Infusion Broth (BHI) (Oxoid Ltd., England), and were transferred to the two sterile flat-bottomed 96-well polystyrene microplates (Greiner bio-one Austria) and incubated for 24 hours at 37 °C. Following incubation, nonadherent cells, and media were removed and wells were washed three times with sterile phosphate-buffered saline (PBS, pH 7.3). Adherent biofilms were fixed with 99% methanol (Merck, Germany) for 15 min. After the solutions were removed, the plate was air-dried. Staining was accomplished by 200 µL of 0.1% (w/v) crystal violet (CV) (Axon Gram, Switzerland) for a period of 5 min at room temperature, Then, rinsed with water and allowed to dry. Destaining was done with 200 µL of 95% ethanol (Merck, Germany) for 30 min. The optical density (OD) was measured at 570 nm using a microtiter plate reader (Biotek-Synergy Ht USA). All experiments were applied in triplicate and repeated three times (31, 32).

Protocol II:

Biofilm formation capacity in hospital wastewater PA was quantified using a sterile flat-bottomed microtiter -plate technique described by Behzadi et al. (33).

Overnight cultures of PA were inoculated into 5 mL of LB-broth (LBB) (CONDA pronadisa, Spain), and Mueller-Hinton broth (MHB) (Oxoid Ltd. England), and were incubated at 37 °C for 24 hours. 180 µL of LB-broth and 20 µL of bacterial suspension (106 CFU/mL) were distributed into two different wells of 96-well flat-bottomed microtiter plates. Microtiter plates were incubated at 37 °C for 24 hours. After the incubation, nonadherent cells, and media were removed and the wells were washed three times with 200 µL of PBS. Fixation was accomplished with 250 µL of 99% methanol (Merck, Germany) for 10 minutes and stained with a 1.0% (w/v) crystal violet (CV) (Axon Gram, Switzerland) dye for 15 minutes. The CV was discarded and washed three times with distilled water. 250 µL of 33% (v/v) glacial acetic acid (Merck, Germany) was applied for solubilization. For measuring the absorbance, a microtiter plate reader (Biotek-Synergy Ht USA) at 570 nm was used. Experiments were performed in triplicate (33).

Calculation and interpretation of the results were done according to Davarzani et al. (36).

The average OD of the three wells for each isolate was ODt. OD average of the three wells for control was ODc. Biofilm formation levels were interpreted according to the following biofilm grouping:

$OD_t < OD_c$ Non-biofilm,

$OD_c < OD_t < 2x\ OD_c$ Weak biofilm,

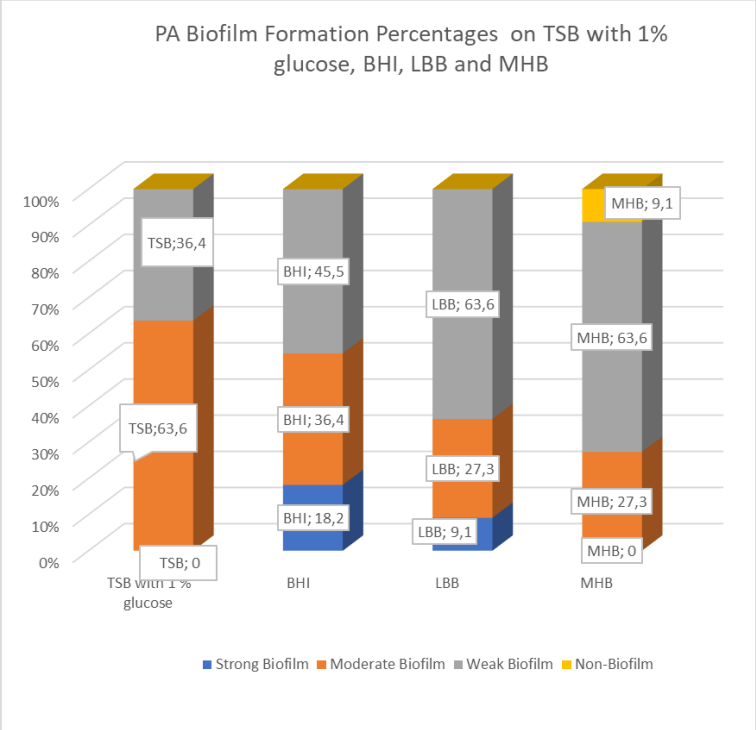

Figure 1: PA Biofilm Formation Percentages on TSB + 1% glucose, BHI, LBB, and MHB culture media

$2x\ OD_c < OD_t < 4xOD_c$ Moderate biofilm,

$OD_t \geq 4xOD_c$ Strong biofilm (36).

RESULT AND DISCUSSION

The biofilm formation capacity of the hospital wastewater isolates was found by a microplate-based assay using flat-bottom polystyrene microtiter plates (Greiner bio-one Austria) with CV-staining, where results were shown after spectrophotometric measurements (OD570). The first biofilm formation protocol was used on TSB with %1 glucose and BHI broth culture media. In all of the experiments, eleven PA strains (n=11) were tested (including PA 1-2 for composite samples and PA 3-11 for samples collected at different time intervals) and the results were as follows: In TSB with %1 glucose no-biofilm (n=0), 63.6% (n=7), and 36.4% (n=4); in BHI 18.2% (n=2), 36.4% (n=4), and 45.5% (n=5) of isolates were strong, moderate, and weak biofilm producers, respectively. The second biofilm formation protocol was used on LBB and MHB culture media. In LBB 9.1% (n=1), 27.3% (n=3), and 63.6% (n=7) of isolates were strong, moderate, and weak biofilm producers, respectively. However, in MHB, 27.3% (n=3), 63.6% (n=7), and 9.1% (n=1) of isolates were moderate, weak, and non-biofilm producers, respectively. (Table 1 and 2, Figure 1 and 2). The decrease in the formation of biofilm

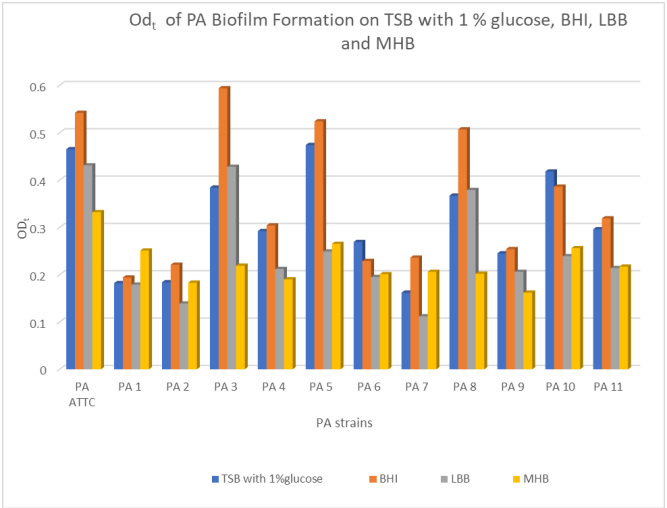

Figure 2: PA Od_t of PA Biofilm Formation on TSB + 1% glucose, BHI, LBB, and MHB culture media.

PA 1-2 = PA strains isolated from composite hospital wastewater samples.

PA 3-11 = PA strains isolated from hospital wastewater samples collected at different time intervals during the day.

Table 1: PA biofilm formation on TSB +1% glucose, BHI, LBB, and MHB culture media

PA 1-2 = PA strains isolated from composite hospital wastewater samples.

PA 3-11 = PA strains isolated from hospital wastewater samples collected at different time intervals during the day.

P.aeruginosa a strains	*TSB + %1 glucose*	*BHI*	*LBB*	*MHB*
PA ATCC 27853	Strong	Strong	Strong	Weak
PA 1	Weak	Weak	Weak	Moderate
PA 2	Moderate	Weak	Weak	Moderate
PA 3	Moderate	Strong	Strong	Weak
PA 4	Moderate	Moderate	Moderate	Weak
PA 5	Moderate	Strong	Moderate	Non
PA 6	Weak	Weak	Weak	Moderate
PA 7	Weak	Weak	Weak	Weak
PA 8	Moderate	Moderate	Moderate	Weak
PA 9	Weak	Weak	Weak	Weak
PA 10	Moderate	Moderate	Weak	Weak
PA 11	Moderate	Moderate	Weak	Weak
Number and % for Biofilm Formation on Culture Media	0 strong (0.0%) 7 moderate (63.6%) 4 weak (36.4%)	2 strong (18.2%) 4 moderate (36.4%) 5 weak (45.5%)	1 strong (9.1%) 3 moderate (27.3%) 7 weak (63.6%)	3 moderate (27.3%) 7 weak (63.6%) 1 Non-biofilm (9.1%)

Table 2: Average Odt of PA biofilm formation on TSB + 1% glucose, BHI, LBB, and MHB culture media

	***Od_t of TSB +1% glucose**	***Od_t of BHI**	***Od_t of LBB**	***Od_t of MHB**
****Od_c**	0,146	0,134	0,104	0,157
PA ATCC 27853	0.465	0,542	0.431	0.332
PA 1***	0.182	0.194	0.179	0,251
PA 2***	0,184	0,221	0,139	0,183
PA 3****	0,384	0,594	0,428	0,219
PA 4****	0,292	0,304	0,212	0,190
PA 5****	0,474	0,524	0,249	0,265
PA 6****	0,269	0,229	0,195	0,201
PA 7****	0,162	0,236	0,112	0,206
PA 8****	0,367	0,507	0,379	0,202
PA 9****	0,245	0,254	0,206	0,162
PA 10****	0,418	0,386	0,239	0,256
PA 11****	0,296	0,319	0,214	0,217

*Od_t = Optical Density of the isolates, **Od_c = Optical Density of the controls ***= PA strains isolated from composite hospital wastewater samples ****= PA strains isolated from hospital wastewater samples collected at different time intervals during the day.

compared to the other three culture media may be because of the polysaccharide type (starch) in MHB, however, in TSB with 1% glucose and BHI contain monosaccharide glucose. The first protocol was more successful in the formation of the biofilms compared with the second protocol. This may be due to supplementary differences in the culture media used or differences in chemical compounds used in procedures and the kind of culture media used.

Strong biofilm formation was the highest in BHI while moderate biofilm formation was the highest in TSB with 1% glucose. An explanation of this result may be the rich nutrients in BHI and TSB with %1 glucose to support the production of PA biofilm. Even though the biofilm production percentages were different, biofilm was produced in all of the four media.

PA ATCC 27853 standard strain was used as a control showing strong biofilm formation in all three media; TSB with 1% glucose, BHI, and LBB (OD) 0.465, 0.542, and 0,431, respectively. However, this strain did not produce strong biofilm formation in the MHB media used.

Previous studies reported that the addition of glucose to TSB (TSB with 0.2 % glucose) and LB (LB with 2% glucose) increased biofilm formation when compared with BHI without the addition of glucose (32). The addition of glucose to TSB and BHI was recommended in Stepanovic et al.'s study for better biofilm formation (32). Commonly used media in biofilm formation assays were reported as LBB, MHB, M9, M63 with magnesium sulfate, casamino acids, glucose, and M63 with arginine (15, 35, 36, 38). Regarding our results, in BHI and LBB strong biofilm formation was higher compared to TSB with %1 glucose, however TSB with %1 glucose formed more moderate biofilms than the other three culture media. The optimal conditions for incubation temperature and time is 37 °C, overnight. The incubation time may change between 30 minutes to 72 hours depending on the purpose and parameters of the experiment (15). It was reported that flat- U or V-bottom microtiter plates could be used in biofilm formation assays and adhesion might change according to the characteristics of the plate material and the mucoid-non-mucoid PA strain (15,39). Since PA is motile, it bonds to the wall and/or bottom of the wells, and non-motile bacteria bond only to the bottom of the wells (36). The stains used were expressed as safranin and crystal violet which bind to the bacterial DNA, proteins, and polysaccharides (15,40). If there is no biofilm in samples, media, incubation temperature, incubation length, inoculation, and evaporation of media are important factors that should be rearranged or checked (15).

According to a meta-analysis, 75–99% of PA were biofilm-producers and, 8–50% were potent biofilm forming isolates in assays (41). Our results showed that 90.9% of PA were biofilm producers. Pseudomonas spp. in "One Health" perspective reports that environmental isolates could be reservoirs of antibiotic resistance genes. In our study, we aimed to assess biofilm formation capacity considering the importance of antibiotic resistance for further studies related to environmental PA isolates.

Chen et al. reported that biofilm formation was affected by many other factors including nutrients and the nutrients in TSB are amino acids, and glucose (2.5 g L− 1) which enhanced the biofilm formation in their study (42). Wijesinghe et al. showed that BHI enhances PA biofilm growth compared to LB. It was also reported that BHI had high amounts of peptones, proteins, and salts, which support biofilm formation. It was explained that the peptone and infusions had the required N, C, necessary growth factors, amino acids, and vitamins; dextrose worked as an energy source and NACl provided the medium osmotic balance. Therefore, the composition of the medium affected biofilm formation. Data from the researchers confirmed the results of the study by Chen et al., who suggested BHI as a good medium for the growth of biofilms. It was also reported that peptone in LB had nutrients and growth factors needed for bacteria; yeast extract provided amino acids, vitamins, and minerals; NaCl maintained an isotonic environment. It was explained that the amount of nutrients was

highest in BHI, followed by LB; and BHI increased the formation of biofilm because of the high nutrients in it. Their results (43) correlated with our results.

4. CONCLUSION

Protocols showed gathered information on the quantification of biofilm by PA and the details of the procedures of biofilm formation by PA isolated from hospital wastewater were given in the study to make the application of PA biofilm quantification easier. Differentiations in test conditions affected PA biofilm formation. In this study, the use of four media to assess biofilm production was evaluated and two protocols were compared. The first protocol was more efficient in the formation of biofilm than the second protocol. Biofilm was produced in all of the four media in different percentages, and strong biofilm formation was the highest in BHI while moderate biofilm formation was the highest in TSB with 1% glucose. The significance of PA as a pathogen in infections was reported widely, while the awareness of PA as an environmental bacterium in hospital wastewater was still increasing. The study results may help in PA biofilm formation quantification in microtiter plates. Further PA biofilm investigations, antimicrobial susceptibility tests, and biofilm formation molecular studies in hospital wastewater would be a major task to understand the nature of biofilm formation and to improve environmental and public health.

Conflicts of Interest: The authors declared that there is no conflict of interest.

Ethical Statement: This study was approved by the Dokuz Eylül University, Ethical Committee (Decision No 2022/03-15, Date 19.01.2022).

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